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The XIth Eucarpia Meeting on Cucurbit Genetics & Breeding

Elżbieta U. Kozik & Harry S. Paris
Editors

July 24–28, 2016

Warsaw, Poland

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PREFACE

Dear Colleagues,

This book of proceedings contains the full-length contributed papers of the lectures and posters presented at Cucurbitaceae 2016, the XIth Eucarpia Meeting on Cucurbit Genetics and Breeding. The date of issue of this book is July 24, 2016, the first day of the conference, following the cucurbit-meeting tradition initiated at the 6th Eucarpia Meeting on Cucurbit Genetics and Breeding (Spain, 1996).

We have edited all of the original contributed papers and contacted all authors for their final approval of the editing. If, nonetheless, some errors have been overlooked, we apologize.

After the meeting, all the communications printed in these proceedings will be available in PDF format on the following web site: www.inhort.pl/en/cucurbit2016

The format was considered by us carefully before the distribution of the call for papers. We selected this format because it is both compact and easily comprehended. We thank all contributing authors for their cooperation in preparing their manuscripts according to the format. We acknowledge with pleasure the attention and professional workmanship of the publisher, Wydawnictwo Sigma, in helping us produce what we believe to be a fine book of proceedings.

July 4, 2016

Editors
Elżbieta U. Kozik
Harry S. Paris

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ORGANIZING COMMITTEE MESSAGE

Dear Colleagues,

We, the Organizing Committee of Cucurbitaceae 2016, the XIth Eucarpia Meeting on Cucurbit Genetics and Breeding, welcome you back to Warsaw, Poland, where the Vth such meeting was held in 1992.

The Eucarpia meetings on Cucurbit Genetics and Breeding were initiated in the 1970s. Since 1988, they have been held on a regular basis every four years, in France (1988), Poland (1992), Spain (1996), Israel (2000), Czech Republic (2004), France (2008), Turkey (2012), and now again Poland. Meetings on cucurbit genetics and breeding have been held in the United States of America since the 1980s. Since 1994, they have been held on a regular basis in the even-numbered years alternating with the Eucarpia meetings, in Texas (1994), California (1998), Florida (2002), North Carolina (2006), South Carolina (2010), and Michigan (2014).

We greatly appreciate the financial support of the EUCARPIA Cucurbitaceae 2016 Sponsors. Please join us expressing thanks to the Sponsors` representatives for their generous contributions.

As of this writing, the following have furnished much-needed financial and material support for Cucurbitaceae 2016: Eucarpia, InHort Skierniewice, City of Skierniewice, Bayer, Amy Goldman Fowler, Bejo, Syngenta, Rijk Zwaan, Polan Kraków, PNOS, PlantiCo, SANLAB, RB, and TORSEED.

We hope Cucurbitaceae 2016 will be the most memorable and enjoyable conference for all participants.

July 4, 2016

Organizing Committee
Elżbieta U. Kozik
Urszula Kłosińska
Marcin Nowicki
Marzena Nowakowska
Wojciech Szczechura

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POSTER PRESENTATIONS

History of Cucumber Breeding in Poland

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Abstract. Cucumber (*Cucumis sativus*) is the most economically important cucurbit in Poland and has been cultivated in this country for centuries. Since the 19th century, companies and growers alike maintained and reproduced seeds of vegetable cultivars of local and foreign origin, mainly from Denmark, Holland, and Germany. Polish cucumber breeding was initiated only at the beginning of the 20th century, by three private companies, "C. Ulrich", "Hoser Brothers", and "E. Freege". Just before World War II (1939), the first Polish register of original vegetable varieties was established; it included both first Polish field cucumbers 'Przybyszewski Hoser' ("Hoser Brothers") and 'Warszawski' ("C. Ulrich"). But, the history of cucumber breeding in Poland resembles the history of our country, turbulent and demanding sacrifices. Unfortunately, in the 1950s, all private breeding companies were nationalized. The successors of the three original privately owned Polish breeding companies underwent numerous administrative transformations, becoming State Treasury-owned commercial enterprises before the new millennium, and they operate in this form today. Herewith presented are the achievements of individual Polish companies and academic institutions in breeding improved cultivars for the cucumber production industry.

Keywords: *Cucumis sativus,* cultivars, F_1 hybrids, genetic resources, processing industry

Introduction

Cucumber (*Cucumis sativus* L.) is the most important cucurbit in Poland, being commonly cultivated in large commercial operations as well as home gardens, for processing and fresh market. According to FAOSTAT (www.faostat.fao.org) statistics for 2012, Poland was first in the European Union for area planted with cucumber (17,100 ha) and second for production (520,868 t). Over the past 10 years, the area planted with cucumber has slightly decreased, while productivity slightly increased. Annual consumption of cucumbers in Poland is about 7 kg/person.

Cucumber adoption and the beginnings of cucumber breeding in Poland

Cucumbers probably first entered Poland by the overland route across southwestern Asia through to eastern Europe in early medieval times (Paris et al. 2012). Cucumbers have been grown in Poland since then. Maintenance and seed reproduction of cucumbers and other vegetables occurred later in Poland than in some other European countries, due to its cool, unfavorable climate and history of political upheaval. During the 19th century, various European companies were established that specialized in the maintenance and reproduction of old, established vegetable cultivars and breeding new, improved ones. Vegetable growers in Poland imported seeds, including cucumber, mainly for their own production and reproduction as they saw fit. Some knowledge of the advanced level of gardening in western Europe did reach Poland, invoking considerable interest among gardening enthusiasts. This spurred increasingly frequent trips to accrue knowledge and skills that would benefit local horticulture. The private seed companies "C. Ulrich", "Hoser Brothers", and "E. Freege", in the 19th century, were the first establishments to promote the introduction and adoption of horticultural plants in Poland. Their present-day successors continue this important function.

"C. Ulrich", founded in 1805 in Warsaw, was the first Polish seed company. Initially, the company specialized in vegetable produce, fruits, and ornamental plants, but starting in 1830 it introduced the sale of vegetable seeds imported from abroad, mainly from Germany, Denmark, and the Netherlands. The company launched actual breeding activities of vegetables, including cucumber, in the 1930s (Korohoda 1984).

"Hoser Brothers" was established in Warsaw in 1848. Early on, it engaged in the production of vegetable produce and ornamental plants. The beginning of the 20th century saw expansion to its actual breeding of vegetable and ornamental plants, and a marked increase in production of its seed varieties.

"E. Freege" was established in 1860 in Cracow. At first it was a horticultural farm, shifting its activities over time towards seed production. This meant, in essence, import of the best foreign varieties and their sale, a model of business similar to English and German seed companies.

In 1917, in the western part of Poland, the oldest of the currently operational major vegetable market companies was founded: "Spójnia Nochowo". Its main activity then was the production of vegetable seeds. During the inter-war period, this

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company was the only breeding facility in western Poland, and its seed reproduction activities contributed greatly to raising the status of gardening in this country.

At the time, plant breeding in the true sense was poorly developed in Poland. Producers of vegetable seeds, including cucumbers, mainly imported cultivars from abroad and reproduced them at their own discretion. Then, in 1937, just before the outbreak of the World War II, the Polish Association of Seed Producers decided to carry out an assessment of seed samples obtained from Polish breeders. Only a small part of these was then selected, after thorough research conducted in the Department of Crop and Vegetable Breeding (Warsaw University of Life Sciences, then in Skierniewice), for the national Preliminary Register of Original Varieties, the first Polish register of original vegetable cultivars. Resulting from this assessment of the seed samples submitted by domestic breeders, 35 cultivars of vegetables were accepted into the Register, including two field cucumbers, 'Hoser Przybyszewski' ("Hoser Brothers") and 'Warszawski' ("C. Ulrich").

Only in the three years 1936–1939 did vegetable cultivation experience intensive development, and it is this period that is considered the birth of the new era of plant breeding in Poland. At this time, many species, including cucumber, began to undergo dynamic improvement. Despite great difficulties, and owing to the erstwhile commitment of various groups of farmers and breeders, Poland managed to almost catch up with the European level of plant breeding. The first export of Polish vegetable seed varieties was planned for the fall of 1939. No one suspected, though, that the outbreak of WWII would almost completely destroy these new achievements and impede plant breeding in Poland for decades.

Cucumber breeding after 1945

World War II hostilities and the Warsaw Uprising (1944) resulted in severe losses, especially for the companies that had facilities located near Warsaw, but also throughout the country. Only small quantities of breeding materials were rescued. Polish plant breeding suffered acutely from the loss of outstanding professionals who died at the hands of the occupiers. The surviving older generation and the young people educated by them had to rebuild Polish seed science and plant breeding almost from scratch, in very difficult post-war conditions. Unfortunately, too, in the 1950s, all private companies were nationalized. Under state aid, they were offered leases of a number of farms, where they could organize new plant breeding stations. On the basis of "E. Freege", decades later and after many administrative and organizational transformations, "POLAN Cracow" was established in 1992, and is operational today. On the basis of the nationalized assets of "Hoser Brothers" and "C. Ulrich", nine horticultural plant breeding stations were created in the 1950s, which eventually became part of "PlantiCo Zielonki" of Warsaw. "PlantiCo Zielonki" is the only survivor of the former nucleus of Warsaw plant breeding. As early as January 1945, "Spójnia Nochowo" re-undertook its activity, but at the end of 1950 it came under compulsory state control and then nationalized. Many organizational transformations followed. Ultimately, in 1994, it became a State Treasury-owned company, functioning in this form today.

The State Register of Original Varieties was established in Poland in 1952. In 1966, the Polish Ministry of Agriculture established the Research Center for Cultivar Testing (Polish acronym COBORU) near Poznań, which operates to this day. Evaluations of cultivars sent to the Register were carried out at the time in different parts of Poland. Cucumber cultivars were evaluated in Puławy, in the east. Comparison standards were cultivars imported from Denmark, Germany, and the Netherlands. Based on the results, the first selection of vegetable cultivars was made at the Research Institute of Vegetable Crops (RIVC) in Skierniewice, led since its inception in 1964 by Prof. Emil Chroboczek. Later on and until now, COBORU publishes annually a list of vegetable varieties registered in Poland. In Poland, as in the other EU countries, *The Common Catalog of Varieties of Vegetable Plant Species* is in effect.

Currently, most cucumber breeding in Poland is conducted at five centers: three seed companies operating as Treasury-owned commercial companies ("POLAN Cracow", "PlantiCo Zielonki", and "Spójnia Nochowo") and two academic institutions (Warsaw University of Life Sciences and the Research Institute of Horticulture Skierniewice, InHort).

"POLAN Cracow"

Field cucumber has been and still is the most important vegetable crop of the company assortment. Its first cultivars, 'Delicius', 'Mikor', and 'Monastyrski', all monoecious, were registered in the 1970s (Żuradzka 2016). For many years (1975–2008), 'Monastyrski' has been widely grown in Poland, being used in processing. Introduction of F_1 hybrids, much more productive than the traditional monoecious cultivars, was a revolution in cucumber cultivation. The first Polish-bred F_1 hybrid cucumber, 'Polan', from the RIVC Skierniewice (currently InHort) was bred by Prof. Bogdan Kubicki and Dr. Ewelina Kłossowska. This cultivar was registered in 1972 and licensed to "POLAN Cracow". 'Polan F_1' is still very popular among growers. Soon thereafter, Dr. Izabela Żuradzka of "POLAN Cracow" led a team of cucumber breeders, in collaboration with Prof. Kubicki, in the development and registration of several high-yielding cucumber hybrids of various fruit types, with resistance to *Cladosporium cucumerinum* (scab): 'Dragon' (1976), 'Mieszko' and 'Racibor' (both 1979). Fifteen to 20 years later, "POLAN Cracow" released seven pickling cucumber F_1 hybrids with resistance to *Pseudoperonospora cubensis* (downy mildew) and *Cucumber mosaic virus* (CMV): 'Krak', 'Julian', 'Borus', 'Zagłoba', 'Hubal', 'Chrobry', and 'Forum'.

An indubitable success of the company was the development of the first Polish gherkin-type F_1 hybrids, 'Anulka', 'Rufus', and 'Tytus'. These were very well received by growers due to high yields, general pathogen tolerance, and high fruit quality. Another major commercial achievement was for pickling cucumbers, 'Julian F_1' (1996) being exceptional because of its very good fruit processing quality.

In the late 1980s, the company started cooperation with the RIVC Skierniewice because of problems with *Pseudoperonospora cubensis* infestations, which decimated cucumber plantings all over Poland. The work focused on methods for screening for resistance. Efforts were conducted under the direction of Prof. Roch W. Doruchowski. After his retirement in 2000, the cooperation continued, coordinated until this day by myself. Common interests cover a search for new sources of resistance to *P. cubensis*, determining the mode of inheritance of resistance, identifying DNA markers for resistance, insights into the genet-

ic diversity of the pathogen in Poland, and generation of new genetic variability in cucumber for low-temperature tolerance. As a result of this cooperation, the company received a wealth of valuable germplasm for their breeding efforts. In 1999, the company undertook cooperation with the Department of Plant Genetics, Biotechnology, and Breeding (Warsaw University of Life Sciences), to obtain parental lines of double haploids (DH) by induced parthenogenesis. Currently, such lines are being developed *in vitro* by the company. Members of the cucumber breeding team of the company include Marta Antos, Eugenia Koterwa, and Aleksander Kopta. In all, "POLAN Cracow" has bred 40 cultivars of field cucumbers, including 37 F_1s, of which 20 are widely cultivated.

"PlantiCo Zielonki"

An heir to the tradition of "C. Ulrich", "PlantiCo Zielonki" took over the pre-war registered field cucumber 'Warszawski' (Karpiński et al. 2016). The company also carried large collections of foreign cultivars, of which 'Delicius' (1970) and 'Wisconsin' (1973) were registered at COBORU. The 1970s mark the origins of breeding of its own cucumber cultivars. During that decade, "PlantiCo" registered four F_1 salad cucumber hybrids: 'Fryko', 'Ikar', 'Kometa', and 'Kuba', that were bred in collaboration with the RIVC Skierniewice. In the 1980s and '90s, the company markedly increased its cucumber breeding activities and released 24 cucumber cultivars, which makes 53% of all those registered by the company. The major economic achievements of the company are its five hybrids for greenhouse production 'Replika', 'Atos', 'Caruso', 'Heros', and 'Ines'. These bridged the gap in the assortment of greenhouse cucumber cultivars and created alternatives to the Dutch semi-long type. Other notable achievements include breeding of new F_1 cultivars of field cucumber: smooth skin, no warts, for salad 'Gracius' (1992) and for pickling 'Soplica' (2001). The main contributors to "PlantiCo's" broad array of cucumber cultivars are: Barbara Chrzanowska, Jadwiga Tomczyk, Teresa Bartłomiejczak, and Maria Guzik. So far, the company has released 45 cucumber hybrids, including 29 of field cucumbers and 16 greenhouse cucumbers.

For many years, "PlantiCo" cooperated with academia in cucumber breeding. Prof. Katarzyna Niemirowicz-Szczytt (Warsaw University of Life Sciences) guided the creation of a range of DH lines with their subsequent takeover by PlantiCo. Dr. Urszula Kłosińska and I at InHort bred cucumbers for increased resistance to *P. cubensis* and for tolerance to low temperatures, receiving new breeding materials that contributed to the expansion of germplasm variation. "PlantiCo Zielonki" has a license for seed production and sale of the 'Cezar F_1', which was bred at InHort. This hybrid is a significant part of "PlantiCo's" total sales of cucumber seeds.

"Spójnia Nochowo"

After the nationalization of the company in the 1950s, a cucumber for greenhouse production, 'Nochowski', was maintained (Pędziński 2016). In 1979, a hotbed hybrid 'Olimp F_1' was released and in 1984 another for greenhouse production was released, 'Lech F_1', which is still active in the register. In the 1980s, thanks to the company president Eng. Marian Pędziński, main business areas were formulated; these included breeding of vegetables, seed production, and seed marketing. Since then, the company has experienced a marked boom. The hit field pickling cucumber 'Śremski F_1' (1988) is the major reason. Popularity of this early variety among processors lasts to this day, keeping the company in good financial condition. 'Śremski F_1' has proven to be an outstanding cultivar; the seed sales in the years 1998 to 2015 amounted to 350 t, representing 24% of the seed sales of field cucumbers in Poland. Notable progress was accomplished during 1994–2004, with the development and release of three new parthenocarpic and genetically devoid of bitterness F_1 hybrid cucumbers for greenhouse production: 'Orion', 'Polonez', and 'Szafir'. Subsequently, the company established its own *in vitro* laboratory, in which they fast-track the breeding with DH lines. They also have set up a resistance-breeding laboratory, working towards the increase in the level of resistance to major cucumber pathogens, mainly *Pseudoperonospora cubensis*. The results of this work are four F_1 hybrids with resistance against *P. cubensis*, 'Śremianin', 'Lider', 'Husar', and 'Meteor', which were released from 2002 to 2008. In 2002, "Spójnia" also released the first two Polish parthenocarpic cucumbers, 'Rubin F_1' and 'Jaspis F_1', for cultivation under low covers and recommended for low-salt souring (fermentation for only a few days). The low-salt souring group was then joined by 'Lazuryt F_1' (2004), another hybrid having short fruits and for cultivation primarily under low tunnels, and 'Bursztyn F_1' and 'Karat F_1' (both in 2007) for open field production. In 2009–2014, the company introduced a series of eight new early cucumbers suitable for pickling, and its latest additions are early, high-yielding, and tolerant to *P. cubensis* 'Atut F_1' and 'Śremski Nowy F_1'. Overall, the company has registered 42 hybrids, including 27 field cucumbers, nine greenhouse type, and six for cultivation under plastic tunnels. The largest contributors to the company breeding efforts have been: Marian Pędziński (40 cultivars), Anna Nowak (15 cultivars), Arkadiusz Labrzycki (15 cultivars), Szymon Frąckowiak (13 cultivars), and Wojciech Matuszak (6 cultivars).

Other companies

Out of the 110 cucumber cultivars currently listed in the COBORU Register (February, 2016), 38 were registered by establishments other than the three described above. These include Polish breeding and seed companies, and some private breeding enthusiasts. It is important to note that foreign companies may also register in the Common Catalog of Varieties of Vegetable Species, and such registration is obligatory for each European Union country.

Academic units

Both the Warsaw University of Life Sciences and InHort Skierniewice are state-sponsored scientific institutions, active mainly in fundamental research in their well-equipped laboratories. They also have greenhouses and experimental fields with an ecological field in Skierniewice. Both institutions have accumulated large collections of cucumber cultigens, used also in their applied activities such as breeding of new cultivars, to increase the range of cucumbers in the domestic market. Both institutions have extensive cooperation with Polish breeding companies. Cucumber is a popular model plant among Polish scholars in fundamental research. Major players involved in research and breeding are named below, but scientists from Warsaw University, the Polish Academy of Sciences, the University of Łódź, the University of

Wrocław, and the Poznań University of Life Sciences have also contributed to various aspects of cucumber biology.

Cucumber breeding activities in Polish academia are supported by various funding sources. So far, the major portion of the funding was through statutory projects of the Polish Ministry of Science and Higher Education. In accordance with the gradual reduction of these subsidies in recent years, faculty search to obtain grants from the National Science Center and the National Center for Research and Development. Grants awarded by the Polish Ministry of Agriculture and Rural Development (PMARD) have proven particularly important for breeding projects in preparation of the starting materials for breeding of vegetable plants and fundamental research for the biological advancement of plant production. Plant breeders of InHort have also been carrying out related applied works under the Multi-Annual Program funded by PMARD. Other funds used for the purposes of breeding include fees from the licenses granted to seed companies.

Warsaw University of Life Sciences, Department of Plant Genetics, Breeding, and Biotechnology

From 1965 to 1969, Prof. Kubicki, while simultaneously working at the Department of Plant Genetics (Polish Academy of Sciences, Skierniewice) and under the leadership of the eminent geneticist Prof. Edmund Malinowski, and part-time at the Department of Plant Breeding and Seed Science (RIVC), developed a method of cucumber F_1 hybrid cucumber seed production based on gynoecious maternal parents (Kubicki 1969, Niemirowicz-Szczytt 1987, 2016). Together with Dr. Kłossowska, they bred the first Polish hybrid cultivar of cucumber, 'Polan F_1'. Together with Dr. Janina Bażant, they developed cultivars for protected cultivation, 'Iwa' and 'Skierniewicki' (both in 1972); 'Skierniewicki' and 'Polan' are still in the COBORU register. After the deployment of this method and the maternal lines to Polish breeding stations, Prof. Kubicki supervised the breeding efforts, and became in the 1970s a co-breeder of nine cucumber hybrid cultivars. As a member of the faculty of the Department of Genetics (the name changed several times) at the School of Horticulture, Warsaw University of Life Sciences, until his untimely death in 1985, he conducted extensive research on plant sexuality, including cucumber. His efforts also focused on heterosis and transgressive segregation for earliness, cold tolerance, pathogen resistance, and nutrient content. Prof. Kubicki also initiated research on interspecific crosses, polyploidy and haploidy, and mutations. The results of his research are documented in numerous publications, as listed by Malepszy (1987).

Prof. Stefan Malepszy, a geneticist and biotechnologist, devoted much time and effort to continue the work of Prof. Kubicki. As a great organizer, he continued the construction of the laboratory, a semi-automatic experimental greenhouse, and other infrastructure projects. He also maintained the high level of experimental work and directed a number of research topics and projects on cucurbit breeding and genetics. Specifically, Prof. Malepszy focused efforts on the effect of genetic background on the ability to regenerate plants *in vitro*, methodological and biological conditions favoring utilization of genetic modifications, and production of genetically modified plants of potential economic value.

Mentoring of the team of vegetable plant breeders has been conducted by Prof. Katarzyna Niemirowicz-Szczytt. Under her guidance, the team has continued improving the induction of cucumber haploids and their diploidization (Malepszy 1987). Three methods of diploidization of cucumber haploids have been developed. The resulting homozygous lines have been applied in breeding by Polish seed companies. Testing for resistance to *Pseudomonas syringae* pv. *lachrymans* (angular leaf spot), in cooperation with seed companies, has received considerable attention (Olczak-Woltman 2008). Currently, cucumber transcriptome analyses in response to infection with this pathogen are under study. Breeding of cucumber has focused on earliness, yield, and tolerance to angular leaf spot and downy mildew. Much effort has been invested in selecting parental lines for F_1 hybrids; this requires knowledge of the genetic basis of parental components, which is crucial for high seed production efficiency. Over the past 25 years, the team has developed a number of cucumber hybrids for field production, the first of which was 'Dar' (1993), a tribute to the activity of Prof. Kubicki. Subsequently, the team of Prof. Niemirowicz-Szczytt released in 1998-2012: 'Tessa F_1', 'Szeryf F_1', 'Lokata F_1', and 'Traper F_1'. The team also maintains the cucurbit collection for InHort's Gene Bank.

Institute of Horticulture (former Research Institute of Vegetable Crops) in Skierniewice (InHort; former RIVC)

Research on heterosis breeding of cucumber in Skierniewice was initiated and led until 1964 by Barbara Miernik-Pinchinat, then continued by Dr. Kłossowska in collaboration with Prof. Kubicki (Malepszy 1987). The resulting breeding materials were used to develop the first Polish cucumber, 'Polan F_1' (Kozik and Doruchowski 2016). Creation of this hybrid resistant against *C. cucumerinum* and CMV was a major breeding achievement, as it yielded two times more than the commonly grown 'Wisconsin SMR-18' or 'Monastyrski' (Kłossowska and Kubicki 1970). The maternal line was handed over to Polish seed companies. "Spójnia Nochowo" used it to breed 'Śremski F_1'. At that time, Dr. Kłossowska and Prof. Kubicki initiated testing for resistance to angular leaf spot (Kłossowska 1976) and Dr. Janina Bażant for resistance to powdery mildew (*Erysiphe* spp.; Bażant 1981).

The year 1985 marked the first recorded occurrence of *Pseudoperonospora cubensis* in Poland, and since then it has been present every year with high severity, often times epidemic. Prof. Doruchowski's team reported the mode of inheritance of resistance to downy milew (Doruchowski and Łąkowska 1992). Later on, results of parallel studies run in the U.S.A., India, and Poland suggested the possibility that there were different races of *P. cubensis* in these countries. Due to the growing impact of downy mildew on cucumber production in our country, Dr. Urszula Kłosińska and I cooperated in screening 1300 cucumber accessions, leading to the identification of cucumbers with resistance levels exceeding those used in previous breeding work (Call et al. 2012). Furthermore, a comprehensive methodology was developed for testing cucumber germplasm under phytotron conditions, using various inoculation methods (Kozik et al. 2013). The results correlated with those of field studies under natural pathogen infection. Resistance in one of the new sources, 'Ames 2354', was found to be polygenically inherited. Currently, the InHort team works toward introgression of genes for *P. cubensis* resistance from new sources into horticulturally elite cucumber germplasm.

One result of efforts by Prof. Doruchowski and his collaborators were the first Polish field cucumber F_1 hybrids resistant to *Pseudoperonospora cubensis*: 'Aladyn', 'Parys', and 'Cezar'

(1993). They bred a series of F_1 cucumbers ('Atlas', 'Pola', 'Cyryl', 'Bazyl', 'Izyd', 'Hermes Skierniewicki', 'Kronos Skierniewicki', 'Odys') in the years 1996–2000, having a high level of *P. cubensis* resistance combined with other useful features. After Prof. Doruchowski's retirement in 2000, I took over the cucumber breeding program together with Dr. Kłosińska, and we have succeeded in releasing more field cucumber hybrids ('Rodos', 'Reja', 'Ozyrys', 'Zefir', 'Edyp', 'Ibis') with high levels of resistance to *P. cubensis*.

From 2009 to 2013, my collaborators and I have searched for a molecular marker for downy mildew resistance in cucumber, which yielded $OPX18_{950}$, a marker that distinguished resistant genotypes from susceptible ones. Subsequently, three quantitative trait loci (QTLs) on cucumber chromosome 5 conferring downy mildew resistance were mapped and three respective DNA markers were identified (Szczechura et al. 2015). This work continues, analyzing the anatomical, biochemical, cellular, and molecular mechanisms of resistance.

Testing cucumber tolerance to low temperatures, led by myself in collaboration with Prof. Todd Wehner of North Carolina State University from 2003 to 2005, showed that this trait at the seedling stage was controlled by a single dominant gene, *Ch* (Kozik and Wehner 2008). In 2014, InHort introduced 'Ibis F_1', the first hybrid cucumber with tolerance to low temperatures during emergence and early seedling development. Currently, we are searching for sources of water-deficit tolerance in cucumber and investigating the genetic, physiological, and biochemical basis of this trait.

Conclusions

This overview of the achievements of Polish cucumber breeding is a reminder of the recent and more distant past, a summary of its current state, and a statement of the future challenges yet to be faced by Polish cucumber breeders. Currently, the COBORU Register lists 92 Polish cucumber varieties (110 total), of which 95% are hybrids; this indicates how much progress has been made in the field of cucumber breeding in Poland over less than 100 years, taking into account the historical tragedies of the nation during this time (WWI and WWII). The richness of the germplasm currently in the possession of Polish cucumber breeders can be expected to provide further success in cucumber improvement. The efforts of Polish cucumber breeders can be expected to stimulate, within Poland and beyond, innovation in cucumber breeding and seed production, and support the implementation of improved cultivars into the farming community.

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Emergence of the Sweet Dessert Watermelon, *Citrullus lanatus*, in Mediterranean Lands During the Roman Era

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Abstract. Watermelons, *Citrullus* species, are native to Africa. Archaeological remains, iconography, ancient literature, and the presence of wild and primitive watermelons in northeastern Africa indicate that the dessert watermelon, *C. lanatus*, is native to that region. The dessert watermelon was nurtured and domesticated there, for water and food, over 4000 years ago. Although the domesticated watermelons of Egypt and Sudan were probably not bitter, there is no evidence to indicate that they were sweet. Indeed, the extant primitive watermelons known as *gurma* in Egypt and *gurum* in Sudan are spherical and small (≤ 14 cm diameter), with watery but bland, white or pale green fruit flesh. Hebrew-language literature from the first centuries CE indicates that, by the time of the Roman Empire, sweet dessert watermelons were esteemed in the Land of Israel, and thus likely were present in other Mediterranean lands as well. The ripe fruit flesh of the dessert watermelons of that time, which was probably distinctly colored rather than pale, was eaten raw and had a sweetness which was comparable to that of figs, grapes, and pomegranates. The seeds were not consumed.

Keywords: *Citrullus lanatus*, evolution under domestication, watermelon

Introduction

The dessert watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, is one of the most cooling, refreshing, and appreciated food items on hot summer days. Watermelons are among the most widely grown vegetable crops in the warmer regions of the world, with over 3,400,000 hectares planted and over 100,000,000 t harvested annually (Wehner 2008). However, the sweet dessert watermelons that are so familiar today are derived from ancestors that, anthropocentrically, were much inferior.

The xerophytic genus *Citrullus* ($2n = 2x = 22$) is native to Africa. In accordance with the classification of Chomicki and Renner (2015), there are seven species in the genus. Three of them, *C. ecirrhosus* Cogn., *C. rehmii* De Winter, and *C. naudinianus* (Sond.) Hooker f., grow wild in southern Africa and have not been introduced to cultivation. The other four *Citrullus* species are cultivated to a lesser or greater extent. The colocynth, *C. colocynthis* (L.) Schrad., is native to northern Africa. The citron watermelon, *C. amarus* Schrad., is native to southern Africa. The egusi watermelon, *C. mucosospermus* (Fursa) Fursa, is native to western Africa. Recently, I presented a view, based on archaeological remains, iconography, ancient literature, and the modern presence of wild and primitive watermelons in Sudan and Egypt, that the familiar, esteemed dessert watermelon, *C. lanatus*, is native to northeastern Africa (Paris 2015).

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However, in spite of the familiarity, dating to 5000 years ago, of ancient Egyptian civilizations with watermelons, I could find no evidence showing that the dessert watermelons of Egyptian antiquity were sweet. Here I will discuss the evidence for the emergence of sweet dessert watermelons during the Roman era.

Materials & Methods

Sources of evidence

A multidisciplinary approach that encompasses botany, horticulture, cookery, philology, and archaeology is necessary to best assess crop plant history (Dalby 2003). For cucurbit crops, archaeological remains, iconography, and literature have provided much information concerning their development under cultivation (Paris 2000, Janick et al. 2007).

Some diagnostic features of Citrullus

Citrullus is readily distinguished from other cucurbit genera by the pinnatifid shape of its leaf laminae (Paris et al. 2013). The flowers are solitary and have five light yellow petals. Most of the flowers are staminate, a pistillate or hermaphroditic flower appearing at every seventh or eighth leaf axil (Rosa 1928). Ovaries and primordial fruits are lanate, becoming glabrous, smooth, and glossy as they grow. Usually 30–40 days ensue from anthesis to fruit maturity. External indications of fruit ripening are subtle (Thompson and Kelly 1957). If stored in a cool, shady place, watermelons can keep for weeks or even months after harvest without serious deterioration of their quality (Keith-Roach 1924, Rushing et al. 2001).

Watermelon fruits can weigh as much as 100 kg but most modern, commercially available watermelons range from 3–13 kg. Fruit shape can be spherical, globular, oval, or oblong. The watermelon rind consists of two layers. The surface of the thin, glossy exocarp is typically boldly striped in two shades of green; the stripes are jaggedly edged and range in breadth from very narrow to very broad. The thick, white mesocarp is wet and hard. Underneath the rind is the watery fruit flesh or endocarp, which is the portion of the fruit that is usually eaten. Early in development, the fruit flesh is hard, white or otherwise pale-colored, and insipid. In citron watermelons, the fruit flesh remains hard, nearly colorless and tasteless to fruit maturity (Xu et al. 2012). In sweet dessert watermelons, the flesh of the maturing fruit becomes soft and accumulates carotenoid pigments and sucrose (Elmstrom and Davis 1981, Brown and Summers 1985, Soteriou et al. 2014). Color begins to accumulate between 2 and 3 weeks after anthesis, first around the developing seeds and thereafter gradually spreading throughout the endocarp (Perkins-Veazie et al. 2012). Depending on the genotype, the flesh color of ripe watermelon fruits can range from red, pink, orange, yellow, or a mixture of these colours, to green or white (Gusmini and Wehner 2006). The range in texture of the ripe fruit flesh can be described as crisp, tender, or liquefied, and coarsely or finely grained. Each fruit can contain several hundred seeds that, to the casual observer, are scattered throughout the flesh and, to the consumer, are of much annoyance. The seeds of dessert watermelons are hard, flat, and oval and, depending on the genotype, range in length from 10 to 16 mm and are black, brown, tan, white, yellow, or red, and can be patterned with a second color.

Dessert watermelons, *C. lanatus*, are sometimes confused with melons, *Cucumis melo* L., as both are often large and sweet. The most salient features distinguishing the two are the shape of the leaf laminae, distribution of staminate and pistillate flowers on the plant, range of fruit shape, fruit surface features, wetness of the fruit, thickness of the fruit rind, fruit flesh color, and shape, color, and distribution of seeds within the fruit (Paris et al. 2012). In the field, watermelons ripen evenly over the course of the harvest season but melons ripen in distinct waves (Rosa 1924, McGlasson and Pratt 1963, Pratt et al. 1977). Watermelons have no well-marked indicators of fruit ripening whereas melons typically become aromatic and yellow, and abscise from the plant upon ripening (Isenberg et al. 1987, Nonnecke 1989). Watermelons have a much longer shelf-life than most melons but are subject to breakage if not handled properly (Whitaker and Davis 1962, Robinson and Decker-Walters 1997). In watermelons, the seeds are distributed within the fruit flesh but in melons the fruit flesh is free of seeds.

Possible pitfalls in interpretation of literature

Although iconography has been the most important source of evidence in understanding cucurbit crop history (Eisendrath 1961), fruit sweetness is not a trait that is readily amenable to illustration or detection in archaeological remains. Literature is a potentially rich source of information concerning possible sweetness of cucurbit fruits (Paris et al. 2012). Generally, though, food items were considered by ancient writers to be familiar to everyone, and thus in no need of description, and were usually discussed only in reference to their supposed dietary or medicinal effects (Dalby 2003). Off-hand, indirect descriptions of foods can sometimes be gathered in other contexts, especially medicine, religion, travel, and cookery. No direct description of the characteristically smooth, glossy, green-striped rind, or the taste, texture, or color of the fruit flesh or seeds of watermelons has been found in literature of antiquity (Janick et al. 2007, Paris 2015).

Caution must be exercised in interpretation of the ancient literature. Adjectives tend to be used differently across languages, contexts, geographic areas, and periods of time (Paris et al. 2012). For example, "sweet" can be synonymous with sugary or not bitter, not sour, not salty, or not spicy. Also, what might have been considered sweet in ancient times might not be considered sweet today, given recent development of cultigens with greater sweetness. The adjective "red" has been variously used for orange, purple, and brown, and "yellow" has been used for orange. Nouns have also been used indiscriminantly. The word "melon" has been used in American English for both *Citrullus* and *Cucumis melo*. Similar examples occur for the medieval Latin *pepo* and *melones*, and Arabic *battikh*. The intention of such words has to be interpreted in the context of time period, language, and geographic area.

Watermelons in Literature of Antiquity

Biblical Hebrew

The Children of Israel, during their sojourn in the Sinai Desert after the exodus, longed for five vegetables they knew from the Land of Egypt; these were, respectively, the *qishu'im* (vegetable melons), *avattihim* (watermelons), *hazir* (leeks), *bezalim* (onions), and *shumim* (garlics) (*Numbers* 11:5). As watermelons were mentioned together with other vegetables, they were considered to be just like the others, to be eaten raw, cooked, or pickled. They were not like modern sweet dessert watermelons, otherwise they would have been craved to be eaten first and immediately in a desert environment.

Centuries later, in the Land of Israel, the word used for a field of cucurbits was *miqsha* (*Isaiah* 1:8). The word *miqsha* is derived from the word *qishu'im*. Indeed, in those times, watermelons must have been of secondary importance to the vegetable melons, and there is no evidence suggesting that the watermelons were sweet.

Greek (400 BCE – 355 CE)

The word *pepon* of classical Greek, literally a sun-ripened fruit, usually referred specifically to watermelon (Liddell and Scott 1948, Andrews 1958, Stol 1987, Grant 2000). The Greek doctor Hippocrates, in approximately 400 BCE, wrote that the *pepones* were easily digestible (Jones 1967). Likewise, Dioscorides, in 70 CE, wrote that the flesh of the *pepon* is easily digestible and diuretic, and prescribed the rind of the *pepon* to be applied on top of the head, for children suffering from heat stroke (Osbaldeston and Wood 2000, Beck 2005). A century later, Galen wrote that the *pepon* was cold and wet, and a diuretic, and the *melopepon* less so (Grant 2000, Powell and Wilkins 2003). Clearly, to all of these doctors, the pepo they were describing was the watermelon, *C. lanatus*. The melopepon, introduced later and of lesser effect, would appear to be the melon, *C. melo*.

Athenaeus, in *The Learned Banqueters* (late second century), quoted thousands of lines of verse written by approximately

1000 Greek authors from various times and localities (Olson 2006). One of them, Phaenias, is quoted as stating that the *pepon* is edible, except for the seeds, when the flesh becomes soft. Another, Diocles, wrote that the best *kolokyntas* were round, very large, *glukeian* (sweet), and easy to digest. The word *kolokyntas* was usually applied to bottle gourds, *Lagenaria siceraria* (Mol.) Standl., but for some ancient Greek speakers the word was applied to watermelons.

Latin (77 – 516 CE)

Pliny, in his *Historia Naturalis* (77 CE), described the *pepo* as a most refreshing and cooling food (Rackham 1950, Jones 1951). This description would fit the watermelon, *C. lanatus*. The *melopepo* (apple-*pepo*) was a new introduction resembling a quince that, upon ripening, turned yellow, became aromatic, and, significantly, spontaneously detached from the plant. Obviously, the *melopepo* was a cultigen of melon, *C. melo*. Quintus Gargilius Martialis, around 260 CE, wrote that the *pepone* were good to eat after removal of the rind and pits (Maire 2007). *De Observantia Ciborum* (510 CE) has a list 101 foods, including three cucurbits (Mazzini 1984). The *pepone* are not listed with the other two cucurbits. Instead, they are among pomegranates, grapes, and figs, which are sweet, juicy fruits that are usually eaten raw when ripe. Anthimus, in *De Observantia Ciborum Epistula* (516 CE), indicates that the *melones* were to be eaten well-ripened, fresh with the seeds still mixed in the flesh (Grant 2007).

Hebrew (150 – 400 CE)

Three large codices of Jewish Law were compiled in Israel during the first centuries CE. They are easily searched at Mekhon Mamre (http://www.mechon-mamre.org). The first of these is the *Mishna*, which was compiled during the latter half of the second century. The second, the *Tosefta*, was compiled approximately a century later and differs in some of its material from the *Mishna*. The third, the *Jerusalem Talmud*, probably compiled during the latter half of the fourth century, includes a text very similar to that of the *Mishna* but with much exposition. All three codices are arranged in *sedarim* (orders) each of which has varying numbers of *massakhtot* (tractates). The *Mishna* has 63 tractates sorted into six orders.

Some of the tractates refer to edible-fruited cucurbits, most notably the ones on *Kil'ayim* (Intermingling), *Ma'asrot* (Tithing), *Terumot* (Contributions), and *'Oqazin* (Peduncles). Four edible cucurbits are often mentioned, these four in Hebrew being the *qishu'im* (vegetable melons, *Cucumis melo*), *delu'im* (bottle gourds, *L. siceraria*), *avattihim*, and *melafefonot*. A fifth edible cucurbit, the *qarmulin*, is mentioned only in the latter two works, apparently being a new introduction with an appearance and use similar to the *delu'im*. The *qarmulin* have been identified as sponge gourds, *Luffa aegyptiaca* Mill. (Janick et al. 2007, Avital and Paris 2014).

The four edible-fruited cucurbits are considered together in the tractate *Kil'ayim* because of the concern given to their vines intermingling with one another. There are some elements of practicality in this prohibition. On the other hand, in the *Jerusalem Talmud* version (*Kil'ayim* 1:2, 2a), there is also a recantation of Mediterranean agricultural folklore: "A person takes a seed from the flesh of an *avattiah* and a seed from the flesh of an apple and puts them together in the same hole and they unite to become an intermingling. This is called in the Greek language *molefefon*." Thus the myth of the apple-*pepo* was carried across three languages, Greek, Latin, and Hebrew. More importantly, it allows definite identification of the Hebrew *melofefonot* as melons, *C. melo*. Another passage from the *Jerusalem Talmud* (*Kil'ayim* 1:8, 4a) has this comment: "Prohibited is the insertion of cuttings from grapevines into an *avattiah* lest it throw its waters into them." The *avattiah*, like the Greek *pepon* and Latin *pepo*, was a large, watery fruit, the watermelon, *C. lanatus*.

The four edible-fruited cucurbits are considered together in the first chapter of the tractate on tithing, *Ma'asrot*. The *qishu'im* and *delu'im* were to undergo *piqqus*, rubbing off of their hairs, prior to eating. Hence, these two cucurbit fruits, vegetable melons and bottle gourds, were eaten young and immature, when they were hairy. In contrast, the *avattihim* were to be tithed after they underwent *shilluq*, smoothing or polishing for removal of the dust that accumulated on them. Therefore the *avattihim*, watermelons, were eaten when ripe. Corroborating evidence for the eating of the watermelons when they were ripe is also found in the *Mishna* tractate on peduncles (*'Oqazin* 2:3), in which it is mentioned that a partially withered pomegranate or watermelon is not eaten. The tractate on tithing in the *Tosefta* indicated that the *melafefonot*, melons, were to be tithed after they were taken out of the field and gathered (*Ma'asrot* 1:6). Specifically, the grower was obliged to tithe each wave (harvest) of ripened melons.

Melon seeds were to be tithed but watermelon seeds were not, because the latter could not be used as food (*Jerusalem Talmud*, *Ma'asrot* 1:2, 2b). This might at first seem strange as, today, watermelon seeds are consumed in many areas. However, watermelon seeds of the time were quite small, only 11 mm long (Cox and van der Veen 2008, Kislev and Simhoni 2009), and given their thick, hard seed coats were not particularly suitable for use as food.

The tractate on contributions, *Terumot*, contains a discussion on the amount of time, post-harvest, that various fruits and vegetables are acceptable as contributions. As would be expected from highly perishable, young cucurbit fruits, the vegetable melons and bottle gourds were suitable for contribution for only one day after harvest (*Tosefta*, *Terumot* 4:5). The melons were acceptable for three days. The watermelons are not mentioned in this context, apparently because they had a long shelf life. Also unlike the other three cucurbits, watermelons could not be gathered for sale in a pile. Instead they had to be laid out one-by-one (*Jerusalem Talmud*, *Ma'asrot* 1:4, 4a), indicating that they were fragile, and thus very different from the modern cultivars of watermelons that have been bred for adaptation to long-distance shipping.

Finally and most significantly, watermelons differed in one more way from the other edible cucurbits. In the tractate on tithing, watermelons are discussed together with figs, table grapes, and pomegranates. Fruits that were picked in the garden or field and eaten there were exempt from tithing (*Mishna*, *Ma'asrot* 2:6; *Jerusalem Talmud*, *Ma'asrot* 2:4, 11a). Figs were simply chosen and eaten, grapes were picked one-by-one from a cluster and eaten, pomegranates were plucked and eaten, and watermelons were sliced and eaten. Evidently, the watermelons of the latter half of the second century in Israel, like figs, table grapes, and pomegranates, were common fruits that were eaten raw without any culinary preparation and, like them, were juicy and sweet.

Discussion

Domesticated plants are derived from small samples of wild source populations, and thus are themselves founder populations that contain only a small fraction of the genetic diversity of their wild ancestors (Ladizinsky 1998). Cultigens have various traits that were selected early and continually in the domestication process, such as lack of bitterness, increased size of the harvested parts, increased yield, and novel coloration (Heslop-Harrison and Schwarzacher 2012). The sweet dessert watermelon, *C. lanatus*, which has relatively little genetic diversity (Levi et al. 2001, Reddy et al. 2015), follows this general pattern of crop-plant domestication. The fruit flesh of wild and primitive *Citrullus* is bitter or insipid, hard, and pale-coloured (Wehner 2008). Non-bitterness of the fruits was probably the first and most important trait to be selected in the process of watermelon domestication. As non-bitterness is conferred by a single recessive gene (Wehner 2007), this trait should have been relatively easy to maintain if isolation from neighbouring wild populations was feasible. The modes of inheritance of hard versus soft and of insipid versus sweet flesh in *Citrullus* have not been illuminated.

Although it is agreed that the genus *Citrullus* is of African origin, there has been major disagreement concerning where in Africa the dessert watermelon originated. Much of the controversy stems from phenotypic variations that overlap among *C. colocynthis*, *C. amarus*, *C. mucosospermus*, and *C. lanatus*, and the weak crossability barriers among these four species. Regardless of species, wild and primitive *Citrullus* fruits typically have hard, bitter or bland, weakly coloured flesh and, as indicated by Wehner (2008), this situation has repeatedly been a source of incorrect taxonomic identifications. Archaeological remains, iconography, and literature, as well as the modern presence of wild and primitive watermelons in Sudan and Egypt, support the idea that the dessert watermelon, *C. lanatus*, is native to northeastern Africa (Paris 2015).

The present investigation, which has focused mainly on ancient literature, has attempted to define a narrower time frame and geographical range within which the dessert watermelons having non-bitter, tender, highly coloured, sweet flesh were developed. The latest possible date for this time frame is provided by illustrations of both red-flesh sweet dessert watermelons and white-flesh citron watermelons in illustrated manuscripts prepared in northern Italy dating to the end of the 14th century (Paris et al. 2009, 2013).

Ancient Greek, Latin, and Hebrew writers clearly distinguished between watermelons and melons. The watermelon, as *pepon*, *pepo*, and *avattiah*, was a large, watery, cooling but fragile fruit, eaten raw when ripe, except for the rind and pits. The melon, as *melopepon*, *melopepo*, and *melafefon*, was the mythological intermingling of apple with watermelon, less watery and less cooling than watermelon, lobed, turning yellow, aromatic, and detaching from the plant when ripe, with a short, 3-day shelf life.

The earliest evidence for the existence of sweet dessert watermelons is primarily from the Hebrew literature. The context is not an appraisal or a praising of their sweetness but, instead, an exemption to the Jewish instruction of tithing. Under particular conditions, several succulent, sweet fruits, figs, table grapes, and pomegranates, are exempt from tithing and, together with them, so are watermelons. Only watermelons and not melons or other cucurbits are treated in this context. Sweet melons emerged later, in Khorasan, Central Asia, during early medieval times, reaching Mediterranean lands in the eleventh century (Paris et al. 2012).

The Hebrew evidence for the existence of sweet dessert watermelons in Mediterranean lands by Roman times is corroborated by a contemporary Greek writing and a Latin writing from just after the fall of the Roman Empire. In the late second century, Athenaeus quoted Diocles as stating that the best *kolokyntas* are *glukeian*, sweet (Olson 2006). The early sixth-century Latin *De Observantia Ciborum* lists the *pepone* among pomegranates, grapes, and figs, confirming the presence of sweet watermelons in what is now Italy (Mazzini, 1984). Thus, sweet watermelons had diffused to Europe centuries before their earliest recorded presence in Moorish Spain, in the Cordoban Calendar of 961 CE (Pellat, 1961).

Conclusion

Sweet dessert watermelons were selected from non-sweet ancestors. By the time of the Roman Empire, sweet dessert watermelons were a familiar and esteemed produce item in Israel, and probably in other Mediterranean lands as well.

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Status of Cucurbit Breeding at AVRDC – The World Vegetable Center

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Abstract. AVRDC – The World Vegetable Center's global cucurbit breeding program started in 2006 at Center headquarters in Taiwan. The program shifted to AVRDC East and Southeast Asia, Thailand, in May 2010. Breeding efforts are focused on two cucurbit species, bitter gourd (*Momordica charantia* L.) and tropical pumpkin (*Cucurbita moschata* Duchesne), with comparatively less emphasis on cucumber (*Cucumis sativus* L.). The Center's bitter gourd breeding efforts have resulted in the development of high yielding open-pollinated lines for different market segments with superior fruit quality, early maturity, ability to set fruit under high temperatures, high concentration of anti-diabetic compounds, and disease and insect resistance. Eight bitter gourd open-pollinated lines of different market segments with yield and fruit quality comparable to commercial hybrids have been released. Bitter gourd lines resistant to powdery mildew (*Podosphaera xanthii*) have been developed and the resistance has been confirmed in multi-country trials in Asia. Bitter gourd lines resistant to leaf spot (*Cercospora* spp., *Alternaria* spp.) and anthracnose (*Colletotrichum* spp.) have been identified. Melon fly (*Bactrocera cucurbitae*) resistance in *Momordica balsamina* L., a close relative of the bitter gourd, has been identified. Populations for further selection through inbreeding for improved yield, fruit quality, and disease resistance have been created at AVRDC for various market segments by crossing genetically unrelated bitter gourd varieties and genebank accessions. Four cycles of recurrent selection have been completed. Pumpkin breeding at AVRDC has led to the development of open-pollinated lines rich in beta-carotene with good eating quality and resistance to *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Papaya ringspot virus* – watermelon strain (PRSV-W), and *Squash leaf curl virus* (SqLCV). AVRDC's cucumber breeding program has developed non-bitter, predominantly female, high yielding tropical lines suitable for the Southeast Asian market.

Keywords: *Momordica charantia, Cucurbita moschata, Cucumis sativus*, breeding, disease/insect-pest resistance, phytonutrient

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Introduction

AVRDC's cucurbit breeding program was started in 2006 at the Center's headquarters in Taiwan and shifted to AVRDC East and Southeast Asia, Thailand in May 2010. Considerations to select the target cucurbit crops included economic importance, nutrient density, access to genetic resources, and AVRDC's comparative advantage versus the private sector. The cucurbit breeding program initially emphasized improvement of cucumber, *Cucumis sativus* L., because of the crop's economic importance, and of tropical pumpkin, *Cucurbita moschata* Duchesne, which is nutritionally rich but has received relatively less attention from the private seed sector. Subsequently, the Center downgraded cucumber breeding and took up improvement of bitter gourd, *Momordica charantia* L., a crop that is economically and nutritionally important.

Bitter Gourd Breeding at AVRDC – The World Vegetable Center

Bitter gourd is an important cucurbitaceous cash crop cultivated mainly by smallholder farmers in Asia, where it is grown on approximately 340,000 ha annually (McCreight et al. 2013). Bitter gourd fruits are high in nutrients such as beta-carotene, vitamin C, folic acid, magnesium, phosphorus, and potassium (Yuwai et al. 1991, Dhillon et al. 2016). It is often used in folk medicine to manage Type 2 diabetes, a rapidly spreading non-communicable disease that affects 347 million people worldwide, with 80% of them living in low-income and middle-income countries (WHO 2015). The current bitter gourd seed market in Asia is valued at about 16 million Euros (Narendra Kumar Singh, HM Clause, personal communication). India is the largest producer of bitter gourd and approximately 350 t of seed (hybrid seed = 200 t and open-pollinated seed = 150 t, M. Anil, Rasi Seeds Pvt. Ltd., personal communication) is produced annually for the Indian bitter gourd seed market. By 2020, the market is expected to grow to 600 t (hybrid seed = 360 t, open-pollinated seed = 240 t, Vimal Chawda, VNR Seeds Pvt. Ltd., personal communication).

Based on a literature survey, Léon (1994) suggested that hybrids of out-crossing crops had greater yield stability. Bitter gourd displays high heterosis for yield ranging from 27 to 86% above the high parent (Behera 2004). This has encouraged the development and adoption of hybrid cultivars in Asia, which have the potential to increase profitability of seed companies as well as growers. However, approximately 57% of the bitter gourd area is planted to open-pollinated cultivars in Asia (Ajay Dyal, Rasi Seeds Pvt. Ltd., personal communication). Average yield of popular open-pollinated cultivars ranges between 12 to 15 $t \cdot ha^{-1}$, whereas commercial hybrids yield 20 to 25 $t \cdot ha^{-1}$ (S.R. Verma, VNR Seeds Pvt. Ltd., Chaiwat Manoworn, Chia Tai Seeds, personal communication). AVRDC has developed bitter gourd inbred lines comparable in yield and fruit quality to popular commercial hybrids (Table 1). Two of these lines (AVBG1310 and AVBG1313) produced stable yields in trials at Kamphaeng Saen (Thailand) in 2013 and 2014. This result was despite the significantly higher temperatures (32 to 38 °C) during the flowering and fruiting stages in 2014, compared to the same period in 2013 (28 to 35 °C). Powdery mildew caused by *Podosphaera xanthii* is a serious fungal disease of bitter gourd, and yield losses of up to 50% have been observed in China and India (Fu Jiqin, Enza Zaden, Vimal Chawda, VNR Seeds Pvt. Ltd., personal communication). The disease can be controlled with fungicides, but genetic resistance provides a more economically sound and environmentally safe approach. After screening 150 accessions of a global collection of bitter gourd against the local isolate of *P. xanthii* at Kamphaeng Saen (Thailand), the cucurbit breeding program developed five inbred lines resistant to this pathogen. A single resistant plant was identified from five segregating populations derived from genebank accessions; multiple cycles of inbreeding and selection led to development of five resistant inbred lines (THMC 113, THMC 143, THMC 153, THMC 167, and THMC 170). These lines were screened against local isolates of *P. xanthii* at 11 locations in six countries (China, Thailand, India, Taiwan, Vietnam, Philippines). THMC 153 was resistant across all locations, and THMC 143 and THMC 167, were resistant at all locations except one in China. The other bitter gourd lines exhibited variable reactions to the pathogen in different regions, which confirms the presence of genetic variants (pathotypes and physiological races) of *P. xanthi* with different abilities to infect and sporulate on bitter gourd. THMC 143, THMC 153, and THMC 167 have the potential to be sources of broad spectrum disease resistance for breeding purposes. The resistance of these bitter gourd lines to the Kamphaeng Saen (Thailand) isolate of *P. xanthii* is controlled by recessive polygenes (N.P.S. Dhillon, unpublished data).

The melon fly, *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae) is an important insect pest of bitter gourd and other cucurbits. Fruit infestation by this pest has been reported to vary between 41 to 89% in Asia (Rabindranath and Pillai 1986) and up to 95% bitter gourd fruit infestation has been reported in Papua New Guinea (Hollingsworth et al. 1997). Chemical control of this pest is expensive and represents a risk to the environment. In addition, this insect can rapidly develop resistance to chemical pesticides. Host-plant resistance is safe, economical, and an effective alternative to chemical pesticides. In a review, Dhillon et al. (2005) listed several cultivated and wild bitter gourd accessions as resistant or highly resistant to *B. cucurbitae.* 'Faisalabad Long' and 'Col-II', originally derived from bitter gourd landraces, were resistant to local strains of *B. cucurbitae* in Pakistan (Gogi et al. 2010), but these two cultivars were highly susceptible to the local strain of *B. cucurbitae* in Taiwan (Table 2). However, THMC 281, an accession of *Momordica balsamina* L., which is closely related to *M. charantia*, was resistant to the Taiwan strain of melon fly in two field screenings during 2012 and 2013. Usually, melon fly-resistant bitter gourd fruits are thick with a tough rind, like the fruits of THMC 281. Hence, it might be worthwhile investigating the basis and mechanisms of melon-fly resistance in this accession, and whether it could be incorporated into resistance breeding programs.

Feeding studies on animals and humans confirmed that bitter gourd whole fruit, juice, or extract can play a role in managing Type 2 diabetes (Krawinkel and Keding 2006). The antidiabetic effect of bitter gourd is due to the complex action of multiple compounds in the fruit. The AVRDC-led research project "A better bitter gourd: Exploring bitter gourd (*Momordica charantia*) to increase incomes, manage type 2 diabetes, and provide health in developing countries" funded by the Federal Ministry for

Economic Cooperation and Development (BMZ), Germany, led to the identification of bitter gourd lines with high and stable concentrations of antidiabetic compounds, and an assessment of the contribution of these antidiabetic effects to human health where bitter gourd is consumed as a part of a balanced diet. A total of 31 single/groups of compounds from LC/ToF-MS (Liquid chromatography/Time-of-flight mass spectrometery) profiles were identified with a molecular weight (M+H±0.05 Da) corresponding to triterpenoids (Yang et al. 2015), and 16 antidiabetic related compounds including seven phosphatidyl cholines, two fatty acid derivatives, five triterpenoids, vicine and tryptophan were identified in the LCMS (Liquid chromatography/mass spectrometery) profiles of methanol extracts of *M. charantia*. Preliminary results of feeding studies with humans conducted by AVRDC in India revealed that the mean initial fasting blood glucose levels of pre-diabetics had reduced significantly ($P < 0.01$) after consuming bitter gourd for an extended time.

For the last two decades, bitter gourd breeders have pursued genetic improvement of the crop through a process of inbreeding and pedigree selection to develop lines, followed by elite × elite crosses and identification of commercilizable hybrids. This approach was adopted to increase the chances of developing lines possessing multiple important traits preferred by growers, such as earliness and high marketable yield, and consumer-preferred traits, such as fruit color, shape, size, and skin pattern. Performance-driven selection to achieve the preferences of value chain actors (growers, distributors, retailers, and consumers) led to repeated use of elite inbred lines for hybrid development, which narrowed the genetic base of the crop and has led to low genetic diversity among commercial cultivars (Duvick 2005). Lack of genetic diversity can enhance vulnerability of the crop to disease and insect outbreaks (Keneni et al. 2012). Breeders' greater use of the bitter gourd diversity stored in genebanks could eventually broaden the genetic base of elite cultivars and reduce the chances of catastrophic outcomes. AVRDC – The World Vegetable Center, Taiwan has a fairly large collection of bitter gourd germplasm from more than 15 countries. The genetic diversity of 114 bitter gourd accessions of the AVRDC collection and of commercial cultivars was assessed based on the genotypes at 50 simple sequence repeat (SSR) loci. Commercial cultivars of similar market segments were closely related, even though they were released by different seed companies, corroborating the assumption that the genetic base of bitter gourd varieties in Asia is very narrow. South Asian accessions originating from India, Bangladesh, and Pakistan were more closely related to each other than to accessions of any other geographic group. Likewise, accessions from Cambodia, Vietnam, and The Philippines clustered into one group, and were distinct from the Taiwan accessions. There is a pressing need to methodically evaluate bitter gourd accessions held by various national and international genebanks for resistance against economically important pests and pathogens, assess their tolerance to abiotic stresses, and evaluate their phytonutrient contents and profiles for micronutrients and antidiabetic compounds. For instance, *Cucurbit aphid-borne yellow virus* also known as *Namamarako* in the Philippines and *Mara Ba* in Thailand, is an aphid-transmitted polerovirus that has become a serious production limitation in bitter gourd (Relevante et al. 2012, unpublished). Bitter gourd accession VI049946 from the AVRDC genebank has segregated for resistance to this virus (Fatkhu Rokhman, East-West Seed Indonesia, personal communication) and the company is using selections from this accession to eventually develop resistant cultivars.

Pumpkin Breeding at AVRDC – The World Vegetable Center

Tropical pumpkin, *Cucurbita moschata*, is an underutilized cucurbitaceous crop with economic potential and nutritional importance. Pumpkin fruits are rich in alpha and beta-carotenes and

Table 1. Mean fruit number/plant, fruit weight and yield, and fruit traits of AVRDC bitter gourd lines.

No.*	Genotype	Fruit number/plant	Fruit weight (g)	Yield (t/ha)	Fruit bitterness	Fruit color	Fruit skin pattern
1	AVBG1304	41	173	35.9	Low	Dark green	Spiny
2	AVBG1310	23	266	33.6	Low	Green	Spiny
3	Palee (Check)	30	194	36.3	Low	Medium green	Spiny
4	BARI Karela 1 (Check)	22	178	23.5	Medium	Medium green	Spiny
5	AVBG1311	18	326	39.4	Low	Light green	Ridged
6	AVBG1313	19	374	41.3	Low	Light green	Ridged
7	AVBG1314	21	350	41.3	Low	Light green	Ridged
8	Benteng (Check)	19	407	40.6	Low	Light green	Ridged
9	AVBG1323	47	138	32.8	Medium	Green	Spiny
10	AVBG1324	59	115	38.6	Medium	Medium green	Spiny
11	AVBG1325	52	129	36.9	Low	Green	Spiny
12	AVBG1301	45	125	34.9	Medium	Medium green	Spiny
13	Noor (Check)	48	134	36.3	Low	Medium green	Spiny
	LSD ($P = 0.05$)	9	70	7.0			

*Market segment: 1-4: medium fruit length segment (South Asian type), 5 to 8: long fruit segment (Southeast Asian/Chinese type), 9 to 13: small fruit length segment (South Asian type).

minerals (Arima and Rodriguez-Amaya 1990). The world production of squash, pumpkins, and gourds in 2012 was estimated at 24.6 million t from 1.78 million ha (FAO 2012). Productivity of these cucurbits is high in North America (23.7 $t \cdot ha^{-1}$) compared to Asia (13.8 $t \cdot ha^{-1}$) and Africa (7 $t \cdot ha^{-1}$). Open-pollinated cultivars still dominate commercial acreage of pumpkin in the tropics. Genetic uniformity and disease susceptibility are two major constraints leading to low pumpkin yields in the tropics (Loy 2011). Viral diseases caused by *Cucumber mosaic virus* (CMV), potyviruses such as *Papaya ringspot virus*-watermelon strain (PRSV-W), *Zucchini yellow mosaic virus* (ZYMV), and begomoviruses such as *Squash leaf curl virus* (SqLCV) and *Tomato leaf curl New Delhi virus* (ToLCNDV), are diseases of major economic importance in the tropics and can cause losses of up to 100%. Use of host plant resistance is the cheapest and most environmentally sound means to control viruses.

The aphid-transmitted ZYMV is a major pathogen of pumpkin. *C. moschata* landrace Nigerian Local carries a single dominant resistance gene to the local isolate of ZYMV (ZYMV-CT) in the U.S.A. (Munger and Provvidenti 1987, Brown et al. 2003). Two genes (*Zym-0* and *Zym-4*) for resistance to a mixture of five Austrian isolates of ZYMV have been identified in Nigerian Local (Pachner et al. 2011). A recessive gene carried by 'Waltham Butternut', *zym-5*, is complimentary with the dominant *Zym-4* of Nigerian Local and the resistance conferred by *Zym-4* is only expressed in *zym-5/zym-5* individuals. We found that resistance in Nigerian Local to the most virulent strain, ZYMV-TN3, which is prevalent in Taiwan, is controlled by two complementary dominant genes. We transferred ZYMV resistance from Nigerian Local into the susceptible local variety PY through backcross breeding and obtained two pumpkin lines completely resistant to ZYMV; these lines are comparable in yield and fruit quality to a popular local variety and to a commercial hybrid susceptible to the virus. Pumpkin crops grown in farmers' fields in the tropics can be infected with multiple virus species and cultivars carrying resistance to only a single virus will not perform well. Potyviruses and begomoviruses are present at high incidences in the pumpkin fields at the Research and Training Station of AVRDC – The World Vegetable Center East and Southeast Asia, Kamphaeng Saen, Thailand, during March and April every year. Plants of the susceptible check variety exhibit 100% susceptibility if planted in the field in this period. Based on field screening in this environment, we have developed eight pumpkin lines with field resistance to multiple unidentified viruses. We are confirming the resistance by artificial screening against local isolates of CMV, PRSV-W, ZYMV, and SqLCV.

Cucumber Breeding at AVRDC – The World Vegetable Center

Cucumber breeding at AVRDC focused on the development of predominantly female high yielding tropical lines suitable for the Southeast Asian market. We have released one completely gynoecious line and five predominantly female lines, which are also high yielding and remain non-bitter in tropical growing conditions.

Future of AVRDC's Cucurbit Breeding Program

We are in the process of developing markers to map powdery mildew resistance genes. Currently more than 5,000 polymorphic markers have been obtained for a segregating bitter gourd population and mapping of the resistance genes is ongoing. Populations of pumpkin are being developed to study the inheritance of resistance to ZYMV, PRSV-W, and SqLCV and molecular markers associated with these resistance gene(s). We are testing the performance of AVRDC-bred improved lines of bitter gourd and pumpkin in various countries in Southeast Asia and South Asia. We are exploring the potential of extending testing of AVRDC bitter gourd and pumpkin lines in parts of Africa. We are also screening AVRDC genebank accessions to find suitable prospects for the development of bitter gourd lines resistant to *Cercospora* spp., downy mildew, and begomoviruses. AVRDC cucurbit lines have been shared with breeders from private seed companies, who have developed hybrids using this breeding material; these hybrids are being tested at multiple locations by product development teams from seed companies (M. Anil, Rasi Seeds Pvt. Ltd., India; Parag Agarwal, VNR Seeds Pvt. Ltd., and Murali Kumar, Sakata Seeds, India, personal communication).

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Superfruiter (*sf*): A New Melon Type with Multiple Small Seedless Fruits and Increased Yield

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Abstract. We screened an EMS mutation library of melon for unusual fruit and plant phenotypes and identified a unique recessive mutant that produces more fruit than the wild-type. Fruits were small, one-third of the wild-type size, and seedless. We called this mutant *superfruiter* (*sf*). Indeed, *sf* decreased fruit size by one-third, but increased fruit number by five times and on the average increased yield by 67%, in various genetic backgrounds. Thus, *sf* is a single recessive gene that significantly breaks yield barriers in melon, without any adverse effect on fruit quality attributes. We are now developing *sf* melon varieties by introducing this gene into selected genetic backgrounds.

Introduction

It is widely accepted that organisms are selected according to fitness in their natural habitats. Some of the initial steps in human civilization included the selection of plant phenotypes that had better performance under cultivation. Thus, the organism became selected for its value to humans, or its ability to fulfill human's personal desires, rather than for fitness "in the wild" (Pollan 2001). These axioms were the fundamental pillars of ancient agronomy and horticulture; and traits that were favorable were selected based on their ability to promote the supply of food under the conditions in which the growers lived. Adaptation to new environments or improvements of these primitive cultivars was based on selection of new phenotypes from the existing gene pool. Soon enough, humankind learned, by mixing and crossing accessions, to create new variations that served as a new pool from which desired phenotypes were further selected (Zamir 2001).

We are therefore constantly looking for methods to increase variation within a crop. The most common method to increase variation within a crop is to go back to its origins, look for beneficial traits that have been left behind during domestication either in wild relatives or in primitive varieties and introduce these traits by hybridization. Modern technologies also enable the transformation of crops with genes from other species; however, this technology has not been accepted for food production in parts of the world. The discovery that genes can be inductively mutated (Muller 1927) opened the gate to practical mutagenesis as a research and breeding tool. Thereafter, thousands of new crop varieties were released that were bred with new traits obtained via induced mutation (Ahloowalia et al. 2004, Mba 2013). In addition, analyses of induced mutants led to the discovery of many genes and gene functions by forward and reverse genetics methods (Dahmani-Mardas et al. 2010, Bahadur et al. 2015). Induced mutagenesis provides a powerful alternative to natural monogenic or polygenic variation for identifying functional pathways and genes involved in complex traits (Varshney et al. 2014).

Melon (*Cucumis melo* L.) is an economically important cucurbit crop. Melons exhibit high polymorphism at the DNA level that is reflected in their high variation of fruit morphology and physiology. Melon diversity encompasses fruit size, weight, external and internal color, shape, and aroma. The large within-species diversity, a small genome size, and a high economic value, make melon a convenient and attractive model plant for research.

Modern markets of agricultural products are constantly looking for novel fruits and vegetables. This is reflected by the demand for personal mini fruit; however, decreased fruit size is usually associated with yield reduction. To induce new variation in melon, we conducted chemical mutagenesis on seeds of CEZ, a 'Charentais'-type cantaloupe breeding line. One of the resulting M_2 families segregated to a unique phenotype easily recognizable by its large number of small, seedless fruits.

Materials & Methods

Plant material

CEZ is a 'Charentais'-type (Cantalupensis Group) inbred line extracted from the F_1 hybrid Cézanne (Clause-Tézier, France). 'Piel De Sapo' (PDS) is a Spanish casaba melon (Inodorus Group), Noy Yizre'el (NY) is an Israeli 'Ha'Ogen'-type melon

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Figure 1. Fruit harvested from wild-type CEZ (A) and from its isogenic line superfruiter (B).

(Cantalupensis Group) and 'En Dor' (ED) is an Israeli melon of the 'Ananas Yoqne'am' type (Ameri Group) (Paris et al. 2013). All of these accessions came from the melon collection maintained by the Cucurbit Section at the Newe Ya'ar Research Center, Israel.

EMS mutagenesis

Five-thousand seeds of the CEZ inbred were treated with ethyl methanesulfonate (EMS, Sigma M-0880). Seeds were placed in water in an Erlenmeyer flask for 12 hours while stirring with a magnetic stirrer and bubbling air into the water with an aquarium pump. After 12 hours, 1.5% EMS was added for an additional 12 hours, after which the seeds were thoroughly washed with running water and dried on paper towels. After this treatment, M_1 seeds remained viable for at least six months. M_1 seeds were planted in the greenhouse and were self-pollinated to create M_2 families, which were phenotyped in the field.

Phenotyping

Phenotyping was by visual inspection of the fruit and the mature plants in the field. Twelve mature plants from each M_2 family were phenotyped based on visual categories (Tadmor et al. 2007).

Results

Screening of 2,000 M_2 families led to identification of approximately 200 families that segregated for one or two phenotypic traits. One family segregated for a unique phenotype: three out of the 12 family members yielded a large number of seedless fruits that were smaller in size as compared to the control "wild type" CEZ (Figure 1). We named this phenotype "superfruiter" (sf). *Sf* plants were characterized by an increase of fruit number, decrease of fruit size and absence of seeds. Pollen of the mutated plants was viable so we crossed it to the progenitor line CEZ, and to PDS, NY, and ED. The F_1 plants were self-pollinated to produce F_2 segregating populations. Segregation and yield components analyses of these *sf* × wild-type F_2 segregating populations confirmed that *sf* was controlled by a single recessive gene designated *sf*. The average fruit number of *sf*/*sf* plants was increased by five-fold, average fruit size of these plants decreased to a third of the wild-type and average total yield of *sf*/*sf* plants increased by 67% compared to wild-type plants in all tested genetic backgrounds (Figure 2). In addition, no difference in fruit quality was found when we compared total soluble solids (TSS) and carotenoids (Figure 3) as well as flavor of fruits of the isogenic *sf*/*sf* and *Sf*/– lines of CEZ.

Discussion

Single genes that significantly break yield barriers in crops have been shown in heterozygous 'single flower truss' (SFT) tomato plants (Krieger et al. 2010) and, potentially, in the recessive homozygous 'multi-flowering' zucchini (Paris and Hanan 2010). Screening of ethyl methanesulfonate (EMS)-induced mutants identified *superfruiter* (*sf*) as a single gene that breaks yield barriers in melon by developing seedless fruit with an average 5-fold increased fruit number per plant and decreased fruit weight of 3-fold only. Thus, on the average, *superfruiter* increases yield by 67%. Such yield increasing genes are "game changers" because they can easily be introgressed into elite material and quickly become commercialized. We are now cloning

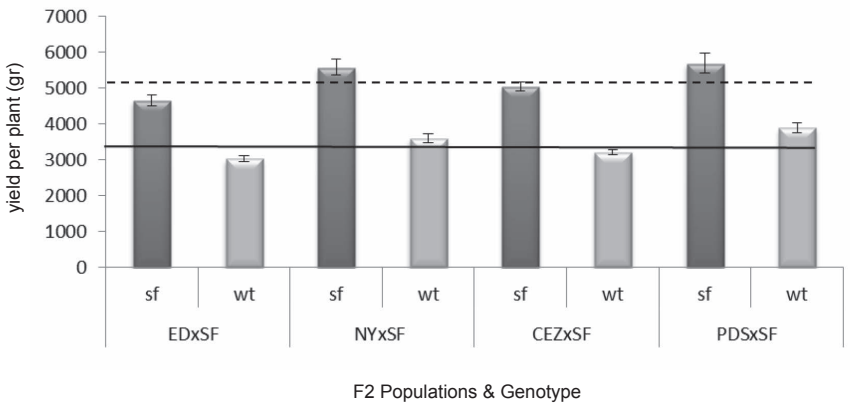

Figure 2. Average yield per plant belonging to the ED, NY, CEZ, and PDS F_2 populations. The horizontal broken and continuous lines represent the average yield (in grams) per wild-type and superfruiter plants in all four F_2 populations.

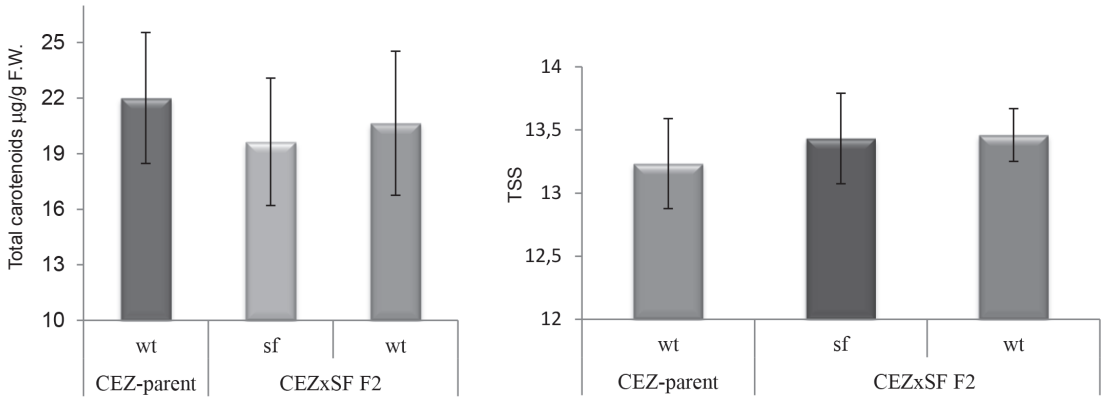

Figure 3. Total carotenoids (left) and TSS (right) of the progenitor line CEZ fruit, *superfruiter* (*sf*), and wild-type segregants in the *sf* × CEZ F_2 population.

the *sf* gene so that we can introduce various melon types with *sf* phenotype into the market.

In summary, the superfruiter trait has high agricultural potential, it can create small, seedless, high quality melon fruit without yield reduction; on the contrary, *sf*/*sf* plants possess an average of 67% higher yield than their wild-type counterparts.

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The Andromonoecious Allele of *CitACS4* Reduces Seed and Fruit Set in Watermelon

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Abstract. The ethylene biosynthesis gene *CitACS4* regulates the abortion of stamens during female flower development in monoecious watermelons. We have found that a mutation in *CitACS4* (allele *A*) reduces the production of ethylene in pistillate flowers, promoting the conversion of female into hermaphrodite flowers, and therefore of monoecy into andromonoecy. Here we have assessed the possibility of this reduction in flower ethylene production affecting other traits during flower and fruit development. To that end, we evaluated each trait in the parental lines and F_1 generations of two independent crosses (monoecious × andromonoecious), and performed segregation analyses between each trait and the *CitACS4* gene in the two F_2 populations. Segregating plants were genotyped, and the traits of interest assessed in homozygous *MM* (monoecious), homozygous *AA* (andromonoecious), and heterozygous *MA* (partially andromonoecious). The data indicated that the andromonoecious allele (*AA* plants) co-segregates with a lower fruit set and a reduced number of seeds. At anthesis, the *AA* plants in both the parental and the segregating populations also showed larger ovary size than *MM* plants, but the fruits of both genotypes reached a similar size at 4 days post anthesis. The growth rate of *MM* fruits was also found to be higher than in *AA* fruits at early stages of development, although equivalent in later stages. At maturity, the *MM* fruits were more elongated and showed a smaller scar. However, other analyzed traits, including external and internal fruit colour and seed size, did not co-segregate with the *CitACS4* gene. The reduced seed and fruit set in andromonoecious watermelon plants is probably caused by the uncoordinated development and maturation of floral organs in hermaphrodite flowers, which prevents pollination and fertilization. We discuss the role of ethylene in the coordination of flower maturity and fruit development in the unisexual flowers of this and other cucurbit species.

Keywords: Fruit and seed set, watermelon, *CitACS4* gene, andromonoecious, flower development

Introduction

Ethylene is an important regulator of sex in watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai. External treatments with this hormone induce the production of male flowers (Rudich and Zamski 1985), while treatments with ethylene inhibitors hasten the appearance of the first female flower and increase the number of female flowers per plant (Rudich and Zamski 1985, Manzano et al. 2014). This is in contrast to what happens in other cucurbit species such as cucumber, melon, and squash. Recently, we have differentiated between two sex-related processes: sex expression, i.e., the earliness and production of female flowers per plant, and sex determination, i.e., the mechanism that leads to the correct development and differentiation of unisexual female and male flowers (Manzano et al. 2014). In contrast to the effect on other cucurbits, ethylene inhibits the transition from male to female flowering in watermelon and reduces the number of female flowers per plant. Nevertheless, as in other cucurbit species, ethylene is necessary for the arrest of stamen development during the normal development of the female flower, and the reduction of ethylene production or action leads to the transformation of female flowers into bisexual and hermaphroditic ones (Manzano et al. 2014).

After pollination, ethylene is involved in floral organ senescence and abscission. Pollination induces ethylene production in the ovaries and petals, which appears to be responsible for coordinating ovary growth and petal senescence (Larsen et al. 1993, Balbi and Lomax 2003, Wang et al. 2005, Stepanova et al. 2008). Recent studies have shown an interconnection between early ovule abortion and the size of the silique in *Arabidopsis* ethylene mutants (Carbonell-Bejerano et al. 2011). In squash, *Cucurbita pepo* L., Martinez et al. (2013) demonstrated that a reduction in ethylene production or signalling in the zucchini flower can induce fruit set and early fruit development, and therefore that ethylene is actively involved in fruit set and early fruit development. Meanwhile, pollination and gibberellin treatments are responsible for downregulating ethylene biosynthesis and gene signaling in tomato immediately after fruit set (Pandolfini et al. 2007, Stepanova et al. 2008).

The ethylene biosynthesis gene *CitACS4*, orthologous to *CmACS7*, *CsACS2,* and *CpACS27A* in melon, cucumber, and

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squash (Boualem et al. 2008, 2009, Martínez et al. 2014), is responsible for the arrest of stamens in female flowers; a mutation in this gene leads to the conversion of female flowers into bisexual or hermaphrodite ones, and monoecy into andromonoecy (Manzano et al. 2016). Here we show that the ethylene controlled by *CitACS4* at very early stages of pistillate flower development is not only able to regulate sex determination and monoecy in watermelon, but may also influence fruit set, early fruit development, and seed numbers per fruit.

Materials & Methods

Plant material, growing conditions and genotyping

Three inbred lines of watermelon (*C. lanatus*), two monoecious lines (P85 and P86) and one andromonoecious line (P87), were characterized in this work. The F_1 and F_2 generations from two independent crosses (P85 × P87 and P86 × P87) were genotyped for *CitACS4* alleles, and then phenotyped for different flower and fruit developmental traits. The phenotyping was carried out in plants grown under standard greenhouse conditions in the province of Almeria (Spain) in the spring-summer of 2014 and 2015.

To genotype the monoecious (*M*) and andromonoecious (*A*) alleles of *CitACS4*, we used the specific primer pair CitACS4M-F (GAATGCCGGTTTATTTTGC) and CitACS4gen-R1 (TTCATCTTCCTTCCTCATCCTC) which were designed specifically to amplify the *M* allele, and also the primer pair CitACS4A-F (GAATGCCGGTTTATTTTGG) and CitACS4gen-R1 (TTCATCTTCCTTCCTCATCCTC), used to amplify specifically the *A* allele. DNA was isolated from young frozen leaves, and used to amplify a 253 or 271 bp PCR fragment of the *CitACS4* gene. PCR reactions were performed in the GeneAmp PCR System 2700 (Applied Biosystems), and consisted of 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 90 s at 72 °C. PCR products were resolved in agarose gels at 1.3 %.

Sex phenotyping

The sexual expression of each genotype was determined by counting the number of staminate and pistillate flowers in the first 20 nodes of the main shoot in at least 10 plants per genotype. To evaluate monoecy and andromonoecy, pistillate flowers were classified and scored according to their stamen development using the Andromonoecy Index (AI, Martínez et al. 2014). The female flowers with no stamen development were scored as AI = 1, while hermaphroditic flowers with complete stamens and pollen were scored as AI = 3. Ovary-bearing flowers with intermediate stamen development and no pollen production were classified as bisexual and scored as AI = 2.

The AI of each plant, genotype, and progeny was then calculated, and used as the average score of each plant with a minimum of five pistillate flowers, using at least 10 plants for each genotype or progeny. Plants and genotypes with an AI = 1 to 1.2 were considered to be monoecious, those with AI = 1.2 to 2.7 to be partially andromonoecious, while those with AI ≥ 2.7 were phenotyped as andromonoecious.

Fruit set and development, and seed productivity

To evaluate fruit set and fruit development, at least 12 pistillate flowers were hand-pollinated. The length and diameter of 12 ovaries/fruits were measured from anthesis to 10 days post anthesis (DPA). Fully mature fruits were harvested at 69 days after transplantation. Length, diameter, and weight, as well as external and internal color, were recorded for all harvested fruit. At least 10 fruits from each genotype were analyzed. The number of viable and non-viable seeds were counted in 1/8 of each fruit, and then estimated for one kg of fresh fruit weight.

Statistical analysis

Simple and factorial analyses of variance (ANOVA) at $P < 0.05$ were performed by the STATISTIX 8.0 software package, and each two means were compared using Fisher's Least Significant Difference (LSD) method.

Results & Discussion

Phenotypic and genetic characterization of monoecious and andromonoecious lines

The P87 andromonoecious line only produced male and hermaphrodite flowers with complete stamens and pollen (AI = 3). The monoecious P85 and P86 lines predominantly produced female flowers, although also produced some bisexual flowers (AI = 1.16). The sexual phenotype of the two F_1 hybrids derived from crosses between monoecious and andromonoecious lines (P85 × P87 and P86 × P87), had an intermediate phenotype between monoecious and andromonoecious (IA = 1.52 and 1.35), and were therefore classified as partially andromonoecious.

The segregation of monoecious, andromonoecious, and partially andromonoecious plants in the two F_2 populations studied, demonstrated that each trait is controlled by a single gene pair, the monoecious allele (*M*) being incompletely dominant over the andromonoecious allele (*A*). As expected, the segregation of monoecious, partially andromonoecious, and andromonoecious plants in the two F_2 populations fit the 1 : 2 : 1 ratio ($\chi^2 = 0.34$, $P = 0.53$; and $\chi^2 = 1.02$, $P = 0.17$). Although it has been reported that monoecy is dominant to andromonoecy and controlled by a single gene with two alleles (Poole and Grimball 1944, Jiang et al. 2007, Ji et al. 2015), our two crosses indicate that the monoecy of P85 and P86 is actually incompletely dominant to andromonoecy.

Given that the andromonoecious phenotype is the result of a mutation in the *CitACS4* gene (Manzano et al. 2016), the parental lines and the F_1 and F_2 plants of the two crosses were genotyped for the two alleles of the gene. In the two F_2 populations, the *M* and *A* alleles co-segregated with the monoecious and andromonoecious phenotypes, respectively, demonstrating that this gene is responsible for sex determination in watermelon. Homozygous *MM* and *AA* plants were monoecious and andromonoecious, respectively, while heterozygous *MA* plants had a partially andromonoecious phenotype.

As the *CitACS4* gene is involved in ethylene production at early stages of female flower development (Manzano et al. 2016), we have studied whether this gene may also be involved in other ethylene-regulated processes, including sex expression, fruit and seed set, and early fruit development, as well as quality parameters of the mature fruit. The expression of each trait was compared in *MM* and *AA* parental lines, in the F_1 generation (*MA*), and in the segregating *MM*, *MA,* and *AA* plants of the two F_2 generations. When the linkage analysis detected a co-segregation between *CitACS4* and the trait, we concluded that this

ethylene biosynthesis gene is most probably involved in the regulation of it.

Involvement of the CitACS4 *gene in sexual expression*

A linkage analysis was performed between *CitACS4* and two sex-expression traits that are known to be regulated by ethylene in watermelon: the number of nodes before the production of the first pistillate flower (pistillate flowering transition) and the number of pistillate flowers per plant (Manzano et al. 2014). The andromonoecious parental line P87 flowered later in comparison with the monoecious lines P85 and P86 (Table 1). The two F_1 generations had an early-flowering phenotype, but in the F_2 generations, the plants with the *A* allele did not flower later than those with the *M* allele. In fact, no significant difference was detected among F_2 plants for three genotypes *MM*, *MA,* and *AA* (Table 1).

Regarding the number of pistillate flowers per plant, no significant differences were detected between andromonoecious (P87) and monoecious (P85 and P86) parental lines, nor between genotypes *MM*, *MA,* and *AA* in the F_2 generation (Table 1). These data indicate that pistillate flowering transition and the percentage of female flowers, although controlled by ethylene, are not regulated by the *CitACS4* gene.

Involvement of the CitACS4 *gene in fruit and seed set*

To compare fruit set and early fruit development in *MM*, *AA,* and *MA* plants, 12 flowers of each genotype and population were pollinated and the fruit development examined from anthesis to 14 DPA. The percentage of fruit set in *AA* plants of the P87 parental line and the two segregating populations was reduced in comparison to that found in *MM* and *MA* plants (Figure 1), suggesting that andromonoecy adversely affects watermelon fruit setting.

Table 1. Sex expression in parental lines, and F_1 and F_2 populations derived from crosses between monoecious (P85 and P86) and andromonoecious (P87).

Generation	*CitACS4* genotype	Sex phenotype	Pistillate flowering transition	Percentage of pistillate flowers/plant
P87	*AA*	Andromonoecious	12.55±4.12 a	16.66±7.9 ab
P85	*MM*	Monoecious	4.77±1.92 b	13.84±5.46 b
P86	*MM*	Monoecious	2.05±0.72 cd	19.44±5.66 ab
F_1 (A)	*MA*	Part. andromonoecious	3.64±2.23 bc	22.05±5.15 a
F_1 (B)	*MA*	Part. andromonoecious	1.56±1.19 d	19.16±4.28 ab
F_2 (A)	*MM*	Monoecious	5.18±3.16 b	16.09±4.25 b
	MA	Part. andromonoecious	4.04±2.63 b	16.85±5.8 ab
	AA	Andromonoecious	4.26±2.54 b	20±7.07 ab
F_2 (B)	*MM*	Monoecious	4.65±2.54 b	15±4.3 b
	MA	Part. andromonoecious	3.83±2.61 b	16.78±5.27 ab
	AA	Monoecious	4.2±3.09 b	18.33±4.49 ab

(A) and (B) are the crosses P85 × P87 and P86 × P87, respectively.

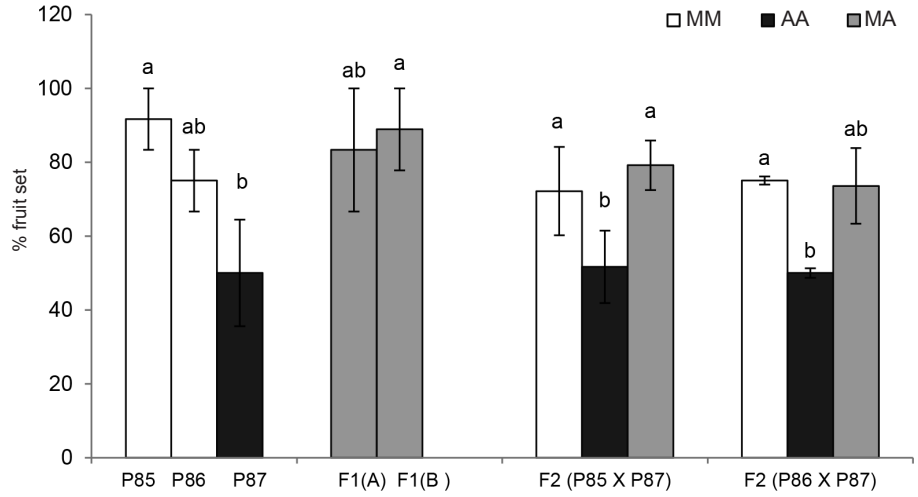

Figure 1. Percentage of fruit set in accessions P85, P86, and P87, and F_1 and F_2 generations of crosses P85 × P87 and P86 × P87. SE of 12 fruits per genotype and generation. (A) and (B) are the crosses P85 × P87 and P86 × P87, respectively. Different letters indicate statistical differences between genotypes ($P \leq 0.05$).

Figure 2 compares the production of seeds in *MM*, *MA,* and *AA* fruits. The *MM* and *MA* fruits from both parental lines and the segregating population from the P85 × P87 cross produced a higher number of viable seeds than those of *AA* plants (Figure 2). As expected, in the fruit of the andromonoecious P87 line and the F_2 *AA* plants, the number of non-viable seeds increased in comparison with those found in *MM* and *MA* plants. In the F_2 populations derived from the cross P86 × P87, differences were not found between the number of seeds in *MM* and *AA* fruits (Figure 2). Although co-segregation between the trait and the gene was not found for the two populations, the data suggest that the reduced ethylene production in the *AA* floral buds may negatively influence seed set in andromonoecious watermelon. The confirmation of this conclusion will require a higher number of *MM* and *AA* fruits to be investigated in the two segregating populations.

Involvement of the CitACS4 *gene in early fruit development and fruit quality parameters*

At anthesis, the *AA* ovaries of both the parental line P87 and the F_2 populations were larger than *MM* ovaries (Table 2), indicating that this trait is under the control of *CitACS4*.

During the days immediately after anthesis (4 to 10 DPA), the fruit growth rate in *AA* fruits was lower than in *MM* and *MA* fruits, which allows the fruits of the different genotypes to reach a similar size at 4 DPA (Table 2). From 4 to 10 DPA, however, no differences were detected between monoecious and andromonoecious parental lines, although the reduced growth rate of *AA* fruits was still observed in the F_2 population of the cross P86 × P87 (Table 2).

The larger ovaries of *AA* flowers at anthesis could be explained by the reduced production of ethylene in *AA* floral buds (Manzano et al. 2016), which could possibly either increase ovary growth rate at pre-anthesis or delay petal maturation, thereby extending the pre-anthesis period of *AA* flowers. The second explanation seems to be more likely, since petals of *AA* flowers remain green for longer than those of *MM* or *MA* flowers. This delay in petal maturation not only increases the size of the ovary at anthesis, but causes the stigma to become overripe before the flower opens (data not shown), which would also reduce the fruit set and the number of seeds as discussed above.

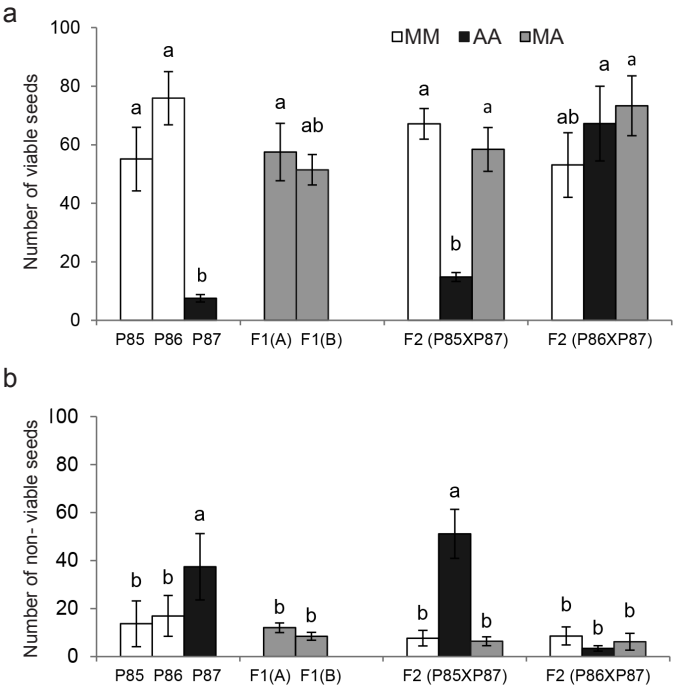

Figure 2. Number of seeds per kg of watermelon fruit in parental lines P85, P86, and P87, as well as in F_1 and F_2 populations of P85 × P87 and P86 × P87 crosses. a) Number of viable seeds. b) Number of non-viable seeds. SE of at least 10 fruits per line and generation. F_1 (A) and F_1 (B) are derived from crosses P85 × P87 and P86 × P87, respectively. Different letters indicate statistical differences between genotypes ($P \leq 0.05$).

The reduced growth rate of *AA* fruits during the days immediately after anthesis could also be related to the reduced recep-

Table 2. Ovary size and longitudinal early growth rates of fruits in lines P85, P86, and P87, and F_1 and F_2 populations derived from the crosses P85 × P87 and P86 × P87.

Population	Genotype	Ovary length at anthesis	Longitudinal growth rate (mm·DPA^{-1})	
			Anthesis to 4 DPA	4 DPA to 10 DPA
P85	*MM*	14.88±0.78d	13.04±0.63a	16.12±1.35b
P87	*AA*	19.95±0.96ab	8.34±1.08b	18.07±1.66b
F_1 (A)	*MA*	17.93±1.21bc	13.06±0.46a	23.96±0.82a
F_2 (A)	*MM*	15.68±0.72cd	11.78±0.95a	17.76±1.19b
	AA	20.79±0.57a	10.54±0.53ab	17.34±1.89b
	MA	17.89±0.74bc	11.46±0.80a	19.51±0.78b
P86	*MM*	15.57±0.95c	11.40±1.21ab	17.34±2.63ab
P87	*AA*	19.95±0.96a	8.34±1.08b	20.02±1.66ab
F_1 (B)	*MA*	19.48±0.82ab	13.66±0.87a	18.07±1.48ab
F_2 (B)	*MM*	17.13±1.24bc	9.89±1.95ab	21.60±2.07a
	AA	19.04±0.66ab	7.52±1.34b	15.70±1.14b
	MA	16.71±0.64c	12.38±1.26a	19.79±1.32ab

SE of 12 flowers/fruits per line and generation. (A) and (B) are the crosses P85 × P87 and P86 × P87, respectively. Different letters indicate statistical differences between genotypes ($P \leq 0.05$).

tivity of the stigma, and the level of ovule fertilization. Similar results were found in partially andromonoecious cultivars and andromonoecious mutants of *Cucurbita pepo*, where the extension of the preanthesis period was found to be correlated with a larger ovary size and parthenocarpic development of the fruit (Martínez et al. 2013, García et al. 2016). In order to verify this last hypothesis, we are now comparing the time taken to reach anthesis and the growth rates of *MM* and *AA* flowers in parental lines, and also segregating populations of watermelon.

At maturity, the *AA* fruits of the P87 line and the segregating populations were rounder and showed a wider scar than *MM* and *MA* fruits, suggesting that fruit shape and scar size are also regulated by CitACS4-produced ethylene in early flower development. In other analyzed traits, including external and internal fruit color and seed size, we found differences between monoecious and andromonoecious parental lines; however, these differences were not maintained between *AA* and *MM* plants of the F_2 populations (data not shown), indicating that these differences are not dependent on the *CitACS4* gene.

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The Rhizosphere and Seed Microbiomes of Oil Pumpkin Breeding Lines, Hybrids, and a Population Cultivar

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Abstract. The specific arrangement of plant-associated microbiomes is supposed to be a consequence of breeding activities and genotype selection. To develop a concept involving beneficial plant-microbe interactions into the plant breeding strategies of Styrian oil pumpkin, the microbiomes of the seeds and the rhizospheres of 14 genotypes showing different morphological and horticultural characteristics were assessed using a 16S rRNA amplicon sequencing approach. One open-pollinated cultivar, three oil pumpkin hybrids and their corresponding inbred lines, five segregating lines, and a zucchini hybrid were evaluated. In general, the diversity of the observed operational taxonomic units (OTUs) in rhizosphere samples was significantly higher than in seed samples. In contrast to the rhizosphere microbiomes, a strong genotype specificity was detected for the seed-associated microbial communities. The seed microbiomes of all genotypes were dominated by Enterobacteriaceae. In the open-pollinated cultivar and in one inbred line, the microbiome was comprised, to a large extent, of the genus *Erwinia* as well as the important pathogen *Pectobacterium carotovorum* (syn. *Erwinia carotovora*), the causal agent of soft rot of fruits. Another prevalent group was Firmicutes, especially in the two three-way cross hybrids and one of the segregating lines. No strong relationships among the hybrids and their pedigree components could be detected. The results regarding the seed microbiomes are of particular interest, as the Styrian oil pumpkin is highly susceptible to various fungal and bacterial pathogens during germination due to the lack of lignification of the seed coat. It remains to be investigated if seed-borne bacteria influence germination and plant development.

Keywords: *Cucurbita pepo*, genotype-specific microbiome, plant-microbe interactions, 16S rRNA amplicon sequencing

Introduction

The traditional Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) has become an important oil crop for international gourmet cuisines. Due to the lack of lignification of the seed coat, this crop is highly susceptible to various fungal and bacterial pathogens during germination. Fruit rot is caused by the consortium of *Didymella bryoniae* and *Pectobacterium carotovorum* (syn. *Erwinia carotovora*). Abiotic stress factors influence the performance of the crop as well. Collectively known as the plant microbiome, plant-associated microbes can help plants to fend off diseases, stimulate growth, and promote stress resistance. Additionally, microorganisms influence crop yield and quality (Berg et al. 2013). As a high genotype-specificity has already been shown for interactions with pathogens (Neupane et al. 2015), it is likely that there also exists a high genotype-specificity for the beneficial plant-microbe interactions. The development of a concept to integrate beneficial plant-microbe interactions into the plant breeding activities in Styrian oil pumpkin should facilitate breeding of new cultivars that are better capable to exploit the beneficial indigenous microbial community as well as artificially added biocontrol agents. Microbiome analyses are a first step to gain a deeper understanding of genotype-dependent differences in plant-microbe interactions.

Materials & Methods

Pumpkin genotypes

As shown in Table 1, the focus was toward coverage of various genotypes and cultigens with a high market share and their pedigree components.

Sampling of seed, rhizosphere, and soil replicates

For the seed microbiome analysis, 40 seeds per genotype were washed five times for one minute with 50 mL sterile deionized water and soaked in 25 mL sterile deionized water for 4 hours on a rotary shaker at 100 rpm. Subsequently, the seeds were divided into four replicates and were ground with a pestle in 10 mL 0.85% NaCl in sterile bags (Nasco Whirl-Pak®). For each sample, a 3 mL suspension was pelleted by centrifugation for 20 min at 4 °C and 13,500 *g*. For the rhizosphere microbiome analysis, 40 seeds per genotype were coated with 0.3 g

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of the fungicide Maxim® XL (Syngenta) and split into four replicates as well. Seeds were sown at a field site near the breeding station of Saatzucht Gleisdorf GmbH in randomized plots. One month after sowing, rhizosphere material from four randomly chosen plants per plot was sampled and pooled. Additionally, four bulk soil samples were taken from random places at the field site. Five to 7 g of each rhizosphere and soil replicate were suspended in 50 mL 0.85% NaCl and homogenized by a 3 min bag mixer (stomacher) treatment, then a 4 mL sample of the homogenized solution was pelleted as described above.

DNA extraction and amplicon sequencing

The DNA extraction was performed using a modified protocol of the FastDNA™ SPIN Kit for Soil (MP Biomedicals). The variable region 4 of the 16S rRNA gene was amplified with 515f (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806r (5′-GGACTACHVGGGTWTCTAAT-3′) primers extended by individual barcodes. For PCR amplification, a modified protocol of Lundberg et al. (2013) including synthetic peptide nucleic acid PCR clamps (PNAs) for blocking the amplification of mitochondrial and plastidal 16S rRNA gene sequences of plants was applied. Three independent PCR amplifications were performed per replicate sample. The triplicate amplification products were pooled and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) protocol. The PCR products were adjusted volumetrically to reach equimolarity of each sample in one common pool for 16S rDNA sequencing. The high-throughput amplicon sequencing was performed with the Illumina MiSeq V2 sequencing platform (2x150 bp paired-end) by GATC Biotech (Germany).

Bioinformatic analysis

De-multiplexing of raw sequencing data (joined reads) and the analysis were performed with the open-source bioinformatics pipeline QIIME 1.9.1 (Caporaso et al. 2010a). The primers and barcodes flanking the reads were removed by the automated pipeline and the chimeric sequences by means of the UCHIME method (Edgar 2010, Edgar et al. 2011). The clustering of sequences was done at the 97% similarity level using the UCLUST clustering method (Edgar 2010). The most abundant operational taxonomic unit (OTU) was picked as a representative, and the assignment of taxonomy was carried out using RDP Classifier 2.2. (Wang et al. 2007) based on the reference database Greengenes release gg_13_8_99 (DeSantis et al. 2006). The alignment of reads was performed using PyNAST (Caporaso et al. 2010b). After filtering, an approximately-maximum-likelihood phylogenetic tree using FastTree 2.1.3. (Price et al. 2010) was generated.

Each replicate was comprised of 11,245 to 276,132 sequences after initial data processing. Prior to diversity analyses, the mitochondrial and plastidal sequences with plant origin and unassigned OTUs (comprising roughly up to one third of each of the mitochondrial sequences, unassigned bacteria, and sequence fragments) were excluded by filtering. Additionally, the replicate with the lowest read number of a sample was removed. As the remaining replicates comprised of 3,758 to 256,248 sequences, the subsequent analyses were performed after normalizing the sequence number per sample to 3,758.

Results & Discussion

Diversity of rhizosphere, seed, and soil samples

Alpha diversity Chao1 and Shannon indices revealed that the species richness in rhizosphere samples was significantly higher than in seed samples (paired t-test, significance level $\alpha = 0.01$, P values 0.003), whereas the richness in the soil was significantly higher than in the rhizosphere samples. The trend that rhizosphere communities comprise less diversity than the surrounding soil is described by Berendsen et al. (2012) in hypothesizing that the plants selectively select beneficial microorganisms.

Table 1. Characteristics of *Cucurbita pepo* genotypes selected for the microbiome analysis.

Denomination	Category*	Pedigree	Geographic origin
Line A	Inbred line (nl)	--	Austria
Line B	Inbred line (nl)	--	Austria
Line C	Inbred line (nl)	--	Austria
Line D	Inbred line (nl)	--	Austria
‘Gleisdorfer Diamant’	Single cross hybrid (nl)	Line A × Line B	Austria
‘GL Opal’	Three-way cross hybrid (nl)	Gl. Diamant × Line C	Austria
‘GL Rustikal’	Three-way cross hybrid (nl)	Gl. Diamant × Line D	Austria
‘GL Classic’	Open-pollinated cultivar (nl)	--	Austria
‘Naxos’	Single cross zucchini hybrid (l)	unknown	Netherlands
Line E	Segregating line (nl)	--	Germany
Line F	Segregating line (l)	--	Slovenia
Line G	Segregating line (nl)	--	Slovenia
Line H	Segregating line (l)	--	China
Line I	Segregating line (l)	--	China

*nl: no lignification of the seed coat, l: lignification of the seed coat.

In addition to the diversity of a plant-associated microbial habitat, its evenness has high importance for maintaining plant vitality (Bakker et al. 2012). The Heip index indicated that the evenness in the seed samples was considerably lower than in the rhizosphere and soil samples, meaning that the relative abundance of taxa in the surrounding habitat was not evenly distributed.

The seed samples of the genotypes Line E, 'Gleisdorfer Diamant', and Line G showed a higher alpha diversity (Shannon diversity index H′ of 8.6, 7.9, and 7.1, respectively) as well as a higher Heip evenness index E′ (0.29, 0.17, 0.16) than the other genotypes investigated (average Shannon diversity index of 4.7, average Heip evenness index of 0.05).

As compared with the seed microbiomes, the diversity and structure among the rhizosphere communities of different *C. pepo* genotypes was similar. The influence of the soil type might have been responsible for a normalization of the root-associated microbiomes as plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere (Berg and Smalla 2008). A possible effect of the chemical stripper on the seed and root-associated microbial community needs to be further investigated.

Genotype specific colonization patterns of seed samples

The microbial communities of all seeds were dominated by Proteobacteria, especially by the family of Enterobacteriaceae. Moreover, the genus *Pseudomonas* was present in all genotypes. *Pseudomonas* contains a number of beneficial species (Avis et al. 2008), but also includes some which infect *C. pepo*, such as *P. viridiflava* (Huss and Mavridis 2007, Grube et al. 2011).

Genotype-specific colonization patterns of the seeds were detected. In the open-pollinated 'GL Classic' and in the inbred Line D, the microbiome contained the genus *Erwinia* to a greater extent, and also the important pathogen *Pectobacterium carotovorum* (syn. *Erwinia carotovora*), the causal agent of soft rot of fruits of the Styrian oil pumpkin (Grube et al. 2011). The genus *Acinetobacter* occurred to a higher extent in the segregating lines, especially in Line H. Further prevalent groups included Firmicutes, especially in the two three-way cross hybrids 'GL Opal' and 'GL Rustikal', as well as in the segregating Line F and the two inbred Lines B and D. Actinobacteria, which are known for the production of antimicrobial compounds (Berg et al. 2013), Bacterioidetes, and Planctomycetes were observed to a higher extent in Line E, Line G, and 'Gleisdorfer Diamant'. These three genotypes had similar communities which were more diverse than those of the other genotypes, congruent with the results of the calculated diversity indices.

According to Berg et al. (2013), the phylum of Proteobacteria includes many plant growth-promoting bacteria. Nevertheless, the strong dominance of the seed-associated microbiomes by Proteobacteria, especially the Enterobacteriaceae, might contribute to the susceptibility to diseases as the microbial richness and evenness of the microbial taxa are important for the maintenance of plant health (Bakker et al. 2012). No specific patterns concerning the field site of origin of studied seeds were observed.

Core microbiome of 'GL Rustikal' seeds and rhizosphere and seed microbiome of 'GL Rustikal' and its pedigree components

The comparison of the 'GL Rustikal' seeds and rhizosphere with the bulk soil microbiome (comprising OTUs which were present in all three replicates of the corresponding samples) shows that the seed and rhizosphere samples shared 8% of the OTUs, whereas the rhizosphere and the bulk soil shared 28% (Figure 1A). Apparently, the seed microbiome has a smaller influence on the rhizosphere microbiome than the soil microbiome.

The seed microbiome analysis of the 'GL Rustikal' pedigree components (Figure 1B) revealed that 'Gleisdorfer Diamant' and Line D as parental components shared 26% and 24% of OTUs with 'GL Rustikal' whereas the genetically more distant components 'Gleisdorfer Diamant' and Line D shared 34%. The core microbiome of the three genotypes investigated comprised of 22% of the taxa.

A detailed analysis of the contribution of Enterobacteriaceae to the communities revealed that major proportions of the OTUs observed were unique for the seed samples. A BLAST analysis in the NCBI nucleotide database revealed that *Pectobacterium carotovorum* was part of the seed microbiome in all genotypes, except 'GL Rustikal'. Transmission of enterobacteriaceal pathogens from the seeds to other plant organs could result in

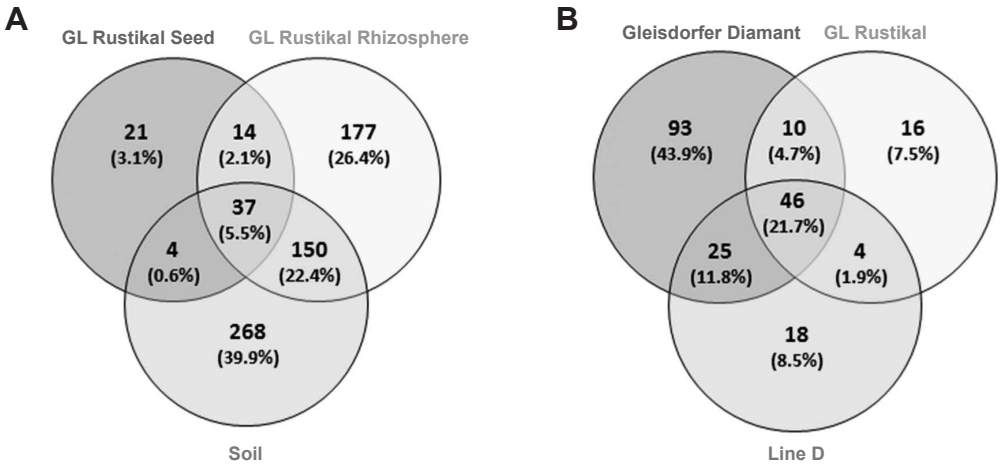

Figure 1. Unique and shared OTUs (A) of 'GL Rustikal' seed and rhizosphere as well as bulk soil microbiomes and (B) seed microbiomes of the three-way cross hybrid 'GL Rustikal' and its pedigree components.

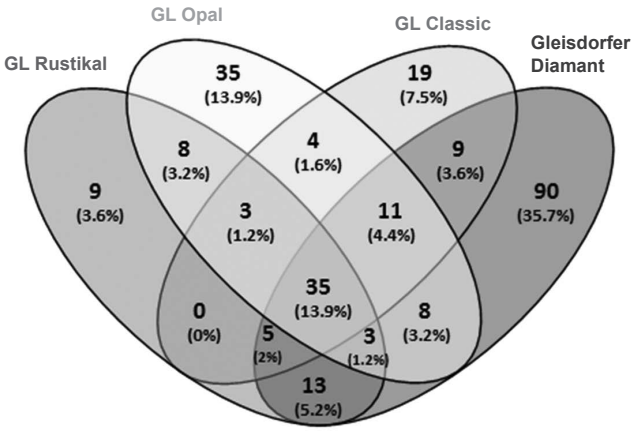

Figure 2. Seed microbiomes (OTU count) of the three-way cross hybrids 'GL Rustikal' and 'GL Opal', the single cross hybrid 'Gleisdorfer Diamant' and the open-pollinated 'GL Classic'.

systemic pathology, such as soft rot disease. A possible implication for breeding programs could be the selection of genotypes enriching less enterobacteriaceal pathogens in their seeds.

Seed microbiomes of 'GL Classic', 'Gleisdorfer Diamant', 'GL Rustikal', and 'GL Opal'

When comparing the four genotypes 'GL Classic', 'Gleisdorfer Diamant', 'GL Rustikal', and 'GL Opal', 14% of the OTUs build up the core microbiome (Figure 2). The 'Gleisdorfer Diamant' seeds were colonized with 90 OTUs unique to the four genotypes investigated.

Conclusions & Outlook

A strong genotype specificity was detected for the seed-associated microbial communities. The results of the seed samples are of particular interest, as the Styrian oil pumpkin is highly susceptible to various fungal and bacterial pathogens during germination. It remains to be investigated whether seed-borne bacteria influence germination and plant development, and if pathogens are transmitted by the seeds. Thus, the results have implications for seed production and could direct the design of tailored biological seed treatment strategies. The conducted experiments and analyses are just the beginning of a versatile project for implementing the knowledge of plant-microbe interactions into an advanced breeding strategy. The next steps will include the 16S rRNA amplicon sequencing of the progeny seeds of some selected genotypes as well as an ITS amplicon sequencing of the seed and rhizosphere samples. Confocal laser scanning microscopic analyses, greenhouse experiments, and field trials for the assessment of differences in plant-microbe interactions of *Cucurbita pepo* genotypes could reveal deeper biological roles of these microbial communities in the development and life cycle of the Styrian oil pumpkin.

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Inheritance of Rind Pattern in Watermelon

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Abstract. Genes for watermelon [*Citrullus lanatus* (Thunb.) Matsumura & Nakai] fruit traits have been identified since the 1930s. We studied the inheritance of fruit stripe width and rind color. Several new genes or alleles were discovered. A series of alleles at the *g* locus is proposed to explain the inheritance of fruit rind pattern: *G* (medium or dark solid green); g^W (wide stripe); g^M (medium stripe); g^N (narrow stripe); and *g* (solid light green or gray). The dominance series is $G > g^W > g^M > g^N > g$.

Keywords: Rind, pattern, color, gene

Introduction

Watermelon is a major vegetable crop cultivated as an annual in warm regions worldwide (Wehner 2008b). Genetic studies since the 1930s have identified more than 100 genes. The genes control traits in seed and seedling, vine, flower, and fruit, as well as resistance to diseases, insects, and stress (Poole 1944, Robinson et al. 1976, Guner and Wehner 2004, Wehner 2008a, Wehner 2012).

The rind of watermelon fruit can be striped or solid colored. The solid rind patterns include solid dark green as in 'Black Diamond', solid medium green as in 'Peacock Shipper', solid light green as in 'King & Queen', gray (medium green reticulations on a light green background) as in 'Charleston Gray', or golden as in 'Royal Golden' (Barham 1956, Guner and Wehner 2003, 2004, Gusmini and Wehner 2006a, 2006b).

The stripes of watermelon can be characterized by stripe width (narrow, medium, wide), stripe color, and background color (dark green, medium green, light green). In this study, we considered the dark colored area to be the stripes. Studies by Porter (1937) and Weetman (1937) identified three alleles at the *g* locus that produce solid dark green (*G*), striped (g^s), or gray (*g*) rind pattern. The gray rind pattern has also been described as light green in some studies. Solid dark green (*G*) is dominant to striped (g^s) and gray (*g*). Striped rind pattern (g^s) is dominant to gray (*g*). Here *G* is from 'California Klondike', *g* is from 'Thurmond Gray', and g^s is from 'Golden Honey'. Recently, Kumar and Wehner (2011) found a second gene controlling the dark green rind versus gray rind pattern. The *g* gene is considered *g-1* and the second gene *g-2,* from type-line 'Minilee'. The *g-1* and *g-2* genes produce the gray rind pattern. The others (*G-1/G-1 G-2/G-2, G-1/G-1 g-2/g-2,* and *g-1/g-1 G-2/G-2*) produce dark green rind pattern.

The objectives of this experiment were to study the inheritance of exterior fruit traits of watermelon, including stripe width and rind color.

Materials & Methods

Seeds of the type-lines were collected based on descriptions in the watermelon gene list (Wehner 2012), as well as the vegetable cultivar list (Wehner 2002). Three families were developed by crossing parents having different stripe widths: (1) 'Crimson Sweet' (medium stripe) × 'Red-N-Sweet' (narrow stripe); (2) 'Allsweet' (wide stripe) × 'Red-N-Sweet' (narrow stripe); and (3) 'Tendersweet Orange Flesh' (wide stripe) × 'Red-N-Sweet' (narrow stripe). Seven families were developed by crossing a parent having striped rind with a parent having solid color rind: (1) 'Red-N-Sweet' (narrow stripe) × 'King & Queen' (solid light green); (2) 'Red-N-Sweet' (narrow stripe) × 'Charleston Gray' (gray); (3) 'Crimson Sweet' (medium stripe) × 'Peacock Shipper' (solid medium green); (4) 'Red-N-Sweet' (narrow stripe) × 'Black Diamond' (solid dark green); (5) 'Crimson Sweet' (medium stripe) × 'King & Queen' (solid light green); (6) 'Allsweet' (wide stripe) × 'King & Queen' (solid light green); and (7) 'Allsweet' (wide stripe) × 'Black Diamond' (solid dark green). Three families were developed by crossing two solid color parents: (1) 'Peacock Shipper' (solid medium green) × 'Charleston Gray' (gray); (2) 'King & Queen' (solid light green) × 'Peacock Shipper' (solid medium green); and (3) 'Black Diamond' (solid light green) × 'Charleston Gray' (gray).

We used two sets (two locations). For each location, there were 10 plants of P_1S_1, 10 of P_2S_1, 10 of F_1, 10 of F_1R, 30 of BC_1P_1, 30 of BC_1P_2, 100 of F_2. The data were analyzed by location for each tested trait and then pooled over locations. Segregation analysis and goodness-of-fit tests were performed based on chi-square testing of the expected segregation ratios for a single gene, using the SAS-STAT statistical package (SAS Institute, Cary, North Carolina) and the SASGene 1.2 statement (Liu et al. 1997). The calculations were done manually for the families involving a heterozygote with a third phenotype (incom-

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plete dominance) other than the two parents, or when two gene loci were involved. All chi-square tests were performed with a 95% confidence level. For the generations F_1 and F_1R, when both had the same phenotype, F_1 and F_1R were combined as a single generation. When the F_1 differed from the reciprocal, they were treated as separate generations. Families with the same traits and segregation ratios were pooled. Gene nomenclature rules for the Cucurbitaceae family (Cucurbit Gene List Committee 1982) were applied for naming the proposed new genes.

Results & Discussion

In the family 'Crimson Sweet' (medium stripe) × 'Red-N-Sweet' (narrow stripe), all F_1 fruit had medium stripes, which indicates the medium stripe is dominant over narrow stripe. F_2 progenies segregated into medium stripe and narrow stripe with a ratio 3 : 1. BC_1P_2 segregated into medium stripe and narrow stripe with a ratio 1 : 1. And all BC_1P_1 were medium stripe (Table 1). This data shows that the medium stripe is controlled by a single gene dominant over narrow stripe, and the BC_1P_1, and BC_1P_2 data confirmed it.

However, in the family of 'PDS 808' (medium stripe) × 'Red-N-Sweet' (narrow stripe) (data not presented), no Mendelian pattern of inheritance was observed. All F_1, F_1R, BC_1P_1, BC_1P_2, and F_2 fruit had stripe width similar to 'Red-N-Sweet'.

In the families with a wide striped parent crossed with a narrow striped parent, 'Allsweet' (wide stripe) × 'Red-N-Sweet' (narrow stripe) and 'Tendersweet Orange Flesh' (wide stripe) × 'Red-N-Sweet' (narrow stripe), all F_1 fruit were wide-striped, indicating that wide stripe is dominant over narrow stripe. The F_2 segregated 3 wide stripe : 1 narrow stripe and BC_1P_2 segregated 1 wide stripe : 1 narrow stripe. The BC_1P_1 were all wide stripe (Table 2). The F_1 and F_2 showed that wide stripe from 'Allsweet' is a single gene dominant over the narrow stripe of 'Red-N-Sweet'.

Two families had a wide striped parent crossed with a solid green parent, 'Red-N-Sweet' (narrow stripe) × 'King & Queen' (light green rind with inconspicuous light narrow stripes, appearing solid light green) and 'Red-N-Sweet' (narrow stripe) × 'Charleston Gray' (gray). Although 'King & Queen' has light green stripes, the rind pattern can be considered solid light green, since the stripes are inconspicuous. In the first family, all F_1 fruit had narrow stripes. That indicates narrow stripe was dominant over solid light green (Table 3). The F_2 segregated 3 narrow stripe : 1 solid light green, indicating that the narrow stripe of 'Red-N-Sweet' is conferred by a single gene that is dominant over solid light green in 'King & Queen'. The BC_1P_1 were all narrow stripe, and the BC_1P_2 had 1 narrow stripe : 1 solid light green, confirming that inheritance pattern.

In the family 'Red-N-Sweet' (narrow stripe) × 'Charleston Gray' (light green with reticulations, called gray), all F_1 had narrow stripe, indicating dominance over gray. The segregation ratios in the F_2 (3 narrow stripe : 1 light green) and BC_1P_2 (1 narrow stripe : 1 light green) further confirmed that narrow stripe of 'Red-N-Sweet' is controlled by a single dominant gene (Table 4). This is a similar pattern to 'Red-N-Sweet' (narrow stripe) × 'King & Queen' (solid light green). In some reports, gray rind has been described as yellowish white or yellowish green (Porter 1937).

In the family with a striped parent crossed with a solid parent, 'Crimson Sweet' (medium-wide stripe) × 'Peacock Shipper' (solid medium green), the F_1 fruit had solid medium green rind and the F_2 segregated 3 solid medium green : 1 medium stripe. The BC_1P_1 had 1 solid medium green : 1 medium stripe fruit, and the BC_1P_2 were all solid medium green (Table 5). Thus, solid medium green rind of 'Peacock Shipper' is a single gene, dominant over medium stripe of 'Crimson Sweet'.

For the other four families involving a striped parent and a solid green parent, the data were more complicated. Intermediate phenotypes were often present in the F_1 and the green shades of F_2 progeny often acted like a quantitative trait making classification difficult. These four crosses were 'Red-N-Sweet' (narrow stripe) × 'Black Diamond' (solid dark green), 'Crimson Sweet'

Table 1. Single locus goodness-of-fit-test for stripe width in watermelon in the cross 'Crimson Sweet' (medium stripe) × 'Red-N-Sweet' (narrow stripe), trials at Kinston and Clinton, North Carolina.

Generation	Total no.†	Medium stripe‡	Narrow stripe§	No. missing	Expected ratio	Chi square	df	Probability
P_1S_1	20	15	0	5				
P_2S_1	20	0	19	1				
F_1	40	34	0	6				
F_2	200	105	45	50	3 : 1	2.00	1	0.15*
BC_1P_1	60	43	1	16	1 : 0	0.02	1	0.88*
BC_1P_2	60	24	20	16	1 : 1	0.36	1	0.54*

*Significant at the 0.05 probability level.
†Data were pooled over the two locations.
‡Medium stripe was dominant and P_1 was the carrier.
§Narrow stripe was recessive and P_2 was the carrier.

Table 2. Single locus goodness-of-fit-test for stripe width in watermelon for crossings involving wide stripe × narrow stripe, trials at Kinston and Clinton, North Carolina.

Generation	Total no.†	Wide stripe‡	Narrow stripe§	No. missing	Expected ratio	Chi square	df	Probability
'Allsweet' (wide) × 'Red-N-Sweet' (narrow)								
P_1S_1	20	8	0	12				
P_2S_1	20	0	10	10				
F_1	40	31	1	8				
F_2	200	98	39	63	3 : 1	0.88	1	0.34*
BC_1P_1	60	42	3	15	1 : 0	0.20	1	0.65*
BC_1P_2	60	15	15	30	1 : 1	0.00	1	1.00*
'Tendersweet Orange Flesh' (wide) × 'Red-N-Sweet' (narrow)								
P_1S_1	20	20	0	0				
P_2S_1	20	0	13	7				
F_1	40	34	0	6				
F_2	200	125	34	41	3 : 1	1.11	1	0.29*
BC_1P_1	60	54	0	6	1 : 0	0.00	1	1.00*
BC_1P_2	60	27	29	4	1 : 1	0.07	1	0.78*

*Significant at the 0.05 probability level.
†Data were pooled over the two locations.
‡Wide stripe was dominant and P_1 was the carrier.
§Narrow stripe was recessive and P_2 was the carrier.

Table 3. Single locus goodness-of-fit-test for stripe in watermelon in the cross 'Red-N-Sweet' (narrow stripe) × 'King & Queen' (solid light green), trials at Kinston and Clinton, North Carolina.

Generation	Total no.†	Narrow stripe‡	Solid light green§	No. missing	Expected ratio	Chi square	df	Probability
P_1S_1	20	17	0	3				
P_2S_1	20	0	15	5				
F_1	40	33	0	7				
F_2	200	143	41	16	3 : 1	0.72	1	0.39*
BC_1P_1	60	42	1	17	1 : 0	0.02	1	0.87*
BC_1P_2	60	36	23	1	1 : 1	2.86	1	0.09*

*Significant at the 0.05 probability level.
†Data were pooled over the two locations.
‡Narrow stripe was dominant and P_1 was the carrier.
§Solid light green was recessive and P_2 was the carrier.

Table 4. Single locus goodness-of-fit-test for stripe width in watermelon in the cross 'Red-N-Sweet' (narrow stripe) × 'Charleston Gray' (gray), trials at Kinston and Clinton, North Carolina.

Generation	Total no.†	Narrow stripe‡	Gray§	No. missing	Expected ratio	Chi square	df	Probability
P_aS_1	20	19	0	1				
P_bS_1	20	0	8	12				
F_1	40	30	0	10				
F_2	200	128	39	33	3 : 1	0.24	1	0.62*
BC_1P_a	60	54	0	6	1 : 0	0.00	1	1.00*
BC_1P_b	60	26	30	4	1 : 1	0.29	1	0.59*

*Significant at the 0.05 probability level.
†Data were pooled over the two locations.
‡Narrow stripe was dominant and P_1 was the carrier.
§Gray was recessive and P_2 was the carrier.

Table 5. Single locus goodness-of-fit-test for stripe width in watermelon in the cross 'Peacock Shipper' (solid medium green) × 'Crimson Sweet' (medium stripe) in trials at Kinston and Clinton, North Carolina.

Generation	Total no.†	Solid medium green‡	Medium stripe§	No. missing	Expected ratio	Chi square	df	Probability
P_1S_1	20	20	0	0				
P_2S_1	20	0	18	2				
F_1	40	39	0	1				
F_2	200	138	36	26	3 : 1	1.72	1	0.18*
BC_1P_1	60	56	0	4	1 : 0	0.00	1	1.00*
BC_1P_2	60	27	29	4	1 : 1	0.07	1	0.78*

*Significant at the 0.05 probability level.
†Data were pooled over the two locations.
‡Solid medium green was dominant and P_1 was the carrier.
§Medium stripe was recessive and P_2 was the carrier.

Table 6. Single locus goodness-of-fit-test for stripe width in watermelon in the cross 'Crimson Sweet' (medium stripe) × 'King & Queen' (solid light green), trials at Kinston and Clinton, North Carolina.

Generation	Total no.†	Medium stripe‡	Solid light green§	No. missing	Expected ratio	Chi square	df	Probability
P_1S_1	20	17	0	3				
P_2S_1	20	0	16	4				
F_1	40	33	0	7				
F_2	200	112	42	46	3 : 1	0.42	1	0.51*
BC_1P_1	60	54	0	6	1 : 0	0.00	1	1.00*
BC_1P_2	60	55	0	5	1 : 1	55.00	1	0.00

*Significant at the 0.05 probability level.
†Data were pooled over the two locations.
‡Medium stripe was dominant and P_1 was the carrier.
§Solid light green was recessive and P_2 was the carrier.

(medium stripe) × 'King & Queen' (solid light green), 'Allsweet' (wide stripe) × 'King & Queen' (solid light green), and 'Allsweet' (wide stripe) × 'Black Diamond' (solid dark green).

In the family 'Red-N-Sweet' (narrow stripe) × 'Black Diamond' (solid dark green), all F_1 fruit had an intermediate phenotype. The color of the F_1 was lighter than 'Black Diamond' and darker than the light green background of 'Red-N-Sweet'. The fruit of the F_1 had inconspicuous stripes that were difficult to see on some individuals. The F_2 segregated into three classes, the P_1 phenotype, the P_2 phenotype, and the intermediate F_1 phenotype, and the green color of the F_2 (disregarding the stripes) was difficult to classify. The goodness-of-fit tests for the F_2, BC_1P_1, and BC_1P_2 data were not significant, probably because of misclassification due to the inconspicuous stripes of the intermediate phenotype. The intermediate F_1 phenotype also indicated that the color shade and stripes are controlled by different gene loci. Porter (1937) investigated two similar families between solid dark green cultivars and striped cultivars, 'California Klondike' (solid dark green) × 'Golden Honey' (striped) and 'Golden Honey' (striped) × 'Angeleno Black Seeded' (solid dark green). In both of the F_1s, fruits were intermediate with faint stripes different from both parents, and the F_2 had a 1 : 2 : 1 segregation ratio. However, it is unclear how that compares with our findings since the F_2 and backcross data were not presented (Porter 1937).

In the family 'Crimson Sweet' (medium stripe) × 'King & Queen' (solid light green), the F_1 fruit had medium stripes that were narrower than the striped parent 'Crimson Sweet'. The F_2 and backcross had fruit with various stripe widths. Disregarding the stripe width, there were two phenotypes in F_2, striped and solid, with a ratio close to 3 : 1 (Table 6). All BC_1P_1 fruit were striped. Unfortunately, the BC_1P_2 fruit also were all striped, although we expected a ratio of 1 striped : 1 light green fruit. The F_2 progenies showed that medium stripe is a single gene, dominant over solid light green, but the BC_1P_2 failed to verify it. Further study is needed.

In the family 'Allsweet' (wide stripe) × 'King & Queen' (solid light green), all F_1 were medium striped. Similar to 'Crimson Sweet' (medium stripe) × 'King & Queen' (solid light green), the F_2 progeny was a mixture of various green shades and stripe widths. The stripes were blended into the background color, and were difficult to classify. All BC_1P_1 were wide stripe and BC_1P_2 were similar to F_2 progenies. So, no Mendelian inheritance was identified in this family.

Table 7. Single locus goodness-of-fit-test for stripe width in watermelon in the cross 'Black Diamond' (solid dark green) × 'Allsweet' (wide stripe), trials at Kinston and Clinton, North Carolina.

Generation	Total no.†	Solid dark green‡	Wide stripe§	No. missing	Expected ratio	Chi square	df	Probability
P_1S_1	20	20	0	0				
P_2S_1	20	0	17	3				
F_1	40	40	0	0				
F_2	200	156	36	8	3 : 1	4.00	1	0.045
BC_1P_1	60	49	4	7	1 : 0	0.30	1	0.58*
BC_1P_2	60	35	24	1	1 : 1	2.05	1	0.15*

*Significant at the 0.05 probability level.
†Data were pooled over the two locations.
‡Solid dark green was dominant and P_1 was the carrier.
§Wide stripe was recessive and P_2 was the carrier.

Table 8. Single locus goodness-of-fit-test for fruit color in watermelon in family 'Peacock Shipper' (solid medium green) × 'Charleston Gray' (gray) in trials at Kinston and Clinton, North Carolina.

Generation	Total no.†	Solid medium green‡	Light green§	No. missing	Expected ratio	Chi square	df	Probability
P_1S_1	20	14	0	6				
P_2S_1	20	0	17	3				
F_1	40	37	0	3				
F_2	200	132	41	27	3 : 1	0.16	1	0.69*
BC_1P_1	60	53	0	7	1 : 0	0.00	1	1.00*
BC_1P_2	60	17	17	26	1 : 1	0.00	1	1.00*

*Significant at the 0.05 probability level.
†Data were pooled over the two locations.
‡Solid medium green was dominant and P_1 was the carrier.
§Gray was recessive and P_2 was the carrier.

In the family 'Allsweet' (wide stripe) × 'Black Diamond' (solid dark green), the F_1 fruit had intermediate, solid medium green rind. The F_2 segregated into striped and solid-colored fruits with different shades of green. If the shades of green were disregarded and the phenotypes classified into stripe and solid green, then the data fit the pattern of a single gene, with solid dark green dominant to wide stripe (Table 7), and confirmed by the BC_1P_1 and BC_1P_2. It appears that some wide stripe fruit were misclassified as solid dark green, indicating that stripes were difficult to distinguish from background color.

Three families were made using solid green parents, 'Peacock Shipper' (solid medium green) × 'Charleston Gray' (gray), 'King & Queen' (solid light green) × 'Peacock Shipper' (solid medium green), and 'Black Diamond' (solid dark green) × 'Charleston Gray' (gray). For 'Peacock Shipper' (solid medium green) × 'Charleston Gray' (gray), the F_1 fruit were solid medium green rind, which indicated that the solid medium green rind is dominant over light green rind. Both parents have reticulations on the rind, but the reticulation was ignored for this trait. The F_2 progeny segregated medium green, light green, and a medium light green color (between the light green of 'Charleston Gray' and the medium green of 'Peacock Shipper'). The segregation ratio was 3 : 1 when combining medium and medium light green fruit and comparing with light green (Table 8). The segregation ratios in the F_2 and BC_1P_2 suggest that the solid medium green rind of 'Peacock Shipper' is a single gene, dominant over the light green rind of 'Charleston Gray'.

In the family, 'King & Queen' (solid light green) × 'Peacock Shipper' (solid medium green), the F_1 had medium green rind with inconspicuous dark narrow stripes. The F_2 progeny segregated into 4 phenotypes: 31 light green with inconspicuous stripes (same as 'King & Queen'), 46 solid medium green (same as 'Peacock Shipper'), 45 medium green with narrow medium green stripes, and 24 light green with narrow medium-green stripes. All BC_1P_1 were like 'King & Queen', while BC_1P_2 segregated into 32 solid medium green and 18 light green with narrow medium green stripes. If all striped fruit were combined, striped was a single gene, dominant over solid.

From the above, we conclude that (1) medium stripe of type-line 'Crimson Sweet' is a single gene, dominant over narrow stripe of type-line 'Red-N-Sweet' (Table 1), (2) wide stripe of type-lines 'Allsweet' and 'Tendersweet Orange Flesh' is a single gene, dominant over narrow stripe of type-line 'Red-N-Sweet' (Table 2), (3) narrow stripe of type-line 'Red-N-Sweet' is a single gene dominant over solid light green of type-line 'King & Queen' (Table 3), (4) narrow stripe of type-line 'Red-N-Sweet' is a single gene, dominant over gray of type-line 'Charleston Gray' (Table 4), (5) solid medium green of type-line 'Peacock Shipper' is a single gene, dominant over medium stripe of type-line 'Crimson Sweet' (Table 5), (6) medium stripe of type-line 'Crimson Sweet' is a single gene, dominant over solid light green of type-line 'King & Queen' (Table 6), (7) solid dark green of type-line 'Black Diamond' is a single gene, dominant over wide stripe of type-line 'Allsweet' (Table 7), and (8) solid medium green of type-line 'Peacock Shipper' is a single gene, dominant over gray of type-line 'Charleston Gray' (Table 8).

The *g* locus was described earlier by Porter (1937) and Weetman (1937): *G* is from 'California Klondike', g^S is from 'Golden Honey', and *g* is from 'Thurmond Gray'. We now add alleles to the locus as follows: *G* (solid medium or dark green) from 'Peacock Shipper', 'Black Diamond', and 'California Klondike'; g^W (wide stripe) is from 'Allsweet' and 'Tendersweet Orange Flesh'; g^M (medium stripe) is from 'Crimson Sweet'; g^N (narrow stripe) is from 'Red-N-Sweet'; and *g* (solid light green or gray) is from 'King & Queen', 'Charleston Gray' and 'Thurmond Gray'. *G* (solid medium or dark green) is the most dominant; g^W (wide stripe) is the second dominant and is only recessive to *G* but dominant over the rest; g^M (medium stripe) is the third dominant and is dominant over g^N and *g*, but recessive to *G* and g^W; g^N (narrow stripe) is the fourth dominant; and *g* (solid light green or gray) is the recessive. The g^S allele from 'Golden Honey' may be the same as g^M from 'Crimson Sweet', but additional crosses are needed to verify that. A series of alleles at the *g* locus is proposed to explain the inheritance of fruit rind pattern and color: *G* (solid medium or dark green); g^W (wide stripe); g^M (medium stripe); g^N (narrow stripe); and *g* (solid light green or gray). Their dominance is $G > g^W > g^M > g^N > g$. The following type-lines are proposed: *G/G* for solid medium or dark green of 'Peacock Shipper', 'Black Diamond', as well as 'California Klondike'; g^W/g^W for wide stripe of 'Allsweet' and 'Tendersweet Orange Flesh'; g^M/g^M for medium stripe of 'Crimson Sweet'; g^N/g^N for narrow stripe of 'Red-N-Sweet';

and *g/g* for gray or solid light green of 'Charleston Gray' and 'King & Queen'. The difference between the solid light green of 'King & Queen' and the gray of 'Charleston Gray' needs further investigation. An allelism test between wide stripe and medium stripe is also needed. Future experiments might include the following: (1) 'Allsweet' (wide stripe) × 'Peacock Shipper' (solid medium green), (2) 'Allsweet' (wide stripe) × 'Crimson Sweet' (medium stripe), and (3) 'King & Queen' (light green) × 'Charleston Gray' (gray).

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Significant Reciprocal-Cross Differences in Cucumber

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Abstract. Cucumber is a useful plant to study organellar effects on plant performance because chloroplasts are maternally and mitochondria paternally transmitted. We generated reciprocal hybrids among doubled haploid (DH) cucumbers in a diallel mating scheme, measured fresh and dry weights 22 to 30 days after planting of progenies, estimated combining abilities and heterosis for these traits, and assessed performance differences between reciprocal hybrids possessing identical nuclear genotypes. Across experiments, general and specific combining abilities and reciprocal effects were highly significant ($P < 0.001$). Average heterosis was 14% to 30% over the over mid-parent values. The MSC3 mitochondrial mutant showed negative effects when used as a male, but not as a female. Reciprocal hybrids among wild-type DH parents were identified that differed significantly for dry and fresh weights, indicating that cucumber breeders should evaluate both directions of crosses when producing hybrid cultivars.

Keywords: Cytoplasmic effects, diallel, heterosis

Introduction

Cucumber, *Cucumis sativus* L., possesses characteristics useful for organellar genetics, including differential transmission of organelles, maternal for chloroplasts and paternal for mitochondria (Havey et al. 1998), and a large mitochondrial DNA with repetitive sequences that undergo recombination to produce structurally rearranged molecules (Lilly and Havey 2001, Bartoszewski et al. 2004a, Alverson et al. 2011), and existence of the mitochondrially associated mosaic (MSC) phenotypes (Malepszy et al. 1996, Lilly et al. 2001, Bartoszewski et al. 2004b). Differential transmission of organelles in cucumber provides a unique system to characterize chloroplast and mitochondrial effects on plant growth and development. We crossed among cucumber DHs to produce a complete diallel and detected significant differences for plant growth between reciprocal hybrids possessing identical nuclear genotypes, indicating potentially beneficial organellar effects on plant performance.

Materials & Methods

DH plants were produced by culturing immature female flowers (Dirks 1988) from GY14, 'Marketmore 76' (MM76), 'Straight 8' (ST8), TMG1, and 9930. MSC3 is a mitochondrial mutant selected from the highly inbred line B (Bartoszewski et al. 2004b). The DH lines and MSC3 were crossed with each other both as the male and female to produce a complete diallel of 30 hybrids. Plants of each hybrid and parental line were grown in greenhouses (blocks) and the experiment was repeated three times. Growth conditions were 16 hour days at 28 °C with light intensity of 270 $\mu mol \cdot s^{-1} \cdot m^{-2}$; nights were at 24 °C. Plants were destructively harvested 22 or 30 days after planting, and fresh and dry weights taken. General combining ability (GCA), specific combining ability (SCA), and reciprocal-cross effects were calculated using a Statistical Analysis Software version 9.3 (SAS Institute, Cary NC) program based on Griffing (1956) method 1; fixed effects model 1. Average heterosis was estimated using the program of Burow and Coors (1994).

Results & Discussion

Genotypes (parents and hybrids) were highly significant ($P < 0.001$) for both fresh and dry weights. The GCA of an inbred is a measure of the average performance of hybrids from crosses with other inbreds; SCA measures the performance of specific hybrid combinations relative to the average performance expected from the inbred parents. GCA, SCA, and reciprocal effects, as well as their interactions with experiments, were all highly significant ($P < 0.001$). The DH lines from TMG1, MM76, and 9930 showed positive GCA effects for both fresh and dry weights; while ST8, GY14, and MSC3 had negative GCA effects for both traits (see Shen et al. 2015 for specific values). The parental DHs and MSC3 showed negative SCA effects for both fresh and dry weights, indicating that hybrids consistently out-performed their parents. Across experiments, average heterosis over the

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mid-parent value was between 14% and 30%. These results agree with other research reporting significant heterosis in cucumber (Singh et al. 1970, Ghaderi and Lower 1979, Rubino and Wehner 1986, Hormuzdi and More 1989).

We detected significant ($P < 0.001$) differences between reciprocal hybrids from crosses among the DH lines and MSC3 for both fresh and dry weights. The mitochondrial mutant MSC3 showed significant negative effects when used as a male, but not when used as a female parent. The TMG1 DH performed significantly better as a female than male; however, the opposite was true for ST8 which performed better as a male than female. Significantly better performance as a male parent could be due to superior mitochondria or poorer chloroplasts. Conversely, better performance as the female parent could indicate better performing chloroplasts or poorer mitochondria. Our results indicate that different organellar types may exist in cucumber, and inbreds possessing specific organelles may perform better as the male or female to produce superior hybrids.

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Isolation and Characterization of Three Recessive Andromonoecious Mutants of *Cucurbita pepo*

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Abstract. Most of *Cucurbita pepo* cultivars are monoecious, as they produce both male and female flowers on the same plant. By using triple-response to ethylene on etiolated seedlings, we have screened a zucchini mutant collection of more than 3,800 M_2 families, and identified three ethylene-insensitive mutants: *andro1*, *andro2*, *andro3*. The three ethylene-insensitive phenotypes segregated as a single recessive gene, and were characterized by the conversion of female into bisexual or hermaphrodite flowers, and of monoecy into andromonoecy. The *andro2* mutant was completely andromonoecious, since it developed hermaphrodite flowers with complete stamens and mature pollen. The *andro1* and *andro3* mutants, however, were partially andromonoecious, developing bisexual flowers with immature stamens and no pollen. Given that andromonoecy is known to be controlled by ethylene biosynthesis genes in other cucurbits, we assessed the production of ethylene in pistillate flowers of sensitive and insensitive plants from each mutant family. The *andro1* mutant showed a reduced production of ethylene throughout pistillate flower development, but ethylene production was not affected in the other two mutants. The three andromonoecious mutants were also altered with regard to sex expression, showing a delayed pistillate flowering transition and a reduced number of pistillate flowers per plant. The mutations also affected the coordination of floral organ growth and maturation. In comparison with wild-type (WT) female flowers, both bisexual and hermaphrodite flowers showed longer ovaries and parthenocarpic fruits, and petals with delayed maturation. These prevented fertilization and seed set in the mutant flowers. The three mutations also had increased internode number and length, which resulted in taller plants than WT. These results indicate that *andro1*, *andro2*, and *andro3* mutations probably occur in genes which alter ethylene response at different phases of plant development.

Keywords: Ethylene, EMS, triple-response, andromonoecious, parthenocarpic

Introduction

Ethylene regulates a number of developmental and physiological processes in plants. It has been demonstrated that ethylene is the key hormone regulating sex determination in cucurbits. This plant hormone is an essential regulator of flower development in *Cucurbita pepo* L. controlling the sexual expression and the differentiation and maturation of floral organs (Manzano et al. 2010, 2011, 2013).

The molecular bases of ethylene-regulated processes have been discerned thanks to the analysis of *Arabidopsis* mutants which were altered in the seedling triple-response to ethylene (Bleecker et al. 1988). At the University of Almeria (UAL), we have generated a mutant collection of about 3,800 M_2 families of the zucchini genetic background MUC-16. The mutant families were produced by using ethyl methane sulfonate (EMS) at two different concentrations: 0.2 and 0.3% (Manzano et al. 2012). By using the triple-response of etiolated seedlings to ethylene, we have been able to identify three ethylene-insensitive mutants (*andro1*, *andro2*, *andro3*) which show a reduction in hypocotyl and root length, and less hypocotyl thickening compared with the genetic background MUC-16 (García et al. 2015).

In this investigation, we observed the effects of these mutations on flower and fruit development. The three mutations suppressed the arrest of stamens during female flower development, promoting a conversion of female into bisexual or hermaphrodite flowers, and therefore of monoecy into andromonoecy. The mutations also altered ovary and fruit growth rates, encouraging the production of parthenocarpic fruits. The identification of the genes responsible for these mutant phenotypes would promote a better understanding of the relationship between ethylene, sex determination, and parthenocarpy in zucchini squash.

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Materials & Methods

Plant material and triple-response test

We have developed a mutant collection of about 3,800 EMS M_2 families in the genetic background MUC-16. To detect mutations in the ethylene-response pathway, we performed a massive screening of the collection by using the ethylene triple-response assay. Ethylene-insensitive plants were detected in three M_2 families (M_2-118, M_2-435, M_2-1717), grown to maturity, and selfed to obtain the respective M_3 generations. The mutants of the three families were partially or completely andromonoecious, and were therefore called *andro1*, *andro2,* and *andro3*, respectively. The three mutants were female-sterile, but they were used as pollinators to backcross with MUC-16 for two generations before the final phenotypic characterization.

Before field evaluation, all backcrossing and selfing generations were checked for ethylene sensitivity by using the triple-response test. Seeds were sown in seedling trays, germinated for two days in the absence of ethylene, and then introduced into a chamber containing 50 ppm of ethylene and kept in darkness for a total of 5 days. The hypocotyls of the seedlings that were insensitive to ethylene did not reduce in length, so that they protruded further than those of the rest of the seedlings in the growth chamber. The rest of the seedlings were either sensitive to ethylene, or showed an intermediate phenotype. Selected ethylene-sensitive and -insensitive plants were grown and evaluated under standard greenhouse conditions in Almería (Spain).

Sex expression and floral organ development

For the study of sex expression, ten ethylene-sensitive and ten ethylene-insensitive plants from each family were evaluated. For each plant, the number of nodes up to the production of the first female flower (pistillate flowering transition), and the number of pistillate flowers per plant were assessed within the first 36 nodes of the main shoot. The growth rates of ovaries/fruits and petals of ethylene sensitive and insensitive plants were determined by measuring the length of these floral organs every two days, starting with flowers of about 4 mm in length.

Ethylene production and CpACS27A *sequencing*

Throughout pistillate flower development within the three families, ethylene production in mutant and wild-type (WT) flowers was determined by using 4 replicates, each one containing 3 flowers at the same stage of development. These three flowers were enclosed in sealed containers for 6 hours, and the level of ethylene produced was determined by gas chromatography. The measurement of ethylene production in each sample was repeated three times in a VARIAN 3900 gas chromatograph equipped with a flame ionization detector (FID).

For *CpACS27A* sequencing, the DNA from WT and *andro1*, *andro2,* and *andro3* plants was isolated and used in PCR reactions with different primer pairs: CpACS27F1 (CCGTCTAAGTTCATCGATGTTA) and CpACS27R1 (AACCCAAGTGTTGAGTTAATGGG); ACSPROM1958 (GGGTTGGTGGGGTCATA) and ACS27 REV RACE (GGGAAGGCCGAGGTCTTTGGATAGGC); ACS27 FWD RACE (AACGGTCCACAATTGAGGAG) and ACS27 3UTRREV (GCAAGAAA AAAGAATCACAATGG). The PCR fragments represented a sequence of 1,997 kb, covering both the promoter and the complete coding region of *CpACS27A* gene. The PCR products were sequenced and the complete sequences of WT and mutant plants of each family were aligned in order to detect possible gene mutations.

Statistical analysis

Simple and factorial analyses of variance (ANOVA) at $P < 0.05$ were performed using the Statgraphics Plus v 5.1 software. The variables follow a normal distribution. Averages were compared using Fisher's Least Significant Difference (LSD) method.

Results & Discussion

Identification and genetic analysis of three ethylene-insensitive mutants

After performing a massive screening of the *C. pepo* mutant collection with the triple-response test, we identified three families that presented a different ethylene response in comparison with the genetic background MUC-16. Seedlings of MUC-16 showed reduced hypocotyl and root length, increased hypocotyl thickness, and a greater curvature in the apical hook. Some plants of three of the mutant families (M2-118, M2-435, M2-1717) showed a reduced response to ethylene. Insensitive plants were unable to self because of a lack of receptivity in the stigma, but they were used as pollinators in backcrosses with MUC-16. After two generations of backcrossing, heterozygous plants were selfed again to obtain the mutant plants. Heterozygous plants from the M_2 were also selfed to obtain the M_3 generations.

Backcrossing and selfing generations were tested for their ethylene triple-response, in order to distinguish between three different phenotypes: seedlings which were sensitive, intermediate, and insensitive to ethylene. The 1 : 1 (sensitive : intermediate), and 1 : 2 : 1 (sensitive : intermediate : insensitive) segregation ratios in backcrossing and selfing generations from plants having an intermediate ethylene triple response indicated that the ethylene-insensitive phenotype of each mutant was conferred by a single recessive gene.

The ethylene-insensitive mutants were andromonoecious plants with altered floral organ development

In cucurbit species, ethylene is involved in the regulation of multiple traits of horticultural interest. These include sex determination, sex expression, fruit set and parthenocarpy, and also postharvest fruit quality parameters such as levels of postharvest chilling injury (Manzano et al. 2010, 2011, 2013, Martínez et al. 2013, 2014, Megías et al. 2014, 2015). We have evaluated the three ethylene insensitive mutants for flower and fruit development. The genetic background MUC-16 and ethylene-sensitive and -intermediate plants from segregating populations were all monoecious, developing both male and female flowers. However, ethylene-insensitive plants of the three mutant families were andromonoecious, and produced male and bisexual or hermaphrodite flowers (Figure 1). We called these mutants *andro1*, *andro2,* and *andro3*. The pistillate flowers of *andro1* and *andro3* usually developed immature stamens (Figure 1b,c), although when they were grown under high-temperature conditions, complete mature stamens were produced. However, under all the environmental conditions that were used during testing,

the pistillate flowers of *andro2* showed complete stamen development with mature pollen (Figure 1d).

Several monoecious cultivars of *Cucurbita pepo* show a partially andromonoecious phenotype when grown under high-temperature conditions (Martínez et al. 2014). In these cultivars, plants develop male and female flowers under standard conditions, but produce male and bisexual flowers when the greenhouse temperature exceeds 30 °C. The andromonoecious phenotypes of *andro1* and *andro3* were also temperature-sensitive; however, they produced no female flowers, only male and bisexual ones. Martínez et al. (2014) reported that the partial andromonoecious phenotype of these *C. pepo* cultivars is conferred by a mutation in the ethylene biosynthesis gene *CpACS27A*, the orthologue of *CmACS7* and *CsACS2* in melon and cucumber, respectively (Boualem et al. 2008, 2009, Li et al. 2009). These ethylene biosynthesis genes are all specifically expressed in the female flowers at very early stages of development, regulating the arrest of stamen development and sex determination in these species. We have sequenced 1,997 kb of the promoter and the complete coding region of the *CpACS27A* gene in *andro1*, *andro2,* and *andro3* mutant plants, but no mutation was detected in comparison with the same gene in the MUC-16 genetic background. Although we cannot exclude the possible downregulation of *CpACS27A* in the three andromonoecious mutants, the mutations resulting in *andro1*, *andro2,* and *andro3* occur neither in the *CpACS27A* coding sequence nor in the proximal promoter region of this ethylene biosynthesis gene. Moreover, although ethylene production was slightly reduced in the pistillate floral buds of *andro1*, no significant differences were detected between pistillate flowers of WT and *andro2* and *andro3* (data not shown).

The *andro1*, *andro2,* and *andro3* mutations also modified the coordinated development and maturation of floral organs in pistillate flowers. In squash monoecious cultivars, the female flowers take twice as long to reach anthesis as male flowers (Peñaranda et al. 2007). This so-called anthesis time was not altered in the male flowers of any of the mutants (Table 1), but the maturation of petals and anthesis time were very delayed in the pistillate flowers of the three mutants (Table 1). In fact, more than 50% of the studied mutant flowers had still not reached anthesis 30 days later than WT. At early stages of pistillate flower development, petals grew at the same rate in WT and mutant plants; however at later stages, the growth rates of mutant petals were reduced in comparison to those of WT ones (Table 1). Meanwhile, the growth rates of ovaries in the three mutant flowers were increased as opposed to those in the WT flowers (Table 1). These data suggest that ethylene biosynthesis and/or

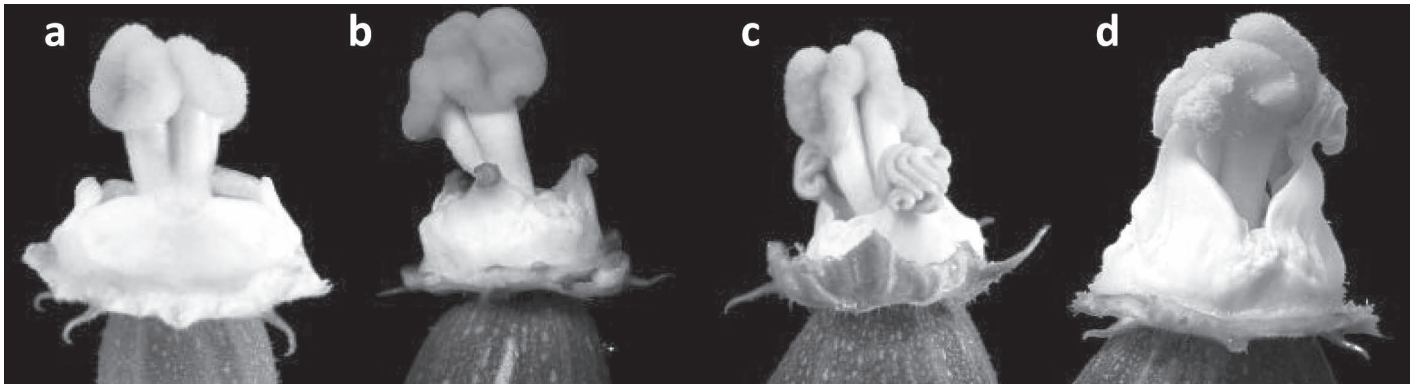

Figure 1. Andromonoecious phenotypes of *andro1*, *andro2*, and *andro3* mutants of *C. pepo*. (a) monoecious WT in MUC-16 and sensitive plants of segregating population; (b) *andro1*; (c) *andro3*; (d) *andro2*. The arrest of stamen development was altered in the three mutants, converting female into bisexual (b and c) or hermaphrodite flowers (d). Note that the degree of stamen development varied among the three identified ethylene-insensitive mutants.

Table 1. Comparison of the development and maturation of male and female floral organs in WT and andromonoecious mutants.

	Anthesis time (days)		Ovary length (mm)			Petal length (mm)	
	female flowers	male flowers	10 days	14 days (anthesis)	20 days	8 days	14 days (anthesis)
WT	13.7 a	29.2 a	35.8 a	64.8 a	114.9 a	18.4 a	82.4 a
andro1	>17.8 b	27.6 a	42.3 a	71.4 a	149.9 b	20.4 a	56.7 b
WT	13.9 a	28.5 a	39.3 a	69.8 b	109.1 a	17.1 a	80.6 a
andro2	>21.8 b	28.8 a	46.4 a	81.3 a	201.5 b	16.3 a	37.7 b
WT	13.2 a	28.4 a	40.6 a	66.6 a	123.6 a	19.2 a	96.6 a
andro3	>19.4 b	27.5 a	43.2 a	76.1 a	173.1 b	15.8 a	43.7 b

Flowers were labeled when they were 4 mm in length, and organ size measured every two days. Average of 15 flowers/fruits per genotype. Different letters indicate statistical differences between WT and mutants flowers ($P \leq 0.05$).

sensitivity is not only responsible for the arrest of stamen development in female flowers, but can also regulate ovary and petal development. If so, the higher ethylene production and/or sensitivity in female flowers could be responsible for the prevention of ovary development before anthesis (i.e., before pollination and fertilization) whilst promoting the development of petals; the female flowers, thereby**,** reach anthesis earlier than bisexual or male flowers. By comparing flowers of the different mutants, however, we observed that the growth rate of petals and ovaries was very dependent on stamen development. In fact, the faster the development of stamens (in *andro2*, for example), the slower the development of the petals, with an increase in ovarian growth. It is also likely, therefore, that the coordination of floral organ growth in hermaphrodite flowers is dependent on stamen development. In monoecious species, the lack of stamens in female flowers would allow the unisexual flower to acquire a new regulating mechanism; however, this mechanism itself could be masked by the presence of stamens in the *andro1*, *andro2,* and *andro3* mutants.

The ethylene-insensitive mutants andro1, andro2, *and* andro3 *are altered in sexual expression and vegetative development*

Cucurbita pepo is a monoecious crop with three flowering phases. In the first phase, the plant only produces male flowers. This is followed by a second phase where the plant produces male and female flowers, and a final phase in which only female flowers are produced (Peñaranda et al. 2007, Manzano et al. 2010, 2013). Since the transition to female flowering and the number of female flowers per plant are both controlled by ethylene (Manzano et al. 2010, 2013), we have studied the possibility of these two traits being altered in the andromonoecious mutants. The three *andro* mutations delayed pistillate flowering transition (Figure 2a), and reduced the number of female flowers per plant (Figure 2b). Therefore we concluded that these mutations not only affected sex determination in female flowers, inhibiting the arrest of stamen development and promoting the conversion of monoecy into andromonoecy, but also altered the production of male and female flowers along the main shoot of the plant.

To compare the vegetative development between WT plants and *andro1*, *andro2,* and *andro3*, plant height and also the number and size of internodes were determined in at least 10 plants of each genotype. The height of the three ethylene-insensitive mutants was greater than in WT plants (Table 2). In *andro1* and *andro2* mutants, the greater height was due to an increase in the number and average size of internodes (Table 2). However, the mutant *andro3* only showed a significant difference from WT plants in the size of internodes; not in their number (Table 2). These observed differences in sexual expression and vegetative development between WT and mutant plants indicate that andro-

Table 2. Comparison of vegetative development in WT and mutant plants.

WT / mutant	Plant height (cm)	Internode length (mm)	Node numbers
WT	70.3 b	12.3 b	41.1 b
andro1	82.3 a	16.7 a	46.5 a
WT	85.4 b	13.5 b	47.2 b
andro2	112.5 a	24.1 a	50.7 a
WT	93.9 b	14.8 b	47.1 a
andro3	113.3 a	28.8 a	48.5 a

Mean of 10 plants per genotype. Different letters indicate statistical differences between WT and insensitive plants ($P \leq 0.05$).

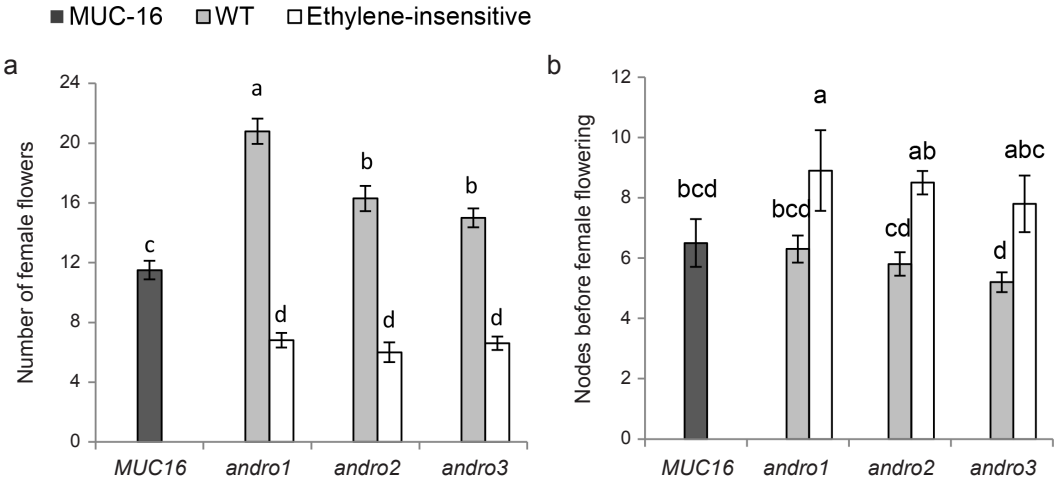

Figure 2. Comparison of pistillate flowering transition (a) and pistillate flower number per plant (b) in WT and andro1, andro2, and andro3 mutant plants. Different letters indicate statistical differences between genotypes ($P \leq 0.05$). Error bars show SE.

monoecious mutations must affect the genes regulating response to ethylene; this not only during female flower development and sex determination, but throughout the different phases of plant development.

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Genome-Wide Association Study of Powdery Mildew Resistance in a Worldwide Collection of Melon (*Cucumis melo* L.) Germplasm

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Abstract. Melon (*Cucumis melo* L.) is one of the most important vegetable crops worldwide. Identification of the genes conferring resistance to powdery mildew at the whole genome level could provide an efficient tool for further melon germplasm improvement and insights into the molecular mechanisms of resistance. To facilitate gene identification and marker-assisted selection (MAS) breeding in melon, 304 accessions were tested. The performance of 13 melon powdery mildew race differential accessions to *Podosphaera xanthii (Px)* was surveyed. According to differential reactions of 13 melon powdery mildew race international differential lines, the strain was identified as race 2F of *Px*. Single nucleotide polymorphisms (SNPs), 12,938 in number, were characterized by the technique of type IIB endonucleases restriction-site associated DNA (2b-RAD). Population structure analysis showed that K-3 was the most appropriate cluster for this population and was used as fixed effects in genome-wide association study (GWAS) of powdery mildew resistance. 12 GWAS signals were detected for powdery mildew resistance traits, 7 of them have been reported in previous research and another 5 loci were novels that need to be further validated. This study provides resources for genomics-enabled improvements in melon breeding for powdery mildew resistance trait.

Keywords: Melon, powdery mildew resistance, GWAS

Introduction

Melon is popular worldwide and is high in carotene and sugar. Melon is of major economic importance and is widely cultivated. The data for 2013 indicated that 29.3 million tonnes of melon were produced on 1.2 million hectares around the world (http://faostat3.fao.org).

Powdery mildew caused by *Podosphaera xanthii* (*Px*) or *Golovinomyces cichoracearum* (*Gc*) is, worldwide, a devastating disease of melon crops. Powdery mildew can decimate the foliage of melon plants, thereby decreasing yield and especially the quality of the fruits. *Px* occurs more frequently in subtropical and tropical areas, while *Gc* is common in temperate and cooler areas. More than 28 physiological races of *Px* have been identified according to their reactions on different melon lines (McCreight 2006). In China, the major pathogen of melon powdery mildew is *Px* (Cheng et al. 2011).

Chemical treatments are commonly used to prevent or slow the spread of powdery mildew. However, long-term use of fungicides has led the pathogens to develop resistance against them. Over time, many chemical fungicides have gradually lost their effectiveness in combatting powdery mildew. Also, most of these chemical fungicides are environmentally unfriendly. The growing issue of the environment and public health has promoted melon breeders to seek other strategies to control powdery mildew. So there is an urgent need for highly efficient, low cost, and eco-compatible approaches against this predominant pathogen. The development of new resistant cultivars offers a promising way to control powdery mildew.

The traditional breeding approach of phenotypic selection is laborious, time-consuming, and not mistake-proof. Marker-assisted selection (MAS) promises a more efficient and rapid selection method of desired phenotypes, which could save much effort, time, and expenses of field work, and eliminate the need for pathogen inoculum. Identification of the target genes and development of molecular markers tightly linked with them are a prerequisite for efficient MAS. However, many of the genes for resistance to powdery mildew carried by approximately 30 resistant cultivars still have not been located and annotated (Zhang et al. 2012, Ning et al. 2013). Recently, a genome-wide association study (GWAS) has been employed to search for more loci conditioning desirable agronomic traits in worldwide collections of rice (Huang et al. 2012) and soybeans (Zhou et al. 2015). Molecular markers more tightly linked with the desired agronomic traits have been developed for MAS. To date, although

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there is only one GWAS on sugar accumulation and climacteric behavior in a broad collection of 175 melon accessions (Leida et al. 2015), GWAS appears to be a powerful tool that could detect regions of the genome containing genes for other desired horticultural traits in melon.

Presently, we applied GWAS to search for molecular markers of genes conferring powdery mildew resistance in melon. A germplasm collection comprised of 304 melon accessions derived from widely different geographical areas was employed. This collection included 13 accessions that are used internationally to differentiate among races of powdery mildew (McCreight 2006). SNP genotype data was obtained by the sequence method of the type IIB endonucleases restriction-site associated DNA (2b-RAD) (Wang et al. 2012). The races of *Px* were surveyed and the population structure of the melon germplasm was analyzed. GWAS was then performed between powdery mildew resistance and SNP genotype.

Materials & Methods

Plant material

The 304 melon accessions included two wild melons (*Cucumis melo* ssp. *agrestis*), 47 landraces, and 255 improved cultivars. Geographic sources of this germplasm included China, U.S.A., Canada, South Korea, France, India, Iran, and Zimbabwe.

DNA sample preparation and sequencing

Melon plants were grown in a plastic greenhouse at the Xiangyang Farm of Northeast Agricultural University, Harbin, China. Young leaves were collected from 2 to 3 week-old plants representing each accession and stored at -80 °C for later DNA isolation. Total DNA was extracted with the cetyl trimethyl ammonium bromide (CTAB) method (Murray et al. 1980). At least 100 ng of genomic DNA from each accession was used to construct a sequencing library following the manufacturer's instructions of the 2b-RAD genotyping method. Single-read sequencing libraries with a length of 50 bp were sequenced on an Illumina Hiseq 2500 sequencer at Berry Genomics (Beijing, China).

Variation calling and annotation

Single-read resequencing reads were mapped to the melon DHL92 reference genome (Garcia-Mas et al. 2012) with BWA (version 0.6.1-r104) using the default parameters. SAMtools (version 0.1.18) (Li et al. 2009) software was used to convert mapping results into the BAM format and to filter the unmapped, non-unique reads and duplicated reads.

SNP calling

SNP detection was performed by using the VarScan (version 2.3.9) (Koboldt et al. 2012). The detailed procedure of SNP identification was followed as described previously (Koboldt et al. 2013). Genotypes of the 304 melon accessions were called at the SNP sites. For the genotype datasets of all the accessions, SNPs with more than 80% missing data and SNPs with minor allele frequency (MAF) < 5% were excluded.

Phenotyping

For phenotyping, the 304 accessions were germinated and planted at the Xiangyang Farm from May 2015 to October 2015. The melon plants were infected with powdery mildew naturally in the field, without artificial inoculation. According to differential reactions of the 13 race-differentiating accessions (McCreight 2006), the strain was identified as race 2F of *Px* (Table 1). Plants without visible sporulation on the leaf were considered to be resistant, while plants with typical mildew colonization were considered to be susceptible.

Genome-wide association study (GWAS)

To minimize false positives and increase statistical power, population structure and cryptic relationships were considered. A mixed linear model (MLM) program, TASSEL (version 5.2.19) (Bradbury et al. 2007) was used for the association analysis. Population structure was analyzed by STRUCTURE (version 2.3.4) (Pritchard et al. 2000) and Structure Harvester (http://taylor0.biology.ucla.edu/structureHarvester) was used for fixed effects in the mixed model to correct for stratification. The random effect was estimated from the groups clustered based on the kinship among all accessions. Kinship was derived from all SNPs and analyzed by GAPIT (version 2) (Lipka et al. 2012). We defined the whole-genome significance, cut off as the Bonferroni test threshold. For SNP GWAS, the threshold was set as 0.01/total SNPs ($\log_{10}$(P) = -6.11). For further interpretation and validation of the GWAS signals associated with powdery mildew resistance, we performed comparative genomics analysis to annotate the GWAS signals by Blastn between cucumber (*Cucumis sativus* L.) and melon, due to the highly conserved gene contents and collinearity orders between these two cucurbits (Zhang et al. 2012).

Results & Discussion

2b-RAD sequencing of the 304 melon accessions by an Illumina HiSeq 2500 sequencer generated 33 billion single reads of 50 bp in length. After mapping against the melon DHL92 reference genome, 12,938 SNPs were identified. The population structure of the 304 melon accessions was analyzed (Figure 1A). Use of Structure Harvester provided mean LnP (K) and Delta K

Table 1. Reaction of melon powdery-mildew race-differential accessions to natural infection by *Podosphaera xanthii* in the field.

Race differential line	Reaction
Iran H	Susceptible
Topmark	Susceptible
Védrantais	Susceptible
Edisto 47	Resistant
MR1	Resistant
WMR 29	Resistant
PMR 45	Susceptible
PMR 5	Resistant
PMR 6	Resistant
PI 414723	Resistant
PI 124111	Resistant
PI 124112	Resistant
Nantais	Susceptible

Table 2. A subset of associated loci and candidate locus in melon and cucumber according to the GWAS and comparative genomics analysis.

Chromosome	Marker	Position	Candidate locus in melon	Candidate locus in cucumber
2	S2_760848	760848	*QTL1*, *Pm-2F*, *Pm-X1*, *Pm-X5*, *Pm-X3*	
2	S2_23109135	23109135		
2	S2_23126537	23126537		
4	S4_26768087	26768087		*Pm3.1*, *Pm5.1*
4	S4_26779834	26779834		*Pm3.1*, *Pm5.1*
4	S4_32260261	32260261		*Pm3.1*, *Pm5.1*
7	S7_983345	983345		
8	S8_16632671	16632671		
8	S8_31402544	31402544		
10	S10_550732	550732		*Pm-h*, *Pm5.2*, *Pm-R*, *Pm5.3*
12	S12_22946137	22946137	*QTL2*	*Pm1.1*
12	S12_22946146	22946146	*QTL2*	*Pm1.1*

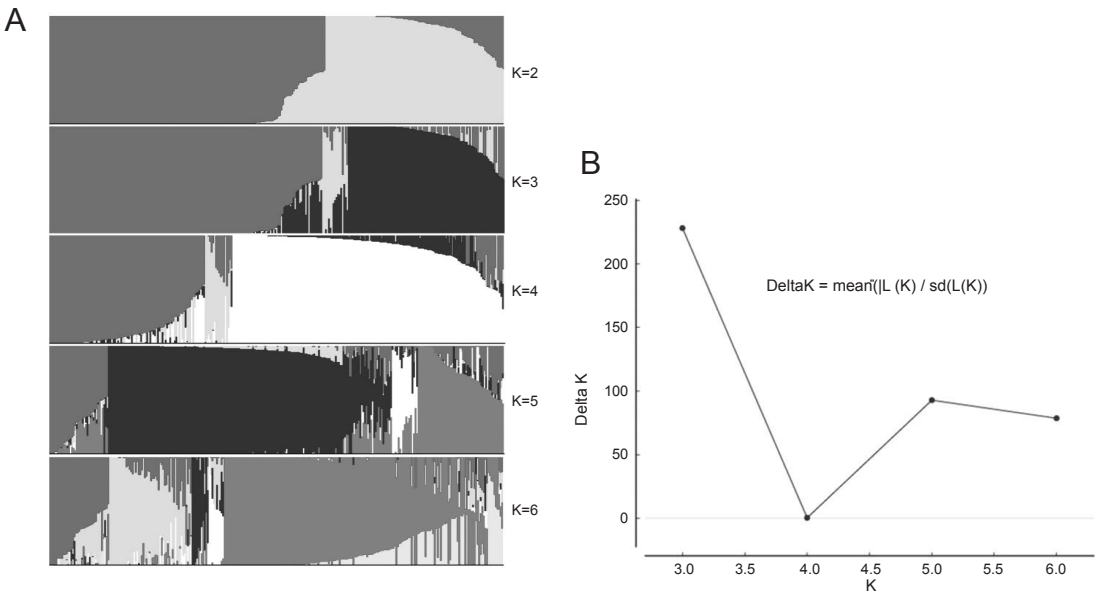

Figure 1. Population structure analysis of a collection of melon germplasm. (A) Population structure analyzed using STRUCTURE with K ranges of 2 to 6. (B) K3 had the highest peak (based on Delta K distribution) indicating that three clusters sufficiently defined the population structure of the melon collection.

values (Figure 1B). K-3 was the most appropriate cluster for this population, with the highest Delta K value of 228 as compared with the other clusters. In the summer of 2015, we investigated the reaction of these melon accessions infected by powdery mildew and also performed GWAS for powdery mildew resistance (Figure 2). Twelve GWAS signals associated with powdery mildew resistance were detected at the previously reported *QTL1* (Fukino et al. 2008, Ning et al. 2013), *Pm-2F* (Zhang et al. 2012), *Pm-x1.5*, *Pm-x3* (Fazza et al. 2013) locus on chromosome 1 and the *QTL2* (Fukino et al. 2008) locus on chromosome 12 (Table 2). In addition, the *Pm3.1* (He et al. 2013) and *Pm5.1* (Liu et al. 2008) loci that related to powdery mildew resistance in cucumber, on chromosome 4 of the melon genome, were identified by comparative genomics analysis between melon and cucumber. Also, the *Pm-h*, *Pm5.2*, *Pm-R*, and *Pm5.3* loci on chromosome 10 (Liu et al. 2008, De Ruiter et al. 2008, Zhang et al. 2011, He et al. 2013) and the *Pm1.1* locus on chromsome 12 (Liu et al. 2008, He et al. 2013) were identified in this way. Moreover, another five new loci related to powdery mildew resistance in melon were identified and found to be distributed on chromsomes 2, 7, and 8.

For melon, practically no GWAS of powdery mildew resistance has been reported. This is the first time, to our knowledge, that a more complete picture of the locations of the resistance loci in the genome has been presented. A more in-depth approach is needed to better understand the molecular mode of action of the genes conferring powdery mildew resistance and also to develop more efficient MAS markers to assist in melon breeding.

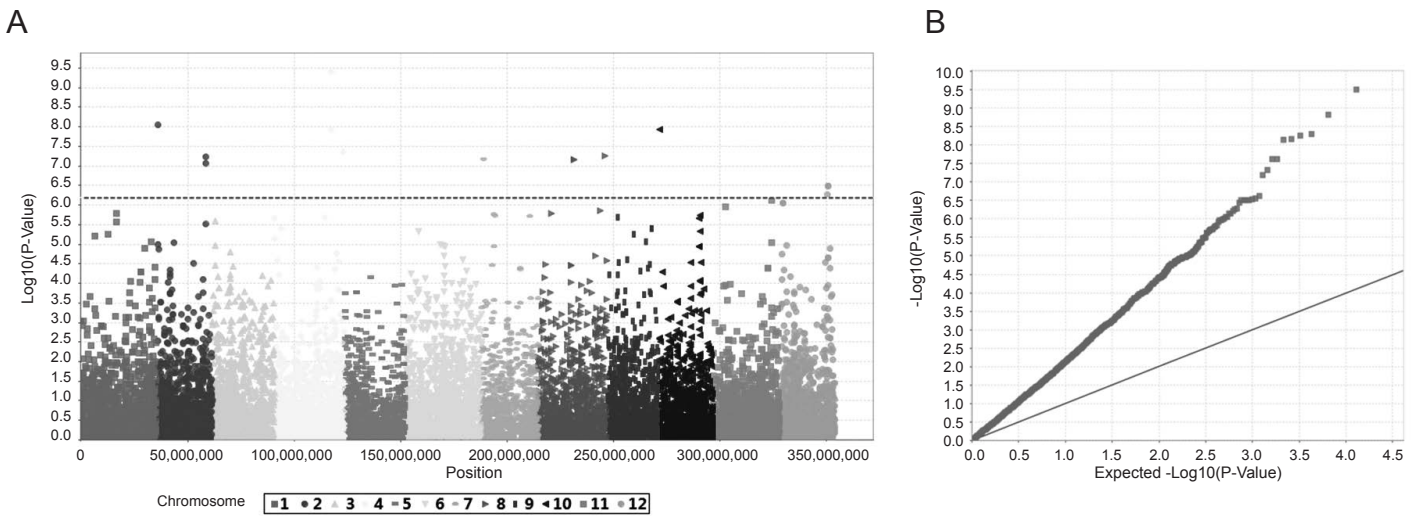

Figure 2. Genome-wide association study of powdery mildew resistance in a collection of melon germplasm using MLM. (A) Manhattan plots for powdery mildew resistance in the melon population. The $-\log_{10}P$ values from a genome-wide scan are plotted against the position on each of the 12 chromosomes. The horizontal dashed grey line indicates the genome-wide significance threshold ($P = 7.73 \times 10^{-7}$). (B) Quantile-quantile plot for powdery mildew resistance in the melon population. The horizontal axis shows $-\log_{10}$ transformed expected P values, and the vertical axis indicates $-\log_{10}$ transformed observed P values.

Acknowledgements

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cmv1 Encodes a Vacuolar Protein Sorting 41 Involved in Transport of *Cucumber Mosaic Virus* in Melon

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Abstract. Infections by *Cucumber mosaic virus* (CMV), the type member of the *Cucumovirus* genus, can cause complete harvest loss in more than 1000 plant species, including important crop plants. CMV is classified into two subgroups (I and II) sharing a 70% of nucleotide sequence. Deployment of naturally resistant cultivars is a successful control strategy against viral infections. In the Korean melon accession PI 161375, 'Songwhan Charmi' (SC), the resistance to CMV is mediated by a complex mixture of qualitative and quantitative genes. One single gene, *cmv1*, confers by itself complete recessive resistance to strains of subgroup II by preventing viral transport, from the bundle sheath cells that surround the vein, to the phloem. To confer resistance to the subgroup I strains, at least two additional QTLs must act together and cooperatively with *cmv1*. Here, we report the fine mapping and cloning of *cmv1*. We have screened a recombinant population of more than 3000 F_2 plants and narrowed the region carrying *cmv1* to 132Kb including a *Vacuolar Protein Sorting* (*CmVPS41*) gene. This gene is conserved among plants, animals, and yeast, and is required for post Golgi vacuolar trafficking in the cell. We have validated the *CmVPS41* as *cmv1* by generating susceptible SC transgenic lines, expressing the *CmVPS41* allele from the susceptible genotype PS. We hypothesize that the virus would recruit some CmVPS41 activity in the bundle sheath cell to be transported to the plasmodesmata and invade the phloem cells to develop a systemic infection.

Keywords: *Cucumber mosaic virus*, *cmv1*, melon, recessive resistance, vesicle trafficking

Introduction

Cucumber mosaic virus (CMV) affects more than 1200 plant species, including important crop plants in the Solanaceae, Cruciferae, and Cucurbitaceae, in which they cause complete harvest loss and severe damage worldwide (Edwardson and Christie 1991). Resistances to CMV are mainly recessive and oligogenic. In melon, *Cucumis melo* L., among the few resistances reported, one of them is present in the Korean accession PI 161375, 'Songwhan Charmi' (SC), one of the parental lines of our populations. It shows a recessive, oligogenic (Karchi et al. 1975), and also quantitative resistance (Dogimont et al. 2000). We studied this trait and found a major QTL in linkage group XII (LGXII), *cmv1/cmqw12.1*, able to provide full resistance to CMV strains of subgroup II, like CMV-LS (Essafi et al. 2009, Guiu-Aragonés et al. 2015), and at least two other QTLs, *cmqw3.1* and *cmqw10.1*, that must act together with *cmv1/cmqw12.1* to provide resistance to strains of subgroup I, like CMV-FNY (Guiu-Aragonés et al. 2014). Characterization of the resistance mediated by the gene *cmv1* showed that it involves a restriction of viral transport from the bundle sheath cells (BS) to phloem cells (Guiu-Aragonés et al. 2016) and that the determinant of virulence is the Movement Protein (MP) of the virus (Guiu-Aragonés et al. 2015). We have addressed the map-based cloning of *cmv1* from an F_2 population between the Near Isogenic Line SC 12-1, which is resistant to CMV-LS, and the Spanish 'Piel de Sapo'. After screening more than 3000 F_2 individuals, we reached an interval with three genes. Among them, we have cloned and validated a Vacuolar Protein Sorting 41 (CmVPS41), a gene involved in vesicle trafficking from the late Golgi to the vacuole, as *cmv1*.

Materials & Methods

Plant and virus material and inoculations

The Spanish Piel de Sapo-type 'T111' (PS) and the Korean accession PI 161375, 'Songwhan Charmi' (SC), were used as susceptible and resistant controls for inoculations. For fine mapping of *cmv1*, an F_2 population from the cross between the susceptible accession PS and the NIL SC12-1 was prepared. NIL SC12-1 carries a single introgression from SC within chromosome XII containing the *cmv1* gene (Essafi et al. 2009). F_2 recombinant plants were selected using molecular markers flanking the *cmv1* interval and self-pollinated. F_3 plants were inoculated for phenotyping. Virus strains used in this study were CMV-LS, belong-

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ing to subgroup II, and CMV-FNY, belonging to subgroup I. For the inoculation experiments, seeds were pre-germinated and the plants were grown as previously indicated (Guiu-Aragonés et al. 2014). Viral inocula were freshly prepared from infected zucchini squash, *Cucurbita pepo* L., and inoculated into melon plants as previously reported (Essafi et al. 2009).

Development of molecular markers and fine mapping of cmv1

Molecular markers were developed from polymorphisms previously detected by re-sequencing PS and SC lines (Garcia-Mas et al. 2012). The polymorphisms were selected based on their physical position in the melon genome (sequence available from http://melonomics.net). For positional cloning of the *cmv1* gene, recombinants inside successive small intervals were selected in the F_2 population. DNA was extracted using the CTAB method (Doyle and Doyle 1990). Plants were genotyped for the two flanking markers of each *cmv1* interval and recombinant plants were selected and self-pollinated. F_2 plants were genotyped with a set of markers inside the interval to determine the exact point of recombination. To locate the *cmv1* gene, 20 F_3 plants from each breakpoint were inoculated with CMV-LS as described above and symptoms were visually scored. A recombinant was considered resistant only when none of the F_3 plants showed symptoms. This procedure was repeated for successive smaller intervals, until less than 0.1% of the F_2 plants screened were recombinant.

Cloning of the cmv1 *candidate from PS.*

The complete coding sequence of the *cmv1* candidate gene from PS was cloned using the GATEWAY system (Gateway®-Gene Cloning, Life Technologies, U.S.A.). Briefly, total RNA was extracted from 100 mg of young leaves using TRI Reagent (Sigma–Aldrich, Saint Louis, U.S.A.) following the manufacturer's instructions. cDNA was synthesized with SuperScript III Reverse Transcriptase Kit (Invitrogen, U.S.A.) and oligo(dT)20 as reverse primer. Complete *cmv1* coding sequence was PCR amplified using primers AC**GTCGAC**TATGGCTCCCATTCTATCGGTA (incorporating *Sal*I restriction enzyme site, in bold) and TC**GATATC**AGTCTTGGAAGCAGCAG (incorporating *Eco*RV restriction enzyme site, in bold), and ligated between *Sal*I and *Eco*RV in pENTR3C (Earley et al. 2006) generating the pENTR CmVPS41 PS construct.

Validation of cmv1 *candidate gene*

From the pENTR CmVPS41 PS, the gene was transferred to a pEARLY expression binary vector under its own promoter. First, *cmv1* PS putative UTR and promoter were amplified by PCR using primers GTCGACGTTCACTGAGACATTCG and AATCGGCCGTTCATCACAGTCGAC and cloned in the *Sal*I site of pENTR CmVPS41 PS, upstream of the starting codon. Then, pENTR CmVSP41 PS carrying its own promoter was recombined using the LR clonase mix enzyme II (Gateway®Gene Cloning, Life Technologies, U.S.A.), into the pEARLY 301 expression vector (Earley et al. 2006) obtaining the pEXPR 301 CmVPS41 PS. Transgenic lines were developed by introducing this construct into the resistant parental accession SC following the protocol previously described (Castellblanque et al. 2008). The expression of the *cmv1* PS allele in leaves of T_0 transgenic plants was confirmed by reverse transcription-PCR (RT-PCR) using primers designed for amplification of exon 5 followed by digestion with the *Bst*EI restriction enzyme, that allows detection of the PS allele mRNA (non-digested) as different from the host SC allele (digested).

The response to the CMV LS and FNY strains was tested in T_0 plants expressing the *cmv1* PS allele. Six newly rooted *in vitro* clones from each of the T_0-SC independent lines, PS and SC were transferred to soil in pots. Plants were kept in growth chambers in long-day conditions (22 °C for 16 h with 5000 lx of light and 18 °C for 8 h of dark) during one week. Then, two newly formed leaves were inoculated with CMV, as described before. Symptoms were scored visually at 14, 21, and 28 days post inoculation (dpi). Plants were considered susceptible when symptoms appeared in non-inoculated new leaves. Viral detection was done by RT-PCR from non-inoculated new leaves as described (Guiu-Aragonés et al. 2015). For CMV-FNY, specific primers F109-3'R (TGGTCTCCTTTTAGAGACCC) and F109-2200F (CGGGACCATTAGTCAAGTTG) were used to amplify a 1147 bp fragment. For CMV-LS, specific primers LS1-1400R (GAAGCATTCCACATATCGTAC) and LS1-1F (GTTTTATTTACAAGAGCGTACG) were used to amplify a 1400 bp fragment.

Results & Discussion

Fine mapping of the cmv1 *interval*

Fine mapping of *cmv1* was addressed using an F_2 population from a cross between PS and the NIL SC12.1, which carries *cmv1* (Essafi et al. 2009). Screening of 3000 F_2 individuals with flanking markers CMN61_44 and CMN21_55 (Essafi et al. 2009) identified 58 recombinant individuals. Genotyping with internal markers identified eight different recombination points, and inoculation of F_3 plants representing these points mapped *cmv1* within a 1.37 Mb interval flanked by markers Sca02308.2 and Sca07080.7. Successive addition of internal markers and phenotyping of plants representing the different recombination points finally reduced the *cmv1* interval to 132 Kb flanked by markers Sca4_345 and Sca_358. According to available *in silico* annotations of the melon genome, the *cmv1* interval includes three genes: a possible Lysosomal Pro-X carboxypeptidase (PCP), a Crooked neck-like protein 1 (CNL) and a Vacuolar protein sorting-associated protein 41 (VPS41). Analysis of the polymorphisms between both parental lines PS and SC in the *cmv1* interval (Sanseverino et al. 2015) revealed five non-synonymous polymorphisms, one in the PCP, one in CNL and three in the VPS41. Since *cmv1* is able to block the movement of the virus from the BS cells to the phloem cells in the resistant plant, our main candidate gene was the VPS41 (Figure 1), since in other organisms it is involved in the transport of vesicles and cargo proteins towards the vacuole (Asensio et al. 2013) and this function could be recruited by the virus for its own transport.

cmv1 *encodes a Vacuolar Protein Sorting 41*

Given that *cmv1*, the SC allele, is sufficient to confer resistance to LS, but also necessary for resistance to FNY (Guiu-Aragonés et al. 2014, Guiu-Aragonés et al. 2016), expression of the CmVPS41 from PS should allow both CMV-LS and CMV-FNY to spread in transgenic SC plants. Thus, we transformed the resistant parental line SC with a construct carrying the susceptible allele of CmVPS41 (from PS) under its own promoter to generate three independent lines expressing the gene. The three transgenic

lines were tetraploid and it was not possible to obtain diploid plants via self-pollination. Therefore, we tested their susceptibility to CMV in the T_0 plants. *In vitro* T_0 clones from the three independent transgenic lines were newly rooted, acclimatized, and then inoculated with either CMV-LS or CMV-FNY. CMV-LS was detected in systemic leaves only in one plant. However, CMV-FNY was able to infect all transgenic lines and was also detected by RT-PCR (Figure 2). Control untransformed and *in vitro* regenerated PS plants were also infected, whereas control untransformed and *in vitro* regenerated SC plants remained uninfected. Thus, our results demonstrate that expression of the susceptible CmVPS41 allele in the resistant line allows CMV systemic infection and, therefore, CmVPS41 is indeed encoded by *CMV1*.

We have identified CmVPS41, a protein involved in membrane trafficking to the vacuole as part of the "Homotypic Fusion and Vacuole Protein Sorting" (HOPS) complex, as the product of the resistance gene *cmv1*. Our previous results have determined that *CMV1/cmv1* is a checkpoint for CMV phloem entry, acting only in the BS cells. The susceptible allele *CMV1* would permit virus transport from BS cells to vascular parenchyma (VP) cells or intermediary cells (ICs) to develop a systemic infection, whereas the resistant allele *cmv1* would prevent this movement (Guiu-Aragonés et al. 2016). For this function, the protein *CMV1* would interact with the viral Movement Protein (MP), the viral factor determining the infection (Guiu-Aragonés et al. 2015). Therefore, CMV could somehow be recruiting part of CmVPS41 activity to mediate its own transport towards the PDs or, alternatively, it might well be needed already in the PDs to gate them and allow CMV movement to the phloem. In the resistant plant, this putative interaction would not happen and the resistant allele of CmVPS41 would not carry out this function. However, the main activity of the CmVPS41 resistant allele in vesicle trafficking would remain unchanged, since the resistant plant shows no phenotype associated to lack of function of CmVPS41, a gene essential for cell viability. VPSs and other components of the

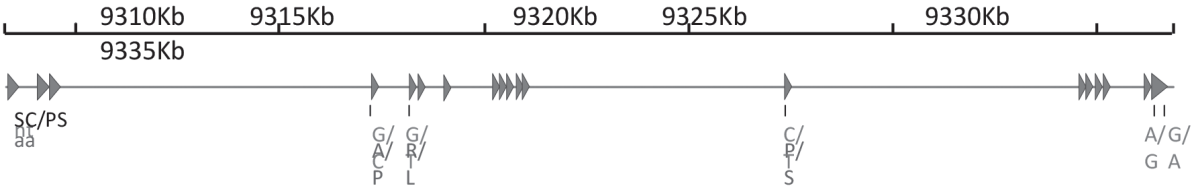

Figure 1. VPS41 structure based on the complete sequence of the CDS. Numbers are given according to annotation on the melon genome. SNPs between PS and SC are detailed below. The arrows indicate exons.

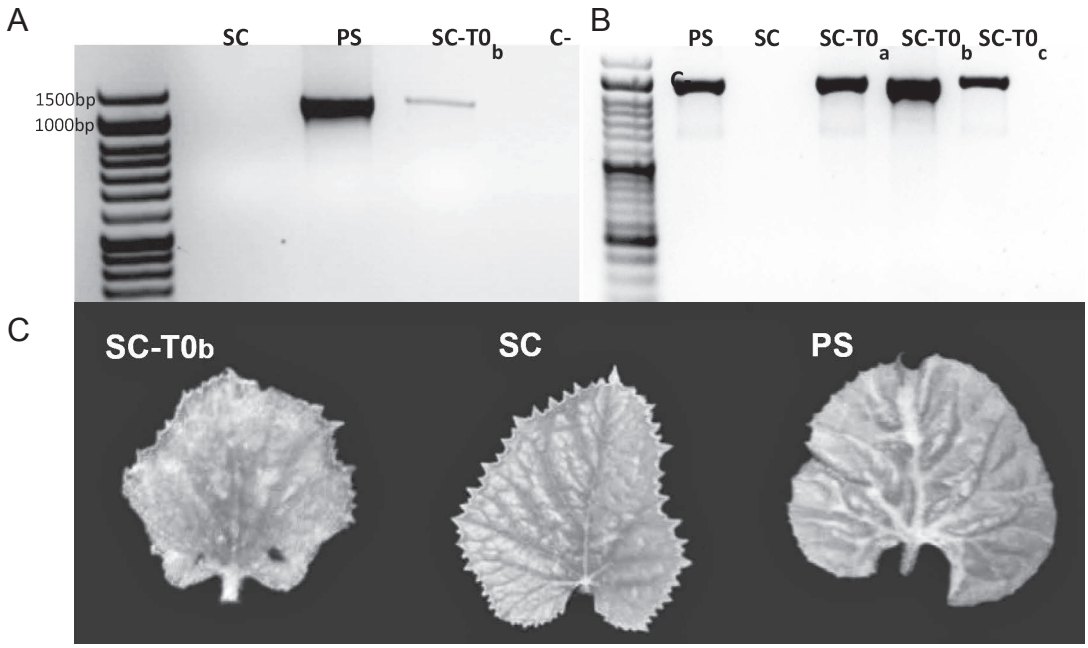

Figure 2. CMV infection of T_0 transgenic plants expressing *CmVPS41* allele from PS. Representative results 21 days after the inoculation with either CMV-LS or CMV-FNY (A) RT-PCR detection of CMV-LS-infected plants. (B) RT-PCR detection of CMV-FNY. (C) Detail of upper leaves from in vitro acclimatized T_0 transgenic plans inoculated with CMV-FNY. PS: non-transgenic, in vitro regenerated susceptible line; SC: non-transgenic, in vitro regenerated resistant line; SC-T_0 transgenic SC lines (a, b, and c) expressing *CmVPS41* from PS under its own promoter; C-: RT-PCR negative control without RNA.

vesicle trafficking machinery have been implicated in virus life cycle, at the replication level (Amano et al. 2013, Barajas et al. 2014). However, no other VPS has been demonstrated to act at the viral transport level.

Recessive genes for resistance cloned to date are mainly eukaryotic translation initiation factors (eIFs) involved in viral replication. Only a few examples involve genes other than eIFs, although they also restrict virus multiplication, like *rwm1* in *Arabidopsis* (Ouibrahim et al. 2014), *rim1-1* in rice (Yoshii et al. 2009), and *rym11* in barley (Yang et al. 2014). *cmv1* is the first natural recessive resistance gene whose function has been located in a particular cell type and is involved in the transport of the virus (Guiu-Aragonés et al. 2015). Identification and cloning of *cmv1* provides insight for a new mechanism of virus resistance based on the virus movement reotriotion and facilitates the designing of new strategies for breeding resistance into elite cultivars.

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Genome Sequence of the Bottle Gourd [*Lagenaria siceraria* (Mol.) Standl.] and Applications to the Study of Hetero-Graft-Conferred Cold Tolerance

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Abstract. Bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] is an edible, medical, container, and grafting stock plant cultivated all over the tropics and sub-tropics. A genetically anchored genome assembly of bottle gourd was established in the current study. The assembled genome is 335 Mb in size, consistent with the size of ~ 330 Mb estimated by flow cytometric analysis. A total of 216.6 Mb (65%) of the genome was annotated as repetitive sequences and 25,502 protein-coding genes were predicted from the coding regions. Macrocollinearity was revealed between bottle gourd and each of the other three sequenced cucurbit genomes: cucumber, watermelon, and melon. The genome sequence of bottle gourd then served as a critical tool to underpin the mechanisms of hetero-grafting-conferred cold tolerance between bottle gourd and watermelon. A bioinformatical pipeline was established to discriminate local transcripts from the transmitted ones, which was on the basis of sequence dissimilarities between species. RNA-Seq analyses facilitated by this analytical pipeline led to the identification of a set of graft-transmitted transcripts in both the scion and rootstock tissues. More intriguingly, not a few transmitted transcripts were found to be related to the biological pathways of cold tolerance, suggesting that the mechanisms of hetero-graft-conferred cold tolerance involve direct movement of effector RNA molecules.

Keywords: Bottle gourd, genome sequencing, hetero-grafting, mobile mRNA

Introduction

Bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] ($2n = 2x = 22$) is an ancient domesticated crop of the Cucurbitaceae. Considered to have originated in Africa, bottle gourd has been cultivated by man for over 100 centuries (Whitaker 1971, Erickson et al. 2005). Unlike its cucurbit relatives cucumber (*Cucumis sativus* L.), melon (*C. melo* L.), and watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) that are consumed predominantly as a vegetable or dessert, bottle gourd is highly versatile in usage. Besides its young fresh fruits being widely consumed as a vegetable, in many areas of China and Africa mature dried bottle gourd rinds are used as crafts, containers, or instruments (Xu et al. 2014). In India, bottle gourd is an important herb with effects on treating baldness and diseases. In modern horticultural practices, bottle gourd is widely used as a rootstock for grafting watermelon and cucumber, conferring the grafted plants increased resistance to biotic and abiotic stresses such as Fusarium wilt, root nematode, low temperatures, high salinity, and drought (Wang et al. 2016).

In this study, we sequenced and annotated the entire genome of bottle gourd. Subsequently, a bioinformatic pipeline was developed to identify mobile mRNAs between the scion and the rootstock in a bottle gourd-watermelon hetero-grafting system, based on sequence dissimilarities. We detected not a few mobile mRNAs and found that some of them may play a role in conferring enhanced cold tolerance.

Materials & Methods

Plant materials

Genomic DNA was extracted from leaves of the inbred bottle gourd line 'Hangzhou Gourd'. For grafting, the bottle gourd 'Y1' and the watermelon '8424' were used.

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Methods

Three paired-end DNA libraries with an insert size of 200 bp, 500 bp and 800 bp, respectively, and four mate-pair DNA libraries with an insert size of 2 kb, 5 kb, 10 kb, and 20 kb, respectively, were constructed. Purified DNA was amplified by PCR, cloned to vector to yield libraries for Illumina Hiseq 2000 sequencing.

To predict gene models in the genome, an integrated predictive pipeline combining *de novo* predictions and homology-based prediction was employed, followed by RNA-Seq verification. A high density genetic map constructed in a previous study (Xu et al. 2014) was used to facilitate anchoring and orienting of the scaffolds and contigs. To assess the completeness and accuracy of the assembled genome, conserved core eukaryotic genes and RNA-Seq data were analyzed (Wu et al. 2013).

For grafting, seeds of each variety were sown in plastic pots. The grafting was made at the two true-leaf stage using a slit-grafting method (Cushman 2006). Leaves of the scion and the rootstock parts were harvested.

Results & Discussion

A genetically anchored reference genome for bottle gourd

We obtained 115.2 Gb of raw WGS sequencing data from sequencing three short-insert (200 bp to 800 bp) paired-end DNA libraries and four large-insert (2 kb to 20 Kb) mate-pair libraries. By analyzing k-mer statistics (k = 17), we estimated the haploid genome size of HZ to be 335 Mb. The genome coverage of the high-quality data in this study was ~ 314.6x (105.4 Gb/335 Mb).

A total of 7,430 scaffolds (N50 = 1,534,212 bp) and 53,603 contigs (N50 = 13,299 bp) were assembled from the clean data, sizing 327.4 Mb (311.3 Mb after subtracting N calls) in total and taking up 97.7% of the genome (327.4 Mb/335 Mb). Approximately 99.8% (326.8 Mb/327.4 Mb) of the assembled genome was represented by the 695 longest scaffolds (> 1 Kb). By anchoring scaffolds/contigs onto a previously constructed genetic map (Xu et al. 2014), we successfully assembled the genomic sequence into 11 psuedo-chromosomes. These psuedo-chromosomes taken together are 295.5 Mb in length and represent 90.3% (295.5 Mb/327.4 Mb) of the assembled genome. We then further evaluated the completeness of the assembled genome by analyzing the discovery rate of conserved core eukaryotic genes (CEGs). Out of the 248 CEGs tested, 239 were uncovered in our genome assembly, suggesting a high coverage (96.37%) of the genome.

Genome annotations and cross-species synteny

We identified 216.6 Mb of repeated sequences (65% of the assembled genome) from the HZ genome. *In silico* prediction led to 27,500 putative gene models. After removing gene models that have no functional annotations and detectable transcripts from RNA-Seq data, a final set of 25,502 gene models with high confidence were retained. Among all gene models, 23,310 (91.4% of the total) were allocated onto the 11 pseudo-chromosomes, and 23,638 (92.7%) were assigned with a putative function. In addition, 141 microRNAs (miRNAs), 982 transfer RNAs (tRNAs), 99 ribosomal RNAs (rRNAs), and 266 small nuclear RNAs (snRNA) were identified from the bottle gourd genome. Through comparing the currently available cucurbit genome sequences, we were able to construct a phylogenetic relationship among the four species and a high resolution synteny map (Figure 1).

Mobilomes in the hetero-grafts under cold stressed and non-stressed conditions

A hetero-graft system, which employed bottle gourd as the rootstock and watermelon as the scion, was used to identify the global set of transcripts (mobilome) that moved across the graft junctions (Notaguchi et al. 2015). By comparing the mobilomes between cold-stressed and non-stressed grafts, we found that the abundances of most of the mobile RNAs, both shoot-ward and root-ward, were decreased in cold-treated hetero-grafts, suggesting a general inhibition of long-distance RNA movement by low temperature in this system. Neverthe-

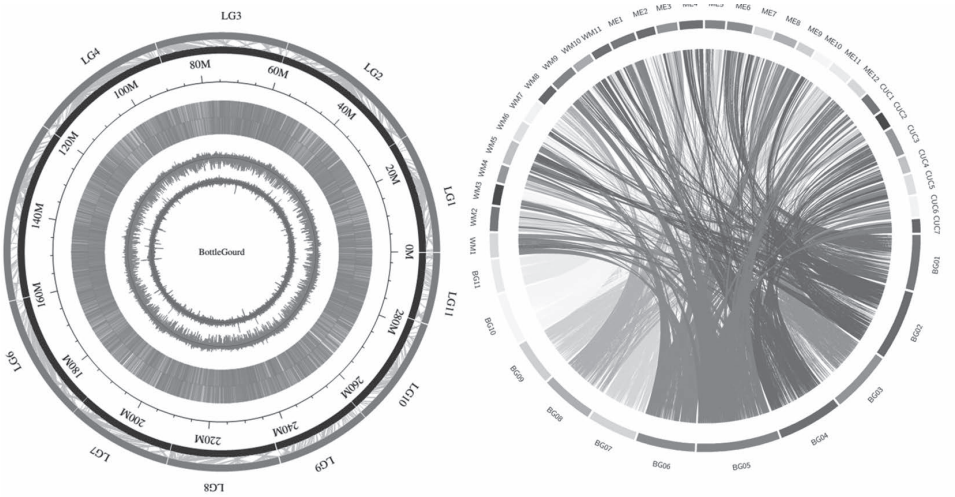

Figure 1. Circos illustration of the genome structure of bottle gourd (A) and the syntenic relationship among four sequenced cucurbits (B).

less, some mobile RNAs exhibited a reverse regulatory mode, they were induced by cold stress. Notable shootward cold-induced mobile RNAs included those encoding polygalacturonase, sterol 3-beta-glucosyltransferase and a variety of transcription factors. Interestingly, the rootward cold-induced mobile RNAs were quite different from the shootward, including, for example, those encoding citrate synthase, transport protein SEC24, and different types of transcription factors. These results suggest that the mechanisms of hetero-graft-conferred cold tolerance involve direct movement of effector mRNA molecules.

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New Sources of Resistance to CYSDV in Melon

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Abstract. *Cucurbit yellow stunting disorder virus* (CYSDV) is a whitefly-transmitted closterovirus that reduces melon (*Cucumis melo*) fruit yield and quality in greenhouse and open-field production systems in the Middle East, the Mediterranean Basin, the Americas, and Asia. Resistance to CYSDV has been reported in melon accessions TGR 1551 (PI 482420) and PI 313970, both members of the *C. melo* ssp. *agrestis* Acidulus Group. Their non-sweet, vegetable-type fruits are similar: small, oval, thin flesh, and extremely hard and bitter at maturity, though slightly aromatic. This poses a challenge to development of sweet, western U.S. shipping-type muskmelon (*C. melo* ssp. *melo* Group Reticulatus) and green flesh honeydew (*C. melo* ssp. *melo* Inodorus Group) cultivars. Neither accession is immune to CYSDV infection. We evaluated, therefore, approximately 500 melon plant introductions from India from 2007 through 2012 in replicated field tests for sources of higher-level resistance to CYSDV. Self-pollinated progenies from single putative resistant plants in two accessions tested in 2009 (one accession) and 2010 (one accession) expressed resistance in 2012. Six Indian accessions planted in 2011 were heterogeneous for reactions to CYSDV, and were selected for self-pollination and re-evaluation in 2013 and subsequent years. Most of these putative sources of resistance proved susceptible in subsequent field tests. None provide higher-level resistance than TGR 1551 or PI 313970, based on ELISA estimates of virus titer. Two accessions of note for plant breeders are PI 123496 and PI 145594. PI 123496 produces fruit with many characteristics of sweet melons: medium-large size (1.5 kg), shallow vein tracts, scattered net, round-oval shape, and comparable flesh thickness. PI 145594 fruits are non-sweet, vegetable type, like TGR 1551, but its F_1 ('Impac' × PI 145594) produced large, oval, dark green, lightly netted fruit, and the F_2 segregated for a high number of sweet melon-like fruit.

Keywords: *Cucumis melo*, *Cucurbit yellow stunting disorder virus*, host plant resistance

Introduction

Cucurbit yellow stunting disorder virus (CYSDV) is a whitefly-transmitted closterovirus that reduces melon (*Cucumis melo* L.) fruit yield and quality in greenhouse and open-field production systems in the Middle East, the Mediterranean Basin, the Americas, and Asia. Resistance to CYSDV is available to melon breeders in melon accessions TGR 1551 (PI 482420) (López-Sesé and Gómez-Guillamón 2000), and PI 313970 (McCreight and Wintermantel 2011), both members of the *C. melo* ssp. *agrestis* Acidulus Group. Their non-sweet, vegetable type fruits are similar: small, oval, thin flesh, and extremely hard and bitter at maturity (fruit abscission), though slightly aromatic. This poses a challenge to development of sweet melons, as neither TGR 1551 nor PI 313970 is immune to CYSDV infection.

While initiating the breeding of sweet melons using these two accessions, we sought, therefore, to identify a higher level of resistance to CYSDV that is, ideally, accompanied by a sweet melon fruit type, *C. melo* ssp. *melo* in the Cantalupensis, Reticulatus, or Inodorus Group. Here we report the results of field screening ca. 500 melon plant introductions from India from 2007 through 2012.

Materials & Methods

Plant materials

Melon accessions were obtained from the USDA, ARS National Plant Germplasm System collection held at the North Central Regional Plant Introduction Station, Ames, Iowa (http://www.grin-global.org/). Approximately 100 unique accessions were planted each fall from 2007 through 2012. The 2012 test was a repeat of the 2011 test that was severely damaged at emergence by ground squirrels, then in succession by hail and rainstorms, though a few survived.

Experimental design

Tests were planted mid-August into dry soil and irrigated via buried (ca. 20 cm depth) subsurface drip lines in raised beds on 203 cm centers, and grown using cultural practices common to the desert southwest U.S. Each experimental plot was ca. 4.0 m long and consisted of five hills spaced ca. 61 cm apart, two seeds per hill, followed by a ca. 1.5 m buffer to provide space for better separation of plots. There were three replications. All tests were

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done at the University of California ANR, Desert Research and Extension Center (DREC), Holtville, CA.

A single application of Admire® was applied at seedling emergence in 2007 and 2008. More aggressive whitefly control measures were implemented in 2009: Admire Pro® at planting (3 Sept.) was followed by single applications of Discipline® (Capture; 14 Sept.) and Widow® (30 Sept.). Plant size and condition were much improved, though the whitefly population was held to a level where feeding damage was reduced or eliminated, transmission of CYSDV was virtually 100% in susceptible 'Top Mark' (data not shown).

Inoculation and disease symptom evaluation

Whitefly populations were abundant each year, and usually swarming at time of planting when the ground was bare. Plants were heavily and uniformly infested from emergence, and under continuous feeding pressure through the test period. CYSDV symptoms were visually assessed twice, at four or five and nine or 10 weeks post-planting (WPP). Symptoms were mild at the first rating, increasing in severity through the end of the test. The disease rating scale changed through the first three years as we sought to improve the assessment (Table 1). A 1 to 4 scale was used in 2007, where 1 = asymptomatic, 2 = mild symptoms, 3 = symptoms restricted to crown leaves, 4 = symptoms extensive from crown to terminal buds. In 2008, a 1 to 9 rating scale was used in 2008, where 1 = asymptomatic, 2 = possible, 3 = faint at crown, 4 = obvious at crown, 5 = bright at crown, 6 = advancing toward tips, 7 = advancing toward tips, bright, 8 = some tips symptomatic, 9 = all tips symptomatic. In 2009, percentage symptomatic foliage was estimated. The scale used from 2010 through the present estimates percent symptomatic foliage without regard to intensity (brightness), where 1, < 10% symptomatic foliage; 2, up to 20%; 3, up to 30%; 4, up to 40%; 5, up to 50%; 6, up to 60%; 7, up to 70%; 8, up to 80%; 9, up to 90%; 10, up to 100%.

Selection of putative CYSDV-resistant plants

Melon accessions are commonly heterogeneous for two reasons. First, many, if not most, collections evaluated were obtained from open-pollinated fruit selected in a market, or from a garden or field (Staub and McCreight 1993). Second, accessions were often obtained as seeds saved from open-pollinated fruit, or were roasters mixes meant to be eaten that often consisted of more than one cucurbit species, e.g., PI 414723 (McCreight et al. 1992). Thus, we expected few accessions to be uniformly resistant for CYSDV; most were uniformly susceptible. Where an individual plant in an accession appeared to exhibit less severe or extensive yellowing, three vegetative cuttings were taken, placed individually in rooting cubes in water, and placed in a clear plastic moist chamber (terrarium) for rooting and subsequent transplanting; survivors were self- and cross-pollinated in a greenhouse at Salinas, CA.

Progenies from self-pollination of the selected putative plants identified in the six field tests were evaluated under similar conditions from 2012 to 2015. Due to limited numbers of seeds and poor seed quality, these tests did not produce much statistically analyzable data, but they served to confirm susceptibility/resistance and revealed other characters of these exotic melon accessions. We noted plant size and condition, and general observations of fruit quality.

Results & Discussion

2007

Mean CYSDV symptom severity rating on the 1 to 4 rating scale was, in October, 1.7 for PI 313970, 2.0 for 'Top Mark', and 2.1 for the 86 accessions; in November the respective means were 2.7, 4.0, and 4.0. Potentially resistant individuals were noted in three accessions in November (Table 1). Cuttings from selected plants of PI 313970 and the three putative resistant individuals

Table 1. Summary, by year from 2007 to 2012, of melon accessions from which cuttings were taken for self- and cross-pollination from one or more plants selected for putative resistance to CYSDV.

Year	n	Rating scale	Mean	Cuttings from accessions
2007	86	1 to 4	4.0	Ames 20203, PI 614185, PI 614213
2008	100	1 to 9	4.2	PI 614278, PI 614291, PI 614295, PI 614301, PI 614305, PI 614320, PI 614324, PI 614332
2009	101	percent symptomatic foliage	55%	PI 614393, PI 614395, PI 614441, PI 614461, PI 614475, PI 614476, PI 614479, PI 614481
2010	98	1 to 10	5.6	PI 614553, PI 614526, PI 614486, PI 614524
2011	100	1 to 10	–[z]	PI 116482, PI 123683, PI 123689, PI 124103, PI 124107, PI 124207, PI 124431, PI 124435, PI 145594
2012	100	1 to 10	8.3	PI 122847, PI 123495, PI 123683, PI 123683, PI 123687, PI 123821, PI 124103, PI 124103, PI 124105, PI 124106, PI 124429, PI 124435, PI 124439, PI 124445, PI 124445, PI 124550, PI 124550, PI 164269

[z]Test was heavily damaged by animals, hail, and rainfall; data were, therefore, not taken, but cuttings were taken from putative resistant survivors.

were rooted in the greenhouse at Salinas for self-pollination and subsequent testing; seed was obtained from the PI 313970 cuttings, but not from the putative CYSDV-resistant individuals.

2008

One hundred plant introduction (PI) accessions were planted in three replications. CYSDV symptoms were evaluated on a plot basis using the 1 to 9 rating scale in October and November. There were statistically significant differences among the accessions in October ($P < 0.0001$) and November ($P = 0.0356$). Overall mean severity in October was 3.0 with accession means ranging from 2.3 to 3.7. Severity increased through November when the overall mean was 4.2 and accession means ranged from 3.1 to 4.7. Most accessions were dead in December. Four accessions were noteworthy, though they were not as good as either PI 3133970 or TGR 1551: PI 614270, PI 614294, PI 614291, PI 614278. Cuttings from single plants in eight of the accessions were taken in November for self- and cross-pollination and subsequent testing (Table 1).

2009

The overall mean CYSDV disease rating for the 101 previously untested accessions tested in 2009 was 55%; accession means ranged from 25% to 90%. Eight accessions with one or more putative resistant plants were identified, and cuttings were taken from the eight accessions for self-pollination in a greenhouse and subsequent testing (Table 1).

2010

Mean symptom severity of 98 accessions was 5.6 and ranged from 2.3 to 7.5. Mean ratings of 'Top Mark' and PI 313970 were 5.6 and 1.8, respectively. Two accessions had one plot rated 1 and one accession had two plants rated 1. Cuttings were taken from five plants of four accessions for self- and cross-pollination in a greenhouse at Salinas and subsequent testing (Table 1).

2011

One hundred accessions were planted in 2011, but extensive hail damage precluded meaningful evaluation, and they were replanted in 2012. Cuttings were, nevertheless, made from chance survivors of eight accessions (Table 1).

2012

This test was a repeat of the 2011 planting. Overall mean CYSDV symptom severity rating of the 100 accessions was 8.3 and accession means ranged from 5.0 to 10. None of the accessions were uniformly resistant, as in previous years, but cuttings were taken from single plants of 18 accessions (Table 1). Cuttings were also taken from three of these accessions in 2011: PI 123683, PI 124103, PI 124435.

Evaluations of progenies from selected plants

None of the cuttings taken in 2007 or 2008 survived to produce seed from either self- or cross-pollination. Most of these putative sources of resistance proved susceptible in subsequent field tests.

The S_1 from a single plant selection of PI 614479 in 2009 exhibited resistance to CYSDV in 2010 comparable to PI 313970 (data not shown), and its F_1 with 'Esteem' was susceptible as has been observed for crosses of PI 313970 and TGR 1551 with susceptible cultivars (McCreight and Wintermantel 2011, McCreight et al. 2016). Limited numbers of plants of PI 614479 and PI 614486, selected in 2010, exhibited resistance to CYSDV in 2011 (data not shown).

Selfed progenies of eight accessions were evaluated in an unreplicated field test in 2012 (Table 2). Most were comparable to PI 313970, but the numbers of plants were too small for statistical comparisons. Recessive resistance was subsequently demonstrated in PI 614479 (unpublished data).

Progenies from five accessions were evaluated in 2013, and several were comparable to TGR 1551 (Table 3). PI 145594⊗ and PI 614486⊗ exhibited milder symptoms than they did in 2012. Plants from remnant seed of PI 122847 exhibited milder symptoms than PI 122847⊗. Several entries were notable for

Table 2. Mean CYSDV symptom severity ratings of susceptible 'Top Mark', resistant PI 313970, and self-pollinated progenies from single plant selections of nine putative sources of resistance to CYSDV in an un-replicated, naturally infected field, test 2012.

Entry	CYSDV symptom severity[z]
'Top Mark'	8
PI 313970	6
PI 116482⊗	7
PI 123496⊗	5
PI 123689⊗	6
PI 123689⊗	4
PI 124107⊗	6
PI 145594⊗	7
PI 614479⊗	3
PI 614486⊗	7
PI 614553⊗	7

[z]Rated using a visual scale from 1 (≤ 10%) to 10 (100%) scale that estimated the percentage leaf area exhibiting CYSDV symptoms.

Table 3. Summary of CYSDV symptom ratings and plant notes of five putative resistant accessions and their F_1 progeny. Caveat: small numbers of plants, 2013.

Entry	CYSDV	Notes
'Top Mark'	8.3	
TGR 1551	1.3	
PI 122847 Remnant seed	2.0	Flowering and excellent plant condition
PI 122847⊗	4.5	
F_1	6.0	
PI 123496⊗	2.0	Round, "marketable" fruit
PI 124550⊗	5.5	Large fruit, uniform plants and fruit
F_1	5.5	Outstanding "marketable" fruit, large, either round or oval
PI 145594⊗	3.0	
PI 614486⊗	2.0	

Table 4. CYSDV symptom severity, and plant size and condition ratings of resistant PI 313970 and five putative sources of CYSDV resistance, 2014.

Accession	CYSDV[z]	Size[y]	Condition[x]
PI 313970	4	9	6
PI 122847	5	9	7
PI 123496	6	9	4 and 5
PI 124550	6	9	4
PI 145594	7	9	6
PI 614486	6	4 and 5	4 to 6

[z]Rated using a visual scale from 1 (≤ 10%) to 10 (100%) that estimated the percentage leaf area exhibiting CYSDV symptoms.
[y]Plant size rated using a 1 to 9 visual scale where 1 = extremely stunted, ca. size of a newly emerged seedling, and 9 = large, dense plant canopy that completely spans and covers the 2-m wide bed. Two ratings or a range of ratings indicate variation among different progenies of the accession.
[x]Plant condition rated using a 1 to 9 visual scale where 1 = dead, and 9 = large, vigorously growing plant canopy free of disease or other types of stress symptoms and healthy terminal buds. Two ratings or a range of ratings indicate variation among different progenies of the accession.

Table 5. Mean CYSDV symptom severity ratings of susceptible 'Top Mark', resistant PI 313970, and selfed progenies of putative resistant plants of three accessions, 2015.

Entry	n	Mean[z]
Top Mark	4	10.0
PI 313970	15	5.8
PI 122847⊗	9	5.2
PI 123496⊗	7	3.7
PI 145594⊗	10	6.9

[z]Rated using a visual scale from 1 (≤ 10%) to 10 (100%) that estimated the percentage leaf area exhibiting CYSDV symptoms.

excellent plant condition, or "marketable" fruit that were more dessert type than those produced by PI 313970 and TGR 1551.

In 2014, progenies of five accessions exhibited slightly more severe CYSDV symptoms than PI 313970, but four were comparable to PI 313970 for plant size and two were comparable to PI 313970 for plant condition (Table 4). Three of the progenies were evaluated in 2015 (Table 5). PI 123496⊗ and PI 122847⊗ exhibited milder symptoms than PI 313970 (Table 5). The CYSDV rating of PI 145594 was higher than PI 313970.

Most of the selfed progenies of the putative single plant selections failed to exhibit resistance to CYSDV in our field tests. None of the better selfed progenies of putative CYSD-resistant V plants provide higher-level resistance than TGR 1551 or PI 313970, based on ELISA estimates of virus titer (data not shown).

Two accessions of note for plant breeders are PI 123496 and PI 145594. PI 123496 had large plants that produced numerous medium-large (1.5 kg) fruit with ivory rind, light net and white flesh, shallow vein tracts, scattered net, round-oval shape (Figure 1), and flesh thickness comparable to western U.S. shipping type muskmelon. Fruit of PI 145594 are non-sweet, vegetable-type, like TGR 1551, but its F_1 ('Impac' × PI 145594) produced large, oval, dark green, lightly netted fruit, and the F_2 segregated for a high number of sweet melon-like fruit (Figure 1). Two F_1 progenies of PI 145594 with 'Top Mark' and 'Impac' were rated 10 and 8, respectively (data not shown), which indicates resistance in this line is recessive in nature.

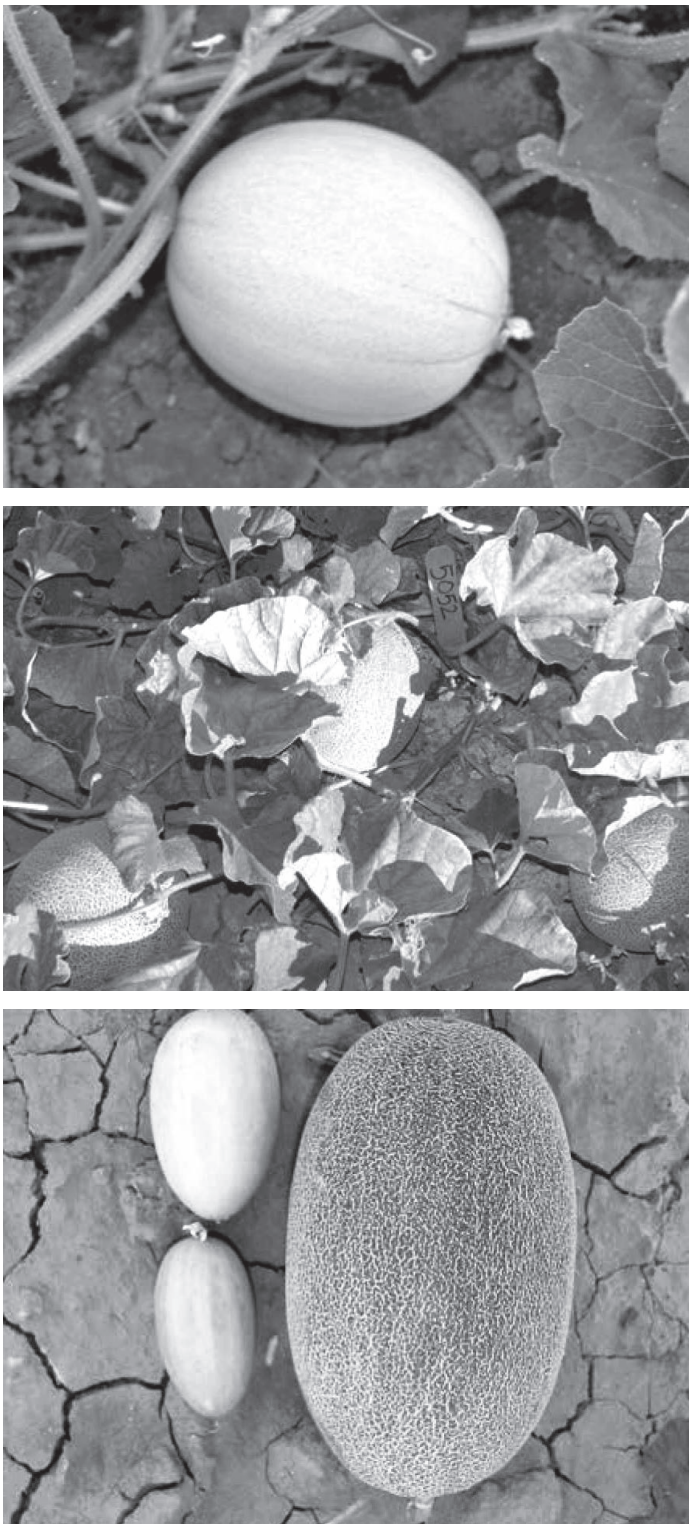

Figure 1. Fruit 9-weeks post-planting (from the top): PI 123496, F_1 'Top Mark' × PI 123496, and PI 145594 and F_1 'Impac' × PI 145594.

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Salt Stress Affects the Chloroplast Ultrastructure of Different Ploidy Watermelon Leaves

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Abstract. Plant characteristics and chloroplast ultrastructure were investigated in different ploidy levels of watermelon seedlings under NaCl stress. The results showed that the degree of damage to watermelon seedlings increased with increasing NaCl concentrations. The index of NaCl damage to seedlings of different ploidy levels varied with NaCl concentrations. After NaCl stress, 3x and 4x plants were affected less than 2x plants. At the relatively low NaCl concentration of 100 mM, the damage to the seedlings was slight. As the NaCl concentration was increased, the degree of plant damage increased. Under 400 mM NaCl, all plants suffered the most serious damage, the index of NaCl damage in 2x was quite high, with some of the 2x plants dead due to desiccation, and the leaf laminae in the 3x and 4x plants became withered. Of the non-treated controls, the tetraploid had the most chloroplasts per cell (9.7) and the largest chloroplasts (10.14 μm^2), whilst the diploid had the fewest and the smallest. As NaCl concentration increased, salt stress appeared to harm the chloroplast membranes, accelerating aging and gradual disintegration. Apparently, this led to changes in the shape of the grana and direction of the lamellae, bringing about lumen expansion, decreasing the number of lamellae, and simplifying the internal structure of chloroplast until total collapse. The three ploidy levels of watermelon were mostly unharmed under salt stress of 100 mM. Under 200 mM, the chloroplasts in the diploid and tetraploid watermelons became round, and the grana in the diploid became loose. At the level of 300 mM NaCl, the chloroplasts were badly damaged in the diploid. Damage to the tetraploid and triploid was markedly lesser, but the grana became loose and the chloroplasts became round. Under 400 mM NaCl, the chloroplasts disintegrated in the diploid, while the grana scattered in the chloroplasts of both the tetraploid and triploid.

Keywords: Watermelon, NaCl stress, chloroplast ultrastructure

Introduction

Watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, is a member of the Cucurbitaceae, a large and diverse family containing several economically important domesticated genera (Harlan 1992). Watermelon is an important horticultural crop, grown on 7% of the agricultural area devoted to vegetables (FAO 2012). China is the largest producer and consumer of this crop, with an annual production of about 68 million tonnes (http://faostat.fao.org/site). The seedless watermelon planting area in China grew to 3.5 million acres by 2010, more than 20% of the total area devoted to watermelons (http://www.wuzixigua.net/). Seedless watermelons are triploids, with more advantages than just seedlessness over their diploid counterparts, including higher yields, improved disease resistances, and superior quality.

Polyploidization is one of the most significant driving forces in higher plant evolution (Masterson 1994). More than 70% of the angiosperms have experienced polyploidization at least once over the course of evolution (Soltis et al. 2003). Polyploid plants always show different physiological phenomena, owing to changes in the composition of the genome and gene expression (Peng et al. 2008). Polyploid breeding has been applied to vegetables, ornamentals, and medicinal plants in order to obtain lines exhibiting new or improved horticultural characteristics. Polyploidization has often resulted in plants without seeds, with significantly larger fruits and flowers, and with longer-lasting flowers, which can overcome hybridization barriers and show enhanced tolerance to pests and abiotic stress (Predieri 2001). There is widespread polyploidy in higher plants because such tolerance tends to be superior to that of the homologous diploid ancestors (Soltis et al. 2000). Triploid bananas have a higher level of disease resistance than the diploid ones, and thus disease resistance seems to be based on a gene dosage effect (Ortiz et al. 1994). The triploid and tetraploid watermelons tend to have better resistance to Fusarium wilt than their diploid counterparts (Liu et al. 2009).

Soil salinization is an increasing worldwide problem. In the northwest and the coastal areas of China, soil salinity is causing much damage to crop production. Most soils are naturally saline,

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but recently a significant proportion of cultivated land is also being affected by secondary salinization (Munns 2002).

In China, greenhouse cultivation is the most common method of vegetable production. Secondary salinization of the soil is primarily attributed to over-irrigation, intensive farming, lack of rain, and concentration of salt through intense evaporation (Ashraf 1994, Liang et al. 2005, Yu et al. 2005). Secondary salinization is an important limitation to crop production in greenhouses in China (Yu et al. 2005). Watermelon cultivation is dependent upon irrigation, so watermelon cultivation can easily result in higher salinity, which could affect plant growth and lead to an annual decrease in production. At present, the research on salt resistance in vegetables is more focused on tomato, *Solanum lycopersicum* L. (Solanaceae) (Liu et al. 2001) and cucumber, *Cucumis sativus* L. (Huanwen et al. 1999, Ling et al. 2012, Sheng et al. 2013). Salt tolerance of watermelon can be affected by grafting, but only Liu et al. (2002) have done a study on salt tolerance of watermelons of different ploidy levels, and then concluded that tetraploids have higher salt tolerance than diploids and triploids.

A major cause of the worldwide decline in crop productivity has been attributed to salt stress (Giri et al. 2003, Al-Karaki 2006). The accumulation of salt in the soil generates a low water potential zone and hinders the plant from acquiring water as well as nutrients (Porcel et al. 2011). High soil salinity generates both hyperionic and hyperosmotic stress. Plant ultrastructural features are strongly modified by the presence of excess salt in the rhizosphere (Pareek et al. 1997, Yamane et al. 2004, Miyake et al. 2006, Andrea and Tani 2009). At high soil salinity, most of the structural components of plants are damaged, leading to loss of function and their subsequent death. After NaCl stress, chloroplast ultrastructure was significantly altered; the thylakoids were dilated and the number of plastoglobules was substantially higher in leaves of *Arbutus unedo* L. (Ericaceae) (Navarro et al. 2007). With increasing NaCl concentration, the chloroplast structure became visibly damaged. In cucumber grown under 50 mM NaCl, the thylakoids in self-grafted plants appeared to swell slightly, and the arrangements of the grana (stacks of thylakoids) in the stroma (aqueous space) were messy. Under 100 mM NaCl, the chloroplast envelope was slightly lost, and the grana and the lamellae (intergranal connections) were thin and obscure, even fractured (Zhen et al. 2011). In *Aeluropus littoralis* (Gouan) Parl. (Poaceae), NaCl affected the ultrastructure of the chloroplasts, especially those of the mesophyll (Zouhaier et al. 2007). Chloroplasts are considered to be the cell organelles most sensitive to NaCl stress (Utrillas and Alegre 1997, Miyake et al. 2006).

Polyploid watermelons have some advantages over their autodiploids, tending to have better low-temperature tolerance (Liu et al. 2004), resistance to Fusarium wilt (Liu et al. 2009), and salt tolerance (Liu et al. 2002). However, there is no ultrastructural evidence showing how and why polyploid watermelons have salt tolerance. The purpose of this study was to examine plant growth changes and ultrastructural changes in the chloroplasts of watermelon leaves under NaCl stress. Another aim was to test the hypothesis that polyploidy in watermelon protects chloroplast ultrastructure against the damage induced by salinity. Some leaf physiological characteristics were observed and anatomical studies performed to test whether triploid and tetraploid watermelons can better accommodate salt stress than the diploid.

Materials & Methods

Plant material and treatments

The experiment was conducted in a climate-controlled greenhouse at the Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences (CAAS), during September and October 2011, to assess the effect of salt stress on growth and chloroplast ultrastructure changes in leaves of diploid, triploid, and tetraploid watermelon, *Citrullus lanatus*, seedlings. Diploid, triploid, and tetraploid watermelon seeds of an accession designated HM were obtained from the Zhengzhou Fruit Research Institute, CAAS. HM is late maturing, bearing fruits having a black-green rind and yellow flesh. Seeds of uniform, accelerated germination were planted in 55 pots (16 cm in diameter) containing a mixture of garden soil and perlite (1 : 1 by volume). They were grown under average day and night temperatures of 28±1 °C and 18±1 °C, day length ranged from 13 to 14 h and the relative humidity between 75% and 85%. The plants were fertilized with Hoagland nutrient solution once a week. When the plants attained the three leaf stage, they were subjected to five levels of NaCl stress (0, 100, 200, 300, and 400 mM NaCl). Plants were irrigated with a prescribed NaCl solution (300 ml·pot^{-1}) the first day and 150 ml NaCl solution the second day, for a total volume of 450 ml of saline solution to each pot in the experiment.

We used a salt-damage index for the watermelon seedlings to quantify the degree to which they were damaged: Salt damage index = ∑(the number of the plants in each level × the value of the level). The classification standards are presented in Table 1.

Table 1. Classification standards of salt-damage symptoms of watermelon seedlings.

Classification	Representative value	Salt-damage symptom
0	0	None
1	1	Leaf margin of cotyledons withered
2	3	Cotyledons withered totally
3	5	Leaf margin of 1 or 2 laminae withered
4	7	2 laminae withered
5	9	Dehydration of the whole plant

Transmission electron microscope

To avoid a differential structure in different parts of leaves, the middle part of the second fully expanded true leaf without midrib was used and cut into small pieces (about 2 × 2 mm) 8 days after NaCl treatment. These small pieces were placed in a bottle with 4% glutaraldehyde buffer solution and the air was pumped out of the bottle with a syringe to keep the leaves totally soaked in buffer solution, according to the method described by Zhang et al. (2010) with some modifications. The leaves were fixed at 4 °C over 24 h, they were then rinsed with phosphate buffer (pH 7.4) for 15 min, and post-fixed in 5% OsO_4 at room temperature. The fixed samples were dehydrated with ethanol gradients of 30, 50, 70, 90, and 100% twice, then soaked with epoxy propane, embedded with Epon-812 epoxy resin, and polymerized. Thin sections were obtained with an LKB-5 ultra-microtome, double-stained with uranium acetate-lead citrate, then observed under a Hitachi-H7500 microscope and photographed. The experiment was

laid out in a completely randomized design with 3 replications. This experiment was conducted at the Institute of Agro-products Processing Science and Technology, CAAS.

Results

Effects of NaCl treatment on watermelon seedling growth

After 8 days of NaCl treatment, the degree of damage to watermelon seedlings was greater according to the increasing NaCl concentrations (Table 2, Figure 1). The index of NaCl damage of different ploidy watermelon seedlings at different NaCl concentrations is presented in Table 2. At the lower NaCl concentration, 100 mM NaCl, damage occurred in 2x plants, the index of NaCl damage being 1.92 (Table 2). The 3x and 4x plants were affected slightly. At 200 mM NaCl, damage was markedly greater. At 300 mM, 2x plants had an index of 4.09, 3x had 3.16, and 4x 2.95. At this level, there was a marked increase in the withering of the leaf laminae in 2x plants, but there was also slight withering in the 3x and 4x seedlings. At 400 mM NaCl, all plants suffered serious damage, the index of NaCl damage in 2x was 5.07, some of the 2x plants desiccated completely, and the laminae in the 3x and 4x were withered.

Effects of NaCl treatment on chloroplast ultrastructure

The sub-cellular structures of the mesophyll cells in the control (untreated) growth watermelon seedling leaves were observed. The electron microscopy analysis revealed that the areas of the cells and chloroplasts of the tetraploid leaves were significantly bigger than those of the diploid leaves, and the area of triploid leaves was in-between. At the same time, significant differences were observed in the number of chloroplasts, grana, and lamella between diploid and polyploid watermelon (Table 3). In the control (untreated) seedlings, the chloroplasts had ellipsoidal shapes with well-developed membrane systems, the grana and stroma were in orderly arrangements, the grana and lamellae were densely stacked, and there were few plastoglobules (Figure 2A,3A,4A).

Chloroplast ultrastructure of the watermelon seedlings was affected by exposure to NaCl. With increasing concentrations of NaCl, the chloroplast structure was visibly damaged, particularly in the 2x plants. At the low salt concentration, 100 mM NaCl, chloroplast structure (Figure 2B,3B,4B) was almost identical to that observed in the control. At 200 mM NaCl, the chloroplast envelope in the 2x watermelon seedlings was slightly lost, and the stacks of grana and intergranal lamellae were thin and obscure (Figure 2C). The damage to the chloroplasts of the 3x and 4x plants, though, was less severe (Figure 3C,4C), the chloroplasts only exhibited a swollen membrane system with large granal stacks, the thylakoids at the ends of the grana were more affected than the internal ones (Figure 4C). At 300 mM NaCl, the thylakoids in all three ploidy levels appeared to swell, the arrangements of the grana and the stroma were messy in both, the 2x and 3x plants, even fractured (Figure 2D,3D); in the 4x plants, the apparent chloroplast damage was slight, the chloroplasts only appeared to swell and were not messy (Figure 4D). At 400 mM NaCl, the chloroplasts in the 2x plants (Figure 2E), came into contact with the vacuolar fluid, swelled and burst, and the cells were thus totally disrupted. A decrease in the amount of grana stacking was observed in the 3x and 4x plants (Figure 3E,4E), the chloroplasts exhibited stacks of grana and lamellae that were thin and obscure. Starch grains and osmiophilic droplets were

Table 2. Index of NaCl damage of different ploidy watermelon seedlings at different NaCl concentrations.

Sample	NaCl concentration (mM)	0	100	200	300	400
Hm	2x	0	1.92 a	3.28 a	4.09 a	5.07 a
	3x	0	1.10 b	2.06 b	3.16 b	4.14 b
	4x	0	0.85 b	1.76 c	2.95 b	3.78 c

The data were analyzed using a factorial analysis of variance. Least significant difference (LSD) at $P \leq 0.05$, the letters a, b, and c denoting the difference at $P \leq 0.05$; only those parts not marked with the same letter significantly differ. The comparisons are under the same NaCl concentration after 8 days.

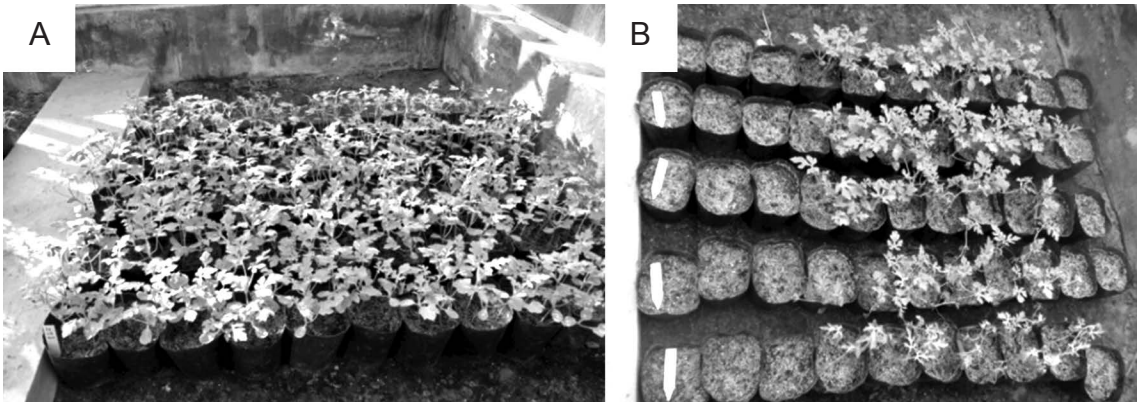

Figure 1. Watermelon seedlings growing in the climate-controlled greenhouse. (A) 2x watermelon seedlings before NaCl treatment. (B) 2x watermelon seedlings after NaCl treatment.

Table 3. Number of chloroplasts, grana and lamellae, areas of cells and chloroplasts per cell in the control (non-saline-treated) watermelon seedlings (n = 10).

Parameter	Diploid	Triploid	Tetraploid
No. chloroplasts/cell	6.70 b	8.90 a	9.70 a
No. grana/chloroplast	12.00 b	13.90 ab	15.20 a
No. lamellae/ grana	10.01 b	13.76 a	13.87 a
Cell area (μm^2)	218.30 a	305.45 b	376.43 c
Chloroplast area (μm^2)	7.85 c	9.62 b	10.14 a

The data was analyzed using factorial analysis of variance. Least significant difference (LSD) at $P \leq 0.05$ was used to distinguish significantly different means, the letters a, b, and c denoting the difference at $P \leq 0.05$; only those parts not marked with the same letter are significantly different.

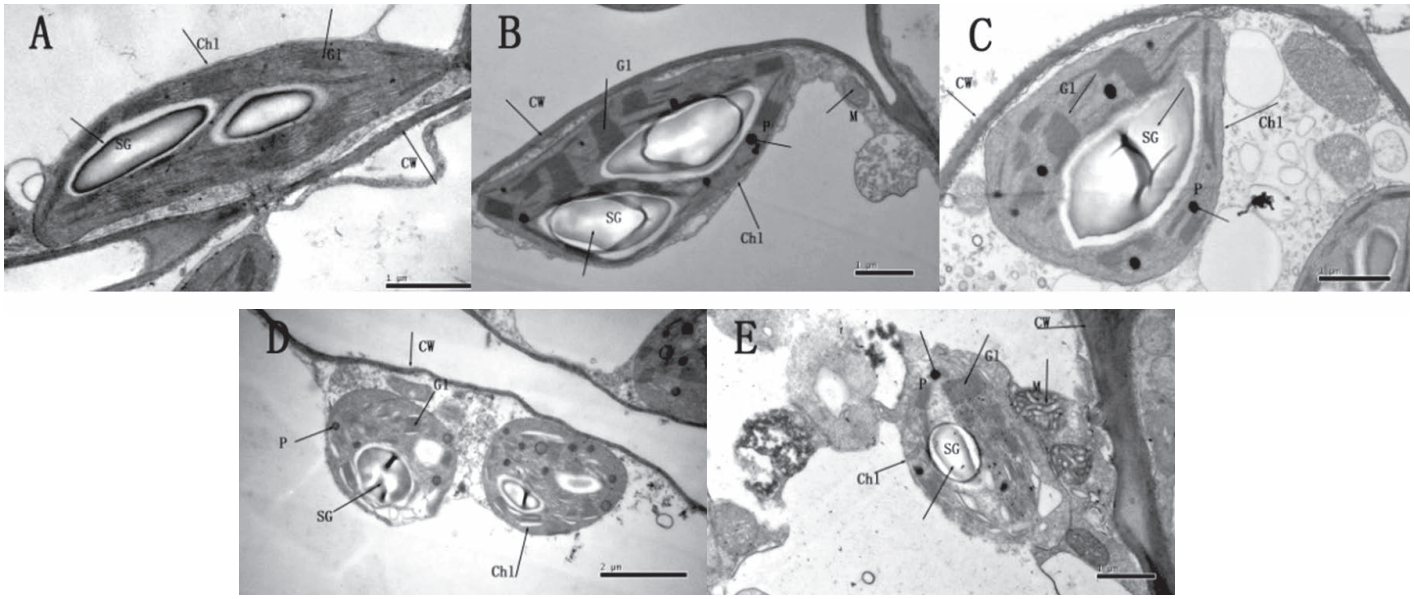

Figure 2. Transmission electron microscopy (TEM) of chloroplast ultrastructure in 2x watermelon seedling leaves treated by 0 (A), 100 (B), 200 (C), 300 (D), and 400 (E) mM NaCl, respectively.

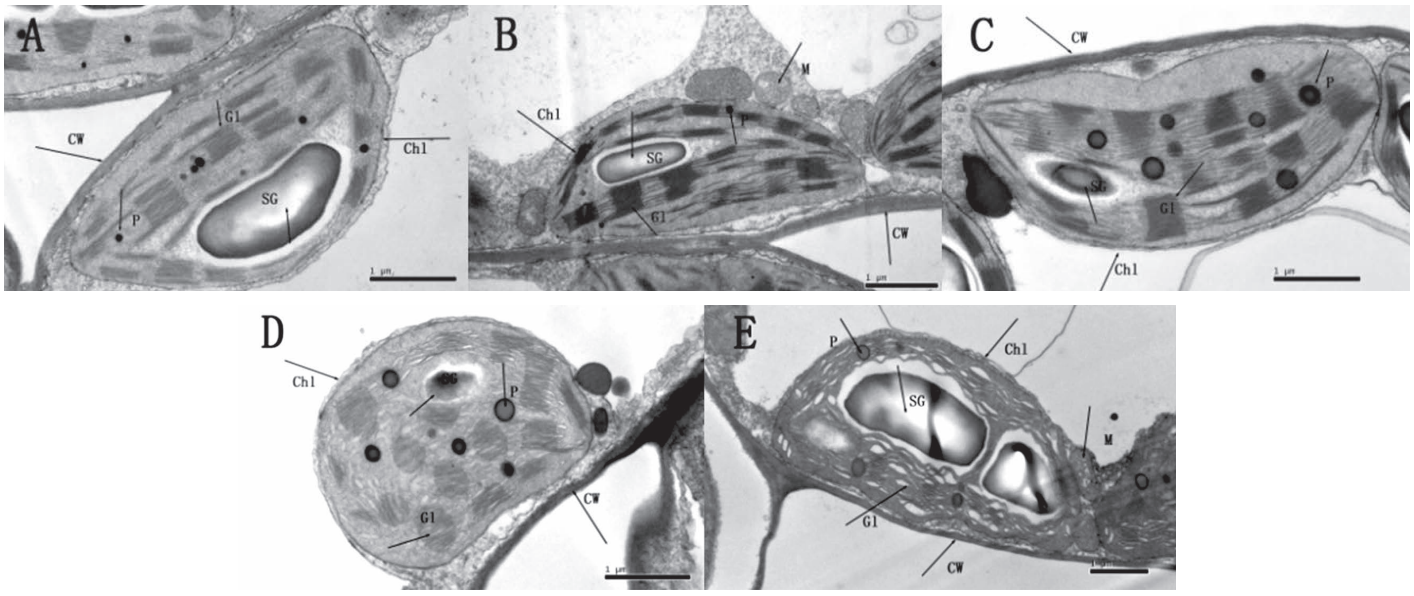

Figure 3. Transmission electron microscopy (TEM) of chloroplast ultrastructure in 3x watermelon seedling leaves treated by 0 (A), 100 (B), 200 (C), 300 (D) and 400 (E) mM NaCl, respectively.

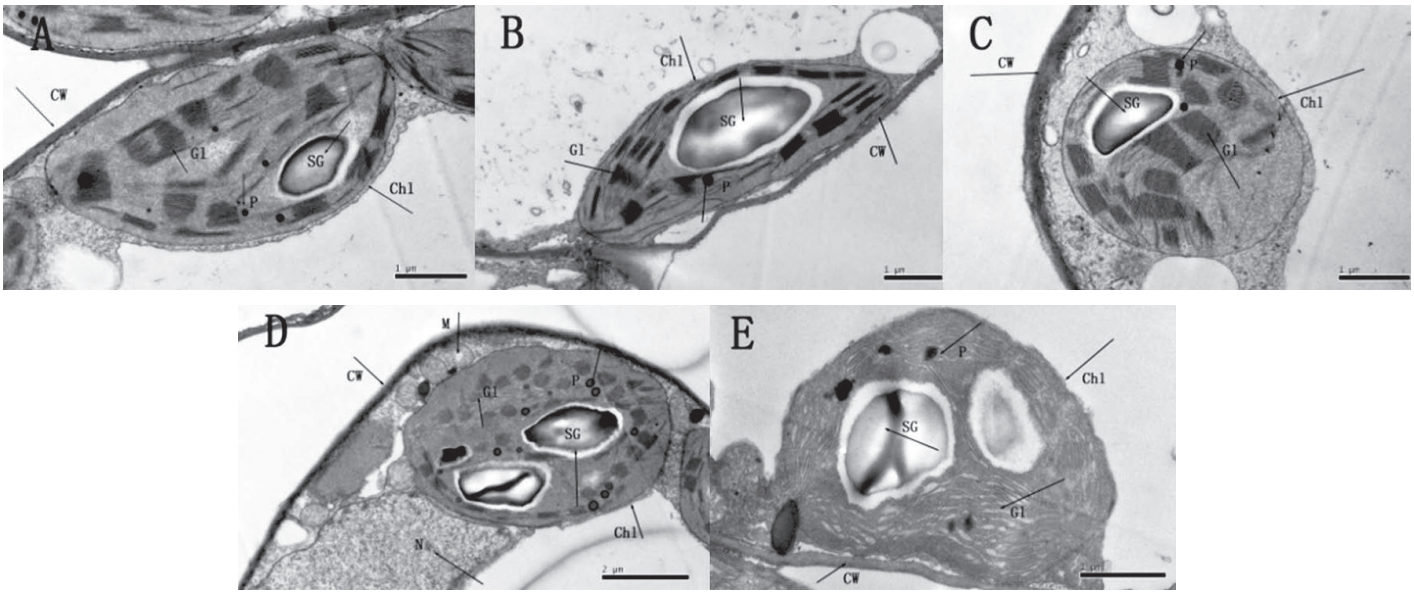

Figure 4. Transmission electron microscopy (TEM) of chloroplast ultrastructure in 4x watermelon seedling leaves treated with 0 (A), 100 (B), 200 (C), 300 (D), and 400 (E) mM NaCl, respectively.

observed in these chloroplasts at all stages of damage and did not significantly change in number or size.

Discussion & Conclusion

Polyploid plants always show different physiological phenomena owing to the changes in the composition of the genome and gene expression. The resistance of polyploids to biotic and abiotic stresses tends to be superior to that of their homologous diploid ancestors. In watermelon, in the adverse situation of salt stress, the polyploid plants displayed obvious superiority. The enhancement of chilling resistance in polyploid watermelon seedlings with cold pretreatment might be related to the enhancement of function of a membrane protective system as compared with the diploid (Liu et al. 2004); after cold treatment, the autotetraploid and triploid watermelon seedlings had better cold tolerance than the diploid cultivars (Liu et al. 2003). The triploid and tetraploid watermelons also had better resistance to Fusarium wilt than their homologous diploid (Liu et al. 2009). Here we observed the same phenomenon with regard to salinity stress, the polyploid plants clearly displayed superiority.

In melon (*Cucumis melo* L.) plants, the number of chloroplasts, starch granules and grana, and the length of the chloroplasts and granules of the tetraploid leaves, are significantly greater than those of the diploid (Zhang et al. 2010). Chloroplasts are the cell organelles most sensitive to salt stress (Utrillas and Alegre 1997, Miyake et al. 2006). In the present study, we observed that exposure of watermelon seedlings of different ploidy levels to NaCl stress resulted in ultrastructural changes in chloroplasts. Under low salt stress, 100 mM NaCl and 200 mM NaCl, the thylakoid membrane showed contraction, indicative of a reduction of thylakoid lumen. Subsequent contraction of the thylakoid may be a result of osmotic collapse due to a change in pH (Murakami and Packer 1970). As the concentration of NaCl increased, to 300 mM NaCl and 400 mM NaCl, the lumen swelled, resulting in destacking of grana. Such ultrastructural changes in other plants due to salt stress have been related to osmotic stress (Taylor and Craig 1971, Ciamporova and Mistrik 1993, Lopez-Carbonell et al. 1994). The decreased thylakoid layers per granum might be an adaptive response to stress (Kasperbauer and Hamilton 1984). The structure of the thylakoid and granal stacking is dependent on the cationic concentrations of the chloroplast. Therefore, an ionic imbalance due to salt stress could affect normal grana stacking in chloroplasts of mesophyll cells (Utrillas and Alegre 1997). Thylakoid swelling and membrane damage have also been related to osmotic stress (Hernandez et al. 1995, Fidalgo et al. 2004, Yamane et al. 2004, Oksanen et al. 2005).

In conclusion, in watermelon seedlings, the salt tolerance of the polyploids is better than of the diploid. The results of this study indicate that ultrastructural alterations due to NaCl stress were consistent with the plant growth, so the chloroplast ultrastructural changes could be a suitable measurement to assess salt tolerance in watermelon.

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Progress in Assembling the Cucumber (*Cucumis sativus*) Borszczagowski B10 Line Genome Using Long Single Molecule, Real-Time Reads

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Abstract. Today, most eukaryotic genome assemblies are in databases as incomplete drafts because they contain repetitive sequences much longer than reads that NGS technologies can generate. Single molecule, real-time sequencing technologies have proven to be useful in overcoming these constraints. Here, we present preliminary data of an upgraded version of the B10 cucumber inbred line genome sequence, assembled with small 47.6x coverage of solely Pacific Biosciences long reads. This is the third higher plant genome sequence generated with this method. Compared with genomes stored in the NCBI databank, the upgraded sequence (GenBank: GCA_001483825.1) is over 42% longer, making approximately 76% of the whole cucumber genome sequence known for this moment. We observed a great improvement in contig N50 and L50 statistics. Third-generation sequencing technology has proven to be of high value in obtaining the best version of cucumber genome contigs. Therefore, this new draft will serve us as a quality material for further comparative analyses among accessible cucumber genomes.

Keywords: Genome assembly, contigs, SMRT reads, PacBio, cucumber

Introduction

High-throughput DNA sequencing technologies have become basic tools in genomics (Pawełkowicz et al. 2016), especially in genome assembly. However, length and quality of reads produced by recently used technologies still need improvement to reach a fully satisfying genome sequence length and accuracy (Shatz et al. 2010). Second-generation reads have a low error rate (< 0.01%) but are too short to overcome many problems posed by long repetitive elements. On the other hand, third-generation sequencing technologies can produce single molecule real-time (SMRT) reads up to 100,000 bp long, but with an error rate of at least 15%. Despite the errors, long reads have already proven to be effective in *de novo* genome assembly projects, both of procaryotic (Koren and Phillippy 2015, Madoui at al. 2015) and eukaryotic (Chen et al. 2014, Berlin et al. 2015, Gordon et al. 2016) origin, helping to surpass assembly gap problems. At present, we have found four plant genome drafts assembled solely from Pacific Biosciences technology (PacBio) reads and nine assembled via hybrid approach (DDBJ/EMBL/GenBank).

Originating from the foothills of the Eastern Himalayas and secondary domestication regions, cucumber (*Cucumis sativus* L.) is a widely cultivated crop. B10 is a highly inbred (20 generations) monoecious accession of 'Borszczagowski', an old field cultivar from Poland. The first published cucumber genomes of accessions 9930 (Huang et al. 2009), Gy14 (Cavagnaro et al. 2010), and B10 (Wóycicki et al. 2011) were highly incomplete. Assembled mostly from second generation sequencing data, their contig sequences share similar sequence length statistics and limitations. We started our project to significantly enhance the genome of accession B10 through the usage of PacBio long reads. Here we report the preliminary results of assembled contigs in comparison with accessible cucumber genomes. Through application of bioinformatic tools, we show that PacBio reads can vastly improve lengths of sequences as well as maintain quality up to the level of the reads produced from second generation reads.

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Materials & Methods

DNA isolation and sequencing

Cucumber B10 line plants were cultivated in a greenhouse during the summer under a controlled photoperiod of 16h/8h (day/night). Young leaves were collected in the morning, frozen in liquid nitrogen and stored at -74 °C. For isolation, 1 g of tissue from each plant was used. The amount and purity of the isolated DNA were determined by using a NanoDrop 2000 and a quality check by performing electrophoresis in 1% agarose gel. Sequencing was done by McGill University and Génome Québec Innovation Centre with the PacBio RS II system (P5C3 chemistry and older).

Genome assembly and analysis

A reference genome was assembled using Celera Assembler software suited for long SMRT reads (Berlin et al. 2015). Raw reads were self-corrected before the assembly by the software tool. With only 1x of suitable read data, assembled contig sequences were corrected and filtered through the Pacific Biosciences pbalign approach. Subsequently, contigs with mitochondrial elements were filtered out. In order to compare new genome draft statistics, three other cucumber genomes were used in this study: (1) The Gy14 genomic sequence (downloaded from a Weng lab website: http://wenglab.horticulture.wisc.edu/), (2) The 9930 reference genome for cucumber (GenBank: GCA_000004075.2) and (3) The CsB10v1 previous B10 draft (GenBank: GCA_001483825.1). The data sets for Gy14 and 9930 were accessible in scaffold structures. Therefore, in order to make contig comparisons, we applied perl script modifying the data by splitting sequences wherever 'N' symbols occurred in any sequence. Genome draft quality assessment was performed through evaluation of gene structure assembly. For this purpose, we used the Core Eukaryotic Genes Mapping Approach (CEGMA) (Parra et al. 2009) and the Benchmarking Universal Single-Copy Orthologs (BUSCO) (Simão et al. 2015) programs, locating in the compared draft sequences core eukaryotic genes and near-universal single-copy orthologs for plants, respectively.

Results

Average length of the PacBio read data was 3,119 bp (min. length = 50 bp, max. length = 35190 bp) and coverage of the genome was 47.6x. Statistics of achieved contigs as compared with other assemblies are presented in Table 1. The newly generated draft covered approximately 25% more of the genome than other versions, being 278.1 Mb long. The N50 value rose nearly 9-fold, to 379.9 Kb, as compared with the actual GenBank reference, 42.3 Kb.

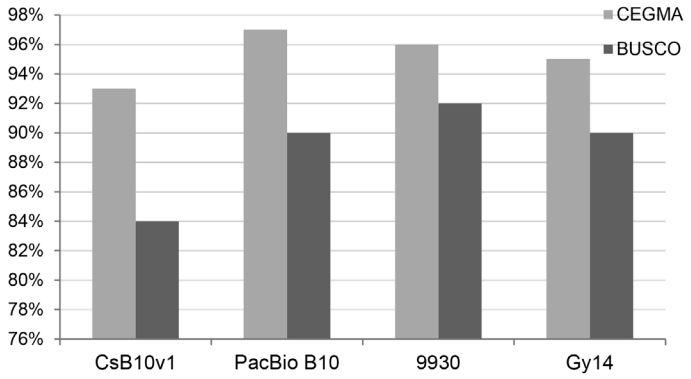

Figure 1. Results from CEGMA and BUSCO tools for compared draft genome assemblies, assessing completeness (%) of draft genome sequence by locating on each genome 248 core eukaryotic genes and 956 single-copy, plant-specific orthologs, respectively.

In terms of assembly quality, the 9930 contigs had the highest (~92%) completeness score using the BUSCO benchmark. The PacBio B10 genome had the highest (~97%) CEGMA score. Overall, PacBio B10, Gy14, and 9930 seem to have assemblies of similar quality, significantly outclassing the first version of the B10 genome (Figure 1).

Discussion

Recent research on the cucumber genome done through short-read analysis showed in detail structural variation and genomic diversity (Qi et al. 2013, Zhang et al. 2015). However, there is still a place for improvement in reading the genome at the structural level because of the bias generated by short-read data. Contiguity of a genome sequence will be an important factor in detailed genomic research, providing better completeness and correctness (Lee et al. 2016). Contiguity seems to be highly correlated with quality of an assembly of genomic elements such as genes, gene clusters, and transposons, providing expanded knowledge about the nearest surroundings. The genome draft reported in this work shows much better contiguity than all previous cucumber genome sequences. PacBio read assembly presents no quality loss, which can be an issue due to the high error rate of long reads. It has been shown that, similar to Illumina, nucleotide read consensus accuracy is achievable in long-read assemblies (Chin et al. 2013). Some of the PacBio plant genome assemblies found in GenBank (*Arabidopsis thaliana*, *Oropetium thomaeum*),

Table 1. Summary data for contig parameters of published draft cucumber genomes.

Genome draft	PacBio B10	CsB10v1	Gy14	9930
No. contigs	6693	16454	13604	11366
Total bases (Mb)	278.1	193.2	192.3	195.7
Genome coverage (%)	75.70	52.64	52.39	53.32
N50 length (Kb)	379.9	23.2	37.6	42.3
L50	175	2417	1476	1290

mentioned earlier in this paper, are of better characteristics than the new B10 draft, however, higher read coverage was used in those instances. This exhibits that, together with mapping information, long reads can serve to raise structural accuracy of cucumber genome drafts. As a reference, such assembly will provide a fine resource to uncover many still unclear regions connected to repetitive elements and will help understanding the dynamics of structures in the genome of cucumber.

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Progress in Cucumber Molecular Breeding

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Abstract. In cucumber breeding, a large range of methods can be used to improve disease resistance, yield, fruit appearance, other fruit quality characteristics, and sex expression. The genome sequence of the cucumber provides an invaluable new resource for genetic improvement, basic biological research, and breeding of cucumbers. We review here the development and use of molecular markers for cucumber breeding.

Keywords: Marker, disease resistance, fruit quality-related genes, QTL, MAS

Constructing a High-Resolution Genetic Map

The development of high-resolution, easy-to-use genetic maps not only provides a platform for gene/quantitative trait loci (QTLs) fine mapping and map-based gene isolation, but also reduces the time required for molecular breeding of cucumber, *Cucumis sativus* L.

We constructed an ultra-high-density cucumber genetic map using RIL (recombinant inbred line) populations derived from a gynoecious line 9110Gt from Europe, and a monoecious line 9930 from North China, which was phenotyped over multiple seasons (Zhou et al. 2015). We generated 73 Gb sequences by barcoded multiplexed sequencing of 147 RILs, equivalent to approximately 500 Mb per RIL, covering about 33% of the assembled 9930 genome for each RIL. Based on the previous re-sequencing data of 9110Gt (five-fold genome coverage), we identified 116,710 SNPs between the parental lines, adding 10,629 novel polymorphic sites into the cucumber genomic variation map (Qi et al. 2013). In the RILs, 99.3% (115,933) of the 116,710 parental SNPs were present and genotyped.

We defined a block of the same genotype across the entire population as a raw bin and constructed a genetic linkage map spanning 1,384.4 cM with bin markers. After lumping successive raw bins with identical genetic positions, 759 unique bins were anchored on seven linkage groups corresponding to the seven chromosomes. These bins had an average physical length of 248.8 kb, ranging from 10.6 kb to 1.6 Mb, representing more than 98% of the 9930 reference genome.

Using the bin map, we also detected 28 QTLs above the permutated likelihood of odd (LOD) thresholds for ten quantitatively inherited traits. The clustering of fruit-associated trait QTLs *fws5* (fruit wart size), *u5* (uniform immature fruit color), *fr5* (fruit ribing), *mfc5* (mature fruit color), and the two Mendelian genes *D* (dull fruit skin) and *H* (heavy netting of mature fruit), suggested the region of C5B496–C5B506, locating to a 17.5 to 20.4 Mb segment on chromosome 5, that plays an important role in cucumber fruit development.

Mapping Genes Conferring Disease Resistance

The most effective way of plant protection is the production of cucumber hybrids with resistance to many diseases. Therefore, breeding of cucumber hybrids with a combination of disease resistances is a major objective (Table 1).

Scab

Scab can cause serious losses for cucumber production, especially in protected culture such as high tunnel production. Resistance to cucumber scab is dominant and is controlled by a single gene, *Ccu*. The population included 148 individuals derived from the cucumber inbred accession 9110Gt crossed with accession 9930. The *Ccu* gene was mapped to chromosome 2. The flanking markers SSR03084 and SSR17631 were linked to the *Ccu* gene with distances of 0.7 and 1.6 cM, respectively. The validity of SSR03084 and SSR17631 was tested using 59 diverse inbred lines and hybrids, and the accuracy rate for the two markers was 98.3% (Zhang et al. 2010). Using a population of 1,944 F_2 plants, the two closest markers, Indel01 and Indel02, were 0.14 and 0.15 cM away from the target gene *Ccu*, respectively, and the physical distance between the two markers was approximately 140 kb. In this region, there are contained four nucleotide binding site (NBS)-type resistance gene analogs (RGAs) (Kang et al. 2010). Using the bin map, we delimited *Ccu* into bin C2B123 that spans 191 kb. Bin C2B123 and the 180-kb region overlapped at an interval of 88 kb, where only one NBS-LRR gene, *Csa2G021710*, is present, which seems to be a strong candidate gene for *Ccu* (Zhou et al. 2015).

Powdery mildew

The cucumber inbreds K8 (resistant) and K18 (susceptible) were used to study the inheritance of resistance to powdery

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mildew. Four QTLs named *pm5.1*, *pm5.2*, *pm5.3,* and *pm6.1* for the gene conferring resistance to powdery mildew were detected in this study. The QTL *pm5.2*, located to chromosome 5, was the major QTL. Four NBS resistance genes were found in the region of *pm5.2* (Zhang et al. 2011).

Downy mildew

Five QTLs for resistance to downy mildew were detected: *dm1.1, dm5.1, dm5.2, dm5.3*, and *dm6.1*. Linked SSR markers for the five QTLs were identified: SSR31116, SSR20705, SSR00772, SSR11012, SSR16882, and SSR16110. Six and four NBS-type RGAs were predicted in the region of *dm5.2* and *dm5.3*, respectively (Zhang et al. 2013a).

Fusarium wilt

A set of 148 F_9 recombinant inbred lines (RILs) derived from the cross 9110Gt × 9930 were used to study the inheritance of Fusarium wilt resistance and detect quantitative trait loci (QTLs) conferring resistance in cucumber. The major QTL was placed in the region of SSR03084~SSR17631 within a genetic distance of 2.4 cM on chromosome 2. The validation of SSR17631 linked to *Foc* was tested using 46 diverse germplasms. The accuracy rate of SSR17631 for selecting resistant genotypes was 87.88% and it could be used to screen cucumber resources for Fusarium wilt resistance in molecular marker-assisted selection (MAS) (Zhang et al. 2014).

Watermelon mosaic virus (WMV)

Inbred lines 65G and 228 were used as the experimental materials for genetic analysis and mapping of the gene conferring *Watermelon mosaic virus* resistance. Genetic analysis showed that the resistance to WMV is recessive and controlled by a single gene, *wmv* (Zhou 2012). The *wmv* gene was mapped to chromosome 6, the loci SSRWMV60-23 and CAPS-W1 were found to flank the *wmv* locus with genetic distances of 0.34 cM and 1.19 cM. The physical distance of this region was 134.7 kb. There were 19 candidate genes in this region. Among them, the candidate gene *Csa6P421660.1* encodes the pathogenesis-related transcriptional factor/ERF DNA-binding (PR-TF) site, which is involved in plant disease resistance (Tian et al. 2015).

Papaya ring spot virus (PRSV)

In the present study, an F_2 population was developed from the cross between the susceptible 65G and resistant 02245 cucumber inbred lines. Genetic analysis of PRSV resistance in 144 $F_{2:3}$-derived F_3 families showed that resistance is controlled by

Table1. Summary of gene mapping results.

Trait	Gene	Chromosome	Population	Reference
Scab	*Ccu*	2	9110Gt × 9930	Zhang et al. 2010, Zhou et al. 2015
Powdery mildew	*pm*	5,6	K8 × K18	Zhang et al. 2011
Downy mildew	*dm*	1,5,6	K8 × K18	Zhang et al. 2013a
Fusarium wilt	*Foc*	2	9110Gt × 9930	Zhang et al. 2014
Watermelon mosaic virus	*wmv*	6	65G × 228	Zhou 2012
Papaya ring spot virus	*prsv*	6	65G × 228	Tian et al. 2015
Bitterness	*bi-1, bi-3*	5,6	9110Gt × 9930	Zhang et al. 2013b
	Bt-2	5	931 × hardwickii	Liu et al. 2014
Glossy fruit skin	*G*	5	1101 × 1106	Dong et al. 2013
White fruit skin	*w*	3	1507 × 1508	Dong et al. 2012
Yellow flesh	*yf*	7	PI200815 × 931	Lu et al. 2015
Glabrous	*gl-2*	2	9110Gt × NCG-042	Yang et al. 2012
	gl-3	6	9930 × NCG157	Cui et al. 2016
Black spine	*B*	4	GY14 × NC76	Liu et al. 2013
Red mature fruit	*R*	4	NCG127 × 9930	Liu et al. 2014
Heavy netting mature fruit	*H*	5	PI205996 × PI263079	Wang et al. 2014
Virescent leaf	*v-1*	6	9110Gt × 9930	Miao 2010
Plant height	*Ph*	1,2,5,6	9110Gt × 9930	Miao et al. 2012a
Cotyledon length	*Cl*	1,3,5,6	9110Gt × 9930	Miao et al. 2012b
Cotyledon width	*Cw*	1,3,5		
Hypocotyl length	*Hl*	5,6		
The first pistillate flower node	*Fpfn*	3,6		
Days to anthesis	*Da*	1		
First true leaf length	*Fll*	5,6	PI 183967 × 931	Wang et al. 2015
First true leaf width	*Flw*	5,6		
Aboveground fresh weight	*Afw*	5		
Aboveground dry weight (Adw)	*Adw*	2,5,6		

a single recessive gene which was designated *prsv*^{02245}. Simple sequence repeat (SSR) markers were employed in polymorphism screening between PRSV-susceptible and -resistant DNA pools. The PRSV resistance gene, *prsv*^{02245}, was mapped to chromosome 6 and was flanked by two SSR markers, SSR11-177 and SSR11-1, which were 1.1 and 2.9 cM away from the *prsv02245* locus, respectively. The accuracy rate of marker-assisted selection of PRSV resistance among 35 cucumber lines using the marker, SSR11-177, was more than 80%. Results from this study provide a valuable tool for fine mapping, gene cloning, and marker-assisted breeding for PRSV resistance in cucumber (Tian et al. 2015).

Mapping cucumber fruit quality-related genes

Cucumber fruit quality includes flavor (bitterness), fruit visual appearance (e.g., size, shape, fruit skin color, spine color, flesh color), and so on. The development and use of molecular markers for cucumber fruit traits in our lab is depicted in Table 1.

Cucumber bitterness

Bitterness in cucumber is due to the presence of cucurbitacins and has a complex inheritance. We were interested in studying the inheritance of cucumber fruit and foliage bitterness and in locating the genes on a current linkage map using a RILs population derived from the cross 9110Gt × 9930. It was concluded from the inheritance analysis that there were two loci controlling fruit bitterness in the population. One locus was in the same position as the location previously identified for *bi-1*, and another locus was for *bi-3*. The locus *bi-1* was located on chromosome 6. The locus *bi-3* was on chromosome 5 (Zhang et al. 2013b).

Glossy fruit skin

Glossy fruit skin is one of the highly valuable appearance quality traits related to the market value of cucumber. In previous studies, the glossy fruit skin gene appeared to be conferred by a single recessive gene, *d*, but in this study, we found it to be conferred by a single dominant gene. *G* gene was mapped to chromosome 5. Flanking markers were CS28 and SSR15818, genetic distances of 2.0 and 6.4 cM, respectively (Dong et al. 2013).

White immature fruit skin

Inbred line 1507 (dark green fruit skin) and 1508 (white fruit skin) were used as the experimental materials for genetic analysis and gene mapping of white fruit skin in cucumber. Genetic analysis showed that it is conferred by a single recessive nuclear gene, *w*, which was mapped to chromosome 3. The markers SSR23517 and SSR23141 flanked *w* (Dong et al. 2012). The *w* gene was fine mapped between markers 3WSSR2-85 and SSR23141 among 2,347 F_2 individuals derived from the cross 1507 × 1508. The genetic distances were 0.6 and 0.5 cM, respectively, and the physical distance was approximately 115.6 kb in 9930 scaffold 000112.

Yellow fruit flesh

Vitamin A deficiency is a major health problem in many countries around the world. Efforts to alleviate this have included adding supplements to wheat and maize flour. China produces over 70% of the world's cucumbers, and any attribute that could increase the beta-carotene content of the fruit has the potential to have a major impact on world health. Cucumbers with yellow flesh contain larger amounts of beta-carotene than those with white and green flesh. Yellow fruit-flesh cucumber inbred line PI 200815 and white fruit-flesh inbred line 931 were used as parents to construct a population for genetic analysis of cucumber fruit-flesh color. The results showed that the yellow fruit-flesh trait of PI 200815 was conferred by a single recessive gene *yf*. The *yf* gene was mapped to chromosome 7. The closest flanking markers linked to *yf* were yfSSR108 and yfIndel29, with genetic distances of 0.6 and 0.3 cM, respectively. The physical distance for the region harboring *yf* was 149.0 kb with 21 predicted candidate genes. The accuracy of marker-assisted selection breeding using the molecular markers, yfSSR108 and yfIndel29, was 92.3 and 84.6%, respectively (Lu et al. 2015).

Trichomes

The genetic analysis showed having trichomes or not is determined by a single nuclear gene, *gl-2*, which was mapped to a linkage group with 11 SSR makers corresponding to chromosome 2. The flanking markers SSR10522 and SSR132751 were linked to the *gl-2* gene with genetic distances of 0.6 and 3.8 cM, respectively. These two markers were tested using a BC_1P_2 population, and the accuracy rate was 94.4% and 91.6%, respectively (Yang et al. 2011).

Previous studies have reported two glabrous mutant plants containing the genes *csgl1* and *csgl2*. A new glabrous mutant, NCG157, was identified showing a gene interaction effect with *csgl1* and *csgl2*. This mutant showed the glabrous character on stems, leaves, tendrils, receptacles, and ovaries, and there were no spines or tumors on the fruit surface. Inheritance analysis showed that a single recessive gene, named *csgl3*, determined the glabrous trait. The *csgl3* gene was mapped to chromosome 6, the physical distance of this region was 19.6 kb. Quantitative real-time PCR showed that the expression of *Csa6M514870* was higher in the tissues of 9930 than in NCG157, and this was consistent with their phenotypic characters. *Csa6M514870* is therefore postulated to be the candidate gene for the development of trichomes in cucumber (Cui et al. 2016).

Black fruit spines

Cucumber inbred lines GY14 with white fruit spines and NC76 with black fruit spines were used as the experimental materials for genetic analysis and gene mapping for black fruit spine. The *B* gene was mapped to chromosome 4. The two closest flanking markers were SSRB-181 and SSRB-130, with genetic distances of 2.0 and 1.6 cM, respectively. The flanking markers can predict spine color for marker-assisted selection (MAS) with 96.8% and 96.2% accuracy, respectively (Liu et al. 2013).

Red mature fruit

Genetic analysis and gene mapping were carried out on red mature fruit in cucumber using NCG127 (red mature fruit) and 9930 (yellow mature fruit) as experimental materials. The results showed that a single dominant nuclear gene, *R*, confers red mature fruit, and *R* was mapped to a linkage group corresponding to chromosome 4. The flanking markers UW019319 and UW019203 had genetic distances of 0.8 and 0.7 cM, respectively (Liu et al. 2014).

Heavy netting of mature fruit

Cucumber inbred lines PI 205996, without heavy netting of mature fruit, and PI 263079, with heavy netting, were used as the experimental materials for genetic analysis and gene mapping. The heavy netting of the mature fruit of PI 263079 was conferred by a dominant nuclear gene (*H*). It was located on chromosome 5 and delimited in a physical distance of 297.7 Kb. Flanking markers SSR13006 and SSR-90 were 3.6 and 1.7 cM away from the *H* gene, respectively (Wang et al. 2014).

Virescent leaf

Based on the cucumber genome sequence, new markers were developed to fine map the *v-1* gene. The *v-1* gene was fine mapped to the short arm of chromosome 6 (Miao 2010). Fine mapping was conducted with draft genome scaffold-assisted chromosome walking, which allowed for assignment of the *v-1* locus to a 50.9 kb genomic DNA region with two flanking markers that were genetic distances of 0.14 and 0.16 cM, respectively. Quantitative RT-PCR revealed significantly higher levels of expression of *CsaCNGCs* in 9110Gt as compared with 9930 at the virescent yellow stage, where the levels of *CsaCNGCs* were significantly lower. This gene is considered to be a candidate gene for the *v-1* locus conditioning chloroplast development and chlorophyll synthesis in cultivated cucumber.

Plant height

Phenotypic data on 148 RILs which originated from the cross 9110Gt × 9930 were investigated four times in different seasons. Eleven QTLs were detected for three plant height-associated traits. These QTLs were mapped to chromosomes 1, 2, 5, and 6, respectively. Five QTLs explained more than 10% of the phenotypic variation. Three QTLs (27.3%) were found to be expressed consistently under four cropping seasons in greenhouse cultivation. A QTL cluster was detected on chromosome 1. Compared with the previous map, the *de* gene controlling determinate habit was found in the region of the QTLs mapped to the long arm of chromosome 6. Based on the results in this study, we speculated that there are at least four genes controlling plant height (Miao et al. 2012a).

Seedling traits

Nineteen QTLs were detected for five traits: cotyledon length (*Cl*), cotyledon width (*Cw*), hypocotyl length (*Hl*), the first pistillate flower bearing node (*Fpfn*), and days to anthesis (*Da*). These QTLs were mapped to chromosomes 1, 3, 5, and 6 (Table 1). Seventeen QTLs (89.5%) were major QTLs which explained more than 10% of the phenotypic variation. Three QTLs were repeatedly detected in different seasons in greenhouse cultivation. Their LOD values varied between 3.28 to 15.25, which explained 6.0% to 37.8% of the phenotypic variation (Miao et al. 2012b).

One hundred sixty recombinant inbred lines (RILs), derived from crossing the wild cucumber PI 183967 (*Cucumis sativus* var. *hardwickii*) with a cultivated cucumber from North China, line 931, were employed to identify quantitative trait loci (QTLs) of seedling traits in cucumber. Thirty-six QTLs associated with the seven traits were detected on chromosomes 1, 2, 3, 5, and 6 in four environments (Spring and Autumn of 2012 and 2013), explaining 6.1 to 23.6% of the observed phenotypic variations. Among the 36 QTLs, 21 were responsible for more than 10% of the observed phenotypic variations. We obtained 2, 2, 1, and 3 QTL loci for the traits of first true leaf length (Fll), first true leaf width (Flw), aboveground fresh weight (Afw), and aboveground dry weight (Adw), respectively. In addition, genes in the genetic region spanned by SSR15321-SSR07711 on chromosome 5 may contribute to *Flw*, *Afw*, and *Adw* (Wang et al. 2015).

From these results, we can derive the locations of genes on chromosomes, and use this information for fine mapping and gene cloning. We have used these markers to create some good inbred lines and to breed commercial cultivars, including 'Zhongnong 18', 'Zhongnong 50', and 'Zhongnong 37'.

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The Plant Breeders' Dream Come True: Editing Non-Transgenic Cucumber for Broad Virus Resistance Using CRISPR/Cas9 Technology

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Abstract. Genome editing in plants has been boosted tremendously by the development of the CRISPR/Cas9 technology. This powerful tool allows substantial improvement of plant traits in addition to those of classical breeding. Here we demonstrate the development of virus resistance in cucumber (*Cucumis sativus* L.) by utilizing Cas9/sgRNA technology to disrupt the recessive *eIF4E* gene function. Cas9/sgRNA constructs were targeted to the N' and C' termini of the *eIF4E* gene. Small deletions and SNPs were observed in the *eIF4E* gene targeted sites of T_1-generation transformed cucumber plants, but not in putative off-target sites. Non-transgenic heterozygous *eIF4E* mutant plants were selected for production of non-transgenic homozygous T_3-generation plants. Homozygous T_3 progeny following Cas9/sgRNA that had been targeted to both *eIF4E* sites exhibited immunity to *Cucumber vein yellowing virus* (ipomovirus) infection and resistance to the potyviruses *Zucchini yellow mosaic virus* and *Papaya ring spot mosaic virus-W*. In contrast, heterozygous-mutant and non-mutant plants were highly susceptible. For the first time, virus resistance has been developed in cucumber non-transgenically, not visibly affecting plant development, and without long-term backcrossing, via a new technology that can be expected to be applicable to a wide range of crop plants.

Keywords: Potyvirus, *eIF4E*, virus resistance, CRISPR/Cas9, cucumber, genome editing

Introduction

Plant viruses cause extensive reductions in crop yields worldwide. There are several paths to the development of virus resistance in crop plants. One path is classical plant breeding, by introgressing genes for virus resistance from crop-plant relatives. Another path is genome editing, which permits the introduction of alleles conferring resistance directly into the crop plants, without the many backcrosses required by classical breeding. As "gene edited crops" do not necessarily include transgenic segments (Xu et al. 2015), they probably would not need extensive regulation (Jones 2015), thereby opening a new publicly acceptable method for breeding of virus-resistant crops. RNA-guided genome editing using *Streptococcus pyogenes* CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) has renewed the concept of genome editing in plants (Belhaj et al. 2015). Cas9-induced double-strand breaks in the plant genome are mainly repaired by Non-Homologous End Joining (Li et al. 2013, Nekrasov et al. 2013). Cas9/sgRNA editing has been demonstrated in various plant families including Cruciferae, Solanaceae, Poaceae and Fabaceae (Belhaj et al. 2013, Bortesi and Fischer 2015).

Plant RNA viruses require host factors to maintain their life cycle. Many genes conferring resistance to viruses are recessive (Truniger and Aranda 2009), including the eukaryotic translation initiation factors *eIF4E* or *eIF(iso)4E* (Lellis et al. 2002). Viruses, especially potyviruses, can associate with one or both of those proteins, through the viral-encoded protein VPg (Duprat et al. 2002, Ruffel et al. 2006). In *Cucumis* spp. (cucumber and melon), one gene each of *eIF4E* and *eIF(iso)4E* have been identified (Rodríguez-Hernández et al. 2012). Both *eIF4E* and *eIF(iso)4E* are recessive when mutated, and are essential for the translation of uncapped viruses having the VPg protein covalently linked to the viral RNA 5′ (Wittmann et al. 1997). The association of natural mutations in the *eIF4E* and *eIF(iso)4E* genes with potyvirus resistance has been observed in various crops and applied to breeding (Gómez et al. 2009). Broad RNA virus resistance has been demonstrated by silencing of the *eIF4E* gene in tomato, *Solanum lycopersicum* L., and melon, *Cucumis melo* L. (Rodríguez-Hernández et al. 2012). Potyviridae cause significant losses in a wide range of crops. The viruses in this family have an RNA genome of approximately 10 kb polyprotein which is cleaved by three viral proteases, resulting in 9 to 11 putative mature pro-

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teins (Revers and García 2015). Mutations in the *VPg* gene have been shown to be associated with breaking natural resistance by a number of viruses (Moury et al. 2004, Hébrard et al. 2006).

Materials & Methods

CRISPR/Cas9 binary construct design

We used the pRCS binary vector which contained 35S:Cas9-AtU6:sgRNA-PDS, where the Cas9 gene was optimized for plant codon usage (Li et al. 2013, Nekrasov et al. 2013). The nptII (kanamycin) was the selection marker gene under the control of the 35S promoter.

eIF4E *sgRNA design and cloning*

The *eIF4E* gene (XM_004147349) of cucumber, *Cucumis sativus* L., was selected as the target gene. Each target sequence of 20 nucleotides was designed according to the criteria described (Mali et al. 2013) upstream of the NGG trinucleotide known as the protospacer adjacent motif (PAM). Two different sgRNA forward primers were designed for the *eIF4E* target gene. The sgRNA were cloned into *Sal*I and *Hind*III sites of the pRCS-35S:Cas9-AtU6:sgRNA-nptII binary plasmid.

Genotyping and mutant verification

The presence of the Cas9/sgRNA1/sgRNA2 transgene in T_0 lines was confirmed by PCR using specific primers. The transgenic lines were genotyped for indel polymorphisms using primers flanking the sgRNA1 or sgRNA2 of *eIF4E* target regions. PCR products were digested with restriction enzymes *Bmg*BI or *Bgl*II for sgRNA1 and sgRNA2, respectively. The digested products were separated on 1.5% agarose gel and the un-digested PCR products were excised, purified and cloned into the pJET1.2/blunt.

Inoculation of plant with viruses

Cucumber seedlings at the cotyledon stage with small true leaves were dusted with carborundum prior to mechanical inoculation with virus-bearing sap of ZYMV, PRSV-W, CMV, and CGMMV. CVYV inoculation was performed with whiteflies (*Bemisia tabaci*).

Evaluation of virus resistance

The response of the tested plants to virus infection was determined by visual monitoring of symptoms from 28 to 45 days post-inoculation following RT-PCR for the presence of viral RNA. Virus accumulation was determined by RT-PCR and reverse transcription – qPCR (qRT-PCR). RNA samples were collected from the second and third leaves of cucumber (2 leaf discs per plant). Total RNAs was extracted by TRI-REAGENT kit and adjusted to the same concentration prior to the RT-PCR, measured by a NanoDrop ND1000 spectrophotometer.

Results

Efficacy of CRISPR/Cas9 in T_0 generation

To test the efficacy of CRISPR/Cas9 in cucumber and develop a new strategy to generate virus resistance, we chose to disrupt *eIF4E*. In cucumber, two *eIF4E* genes have been identified, *eIF4E* (accession no. XM_004147349) (236 amino acids) and *eIF(iso)4E* (accession no. XM_004147116.2), which share 56% nucleotide and 60% amino acid homology, respectively. We targeted two regions in the cucumber *eIF4E* gene by Cas9/sgRNA, which have no homology in the *eIF(iso)4E* gene. The Cas9/sgRNA1 construct was designed to target the sequence in the first exon of *eIF4E* (Figure 1A). The Cas9/sgRNA2 construct was designed to target the third exon to allow translation of approximately two-thirds of the protein, perhaps without disrupting all of its functions (Figure 1A). Five independent T_0 transgenic lines were generated by *Agrobacterium*-mediated transformation. The presence of the transgene (Cas9/sgRNA) was confirmed by kanamycin resistance and PCR using sgRNA specific primers (Figure 1B). Three lines (1, 3, 4) were identified as transgenic with Cas9/sgRNA1. To evaluate the types of mutations generated in sgRNA1 transgenic plants, PCR was performed in T_0 plants using primers flanking the sgRNA1 target and subsequently digested with *Bmg*BI (a site that would disappear if CAS9 and NHEJ were active in this location). In line 1, a distinct undigested fragment was observed following *Bmg*BI restriction (Figure 1B). The partial digestion observed indicated a heterozygous genome with both wild-type and mutant *eIF4E* alleles. Cloning and sequencing of the uncut *Bmg*BI fragment showed two types of mutations, a 20 nt deletion around the PAM sequence in seven colonies and 1 nt deletion 3 bp upstream of the PAM sequence in two colonies (Figure 1C). Whereas in lines 4 (Figure 1B) and 3 (data not shown) the amplified PCR was completely digested by *Bmg*BI, two additional Cas9/sgRNA2 transgenic plants (2 and 5) did not show genome editing in T_0 as determined by PCR and restriction analysis with *Bgl*II (Figure 1B and data not shown). We continued our study with two sgRNA1 lines (1 and 4) designated CEC1-1 and CEC1-4, respectively, and one sgRNA2 line (no. 5) designated CEC2-5.

Genotypes and segregation of T_1 mutants of CEC1-1

For propagation by seeds, a *Cucumis sativus* CEC1-1 T_0-mutant plant derived from 'Ilan', a multi-pistillate, parthenocarpic greenhouse cucumber, was cross-pollinated with 'Bet Alfa', a monoecious, non-parthenocarpic, open field cucumber. Indel polymorphisms were genotyped by PCR restriction analysis with *Bmg*BI of the *eIF4E* gene in representative CEC1-1 T_1 plants (Figure 2). The T_1 progeny segregated into three groups: (a) heterozygous plants that contained about equal amounts of undigested and digested DNA (plants 5, 8, 12, 16); (b) plants with undigested DNA of intensity stronger than that of the digested DNA intensity (plants 2, 9, 7, 20); (c) non-mutants (wild type), with most of the DNA digested (plants 3, 10). To evaluate the types of mutations generated in CEC1-1 T_1 plants, four representative plants (nos. 1, 4, 7 non-transgenic lacking Cas9 transgene) and no. 5 (transgenic) were chosen and the undigested DNA was cloned and sequenced. Plant no. 1 had a 20 nt deletion and plant nos. 4 and 5 had 1 nt deletions (Figure 2B). Plant no. 7 had both the 20 nt and 1 nt deletions as observed in the T_0 (Figure 1C). The non-transgenic CEC1 T_1 plant no. 7 (CEC1-1-7) was grown to produce seeds for the production of homozygous *eIF4E* mutant alleles. The all-pistillate CEC1-1-7 plant was cross-pollinated once again with the monoecious 'Bet Alfa'. The resulting T_2 progeny were genotyped and plants hemizygous for a 20 nt deletion (plant no. 1, Figure 2B) (CEC1-1-7-1) and 1 nt deletion (plant no. 4, Figure 2B) (CEC1-1-7-4) were

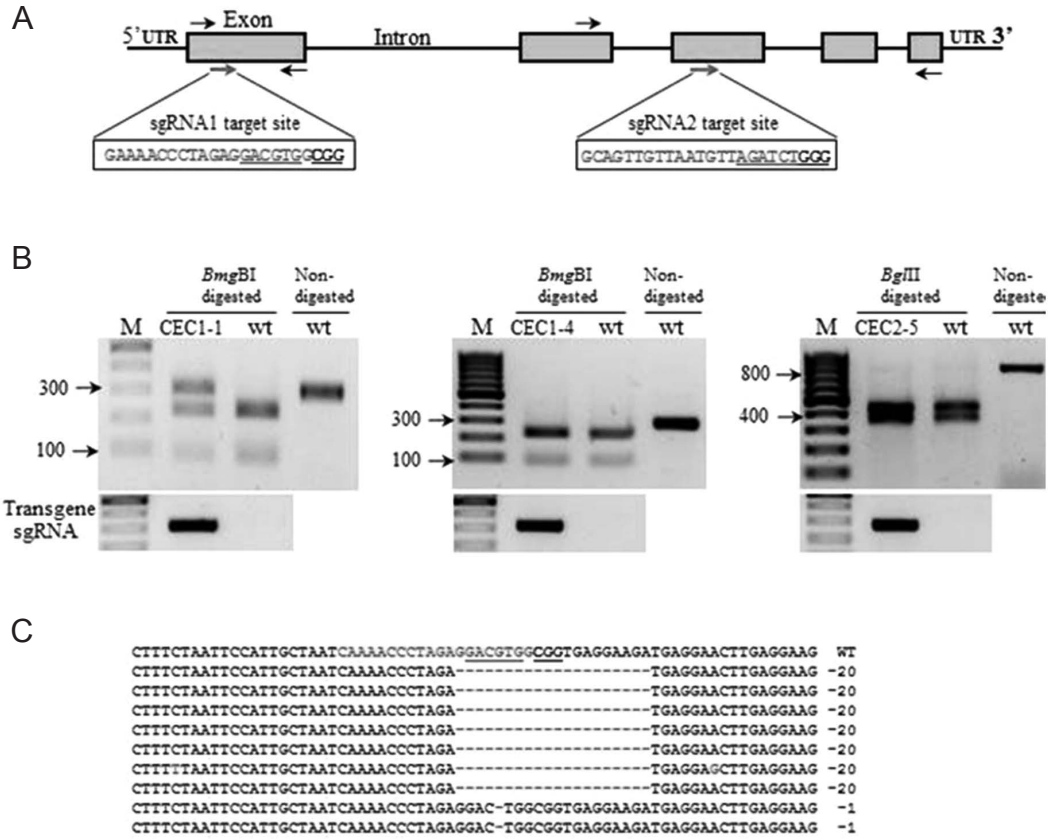


Figure 1. Gene editing of *eIF4E* mediated by CRISPR/Cas9 in transgenic cucumber plants.
(A) Schematic representation of the cucumber *eIF4E* genomic map and the sgRNA1 and sgRNA2 target sites (light grey arrows). The target sequence is shown in light grey letters together with the restriction site (underlined). The black arrows indicate the primers flanking the target sites used to detect the mutations. (B) Restriction analysis of T_0 PCR fragments of CEC-1, CEC1-4, and CEC2-5. (C) Alignment of 9 colony sequences from the undigested fragment of the top line with the wild-type (wt) genome sequence. DNA deletions are shown in grey dashes.

self-pollinated to obtain a T_3 generation. The homozygous, non-transgenic T_3 plant designated CEC1-1-7-1, heterozygous, and non-mutant (wt) plants were tested for virus resistance.

Virus resistance analysis

To test whether CRISPR/Cas9-mediated mutations in *eIF4E* confer virus resistance, T_3 progenies of CEC1 and CEC2 seedlings were inoculated with CVYV-*ipomovirus*, two potyviruses ZYMV and PRSV-W, *Cucumber mosaic cucumovirus* (CMV), and *Cucumber green mottle mosaic tobamovirus* (CGMMV). T_3 non-transgenic progenies of CEC1-1-7-1 showed a Mendelian segregation ratio of 1 : 2 : 1 for the homozygous mutant allele (*eif4e*), heterozygote mutant allele, and homozygous non-mutant. In the case of CEC2-5-M-8,9,16,21 (mixture of 4 lines: 8,9,16,21) (designated CEC2-5-M-8,9,16,21), the T_3 progenies of plants 9 and 16 segregated 1 : 1 (homozygous : heterozygous) and plants 8 and 21 segregated 1 : 2 : 1 (homozygous : heterozygous : non-mutant).

CVYV resistance analysis

Whitefly inoculation of the T_3 generation with CVYV showed that both CEC1-1-7-1 and CEC2-5-M-8,9,16,21 homozygous mutant plants were immune to CVYV infection (0/20 and 0/32), whereas in the heterozygous mutant and wild-type plants severe symptoms were observed 7 to 10 days past inoculation (dpi) (Table 1, Figure 3A). The mutant homozygous plants remained healthy through 45 dpi. The experiment was repeated four times with consistent results (Table 1). RT-PCR analyses revealed no viral RNA accumulation in the homozygous mutant plants, whereas in the heterozygous plants the amount of viral RNA accumulation was similar to that of the wild-type (Figure 3B).

ZYMV resistance analysis

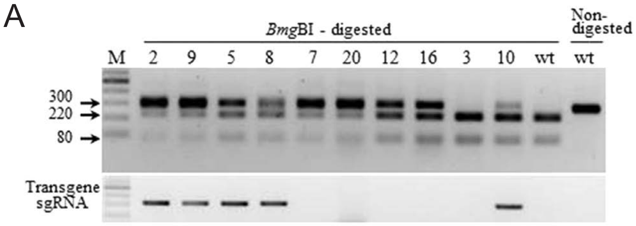
Following ZYMV inoculation (mechanical and by aphids) of CEC1-1-7-1 and CEC2-5-M-8,9,16,21 T_3 lines, seedlings showed mosaic symptom development 7 to 10 dpi that exacerbated to severe symptoms of leaf deformation and stunting 20 dpi in heterozygous and non-mutant plants (Figure 4A), whereas, the *eif4e* mutant plants did not display disease symptoms 25 dpi. Accordingly, resistance to ZYMV systemic infection was observed (0 of 28 plants were infected) in four separate biological repeat experiments (Table 1). However, at 25 to 45 dpi, mild symptoms could be observed in 48% (10 out of 21 plants) of the T_3 homozygous plants in three biological experiments with CEC1-1-7-1 and in 25% of the CEC2-5-M-8,9,16,21 plants (16 out of 63 plants, Table 1). However, the mild symptoms appeared only in patches (Figure 4A) and the plants developed normally similar to the non-infected plants, compared to the stunted

plants with deformed fruit of the infected heterozygous and wild-type plants. ZYMV RNA was not detected in the immune homozygous resistant plants (Figure 4B). The late appearance of mild symptoms was accompanied by accumulation of ZYMV RNA in the upper leaves (plants 4 and 10, Figure 4B). The level of ZYMV RNA accumulation in mild symptomatic plants was lower in CEC1-1-7-1 and CEC2-5-M-21 homozygous plants than in the wild type (Figure 4C). Interestingly, resistance breaking was not observed when inoculation was made by the natural vector *Aphis gossypii* (Table 1).

Discussion

A major goal of plant biotechnology is to improve crop yield and quality in a sustainable manner, more rapidly than classical breeding that may require many generations. However, difficulties meeting regulatory requirements and the public opposition to transgenic plants have limited the implementation of these biotechnology methods. For this purpose, the development of an efficient plant genome editing by CRISPR/Cas9 opens many opportunities for crop improvement. Here, for the first time using the CRISPR/Cas9 editing technology, we have developed *eIF4E* non-transgenic cucumber mutants that exhibited resistance to three economically important viruses. We designed two Cas9/sgRNA constructs to target the cucumber *eIF4E* gene; one (sgRNA1) was expected to disrupt the intact eIF4E protein, and the other (sgRNA2) permit translation of 2/3 of the protein product. In *Agrobacterium*-transformed T_0 lines, we found deletions in the *eIF4E* target gene in one line (CEC1-1) out of five (Figure 1 and data not shown). In the transgenic T_0 of the CEC2-5 line, a mutation was not detected by PCR or restriction analysis (Figure 1B), although in the T_1 generation (data not shown) homozygous, heterozygous, and non-mutant plants were observed; mutations in homozygous plants were bi-allelic, with two mutations in the same plant. The differences in Cas9 targeting between the three T_0 lines (CEC1-1, CEC1-2, and CEC2-5) may be due to differential

A



B

Plant no. 1
```
CTAATTCCATTGCTAATCAAAACCCTAGAGGACGTGGCGGTGAGGAAGATGAGGAACTTGAGGAAG WT
CTAATTCCATTGCTAATCAAAACCCTAGA--------------------TGAGGAACTTGAGGAAG -20
CTAATTCCATTGCTAATCAAAACCCTAGA--------------------TGAGGAACTTGAGGAAG -20
CTAATTCCATTGCTAATCAAAACCCTAGA--------------------TGAGGAACTTGAGGAAG -20
CTAATTCCATTGCTAATCAAAACCCTAGA--------------------TGAGGAACTTGAGGAAG -20
```
Plant no. 4
```
CTAATTCCATTGCTAATCAAAACCCTAGAGGACGTGGCGGTGAGGAAGATGAGGAACTTGAGGAAG WT
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
```
Plant no. 5
```
CTAATTCCATTGCTAATCAAAACCCTAGAGGACGTGGCGGTGAGGAAGATGAGGAACTTGAGGAAG WT
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
```
Plant no. 7
```
CTAATTCCATTGCTAATCAAAACCCTAGAGGACGTGGCGGTGAGGAAGATGAGGAACTTGAGGAAG WT
CTAATTCCATTGCTAATCAAAACCCTAGA--------------------TGAGGAACTTGAGGAAG -20
CTAATTCCATTGCTAATCAAAACCCTAGA--------------------TGAGGAACTTGAGGAAG -20
CTAATTCCATTGCTAATCAAAACCCTAGA--------------------TGAGGAACTTGAGGAAG -20
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
CTAATTCCATTGCTAATCAAAACCCTAGAGGAC-TGGCGGTGAGGAAGATGAGGAACTTGAGGAAG -1
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Figure 2. Genotyping of *eIF4E* mutants in representative T_1 progeny plants of CEC1-1. (A) PCR restriction analysis of Cas9/sgRNA1-mediated mutations (upper-panel) and transgene insertion (lower panel) in 10 representative T_1 cucumber plants and non-mutant wild-type (wt). (B) Alignment of 4 representative *eIF4E* mutant plants with wild-type sequence. Sequences of each plant represented clones from undigested fragments. The target sequence is shown in light grey letters and the PAM motif marked by bold underlined letters. DNA deletions are marked in ligth grey dashes and deletion sizes (nt) are indicated on the right side of the sequence.

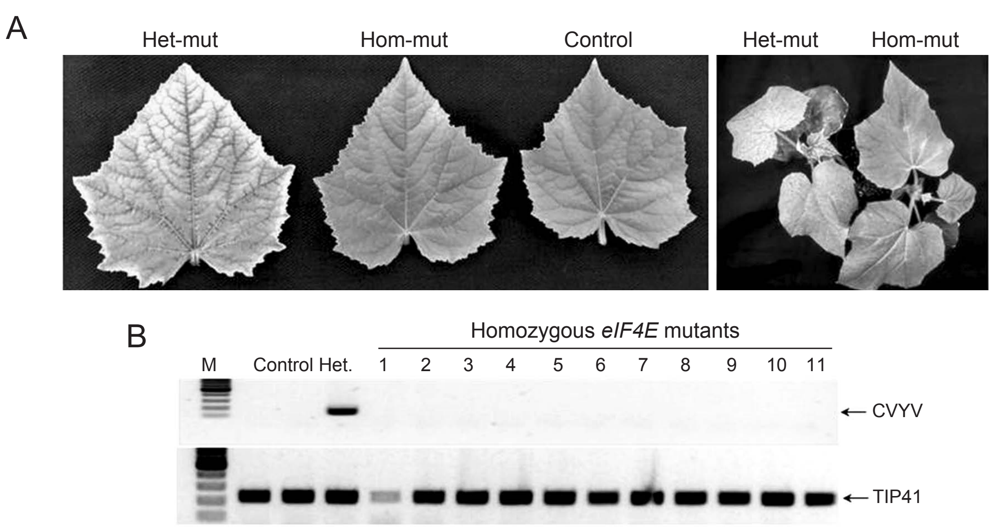

Figure 3. Homozygous *eif4e* mutant plants exhibited immunity to CVYV infection. (A) Disease symptoms of heterozygous (Het-mut), homozygous (Hom-mut), and non-inoculated plants (control) of CEC1-1-7-1 T_3 generation, 10 dpi. (B) RT-PCR analysis of CVYV RNA accumulation 14 dpi in a homozygous *eIF4E* mutant plant and a non-inoculated plant (control), 14 dpi. The *TIP41* was used as a reference gene for RT-PCR amplification.

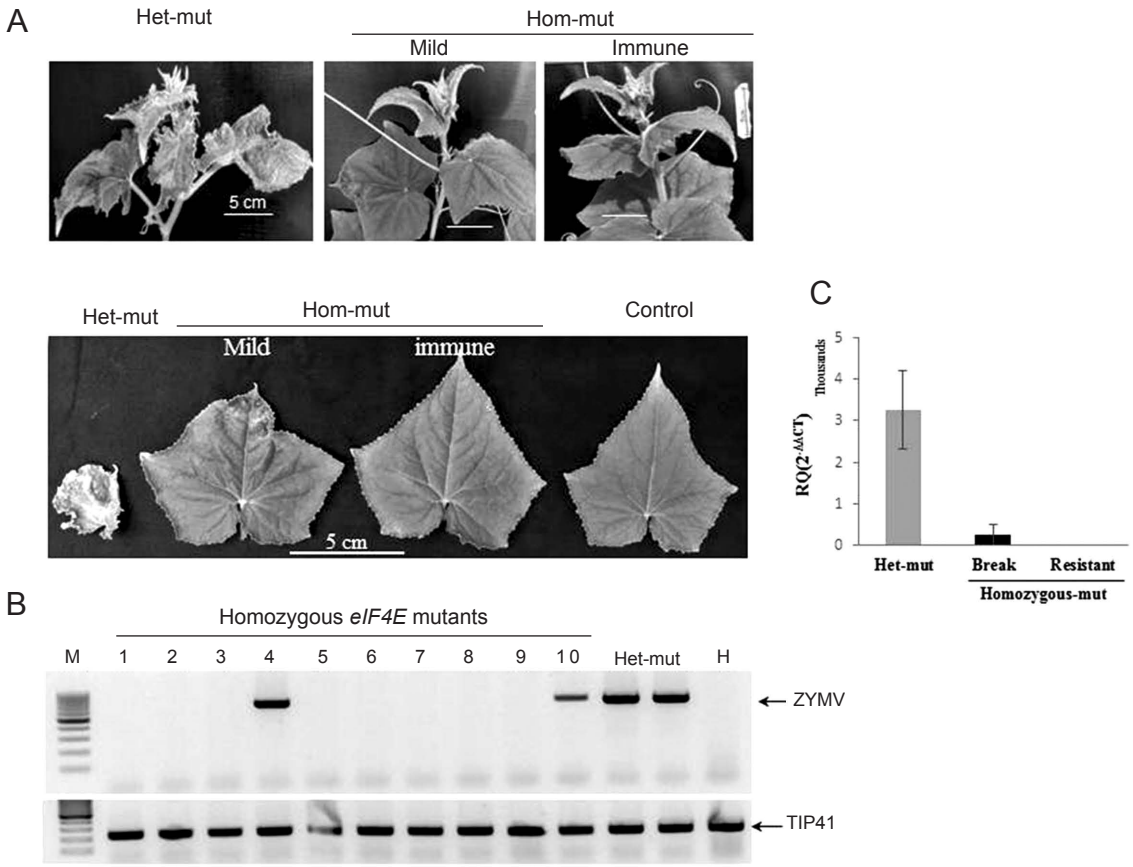

Figure 4. Homozygous *eIF4E* mutant plants exhibited resistance to ZYMV infection. (A) Disease symptoms of heterozygous (Het-mut), homozygous (Hom-mut) and non-inoculated plants (control) of the CEC1-1-7-1 T_3 generation 25 dpi. (B) RT-PCR analysis of ZYMV RNA accumulation in homozygous *eIF4E* mutant plants (1 to 10), heterozygous plants (Het-mut) and non-inoculated plants (H) at 14 dpi. *TIP41* was used as a reference gene for RT-PCR amplification. (C) Relative (qRT-PCR) ZYMV RNA accumulation in CEC1-1-7-1 heterozygous (Het-mut) and 2 classes of homozygous mutants: resistant (resistant) and breaking (Break). RNA was extracted from three plants (third top leaf) and the ZYMV level was calculated using the ΔΔCt method normalized to *F-box* gene expression level.

activities of Cas9 in different transgenic lines, depending on transgene insertion sites. The T_3 progeny plants of the non-transgenic CEC1-1-7-1 and CEC2-5-M-8,9,16,21 lines segregated for *eIF4E* mutants into homozygous, heterozygous, and non-mutant. This allowed an evaluation of virus resistance with internal susceptible controls. Progeny from the two independent lines CEC1-1-7-1 and CEC2-5-M-8,9,16,21 showed immunity to CVYV infection by the natural whitefly vector. As CVYV-RNA was not detected in inoculated and systemic leaves at different times post inoculation, we assume that initial viral translation probably could not be established in the cells containing virus particles. Similar resistance to CVYV has been shown in transgenic melon in which the *eIF4E* gene was silenced (Rodríguez-Hernández et al. 2012). This demonstrates that *eIF4E* has a crucial function in the CVYV life-cycle in *Cucumis* spp. (cucumber and melon); notably, the eIF4E protein of cucumber shares 99% similarity with that of melon. Resistance to ZYMV and PRSV-W were detected in the two *eIF4E* homozygous lines CEC1-1-7-1 and CEC2-5-M-8,9,16,21 (Table 1). For ZYMV, resistance breaking was observed late in infection (25 dpi) in some T_3 progenies, which was associated with low virus titer, mild symptoms, and plant development similar to uninfected cucumber. Two explanations may account for the breaking of resistance in *eif4e* homozygous mutant plants: (a) ZYMV can use *eIF(iso)4E* with less efficiency than *eIF4E* for replication and systemic movement, and therefore is able to cause mild symptoms, and (b) a mutation occurred in the ZYMV genome which allowed interaction of ZYMV with *eIF(iso)4E*. Interestingly, resistance overcome by ZYMV was not observed by inoculation with the natural aphid vector. This suggests that the few virus particles transmitted by aphids may not be enough to overcome the *eIF4E* mutation.

Conclusions

Here we show for the first time that CRISPR/Cas9 is an efficient tool for genome editing in cucumber. Disruption of the *eIF4E* gene in cucumber by CRISPR/Cas9sgRNA led to development of virus-resistant plants without otherwise affecting the plant genome. Three generations of backcrossing produced virus-resistant plants free of genetic modification, and thus would be considered safe for human consumption and for release into the environment. We believe that this novel technology has the potential for expediting development of pest resistance in many

Table 1. Response of T_3 generation plants of non-transgenic CEC-1-7-1 and CEC2-5-M-8,9,16,21 lines to CVYV, ZYMV, PRSV-W, CMV, and CGMMV infection at different days post infection (dpi).

Virus	CEC1-1-7-1		
	Homozygous[¤]		Non-homozygous[§]
	14 dpi	25-45 dpi*	14 dpi
CVYV	0/20	0/12	60/60
ZYMV	0/28	10/21 (0.48 ± 0.04)[¤]	97/97
PRSV	1/14	n.t[◊]	40/41
CMV	11/11	-	9/9
CGMMV	10/10	-	10/10
	CEC2-5-M-4n		
	Homozygous		non-homozygous
CVYV	0/32	0/10	42/43
ZYMV	0/64	16/63 (0.26 ± 0.05)[¤]	67/69
ZYMV[º]	0/8	0/8	6/7
PRSV	1/55	7/18	33/37

crops without the need for extensive backcrossing and genetic manipulation with wild sources of resistance.

Acknowledgements

The authors are grateful to Dr. Moshe Lapidot for performing the CVYV inoculations by whitefly, and Drs. Victor Gaba and Harry S. Paris for critical reading of the manuscript. This research was supported by the Chief Scientist of the Israeli Ministry of Agriculture and Rural Development (Project 20-10-0039).

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QTL Mapping of Cucumber Parthenocarpy by QTL-Seq

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Abstract. Cucumber is one of the most important vegetable crops worldwide. Parthenocarpy means fruit setting and development without pollination, fertilization, or any other treatment, and it plays an important role in determining yield and quality in the production of cucumber, especially in greenhouse conditions. However, understanding the molecular genetic mechanisms underlying parthenocarpy of cucumber was limited up to now. In this study, QTL-seq, a rapid high-resolution genome-wide strategy, combining BSA (bulk segregant approach) with whole genome sequencing, was applied to identify the QTLs of parthenocarpy in cucumber. An F_2 genetic population derived from a cross between DDX (gynoecious and parthenocarpic) and Zaokang (gynoecious but non-parthenocarpic) was used for the QTL mapping of cucumber parthenocarpy. We detected a 1.62-Mb-long quantitative trait locus (QTL) on chromosome 2 containing 296 genes. Based on the cucumber genome and function analysis, nine genes including *Csa2M349050.1*, *Csa2M350390.1*, *Csa2M351550.1*, *Csa2M354670.1*, *Csa2M354750.1*, *Csa2M355020.1*, *Csa2M353460.1*, *Csa2M354910.1*, and *Csa2M354980.1* are predicted as candidate genes for parthenocarpy of cucumbers. Eight of the nine genes are involved in hormone synthesis, regulation, or signal transduction, and the other one was shown to be involved in cell division, the same as in previous studies. Our results are an important step forward in deepening the understanding of the genetic mechanisms for parthenocarpy in cucumber.

Keywords: Cucumber, parthenocarpy, QTL-seq, candidate genes

Introduction

Cucumber (*Cucumis sativus* L.; 2n = 2x = 14) is one of the most agriculturally and economically valuable vegetable species of the Cucurbitaceae (Yang et al. 2012). However, the fruit setting of cucumber depends on the successful completion of pollination and fertilization which is affected by many environmental factors (Picken 1984). Parthenocarpy is the process of development of seedless fruits without pollination, fertilization, or any other treatment, and many species or varieties have natural parthenocarpy capacity such as cucumber (Cui et al. 2014), zucchini (Martinez et al. 2014), tomato (Marti et al. 2007), and pear (Nishitani et al. 2012).

A few attempts have been made to detect QTLs related to parthenocarpy in cucumber. Sun et al. (2006a,b) reported that parthenocarpy in cucumber under open-field conditions is quantitatively controlled by at least two genes. Furthermore, they detected 10 QTLs for parthenocarpy in four genomic regions, three of which also mapped to the same genomic regions as QTLs detected for fruit yield at first harvest (Sun et al. 2006c). Li et al. (2014) investigated the transcriptome of cucumber fruits and compared the transcriptional events occurring in fruits of different cucumber accessions based on the 14 genes that were predicted as putative parthenocarpic genes.

Conventional QTL mapping is labor-intensive, time-consuming, and costly (Salvi and Tuberosa 2005). Recently, Takagi et al. (2013) described the QTL-seq approach for rapid mapping of quantitative trait loci by whole-genome resequencing of DNA bulks of phenotypic extremities in rice. In this study, we used the QTL-seq approach to detect a genomic region in cucumber harbouring the major QTL for parthenocarpy and have made an important step forward in deepening the understanding of the genetic mechanisms for parthenocarpy in cucumber.

Materials & Methods

Two cucumber inbred lines, 'DDX' (gynoecious and parthenocarpic) and 'Zaokang' (gynoecious but non-parthenocarpic) were chosen as parents. We used P_1, P_2, and an F_2 population of 762 individuals for the QTL mapping of the parthenocarpy of cucumber in March 2015. All materials were planted on the experimental farm at the School of Horticulture and Plant Protection at Yangzhou University in China.

The parthenocarpic percentage of each plant was calculated by the number of fruits fully developed/total number of female flowers. Young healthy leaves from the two parents and F_2 individuals were collected for DNA extraction. For QTL-seq, by mixing an equal amount of DNA from 50 of the 762 F_2 plants, two DNA pools, the P-pool (parthenocarpic pool) and the

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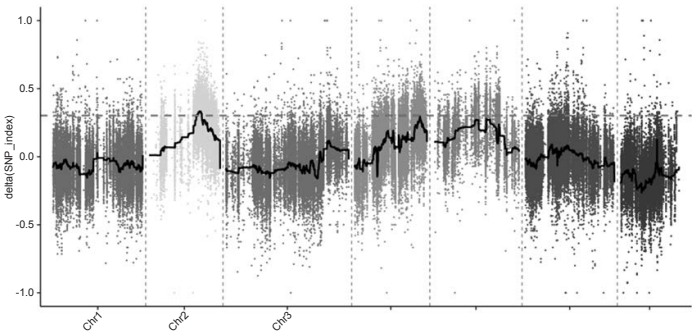

Figure 1. ΔSNP-index in the whole genome.

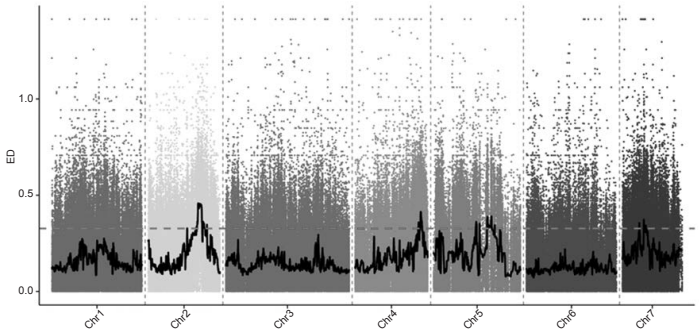

Figure 2. ED value in the whole genome.

NP-pool (non-parthenocarpic pool) were constructed. Finally, the two bulks were pooled per lane on the Illumina HiSeq 2500 at the Biomarker Technologies Corporation (Beijing, China).

Results

Mapping of a major QTL using QTL-seq

High-throughput sequencing resulted in 126 Gb of data and all the values of Q30 were above 87%, with an average depth of 33x to 64x, covering 99% of the reference genome. As shown in Figure 1, the x-axis represents the chromosome position, and the y-axis represents the Δ(SNP_index) values. Black lines are the average values of Δ(SNP_index). The dotted line is the threshold value, which was calculated by Loess regression. Peak regions are defined as regions where the Loess fitted values are greater than the threshold value. Similar to Δ(SNP_index), the value of ED was figured and regions over 99% of its threshold value were also chosen as candidate QTLs (Figure 2). According to the intersection of the two arithmetics, a QTL named *cp1* containing 296 genes was located, spanning 1.6 Mb from 15,589,893 to 17,206,312 bp between two SNP markers, SNP9893 and SNP6312, on chromosome 2 in cucumber.

Classification and preliminary prediction of genes for parthenocarpy

A COG (Clusters of Orthologous Groups) database was constructed based on the evolutionary relationships of bacteria, algae, and eukaryotic phylogeny. By COG functional annotation,

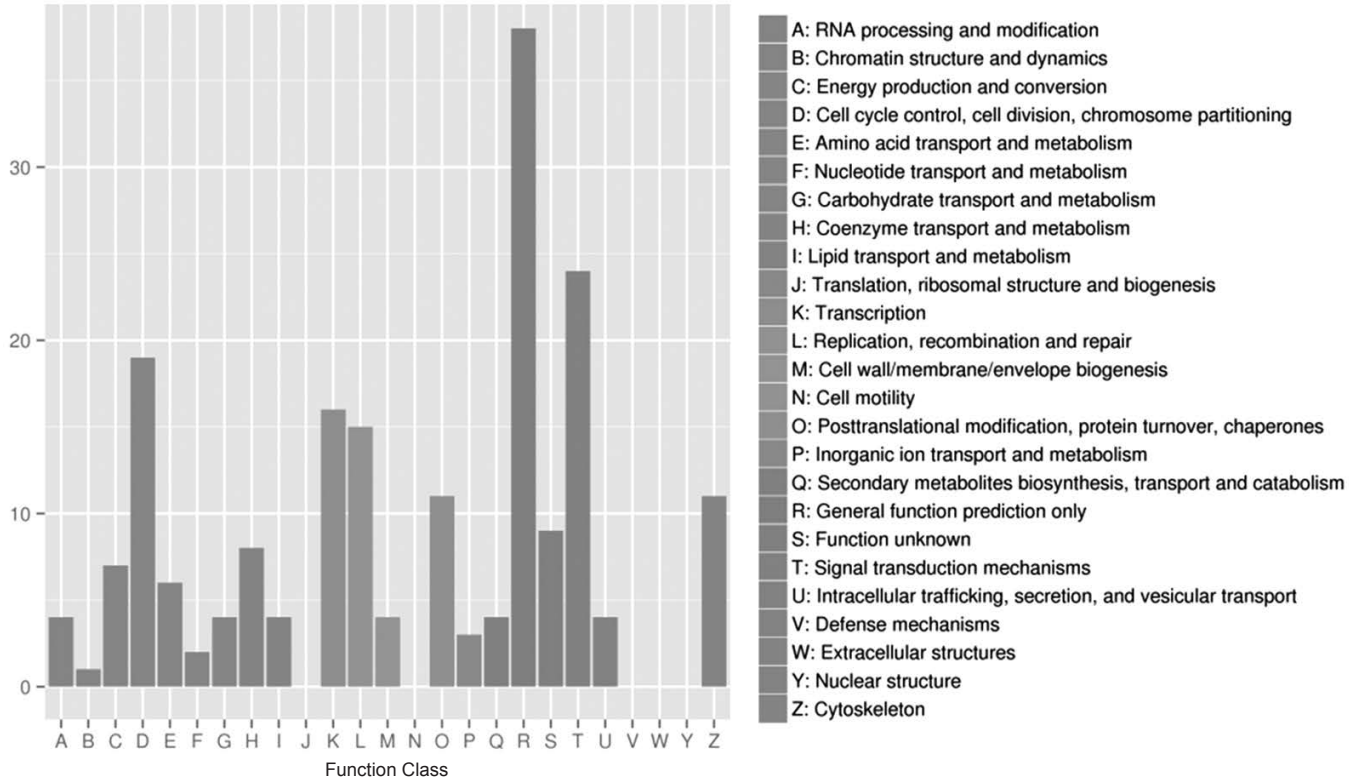

Figure 3. Gene COG annotation classification.

the 296 genes contained in *cp1* were classified into 25 groups such as group A (RNA processing and modification), group B (Chromatin structure and dynamics), group C (Energy production and conversion) and so on (Figure 3).

Except the group R without clear gene function annotations, a total of 24 genes were divided into group T, which play an important role during hormone perception and signal transduction. Among these genes, *Csa2M349050.1*, *Csa2M350390.1*, *Csa2M351550.1*, *Csa2M354670.1*, *Csa2M354750.1*, and *Csa2M355020.1* belong to the F-box gene family, which influences self-incompatibility, phytochrome signaling, and responses to plant growth regulators like auxins, ethylene, and GAs (Yang et al. 2008). Moreover, *Csa2M353460.1* regulates rate-limiting enzymes catalyzing the biosynthesis of ethylene, and *Csa2M354910.1* is one of the members of the gibberellin-regulated gene family. Group D followed group T and has almost 20 genes that are related to cell-cycle control, cell division, and chromosome partitioning. Out of these genes, *Csa2M354980.1* belongs to the cyclin gene family which has been shown to be involved in cell division and growth in *Arabidopsis*. The cyclin family of proteins is thought to regulate the cell cycle by interacting with cyclin-dependent kinases (Dong et al. 2011). In summary, we suggest that *Csa2M349050.1*, *Csa2M350390.1*, *Csa2M351550.1*, *Csa2M354670.1*, *Csa2M354750.1*, *Csa2M355020.1*, *Csa2M353460.1*, *Csa2M354910.1*, and *Csa2M354980.1* may be candidate genes for the major QTL controlling parthenocarpy in cucumber.

Discussion

In the present study, we identified and mapped one major genomic region for parthenocarpy in cucumber on chromosome 2, using BSA combined with QTL-seq. There are 296 genes in *cp1*. Eight of the candidate genes are involved in hormone synthesis, regulation, or signal transduction, and the other one has been shown to be involved in cell division. Previous studies have showed that phytohormones including auxins, GAs, cytokinins (CKs), and others induce parthenocarpy in various plant species (Boonkorkaew et al. 2008, Vriesen et al. 2008, de Jong et al. 2009, Zhang et al. 2010, Ding et al. 2013). Auxins were considered to be the primary regulatory hormone for cucumber parthenocarpic fruit set, and the transcription of cytokinin-related and gibberellin-related genes can be regulated by the auxin-related genes (Li et al. 2014). Gibberellins are closely associated with parthenocarpic fruit development in Japanese pears (Nishitani et al. 2012). Additionally, the application of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) to pollinated ovaries reduced fruit setting in tomato (Shinozaki et al. 2015).

In our study, QTL-seq delimited the parthenocarpy QTL *cp1* to a 1.6 Mb physical interval with 296 genes on chromosome 2 in cucumber and we finally found nine genes that most likely confer parthenocarpy. But the current detected QTL interval is still too large to pinpoint the key gene determining parthenocarpy in cucumber. So, further fine mapping of the QTL interval will soon continue.

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A Chromosome Translocation Leads to Gynoecy in Watermelon

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Abstract. The Cucurbitaceae have become model plants in sex determination research, as this family has all seven sex forms found in angiosperms. With the Chinese discovery of a watermelon *Citrullus lanatus* gynoecious mutant, designated XHBGM, we report here the inheritance of watermelon sex expression. In this research, we used map-based cloning to characterize the gene for gynoecy (*gy*) from gynoecious mutant XHBGM. The results show that polymorphism markers associated with the *gy* locus are positioned on Chromosome 2 (Chr. 2) and Chromosome 3 (Chr. 3). As suggested by a bioinformatics study and verified by PCR and FISH analyses, a reciprocal chromosome translocation in the XHBGM genome was identified. The translocation breakpoints were located at the genomic positions Chr. 2: 33348296 and Chr. 3: 4043234..4043236. The translocation breakpoint on Chr. 2 is in the coding region of a C_2H_2 zinc finger transcriptional factor gene (gene ID: *Cla008537*), which was the orthologous to the melon gene *g*, *CmWIP1*, and thus was named *ClWIP1*. Functional studies showed that *ClWIP1* is expressed specifically in carpel primordia and is related to the abortion of carpel primordia in early developmental stages. The chromosome translocation in XHBGM suppressed the transcription of *ClWIP1*, hampered the abortion of carpel primordia, and led to gynoecy.

Keywords: *Citrullus lanatus*, sex determination, chromosome translocation, gynoecious

Introduction

The polymorphism of sex determination in angiosperms is determined by the presence/absence of the three sexual type flowers, male, female, and hermaphroditic, and their distributions (Dellaporta and Calderon-Urrea 1993). The Cucurbitaceae have currently become the model plants in sex determination research, as cucurbits can have all seven sex forms in angiosperms. The seven sex forms in angiosperms are as follows: monoecious, andromonoecious, trimonoecious, gynoecious, gynomonoecious, hermaphrodite, and androecious. In watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, monoecy is the common sex form, though five other sex forms, all except androecious, have been found (Ji et al. 2015).

The formation of gynoecy in cucumber, *Cucumis sativus* L., is controlled by the *F* gene *CsACS1G*, the paralog gene of *CsACS1* (Mibus and Tatlioglu 2004). A zinc finger transcription factor, *CmWIP1*, was also reported to control gynoecy formation in melon, *C. melo* L. (Martin et al. 2009). In a recent study, *CsWIP1*, the orthologous gene of *CmWIP1*, was also shown to be associated with gynoecy in cucumber (Boualem et al. 2015). The mutation in *CsACS11* and *CmACS11* lead to the androecy in cucumber and melon (Boualem et al. 2015). The sex determination systems in melon have been suggested as follows: *CmACS11*, the gene for androecy, acts upstream and negatively regulates the expression of *CmWIP1*, and the stamen inhibitor *CmACS-7* is expressed while *CmWIP1* is not being expressed. Also, it seems that melon and cucumber possess a conserved sex determination system (Boualem et al. 2015).

Gynoecy in watermelon was unknown until a natural gynoecious mutant, designated XHBGM, was found in China (Jiang and Lin 2007). A recessive allele, symbol *gy*, confers gynoecy (Jiang and Lin 2007, Ji et al. 2015). In this study, we used map-based cloning to identify the gene for gynoecy (*gy*) in XHBGM. The *gy* gene encoded a transcription factor. Also the mechanism of watermelon sex determination will be discussed, as will the expectations of the usefulness of the gynoecious trait for watermelon breeding.

Materials & Methods

Plant material and segregating populations

The andromonoecious accession AKKZW (*C. lanatus* subsp. *mucosospermus* Fursa) was crossed with accession XHBGM (*C. lanatus* subsp. *vulgaris* Schrad.), and the F_1 was backcrossed to XHBGM to obtain a first-generation backcross (BC_1)

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population, in order to map *gy*. Sex of the flowers on the first 30 nodes of all plants was observed (Ji et al. 2015). Leaves of individual plants were sampled, and DNA was isolated.

Genomic mapping for the gy *locus*

Ten monoecious plants and 10 gynoecious plants were selected randomly for preliminary mapping of the *gy* allele. Insertion-deletion (InDel) markers were developed by the genomic difference between accessions AKKZW and XHBGM. On the basis of the preliminary mapping of *gy*, more single nucleotide polymorphisms (SNPs) or Indel markers, which also used genomic comparison between AKKZW and XHBGM, were developed, for a chromosome walking experiment.

Fluorescent in situ hybridization and image processing

Bacterial artificial chromosomes (BAC) library screening, DNA probe preparation, and chromosome preparation were conducted according to Ren et al. (2012). Global image adjustments for contrast, brightness, and color saturation were done with Photoshop 6.0.

Quantitative RT-PCR

Total RNA was extracted from frozen leaves, stems, fruits, and flowers by using the EASY spin Plus Plant RNA Kit (Qiagen). First-strand cDNA was synthesized and PCR was performed in a LightCycler 480II apparatus. PCR conditions were as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s. Three independent biological experiments were performed. The primers are listed in Table 1.

RACE-PCR experiment

The 5' end of the *ClWIP1* cDNA was determined with the 5'-Full RACE Kit with TAP and the 3' end was determined with the 3'-Full RACE Core Set with PrimeScript™ RTase (Takara). The primers used in the RACE-PCR are listed in Table 1. In accordance with the manufacturer's instructions, two nested PCRs were performed. RACE reaction products were gel purified and sequenced.

In situ *hybridization*

ClWIP1 in situ hybridization was performed using the method reported by Liu et al. (2013). The primers used for this experiment are listed in Table 1.

Results

Map-based cloning of the gy *gene in XHBGM*

A spontaneous gynoecious mutant, XHBGM, was found in accession XHB (*Citrullus lanatus* subsp. *vulgaris* Schrad.). This mutant displayed female flowers at all early (25 to 30) nodes and developed a few male flowers later in the growing period. The normal type XHB was monoecious, having separate male and female flowers on the same plant. The normal and mutant phenotypes segregated at an approximately 3 : 1 ratio, respectively, in the F_2 population (n = 271) obtained by crossing XHB and XHBGM (199 monoecious : 72 gynoecious, chi-square = 0.355, P = 0.55). This result confirms that the mutant phenotype is indeed conferred by a single recessive gene, *gy*.

To isolate *gy*, a mapping population was constructed by crossing XHBGM with an andromonoecious accession, AKKZW. In order to make a preliminary map for the *gy* locus, 10 monoecious plants and 10 gynoecious plants were chosen randomly in the BC_1 population. The markers which were used for mapping were obtained from the re-sequencing results of the parent accessions compared with the watermelon genomic sequencing drafts (Guo et al. 2013). There were 6,854 SNP/InDel positions between accessions AKKZW and XHBGM. Ten markers per chromosome were chosen to map the *gynoecious* (*gy*) locus (Figure 1A). The polymorphism analysis showed that two InDel markers were associated with gynoecious sex form, one on chromosome 2 (Chr. 2) and the other on Chr. 3 (Figure 1A). Furthermore, we developed more SNP/InDel markers in these two regions to target the *gy* locus in all individual plants from the BC_1 population. The results showed that the markers wmgy_indel_1 and wmgy_CAPs_MboII co-segregated with gynoecy in the backcross population, and both of them were located on Chr. 2 (Figure 1B). However, the marker wmgy_indel_3 was located on Chr. 3 and associated with gynoecy closely, having only one recombinant plant. This result suggested that *gy* might be located on Chr. 2 and Chr. 3 (Figure 1B).

XHBGM has a translocation between Chromosome 2 and Chromosome 3

In order to clarify the *gy* gene localization and sequence, we analyzed the genomic re-sequence data of XHB and XHBGM, focusing on these two candidate regions. The results

Table 1. Primers used in this study.

Primer	Sequence (5' to 3')	
gy_5'RACE	GCCCTAAATGCAAAGCAACAGTAACAC	
gy_3'RACE	TACTGTTGCTTTGCATTTAGGGCTTCC	
wmgy_qPCR	ACTCCTGCTCAGATTCTTATTGGTC	CTCTTAGTGATTGTGGCCCTTTT
ClActin_qPCR	CCTACAACTCAATTATGAAGTGTG	GAAATCCACATCTGCTGGAAGGTG
wmgy_TL_1	CACTTCCCATAACCATTTTTCTCAC	TATTGGAATTAAATCGTTACAGACC
wmgy_TL_2	ATGACTGATCCTTACCCCATCAACA	ATCAAGTCTTTGGAAAATGAGTGAT
wmgy_TL_3	TGGGAATTAAAGATGTGACAGGCAG	GCCCTAAATGCAAAGCAACAGTAAC
wmgy_TL_4	AAACCACATCTCATTAGCTCCCT	TCTTCATTCCTAAGTGGAGCAG
wmgy_in situ	GATTTAGGTGACACTATAGAAT-GCTCTTTGGCAATTTATACCCTCTTC	TGTAATACGACTCACTATAGGGTATT-GAGATCCATGCCCCCACAT

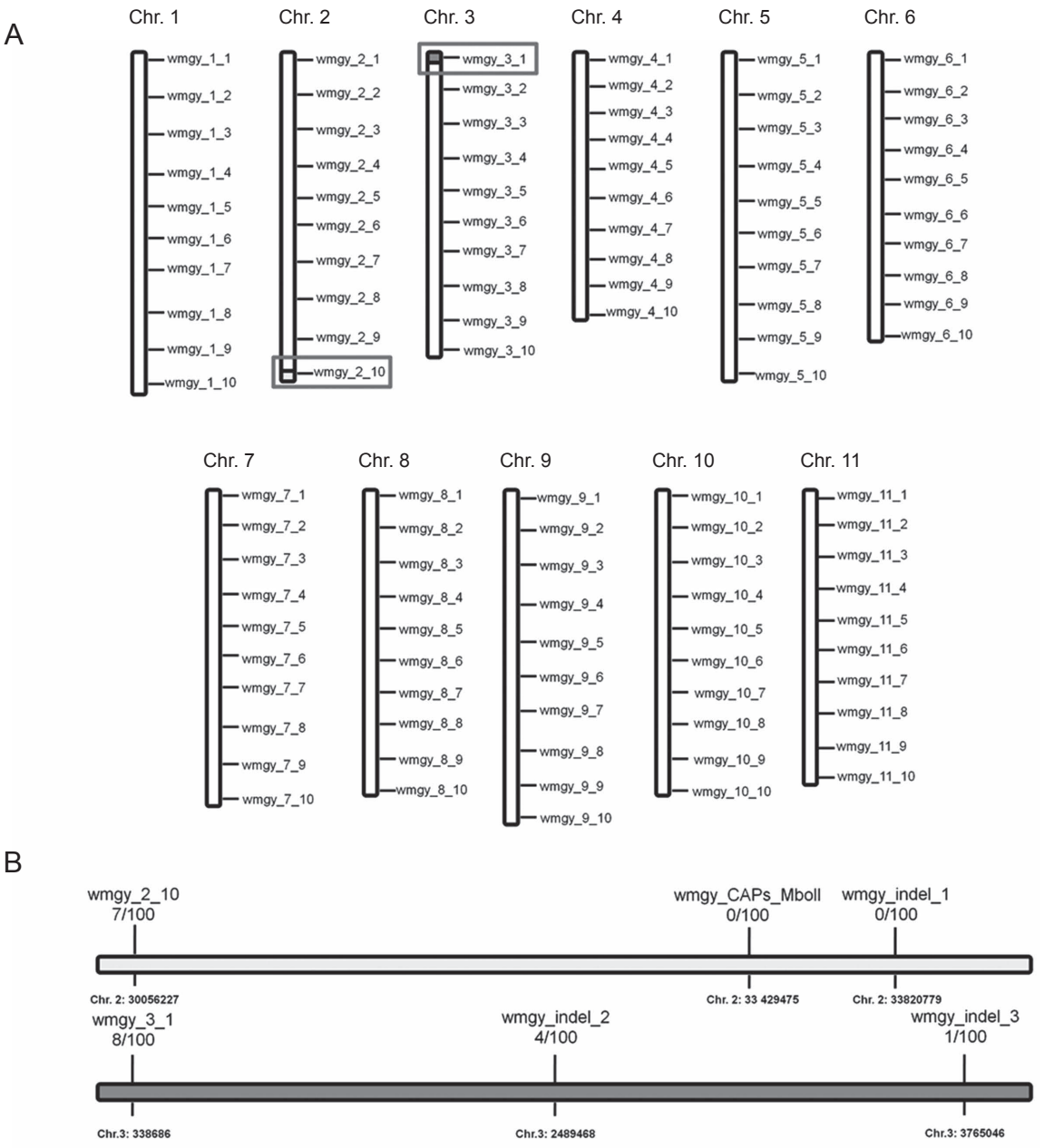

Figure 1. Genomic mapping for the *gy* allele.
(A) The genomic position of markers used in gene mapping. The grey boxes refer to markers and genomic regions that are associated with gynoecy, the genomic region on Chromosome 2 is indicated as light grey and the genomic region on Chromosome 3 is indicated as dark grey. (B) Chromosome walking analysis of *gy* allele positioning.

showed that an approximately 1 Mb reference genomic region after Chr. 2: 33348295..33348298 was assembled to Chr. 3 of XHBGM, while a 4 Mb reference genomic region before Chr. 3: 4043236..4043237 was assembled to Chr. 2 of XHBGM. On the contrary, the genomic of XHB was similar to the reference genome (Figure 2A). This bioinformatics analysis indicates that a reciprocal chromosome rearrangement, with translocation between Chr. 2 and Chr. 3, occurs in XHBGM, specifically, the distal region of Chr. 2 and the start of Chr. 3 were exchanged. The chromosome translocation breakpoints might be located on Chr. 2: 33348295..33348298 and Chr. 3: 4043236..4043237.

Fluorescence *in situ* hybridization (FISH) was performed to verify the chromosome translocation cytologically. Four pairs of primers were designed to screen BACs containing the genomic sequence in or out of the translocation breakpoint on Chr. 2 and Chr. 3, respectively. Four BACs were forwarded to the FISH experiment, which showed that signals of BAC 68K05 and 76E01 were co-localized on some chromosomes, and BAC 75K12 and 51F12 were also co-localized on some other chromosomes in XHB. However, in the mutant type XHBGM, the co-localization pattern of the four BAC signals were changed, of which 68K05 and 75K12 were on the same chromosome while 76E01

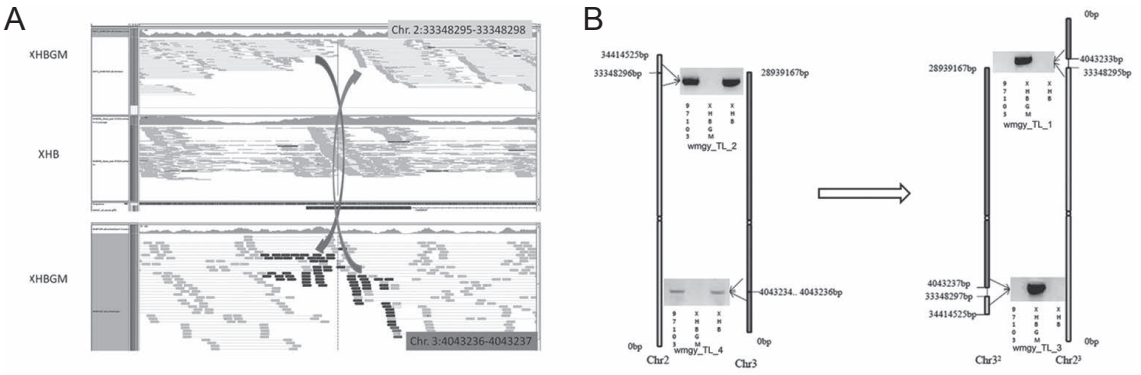

Figure 2. Chromosome translocation identified on accession XHBGM.
(A) Bioinformatics analysis of chromosome translocation between XHB and XHBGM. A 1 Mb reference genomic region after chromosome 2: 33348298, was assembled to chromosome 3, while about 4 Mb of the reference genomic region before chromosome 3: 4043236, was assembled to chromosome 2 of XHBGM. (B) Schematic diagram of the chromosome translocation between XHB and XHBGM. PCR amplification analysis of chromosome translocations are indicated as the translocation point on chromosomes 2, 3, 2^3, and 3^2, which are indicated by wmgy_TL_1~4.

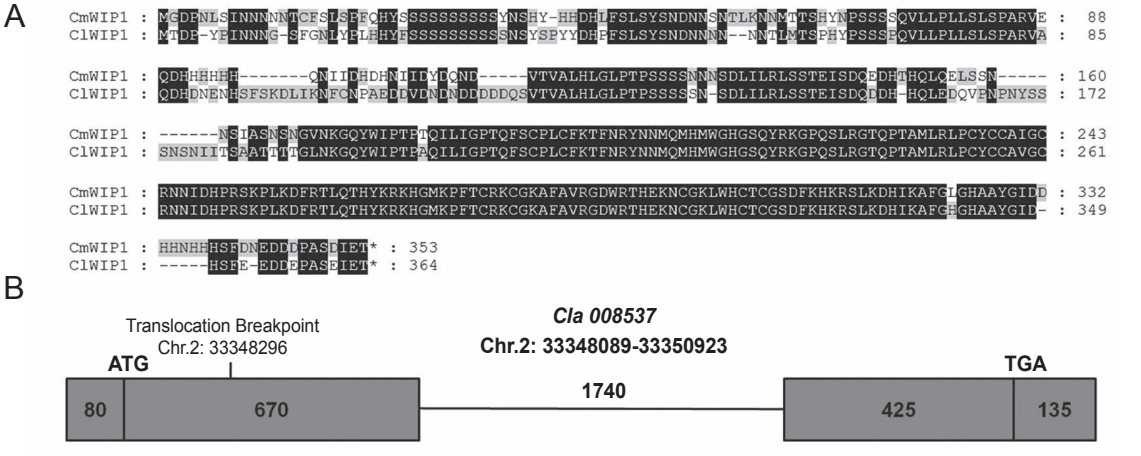

Figure 3. Cloning and identification of the candidate gene.
(A) The protein sequence comparison between ClWIP1 and CmWIP1. (B) The genomic structure of the candidate gene for *gy*, *Cla008537*.

and 51F12 were co-localized on another chromosome. The FISH analysis showed that a translocation between Chr. 2 and Chr. 3 had occurred in XHBGM.

To identify the translocation breakpoint, we developed four pairs of PCR primers, wmgy_TL_1 to 4, to amplify the genomic sequences containing the translocation breakpoints on Chr. 2 and Chr. 3. The primers wmgy_TL_1 and wmgy_TL_3 were used to amplify the genomic sequence of Chr. 2^3 and Chr. 3^2, respectively, from XHBGM. And primers wmg_TL_2 and wmg_TL_4 were used to amplify the genomic sequence of Chr. 2 and Chr. 3, respectively, from XHB. The results are shown in Figure 2B. The BLAST results showed that the translocation breakpoints were located on Chr. 2: 33348296 and on Chr. 3: 4043234..4043236 in XHBGM (Figure 2B).

Characterization of the candidate gy gene

The watermelon genomic data showed that the translocation breakpoint on Chr. 2 was located in the first exon of *Cla008537* (Chr. 2: 33348089... 33350923). *Cla008537* is a C_2H_2 zinc finger transcriptional factor gene, sharing 75% identity with *CmWIP1* at the protein level (Figure 3A). So we suggest that *Cla008537* is the candidate gene for *gy* allele in watermelon, and named it *ClWIP1*. On the basis of the genomic data, *ClWIP1* contains two exons and one intron. Amplified RACE-PCR showed that the length of the first exon was 670 bp and the second exon was 425 bp, with 80 bp 5'-UTR and 135 bp 3'-UTR (Figure 3B). The translocation breakpoint was located in the genomic region of the first exon of *ClWIP1* (Chr. 2: 33348296).

In order to identify whether *ClWIP1* was associated with gynoecious phenotype or not, the F_2 segregating population obtained by crossing the normal XHB (*Gy*/*Gy*) with the mutant XHBGM (*gy*/*gy*) was tested. On the basis of the chromosomal translocation, the primers used in this experiment were wmg_TL_1 and wmg_TL_2. Among the 271 F_2 plants, 68 were *Gy*/*Gy*, 131 were *Gy*/*gy*, and 72 were *gy*/*gy*. All *gy*/*gy* plants were gynoecious, and no recombinant plants were observed, suggesting that the chromosome translocation between Chr. 2 and Chr. 3 in XHBGM co-segregated with gynoecy in the F_2

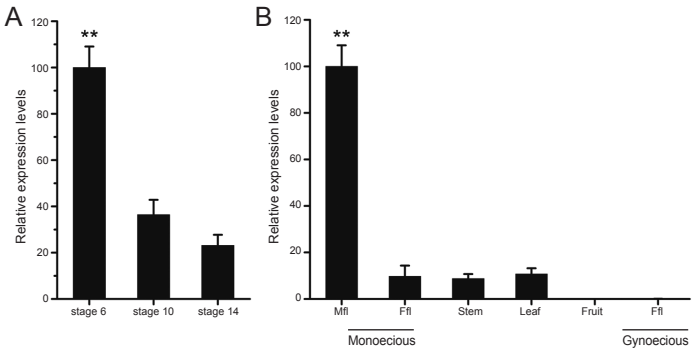

Figure 4. The expression analysis of *ClWIP1*.
(A) Expression analysis of *ClWIP1* in male flowers. In stage 6, *ClWIP1* expression in male flowers is significantly higher (indicated with **) than stage 10 and stage 14 buds. (B) Expression analyses of *ClWIP1* in different organs. *ClWIP1* expression in stage 6 male (Mfl) buds is significantly higher (indicated with **) than that in female flower buds (Ffl) and other organs.

population. Thus, we conclude that *ClWIP1* is the *gy* gene in watermelon.

ClWIP1 *is expressed in the carpel primordia of male flowers*

To detect the expression of *ClWIP1* during flower development, total RNA was extracted from flower buds of the monoecious and gynoecious watermelons at developmental stages 6, 10, and 14, according to the definition of developmental stages used previously for cucumber (Bai et al. 2004). Quantitative real-time PCR (qPCR) assays were performed. The results showed that the transcript of *ClWIP1* reached its suggested level at stage 6 of male flower development (Figure 4A). The transcript levels of *ClWIP1* at other stages of male flower development were significantly lower than at stage 6. To our surprise, no *ClWIP1* expression was detected in female flower buds from both, the monoecious and gynoecious accessions (Figure 4B). RNA *in situ* hybridization analysis verified the male-specific expression of *ClWIP1* in the carpel primordia of male flower buds at stage 6, while no expression was detected in female flower buds and hermaphroditic buds at the same stage.

Discussion

The sex determination system in Cucurbitaceae is conserved

Our data showed that *ClWIP1*, the *gy* gene in watermelon, is orthologous to *CmWIP1*, the *g* gene in melon. Our data also showed that loss of *ClWIP* function leads to gynoecy, which is similar to the mechanism of *CmWIP1* and *CsWIP1*. Previous studies showed that cucumber and melon share the same sex determination system, which is controlled by three major genes, two ACS family members and one zinc-finger transcription factor (Martin et al. 2009, Boualem et al. 2015). The model of sex determination in melon has *ACS11* controlling female flower development, *WIP1* controlling the male flower development, and *ACS-7* controlling development of staminate primordia on pistillate flowers (Boualem et al. 2015). We believe that the sex determination system of the Cucurbitaceae is conserved, and that further research will verify this.

Probable function of ClWIP1 *in watermelon*

According to the sex expression analysis in watermelon, *ClWIP1* mainly inhibited the development of female organs during the early period of floral development. In a previous study with cucumber, both male and female floral primordia were observed in the early period of floral development (Bai et al. 2004). The expression of *ClWIP1* mainly inhibited the development of female floral organs, resulting in the development of male flowers.

Chromosome translocation is an important event in watermelon evolution

The 11-chromosome genome of extant watermelons was shaped from 7-chromosome eudicot ancestors through 21 paleo-hexaploid intermediates, as reported from our previous research (Guo et al. 2013). And we suggest that the transition from the 21-chromosome eudicot intermediate ancestors involved 81 fissions and 91 fusions to reach the modern 11-chromosome structure of watermelon, which is represented as a mosaic of 102 ancestral blocks (Guo et al. 2013). Chromosome translocation is one type of mutation which includes both, chromosome fission and fusion. Our data show that the appearance of the gynoecious watermelon mutant resulted from a translocation between Chr. 2 and Chr. 3.

Using the gynoecious line in breeding is expected

For reducing the cost of production, yield improvement, and enhanced seed quality, gynoecious germplasm is a potentially valuable resource for cucurbit breeding. XHBGM is the only gynoecious sex type found to date in watermelon, and can be selected by a gene marker designed around a chromosome translocation. Using this marker, improved gynoecious watermelon germplasm can be bred quickly. We are now attempting to introgress this gynoecy into horticultural valuable watermelon germplasm.

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S-Gene and *R*-Gene Candidates for Disease Resistance in Watermelon

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Abstract. The mainstream research on plant genes for disease resistance has focussed on different classes of resistance genes (*R*-genes), pathogen effectors directly or indirectly recognized by proteins encoded by *R*-genes, and downstream defence cascades. Most of the *R*-genes belong to the NB-LRR family. Whole genome sequences allow readily positioning of putative resistance genes on chromosomes of plants. Watermelon has a relatively low frequency (66) of NB-LRR-like *R*-genes. An emerging topic is resistance provided by impaired susceptibility genes (*S*-genes). *S*-genes are plant genes that are "abused" by a pathogen for its own benefit during the infection process. Loss of a functional *S*-gene can lead to durable resistance. Whereas resistance caused by *R*-genes is dominantly inherited, resistance due to impaired *S*-genes inherits recessively. The most well-known group of impaired *S*-genes consists of *mlo*-genes, providing durable resistance to powdery mildew in a series of plant species. Three groups of *S*-genes have been distinguished, differing in time of their action: (1) those that provide early pathogen establishment; (2) those that interfere with defence responses by the host; (3) those involved in feeding of the pathogen. We listed amino-acid sequences of 121 proteins, encoded by genes that were functionally characterized as *S*-genes in several plant species, mainly *Arabidopsis thaliana.* We searched for genes in the watermelon genome, encoding similar proteins. We positioned these *S*-gene candidates on the published genome of watermelon, adhering to the mentioned division in three *S*-gene groups. Finally, we compared published QTL regions for disease resistances in watermelon with *R*-gene and *S*-gene candidate positions. We have applied this approach to different cucurbits.

Keywords: Resistance genes, susceptibility genes, QTLs, watermelon, *Citrullus lanatus*

Introduction

Disease resistances in plant cultivars are commonly obtained by introgression of resistance genes (*R*-genes) from wild relatives. The most well-known class of *R*-genes belong to the NB-LRR family (Belkhadir et al. 2004). The conserved nature of this domain allows recognition of the NB-LRR group of *R*-gene candidates in plant genomes.

Another group of genes that can provide disease resistance in plants consists of impaired susceptibility genes (*S*-genes). Pathogens and parasites can "abuse" plant genes, thus allowing entrance into the plant, or suppressing defence responses, or stimulating e.g., transport of nutrients from the plant cells to the pathogen. Loss or down-regulation of a functional *S*-gene can lead to durable resistance (Pavan et al. 2010). As this type of resistance is obtained through loss of a functional gene, this resistance usually inherits recessively. In contrast, *R*-genes are dominantly inherited. Loss of a functional *S*-gene may lead to negative fitness effects in the plants (van Schie and Takken 2014).

Watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, belongs to the cucurbit family (Cucurbitaceae) and suffers from diseases such as downy (*Pseudoperonospora cubensis*) and powdery (*Podosphaera xanthii*) mildews, gummy stem blight (*Didymella bryoniae*), Fusarium wilt (*Fusarium oxysporum* f. sp. *niveum*), anthracnose (*Colletotrichum orbiculare*), fruit rots (*Pythium aphanidermatum*, *P. debaryanum* and *Phytophthora capsici*), and leaf spot (*Alternaria cucumerina*). Other cucurbit crops such as cucumber suffer from most of these diseases too. Co-localization of QTLs with known *S*-genes in cucumber has facilitated the cloning of a mutant of *CsaMLO8* providing hypocotyl resistance to powdery mildew (Berg et al. 2015). Before the genome of watermelon was sequenced, Harris et al. (2009) cloned conserved fragments of 66 NB-LRR-like genes in watermelon. In this study, we used the published draft genome sequence of watermelon (Guo et al. 2012), and searched for NB-LRR like *R*-genes as well as homologues of *S*-genes published by van Schie and Takken (2014). The genomic positions of candidate *R*-genes and candidate *S*-genes are compared with published QTLs for disease resistances in watermelon.

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Materials & Methods

R-*genes*

We downloaded the file <watermelon_v1_gene_anno.gz> from the Cucurbit Genomics Database, using the web address ftp://www.icugi.org/pub/genome/watermelon/97103/v1/watermelon_v1_gene_anno.gz. This file had two columns, i.e., a column containing identifiers for putative genes (*Cla000001* to *Cla016411*) predicted from the whole genome assembly (Guo et al. 2012), and a second column, giving annotations to these predicted genes, based on similarity to genes that were functionally characterized before. We imported this file into Excel, and selected genes that had in the annotation the text string 'resistan', 'disease', and/or 'NB-ARC'. During manual curation of this list, we selected only resistance genes (*R*-genes) belonging to the NB-LRR (NB-ARC) family, although several other genes can be involved in defence and defence cascades too (Jones and Dangl 2006).

Subsequently, we extracted the genomic positions of the NB-LRR-like genes, according to the draft genome sequence (Guo et al. 2012), using the file watermelon_v1.cds from the Cucurbit Genomics Database. We imported this list of genes and their genomic positions into MapChart (Voorrips 2002), for a graphical representation of the locations of these candidate *R*-genes on the 11 chromosomes of watermelon.

S-*genes*

The 182 *S*-genes listed in the appendix of the mentioned review paper on susceptibility genes in plants (van Schie and Takken 2014) were used as starting point to search for candidate *S*-genes in watermelon. We looked for the amino acid sequences, encoded by these *S*-genes according to the NCBI database, and compiled a FASTA file, containing the predicted peptide sequences of the functionally characterized *S*-genes. The list of 182 *S*-genes was manually curated to remove redundant entries, i.e., different paralogs within the same gene family in a species, or orthologs in multiple plant species, to obtain a list of 121 non-redundant *S*-genes. We searched for homologous predicted proteins in watermelon, by downloading the 23.440 predicted watermelon protein sequences from the Cucurbit Genomics Database (ftp://www.icugi.org/pub/genome/watermelon/97103/v1/watermelon_v1.pep.gz). On a local Linux server, we made a blast database for these proteins of watermelon, using the 'makeblastdb' command from the BLAST+ package, and performed blastp searches against this database, using the protein sequences encoded by functional *S*-genes as query, at threshold E-values of 1.0E-100, 1.0E-50, and 1.0E-10. This yielded lists of candidate S-proteins in watermelon at three levels of stringency. The resulting lists were manually combined and curated to obtain for each *S*-gene if possible the first till fifth best hits, based on E-value. The downloaded watermelon_v1.pep file contained the genomic positions of the genes encoding the protein. These genomic positions were extracted for the *S*-gene candidates, using the grep-command in Linux.

Results

R-*genes*

Sixty-two predicted NB-LRR-like genes were found (Figure 1). Especially chromosomes 2 and 8 harbour large clusters of predicted NB-LRR genes, whereas most other chromosomes contain smaller clusters or single genes belonging to this family. Chromosomes 4 and 6 do not harbour any predicted NB-LRR-like gene, and chromosome 3 only one.

There are other resistance gene families, not belonging to the NB-LRR family (Jones and Dangl 2006), but these are not displayed in Figure 1.

S-*genes*

Using E-value thresholds of 1E-100, 1E-50, or 1E-10, we found 376, 936, and 4001 putative watermelon homologs of *S*-genes, respectively. As for some *S*-genes more stringent thresholds were required than for others, we manually curated the list to obtain the closest related watermelon homologs for each of the 121 *S*-genes. The resulting list comprises 364 candidate *S*-genes in watermelon.

Figure 1 shows, in addition to *R*-genes, also the positions of the identified putative *S*-genes. For the first group of *S*-genes, which plays a role in the early infection process before and during penetration, we found 118 candidate genes. For the second group, consisting of defence suppression genes, we found 184 candidates. For the third group, 61 candidate genes were detected. This third group, being the smallest group, contains genes that allow sustained compatibility and pathogen proliferation after penetration, fulfilling metabolic or structural needs (van Schie and Takken 2014). An example is a sugar transport gene. Apparently, the second group of candidate genes, encompassing the putative defence suppressor genes, is approximately nearly as large as the first and third group together. We have to mention that the division in these three groups is helpful, but the assignment of *S*-genes to these groups is not always unequivocal (van Schie and Takken 2014).

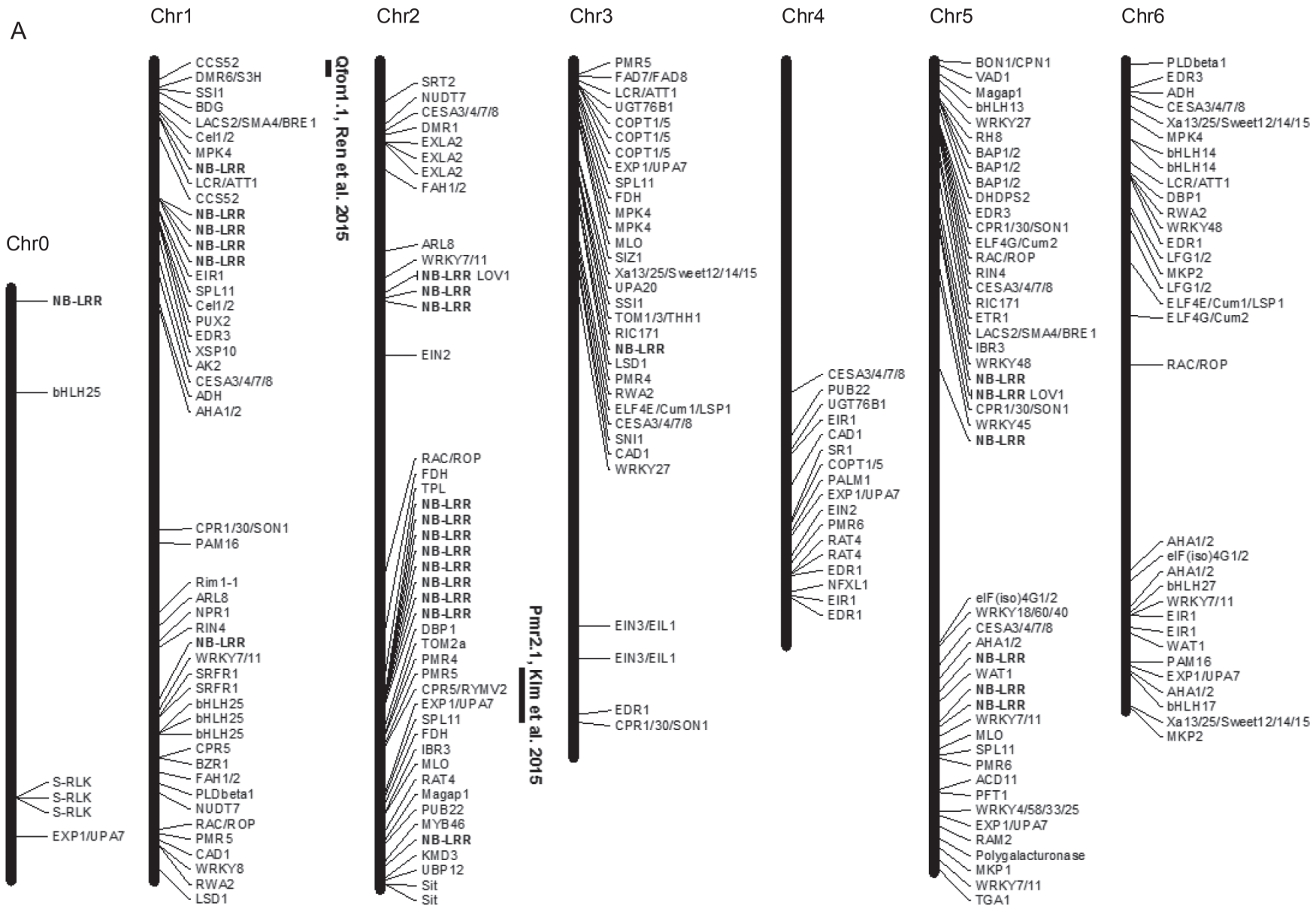
The most well-known group of susceptibility genes is the *Mildew Locus O* (*MLO*) gene family. Impaired *mlo* genes may lead to resistance to powdery mildew. We detected a series of putative *MLO* homologs in watermelon, but we display in Figure 1 only the three *MLO*-like genes with the lowest E-value (E=0). These three genes belong to Clade V of the *MLO* gene family (Iovieno et al. 2015), which makes sense as only *MLO* genes that belong to this clade have been found to be functional *S*-genes for powdery mildew in many species, whereas *MLO*-genes residing in other clades are thought to have other functions (Devoto et al. 2003, Schouten et al. 2014, Appiano et al. 2015, Berg et al. 2015). Clade V *MLO*-like genes are present on chromosomes 2, 3, and 5.

Discussion

R- *and* S-*gene candidates in reported QTLs*

A few papers are published on QTLs for disease resistances in watermelon. We focussed on the papers that provided marker sequences that could be aligned to the genome sequence of watermelon. We could not include QTLs that lacked sufficient DNA sequence information for positioning on the genome (Kim et al. 2013, Tetteh et al. 2013).

A QTL for powdery mildew resistance in watermelon was positioned on chromosome 2 (Kim et al. 2015). In this QTL region, one putative *S*-gene is present, i.e., a homolog of the *Arabidopsis TPL* gene. However, also a cluster of eight NB-LRR genes is present in the QTL region, as was already mentioned by the authors, and also appears from Figure 1. Kim et


A
Chr0
NB-LRR
bHLH25
S-RLK
S-RLK
S-RLK
EXP1/UPA7
Chr1
CCS52
DMR6/S3H
SSI1
BDG
LACS2/SMA4/BRE1
Cel1/2
MPK4
NB-LRR
LCR/ATT1
CCS52
NB-LRR
NB-LRR
NB-LRR
NB-LRR
EIR1
SPL11
Cel1/2
PUX2
EDR3
XSP10
AK2
CESA3/4/7/8
ADH
AHA1/2
CPR1/30/SON1
PAM16
Rim1-1
ARL8
NPR1
RIN4
NB-LRR
WRKY7/11
SRFR1
SRFR1
bHLH25
bHLH25
bHLH25
CPR5
BZR1
FAH1/2
PLDbeta1
NUDT7
RAC/ROP
PMR5
CAD1
WRKY8
RWA2
LSD1
Qfon1.1, Ren et al. 2015
Chr2
SRT2
NUDT7
CESA3/4/7/8
DMR1
EXLA2
EXLA2
EXLA2
FAH1/2
ARL8
WRKY7/11
NB-LRR LOV1
NB-LRR
NB-LRR
EIN2
RAC/ROP
FDH
TPL
NB-LRR
NB-LRR
NB-LRR
NB-LRR
NB-LRR
NB-LRR
NB-LRR
NB-LRR
DBP1
TOM2a
PMR4
PMR5
CPR5/RYMV2
EXP1/UPA7
SPL11
FDH
IBR3
MLO
RAT4
Magap1
PUB22
MYB46
NB-LRR
KMD3
UBP12
Sit
Sit
Pmr2.1, Klm et al. 2015
Chr3
PMR5
FAD7/FAD8
LCR/ATT1
UGT76B1
COPT1/5
COPT1/5
COPT1/5
EXP1/UPA7
SPL11
FDH
MPK4
MPK4
MLO
SIZ1
Xa13/25/Sweet12/14/15
UPA20
SSI1
TOM1/3/THH1
RIC171
NB-LRR
LSD1
PMR4
RWA2
ELF4E/Cum1/LSP1
CESA3/4/7/8
SNI1
CAD1
WRKY27
EIN3/EIL1
EIN3/EIL1
EDR1
CPR1/30/SON1
Chr4
CESA3/4/7/8
PUB22
UGT76B1
EIR1
CAD1
SR1
COPT1/5
PALM1
EXP1/UPA7
EIN2
PMR6
RAT4
RAT4
EDR1
NFXL1
EIR1
EDR1
Chr5
BON1/CPN1
VAD1
Magap1
bHLH13
WRKY27
RH8
BAP1/2
BAP1/2
BAP1/2
DHDPS2
EDR3
CPR1/30/SON1
ELF4G/Cum2
RAC/ROP
RIN4
CESA3/4/7/8
RIC171
ETR1
LACS2/SMA4/BRE1
IBR3
WRKY48
NB-LRR
NB-LRR LOV1
CPR1/30/SON1
WRKY45
NB-LRR
eIF(iso)4G1/2
WRKY18/60/40
CESA3/4/7/8
AHA1/2
NB-LRR
WAT1
NB-LRR
NB-LRR
WRKY7/11
MLO
SPL11
PMR6
ACD11
PFT1
WRKY4/58/33/25
EXP1/UPA7
RAM2
Polygalacturonase
MKP1
WRKY7/11
TGA1
Chr6
PLDbeta1
EDR3
ADH
CESA3/4/7/8
Xa13/25/Sweet12/14/15
MPK4
bHLH14
bHLH14
LCR/ATT1
DBP1
RWA2
WRKY48
EDR1
LFG1/2
MKP2
LFG1/2
ELF4E/Cum1/LSP1
ELF4G/Cum2
RAC/ROP
AHA1/2
eIF(iso)4G1/2
AHA1/2
bHLH27
WRKY7/11
EIR1
EIR1
WAT1
PAM16
EXP1/UPA7
AHA1/2
bHLH17
Xa13/25/Sweet12/14/15
MKP2

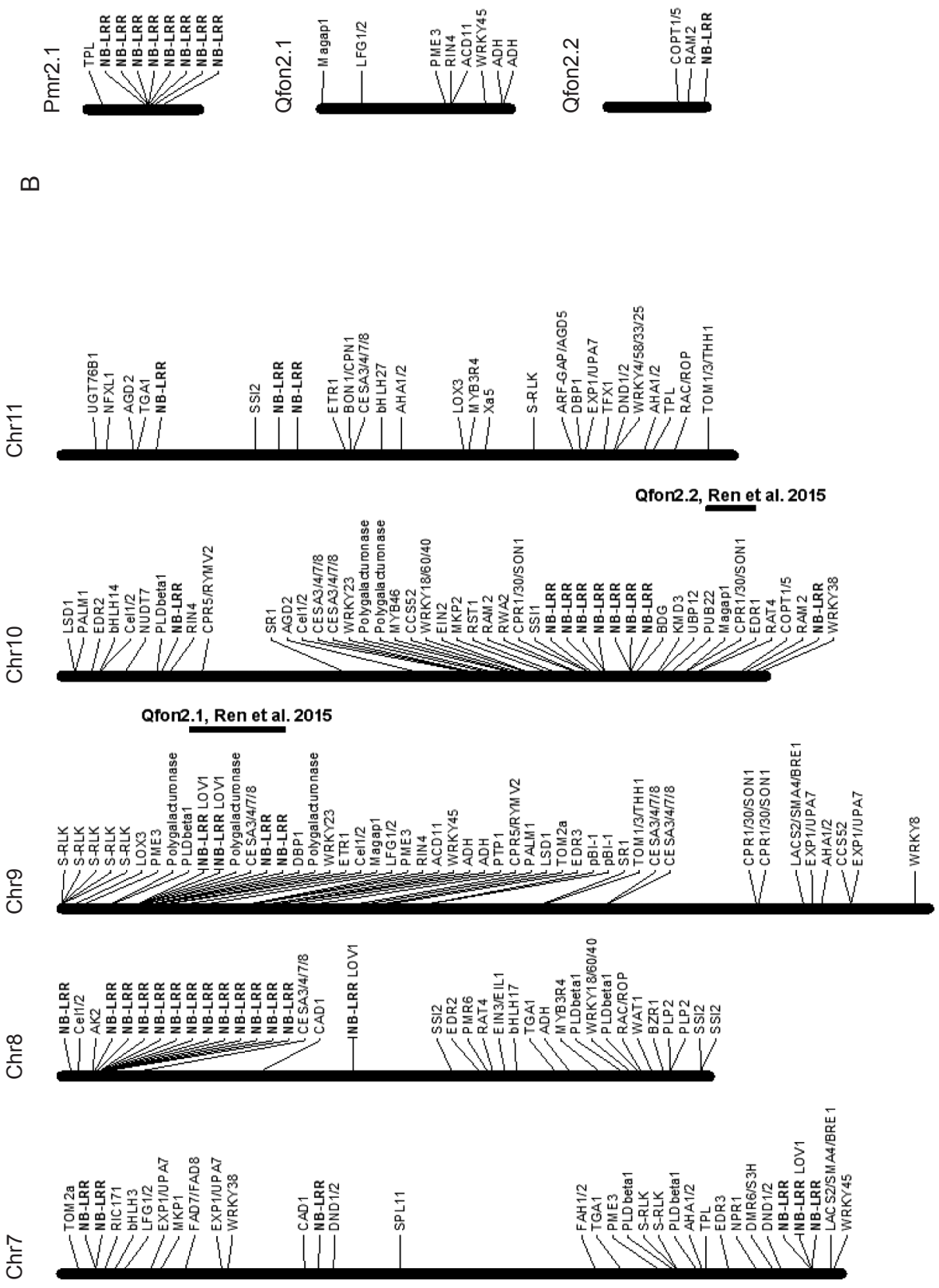


Figure 1. (**A**) Positions of candidate *R*-genes and *S*-genes on the 11 chromosomes of watermelon. In bold, candidate *R*-genes are shown, belonging to the NB-LRR gene family according to the annotation of the predicted genes in the watermelon genome (Guo et al. 2012). The names of the candidate *S*-genes refer to genes that are functionally characterized in other plant species. S-RLK refers to receptor like kinases with *S*-gene function, which are homologs of several genes listed by van Schie and Takken (*IOS1*/*FER*/*PSKR*/*PSY1R*/*BIR1*/*RHG4*/*BIK1*/*LecRK-V.5*).
(**B**) The inset shows in more detail the locations of the candidate genes on QTLs *Qfon2.1* and *Qfon2.2* described by Ren et al. (2015) and QTL *Pmr2.1* described by Kim et al. (2015).

al. (2015) mentioned that the QTL harboured a major gene with an incompletely dominant effect. This makes an *R*-gene, such as a NB-LRR gene, a more likely candidate than an impaired *S*-gene.

In another study, three QTLs for resistance of watermelon to *Fusarium oxysporum* f. sp. *niveum* (FON) were mapped (Ren et al. 2015). A QTL on chromosome 1 was effective against race 1 but not against race 2, and two QTLs for resistance to race 2 were found on chromosomes 9 and 10. The authors reported that plants with the two QTLs for race 2 resistance were often also resistant against race 1, indicating that those QTLs are presumably rather broad-spectrum than race-specific.

The QTL on chromosome 1 was detected at the top of this chromosome, a region harbouring no known *S*-gene or NB-LRR-gene candidates according to our analysis (Figure 1). The 84 genes within the QTL interval contain a receptor kinase, a glucan endo-1,3-β-glucosidase, and three different chitinases as possible candidate genes (Ren et al. 2015). Given the dominant inheritance and race-specific resistance of this QTL, it is more likely that the observed resistance is due to an *R*-gene than an *S*-gene.

The QTL for FON race 2 resistance on chromosome 9 does not contain NB-LRR-like genes according to the reference genome, but harbours at least eight *S*-gene candidates (Figure 1). Race 2 resistance was previously shown to be recessively inherited (Martyn 2012), and this QTL seems to give a broad spectrum resistance against both FON races. This indicates that likely an impaired *S*-gene causes the QTL. One of the *S*-gene candidates is a *PME3*-like gene. *PME3* can be induced in *Arabidopsis* upon infection, and is necessary for a successful colonization by necrotrophic pathogens (Raiola et al. 2011, Kohorn et al. 2014). Other candidate genes are homologs of the *S*-genes *Magap1, LFG, RIN4, ACD11, WRKY45,* and two *ADH* homologs. Screening for potential loss-of-function mutations in those genes in resistant material could facilitate the identification of the causal gene. A lipoxygenase (*LOX*) gene, five receptor-like kinase (*RLK*) genes, and four gluthathione *S*-transferase genes have been mentioned as possible candidates (Ren et al. 2015). In our analysis, these homologs of the *LOX* and *RLK* genes have also been found, but were discarded as candidate *S*-genes, as other watermelon genes were more similar to *LOX* and *RLKs* with known *S*-gene function. We do not consider gluthathione *S*-transferase genes as candidate *S*-genes. However, they might have an effect on pathogenicity by detoxifying fungal xenobiotics (Marrs 1996).

As mentioned, Ren et al. (2015) detected a third QTL for race 2 FON-resistance on chromosome 10. At the border of the QTL, a NB-LRR-like gene is present, whereas more to the centre two *S*-gene candidates were found, i.e., homologs of the *COPT1/5* and *RAM2* genes. *COPT* genes encode copper transporters, which have been reported as susceptibility genes towards the bacterial pathogen *Xanthomonas oryzae* in rice (Yuan et al. 2010), and *RAM2* encodes a glycerol-3-phosphate acyl transferase, which has been found to be a susceptibility gene towards *Phytophthora palmivora* in *Medicago* (Wang et al. 2012). Ren et al. (2015) mention also an *LTP*-like gene (*lipid-transfer protein*) as a possible causal candidate gene for this QTL. *LTP* has indeed been reported as an *S*-gene for Fusarium wilt in tomato, as mentioned by Ren et al. However, the homology to LTP, referred to as *XSP10* by van Schie and Takken (2014), was low at the protein level, and therefore this candidate is not mentioned in Figure 1.

These examples illustrate that Figure 1 can be helpful for looking for candidate genes for QTLs for resistance.

R-*genes*

We found in the list of annotated genes of watermelon 62 NB-LRR-like genes. Remarkably, the authors of the draft genome paper (Wu et al. 2012) mentioned that they detected only 44 NB-LRR genes in the genome sequence, and listed these 44 in Supplementary Table 20 of their paper. We compared these two lists, but do not know why 18 NB-LRR-like genes were not included in the list together with the other 44 NB-LRR-like genes.

Even if the number of NB-LRR-like genes in watermelon is not 44 but 62, still the number is small compared to the approximately 149 NB-LRR-like genes in *Arabidopsis thaliana* (Meyers et al. 2003), 459 in grape (Yang et al. 2008), 500 in rice (Monosi et al. 2004), and even 1015 in apple (Arya et al. 2014). Although these numbers are estimates, and depend on selection criteria, they still illustrate clearly the wide variation in numbers of NB-LRR family members between plant species. In another cucurbit, *Cucumis sativus* L. (cucumber), the number of NB-LRR-like genes is approximately 57 (Wan et al. 2013), thus similar to the number in watermelon. Guo et al. (2014) suggested that the low number in the reference genome of watermelon is due to neglecting disease resistance during breeding. However, we doubt whether the attention for disease resistances in watermelon and cucumber was much lower than in other crops. We do not have a convincing theory either to explain the low amount of NB-LRR genes in the Cucurbitaceae. The fact that the amount of NB-LRR genes is low in these species leads to the conclusion that looking for impaired *S*-genes could be a suitable alternative strategy to obtaining resistances to various pathogens of cucurbit crops.

We conclude that the number of *S*-gene candidates is higher than the number of *R*-gene candidates belonging to the NB-LRR family. The positions of these *S*-gene candidates are given in Figure 1, and may be helpful for finding candidate genes in QTLs for disease resistance.

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Dissection of Climacteric and Non-Climacteric Ripening in Melon by Using Genetic, Analytic, and Genomic Resources

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Abstract. Melon (*Cucumis melo* L.) is a useful plant model to elucidate the mechanism of ripening behaviour given the coexistence of climacteric and non-climacteric cultivars. In addition, useful genetic and genomic resources are available, such as extensive transcriptome data, an annotated genome sequence, resequenced cultivars, and several mapping populations. Among them, a near isogenic line population, obtained by introgressing the exotic PI 161375 (aka SC, non-climacteric) genome into 'Piel de Sapo' (PS, non-climacteric) background, facilitated the identification of two quantitative trait loci (QTLs) that confer climacteric ripening to PS: the recently cloned *eth6.3* and *eth3.5*, which have been mapped in a short genomic interval. In order to elucidate additional mechanisms and genes involved in melon ripening behaviour, a new recombinant inbred line (RIL) population has been obtained by crossing 'Védrantais' (Ved, a typical climacteric melon) with PS. Phenotypic data related to fruit ripening was collected from the RIL population, such as fruit abscission, external color change, and ethylene production. For the latter, we developed a high throughput gas chromatography-mass spectrometry head space methodology that quantifies the production of ethylene in attached fruit with a limit of quantification near one part per billion (1 nL·L^{-1}). These phenomic and analytical data will be correlated to a genotyping by sequencing analysis of the RIL population in order to identify new genes/QTLs involved in ripening in melon. These combined genetic, phenomic, and analytical approaches will offer significant advances in the characterization of melon fruit ripening.

Keywords: Genetic and genomic resources, ethylene, fruit ripening, melon

Introduction

Plant hormones are signaling molecules that regulate many aspects of growth and development. Ethylene is a hormone involved in many plant processes, among them the regulation of fruit ripening. The presence or absence of a peak of ethylene and a subsequent increase of respiration at the onset of ripening (Lelievre et al. 1997) permits classification of fleshy-fruits into two different categories: climacteric and non-climacteric. While the ripening process includes some structural, physiological, and biochemical changes that are common to both ripening types, others are specific to climacteric fruits or even to a particular species. Some of the most important fruit organoleptic properties, as sweetness, color, firmness, and aroma production, are usually related to ethylene production (Seymour et al. 2013).

The main plant model to understand climacteric fruit ripening is tomato, a species in which many key genes have been described and cloned. Two enzymes participate in the biosynthesis of ethylene, ACC synthase (ACS) and ACC oxidase (ACO), and only some isoforms are active in fruit tissues (Giovannoni 2004). In the past decades, tomato mutants have been used to figure out the ethylene pathway, including many transcription factors and ethylene receptors as *rin* (ripening-inhibitor) (Vrebalov et al. 2002), *Cnr* (colorless non-ripening) (Manning et al. 2006), *SlAP2a* (*APETALA2* transcription factor) (Chung et al. 2010), and *Nr* (Never-ripe) (Lanahan et al. 1994), among others.

Over the past few years, melon has become an alternative model to study the differences between the two types of ripening given the coexistence of climacteric and non-climacteric varieties (Ezura and Owino 2008) and the large amount of genomic and genetic resources available (Diaz et al. 2011, Garcia-Mas et al. 2012). Previous studies determined which aspects of ripening are ethylene-dependent, as chlorophyll degradation, volatile production, abscission layer formation, and part of flesh softening. However, some components of ripening that are a consequence of climacteric ripening in other species, as color flesh and sugar production, are ethylene-independent in melon (Pech et al. 2008).

In this favorable context, some studies have improved the knowledge of melon fruit ripening. The silencing of *Aco1* in a climacteric variety using an antisense construction proved the tight relationship between ethylene production and the physio-

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logical aspects of fruit ripening (Ayub et al. 1996). Later, the analysis of a RIL population derived from a cross between the *cantalupensis* melon Ved, a typical climacteric, and the non-climacteric line SC, implicated two major genes and four QTLs in ethylene production and fruit abscission (Périn et al. 2002). Besides, a NIL population constructed using the same exotic parental, SC, and the *inodorus* non-climacteric PS, revealed two new QTLs that allow recovery of a climacteric phenotype when the SC alleles are introduced into the PS background (Vegas et al. 2013).

Nowadays, the increase of big data, the decrease in the pricing of sequence technologies, and the enormous advances in bioinformatics methods facilitate the use of massive genotyping. Genotyping-by-sequencing (GBS) is a robust and relatively simple technique to genotype an entire population with thousands of SNPs well-distributed across the genome, without a significant cost in terms of economic and bioinformatic resources (Elshire et al. 2011).

The aim of the work presented here is to contribute to identifying new major genes or QTLs involved in climacteric ripening, including ethylene production and its physiological consequences. A RIL population segregating for many traits, including climacteric ripening, was developed by crossing Ved and PS, two popular sweet-fleshed cultivars. A new method, based on gas chromatography-mass spectrometry (GC-MS), to measure fruit ethylene production *in planta* was tested, obtaining promising results. The present study will significantly contribute to the knowledge of climacteric ripening and ethylene production in melon.

Materials & Methods

Plant material

The parental accessions used to develop the RIL population were Ved, a typical climacteric cantaloupe cultivar, with sweet aroma, presence of abscission layer and change of skin color from white to cream, and PS, a typical non-climacteric cultivar, without any of the above-mentioned properties. The two lines differ also in many interesting traits related to fruit quality: Ved has depressed vein tracts, orange flesh, and medium sugar content, whereas PS has green skin, white-cream flesh, and high sugar content.

A RIL population of 91 individuals was developed in collaboration with Semillas Fitó. After the initial cross, the hybrid was self-pollinated by hand. The F_2 generation was entered in a single seed descent scheme, until F_7-F_8 generations.

Three replicates of the RIL population were grown under greenhouse conditions during the summer of 2015, each replicate separated by three weeks. The plants were hand-pollinated in order to obtain one melon per plant.

Ethylene production

To register the ethylene production with the fruit attached to the plant, a novel system was tested. We used a plastic bag (polyethylene-polyamide) to enclose the fruit, producing a hermetic seal around the pedicel of the fruit (Figure 1A,B). A PVC tube with a two-point valve was added to the system, to enable a flux of air between the inside and the outside of the bag. The bag was filled with air using a vacuum pump and closed with the valve to accumulate the ethylene that the fruit was producing (Figure 1C). After one hour, 60 mL of the inner air was extracted with a syringe and collected in a headspace vial of 10 mL (Figure 1D).

Once all the samples were collected, the ethylene content was measured with GC-MS; 500 µL were injected in a gas chromatograph (Agilent Technologies 7890A) coupled with a mass spectrometer detector (Agilent 5975C inert MSD). Two standards (5 and 20 ppm) were collected in the greenhouse and utilized to produce a calibration curve. The air volume inside the bag was estimated to calculate the absolute ethylene production per fruit.

The ethylene peak was characterized in 72 RILs in one replicate, using the parents and the hybrid as controls. For each melon fruit, the measurements started before the beginning of ethylene production and were recorded every 1 or 2 days until the ethylene peak was profiled. The non-climacteric lines were also monitored over ripening to confirm that there was no ethylene production.

Phenotypic evaluation

Phenotypic traits related with climacteric ripening were evaluated in 91 RILs in three replicates. During the season, formation of the abscission layer, aroma, and external color change were recorded as qualitative: presence or absence, and as quantitative: days after pollination (DAP) when the trait appears. Abscission and flesh softening were registered at harvest. Abscission was evaluated visually on a scale from 0 (no scar) to 3 (scar totally formed/abscission). Flesh softening was measured with a penetrometer.

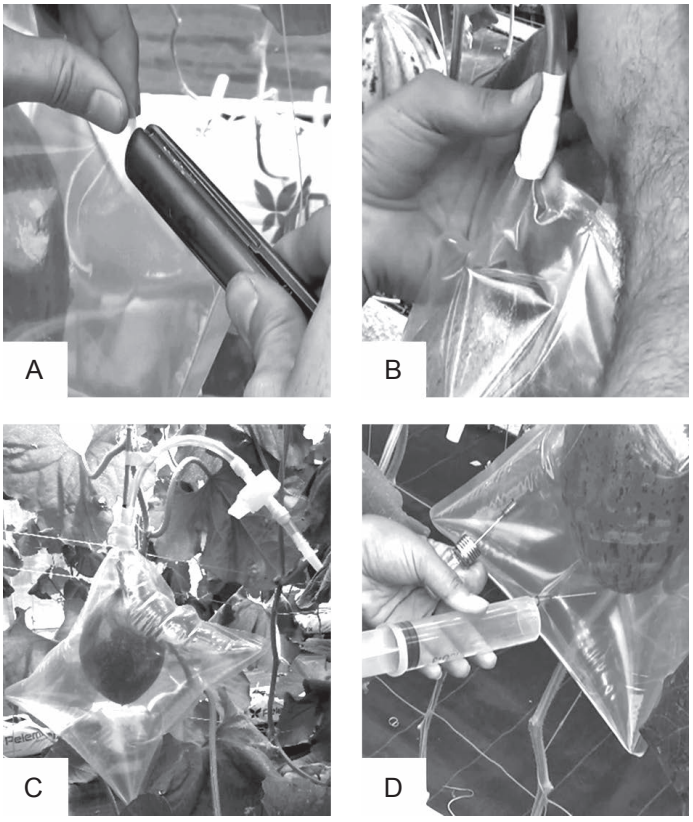

Figure 1. Method of collecting fruit ethylene production *in planta* for GC-MS analysis. Thermo-sealing of the bag in the area next to the pedicel (A). Hermetic seal with Teflon film around the pedicel (B). Bag is filled with air with a pump (C). Collection of the air with a syringe (D).

Results

Estimation of ethylene production

To characterize the ethylene peak, we defined four different parameters related to ethylene production: maximum ethylene production, DAP to initial ethylene production, DAP to maximum ethylene production, and width of the increase of ethylene production, which is measured by counting the number of days between the DAP at which ethylene is detected and the DAP at which the maximum level is reached.

In order to study the suitability of the new method, we calculated the limit of detection (0.4 $nL \cdot L^{-1}$) and the limit of quantification (1.3 $nL \cdot L^{-1}$). We also tested the range of linearity, which was maintained between 0 to 20 nL (data not shown).

The results obtained for the parental lines, the hybrid (F_1), and the RILs are presented in Table 1. The experiment confirmed the climacteric and non-climacteric behavior of the parental lines, as Ved showed an initial production of ethylene of 0.71 $\mu L \cdot kg^{-1} \cdot h^{-1}$ at 34 DAP, reaching a maximum ethylene production of 72.6 $\mu L \cdot kg^{-1} \cdot h^{-1}$ at 36 DAP, whereas PS did not show ethylene production during ripening.

The climacteric parent Ved started to produce the hormone at 34 DAP, while the hybrid did it earlier, at 28 DAP. The RIL population showed a large range of DAP of ethylene production, from 28 to 51 DAP, and the peak appeared between 32 and 53 DAP.

Once ethylene was detected, the levels of the hormone increased progressively over the next few days, until reaching maximum ethylene production. This parameter, defined as width of the increase of ethylene production, varied in the population between zero and nine days, showing a tendency to be higher for lower ethylene peaks (Figure 2). All of the traits described showed transgressive segregation, the maximum ethylene production being the most striking with the highest value of 239.6 $\mu L \cdot kg^{-1} \cdot h^{-1}$, more than three times the climacteric parental line value.

Climacteric behavior

Various components of climacteric behavior, including external color change, presence of characteristic aroma, and abscission layer formation, were registered in the 91-RIL population in three replicates, with the parents and the hybrid as controls. The qualitative data is presented as a consensus (Table 2) and the quantitative data as a mean of three replicates (Table 3).

In the RIL population, each trait presented a particular segregation; 72 lines of the population showed the characteristic aroma (segregation 3 : 1, $\chi^2 = 1.56$), the external color change was observed in 48 lines (segregation 1 : 1, $\chi^2 = 1.06$) and the abscission layer, with more or less intensity, appeared in 27 lines (segregation 1 : 3, $\chi^2 = 1.77$).

In addition to the most typical qualitative phenotypes, we evaluated other quantitative parameters to characterize the

Table 1. Values of the controls (parental lines Ved and PS, and hybrid F_1) and range observed in the RIL population. The width of the increase of ethylene production is expressed in days, and the maximum ethylene production in $\mu L \cdot kg^{-1} \cdot h^{-1}$.

Trait	Ved	PS	F_1	Population
DAP ethylene production	34	-	28	28 – 51
Width increase ethylene production	2	-	1	0 – 9
Maximum ethylene production	72.6	0	33.3	0 – 239.6
DAP maximum ethylene production	36	-	29	32 – 53

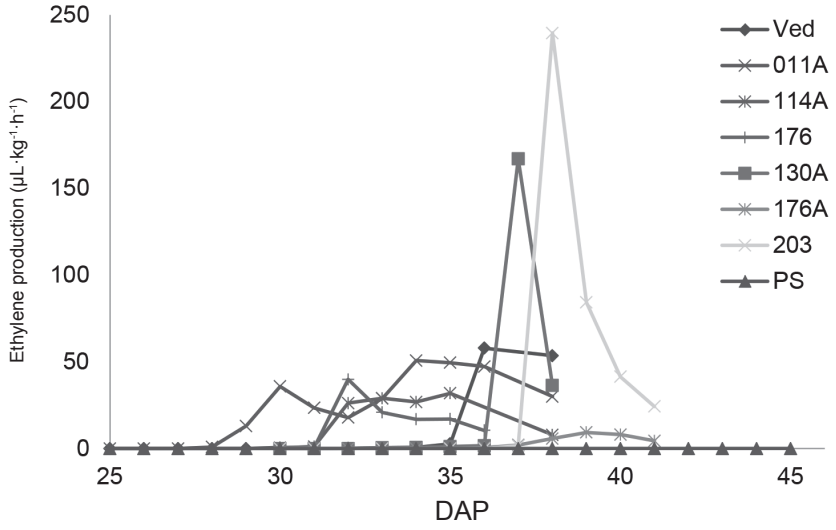

Figure 2. Ethylene production during fruit ripening in the parents (Ved and PS) and some representative examples from the RIL population.

climacteric behavior in the RIL population (Table 3). A wide range of DAP was observed for aroma production, external color change and abscission layer formation. In Ved, the three first effects are almost simultaneous. In the hybrid, the aroma production was the initial symptom, followed by external color change and, later, abscission layer formation. In both cases, fruit was harvested around one week later and the abscission was not total. PS did not show any of the traits related to climacteric ripening and the fruit was harvested at 61 DAP. The RIL population revealed transgressive segregation in all traits, obtaining both extreme early and delayed climacteric lines in comparison with Ved. The penetrometer values did not show a big difference between the parental lines, even though in the population we observed there were values almost four times higher than that of PS.

Discussion

The molecular control of fruit ripening, discerning between the ethylene-dependent and independent components, is an important topic in plant research (Gapper et al. 2014) and melon is considered as an alternative model to study its genetic regulation (Ezura and Owino 2008). The analysis of the current RIL melon population derived from a cross between two commercial cultivars with opposite and extreme ripening behavior may reveal new genes involved in this trait.

Over the past few decades, the ethylene fruit production has mostly been estimated by cutting the immature fruit and putting it in jars, using either static or dynamic methods (Bassi and Spencer 1982, Trebitsh et al. 1987, Vegas et al. 2013). The analysis of ethylene fruit production with the fruit *in planta* developed in this work offers some advantages. Traits other than ethylene can be evaluated on the same day, the fruit is conserved in optimal conditions, and the physiological responses are more accurate. The quantity of ethylene registered in our assay for Ved is much greater, around 10 times more, than the one described before with detached fruits (Saladié et al. 2015), probably due to the different methodology (detached fruits and GC-FID measurement). This new approach, with higher sensitivity, allowed us to detect some lines that produced very low levels of ethylene during the last days of the ripening period. Besides, due to the continuous and non-destructive sampling, we confirmed that most of the lines showed the external color change and the production of aromas almost simultaneously with the ethylene production, and sometimes even preceding the peak (Figure 3). Depending on the genetic background of the RILs, low levels of the hormone produced before the peak can be enough to trigger the climacteric ripening-associated phenotypes, including external color change, aroma, and abscission layer formation.

The RILs showed differences in ethylene levels, precocity, and in the correlation between the physiological consequences of climacteric ripening. Ethylene is the signal that activates secondary pathways determining chlorophyll degradation, abscission layer formation, aroma production, and flesh softening (Pech et al. 2008). Genes implicated in these secondary pathways, but not connected with ethylene, could be segregating in our population. In this way, the qualitative and quantitative differences between climacteric lines could be due to quantitative segregation in ethylene production or to differences in other regulators implicated in only one of the traits.

The results of a GBS experiment that is being performed will allow the characterization of QTLs or genes implicated in climacteric ripening, contributing substantially to our current knowledge of ripening in melon.

Acknowledgements

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Table 2. Phenotypic evaluation of qualitative traits related to ripening in the controls (Ved, PS, F_1) and the segregation in the RIL population.

Trait	Ved	PS	F_1	Population*
Presence of characteristic aroma	Yes	No	Yes	72 : 19
External color change	Yes	No	Yes	48 : 43
Abscission layer formation	Yes	No	Yes	27 : 64

*Population (Yes : No).

Table 3. Means of the quantitative traits related to ripening in the controls and the range in the RIL population.

Trait	Ved	PS	F_1	Population
DAP aroma production	36.0	-	34.0	29 – 62
DAP external color change	36.5	-	35.5	31 – 59
DAP abscission layer formation	35.5	-	38.5	28 – 56
Abscission evaluation at harvest	2.7	0	2.2	0 – 3
Penetrometer ($kg \cdot cm^{-2}$)	1.7	2.2	0.2	0.1 – 7.5

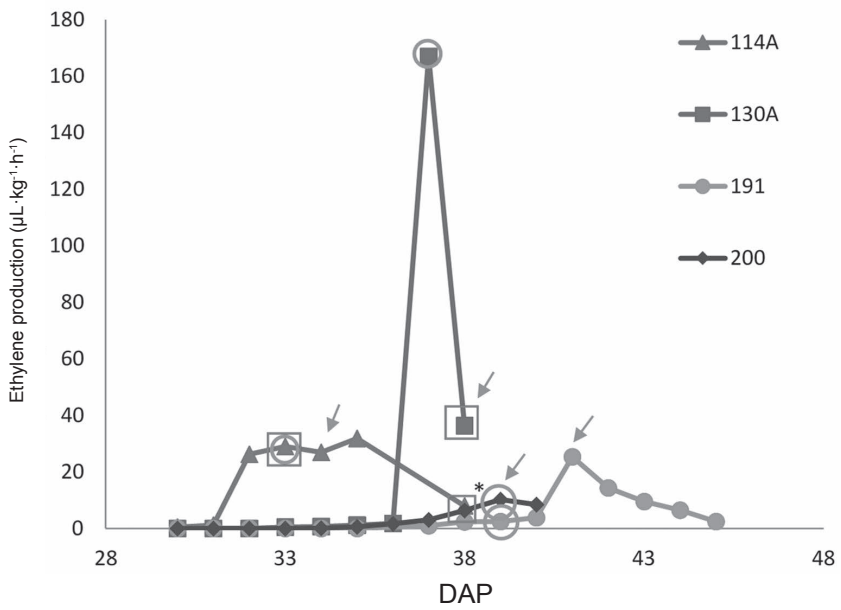

Figure 3. Ethylene production and climacteric symptoms: circles represent the presence of aromas; squares, the external color change, and arrows the abscission layer formation. *Line 191 did not show external color change.

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Utilizing Genetic Diversity in the Desert Watermelon *Citrullus colocynthis* for Enhancing Watermelon Cultivars for Resistance to Biotic and Abiotic Stress

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Abstract. Wide genetic diversity exists among the desert watermelon *Citrullus colocynthis* L. Schrad. (*CC*) accessions collected in the deserts of northern Africa, the Middle East, and Asia. Because of their resistance to biotic and abiotic stresses, they can be a viable source of genes used for enhancing watermelon cultivars. Here, we examined genetic diversity and relationships among 29 accessions of *CC* collected in northern Africa, the Middle East, and Asia. Also, we examined their relationships to watermelon cultivars and accessions representing *Citrullus lanatus* var. *lanatus* (*CLL*), *C. lanatus* subsp. *lanatus* var. *citroides* (*CLC*), and an accession representing the desert perennial *C. ecirrhosus* (*CE*). Twenty-three high frequency oligonucleotides-targeting active gene (HFO-TAG) primers were used to produce a total of 431 polymorphic fragments representing coding regions of the genome. Cluster and multidimensional scaling plot analysis separated the *CC* accessions into five distinct clades which is in general agreement with geographic origins. The analyses indicated closer genetic relationships of *CC* to *CLL* than to *CLC*. The desert perennial *CE* contained alleles present in *CC, CLL,* and *CLC*, indicating that it may have evolved from an ancestor common to *CC*, *CLL,* and *CLC*. Several *CC* PIs were identified as resistant to *Papaya ringspot virus* and/or whiteflies, and are being crossed with watermelon cultivars (*CLL*) and the F_1 or BC_1 seeds are viable, indicating that *CC* can be used to introduce biotic or abiotic stress-resistance genes into cultivated watermelon.

Introduction

Watermelon is an important vegetable crop throughout the world. It belongs to the xerophytic genus *Citrullus* Schrad. ex Eckl. & Zeyh. and is cultivated in temperate and tropical regions of the world (Jarret et al. 1997, Paris 2015). The genus *Citrullus* includes several diploid (n = 11) species, including *Citrullus lanatus* (Thunb.) Matsum. & Nakai that gave rise to the red-fleshed sweet dessert watermelon (*CLL*), as well as the "egusi" type (also referred to as *C. mucosospermus*; Fursa 1972, Chomicki and Renner 2014) cultivated for its large oily seeds. *C. lanatus* also includes the "tsamma" or preserving melon that is common in southern Africa and is known in the literature as *C. lanatus* var. *citroides* (*CLC*) (Whitaker and Bemis 1976, Jeffrey 2001). This taxon is also referred to as *C. lanatus* subsp. *lanatus* var. *lanatus* (Jeffrey 2001) and *C. amarus* Schrad. (Chomicki and Renner 2014). *C. ecirrhosus* Cogn. (*CE*) is a perennial native to deserts in southern Africa, and is not cultivated.

C. colocynthis (L.) Schrad. (*CC*) is a perennial watermelon species, also known as 'bitter apple', that is endemic to desert soils throughout northern Africa, the Middle East, and south-western Asia (Jarret et al. 1997, Dane et al. 2006, Paris 2015). Although there are reproductive barriers resulting from wide genomic differences between *CC* and *CLL* (Levi et al. 2006, 2013), *CC* can be crossed with dessert watermelons (Levi et al. 2010) and could be a useful source of genes for enhancing biotic and abiotic stress resistance in watermelon cultivars (Levi et al. 2016). Several *CC* PIs have been identified as having resistance to whiteflies, *Bemisia tabaci* Gennadius (Coffey et al. 2015), spider mites, *Tetranychus urticae* C. L. Koch (Cantu 2014), powdery mildew, *Podosphaera xanthii* race 2W (Tetteh et al. 2010), or *Zucchini yellow mosaic virus* (ZYMV) (Guner and Wehner 2008). As a desert plant species, *CC* is endemic to arid environments and could be a useful source for enhancing drought tolerance in watermelon cultivars (Si et al. 2009, Wang et al. 2014).

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A previous study using randomly amplified polymorphic DNA (RAPD) markers (Levi et al. 2001a) indicated that high levels of genetic diversity exist among *CC* PIs. Dane and Lang (2004) used chloroplast and a single-copy nuclear gene sequences to differentiate among *CC* PIs. In this study, we used polymerase chain reaction (PCR) markers referred to as "high frequency oligonucleotide-targeting active genes (HFO-TAG)" (Levi et al. 2010) to (1) examine genetic relationships and diversity among *CC* PIs collected in northern Africa, Asia, and the Middle East, and (2) identify *CC* PIs that are variable in their genetic distances from *CLL* and *CLC*. Also here, we report the evaluating of *CC* PI collections and identified significant levels of resistance to *Papaya ringspot virus* (PRSV) in several accessions collected in northern India and in northern Africa, indicating that *CC* could be a viable source of resistance to potyviruses (Levi et al. 2016) and to whiteflies (Coffey et al. 2015).

Materials & Methods

Plant material and isolation of DNA

A total of 48 *Citrullus* genotypes were used for analysis, including 29 *CC* Plant Introductions (PIs), 12 *CLC* PIs, three *CLL* PIs, three *CLL* cultivars ('Charleston Gray', 'Sugar Baby', and 'Black Diamond'), and one *CE*. Five seedlings of each genotype were grown in the greenhouse at 26/20 °C (day/night). DNA was extracted using the method described by Levi and Thomas (1999).

PCR amplification and analysis using HFO-TAG primers

The PCR amplification conditions and scoring using the 23 HFO-TAG primers were as described by Levi et al. (2013). A dendrogram (Figure 1) was created based on the un-weighted pair-group method with arithmetic average (UPGMA) using the SAHN module in NTSYS-PC version 2.02j (Rohlf 1998). Bootstrap support for clusters was conducted in FreeTree using 5,000 permuted datasets (Pavlicek et al. 1999). A cophenetic matrix was generated from the dendrogram by using the COPH module in NTSYS-PC. A population structure analysis procedure for clarifying genotypic ambiguity was also used (Figure 2) by means of the computer program ''Structure version 2.200" (Falush et al. 2007).

Plant material for cross pollination attempts of CC *with watermelon cultivars*

U.S. PIs of *CC* and heirloom watermelon cultivars ('Charleston Gray' and 'Sugar Baby') were chosen for the interspecific hybridization experiment. Reciprocal cross attempts were carried out in a greenhouse using plants of heirloom cultivars and *CC* PIs. The greenhouse was maintained at a temperature of 26/20 °C (14 hours day/10 hours night), with a light intensity of ≈140 to 175 µmol/m^2/s^1 with LED lights (PRO 325; LumiGrow, Inc. Novato, CA). Pollination attempts were carried out between 0700_{HR} and 1000_{HR}, immediately following the collection of three male flowers from the pollen donor parent plant that were used to pollinate female flowers on the recipient plant. The ripe fruits of each plant of the heirloom cultivars were harvested at the 35th to 45th day post pollination.

Virus inoculation

The PRSV-W isolate, derived from strain 2052 described by Baker et al. (1991), was obtained from Sakata Seeds, Ft. Meyers, Florida and maintained on squash plants (*Cucurbita pepo* L.) in the USVL virology greenhouse. Virus inoculum was prepared as described by Ling et al. (2009). Three and four weeks after the first inoculation, plants were evaluated for virus symptoms, as described by Levi et al. (2016).

Results & Discussion

Genetic relationships and diversity within and among *CC* PIs and in relation to PIs *CLC* and *CLL* subgroups are based on 431 HFO-TAG markers and are shown in Figure 1. The HFO-TAG fragments ranged in molecular weight from 80 to 420 bp and a large number of these fragments differed in molecular size by only one or a few nucleotides and could represent similar sequences or the same locus (Levi et al. 2010). As in previous studies (Levi et al. 2013, Paris et al. 2015), the HFO-TAG markers proved useful for population structure analysis and for differentiating among closely related genotypes. As depicted in Figure 1, wide genotypic diversity exists among the *CC* PIs collected in the deserts of northern Africa, the Middle East, and Asia.

The population structure and genetic analyses differentiated the *CC* PIs into five distinct groups (Figure 1) and identified transitional genotypes that fall between the *CC* and *CLL* or *CLC* species. The first major *CC* group contains predominantly PIs that were collected in northern Africa or in the adjacent Negev Desert, Israel. Several of the accessions in this group have a set of alleles that are unique to *CC* group 1. Several of the PIs in *CC* group 1 share alleles with *CLL* PIs collected in the same geographic region. The second and third *CC* groups include PIs collected mainly in the Middle East (the Negev Desert, Israel, Jordan, and Iran). The third group includes *CC* PIs collected in Iran and Afghanistan. The fourth and the fifth groups are represented by *CC* PIs 386024 and 525082 that were collected in Iran and Egypt, respectively (Table 1). Each of these two PIs has a unique set of alleles and may represent isolated *CC* populations that have undergone fixation as a result of sexual or geographic barriers. In addition to these *CC* PIs with unique alleles, PIs in a transitional position that contained alleles from the different *CC* groups were identified. The genetic analysis presented here suggests that the *CC* genotypes are (in general) more closely related to *CLL* than to *CLC*. Several of the *CC* PIs collected in northern Africa or in the Middle East share many alleles with the *CLL* genotypes.

The narrow and pointed sharp leaves of *CC* more closely resemble those of xerophytic *CLL* than *CLC* leaves which have wider and rounder lobes (Figure 2). *CLC* and *CLL* share the same reproductive features and are readily crossed with each other to produce fertile progeny using traditional breeding procedures (Levi et al. 2011, 2012), in contrast to crosses with *CC* that show some directionality and frequently result in significantly reduced fertility of progeny (Levi et al. 2006).

Our previous study using HFO-TAG markers indicated possible close relationships of *CC* to watermelon cultivars (*CLL*) (Levi et al. 2013). For this reason, we have been investigating the possibility of crossing *CC* PIs that show resistance to whiteflies (Coffey et al. 2015), spider mites, or PRSV (Levi et al. 2016) with watermelon cultivars, including 'Charleston Gray' and 'Sugar Baby' (Table 1).

Our cross-pollination attempts of watermelon cultivars ('Charleston Gray' or 'Sugar Baby') with *CC* accessions representing the different *CC* groups (Table 1, Figure 2) produced

Table 1. The *Citrullus colocynthis* PIs (and the group they belong to in Figure 1) and watermelon cultivars used for producing interspecific F_1 hybrids and consequent F_2 and BC_1 seeds in a greenhouse at the USDA, ARS, U.S. Vegetable Laboratory, Charleston, South Carolina. Listed are the approximate number of viable F_1, F_2, and BC_1 seeds per fruit produced in plants derived from the crosses. Approximate number of seeds is based on 2–6 fruits collected from 2–4 plants of each cross in the greenhouse.

CC accession crossed with cultivar	Male parent	Female parent	F_1 seeds per fruit	F_2 seeds per fruit	[z]BC_1 seeds per fruit (M)	[z]BC_1 seeds per fruit (F)
PI 525080 (CC Group 1)	'Sugar Baby'	PI 525080	40-75 seeds	30-70 seeds	50-90 seeds	20-40 seeds
	PI 525080	'Sugar Baby'	Few seeds[y]	------	------	------
PI 386024 (CC Group 4)	PI 386024	'Charleston Gray'	25-40 seeds	------	30-70 seeds	25-40 seeds
	'Charleston Gray'	PI 386024	25-40 seeds	------	20-40 seeds	25-40 seeds
PI 386026 (CC Groups 3, 1 and 4)	PI 386026	'Charleston Gray'	20-50 seeds	------	30-60 seeds	25-40 seeds
	'Charleston Gray'	PI 386026	25-40 seeds	------	25-40 seeds	25-40 seeds
PI 537277 (CC Groups 5, 1, 3 and CLL)	'Sugar Baby'	PI 537277	Few seeds[y]	No Seeds	Few seeds	No seeds
	PI 537277	'Sugar Baby'	60-100 seeds	------	------	40-70 seeds
PI 386021 (CC Groups 3, 1, 5 and CLL)	'Sugar Baby'	PI 386021	20-35 seeds	------	20-45 seeds	20-35 seeds
	PI 386021	'Sugar Baby'	Few seeds[y]	------	20-35 seeds	20-40 seeds

[z]BC_1 using the watermelon cultivar as a male (M) or female (F) recurrent backcross parent.
[y]Few Seeds = 4 to 10 seeds

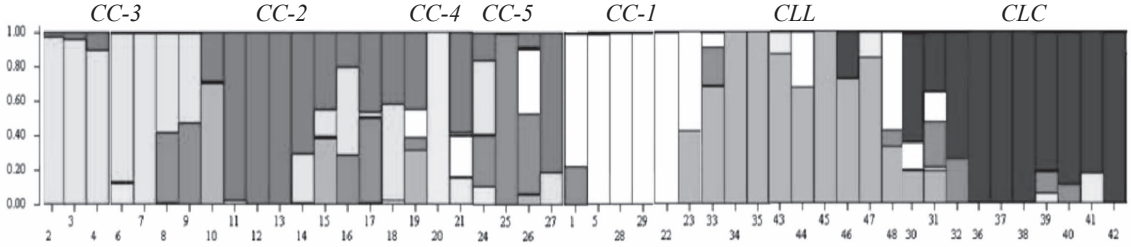

Figure 1. Population structure analysis resolving several subpopulations (K = 7). Scale of Y axis represents probability of log likelihood. Ancestry of the 48 *Citrullus* spp. genotypes, estimated based on the "Structure version 2.200" (Falush et al. 2007). The ancestry from the inferred *C. lanatus* var. *citroides* (*CLC*) and *C. lanatus* var. *lanatus* (*CLL*) gene pools are shown in dark blue and brown, respectively. The ancestry from the inferred *C. colocynthis* (*CC*) gene pools are shown in yellow (Group 1), red (Group 2), green (Group 3), navy blue (Group 4), and purple (Group 5).

variable numbers of seeds in fruits of the F_1, F_2, and BC_1 plants (Table 1), indicating that reproductive barriers exist between *CC* PIs and watermelon cultivars. The interspecific pollination attempts produced viable F_1 and BC_1 plants in all cross-pollinations of watermelon cultivars and *CC* accessions (Table 1). Additional studies are needed to estimate pollen viability and compatibility of plants representing the different *CC* groups, in order to better assess their use in efforts to enhance genetic diversity within and among watermelon cultivars.

Overall, *CLC* and *CLL* are distinct from the *CC* groups (Figure 2). Each of the five *CC* groups contains unique alleles, but also shares alleles with *CLL*, indicating a possible evolutionary relationship. Phenotypic variation in leaf shape, leaf size, fruit shape, and fruit color is also present among the *CC* PIs (Figure 2). However, the serrated leaves and sharp lobes of *CC* more closely resemble those of *CLL* than *CLC*. The *CLC* collected in southern Africa have leaves with generally wider-rounded lobes (Figure 2). The results presented in this study clarify the relationship of *CC* to *CLL* and possibly *CE.* However, the question of whether *CC* evolved from *CLL* or from a common ancestor of *CE, CLL*, and *CLC*, remains to be determined. Rapid advances in next generation sequencing technologies (NGS) and the possibility of sequencing and assembling the genomes of a large number of genotypes will provide new opportunities for more accurately determining genetic relationships among *Citrullus* groups and species. Here, we also identified several *CC* PIs with high resistance to potyviruses (Figure 3) and whiteflies that could be used in genetic and genomic studies aiming to identify the gene loci conferring resistance and furthermore in breeding programs aiming to enhance resistance in watermelon cultivars.

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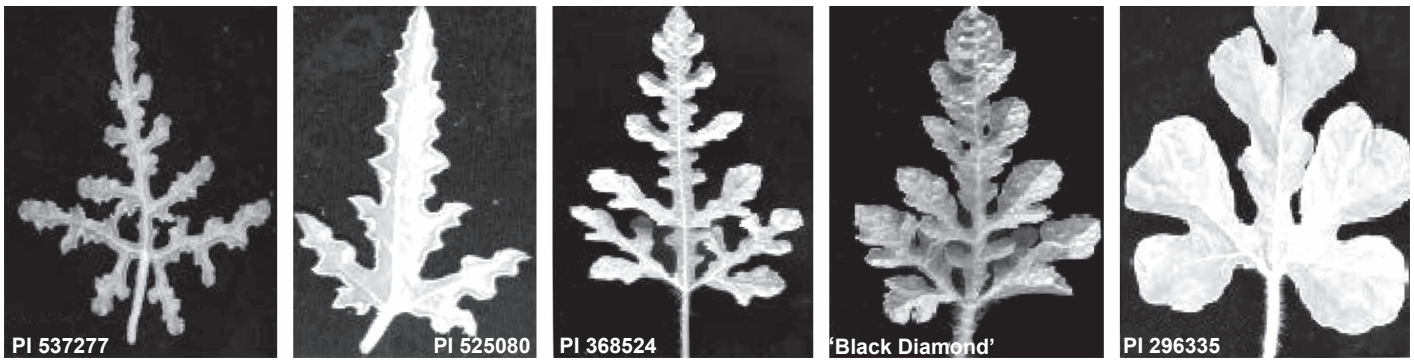

Figure 2. Leaf samples of plants representing *Citrullus colocynthis CC* (PI 537277 and PI 525080), *Citrullus lanatus* var. *lanatus CLL* (PI 368524 and the 'Black Diamond'), and *Citrullus lanatus* var. *citroides CLC* (PI 296335). Leaves of *CC* have a narrow and pointed shape and serrated lobes with a dry waxy surface. Leaves of *CLL* are narrow and pointed but with larger serrated lobes. The *CLC* leaves have wide and round lobes.

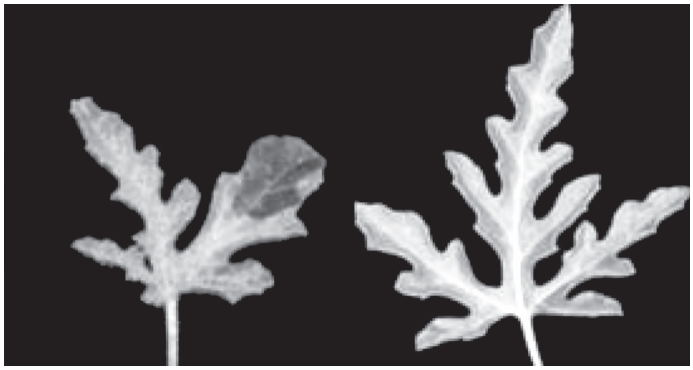

Figure 3. A distorted leaf with mosaic patterns of the *Papaya ringspot virus* (PRSV)-susceptible *Citrullus colocynthis* PI 549161 (left) *versus* a healthy leaf of *C. colocynthis* PI 537277 (right), following inoculation with a PRSV-watermelon strain isolate. The photographs were taken 3 weeks after plants were inoculated.

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Inheritance and QTL Mapping of Resistance to Gummy Stem Blight in Cucumber (*Cucumis sativus* L.)

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Abstract. Gummy stem blight (GSB) is an important disease of cucumber worldwide. There are no reports on accurate simple sequence repeat (SSR) markers for use in molecular breeding of GSB resistance and no studies on chromosomal mapping of this resistance in cucumber. In this paper, a set of 160 F_9 recombinant inbred lines (RILs) derived from the cross PI 183967 × 931 and a total of 2,112 pairs of SSR primers were used to study the inheritance of GSB resistance and to detect quantitative trait loci (QTLs) conferring resistance in the cucumber stem. Genetic analysis indicated that the resistance to GSB in PI 183967 was quantitative and mainly governed by three pairs of additive-epistatic major genes. Five QTLs, *gsb-s1.1*, *gsb-s2.1*, *gsb-s6.1*, *gsb-s6.2*, and *gsb-s6.3*, for resistance to GSB in cucumber stems were detected. The loci *gsb-s1.1* and *gsb-s2.1* with phenotypic variation of 8.7% and 6.7% were mapped to chromosomes (Chr.) 1 and 2, respectively. The loci *gsb-s6.1*, *gsb-s6.2*, and *gsb-s2.1,* were on Chr. 6 and were linked. Locus *gsb-s6.2* accounted for the highest phenotypic variation, 22.7%, and was flanked by SSR04083 and SSR02940 with genetic distances of 5.0 and 1.8 cM, respectively. There were 117 candidate genes predicted between SSR04083 and SSR02940, of which 14 were related to disease resistance.

Keywords: *Cucumis sativus*, stem, gummy stem blight, molecular mapping, SSR markers, candidate gene

Introduction

Gummy stem blight (GSB) is a destructive disease of cucumber (*Cucumis sativus* L.). It is caused by *Didymella bryoniae*, and it affects root, stems, and leaves of cucumber. GSB often causes 15 to 30% production losses and severe infection can result in 80% or greater production losses. Understanding the inheritance of GSB resistance and mapping of genes associated with GSB-resistance will facilitate fine mapping and molecular cloning of resistance genes and molecular marker-assisted breeding. This is important for the development of new GSB-resistant cultivars.

Reports on the inheritance of cucumber GSB resistance are limited and somewhat controversial. Norton (1979) generated genetic populations using GSB-resistant PI 189225 and GSB-susceptible 'Charleston Gray' watermelons (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), and discovered that GSB resistance is controlled by a single recessive gene (*db*/*db*). Amand and Wehner (2001) reasoned that the major effect conferred by *db*/*db* is complemented by the expression of other modifier genes for GSB resistance in stems and leaves. They also found that the genetic factors are weaker than the environmental factors. In melons (*Cucumis melo* L.), five relatively independent single genes conferring resistance to GSB were identified from different cultigens, among which four were dominant and one was recessive (Zuniga et al. 1999, Wako et al. 2002, Frantz and Jahn 2004).

So far, we did not find reports on molecular markers and genetic mapping of cucumber GSB resistance. However, there has been research on molecular markers and genetic mapping of GSB resistance in melon. Joseph (2009) identified four amplified fragment length polymorphism (AFLP) markers linked with the GSB resistance in PI 420145. These had genetic distances of 2.0 cM, 6.0 cM, 5.4 cM, and 6.0 cM. Molecular markers CMCT505, CMTC160a+b220 and ISSR-57560, ISSR-100900, and CMTA170a have been closely linked with the melon GSB resistance genes *Gsb-1*, *Gsb-2*, *Gsb-3*, and *Gsb-4*. Among these markers, CMTA170a and Gsb-4 had the shortest genetic distance, 5.14 cM. The *Sb-x* GSB-resistance gene in melon was identified and mapped to the LG4 linkage group using simple sequence repeats (SSR) markers and a double haploid (DH) melon population. Ha et al. (2010) mapped *Sb-1*, a gene for resistance, to the melon LG1 linkage group. These results provide valuable references for the identification of GSB-resistance genetic markers in cucumbers.

In this study, we used wild-type cucumbers and recombinant inbred lines (RILs) with distinct resistance to GSB. Cucumber stems were inoculated with spore suspensions and then scored for susceptibility. Genetic analysis and mapping of quantitative trait loci (QTLs) were performed to provide a basis for fine mapping and molecular cloning of genes for GSB-resistance and for future marker-assisted selection (MAS).

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Materials & Methods

Plant materials

A wild-type GSB-resistant cucumber accession PI 183967 (*Cucumis sativus* var. *hardiwickii* (Royle) Alef.) was crossed with a cultivated GSB-susceptible cucumber accession designated 931 (*C. sativus* var. *sativus*). After single-seed descent (SSD) reproduction, a population was developed that consisted of 160 F_9 RILs.

Disease-resistance screening and symptom assessment

In autumn 2012 and spring 2013, the parents, F_1, and RILs were planted. The experiments were repeated three times and a total of 18 plants were grown out for each line. All of the field and greenhouse experiments were conducted at the Nankou Farm, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences. The distances between plants and rows were 25 cm and 55 cm, respectively. The plants were grown using standard cultivation practices. Well-grown and healthy stems from the parents, F_1, and RILs were trimmed to 15 cm. The stems were arranged randomly with three replications of six stems each.

GSB resistance of excised stems was analyzed by inoculation with a *Didymella bryoniae* spore suspension. First, *D. bryoniae* was grown on cucumber fruits for 7 to 10 d to allow pycnidia formation. Spores were collected by scraping the infested fruits. The spores were then suspended in 10 ml of sterile water. Spore concentration was measured with a hemocytometer and adjusted to 10^6 spores/ml. Trimmed stems were soaked in spore suspension for 30 min and then placed in a humidified chamber (Note: the soaked ends of all the stems were placed in the same direction). Disease resistance was rated 3 to 5 d after inoculation, and a disease index (DI) was calculated as a weighted mean according to the formula:

$$DI = 100 \times \frac{\sum(\text{Number of plants with disease rating} \times \text{Disease rating})}{(\text{Total number of plants} \times \text{Highest disease rating possible})}$$

The disease rating scale for each seedling was as follows: 0 – no symptoms; 1 – infected part of the stem was less than ¼ of the total stem; 2 – infected stem was ¼ to ½ of the total stem; 3 – infected stem was ½ to ¾ of the total stem; 4 – infected stem was > ¾ of the total stem. Stem resistance grading was defined using DI. High-resistance (HR): $0 < DI \leq 15$; resistance (R): $15 < DI \leq 35$; medium resistance (MR): $35 < DI \leq 55$; susceptible (S): $55 < DI \leq 80$; and highly susceptible (HS): $DI > 80$.

Genetic analysis software and statistical analysis

The DI of each stem was recorded and summarized in Microsoft Excel 2003 and SAS 9.0 software. The mean DI of each line was calculated, and the genetic parameters of the parents, F_1, and RILs were calculated. A joint analysis assuming two major genes plus the polygene model of RILs (Gai et al. 2003) was used for the GSB-resistance analysis. The steps were to establish the genetic models, estimate the iterated expectation and conditional maximization algorithm, select the best genetic model through AIC (Akaike information criterion) value, and evaluate goodness-of-fit through the least squares method. The optimized genetic model was then used for estimation of other genetic parameters.

SSR marker analysis

DNA was extracted from young leaf tissue of the parents, the F_1, and each plant in the RILs population by using a CTAB extraction procedure. DNA concentration was estimated on a 1% agarose gel with 1× TEA buffer, stained with ethidium bromide. Each 15 µl of the PCR reaction mix contained double distilled water (ddH_2O) 8.02 µl, 10 × buffer 1.5 µl, dNTPs (10 mM) 0.2 µl, Taq DNA polymerase (10 U/µl) 0.08 µl, primer F (50 ng/µl) 0.6 µl, primer R (50 ng/µl) 0.6 µl, DNA (10 ng/µl) 4.0 µl. The PCR amplifications were performed using a GeneAmp PCR system 9700 (Applied Biosystems Foster City, California) as follows: 94 °C/4 min, 35 cycles of 94 °C/15 s, 55 °C/15 s, 72 °C/30 s, and 72 °C/5 min, 16 °C. Subsequently, 3 µl of the PCR product was employed for electrophoresis in 6% polyacrylamide gel according to the method used by Sambrook and Russell (2001).

A total of 2,112 pairs of SSR primers were screened to identify polymorphisms between the parental lines (PI 183967 and 931) of the RILs populations. The development of the SSR primers used in this study was described by Ren et al. (2009). Polymorphic SSR primers were identified from individual plants of the RILs for linkage construction and QTL analysis.

Linkage construction and QTL mapping

JoinMap 4.0 (Van Ooijen 2006) was used to develop linkage groups. Chromosomal assignment of the linkage groups (LGs) was based on common markers between the present map and the high-resolution cucumber genetic map produced by Ren et al. (2009). Marker data were assigned to LGs by using a minimum logarithm of odds (LOD)-likelihood score of 2.5. The Kosambi map function (Kosambi 1944) was used to calculate the genetic distance between markers. An interval mapping analysis was conducted by using MapQTL 4.0 (Van Ooijen et al. 2000) to detect QTLs. Permutation tests were conducted to assess LOD threshold at $\alpha = 0.05$ level. Each locus was named by an abbreviation of the trait followed by the chromosome (Chr.) number and a locus number.

Sequence annotation and gene prediction in genomic regions harboring the major QTLs

Based on the whole genome sequence of the cucumber inbred line 9930 (Huang et al. 2009), genomic regions for the locations of the major QTLs were annotated. The DNA sequences were aligned to the cucumber genome sequences using BLASTN. Only matches with an identity of ≥ 95% were retained. Gene prediction was performed as reported by Zhang et al. (2013).

Results

GSB-resistance in cucumber stem for the progeny of PI 183967 × 931

In autumn 2012, the DI of the GSB-resistant parent PI 183967 was 27.09 (R), while the DI of the GSB-susceptible parent 931 was 77.38 (S). The DI of the F_1 was 96.43 (HS), a transgressive segregation phenotype. In spring 2013, the DI of the GSB-resistant parent was 21.88 (R), while that of the GSB-susceptible parent was 89.60 (HS). The DI of the F_1 was 66.29 (S), intermediate between the two parents with a trend toward susceptibility. The mean DI of the RIL populations was intermediate between the

Table 1. Disease index of the parents and F_1 and some genetic parameters in RILs populations in two seasons.

Season	Parental lines		F_1	RILs populations			
	PI183967	931		Mean	SD	Kurtosis	Skewness
Autumn 2012	27.08±1.78	77.38±19.04	96.43±3.12	62.77	30.60	-0.56	-1.11
Spring 2013	21.88±0.51	89.60±3.85	66.29±19.41	46.76	29.03	0.22	-1.13

Table 2. Fitness test of alternative genetic models for resistance to gummy stem blight in the cucumber stem.

Season	Model	Generation	$U1^2$	$U2^2$	$U3^2$	${}_nW^2$	D_n
Autumn 2012	F-4	P_1	0.812(0.36767)*	1.175(0.27829)*	0.719(0.39660)*	0.14	0.482
		P_2	0.224(0.63619)	0.000(0.99986)	3.359(0.06684)*	0.105	0.36
		RILs	8.738(0.00312)*	13.137(0.00029)*	9.296(0.00230)*	1.347*	0.261
	F-1	P_1	5.661(0.01735)*	7.693(0.00554)*	3.533(0.06015)*	0.616	0.78
		P_2	0.020(0.88696)	0.082(0.77526)	2.865(0.09054)*	0.076	0.32
		RILs	0.017(0.89689)	0.003(0.95358)	0.072(0.78788)	0.025	0.058
	G-2	P_1	0.119(0.73003)	0.476(0.49041)	2.022(0.15499)*	0.098	0.415
		P_2	0.041(0.83895)	0.012(0.91219)	1.509(0.21936)*	0.06	0.297
		RILs	8.216(0.00415)*	9.221(0.00239)*	1.092(0.29606)*	1.012*	0.216
Spring 2013	F-1	P_1	0.044(0.83308)	0.048(0.82675)	2.862(0.09070)*	0.08	0.314
		P_2	1.326(0.24945)*	1.395(0.23755)*	0.070(0.79175)	0.164	0.488
		RILs	0.080(0.77688)	0.068(0.79462)	0.003(0.95510)	0.03	0.059
	F-4	P_1	2.602(0.10676)*	3.597(0.05788)*	1.795(0.18034)*	0.275	0.572
		P_2	1.041(0.30763)*	1.263(0.26115)*	0.295(0.58679)	0.148	0.483
		RILs	4.121(0.04236)*	2.771(0.09599)*	1.448(0.22878)*	0.512	0.173
	G-2	P_1	0.256(0.61267)	0.629(0.42762)*	1.470(0.22539)*	0.076	0.322
		P_2	1.009(0.31504)*	0.800(0.37124)*	0.099(0.75310)	0.144	0.429
		RILs	1.302(0.25393)*	1.276(0.25860)*	0.010(0.92007)	0.217	0.124

Note: $U1^2$, $U2^2$, $U3^2$ are the statistic of Uniformity test; ${}_nW^2$ is the statistic of Smirnov test; D_n is the statistic of Kolmogorov test.
${}_nW^2_{0.05}$, = 0.4610, $D_{n0.05}$ = 1.3600
The relevant probability of U_1^2, U_2^2, U_3^2 in the bracket.
*indicates that the value is significant at $\alpha = 0.05$.
P_1, P_2: parents; RILs: recombinant inbred lines.

two parents, but skewed toward the susceptible parent (Table 1). Genetic parameter analysis produced negative RIL population skewness values, further indicating that the DI of the RIL populations was inclined toward the 931 parent. There was a continuous distribution of DI values in the RIL populations, suggesting that the GSB-resistant phenotype was not conferred by a single major gene. These results show that the GSB-resistant phenotype of parental line PI 183967 has quantitative-trait heritability characteristics.

Inheritance of GSB resistance in the cucumber stem

We used the RILs populations and major gene plus polygene mixed genetic model analysis, together with the DI measurement in cucumber stems during autumn 2012 and spring 2013, to generate 35 mixed genetic models. The maximum likelihood (ML) and AIC of these models were estimated. Based on the principle that the smallest AIC value is the best-fitting genetic model, three models (F-4, F-1, and G-2) were prioritized based on the 2012 autumn data and the lowest AIC was selected and further tested for goodness-of-fit (Table 2,3). The number of statistically different parameters was 8, 4, and 6 for F-4, F-1, and G-2, respectively. Since model F-1 had the lowest number, it was considered the best fit for the autumn 2012 GSB stem-resistance inheritance analysis. The AIC values of F-1, F-4, and G-2 for the spring 2013 data were relatively lower than other models, and therefore they were selected for goodness-of-fit testing (Table 3). The number of statistically different parameters was 3, 8, and 6 for the F-1, F-4, and G-2 models, respectively. Since model F-1 had the lowest number, it was considered the best fit model for analysis of the 2013 spring GSB stem-resistance inheritance. Taken together, the F-1 model (three pairs of additive - epistatic major genes, no polygenes) appeared to be the best model for both seasons. The first order genetic parameters are shown in Table 4. The test of the best-fit model indicated that the three major genes controlling the cucumber GSB-resistance phenotype all had additive and epistatic effects. The inheritance of these major genes was 98.63% and 97.34%, respectively, for autumn and spring.

QTL mapping of GSB resistance in cucumber stems

Molecular analysis performed on cucumber lines PI 183967 and 931 using the SSR method resulted in identification of 1,125 primers generating polymorphic amplicons from a total of 2,112 pairs of SSR primers. The polymorphism rate was 53.2%. From 1,125 primer pairs, 350 SSR primer pairs, which were evenly distributed on the 7 cucumber chromosomes, were selected for RIL population analysis. The SSR primers gave good polymorphism results and clear stable bands. A genetic linkage map including 7 linkage groups (corresponding to the 7 chromosomes) was obtained. The linkage map included 307 SSR markers, had a total genome length coverage of 993.3 cM, and an average distance of 3.23 cM (Figure 1).

QTL mapping was conducted using this linkage map. In autumn 2012, two QTLs for GSB resistance in the cucumber stem, *gsb-s1.1* and *gsb-s6.1*, were detected. They were located on Chr. 1 and Chr. 6, respectively. QTL *gsb-s1.1* was between SSR12157 and SSR31116, with an LOD of 2.50, phenotypic variation of 8.7%, and additive effect of −9.54. The phenotypic variation of *gsb-s6.1* was 10.3%, with an LOD of 2.56, and two flanking markers SSR01012 and SSR03527 with genetic distances of 0.4 cM and 0.3 cM, respectively (Table 5, Figure 1). Both QTLs had negative effects, while the genes with positive effects were inherited from the GSB-susceptible parent, 931.

In spring 2013, three QTLs for GSB resistance were detected, which were *gsb-s2.1, gsb-s6.2,* and *gsb-s6.3*. Among

Table 3. AIC values of different genetic models for the resistance to gummy stem blight in cucumber stems.

Model	Autumn 2012	Model	Spring 2013
F-4	705.865662	F-1	718.553345
F-1	711.302551	F-4	718.754395
G-2	713.326843	G-2	722.976257

Table 4. Parameter estimation of the first order for the optimal model in different seasons.

Season	Model	Content	Mean	Weight	Content anava
Autumn 2012	F-1	1	13.04	0.14	27.15
		2	31.15	0.11	
		3	65.91	0.15	
		4	93.58	0.12	
		5	52.07	0.12	
		6	81.96	0.12	
		7	93.65	0.12	
		8	78.39	0.12	
Spring 2013	F-1	1	23.94	0.14	21.85
		2	6.92	0.13	
		3	44.67	0.11	
		4	59.36	0.16	
		5	25.02	0.14	
		6	44.94	0.11	
		7	75.45	0.12	
		8	94.75	0.11	

Table 5. QTL analysis of gummy stem blight resistance in cucumber stems in different seasons.

Season	QTL	Chr.	Position	Marker interval	LOD	Phenotypic variation % R^2	Additive effect
Autumn 2012	*gsb-s1.1*	1	16.4	SSR12157-SSR31116	2.50	8.7	-9.54
	gsb-s6.1	6	14.9	SSR01012-SSR03527	2.56	10.3	-10.17
Spring 2013	*gsb-s2.1*	2	74.8	SSR13275-SSR10064	2.53	6.7	7.61
	gsb-s6.2	6	30.7	SSR04083-SSR02940	7.30	22.7	-15.92
	gsb-s6.3	6	179.0	SSR13251-SSR15516	3.24	8.7	-8.70

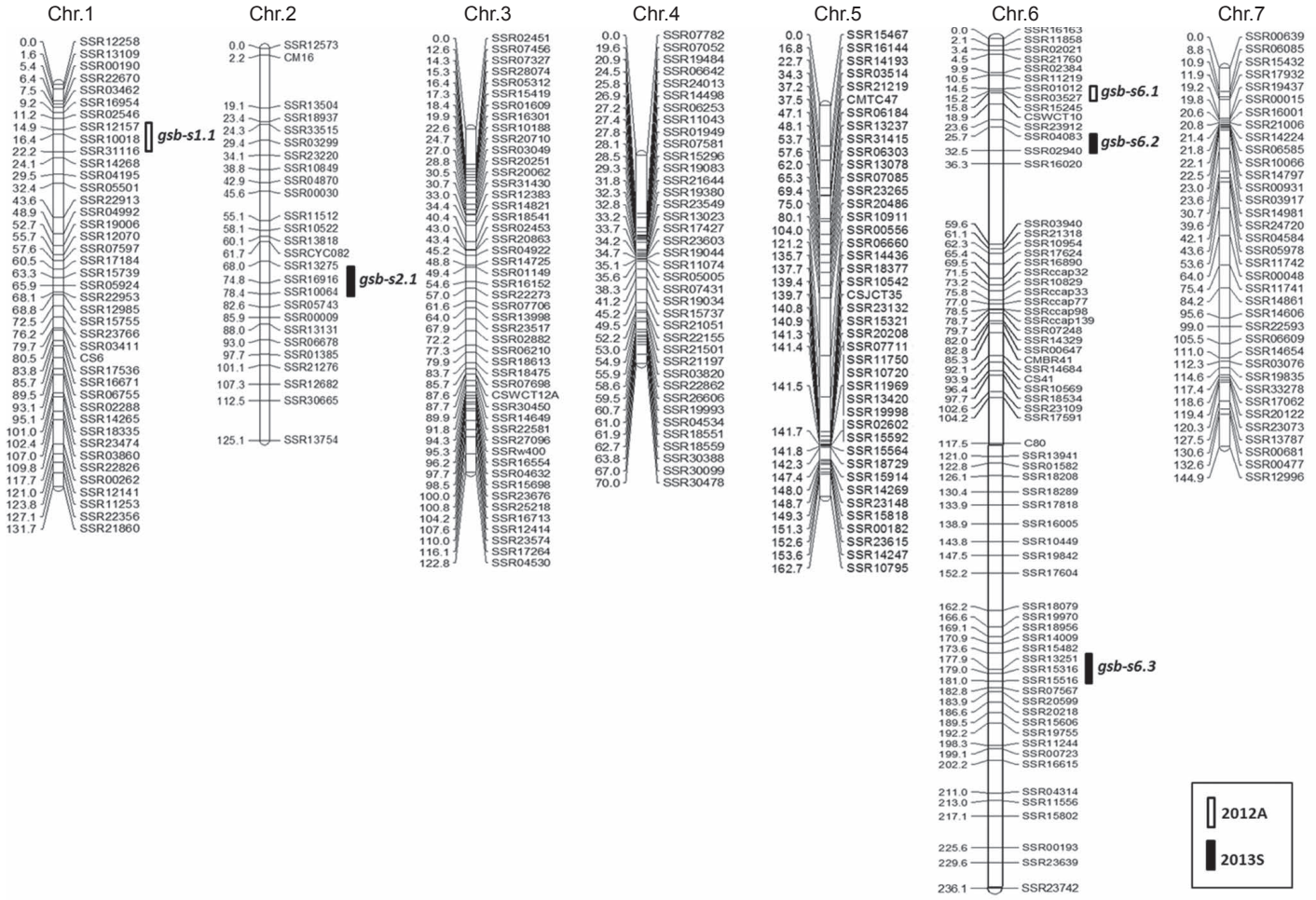

Figure 1. QTL mapping of gummy stem blight resistance in the cucumber stem.

these, *gsb-s2.1* was located on Chr. 2 between SSR13275 and SSR10064, with an LOD value of 2.53 and phenotypic variation of 6.7%. QTL *gsb-s2.1* was inherited from the GSB-resistant parent PI 183967, and promoted the GSB resistant phenotype. QTLs *gsb-s6.2* and *gsb-s6.3* were located on Chr. 6 at 30.7 cM and 179.0 cM locations, between SSR04083-SSR02940 and SSR13251-SSR15516, respectively. QTLs *gsb-s6.2* and *gsb-s6.3* provided additive effects of −15.92 and −8.70, and contributed to the GSB-resistance phenotype by 22.7% (LOD = 7.30) and 8.7% (LOD = 3.24), respectively. Both *gsb-s6.2* and *gsb-s6.3* were inherited from the GSB-susceptible parent and can inhibit the GSB-resistant phenotype. Based on contribution level and LOD, *gsb-s6.2* was considered a major QTL, and its genetic distances to nearby markers SSR04083 and SSR02940 were 5.0 cM and 1.8 cM, respectively.

Sequence annotation and gene prediction in genomic regions harboring QTL

The QTLs *gsb-s6.1* and *gsb-s6.2* had greater contributions, shorter genetic distances, and they were detected in both seasons (Figure 1). We therefore postulated that GSB-resistance QTL sites exist in these two regions. We performed biological analysis and predicted GSB resistance-related genes in the SSR01012-SSR02940 region. BLAST analysis showed that this region has a physical distance of 1.27 Mbp and contains 117 annotated genes, including 14 disease-resistance related genes, ten kinase domains (*Csa6P052030.1*, *Csa6P052080.1*, *Csa6P052130.1*, *Csa6P052820.1*, *Csa6P055410.1*, *Csa6P058190.1*, *Csa6P067340.1*, *Csa6P061230.1*, *Csa6P074020.1*, *Csa6P076750.1*), three Zn-finger motifs (*Csa6P055930.1*, *Csa6P067930.1*, *Csa6P074560.1*), and one leucine-rich domain (*Csa6P062270.1*). The large number of genes in this region makes it difficult to determine the specific cucumber gene(s) which have large effects on GSB resistance. However, these predicted GSB resistance-related genes will be key candidates for further study.

Discussion

GSB is an important disease of cucumber worldwide. GSB can cause significant reductions in yields, especially in protected culture such as greenhouse production. Probably the best method for controlling GSB in cucumber is the use of resistant cultivars. As a result, GSB resistance is one of the main objectives in cucumber breeding programs (van der Meer et al. 1978, Wehner et al. 1996). Two patterns of inheritance have been reported for

resistance to GSB in cucurbits, quantitative resistance controlled by multiple genes (Amand and Wehner 2001), and qualitative resistance controlled by a single gene (Norton 1979). In this study, resistance to GSB in PI 183697 was concluded to be quantitative and controlled by three pairs of additive-epistatic major genes based on the disease index analyses for genetic populations derived from PI 183697 × 931. The two different patterns of inheritance that have been reported may be explained by several factors. First, different species, cultivars, and breeding lines have been used as resistance sources. Second, different disease identification methods and rating scales make it difficult to compare studies. Third, development of cucumber GSB is strongly affected by environmental conditions, as for many diseases, and maintenance of uniform environmental conditions during testing can be difficult. Fourth, the inheritance of GSB resistance appears to be complex.

In a conventional cucumber resistance-breeding program, phenotypic selection for GSB resistance is difficult due to the large influence of environmental factors on the development of the disease. Multiple tests with several replications and years are required. It may be possible to select for resistance more efficiently using molecular, marker-assisted selection (MAS), than using just disease ratings. However, we did not find a report on the molecular biology of GSB resistance in cucumber. This study used 2,112 pairs of SSR primers to map resistance genes for GSB in the cucumber stem. Five QTLs for GSB resistance were detected: *gsb-s1.1* was mapped into the marker interval between SSR12157 and SSR31116 with genetic distances of 7.3 cM; *gsb-s6.1* was flanked between SSR01012 and SSR03527 within 0.7 cM; *gsb-s2.1* was located in the marker interval between SSR13275 and SSR10064 with genetic distances of 510.4 cM; *gsb-s6.2* was flanked by SSR04083 and SSR02940 within 6.8 cM; *gsb-s6.3* was mapped into the marker interval between SSR13251 and SSR15516 with genetic distances of 3.1 cM. These ten SSR markers will benefit MAS for gummy stem blight resistance and will be useful for future genetic studies in cucumber.

Many genes conferring resistance (*R* genes) to a diverse array of pathogens, including bacteria, fungi, oomycetes, viruses, and nematodes, have been isolated in plants. In the cucumber 9930 draft genome, 61 nucleotide binding site (NBS) type resistance gene analogs (RGAs) were identified and were distributed mostly in 11 clusters (Huang et al. 2009). In this study, the genomic regions bearing the major QTLs of *gsb-s6.1* and *gsb-s6.2* were predicted. Of the 117 genes predicted in this region, there were 14 disease resistance-related genes and one (*Csa6P062270.1*) belonged to the NBS gene. The one NBS-type RGAs will be studied for GSB-resistance candidate genes.

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Global Occurrence of the A2 Mating Type of *Pseudoperonospora cubensis*, the Causal Agent of Cucurbit Downy Mildew

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Abstract. The A2 mating type of *Pseudoperonospora cubensis* is a newcomer to the western hemisphere. It was first detected in Israel in 2002 among pathotype 6 isolates collected from *Cucurbita* species. A survey made in Israel from 2002 to 2016 showed that most A2 isolates occur on *Cucurbita* whereas most A1 isolates occur on *Cucumis*. In a global survey conducted from 2011 to 2014, A2 isolates were found in Angola, China, Crimea, France, Germany, India, Kenya, Russia, and Vietnam. A2 was recently reported in the U.S.A. In India, nine out of 21 isolates were A2. Similarly, in Vietnam, 12 out of 21 isolates were A2. We hypothesize that the recent migration of A2 to the western hemisphere originated from India and Vietnam.

Keywords: Migration, oospores, seed transmission, sexual recombination, population structure

Introduction

Downy mildew is a major disease of cucurbits with a global distribution (Palti and Cohen 1980). The causal agent, *Pseudoperonospora cubensis* (Berk. & Curt.) Rost., is an obligate biotrophic pathogen that infects over 50 host plant species belonging to 20 genera of the family Cucurbitaceae (Cohen et al. 2015). This oomycete is known to reproduce asexually by aerially dispersed sporangia. Oospores were observed in nature (Zhang et al. 2012) and recently were produced by artificial inoculations (Cohen and Rubin 2012). Major changes in the population structure of *P. cubensis* have been described since 2002. New pathotypes and mating types were reported from around the globe (Cohen et al. 2015). Possible mechanisms may involve mutation, migration, and sexual recombination of the pathogen. In this paper we provide evidence that the emergence of A2 allowed for sexual recombination of the pathogen and is thus responsible for the changes in the population structure of *P. cubensis*.

Materials & Methods

Biological and molecular determination of mating type

Mating type was determined as described earlier (Cohen et al. 2011a,b, Cohen and Rubin 2012). An isolate was considered A1 if it produced oospores when mixed-inoculated with an A2 tester isolate; it was considered A2 if it produced oospores when mixed-inoculated with an A1 tester isolate; and it was considered A1A2 if it produced oospores when mixed-inoculated with either tester isolates but not when inoculated alone; it was considered homothallic if it produced oospores when inoculated alone. Molecular determination of mating type was done by using the SSR primer set PC17 of Kanetis et al. (2009): F: CTTAGTAAGCGGAAGGTCGGTAGTT; R: CGGTCGATAATATGACATACACAAG. PCR products were separated on 8% acrylamide gels and stained with ethidium bromide.

Clade determination

DNA was extracted from sporangia. For amplification of the mitochondrial *cox2* gene, primers designed by Hudspeth et al. (2000) were employed as described by Choi et al. (2007), but using the RedTaq Mastermix (Genaxxon, Germany). Amplicons were sequenced using the forward primer used for amplification. Sequences were edited using the DNAStar software package (DNAStar, Madison, WI, U.S.A.). After removal of leading and trailing gaps, an alignment of 445 bp was available. In this alignment, nine polymorphic sites occurred of which six were diagnostic for a split between two *P. cubensis* clusters. Comparison of the present sequences with those described by Runge et al. (2011), in which *cox2* was the most informative locus, indicated that the two clusters found here perfectly matched the two clades of the phylogenetic analyses done by Runge et al.

Pathotype determination

Pathotype was determined according to Thomas et al. (1987), with the modifications described in Cohen et al. (2015).

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Fitness of A1 and A2 isolates

The fitness of the A1 and A2 isolates was estimated in laboratory and field experiments. In the laboratory, 13 A1 and 9 A2 isolates were each inoculated onto ten 5-cm diameter leaf discs of *Cucumis sativus* L. (cucumber), *C. melo* L. (melon), and *Cucurbita moschata* Duchesne (butternut squash). Each disc was inoculated with a single 20 µl inoculum droplet containing a mean of 1, 10, or 100 sporangia of *Pseudoperonospora cubensis*. Seven days after inoculation, the proportion of tissue infected (0 to 10) and sporulation intensity (0 to 3) were estimated. The two figures for each leaf disc were multiplied to obtain disease intensity (0 to 30).

In the first field experiment, 295 single-leaf isolates were randomly sampled from April to July, 2012, from 800 naturally infected cucumber, melon, and three *Cucurbita* species growing in equal proportions in three adjacent net-houses at the Bar-Ilan University (BIU) Farm and examined for mating type as described above to determine their distribution among the five hosts.

In the second field experiment, eight cucurbit species (120 plants/species) were planted at the BIU Farm in a 500 m^2 net-house on September 20, 2013 and inoculated on November 3, 2013 with a sporangial mixture (1 : 1) of A1+A2 single-sporangium isolates. Frequent visual estimates of disease development were taken and a total of 93 single-leaf isolates were periodically collected and examined for mating type.

Results & Discussion

A2 isolates of *P. cubensis* were first detected in Israel among pathotype 6 (P6) isolates of *P. cubensis* (Cohen and Rubin 2012). P6 isolates were first collected in Israel in 2002 (Cohen et al. 2003) from downy mildew-infected *Cucurbita* species (*C. moschata, C. pepo* L., and *C. maxima* Duchesne) which had never been attacked before. Eight years later, P6 isolates were found to belong to the A2 mating type. Co-inoculation with P6+P3 sporangia (from butternut squash and cucumber, respectively) resulted in the formation of abundant oospores inside the mesophyll of detached melon leaves (Cohen et al. 2011a,b). This confirmed for the first time that *P. cubensis* is heterothallic, requiring two opposite mating types for sexual reproduction (Cohen and Rubin 2012).

A long-term study revealed that 47 of the 50 isolates collected in Israel from 2002 to 2016 from *Cucurbita* species belonged to the A2 mating type, whereas 90 out of the 102 isolates collected from cucumber or melon belonged to the A1 mating type, confirming the preferential compatibility of A2 to *Cucurbita* and of A1 to *Cucumis* (Cohen et al. 2013a and unpublished data). All isolates collected in Israel from 2002 to 2016 from *Cucurbita* species belonged to P6 whereas almost all isolates collected from cucumber or melon belonged to P3 (Cohen et al. 2013a and unpublished data). Resistance to mefenoxam was frequent among isolates collected from *Cucumis* but rare among isolates collected from *Cucurbita* (unpublished data).

A1 and A2 isolates could be distinguished by PCR using the SSR primers PC17 (Kanetis et al. 2009). A1 isolates produced three bands with approximate sizes of 240, 350, and 450 bp, whereas A2 isolates produced three bands with approximate sizes of 240, 450, and 500 bp.

Leaf disc assays showed that A1 isolates were better fit to cucumber than A2 isolates. Mean disease intensity in leaf discs of cucumber inoculated with 1, 10, or 100 sporangia/disc of A1 and A2 isolates was 6.7, 13.8, and 22.6, respectively, for A1 isolates as compared to 1.3, 3.9, and 7.9, respectively, for A2 isolates. On the other hand, A2 isolates were compatible with butternut squash while A1 isolates were incompatible with this host. Mean disease intensity in leaf discs of butternut squash inoculated with 1, 10, or 100 sporangia/disc of A2 isolates was 3.2, 6.6, and 15.9, respectively, as against 0.0, 0.0, and 0.0 in leaf discs inoculated with A1 isolates (Falach 2014).

Monitoring the frequency of A1 and A2 isolates in naturally infected cucurbits in our field resulted in 147 and 5 A1 isolates, as against 18 and 111 A2 isolates, collected from *Cucumis* and *Cucurbita* species, respectively. Interestingly, 11 isolates from *Cucumis* and 3 isolates from *Cucurbita* were bisexual, A1A2.

Table 1. Occurrence of the various mating types of *Pseudoperonospora cubensis* in 10 countries from 2002 to 2015. Abbreviation for hosts: B = Ash gourd, *Benincasa hispida* (Thunb.) Cogn.; C = cucumber, *Cucumis sativus*; D = Butternut squash, *Cucurbita moschata*; L = Ridge gourd, *Luffa acutangula*; M = melon, *C. melo*; P = pumpkin, *C. maxima*; S = squash, *C. pepo*; T = Snake gourd, *Trichosanthes cucumerina* L.; Lag = Bottle gourd, *Lagenaria siceraria*.

Country	Year	Hosts	Total	A1	A2	Homo	Sterile
Angola	2014	P*	1	-	1	-	-
China	2011-2012	C	29	22	6	-	1
Crimea	2011	C, S*	16	15	1	-	-
France	2014	P*	1	-	1	-	-
Germany	2015	P*	1	-	1	-	-
Kenya	2012	P*	1	-	1	-	-
India	2014	C*, P*, T*, B*, Luf	21	1	9	-	11
Israel	2002-2015	C, M, S*, P*, D*	152	-	47	-	-
Russia	2013	C, S*	34	30	1	-	3
Vietnam	2013	C*, Lag**	21	1	12	5	3

*A host harboring A2 isolates.
**A host harboring Homo = homothallic isolates.

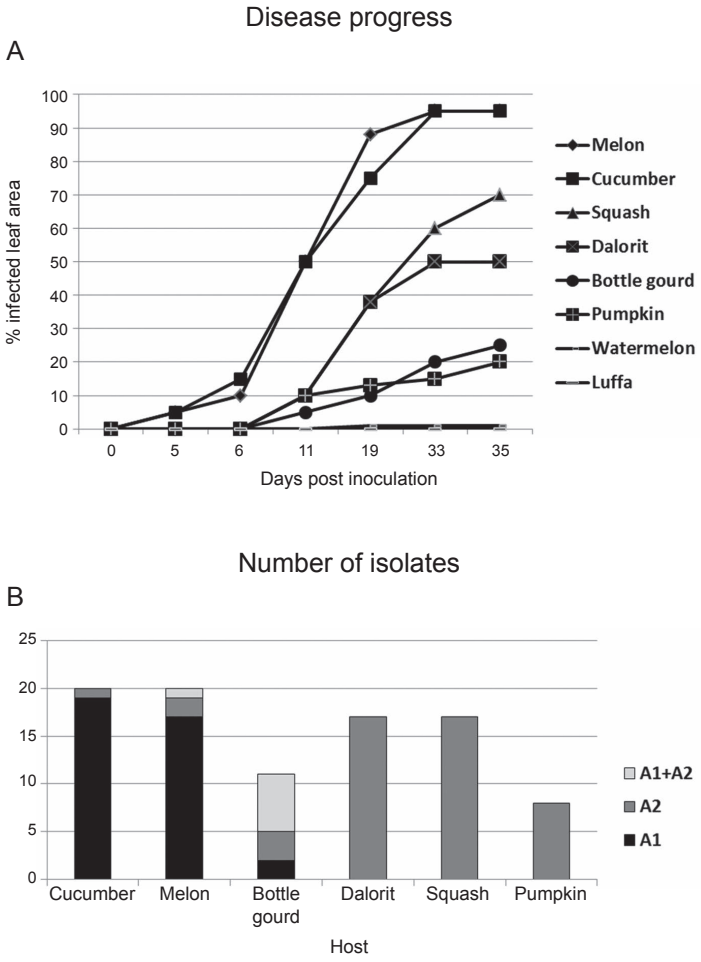

Figure 1. Mating types of 93 isolates of *Pseudoperonospora cubensis* collected from artificially infected cucurbits at the BIU Farm during October and November 2013. Plants were inoculated with a sporangial mixture (1 : 1) of single sporangium A1 (from cucumber) and A2 (from squash) isolates (squash = *Cucurbita pepo*, dalorit = butternut squash (*C. moschata*), pumpkin = *C. maxima*).

Disease progress in various cucurbits that were inoculated with mixed A1+A2 sporangia in the field is shown in Figure 1A. It was fastest in cucumber and melon, moderate in *C. pepo* and *C. moschata*, and slowest in *C. maxima* and *Lagenaria siceraria* (Mol.) Standl. (bottle gourd). *Citrullus lanatus* (Thunb.) Matsum. & Nakai (watermelon) and *Luffa acutangula* (L.) Roxb. (ridge gourd) showed no disease due to the fact that the isolates used belonged to P3 and P6, which are incompatible with these taxa. Of the 93 isolates collected during the season, 38 A1 and 3 A2 isolates were collected from *Cucumis* as against 0 A1 and 42 A2 isolates that were collected from *Cucurbita* (Figure 1B). Interestingly, 6 out of the 11 isolates collected from bottle gourd were A1+A2. These results reaffirm the differential host preference of A1 to *Cucumis*, A2 to *Cucurbita,* and both mating types to *Lagenaria*.

Molecular analysis of mitochondrial *cox2* revealed that most A2 isolates belonged to the relatively new (post-2004) Clade 1 whereas most A1 isolates belonged to the old (pre-2004) Clade 2, confirming that the A2 mating type of *Pseudoperonospora cubensis* is a newcomer to the western hemisphere (Runge et al. 2011).

In a recent survey, conducted from 2011 to 2014, we discovered the occurrence of A2 in the following nine countries: Angola, Kenya, Crimea, Russia (Y. Cohen, A. E. Rubin, and N. Bibi, unpublished data), China (Cohen et al. 2013b), Vietnam (Y. Cohen, H. Nguyen, and A.E. Rubin, unpublished data), India (Cohen et al. 2016), France (Y. Cohen, M.Waldner-Zulauf, and A. E. Rubin, unpublished data), and Germany (Y. Cohen, D. Hermann, and A. E. Rubin, unpublished data). A2 was also reported in the U.S.A. (Thomas et al. 2013). Interestingly, most isolates collected in India and Vietnam were A2 (Cohen et al. 2016). Ridge gourd, *Luffa acutangula*, in India was often seen heavily infected with downy mildew. Some isolates collected from bottle gourd in Vietnam were homothallic, producing oospores when inoculated singly onto detached melon leaves (Y. Cohen and A. E. Rubin, unpublished data). The global occurrence of A2 is summarized in Table 1.

The post-2004 extensive global changes in the population structure of *Pseudoperonospora cubensis* (reviewed by Cohen et al. 2015) may have resulted from the appearance of A2. Apparently, the new immigrant A2 mated with the prevailing A1 so as to produce descendent recombinant offspring progenies with altered traits. Indeed, oospores produced in our laboratory in detached melon leaves produced F_1 offspring isolates with altered host range and sensitivity to mefenoxam and CAA fungicides (Falach 2014). Although A2 isolates were not reported in the Czech Republic, enhanced heterozygosity was reported in the population of the pathogen since 2009 (Kitner et al. 2015) probably due to the presence of the two mating types

From where has the A2 form of *P. cubensis* emigrated? Runge et al. (2011) suggested that the post-2004 change in the population structure in the U.S.A. is likely due to the introduction by man of strains from East Asia (Japan, Korea), first to Europe and then to the U.S.A. We suggest here that India and Vietnam served as another source of A2 Clade 1 *P. cubensis.* Possible migration routes were from (i) India to Israel and Europe and (ii) India and Vietnam to China. The pathogen could have migrated with people (Runge et al. 2011), fruit or seed (Cohen et al. 2014), or wind or birds (Cohen et al. 2015).

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Genetic Structure of *Pseudoperonospora cubensis* Populations Infecting Commercial and Non-Commercial Hosts in North Carolina

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Abstract. *Pseudoperonospora cubensis* is an obligate oomycete pathogen of plants in the Cucurbitaceae and the causal agent of cucurbit downy mildew. In 2004, the pathogen overcame cucumber resistance in the United States and since then cucurbit downy mildew causes major losses each year, particularly on cucumber. Understanding how populations of *P. cubensis* are structured by the host can help optimize management strategies by establishing which genotypes of the pathogen are infecting particular hosts and possible diversifying effects that the host can have on pathogen populations. Microsatellites were developed to analyze the population structure of *P. cubensis* infecting commercial and non-commercial cucurbits in North Carolina, a state with wide host availability. Microsatellites were identified from the predicted transcriptome of *P. cubensis* and primers flanking these sequences were designed. Population structure analysis using 10 markers showed the highest genetic differentiation between isolates from *Cucumis* species and isolates from *Citrullus* and *Cucurbita* species. When isolates from particular cucurbit species were analyzed, the highest genetic differentiation was between isolates from *Cucumis sativus* and *Cucurbita pepo*. No differentiation was detected between isolates from *Cucumis melo* and *Cucurbita foetidissima*, *C. pepo* and *Cucurbita moschata*, and *Momordica balsamina* and *C. pepo* and *C. moschata*. Using these informative markers, *P. cubensis* population structure in North Carolina can be evaluated based on time, geography, and host.

Keywords: Watermelon, melon, cucumber, squash, downy mildew

Introduction

Pseudoperonospora cubensis, the causal agent of cucurbit downy mildew, is an obligate oomycete plant pathogen that re-emerged in 2004 in the United States (Holmes et al. 2015). Cucurbit downy mildew was once controlled in the United States primarily via host resistance in cucumber and with modest fungicide applications in other cucurbits, but a population shift rendered resistant varieties and commonly used fungicides ineffective. Devastating economic losses occurred in 2004 and, since then, disease management requires substantial and frequent fungicide applications (Holmes et al. 2015). The pathogen remains a high risk for developing resistance to fungicides and overcoming host resistance, in part, because of its high rate of asexual reproduction (Cohen et al. 2015). Sporangia are readily produced on the abaxial surface of the leaf and can be widely transported via air currents from field to field. The pathogen survives year-round in southern regions, such as Florida, where winter weather does not eliminate the host. Then, as cucurbit hosts are planted and temperatures become conducive for infection, *P. cubensis* is dispersed northward. Epidemiological research reports that sporangia can travel approximately 1,000 miles (Ojiambo et al. 2015).

In contrast with most downy mildew pathogens that typically have a narrow host range only causing disease on a few plant species, *Pseudoperonospora cubensis* has a very broad host range and it is capable of infecting over 60 different species of plants in the Cucurbitaceae from diverse genera (Savory et al. 2011). Differences in virulence of *P. cubensis* on diverse hosts has resulted in isolates of the pathogen being classified into different races and pathotypes depending on an isolate's ability to infect a panel of cucurbits (Lebeda and Widrlechner 2003). It has also been reported that the morphological characteristics of a particular *P. cubensis* isolate can change depending on the host that is currently infecting (Runge and Thines 2011). Population genetics studies have shown genetic differentiation of *P. cubensis* isolates by both host and geographical location (Quesada-Ocampo et al. 2012). Analysis of five nuclear and two mitochondrial genes revealed that isolates from Europe were more likely to belong to different genetic clusters than isolates from North America. The study also found that isolates belonging to one particular genetic cluster occurred more frequently in hosts other than cucumber, and that other genetic clusters primarily contained isolates sampled from cucumber. Finally, results indicated that sources of inocula other than southern Florida may exist for certain geographic regions in the United States. For example, isolates from North Carolina have a relatively high genetic diversity and show genetic differences with

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Florida isolates (Quesada-Ocampo et al. 2012). Nonetheless, that study was not able to determine if the observed geographic structure was an artifact of population stratification driven by host due to differences in host availability on the diverse regions sampled.

Another aspect that remains open to question is the role of non-commercial and wild cucurbit hosts as inoculum reservoirs or diversifying factors of *P. cubensis*. Previous reports found that the perennial wild hop, *Bryonia dioica* Jacq., is a host for *P. cubensis* in Europe but a role in overwintering was not established (Runge and Thines 2009). Another study that evaluated wild cucurbits for resistance determined that most of the 56 accessions were susceptible to the pathogen (Lebeda 1992). Recent reports in the U.S.A. revealed that hosts such as *Momordica balsamina* L., *M. charantia* L., and *Cucurbita foetidissima* Kunth become infected with *P. cubensis* in field settings (Wallace et al. 2014, 2015). As dozens of reported hosts for *P. cubensis* are found in the wild or are grown on a small scale in areas with high cucumber production in the United States, studies that examine the potential of wild and non-commercial cucurbits to contribute to the yearly downy mildew epidemic are needed. Non-commercial cucurbits could be involved in overwintering, survival, and genetic diversification of the pathogen. Thus, in this study we aimed to evaluate how different cucurbit hosts structure North Carolina *P. cubensis* populations by using standardized field plots with different commercial and non-commercial cucurbits and previously developed microsatellite markers (Naegele et al. 2016, Wallace and Quesada-Ocampo 2016). Specifically, we aimed to determine if *P. cubensis* isolates are genetically differentiated according to the host genera *Cucumis*, *Cucurbita*, *Citrullus*, and *Momordica*, or to the host species *Cucumis sativus* L., *C. melo* L., *C. pepo* L., *C. moschata* Duchesne, *C. maxima* Duchesne, *C. foetidissima*, *M. charantia*, and *M. balsamina*.

Materials & Methods

Isolate collection and DNA extraction

P. cubensis isolates were collected from three plots in three distinct geographic regions in North Carolina: the Coastal Plains (Kinston), the Piedmont (Salisbury), and the Mountains (Waynesville). Plots were part of the CDM IPM-PIPE sentinel plot network and contained ten plants of each commercial cucurbit crop including *C. sativus* 'Straight 8' (cucumber), *C. melo* 'Hale's Best Jumbo' (cantaloupe), *C. pepo* 'Table Ace' (acorn squash), *C. maxima* 'Big Max' (pumpkin), *C. moschata* 'Waltham Butternut' (butternut squash), and *Citrullus lanatus* (Thunb.) Matsum. & Nakai 'Micky Lee' (watermelon) (Ojiambo et al. 2011). The plots also contained five plants each of non-commercial or wild cucurbits including *M. charantia* (bitter melon), *M. balsamina* (balsam apple), and *C. foetidissima* (buffalo gourd). As plots were monitored for disease, individual sporulating lesions on symptomatic leaves were collected in the summer and fall of 2013 and 2014, excised, and stored in microfuge tubes at -80 °C.

Infected tissue was disrupted with 425 to 600 µM acid-washed glass beads (Sigma Life Sciences) and 2.3 mm Zircona/Silica beads (BioSpec Products, Bartlesville, OK) in an Omni Bead Ruptor 24 (Omni International, Kennesaw, GA). SDS-page extraction buffer was used to extract DNA and was purified via phenol-chloroform extractions and ethanol washes adapted from previous work (Ahmed et al. 2009). DNA quantity (ng/µL) and quality $A_{260/280}$ were measured with Nanodrop ND 1000 spectrophotometer and NanoDrop 2.4.7c software (NanoDrop Technologies, Wilmington DE).

Genotyping and population analysis

DNA from each *P. cubensis* isolate was amplified with 10 microsatellite markers as previously described (Wallace and Quesada-Ocampo 2016). PCR products labeled with different fluorescent dyes were pool-plexed and diluted 20-fold in 96-well plates. Samples were prepared for the 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) by adding HiDi Formamide and Liz600 size standard to each well. Samples were submitted to the North Carolina State University Genomic Science Laboratory for genotyping. Raw data generated by the 3730xl DNA Analyzer and the Geneious Microsatellite Plug-In (Kearse et al. 2012) were used to assign alleles for each locus and isolate. GenAlEx (Peakall and Smouse 2006) was used to generate pairwise population matrices of Nei unbiased genetic distance.

Results

Three hundred forty-four *P. cubensis* isolates were genotyped with 10 microsatellites. Two hundred ninety-eight of the isolates were from commercial hosts, with 62 from *C. sativus*, 58 from *C. melo*, 47 from *C. pepo*, 46 from *C. maxima*, 47 from *C. moschata*, and 38 from *C. lanatus*. The remaining 46 were comprised of 30 isolates from *M. charantia*, 10 isolates from *M. balsamina*, and 6 isolates from *C. foetidissima*.

When isolates were grouped by the genus of the host of origin, Nei's unbiased genetic distance (D) revealed the greatest genetic differentiation between isolates from *Cucumis* and isolates from *Citrullus* at 0.247, followed by isolates from *Cucumis* and isolates from *Cucurbita* at 0.220 and isolates from *Cucumis* and isolates from *Momordica* at 0.117 (Table 1). The lowest value of differentiation was between *Cucurbita* isolates and *Citrullus* isolates with 0.019, followed by isolates from *Citrullus* and *Momordica* with 0.044, and isolates from *Cucurbita* and *Momordica* with 0.053.

Table 1. Pairwise population matrix of Nei unbiased genetic distance (D) of *P. cubensis* isolates grouped by host genera.

	Cucumis	*Cucurbita*	*Citrullus*
Cucumis	0.000		
Cucurbita	0.220	0.000	
Citrullus	0.247	0.019	0.000
Momordica	0.117	0.053	0.044

Table 2. Pairwise population matrix of Nei unbiased genetic distance (D) of *P. cubensis* isolates grouped by host species.

	Cucumis sativus	*Cucumis melo*	*Cucurbita pepo*	*Cucurbita maxima*	*Cucurbita moschata*	*Citrullus lanatus*	*Cucurbita foetidissima*	*Momordica charantia*
Cucumis sativus	0.000							
Cucumis melo	0.012	0.000						
Cucurbita pepo	0.344	0.239	0.000					
Cucurbita maxima	0.196	0.115	0.019	0.000				
Cucurbita moschata	0.337	0.228	0.000	0.014	0.000			
Citrullus lanatus	0.303	0.189	0.026	0.021	0.021	0.000		
Cucurbita foetidissima	0.010	0.000	0.153	0.065	0.146	0.139	0.000	
Momordica charantia	0.127	0.069	0.136	0.071	0.131	0.083	0.071	0.000
Momordica balsamina	0.275	0.180	0.000	0.005	0.000	0.009	0.108	0.085

When isolates were grouped by the host species of origin, genetic differentiation values were higher, revealing a higher degree of differentiation at the species level (Table 2). Some of the greatest differentiation exists between isolates from *C. sativus* and *C. pepo* (D = 0.344), *C. sativus* and *C. moschata* (D = 0.337), and *C. sativus* and *C. lanatus* (D = 0.303). There was also high differentiation between isolates from *C. melo* and *C. pepo* (D = 0.239) as well as between isolates from *C. sativus* and *M. balsamina* (D = 0.275). Moderate differentiation was detected between isolates from *C. sativus* and *C. maxima* (D = 0.196) and *M. charantia* (D = 0.127), *C. melo* and *C. moschata* (D = 0.228), *C. lanatus* (D = 0.189), and *M. balsamina* (D = 0.180). Moderate differentiation was also seen between isolates from *C. pepo* and *C. foetidissima* (D = 0.153) and *M. charantia* (D = 0.136), *C. moschata* and *C. foetidissima* (D = 0.146) and *M. charantia* (D = 0.131), and *C. lanatus* and *C. foetidissima* (D = 0.139). Lower differentiation was detected between isolates from *C. sativus* and *C. melo* (D = 0.012) and *C. foetidissima* (D = 0.010), *C. melo* and *C. maxima* (D = 0.115) and *M. charantia* (D = 0.069), *C. pepo* and *C. maxima* (D = 0.019), and *C. lanatus* (D = 0.026), *C. maxima* and *C. moschata* (0.014), *C. lanatus* (D = 0.021), *C. foetidissima* (D = 0.065), *M. charantia* (D = 0.071), and *M. balsamina* (D = 0.005), *C. moschata* and *C. lanatus* (D = 0.021), *C. lanatus* and *M. charantia* (D = 0.083), and *M. balsamina* (D = 0.009), *C. foetidissima* and *M. charantia* (D = 0.071), and *M. balsamina* (D = 0.108), and *M. charantia* and *M. balsamina* (D = 0.085). No differentiation (D = 0.000) was found between isolates from *C. melo* and *C. foetidissima*, *C. moschata* and *M. balsamina*, *C. pepo* and *M. balsamina*, and *C. moschata* and *C. pepo*.

Discussion

Differences in pathogenicity of *P. cubensis* isolates infecting different cucurbit hosts have been observed (Cohen et al. 2003). Changes in morphology of *P. cubensis* isolates depending on the host being infected have also been documented (Runge and Thines 2011). Previous population studies have shown evidence of genetic differentiation in *P. cubensis* based on the cucurbit host of origin (Quesada-Ocampo et al. 2012). Differentiation was detected among isolates from cucumber and other commercial cucurbit hosts, melon, squash, and pumpkin. However, isolates from some commercial cucurbit hosts such as watermelon were not included in that study and because of differences in host availability in the different regions sampled, the effect of host vs. geography in structuring *P. cubensis* populations was not determined. More recent studies also observed different genetic cluster patterns in isolates from spaghetti squash (*Cucurbita pepo*) as compared with isolates from cucumber; however, this study only examined two host species (Naegele et al. 2016). Quesada-Ocampo et al. (2012) determined relatively high genetic diversity of *P. cubensis* in North Carolina. This was likely due to the fact that all commercial cucurbits are grown in the state, and many wild and non-commercial cucurbit hosts are also found in the region, making North Carolina an ideal location to study the role of commercial and non-commercial hosts in structuring *P. cubensis* populations.

When isolates were grouped by the genus of the host of origin, Nei's genetic distance (D) values showed low genetic differentiation. Genera of commercial and non-commercial hosts were included for comparison with *Cucurbita*. These comparisons show the greatest differentiation between isolates from *Cucumis* and isolates from *Citrullus.* The lowest differentiation was between isolates from *Cucurbita* and *Citrullus,* isolates from *Cucurbita* and *Momordica,* and also isolates from *Citrullus* and *Momordica*. These low genetic distance values (D = 0.019, D = 0.053, and D = 0.044, respectively) suggest that genetically similar isolates occur in these hosts, therefore, *Momordica* could contribute inoculum for infection of *Cucurbita* and *Citrullus*. Isolates from *Cucumis* and *Cucurbita,* and isolates

from *Cucumis* and *Momordica*, showed moderate differentiation (D = 0.220 and D = 0.117, respectively). However, in this analysis, isolates from the non-commercial host *C. foetidissima* were grouped with isolates from commercial *Cucurbita* hosts, so evaluation of each cucurbit species independently was necessary to establish inoculum contribution of non-commercial cucurbit hosts.

When isolates were grouped by the host species of origin, high to low values of genetic distance were seen in a pairwise population matrix. The greatest genetic distance was between isolates from *C. sativus* and isolates from *C. pepo*, *C. moschata*, *C lanatus*, and *M. balsamina* (Table 2). Medium to high genetic differentiation was also observed between isolates from *C. melo* and isolates from *C. pepo*, *C. moschata*, *C. lanatus*, and *M. balsamina*. These findings indicate that isolates from *Cucumis* are more genetically different to isolates from other commercial cucurbit hosts and *M. balsamina*. A similar observation has been made in previous studies of genetic differentiation of isolates infecting cucumber in comparison to isolates from *Cucurbita* commercial hosts (Quesada-Ocampo et al. 2012).

This study was also able to determine that wild and non-commercial cucurbits are playing a role in genetic differentiation of *P. cubensis*. Isolates from *Momordica* are moderately differentiated from *Cucumis* isolates, but have lower differentiation with isolates from *Cucurbita* and *Citrullus* species. Thus, *Momordica* infections could become a source of inoculum for *Cucurbita* and *Citrullus*. Interestingly, *C. foetidissima* isolates show higher differentiation with isolates from commercial *Cucurbita* hosts, but lower differentiation from isolates from *Cucumis* hosts. This finding may have implications for early arrival of disease in temperate regions because *Cucurbita foetidissima* is a perennial and it is not clear whether the pathogen can overwinter in this host (Wallace et al. 2015). As *Cucumis* species usually become infected earlier in the season than commercial *Cucurbita* species, *C. foetidissima* may pose a threat for the disease to occur earlier than anticipated. This can be a significant management hurdle when many growers rely on forecasting data from the CDM-IPM Pipe, which does not account for overwintering sources (Ojiambo et al. 2011). Overall, wild and non-commercial cucurbits evaluated in this study can be infected by the same isolates as some commercial cucurbits according to genetic evidence examined. In addition, findings presented here indicate that host species of origin is a major factor contributing in the genetic structuring of *P. cubensis* populations.

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Interactions of *Cucumis melo* and *Cucurbita* spp. Accessions with *Pseudoperonospora cubensis* Are Race- (Pathotype-) Specific

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Abstract. Cucurbitaceous vegetables are seriously affected by *Pseudoperonospora cubensis*, the causal agent of cucurbit downy mildew. Experiments focused on deciphering variation in interaction of 115 accessions of *Cucumis melo* (melon) with 12 isolates of *P. cubensis* representing 10 distinct pathotypes, and of 97 accessions of wild and weedy *Cucurbita* spp. (gourd) to 11 *P. cubensis* isolates, were carried out under controlled conditions. Within the melons, 45 different reaction patterns were recorded. Most of the melon accessions (67) were highly susceptible to all isolates. The reaction of 46 accessions was clearly race- (pathotype-) specific. None of the screened melon accessions expressed complete resistance to all isolates. Accession PI 315410 from India was resistant to 5 isolates and incompletely resistant to 2 others. Within the melon populations from India and southern Africa, there are accessions completely or incompletely resistant to various European races of *P. cubensis*. Within the *Cucurbita* accessions (representing 10 species), 57 different reaction patterns were recorded; 13 accessions were completely resistant and 12 completely susceptible. Wild *Cucurbita argyrosperma* has potential field resistance to a number of races of *P. cubensis*. This study established the background for future development of methodology for *P. cubensis* racial determination and denomination.

Keywords: Cucurbitacae, *Cucumis melo*, wild *Cucurbita*, cucurbit downy mildew, host-pathogen interaction, germplasm

Introduction

Cultivation of cucurbits in major production areas is complicated by the occurrence of cucurbit downy mildew, *Pseudoperonospora cubensis* (Berk. et Curt.) Rostov. (Peronosporales, Chromista) (Lebeda and Cohen 2011, Savory et al. 2011). This pathogen exclusively attacks members of the Cucurbitaceae and 50 to 60 species, including both wild and cultivated forms (e.g., cucumbers, gherkins, melons, watermelons, squash, and pumpkins) from about 20 genera, are known to be natural hosts (Runge et al. 2012).

Virulence variation in *P. cubensis* can be represented by pathotypes (Lebeda and Widrlechner 2003) as determined on a standard, differential set of cucurbitaceous taxa (Lebeda and Gadasová 2002, Lebeda and Widrlechner 2003), or by races (Lebeda et al. 2006). Although the existence of distinct races of *P. cubensis* on *Cucumis sativus* L. is uncertain (Lebeda and Schwinn 1994), and distinct responses to *P. cubensis* were not observed within 20 wild *Cucumis* species (Lebeda 1992, Lebeda and Widrlechner 2003), racial differentiation has been demonstrated for *Cucumis melo* L. (Thomas et al. 1987, Lebeda 1991), *Cucurbita pepo* L., and other *Cucurbita* spp. (Lebeda and Křístková 1993, Lebeda and Widrlechner 2004). Our understanding of the genetic basis of this virulence variation in *P. cubensis* is fragmentary (Savory et al. 2011), and the role of sexual reproduction in generating such variation has not yet been adequately assessed (Lebeda and Cohen 2011, Polat et al. 2014, Kitner et al. 2015). The genetic basis for resistance to *P. cubensis* is known for a few host species (Lebeda and Widrlechner 2004). Sources of resistance to downy mildew have been reported in several Indian accessions of *Cucumis melo* (Dhillon et al. 2007, Fergany et al. 2011).

Knowledge of relations among species and/or genera of the Cucurbitaceae and adequate taxonomic concepts are essential for genetic studies and also for practical breeding. Approximately 95 genera and 950 to 980 species are recognized in the latest taxonomical concept of this family (Schaefer and Renner 2011). In contrast to the wide range of genetic and phenotypic diversity found among melons, Nesom (2011) concurred with Kirkbride

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(1993) in recognizing only two formal infraspecific taxa within *Cucumis melo*, following an earlier informal proposal by Jeffrey (1980), subsp. *melo* and subsp. *agrestis* (Naudin) Pangalo.

The *Cucurbita* species are, according to ecological requirements, divided into mesophytic (annuals) and xerophytic (perennials). Recent understandings of the genus *Cucurbita* elaborated by Nee (1990) and Sanjur et al. (2002) distinguish 12 to 14 species. The group of mesophytes includes the wild species *C. ecuadorensis* H.C. Cutler & Whitaker, *C. lundelliana* L.H. Bailey, *C. okeechobeensis* (J.K. Small) L.H. Bailey, the cultivated *C. maxima* Duchesne, *C. ficifolia* Bouché, *C. moschata* Duchesne, *C. argyrosperma* C. Huber, and *C. pepo*. (Lira Saade et al. 1993, Sanjur et al. 2002). Closely related wild progenitor/domesticate of *Cucurbita* are treated as subspecies. Hence, *C. argyrosperma* includes several cultivated subspecies as well as the wild *C. argyrosperma* subsp. *sororia, C. maxima* subsp. *maxima* is the cultivated counterpart of the wild *C. maxima* subsp. *andreana* (Naudin) Filov, which is native to Argentina and Bolivia. Cultivated *C. pepo* is extremely variable and is comprised of two subspecies, *pepo* and *ovifera* (L.) D.S. Decker (syn. *texana* (Scheele) Filov); the former has no known wild equivalent whilst wild populations of the latter are widely dispersed in the United States. A third subspecies, *fraterna* Lira, Andres & Nee, grows wild in northeastern Mexico, but does not seem to have any cultivated equivalents. The group of perennial xerophytic *Cucurbita* species includes *C. digitata* A. Gray, *C. cordata* S. Watson, *C. palmata* S. Watson, *C. pedatifolia* L.H. Bailey, *C. radicans* Naudin, *C. foetidissima* Kunth (Lira Saade et al. 1993, Sanjur et al. 2002).

The aim of our experiments was to study the interactions between germplasm accessions of *C. melo* and *Cucurbita* spp. and isolates of *Pseudoperonospora cubensis* with exactly defined virulence, at the level of pathotypes under controlled conditions, and to characterize reaction patterns.

Material & Methods

Plant material, origin, cultivation, and preparation for tests

A set of 115 germplasm accessions of *Cucumis melo* and of 97 accessions of *Cucurbita* spp. were kindly provided by North Central Regional Plant Introduction Station, Ames, Iowa (USDA). The accessions of *C. melo* were originally collected or donated to the U.S. National Plant Germplasm System (NPGS) between 1934 and 1994, the accessions of *Cucurbita* spp. were acquired between 1951 and 1994 (USDA, ARS, National Genetic Resources Program, 2015).

Within the set of *Cucumis melo* accessions, there were three of subsp. *agrestis* and 110 of subsp. *melo in sensu* Nesom (2011). Infraspecific classification of the two remaining accessions was not determined. They originated from 28 countries in Europe, Asia, Africa, and the Americas, and represented wild collections, landraces, local cultivars, and cultivated or breeding lines (Lebeda et al. 2016). The set of 97 *Cucurbita* spp. accessions was composed predominantly of wild and weedy *C. argyrosperma*, *C. cordata*, *C. digitata*, *C. ecuadorensis*, *C. ficifolia*, *C. foetidissima*, *C. maxima, C. okeechobeensis*, *C. pedatifolia*, and *C. pepo*, and five landraces *C. argyrosperma*, one of *C. ficifolia*, six of *C. pepo*, and three of *C. maxima*. The improvement status of nine accessions was not determined in the passport data of the donor. The majority of the *Cucurbita* accessions originated from the U.S.A., Mexico, and South America (Argentina). Two accessions *C. ficifolia* were acquired from Europe (U.K. and Spain) and six landraces *C. pepo* originated from Asia

Table 1. Stucture of the set of *Cucurbita* accessions under study.

Cucurbita taxon*	No. accessions
Mesophytic (annual)	
C. argyrosperma C. Huber subsp. *argyrosperma* var. *palmeri* (L. H. Bailey) L. Merrick & D. M. Bates	18
C. argyrosperma C. Huber subsp. *sororia* (L. H. Bailey) L. Merrick & D. M. Bates	25
C. pepo L. subsp. *ovifera* (L.) D. S. Decker var. *texana* (Scheele) Filov	9
C. pepo L. subsp. *ovifera* (L.) D. S. Decker var. *ozarkana* D. S. Decker	9
C. pepo L. subsp. *ovifera* (L.) D. S. Decker	1
C. pepo L. subsp. *fraterna* (L. H. Bailey) Lira et al.	4
C. pepo L. subsp. not specified	6
C. maxima Duchesne subsp. *andreana* (Naudin) Filov	3
C. ficifolia Bouché	4
C. ecuadorensis H. C. Cutler & Whitaker	3
C. okeechobeensis (Small) L. H. Bailey subsp. *martinezii* (L. H. Bailey) T. C. Andres & Nabhan ex T. W. Walters & D. S. Decker	7
Xerophytic (perennial)	
C. foetidissima Kunth	5
C. cordata S. Watson	1
C. digitata A. Gray	1
C. pedatifolia L. H. Bailey	1

*Taxonomy and nomenclature of *Cucurbita* spp. accessions in accordance with U.S. National Plant Germplasm System https://npgsweb.ars-grin.gov/gringlobal/taxonomybrowse.aspx (accessed 15 March 2016).

(Turkey, Iran, and India) and Europe (Macedonia and Spain). The structure of the whole set of *Cucurbita* spp. accessions according to their taxonomical classification is presented in the Table 1.

Cucumis sativus accession 09H3900121, 'Marketer 430', was obtained from the Czech National Plant Germplasm Collection (Crop Research Institue 2015a), and was used as a highly susceptible control in all experiments as well as serving as the host for pathogen cultivation and multiplication.

Each accession was represented by three plants in our experiments. Plants were grown in a greenhouse as described by Lebeda (1986) and Lebeda and Urban (2010). Leaf discs (15 mm in diameter) were cut from well-developed true leaves of 5 to 12-week-old plants and placed, with their abaxial surface up, on wet filter paper in petri dishes. Each accession was represented by 5 leaf discs in three replicates (one replicate per plant) to evaluate its interactions with each *P. cubensis* isolate under study (Lebeda and Urban 2010).

Pathogen isolates, origin, character

Twelve isolates *P. cubensis* collected during 1988–2001 from infected *C. sativus* plants in the Czech Republic, France, the Netherlands, and Spain were obtained from the National Collection of Microorganisms (Crop Research Institute 2015b) maintained by the Department of Botany, Palacký University in Olomouc, Czech Republic. These isolates represent 10 different pathotypes as determined from a standard set of cucurbitaceous taxa, denominated by triple-part tetrade codes following Lebeda and Widrlechner (2003), with low (isolates 3/00, 6/97, 1/88), medium (isolates 11/00, 2/95, 6/96, 14/00, 1/00), and high (isolates 2/00, 12/00, 1/97, 39/01) levels of pathogenicity (Table 2). Seven isolates were tested to both host plant genera, four isolates were tested to *Cucurbita* spp. exclusively, and one isolate to *Cucumis melo* (Table 2).

In vitro *experiments*

Leaf discs of each accession were inoculated with an aqueous suspension of *P. cubensis* spores and then incubated following Lebeda and Urban (2010). Reactions of leaf discs (sporulation intensity of each respective *P. cubensis* isolate) were evaluated visually on the 6th, 8th, 10th, 12th, and 14th day after inoculation by using a 0 to 4 scale (Lebeda 1986, Lebeda and Urban 2010). For each accession, the degree of infection (DI) was expressed as a percentage of maximal potential sporulation at the time of the final evaluation, 14 days after inoculation (Lebeda 1986, Lebeda and Gadasová 2002). Four types of reactions, based on DI value and variation in leaf-discs responses, were assigned: +, susceptible (DI = 35 to 100%); (+), heterogenous; (-), partially resistant (DI = 1 to 34%); and -, resistant (DI = 0%) (Lebeda 1986, Lebeda and Urban 2010). Heterogeneous response (+) indicates the mixture of completely susceptible together with at least one resistant plant in the tested sample.

Table 2. List of isolates of *Pseudoperonospora cubensis* studied, their pathotypes and virulence to the set of 115 accessions of *Cucumis melo* and 97 of *Cucurbita* spp.

Isolate number	Country of origin	Pathotype*	Interaction studied	Number of compatible reactions** to *C. melo*	Number of compatible reactions ** to *Cucurbita* spp.
3/00	France	3.0.0	*Cucumis melo, Cucurbita* spp.	109	55
6/97	Czech Republic	1.2.10	*Cucumis melo, Cucurbita* spp.	103	45
1/88	Czech Republic	3.2.10	*Cucumis melo, Cucurbita* spp.	103	47
11/00	Czech Republic	1.10.10	*Cucumis melo, Cucurbita* spp.	104	45
2/95	Czech Republic	1.10.10	*Cucurbita* spp.	nt	42
6/96	Czech Republic	3.10.10	*Cucumis melo, Cucurbita* spp.	106	50
14/00	Czech Republic	3.10.10.	*Cucurbita* spp.	nt	52
1/00	The Netherlands	11.0.10	*Cucurbita* spp.	nt	33
2/00	Spain	11.10.11	*Cucurbita* spp.	nt	49
12/00	Czech Republic	11.10.14	*Cucumis melo, Cucurbita* spp.	101	53
1/97	Czech Republic	15.10.14	*Cucumis melo, Cucurbita* spp.	108	36
39/01	Czech Republic	15.15.15	*Cucumis melo*	107	nt

*Pathotype determined and denominated according to Lebeda and Widrlechner (2003).

**Number of compatible reactions includes susceptible + and heterogeneous (+) reactions determined according to Lebeda and Urban (2010).

nt - interaction not tested.

Results

Reactions of C. melo *accessions to* P. cubensis *isolates*

Collectively, 45 different reaction patterns to eight isolates *P. cubensis* were recorded within this set of 115 *C. melo* accessions (Lebeda et al. 2016). Most *C. melo* accessions (in total 67) were highly susceptible to all isolates. Two accessions were incompletely resistant to all isolates. The remaining 46 accessions developed race-specific response to eight isolates; only three pairs of accessions showed identical reaction patterns. Heterogeneous reactions to one or more isolates were recorded for 32 accessions. About half of these accessions were also incompletely resistant to at least one isolate. None of the screened accessions expressed complete resistance to all isolates. The broadest resistance pattern was observed in accession PI 315410, which was completely resistant to five isolates and incompletely resistant to two more (Lebeda et al. 2016).

None of the accessions from Europe, Central Asia, East Asia, or Africa were completely or incompletely resistant to isolate 39/01 having the highly virulent "superpathotype" (15.15.15). Only one accesion from the Americas and seven from India were incompletely resistant to it, and only a single accession, PI 315410, was fully resistant. This exceptional accession was also resistant also to the highly virulent isolate 1/97 (pathotype 15.10.14), but it was susceptible to isolate 12/00 (pathotype 11.10.14). Notably, isolate 12/00 was virulent to the fewest accessions of *C. melo* in our experiment. In contrast, isolate 3/00 with a very weak pathotype, was virulent to the largest number of accessions (Table 2).

Reaction of Cucurbita *spp. accessions to* P. cubensis *isolates*

Within 97 *Cucurbita* accessions representing 10 species, 57 different reaction patterns were recorded. A group of 13 accessions resistant to all isolates were found in different subspecies of *C. argyrosperma*. Twelve accessions completely susceptible to all isolates were represented by 10 accessions of the different subspecies of *C. pepo* and by two accessions of *C. maxima* subsp. *andreana*. We cannot make a general conclusion on reactions of different *Cucurbita* spp. to *P. cubensis* because each species was represented by a differing number of accessions in our experiment.

There was no positive relationship between pathotype of *P. cubensis* isolates and its virulence on the set of *Cucurbita* spp. accessions under study. Isolate 3/00 with lowest level of pathogenicity determined according to Lebeda and Widrlechner (2003) reached the highest number (55) of compatible reactions with *Cucurbita* spp. accessions under study. On the contrary, isolate 1/97, with highly virulent pathotype, was virulent to only 36 accessions *Cucurbita* spp. (Table 2).

Discussion

Interactions C. melo – P. cubensis

The resistant (or incompletely resistant) reactions of 46 *C. melo* accessions observed here could be considered as race/pathotype-specific, as none of them expressed resistance against the entire set of *P. cubensis* isolates. This especially holds true for PI 315410 (item 34), which displayed high resistance against seven isolates, but clear susceptibility to a single isolate (PC 12/00). Such a distinct reaction pattern, when compared to other *C. melo* genotypes, suggests the possible use of PI 315410 as a differential line for racial or pathotype identification and as a good model system for further research. The full potential of PI 315410 in breeding melons for resistance to *P. cubensis* should be investigated by testing it against a larger set of *P. cubensis* isolates (pathotypes) originating from different geographical regions and by hybridization experiments involving susceptible genotypes to determine the heritability of resistance and define its genetic control.

Interaction Cucurbita *spp.* – P. cubensis

The reaction of different wild and weedy species within the genus *Cucurbita* to *P. cubensis* is race-specific. This type of interaction was proved by Lebeda and Křístková (1993) at the infraspecific level, on a set of cultivars of *C. pepo*. This phenomenon is in relation to the high genetic diversity of this species (Ferriol et al. 2003, Lebeda et al. 2007, Gong et al. 2012) and polymorphism in the fruit shape (Paris 1986, Paris et al. 2012). Pathotype- and race-specific interactions were described by Lebeda and Widrlechner (2003) also for the other cultivated and highly variable species, *C. maxima* and *C. moschata*. There is no evidence in the literature for the study of interactions between cucurbit downy mildew and wild *Cucurbita* species. The data obtained in our experiment aimed almost exclusively at wild and weedy *Cucurbita* species, and this information could be useful in breeding for resistance.

There appeared to be no relation between the virulence of *P. cubensis* isolates, expressed as their pathotype according to Lebeda and Widrlechner (2003), and their virulence on the set of *Cucurbita* spp. accessions. High variability in the virulence of *P. cubensis* populations reported from different parts of the world (Shetty et al. 2002, Salati et al. 2010, Lebeda and Cohen 2011, Polat et al. 2014) should be taken in consideration for resistance breeding. A system for determination and denomination of races *P. cubensis* needs to be elaborated.

Conclusions

Broad race-specific variation occurs in interactions of the hosts *C. melo* and *Cucurbita* spp. with the obligate parasite *P. cubensis*. *C. melo* subsp. *agrestis* and wild *C. argyrosperma* are taxa with potential field resistance to a number of races of *P. cubensis*. Within *C. melo* populations from India and southern Africa there are accessions completely or incompletely resistant to various European races of *P. cubensis*. The results establish the background for future development of methodology for *P. cubensis* race determination and denomination.

Acknowledgments

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Cellular and Biochemical Mechanisms of Cucumber Resistance Against *Pseudoperonospora cubensis*

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Abstract. Downy mildew caused by the oomycete *Pseudoperonospora cubensis* generates significant yearly losses of cucumbers and related crops in Poland. We chose five cucumber cultigens showing a broad range of *P. cubensis* reactions, and submitted them to laboratory bio-assays with an aggressive local pathogen isolate, to observe the important biochemical and subcellular reactions. Leaf samples taken daily (0 to 5 days after inoculation) served for subsequent analyses of generation of reactive oxygen species, activities of plant red-ox enzymes, and contents of defense substances. Our observations indicate that, regardless of their apparent *P. cubensis* resistance evaluated macroscopically, the tested cultigens invoke penetration resistance as their primary reaction. Simultaneous increase of polyphenol oxidase activity and drop of free phenol levels during pathogen infection suggest their involvement in the defense reaction, likely by phenolic deposition at the perceived infection sites.

Keywords: *Cucumis sativus*, downy mildew, primary defense, biochemical assays

Introduction

Pseudoperonospora cubensis [(Berk. et Curt.) Rostov.], the causal agent of downy mildew, limits cucurbit production in Poland and elsewhere (Savory et al. 2011, Call et al. 2012, Kłosińska 2013). Plants display a range of reactions to the perceived pathogen infection; the first and primary line of defence is the "surface protection", which aims to restrict pathogen infection through the external plant tissues. This primary defense mechanism involves numerous metabolic changes, and manifests itself as focused fortifications of cell walls (papilla and callose coating) under the perceived pathogen attack site (appresorium) (Jacobs et al. 2003, Nowicki et al. 2012, Nowakowska et al. 2016). Other ubiquitous plant cell responses include cell wall lignification and deposition of phenolics, the latter catalyzed by polyphenoloxidases (PPOs), thereby strengthening the defensive structures (Torres et al. 2006, Korgan et al. 2011). All these responses undergo regulation by reactive oxygen species (ROS), amply generated upon perceived infection (Torres et al. 2006, Iakimova et al. 2013, Mandal et al. 2014). PPOs are involved in the oxidation of toxic polyphenols into quinones (antimicrobial compounds) and lignification of plant cells during microbial invasion. They may also participate in the inducible defense reactions and the hypersensitivity that induces resistance of plants to fungi, viruses, and bacteria (Torres et al. 2006, Hanifei et al. 2013). Peroxidases (POXs) interact with the plant defense systems in response to pathogen attack through production of H_2O_2, lignin biosynthesis, and participate in the cell-wall polysaccharide processes such as oxidation of phenols, suberization, and lignification of host plant cells (Hanifei et al. 2013).

The overall goal of this study was to observe the important biochemical and subcellular events underlying cucumber reactions to *P. cubensis*. We addressed this by assessing numerous biochemical responses (ROS generation; red-ox enzyme activities; synthesis of defensive substances) on an array of cucumber cultigens differing in their reaction to the pathogen, at 24 h intervals, 0 to 5 dpi (days post inoculation). This could allow attribution of a particular reaction type (induced resistance; HR) to each particular cucumber cultigen.

Materials & Methods

We used our cucumber collection (InHort, Skierniewice, Poland) and chose three cultigens with resistance against *Pseudoperonospora cubensis* ('Ames 2354', PI 197088, PI 330628) and two susceptible ones (PI 175695, 'Coolgreen'), as per previous exhaustive experimentation (Call et al. 2012, Kozik et al. 2013). Plants were inoculated at the stage of two to three leaves, with a local aggressive pathogen isolate, using 50,000 sporangia × ml^{-1}. Inoculated plants were incubated at 20 °C, 100% RH, in darkness for 48 hours, then under 24 °C, 60% RH, 12h/12h photoperiod. Symptoms typical for downy mildew became apparent on the susceptible plants at 5 dpi. For the biochemical assays, collected leaves were immediately snap-frozen in liquid nitrogen, and then ground mechanically. Powdered leaf samples (from 5 plants/cultigen) were extracted as per the respective analytical method required (see below), and assayed in a microtiterplate spectrophotometer (3 measurements/sample).

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We considered the respective 0 dpi samples as baseline, as well as those from susceptible cultigens.

Generation of ROS species (H_2O_2, $HO^{\bullet}$)

To assess generation of either ROS species, we adapted the published methods (Loreto et al. 2001, Loreto and Velikova 2001, Habu and Ibeh 2015, Moukette et al. 2015). Methanol extracts (5:1 [v:w], twice 15 min at 4 °C) were assayed at A_{390} or A_{560}, respectively. We used H_2O_2 as one of the standards; in the $HO^{\bullet}$ assay, we calculated the absorbance readout per mg of fresh tissue.

Activities of plant red-ox enzymes

To analyze the dynamics of PPO and POX, we adapted the method of Korgan et al. (2011). Buffer-extracted samples (50 mM potassium phosphate, pH 7.2, 30 min at 4 °C) were assayed for protein according to Bradford (1976), and analyzed for linear assay range on a randomly chosen sample (0 dpi). Proper assays were run for 3 minutes, while absorbances were read out every minute. Protein activity was calculated as change in absorbance per minute and per mg of protein (PPO) or mg metabolized guaiacol/min/mg protein (POX).

Deposition of defensive substances

Levels of lignins and phenolics deposition were recorded using established spectrophotometric methods (Campbell and Ellis 1992, Cvikrova et al. 1992). Methanolic extracts (see above) and pellets were used, and the assays used technical lignins and gallic acid as the respective standards.

Results & Discussion

Generation of ROS upon Pseudoperonospora cubensis *infection*

The methods reported in the literature were successfully adapted for the cucumber – *P. cubensis* pathosystem. Moreover, H_2O_2 levels generally slightly decreased in the susceptible cultigens and showed a bell-shaped activation curve over time in the resistant ones (Figure 1A). The $HO^{\bullet}$ contents tended to increase from 0 to 5 dpi, irrespective of the cultigen's apparent resistance (Figure 1B).

Bursts of ROS, generated primarily as plant signaling molecules, but also used as chemical defense, are ubiquitously produced as initial cellular responses following successful pathogen recognition (Torres et al. 2006). Another line of evidence proves this function: The transcription profiling of this pathosystem revealed that suppression of the genes encoding the ROS-scavenging system and photosynthesis pathways may inhibit disease development in the host tissue (Li et al. 2011). H_2O_2 generation is of importance due to its involvement in the induction and/or execution of HR (Torres et al. 2006). Our data are in accordance with both of these observations; in particular, the differing generation of H_2O_2 in the susceptible vs. resistant cultigens seems of interest, complementing the data of another study of up to 20 hours post inoculation (Hao et al. 2013). Similar H_2O_2 increases were observed in a muskmelon pathosystem (Ge et al. 2014).

Activities of cucumber red-ox enzymes under Pseudoperonospora cubensis *stress*

Adaptation of the activity assays from the literature (Korgan et al. 2011, Moukette et al. 2015) and the linear dynamic range of PPO and POX assays were checked on a randomly chosen 0 dpi sample. The protein range checked for both enzymes was 3 to 60.5 µg/assay, and the linear activity range was: for PPO 3 to 12 µg/assay; for POX 3 to 9 µg/assay.

The recorded data for POX activity (Figure 2A) lacks any particular trend for the analyzed cultigens, from 0 to 5 dpi *Pseudoperonospora cubensis*. Some increase in POX activity was seen in the late-stage samples of the resistant cultivars.

On the other hand, the PPO activity (Figure 2B) increased along with the bio-assay duration, irrespective of the cultigen's resistance. But, rate of PPO activation was higher in the resistant cucumber cultigens.

Our data trends confirm those obtained in the same pathosystem (Anand et al. 2007), as well as in the related cucumber pathosystem (Hanifei et al. 2013), both with only one cucumber line tested. The seemingly higher POX activation in the resistant versus the susceptible cultigens reported here was also observed in a melon pathosystem (Madadkhah et al. 2012).

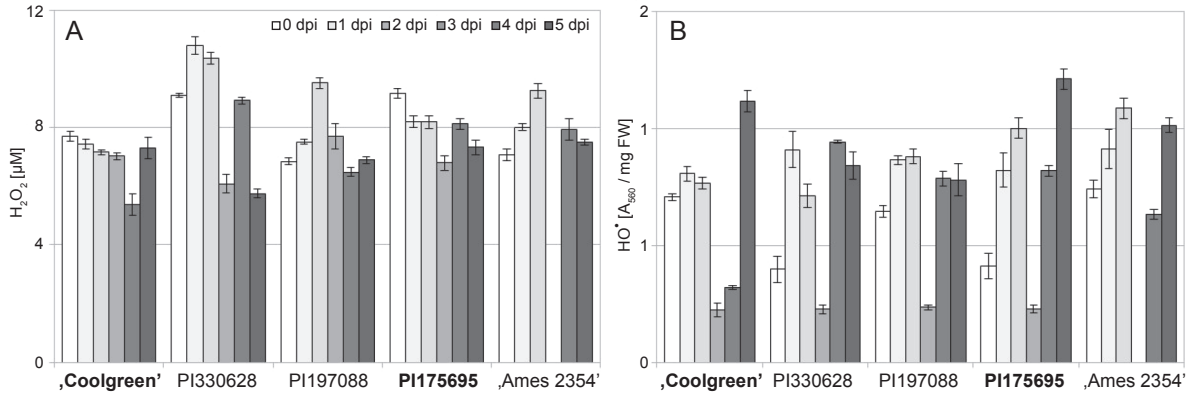

Figure 1. Dynamics of ROS generation by cucumber cultigens under stress from *Pseudoperonospora cubensis* (0 to 5 dpi). Spectrophotometric assays of the methanolic leaf extracts from given stages show changes in H_2O_2 (A) and $HO^{\bullet}$ (B) generation. ROS concentration was read out from the H_2O_2 standards (A), or calculated as Abs_{560}/mg FW (B). Data means (bars) and respective SDs are shown. Susceptible cultigen labels are in bold.

Deposition of defense substances: phenolics and lignin

To assay the contents of the phenolics and lignins synthesized in response to *P. cubensis* inoculation, we adapted the methods developed for other plant systems (Campbell and Ellis 1992, Korgan et al. 2011). Free phenolics content in cucumbers tested here showed a general decreasing trend, irrespective of the cultigen's resistance (Figure 3A). A decreasing trend, yet less marked than this for the phenols, was also observed for lignins (Figure 3B).

Phenol content dynamics observed in this study differed from the trends observed in the related cucurbit pathosystems (Madadkhah et al. 2012, Hanifei et al. 2013, Ge et al. 2014). In particular, we considered our methanolic extracts to only reflect the contents of "free phenols" due to the methodology used, while the studies cited here reported the total phenols. Another reason for the observed differences may be the pathosystems analyzed. Irrespectively, phenolics deposition as a means for strengthening plant cell walls, suggested by the simultaneous PPO activation and decrease of free phenols, calls for an experimental confirmation.

Quick deposition of phenolic compounds or lignin in the infection spots is a defense mechanism against pathogen attack (Cohen et al. 1989). In contrast, our data indicate a variation in lignin deposition trends which could not fully support the data of the microscopic study of the muskmelon – *P. cubensis* pathosystem (Cohen et al. 1989).

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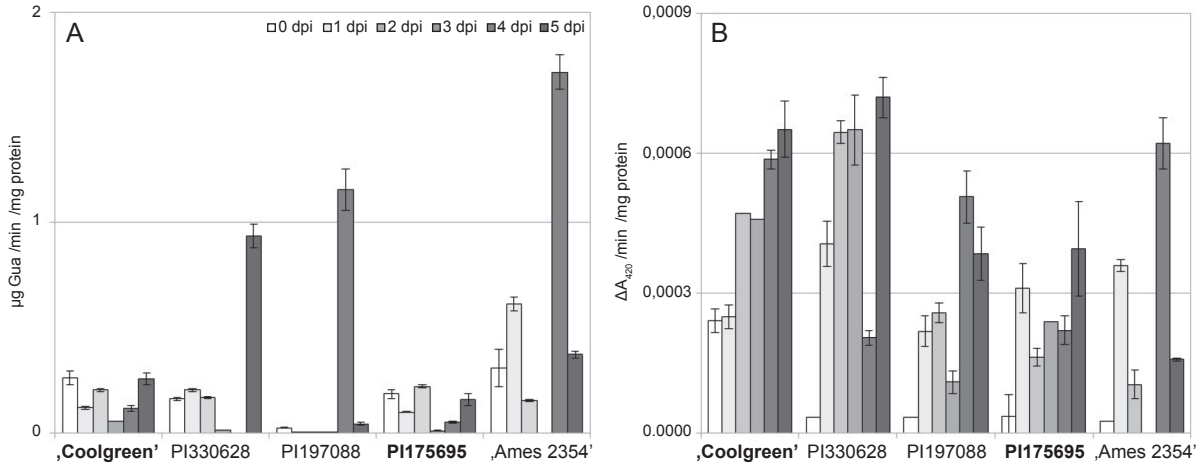

Figure 2. Dynamics of plant red-ox enzymes activities under stress from *Pseudoperonospora cubensis* (0 to 5 dpi): POX (A) and PPO (B). Data means (bars) and respective SDs are shown. Susceptible cultigen labels are in bold.

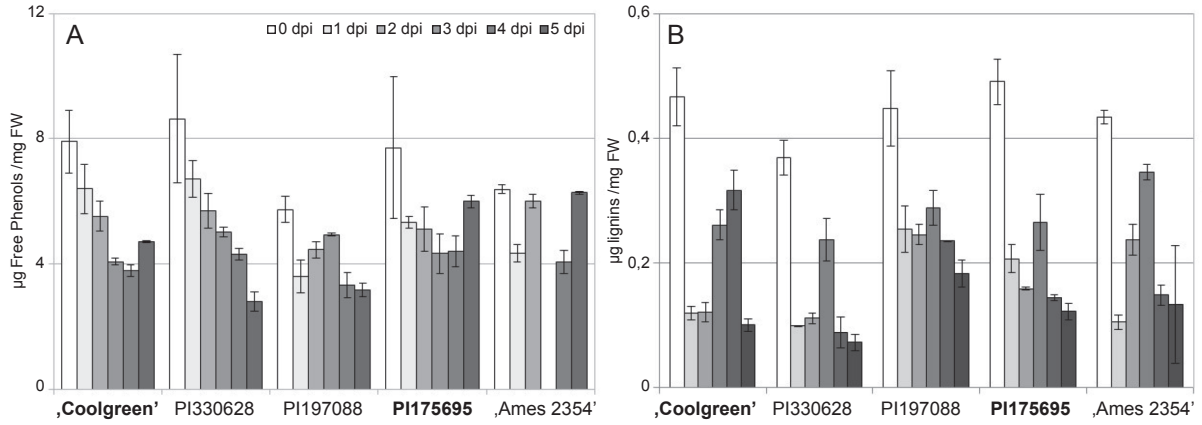

Figure 3. Contents of free phenols (A) and lignins (B) in cucumbers under stress from *Pseudoperonospora cubensis* (0 to 5 dpi). Free phenols and lignins were assayed in the methanolic leaf extracts, according to the standard curves of gallic acid (A) or technical lignin (B), respectively. Data means (bars) and respective SDs are shown. Susceptible cultigen labels are in bold.

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Loss-of-Function Mutations in a Cucumber *MLO* Gene Lead to Hypocotyl Resistance to Powdery Mildew

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Abstract. Powdery mildew (PM) is an important disease of cucumber (*Cucumis sativus*). We are interested in the identification of susceptibility genes in cucumber. Mutations leading to loss of function or silencing of susceptibility genes can lead to durable, broad range resistance against PM. *CsaMLO8*, a cucumber homolog of the well-known barley *MLO* gene, was previously identified as a candidate susceptibility gene for PM in cucumber. *MLO* genes form gene families, in which two clades have previously been shown to harbour susceptibility genes: clade IV in monocot species and clade V in dicot species. *CsaMLO8* belongs to clade V and co-localizes with a QTL on chromosome 5 for hypocotyl-specific resistance to PM in cucumber. Recently, two research groups published about the functional characterization of *CsaMLO8* and the identification of mutant alleles of this gene. In this review we compare the findings from these two groups, and discuss the current knowledge about *MLO* genes in PM susceptibility.

Keywords: Cucumber, powdery mildew, *MLO* genes, hypocotyl resistance

Powdery mildew resistance in cucumber

With an annual worldwide production value of over 33 billion US dollar, cucumber (*Cucumis sativus* L.) is an economically important crop (FaoStat 2016). The genome of cucumber ('Chinese Long' inbred line 9930) was sequenced as one of the first vegetable crops, and is publicly available since 2009 (Huang et al. 2009). Furthermore, several cucumber accessions have been resequenced (Woycicki et al. 2011, Qi et al. 2013). This wealth of information can make cucumber a model species for other cucurbit crops, such as melon, watermelon, and pumpkin.

Powdery mildew (PM) is a devastating fungal disease in many crop species including cucurbits such as cucumber. Two species of fungi have been reported to cause PM in cucumber, *Podosphaera xanthii* (synonymous with *P. fusca*, previously named *Sphaerotheca fuliginea*) and *Golovinomyces cichoracearum* (previously named *Erysiphe cichoracearum*). In recent decades, *P. xanthii* is considered to be the main causal agent of PM in cucurbits, especially under greenhouse conditions (Block and Reitsma 2005, Perez-Garcia et al. 2009). Breeding of resistant cucumber varieties has been undertaken for several decades (for example, Shanmugasundaram et al. 1971, Sitterly 1972), but underlying resistance genes have to date not been functionally characterized.

Several sources of resistance to PM in cucumber have been identified, especially from Asia, such as the Indian accessions PI 197087, PI 197088, and the Japanese 'Natsufushinari' (Kooistra 1968). Resistance in those materials was shown to be under the control of multiple genes, most of which show recessive inheritance (Kooistra 1968). Shanmugasundaram et al. (1971) showed that one major recessive gene, *s*, was the most important factor contributing to PM resistance. This recessive gene *s* confers intermediate or hypocotyl resistance, characterized by completely resistant hypocotyl, stem, and petiole tissue, and partially resistant leaves and cotyledons. Combinations of this gene with other sources of resistance led to full resistance (Shanmugasundaram et al. 1971). In a more recent study, He et al. (2013) mapped several QTLs for PM resistance, scoring resistance of hypocotyl, cotyledon, and true leaf tissue separately. The authors reported three QTLs for hypocotyl resistance, one with a major effect and two with a minor effect. They conclude that the major effect QTL, *pm5.2*, is probably the same as the gene *s* described by Shanmugasundaram et al. (1971).

Introgression of dominantly inherited resistance (*R*) genes, usually from wild relatives, is the traditional approach to obtain resistance in a crop. *R*-genes encode proteins, often of the nucleotide-binding, leucine-rich-repeat (NB-LRR) type, which are able to recognize a pathogen and trigger a strong defense response, usually associated with a hypersensitive response (HR) leading to cell death. The *R*-gene product either directly recognizes corresponding avirulence (*Avr*) gene products of the pathogen, or modifications of host factors by *Avr* gene products (Jones and Dangl 2006). The direct relation of plant *R*-genes with their cognate *Avr* genes in the pathogen is known as the gene-for-gene relationship (Flor 1971). Whereas introgression of a new *R*-gene initially gives good resistance against a pathogen, it puts selective pressure on the pathogen to evolve the corresponding

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Avr gene in such a way that it is no longer recognized by the host plant. Therefore, *R*-gene based resistance is often breached by new, virulent races of the pathogen quite soon, especially for versatile pathogens, such as powdery mildew fungi (Jones and Dangl 2006). The sequenced genome of cucumber harbours only 61 *NB-LRR* genes (Huang et al. 2009), a surprisingly low number compared to, for example, the 149 *NB-LRR* genes in the model plant *Arabidopsis thaliana* (Meyers et al. 2003), 500 in rice (Monosi et al. 2004), or even 1015 in apple (Arya et al. 2014). Similar low numbers have been reported in other cucurbit taxa, such as 44 *NB-LRR* genes in watermelon (Guo et al. 2013) and 81 in melon (Garcia-Mas et al. 2012).

During the last decades, a novel alternative for *R*-gene mediated resistance has grown in popularity, which is the identification of impaired susceptibility (*S*) genes (Vogel and Somerville 2000, Eckardt 2002, Pavan et al. 2010, van Schie and Takken 2014). In contrast to *R*-genes recognizing a pathogen, *S*-genes are plant genes facilitating compatible interactions between the plant host and its pathogen, for instance by negative regulation of defense responses, facilitating host recognition by the pathogen, or being essential for the uptake of nutrients by the pathogen (van Schie and Takken 2014). The fact that most pathogens are able to infect only a limited amount of plant species suggests that there must be specific genes in those plant species that are essential for the pathogen. This is especially true for biotrophic pathogens such as mildew-causing fungi taxa, which rely on a long-lasting interaction with living host cells (Eckardt 2002). Loss-of-function mutations in an *S*-gene are thought to lead to durable, broad-spectrum, recessively inherited resistance (Eckardt 2002, Pavan et al. 2010). As inheritance of resistance against powdery and downy mildews in cucumber has often been reported to be recessive (Kooistra 1968, Shanmugasundaram et al. 1971, van Vliet and Meijsing 1977), it is likely that loss-of-function mutations in *S*-genes rather than (dominantly inherited) *R*-genes are causal to many known sources of resistance in cucumber.

MLO susceptibility genes

One of the oldest and best-known examples of impaired *S* genes is the *MLO* gene family. Mutant alleles of the "Mildew Locus O" (*mlo*) were originally found in the 1940s in mutagenized barley populations (Freisleben and Lein 1942), which became resistant against *Blumeria graminis* f. sp. *hordei,* the causal agent of PM in barley. Barley varieties with *mlo* resistance have since been grown in the field for several decades without breaching of resistance by virulent new mildew races to date, providing evidence for the durability of *S*-gene based resistance (Jorgensen 1992). After the barley *MLO* gene was cloned (Büschges et al. 1997), it was found that *MLO* genes are conserved throughout the plant kingdom and occur in plants as a multi-copy gene family (Devoto et al. 1999, Devoto et al. 2003). Recently, Kusch et al. (2016) provided evidence for the occurrence of *MLO*-like genes in representatives of all land plants, including mosses and gymnosperms, in related unicellular algae, and even in distantly related eukaryotes such as *Amoebozoa* and *Chromalveolata*, the latter intriguingly including plant pathogens such as *Phytophthora infestans* and *Hyaloperonospora arabidopsidis*.

In several plants, some, but not all, *MLO* genes have been found to be involved in PM susceptibility, such as *Arabidopsis*, tomato, pea, pepper, tobacco, bread wheat, and potentially also grapevine and peach (Consonni et al. 2006, Bai et al. 2008, Feechan et al. 2008, Jiwan et al. 2013, Zheng et al. 2013, Ning et al. 2014, Appiano et al. 2015a). It has been found that in phylogenetic trees of the *MLO* gene family, all *S*-genes for PM cluster in two clades, namely clade IV for *S*-genes in monocot species and clade V for dicot species. The other five clades harbour *MLO*-like genes that have not been found to be *S*-genes (Devoto et al. 2003, Kusch et al. 2016).

Recently, Appiano et al. (2015b) showed that despite the occurrence of clade-specific molecular features, overexpression of a clade IV *MLO* gene (i.e., *HvMLO*) was able to restore susceptibility in a tomato mutant that is PM resistant because of a loss-of-function mutation in a Clade V *MLO* gene (*SlMLO1*). This proves that clade IV and clade V *MLO* genes are functionally conserved in the plant-pathogen interaction. Additionally, amino acid substitutions in *MLO* genes leading to PM resistance in either the monocot barley or the dicot *Arabidopsis* were reviewed, and it was found that amino acids required for susceptibility were usually conserved between clade IV and clade V *MLO* genes (Appiano et al. 2015b). In addition to single nucleotide variants leading to amino acid substitutions, other types of natural loss-of-function mutations have been identified in *MLO* genes. In tomato, a 19 bp frameshift deletion in the gene *SlMLO1* was found to be causal to recessive *ol-2* resistance (Bai et al. 2008). In pea, several allelic variants of the *MLO* gene *PsMLO1* were found to be causal to the *er1* locus, including small deletions and a transposable element insertion leading to aberrant splicing at the transcript level (Humphry et al. 2011). In the PM resistant tobacco 'Kobuku', nucleotide substitutions were identified in splice sites of introns of both *NtMLO1* and *NtMLO2* genes, leading to aberrant splicing of both genes at the transcript level (Fujimura et al. 2016).

With the cucumber genome sequence publicly available, two groups searched for *MLO* homologs in cucumber. Zhou et al. (2013) reported that they identified 14 *MLO*-like genes in the first version of the cucumber genome. Schouten et al. (2014) used the updated version of the cucumber genome (v2) and identified 13 *MLO*-like genes, the difference being that in the first version of the genome one *MLO* gene on chromosome 1 was erroneously annotated as two shorter neighbouring genes. Both groups agree that three cucumber *MLO* genes cluster in clade V of the *MLO* gene family, and can therefore be considered to be candidate *S*-genes. In the nomenclature proposed by Schouten et al. (2014), which we shall follow in this article, the names of those clade V *MLO* genes are *CsaMLO1*, *CsaMLO8,* and *CsaMLO11*.

Recently, *MLO*-like genes were also identified in other cucurbit crops, 14 *MLO* in watermelon, 16 in melon, and 18 in pumpkin (Iovieno et al. 2015). However, it should be noted that the lists of melon and pumpkin homologs contain several very short genes, some of which are adjacent to one another. Potentially those are errors in gene prediction, reflecting the poor quality of the current genome annotations for these species, so the actual number of *MLO* genes could be lower, reminiscent of the case in cucumber where at first 14 homologs were identified due to annotation problems. In melon and watermelon, three clade V *MLO* genes were identified, versus four clade V *MLO* genes

for pumpkin. In the phylogenetic analysis, three orthologous groups of cucurbit clade V *MLO* genes can be distinguished (Iovieno et al. 2015), indicating that the last common ancestor of cucurbits probably already had three clade V *MLO* genes, with one additional gene duplication in the branch leading to pumpkin.

CsaMLO8 is a cucumber *S*-gene for PM

Interestingly, one of the three clade V *MLO* genes identified in cucumber, *CsaMLO8*, is located on the interval of a major QTL for hypocotyl resistance to PM described by He et al. (2013). This led us to the hypothesis that a mutation in this *MLO* gene might be causal to the hypocotyl resistance that was reported more than 40 years ago (Shanmugasundaram et al. 1971), and which is still frequently used in cucumber breeding, indicating the durability of this resistance. In our recent publication (Berg et al. 2015), we reported the cloning and functional characterization of *CsaMLO8* from both susceptible and (hypocotyl) resistant cucumber genotypes. Simultaneously, another group described the fine-mapping of a major PM-resistance QTL from a highly resistant North China type cucumber inbred line to a 170 kb interval on chromosome 5 containing 25 genes, one of which was *CsaMLO8* (Nie et al. 2015a). They subsequently characterized this gene, to which they refer in their publication as *CsMLO1*, the results of which confirmed our analysis and added additional information (Nie et al. 2015b). In this review we compare the findings of the two groups, summarizing the current knowledge about *CsaMLO8*.

Both Berg et al. (2015) and Nie et al. (2015b) cloned the coding sequence of *CsaMLO8* from cDNA of susceptible cucumber genotypes, and confirmed the predicted wild-type sequence of *CsaMLO8*. Berg et al. (2015) overexpressed *CsaMLO8* in a tomato mutant that is PM resistant because of a loss-of-function mutation in a Clade V *MLO* gene (*SlMLO1*). Complementing this mutant with the wild-type *CsaMLO8* gene from cucumber restored susceptibility in six out of ten transformants. Nie et al. (2015b) overexpressed *CsaMLO8* in PM-resistant double *atmlo2/atmlo12 Arabidopsis* mutants, and observed restoration of susceptibility in seven transformants. The results of both groups are in agreement with each other. The fact that *CsaMLO8* is able to functionally complement loss-of-function mutations in unrelated species strengthens the conclusion that *CsaMLO8* is a functional susceptibility gene for PM.

Both research groups studied the expression of *CsaMLO8* in various cucumber tissues, although the experimental set-ups were different from each other. Berg et al. (2015) quantified the expression of *CsaMLO8* in leaf, hypocotyl, and cotyledon tissue before and at several time-points after inoculation with *Podosphaera xanthii* and found that, whereas in the hypocotyl the expression significantly increased at four and six hours post inoculation, the expression in leaves and cotyledons remained constant over the time course. This supported the notion that mutations in *CsaMLO8* give full resistance in hypocotyl tissue, and only partial resistance in leaf tissue.

Nie et al. (2015b), however, quantified *CsaMLO8* expression in more tissues, including hypocotyl, cotyledon, and leaf, but also root, stem, male and female flowers, and fruits, without inoculating the plants with *Podosphaera xanthii*. They concluded that in non-inoculated plants, *CsaMLO8* is highly expressed in leaves, cotyledons, and flowers, and lower in hypocotyl and stem tissue. *CsaMLO8* was found to be barely expressed in roots and fruits. Although it looks at first glance like the data presented by Berg et al. (2015) and by Nie et al. (2015b) contradict each other, one should notice that Berg et al. (2015) did not directly compare the expression in the different tissues with one another but instead analyzed the (normalized) expression within the different tissues over a time course. Re-analysis of the data presented by Berg et al. (2015) confirms the finding by Nie et al. (2015b) that, in non-inoculated cucumber plants, *CsaMLO8* is more highly expressed in leaf and cotyledonary tissue than in hypocotyl tissue. This does not exclude the finding by Berg et al. (2015) that, in hypocotyl tissue, *CsaMLO8* expression is induced by PM inoculation.

Nie et al. (2015b) furthermore quantified *CsaMLO8* expression in leaves of *P. xanthii* inoculated cucumbers over a time course, and found that *CsaMLO8* expression was induced, particularly at 12 hours post inoculation. This contradicts the finding of Berg et al. (2015) that *CsaMLO8* expression is not significantly induced in leaf tissue upon inoculation with powdery mildew, although it should be noted that Berg et al. (2015) did not quantify the *CsaMLO8* expression at 12 hours post inoculation, so it is possible that a significant upregulation was missed.

Nie et al. (2015b) also made fusion constructs of *CsaMLO8* with *GFP*, to observe the subcellular localization of the protein. As was expected, the CsaMLO8 protein localized to the plasma membrane.

Mutant alleles of *CsaMLO8*

Berg et al. (2015) cloned the coding sequence of *CsaMLO8* from a hypocotyl-resistant cucumber line, and observed two different splicing variants, resulting in deletions of either 72 bp or 174 bp compared to the wild-type gene. qRT-PCR using splice junction spanning primers revealed that the 174 bp deletion product was the most abundant isoform. Sequencing of the deletion region from genomic DNA revealed the presence of a 1449 bp insertion in exon 11 of the gene. Characterization of this insertion showed that it had long terminal repeats (LTR) with a length of 184 bp, as well as a 5 bp target site duplication (TSD). In the sequenced cucumber genome, at least 44 other insertions of near-identical sequences could be identified, leading to the conclusion that this is a family of transposable elements (TE) of the LTR type. During splicing of the pre-mRNA of *CsaMLO8*, the TE is apparently spliced out together with either the complete exon 11 (174 bp), or a smaller part of exon 11 (72 bp). Berg et al. (2015) overexpressed the 174 bp deletion variant of *CsaMLO8* in *Slmlo1* tomato, and showed that it is unable to restore susceptibility in tomato. This underlines that the TE insertion in the gene, and subsequent loss of a part of the gene at the RNA-level, led to a non-functional allele of *CsaMLO8*, and therewith to hypocotyl resistance.

Nie et al. (2015a) identified the exact same 1449 bp insertion in their resistant material during fine-mapping of a recessively inherited QTL for PM resistance. They reported that it leads to the loss of exon 11 (174 bp) on the cDNA level, in agreement with the findings of Berg et al. (2015). They do not report the 72 bp deletion isoform, although the picture of their gel clearly

Table 1. Cucumber genotypes among the 115 lines resequenced by Qi et al. (2013) homozygous or heterozygous for the *CsaMLO8* T to C point mutation at position 1301.

Individual Code	Zygosity	Accession name	Origin	Ecotype
CG4210	Homozygous	71 Hao Huang Gua	China, Chongqing	East Asian
CG6508	Homozygous	Puerta-Rico #6	Puerto Rico	Eurasian
CG7704	Homozygous	N2/81	Dem. Rep. of the Congo	Eurasian
CG1811	Heterozygous	Qing Dao Qiu Ye Er San	China, Shandong	East Asian
CG1077	Heterozygous	Qing Pi Ba Cha	China, Jilin	East Asian
CG1373	Heterozygous	Ye San Bai	China, Hebei	East Asian

shows two bands, suggesting that in their material there are indeed two isoforms as well.

In addition to characterizing this particular mutation in their resistant material, Nie et al. (2015b) amplified the genomic *CsaMLO8* sequence of 27 additional cucumber inbred lines of diverse adaptation and origin, including well-known sources of PM resistance such as WI 2757 (He et al. 2013) and (descendants of) PI 197088. Whereas they found the same 1449 bp insertion allele in nine of the additional resistant cucumber lines, including WI 2757, they also characterized two additional natural variants in other resistant lines. In four instances, they found a T to C point mutation with respect to their susceptible allele at position 1301, leading to erroneous splicing of exon 5. In one case, they found a 1 bp frameshift insertion at position 3703, leading to an early stop codon. Whereas most of the susceptible lines (11 out of 14) were found to have the wild-type *CsaMLO8* allele, there were some exceptions: one susceptible line, "S49", was found to have the 1449 bp transposable element insertion, and another susceptible line, "9930", which is the genotype of the cucumber reference genome, was found to have the T to C point mutation leading to aberrant splicing of exon 5. Apparently, in these two inbred lines, the *CsaMLO8* mutation is not sufficient for obtaining resistance, which might be explained by the fact that hypocotyl resistance is known to be only partially effective (Shanmugasundaram et al. 1971), so having the *CsaMLO8* mutation in a particularly susceptible background does not lead to resistance. Alternatively, it could be possible that those genotypes have mutated alleles of genes required for *mlo* resistance, like the *ror* genes described in barley (Freialdenhoven et al. 1996) and *pen* genes in *Arabidopsis* (Consonni et al. 2006).

Interestingly, another susceptible line was found to have a 1451 bp insertion with a sequence very similar (95.6% identical) to the 1449 bp insertion, but at a different location, intron 9 instead of exon 11. This insertion in the intron was found not to alter the coding sequence nor the transcript abundance of *CsaMLO8*, explaining the susceptibility of this genotype. However, it indicates that insertions of this TE did occur frequently in the cucumber genome.

Berg et al. (2015) did not attempt sequencing *CsaMLO8* alleles from other cucumber genotypes. Instead they examined the occurrence of the 1449 bp insertion in the publicly available dataset of 115 resequenced cucumber genotypes (Qi et al. 2013), and found that the TE-allele of *CsaMLO8* occurred frequently, in 31 out of the 115 genotypes. This is in accordance with the fact that Nie et al. (2015b) found this allele to be the most common among resistant genotypes, and indicates that it has been actively selected for during cucumber breeding. One of the 31 genotypes with the TE-allele was found to be a semi-wild, *C. sativus* var. *hardwickii* (Royle) Alef. accession. Possibly, a *C. sativus* var. *hardwickii* accession has been the donor of this allele, introgressed into commercial cultivars.

For the current review, we tried to identify lines among the 115 resequenced cucumber genotypes (Qi et al. 2013) with the other loss-of-function alleles discovered by Nie et al. (2015b). For the T to C point mutation at position 1301 described by Nie et al. (2015b), we should note that as pointed out by the authors, the reference genome "9930" has this mutation. We could identify three other genotypes homozygous for a C at this position, and additionally three heterozygous genotypes (Table 1), confirming the finding by Nie et al. (2015b) that this allele is less common than the 1449 bp insertion. Interestingly, one of the three genotypes homozygous for this allele originated from Puerto Rico, and might therefore be related to accessions Puerto Rico 37 and Puerto Rico 40, the first reported sources of resistance to PM in cucumber (Kooistra 1968). We could not identify any lines with the one bp insertion at position 3703 reported by Nie et al. (2015b), which is remarkable as the accession in which Nie et al. (2015b) identified this mutation, PI 197088, is a well-known and often-used source of resistance to both powdery and downy mildew.

Conclusions

Summarizing, the combined works of Berg et al. (2015) and Nie et al. (2015b) show that *CsaMLO8* is a functional susceptibility gene in cucumber, and that several natural loss-of-function mutations of this susceptibility gene have been used in breeding to obtain powdery mildew resistance. The most commonly occurring mutation of this gene in cucumber breeding material is an insertion of a 1449 bp transposable element in exon 11. The expression of *CsaMLO8* is highest in leaves, cotyledons, and flowers, and lower in hypocotyl and stem tissue. However, inoculation with powdery mildew pathogen induces expression of *CsaMLO8* in the hypocotyl. Whether or not *CsaMLO8* expression is also induced in leaf tissue is still under debate.

The mutant alleles of *CsaMLO8* are to our knowledge the first known example of impaired *S*-gene alleles in a cucurbit species. As resistances in cucurbits have previously often shown to inherit recessively, and cucurbits have a low number of *NB-LRR* genes, it can be expected that in the future more impaired susceptibility genes in cucurbits will be discovered. *MLO*-like

genes have also been identified in melon, watermelon, and pumpkin (Iovieno et al. 2015). The information presented about *CsaMLO8* might be useful in identifying orthologous susceptibility genes in those other cucurbit crops.

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Expression Analysis on Melon *MLO* Family Genes and Identification of *CmMLO2* Mutants Resistant to Powdery Mildew

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Abstract. Using the method of homologous sequences, 3 *CmMLO*s have been cloned, which are named *CmMLO1*, *CmMLO2,* and *CmMLO3*. Sequence analysis showed that these *CmMLO*s belong to a gene family that is homologous to *Arabidopsis AtMLO*s. The expression of the *CmMLO* genes was analyzed in leaves symptomatic for powdery mildew, and it was found that *CmMLO2* was mainly expressed in melon leaves in a non-tissue-specific pattern. There is an 85 bp difference in the *CmMLO2* gene between two melon accessions, G24 and C18. The *CmMLO2* mutation in C18 confers resistance to powdery mildew. Therefore *CmMLO2* appears to play a crucial role in the pathogenesis of powdery mildew.

Keywords: Melon, powdery mildew, *MLO* family genes, *CmMLO2* mutation

Introduction

Powdery mildew is one of the major diseases damaging melon production (Kusuya 2003). The normal transcription and expression of a barley mildew resistance locus *o* gene (*MLO*) enables the invasion of powdery mildew spores (Bhat 2005). Mutant loss-of-function alleles at this locus, induced and naturally occurring, are resistant to all known races of powdery mildew (Bueschges et al. 1997). Barley with the mutated *mlo* gene not only resists all 30 known races of powdery mildew pathogen, but has stronger resistance. Its reaction type is immune or nearly immune (Jφregensen et al. 1992). Besides barley (Jφrgensen et al. 1992) and *Arabidopsis* (Devoto et al. 1999), *MLO* genes have been found in other plants, including tomato (Bai et al. 2008), rose (Kaufmann et al. 2012), rice (Elliott et al. 2002), and soybean (Shen et al. 2012). No matter whether the normal *MLO* gene was prevented from expressing through antisense interference or a new *MLO* allele was derived from mutagenesis or natural mutation, the *MLO* locus can be a source of a wide-spectrum resistance to powdery mildew (Panstruga 2005b).

One melon accession was observed in the field to be resistant to powdery mildew. In order to verify whether its resistance is related to *MLO* mutant genes, *MLO* family genes of infected melons were cloned where the main-effect *MLO* genes related to powdery mildew were identified through semi-quantitative gene expression. The reactions to artificial inoculation with powdery mildew pathogen of this resistant accession and a susceptible accession of melon were compared. Based on the sequence differences of the *CmMLO2* gene, a *CmMLO2-GFP* fusion gene transformation carrier was built and the functions of *MLO* in the resistant melon were identified, aiming to provide guidance in breeding melon resistant to powdery mildew.

Materials & Methods

Plant material

Two melon accessions were used, one susceptible to powdery mildew, designated G24, and the other resistant to powdery mildew, designated C18. Seeds of both were planted in pots in an artificial climate box. The radicle, stem, blossom, leaves, and young fruits were used for constitutive expression analysis and were frozen in liquid nitrogen for later use. When the true leaves of the plants appeared, they were inoculated with the pathogen, and leaves 0, 1, 3, 5, and 7 days after inoculation were collected and frozen in liquid nitrogen.

Cloning of CmMLOs

A primer was designed according to the amino acid sequences of known homologous genes in barley and *Arabidopsis* to get the target fragment of RT-PCR amplification. The sequence of the primer is as follows: P3: CA[C/T]CAGCTGCA[C/T/G]AT[A/C/T]TTCATCTT; P4: CCCATCTGAGT[A/T/G]AC[A/T/G]AG[T/A/C/G] GC[A/G/C]TA. The fragment was amplified by the first strand synthetic product of cDNA.

Sequence analysis and full-length sequence assembly

DNAman (Lynnon Biosoft, San Ramon, CA) was used in full-length cDNA sequence assembly; then, a F-primer and

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a R-primer of full-length cDNA were designed and connected to pGEM-Teasy for sequence testing. DNAman and Blast were used in sequence analysis. Analysis on Open Reading Frames was done through on-line ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) from NCBI (The National Center for Biotechnology Information).

Semi-quantitative RT-PCR analysis

One pair of specific primers (F and R) was used in the analysis: *CmMLO1* (F:GGGGTTCATTTCACTTCTGC; R:GAGTGATAGCAATGGCACCT), *CmMLO2* (F:CTTGCCTTCTTTGCTTGG; R:TCTTCTTGGCGGAGTGGT), *CmMLO3* (F:TTTCAAGTCCGTTGGTAAGG; R:AAGCGAAGTGGATGAGGTAA), Actin (F:TGCCCAGAAGTTCTATTCCAGC; R:CATAGTTGAACCACCACTGAGGAC).

An amplified β-actin gene of melon (GenBank Acc: AY859055) was selected as the control; 2 μg root, stem, leaves, blossom, and young fruit RNA were taken for the synthesis of the first-strand cDNA. Then, 1 μl of each of them was used as a template for amplification; 1.2% AGE (agarose gel electrophoresis) and bandscan were used to analyze the relative transcript level of *CmMLO*s in each tissue of melon. SigmaPlot 10.0 was used to draw figures. Referring to the method of analyzing constitutive expression of each family, plants were inoculated with powdery mildew pathogen, and then RNA in leaves 0, 1, 3, 5, and 7 days after inoculation was used to synthesize the first-strand cDNA. Then 1 μl of them was used as template for amplification; 1.2% AGE and Bandscan were used to analyze the relative transcript level of *CmMLO*s induced by powdery mildew at different times.

Establishment of transformation vector with fluorescence labeling

The building of the transformation vector of *CmMLO2* was accomplished by using pROK2 and green fluorescent protein pjit163GFP vectors. At first, primers were designed to amplify the coding region of *CmMLO2*, and restriction enzyme cutting sites of *Bam*HI and *Sal*I were added. In order to form the fused fluorescent protein, the termination codon TGA was mutated as GGA, CmMLO2-F:5′-TAGGATCCATGGCTGAATGTGGAACAG-3′, CmMLO2-R:5′-CTGTCGACTCCTTTGGCAAATGAGAAG-3′. Then, the plasmid pjit163GFP as a template was used to amplify the coding region of GFP, and restriction enzyme cutting sites of *Sal*I and *Kpn*I were added, GFP-F:5′-TAGTCGACATGGTGAGCAAGGGCGAGG-3′, GFP-R:5′-GCGGTACCTTACTTGTACAGCTCGTCC-3′. Finally, *CmMLO2* and segment GFP were amplified by using PCR, and then respectively ligated into pGEM-Teasy vector. After verification through sequencing, the plasmid was extracted and restriction enzyme digestion was carried out by *Sal*I. T4 ligase overnight, and again shifted to the pGEM-Teasy vector. After double restriction enzyme digestion by *Bam*HI and *Kpn*I, the pROK2 plasmid was joined through restriction enzyme digestion by *Bam*HI and *Kpn*I, to complete the establishment of the fused vector.

Genetic transformation and resistance identification of mutant C18

Based on leaf-disc transformation mediated by *Agrobacterium tumefaciens*, the fusion expression vector pROK-CmMlo2-GFP was transformed into melon accession C18. Resistance selection on culture medium was conducted with kanamycin of 50 $mg \cdot L^{-1}$ to obtain callus, and a few tissues were selected to conduct the secondary screening under a fluorescence microscope. Positive tissue was selected for regeneration, and then transplanted to the greenhouse. Plants were self-pollinated and their seeds saved, then planted in a controlled climate chamber. Resistance identification was implemented by inoculating seedlings with powdery mildew pathogen.

Results

Cloning MLO *family genes*

A 800 bp PCR product was obtained from the RT-PCR of the cDNA prepared from melon using primers P3 and P4. The sequence analysis indicated that this fragment shared high identity with the *MLO* genes in barley and *Arabidopsis*, which implies that it is probably a part of the *MLO* gene. The novel three genes were amplified using the RACE method and these were designated *CmMLO1*, *CmMLO2*, and *CmMLO3*. *CmMLO1* is 1551 bp long, coding 516 amino acids, and its Genbank number is FJ713541; *CmMLO2* is 1713 bp long, coding 570 amino acids, and its Genbank number is FJ713542; and *CmMLO3* is 1464 bp, coding 487 amino acids, and its Genbank number is FJ713543.

Analysis on sequences of MLO *family genes*

The alignment was generated by DNAman using the default parameters. Positions of the seven transmembrane regions (TM1 through TM7) inferred from the experimentally determined topology of the barley *MLO* are indicated by bars above, and below the sequences (Figure 1).

Comparisons among the three *MLO* sequences of melon in Genbank revealed the homology is about 70%. The homology of *CmMLO1* to *AtMLO1* of *Arabidopsis* (Acc: NM_116494.4), *MLO1* of corn (Acc: EU971076.1), *AtMLO15* of *Arabidopsis* (Acc: NM_201957.1), and *MLO* of barley (Acc: AK248626.1) was found to be 73%, 72%, 69%, and 72%, respectively. The homology of *CmMLO2* to *AtMLO12* of *Arabidopsis* (Acc: NM_129478.4), *AtMLO2* (Acc: NM-101004.3), *MLO1* of lotus root (Acc: AY967410.1), and *MLO* of pepper (Acc: AY934528.1) was 69%, 68%, 69%, and 69%, respectively. The homology of *CmMLO3* to *AtMLO8* of *Arabidopsis* (Acc: NM_129478.4), *AtMLO7* (Acc: NM-101004.3), *MLO1* of lotus root (Acc: AY967410.1), and *MLO* of pepper (Acc: AY934528.1) was 73%, 72%, 64%, and 63%, respectively.

Chen (2006) divided the 15 family genes of *Arabidopsis* into four groups. It was observed that melon *CmMLO3* has a closer evolutionary relationship with *AtMLO5, 7, 8,* and *9* of *Arabidopsis*, so they are in the first group and that *CmMLO2* together with *AtMLO2, 3, 6* and *12* of *Arabidopsis* is in the second group while *CmMLO1* along with *AtMLO1, 13,* and *15* of *Arabidopsis* is in the third group (Figure 2).

RT-PCR analysis on MLO *family genes*

The results show that the three *MLO* family genes that were discovered were expressed in the root, stem, cotyledon, leaf, blossom, and fruitlet of melon, however, their level of expression obviously varied (Figure 3A). The density of each band was quantitated with Bandscan to calculate the expression levels compared to that of Actin. The results showed that the

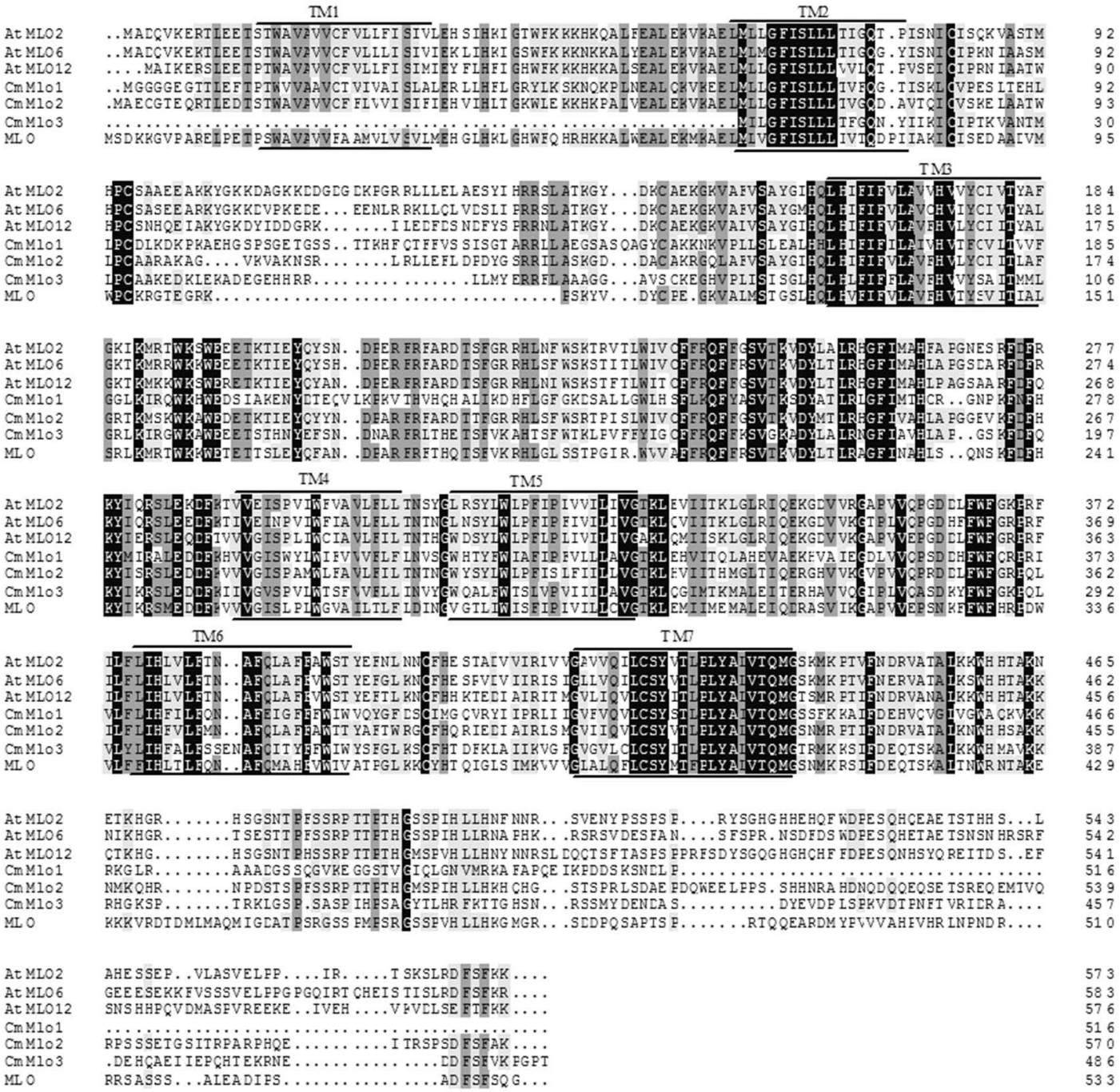

Figure 1. Alignments of the deduced amino acid of *CmMLO*s and other *MLO*s.

expression of *CmMLO1* in the cotyledons, and flowers reached its peak, followed by that in the root, stem, leaf, and fruitlet, and that the expression of *CmMLO2* in the leaf is the higher than that in other tissues while *CmMLO3* mainly expresses in the fruitlet, root, and blossom (Figure 3C). The *MLO* family genes of melon belong to the tissue-specific expression mode.

The expression of each gene at 0, 1, 3, 5, and 7 days after inoculation with pathogen was compared using the semi-quantitative RT-PCR method and the Actin gene was also used as the internal reference to obtain uniform amounts of signals. The results showed that expression pattern of *CmMLO2* changes more than *CmMLO1* and *CmMLO2* (Figure 3B) and the calculated result of relative transcript level indicated that in tissue stressed by powdery mildew, the expression of *CmMLO1* slightly increased before resuming the original level, that the expression of *CmMLO2* increased but decreased by the seventh day and that the expression of *CmMLO3* changed little during the whole process (Figure 3D). These observations indicate that *CmMLO2* likely confers the powdery mildew resistance.

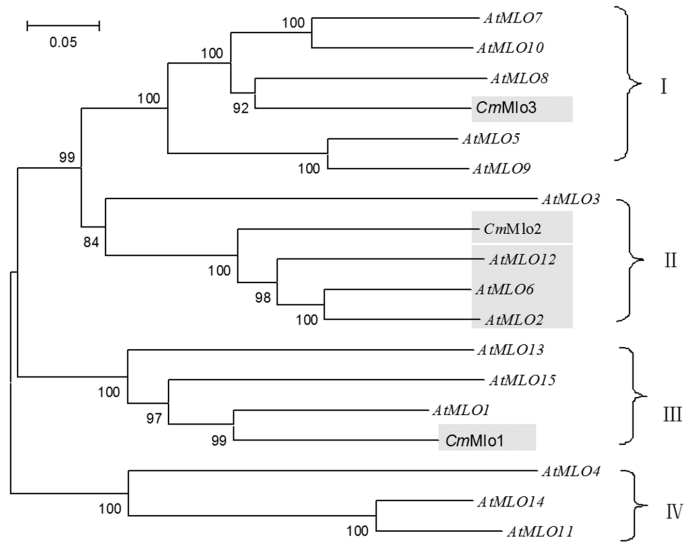

Figure 2. A neighbor-joining phylogenetic tree of *Arabidopsis* and selected CmMLO proteins.

Differences of MLO *genes in resistant and susceptible melon*

The sequencing results indicate that the full length of *CmMLO2* of the susceptible accession G24 is 1,713 bp, encoding 570 amino acids and 7 transmembrane helices. On the other hand, the full length of *CmMLO2_1* of the resistant accession C18 is 1,798 bp. The two sequences were compared using DNAman, and it can be seen that the middle part of C18 has 85 more base pairs than G24 (TGCAAGCACAGGGTGGATGTCATTTTCTCAGGAAGGAAAATAAATTATTGGTGGAAACATAAATAACGTTGTTAAATATAGTACC).

Disease resistance of genetic and transformed plant of the resistant mutant

After establishing pROK2-CmMlo2-GFP, it was shifted into C18 using the *Agrobacterium*-mediated method, and cultivated on MS culture medium containing kanamycin, to get kanamycin-resistant callus tissue. After continuous cultivation, some callus was examined under a fluorescence microscope. Transformed callus is marked by clear fluorescent signals (Figure 4Aa), while negative-control (non-transformed) callus has no fluorescence (Figure 4Cc). After conducting induced differentiation on callus and cultivation for rooting, the genetically transformed F_0 generation plant of C18 was accrued. The F_1 generation, five days after inoculation with pathogen, showed powdery mildew symptoms on the transformed plant (Figure 4B) and no powdery mildew symptoms on the control plant (Figure 4D), showing that its resistance against powdery mildew stays the same.

Discussion

Since *MLO* genes were discovered in barley (Bueschges et al. 1997), *MLO* genes have been successively found in many crops (Zhao 2003). The *MLO* genes of many plants are like a family with many members. For example, there are 15 family members in *Arabidopsis* (Chen et al. 2006); so far, nine have been found in corn and 12 in rice (Liu et al. 2008). Because the whole-genome sequencing of other plants lags behind, few members of the *MLO* family have been discovered in crops such as barley, wheat, lotus, pepper, and tomato.

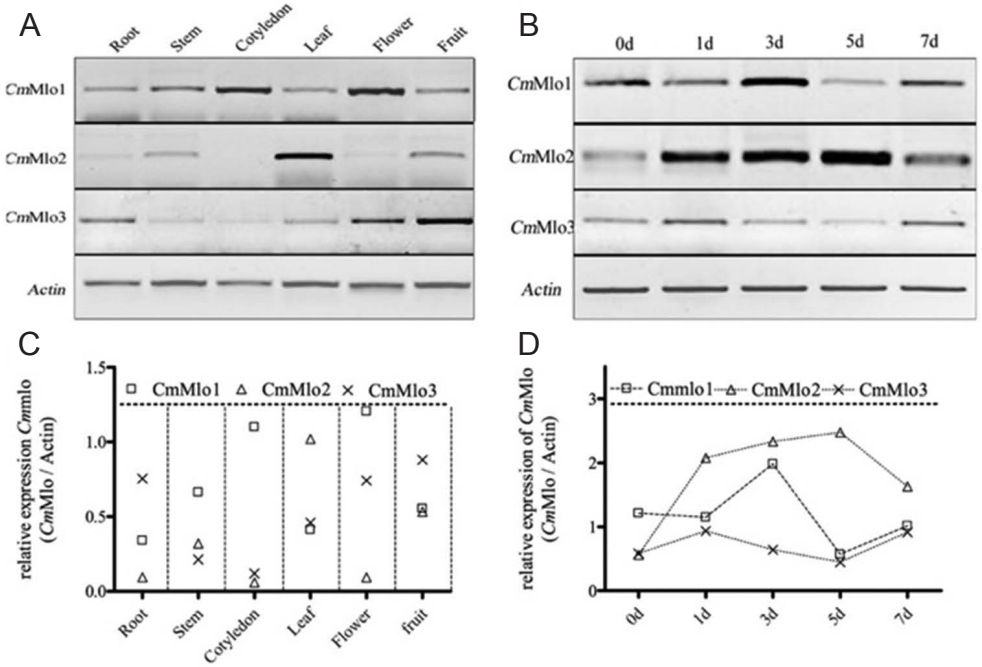

Figure 3. (A) RT-PCR analysis of the expression of *CmMLO*s genes in different tissues. (B) The expression of *CmMLO*s in leaves induced by powdery mildew 0, 1, 3, 5, 7 days after inoculation. (C) Relative expression of *CmMLO*s in melon tissues. (D) Relative expression of *CmMLO*s in leaves induced by powdery mildew 0, 1, 3, 5, 7 days after inoculation.

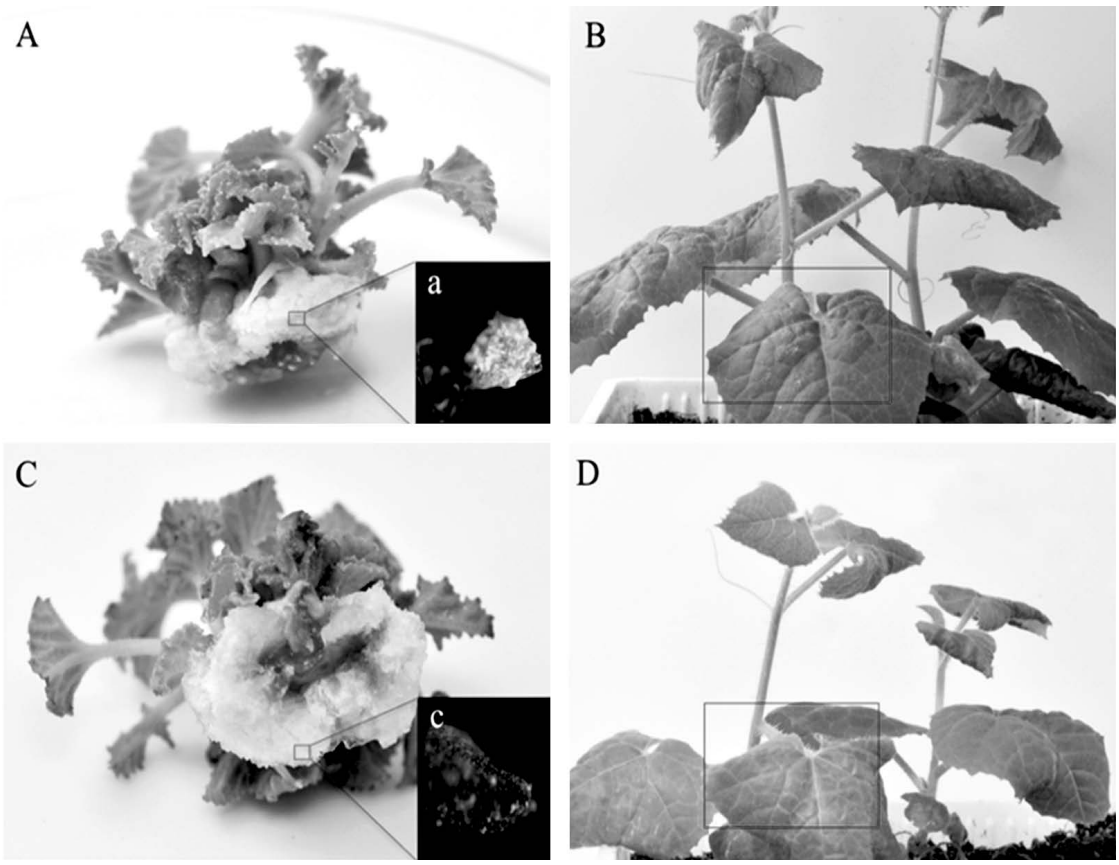

Figure 4. Fluorescence screening of callus on mutant C18 and identification of a positive plant resistant to powdery mildew. (A) Transformed callus; a. transformed callus under fluorescence. (B) Transformed plant five days after inoculation with powdery mildew pathogen. (C) Untransformed callus; c. control callus under fluorescence. (D) Control plant five days after inoculation with powdery mildew pathogen.

After inoculation with powdery mildew, increased expression of *CmMLO2* was found mainly in melon leaves, increasing to as much as 3.5 times of the original level, belonging to the up-regulated pattern. However, the expression of *CmMLO1* and of *CmMLO3* decreased, belonging to the down-regulated pattern. Based on the expression patterns in *Arabidopsis* and barley, we speculate that *CmMLO2* is the major gene for occurrence of powdery mildew resistance.

The normal transcription and expression of the *MLO* genes are necessary for the successful invasion of powdery mildew pathogen (Freialdenhoven et al. 1996, Lyngkjaer et al. 2000, Zellerhoff et al. 2010). By inducing mutations, the coding sequence of *MLO* can be changed to induce plant resistance to powdery mildew (Bueschges et al. 1997, Panstruga 2005a). Additional research results have indicated that the deficiency of genetic transcription or mutation of the encoded cDNA sequence can result in the change of sequence of the polypeptide, which will then cause the transformation for resistance to powdery mildew. In the powdery mildew-resistant wild tomato R26, the cDNA sequence of *SlMlo1* showed the loss of 19 bp, which caused a shift in the *SlMlo1* coding region (Bai et al. 2008). This shift may cause premature termination of translation, leading to resistance to powdery mildew. The up-regulation of *MLO* may prevent necrosis of mesophyllic cells, as well as provide enough nutrition for the development of pathogens, allowing further invasion. The analysis of the signal peptide also proves that the mutated *CmMLO_1* failed to normally encode the CmMLO2 protein. To verify that the resistance of the mutant to powdery mildew is caused by a mutation in the *CmMLO2* gene, a genetically transformed expression vector that was tagged to a fluorescence label was established in the mutated plant. The results of this transformation experiment suggest that the mutation of *CmMLO2* is connected to the resistance of C18 to powdery mildew. The mutated strain of the *MLO* gene of C18 opens the possibility for exploring the functions of genes related to powdery mildew resistance and susceptibility in melon. C18, a naturally mutated accession, offers valuable germplasm for breeding melons with resistance to powdery mildew.

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Sensitivity to Fungicides in *Podosphaera xanthii* and Impact on Managing Cucurbit Powdery Mildew in New York, USA

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Abstract. Effectively managing powdery mildew in cucurbit crops necessitates applying fungicides with risk for resistance development (due to their single-site mode of action) because their mobility enables movement to lower leaf surface where the pathogen develops best. Efficacy of such fungicides is being examined annually by applying them at highest label rate weekly with a tractor-sprayer to field-grown pumpkin (*Cucurbita pepo*) in replicated experiments conducted in New York. A leaf disk bioassay is used to determine fungicide sensitivity of isolates collected after last application in experiments and also commercial crops. Efficacy of some fungicides appeared to be affected by resistance. Resistance to MBC (FRAC code 1) and QoI (FRAC 11) fungicides continues to be common. Isolates have exhibited a range in sensitivity to the DMI fungicide myclobutanil (FRAC 3), quinoxyfen (FRAC 13), and also boscalid, an SDHI (FRAC 7) fungicide. Isolates tolerating 500 ppm boscalid are resistant and associated with poor fungicide efficacy. Boscalid-resistant isolates have been detected regularly since 2008; however, in most years boscalid has provided some control of powdery mildew. Quinoxyfen has consistently provided excellent control when applications were started early in powdery mildew development. However, isolates were collected in 2015 able to tolerate 200 ppm quinoxyfen, which is indicative of resistance as it is similar to the dose in a fungicide application. Evidence was obtained that using resistance-prone fungicides like boscalid alone during a growing season can select for strains with reduced sensitivity or resistance. Cross resistance was documented among SDHI fungicides except fluopyram. To manage powdery mildew in cucurbit crops in the U.S.A., it is recommended to use metrafenone (FRAC U8) and cyflufenamid (FRAC U6), which are the fungicides most recently registered for this disease, in an alternation program along with fluopyram, with all of these targeted fungicides combined with a protectant fungicide such as chlorothalonil. Myclobutanil and quinoxyfen are recommended for use on a limited basis if at all.

Keywords: Cucurbitaceae, powdery mildew, *Podosphaera xanthii*, fungicide resistance

Introduction

Powdery mildew caused by *Podosphaera xanthii*, is the most important disease affecting cucurbit crops, occurring every year throughout most production areas. It occurs commonly due to the quantity of easily wind-dispersed spores that the pathogen produces and the breadth of conditions under which it can develop (no high moisture requirement). Management is needed to avoid loss in yield and/or fruit quality.

Fungicides and resistant varieties are the only management tools. An integrated management program with both tools is recommended because resistant varieties in most cucurbit crop groups do not provide sufficient disease suppression to avoid impact on yield and to minimize selection pressure for pathogen strains able to overcome either tool.

Fungicide resistance is an important issue with managing cucurbit powdery mildew. *Podosphaera xanthii* has already demonstrated high propensity for developing resistance (McGrath 2001, 2015). The most effective chemistry is at risk of resistance developing. The goals of this study were to determine the efficacy of at-risk fungicides, with the objective of identifying when efficacy was below expectation, potentially due to resistance, and to examine sensitivity to fungicides of pathogen strains.

Materials & Methods

Fungicide efficacy

Pumpkin (*Cucurbita pepo* L.) was grown under field conditions following recommended production practices for the region except that fungicides with activity for powdery mildew were not applied. A randomized complete block design with four replications was used. Plots were three 4.6-m rows spaced 1.7 m apart. Treatments were applied typically six times on a 7-day IPM schedule (starting after disease detection) using a tractor-mounted

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boom sprayer. Tested fungicides were applied individually and in alternation as would be done in production fields. Powdery mildew severity was assessed weekly on upper and lower leaf surfaces.

Sensitivity of pathogen isolates to fungicides

Isolates of *P. xanthii* were collected at the end of the growing season from research and commercial plantings of cucurbits that had or had not been treated with fungicides, including from the plots in the Fungicide Efficacy experiments described above. Isolates were maintained on cotyledons on water agar media in Petri dishes.

A leaf disk bioassay was used to determine ability of isolates to tolerate fungicides (McGrath 2001) (Figure 1). Pumpkin seedlings at the cotyledon leaf stage (about 7-days-old) were sprayed with various fungicide doses in a laboratory fume hood, the treated plants dried overnight, then disks were cut from the cotyledons and placed on water agar in Petri plates with four sections. Six disks with the same treatment were placed in each section. Each plate used to test an isolate had three treatments plus a nontreated control. Disks were inoculated by transferring spores from culture plates to each disk center. Amount of pathogen growth on the disks was assessed 10 and 14 days later when the control treatment usually had good growth of the pathogen. Percent leaf disk area with symptoms of powdery mildew was recorded for each disk. An isolate was considered to be insensitive (tolerant) to a particular fungicide concentration if it was able to grow and produce spores on at least half of the disks. Due to limitations in the number of isolates and fungicide doses that can be done in each bioassay, the procedure was conducted multiple times over many weeks to obtain information on sensitivity to several fungicides.

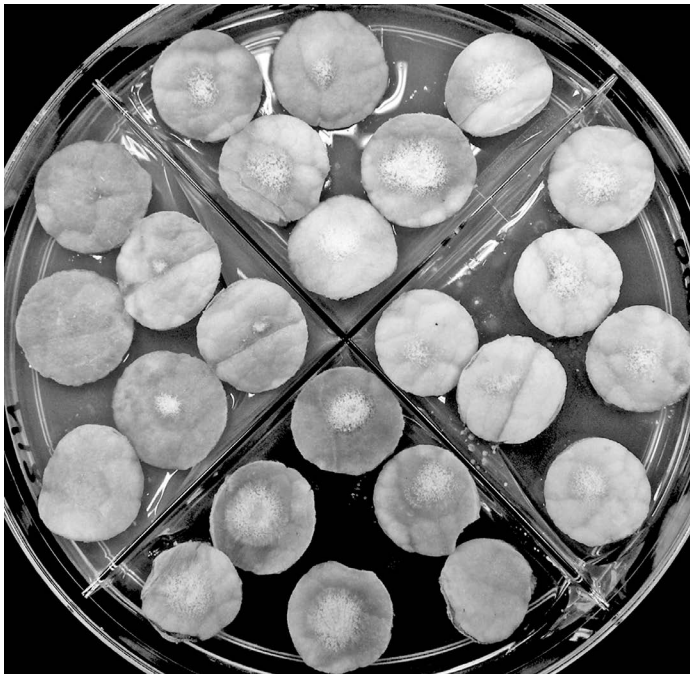

Figure 1. Leaf-disc bioassay conducted in segmented Petri dish with isolate tolerating 120 ppm quinoxyfen (disks in top section) and 200 ppm quinoxyfen (right section). Growth reduced on disks treated with 120 ppm myclobutanil (left section). Non-treated discs are in bottom plate section. Photograph taken ten days after inoculation.

Cross resistance among SDHI (FRAC 7) fungicides was examined using isolates found through the bioassay to be sensitive to the SDHI fungicide boscalid and resistant (tolerating 500 ppm). They were tested with penthiopyrad (formulated as Fontelis), fluopyram (Luna Privilege), fluxapyroxad (Merivon), and benzovindiflupyr (Aprovia).

Results & Discussion

Fungicide efficacy results, 2013

Pristine (containing the active ingredients boscalid, FRAC Code 7, and pyraclostrobin, FRAC 11) applied at its highest label rate was effective, providing 93% control on lower leaf surfaces based on AUDPC values (Table 1). Procure (triflumizole, FRAC 3) applied at its highest label rate was equally effective (95%). Quintec (quinoxyfen, FRAC 13) was not significantly more effective (99%). The grower standard program with these fungicides (Quintec, Procure, Quintec, Pristine, Quintec) also performed very well.

Sensitivity of pathogen isolates to fungicides, 2013

The bioassay revealed that all isolates tested were resistant to thiophanate methyl (FRAC 1) and trifloxystrobin (FRAC 11). Of the 26 isolates from the research field where fungicides were evaluated, 23% were resistant to boscalid (FRAC 7 ingredient in Pristine); 73% were able to grow on leaf disks treated with 40 ppm myclobutanil (FRAC 3); 27% were able to grow on leaf disks treated with 80 ppm myclobutanil; and 81% on disks treated with 40 ppm quinoxyfen (FRAC 13). All four isolates from pumpkin treated with Pristine alone in the fungicide efficacy experiment were resistant to boscalid (tolerated 500 ppm), whereas all isolates from non-treated plots were sensitive. This indicates selection for this resistance occurred during the experiment, and adds to previous observations that using at-risk fungicides alone during a growing season can select for resistant strains (McGrath 2001). In this case, product efficacy was not impacted. Boscalid resistance was detected in one of three isolates from pumpkin treated once with Pristine applied in alternation with Quintec and Procure. An isolate tolerating 80 ppm quinoxyfen was obtained from a Quintec plot.

Of the 27 isolates from the six commercial plantings of pumpkin, 37% were resistant to boscalid; 44% were able to grow on leaf disks treated with 40 ppm myclobutanil; 3% were able to grow on leaf disks treated with 80 ppm myclobutanil; and 56% on disks treated with 40 ppm quinoxyfen. Both boscalid-resistant and sensitive isolates were found in most plantings; however only resistant ones were found in one planting and only sensitive ones in another, which might reflect how much Pristine was applied.

Fungicide efficacy results, 2014

Pristine was moderately effective (Table 1). Pristine was ineffective at the last assessment date based on powdery mildew severity on lower leaf surfaces (71% on September 19 versus 73% for non-treated control), which suggests resistant strains were selected for during the experiment (McGrath and LaMarsh 2015). It was the least effective treatment tested, based on defoliation

on September 19. Pathogen isolates resistant to both components of this fungicide have been detected at this location, including in 2013, and the fungicide has exhibited variable performance including failure in 2010 and 2012 (Table 1). Procure applied at its highest label rate was as effective as Quintec except for the last assessment on lower leaf surfaces (43% versus 9% severity, respectively). Procure was ineffective when applied at its middle label rate in 2009 and 2010. Quintec was highly effective (96% control; Table 1). Similar control (95%) was achieved by alternating among these three products with Quintec applied at its lowest labeled rate. Vivando (metrafenone, FRAC U8), a new fungicide registered in the U.S.A. for this use in 2014, was as effective as Quintec (98% control).

Sensitivity of pathogen isolates to fungicides, 2014

Similar to 2013, all isolates tested were determined to be resistant to FRAC code 1 and 11 fungicides (not all of the isolates were tested with these chemicals). Boscalid (FRAC 7) resistance was detected in 30% of the isolates (all 59 isolates were tested for this trait). Almost all of the resistant isolates were from research fields. All resistant isolates were from research plots that were treated with Pristine in the fungicide efficacy experiment. This suggests selection for this resistance occurred during the experiment, and explains why Pristine was ineffective at the last assessment in this experiment (all isolates from the Pristine-treated plots were also resistant to QoI fungicides, the other active ingredient in Pristine). Only two of the 36 isolates from commercial pumpkin fields were resistant; they were from the same field. These results support use of Pristine on a limited basis in a fungicide program for powdery mildew. Boscalid resistance was at higher levels in commercial fields in 2013. Infrequent occurrence in 2014 may reflect increased use of other chemistry by growers. Most isolates (68%) were able to grow on leaf disks treated with 40 ppm myclobutanil (FRAC 3). Thirteen isolates tested at 120 ppm myclobutanil were able to tolerate this dose. The dose a farmer applies is 300 ppm with Rally applied at the highest label rate (0.35 kg/ha) at 50 gallons/A. Quinoxyfen (FRAC 13) at 40 ppm was tolerated by 29% of the isolates. Six isolates tested at 120 ppm quinoxyfen were able to tolerate this dose. The dose a farmer applies is 212 ppm with Quintec applied at the highest label rate (177 ml/ha) at 468 L/ha.

Fungicide efficacy results, 2015

Pristine was as effective as Quintec and Vivando (Table 1) and also the other eight fungicide treatments evaluated (McGrath 2016).

Sensitivity of pathogen isolates to fungicides, 2015

Four isolates were sensitive to QoI fungicides. They were also sensitive to boscalid and to 40 ppm myclobutanil. Boscalid resistance was more common than in previous years (87% of isolates). Most of these (92%) were able to grow on leaf disks treated with 40 ppm myclobutanil; 80% of those tolerating 40 ppm were able to grow on leaf disks treated with 120 ppm myclobutanil, but often at a reduced rate, and also on disks treated with 200 ppm quinoxyfen (Figure 1).

Cross resistance among SDHI (FRAC 7) fungicides

All six boscalid-resistant isolates collected in 2012 were resistant to penthiopyrad and fluxapyroxad but not fluopy-

Table 1. Efficacy for cucurbit powdery mildew of fungicides at risk for resistance development determined in replicated experiments conducted under field conditions with pumpkin in New York from 2006 to 2015[a].

Fungicide	Product	Control of powdery mildew on abaxial leaf surfaces (%)[b]									
		2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
Pyraclostrobin	Cabrio	20 ab[c]									
Pyraclostrobin + boscalid	Pristine	89 efg	58 e	94 d-g	80 bcd	50 abc		40 abc	93 bcd	54 b	73 b
Boscalid	Endura	90 fg	60 ef								
Penthiopyrad	Fontelis			82 b-e		50 abc	60 ab	33 abc			
Triflumizole	Procure M[d]	35 b	75 fg		40 ab	50 abc					
Triflumizole	Procure H[d]		78 g	93 d-g	72 cd	69 bcd	22 ab	57 bc	95 bcd	70 b	
Quinoxyfen	Quintec	89 efg	81 g	99 g	86 d	95 d	41 ab	96 e	99 d	96 cd	69 b
Cyflufenamid	Torino						62 ab				
Metrafenone	Vivando					82 cd			97 bcd	98 bc	70 b
Alternation[e]	*Alternation*							89 de	98 cd	95 cd	

[a]Reports on these experiments have been published (McGrath and Davey 2007; McGrath and Fox 2008, 2009, 2010; McGrath and Hunsberger 2011, 2012; McGrath and LaMarsh 2013, 2014b, 2015; McGrath 2016). Fungicides were applied on 7-day intervals in all experiments.

[b]Control of powdery mildew on lower leaf surfaces based on comparing Area Under Disease Progress Curve (AUDPC) values for the fungicide to the non-treated control. AUDPC is a summation measure of severity ratings taken on several dates over the assessment period.

[c]Values with an 'a' indicate the AUDPC value was not significantly different from the non-treated control for all years except 2011, when mean separation for the control was 'ab'. Efficacy of all fungicides tested in 2011 was likely affected by applications being started after powdery mildew was well established.

[d]M = middle label rate. H = high label rate. Other products were applied at the highest label rate when labeled for use over a rate range.

[e]Quintec was applied on odd application numbers alternated with Procure and Pristine applied on the even times except in 2015 when it was alternated with Vivando and Torino.

ram. There was one boscalid-sensitive isolate that was tolerant of 50 and 500 ppm penthiopyrad and fluxapyroxad. All nine boscalid-resistant isolates collected in 2013 were resistant to 50 ppm penthiopyrad, eight were also resistant to 50 ppm benzovindiflupyr, and four were also resistant to 50 ppm fluxapyroxad. Isolates collected in 2015 are also exhibiting cross resistance with all SDHI fungicides except fluopyram.

Conclusions

Resistance to MBC (FRAC code 1) and QoI (FRAC 11) fungicides continues to be common in *P. xanthii*. These fungicides are not recommended for powdery mildew in cucurbits, or some other diseases (e.g., gummy stem blight) also because of resistance in the U.S.A. They remain labeled for these uses. Resistance to these chemistries was first detected in the U.S.A. in 1967 and 2002, respectively (McGrath 2015). It is a qualitative type of resistance. Resistance to MBC fungicides in *P. xanthii* has been maintained despite very limited use on cucurbit crops. Thiophanate methyl is still recommended for anthracnose. The first SDHI fungicide marketed in the U.S.A., boscalid, has only been available formulated with a QoI fungicide, therefore there has continued to be selection pressure for maintaining QoI resistance in the U.S.A.

P. xanthii continues to be sufficiently sensitive to currently available DMI (FRAC 3) fungicides that usually have provided effective control at high label rates when applied alone in fungicide evaluations. Control failure due to resistance to triadimefon, the first DMI fungicide registered for this use, was documented in the U.S.A. beginning in 1986 (McGrath 2001). However, strains of *P. xanthii* are less sensitive now to DMI fungicides than in the 1990s. Resistance to this chemistry is quantitative.

Strains of *P. xanthii* resistant to the SDHI (FRAC 7) fungicide boscalid (tolerating 500 ppm) have been detected every year in New York since 2008. Pristine, the product containing boscalid, has continued to provide control most years, suggesting the frequency of boscalid-resistant strains is sufficiently low. High frequency in Pristine-treated research plots documents potential for shift in the pathogen population over a single growing season. This was associated with loss of control at the end of the season. Cross resistance was documented among the SDHI fungicides boscalid, penthiopyrad, fluxapyroxad, and benzovindiflupyr, but not fluopyram. Fluopyram evidently binds at the target site sufficiently differently. It is in a different chemical group from the other SDHIs (pyridinyl-ethyl-benzamides). Therefore, fungicides containing fluopyram are recommended over other SDHI fungicides for managing powdery mildew.

Quinoxyfen has continued to provide a high level of control in fungicide evaluations conducted in New York. However, isolates have been detected that are able to grow on leaf disks treated with 200 ppm quinoxyfen, which is indicative of resistance as it is similar to the dose in a fungicide application. These strains are expected to impact control.

Existence of *P. xanthii* isolates with resistance and insensitivity to multiple chemistries is a major concern for managing cucurbit powdery mildew.

Current recommendation for control of powdery mildew in cucurbit production fields in the U.S.A. is to select resistant varieties when possible and to apply at-risk fungicides in alternation and mixed with a protectant fungicide like chlorothalonil. The program should include two recently-registered fungicides, metrafenone (FRAC U8) and cyflufenamid (FRAC U6), plus fluopyram and limited use of quinoxyfen and a DMI fungicide.

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Initiative for International Cooperation of Researchers and Breeders Related to Determination and Denomination of Cucurbit Powdery Mildew Races

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Abstract. Cucurbit powdery mildew (CPM) is caused most frequently by two obligate erysiphaceous ectoparasites, *Golovinomyces orontii s.l.* and *Podosphaera xanthii*, that are highly variable in virulence. Various independent systems of CPM race determination and denomination cause a chaotic situation in cucurbit research and resistance breeding. We developed new tools to enhance research, communication, and management of CPM races, and proposed them for use by the international CPM research, breeding, seed, and production community. The tools are: (1) a set of 21 differential genotypes of melon, *Cucumis melo*, for the identification of CPM races; (2) a triple-part, septet code for meaningful, concise designation of CPM races; (3) protocols for maintenance of CPM isolates, differential genotypes, and laboratory assays; (4) rules and principles of practical application of this system to breeding, seed production, and cucurbit growing, and a proposal for a race denomination suitable for practical application; (5) crucial activities leading to the implementation and running of new tools for CPM research and management. The adoption of the proposed system is based on the following three equally important components that form a complete system, none of which can be omitted: (1) adoption of differential host genotypes; (2) equal consideration of the two major CPM pathogens and acceptance of the unified system of determination, description, and denomination of races for each CPM species; (3) establishment of a cucurbit crop and CPM expert panel as a coordination group, as well as an international CPM race network. There remains a need for urgent additional discussion among the international community of CPM researchers and cucurbit breeders about these proposals.

Keywords: Cucurbitaceae, *Cucumis melo*, race, differential set, septet code

Recent Status of Determination and Denomination of CPM Races

Golovinomyces orontii (Castagne) Heluta (Braun and Cook 2012) (*Go*) and *Podosphaera xanthii* (Castag.) U. Braun & N. Shish. (Shishkoff 2000) (*Px*) are two fungal obligate ectoparasites from the order Erysiphales causing cucurbit powdery mildew (CPM), a disease on field and greenhouse cucurbit crops worldwide. Highly variable pathogenicity and virulence of both pathogens are manifested by the existence of large number of different races. Various independent systems of CPM race determination and denomination are used worldwide, having been based on different cultivars or lines of melon (*Cucumis melo* L.), as reviewed by Lebeda et al. (2011). The heterogeneity of these systems and the lack of clear and uniform descriptions of the genetic variation in the virulence of the CPM pathogens is blocking the study of genetics of resistance to *Go* and *Px* in cucurbits (Pitrat et al. 1998, Montoro et al. 2004, McCreight 2006), resistance breeding of cucurbits (Bardin et al. 1997, 1999, McCreight 2003, 2006, Lebeda and Sedláková 2006, Lebeda et al. 2007), and practical growing of cucurbits as well.

The need to develop a standardized, uniform system facilitating the development and exchange of information on CPM races, the elaboration of optimal and strategic control of CPM, and production of suitable CPM-resistant germplasm has been underscored to the scientific and breeding communities (McCreight and Pitrat 1993, Pitrat et al. 1998, McCreight 2006, Lebeda and Sedláková 2006, Lebeda et al. 2007, 2008, 2010).

Newly Proposed System for Determination and Denomination of CPM Races

The system for CPM race determination and denomination proposed by Lebeda et al. (2016) follows the principles and

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guidelines postulated by Limpert et al. (1994) and Limpert and Müller (1994). This system of CPM race determination and denomination is based upon three components: (1) a standard set of race differentials (Lebeda et al. 2008), (2) a uniform screening methodology (Lebeda and Sedláková 2010), and (3) a uniform code for the host-CPM interactions/scores (Lebeda et al. 2016).

Standard set of race differentials

The system is based on the intraspecific genetic variability of melon (*Cucumis melo*), i.e., race-specific resistance. A set of 21 differential melon genotypes (Table 1) is proposed for differentiation of *Go* and *Px* races because they show enough variation and differentiation capacity for both CPM species (Lebeda and Sedláková 2006, McCreight 2006, Lebeda et al. 2007, 2008, 2012, Sedláková et al. 2014). This enlarged set is based on the set of melon CPM differentials developed by French investigators (Bertrand 1991, Bertrand et al. 1992) (Table 1), and is supplemented with 10 additional melon genotypes that revealed new *Px* and *Go* races (Hosoya et al. 2000, Bertrand 2002, Lebeda and Sedláková 2006, McCreight 2006, Lebeda et al. 2007, 2008, 2011, Longzhou et al. 2008, McCreight et al. 2012, Sedláková et al. 2014). CPM race differentials within this system are arbitrarily divided into three groups, called triplets, each containing seven differentials (Tables 1,2). The differentials are assigned an arbitrary, permanent order within a respective group. A value of 1, 2, 4, 8, 16, 32, or 64 is attributed to the differentials in relation to their order within each group (Table 2).

Uniform screening methodology

Screening and evaluation protocols have been described in detail by Lebeda (1984) and Lebeda and Sedláková (2010). Virulence of isolates to the set of differentials is determined by using the leaf disc method. Leaf discs, 15 mm in diam., are taken from 6 to 8 week-old, CPM-free plants of the differentials and placed on moist filter paper in plastic boxes, inoculated, and incubated in a growth chamber at 24/18 °C day/night and 12 h photoperiod. Sporulation intensity (also called degree of infection, DI) is evaluated using a 0 to 4 scale. Genotypes with no or low sporulation (DI = 0 or 1) at the last evaluation (14 days after inoculation) are considered to be resistant, whereas genotypes with a DI = 2 to 4 are considered to be susceptible (Lebeda 1984, Lebeda and Sedláková 2010).

Uniform code for the host-CPM interactions

The proposed numerical system for designation of races is derived from "coded triplets", a system for designation of pathotypes of plant pathogens (Limpert and Müller 1994, Limpert et al. 1994). The binary results of any CPM assay, i.e., compatible (+) and incompatible (−) reactions, are then translated into a triplet-septet code. Each differential that is susceptible (compatible) to a given CPM isolate contributes to this code by a defined value of 1, 2, 4, 8, 16, 32, or 64, according to its position in the group, and resistant (incompatible) differentials are assigned a score of zero (0). Obtained values are added separately for each of three groups. The three sums are then combined to form a unique triplet code in the format: group 1 sum.group 2 sum.group 3 sum. Examples of the translation of disease reactions (compatible and incompatible) to triplet values composition (construction) of triplet-septet codes for four hypothetical CPM isolates are presented in Table 2.

Application of the Proposed System to Practical Breeding

Practical application of the proposed system by breeders, seed producers, and growers has been inspired by the approach of the International Bremia Evaluation Board (2016). CPM isolates being collected each year in the most important cucurbit production areas should be tested on the differential set in order to confirm the novelty of their reaction patterns. When a specific and novel reaction pattern appears repeatedly in several countries and over several years, the international board (committee) for CPM (to be established) will: (i) identify isolates with this pattern as a threat to the cucurbit industry, and (ii) define a new race and denominate an isolate for this new race.

Denomination of Races (Construction of Formulas)

We propose the following formulae for the two pathogens, *P. xanthii* and *G. orontii*, starting with Pxmel 1 and Gomel 1. It is evident that reaction patterns expressed by triple-part septet code of Pxmel 1 will differ from Gomel 1, and both series of CPM races, i.e., Pxmel and Gomel, will be developed independently of each other. This new numbering (1, 2, 3, …) of CPM races has no relationship to previously used designations.

Actions Needed to Implement a Proposed System for CPM Race Determination and Denomination

Progress in CPM race determination and denomination is based on three pillars: scientific, organizational, and financial. The most important principles of the work with the host plants and pathogens must be agreed upon by the international scientific and breeding community. The responsibility and competence must be clearly defined and co-ordinated. Financial support for scientific activities must be balanced by benefit to donors (breeding and seed companies).

The most important activities related to the host plants (Cucurbitaceae) are:

a. Adopt a clearly described set of differential host genotypes (recently *Cucumis melo*) in relation to taxonomic identity, origin, and genetics (resistance factors/genes, markers, other genetic characters);
b. Conserve these genotypes in a "germplasm collection" under standard conditions;
c. Share them (freely?) and encourage their use throughout the research and breeding community.

The most important activities related to pathogens (*P. xanthii, G. cichoracearum*) include:

a. Adopt unified system of determination, description, and denomination of races of CPMs, including standardization of screening methodology;
b. Establish a standard collection of well-characterized (geographic origin, country and place of origin, host species and genotype, pathogenic variation, etc.) isolates of CPMs;
c. Conserve these isolates under standard and controlled conditions;

d. Share them (freely?), within the limits of phytosanitary control and appropriate restrictions to reduce the risk of the evolution and spread of new races, and encourage their use throughout the research and breeding community.

International Network (Platform) for CPM Races

An international network should be established among public and commercial institutions responsible for maintaining collections of host-differential genotypes and pathogen isolates, for investigating this pathosystem and related topics, for Cucurbitaceae breeding, seed production, and growing. This platform will help (i) establish efficient linkages and information exchange among research groups involved in this topic; (ii) advance our understanding by extensive testing of the widest possible array of host and pathogen genotypes; and (iii) efficiently develop disease-resistance breeding.

All of the above-mentioned aspects must be based on broad international cooperation and coordination. Experts, scientists, breeders, and seed producers are encouraged to meet and discuss the following questions:

1. Who will create and coordinate the contacts among researchers on this topic?
2. Who will coordinate the practical application of this system (researchers, breeders, or both together); e.g., ICPMB (International Cucurbit Powdery Mildews Board)?
3. Who will decide about inclusion of new differential host genotypes?
4. Who will maintain, multiply, and distribute the differential host genotypes?
5. Who will identify, maintain, multiply, and distribute CPM races?
6. All of these activities will be on a continental (e.g., Europe, America, Australia) or a global scale?
7. Who will give financial support to these activities?
8. Results of all these activities will be freely available for everybody or only for "members" and those who are paying (e.g., selected breeding and seed companies)?

Table 1. Set of CPM race differential genotypes (*Cucumis melo*) by group number, as proposed by Lebeda et al. (2016).

Differential genotype			Origin	
group. No	cultigen/accession	other designation(s)*	source	country
1.1	Iran H	–	INRA	Iran
1.2	Védrantais	M 319	INRA	France
1.3	PI 179901	Teti	USDA	India
1.4	PI 234607	Sweet Melon	USDA	South Africa
1.5	AR HBJ	AR Hale's Best Jumbo	USDA	USA
1.6	PMR 45	M 321	USDA	USA
1.7	PMR 6	Ames 26810	USDA	USA
2.1	WMR 29	M 322	USDA	USA
2.2	Edisto 47	NSL 34600	Clemson Univ.	USA
2.3	PI 414723	LJ 90234	USDA	India
2.4	PMR 5	Ames 26809	USDA	USA
2.5	PI 124112	Koelz 2564	USDA	India
2.6	MR-1	Ames 8578	USDA	USA
2.7	PI 124111	Koelz 2563	USDA	India
3.1	PI 313970	90625 VIR 5682 PI 315410	USDA	India
3.2	Noy Yizre'el	–	Bar-Ilan Univ.	Israel
3.3	PI 236355	–	USDA	England
3.4	Negro	–	Univ. Zaragoza	Spain
3.5	Amarillo	–	Univ. Zaragoza	Spain
3.6	Nantais Oblong	M 320	INRA	France
3.7	Ames 31282	PI 134198	USDA	China

Differential genotypes of *C. melo* listed are maintained by Dept. Botany, Palacký University, Olomouc (Czech Republic), and by ARS, USDA, Salinas, California (USA).
* = name or additional accession number in the germplasm database or in working collection.
M 319 to M 322 = original designation by M. Pitrat, INRA, Montfavet, France; provided to A. Lebeda in 1997
INRA = L'Institut National de la Recherche Agronomique, Montfavet (France).
USDA = United States Department of Agriculture, Agricultural Research Service; info on USDA accessions available on the website of the National Genetic Resources Program, Germplasm Resource Information Network (GRIN), http://www.ars-grin.gov/npgs/acc/acc_queries.html.

Table 2. Examples of triplet-septet codes for four CPM (*G. orontii*, *P. xanthii*) hypothetical isolates based on their reactions of CPM race differentials in three septet groups (adapted from Lebeda et al. 2016).

Isolate	Triplet																				Triplet -septet code
	1							2							3						
	Differential genotype no.							Differential genotype no.							Differential genotype no.						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
	Values							Values							Values						
	1	2	4	8	16	32	64	1	2	4	8	16	32	64	1	2	4	8	16	32	64	
#1																						
Reaction	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Score	1	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.0.0
#2																						
Reaction	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	
Score	1	2	4	8	16	0	0	0	0	0	0	0	0	64	1	0	0	0	0	32	0	31.64.33
#3																						
Reaction	+	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	+	
Score	1	2	0	0	16	0	0	0	0	0	0	16	0	0	1	0	4	8	16	32	64	19.16.125
#4																						
Reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Score	1	2	4	8	16	32	64	1	2	4	8	16	32	64	1	2	4	8	16	32	64	127.127.127

Compatible (+) reactions result in a score equal to the code value for the respective differential in each septet group; incompatible (-) reactions are scored zero. The sum of the seven scores in each group is the code for the respective triplet, and the septet scores are combined to form a triplet code for the isolate.

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Survival of *Xanthomonas cucurbitae*, Cause of Bacterial Spot of Cucurbits, in the Field

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Abstract. Bacterial spot of cucurbits, caused by *Xanthomonas cucurbitae*, is an emerging disease in the United States. To develop effective crop rotations for management of the disease, survival of the pathogen in fields was studied. Leaves and fruits infected with *X. cucurbitae* were collected from two commercial fields in September 2011 and used in this study. A factorial experiment was conducted at the University of Illinois Research Farm in Urbana, Illinois. The experimental factors were field location where infected samples were collected; plant tissue (leaf and fruit); burial depth (5 and 15 cm); and recovery date (during 24 months). The experimental unit was either five leaves or two fruit pieces (10 cm × 10 cm) which were placed in a fiberglass mesh and buried in the field on 7 October 2011. Recovered samples were tested on kasugamycin-cephalexin agar in Petri plates for presence of *X. cucurbitae. Xanthomonas*-like colonies were sub-cultured on yeast dextrose agar (YDC). Identification of *X. cucurbitae* isolates was based on the colony characteristics on YDC, PCR test, 16S rRNA gene sequence, and pathogenicity test. Mean number of colony forming units (CFU) of *X. cucurbitae* per g of dry plant tissue was calculated. *X. cucurbitae* was isolated from the samples for 24 months after placing them in the field, and isolated bacteria were pathogenic. The location of the original fields did not significantly ($P = 0.7895$) affect the number of *X. cucurbitae* CFUs recovered from the samples. However, survival of *X. cucurbitae* was significantly affected by the plant tissue ($P = 0.00001$) and time period of plant tissue in soil ($P = 0.0001$).

Keywords: *Xanthomonas cucurbitae,* pathogen survival, cucurbits, pumpkin

Introduction

Bacterial spot disease of pumpkin (*Cucurbita* spp.), caused by *Xanthomonas cucurbitae*, has become a serious threat to pumpkin production in Illinois and other Midwestern states of the United States (US). No strategies for effective management of bacterial spot of cucurbits are available because very little is known about the biology of *X. cucurbitae* and epidemiology of the bacterial spot disease. *X. cucurbitae* is reported as a seed-borne pathogen (Williams and Zitter 1996). It also survives in plant debris (Babadoost and Zitter 2009). There is no published report on survival of *X. cucurbitae* in soil. The objective of this study was to assess the survival of *X. cucurbitae* in plant debris in soil in the field.

Materials & Methods

Survival of Xanthomonas cucurbitae *in the field*

An experiment was conducted at the University of Illinois Fruit and Vegetable Research Farm, Urbana, IL (latitude: 40°04'.7" N; longitude: 88°12'.9" W; elevation: 236 m) to assess survival of *X. cucurbitae* in plant tissues in the field. The soil texture was silt loam with pH 6.7 and 4.5% organic matter. The field had been planted with soybeans prior to setting up the experiment. An area of 20 m × 15 m was plowed and disked on 18 September 2011 to prepare for the experiment.

Leaves and fruits infected with *X. cucurbitae* were collected from two commercial fields; one field in Putnam County (designated as northern field) and another field in Champaign County (designated as central field). All samples were from the *Cucurbita pepo* L. pumpkin 'Howden'. The infected leaves and fruit samples were collected from 25 to 30 September 2011 and were kept at 4 °C until being prepared for experimental samples. Each experimental sample was either five infected leaves or two infected fruit pieces (10 cm × 10 cm each). Samples were prepared from 5 to 6 October. Asymptomatic leaves from pumpkin 'Howden' plants grown in a greenhouse were included as controls. Each leaf or fruit sample was placed in a fiber glass mesh bag (35 cm × 35 cm) and the bags were tied with non-biodegradable nylon string. The samples were kept at 4 °C until placed in the experimental site in the field on 7 October 2011.

The experiment was performed in a factorial design with four replications. The factors were: (i) location of the fields where plant samples were collected from (northern and central fields); (ii) plant tissue (leaf and fruit); (iii) burial depth of tissue (0 to 10 and 10 to 20 cm); and (iv) recovery date (time) of the samples.

Isolation of Xanthomonas cucurbitae *from leaf and fruit samples*

For the isolation of the baseline samples, each sample was ground in a blender with 500 ml water. Then, a 1 ml suspension

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was transferred to a 30 ml glass tube with 9 ml of sterile distilled water (SDW) to make a 10^{-1} dilution. Similarly, 10^{-2} and 10^{-3} dilutions were prepared.

Buried samples were recovered on 7 April, 7 July, and 7 October 2012; and 7 January, 7 April, and 7 October 2013. At each recovery time, 40 sample bags were removed and processed. The processed bags included four control leaf samples buried at 0 to 10 cm and four control leaf samples at 10 to 20 cm; four leaf samples at 0 to 10 cm, four leaf samples at 10 to 20 cm, four fruit samples at 0 to 10 cm, and four fruit samples at 10 to 20 cm from northern field; and four leaf samples at 0 to 10 cm, four leaf samples at 10 to 20 cm, four fruit samples at 0 to 10 cm, and four fruit samples at 10 to 20 cm from central field. Suspensions of 10^{0}, 10^{-1}, 10^{-2}, and 10^{-3} from each sample were prepared using the following procedure. One gram of the processed tissue was added to 9 ml to sterile distilled water (SDW) in a 30 ml glass tube, which was considered as the 10^{0} dilution. The tube was shaken by hand for 5 sec. Then, 1 ml of suspension was transferred to a new tube with 9 ml of SDW to make 10^{-1} dilution. Similarly, 10^{-2} and 10^{-3} dilutions were prepared.

Aliquots of 100 µl of each diluted suspension in SDW were added onto the surface of the kasugamycin-cephalexin agar (KC) in petri plates. KC medium contained 7 g yeast extract, 7 g peptone, 7 g glucose, 18 g agar, 20 mg propiconazol, 40 mg cephalexin, and 20 mg kasugamycin in 1,000 ml distilled water. KC is a semi-selective medium for *Xanthomonas* spp. (Schaad et al. 2001). The plates were incubated in the dark at 28±1 °C. After 4 days, *Xanthomonas*-like yellow colonies were sub-cultured by streaking onto yeast extract dextrose calcium carbonate (YDC) medium (Schaad et al. 2001). YDC medium contained 10 g yeast extract, 20 g dextrose, 20 g calcium carbonate, and 15 g agar in 1,000 ml of distilled water. The YDC culture plates were incubated in the dark at 28±1 °C (Mohammadi et al. 2001, Schaad et al. 2001). Developing colonies were sub-cultured twice on Luria Bertani agar (LB) medium to produce single cell colonies. After 2 days, single-cell colonies were transferred into 1.2 ml cryogenic vials (Corning Incorporated, Life Sciences, Acton, MA, U.S.A.) containing 1 ml solution of 15 or 30% glycerol for storage at -20 °C and -80 °C, respectively (Goto et al. 1980, Schaad et al. 2001) for the future studies.

Identification of Xanthomonas cucurbitae

Xanthomonas cucurbitae was identified based on morphological characteristics of bacterial colonies, polymerase chain reaction (PCR) test, and pathogenicity in pumpkin plants. Colonies of *Xanthomonas* spp. are mucoid, convex yellow on YDC (Schaad et al. 2001).

Isolates with the characteristics of *Xanthomonas* spp. on YDC were selected for PCR test. *Xanthomonas* genus-specific primers RST2 (5'AGGCCCTGGAAGGTGCCCTGGA3') and RST3 (5'ATCGCACTGCGTACCGCGCGCGA3') were used in the test (Schaad et al. 2001). PCR test was conducted using a thermal cycler machine (PCT-200, MJ Research Inc., Waltham, MA). A 25 µl reaction volume used contained 12.5 µl Gotaq Green Master Mix 2x (Promega Corporation, Madison, WI, U.S.A.), 1 µl of each primer, 10 µl of nuclease-free water, and 0.5 µl of bacterial suspension (10^{8} CFU·ml^{-1}). ATCC strain 23378 of *X. cucurbitae* was included as a reference isolate. PCR cycling included initial denaturation at 95 °C for 10 min; 32 cycles of denaturing at 95 °C for 40 s, annealing at 66 °C for 40 s, and extension at 72 °C for 1 min; followed by a final extension step at 72 °C for 10 min. The resulting product was preserved at 4 °C until analysis. The PCR product was run in 1% agarose gel containing SYBR® safe DNA gel stain at 90 volts for 40 min, and visualized under safe imager™ blue light transilluminator.

The isolates with mucoid, convex, yellow colonies on YDC were tested for their pathogenicity in the susceptible pumpkin 'Howden' in a greenhouse. From each recovery date, 22 to 28 *Xanthomonas* isolates were tested for their pathogenicity. Each isolate was cultured on LB agar at 24±1 °C for 3 days. Bacterial inoculum was prepared by washing bacterial colonies with SDW into a 15 ml conical tube (Falcon 352097). The density of bacterial cells was adjusted to 5×10^{7} CFU·ml^{-1} using a spectrophotometer (Smart Spec 3000; Bio-Rad, Philadelphia, PA, U.S.A.) at OD_{600} = 0.5. Three-week-old pumpkin plants were inoculated by infiltration of the bacterial suspension into the abaxial side of leaves, using a 10 ml syringe. ATCC strain 23378 of *X. cucurbitae* was used as a positive control and SDW as a negative control. For each isolate, two leaves from the same plant were inoculated by three infiltrations per leaf. Inoculated plants were placed in a greenhouse maintained at 26±2 °C. Development of the disease symptoms (water-soaked, necrotic lesions) was recorded every day from 5 to 10 days post inoculation. Symptomatic tissues were processed to isolate *X. cucurbitae.* Isolated bacterial colonies were identified based on the colony characteristics on YDC (Barak et al. 2001). The experiment for each isolate was repeated once.

Results

Survival of Xanthomonas cucurbitae *in the field*

There was no significant effect (P = 0.79) in the survival of *X. cucurbitae* of samples collected from northern and central fields. However, survival of *X. cucurbitae* in soil was significantly affected by plant tissue (P = 0.00001) and time period of plant tissue in soil (P = 0.0001). The interaction of the plant tissue and the time period of plant tissue in soil on the survival of *X. cucurbitae* was significant (P = 0.0018).

There were significant differences in the number of CFUs recovered from plant samples at different recovery dates. Recovered numbers of CFUs after 6 months were significantly greater than those recovered after 9 months (Figure 1). Similarly, the numbers of CFUs recovered after 9 months were significantly greater than those recovered after 12 months. There were no significant differences between numbers of CFUs recovered after 12 months and 18 months, but numbers of CFUs recovered after 12 months and 18 months were significantly greater than those recovered after 15 months and 24 months. Numbers of CFUs recovered after 24 months were significantly lower than those recovered before 18 months (Figure 1).

The numbers of CFUs recovered from fruit samples were significantly higher (P = 0.05) at 6, 9, and 18 months of recoveries, compared to the numbers of CFUs recovered from leaf samples (Figure 1). The numbers of CFUs recovered from fruit and leaf samples at 0, 12, 15, and 24 months were not significantly different from each other (Figure 1).

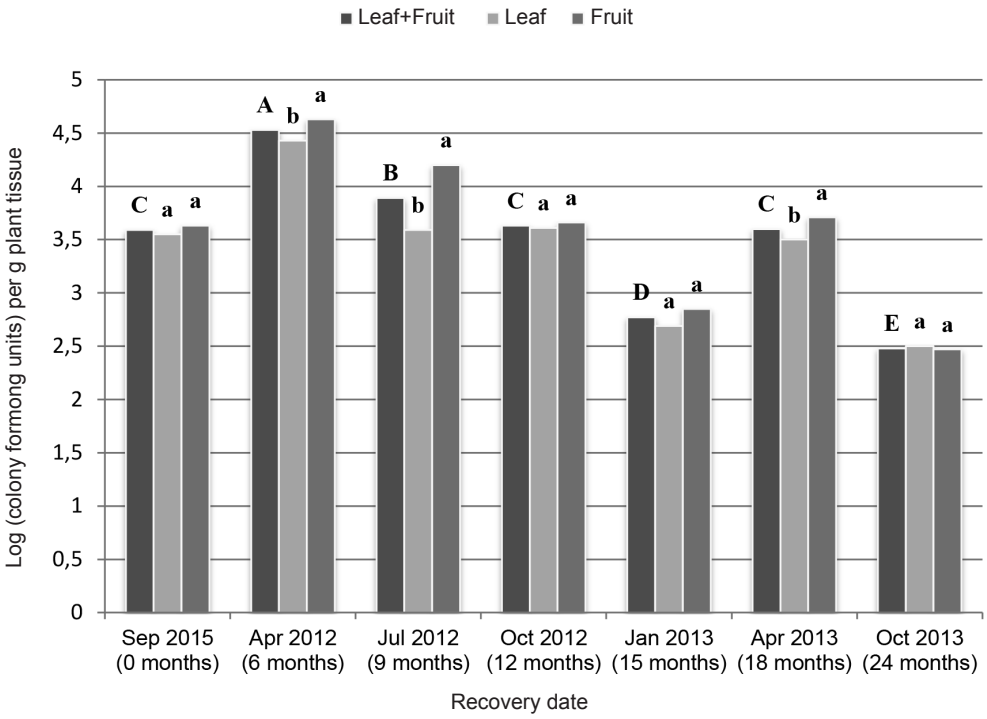

Figure 1. Survival of *Xanthomonas cucurbitae* in infected leaves and fruit of pumpkin in soil at the University of Illinois Fruit and Vegetable Research Farm in Urbana, IL during 2011-2013. Baseline data processed during September 2015. Colony forming units (CFUs) of *X. cucurbitae* were determined by culturing plant tissues on kasugamycin-cephalexin agar. Bars with average $\log_{10}$ CFUs of leaves plus fruit with a letter in common are not significantly different from each other according to Fisher's protected LSD ($\alpha = 0.05$). At each recovery date, bars with CFU numbers of leaves and fruit with the same letter are not significantly different from each other at $\alpha = 0.05$.

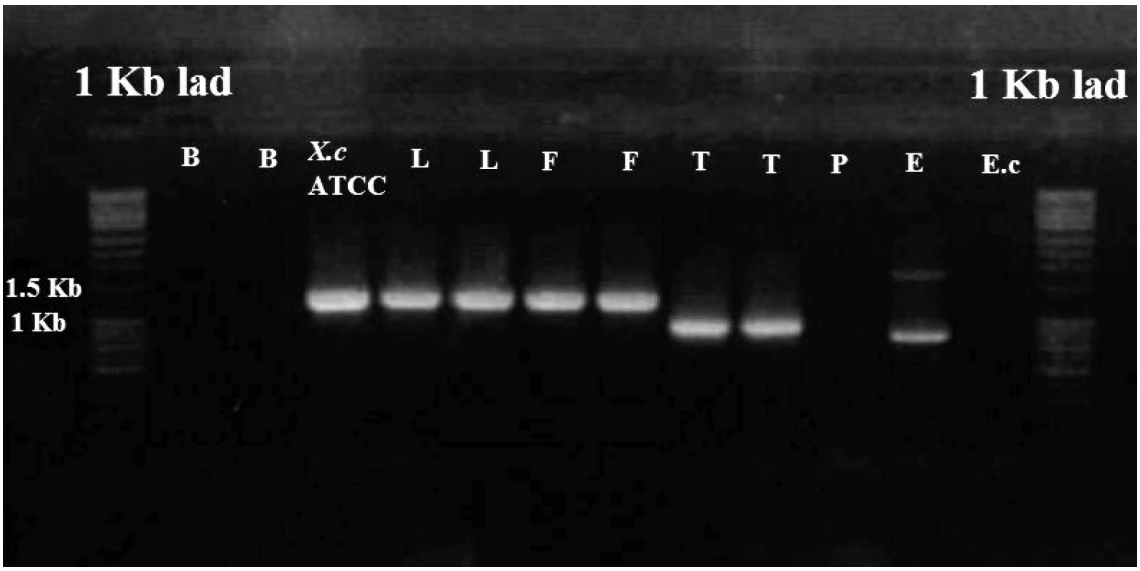

Figure 2. PCR product of *Xanthomonas cucurbitae* isolated from samples buried for 24 months in the field, following amplification with primers RST2 (5'AGGCCCTGGAAGGTGCCCTGGA3') and RST3 (5'ATCGCACTGCGTACCGCGCGCGA3'). B = blank sample; *X.c* ATCC = ATCC strain 23378 of *X. cucurbitae*; L = isolates from originally *X. cucurbitae* infected leaves; F = isolates from originally *X. cucurbitae* infected fruit; T = *Xanthomonas* sp. isolated from tomato; P = *Pseudomonas* sp.; E = *Erwinia amylovora*; and E.c = *Escherichia coli*.

Figure 3. Lesions on pumpkin 'Howden' leaves 7 days after inoculation with *Xanthomonas cucurbitae* isolated from infected pumpkin leaves buried 10 to 20 cm deep in the field for 24 months.

Isolation of Xanthomonas cucurbitae *from field samples*

X. cucurbitae was isolated from the samples collected for baseline data. *X. cucurbitae* was also isolated from both originally infected leaf and fruit samples 6, 9, 15, 18, and 24 months from the time they were placed in the field. Colonies of the isolated bacteria were mucoid, convex yellow on YDC. None of the bacterial isolates from originally asymptomatic leaf samples produced mucoid, yellow colonies on YDC.

The isolates with a mucoid, convex yellow colony on YDC also produced a 1.4 Kb amplicon (Figure 2) in the PCR test, identical to the amplicon produced by ATCC strain 23378. Also, all of the isolates with mucoid, yellow colonies on YDC produced water-soaked brown lesions with yellow halos (Figure 3) on pumpkin 'Howden', like the lesions produced by the ATCC strain 23378. No lesions developed on SDW-infiltrated leaves. The bacterium was re-isolated from the inoculated plants, and the isolated bacteria produced mucoid, yellow colonies on YDC and had a 1.4 Kb amplicon. Based on the characteristics of the isolates on YDC, production of a 1.4 Kb amplicon in the PCR tests, and development of water-soaked brown lesions on inoculated pumpkin leaves, the isolated bacteria were identified as *X. cucurbitae*.

Discussion

Survival of *Xanthomonas* spp. in soil has been studied by several researchers who have reported relatively rapid decline of the bacteria in the field (Scroth et al. 1979, Bashan 1982, Torres et al. 2009). *X. axonopodis* pv. *phaseoli* var. *fuscans (Xap)* survived up to 180 days in leaves placed on the soil surface, but up to 120 days in leaves buried in the soil to depths of 10 and 15 cm (Torres et al. 2009). Only a few CFUs of *X. campestris* pv. *vesicatoria* were isolated from pepper debris after 18 months (Bashan 1982). The result of our research showed that *X. cucurbitae* survived 24 months in infected leaf and fruit tissues of pumpkin in the soil.

The possible reasons for relatively longer survival period of *X. cucurbitae* in the soil as compared with other *Xanthomonas* spp. could be: (i) *X. cucurbitae* may survive longer in the plant tissue in the soil than other *Xanthomonas* spp.; (ii) the decomposition of pumpkin leaf and fruit tissues takes a longer time, thus these tissues support a longer period of survival of *X. cucurbitae*; and (iii) *Xanthomonas* spp. survival may differ in different soil textures. Additional research is needed to assess the survival of *X. cucurbitae* in different host tissues, in different soil textures, and under different climatic conditions.

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Molecular Characterization of the P1 Protein of the *Zucchini Yellow Mosaic Virus* in Turkey

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Abstract. *Zucchini yellow mosaic virus* (ZYMV) is one of the most important viruses in cucurbit plantings in Turkey. In this research, samples from symptomatic cucurbit plants were collected from fields and greenhouses in Ankara, Antalya, and Burdur provinces. Collected samples were tested with double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) against ZYMV antiserum and total RNAs were isolated from infected plants and subjected to RT-PCR amplifications with ZYMV protein-coding regions. After nucleotide sequence analysis, the full nucleotide sequences of the P1 coding region of Turkish isolates of ZYMV were compared with those of other ZYMV isolates in Genebank and phylogenetic tree analysis was performed on the basis of the P1 gene sequences. Sequence analyses and comparisons were performed using MEGA6. The results showed that most isolates belonged to a major molecular subgroup, A1, the most widespread worldwide. However, several isolates from Antalya province belonged to a different molecular subgroup, A5, which has been observed in Korea. The P1 gene region among the ZYMV isolates seems to be very divergent, based on nucleotide and amino acid comparisons. Based on the computer analysis, positive selection was found among the amino acid sequences.

Keywords: ZYMV, P1 protein gene, *Cucurbitaceae*

Introduction

Ankara and Antalya provinces are main vegetable-growing areas of Turkey. The most widely cultivated cucurbits are watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), melon (*Cucumis melo* L.), cucumber (*Cucumis sativus* L.), and squash (*Cucurbita pepo* L.) (TUIK 2015). Virus diseases are a major cause of economic losses in commercial cucurbit production in Turkey and around the world. *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV) are the most widespread cucurbit viruses in Turkey. ZYMV was first isolated in 1973 from a zucchini squash plant in a family garden in Northern Italy (Lisa et al. 1981). Subsequently, ZYMV was reported as a major cucurbit virus around the world. ZYMV is a member of the Potyviridae, which cause major losses of cucurbitaceous crops in Turkey and worldwide. They cause mosaic, yellowing, shoestring, leaf distortion, stunting, and fruit deformation.

Like other potyvirus genomes, the genome of ZYMV consists of a single-stranded plus-sense RNA (+ssRNA) of approximately 9.6 kb, coding a single large polyprotein that is proteolytically processed by virus-encoded proteases to yield as many as 10 functional proteins (Revers et al. 1999). The biological, serological, and molecular variability among ZYMV isolates has been described (Lisa and Lecoq 1984, Lecoq and Purcifull 1992, Wang et al. 1992). A knowledge of ZYMV variability is essential for an understanding of the complexity of this virus and, therefore, for designing effective control strategies (Glasa and Pittnerova 2006). Today, both biological and serological variations have been reported for ZYMV isolates (Lecoq and Desbiez 2012). Also, many of the traditional and molecular studies involved in distinguishing potyviruses have been confined to the coat protein (CP) and to sequences at the 3' end (Shukla et al. 1991). Also, the high sequence variability of P1 in potyviruses makes it an interesting protein-coding region for study and a target for the development of probes for distinguishing potyviruses. More than 500 ZYMV partial sequences are available in GenBank, mostly from parts of the CP-coding region. Worldwide, three major groups of ZYMV populations, namely Group A, Group B and, more recently, Group C were defined. Only Group A has been detected so far in Turkey (Lecoq and Desbiez 2012).

The protein encoded by the 5'-terminal region of the potyvirus genome is the most variable of those that have been sequenced (Shukla et al. 1991) and shows the greatest molecular mass variation, in over 30 potyviruses which have been studied by *in vitro* translations (Hiebert and Dougherty 1988). The C terminus of P1 has been identified as a serine-type protease responsible for cleavage between P1 and the helper component protease (HC/Pro; P2) (Verchot et al. 1991).

Phylogenetic analysis based on the P1 protein was performed in order to study the evolutionary relationships among potyviruses. The variable sequence information of the P1 protein is useful for strain differentiation (Lee and Wong 1998).

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The primary objective of this study was to compare the P1 protein and coding region of ZYMV isolates collected from Ankara, Antalya, and Burdur provinces of Turkey with those of other ZYMV isolates.

Materials & Methods

Plant material

In this study, symptomatic samples from squash plants showing mosaic and leaf deformation were collected from fields in Ankara, Antalya, and Burdur provinces (Figure 1).

Figure 1. Locations (in dark grey) in Turkey of the provinces of collected samples.

Serological Test

Collected samples were tested for the presence of ZYMV by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams 1977) and the test was applied according to the manufacters' instructions (Loewe).

RNA extraction and RT-PCR amplification

The study of molecular variability was conducted using a set of 16 ZYMV isolates and two different host species, *Cucurbita maxima* Duchesne and *C. moschata* Duchesne. Total RNAs were extracted from the leaves and fruits of ZYMV-infected plants that were positive in DAS-ELISA. Total RNA extraction was done according to Astruc et al. (1996). The RNA was resuspended in 50 µl diethylpirocarbonate (DEPC)-treated water and was heated at 65 °C for 5 min before reverse transcription. Two microlitres of the RNA were submitted to reverse transcription in a final volume of 20 µl, using the hexamer primer for 1 h at 42 °C with M-MuLV reverse transcriptase (Fermentas); 2.5 µl of the RT reactions were used for PCR, using the forward primer P1 5'-CATGAGAATTCAAGCTTACATGGCCTC-TATCATG-3' and reverse primer 5'-CTGACTTCTAGACCT-GTTCCAGCGCGT-3. The denaturation at 94 °C for 5 min was followed by 35 cycles of 94 °C for 30 s, 55 °C for 40 s, 72 °C for 30 s, and a final elongation step at 72 °C for 7 min.

Phylogenetic analysis

The RT-PCR products were sent for sequence analysis. After nucleotide sequence analysis, the full nucleotide sequence of the P1 coding region of the Turkish isolates of ZYMV were compared with the other ZYMV isolates in Genbank. A phylogenetic tree analysis was performed on the basis of P1 gene sequences. Sequence analyses and comparisions were performed using MEGA6 software (Tamura et al. 2013).

Results

Full nucleotide sequences coding for the P1 protein region of 16 ZYMV strains from Turkey, including 5 isolates from Antalya, 8 isolates from Ankara, and 3 isolates from Burdur, were obtained during this work. The P1 coding regions of Turkish ZYMV were 930 nt long and encoded 310 amino acids (aa).

Sequence analyses of the P1 coding regions of the Turkish ZYMV isolates were compared with the 10 reference sequences listed in Table 1. Four of the references were from subgroup A1, one each was from A2, A3, and A6, and three were from A5.

A phylogenetic tree was constructed based on the reference sequences and sequences obtained from the Turkish isolates (Figure 2). Most of the isolates clustered into the A1 subgroup, the most prevalent subgroup worldwide. Nine samples clustered with the ZYMV-Fars isolates (from Iran) and five samples clustered with other subgroups, Kuchyna and ZYMV-H. Only the two samples (Y4, Y23) from Antalya province clustered with a different subgroup, the Korean isolates.

According to nucleotide and amino acid comparisons, the P1 gene among the isolates of ZYMV has a very high variability.

Table1. Reference isolates from Genbank.

Number	Reference name	Accession number	Group	Country
1	ZYMV KUCHNA	DQ124239	A1	Slovakia
2	ZYMV-NAT	EF062582	A1	Israel
3	ZYMV-FARS	JN183062	A1	Iran
4	ZYMV-H	KF976712	A1	Czech Republic Re[pu
5	ZYMV-SG3	KC665633	A2	USA
6	ZYMV-Florida	L35590	A3	Florida, USA
7	ZYMV-RDA	AB369279	A5	Korea
8	ZYMV-KR-PA	AY278998	A5	Korea
9	ZYMV-KR-PS	AY279000	A5	Korea
10	ZYMV-CU	AJ307036	A6	China

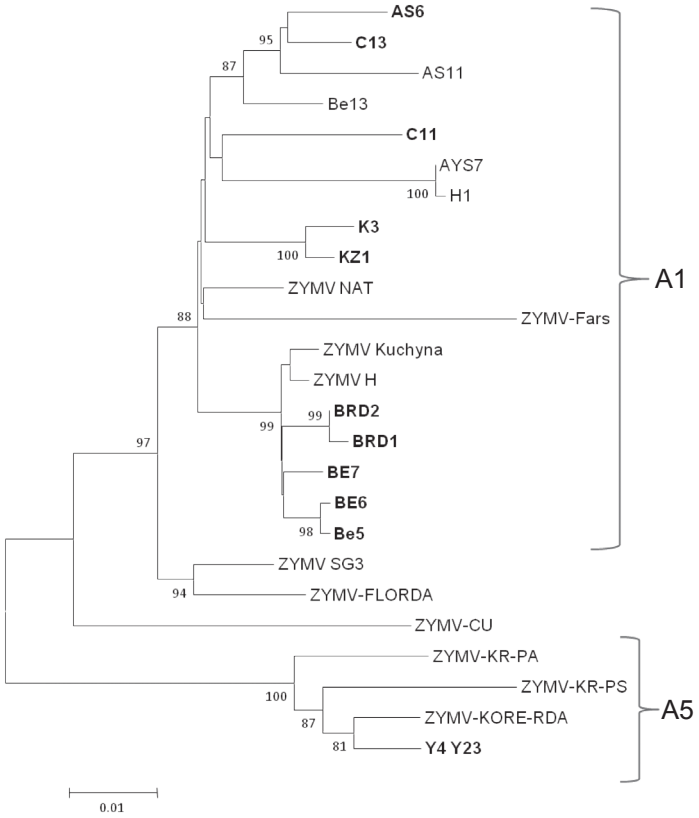

Figure 2. Phylogenetic tree of the P1 coding region of ZYMV isolates from Turkey.

When the 16 full-length ZYMV P1 nt sequences were analyzed, they clustered into two subgroups (A1 and A5). Sequence analysis showed that most isolates belonged to a major molecular subgroup (A1), the most widespread worldwide. However, several isolates from Antalya region belonged to a different molecular subgroup (A5) that has been observed in Korea. The P1 gene region among the ZYMV isolates seems to be very divergent by nucleotide and amino acid comparisons.

The serine protease active site motif, GCSG, was found in all ZYMV isolates. The P1 consensus motif "LVIRG" for all reported ZYMV isolates was also found at amino acid positions 284 to 288. Based on the computer analysis (IFEL analysis), positive selection was found among the 96, 14, and 190 amino acid sequences.

Discussion

According to DAS-ELISA and RT-PCR results, ZYMV is a widespread pathogen in Turkey, as has been reported previously (Yılmaz et al. 1994, Sevik and Sökmen 2003, Dag 2005, Karamanlı 2007, Yesil 2013).

From our results, phylogenetic analysis of ZYMV isolates revealed that at least two genetic subgroups coexist in the field in Turkey. These results were also similar to CP analysis (unpublished results). Lin et al. (2001) proposed that the diversity of P1 protein of the genus Potyvirus is useful as a standard for distinguishing different isolates of viruses and to investigate evolutionary relationships.

Significant differences were found in nucleotide and amino-acid sequences among the ZYMV isolates from Turkey. There is no geographically associated variation based on the clustering of Turkish ZYMV isolates and there is no correlation related to the time of isolation. Bananej et al. (2008) have reported that variation was not related to the region and the collection date of samples.

Phylogenetic analysis using the P1 protein of ZYMV isolates showed that highly variable proteins in the viral genome could also be used in the study of potyvirus taxonomy. This could be useful to distinguish strains of potyviruses originating from different geographical regions (Lee and Wong 1998). Our results are the first to describe the P1 region sequences of Turkish ZYMV isolates.

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Sensitivity of *Phytophthora capsici* Isolates from Illinois to Fungicides

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Abstract. Phytophthora blight, caused by *Phytophthora capsici*, is a destructive disease of cucurbits and peppers in Illinois and worldwide. Fungicide use is a significant component of management strategies of Phytophthora blight of cucurbits and peppers in the United States. Studies were conducted in the laboratory and field to evaluate efficacy of selected fungicides for control of Phytophthora blight of pumpkin in Illinois. In the laboratory, sensitivity of five *P. capsici* isolates from Illinois was evaluated to the fungicides cyazofamid, dimethomorph, and mandipropamid. Development of colonies and production of sporangia of the isolates were assessed at concentrations of 0.0, 0.1, 0.5, 1.0, 5.0, and 10.0 µg/mL of cyazofamid; 0.0, 0.1, 0.5, 1.0, 2.5, and 5.0 µg/mL of dimethomorph; 0.0, 0.05, 0.1, 0.5, 1.0, and 5.0 µg/mL of mandipropamid; and 0, 1, 10, 50, 100, and 200 µg/mL of A20941OD in V8 juice agar in Petri plates. In 2014 and 2015, effectiveness of the fungicides cyazofamid, dimethomorph, and mandipropamid for control of *P. capsici* in pumpkin was investigated. In 2014, incidence of fruit rot caused by *P. capsici* was 31% in control plots and 5, 10, and 2% in the plots treated with cyazofamid, dimethomorph, and mandipropamid, respectively. In 2015, incidence of fruit rot caused by *P. capsici* was 69% in control plots and 42, 21, and 18% in the plots sprayed with cyazofamid, dimethomorph, and mandipropamid, respectively.

Keywords: *Phytophthora capsici*, oomycete, soilborne pathogen, fungicide resistance, fruit rot

Introduction

Phytophthora blight, caused by the oomycete *Phytophthora capsici*, has become one of the most serious threats to cucurbit and pepper production worldwide (Erwin and Ribeiro 1996, William and Zitter 1996, Ristaino and Johnston 1999, Hausbeck and Lamour 2004, Babadoost and Pavon 2013). *P. capsici* can attack the host plant at any growth stage causing up to 100% crop loss (Babadoost and Islam 2003, Hausbeck and Lamour 2004). The pathogen causes seedling death, crown rot, foliar blight (Figure 1A,B) and fruit rot (Figure 1C,D) in cucurbits (Hausbeck and Lamour 2004, Islam et al. 2005, Kousiki and Keinath 2008), and causes root rot, crown rot, foliar blight, and fruit rot in peppers (Ristaino and Johnston 1999, Matheron and Porchas 2000, Ristaino 2003). *P. capsici* can also infect plant species in 15 families (Hausbeck and Lamour 2004, Tian and Babadoost 2004).

Phytophthora capsici is a soil-borne pathogen and produces oospores, sporangia, and zoospores (Figure 2). The pathogen survives between crops as oospores in soil (Hausbeck and Lamour 2004, Babadoost and Pavon 2013). Oospores are resistant to desiccation, cold temperatures, and other extreme environmental conditions, and can survive in the soil, in the absence of a host plant, for several years. Oospores germinate and produce sporangia and zoospores (Erwin and Ribeiro 1996, Islam et al. 2005). Zoospores are released in water, dispersed by irrigation or surface water, and infect plant tissues. If the environmental conditions are conducive (moist and warm), the disease develops rapidly (William and Zitter 1996, Hausbeck and Lamour 2004, Islam et al. 2005, Babadoost and Pavon 2013).

The recommended management strategies for *P. capsici* in vegetables include selecting well-drained sites, planting resistant cultivars (for peppers), crop rotation, and application of fungicides (William and Zitter 1996, Hausbeck and Lamour 2004, Babadoost 2013). Since there is no cucurbit resistant to *P. capsici*, use of fungicides is essential for controlling this pathogen in cucurbit crops. *P. capsici* is a multi-cycle pathogen and quickly develops resistance to fungicides. Thus, in each area, the efficacy of the fungicides for management of *P. capsici* should be assessed and the use of fungicides should be monitored carefully.

Over the past 14 years, several fungicides have been developed for management of oomycete pathogens, including *Phytophthora* spp. Some of these fungicides are effective against *P. capsici*. Cyazofamid, dimethomorph, and mandipropamid are

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commonly used fungicides to manage *P. capsici* on cucurbits in Illinois and other vegetable growing areas. These fungicides have different modes of action and can be used together. This study was conducted to determine the sensitivity of *P. capsici* isolates from Illinois to cyazofamid, dimethomorph, and mandipropamid.

Materials & Methods

Phytophthora capsici *isolates*

Five isolates of *P. capsici* were selected from the collection maintained in Dr. Babadoost's laboratory at the University of Illinois for this study. The isolates were grown on V8-juice agar in

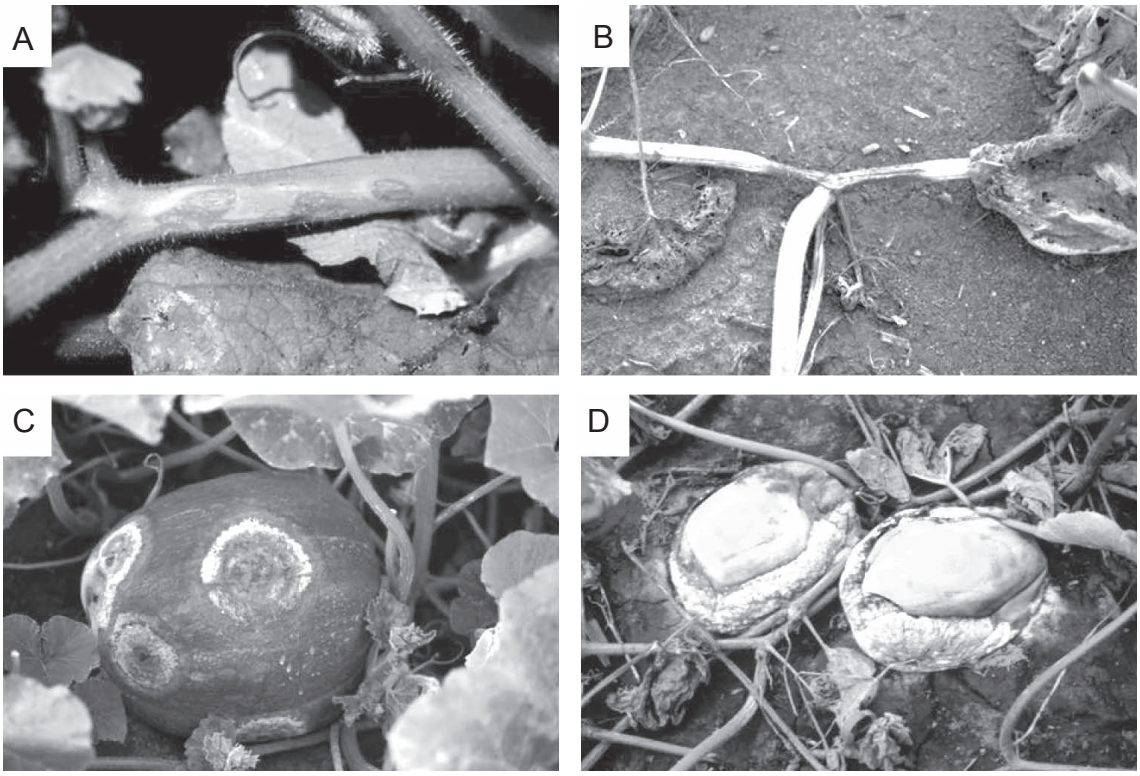

Figure 1. Pumpkin vines and fruit infected by *Phytophthora capsici.* (A) a newly infected vine; (B) an advanced infection in a vine; (C) a newly infected fruit; (D) advanced infection of fruit.

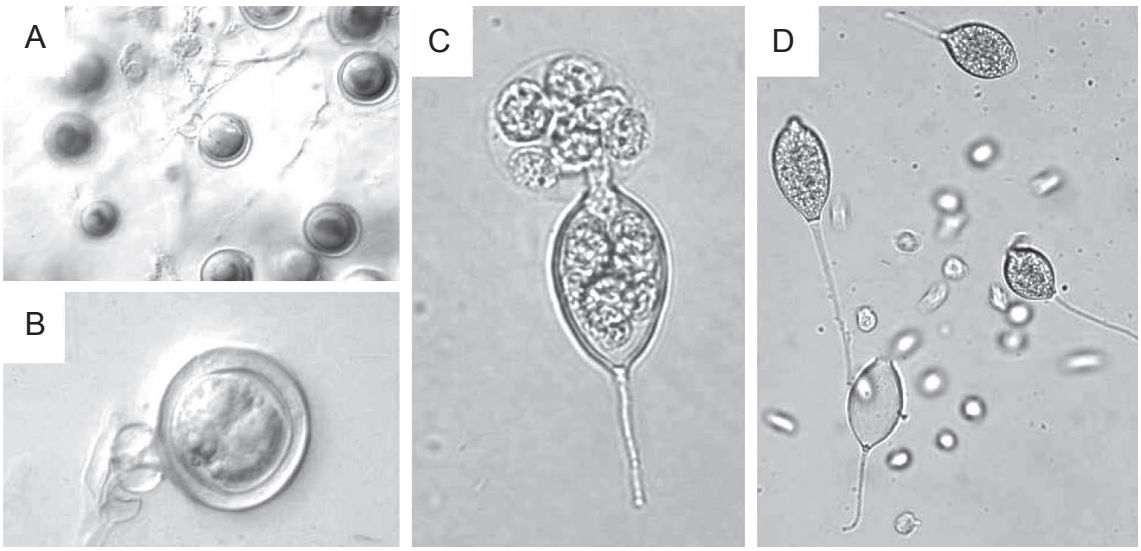

Figure 2. Reproductive bodies of *Phytophthora capsici.* (A) oospores; (B) an oospore; (C) a sporangium releasing zoospores; (D) sporangia and zoospores.

Petri plates. V-8 juice agar was prepared by adding 50 ml of V8 juice, 1 g of $CaCO_3$, and 16 g of agar to 950 mL of distilled water.

Fungicides

Four fungicides were used in this study, including cyazofamid (FRAC code 21), dimethomorph (FRAC code 40), mandipropamid (FRAC code 40), and A20941OD (FRAC code U15). Commercial names of the fungicides in the United States (US) are Ranman 400SC, Forum 4.16SC, and Revus 2.09SC for cyazofamid, dimethomorph, and mandipropamid, respectively. A20941OD was an experimental fungicide.

Inhibition of mycelia growth

An *in-vitro* mycelial growth assay was conducted to determine the effective fungicide concentration at which 50% of the fungal growth was inhibited (EC_{50}) for each isolate-fungicide combination. Plugs of 7-mm diameter from actively growing cultures of *P. capsici* were transferred onto V8 juice agar in Petri plates amended with technical grades of the fungicides: cyazofamid at 0.0, 0.1, 0.5, 1.0, 5.0, and 10.0 µg/mL; dimethomorph at 0.0, 0.1, 0.5, 1.0, 2.5, and 5.0 µg/mL; mandipropamid at 0.0, 0.05, 0.1, 0.5, 1.0, and 5.0 µg/mL; and A20941OD at 0, 1, 10, 50, 100, and 200 µg/mL. For assays, the stock solutions of fungicides were diluted with methanol, acetone, or water. Four plates were used for each fungicide-concentration combination. Culture plates were incubated at 25 °C in darkness. Two perpendicular diameters of colonies were measured 4 days after incubation. The experiment was repeated once under the same conditions.

Inhibition of sporangium formation

P. capsici isolates were grown on V8 juice agar in Petri plates at 25 °C in the dark for 4 days. Then, plugs of 10 mm × 10 mm were cut from the edge of the colonies. Three 100-mm^2 plugs were placed with the mycelium side up in a Petri plate containing sterilized distilled water (SDW) amended with fungicides cyazofamid at 0.0, 0.1, 0.5, 1.0, 5.0, and 10.0 µg/mL; dimethomorph at 0.0, 0.1, 0.5, 1.0, 2.5, 5.0, and 10.0 µg/mL; mandipropamid at 0.0, 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 µg/mL, and A20941OD at 0, 1, 10, 50, and 100 µg/mL. The Petri plates were incubated at 25 °C under continuous light (Keinath 2007). After 48 h, agar plugs with the colonies were placed on glass slides, stained and fixed with 0.01% (w/v) fuchsin acid in 85% lactic acid. The number of sporangia was counted on two of the agar plugs using a light microscope. The fungicide concentration at which 50% of sporangia production was reduced (EC_{50}) was determined. The experiment was repeated once. Percent of suppression of sporangium formation was calculated from the ratio of the number of sporangia produced in the fungicide-amended plates to the number of sporangia in the control plates.

Table 1. Occurrence of Phytophthora infection, caused by *Phytophthora capsici*, in vines and fruit following applications of the fungicides, Illinois, 2014.

Fungicide[z] treatment and rate/ha (application timing)[y]	Phytophthora infection	
	infected vines (%)[x]	infected fruit (%)[w]
Untreated check	38.75 a[v]	30.68 a
Ranman 400F, 194 mL + Silwet L-77, 140 mL (1-9)	10.00 c	9.61 b
Ranman 400F, 194 mL + Silwet L-77, 140 mL (1,3,5,7,9) *alt* Revus 2.09SC, 563 mL + Activator-90, 0.25% (2,4,6,8)	6.25 c	5.72 b
Revus 2.09SC, 563 mL + Kocide-3000 46.1DF, 1.12 kg + Activator-90 0.125% (1,3,5,7,9) *alt* Ranman 400F, 194 mL + Silwet L-77, 140 mL (2,4,6,8)	13.75 c	4.45 b
Revus 2.09SC, 563 mL + Kocide-3000 46.1DF, 1.12 kg (1,3,5,7,9) *alt* Forum 4.16SC, 423 mL + Kocide-3000 46.1DF, 1.12 kg (2,4,6,8)	10.00 c	10.14 b
Revus 2.09SC, 563 mL + Kocide-3000 46.1DF, 1.12 kg + Activator 90, 0.125% (1,3,5,7,9) *alt* Forum 4.16SC, 423 mL + Kocide-3000 46.1DF, 1.12 kg + Activator-90, 0.25% (2,4,6,8)	25.00 b	4.89 b
LSD ($\alpha = 0.05$)	10.25	9.38

[z]Ranman 400F = cyazofamid; Forum 4.16SC = dimethomorph; Kocide-3000 46.1DF = copper hydroxide; Revus 2.09SC = mandipropamid; Silwet L-77 = a spreader-sticker adjuvant; and Activator-90 = a nonionic spray adjuvant.

[y]Application date: 1 = 12 June; 2 = 19 June; 3 = 26 June; 4 = 3 July; 5 = 10 July; 6 = 17 July; 7 = 24 July; 8 = 31 July; and 9 = 7 August 2014.

[x]Vine infection was assessed on 18 August 2014.

[w]Fruit infection was assessed on 29 August 2014 (at harvest).

[v]Values are averages of four replications. Values within each column with a letter in common are not significantly different ($\alpha = 0.05$) from each other according to Fisher's protected LSD test.

Table 2. Occurrence of Phytophthora infection, caused by *Phytophthora capsici*, in vines and fruits following applications of fungicides, Illinois, 2015.

Fungicide[z] treatment and rate/ha (application timing)[y]	Phytophthora infection	
	infected vines (%)[x]	infected fruit (%)[w]
Control	10.00 a[v]	69.17 a
Revus 2.09SC, 563 mL + Kocide-3000 46.1DF, 1.12 kg (1,3,5,7) *alt* Ranman 400F, 194 mL + Kocide-3000 46.1DF, 1.12 kg (2,4,6,8)	0.00 b	26.07 cd
Revus 2.09SC, 563 mL (1-8)	0.00 b	25.56 cd
Revus 2.09SC, 563 mL + Kocide-3000 46.1DF, 1.12 kg (1-8)	5.00 ab	28.69 bcd
Revus 2.09SC, 563 mL + Activator-90, 0.25% (1-8)	0.00 b	31.25 bcd
Revus 2.09SC, 563 mL + Kocide-3000 46.1DF, 1.12 kg + Activator-90, 0.25% (1-8)	1.25 b	17.53 d
Forum 4.16SC, 423 mL (1-8)	2.50 b	21.07 d
Ranman 400F, 194 mL (1-8)	2.50 b	41.74 b
Ranman 400F, 194 mL Kocide-3000 46.1DF, 1.12 kg + (1-8)	3.75 ab	39.09 bc
LSD ($\alpha = 0.05$)	6.67	15.30

[z]Ranman 400F = cyazofamid; Forum 4.16SC = dimethomorph; Kocide-3000 46.1DF = copper hydroxide; Revus 2.09SC = mandipropamid; and Activator-90 = a nonionic spray adjuvant.
[y]Application date: 1 = 20 August; 2 = 27 August; 3 = 3 September; 4 = 10 September; 5 = 17 September; 6 = 24 September; 7 = 31 September; and 8 = 7 October 2015.
[x]Vine infection was assessed on 9 October 2015.
[w]Fruit infectio n was assessed on 30 October 2015 (at harvest).
[v]Values are averages of four replications. Values within each column with a letter in common are not significantly different ($\alpha = 0.05$) from each other according to Fisher's protected LSD test.

Field trials

In 2014 and 2015, trials were conducted in an irrigated field near Green Valley City (Tazewell County), Illinois to evaluate efficacy of selected fungicides for control of Phytophthora blight of pumpkin, caused by *P. capsici* (Tables 1,2). In 2014, seeds were sown on 7 May. In 2015, seeds were sown on 14 May. But due to continuous heavy rains, the plots were lost and the trial was re-established using 2-week old transplants on 1 August 2015. Seeds of the processing pumpkin *Cucurbita moschata* Duchesne 'Dickinson' were sown 45-cm apart in single-row plots, 6 m long. The plots were spaced 9 m apart in a randomized complete block design with four replications. Weeds were controlled by herbicide applications and hand-weeding. Cucumber beetles (*Acalymma vittatum* and *Diabrotica undecimpunctata*) and other insects were managed by applying permryhrin (Pounce 25WP at 740 mL/ha) on 9 July and acetamiprid (Assail 30SG at 358 g/ha) on 23 July. Spray applications of fungicides were at 7-day intervals, as shown in Tables 1 and 2. Fungicides were applied with a backpack sprayer using 470 L of solution per ha. The plots were irrigated as needed, using a central pivot irrigation line, which delivered 7.6 ml water each time. Incidence of Phytophthora blight in the plots were assessed by examining five vines per plot prior to harvest and all of the fruit at harvest.

Results

In-vitro sensitivity of Phytophthora capsici *to fungicides*

Mycelial growth and sporangia production were significantly ($\alpha = 0.05$) reduced by all four fungicides tested in the laboratory. The EC_{50} values of the fungicides for mycelial growth ranged from 0.34 to 3.63 µg/mL (average 1.03 µg/mL); from 0.75 to 4.66 µg/mL (average 2.98 µg/mL); from 0.13 to 7.18 µg/mL (average 4.03 µg/mL); and from 0.03 to 0.97 µg/mL (average 0.28 µg/mL) for cyazofamid, dimethomorph, mandipropamid, and A20941OD, respectively. The EC_{50} value for sporangia production was 10, 2.5, 0.05, and 100 µg/mL for cyazofamid, dimethomorph, mandipropamid, and A20941OD, respectively.

Field trials

In 2014, vine and fruit infection was first observed in untreated plots on 1 July. Percentages of vines and fruits infected by *P. capsici* were significantly higher in untreated (check) plots than those in treated plots (Table 1). The lowest incidence of infected vines by *P. capsici* (6.25%) was in the plots sprayed with cyazofamid (Ranman 400F) plus Silwet L-77 alternated with mandipropamid (Revus 2.09SC) plus Activator-90. The lowest incidence of infected fruit by *P. capsici* (4.45%) was in the plots sprayed with mandipropamid (Revus 2.09SC) plus copper hydroxide (Kocide-3000 46.1DF) plus Activator-90 alternated with cyazofamid (Ranman 400F) plus Silwet L-77 alternated.

In 2015, vine and fruit infection was first observed in untreated plots on 15 September. Percentages of vines and fruits infected by *P. capsici* were significantly higher in untreated (control) plots than those of treated plots (Table 2). Incidence of vines infected by *P. capsici* was 5% or less in the treated, while incidence of vine infection was 10% in untreated plots. Incidence of fruit infection in treated plots ranged from 17.53 to 31.25% compared with untreated plots with 69.17% fruit infection (Table 2). Incidence of fruit infection was the lowest (17.53%) in the plots sprayed with Mandipropamid (Revus 2.09SC) plus copper hydroxide (Kocide-3000 46.1DF) plus Activator-90.

Discussion

Phytophthora blight, caused by *P. capsici*, is one of the most destructive diseases of cucurbits in the world. Management of this pathogen in cucurbits is a challenging task because no resistant cultivar of cucurbits to *P. capsici* is available. An important component of strategies for management of *P. capsici* in cucurbits is fungicide application. Babadoost and Islam (2003) developed an effective seed treatment of cucurbits with mefenoxam (Apron XL LS) that prevents plant infection for five weeks from sowing seed in the soil. Over the past 14 years, several effective fungicides were developed for management of *P. capsici* in cucurbits. Among the newly developed fungicides are cyazofamid, dimethomorph, and mandipropamid.

Virulence of *P. capsici* isolates in cucurbits varies among cucurbit producing areas (Hausbeck and Lamour 2004, Islam et al. 2005). Also, *P. capsici* can quickly develop resistance to fungicides. Therefore, testing efficacy of fungicides for management of *P. capsici* in each cucurbit growing areas is essential.

Our *in-vitro* and field tests showed that all fungicides (cyazofamid, dimethomorph, and mandipropamid) tested are effective against *P. capsici* isolates from Illinois. Also, separate studies have shown that A20941OD fungicide effectively controls *P. capsici* in pumpkin, squash, and pepper (Babadoost, unpublished data). But applications of these fungicides should be alternated to prevent resistance development in the pathogen. Among the four fungicides tested in fields over the past 4 years, mandipropamid was the most effective for management of *P. capsici* in pumpkin. Similar results have been observed in squash and pepper trials in fields in Illinois (Babadoost, unpublished data).

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A Point Mutation of the Coat Protein of *Cucumber Green Mottle Mosaic Virus* Alters the Symptoms from Mosaic to Yellow Spots

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Abstract. *Cucumber green mottle mosaic virus* (CGMMV), a member of the genus *Tobamovirus*, causes great damage to Cucurbitaceae. In this study, we constructed the infectious clone of CGMMV-hn, and the virulence and symptom characteristics of the modified CGMMV were investigated. A mutant of CGMMV CP, named L25A, which resulted in an amino acid replacement, Leu→Ala, a change in amino acid 25, induced severe chlorotic, yellow symptoms in *Nicotiana benthamiana* instead of the light green/dark green mosaic symptoms induced by CGMMV-hn. Similar symptoms were also produced in cucumber, watermelon, melon, and bottle gourd. The acquisition of the mutant L25A provides an approach to reveal the mechanism of how CGMMV causes the different symptoms and which gene may play an important role in this process.

Keywords: CGMMV, infectious clone, mutant, L25A, virus symptoms

Introduction

The Cucurbitaceae consist of around one hundred genera, the most important of which are *Cucurbita*, *Citrullus*, *Cucumis*, and *Luffa.* The family ranks among the highest of plant families for number and percentage of species used as human food (https://en.wikipedia.org/wiki/Cucurbitaceae). *Cucumber green mottle mosaic virus* (CGMMV), a member of the genus *Tobamovirus*, causes serious damage to Cucurbitaceae.

The genome of CGMMV consists of a 6,423-nucleotide, single-strand positive-sense RNA which encodes at least four proteins: a 129-kDa replicase (129K), a 186-kDa replicase (186K), a 29-kDa movement protein (MP), and a 17.4-kDa coat protein (CP) (Ugaki et al. 1991). Like other *Tobamoviruses*, CGMMV can be efficiently transmitted by mechanical ways (e.g., farmers' hands and farm tools) (Gooding and Hebert 1967, Reingold et al. 2014), and spread widely by seeds and soil contamination (Medvedskaya 1981, Li et al. 2015). It can cause mottling and mosaic symptoms on leaves and different degrees of mottling or deterioration on fruits (Park 2001, Reingold et al. 2014). CGMMV was first reported in the United Kingdom in 1935 (Ainsworth 1935). Subsequently, it spread among cucurbit crops worldwide (Wang and Chen 1985, Al-Shahwan and Abdalla 1992, Rudnieva et al. 2005, Yoon et al. 2008, Reingold et al. 2013, Asad et al. 2014). CGMMV was listed as a quarantine disease in China in 2006 (http://www.moa.gov.cn/ztzl/zwjy_1/200612/t20061225_745522.htm). Even so, there has been no effective method for preventing infection by CGMMV until now.

Unlike some pathogens such as bacteria and fungi which can survive detached from the host, the virus depends upon its host for replication and reproduction. Infectious cDNA cloning is a powerful tool (Zheng et al. 2015) and constructing infectious cDNA clones allows maintaining the molecular and biological characteristics of the virus without mutation accumulation (Kang et al. 2015). Infectious cDNA clones have been obtained for several species of the genus *Tobamovirus*, including *Tobacco mosaic virus* (TMV), *Pepper mild mottle virus* (PMMoV), and *Hibiscus latent Singapore virus* (HLSV) (Saito al. 1987, Hagiwara et al. 2002, Niu et al. 2014). For CGMMV, two infectious clones, which are respectively under the control of a 35S promoter and a T7 promoter, have been already constructed (Ooi et al. 2006, Kang et al. 2015, Zheng et al. 2015, Zhong et al. 2015). However, there is no further research on host-pathogen interactions.

In this study, we successfully developed the infectious cDNA clone of CGMMV-hn (GenBank accession: KC851866), coupled with site-directed mutagenesis, which facilitates reverse genetic studies to better understand the pathogen (Delfosse et al. 2013). The virulence and symptom characteristics of the modified CGMMV were investigated, to reveal the mechanism of how CGMMV causes the difference in symptoms and which gene may play an important role in host-pathogen interaction.

Materials & Methods

The construction of the full-length CGMMV clone

The CGMMV-hn isolate used in this study was obtained from the leaves of CGMMV infected watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, in Zhengzhou. Firstly, the virion was purified from the virus-infected leaves according to the protocol

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of TMV (Gooding and Hebert 1967), and then viral genomic RNA was extracted from the virion by RNAiso plus (TAKARA). According to the protocol of M-MLV Reverse transcriptase (TAKARA), the first-strand cDNA was synthesized using the 3UTR antisense primer 5'-TGGGCCCCTACCCGGGGAA-3').

To clone the full-length of CGMMV downstream of the *Cauliflower mosaic virus* (CaMV) 35S promoter, and upstream of HDV-RZ in plant expression binary vector pXT1, the full-length cDNA was amplified as two fragments using Phusion High-Fidelity DNA Polymerase (New England Biolabs). First, the 3'terminal sequence of CGMMV was amplified using the 3UTR antisence primer and the 2083 sense primer 5'-TGGGCAGAGAAAGGTAAGGCTGTCTT-3', and the PCR fragment (~4.6 Kb) was digested by *Sma*I, and then the product was inserted into the vector pXT1, which was digested by *Sal*I and *Sma*I, to generate pXT-CG-B. Second, the 5' terminal sequence of CGMMV was amplified using the 5UTR sense primer 5'-GTTTTAATTTTTATAATTAAACAAAC-3' and the 2758 antisense primer 5'-GGCTCTCCGCCTAATCATAGCAGC-3', and the PCR fragment (~2.7 Kb) was digested by *Stu*I, and then the product was inserted into pXT-CG-B which was digested by *Stu*I and *Sal*I, to generate the full-length of CGMMV clone, named here pXT-CGMMV. Finally, the accuracy of the pXT-CGMMV clone was confirmed by sequencing (Invitrogen).

Mutagenesis of CGMMV

Mutations were introduced sequentially into the ORF of 129K, MP, and CP of CGMMV by site-directed mutagenesis. Each mutant contained only one amino acid change by either of two methods. The first method involved the overlapping pairs of primers, which could be designed according to the protocol of the Fast mutagenesis system (Transgen) and the PCR products were transformed directly without phosphorylation and ligation, while the second method did not involve overlapping pairs of primers, which would be designed according to the protocol of the Q5 site-mutagenesis kit (New England Biolabs) and the PCR products would be phosphorylated and ligated before transformation.

Regardless of which method was used, the plasmid of pXT-CGMMV as the template and Phusion High-Fidelity DNA Polymerase (New England Biolabs) were mixed in the PCR reaction system and the PCR products were digested by *Dpn*I overnight to remove the template before the next step. Finally, the accuracy of the mutants' clones was confirmed by sequencing (Invitrogen).

Agrobacterium-*mediated inoculation*

The expected plasmids were transfermed into *A. tumefaciens* strain GV3101 by freeze-thaw method. Then, a single colony was picked from a transformed plate of Luria-Bertani (LB) broth containing rifampicin (50 $\mu g \cdot mL^{-1}$) and kanamycin (50 $\mu g \cdot mL^{-1}$), and was grown at 28 °C overnight in vigorous shaking. On the following day, the bacterial cells were harvested by centrifugation at 6000 *g* for 5 min and were resuspended into induction buffer (10 $mmol \cdot L^{-1}$ $MgCl_2$, 10 $mmol \cdot L^{-1}$ MES, 100 $\mu mol \cdot L^{-1}$ Acetosyringone), and adjusted to a final OD_{600} of 1.0 to 2.0 and kept at room temperature for 2 h without shaking before inoculation. The cell suspension was injected by syringe into the abaxial intercellular space of cotyledons of watermelon, muskmelon (*Cucumis melo* L.), and bottle gourd (*Lagenaria siceraria* (Mol.) Standl.). Agroinoculation of *Nicotiana benthamiana* Domin plants (6 to 8 leaf stage) was performed by infiltration on the top of two or three expanded leaves.

Double-antibody sandwich enzyme-linked immunosorbent assay

Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was carried out according to the commercial ELISA kit (ADGEN). For every treatment, three plants were chosen and the symptomatic leaves were collected at four weeks post-inoculation separately. The results were recorded by measuring the absorbance at 405 nm with an ELISA plate reader (Model 550, Bio-Rad). The raw ELISA readings were then averaged and converted to relative values using the following formula: (sample - blank) / (negative control - blank) (Chewachong et al. 2015). The samples with an adjusted value of 3.0 or above were considered positive for the corresponding virus.

Reverse-Transcription PCR

Total RNAs were extracted from symptomatic leaves at four weeks post-inoculation by RNAiso plus (TAKARA) and the first-strand cDNA was synthesized using 3UTR antisense primer, according to the protocol of PrimescriptII Reverse transcriptase (TAKARA). Reverse-transcription PCR (RT-PCR) was performed using the MP sense primer 5'-ATGTCTCTAAGTAAGGTGTCAG-3' and the 3UTR antisense primer which was designed to amplify a 1,430 bp fragment, according the to protocol of Taq polymerase (TAKARA).

Results

Infectivity of the full-length clone of CGMMV

To investigate the infectivity of the CGMMV full-length clone, *A. tumefaciens* strain GV3101 carrying pXT1-CGMMV was infiltrated into the leaves of *N. benthamiana,* watermelon, muskmelon, and bottle gourd. At 15 days post-inoculation (dpi), *N. benthamiana* and bottle gourd began to show systemic mosaic symptoms in the leaves, while watermelon and muskmelon showed symptoms later. The infection of CGMMV was confirmed in systemic leaves by RT-PCR and DAS-ELISA (data not shown).

To fully test the biological activity of CGMMV clones, *N. benthamiana* was mechanically inoculated with the sap from infected *N. benthamiana*, which was prepared from 0.1 g of infected leaves using 1 mL sodium phosphate buffer (10 mM, pH 7.2). After seven days, systemic symptoms appeared in the leaves. Equally, the infection of CGMMV was confirmed by RT-PCR and DAS-ELISA (data not shown). The results showed that the full-length clone of CGMMV was able to generate viral progeny, the behavior of which was consistent with the wild-type isolate.

Infectivity of the mutants of CGMMV clone

To investigate the infectivity and observe symptoms induced by the mutants, *A. tumefaciens* strain GV3101 carrying the mutants was infiltrated into the leaves of *N. benthamiana* and watermelon, respectively. After inoculation, we recorded the symptoms the mutants induced on host plants and the systemic leaves of the mutants were analyzed by DAS-ELISA and

RT-PCR. The results showed that the replicase mutants were highly diverse, dependent on whether the mutants infected the plants and whether the symptoms appeared. However, the mutants of the MP seemed not to affect the biological characteristics of CGMMV, which was capable of infecting the plants and the symptoms were consistent with the CGMMV clone.

Surprisingly, the mutations of the CP affected not only the infectivity but also the symptoms of CGMMV. Actually, both mutants, Q37N and R114K, did not induce visible symptoms, though the results of DAS-ELISA and RT-PCR were positive. However, both L25A and V97A induced visible symptoms and changed the typical mosaic symptoms into severe chlorotic, yellow symptoms.

Mutant L25A causes severe chlorotic symptoms in cucumber, watermelon, muskmelon, and bottle gourd, similar to Nicotiana benthamiana

Though both of the mutants, L25A and V97A, could induce chlorotic symptom development in *N. benthamiana* and watermelon, it is not certain that CP is a symptom determinant of CGMMV. Therefore, it was necessary to observe the symptoms induced in other host plants. Furthermore, for the reason that the older leaves became necrotic after infiltration with the mutant L25A, watermelon was also mechanically inoculated to ensure the same necrotic appearance induced by L25A. Here, we just studied the mutant L25A as an example.

Similar to the above, cucumber, muskmelon, bottle gourd, and watermelon were mechanically inoculated with the sap of infected *N. benthamiana*. After 10 days, systemic chlorotic spots began to appear in leaves of host plants, and then some of spots coalesced into small patches, which were distributed in the leaves unevenly. Again, along with the growth of the watermelon plants, the chlorotic spots began to coalesce and became necrotic, and eventually the plants died.

Discussion

In this study, the full-length clone of CGMMV, which was inserted between the CaMV 35S promoters and the HDV ribozyme, was constructed by the restriction-ligation reaction. On the basis of the CGMMV infectious clone, a series of mutants were obtained by site-directed mutagenesis. Through the analysis of these mutants, we learned that the infectivity of the mutants of replicase was easily affected and the mutants of MP maintained the infectivity similar to that of the CGMMV clone.

According to results of a study on *Tobamovirus*, we know the replicase protein has a multifunctional role during infection by the virus (Foster 2008). Initiation of infection requires translation of genomic RNA into a 130K protein and its readthrough product, a 180K protein. The 130K protein is comprised of a methyltranferase (MT) domain, an interval region (IR) and a helicase (HEL) domain. While MT domain plays a role in 5' capping of the viral RNA, the HEL domain is capable of unwinding duplexed RNA in an ATP-dependent manner (Goregaoker and Culver 2003, Ishibashi et al. 2010). Additionally, some researchers showed that replicase protein or HEL domain alone can suppress post-transcriptional gene silencing (PTGS) (Ishibashi et al. 2010). Furthermore, the other proteins, MP and CP, are both encoded by subgenomic RNAs synthesized during viral RNA replication and mediate cell-to-cell and long-distance movement, respectively (Ishibashi et al. 2010). Therefore, any change of the replicase probably influences not only the activity of the replicase but also the expression of MP and CP, while the change of MP and CP probably affects virus accumulation by controlling the speed of virus movement. Altogether, the results of the mutations in the replicase and MP are consistent with their roles.

Surprisingly, the mutations of the CP affected not only the infectivity but also the symptoms of CGMMV. Similar to L25A and V97A, the amino acid change in the CP of TMV, 2b of *Cucumber mosaic virus* (CMV), and 3A domain of *Tomato torrado virus* (ToTV) induced chlorosis (Banerjee et al. 1995, Mochizuki et al. 2014, Wieczorek and Obrepalska-Steplowska 2016). In addition, some other amino acid changes in the CP of TMV and 2a of CMV induced the hypertensive reaction (HR), and a study showed the N' gene in *Nicotiana sylvestris* Speg. & Comes conferred resistance to TMV (Knorr and Dawson 1988, Culver 1991, Hu et al. 2012). Therefore, it is very possible that CP plays an important role in virus infection, acting as a symptom determinant in *Tobamovirus* (Saito 1987, Dawson 1988).

Furthermore, there is no doubt that the mutations which caused HR in the host plants would be very useful for exploring the resistance gene. To date, neither the CGMMV-encoded necrosis determinant nor the gene of Cucurbitaceae conferring resistance to CGMMV have been characterized (Wieczorek and Obrepalska-Steplowska 2016). Therefore, the acquisition of the mutant L25A provides an approach to carry out the study. Besides, it could help us reveal the mechanism of how CGMMV causes the different symptoms and can lead us to a method for preventing infection by CGMMV.

Conclusions

We constructed the infectious clone of CGMMV-hn successfully and obtained a series of mutants by site-directed mutagenesis, which were all investigated. The mutant L25A changed the host-pathogen interaction, inducing chlorotic symptoms instead of the typical mosaic symptoms in five species of plants. It provides us an approach to better understand CGMMV and possibly toward a method for controlling it.

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Sources of Variation in ELISA Tests Used to Quantify ZYMV and PRSV Resistance in *Cucurbita moschata*

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Abstract. Phenotyping for potyvirus resistance can be based on symptoms, on ELISA, RT-PCR, qPCR, or a combination of methods. ELISA is semi-quantitative and affordable, and can be high throughput if plate washers, multiple channel pipettes, etc., are used. Since virus titer is not necessarily uniform throughout infected plants, within-plant variation should be considered when evaluating genotypes. Our objective was to determine if a single sample can represent the degree of resistance in an entire plant, and if so, what leaf should be sampled. In separate experiments, *Cucurbita moschata* genotypes known to vary from resistant to susceptible were inoculated (cotyledons of 7-day-old seedlings) with two potyviruses: *Zucchini yellow mosaic virus* (ZYMV) or *Papaya ringspot virus* (PRSV). The 1st to the 4th leaves were sampled at 21 days post-inoculation. ANOVA was used to test the relative importance of variation among genotypes, plants within genotypes, leaf position within plants, quadrants within leaves, and subsamples within quadrants. Leaves were treated as subplots and quadrants were considered nested within leaves. For both ZYMV and PRSV, there were differences in readings among the tested genotypes, and variation in readings among the four leaf quadrants was no greater than among the two subsamples per quadrant. For both potyviruses, there were differences in ELISA readings among the four leaf positions sampled in each plant, but also a highly significant leaf × genotype cross-over-type interaction. In the case of ZYMV, using ELISA readings from any of the four leaves produced the similar genotype rankings for resistance. For PRSV, ELISA from the 4th leaf ranked the genotypes in the expected manner. We conclude that a single tissue sample from the 4th leaf of an approximately 4-week-old seedling that was inoculated when plants were 7 days old can be used to compare resistance to ZYMV and PRSV in *C. moschata* using ELISA.

Keywords: *Zucchini yellow mosaic virus*, *Papaya ringspot virus*, disease resistance, enzyme-linked immunosorbent assay, phenotyping

Introduction

While plant genotyping has become more and more routine, phenotyping can still present a challenge to the plant breeder. A reliable, reproducible and efficient method of phenotyping is important not only for traditional phenotypic selection but also for the development of molecular markers used in marker assisted selection. Phenotyping of potyvirus resistance can be based on plant symptoms, on methods that determine virus presence and/or concentration such as enzyme-linked immunosorbent assays (ELISA), RT-PCR, and qPCR assays, or on a combination of methods. ELISA has the advantage of being a semi-quantitative assay method that is relatively economical and can also be high-throughput if automated plate washers, automated multiple channel pipettes, etc., are used.

As virus distribution and titer are not necessarily uniform throughout an infected plant, it is important to consider within-plant variation when using ELISA to compare virus resistance among various genotypes. Our objective was to determine an efficient protocol for quantifying resistance to two potyviruses, *Zucchini yellow mosaic virus* (ZYMV) and *Papaya ringspot virus* (PRSV) in seedlings of *Cucurbita moschata* Duchesne. Specifically, we wanted to know if a single tissue sample is sufficient to represent the degree of resistance/susceptibility in an entire plant and, if so, what leaf should be sampled.

Materials & Methods

Cucurbita moschata cultigens (genotypes) known from previous studies to vary from resistant to susceptible were selected for this study. Cultigens 'Taina Dorada', 'Waltham', 'Nigerian Local', PR-V-425, and PR-V-410 were mechanically inoculated with *Papaya ringspot virus* (PRSV). Cultigens 'Taína Dorada', 'Waltham', PR-V-408, PR-V-410, and PR-V-424 were

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inoculated with *Zuccchini yellow mosaic virus*. Fresh tissue from virus-infected plants was macerated in 0.2 M phosphate buffer (1 g tissue in 10 mL buffer) and the sap was gently rubbed on the carborundum-dusted cotyledons of 7-day-old seedlings. The 1st to the 4th leaves (oldest to youngest) were sampled at approximately 21 days post-inoculation (as the 5th leaf was beginning to emerge) (Figure 1). Within each leaf, tissue from each of four leaf quadrants was sampled. Two subsamples were taken from each macerated tissue sample and tested with commercial ELISA kits (Agdia, Elkhart, IN, U.S.A.) for either PRSV or ZYMV. Absorption readings at 405 nm were taken with a Multiskan™ FC microplate photometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Analysis of variance (ANOVA) was used to test the relative importance of variation among genotypes, plants within genotypes, leaf position (1st to 4th) within plants, quadrants within leaves, and subsamples within quadrants. Leaf positions were treated as subplots (fixed) and quadrants (random) were considered to be nested within a particular combination of genotype, plant, and leaf. Fisher's Least Significant Difference was used to test differences between means of genotypes. Pearson's correlation was used to test the association between various variables. All analyses were run using InfoStat (Di Rienzo et al. 2014).

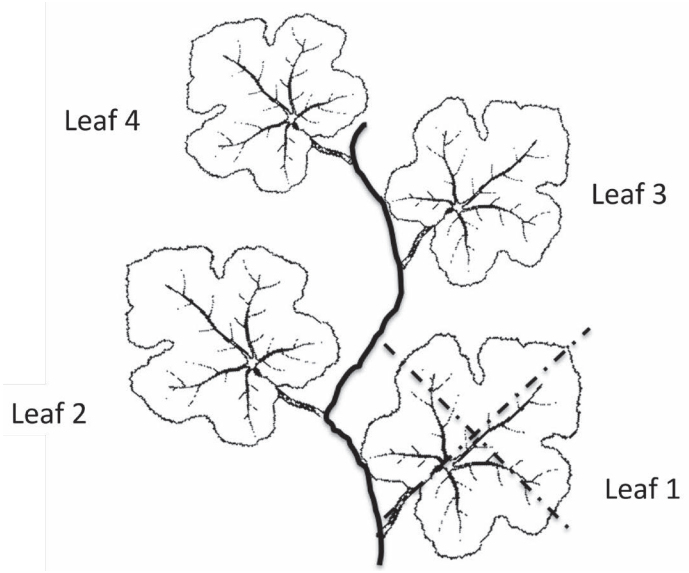

Figure 1. Schematic representation of the 1st (base) to the 4th (top) sampled leaf, and the four quadrants sampled within each leaf.

Results & Discussion

For plants inoculated with either ZYMV or PRSV, very little variation was observed between the two subsamples tested within each sample. The correlation between subsamples within a sample was r = 0.98 ($P < 0.0001$) for ZYMV and $r = 0.90$ ($P < 0.0001$) for PRSV. Our results indicate that the additional readings from ELISA testing of multiple subsamples from the same tissue provide no additional information for the breeder interested in assessing resistance or susceptibility to ZYMV or PRSV. However, researchers may want to include multiple subsamples for other reasons, such as verifying that a protocol has been correctly followed.

Table 1. Sources of variation in ELISA readings (absorbance at 405 nm) in plants of *Cucurbita moschata* inoculated with *Zucchini yellow mosaic virus* (ZYMV).

Sources of variation	Sums of squares	Degrees of freedom	Mean squares	F	p-value
Genotype	512.21	3*	194.65	194.65	0.0001
Plant/genotype	3.51	4	1.50	1.50	0.2087
Leaf position	6.62	3	5.48	5.48	0.0016
Genotype × leaf position	38.29	9	7.27	7.27	<0.0001
Quadrant/genotype × plant × leaf	56.19	96	0.93	0.93	0.6363
Error	87.68	140			

*Only four genotypes were included in the ANOVA due to missing data in the fifth genotype.

Table 2. Sources of variation in ELISA readings (absorbance at 405 nm) in plants of *Cucurbita moschata* inoculated with *Papaya ringspot virus* (PRSV).

Sources of variation	Sums of squares	Degrees of freedom	Mean squares	F	p-value
Genotype	147.49	4	36.87	5.46	0.0454
Plant/genotype	33.75	5	6.75	41.19	<0.0001
Leaf position	43.83	3	14.61	89.17	<0.0001
Genotype × leaf position	85.89	12	7.16	43.68	<0.0001
Quadrant/genotype × plant × leaf	19.66	120	0.16	1.19	0.1429
Error	24.03	175	0.14		

For both viruses, the variation among the four leaf quadrants sampled within each combination of leaf position × plant × genotype was not significant (Tables 1,2). For both ZYMV- and PRSV-inoculated plants, the correlations between quadrants were very high. For ZYMV, the correlations among the four quadrants ranged from $r = 0.82$ to 0.89 and for PRSV the correlations ranged from $r = 0.88$ to 0.95 (all significant at $P < 0.0001$). Therefore, a single tissue sample from a leaf should be adequate for assessing resistance.

The principle source of variation in ELISA tests for either ZYMV or PRSV was between genotypes, but there was also significant variation due to differences among leaf positions, and importantly, the genotype × leaf position interaction was also significant (Tables 1,2). For PRSV, there was also significant variation among plants within genotypes (Table 2). For ZYMV (Table 3), ELISA readings between leaves at different positions within the same plant were strongly correlated ($r = 0.69$ to 0.95), although the correlation between the first leaf versus the third or fourth leaf was much smaller ($r = 0.69$ and 0.71, respectively) than between the second and third leaf ($r = 0.98$) or the second and fourth leaf ($r = 0.86$). There was an extremely high correlation ($r = 0.95$) between the third and fourth leaf. For PRSV (Table 4), correlations were very strong between the first vs. the second leaf and the first vs. the third leaf ($r = 0.99$ and 0.96, respectively) and the second vs. third leaf ($r = 0.98$), but ELISA readings in the fourth leaf were not correlated with readings in the three older leaves ($P > 0.05$).

For both viruses, the average ELISA reading was lower in younger leaves compared to older leaves, and certain genotypes were, on average, more susceptible or resistant than other genotypes (Tables 5,6). However, these main effects were not necessarily consistent among leaf positions or among genotypes. The significant genotype × leaf position interaction observed can be studied by considering the rankings of genotypes based on ELISA readings for each leaf position. Although there was a significant genotype × leaf position interaction for both viruses, in the case of ZYMV-inoculated plants the ranking of genotypes did not substantially change when ELISA readings were taken from different leaf positions (Table 5). For ZYMV, only the magnitude of the relative differences among genotypes varied among

Table 3. Pearson's correlations between ELISA readings (absorption at 405 nm) in leaves sampled from different positions (leaf 1 = oldest leaf) within plants of *Cucurbita moschata* inoculated with *Zucchini yellow mosaic virus* (ZYMV).

	Leaf 1	Leaf 2	Leaf 3
Leaf 2	0.83 ($p < 0.01$)		
Leaf 3	0.69 ($p < 0.01$)	0.86 ($p < 0.01$)	
Leaf 4	0.71 ($p < 0.01$)	0.80 ($p < 0.01$)	0.95 ($p < 0.01$)

Table 4. Pearson's correlations between ELISA readings (absorption at 405 nm) in leaves sample from different positions (leaf 1 = oldest leaf) within plants of *Cucurbita moschata* inoculated with *Papaya ringspot virus* (PRSV).

	Leaf 1	Leaf 2	Leaf 3
Leaf 2	0.99 ($p < 0.01$)		
Leaf 3	0.96 ($p < 0.01$)	0.98 ($p < 0.01$)	
Leaf 4	-0.03 ($p = 0.94$)	0.08 ($p = 0.82$)	0.15 ($p = 0.68$)

Table 5. ELISA readings (absorbance at 405 nm) of plants of *Cucurbita moschata* inoculated with *Zucchini yellow mosaic virus* (ZYMV). Within a leaf position (leaf 1 = oldest leaf), genotypes are ranked from the lowest to the highest ELISA reading.

Leaf 1		Leaf 2		Leaf 3		Leaf 4		Mean of four leaves	
Genotype	A_{405}	Genotype	A_{405}	Genotype	A_{405}	Genotype	A_{405}	Genotype	A_{405}
408	1.27a	410	0.25a	410	0.15a	410	0.15a	408	0.43a
410	1.69a	408	0.48a	408	0.15a	408	0.15a	410	0.56a
425	3.18b	TD	3.58b	425	3.17b	425	2.11b	425	3.14b
TD	3.29bc	Walt	3.97b	TD	4.12c	TD	3.43c	TD	3.60c
Walt	3.75c	425	4.08b	Walt	4.26c	Walt	4.34d	Walt	4.08d
Mean	2.636	Mean	2.472	Mean	2.370	Mean	2.036	Mean	2.362

Within a column, means followed by a common letter are not significantly different at the 0.05 significance according to Fisher's Least Significant Difference.

TD=Taína Dorada, Walt=Waltham. Other genotypes are experimental lines PR-V-408, PR-V-410, and PR-V-425.

Table 6. ELISA readings (absorbance at 405 nm) of plants of *Cucurbita moschata* inoculated with *Papaya ringspot virus* (PRSV). Within a leaf position (leaf 1 = oldest leaf), genotypes are ranked from the lowest to the highest ELISA reading.

Leaf 1		Leaf 2		Leaf 3		Leaf 4		Mean of four leaves	
Genotype	A_{405}	Genotype	A_{405}	Genotype	A_{405}	Genotype	A_{405}	Genotype	A_{405}
TD	0.95a	TD	1.06a	TD	1.23a	NL	0.63a	TD	1.08a
Walt	1.52b	NL	1.56b	NL	1.42ab	410	1.00b	NL	1.37a
425	1.80bc	Walt	1.67b	425	1.53b	TD	1.11bc	425	1.59a
NL	1.86c	425	1.68b	Walt	1.60b	425	1.33c	Walt	1.62a
410	4.29d	410	4.16c	410	2.73c	Walt	1.70d	410	3.04b
Mean	2.084	Mean	2.026	Mean	1.702	Mean	1.154	Mean	1.740

Within a column, means followed by a common letter are not significantly different at the 0.05 significance according to Fisher's Least Significant Difference.

TD=Taína Dorada, Walt=Waltham, NL=Nigerian Local. Other genotypes are experimental lines PR-V-410 and PR-V-425.

different leaf positions. PR-V-408 and PR-V-410 had consistently low ELISA readings for ZYMV no matter the leaf sampled, while 'Taína Dorada' and 'Waltham' had consistently high readings. 'Waltham' is known to be susceptible to ZYMV (Brown et al. 2003, Pachner et al. 2011). In contrast, for PRSV-inoculated plants, the ranking of genotypes changed dramatically, depending on which leaf position was considered (Table 6). 'Waltham' is known to be highly susceptible to PRSV (Brown et al. 2003), yet when the first leaf of 'Waltham' was sampled, the ELISA reading was quite low. In contrast, ELISA readings for PRSV in leaf 4 ranked 'Waltham' as the most susceptible genotype. As plants of 'Waltham' developed, virus symptoms in the third, fourth or later leaves were more extreme than in older leaves (leaf 1 or leaf 2) (data not shown). Rankings of other genotypes also changed substantially as leaves higher up on the plant were sampled. Plants of 'Nigerian Local', which is known to be resistant to PRSV (Brown et al. 2003), exhibited few or no virus symptoms in leaf 1, 2, 3, or 4 (nor on later leaves), yet these plants initially had higher ELISA readings in leaf 1 than even the susceptible 'Waltham'.

Ideally, the plant breeder would like to be able to reliably phenotype a seedling for disease resistance as soon as possible after inoculation with ZYMV and PRSV. Based on the results of this study, we recommend that ELISA readings be taken on a single tissue sample taken from the fourth leaf of a seedling. In future studies, we hope to better document the association between ELISA readings and virus symptoms, which may depend on the source of resistance and virus virulence. ELISA readings are not always correlated with severity of virus symptoms (McPhail-Medina et al. 2010). We also plan to sample leaves as they emerge, rather than sample all leaves at once as was done in this experiment. In addition to the within-plant variation we studied here, other factors may impact the reliability of ELISA readings for phenotyping a large number of genotypes for virus resistance, including lack of uniformity in plant-to-plant inoculation technique and plate-to-plate variation.

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Metabolomic Plasticity of Cucumber Fruit Peel - Effects of Developmental Stage and Market Class

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Abstract. Fruit peel is the first tissue to interact with the fruit's environment and serves as a first line of defense. Consistently, transcriptome analysis of cucumber (*Cucumis sativus*) fruit peel and pericarp has revealed that peel tissue is enriched with genes associated with defense, including expression of genes involved in synthesis and decoration of specialized metabolites. Furthermore, there is evidence to show that many of these genes are under strong developmental regulation. In some cases, the expression of these genes was shown to be genotype specific, suggesting metabolomic variability. In this study, we analyzed methanolic peel extracts of six cucumber cultivars, from two market classes, slicing and pickling, using ultra-high-performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QToF-MS). The metabolome of the cucumber peel was characterized from greenhouse-grown, hand-pollinated fruit at two stages of development – exponential growth, 8 days post-pollination (dpp); and post-exponential growth, 16 dpp. The fruit peels exhibited striking metabolome-wide developmental differences, i.e., the majority of metabolites changed with stage of fruit growth. The developmentally associated changes were consistent among biological replicates and across experiments grown at different times in the greenhouse. We further observed that metabolomes of young fruit from all cultivars were similar to each other. However, the metabolite profiles appeared to diverge as the fruit approached full size resulting in differentiation by market class. Statistical identification of peaks differing among samples and classification of compounds that define these differences can be achieved by employing principal component interpretation, combined with relative mass defect (RMD) filtering. These results indicate that cucumber fruit peel metabolome is highly dependent on fruit age and undergoes genotype-dependent metabolic differentiation during fruit development.

Keywords: Cucumber, fruit, peel, metabolomics, specialized metabolism, development

Introduction

The fruit surface is covered by a distinct peel tissue that acts as the first layer of interaction with the environment. As such, the peel serves multiple functions including photosynthesis, protection of the underlying mesocarp and developing seeds from biotic and abiotic stresses, and ultimately signaling ripeness to attract frugivores. Thus, fruit peels in many species have evolved to produce a wide variety of specialized metabolites, many of which have great importance either as attractive or defensive compounds (Seymour et al. 2013). In the context of human consumption, there are several examples of identification of abundant, peel specific, antioxidants, or bioactive compounds that may be beneficial to human health (Okonogi et al. 2007, Deng et al. 2012, Shashirekha et al. 2015).

Cucumber (*Cucumis sativus* L.) peels can vary extensively for a variety of morphological features such as thickness, color, smoothness, glossiness, and the presence or absence of ribs, netting, warts and spines, as well as for biochemical components influencing flavor, aroma, and protection against herbivory (Pierce and Wehner 1990, Li et al. 2013). Many of these peel traits undergo marked changes during the course of fruit development (Ando et al. 2012, Ando et al. 2015). They also can differ among cucumber market classes, influencing consumer preferences (Li et al. 2013). Within the U.S. the two predominant market classes, pickling and slicing cucumbers, typically differ for surface morphology including depth and uniformity of color, glossiness, warts, and thickness of peel.

Cucumber fruit development follows a canonical sigmoidal growth pattern. Unlike many fruits, however, they are typically harvested immature, toward the end of the exponential growth stage, at approximately 10 to 12 days post-pollination (dpp). Our previous transcriptomic investigations of the initial stages of fruit development (0 to 16 dpp) identified two transcriptomic shifts: at the end of cell division and at the end of exponential growth (Ando et al. 2012). Fruit at late and post exponential stages, 12 and 16 dpp, showed enrichment for defense-associated genes, including a significant enrichment for transcription factors (Ando et al. 2012). Subsequent comparisons of fruit organ transcriptomes revealed that, as expected, peel and pericarp tissue differed transcriptomically (Ando et al. 2015). Peel transcripts were

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enriched for genes associated with surface functions (e.g., photosynthesis, cuticle production), and response to the environment and defense, including synthesis of specialized metabolites. This is consistent with the function of the peel as a defense barrier to both biotic and abiotic threats. Furthermore, this study showed that methanolic cucumber peel extracts from 16 dpp fruit had inhibitory effects on the growth of the pathogen *Phythophthora capsici* suggesting changes in the chemical profile of the peel.

Interest in specialized metabolism in cucumber has primarily been focused on phytoalexin production. Past studies have shown synthesis of glycoside-linked phenolics, including C-glycosyl flavonoids as well as sphingolipids, in cucumber vegetative tissues in response to infection (McNally et al. 2003, Fofana et al. 2005, Lin et al. 2009). In regard to fruit defense, perhaps the most infamous compounds in the cucurbit family are the cucurbitacins, bitter tetracyclic triterpenes involved in defense from herbivory, insects, and pathogens (Chen et al. 2005, Shang et al. 2014). Due to their medicinal value and association with cucurbit domestication, these compounds and the genetic mechanisms involved in their production have been studied thoroughly and recently elucidated (Qi et al. 2013, Shang et al. 2014). The majority of research of other specialized metabolites in cucumber fruit was mainly focused on volatile organic compounds (VOC) derived from lipoxygenase (LOX) activity that influence aroma and flavor (Guler et al. 2013), while a more recent study utilized HPLC-MS to analyze flavonoids and polyphenolics and their glycosides (Abu-Reidah et al. 2012). However, despite morphological and biochemical differentiation between peel and flesh tissue, and differences among varieties, the majority of these studies were performed on whole fruit, from a single cultivar, at a single stage of development. One early study showed a two-fold increase of LOX activity in cucumber peel compared to flesh (Wardale and Ambert 1980), and a recent exploration of VOCs in the peel found that a majority of VOCs identified were higher in concentration or uniquely found in peel compared to flesh tissue (Guler et al. 2013).

The recent improvement in mass spectrometry methods as well as computational analysis capabilities has allowed for whole metabolome analysis on complex plant extract matrices (Last et al. 2007). These types of untargeted analyses may yield several thousands of mass-to-charge ratio (*m/z*)-retention time pairs, often corresponding to thousands of compounds contained within the extracts. The limitations of these experiments are, however, in the interpretation and deconvolution of these datasets, owing to the vast diversity of plant metabolites, most of which have yet to be identified. Recently, relative mass defect (RMD) filtering, a method for screening, classification, and mining of LC-MS data was developed (Ekanayaka et al. 2015). This method allows for rapid, fairly accurate assignment of unknown metabolites to biosynthetic classes of compounds identified in metabolomic experiments and removal of signals that do not correspond to metabolites of interest. In this study, initial examinations of the age-related transitions of the peel transcriptome prompted an investigation into the metabolome of the cucumber peel. Cucumber peel methanolic extracts form six cucumber varieties, representing U.S. pickling and slicing market classes, were examined at two ages using ultra-high-performance liquid chromatography paired with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QToF-MS). The metabolome-wide changes in these samples were assessed using multivariate statistical analyses and RMD filtering. We observed a drastic metabolome-wide transition with age driven primarily by compounds with RMD values consistent with terpenoid glycosides. A differentiation based on market class that manifested at 16 dpp was also observed.

Materials & Methods

Plant material and library preparation

Greenhouse production of pickling cucumber fruit of 'Vlaspik' and 'Gy14' was as described in Ando and Grumet (2010). Flowers were hand pollinated in a staggered manner, such that 8 and 16 dpp fruit were harvested on the same day. Fruits for RNA extraction were peeled using a vegetable peeler and immediately frozen in liquid nitrogen. RNA was extracted using the Trizol procedure, samples were DNase treated and RNA concentration and quality were measured using Qubit 2.0 Fluorometer and The Agilent 2100 Bioanalyzer system. For each genotype and age, three biological replicates were prepared; each replicate included equal quantities of RNA pooled from two fruits. Single-end libraries were prepared according to the Illumina protocol.

RNA sequencing and analysis

Sequencing was performed using an Illumina HiSeq 2500 at 50 nt single end (SE) reads. Reads were trimmed and cleaned using Trimmomatic (Bolger et al., 2014) and mapped to the 'Chinese Long' (v2) genome (Huang et al. 2009, Li et al. 2011) using Bowtie and TopHat v1.4.1 (Trapnell et al. 2012). Raw read counts were generated using HTseq (Anders et al. 2015) and differential expression was calculated using the DEseq2 (Love et al. 2014) accounting for fruit age, genotype, and their interaction in the model. Differentially expressed genes were called significant using an adjusted p-value (Benjamini-Hochberg adjustment) and a false discovery rate of less than 5%. A cutoff expression change of above two-fold was used. GO term analysis was performed using the *Arabidopsis* best protein hits and the agriGO tool (Du et al. 2010) using the default settings. Reduction and visualization of GO terms was performed using REVIGO (Supek et al. 2011).

Untargeted metabolomic profiling of peel extracts

Methanolic extracts were prepared from fruit peels of cucumbers grown in two seasons in the greenhouse as described above. Tissues used in RNAseq were also used in metabolome experiment 1. Cucumber fruit from the pickling varieties and the slicing varieties 'Ashley's', 'Long Green', 'Poinsett 76', and 'SpaceMaster80' were included in experiment 2. Due to greenhouse conditions, 8 dpp samples of 'Ashley's', and 'Long Green' were not collected. Peels were lyophilized, milled, and extracted for 3 hours in 80% HPLC-grade methanol at a 1 g : 20 mL ratio on a rotary shaker at room temperature. Samples were then spun in a microfuge at 15000 × *g* and the supernatant was transferred to amber vials. An aliquot from each sample was transferred to a new vial and diluted with water to 25% methanol. A pool of all collected samples was used as a reference for peak alignment.

Chromatography was performed over 7 minutes with a 2.1 × 100 mm (1.7 µm) BEH C18 UPLC column (Waters Corp., Milford) using a gradient of acidified water (0.1% acetic acid, v/v) and acetonitrile as mobile phases A and B respectively

(0 min, 5% B; 0.5 min, 5% B; 5 min, 95% B; 6 min, 95% B; 6.1 min, 5% B; 7 min, 5% B). Column temperature was set at 40 °C and flow rate was 0.4 mL/min. After chromatographic separation samples were analyzed by ESI (negative ion mode)-QToF-MS on a Xevo G2-XS mass spectrometer using MS^E mode and continuum data acquisition (Waters Corp., Milford). MassLynx RAW files were imported into the Progenesis QI software (Nonlinear Dynamics, Newcastle) for pre-processing, peak alignment and picking, and abundance normalization. The following ions were grouped into a single measure for each metabolite: $[M-H]^-$, $[M+Cl]^-$, [M+formic acid-H]$^-$, $[M+H_2PO_4]^-$, $[2M+Cl]^-$, [2M+formic acid-H]$^-$, $[2M+H_2PO_4]^-$. Finally, multivariate statistical analysis and PCA were performed using the R statistical software. Chromatograms were trimmed to a retention time range of 0.5 to 4 min, where most specialized metabolites eluted. Ions with negative absolute mass defects were filtered from analysis as these are often inorganic substances. Correlation loadings thresholds of 0.4 and 0.5, for age and market class respectively, were used for determining ions of interest. RMD values were calculated as in Ekanayaka et al. (2015).

Results & Discussion

Transcriptomic and metabolomic changes in cucumber fruit peel with age

RNA-seq analysis was performed on peel samples from pickling cultivars 'Vlaspik' and 'Gy 14' at exponential (8 dpp) and post-exponential (16 dpp) stages of fruit growth. Over 300 M reads were generated, of which approximately 85% mapped uniquely to the reference genome (Huang et al. 2009, Li et al. 2011). Principal component analysis of the peel transcriptomes showed that age was the key component differentiating samples, explaining 82% of the variance, with a secondary effect of genotype (Figure 1A,B). Consistent with our previous findings indicating that cucumber fruit tissue undergoes a transcriptomic reprogramming during development, with a downregulation of gene involved in growth, cell division, and metabolic processes as it reaches full size (Ando et al. 2012, Ando et al. 2015), GO term enrichment analysis (Figure 1C) of differentially expressed genes revealed that age-downregulated genes were enriched for 'photosynthesis', 'metabolic processes', 'cellular processes', 'post-embryonic development', 'cell cycle', 'response to stimulus', and 'lipid metabolism'. Of the genes that were significantly upregulated with age, the most significantly enriched GO terms were 'response to stimulus' (adjusted $P = 1.9e^{-19}$) and 'response to stress' (adjusted $P = 2.2e^{-08}$). There also was enrichment of several terms associated with specialized metabolism, including 'secondary metabolic process' and 'phenylpropanoid metabolic process'.

Based on the observation that specialized metabolism genes were strongly age-regulated, metabolomic analyses were performed by untargeted UPLC-ESI-QToF-MS on methanolic peel extracts of the two pickling cucumber cultivars. PCA of the metabolome data showed tight clustering of replicate samples both within experiments and between experiments grown at different seasons in the greenhouse, confirming reproducibility of the results (Figure 2A). PCA further revealed that, similar to the transcriptome data, age is a strong factor influencing the metabolome of the cucumber fruit peel, explaining ~30% of the variance. Heat map visualization of all metabolites analyzed (Figure 3) showed widespread change in metabolome levels with fruit age, and distinct hierarchical clustering of the samples by age. Notably, the great majority of compounds showed age-related changes in quantity, either increasing or decreasing with age.

Metabolomic differences separating cucumber market classes

An additional experiment was performed comparing metabolomes of pickling and slicing cucumbers. Once again, a strong age effect was also observed for all cultivars (Figure 2B). At 8 dpp there was not a clear separation based on market class, however at 16 dpp the two types of fruit clustered separately, suggesting market class-dependent metabolic differentiation during fruit development. Principal component interpretation coupled with RMD analysis was employed to examine what types of compounds were driving these differences. Compounds negatively correlated with PC1 drove separation of 8 dpp *vs*. 16 dpp samples. This group, containing 530 compounds, displayed a broad RMD value distribution, revealing numerous polyphenols (100 to 350 ppm) and terpenoid glycosides (400 to 650 ppm) (Figure 4A). The 860 compounds positively correlated with PC1 drove separation of the 16 dpp samples. These were more abundant than those driving 8 dpp separation. Among those, was a distinct peak of compounds with RMD values of 400 to 450 ppm, consistent with terpenoid glycosides (Figure 4B). Market class differences at 16 dpp were based on positive correlation to PC1 and negative and positive correlations to PC2 for slicing and pickling respectively (Figure 4C,D). These differences were largely defined by the high abundance of compounds that are putatively terpene glycosides in the slicing market class. Also identified within this group, was a compound with an *m/z* and fragmentation pattern matching that of the phyllobilin, NCC-1. Conversely, histograms of RMD values driving separation of pickling cultivars showed higher diversity, with RMD values reaching 600 to 700 ppm, indicating compounds with greater hydrogen content.

Collectively, these results suggest extensive metabolic plasticity of the cucumber fruit peel, both as a result of fruit development and market class. Developmentally regulated changes in fruit metabolome have been studied in other fruit tissues, for example melon (Moing et al. 2011) and tomato (Mintz-Oron et al. 2008, Mounet et al. 2009). These studies compared different stages of fruit development with an emphasis on phases of ripening. Interestingly, a recent meta-analysis approach to find conserved developmental changes across multiple climacteric and non-climacteric fruit species found that at least as many metabolomic changes occurred during developmental stages prior to ripening than during ripening per se (Klie et al. 2014). Here, we analyzed peels from early stages of cucumber fruit development, as these are more pertinent to cucumber production. These initial results reveal that early cucumber fruit development represents a dynamic period in the cucumber peel metabolome which, in turn, may have a direct impact on flavor, aroma, and defense. Furthermore, multiple putative terpenoid glycosides were shown to be produced in cucumber peels. Apart from glycosides of cucurbitacins, knowledge of terpenoid glycosides in cucumber fruit is extremely limited, thus identification of discriminating metabolites awaits more detailed characterization. Additionally, we potentially identified the increased abundance of the

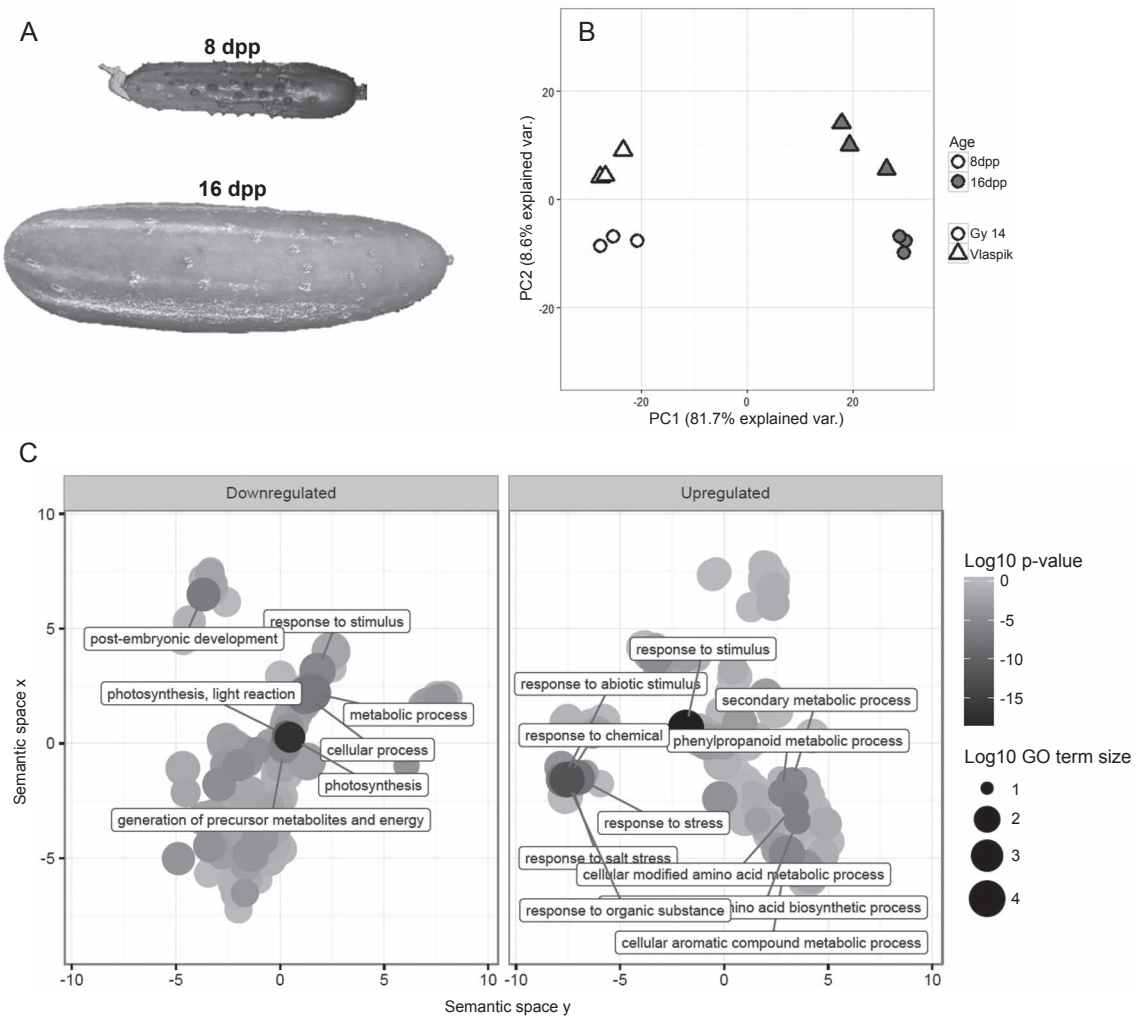

Figure 1. Transcriptome analysis reveals developmental transition in cucumber fruit peel. (A) Cucumber fruit surface ('Vlaspik') at 8 and 16 days post pollination (dpp). (B) Principal component analysis of transcripts expressed in peels of two pickling cucumber cultivars ('Vlaspik' and 'Gy 14') at 8 and 16 dpp. (C) REVIGO term analysis indicating the most significantly enriched GO terms down- and up-regulated with age. Labeled terms have Log_{10} adjusted P-values of less than -5.

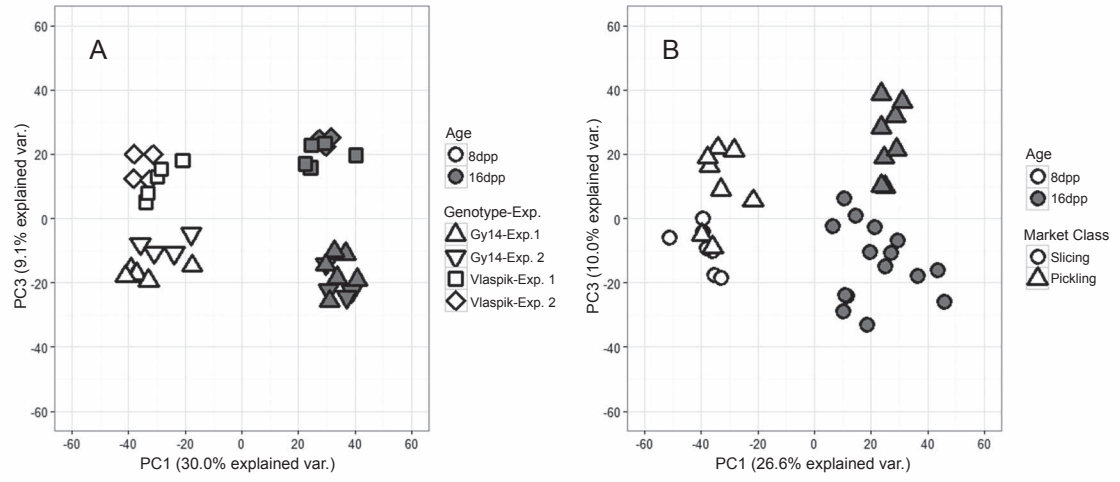

Figure 2. Fruit peel metabolome changes with age. (A) Principal component analysis of methanol soluble metabolome of peels of two cucumber pickling cultivars at different fruit ages, 8 days post-pollination (dpp) and 16 dpp. (B) PCA of peel metabolomes of two pickling and four slicing cucumber cultivars at 8 and 16 dpp.

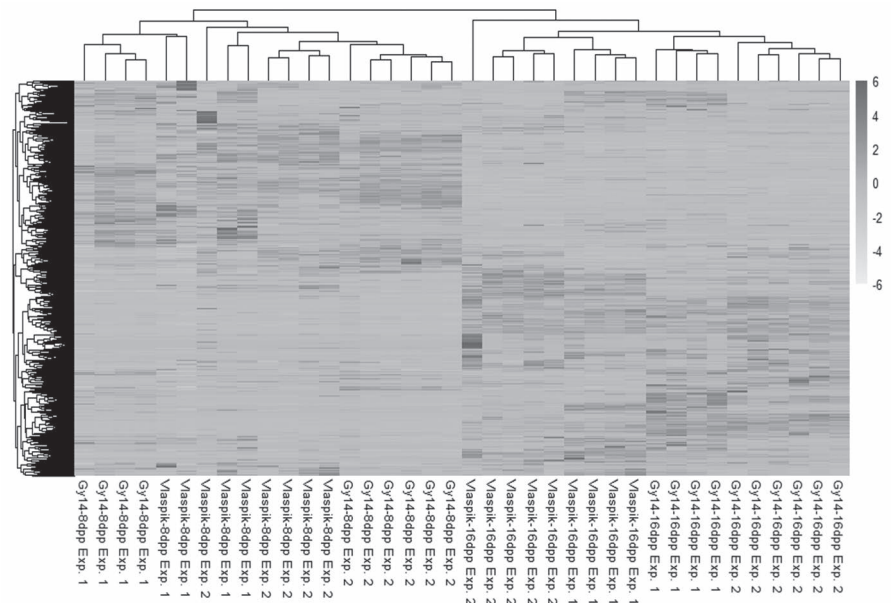

Figure 3. Heat map of the pickling cucumber peel metabolome. Shown are all (m/z)-retention time pairs identified in peel extracts from 8 and 16 dpp fruit from two separate experiments. Rows are scaled to row mean.

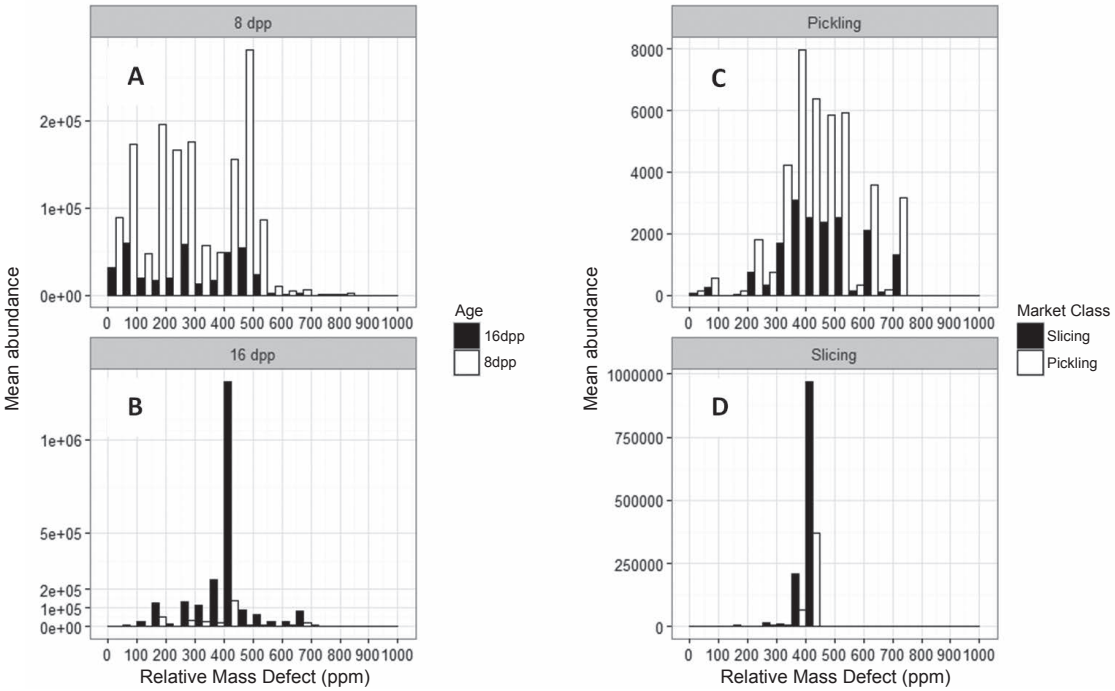

Figure 4. Compounds driving age and market class separation. Abundance-weighted histograms of compounds identified in principal component interpretation binned by relative mass defect (RMD). Compounds driving separation by age: 8 dpp peels (A), 16 dpp peels (B). Compounds separating market classes at 16 dpp: Pickling (C), Slicing (D).

chlorophyll catabolite NCC-1 in 16 dpp slicing cucumber fruit peel. This compound is found in senescing vegetative tissue and identified in peels of a few ripening fruits (Kräutler 2014). There is increased interest in the identification of phyllobilins in fruit due to their antioxidant capabilities as well as function as potential signaling molecules (Müller et al. 2007, Kräutler 2014).

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Towards Whole-Genome Association Mapping of Multiple Fruit Quality Traits in Melon and Squash

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Abstract. The use of germplasm collections for genetic mapping of traits has recently become a common practice in plant research. The immediate incentives are the ease of constructing a mapping set and the expected high level of accumulated historical recombinations across the tested panel. Availability of whole-genome sequencing-based genotyping platforms and statistical tools to account for the inherent population structure effects are promoting the use of association mapping in plants. Melon (*Cucumis melo*) and squash (*Cucurbita pepo*) are highly polymorphic for fruit traits which makes them suitable for implementation of a multi-allelic mapping approach. The first step in the performance of a Genome-wide Association (GWAS) study for melon and squash is presented; 180 diverse melon accessions (representing the main sub-groups for this species) were grown in a replicated trial and phenotyped for diverse fruit-quality traits. The phenotyping made use of digital imaging and image-analysis tools. Among the measured traits were fruit weight, fruit length, fruit width, flesh color, rind color, netting, stripes and suture patterns, Brix, and sugar content. Similar design and phenotypic characterizations were performed across a preliminary core panel of 60 *C. pepo* accessions. Extensive variation was observed for all measured traits in both species. Significant genetic effects and high heritability values were calculated. Preliminary analyses of associations using SNPs at known genes for flesh and rind color in melon validated the quality of the phenotypic data and the experimental setup. The tested melon panel is being subjected to Genotyping-By-Sequencing (GBS) analysis allowing for a comprehensive GWAS study of *C. melo*. The potential of using diverse germplasm collections for genetic mapping and introduction of favorable alleles to breeding programs are discussed.

Keywords: Genome-wide association (GWAS), *Cucumis melo*, *Cucurbita pepo*, genotyping-by-sequencing (GBS), quantitative trait loci (QTLs)

Introduction

Plant breeding is a circular process that is based on the creation of genetic variation and selection of plants showing phenotypes that match agronomic or horticultural specifications and market demands. As an inherent part of the breeding process, the available genetic variation is continuously narrowed down, resulting in reduced ability to develop new products that demonstrate unique sets of traits and outperform current varieties. An important condition for the maintenance of an effective breeding program is the continuous infusion of new variation into the breeding cycle. The challenge is in the exposure, characterization, and construction of the new variation available for the breeding practice. Germplasm collections are a useful resource for that purpose. Metaphorically, diverse collections could be compared to a pile of phenotypes that needs to be sorted and catalogued. The ultimate tool for that purpose is genetic mapping that essentially dissects phenotypic variation into discrete components.

Next generation sequencing (NGS) technologies have been revolutionizing crop genomics by promoting the efficient and detailed characterization of genetic variation. Genotyping by sequencing (GBS) and other NGS-based methods have promoted the ability to carry out high resolution mapping studies in crop plants (Elshire et al. 2011, He et al. 2014). In parallel to that, methodologies and tools were developed and implemented in recent years to allow genome-wide association (GWAS) in plants (Flint-Garcia et al. 2003, Ersoz et al. 2007, Zhu et al. 2008, Gupta et al. 2014). This approach is opening the opportunity for using germplasm collections for performance of multi-allelic mapping studies. Among the advantages of GWAS

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are the ability to simultaneously screen multi-allelic panels and the expected high level of historical recombinations that are captured (Flint-Garcia et al. 2003, Gupta et al. 2014). Downsides of the association approach include the lack of power to detect and map effects of low-frequency alleles. Another possible drawback is the excess in spurious associations that may result from population structure effects. However, this can now be controlled using statistical approaches that account for population structure and relatedness between individuals in the tested panel (Yu et al. 2006).

The Cucurbitaceae are an economically important plant family. Cucurbits are grown in almost all regions around the globe. The family is characterized by extreme diversity in fruit types, in part attributable to their being domesticated and bred for diverse uses (Paris 2000, Burger et al. 2009, Gong et al. 2012). Melon, *Cucumis melo* L., is among the most important fleshy fruits for fresh consumption. It is extremely diverse for phenotypic traits and in particular fruit traits (Burger et al. 2006). Squash and pumpkins, *Cucurbita* spp., are widely cultivated, particularly *C. pepo* L. (Paris et al. 2006). They are used mostly for the consumption of their cooked fruits which are known as summer squash. Cooked, mature fruit flesh is also an important use. The seeds are consumed in some regions and the oil pressed from the seeds is highly valued (Andres 2000).

Considerable research has been performed in recent years aimed at the characterization and genetic dissection of multiple fruit-quality traits in melon (Harel-Beja et al. 2010, Diaz et al. 2011, Cohen et al. 2014, Feder et al. 2015, Freilich et al. 2015, Tzuri et al. 2015, Zhang et al. 2016). Most of these studies were based on bi-parental populations of various genetic designs. So far, a relatively limited number of studies have used diversity panels for GWAS in melon (Tomason et al. 2013, Leida et al. 2015). The genetic architecture of fruit quality traits in squash is largely uncharacterized and a relatively small number of studies report on mapping of QTLs for these traits (Esteras et al. 2012, Zhang et al. 2015). With the recent advances in genomics and the availability of the reference genome sequence for melon (Garcia-Mas et al. 2012) and a draft genome for squash (https://cucurbigene.upv.es/genome-v3.2/), the focus is shifted to germplasm, phenotyping, and data analysis.

Here we present a proposal and describe an ongoing project that is aimed at using two diverse cucurbit collections for genetic mapping of fruit- and seed-quality traits in melon and squash.

Proposal & Preliminary Results

We propose to use existing diverse germplasm collections from the long-standing Newe Ya‘ar melon and squash breeding programs (Burger et al. 2006, Paris et al. 2006) for characterization of their phenotypic variation and mapping of fruit quality traits. The collections are based on historical as well as elite breeding lines and are designed to represent the different subspecies and cultivar-groups as illustrated in Figure 1, and to reflect the extensive variation in a wide array of breeding-relevant traits.

The collections are being extensively phenotyped for multiple fruit-quality traits, as listed in Table 1. The phenotypic analyses include image-based analyses for morphological and color-related traits, and biochemical analyses using different platforms (GC-MS, HPLC, LC-MS). Following and in parallel with the phenotypic characterization, the collections will be subjected to high-density genotyping through GBS. The derived data sets will allow the performance of GWAS and mapping of major effect genes and QTLs for a wide array of fruit characteristics.

Preliminary Results

Heritable variation in fruit traits in the melon diversity panel

One hundred-eighty diverse melon lines were grown in a replicated trial during summer 2015 at Newe Ya‘ar. The lines were phenotyped for fruit size, weight, shape, internal and external color, rind netting, rind stripes, Brix, and sugars. For all measured traits, we found extensive variation. For example, average fruit weight ranged ~100 fold, from 50 g for the smallest lines to 5 kg for the largest. Brix range was ~5 fold, between non-sweet lines (Bx = 3) to very sweet melons (Bx = 16) (Figure 2). For all measured traits, we found a very significant genetic effect, and the broad-sense heritability was 0.70 to 0.95.

Image-analysis was used to characterize fruit morphology and color. More than 2,500 melon fruit scans were automatically analyzed using a dedicated image-analysis software (Rodríguez et al. 2010) resulting in high quality phenotypic data. Figure 3 shows an example of fruit images and the resulting distribution of flesh color (hue) as extracted from the images.

To demonstrate the potential of the melon germplam collection for trait mapping, we have tested the flesh color variation (Figure 3) against genotypic data at a SNP in the *CmOr* gene (Tzuri et al. 2015) that was recently found to be the main factor controlling flesh color in melon. As expected, the association was very strong with an R^2 of 0.80 for the effect of the *CmOr* SNP on color variation (Figure 4).

Heritable variation in fruit and seed traits in Cucurbita pepo *across a core diversity panel*

A preliminary core panel of 60 *C. pepo* accessions was grown and subjected to fruit-trait phenotyping. For all measured traits, a highly significant genetic effect was found. Broad-sense heritabilities for the measured traits ranged from 0.75 to 0.95. Figure 5 depicts internal fruit images and the derived quantitative data analysis that is based on values extracted from these images for two traits (fruit size and fruit color). For both traits, genetics explains more than 90% of the phenotypic variation, providing a good indication of the extensive phenotypic diversity and the accuracy of the phenotypic assay.

Conclusions & Prospects

The above proposal and preliminary results provide examples for the potential use of diverse cucurbit germplasm collections for mapping quantitative traits and discovery of favorable alleles to be used in breeding programs. This approach, if combined with complementary bi-parental linkage population mapping, can promote efficient dissection of the genetic variation that underlies fruit quality traits in highly polymorphic cucurbit crops such as melon and squash.

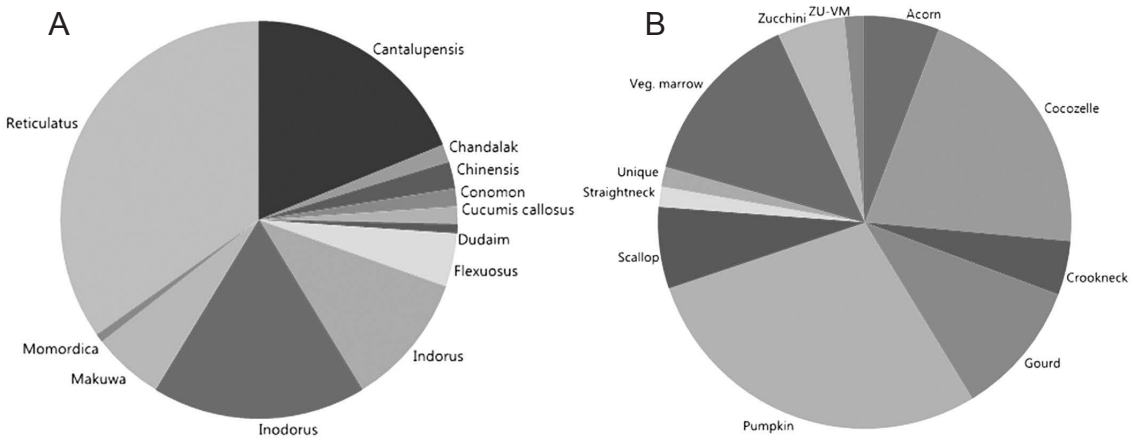

Figure 1. Division to taxonomic groups within the melon (A) and squash (B) collections.

Table 1. List of fruit and seed traits to be measured in the melon and squash collections.

Stage / Organ	Trait	Phenotypic Assay	Crop
Young Fruit	Young fruit rind color	Field, Image	*melo, pepo*
	Young fruit rind color intensity	Field, Image	*melo, pepo*
	Young fruit stripes	Field, Image	*melo, pepo*
	Young fruit soluble solids content	Refractometer	*pepo*
	Young fruit shape index (L/W)	Field, Image	*melo, pepo*
Mature Fruit	Mature fruit shape/cultivar-group	Field, Image	*melo, pepo*
	Mature fruit length	Image-Analysis	*melo, pepo*
	Mature fruit width	Image-Analysis	*melo, pepo*
	Mature fruit shape index (L/W)	Image-Analysis	*melo, pepo*
	Mature fruit weight	Scale	*melo, pepo*
	Mature fruit stripes	Image	*melo, pepo*
	Mature fruit net density	Image	*melo*
	Mature fruit net coverage	Image	*melo*
	Mature fruit rind color	Image	*melo, pepo*
	Mature fruit rind pigments: carotenoids, flavonoids, chlorophyll	HPLC	*melo*
	Mature fruit flesh color and intensity	Image-Analysis	*melo, pepo*
	Mature fruit flesh pigments: carotenoids, chlorophyll	HPLC	*melo*
	Mature fruit total soluble solids content (Brix)	Refractometer	*melo, pepo*
	Mature fruit sugars profile	HPLC	*melo*
	Mature fruit flesh targeted metabolic profile: aroma volatiles	GC-MS	*melo*
	Mature fruit flesh non-targeted metabolic profile	LC-MS	*melo*
	Climacterism: abscission zone formation and detachment	Field, Image	*melo*
	Climacterism: ethylene emission	GC	*melo*
Seed	Average seed weight	Scale	*pepo*
	Seed length	Image-Analysis	*pepo*
	Seed width	Image-Analysis	*pepo*
	Seed shape index	Image-Analysis	*pepo*
	Seed area (size)	Image-Analysis	*pepo*
	Seed color	Image-Analysis	*pepo*

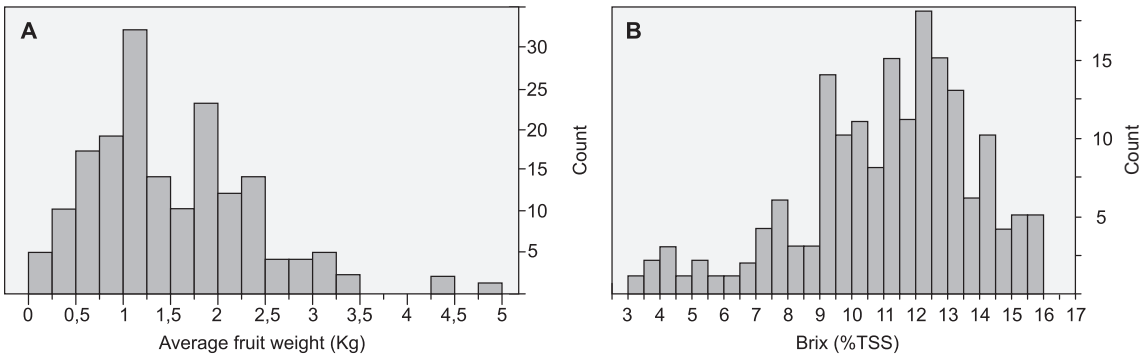

Figure 2. Frequency distributions for fruit weight (A) and Brix (B) for means of 180 diverse melon accessions that were analyzed during the Summer 2015 experiment.

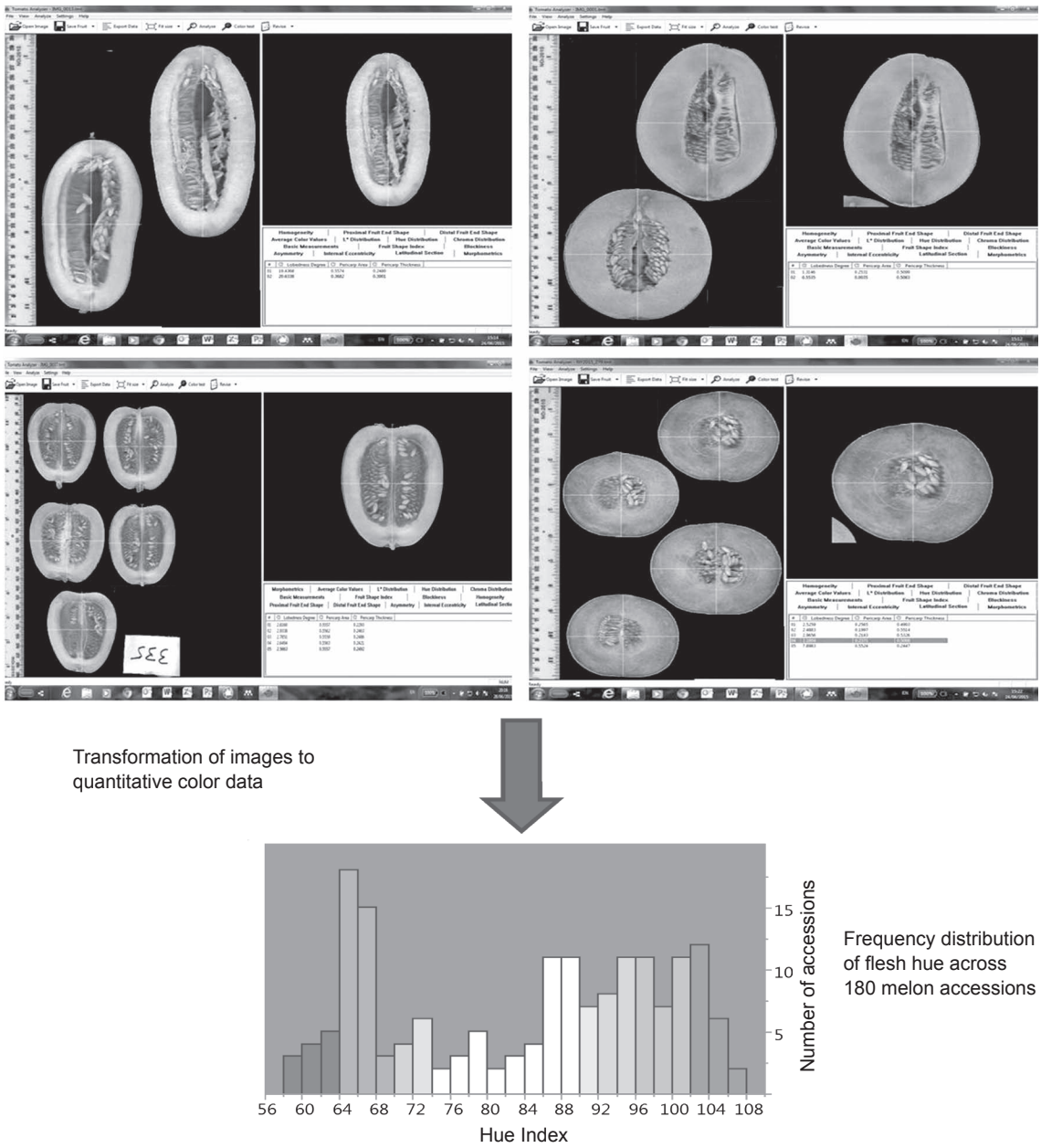

Figure 3. Examples of fruit scans from the summer 2015 experiment, phenotypically analyzed by the 'Tomato-Analyzer' software (Rodríguez et al. 2010). The application is built for batch analysis of multiple image files. This is an illustration of the extraction of color quantitative data from fruit images.

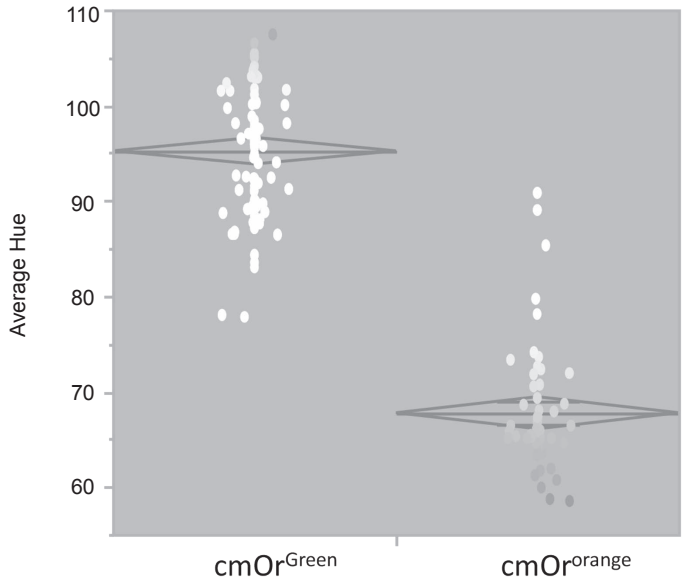

Figure 4. Association analysis between the SNP at the *CmOr* gene and fruit flesh color, measured across 177 melon accessions (Newe Yaʿar 2015). The phenotypic data for each flesh color is the average of 15 fruit scans, analyzed using the 'Tomato Analyzer' software.

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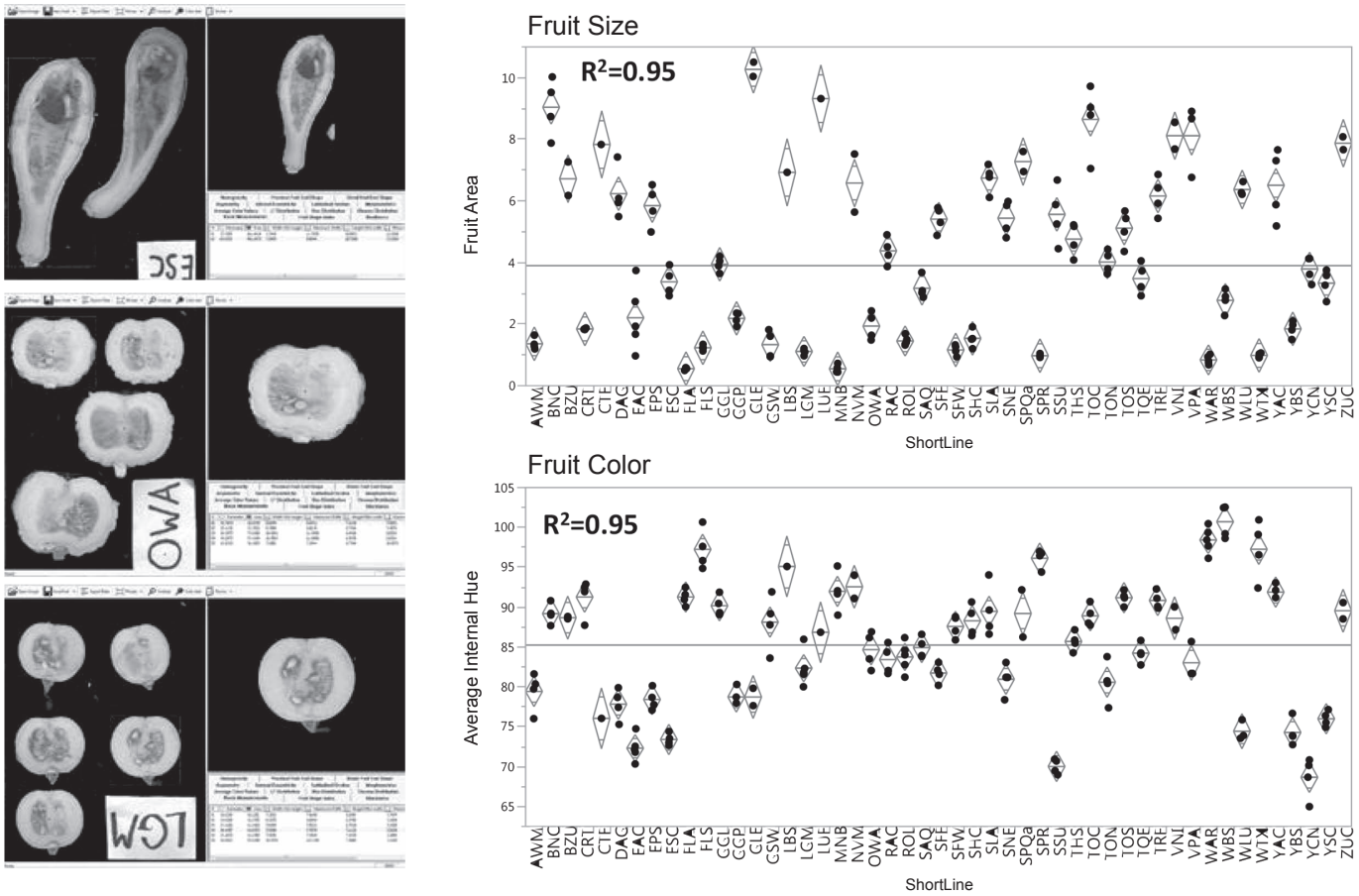

Figure 5: Left box: Fruit scan examples of three *Cucurbita pepo* accessions. Right boxes: One-way analysis of variance across 50 accessions for image-based fruit section area (top) and image-based flesh color (bottom).

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Mapping the Major Genes Related to Lycopene Content and Flesh Color Traits in Watermelon (*Citrullus lanatus*)

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Abstract. Lycopene content and flesh color are important traits in watermelon, determined by a series biochemical reactions in the carotenoid metabolic pathway. Based on our previous study of inheritance and initial mapping, flesh color was observed to be controlled by two major genes, on the genetic basis of red or pale yellow background. A candidate region for lycopene content was detected in a range of about 250 kb on chromosome 4. Herein, we successfully narrow down the preliminary region of the lycopene content locus into a range of 19,731 bp, in an F_2 population developed from a cross between two accessions of watermelon, LSW-177 (red flesh) and 'Cream of Saskatchewan' (pale yellow flesh). Based on the specific markers flanking the target locus region, five candidate genes were detected in this short segment according to the results of BLAST analysis and open reading frames predicted. One of the candidate genes, *Cla0050011*, was highly homologous to lycopene β-cyclase (*LCYB*) sequences and was regarded as the main candidate gene related to lycopene content in watermelon. Two major-effect flesh-color loci on chromosome 4 were also detected and one of these loci shared the same region with the lycopene-related candidate gene, considered to be the major red-flesh QTL. Another major flesh-color locus was found in about a 120 kb region and with the position near the red flesh locus, speculated to be the pale yellow flesh color QTL. The 11 CAPS markers developed based on the sequence of parental materials in the lycopene and flesh-color target region also co-segregated with red and yellow flesh color in a population of 53 watermelon cultivars.

Keywords: Watermelon, lycopene, flesh color, QTL mapping, molecular marker-assisted selection system

Introduction

The watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, is one of the most important cucurbit crops worldwide and has an approximately 6% share of the cultivated area of all vegetables. The main pigment of red flesh-color watermelon is lycopene, which is considered to be one of the most important natural carotenoids found in fruits. Lycopene has been a focus of international research in various fields including nutrition, health care products, and cosmetics, and serves some physiological functions in the human body. Mortensen et al. (1997) reported that lycopene could protect DNA and protein from free radical injury, thereby circumventing health problems caused by free radical scavenging damage. Lycopene can also enhance immunity, prevent cancer, and protect the cardiovascular system (Feng et al. 2010). Compared with tomato, another fruit which is also rich in lycopene, the lycopene in watermelon could be absorbed by the human body directly, but for tomato it is absorbed upon heating and cooking.

Some major genes or QTLs associated with flesh color in cucurbit crops have been described. In watermelon, the first research on QTL mapping of flesh color was by Hashizume et al. (2003), who reported two red flesh color-related QTLs, which were mapped to linkage groups (LGs) II and VIII in an integrated linkage map. In melon (*Cucumis melo* L.), *CmOr* is a key gene regulating the accumulation of β-carotene. A major effective QTL associated with β-carotene in melon was detected on chromosome 9 in the melon genome in a high-density linkage map by Harel-Beja et al. (2010). Based on this research foundation,

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Tzuri et al. (2015) found that the sequence of the *CmOr* gene in the effective QTL region was homologous to the *BoOr* gene in cauliflower (*Brassica oleracea* L.) and controlled the accumulation of β-carotene (Lu et al. 2006). Six SNPs caused the difference between the *CmOr* gene of orange flesh and green flesh with only one functional mutation of histidine into lysine at the position of the 353th base. The differences in *CmOr* expression between green- and orange-flesh melons were not significant throughout growth stages, as observed by RT-PCR, implying that the allelic variation of *CmOr* does not affect transcription or protein level for β-carotene accumulation. The β-carotene could be detected in callus, when the point mutation of the homologous *CmOr* gene in green flesh (histidine to lysine) was transformed into *Arabidopsis* by Tzuri et al. (2015). Gene *ore*, located to cucumber (*Cucumis sativus* L.) chromosome 3DS in a RIL population, is the key gene for β-carotene accumulation in cucumber, similar to the action of *CmOr* in melon (Bo et al. 2011). Pale yellow flesh color in cucumber is conferred by a single recessive gene, *yf*, fine-mapped to a 149 kb region on chromosome 7 together with 22 candidate genes (Lu et al. 2015).

The objective of this research was to locate the major gene controlling lycopene content and flesh color in watermelon. We constructed a molecular marker-assisted selection system for the target traits. The results could provide useful information and efficient tools for molecular breeding of watermelon flesh color.

Materials & Methods

Plant materials

A total of 352 F_2 individuals derived from the cross of accession LSW-177 (the female parent with a red flesh color) and 'Cream of Saskatchewan' (COS, the male parent with a pale yellow flesh color) (Pop.1) were used as the population for the target gene mapping. Another two populations, the F_2 of LSW-177 crossed with PI 186490 (white flesh color) (Pop.2) including 359 plants and a backcross derived from crossing 'Garden Parent' (red flesh) × PI 186490 (BC_1 using the red-flesh 'Garden Parent' as the recurrent parent) (Pop.3, 222 plants) segregating for fruit flesh color were used for genetic analysis and marker identifications tightly linked with lycopene content and flesh color. Seeds of LSW-177, COS, and PI 186490 were maintained by A.R. Davis. A panel of 53 watermelon cultivars with various flesh colors was used for marker assisted selection system verification of watermelon lycopene content and flesh color.

Methods

Mature fruits were cut longitudinally, photographed, and classified into flesh color groups by visual observation and colourimetric cards. Flesh samples were taken from the central tissue and four other points of the outer tissue and in mixed equal proportions for high-performance liquid chromatography (HPLC) analysis.

DNA was extracted from young leaves of 15 plants from each of the parental lines, and their F_1s. DNA of Pop.1, Pop.2, Pop.3 plants, and 53 accessions were extracted individually using a modified CTAB (Hexadecyl Trimethyl Ammonium Bromide) method as previously reported (Luan et al. 2008). Both CAPS (Cleaved Amplified Polymorphism Sequences) markers and SSR (Simple Sequence Repeats) markers were used for linkage map construction and QTL analysis. The CAPS markers were developed both in the preliminary mapping region and the whole genome for the secondary mapping, and the major effective locus detection based on the SNP variations of the sequences between LSW-177 and COS re-sequencing data. The sequences of SSR markers were derived from the literature.

Genetic linkage map construction and QTL analysis were conducted using the IciMapping V3.3 software (Institute of Crop Science Chinese Academy of Agricultural Sciences, Beijing, China). The software package Map Chart 2.1 (Plant Research International, Wageningen, Netherlands) was used to graphically represent the linkage groups in the map. SPSS 19.0 (SPSS Inc., U.S.A.) was used to perform statistical analyses of means, standard deviations, trait distributions, and pairwise correlations.

Results & Discussion

Segregation phenotype analysis of flesh color and lycopene content

Fruit flesh colors of LSW-177 and COS were red and pale yellow, respectively. For the F_1 generation, the flesh color was canary yellow, indicating partial dominance of pale yellow. In the F_2 generation, there were 5 categories of flesh color: red (87 plants), pale yellow (48 plants), canary yellow (173 plants), and two irregular color patterns consisting of red mixed pale and canary yellow, or red swirled together in separate sectors or in mixed patterns in the heart and placental tissues of the fruit (18 and 26 plants, respectively). Fruits with mixed pale and canary yellow were classified according to the majority of the flesh, as canary yellow and pale yellow. Accordingly, 199 (173+26), 66 (48+18), and 87 plants were judged to have canary yellow, pale yellow, and red flesh color in the F_2 generation, fitting the 9 : 3 : 4 two-gene ratio (Table 1). Canary yellow and pale yellow were combined to test segregation to yellow and red. Segregation fit the 3 : 1 ratio for yellow versus red (265 : 87, $\chi^2 = 0.015$, $P = 0.90$). These results indicate that a major single recessive gene determined the red versus yellow color in the cross of LSW-177 with COS. Similar results were

Table 1. Fruit flesh color in parental and filial generations of the cross between LSW-177 and COS.

Generation	Fruit flesh color			Excepted Ratio	χ^2	*P*
	canary yellow	pale yellow	red			
P_1	0	0	30	--	--	--
P_2	0	25	0	--	--	--
F_1	30	0	0	--	--	--
F_2	199	66	87	9 : 3 : 4	0.02	0.99

also observed by Bang et al. (2010), who reported that gene *C* for canary yellow flesh was dominant, and that it was epistatic to the red flesh conferred by gene *Y*.

HPLC analysis of the lycopene content of the mature fruit flesh showed that LSW-177 was rich in lycopene, containing an average of 41.72±2.82 $\mu g \cdot g^{-1}$, which was much more than the 0.24±0.03 $\mu g \cdot g^{-1}$ and 0.42±0.05 $\mu g \cdot g^{-1}$ of the yellow-fleshed COS and the F_1, respectively. When visually comparing flesh color and lycopene content, we observed that the flesh color was red when the lycopene content was higher than 13.57 $\mu g \cdot g^{-1}$. The F_2 generation could be divided into two groups (high-lycopene and low-lycopene) at this threshold value.

The segregation to low lycopene (265 plants) and high lycopene (87 plants) in the F_2 progeny fits the same 3 : 1 ratio as flesh color between the non-red and red groups, indicating the action of one major gene for red flesh color and high-lycopene content. Previous reports suggested that a single *LCYB* gene was the determinant of canary yellow or red flesh color (Bang et al. 2007). The results obtained herein agree in principle with the single recessive inheritance model.

In Pop.2, three different kinds of flesh color (red, yellow, and white) were observed in the F_2 generation, the number of the red flesh plants to non-red flesh (yellow and white) was 97 and 262, respectively. Four kinds of flesh color (red, yellow, orange, and yellow mixed white) segregated in Pop.3. Segregation of red to non-red group was 116 : 106, fit for the ratio of 1 : 1. The flesh color of F_1 generation in Pop.2 and Pop.3 was yellow, implying that the red flesh color trait in watermelon was conferred by one recessive gene of major effect. The separate proportion of red and non-red plants in the three genetic populations is summarized in Table 2. Both LSW-177 and 'Garden Parent' had red flesh color in the mature fruit, but the segregation was different when they were crossed with the white fleshed PI 186490, suggesting that the genetic basis of red in LSW-177 and 'Garden Parent' was different, and some carotenoid resulting in orange color is accumulated in the mature flesh of 'Garden Parent'.

Secondary mapping using genome sequence data

A map consisting of 11 linkage groups (corresponding to the 11 pairs of chromosomes in the watermelon genome) and 311 markers (274 CAPS, 37 SSR) was constructed based on the 352 F_2 plants derived from the cross of LSW-177 with COS, with eight new CAPS markers developed in the region of initial mapping (Figure 1). The linkage map covered a genetic distance of 1836.51 cM, with a mean distance between markers of 5.90 cM. The order of most marker locations corresponded to the physical map. One major QTL related to both, red flesh color and lycopene content, shared the same candidate region and was localized on chromosome 4. This QTL may account for most of the phenotypic variation between the newly developed CAPS markers WII04E08-38 and WII04EBsaHI-6, and is just 0.15 cM and 0.05 cM away from each marker, with a high R^2 (81.45%) and LOD score of 91.21. The two CAPS markers perfectly co-segregated with the red, pale yellow, and canary yellow groups in the F_2 population. Using the re-sequencing data, we obtained the sequences between the CAPS markers WII04E08-38 and WII04EBsaHI-6; 19,731 bp were present in this region and five candidate genes (which can encode the protein normally) were detected by consulting the the Cucurbit Genomics Database (http://www.icugi.org/cgi-bin/ICuGI/index.cgi). With the results of the open reading frame (ORF) and Basic Local Alignment Search Tool (BLAST) analysis, the lycopene beta-cyclase (*LCYB*) mRNA, the LCYB-red allele, and the Complete Coding Sequence (CDS) were in this region, with a high sequence similarity with candidate gene *Cla0050011*. The CAPS marker WII04EBsaHI-6 was located in gene *Cla005011*. Prior to this study, little research was focused on lycopene content QTL analysis in watermelon and the carotenoid biosynthetic pathway of watermelon was extrapolated from the carotenoid biosynthesis pathways in plants. Grassi et al. (2013) reported that the developmental origins of watermelon were similar to tomato; however, they still suggested complex and different regulatory systems for carotenoid biosynthesis pathways between these two species. As a result, a likely candidate gene for color determination between red and other flesh color in watermelon could be the gene immediately downstream of the lycopene synthesis pathway (Bang 2005). In *Cucumis*, seven genes from the carotenoid biosynthetic pathway have been previously mapped by Cuevas et al. (2008, 2009), and five were re-mapped to similar locations with different markers, as reported by Harel-Beja et al. (2010).

Another major QTL, with an R^2 value of 56.24% (*Py4.1*), was also detected in chromosome 4, in the region between CAPS marker WII04E07-40 and WII04EKpnI-1. This QTL was associated with the pale yellow flesh color, with a genetic distance of 0.8 cM. According to the re-sequencing data, the physical distance of the two markers was about 120 kb. Bang et al. (2010) reported that a *py* gene controlled pale yellow flesh color in watermelon. In cucumber, the location and the tightly related markers for a gene of major effect for pale yellow flesh color (*yf*) were observed by Lu et al. (2015) in a 149 kb region on chromosome 7. Using these markers, we observed only one SSR marker (SSR17292) associated with the polymorphism between LSW-177 and COS. As the result of linkage analysis and BLAST alignment, the amplicon of SSR17292 was located on chromosome 10 in the watermelon genome; no pale yellow-related QTL locus of major effect was detected in this region. The SSR17292 marker also did not co- segregate with flesh color in the 53 watermelon accessions, suggesting that different pigments cause pale yellow flesh color in cucumber and watermelon. The pale yellow flesh color would be conferred by low accumulation of β-carotene in *Cucumis* (Lu et al. 2015), while in watermelon it would be the neoxanthin (Bang et al. 2010).

Table 2. Segregation to plants having red- and non-red-fruit flesh in the three genetic populations.

Population	Red	Non-red	Expected Ratio	χ^2	*P*
Pop.1	87	265	1 : 3	0.015	0.90
Pop.2	97	262	1 : 3	0.780	0.38
Pop.3	116	106	1 : 1	0.450	0.50

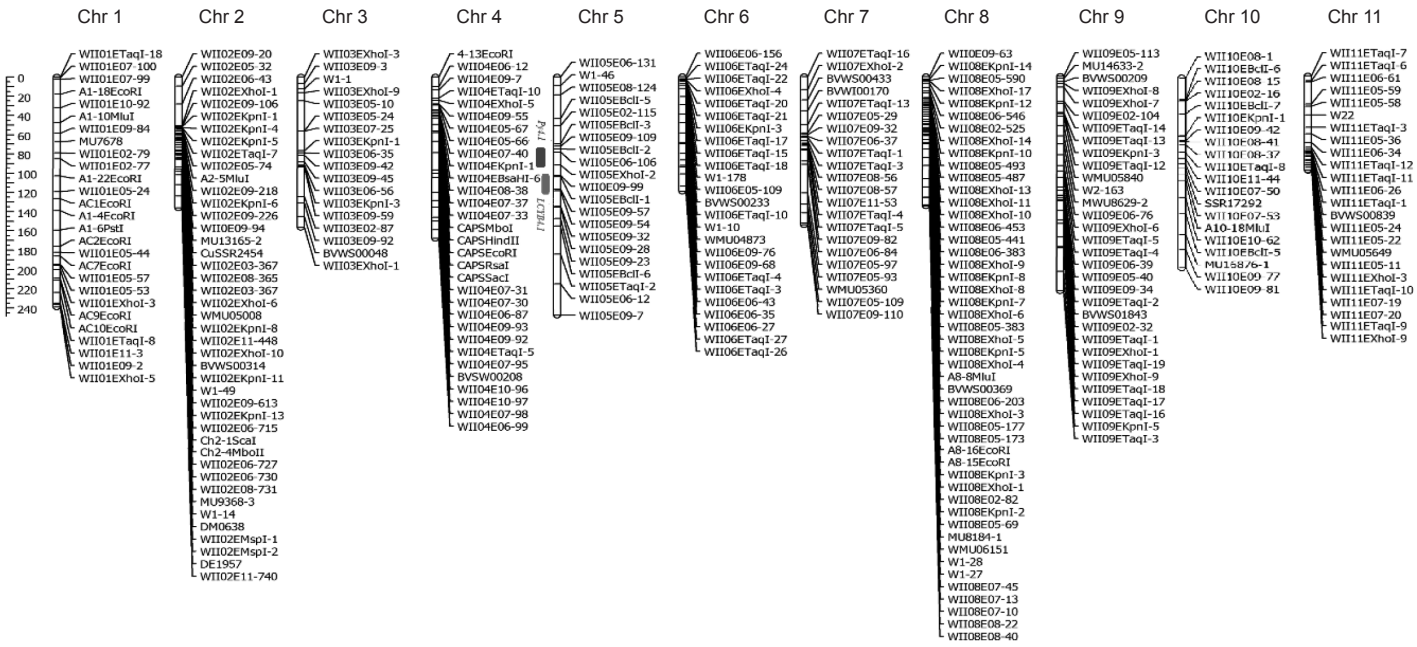

Figure 1. The linkage map of watermelon chromosomes constructed based on the F_2 population derived from the cross between LSW-177 and COS.

Genetic effect of QTLs for lycopene content in the F_2 population

According to the genotyping data, the 11 CAPS markers (from WII04E07-40 to CAPSSacI in chromosome 4, Figure 1) perfectly co-segregated with flesh color and lycopene content in the F_2 population. In order to further validate the results of QTL mapping, we also tested the relations between the lycopene content and the allele pairs of CAPS markers (WII04EBsaHI-6 and WII04E08-38) for all the individuals in the F_2 population. The average content of lycopene with the homozygous allele of LSW-177 was 37.38 $\mu g \cdot g^{-1}$ while the plants with the allele homozygous of COS had a value of 0.23 $\mu g \cdot g^{-1}$. The heterozygous individuals accumulated an average content of 2.62 $\mu g \cdot g^{-1}$ of lycopene. The higher lycopene content of the heterozygous individuals as compared with the homozygous COS allele can be attributed to some heterozygous-allele plants having a mixed fruit-flesh color, both with pale/canary yellow and red.

Marker testing with segregating populations and 53 watermelon accessions

In order to verify the applicability between the segregating populations and the various watermelon accessions, the 11 CAPS markers (from WII04E07-40 to CAPSSacI in chromosome 4, Figure 1) in the effective region were used to genotype Pop.2 and Pop.3. Among the 11 CAPS markers, four (CAPSMboI, CAPSHindII, WII04EBsaHI-6, and W04EII08-38) were polymorphic between LSW-177 and PI 186490. The genotype data of these four CAPS markers corresponded with flesh color in Pop.2. Only two CAPS markers (WII04EBsaHI-6 and W04EII08-38) showed polymorphism between 'Garden Parent' and PI 186490. The allele of the red-flesh plants in Pop.3 was homozygous as in 'Garden Parent' while the white-flesh plants were homozygous as in PI 186490. Both, the yellow and orange individuals had the same heterozygous alleles as F_1 generation.

For the 53 watermelon cultivars, all 11 markers were polymorphic among the different flesh-color accessions. Based on the results of the genotyping results, the 11 CAPS markers could be divided into two categories for marker-assisted selection in yellow flesh color and lycopene accumulation. One group consisted of seven markers (WII04E07-33, CAPSMboI, CAPSRsaI, WII04E07-37, WII04EKpnI-1, CAPSSacI, and WII04E07-40) separated according to the yellow-flesh plants (include canary yellow and pale yellow) and other flesh color accessions (red, pink, white, and orange). The other group consisted of four markers (CAPSEcoRI, WII04EBsaHI-6, CAPSHindII, and WII04E08-38) which could distinguish the red-, pink-, and orange-, flesh-color individuals from the yellow-, and white-flesh individuals. Considering the HPLC analysis (data not shown), the red-, pink-, and orange-flesh plants could obviously undergo lycopene accumulation in the mature fruit, so these CAPS markers could be used in marker-assisted selection for selecting lycopene accumulation in watermelon. For all three genetic populations and the 53 accessions, the homozygous allele of LSW-177 and 'Garden Parent' with CAPS markers WII04EBsaHI-6 and WII04E08-38 were associated with significantly increased lycopene content. Two kinds of watermelon flesh-color marker-assisted selection systems were generated by Bang et al. (2007, 2014), one being the CAPS marker Phe226 located in gene *LCYB*, and the other is the PCR marker based on the promoter region between the red and canary yellow *LCYB* alleles. This polymorphism did not change the expression of *LCYB*, but did provide a reliable marker for discriminating *LCYB* alleles for red and canary yellow flesh. Our results concerning the CAPS markers WII04EBsaHI-6 and Phe226, which are located the same region as *LCYB*, underscore the importance of *LCYB* for lycopene accumulation in watermelon fruit-flesh. The mode of inheritance and the gene interactions affecting watermelon flesh color is complex and there is still much research

needed in this area. For further flesh-color gene mapping, some other genetic populations having different flesh colors should be constructed to better appreciate fruit-flesh color evolution in watermelon.

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Effects of Grafting on Carbohydrate Metabolism in Melon (*Cucumis melo* L.) During Stages of Fruit Development

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Abstract. We studied the effects of grafting on plant growth, photosynthesis, and carbohydrate metabolism in melon (*Cucumis melo*) 'IVF198' during stages of fruit development, using 'Jingxin 3' pumpkin (*Cucurbita maxima* × *Cucurbita moschata*) as the rootstock. The results showed that grafting was associated with significantly increased plant growth and fruit yields, and enhanced photosynthetic capacities. The primary contributors to soluble sugar pools in leaf and flesh tissue were sucrose and hexoses (glucose and fructose). Raffinose was the most important component in the phloem sap, followed by sucrose and stachyose. Grafting led to a reduction in the concentrations of fructose, glucose, and sucrose on a fresh-weight basis, while the levels of all sugars were significantly higher on a whole fruit basis, which was correlated with an increase in levels of sucrose and raffinose in the phloem sap of grafted plants. The decline in sugar concentrations in fruits from grafted plants was mainly due to their excessive growth. The balance between lower levels of invertase transcriptions (both acid and neutral) and higher transcriptions of sucrose synthase and sucrose phosphate synthase accounted for the greater amounts of sucrose stored in mature fruits.

Keywords: Melon, grafting, carbohydrate metabolism, gene expression

Introduction

Melon (*Cucumis melo* L.) is one of the most economically important and widely cultivated horticultural crops in the world. The kinds and amounts of various carbohydrates directly influence fruit quality (Gross and Pharr 1982). The process of carbohydrate accumulation is closely related to leaf-carbohydrate production, the capacity to translocate photosynthates in the phloem, and sucrose metabolism in the fruits (Taji et al. 2002). Changes in chlorophyll concentrations, photosynthetic rates, and carbohydrate partitioning in source leaves can alter photoassimilate export rates, thereby directly affecting the amount of carbohydrates accumulated in the fruits (Madore 1990). Meanwhile, carbohydrate accumulations in fruits provide feedback to regulate photosynthesis and the activities of carbohydrate metabolism-related enzymes in the leaves (Pharr et al. 1985). Sucrose is the most commonly translocated sugar in many plant species, while melon is a typical raffinose (RFO) - transporting plant, and its fruits are economically valuable sink organs (Hu et al. 2009).

Grafting is a useful agricultural technique widely used for producing melon crops. Grafted plants can exhibit increased growth, yields, and tolerance to abiotic and biotic stresses (Colla et al. 2010). However, when rootstocks are used with melon, grafting can alter melon fruit qualities, leading to either enhanced or inferior traits (Colla et al. 2006, Proietti et al. 2008). Little is known about how grafting influences sugar metabolism simultaneously in both leaves and fruits of melon. Therefore, we conducted a comprehensive examination of plant growth, photosynthesis, sugar metabolism, and anatomical observations during various stages of fruit development. The objective of this work was to gain better insight into carbohydrate metabolism of grafted melon.

Materials & Methods

Plant materials and growth conditions

The experiments were conducted during the spring and summer seasons of 2012 and 2013 in the greenhouse at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing. As the scion, *Cucumis melo* L. 'IVF198' (var. *cantalupensis*), was grafted onto the commercial 'Jingxin 3' pumpkin rootstock (*C. maxima* Duchesne × *C. moschata* Duchesne), using the 'insertion grafting' procedure described by Lee et al. (2010). Ungrafted plants of 'IVF198' were used as the control. At the two-true-leaf stage, grafted and ungrafted plants were transplanted into the greenhouse. Flowers were tagged at anthesis and one fruit was allowed to develop per plant. Mature leaves

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and fruits from the same node were collected at 0, 10, 20, 30, and 40 days after anthesis (DAA).

Photosynthetic measurements

The net photosynthetic parameters were measured with a portable photosynthesis system (Li-Cor 6400, Li-Cor Inc., Lincoln, NE, USA), between 9:00 am and 11:00 am.

Carbohydrate extraction and analysis

The phloem sap was sampled as presented by Hu et al. (2009).

Leaf and mesocarp samples (each 1 g FW) were finely ground and then extracted three times in 5 mL of 80% (v/v) ethanol for 30 min at 80 °C. The residues were re-dissolved in 1 mL of distilled water and passed through a 0.45 μm filter. Afterwards, 10 μL of each sample was injected into a high performance liquid chromatograph (HPLC), as described by Miron and Schaffer (1991). The phloem sap samples were centrifuged for 5 min at 3000 × *g*, and the resulting supernatant was analyzed for soluble sugar concentrations by the same method used for the leaf and mesocarp samples.

Enzyme extraction and assays during stages of fruit evelopment

Sucrose phosphate synthase (SPS, E.C.2.4.1.14), sucrose synthase (SS, E.C.2.4.1.13), and acid and neutral invertases (AI and NI, E.C.3.2.1.26) were assayed according to the methods of Lowell et al. (1989).

Total RNA extraction and real-time quantitative RT-PCR analysis

Total RNA was isolated from roots with Trizol reagent (Toyobo, Osaka, Japan). Gene-specific primers used for real-time quantitative RT-PCR are shown in Table 1. All reactions were performed with SYBR PrimeScript™ RT-PCR Kits (Takara) according to the manufacturer's instructions.

Statistical analysis

All data were statistically evaluated by ANOVA, using the SPSS software package (SPSS 10 for Windows, 2001). Duncan's multiple range tests were performed at $\alpha = 0.05$.

Table 1. Primer sequences used for qRT-PCR.

Gene	Forward primer	Reverse primer
C_mSPS	GGAGAAAGTGGGGACACAGA	TGGCATGGAGTTGGTTTACA
C_mSS	GACGAGGAATGGGCAAA	GGGAGGCTTAAGTGAGGAGT
C_mAI	GCTCGGGTCAGAGAATGGTA	AACTCGGAAAGCGATTGATG
C_mNI	CCTGAGGAGGGCGGTTATTT	CAAAGATTCCCGAGCGTAAA
Actin	TCGTTCCTTCCTTCCTTCATTC	AGCCTTCACCATTCCAGTTC

Table 2. Effects of grafting on chloroplast ultrastructure in leaves and stems from ungrafted (CK) and grafted (T) melon plants. Data are means±SE; $n = 10$. Within a row, values not followed by the same letter are significantly different at $\alpha = 0.05$, based on Duncan's multiple range tests.

Trait	Location	CK	T
Number of chloroplasts (cell numbers profile^{-1})	Leaf	12.40±1.40b	15.70±2.30a
	Stem	3.30±0.68a	1.50±0.71b
Chloroplast dimensions (μm)	Length (in leaf)	5.22±0.55b	5.56±0.37a
	Width (in leaf)	1.50±0.11b	2.06±0.23a
	Length (in stem)	2.77±0.44b	4.17±0.32a
	Width (in stem)	1.13±0.10b	1.50±0.13a
Number of grana	Leaf	15.40±2.00b	21.60±1.60a
	Stem	2.00±0.27b	2.50±0.14a
Number of lamella in grana	Leaf	22.50±2.20b	30.40±2.60a
	Stem	27.00±1.25b	60.25±3.86a
Thickness of grana (nm)	Leaf	258.74±15.20b	337.27±14.25a
	Stem	338.54±16.30b	1016.37±21.86a
Number of osmiophilic multilamella	Leaf	7.20±0.14a	1.20±0.08b
	Stem	3.40±0.44a	1.50±0.09b

Results

The use of grafted rootstock significantly enhanced photosynthesis parameters. When compared with control, values for P_N, g_S, C_i, and E in grafted plants were increased by 57.46%, 133.93%, 15.64%, and 35.35%, respectively, at 40 DAA (Figure 1).

Fruit characteristics were also significantly affected by grafting (Figure 2). For example, at 40 DAA, values for transverse diameter, longitudinal diameter, single fruit weight, and pulp thickness were 12.18%, 15.84%, 49.82%, and 35.05% greater, respectively, from grafted plants than from the self-rooted samples.

We monitored soluble sugars in source leaves and found that levels showed distinct decreases over time (Figure 3A,B). Sucrose and hexoses (glucose plus fructose) comprised the bulk of those soluble sugars. At 40 DAA, their levels plus the accumulations of total carbohydrates were 37.97% lower in leaves from grafted plants.

The concentrations of various sugars also changed in the phloem exudates over time (Figure 3C,D). Initially, the levels of total soluble sugars increased gradually before decreasing as the fruits matured. At 30 DAA, the levels of total carbohydrates were 16.17% higher from grafted plants.

In the mesocarp tissues, the levels of total soluble sugars increased gradually over time, mainly due to the accumulations of glucose and fructose (Figure 3E,F). Glucose, fructose, and sucrose accounted for 17.90%, 16.83%, and 65.26% of all soluble sugars in mature fruit from grafted plants. On a fresh-weight basis, grafting resulted in a slight reduction in the concentrations of fructose, glucose, and sucrose, while on a whole-fruit basis,

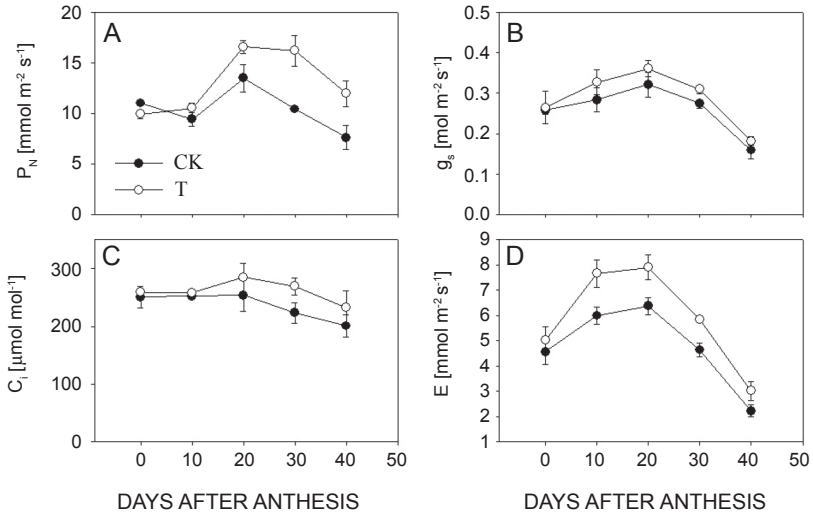

Figure 1. Comparisons between leaves from self-rooted (CK, control) and grafted plants (T) for (A) photosynthesis rate, P_N; (B) stomatal conductance, g_s; (C) intercellular CO_2 concentration, C_i; (D) and transpiration rate, E. Data are means±SE of 5 replicates.

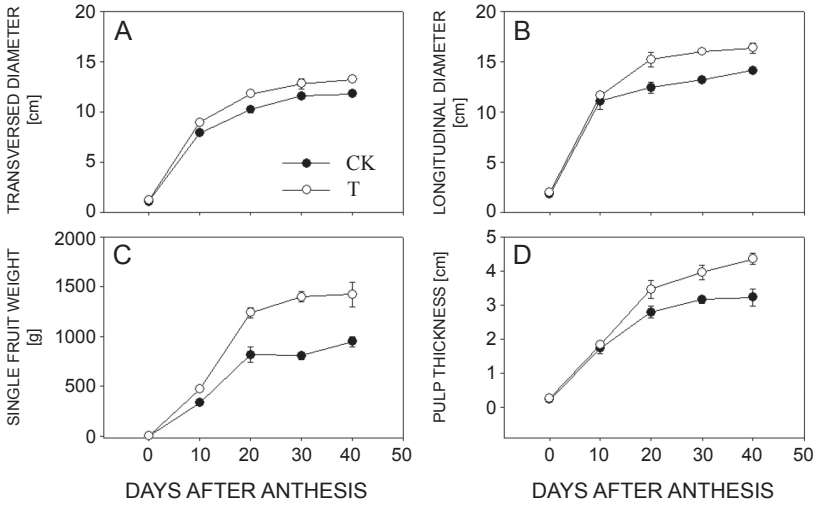

Figure 2. Characteristics of fruits from grafted plants (T) and ungrafted control (CK); (A) transverse diameter; (B) longitudinal diameter; (C) single fruit weight; (D) pulp thickness. Data are means±SE; $n = 10$.

the amounts of all sugars in fruit from grafted plants were significantly increased over those measured in fruits from self-rooted plants (Figure 3G,H).

Expression profiles were examined for the transcripts of four genes that encode key enzymes for sucrose metabolism (Figure 4). From 20 DAA, the expression of genes associated with SPS and SS was lower in samples from the grafted plants, while levels of AI- and NI-related transcripts were higher in those grafted samples beginning at 10 DAA.

The internal structure of leaf and stem chloroplasts was significantly changed by grafting (Figure 5, Table 2). For example, samples from grafted plants showed larger chloroplasts with more grana and lamella. Those tissues also had significantly fewer plastoglobuli per chloroplast, specifically, decreases of 85.71% for leaves and 50.00% for stems. For both grafted and control plants, the stems had significantly fewer chloroplasts and grana when compared with the leaves, but significantly more lamella.

Discussion

Pumpkin, *C. maxima* × *C. moschata*, has a vigorous root system, and it is widely used as a rootstock to promote overall plant growth. For example, yields from grafted watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, plants can be double those from self-rooted plants due to larger fruit sizes and more fruits produced per plant (Yetisir et al. 2003). Comparable findings have been described for tomato (Fernández-García et al. 2004), *Solanum melongena* L. (Pogonyi et al. 2005), and *Cucumis sativus* L. (Rouphael et al. 2012). Consistent with those earlier studies, we found that rootstock-grafting had a significant impact on growth and yield (Figure 2). These responses might be explained by interactions among the following: increased uptake of water and nutrients, enhanced hormone production, tolerance to low soil temperatures, and capacity to grow under saline conditions (Estañ et al. 2005). High rootstock vigor means that scions can benefit from stronger root systems.

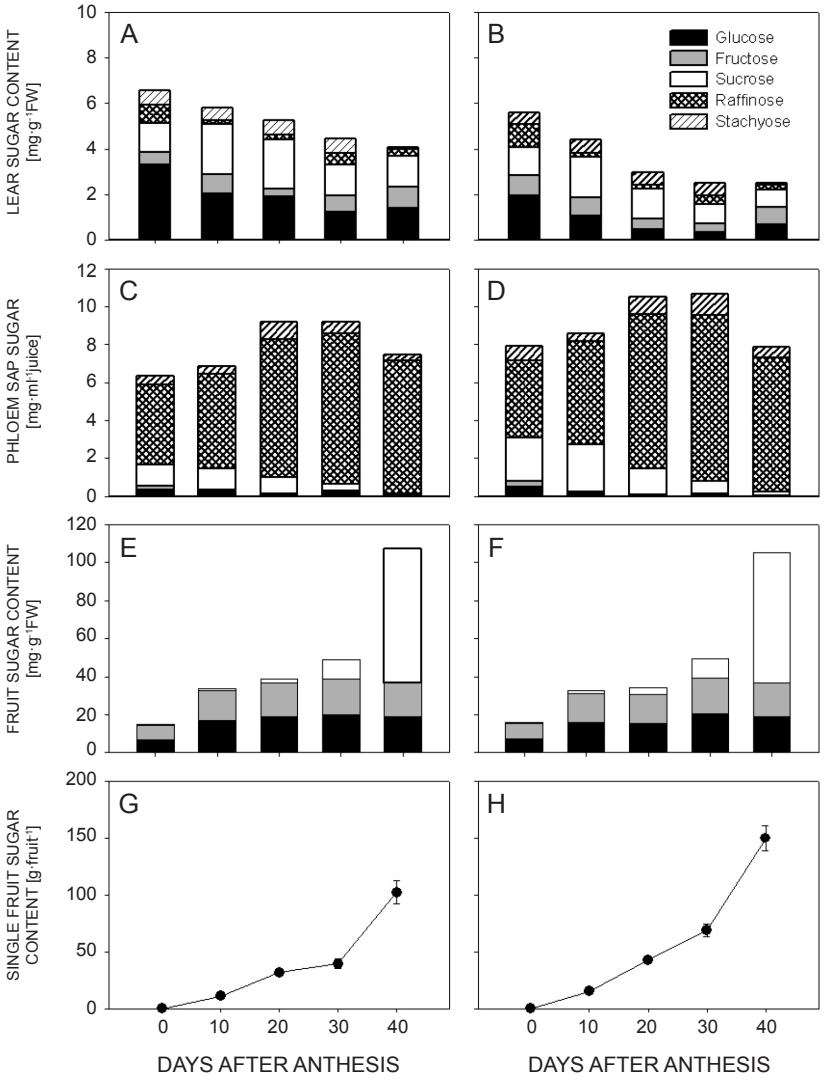

Figure 3. Changes in levels of soluble sugars in leaves, phloem sap, and mesocarp tissues during stages of fruit development in grafted (B,D,F,H) and ungrafted (A,C,E,G) melon plants. Data are means±SE (G,H) of 3 samples per time point.

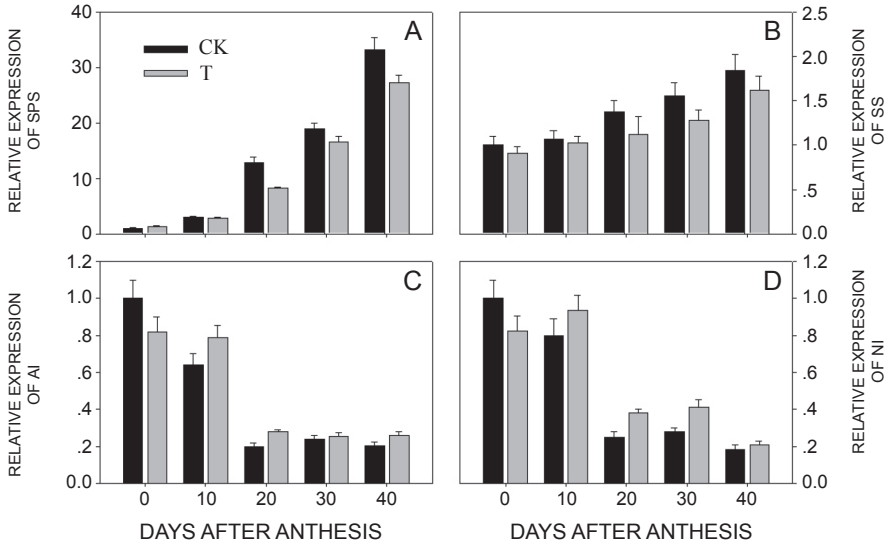

Figure 4. Changes in relative expression of genes related to sucrose-metabolizing enzymes in mesocarp tissues from grafted (T) and ungrafted (CK) plants during melon fruit development. Data are means±SE of 3 samples per time point.

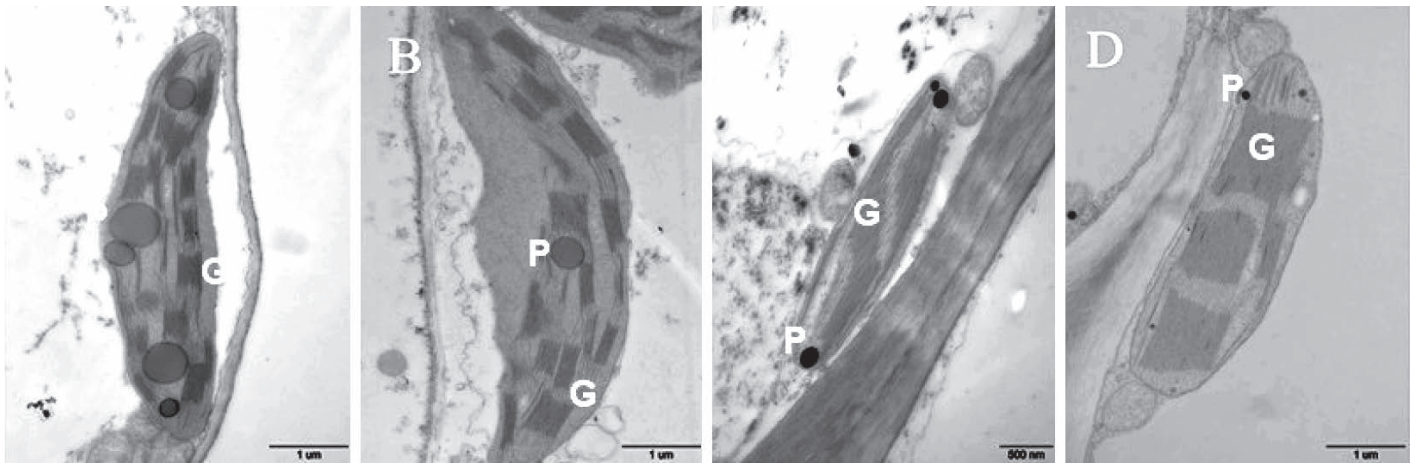

Figure 5. Comparisons of chloroplast ultrastructures in leaf (A,B) and stem (C,D) between ungrafted (A,C) and grafted (B,D) plants. G, Grana; P, Plastoglobuli. Bars represent 1 μm (A,B,D) or 500 nm (C).

We also observed that grafted plants had significantly higher photosynthetic capacities. Similar findings have been reported for grafted plants of oriental melon, watermelon, and various *Citrus* species (Qi et al. 2006).

Grafting can have positive or negative effects on fruit quality. Therefore, it is critical that the best rootstock/scion combinations be used when trying to improve external and/or internal fruit traits. Liu et al. (2006) reported that the fruits from scions grafted onto a bottle gourd rootstock differed only slightly from those produced by ungrafted control plants. We determined here that carbohydrate metabolism was influenced by grafting. Total sugar concentrations in both the leaves and fruit were lower for grafted plants than for self-rooted plants (Figure 3). This response was correlated with lower transcriptions of SPS and SS but higher transcriptions of invertase activities (Figure 4).

The success of fruit development depends largely upon the supply of photoassimilates imported from the leaves into the fruit via the phloem. On a fresh-weight basis, concentrations of fructose, glucose, and sucrose were reduced in fruits from grafted plants, while on a whole-fruit basis, the amounts of all sugars were significantly higher in fruits from grafted plants than in those from the control plants. This phenomenon was correlated with an increase in sucrose and raffinose levels in the phloem sap of grafted plants. Therefore, leaves from grafted melon plants appeared to produce and accumulate more translocating sugars such as sucrose and raffinose, because of their larger leaf areas and greater capacity for photosynthesis. The reason for the decline in sugar levels for those fruits was mainly due to their excessive growth as compared with fruits from ungrafted plants.

Acknowledgments

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Effective Long-Term Storage Methods for Pollen in Bitter Gourd (*Momordica charantia* L.)

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Abstract. Bitter gourd (*Momordica charantia*) is the one of the most important cucurbit vegetables cultivated in Japan, especially in Okinawa. Although Okinawa is in the sub-tropical region and can therefore produce bitter gourd over a long season, a major production problem is that pollen is not produced and/or is ineffective for some weeks during the rainy and winter-spring seasons. So, an effective storage method for the pollen of bitter gourd by using gas treatment was devised. The collected pollen was stored for one year under N_2 gas, air, or vacuum, at either 4 or -25 °C. The pollen stored under N_2 or under vacuum for one year had a high germination ability. Fruits produced by pollen from the control treatment and pollen stored for one year were not significantly different in weight, shape, and flesh color. These results indicate that pollen stored at -25 °C under N_2 gas or under vacuum for one year had a sufficient ability to produce fruit set.

Keywords: N_2-storage, pollen storage for one year, vacuum-storage

Introduction

Bitter gourd (*Momordica charantia* L.) is a tropical and sub-tropical vine of the family Cucurbitaceae. Bitter gourd is widely grown in Asia, Africa, and South America (Raj et al. 1993, Singh 1990). It is the one of popular vegetables in Japan and, being a fast-growing climbing vine, is also used to provide shade against strong summer sunlight. In Japan, it can be grown the year round only in Okinawa, the most southern area. However, in Okinawa, bitter gourd does not produce pollen for one to two months during the winter-spring season.

For some plants, pollen storage in organic solvents has been investigated (Kodani and Omura 1981, Sugiyama and Morishita 2000, Sugiyama et al. 2003). Until now, using an organic solvent has been the most suitable method for long-term storage of pollen. However, it is difficult to use pollen stored in an organic solvent for horticultural purposes because it is necessary to extract the pollen from the organic solvent solution and to dry it, and the pollen has a short life.

On the other hand, Akutsu and Sugiyama (2008) reported that watermelon pollen can be successfully stored for a year in frozen conditions (-25 °C) under N_2. This method can be used to provide watermelon pollen for production in all seasons. It might be possible to use this method to store pollen of bitter gourd.

In this study, the most effective parameters for storage duration were determined, considering temperature and vacuum, N_2, or air. The qualities of the fruit after pollination with stored pollen were also assessed.

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Materials & Methods

Plant materials and cultivation methods

Seeds of the Japanese bitter gourd 'Shiokaze' were sown in pots on 21 September, 2013 in a glasshouse. Seedlings with 3 leaves were transplanted to bigger pots (30 cm) in a greenhouse on 14 October. The potting soil used was 'Wakanatsu' (Kyusyu NSK, Japan). Flowers were pollinated starting at about the 15th node of the lateral branches.

Experiments were performed at the Okinawa Prefectural Research Center in Itoman, Japan.

Pollen storage methods

'Shiokaze' staminate flowers were harvested in the morning to obtain pollen that would be stored for 14, 28, 90, and 365 days. Pollen was collected from the flowers by a brush. The pollen was packaged in paraffin paper. A vacuum packaging machine ('TOSPACK' TOSEI, Shizuoka, Japan) was used to package under N_2, air, or vacuum, and the pollen was then kept at either 4 or -25 °C in order to observe which storage method was the best for the long term. Pollen was assayed for germination ability on a germination medium which contained 14% sucrose, 0.1% boric acid, and 1.5% agar (WAKO, Osaka, Japan). Germinating pollen was incubated at 25 °C in the dark for about 3 h and counted using an optical microscope. All treatments had 10 replicates for each period and for each temperature (4 or -25 °C). Germination ability was assayed by counting 100 or more pollen grains for each treatment (e.g., 4 °C, 28 days).

Fruit set and quality of fruit produced from pollen stored for 365 days

The pollen for use as a control was collected the day of pollination and processed on the morning of the experiment. The pollen

stored under vacuum, N_2, or air at either -25 or 4 °C for 14, 28, 90, and 365 days was used. Before pollination, pollen germination ability on germination medium was observed under a microscope. This pollen was used to pollinate pistillate flowers. The pistillate flowers were bagged with cellophane before anthesis. Prepared pollen was applied with a brush. After pollination, pistillate flowers were again covered with cellophane bags for about 3 days to prevent contact with insect-transferred pollen. On each plant, less than 20 pistillate flowers were pollinated with pollen from each storage treatment, and fruit set was observed about 7 days later. The fruit set percentage of each plant was calculated.

Mature fruits were harvested 20 days later. The fruits were then weighed and assessed for shape and flesh color. Fruit shape was expressed as the ratio of length from peduncle to stylar end, to equatorial diameter. The flesh color of the mature harvested bitter gourds was assessed with a colorimeter [a*: hue relates to red (+60) – green (-60) color axes, SPAD-503, Konica, Tokyo, Japan]. All treatments were arranged as 20 single-plant replicates.

Results & Discussion

Before pollination took place, pollen germination ability on germination medium was observed under a microscope (Table 1). During the investigation of pollen germination before pollination, it was found that pollen stored at 4 °C for about one year with either N_2, air, or vacuum treatment did not germinate on germination medium. On the other hand, germination took place in pollen stored at -25 °C for about one year with either N_2 or a vacuum (Table 1).

The germination rate of pollen stored at -25 °C under vacuum was higher than that stored with N_2. Flowers pollinated with pollen stored at 4 °C for about a year did not set fruit, whereas pollen stored at -25 °C for about a year set fruit for both N_2 and vacuum atmospheric conditions, under vacuum conditions higher than under N_2 conditions. The pollen stored under N_2 or under a vacuum for a year had high pollen germination and fruit set abilities. Fruits of control and pollen stored for a year under N_2 or under vacuum were not significantly different in weight, shape, and flesh color (Table 2).

These results also indicate that pollen stored at -25 °C under N_2 gas or under vacuum for one year had a sufficient ability to produce normal fruit set. And this storage method eliminates the need for organic solvents and chemicals, thus providing an eco-friendly storage system.

Acknowledgements

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Table 1. The relationship between pollen storage condition and fruit set.

Storage treatment	Storage temperature (°C)	Storage period (days)	Germinating ability (%)[y]	Fruit set (%)
Control[z]		0	88.5±4.0	92.6
Nitrogen	4	360–365	0	0
Nitrogen	-25	360–365	32.4±2.1	83.5
Vacuum	4	360–365	0	0
Vaccum	-25	360–365	53.1±5.3	88.2
Air	4	360–365	0	0
Air	-25	360–365	0	0

[z]Control pollen was collected during the morning hours.
[y]Mean ± standard error.

Table 2. Quality of bitter gourd fruit set as a result of pollination with pollen stored in differing conditions.

Storage treatment	Storage temperature	Storage period (days)	Fruit weight (g)[y]	Flesh color[z]	Outside fruit shape (length:width)	Inside fruit shape (length:width)
Control	--	0	78.2±3.1	36.2±0.2	1.9±0.0	3.0±0.0
-25	Nitrogen	300-365	81.9±4.3	36.3±0.4	1.9±0.1	3.1±0.1
-25	Vacuum	300-365	79.6±2.9	35.8±0.3	1.9±0.1	3.0±0.1

[z]a*: Hue relates to red (+60) - green -(-60) color axes.
[y]Mean±standard error.

Notes on *in vivo* and *in vitro* Tetraploidization of Bottle Gourd (*Lagenaria siceraria*), Citron Watermelon (*Citrullus lanatus* var. *citroides*), and Colocynth (*C. colocynthis*)

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Abstract. The objective of this research was to develop tetraploid plants of bottle gourd and watermelon by using different doses of colchicine under *in vivo* and *in vitro* conditions, in order to obtain strong rootstocks for watermelon grafting. One genotype of bottle gourd (*Lagenaria siceraria*), 'Sk 10', and two taxa of watermelon, *Citrullus lanatus* var. *citroides* and *C. colocynthis*, were treated with four doses of colchicine for the *in vivo* experiment (0.2%, 0.3%, 0.5% 1.0%, and 0.0% control) and two doses of colchicine for the *in vitro* experiment (0.5% and 1.0%, and 0.0% control, for 1h, 2h, and 4h). The experiment was conducted at the Faculty of Agriculture Research and Experimental Area and Laboratory of Biotechnology Centre of Cukurova University in Turkey. From the *in vivo* experiment, 9.2% of the colchicine-treated plants were tetraploid. Four tetraploid plants were obtained from each of the three taxa; four, three, three, and two tetraploids were obtained from plants treated with 1.0%, 0.2%, 0.3%, and 0.5% colchicine, respectively. From the *in vitro* experiment, 5.4% of the colchicine-treated plants were tetraploid. Four tetraploids were obtained from *Citrullus lanatus* var. *citroides* and one tetraploid from *Lagenaria siceraria*. In both experiments, colchicine doses of 0.5% and 1.0% resulted in the most tetraploids. In both experiments, most plants were observed to have chloroplast numbers in the stoma ranging from 9 to 20.

Keywords: Tetraploid, rootstock, colchicine, chloroplast counting

Introduction

Grafting has become widely used for vegetable production to control soil-borne diseases, abiotic stresses, and to increase yield and quality (Lee 1994, Lee and Oda 2003, Yetisir and Sari 2003). Indeed, grafting increases low-temperature tolerance, and enhances water and nutrient absorption (Masuda et al. 1981, Oda 1995). It can also increase fruit-flesh firmness and lycopene content (Kyriacou and Soteriou 2015).

Cucurbita plants have been used for a long time as rootstocks for grafted watermelons, *Citrullus lanatus* (Thunb.) Matsum. & Nakai. However, there are problems associated with *Cucurbita* rootstocks. Inferior fruit-flesh quality was reported for watermelons grafted onto *Cucurbita*, especially interspecific hybrid *Cucurbita* rootstocks (Ryu et al. 1973, Lee and Oda 2003). Kyriacou and Soteriou (2015) observed that *Cucurbita* rootstocks reduced soluble solids content of watermelon fruit flesh by 0.8 to 1.0 °Brix. Similar findings were also reported by Zhu et al. (2006). Davis and Perkins-Veazie (2005) observed reductions in fruit size when watermelons were grafted on squash rootstocks. Cucumbers, *Cucumis sativus* L., were reported as having shorter fruits and reduced fruit production when grafted on *Cucurbita* rootstocks (Muramatsu 1981).

Due to the quality problems experienced from the use of *Cucurbita* rootstocks, bottle gourd, *Lagenaria siceraria* (Mol.) Standl., became a preferred rootstock for watermelon grafting (King et al. 2010). *Lagenaria* is more closely related to *Citrullus*, both being in the cucurbit tribe *Benincaseae*, whilst *Cucurbita* is distant, in the tribe *Cucurbiteae* (Schaefer and Renner 2011). *Lagenaria* has been used in many countries in Asia as a source of rootstocks with high compatibility to other cucurbits, especially with watermelon (Lee 1994, Oda 1995, Lee and Oda 2003), and high survival rate, yield, and quality (Yetisir and Sari 2003). According to Yetisir et al. (2007), there have been no rootstocks bred for watermelon selected in Turkey, all rootstocks that are used for watermelon grafting in this country are imported from other countries. *L. siceraria* has been considered to have great potential as a rootstock for watermelon and much untapped variation, in all a good resource for rootstock breeding programs (Yetisir et al. 2007, Karaca et al. 2012, Shahidul Islam et al. 2013). Salam et al. (2002) obtained larger main stems, more lateral roots, and a higher yield from watermelon 'Top Yield' grafted onto a *L. siceraria* rootstock. However, *Lagenaria* and *Citrullus lanatus* var. *citroides* (syn. *C. amarus* Schrad., citron watermelon) type of rootstocks are also associated with some problems, including susceptibility to *Fusarium* (Sakata et al. 2007), root-knot nematodes (Giannakou and Karpouzas 2003),

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and a shallow root system (Davis et al. 2008). *Lagenaria* and *Citrullus lanatus* var. *citroides* rootstocks are relatively weak, hence the current research aims to obtain tetraploids of these cucurbit taxa, to furnish strong rootstocks that would be more compatible for watermelons.

Materials & Methods

For the *in vivo* experiment, seeds of bottle gourd (*L. siceraria*) genotype 'SK 10' and two watermelon taxa, *Citrullus lanatus var. citroides* and *Citrullus colocynthis* (L.) Schrad. (colocynth), were sown in trays with 2 : 1 v/v peat : perlite mixture with one seed per cell on 01 April 2015. Forty five seeds (15 seeds × 3 repetitions) were sown for every genotype and every treatment (however, not all seeds germinated). When the seedlings had one true leaf, each was treated with drops of colchicine on the growing point, the apex. Four doses of colchicine (0.2%, 0.3%, 0.5%, 1%) and the control (0.0%, only water) were used. Five drops per plant were applied, one drop at a time, three times on the first day and twice on the second day, at intervals of 4 h between applications on both days. The treated seedlings were maintained in the glasshouse until they were ready for transplanting. Four weeks after colchicine treatment, putative tetraploid plants (Simsek et al. 2013) were transplanted to the field and, three weeks later, a leaf sample was taken from each plant for assessment of tetraploidy by chloroplast counting.

For the *in vitro* experiment, 80 seeds from each taxon (*L. siceraria*, *Citrullus lanatus* var. *citroides* and *C. colocynthis*) were surface sterilized by soaking them in a 1-L beaker with 500 ml 1% NaOCl for 5 min followed by rinsing 3 to 5 times with distilled water. The hard seed coats were removed by using a new clean blade, then each seed was placed into a test tube with MS media 4.4 g/L, sucrose 20 g/L, IAA 0.1 ml/L, agar 8 g/L, pH 5.9, on March 31, 2015. Three weeks later, germinated and non-germinated seeds were counted (Table 1). Seedlings were multiplied by making micro-cuttings which were placed individually in test tubes on MS media. Three weeks later, the seedlings were multiplied for the second time, again on MS media. Four weeks of the later, seedlings were taken out from the test tubes, the agar on the root systems was removed, and the plants were immersed into 0.5% and 1.0% colchicine for 1 h, 2 h, or 4 h, before cuttings were made and then put into test tubes (Sari and Abak 1996, Inan and Sari 2010) having MS media (Figure 1).

For screening ploidy level, chloroplasts were counted in randomly selected stomata of the lower epidermal part of the leaf, by using a microscope (McCuistion and Elmstrom 1993, Sari et al. 1999). Also, the size of the leaves and flowers was observed.

Results & Discussion

For the *in vivo* experiment, in the field, considerable variation was observed in the size of the plant organs, especially the leaves and flowers of *C. lanatus* var. *citroides* and *C. colocynthis*. In *Lagenaria*, no marked differences of plant organs were observed.

Diploid plants usually had 9 to 20 chloroplasts per stoma, while tetraploid plants had between 17 to 20 (numbers with asterisks) (Table 2 for *in vivo*, Table 3 for *in vitro*). Similar numbers of chloroplasts per stoma were observed by Compton et al. (1994) and Simsek et al. (2013). Of the 131 colchicine-treated plants, 12 were tetraploids (9.2%). In *L. siceraria*, out of 47 plants, four tetraploids were obtained, the same number of tetraploids also obtained from *C. lanatus* var. *citroides* and *C. colocynthis* by chloroplast counting. Of the plants treated with 1.0% colchicine, four tetraploids resulted, followed by three tetraploids from plants treated with 0.2% and 0.3% colchicine, while those treated with 0.5% colchicine resulted in two tetraploids (Tables 2,4). McCuistion and Elmstrom (1993) identified polyploids of various cucurbits by chloroplast counting in stomata guard cells and found that average stomatal chloroplast number of the tetraploid breeding line was 19.7. Also Kumar et al. (2015) found the maximum number of tetraploids, from 41.13 to 44.41%, for four watermelon accessions, 'Arka Manik', 'Arka Muthu', 'Sugar Baby', and IIHR-14) at 0.2%, 0.3%, and 0.4% colchicine doses. Indeed, results obtained from this research are similar to those obtained by Jaskani et al. (2005), Ahmad et al. (2013), and Thayyil et al. (2016).

For the *in vitro* experiment, of the 93 treated plants, only five (5.4%) became tetraploids, four of *C. lanatus* var. *citroides* and one of *L. siceraria* (Tables 3,5). The longer the time the plants were exposed to colchicine, the fewer the tetraploids resulted. Three tetraploids resulted from plants treated with 0.5% colchicine for 1 h, while those treated with 1% colchicine for 2 h and 4 h resulted in one tetraploid each. These results are in accordance with those of Sari and Abak (1996), who studied the effect of dosage of colchicine and exposure time on *in vitro* chromosome doubling in watermelon; they suggested the use of a long exposure at a low dosage (4 h at 0.5%) and a short exposure at a high dosage (2 h at 1%). The obtaining of a very low number of tetraploids and the death of most of the treated seedlings can be explained as a negative effect of colchicine. Vajrabhaya (1983) described colchicine as a toxic chemical, the exposure of young plant cells at high doses can be lethal.

Colchicine dosages of 0.5% and 1.0% resulted in five and six tetraploids each, respectively, more than any other dosage level used in this research.

Table 1. Number of germinated seeds after sterilization, germination percentage, and cuttings made from seeds in the *in vitro* experiment.

Species	Number of seeds sown	Number of germinated seeds	Seed germination percentage (%)	Number of micro cuttings
Lagenaria siceraria	46	20	43.48	43
C. lanatus var. *citroides*	64	8	12.50	26
Citrullus colocynthis	45	7	15.56	8

Table 2. Number of chloroplasts counted in stomata guard cells of sampled plants from *in vivo* experiment.

Species	Treatment	Number of plants	Number of chloroplasts
Lagenaria siceraria	Control	1	15
		2	13
		3	14
		4	13
	0.2%	**1**	**19***
		2	12
		3	11
		4	11
		5	13
		6	10
	0.3%	1	13
		2	11
		3	13
		4	12
		5	13
	0.5%	1	12
		2	10
		3	15
		4	11
		5	12
		6	11
	1%	1	13
		2	9
		3	9
		4	**20***
		5	**19***
		6	9
		7	**18***
		8	10
Citrullus colocynthis	Control	1	11
	0.2%	**1**	**18***
	0.3%	1	10
		2	**19***
		3	**20***
	0.5%	1	9
		2	**19***
	1%	1	10
C. lanatus var. *citroides*	Control	1	12
		2	10
		3	11
		4	12
	0.2%	1	11
		2	13
		3	10
		4	**17***
		5	13
		6	16
	0.3%	**1**	**17***
		2	12
		3	12
		4	11
	0.5%	1	12
		2	12
		3	16
		4	14
		5	**17***
		6	12
		7	12
	1%	1	11
		2	10
		3	**17***
		4	14
		5	12

*tetraploid plant

Table 3. Number of chloroplasts counted in stomata guard cells of sampled plants from the *in vitro* experiment.

Taxon	Treatment	Application time (h)	Number of plants	Number of chloroplasts
Lagenaria siceraria	Control		1	9
			2	8
			3	9
	0.5%	1	1	13
			2	12
		2	1	10
			2	10
			3	12
			4	12
			5	14
		4	1	13
			2	**19***
	1%	1	1	11
C. lanatus var. *citroides*	0.5%	1	1	11
			2	**18***
			3	**20***
			4	**20***
	1%	1	1	11
			2	10
		2	**1**	**17***
		4	1	14
			2	12

*tetraploid plant

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Table 4. Percentage of diploids, percentage of seedlings likely to be tetraploids before analysis, number of sampled plants, number of tetraploid plants, and percentage of tetraploids obtained from application of different colchicine doses in the *in vivo* experiment.

Species	Colchicine doses (%)	Percentage of diploid (2n) plants (%)	Percentage of tetraploid (4n) likely seedlings (%)	Number of colchicine treated plants	Sampled plants	Number of tetraploid plants	Percentage of tetraploids (%)
Lagenaria siceraria	Control	100.00	0.00	0	4	0	0.00
	0.2	50.00	50.00	12	6	1	8.33
	0.3	58.33	41.67	12	5	0	0.00
	0.5	45.46	54.54	11	6	0	0.00
	1	33.33	66.67	12	8	3	25.00
	Subtotal			**47**	**29**	**4**	**8.51**
C.lanatus var. citroides	Control	100.00	0.00		4	0	0.00
	0.2	66.67	33.33	18	6	1	5.56
	0.3	66.67	33.33	12	4	1	8.33
	0.5	68.18	31.82	22	7	1	4.55
	1	80.00	20.00	25	5	1	4.00
	Subtotal			**77**	**26**	**4**	**5.19**
Citrullus colocynthis	Control	100.00	0.00		1	0	0.00
	0.2	0.00	100.00	1	1	1	100.00
	0.3	0.00	100.00	3	3	2	66.67
	0.5	0.00	100.00	2	2	1	50.00
	1	0.00	100.00	1	1	0	0.00
	Subtotal			**7**	**8**	**4**	**57.14**
Total				**131**	**63**	**12**	**9.16**

Table 5. Percentage of diploid plants, percentage of seedlings likely to be tetraploids before analysis, number of tetraploid plants and percentage of tetraploid plants obtained from application of different colchicine doses, and time in *in vitro* experiment.

Taxon	Colchicine doses (%)	Time (hrs)	Diploid (2n) plants (%)	Tetraploid (4n) likely seedlings (%)	Number of colchicine treated plants	Sampled plants	Number of tetraploid plants	Tetraploids (%)
Lagenaria siceraria	Control	0	100.00	0.00	0	3	0	0.00
	0.5	1	100.00	0.00	26	2	0	0.00
		2	100.00	0.00	20	5	0	0.00
		4	90.91	9.09	11	2	1	9.09
	1	1	100.00	0.00	11	1	0	0.00
		2	100.00	0.00	4	0	0	0.00
		4	100.00	0.00	5	0	0	0.00
	Subtotal				**77**	**10**	**1**	**1.30**
C.lanatus var. citroides	Control	0	100.00	0.00	0	3	0	0.00
	0.5	1	25.00	75.00	4	4	3	75.00
		2	0.00	0.00	0	0	0	0.00
		4	0.00	0.00	0	0	0	0.00
	1	1	100.00	0.00	4	2	0	0.00
		2	75.00	25.00	4	1	1	25.00
		4	100.00	0.00	4	2	0	0.00
	Subtotal				**16**	**9**	**4**	**25.00**
Total					**93**	**19**	**5**	**5.38**

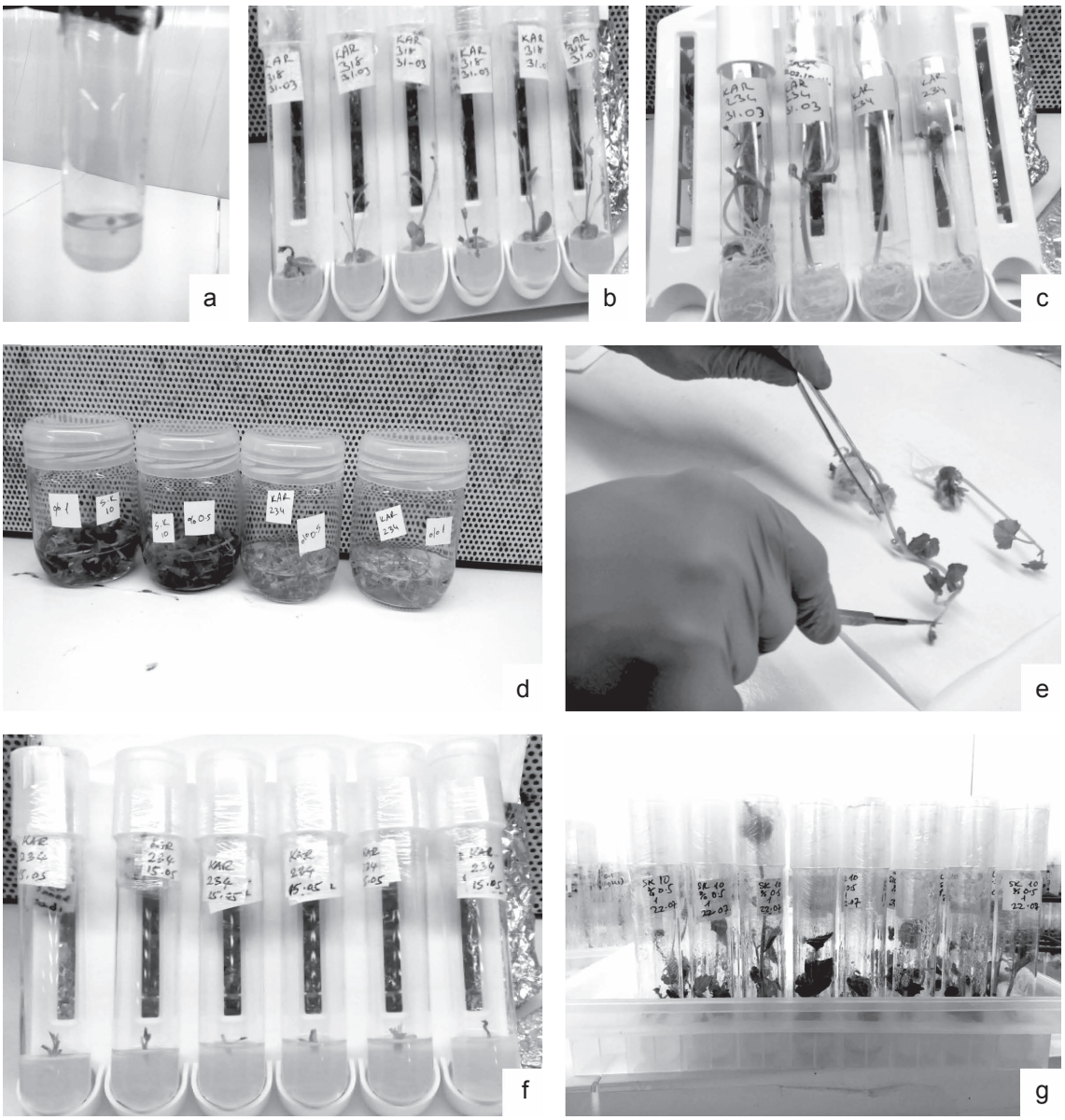

Figure 1. (a) Sowing seed in test tube; (b) *Citrullus colocynthis*; (c) *Citrullus lanatus* var. *citroides* growing seedlings in test tubes; (d) plants soaked in colchicine; (e) multiplication – making cuttings; (f) cuttings in growth media; (g) growing seedlings in the *in vitro* experiment.

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Double-Haploid Pure-Line Development in Galia-Type Melons Having a Long Shelf Life

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Abstract. Melons, *Cucumis melo*, belonging to vars. *cantalupensis* and *reticulatus*, ripen early and are aromatic, sweet, and have a smooth or netted rind. They are widely grown in Turkey, both under tunnels and in greenhouses. These melons, unlike winter melons (var. *inodorus*), have a short shelf life, which limits transport and export opportunities. In this context, the development of cantaloupes and muskmelons having a long shelf life is a most important breeding goal. *In vivo* parthenogenesis induction using irradiated pollen has provided successful results in melon and can accelerate breeding by shortening the time required for obtaining 100% homozygous lines. In this project, we used 84 BC_3 populations. A total 1,104 flowers were pollinated and 700 parthenogenetic fruits were obtained. Fruit setting ratio was 63.4%. The highest fruit setting success, 100%, was obtained from 18 genotypes. The least success, 16.7%, was obtained in one genotype. Mean parthenogenetic fruit weight was 1625.5 g. A total of 1,813 embryos was obtained, of which 1,142 transformed into plants. Mean number of embryos per fruit was 2.5 and the average regeneration rate was 63%. The highest haploid plant numbers, 46 to 53 plants, were obtained from three of the genotypes. A regeneration success of 100% was obtained from 11 genotypes and the lowest regeneration success, 14.3%, was from one genotype.

Keywords: Melon, fruit setting, embryo rescue, regeneration

Introduction

Melon (*Cucumis melo* L.) is cultivated in most countries and is highly polymorphic (Pitrat 2012). Pitrat et al. (2000) classified melons into 16 botanical varieties, five of which (var. *conomon, makuwa, chinensis, momordica*, and *acidilus*) can be assigned to subsp. *agrestis*, and eleven (var. *cantalupensis, reticulatus, adana, chandalak, ameri, inodorus, flexuosus, chate, tibish, dudaim*, and *chito*) to subsp. *melo*. World melon production for 2014 was 31,925,787 tonnes on 1,339,006 ha area. Turkey is the second leading country after China with 1,708,415 tonnes on 102,000 ha (FAO 2014).

Melons belonging to vars. *cantalupensis* and *reticulatus* (cantaloupes and muskmelons) are mainly produced in greenhouses or under tunnels in Turkey. These melons usually weigh 1 to 2 kg and are round, sweet, aromatic, and early ripening. However, their short shelf life limits distant transport and export. In this context, it is important to develop new varieties of cantaloupes and muskmelons with longer shelf life. They can be crossed with long shelf-life, sweet melons, var. *inodorus*, but the obtaining of true-breeding, homozygous lines requires many generations of selfing.

Dihaploidization is a technique which facilitates development of 100% homozygous lines quickly (Forster and Thomas, 2005). The use of irradiated pollen has proven successful in melon (Sari et al. 1992, Abak et al. 1996, Lotfi et al. 2003, Gursoy et al. 2010, Sari et al. 2010, Solmaz et al. 2011) and inbred lines can be obtained within 18 months.

Cantaloupes and muskmelons are typically climacteric, ripening quickly and having a shelf life of several days. If not transported immediately upon harvest to the marketplace, significant losses occur. Therefore, long shelf life has become an indispensable feature of modern varieties (Manohar and Murthy 2012).

The objective of this study was to develop pure lines of Galia-type melons having long shelf life using irradiated pollen and to observe the performance of the GM_3 population on fruit setting, haploid embryo induction and haploid plant obtention.

Materials & Methods

This study was conducted at the Manier Seed Research Station and Biotechnology Center of Cukurova University in Adana. A total of 84 genotypes were used as plant material. Twenty-five

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Table 1. The number of pollinated flowers, number of fruit set, and percent fruit set of Galia-type melons.

Genotype	Number of pollinated flowers	Number of fruit set	Percent fruit set
1-1	11	10	90.9
1-3	6	3	50.0
1-8	13	9	69.2
1-11	7	2	28.6
1-13	21	21	100.0
1-16	11	6	54.5
1-20	18	17	94.4
1-24	11	5	45.5
1-25	5	2	40.0
1-26	14	12	85.7
1-27	17	13	76.5
1-28	19	15	78.9
1-29	2	1	50.0
1-30	4	4	100.0
1-32	15	13	86.7
1-33	14	12	85.7
1-36	22	14	63.6
1-37	15	11	73.3
1-39	1	1	100.0
1-40	5	5	100.0
1-41	9	2	22.2
1-43	33	13	39.4
1-46	2	1	20.0
1-47	13	8	61.5
1-49	17	12	70.6
1-54	19	13	68.4
1-55	15	14	93.3
1-59	3	3	100.0
1-61	15	10	66.7
1-63	7	5	71.4
1-67	19	19	100.0
1-68	3	3	100.0
1-69	18	12	66.7
2-2	22	11	50.0
2-3	23	14	60.9
2-6	20	14	70.0
2-7	25	12	48.0
2-11	24	13	54.2
2-12	24	15	62.5
2-13	17	15	88.2
2-14	1	1	100.0
2-15	21	13	61.9
2-18	1	1	100.0
2-19	20	14	70.0
2-21	18	11	61.1
2-22	11	7	63.6
2-24	21	11	52.4
2-26	21	14	66.7
2-28	22	14	63.6
2-33	23	12	52.2
2-34	22	8	36.4
2-36	25	10	40.0
2-40	26	15	57.7
2-41	19	16	84.2
2-42	19	12	63.2
2-45	3	2	66.7
2-46	17	13	76.5
2-47	24	15	62.5
2-48	5	1	20.0
4	10	9	90.0
9	25	14	56.0
16	23	9	39.1
17	5	4	80.0
20	6	5	83.3
22-2	6	3	50.0
22-4	8	3	37.5
22-5	15	6	40.0
25-1	24	4	16.7
25-6	7	4	57.1
27-8	7	4	57.1
28-1	8	7	87.5
28-6	6	6	100.0
28-10	4	4	100.0
29-3	2	2	100.0
29-6	6	6	100.0
31	20	13	65.0
81-4	20	4	20.0
83-13	2	2	100.0
84-1	3	3	100.0
85-1	2	2	100.0
86-4	2	1	50.0
88-1	3	3	100.0
90-1	2	2	100.0
HT-1	10	5	50.0
Total/ mean	1104	700	63.4

seeds of each genotype were sown in a peat and perlite mixture (2 : 1 v/v), in multipots on 14 February 2015 at Manier. When seedlings had 2 or 3 true leaves, on 14 March 2015, 20 from each genotype were transplanted to a greenhouse and spaced 1 m × 0.5 m apart. Plants were trained on their main stem beginning on April 7, 2015 and pruned regularly. Drip irrigation was used and the plants were treated with fungicides, insecticides, and acaricides against diseases and pests during cultivation period.

Irradiations and pollinations were begun at the onset of flowering. Male flower buds were irradiated seven times, at 2 to 6-day intervals beginning on 28 April 2015. On the day before anthesis, male flowers were collected and their petals and sepals were partially removed. Then they were put into 9-cm glass petri dishes and on the same day irradiated with 300 Gy of Cobalt^{60} for 150 min, at the Akdeniz University, Science Faculty, Department of Physics. Irradiated pollen was stored at room tempera-

Table 2. Mean fruit weight, total haploid embryo number, haploid embryo number per fruit, total haploid plant number, and percent regeneration to plant in local Galia-type melons.

Genotype	Mean fruit weight (g)	Total haploid embryo number	Haploid embryo number per fruit	Total haploid plant number	Regeneration to plant (%)
1-1	1148.6	12	1.2	7	58.3
1-3	1400.2	7	2.3	2	28.6
1-8	1296.7	12	1.3	5	41.7
1-11	1015.0	6	3.0	2	33.3
1-13	1467.7	46	2.2	21	45.7
1-16	1766.0	14	2.3	9	64.3
1-20	1595.1	46	2.7	15	32.6
1-24	1460.8	20	4.0	3	15.0
1-25	1167.0	2	1.0	2	100.0
1-26	1491.4	19	1.6	13	68.4
1-27	1197.5	14	1.1	7	50.0
1-28	1345.1	34	2.3	32	94.1
1-29	702.0	1	1.0	1	100.0
1-30	1158.0	5	1.2	1	20.0
1-32	1337.8	17	1.3	13	76.5
1-33	1461.5	25	2.1	15	60.0
1-36	1209.3	23	1.6	11	47.8
1-37	1230.7	28	2.5	14	50.0
1-39	1200.0	12	12.0	5	41.7
1-40	1646.4	3	0.6	3	100.0
1-41	1322.5	1	0.5	1	100.0
1-43	1546.9	48	3.7	23	47.9
1-46	1483.0	-	-	-	-
1-47	1496.8	17	2.1	9	52.9
1-49	1155.5	65	5.4	18	27.7
1-54	1501.6	20	1.5	8	40.0
1-55	1583.2	16	1.1	11	68.8
1-59	392.6	-	-	-	-
1-61	1516.9	4	0.4	3	75.0
1-63	1280.0	6	1.2	6	100.0
1-67	1382.2	25	1.3	18	72.0
1-68	1327.3	4	1.3	3	75.0
1-69	1211.7	5	0.4	4	80.0
2-2	1806.8	43	3.9	32	74.4
2-3	1638.7	55	3.9	43	78.2
2-6	1529.7	45	3.2	37	82.2
2-7	1745.0	45	3.7	34	75.6
2-11	1497.2	15	1.6	10	66.7
2-12	1286.8	36	1.7	30	83.3
2-13	1608.5	55	3.6	46	83.6
2-14	1099.0	1	1.0	1	100.0
2-15	1175.6	35	2.7	20	57.1
2-18	1555.0	4	4.0	4	100.0
2-19	1179.2	18	1.3	14	77.8
2-21	1726.5	15	1.4	14	93.3
2-22	1767.1	50	7.1	31	62.0
2-24	1728.6	42	3.8	20	47.6
2-26	1317.7	17	1.2	12	70.6
2-28	1504.7	76	5.4	53	69.7
2-33	988.3	12	1.0	7	58.3
2-34	1383.6	32	4.0	15	46.9
2-36	1078.8	19	1.9	15	78.9

Continued **Table 2.**

Genotype	Mean fruit weight (g)	Total haploid embryo number	Haploid embryo number per fruit	Total haploid plant number	Regeneration to plant (%)
2-40	1960.0	27	1.8	26	96.3
2-41	1570.7	101	6.3	81	80.2
2-42	1646.4	33	2.7	23	69.7
2-45	1051.0	14	7.0	8	57.1
2-46	1804.9	55	4.2	48	87.3
2-47	1459.1	44	2.9	35	79.5
2-48	1362.0	2	2.0	2	100.0
4	1077.7	12	1.3	8	66.7
9	1532.7	23	1.6	12	52.2
16	926.3	19	2.1	16	84.2
17	1133.0	9	2.2	7	77.8
20	662.4	26	5.2	16	61.5
22-2	1063.0	13	4.3	8	61.5
22-4	1339.3	6	2.0	6	100.0
22-5	1306.1	8	1.3	8	100.0
25-1	789.0	11	2.7	8	72.7
25-6	862.2	4	1.0	2	50.0
27-8	865.7	14	3.5	3	21.4
28-1	1197.0	30	4.3	11	36.7
28-6	1050.8	20	3.3	12	60.0
28-10	1057.7	19	4.7	8	42.1
29-3	1985.0	19	9.5	6	31.6
29-6	1157.5	19	3.2	9	47.4
31	1610.2	16	1.2	13	81.3
81-4	934.0	5	1.2	4	80.0
83-13	1319.5	9	4.5	3	33.3
84-1	1518.6	17	5.6	5	29.4
85-1	1681.0	14	7.0	2	14.3
86-4	1966.0	4	4.0	4	100.0
88-1	1548.0	22	7.3	22	100.0
90-1	1332.5	23	11.5	9	39.1
HT-1	1912.0	13	2.6	6	46.2
Total/mean	1625.5	1813	2.5	1142	63.0

ture overnight. Also on the day before anthesis, hermaphroditic flowers were emasculated and enclosed in gelatin bags to avoid uncontrolled pollination. On the following day, the female flowers were pollinated with at least three irradiated male flowers, in the early morning (between 06:00 and 09:00). After pollination, the pollinated flowers were covered with a gelatin bag for 3 to 5 days and labeled individually. Fruits were harvested 3 to 4 weeks after pollination and weighed.

Immature parthenogenetic fruits were taken to the tissue culture laboratory of Biotechnology Center of Cukurova University. Fruits were washed with running tap water followed by washing with 1% NaOCl solution and dried on sterile filter paper. Then they were surface sterilized with 96% alcohol by "dry burning". Fruits were cut into two pieces, seeds were extracted, and dissected individually under a flourescent light in a laminar flow cabin. The haploid embryos were cultured in glass jars containing MS medium and incubated in a growth chamber at 25 to 26 °C with a 16/8 h day/night photoperiod. The cultured embryos were transferred to glass tubes containing the same medium when they turned from white to green and the roots and cotyledons were visible. After 7 to 10 days, the cultured embryos had developed into plantlets and were transferred to bigger glass jars containing the same medium until they had 6 to 8 nodes. The regenerated plantlets were then propagated by *in vitro* nodal cuttings. Plants were acclimatized in a plastic greenhouse when reached about 10 cm length. The number of pollinated flowers by irradiated pollen, the number of fruit set after pollination by irradiated pollen, ratio of fruit set (%), mean fruit weight (g), number of haploid embryos, haploid embryo number per fruit, total haploid plant number, and the ratio of regeneration to plant (%) were observed and recorded.

Results & Discussion

A total of 1,104 flowers were pollinated with irradiated pollen and 700 parthenogenetic fruits were obtained (Table 1). The average fruit setting ratio was 63.4%. The highest fruit setting ratio (100%) was obtained from genotypes 1-13, 1-30, 1-39,

1-40, 1-59, 1-67, 1-68, 2-14, 2-18, 28-6, 28-10, 29-3, 29-6, 83-13, 84-1, 85-1, 88-1, and 90-1 while the lowest, 16.7%, occurred in genotype 25-1. Our mean fruit setting ratio is higher than that reported by Sauton and Dumas de Vaulx (1987), who observed 35%. Sari et al. (1999) observed fruit setting from pollen irradiated with 300 Gy of Cobalt^{60} between 25% and 54%. Similarly, Sari et al. (2010) used 300 Gy of Cobalt^{60} for irradiation and obtained fruit set of 42% in 'Kirkagac' and 46% in 'Yuva-Hasanbey' melons, respectively. Zhang et al. (2006) obtained haploid plants from two genotypes, one of *C. melo* subsp. *melo* and one of *C. melo* subsp. *agrestis* var. *conomon*. Observed average fruit set was 50% and 10%, with γ-radiation doses of 300 and 600 Gy, respectively. A high rate of fruit set (71.6%) was observed by Ari et al. (2010) using a 300 Gy dose of Cobalt^{60}. Others (Sauton and Dumas de Vaulx 1987, Abak et al. 1996) reported that the success of haploid embryo induction was dependent on genotype.

Mean fruit weight of 84 melon genotypes, total haploid embryo number, embryo number per fruit, and rate of regeneration to plant are presented in Table 2. Mean weight of 700 immature fruits was 1625.5 g. A total of 1,813 embryos were rescued and the mean number of embryos per fruit was 2.5. The highest embryo number per fruit was 12, and obtained from the genotype 1-39. Among the cultured embryos, 1,142 transformed into plants and the average regeneration rate was 63%. The highest haploid plant number was obtained from genotypes 2-28, 2-46, and 2-13 (53, 48, and 46 plants respectively). The highest transformation rate, 100%, of haploid embryos to haploid plants, was obtained from genotypes 1-25, 1-29, 1-40, 1-41, 1-63, 2-14, 2-18, 2-48, 22-4, 22-5, 86-4, and 88-1, and the lowest was 14.3% in genotype 85-1. Sari et al. (1999) reported that they rescued 552 embryos from 147 parthenogenetic melons. Zhang et al. (2006) found the induction rate of haploids as 0.55 % and 0.63%, respectively, from a subsp. *melo* and a subsp. *agrestis* var. *conomon* melon. Sari et al. (2010) rescued 1,255 embryos from 625 parthenogenetic melon fruits, of which 860 embryos transformed into plants. The difference in haploid embryo induction rate observed among different genotypes, as reported by Zhang et al. (2006), the maternal genotypes, and irradiation doses had differential effects on both fruit setting and haploid production.

The use of irradiated pollen in applied breeding has been limited by the low percentage of haploid embryos (Lotfi et al. 2003). However, the rate of success with melon is higher than with other Cucurbitaceae, and an efficient methodology has been developed by us for application to our melon breeding programmes.

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Genetic Diversity Analysis of Winter Squash (*Cucurbita maxima* Duchesne) Accessions Using SSR Markers

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Abstract. Genetic diversity studies are important to determine the level of within-species variation, to identify varieties, and to help select lines for breeding programs. Despite the fact that winter squash is a highly diverse cultivated cucurbit, its genetic diversity is poorly characterized at the molecular level. SSR markers are the most popular and reliable markers used for this purpose. We used a set of 23 SSR markers derived from *Cucurbita pepo* and *C. moschata* to assess genetic diversity within a collection of 85 *C. maxima* accessions. This collection comprised of cultivars, breeding lines, and landraces from Europe, North America, Asia, and Australia. Using neighbor joining analysis, two major clusters of 26 and 55 accessions were clearly distinguished. The former contained accessions from Asian and American groups (kabocha, hubbard, butternut, and banana) while the second one contained accessions from Europe and North America (mammoth pumpkins and landraces). Ornamental and Australian accessions were located outside the two major clusters. Principal coordinate analysis did not group accessions according to the type of growth habit or country, instead grouping them according to cultivar group and region of origin. This work shows that SSR markers can be successfully applied to study genetic diversity with *C. maxima*.

Keywords: *Cucurbita maxima,* genetic diversity, pumpkin, SSR markers, winter squash

Introduction

Cucurbita maxima Duchesne (winter squash, pumpkin) is one of the most important and diverse species of the genus *Cucurbita*. *Cucurbita* is native to the Americas and was introduced to Europe in the 16th century (Paris 2001). Pumpkins and squash spread rapidly from Europe to other continents (Decker-Walters and Walters 2000). Asia is considered to be secondary center of diversity for *C. maxima* (Esquinas-Alcazar and Gulick 1983). Winter squash fruits are a great source of carbohydrates, vitamins, minerals, and fatty acids, and are used in the food processing industry, also for feeding animals and as a fresh product for human consumption. *C. maxima* shows adaptation to different agroecological conditions and exhibits great variability for potentially valuable horticultural traits. Despite its economic and nutritional importance, there are still but few molecular genetic characterizations of *C. maxima*. Molecular markers and genetic diversity studies are important for increasing precision and efficiency of breeding new, potentially valuable cultivars by accelerating the selection process, helping to determine the level of variation, and selecting parental lines in breeding programs. So far, genetic diversity among *C. maxima* accessions at the molecular level has been investigated only by Ferriol et al. (2003, 2004). Those authors used dominant marker systems to assess genetic diversity of *C. maxima* accessions limited to particular geographic regions. Kong et al. (2014) used SSR markers to study genetic diversity of *Cucurbita* rootstock germplasm that included *C. maxima* accessions. SSR markers are among the most popular and reliable markers for genetic diversity studies (Zhang et al. 2012, Raghami et al. 2014, Yildiz et al. 2015). Therefore, there is a need to explore broad collection of winter squash accessions using codominant markers such as SSRs. In the present work, we assessed genetic diversity of *C. maxima* accessions using SSR markers. The results should help facilitate the designing of *C. maxima* breeding programs.

Materials & Methods

Plant material and DNA extraction

A total of 85 *C. maxima* accessions and a single *C. pepo* accession (outgroup) were used in this study (Table 1). The accessions included 28 cultivars, 36 landraces, and 19 breeding lines of *C. maxima* from Europe, North America, Asia, Australia, and

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New Zealand. All accessions came from the collection of the Department of Plant Genetics, Breeding, and Biotechnology, Warsaw University of Life Sciences, Poland. GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) was used to extract DNA from leaves collected from 3- to 4-weeks-old greenhouse-grown plants, according to the manufacturer's instruction. DNA was qualified with a NanoDrop 2000 (Thermo Scientific) spectrophotometer and diluted in water to a final concentration of 30 ng/µl.

SSR markers analysis

A set of 23 SSR markers, derived from *C. pepo* and *C. moschata* by Gong et al. (2008), was selected and used in this study. PCR reactions were performed in a total volume of 15 µl, according to DreamTaq polymerase (Thermo Scientific) manufacturer's instruction. The SSR PCR reactions were performed using Mastercycler EP Gradient S (Eppendorf). The cycling program described by Pillen et al. (2000) was used. The PCR products were analyzed using 6% polyacrylamide gel electrophoresis in 1 × TBE buffer. The gels were stained using silver-staining method described by Benbouza et al. (2006).

Data analysis

The polymorphic bands of each SSR marker were scored for their presence (1) or absence (0). The binary matrix was converted into the required data input format based on the manuals of the software used. Calculations for each marker included: the number of alleles per locus, expected heterozygosity (He) by Nei (1978), and polymorphism identification content (PIC) by Botstein et al. (1980). Cluster analysis was performed on the genetic distance matrix by using the neighbor-joining method to determine the relationships among accessions based on estimated similarity by Dice coefficient using the NTSYS 2.21 program (Exeter Software). Principal coordinate analysis (PCoA) was carried out using the Dice genetic distance matrix and DCENTER, EIGEN and MXPLOT procedures of NTSYS 2.21.

Results & Discussion

The 23 SSR markers generated a total of 99 alleles, an average of 4.3 alleles per marker (Table 2). This average is similar to that observed by Ferriol et al. (2003) using RAPD and

Table 1. List of the 85 *Cucurbita maxima* accessions and single *C. pepo* accession used for SSR genotyping.

No.	Name	Country	No.	Name	Country	No.	Name	Country
1	*C. pepo*	Romania	30	WUKR 06-1030	Ukraine	59	Sweetmeat	USA
2	L 801	Ukraine	31	WUKR 06-1094	Ukraine	60	474/12	China
3	L 802	Japan	32	WUKR 06-1204	Ukraine	61	476/12	China
4	RCA TO 57226	Hungary	33	WUKR 06-0868	Ukraine	62	POLSOK 00- 49	Poland
5	RCA TO 35431	Hungary	34	Dovleac placintar	Hungary	63	POLAUG 01-46	Poland
6	RCA TO 54832	Hungary	35	Cha-cha	USA	64	POLTAR 00 - 87	Poland
7	RCA TO 72875	Hungary	36	Red Warty	USA	65	POLKILO7-068	Poland
8	Acmariu (Alba)	Romania	37	Vesuv	unknown	66	POLKILO7 - 071	Poland
9	Bixad Satumare	Romania	38	Blue Ballet	USA	67	POLSAN11 - 21	Poland
10	ROMTRA 03-73	Romania	39	Delica	Japan	68	12011/00243	Poland
11	LIT2AP 12-123	Lithuania	40	Crown Prince	Australia	69	12011/00257	Poland
12	LIT2AP 12-124	Lithuania	41	Blue Ballet	USA	70	12011/002171	Poland
13	LITLIT 11- 096	Lithuania	42	Gelber Zentner	Germany	71	L317/98	Poland
14	LITLIT 11- 180	Lithuania	43	Big Max	USA	72	L333/98	Poland
15	LITLIT 11- 181	Lithuania	44	Big Moon	USA	73	L330/98	Poland
16	LITLIT 11- 214	Lithuania	45	Rouge vif d'Etampes	France	74	Ambar	Poland
17	LITLIT 11- 280	Lithuania	46	Lumina	USA	75	Karowita Bis	Poland
18	LITLIT 11- 196	Lithuania	47	Pink Banana	USA	76	R_o L770/06	Poland
19	LITLIT 11- 076	Lithuania	48	Buttercup	USA	77	R_o L 772/06	Poland
20	LITLIT 11- 077	Lithuania	49	Blue Ballet	USA	78	R_m L 771/06	Poland
21	LITLIT 11- 093	Lithuania	50	Bambino	Poland	79	L672/07	Poland
22	Wołżskaja seraja	Lithuania	51	Melonowa Żółta	Poland	80	L673/2007	Poland
23	Załuszka	Lithuania	52	771/1	USA	81	674/2007	Poland
24	Apunha	Lithuania	53	Uchiki Kurii	Japan	82	L 341/98	Poland
25	Zimnaja słodkaja	Lithuania	54	Orange Hokkaido	Japan	83	POLSOK 00- 52	Poland
26	Liczebnaja	Lithuania	55	Prizewinner	USA	84	Rouge tres hatif d'Etampes	France
27	Kustowaja oranżewaja	Lithuania	56	Whanga Crown	New Zealand	85	Gele Renzen	Germany
28	Odeski	Ukraine	57	Queensland Blue	Australia	86	Atlantic Giant	USA
29	WUKR 06-0911	Ukraine	58	Uchiki Kurii	Japan			

Table 2. Properties of SSR markers used for *C. maxima* accessions genotyping. PIC value = polymorphism identification content value, calculated as described by Botstein et al. (1980); He = expected heterozygosity value, calculated as described by Nei (1987).

Marker name	Number of alleles	PIC value	He value
SSR1	4	0.59	0.53
SSR2	10	0.67	0.70
SSR3	3	0.48	0.49
SSR4	6	0.45	0.51
SSR5	5	0.39	0.51
SSR6	4	0.62	0.68
SSR7	4	0.27	0.36
SSR8	4	0.52	0.6
SSR9	5	0.71	0.74
SSR10	4	0.53	0.59
SSR11	4	0.64	0.66
SSR12	4	0.47	0.53
SSR13	3	0.39	0.42
SSR14	4	0.60	0.64
SSR15	3	0.35	0.46
SSR16	3	0.23	0.27
SSR17	3	0.46	0.56
SSR18	6	0.75	0.77
SSR19	6	0.42	0.52
SSR20	4	0.56	0.58
SSR21	3	0.53	0.58
SSR22	3	0.51	0.57
SSR23	4	0.58	0.59

SBAP markers. However, the mean genetic distance obtained by Ferriol et al. (2003) was much lower than in the present study. The range of dissimilarity conducted in our research varied from 0.0 (between accessions 41 and 49, and between accessions 58 and 71) to 1.0 (between accessions 2 and 3). The mean genetic distance among the *C. maxima* accessions was 0.56. The mean distance among accessions of *C. maxima* was lower than the mean distance between them and the *C. pepo* accession used as an outlying genotype. The majority of the SSR markers produced unique bands for the *C. pepo* accession, in accordance with the observations of Ferriol et al. (2003), who also observed *Cucurbita* species-specific bands. Hence the usefulness of this set of SSR markers to assess genetic diversity and species identity within *Cucurbita* genera.

The average Nei's expected heterozygosity (He, gene diversity across alleles) values, that can be considered as a measure of genetic variability, ranged from 0.27 to 0.74 and on average 0.56 per marker. The 23 SSR markers recorded a mean PIC value of 0.51, indicative of the discriminating nature of these markers. Similar results showing high PIC values were reported by others (Zhang et al. 2012, Raghami et al. 2014). Markers with PICs of more than 0.5 are efficient in discriminating genotypes and extremely useful in detecting the polymorphism rate at a particular locus (DeWoody et al. 1995).

Cluster analysis divided the *Cucurbita maxima* accessions into two groups according to geographical origin and type of varietal group, described by Ferriol and Pico (2008). Group I contained the majority of the cultivars originating from Asia and the U.S.A.; these belong to the varietal groups Kabocha, Buttercup, Hubbard, and Banana, and related accessions from Poland. The connections among these varietal groups are not clear except between the Kabocha and Buttercup varietal groups; according to Ferriol and Pico (2008), the Kabocha was developed by Japanese breeders by crossing Buttercup varieties with Japanese varieties. Group II contained two sub-clusters, IIa and IIb. Sub-cluster IIa included landraces from Central and Eastern Europe. Cluster IIb consisted mostly of cultivars from the Mammoth group that originated from the U.S.A. and Western Europe, and other accessions with large fruit size, probably with a common origin. Ornamental accessions with turban shaped fruit separated from the other *C. maxima* accessions. Two Australian cultivars and one New Zealand cultivar, belonging to the Australian Blue group, were located at the greatest distance from the other *C. maxima* cultivars.

The principal coordinate analysis also grouped the *Cucurbita maxima* accessions according to varietal group and region of origin. PCoA analysis clearly divided *C. maxima* cultivars into three groups. One group included cultivars from the Banana, Hubbard, Buttercup, and Kabocha varietal groups. Another group consisted of two cultivars from the Mammoth varietal group. The third group consisted of three cultivars from the Australian Blue varietal group. In a previous study (Ferriol et al. 2004), PCoA analysis also grouped *C. maxima* landraces according to geographical origin and additionally according to type of use.

Introduction of F_1 hybrids have played a significant role in winter squash breeding. Winter squash exhibits heterosis for traits such as earlier flowering, earlier fruit maturity, increased fruit number per plant, and increased fruit size (Ferriol and Pico 2008). Selection of the most distant parental varieties is extremely important for hybrid breeding. This study provides insights into the genetic relationships among commercial cultivars, landraces and breeding lines of *C. maxima*, of potential use in hybrid breeding.

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Inheritance of Tolerance to *Tomato Leaf Curl New Delhi Virus* (ToLCNDV) in Melon

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Abstract. Since 2012, a new strain of *Tomato leaf curl New Delhi virus* (ToLCNDV) has been responsible for severe crop losses in cucurbits grown in southeastern Spain. Zucchini (*Cucurbita pepo*) and melon (*Cucumis melo*), two of the most valuable crops in the area, are severely affected. We screened a core collection of *C. melo* (including accessions of subspecies *melo* and *agrestis*) using mechanical inoculation to evaluate their response to ToLCNDV. Tolerance to the virus infection was found in three accessions of variety *momordica* (*C. melo* subsp. *agrestis* var. *momordica*) and in two wild subsp. *agrestis* accessions, all from India. Plants of the tolerant accessions were crossed with plants of the cultivar Piñonet Piel de Sapo (PS) (*Cucumis melo* subsp. *melo* var. *inodorus*), highly susceptible to the virus. Hybrids with three of these tolerant sources had a behavior similar to that of their tolerant parents. Segregating generations were produced to study the genetic control of tolerance. The F_2 population, obtained from the cross between the subsp. *agrestis* accession WM-7, the one with the highest level of tolerance, and PS, was evaluated. All plants were rated for tolerance at 15 and 30 days after inoculation (DAI), and the level of virus was analyzed by quantitative PCR. At the end of the assay, a 3 : 1 ratio of tolerant : susceptible plants was found, suggesting a single gene with dominance for tolerance. This population is now being genotyped with a SNP panel to map the tolerance gene and identify markers useful for MAS, which could facilitate the introgression of tolerance into cultivars.

Keywords: *Cucumis melo*, hybrid, genetic control, segregation, ToLCNDV, tolerance.

Introduction

Tomato leaf curl New Delhi virus (ToLCNDV) was first detected in southeastern Spain in 2012 and 2013 in the infected zucchini crops in Andalucía and Murcia. Later, many cucurbit crops, including squash, melon, cucumber and watermelon, were described as hosts for the Spanish ToLCNDV isolate in the same region (Font and Alfaro 2015, López et al. 2015). Initially, ToLCNDV was most damaging on zucchini crops, but recently losses of melon have been catastrophic in both intensive production areas of southeastern Spain and traditional open field cultivation areas of central Spain, such as Castilla la Mancha, where the virus affected up to 80% of the late melon crop (Promelón 2016).

The most efficient way of reducing virus incidence is the establishment of integrated control measures in the production areas and the use of resistant cultivars. However, resistant melon varieties are not available.

Fortunately, large variability is known in *C. melo,* a species that is divided into two subspecies, *melo* and *agrestis* (Esteras et al. 2013). Sources of resistance to viruses have been identified, mainly among Indian accessions of subsp. *agrestis*, the set with the highest genetic diversity (Pitrat 2008, Dhillon et al. 2012).

López et al. (2015) developed a mechanical inoculation method for ToLCNDV that was used to screen a core collection of melons, including a set of accessions representative of the Indian variability. This screening provided five sources of tolerance to the virus, all belonging to *C. melo* subsp. *agrestis*, three accessions of the cultivated *momordica* variety and two wild types.

In this paper, we started the study of the genetics of the tolerance found in some of these selected sources. We inoculated mechanically, rated symptoms, and used quantitative PCR to characterize the response of the segregating populations.

Materials & Methods

Seeds of the tolerant accessions (López et al. 2015) were initially supplied by USDA-NPGS and COMAV genebanks (PI 414723 and 'Kharbuja') and by the Indian collection of N. Dhillon (WM7) (Dhillon et al. 2012). Seeds were multiplied by selfing in greenhouses of the COMAV. Six plants of each genotype were grown in a greenhouse and F_1 hybrid progenies were generated by crossing plants of the tolerant accessions to plants of

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the susceptible PS (Piñonet Piel de Sapo) control. Seeds of the different F_1 generations were also cultivated in the greenhouse and self pollinated to generate the F_2 segregating populations.

Seeds of the parents and the three F_1 populations were germinated in Petri plates, along with the PS control, and eight plants per parent and hybrid were mechanically inoculated with an infectious clone of ToLCNDV, as described in López et al. (2015). One plant of each parent and F_1 was left as a negative control. Plants of PS were inoculated with ToLCNDV and used as positive controls.

The response of the parents, previously reported in López et al. (2015), and the corresponding F_1, was confirmed by symptoms and by measuring viral titer with quantitative PCR. Symptoms were rated on a visual scale between 0 (absence of symptoms) and 4 (highly severe symptoms) at 15 and 30 days after inoculation (DAI). At 30 DAI, the relative accumulation of ToLCNDV was obtained by quantitative PCR using the comparative Ct (Cycle Threshold) method, with the CmPEROX gene as internal standard and the amplification protocol described by Sáez et al. (2016). The relative accumulation of the virus was calculated using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ is the difference between the ΔCt of each sample and the ΔCt of the calibrator sample. The tolerant parental WM7 was used as calibrator in all assays.

After the screening of parents and F_1 hybrids, the offspring of WM7, the accession with one of the best responses to ToLCNDV infection, was selected for the study of the genetics of tolerance. A population of 160 F_2 plants was assayed using the same methodology described above.

Results & Discussion

Response of the parents and F_1 progenies

Mechanical inoculation caused a very severe infection in the susceptible control, as expected. The 'Piel de Sapo' cultivar displayed very severe ToLCNDV symptoms (3 to 4) as early as 15 DAI (Figure 1A,B;2). The plants remained severely affected and with high viral titers at 30 DAI (Figure 3). Different levels of tolerance were found in the assayed sources and their F_1 progenies (Figure 1C-F;2).

There were differences in the response of the two *momordica* accessions. Kharbuja was less tolerant than PI 414723, with higher symptoms scores at 30 DAI and much higher viral accumulation. Differences between these two *momordica* accessions were also clear in their F_1 hybrids with PS (Figures 1C,D;2;3).

The hybrid 'Kharbuja' × PS reached moderate symptoms (Figure 1D), whereas some plants of PI 414723 displayed moderate symptoms at the beginning of the assay, but recovered and remained symptomless at 30 DAI. Both hybrids had viral titers about 2 to 4 times lower than those found in the susceptible control (Figure 3).

In general, the wild type *agrestis* WM7 was the most tolerant to the virus infection, with only some plants having mild symptoms at 30 DAI. Its F_1 progeny had a similar behavior with all plants symptomless during the assay (Figures 1E,F;2;3).

According with the lower level of symptoms, this genotype showed viral titers more than 50 times lower than that of the PS control (Figure 3).

Our results confirmed the existence of different levels of tolerance to the virus in these three sources (López et al. 2015). Other wild melon genotypes from India have been reported to be highly resistant to viruses (Roy et al. 2012). ToLCNDV was first detected in India, and all tolerant accessions studied here are from India, suggesting a plant-virus co-evolution in this area (Dhillon et al. 2012).

The tolerant behavior of F_1 progenies suggests dominance for tolerance. Due to the more tolerant response of the WM7 × PS hybrid, this was selected for the inheritance study.

F_2 segregating generation

The F_2 progeny of WM7 × PS was, as expected, segregating for tolerance to ToLCNDV. Figure 4 shows the infection progress from 15 to 30 DAI. At the end of the assay most of

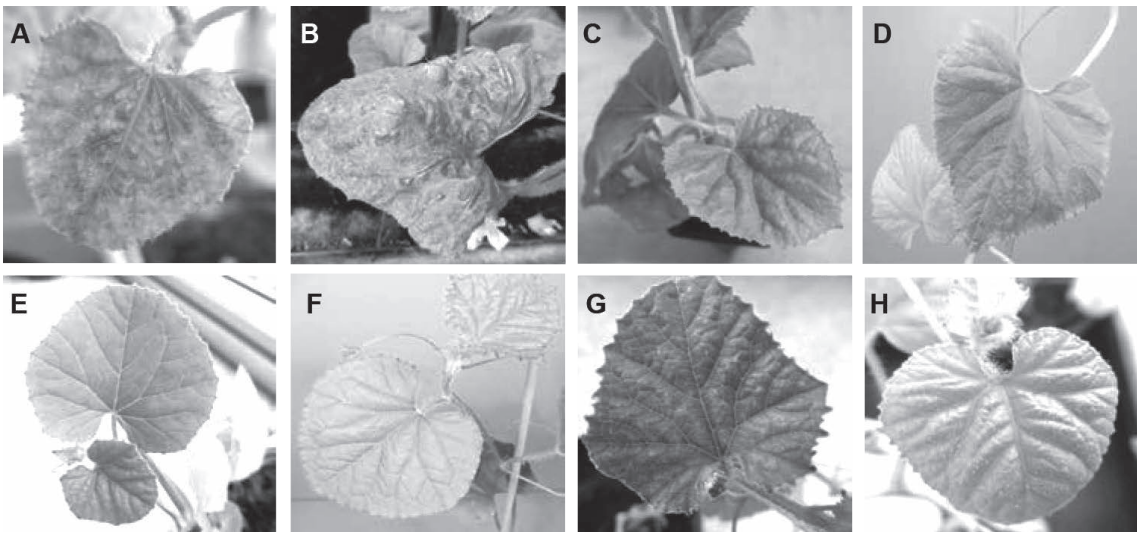

Figure 1. (A,B) severe symptoms of ToLCNDV in plants of PS; (C) asymptomatic plant of 'Kharbuja'; (D) asymptomatic plant of 'Kharbuja' × PS; (E) asymptomatic plant of *C. melo* subsp. *agrestis* WM7; (F) asymptomatic plant of WM7 × PS; (G) susceptible plant of the F_2 population from a WM7 × PS hybrid; (H) tolerant plant of F_2 population from a WM7 × PS hybrid.

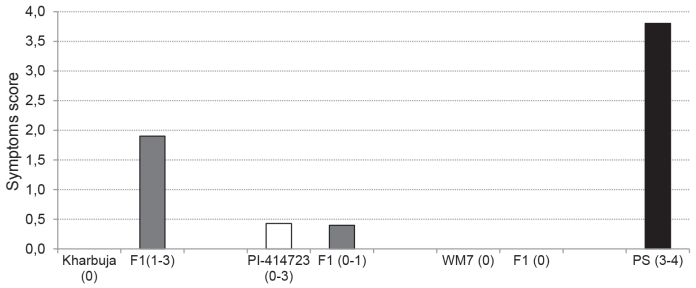

Figure 2. Mean symptom rating of the susceptible control PS, the tolerant accessions of subspecies *agrestis* ('Kharbuja', PI 414723, and WM7) and their F_1 progenies, 15 days after mechanical inoculation with ToLCNDV. The range of symptoms for each genotype is shown in parentheses.

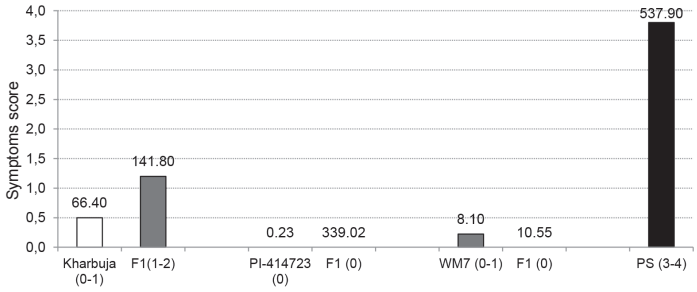

Figure 3. Mean symptom ratings of the susceptible control PS, the tolerant accessions of subspecies *agrestis* ('Kharbuja', PI 414723, and WM7) and their F_1 progenies 30 days after mechanical inoculation with ToLCNDV. The range of symptoms for each genotype is showed in parentheses. The numbers over the bars represent the viral accumulation after quantitative PCR analysis.

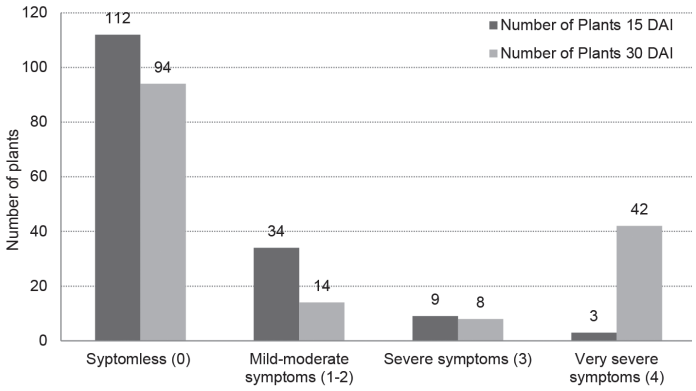

Figure 4. Number of plants of F_2 population in each rating category of symptoms 15 and 30 days after mechanical inoculation with ToLCNDV.

the plants were clearly symptomless (94 plants with rating 0) (Figure 1G), or highly susceptible with very severe symptoms (42 plants with rating 4) (Figure 1H). The severely affected plants developed symptoms of curling and yellow spotting typical of ToLCNDV. However, these were not as severe as typical symptoms of PS (Figure 1A,B), suggesting an influence of genetic background in symptom triggering.

Some plants developed mild symptoms (14 plants with ratings 1 to 2) and some others do not reach very severe, but severe, symptoms at 30 DAI (8 plants with rating 3).

A preliminary analysis of the population showed that symptomless and highly susceptible plants clearly differed in viral titer estimated by quantitative PCR ($\Delta\Delta$Ct = 0.2 *versus* 34.1 in symptomless and plants with very severe symptoms, respectively).

The observed segregation (108 tolerant : 50 susceptible, with symptoms ≤ 2 and ≥ 3 respectively) fits a tolerant : susceptible ratio of 3 : 1 ($\chi^2 = 3.35$, $p = 0.016$) that suggests a monogenetic control with dominance for tolerance. The availability of dense genetic maps and SNP collections in melons (Esteras et al. 2013, Leida et al. 2015) is allowing the identification of molecular markers linked to tolerance. That will facilitate the use of marker assisted selection of ToLCNDV in breeding programs.

Acknowledgements

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Differential Effects of Drought Stress on Germination and Seedling Growth of Cucumber Accessions

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Abstract. Development of new cucumber cultivars with tolerance to water deficit is one of the ultimate methods to overcome the problem with this abiotic stress, and could be assisted by screening the germplasm for higher drought tolerance. The aim of this study was to evaluate the effects of drought stress on germination ability and growth of cucumber seedlings, and identify promising germplasm for further use in cucumber breeding programs. We assessed 16 accessions for germinability and early seedling growth using 18% PEG (polyethylene glycol 8000) to induce drought stress. The results showed that treatment with 18% PEG significantly decreased germination percentage, total root length, hypocotyl length, and fresh weight in comparison with control treatment (H_2O). Among the tested accessions, differences in germination ability were noticed regarding following parameters: energy of germination (GE), germination index (GI), and mean number of days to germination (DTG). The fastest and highest germination in 18% PEG was observed in PW 2A, PW 2, and Ames 4759, which had GEs of 48, 32, and 20%, respectively, on the second day. These accessions also showed the highest germination index (GI = 7.06 to 8.55), and the lowest average time to germination (DTG = 3.29 to 3.68). No correlation between hypocotyl length and total root length under control conditions was observed. However, a high correlation between these two parameters was noticed under PEG treatment (r = 0.69), indicating that drought stress reduced growth of whole seedlings. Two accessions, SU 2 and DM 97, exhibited the smallest losses in root length, hypocotyl length, and fresh weight of drought-stressed in comparison with non-stressed seedlings.

Keywords: *Cucumis sativus*, germination ability, seedling stage, water deficit

Introduction

Drought stress is a common problem occurring in different geographical regions worldwide. Cucumber (*Cucumis sativus* L.), one of the most popular vegetables, is a shallow-rooted crop that has been reported to have little tolerance for water deficiency (Li et al. 2011). Drought, due to its osmotic effect, has been widely studied in many crops, but not in cucumber, although cucumber seedlings are more sensitive to water fluctuations as compared with older plants (Liu et al. 2009, Li et al. 2011). Therefore, germination and early seedling growth might be a useful criterion in screening for drought stress tolerance. Some authors showed that genotypes that were tolerant to drought during *in vitro* germination tests were also tolerant under field conditions (Khakwani et al. 2011, Agili et al. 2012). In addition, Muscolo et al. (2014) found that seed germination and root length can be used as traits for rapid selection of drought tolerance in lentils. There are many reports that connect exposure to polyethylene glycol (PEG) and water deficiency in plants (Hohl and Schopfer 1991, Foolad et al. 2003, Wang et al. 2003, Türkan et al. 2005, Zhu et al. 2006, Ahmad et al. 2009, Muscolo et al. 2014). However, in cucumber, drought-stress tolerance is still a challenge to identify, especially in seedlings, as an aid to cucumber breeders in their breeding programs.

The objective of this study was to search for and identify sources of drought tolerance in cucumber germplasm, based on seed germination and growth of young seedlings subjected to drought stress.

Materials & Methods

We screened 16 cucumber accessions for germination and seedling development under 18% PEG_{8000} (polyethylene glycol) in order to identify drought-tolerant germplasm (Table 1, Figure 1). Four of the accessions, PW 2, PW 2A, SU 2, and DM 97, were bred at the Research Institute of Horticulture, Skierniewice, Poland and the remaining ones were obtained from the North Central Regional Plant Introduction Station, Ames, Iowa, U.S.A. A concentration of 18% PEG was used because it was the most differentiating concentration (15, 18, 25%) in our previous tests of cucumber germination and early seedling development (Kłosińska et al. 2016). Germination testing was conducted in an unlighted incubator set at 25±1 °C. For each of the 16 accessions, 200 seeds in four replicates (50 seeds/replication/dish) were placed in square dishes (10 × 10 cm), lined with two layers of absorbent filter paper saturated with 4 mL of 18% PEG_{8000} or 4 mL of ddH_2O (control). The dishes were sealed with parafilm

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to prevent evaporation. Seeds were considered germinated when the radicle had extended for at least 3 mm. Seed germination was observed and recorded daily for seven days. Data were expressed as energy of germination (GE), germination index (GI), and mean number of days to germination (DTG) (Kłosińska et al. 2013). Seedling growth tests were conducted in a growth chamber set at 24±1 °C under a 12 h photoperiod. For each of the 16 accessions, 50 water-germinated seeds (radicle ≥10 mm) were placed on filter paper in each dish saturated with 5 mL 18% PEG_{8000}, while for the control 5 mL ddH_2O were added. For each accession under each condition (stress or control), three independent biological replicates of one dish were scored after seven days using a camera image and the WinRHIZO program (Regent Instruments, Québec, Canada). Measurements of entire root systems (main and lateral) and hypocotyl length were provided on scanned images of seedlings.

The results of the tests were analyzed using the program STATISTICA 12.0 (StatSoft). Significant differences among means were verified using the Newman-Keuls test at $\alpha = 0.05$.

Results & Discussion

Germination test

Seeds exposed to unfavorable environmental conditions such as drought may compromise subsequent seedling establishment (Albuquerque and Carvalho 2003, Soleymani et al. 2012). In our tests, all cucumber accessions had 100% germination under control conditions (H_2O) within the first or second day (data not presented). However, under drought stress, differences in germination ability among tested lines were noticed regarding GE, GI, and DTG (Table 1). On the second day, half of the accessions did not yet germinate and therefore the smallest differences in GE among the cultigens were observed on the following days of the test. The fastest and highest germination in 18% PEG was observed for accessions PW 2A, PW 2, and Ames 4759, with a GE of 48, 32 and 20% on the second day, respectively. These lines also showed the highest germination index (GI = 7.06 to 8.55), and the lowest average time to germination (DTG = 3.29 to 3.68) (Table 1). In contrast, PI 271326, PI 379282, and PI 391573 had the lowest germinability under drought stress: PI 379282 and PI 391573 had 7.3% of GE on the seventh day of the experiment and PI 271326 did not germinate at all in 18% PEG (Table 1). Zhang et al. (2013) also applied 18% PEG to assess the impact of melatonin on cucumber seed germination under water deficit and found that PEG treatment reduced the germination energy of a Chinese cucumber in comparison with the control treatment. Our results also showed a negative impact of water deficit on the germination ability of all 16 cucumber accessions. PEG in concentrations above 15% has been observed to significantly decrease germination in tomato, rice, corn, lentil, and wheat (Hohl and Schopfer 1991, Basha et al. 2003, Wang et al. 2003, Muscolo et al. 2014).

To the best of our knowledge, our results constitute the first report of differences among cucumber accessions in germination ability under drought stress. Among 16 cucumber accessions, three (PW 2A, PW 2, Ames 4759) showed a high level of drought tolerance, confirmed by high GE, large GI, and low DTG. In contrast, PI 271326 failed to germinate under 18% PEG and might be useful as a control in further work. The higher germination rates of the tolerant genotypes may be due to their capacity to absorb water even under PEG-induced drought stress.

Seedling test

Seedling growth and development of all 16 cucumber accessions were inhibited with 18% PEG treatment. All cultigens had

Table 1. Germination ability at 18% PEG of 16 cucumber accessions.

Accession	Country of origin	Energy of germination (GE) in %			Germination index (GI)	Days to germination (DTG)
		2nd day	4th day	7th day		
PW 2A*	Poland	47.92 a	84.38 a	98.96 a	8.55 a	3.29 d
PW 2*	Poland	32.29 b	72.92 c	90.63 c	7.06 b	3.68 bcd
Ames 4759	USA	19.79 c	72.92 c	95.83 ab	7.23 b	3.42 d
PI 197086	India	10.42 d	71.88 c	89.58 cd	6.74 b	3.50 cd
Ames 2353	USA	9.38 d	76.04 b	87.50 d	6.16 b	3.71 bcd
PI 267086	Russia	3.13 e	42.71 e	67.71 g	3.95 c	4.66 bcd
PI 212233	Japan	1.04 f	38.54 f	44.79 h	2.72 d	4.07 bcd
DM 97*	Poland	1.04 f	12.50 i	30.21 j	1.73 de	4.55 bcd
PI 330628	Pakistan	0.00 f	78.13 b	93.75 b	6.25 b	3.88 bcd
Ames 7752	USA	0.00 f	63.54 d	81.25 e	4.80 c	4.38 bcd
SU 2*	Poland	0.00 f	29.17 g	75.00 f	3.79 c	5.22 b
PI 197088	India	0.00 f	17.71 h	28.13 j	1.66 de	4.26 bcd
PI 188807	Philippines	0.00 f	14.58 i	37.50 i	1.79 de	5.27 b
PI 379282	Yugoslavia	0.00 f	2.08 j	7.29 k	0.35 f	5.14 bc
PI 391573	China	0.00 f	2.08 j	7.29 k	0.35 f	5.14 bc
PI 271326	India	0.00 f	0.00 j	0.00 l	0.00 f	7.00 a

*Bred at the Research Institute of Horticulture (RIH), Skierniewice, Poland.
Means followed by the same letter within each column are not significantly different at $\alpha = 0.05$.

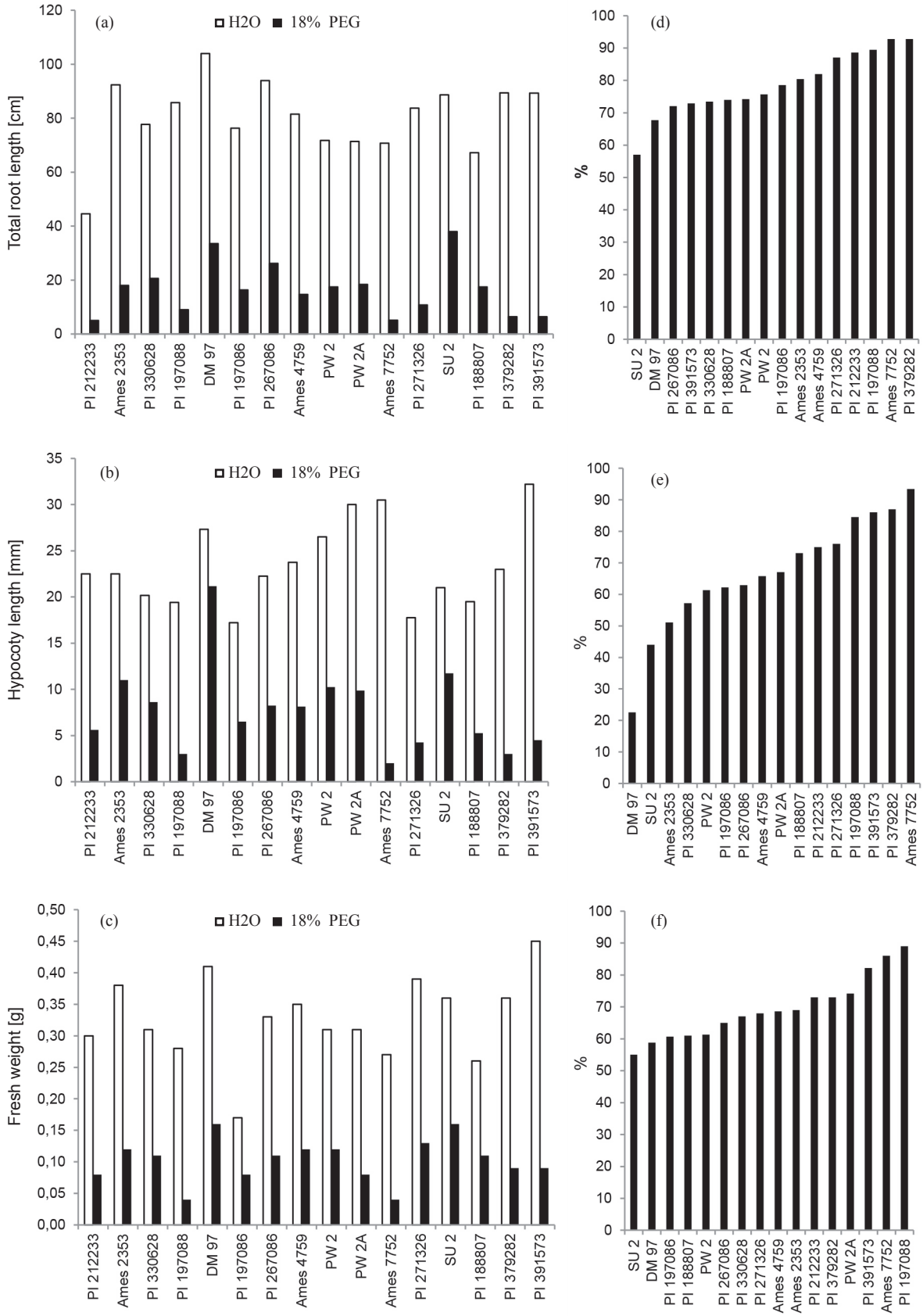

Figure 1. Total root length (a), hypocotyl length (b), and fresh weight (c) of seedlings of 16 cucumber accessions under the control treatment (H_2O) and under 18% PEG treatment; and percent decrease in total root length (d), hypocotyl length (e), and fresh weight (f) under the 18% PEG treatment in comparison with the control treatment (H_2O).

significantly lower total root length in 18% PEG than in the control treatment (Figure 1a). Seven accessions, DM 97, PI 267086, Ames 2353, SU 2, PI 379282, PI 391573, and PI 271326 had the longest total root system (85 to 105 cm) under the control treatment. Four of them, SU 2, DM 97, PI 267086, PI 391573, also had the longest root system under 18% PEG. The shortest root system was observed in PI 212233, in both the control and drought-stress treatments. Also, this genotype had the highest reduction of root length in comparison to the control (Figure 1d). Drought stress limited total root length more than 75% as compared with the control in nine of the cucumber accessions. The smallest impact of 18% PEG treatment on limiting root growth, 55%, was observed in accession SU 2 (Figure 1d). These results are consistent with findings obtained for a Chinese cucumber (Zhang et al. 2013). Moreover, those authors reported a limiting effect of drought stress on lateral root formation, which we also observed. On the contrary, Turkan et al. (2004) reported that PEG treatment increased fresh weight and root length of bean seedlings grown in perlite. This phenomenon could be explained by water absorption of the perlite that might have stimulated better seedling growth.

We also studied the influence of PEG on hypocotyl length of the cucumber accessions (Figure 1b,e). The results showed significant differences among accessions for this trait. Under the control conditions, PI 391573, Ames 7752, and PW 2A had the longest hypocotyls whilst PI 197086 and PI 271326 had the shortest (Figure 1b). In the case of seedlings treated with PEG, the longest hypocotyl was reported for DM 97, with hypocotyl growth inhibited 22% in comparison to the control (Figure 1e). No correlation between the hypocotyl length and total root length under the control treatment was observed (Figure 2). However, a high correlation was observed under the PEG treatment ($r = 0.69$), indicating that drought stress reduced growth of whole seedlings, as has been observed in melon (Kavas et al. 2013) and tomato (Basha et al. 2015). Furthermore, comparative analysis of hypocotyl and root growth under PEG-induced drought stress and under the control treatment showed that this stress limited root growth more than it did hypocotyl growth. Therefore, root length might be a reliable indicator of tolerance/sensitivity of plants to drought stress, as postulated by Basha et al. (2015).

Among the 16 evaluated accessions, SU 2 and DM 97 had the highest fresh weight of seedlings under drought stress conditions (Figure 1c). The smallest degree of fresh weight reduction as compared with non-stressed was observed for accession SU 2 (55%), while the highest observed was for PI 197088 (89%) (Figure 1f).

Our findings suggest that seed germination, fresh weight, and root and hypocotyl lengths can be used as traits for rapid selection of drought-tolerant genotypes of cucumber. We identified three accessions, Ames 4759, PW 2, and PW 2A, expressing high germination ability under PEG-induced drought stress. We also identified the accessions SU 2 and DM 97 as having the highest drought tolerance levels at the seedling stage. These results indicate that mechanisms mediating drought-stress tolerance

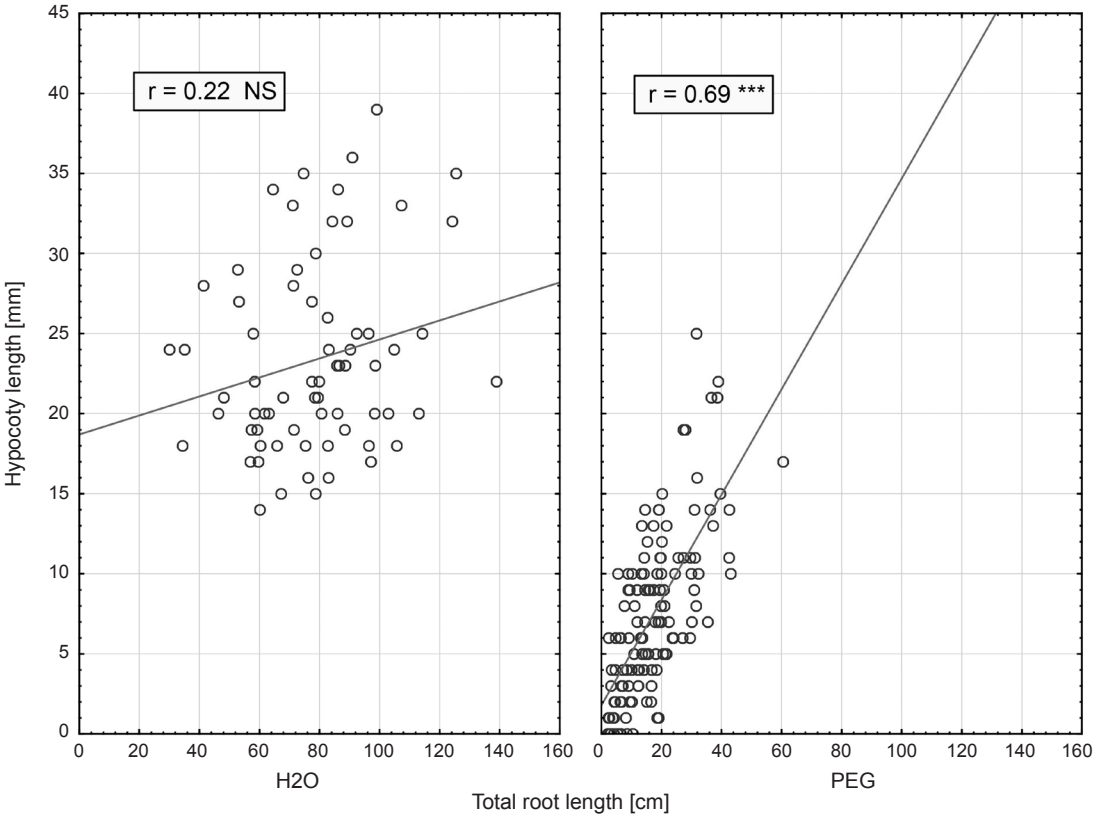

Figure 2. Correlation between total root and hypocotyl length for 16 cucumber accessions grown under control treatment (H_2O) and under 18% PEG treatment.

during germination are probably different from those that mediate drought-stress tolerance of seedlings. The most tolerant and most sensitive genotypes might prove useful for elucidating the genetic mechanism(s) of drought tolerance in cucumber. Moreover, the accessions identified as possessing drought tolerance are potentially useful in breeding programs directed toward drought tolerance in cucumber.

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CucCAP - Developing Genomic Resources for the Cucurbit Community

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Abstract. The United States cucurbit community has initiated a U.S. Department of Agriculture-funded cucurbit genomics project, CucCAP: Leveraging applied genomics to increase disease resistance in cucurbit crops. Our primary objectives are: develop genomic and bioinformatic breeding tool kits for accelerated crop improvement across the Cucurbitaceae; use these tools to facilitate introgression of disease resistance into commercially valuable cucurbit cultivars; perform economic analysis and provide readily accessible information to facilitate disease control. We will develop genomic and phenotypic databases and bioinformatics tools for watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), cucumber (*Cucumis sativus*), and squash (*Cucurbita* spp.). Genotyping by sequencing (GBS) will be performed on ~1000 accessions from the plant introduction collections for each crop. These data will be used to develop genome-informed core collections of 384 accessions for the four crops. Individual plants from the core collections will be self-pollinated and re-genotyped by GBS, providing a set of diverse lines and their sequence data, SNP datasets, and genetic maps. These resources will be available for future phenotypic and genome wide association analysis (GWAS) of any traits of interest. Genomic and phenomic data and bioinformatic tools will be publicly available through the International Cucurbit Genomics Initiative website, along with workshops and webinars to facilitate use. The CucCAP project will focus on resistance to key diseases for each crop as identified by cucurbit producers. GWAS and bi-parental populations will be used to identify resistance-associated quantitative trait loci (QTL) and initiate marker development. Breeding for resistances to downy mildew, *Fusarium*, gummy stem blight, *Phytophthora capsici*, powdery mildew, and several viruses will be performed in parallel with genomic analyses and tool development. Genomic and breeding efforts will be complemented by a centralized cucurbit disease website with content in English and Spanish providing diagnostic information, disease control recommendations, disease alerts, and links to disease clinics and forecasting tools.

Keywords: Watermelon, melon, cucumber, squash, genotyping by sequencing, disease resistance

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Introduction

The Cucurbitaceae include many high-value, flavorful, and nutritious crops, consumed as vegetables and fruits. Successful marketing of these crops requires high quality products that are grown in an economically viable and environmentally sustainable manner. When U.S. watermelon, melon, and cucumber growers, shippers, and processors were surveyed to identify their primary production challenges, losses due to fungal/oomycete pathogens and insect-transmitted viral diseases were consistently identified as their major constraints. The impacts of these diseases include severe reductions in both yield and quality, increased expenses for labor and disease control, environmental impacts from application of pesticides, and potential outright loss of the crop in the field or at point of sale.

The need for disease resistant cultivars is clear, as they provide the most cost-effective and environmentally desirable solution to these problems. Since acceptance and value of new cultivars depends on successfully combining yield and ease of production for the grower, with acceptable price and desirability for the consumer, it is imperative that breeding programs effectively incorporate multiple critical disease resistances without sacrificing yield or quality. Thus, cucurbit breeders are faced with multiple challenges to find new sources of resistance, identify genetic loci conferring resistance, efficiently incorporate new resistances into elite cultivars, and combine resistances to multiple diseases, while at the same time, retaining superior productivity and fruit quality.

Severity of the diseases and complexity of the breeding challenges require that we use the most effective and efficient technologies to develop disease-resistant cultivars. Next generation sequencing technologies have ushered in an array of genomic and bioinformatics tools that can be used for crop improvement, including more rapid and accurate identification of relevant quantitative trait loci (QTL), and development of molecular markers for marker-assisted selection (MAS), which allows for direct DNA screening in early generations or growth stages. MAS is of particular value for disease resistance as screening for one resistance may preclude screening for another, and disease response can be strongly influenced by environment. Fortunately, as the costs of genomic technologies have dropped dramatically, they have become accessible for applications to specialty crops.

The recent assemblies of draft genome sequences for the five major cucurbit crops, cucumber (*Cucumis sativus* L.) (Huang et al. 2009, Li et al. 2011, Yang et al. 2012), melon (*Cucumis melo* L.) (Garcia-Mas et al. 2012), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) (Guo et al. 2013), and pumpkin and squash (*Cucurbita pepo* L., *C. maxima* Duchesne, and *C. moschata* Duchesne) (Fei et al. in preparation, Pico et al. in preparation) will make it feasible to identify, incorporate, and combine genes using advanced genomic approaches that were not possible even a few years ago. The diploid genomes and small genome sizes of cucurbit species, ~ 367, 450, 385, and 400 Mbp for cucumber, melon, watermelon, and squash/pumpkin, respectively, make them highly amenable to genomic approaches. Close genetic relationships among the cucurbit species also allow for synergistic approaches, as cucurbit genomes exhibit extensive synteny, facilitating comparative mapping.

These needs and opportunities have led to the recent funding of a major project for cucurbit genomics, bringing together 21 scientists from 11 institutions, "CucCAP: Leveraging applied genomics to improve disease resistance in cucurbit crops". The goals of the CucCAP project are to: (1) Develop genomic and bioinformatic breeding tool kits for accelerated crop improvement across the Cucurbitaceae; (2) Use these tools to facilitate efficient introgression of disease resistance into commercially valuable cucurbit cultivars; and (3) Perform economic impact analyses with respect to cost of production and disease control, and provide readily accessible information to facilitate disease control.

Expanding the genomic toolkit for cucurbit species

A primary objective of this project is to use linkage analysis from segregating bi-parental populations and genome wide association studies (GWAS) to identify QTLs associated with disease resistance in cucurbit crops. To this end, we seek to increase our ability to perform highly informative QTL analyses. Recent advances in genomic technologies, particularly the development of genotyping by sequencing (GBS) technology for identification of single nucleotide polymorphisms (SNPs), have revolutionized the quantity of data and number of markers that can be deployed for genetic and physical mapping of QTLs (Elshire et al. 2011). The availability of high quality assembled cucurbit genomes, diploid nature, and relatively small genome size, coupled with continually dropping cost of DNA sequencing, make large-scale GBS realistic for SNP genotyping for these species. GBS is quick, extremely specific, highly reproducible, and based on our recent work (Lambel et al. 2014), can reach genomic regions not readily accessible using other methods. The use of methylation-sensitive restriction enzymes simplifies computational alignment by reducing repetitive regions and targeting low copy regions (Elshire et al. 2011). GBS procedures have been optimized for watermelon (Lambel et al. 2014, Nimmakayala et al. 2014, Reddy et al. 2014), melon (Tomason et al. 2013), and squash (Holdsworth and Mazourek 2014), and GBS has been successfully applied to identify QTLs in watermelon for fruit quality and resistance to *Fusarium oxysporum* race 1 (Tomason et al. 2013, Lambel et al. 2014).

Two of the primary tools that we seek to develop for the cucurbit community include GBS analysis of the U.S. plant introduction (PI) collections for each of the five major cucurbit crops, and development of genomic and bioinformatic platforms for GBS sequence data processing and analysis and allied databases for public use. The U.S. National Plant Germplasm System maintains seeds of 1,314 cucumber, 2,043 melon, 1,311 watermelon, and 1,580 squash/pumpkin (*Cucurbita pepo, C. moschata,* and *C. maxima*) accessions. Within each crop is a core collection intended to represent diversity of the crop, provide a common set of material for research, and help streamline maintenance of seed supplies for distribution. The criteria used to establish current core collections predated availability of molecular data. This project will allow us to create core collections that better represent the available diversity based on genome information. Diversity in the collection will be characterized by GBS for 1,000 to 1,600 accessions of each crop. A set of 384 accessions that best represent each crop at the genome level will be selected to maximize diversity and manage substructure. The intra-accession diversity will be managed in this

genome-informed core collection by self-pollination to create a more uniform seed stock. The parents for the self-seed stock will be re-genotyped by GBS as described above. These genome-informed core collections will create a germplasm resource for GWAS, not only for the disease resistance phenotypes prioritized within the context of this project, but any traits that may be of interest for future studies, such as fruit quality.

In concert with GBS characterization of the cucurbit PI collections, we will develop tool kits for sequence data processing and analysis, gene mapping and identification of QTLs, and breeder-friendly databases and visualization tools for sequences, maps, syntenic relationships, markers, QTLs, population and accession phenotypic information, and their interrelationships. These tools will be made publically accessible through the Cucurbit Genomics Database website, ICuGI (http://www.icugi.org). Currently the database stores the genome sequences and gene annotations for watermelon and cucumber. The genome sequences of melon, which recently became publicly available without any restrictions, and of *Cucurbita* species, which are being currently generated and will be publicly available in the near future, will also be included in the database. The database also contains extensive transcriptome sequence, annotation information, marker data, and genetic maps for the five cucurbit crops.

These resources ultimately will have greater value if the information is more readily transferrable across research teams and crop systems. At present, publications commonly have the same gene/QTL names for different traits, or different names for the same traits. To avoid further confusion and provide an important common language for comparative genetic analysis, we seek to establish nomenclature guidelines across the cucurbit crops. We hope that this project can provide a platform to develop guidelines to standardize and simplify description of genetic entities in the four cucurbit crops as has been done for other plants including *Arabidopsis*, rice, grape, and some Solanaceae.

Facilitating disease resistance for cucurbit crops

Given the variety of crops, coupled with the diverse environmental conditions in which they are grown, ranging from short season to long, humid to dry, with variable temperature ranges and day lengths, it is not surprising that the primary diseases affecting cucurbits in U.S. production systems vary for each crop. The major disease threats as identified by each commodity group are summarized in Table 1. Despite variation for the primary diseases for each crop, most of the pathogens infect the other cucurbit crops to varying degrees, as would be expected for this group of related species.

Breeding strategies for each commodity and disease problem are based on knowledge of the pathogens, sources of resistance, cross compatibility between sources of resistance and cultivated materials, inheritance of resistance, availability of breeding populations, and state of breeding progress with regard to introgression of resistance, identification of QTLs, and marker development. Genetic populations (including F_1, F_2, BC_1R and BC_1S, F_3 families, and/or RILs) are available or under development for the different cucurbit crop-disease combinations, allowing for phenotypic and GBS analysis to identify disease-associated QTLs. In addition, for many of the crop-disease combinations, disease resistance screening has been performed on large numbers of PIs or full PI collections, such as: downy mildew in cucumber (Call et al. 2012a,b); Fusarium wilt (*F. oxysporum* f. sp. *niveum*, races 1 and 2) (Netzer and Martin 1989, Boyhan et al. 2003, Wechter et al. 2012), *Phytophthora capsici* (Kousik et al. 2012), powdery mildew (*Podosphaera xanthii*, races 1 and 2) (Davis et al. 2007, Tetteh et al. 2010), gummy stem blight (Boyhan et al. 1994, Gusmini et al. 2005), and PRSV and ZYMV in watermelon (Boyhan et al. 1992, Guner 2004, Levi et al. 2016), and CYSDV in melon (McCreight et al. 2013), providing phenotypic data to perform GWAS for disease-resistance QTLs.

Disease resistances are often controlled by a single QTL with very large effect, similar to a simply inherited gene, and this is the case for several of the cucurbit disease/crop systems. Resistances to Fusarium wilt (*F. oxysporum* f. sp. *niveum*, race 1) (Lambel et al. 2014), powdery mildew (Tetteh et al. 2013a,b) and PRSV-W in watermelon (deAzevedo et al. 2012), and Fusarium wilt (Zink and Thomas 1990), powdery mildew (Thomas 1986), and CMV (Karchi et al. 1975) in melon, and powdery mildew and PRSV in squash (Contin and Munger 1977, Brown et al. 2003, Holdsworth and Mazourek 2014) are simply inherited. In other cases, the resistance may be under control of a few (2 or 3) major-effect ($R^2 > 20\%$) QTLs

Table 1. Major disease threats to cucurbit crop production as identified by cucurbit industry stakeholders.

Disease	Identified as commodity priority	Also affects
Downy mildew	Cucumber	Melon, watermelon, squash/pumpkin
Fusarium wilt	Watermelon	Melon, cucumber
Gummy stem blight	Watermelon	Melon, cucumber, squash/pumpkin
Phytophthora rot	Cucumber, watermelon, squash/pumpkin	Melon
Powdery mildew	Melon, watermelon, squash/pumpkin	Cucumber
Viruses (CMV[z], CYSDV[y], PRSV-W[x], CGMMV[w])	Melon[z,y], Watermelon[x,w]	Cucumber[y,w], squash/pumpkin[z,x]

[z]CMV – *Cucumber mosaic virus;* [y]CYSDV – *Cucurbit yellow stunting disorder virus;* [x]PRSV-W – watermelon strain of *Papaya ringspot virus;* [w]CGMMV – *Cucumber green mottle mosaic virus*

with additive effects, such as powdery mildew (He et al. 2013) and downy mildew in cucumber (Weng et al. in preparation). Depending on complexity of the trait and co-segregation of the QTL with disease resistance, a QTL can serve as the starting point for marker development for use in marker-assisted selection.

The genomic resources for the cucurbit species also will be integrated to anchor consensus physical and genetic maps, allowing us to search for regions of synteny and orthologous disease resistance regions or gene islands across the sister species. While resistance QTLs in one crop may not exist in the target crop, the large association panel for each crop and the association analysis results may help identify homoeologous resistance loci in syntenic regions.

Finally, the genomic and breeding efforts will be complemented by economic analysis of the costs of production and disease control and the development of a centralized, publically accessible, cucurbit disease website providing information for cucurbit disease diagnosis and control. Risk simulation models developed from representative farm panels will be used to evaluate the economic profitability of using disease resistant cultivars compared to current cultivars with respect to production savings in reduced chemical use, labor, and other inputs. The cucurbit disease website will provide links to plant disease clinics, extension production guides, and disease control recommendations, disease fact sheets, with pathogen biology, diagnostic guides, and symptom and pathogen pictures at the macro and micro level. Disease fact sheets will be generated in English and Spanish for the priority cucurbit diseases.

Concluding Remarks

Development of genomic tools for the cucurbit community has been a worldwide effort with major contributions from Europe and China leading the effort for sequencing and genomic analysis of cucumber, melon, and watermelon. We look forward to ongoing and expanding collaborations to continue to develop valuable genomic resources for the study of the Cucurbitaceae and genetic improvement of cucurbit crops.

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Germination of Wild *Cucumis* Species and Interspecific Hybrids

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Abstract. Orthodox seeds may present differences in germination due to possible dormancy, low viability or loss of viability during conservation conditions, or aging. In the context of grafting, poor germination is a limiting factor. In this work, we have evaluated various conditions that include standard conditions, stratification, and *in vitro* culture in order to obtain high and uniform germination rates in accessions of *Cucumis metuliferus*, *C. anguria*, *C. ficifolius,* and *C. myriocarpus*, as well as in interspecific hybrids obtained using *C. ficifolius* as the female parent. Development of new rootstocks of the genus *Cucumis* is of great interest because variable scion performance, including the occurrence of negative impacts on fruit quality, is observed when the common interspecific *Cucurbita* rootstocks are used for grafting melon. Germination rates varied among *Cucumis* species and treatments. Germination was observed in all treatments with the exception of seeds from the interspecific hybrid *C. ficifolius* × *C. metuliferus*, which had poor embryo development. For the rest of the genotypes, the best treatments were a pretreatment at 4 °C for 10 days prior to sowing in substrate. With these conditions, maximum germination rates were obtained as soon as 10 days after sowing. In general, better germination rates were obtained in the hybrids in comparison with their corresponding parents, supporting the use of interspecific *Cucumis* hybrids to improve grafting efficiency.

Keywords: Rootstocks, grafting, incompatibility, stratification

Introduction

The use of grafting is a common practice to deal with soil-borne diseases and other soil-stressful conditions. This practice has been commonly used in watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) and tomato (*Solanum lycopersicum* L.) cultivation. Its use has spread more recently to other Cucurbitaceae and Solanaceae, including melon (*Cucumis melo* L.), cucumber (*C. sativus* L.), eggplant (*S. melongena* L.), and pepper (*Capsicum annuum* L.).

The most common commercial rootstocks for Cucurbitaceae are *Cucurbita* interspecific hybrids (*C. moschata* Duchesne × *C. maxima* Duchesne) and some accessions of *Lagenaria siceraria* (Mol.) Standl. (bottle gourd). Both types of rootstocks confer resistance to most of the soil-borne fungi affecting watermelon and melon (King et al. 2010). However, due to the susceptibility of these rootstocks to root-knot nematodes (RKN, *Meloidogyne* spp.) (Ozarslandan et al. 2011) and to their negative impact on fruit quality that has been observed in some rootstock-scion combinations (Rouphael et al. 2010), there is an urgent need to develop new rootstocks able to deal effectively with RKN and that have reduced or no impact on fruit quality.

Various wild species of the genus *Cucumis* have been reported as unexploited sources of resistance to soil-borne organisms, including nematodes (Siguenza et al. 2005) and *Fusarium* spp. (Trionfetti et al. 2002). Accessions of these species with a high level of resistance could be a good alternative to the current rootstocks. However, their success as new rootstocks depends on having good compatibility with different scions, facility with which they can be grafted, and a low or no negative impact on fruit quality.

One of our research approaches has focused on the development of new rootstocks for melons, for use in management of soil-borne pathogens and with various scions. In this context, we have evaluated as new rootstocks one hybrid between a commercial melon (*C. melo* subsp. *melo* var. *inodorus* market class Piel de Sapo) and one exotic accession (*C. melo* subsp. *agrestis* var. *chinensis* accession Pat 81) resistant to *Monosporascus* vine decline, and one accession of *C. metuliferus* E. Mey. ex Naud., which is highly resistant to RKN (Gisbert et al. 2016). Additionally, and despite the difficulty, we have generated three interspecific *Cucumis* hybrids, *C. ficifolius* A. Rich × *C. anguria* L., *C. ficifolius* × *C. myriocarpus* Naud., and *C. ficifolius* × *C. metuliferus*, that are also under evaluation (Cáceres et al. 2016). Interspecific hybridization is a strategy to improve vigor and hypocotyl diameter of seedlings to facilitate the management of the plants during grafting as well as to combine resistance to soil-borne pathogens and pests, which might improve field performance.

The first step for the generation of grafted plants is the production of rootstock and scion seedlings suitable for grafting. In this process, two essential parameters are the percentages of germination and the time needed to develop plantlets with uniform hypocotyls that facilitate the join. Orthodox seeds may present differences in germination due to dormancy or loss of

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viability during conservation conditions or aging. Thus it is important to evaluate the germination of accessions or hybrids to be tested as putative rootstocks ahead of time. For instance, for *S. torvum* Sw., a RKN resistant eggplant rootstock, it is difficult to get rapid and homogeneous seed germination, which limits its use as a rootstock (Ginoux and Laterrot 1991).

In this work, we have evaluated different germination procedures, including standard conditions, cold stratification, and *in vitro* culture, in order to obtain knowledge about the germination ability of a promising *C. metuliferus* accession and three interspecific *Cucumis* hybrids, and their respective *Cucumis* parents, as a first step toward evaluating their possible usefulness as new rootstocks for melons.

Materials & Methods

Seeds of *C. anguria*, *C. metuliferus*, *C. myriocarpus*, and *C. ficifolius* (accessions BGV12795, BGV11135, BGV8535, BGV12786; held at the genebank of COMAV-UPV) as well as of three interspecific hybrids generated by crossing them, always using *C. ficifolius* as the female parent, were used for testing germination ability. Plants derived from the seeds initially conserved at the COMAV-UPV Genebank were transplanted to the greenhouse and selfed or crossed to obtain the seed used in the present study. The seeds, collected in the summer of 2015, were conserved at room temperature until the germination assay.

Each seed was carefully opened and its coat removed, then disinfected by immersion for 10 min in a solution of 25% commercial bleach (40 $g \cdot L^{-1}$ active chlorine), being then washed twice with sterile deionized water for 5 min before sowing, which was performed in substrate or *in vitro* culture (in petri dishes containing as nutrient medium Murashige and Skoog salts, including vitamins, 2% sucrose, and 0.7% plant agar; DUCHEFA, the Netherlands). The pHs of the media were adjusted to 5.8 before sterilization at 121 °C for 20 min. Cultures were incubated in a growth chamber at 26±2 °C for 2 days in darkness with a 16 h photoperiod for the rest of the culture. Cool white light was provided by Sylvania cool white F37T8/CW fluorescent lamps (90 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

For both germination strategies (substrate and *in vitro* culture), seeds were sown directly or after incubation at 4 °C (stored in the fridge) for 10 days (cold stratification). Additionally, in the substrate assay, incubation at 37 °C for 2 days of non-stratified seeds or after stratification at 4 °C was also tested. Then, a total of 6 treatments were compared for each genotype: T1 (*in vitro*); T2 (4 °C+*in vitro*); T3 (substrate); T4 (4 °C+substrate); T5 (37 °C +substrate; our standard laboratory protocol) and T6 (4 °C+37 °C +substrate). Ten seeds per treatment with two repetitions per treatment and genotype were sown. Seed germination was noted every 5 days from 5 to 20 days after sowing (DAS). ANOVA was conducted to evaluate the effect of genotype and treatment in early and final germination (5 and 20 DAS). *C. myriocarpus* was excluded from this analysis because we have no data for T6.

Results

Germination was observed for all genotypes in all treatments with the exception of the interspecific hybrid *C. ficifolius* × *C. metuliferus* (F×M), for which no germination was obtained in any of the treatments (Figure 1). Maximum rates of germination were found as 10 days after sowing in the rest of genotypes in all the treatments (data not shown). Germination of 100% was obtained only in treatments with cold stratification: in T4 (for *C. ficifolius* and *C. metuliferus*), T6 (for *C. anguria*, and *C. ficifolius* × *C. myriocarpus* (F×My)) and T2 (for *C. ficifilious* × *C. anguria* (F×A)). The maximum germination rate for *C. myriocarpus* was 85.7%, achieved in T4.

The ANOVA analysis supported the existence of a significant effect of genotype for early (at 5 DAS) germination (*P* value: 0.0001) and of the treatment for both, early (0.0038) and final germination (0.0001) (at 5 and 20 DAS). A low, but significant genotype × treatment interaction (0.0420) was observed, but only at 5 DAS.

Figure 1 shows the percentage of germination at 5 days and 20 days averaged per genotype (Figure 1A) and per treatment (Figure 1B). Both hybrids (F×A and F×My) had the highest percentages of early germination (around 60% for both), significantly higher than that observed in at least one of their parents. Conversely, the percentage of germination at 20 days did not significantly differ among genotypes (Figure 1A).

A positive effect of cold stratification (seed incubation at 4 °C for 10 days) on germination was observed for all the geno-

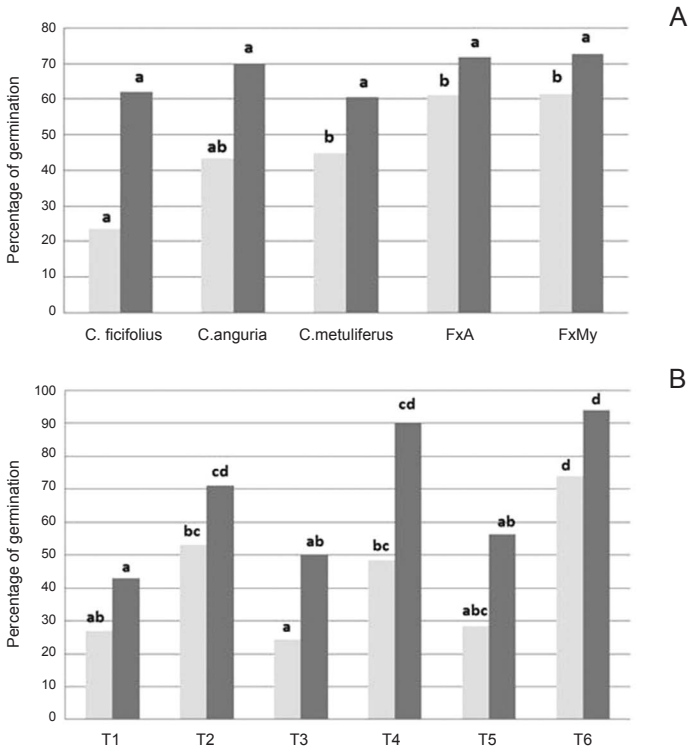

Figure 1. Percentage of germination for genotypes (A) and treatments (B) noted after 5 and 20 days of culture (grey and dark grey, respectively). A. Genotypes: *Cucumis ficifolius*, *C. anguria*, *C. metuliferus*, *C. ficifolius* × *C. anguria* (F×A); *C. ficifolius* × *C. myriocarpus* (F×My). B: T1 (sowing in petri dishes: *in vitro* culture), T2 (stratification at 4 °C for 10 days and *in vitro* culture), T3 (sowing directly in substrate); T4 (stratification 4 °C for 10 days and sowing in substrate); T5 (incubation for 2 days at 37 °C and sowing in substrate); T6 (stratification at 4 °C for 10 days, 2 days incubation at 37 °C and sowing in substrate).

types, in both, substrate and *in vitro* culture procedures (Figure 1B). This increase of the percentage of germination in stratified seeds was clearly observed at early and late germination. For example, for *in vitro* treatments, the percentage of early and late germination in 4 °C treated-seeds was nearly twice that obtained in seeds directly cultured: 53 *vs*. 27% at 5 days and 71% *vs*. 43% at 20 days (Figure 1B). This positive effect of cold stratification was general, occurring in most genotypes, with the exception of *C. metuliferus*, the only genotype that did not have improved germination under the cold treatment (data not shown).

For the substrate treatments, cold incubation also caused a two-fold increase in the germination percentage (from 24 to 49% at 5 days and from 50 to 90% at 20 days), observed in all genotypes. Whereas the incubation at 37 °C itself did not improve significantly germination (24 *vs*. 28% and 50 *vs*. 56%, for T3 and T5 at 5 and 20 DAS, respectively) the combined treatment of 10 DAS at 4 °C and 2 days of incubation at 37 °C followed by a direct sowing under standard conditions gave the highest germination average (Figure 1B).

Discussion

In order to test the germination ability in a promising rootstock (*C. metuliferus* accession BGV11135) and three interspecific *Cucumis* hybrids (putative rootstocks) and their respective *Cucumis* parents, we compared different treatments that included germination in substrate and *in vitro* culture. The latter has been used for germinating mature or immature seeds and for embryo rescue in various species (Dolce et al. 2011, Gisbert et al. 2011, Díez et al. 2014). Given the difficulty in obtaining successful crosses among *Cucumis* species (Deakin et al. 1971, Fassuliotis 1977, Singh and Yadav 1984, Beherav and Cohen 1995), we considered it of interest to include *in vitro* culture in our treatments.

Germination was obtained in all the tested genotypes and treatments with the exception of F×M, and despite its seeds having a similar aspect and which imbibed water like those of the rest of the genotypes. At the end of the assay, a sample of seeds was completely opened and, in a high percentage of seeds, embryos were not found. Therefore, *C. ficifolius* was much more incompatible with *C. metuliferus* than with the other two *Cucumis* species. This result was in agreement with that obtained by Kho et al. (1980), who found unseeded fruits in a cross F×M and seeded fruits in a cross F×A. On the other hand, we did obtain seeded fruits in the cross F×My. Presumably, differences in the accessions of the species involved and/or in the environmental conditions explain our contrasting result.

The best germination percentages for most of the genotypes assayed were obtained in treatments T4 and T6, both including cold stratification at 4 °C for 10 days, followed or not by two days incubation at 37 °C. Seed stratification has been used to induce or increase seed germination (Tezuka et al. 2013, Eckberg et al. 2015). Using these conditions, we were able to double the percentage of germination, reaching 100% in most genotypes from 10 days after sowing. This result is important for grafting, as high and uniform germination is required for efficient, economical grafting.

The best responding genotype to *in vitro* culture was F×A, which had 100% germination in T2, a similar result to that obtained in T6. Changes that occurred after imbibition which are regulated by temperature, light conditions, and plant hormones (Weibrecht et al. 2011), in addition to genotype, could explain this differential response to *in vitro* culture.

In conclusion, stratifying seeds at 4 °C gives fast and uniform germination rates for the promising rootstock *C. metuliferus* BGV 11135 and two of three hybrids that we evaluated as potential rootstocks (F×A and F×My). The higher percentage of germination found in the hybrids compared to their parents confirms the advantage of using interspecific hybrids instead of single *Cucumis* genotypes for grafting purposes.

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Evaluation of a Cucumber RILs Population for Resistance to Angular Leaf Spot

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Abstract. One of the important diseases of cucumber, *Cucumis sativus*, is angular leaf spot (ALS) caused by *Pseudomonas syringae* pv. *lachrymans* (*Psl*). Increased occurrence of this disease in cucumber field production in Poland has caused significant losses over the last few years. The objective of this study was to estimate the ALS resistance level in a RILs population under growth chamber conditions. The tested RILs population was developed by crossing two inbred lines: Gy14 which shows tolerance to ALS and B10 which is susceptible to ALS. Plants were inoculated by spraying with the highly virulent *Psl* strain 814/98, scored (1 to 9 scale), and disease severity index (DSI) was calculated. Two types of reaction to *Psl* were observed, chlorosis on the leaves indicating susceptibility and necrosis with a chlorotic halo indicating tolerance. The results showed that the RILs segregate for ALS resistance/tolerance and thus the Gy14 × B10 RILs population is useful for further inheritance studies and mapping of ALS resistance/tolerance genes.

Keywords: Angular leaf spot disease, *Cucumis sativus*, *Pseudomonas syringae* pv. *lachrymans*, resistance, RILs

Introduction

Angular leaf spot (ALS) caused by the bacterium *Pseudomonas syringae* pv. *lachrymans* (*Psl*) is one of the most important diseases limiting production of field cucumber, *Cucumis sativus* L. Typical ALS symptoms include vein-limited, water-soaked lesions (with or without chlorotic halos) on leaves, which later become necrotic. Water-soaked lesions occur also on fruits, causing the fruits to become misshapen thus resulting in yield reduction (Bradbury 1986, Sherf and Macnab 1986). The first reports describing ALS resistance in cucumber were published by Chand and Walker (1964) and by Dessert et al. (1982). Disease severity, observed in accordance with the number and size of the lesions, differs among cucumber genotypes and has been reported to be a polygenically inherited trait (Chand and Walker 1964, Dessert et al. 1982). By self-pollination and selection of the most resistant plants, Chand and Walker (1964) accumulated genes conferring reduced severity, thereby increasing the level of tolerance to *Psl*. Dessert et al. (1982) distinguished two types of reactions by cucumber plants to ALS based on the symptoms: (1) the occurrence of chlorotic halos around necrotic lesions typical of susceptibility and (2) the lack of chlorotic halos typical of resistance or tolerance. The absence of chlorotic halos was conferred by a single recessive gene *psl* (*pl*). Attempts to find molecular markers associated with ALS resistance gene(s) were ineffective. The RAPD marker, OP-AO07, linked to the locus responsible for the presence/absence of chlorotic halos at a distance of 13 cM, was identified by Olczak-Woltman et al. (2009). The knowledge about inheritance of tolerance/resistance to *Psl* and molecular markers linked to resistance gene(s) are still limited. The objective of this study was to estimate the *Psl* resistance level in a RILs population. The results of the phenotypic evaluation in relation to ALS symptom type will be useful for mapping cucumber gene(s) associated with resistance or tolerance to this disease.

Materials & Methods

The bacterial strain and inoculum preparation

Previously, both virulence and genetic diversity of *Pseudomonas* sp. strains, collected at the Department of Plant Genetics Breeding, and Biotechnology (Warsaw University of Life Sciences, Warsaw, Poland), were studied (Olczak-Woltman et al. 2007, Słomnicka et al. 2015a,b). Based on these studies, the highly virulent *Psl* strain 814/98 was selected and used in this study. *Psl* 814/98 was obtained from the Institute of Plant Protection in Poznań (Poland). To prepare the inoculum, bacteria were grown on King B agar plates at 28 °C for 24 hours. Bacterial colonies were suspended in sterile distilled water and adjusted to OD_{600}= 0.05 to be equal to a concentration of 10^7 CFU·ml^{-1}.

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Plant materials and plant inoculation

Recombinant inbred lines (RILs) of the F_6 generation were tested for *Psl* resistance. The RILs population was developed by crossing two inbred lines: Gy14 that exhibits tolerance to *Psl* and B10 that is susceptible to *Psl*. The F_2 seeds were kindly provided by Prof. M.J. Havey (Univ. of Wisconsin, Madison, WI, U.S.A.) and used further to develop F_6 generation progeny by self-pollination of individual plants at the Wolica experimental field (Department of Plant Genetics, Breeding, and Biotechnology, Warsaw University of Life Sciences, Warsaw, Poland). Cucumber plants were grown in a growth chamber at 25 °C during the day and 22 °C at night, with a 16 h photoperiod. For *Psl*-resistance testing, plants at the 2nd to 3rd leaf stage were inoculated by spraying the abaxial side of each leaf with inoculum, according to Klement et al. (1990). As a control, sterile water was used. Inoculated plants were kept in darkness for 24 hours at 22 °C and relative humidity of 100%, and then for 6 days under typical growth conditions. After 7 days, inoculated leaves were visually scored using a nine-degree rating scale (Jenkins and Wehner 1983) and disease severity index (DSI) was calculated. Each tested line was represented by 16 plants (four replications of four plants in each replication).

Results & Discussion

Parental lines and 110 RILs of the Gy14 × B10 population were characterized for *Psl* resistance. Two types of reaction were observed. B10 was characterized by widespread, water-soaked, angular chlorosis that later became necrosis on leaves. Bacterial exudates on petioles and leaves were also observed. The symptoms covered 50 to 75% and very often even 87% of the leaf surface. The mean DSI of B10 was 4.0. On the leaves of Gy14, small necrotic spots and very limited bright chlorosis were observed. The symptoms appeared on 8 to 25% of the leaf surface and mean DSI was 6.4 (Table 1, Figure 1).

The ALS symptoms observed on Gy14 plants were similar to a hypersensitivity response (HR). Generally, HR is initiated in plants as necrotic lesions in the locally infected tissue and is accompanied by the accumulation of salicylic and jasmonic acids (Yang et al. 2013). The obtained results corresponded well with a previous study aimed at identifying susceptible and tolerant standard lines for *Psl* testing, the DSI for B10 and Gy14 lines being 3.5 to 3.8, and 6.2 to 6.3, respectively (Olczak-Woltman et al. 2008). Among the RILs, two groups were distinguished: tolerant RILs resembling Gy14 ("Gy14 type") and susceptible

Table 1. Disease severity index (DSI) in the Gy14 × B10 F_6 RILs cucumber population and its parents infected with *Pseudomonas syringae* pv. *lachrymans*.

ALS resistance type	Number of lines	DSI range of mean		DSI mean	Standard deviation
		minimum	maximum		
Gy14	Maternal line	6.0	6.8	6.4	0.49
B10	Paternal line	3.3	4.5	4.0	0.61
Gy14 type	58	5.5	6.8	6.1	0.29
B10 type	52	3.0	5.0	4.3	0.45

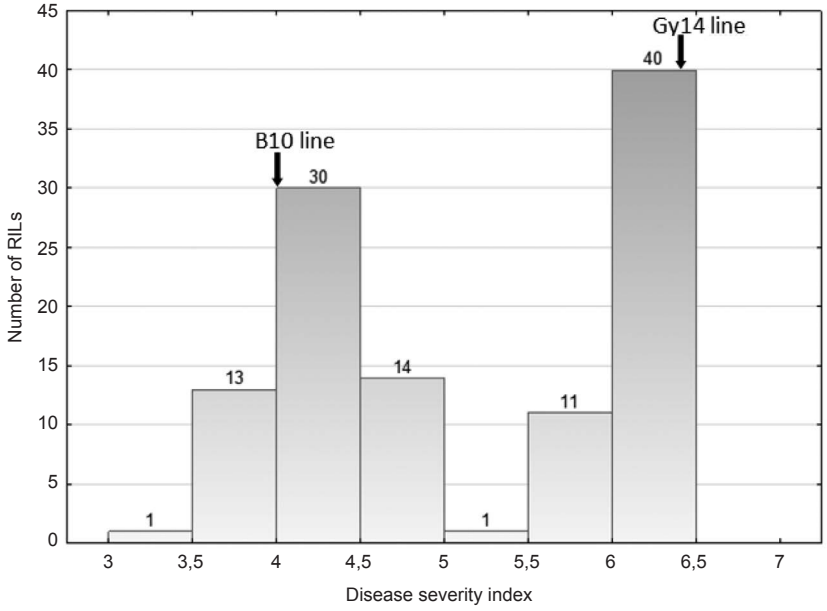

Figure 1. Distribution of disease severity index (DSI) in the Gy14 × B10 F_6 RILs population infected with *Pseudomonas syringae* pv. *lachrymans*.

RILs resembling B10 ("B10 type"). The DSI of lines characterized as "Gy14 type" and "B10 type" ranged from 5.5 to 6.8 and from 3.0 to 5.0, respectively (Table 1, Figure 1). A similar distribution of DSIs in this population was estimated previously at the F_5 generation level (Słomnicka et al. 2015a,b), and it indicates that the RILs of the Gy14 × B10 population are stable and possess a high degree of homozygosity.

The second important indication of cucumber resistance to *Psl* is presence or absence of a chlorotic halo. The chlorotic halo in B10 and "B10 type" RILs was always present. In Gy14 and "Gy14 type" RILs, limited chlorosis and lack of halos were observed. Among 110 RILs, 51 did not possess chlorotic halos, like Gy14, while 58 lines classified as "B10 type" were susceptible, with intense chlorotic halos. One RIL possessed intermediate symptoms. Earlier, Dessert et al. (1982), based on genetic analysis (F_1, F_2, BC, and F_3), indicated that the lack of halos in resistant plants is conferred by a single recessive gene, *psl* (*pl)*, but the number and size of lesions may be controlled by two or more genes (Dessert et al. 1982). Olczak-Woltman et al. (2009), from the cross of B10 × H603 (F_2, BC_1 and BC_2), confirmed this type of inheritance. A recessive mode of inheritance of disease resistance in cucumber was also reported for downy mildew (*dm* gene) and powdery mildew (*pm* gene) (Fujieda and Akiya 1962, van Vliet and Meysing 1977). However, according to recent studies, resistance to downy mildew and powdery mildew is determined by multiple recessive genes (He et al. 2013, Zhang et al. 2013). The phenotypic characterization of the mapping population Gy14 × B10 showed that RILs segregate for ALS resistance type and thus this population can be useful for further inheritance studies and mapping of *Psl* resistance/tolerance genes.

Acknowledgements

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Utilization of a Strongly Female Mutant in Seed-Pumpkin Germplasm of *Cucurbita maxima* Duchesne for Increased Production and to Facilitate Hybrid Seed Production

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Abstract. The strongly female strain LC-03 was used as a female parent, and three inbred lines, 'Duozi 265', 1-14, and Qiang 8-1 with excellent horticultural characteristics, were chosen as male parents to produce hybrids of white-seeded pumpkins, *Cucurbita maxima*. The three hybrid combinations showed many good traits including early maturity, strong femaleness, increased fruit production and fruit size, and increased seed yield. The discovery of this strongly female mutant and its application can play an important role in pumpkin breeding for improved seed pumpkin production, reduced hybrid seed production costs, and enhanced hybrid seed purity.

Keywords: Seed pumpkin, *Cucurbita maxima*, strong femaleness, horticultural traits

Introduction

Seed pumpkins of *Cucurbita maxima* Duchesne are one of the major cash crops in Northeast China (Qu et al. 2013). There is an ever-increasing demand for white pumpkin seeds in the domestic and foreign markets, consequently, the income of farmers and planting enthusiasm are enhanced (Xu 2004). Compared with other cucurbit crops, seed pumpkin breeding lags behind. Only lately has seed-pumpkin breeding been initiated, and germplasm resources and funding have been lacking in China (Wei et al. 2013). The conventional varieties used for seed production show some shortcomings including poor uniformity, disease susceptibility, and inconsistency of yields. Farmers do need better seed pumpkins varieties having early maturity, disease resistance, high yield, and good quality (Xu and Yu 2009).

At present, only part of the market of seeds is furnished by F_1 hybrids of white-seed pumpkins. During the production of hybrid seeds, it is difficult to ensure the purity of the F_1 hybrids, because removal of the male flowers is labor-consuming. Production of hybrid seeds could be facilitated by using female or strongly female lines as female parents, which can be expected to not only reduce the cost of labor, but also improve the purity of the hybrid seeds.

The discovery of a strongly female pumpkin plant

Yu Zhenhua of Heilongjiang Province, now retired, observed a strongly female pumpkin plant in his garden in the early 1990s. He purified the trait of strong femaleness by self-pollination and selection, and named the resulting new, strongly female pumpkin 'Duozi 265'. The proportion of leaf axils bearing female flowers is 2 to 3 times higher than normal. The more apical stem nodes have an even higher proportion of female flowers, but nonetheless the plants do produce a few male flowers.

The oblate fruits are greyish-green in rind color with white seeds. A single fruit usually yields approximately 250 seeds.

A mutant in the strongly female inbred line of pumpkin

In 2003, a mutant plant from a highly inbred line of 'Duozi 265' was observed which had only male and bisexual flowers in the first ten leaf axils of the plant, thereafter only female flowers, without any more male flowers. This mutant was self-pollinated and, in 2004, 150 of its progeny were grown, of which 76% were very strongly female. As a result of four subsequent years of self-pollination and selection, a relatively true-breeding, very strongly female inbred line was obtained, which was named LC-03. This inbred has a combination of four advantageous traits: (1) medium plant size, 3 to 4 meters in length and 2 to 3 branches; (2) leaves light green, rounded, shallow incisions, each leaf axil possesses a tendril; (3) generally, on the first 8 to 10 nodes of the vine the same number of male flowers appears and subsequently, only female flowers appear, at each axil on the

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Table 1. Flowering and sexuality of inbreds and hybrid combinations of crossing a strongly female inbred with three normal-flowering inbreds.

		Inbred lines					F_1 hybrids							
		time of first female flower (month /day)	node number of the first to fourth female flowers				time of first female flower (month /day)	node number of the first to fourth female flowers				strongly female, semi-female and normal (%)		
			first	second	third	fourth		first	second	third	fourth	strongly female	semi-gynoecious	sub-gynoecious	normal
2008	Strongly female LC-03	7/18	8.5	/	/	/	/	/	/	/	/				
	♂'Duozi265'	7/20	10.0	17.0	23.0	28.0	7/17	10.0	16.5	22.6	26.8	27.3	10	22.7	40.9
	♂1-14	7/17	8.4	14.0	18.3	23.2	7/16	7.6	13.0	17.5	22.0	23			77
	♂8-1	7/18	10.2	16.0	21.0	26.0	7/17	9.5	15.4	19.7	24.0	24			76
2008 Hanan Province	Strongly female LC-03	12/13	8.2	/	/	/	/	/	/	/	/				
	♂'Duozi265'	12/13	10.2	15.8	22.2	25.4	12/11	9.2	14.9	19.5	23.6	26	11	22	41
	♂1-14	12/11	8.4	13.4	19.3	24.5	12/10	7.4	12.7	17	21.8	25			75
	♂8-1	12/12	10	15.6	21.0	25.4	12/10	9.0	14.7	19.2	23.0	26			74
2009	Strongly female LC-03	7/21	8.4	/	/	/	/	/	/	/	/				
	♂'Duozi 265'	7/21	10.4	16.9	22.9	27.3	7/20	10.0	16.7	22.6	26.4	30	10	25	35
	♂1-14	7/20	7.9	13.5	18.4	24.0	7/19	7.6	13.1	17.6	22.2	25			75
	♂8-1	7/22	10.0	15.4	19.8	25.0	7/.20	9.5	15.4	19.7	24.4	24			76

main stem and all of the lateral branches. The female flowers have longer pedicels than usual and the ends of the sepals are strongly attenuate, needle-like; (4) the color of the young fruits is light or greyish green, ripe fruit external color is white with light gray or green. The fruits are nearly spherical to oblate and weigh 3 to 4 kg. A single fruit can yield 100 to 150 grams of seeds. The yield of the seeds from the experimental field was about 70 kg/667 square meters with the thousand-seed weight 350 to 400 g. The seed width and length is about 1.10 to 1.25 and 2.10 to 2.30 cm, respectively, with a narrow margin.

F_1 combinations evaluation by field trial

The strongly female inbred line LC-03 was used as the female parent for crossing with three other inbred lines, 'Duozi 265', 1-14, and Qiang 8-1, which have excellent horticultural characteristics. In 2008, we planted the three F_1 hybrids in the fields, and found that they had many good traits, including early maturity, many more female flowers, enhanced fruit production, larger fruit, and higher seed yield. In 2009, we planted these hybrids in Hainan and Heilongjiang Provinces again. The seed yields of LC-03 × 'Duozi 265', LC-03 × 1-14, and LC-03 × 8-1 were 35.9%, 30.4%, and 28.8%, respectively, greater than that of the control, 'Hunan Wucha'.

The first female flower appeared, on average, after the eighth leaf in the strongly female line LC-03, and thereafter each leaf axil had a female flower, no further male flower being produced. The first female flower appeared, on average, after the tenth, eighth, and eleventh leaf in the male parents 'Duozi 265', 1-14, and 8-1, and then another female flower occurred on average at

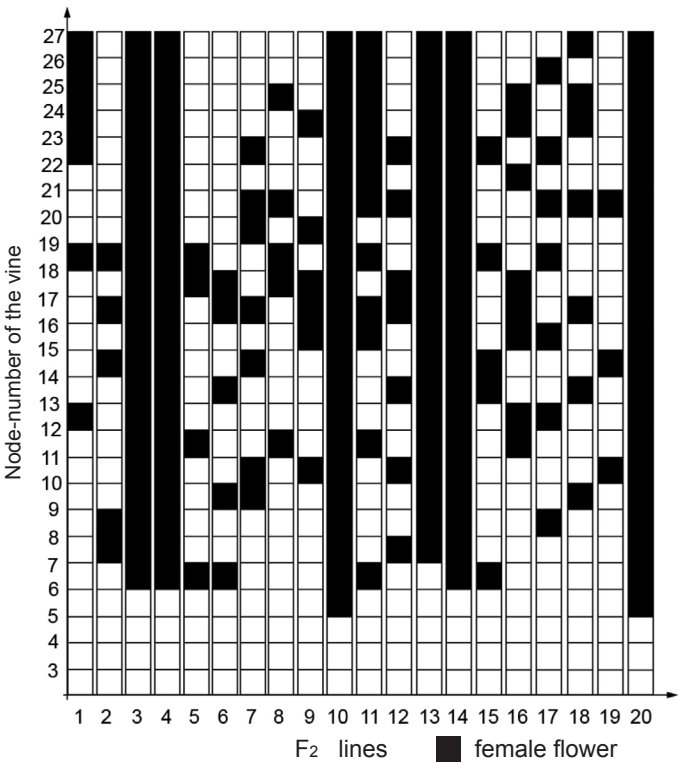

Figure 1. Distribution of male and female flowers on F_2 plants derived from the cross LC-03 × 'Duozi 265'.

intervals of 5.6, 5.3, and 5.1 leaves. The first female flower of all the hybrids of these three combinations appeared earlier than that of the male parents. The first female flower appeared after the tenth, eighth, and ninth leaf, on average, and again after an interval of five leaves. Flowering time is shown in Table 1. Thus, the F_1 hybrids have good horticultural traits, including early and concentrated maturity and increased production.

Characterization of the F_2 plants

We further investigated the inheritance of the trait of strong femaleness in F_2 populations. In 2009, we planted in the field three F_2 populations, derived from the three crosses LC-03 × 'Duozi 265', LC-03 × 1-14, and LC-03 × 8-1. In the F_2 population derived from the strongly female 'Duozi 265', there were 27.3% strongly female, 10% semi-subgynoecious, 22.7% subgynoecious and 40.9% normal plants (Figure 1). In the other two F_2 populations, segregation of strong femaleness to normal fit a 1 : 3 ratio. The backcross progeny plants were all normal plants when 1-14 or 8-1 was the recurrent parent, whereas segregation of strongly female plants to normal plants was in agreement with a 1 : 1 ratio when LC-03 was the recurrent parent. The results suggest that the strong femaleness in LC-03 is conferred by a single recessive gene.

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Searching for Candidate Genes Controlling Flower and Fruit Development in *Cucurbita pepo*

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Abstract. Sex expression, flower development, and fruit growth are traits of main interest for breeding as they are involved in plant production. In cucurbits, sex expression and determination are regulated by ethylene. Particularly, in the monoecious *Cucurbita pepo*, *CpACS27A*-gene expression controls stamen arrest during early development of female flowers. Apart from flower development, the early production of ethylene in female flowers might regulate other processes, such as fruit set and growth. The recent availability of genetic and genomic tools in *Cucurbita* can accelerate the study of these processes. Using a RILs population derived from the cross of two accessions of *Cucurbita pepo*, MU-CU-16 representing the subsp. *pepo* Zucchini Group and V-CU-196 representing the subsp. *ovifera* Scallop Group, we are studying the development of female and male flowers, and parthenocarpic and non-parthenocarpic early fruit growth. The parental accessions show contrasting flowering and fruit phenotypes. V-CU-196 produces more male flowers than MU-CU-16, and both male and female flowers that reach anthesis before those of MU-CU-16. Both parents show differences also in peduncle and petal rate of growth and in pre- and post-anthesis ovary development in pollinated and unpollinated fruits. The expression of ethylene-pathway genes is being analyzed during flower and fruit growth in both genotypes, looking for candidates that explain the observed phenotypic differences. These candidates will be mapped in the derived RILs population, already genotyped and used to construct a SNP-based genetic map with 7,000 SNPs to anchor the *Cucurbita* genome. The co-segregation of candidate variants and their expression levels with the phenotypic traits of interest will be further studied in the RILs population, providing information about the genes involved in the variation of these important traits.

Keywords: Flower growth and maturation, fruit set and development, hormones, ethylene, RILs

Introduction

Flower development is controlled by hormonal balance. In *Arabidopsis* and other genera, a number of mutants have been detected with alterations in phytohormone biosynthesis or signaling pathways which affect floral organ development. Auxins, giberellins, cytokinins, jasmonic acid, and brassinosteroids are involved in stamen development and maturation, perianth size, and pistil development (Chandler 2011). The action of ethylene at early stages of floral organ development, particularly primordial stamen arrest in female flowers, is well characterized in several cucurbits, including *Cucumis sativus* L. (Duan et al. 2008), *Cucumis melo* L. (Boualem et al. 2008), and *Cucurbita pepo* L. (Martínez et al. 2014a). Ethylene also regulates other flowering-related processes, being important for proper flower opening and maturation in *C. pepo* (Manzano et al. 2010, 2013). In this species, ethylene seems to be involved in the development of bisexual flowers. Bisexual flowers often have a delayed maturation of petals and pistil, a delayed flower opening and an increase of ovary size. In this type of flower, downregulation of ethylene biosynthesis and signaling genes are observed throughout development when compared with female flowers (Martínez et al. 2013, 2014a).

After pollination and fertilization, various changes occur that lead to fruit set and growth. Ethylene plays a negative role in this process as the upregulation of ethylene biosynthesis and signaling genes has been associated with prevention of fruit set and development (Vriezen et al. 2008, Pascual et al. 2009, Carbonell-Bejerano et al. 2011). This ethylene function has also been shown in *C. pepo*, in which parthenocarpic development of the fruit was observed to be correlated with downregulation of genes involved in ethylene biosynthesis and signaling, and therefore with reduced ethylene production (Martínez et al. 2013).

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C. pepo is highly polymorphic (Ferriol et al. 2003). Cultigens are quite variable in flowering and fruit development patterns. In this work, we observed and compared flower and fruit development in two contrasting inbred lines, parentals of a RIL population. One of the lines, MU-CU-16, belongs to Zucchini Group, producing uniformly cylindrical fruits of dark green color. The other parental line, V-CU-196, belongs to Scallop Group, producing flat fruits that are white in color. The identification of differences between these lines and their F_1, resulting from the cross MU-CU-16 (female parent) × V-CU-196 (male parent), is the first step toward studying the genetic basis of developmental traits.

Material & Methods

Plant material

MU-CU-16 and V-CU-196 represent one cultivar-group each of the two cultivated subspecies of *C. pepo*: Zucchini (subsp. *pepo*) and Scallop (subsp. *ovifera*). They have contrasting phenotypes for foliar, flowering, and fruit traits (Esteras et al. 2012). After a period of 10 to 15 days in a nursery, 30 plants of each parental line and their corresponding F_1 were transplanted and grown in a greenhouse under standard growing conditions for squash in Valencia (Spain).

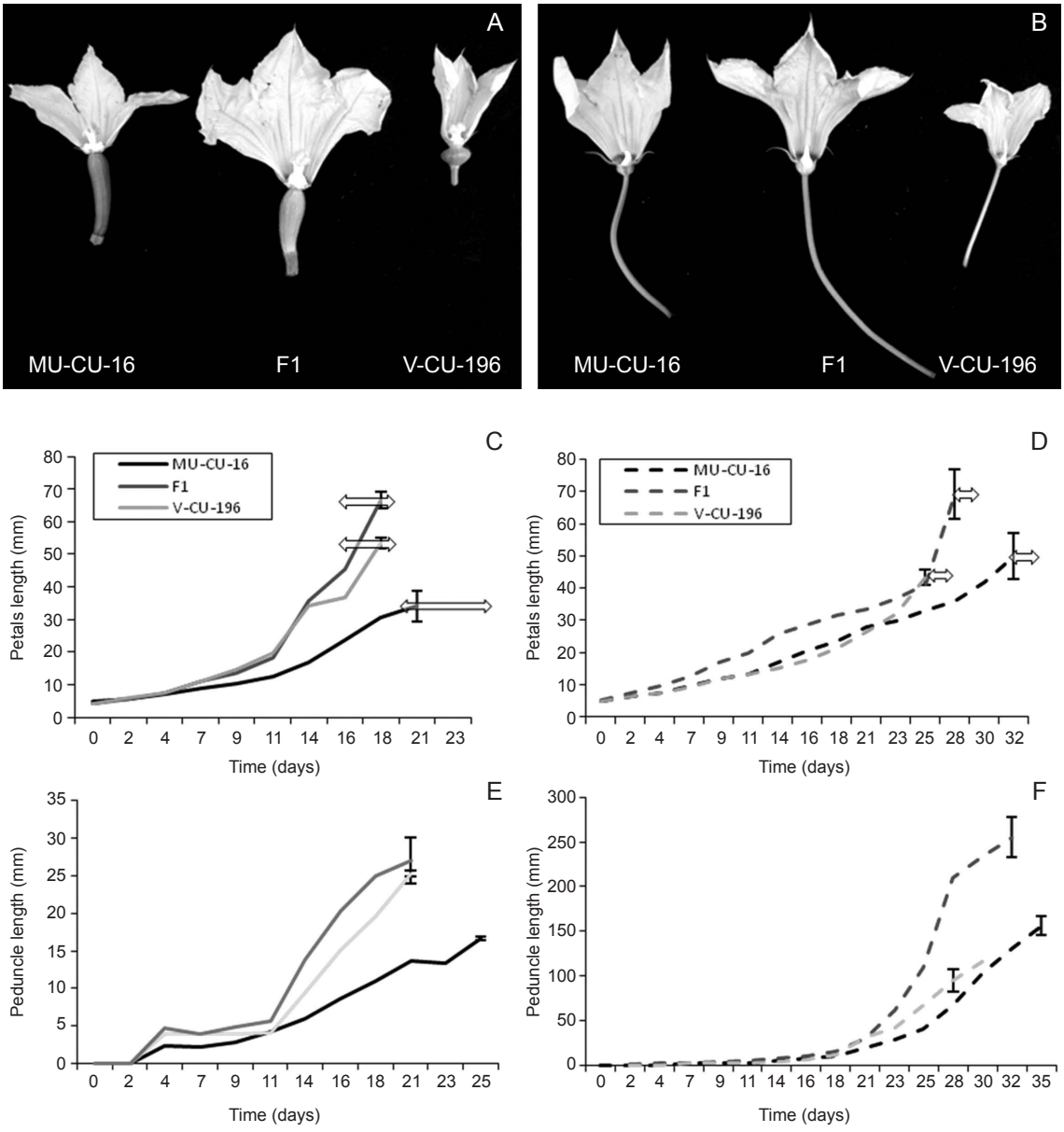

Figure 1. Growth rates of petals and peduncles of male and female flowers of lines MU-CU-16 (Zucchini) and V-CU-196 (Scallop), and their F_1. Female flowers at anthesis (A). Male flowers at anthesis (B). Comparison of petal growth rates of the parental lines and their F_1 in female (C) and male flowers (D). White arrows show petal maturation and flower opening time. Comparison of peduncle growth rates of parental lines and the F_1 of female (E) and male flowers (F). Standard errors of 10 flowers per line and sex is shown at the latest stage of organ maturation (preanthesis in petals and anthesis in peduncle).

Phenotyping and material collection

Starting with flowers of 4 mm of bud length, the growth pattern of male and female flowers was followed during a period of 35 days. Measurements were taken 3 times per week on 10 flowers of each sex type and genotype. Petals and peduncle length and ovary length and diameter were measured in each individual flower until anthesis.

Fruit growth was compared in pollinated and non-pollinated fruits. The length and diameter of at least 12 ovaries per genotype and developmental stage were measured at anthesis, and 2, 4, and 7 days post anthesis (DPA). A set of fruits were marked and pollinated and another set was closed prior to anthesis stage to prevent pollination. The phenotyped flower organs and fruits from the different genotypes and at the different stages of development were sampled (3 replicates) and frozen for future RNA isolation and gene expression analysis.

Statistical analysis

Simple analyses of variance (ANOVA) at $\alpha = 0.05$ was performed using the Statgraphics Plus v 5.1 software.

Results

Flower growth and maturation

We compared the longitudinal growth rate of petals and peduncles in male and female flowers in both parental lines, MU-CU-16 and V-CU-196, and their F_1 (Figure 1A–F).

Female flowers reached anthesis earlier than male flowers in both parental lines and the F_1. Both female and male flowers of MU-CU-16 showed a delay in flower maturation and opening, reaching anthesis 4 to 5 days later than those of V-CU-196 and the F_1 (Figure 1C,D). The growth rates of petals and the final size of the corolla were lower in MU-CU-16 female flowers than in V-CU-196 and the F_1 (Figure 1A,C). In male flowers, the growth rate was higher only in the F_1 from day 21 to anthesis, developing therefore the largest flowers (Figure 1B,D).

Differences in the peduncle growth rates were similar to that observed in petals. The peduncle of female flowers showed a higher growing rate from day 11 to anthesis in V-CU-196 and the F_1 in comparison to that in MU-CU-16 (Figure 1A,E). In male flowers, the growth rates of peduncles were similar in V-CU-196 and MU-CU-16, but size at anthesis was smaller in V-CU-196 because it had an earlier flower opening. The F_1 male flowers showed an increase in the growth rate from day 21, having the largest peduncles at flower opening (Figure 1B,F).

Ovary/fruit development

The growth rate of ovaries was measured in the same flowers described above. Since both parents differed in fruit shape, we observed clear differences in length and diameter during ovary development. Ovaries of MU-CU-16 grew mainly in length, whereas ovaries of V-CU-196 grew mainly in width (Figure 2A,B). The F_1 showed an intermediate profile of growth. A similar progression in ovary length was observed in MU-CU-16 and the F_1 generation (Figure 2A), but the delay of 5 days in the anthesis of MU-CU-16 resulted in longer ovaries (90 *versus* 75 mm in MU-CU-16 and the F_1 respectively). On the other hand, ovary diameter growth pattern was similar in V-CU-196 and the F_1, with accelerated growth from day 11 to anthesis. F_1 ovaries showed an ovary diameter intermediate at anthesis between both parents (Figure 2B).

For observing ovary development, a set of flowers was pollinated at anthesis stage, and the growth of pollinated and unpollinated ovaries was measured from anthesis to 7 DPA. Differences in fruit shape also determined the relative length/width growing pattern in unpollinated and pollinated fruits as previously described for ovaries prior to anthesis (Figure 2C,D). Unpollinated fruits of MU-CU-16 showed an early arrest of development, with a higher effect in width (between 2 to 4 DPA). Similar growth arrest was observed in unpollinated fruits of the Scallop type, but with a higher effect in length. F_1 unpollinated fruits continued to develop for longer (7 DPA and 2 to 4 DPA, in length and diameter, respectively).

Discussion

The parents of the RIL population, MU-CU-16 and V-CU-196, differ in the development of floral organs (petals and peduncles) in both female and male flowers. These differences are attributable to differences in the process of flower development, maturation, and opening. The behaviour of both parents and their F_1 suggests a differential genetic control of these traits in flowers of the two sex types. In female flowers, the behaviour of the F_1 generation is similar to that of the Scallop parent, both showing a higher petal and peduncle growth rate and an earlier flower opening as compared to the Zucchini parent, thus suggesting dominance of the Scallop alleles for these traits. In the male flowers, however, both parental lines showed a similar petal and peduncle growth rate, much lower than that of the F_1, suggesting heterosis. Despite the similar development of the organs of the male flowers in both parents, the parents showed a significant difference in flower opening time. The F_1 generation showed an intermediate response, suggesting partial dominance of this trait.

These floral developmental processes have been reported to be influenced by phytohormones, such as auxins and gibberellins (Ruan et al. 2012), however less is known about the influence of ethylene. Previous studies performed with bisexual flowers of *Cucurbita pepo* show changes in petals and ovary growth, and delayed opening time (Martínez et al. 2013, 2014a) as compared with female flowers, associated with downregulation of ethylene pathway genes. Our results show variability between parents in these traits, indicating potential differences in ethylene balance. The availability of the *C. pepo* genome and of the large collection of SNPs that has been used to construct a high density genetic map, using the RILs population of these two parents, provide the sequence of ethylene pathway genes whose expression will be evaluated through qPCR in the RILs population (Blanca et al. 2015).

One of the most important parameters controlling plant production is fruit set and development (Martínez et al. 2014a). MU-CU-16, UPV-196, and their F_1 also differ in ovary development. These differences are clearly associated with differences in fruit shape. The uniformly cylindrical ovaries of Zucchini mainly grow in length, whereas the flat Scallop ovaries mainly grow in diameter. The F_1 ovaries show a length growing pattern similar to the Zucchini parent. However, the delay of the opening of the female Zucchini flowers results in their longer ova-

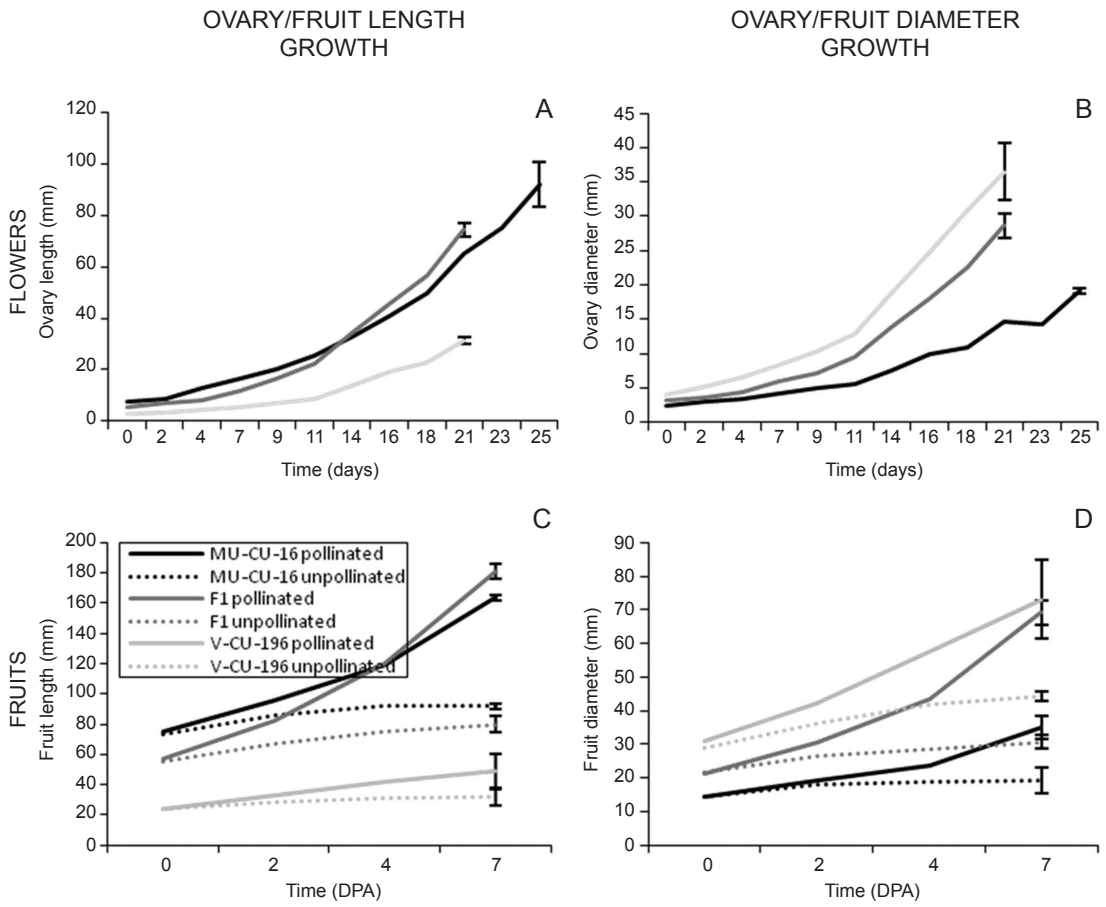

Figure 2. Growth rates of ovaries/fruits of lines MU-CU-16 (Zucchini) and V-CU-196 (Scallop), and their F_1. Comparison of ovary lengthening during flower development until anthesis (A). Comparison of ovary broadening during flower development until anthesis (B). Comparison of pollinated and unpollinated fruit lengthening from anthesis to 7 days post-anthesis (C). Comparison of pollinated and unpollinated fruit broadening from anthesis to 7 days post-anthesis (D). Standard errors of 10 flowers and 12 fruits per line and treatment are shown at the latest stages of development.

ries than in F_1. The hybrid ovaries show a width growing pattern more similar to the Scallop than to the Zucchini parent, although with a slower growing ratio that results in narrower ovaries of the F_1 than in UPV-196. These results suggest a different genetic control for these two traits. As many as five major and minor QTLs have been found to be involved in fruit shape, controlling the length of immature and mature fruits and mature-fruit width and cavity thickness (Esteras et al. 2013). Zucchini-type alleles contributed to produce elongated fruits, while the Scallop-type alleles produced wider fruits with wider cavities. Our results support this observation. Although several genes have been reported to be related to fruit shape, a major dominant gene (*Di*) seems to determine wider shape of fruits in the Scallop type. This gene was reported to be dominant over spherical or pyriform shapes (Paris and Kabelka 2009). However, results by Esteras et al. (2013) indicate the existence of a major gene and several modifiers.

It has been shown that the fruits of many Zucchini cultivars can initiate their growth in the absence of pollination and hormone application. After a few days, if no ovules are fertilized, fruits stop growing and a peak of ethylene is observed at 3 DPA in non-parthenocarpic cultivars (Martínez et al. 2013, 2014b). In our work, unpollinated fruits stopped lengthening (observed in MU-CU-16 unpollinated fruits) and broadening (observed in V-CU-196 unpollinated fruits) between days 2 and 4. F_1 unpollinated fruits kept lengthening until 7 DPA, although at a very slow rate, suggesting some kind of parthenocarpy. F_1 behaviour could be explained by hybrid vigor.

Differences in ovary development and in the growth rate of unpollinated and pollinated fruits will be also studied through gene expression analysis in the RILs population to identify genomic regions implicated in the observed changes. The results of Esteras et al. (2013) will be used to detect potential genes for evaluation of their expression throughout ovary/fruit development.

Acknowledgements

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Identification and Mapping of a QTL in Cucumber Controlling Resistance to *Pseudoperonospora cubensis*

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Abstract. The aim of this study was to map quantitative trait loci (QTLs) conferring cucumber resistance to *Pseudoperonospora cubensis*, the oomycete causing downy mildew. The mapping population consisted of 115 F_2 plants, descendants of a cross between the resistant PI 197085 and the susceptible PI 175695. For identification of QTLs, random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), sequence characterized amplified region (SCAR), and simple sequence repeat (SSR) markers were used, for a total of 238. QTL analysis indicated the presence of three quantitative trait loci, *DM1*, *DM2*, and *DM3*, which were mapped to chromosome 5 of the cucumber genome.

Keywords: *Cucumis sativus,* QTL mapping, downy mildew, DNA markers, disease resistance

Introduction

Downy mildew, caused by *Pseudoperonospora cubensis* (Berk. et Curt.) Rostov., is one of the most economically important foliar diseases in cucumber, *Cucumis sativus* L. This obligate oomycete pathogen attacks leaves of cucurbit plants (Lebeda and Urban 2004). Disease symptoms are manifested on the adaxial surface of leaves as chlorotic lesions limited by veins that cause a decrease in efficiency of photosynthesis. Sporulation occurs on the underside (abaxial surface) of the leaves. The entire leaf, in the most susceptible cultigens, can become necrotic. Cucumber resistance can be mono- or polygenic (van Vielt and Meysing 1974, Epinat and Pitrat 1994). Information about several quantitative trait loci, one or two major and several minor loci, and one or three recessive genes, have been variously reported in the literature (Jenkins 1946, Doruchowski and Łąkowska-Ryk 1992, Epinat and Pitrat 1994, Zhang et al. 2013). In this study, we performed QTL analysis for cucumber resistance to *P. cubensis* by using an F_2 population derived from crossing resistant and susceptible cucumber accessions.

Materials & Methods

Biological material and disease evaluation

The cucumber material for QTL mapping consisted of 115 plants of an F_2 population derived from the cross of the resistant PI 197085 with the susceptible PI 175695. Plants were grown from seeds in 10 cm plastic pots containing a peat substrate. Seeds were pregerminated on petri dishes followed by planting in pots (one seed per pot) and placed in a growth chamber. Seedlings were grown at 24/18 °C (day/night) under 12 h of light.

The inoculum was a sporangial suspension that was washed off, using distilled water, from sporulating lesions on leaves of PI 175695. Sporangia counting was done with a hemocytometer, and final inoculum concentration was adjusted to 5×10^4 sporangia $\times$ mL^{-1}.

Plants were inoculated at the one or two leaf stage by misting both sides of the leaves with the sporangial suspension. The inoculated seedlings were incubated in the dark for 48 h at 20 °C and 100% relative humidity. Thereafter, the plants were grown at 24 °C (day/night) with 12 h of light.

Disease assessments were made 7 days after inoculation using ten-point scale, where 0 = no disease, 1 to 2 = trace, 3 to 4 = slight, 5 to 6 = moderate, 7 to 8 = severe, and 9 = dead (Jenkins and Wehner 1983).

DNA isolation and PCR conditions

Total DNA was isolated from young leaves before inoculation. Genomic DNA was isolated with the NucleoSpin Plant II (Macherey-Nagel) kit. DNA purity and concentration were measured spectrophotometrically, and on 0.8% agarose gel after ethidium bromide staining. For QTL identification, amplification of 169 random amplified polymorphic DNA (RAPD), 3 inter simple sequence repeat (ISSR), 8 sequence characterized amplified region (SCAR), and 58 simple sequence repeat (SSR) markers were used, a total of 238 DNA markers. Primer sequences were obtained from internet databases (Cucurbit Genomics Database, USDA Cucumber SCAR Markers Database, VegMarks), literature, and our previous experiments (seven series of RAPD primers). From the 238 markers, only 36 (22 RAPD, 1 ISSR, 2 SCAR

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and 11 SSR) were polymorphic on the parental genotypes PI 197085 and PI 175695, resistant and susceptible, respectively.

The RAPD-PCR reaction was performed in 20 µl volume containing: 1x PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM dNTP, 0.3 µM primer, 1 U Taq DNA polymerase, 0.01% gelatin and 20 ng genomic DNA. Thermal profile of the PCR reaction was as follows: 94 °C for 1 min, 45 cycles of: 92 °C for 15 s, 36 °C for 25 s, 72 °C for 75 s, and 1 cycle of 72 °C for 5 min. Amplicons were separated on 1.4% agarose gel after ethidium bromide staining.

ISSR-PCR analysis was carried out in 20 µl containing: 1x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM each of primers, 0.8 U Taq DNA polymerase, and 30 ng genomic DNA. DNA was amplified under the following thermal conditions: 94 °C for 1 min, 40 cycles of 93 °C for 20 s, 42 °C for 25 s, 72 °C for 75 s, and 1 cycle of 72 °C for 5 min. PCR products were separated on 1.4% agarose gel after ethidium bromide staining.

SCAR analysis was carried out in 20 µl. The reaction mixture contained: 1x PCR buffer, 0.2 mM dNTP, 3 mM $MgCl_2$, 0.4 µM primers, 2U Taq DNA polymerase, and 20 ng DNA. DNA was amplified under the following conditions: 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 55–60 °C for 60 s, 72 °C for 90 s, and 1 cycle of 72 °C for 5 min. PCR products were separated on 1.6% agarose gel after ethidium bromide staining.

SSR analysis was performed in a 20 µl volume consisting of: 1x PCR buffer, 0.13 mM dNTP, 1.5 mM $MgCl_2$, 0.3 µM each of primers, 1 U Taq DNA polymerase and 40 ng genomic DNA. The PCR thermo profile was as follows: 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s, and final extension 72 °C for 5 min. The amplicons were separated in 8 to 10% polyacrylamide gel electrophoresis and visualized after silver staining.

QTL analysis

JoinMap 4.1 was used for linkage group (LG) construction. QTL analysis was performed using interval mapping and WinQTL Cartographer 2.5. The LOD (minimum logarithm of odds) threshold for QTL detection in interval mapping was determined by permutation test. LG mapping was done on the basis of SSR markers in common with the high resolution cucumber map developed by Ren et al. (2009).

Results & Discussion

PI 197085 showed a low degree of infection by *P. cubensis* whereas PI 175695 was highly susceptible. The F_2 population exhibited continuous phenotypic segregation from classes 1 to 8 (data not shown). In the mapping population, moderately resistant and moderately susceptible genotypes predominated. The normal distribution of phenotypes bins in the F_2 generation suggests that inheritance of *P. cubensis* resistance in cucumber is quantitative. Similar results were observed in the *P. cubensis* resistant 'Ames 2354', K8, and CS-PMR1 (Kozik et al. 2013, Zhang et al. 2013, Yoshioka et al. 2014).

Out of the 36 markers used for linkage map construction, 32 formed eight small linkage groups, while 4 markers remained unassigned. The largest linkage group was LG5, mapped to chromosome 5, spanning 205.4 cM with 8 markers (Figure 1). LG1 (five markers), LG6 (five markers), LG7 (four markers) were mapped to chromosomes 1, 6, and 7, respectively. The chromosomal localization of the remaining four linkage groups with, respectively, three and two markers awaits anchoring to the cucumber genome with additional markers.

Three QTLs, namely *DM1*, *DM2*, *DM3*, with LOD scores of 2.96, 4.11, and 3.89, respectively, were detected with interval mapping only in LG5. All identified loci showed negative additive effects, indicating that QTLs increase resistance to *P. cubensis*. The major QTL, *DM2*, was linked to SSR16110 and accounted for 27.08% of the phenotypic variance. Two minor QTLs, *DM1* and *DM3*, explained 11.41% and 18.03% of the variance of resistance, respectively. *DM1* was flanked by markers OPX06_850 and OPAM14, while *DM3* was linked to OPG12_1000 (Table 1). Previously, five QTLs for this trait were detected using the F_2 families derived from the cross K8 × K18 (Zhang et al. 2013). The loci *dm1.1* and *dm6.1* were on chromosomes 1 and 6, respectively. The loci of *dm5.1*, *dm5*.2, and *dm5*.3 were on chromosome 5, and were linked. One of these loci (*dm5.1*) and *DM2* identified in our research are probably the same, as they shared a similar position to the SSR16110 marker on chromosome 5. Moreover, both of these QTLs showed a negative additive effect and have the highest impact in conferring resistance to *P. cubensis* in cucumber. Other QTLs for this trait, *DM_5.1*, *Necr_5.1*, and *DM_5.2*, were mapped to chromosome 5 in introgression line 52 derived from a cross with *C. hystrix* (Pang et al. 2013). Nevertheless, comparison of the QTLs detected in these studies may not be effective, because there are no common molecular markers, and the mapping population is different. The high resistance of another line, CS-PMR1, was associated with many QTLs with relatively small effects located on chromosomes 3, 5, 6, and 7, whereas the moderate resistance of 'Santou' was linked with one major QTL mapped on chromosome 1 (Yoshioka et al. 2014). Notably, cucumber resistance to *Podosphaer*a *fusca* (Fr.) Braun & Shishkoff (powdery mildew) is associated with a locus located close to *dm5.3* on chromosome 5 (Zhang et al. 2011).

Table 1. QTL identification of *Pseudoperonospora cubensis* resistance in cucumber.

QTL	Chromosome	Spanning markers	LOD*	R^2	Additive effect	Dominant effect
DM1	5	$OPX06_{850}$ – OPAM14	2.96	11.41	- 0.34	0.51
DM2	5	SSR16110	4.11	27.08	- 0.59	0.13
DM3	5	OPG12_1000	3.89	18.03	- 0.47	0.06

*LOD = minimum logarithm of odds.

R^2 = the percentage of the variance explained by the QTL.

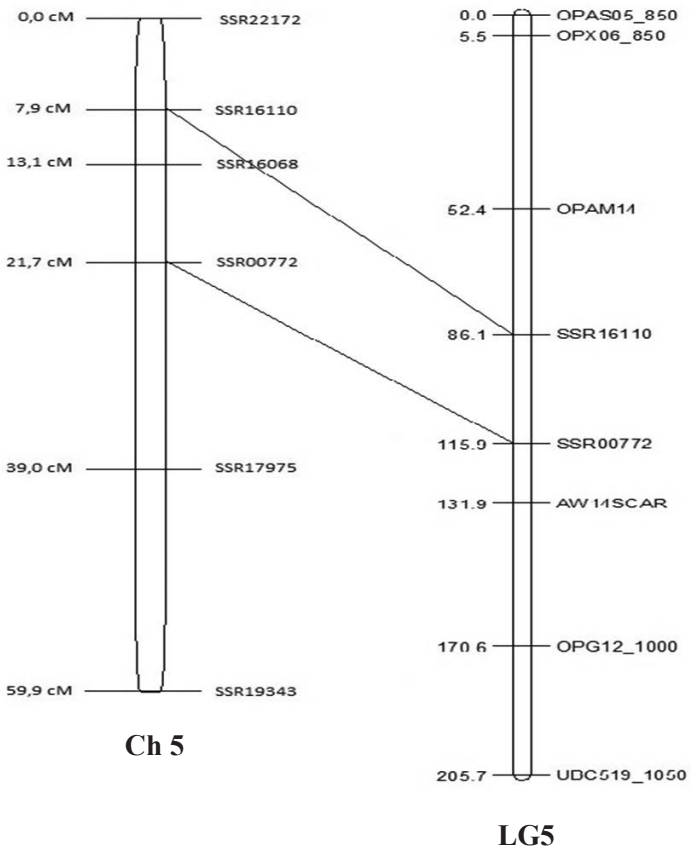

Figure 1. Localization of LG5 on cucumber chromosome 5.

In plants, a ubiquitous response to pathogen infection by the action of the *R* genes (resistance genes) plays an important role. These genes encode disease-resistance proteins, leucine-rich repeat proteins, Toll-Interleukin receptors, NB-ARC (nucleotide binding adaptor shared by APAF-1, R proteins, and CED-4), and others. In the K8 inbred line, ten NBS (nucleotide binding site proteins) were identified. Six were located near the *dm5.2* and four near the *dm5.3* loci. Their function is associated with resistance to *Pseudoperonospora cubensis* (Zhang et al. 2013). In lines Gy14 (U.S.A.) and 9930 ('Chinese Long'), several R genes (encoding leucine-rich repeat receptor-like proteins) responsible for *P. cubensis* resistance were also identified, mainly on chromosomes 4 and 5 (Wang et al. 2014).

The present results overlap with those cited above and confirm a particularly important role of some regions of chromosome 5 in cucumber resistance to *P. cubensis* and possibly other pathogens, such as that causing powdery mildew.

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Effects of Some Plant Activators on Yield, Plant, and Fruit Characteristics of Summer and Winter Melons

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Abstract. The effects of plant activators on plant growth, yield, and fruit quality are well known. An experiment was conducted in southeastern Turkey with 'Galia C8' and 'Kirkagac 637' melons during two spring seasons. OSU 142, M3, SP 245, Bioplin, Phosfert, Em1, Bio-One, Endoroots, Sim-Derma, and *Spirulina* spp., plant activators were used alone and with the organic fertilizers Camlibesi Bioaktif Compost, Camlibesi Liquid Organic Fertilizer, and K-Humat for comparing with conventional fertilizers and control treatments. The main shoots of 'Galia C8' were longer than those of 'Kirkagac 637'. The Spirulina+OF treatment was the earliest. Total yield was 23.59 tonnes/ha in 'Galia C8' and 22.84 tonnes/ha in 'Kirkagac 637'. Mean fruit weight was 1.25 kg in 'Galia C8' and 2.24 kg in 'Kirkagac 637'. Mean total soluble solids was 10.3% in 'Galia C8' and 8.3% in 'Kirkagac 637'. Except for TSS, plant activator effects on fruit quality were non-significant.

Keywords: Plant activators, yield, plant growth, fruit quality

Introduction

Melon (*Cucumis melo* L.) production is around 31.225 million tonnes in the world and 1.69 million tonnes in Turkey (FAO 2013). Melon is the third most important crop after watermelon and cucumber in the vegetable production of Adiyaman (TUIK, 2015). Today, although there is no organic melon production in Adiyaman, farmers appear to be using a minimum of synthetic pesticides and use good agricultural practices.

Benefits of microorganisms for plants can be classified into three groups: (a) nitrogen fixing organisms, (b) mycorrhizal fungi, and (c) plant growth promoting rhizobacteria (PGPRs) (Arcak and Guven 2004). In recent years, PGPR bacterial isolations have been used on various plants as activators and have great potential as bio-fertilizers. Also, PGPRs can be colonized by microflora of roots (Van Loon et al. 1998). Microalgae are one of the organism groups which contain high levels of protein, vitamins, minerals, fatty acids, and pigments, and have been one of the most focused on recently (Kendirli 2010).

The negative effects of conventional production practices on ecology and health are well-known. A major cause of these negative effects is the use of lots of pesticides. For this reason, organic agriculture is rapidly expanding as an alternative. The climate of Adiyaman has changed in recent years. Because of this, lots of vegetables and fruits can now be cultivated organically. This study sheds light on organic production and the rapid development of organic agriculture in Adiyaman. In this study, effects of plant activators are documented for field production of summer and winter melons.

Materials & Methods

This study was carried out in the experimental field of Adiyaman University, Agricultural and Field Management Research Centre at Hasancik, Adiyaman (37° 46' 39.11" N, 38° 25' 39.63" E). Soil texture was loamy and no pesticides were used. 'Galia C8' (*Cucumis melo* var. *cantalupensis*) summer melon and 'Kirkagac 637' (*Cucumis melo* var. *inodorus*) winter melon cultivars were used as plant material.

Seedlings were transplanted to the open field on May 5, 2011 and 2013, between-row and within-row spacings of 1.8 m and 0.5 m, respectively. The experiment had 4 replications and each replicate consisted of 10 plants. The plants were drip-irrigated.

Three plant activators, *Bacillus subtilis* (OSU 142), *B. megatorium* (M3), and *Azospirillum* spp. (SP 245) were obtained from Prof. Dr. Fikrettin Sahin, Yeditepe University of Turkey. They have great potential as bio-control agents, and have a positive effect on fruit quality and element content of leaves (Esitken et al. 2006, Karlidag et al. 2007, Pirlak and Kose 2009). OSU 142, M3, and SP 245 were augmented with a broth medium for the isolated, young population, which had been developed on agar medium, and applied by saturating the plant roots at 10^9 cell·mL^{-1}. Isolates were kept at -80 °C. The seedling roots were dipped into the preparate for 20 minutes

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before being transplanted to the field. For Bioplin (*Azotobacter vinelandii* × *A. chroococcum)* and Phosfert (*A. vinelandii* × *A. chroococcum* × *Bacillus polymyxa*), seedling roots were dipped into 100 mL/7L water, for one time only, and kept for 20 minutes and then the seedlings were transplanted to the field. 500 mL Em1 (*Sacchoromyces cerevisiae* + lactic acid, local bacteria) was mixed with 45 L water, and 200 mL of this solution was applied to the soil per plant every two weeks. Bio-One (*A. vinelandii* × *Clostridium pasteurianum*) was mixed with 1.3 L water, in addition to 154 g refined sugar mixed with 7.2 L water in one place, and these two mixtures were then mixed and applied to the soil in 21.6 mL quantities once per plant. Endoroots [*Glomus intraradices* (25%), *G. mosseae* (24%), *G. aggregatum* (1%), *G. monosporum* (1%), *G. deseticola* (1%), *G. brasilianum* (1%), *G. etunicatum* (1%), *G. margarita* (1%)], are mycorrhizal fungi that were applied to the soil at 2.25 g/9 m^2. Sim-Derma (*Trichoderma harzianum* KUEN1585), 5 g, was mixed with 5 L of water and the roots of the seedlings, ready for transplanting, were kept in this mixture for 30 minutes, and then were transplanted to the field. Lastly, 45 $g \cdot L^{-1}$ *Spirulina* spp. solution was prepared, and 200 mL of this solution was applied to the plants near the seedling roots.

As for the organic fertilizers (OF), Camli Bioactive Organic Fertilizer contains 60% organic matter, 3% nitrogen (N), 2.5% P_2O_5, 2.5% K_2O, and 25% humic and fulvic acids. Biofarm Liquid Organic Fertilizer contains 35% organic matter, 4% nitrogen (N), 2% P_2O_5, 3.5% K_2O, 5% humic acid, and 19% fulvic acid. The pH value is 6. K-Humate's total organic matter content is 16%, total humic and fulvic acid content is 15%, water soluble K_2O content is 2.5% and pH is 9 to 11. Each one of the plant activators was applied separately along with the organic fertilizer applications.

DAP and Urea were used conventionally. In the control application, no plant activator and/or organic/conventional fertilizers were applied.

Main shoot length of plants was measured twice, at 2 months and 3 months after planting. Fruit harvest began on 5 July and continued to 10 August in both years. Daily harvests were made and the fruits weighed. Early harvest was considered as the first ten-day period. Five fruits from each plot were weighed, measured, and checked for total soluble solids (TSS) by digital refractometer.

Statistical analyses were conducted by the use of the JMP 5.1 program and the LSD test was used to compare means. The formula used for the calculation of the D value was:

$$D = \sqrt{2 \times Mean\ squared\ error \div n} \times t$$

Table 1. Main shoot length, 2 and 3 months after planting, of 'Galia C8' and 'Kirkagac 637'.

Applications	Main shoot length (after 2 months from planting)					Main shoot length (after 3 months from planting)				
	'Galia C8'		'Kirkagac 637'		mean	'Galia C8'		'Kirkagac 637'		mean
	2011	2013	2011	2013		2011	2013	2011	2013	
OSU 142	117.1	158.9	102.0	164.2	135.6	131.5	169.4	117.0	186.9	151.6
M3	118.9	150.6	99.6	172.6	135.4	133.9	172.4	114.1	187.5	152.0
SP245	113.4	149.6	99.0	162.4	131.1	128.4	164.8	114.6	197.9	151.4
Bioplin	111.9	146.2	107.6	165.4	132.8	124.8	162.4	128.1	184.9	150.0
Phosfert	123.6	147.9	104.6	161.1	134.3	138.1	175.8	119.6	180.1	153.4
Em1	122.4	155.6	103.8	148.2	132.5	137.5	161.9	118.8	167.5	149.4
Bio-One	124.0	148.0	99.4	155.2	131.7	139.0	173.9	114.4	182.2	152.4
Endoroots	117.0	151.6	108.9	155.6	133.3	131.9	172.0	123.9	183.8	152.9
Sim-Derma	115.9	149.1	100.2	147.1	128.1	130.5	165.2	115.2	178.0	147.2
Spirulina	117.4	148.9	104.0	155.9	131.5	133.2	171.9	119.0	178.2	150.6
OF	119.2	150.1	106.3	168.1	135.9	131.3	168.5	121.3	184.6	151.4
OSU142+OF	111.6	160.9	97.8	155.2	131.4	129.1	172.5	112.2	182.8	149.1
M3+OF	118.5	146.5	106.9	143.2	128.8	133.5	156.8	121.4	166.2	144.5
Sp245+OF	118.6	149.9	103.3	149.1	130.2	133.6	159.6	117.7	179.0	147.5
Bioplin+OF	115.1	144.6	102.3	146.6	127.1	130.0	166.4	115.4	185.6	149.3
Phosfert+OF	121.4	148.2	103.8	154.0	131.9	284.8	165.8	118.1	193.8	190.6
Em1+OF	117.1	166.1	104.1	157.2	136.1	126.6	173.1	118.4	186.2	151.1
Bio-One+OF	117.1	163.2	102.0	156.1	134.6	131.9	188.2	117.0	202.5	159.9
Endoroots+OF	114.6	171.9	103.2	149.0	134.7	129.6	198.9	123.3	199.4	162.8
Sim-Derma+OF	114.9	158.9	100.4	164.1	134.6	128.1	180.2	121.3	191.8	155.3
Spirulina+OF	111.9	156.6	97.9	162.2	132.2	126.8	185.5	112.8	203.5	157.2
DAP+Urea	117.6	153.4	106.7	159.5	124.4	131.7	180.2	123.3	205.4	160.2
Control	118.7	113.9	101.3	162.9	134.0	132.6	170.9	116.3	181.2	150.3
Variety mean	134.5 a		130.5 b			155.1		152.4		
Year mean	2011 = 110.1 b		2013 = 154.5 a			2011 = 128.3 b		2013 = 179.2 a		
D 5% (App.) = ns	D 1% (Var.) = 3.07		D 1% (Year)= 3.07			D 5% (App.) = ns		D 5% (Var.) = ns	D %1 (Year) = 2.2	
D 1% (Var. x Year) = 4.3	D 5% (App. x Year) = ns					D 1% (Var. x Year) = 3.13		D 5% (App. x Year) = ns		
D 5% (App. x Var.) = ns	D 5% (App. x Var. x Year) = ns					D 5% (App. x Var.) = ns		D 5% (App. x Var. x Year) = ns		

Table 2. Early and total yield in 'Galia C8' and 'Kirkagac 637'.

Applications	Early yield (tonnes/ha)					Total yield (tonnes/ha)				
	'Galia C8'		'Kirkagac 637'		mean	'Galia C8'		'Kirkagac 637'		mean
	2011	2013	2011	2013		2011	2013	2011	2013	
OSU 142	3.25	10.40	2.79	5.04	5.37 c-f	22.30	30.85	12.63	27.12	23.22
M3	3.07	6.86	5.24	9.35	6.13 b-f	14.61	28.68	14.42	34.98	23.18
SP245	4.37	9.51	3.76	1.10	7.17 a-e	18.72	31.04	10.57	32.15	23.12
Bioplin	0.70	14.48	0.73	9.20	6.28 b-f	12.75	36.95	14.34	36.98	25.25
Phosfert	2.37	11.41	2.51	5.45	5.43 c-f	20.14	35.28	11.99	31.73	24.79
Em1	1.34	12,57	1.22	4.10	4.81 ef	13.82	40.09	16.61	31.95	25.62
Bio-One	1.55	11.73	0.42	2.76	4.11 f	17.95	29.96	14.33	24.83	21.77
Endoroots	2.67	9.02	1.54	5.29	4.64 de	15.92	33.12	15.06	31.83	23.98
Sim-Derma	0.93	11.48	2.95	6.69	5.51 c-f	13.00	29.64	17.25	31.94	22.96
Spirulina	0.80	15.97	0.54	3.47	5.20 def	10.50	37.65	8.92	35.59	23.17
OF	5.29	11.37	4.36	5.50	6.63 b-f	15.39	28.56	19.37	32.77	24.02
OSU142+OF	4.43	16.88	3.47	3.88	7.16 a-e	12.72	36.65	12.23	29.44	22.76
M3+OF	7.34	12.41	2.67	9.62	8.01 a-d	15.40	28.61	14.40	29.46	21.97
Sp245+OF	4.78	11.97	0.71	8.11	6.39 b-f	11.71	29.76	14.51	35.26	22.81
Bioplin+OF	4.99	9.49	1.57	8.69	6.18 b-f	13.79	27.11	19.73	34.08	23.68
Phosfert+OF	3.23	7.80	0.93	6.39	4.59 ef	13.70	26.73	13.44	29.71	20.96
Em1+OF	7.60	12.07	2.91	6.31	7.22 a-e	15.12	34.12	16.45	35.94	25.41
Bio-One+OF	5.54	10.41	2.00	3.27	5.30 def	13.17	29.32	14.31	29.24	21.51
Endoroots+OF	6.93	16.12	4.09	6.09	8.31 abc	18.22	36.38	10.38	30.59	23.89
Sim-Derma+OF	3.13	12.08	4.89	6.82	6.73 b-f	16.07	33.47	12.98	26.76	22.32
Spirulina+OF	4.91	18.18	0.86	14.95	9.73 a	13.73	42.19	12.59	34.37	25.72
DAP+Urea	12.2	1.80	4.34	12.87	8.78 ab	14.49	35.26	11.31	38.28	24.84
Control	1.68	9.51	2.42	9.05	4.61 ef	12.29	18.02	9.42	28.41	17.04
Variety mean	7.75 a		4.80 b			23.59		22.84		
Year mean	2011 = 3.26 b		2013 = 9.29 a			2011 = 14.41 b		2013 = 32.02 a		
D 1% (App.) = 2.94 D 1% (Var.) = 0.87 D1% (Year) = 0.87 D 1% (Var. x Year) =1.23 D 5% (App. x Year) = ns D 5% (App. x Var.) = 4.16 D 5% (App. x Var. x Year) = 1.23						D 5% (App.) = ns D 1% (Var.) = ns D 5% (Year) = 19.70 D 5% (Var. x Year) = ns D 5% (App. x Year) = ns D 5% (App. x Var.) = ns D 5% (App. x Var. x Year) = ns				

Results

Main shoot lengths are presented in Table 1. Shoots were longer in 2013. The shoots, at the first measurement, of 'Galia C8' (134.5 cm) averaged 4 cm longer than those of 'Kirkagac 637' (130.5 cm). At the second measurement, the average shoot length of 'Galia C8' (155.1 cm) was 3 cm longer than 'Kirkagac 637' (152.4 cm).

Data for early and total yields are presented in Table 2. Early yield from the Spirulina+OF treatment was the highest (9.73 tonnes/ha) followed by DAP+Urea (8.78 tonnes/ha) and Endoroots+OF (8.31 tonnes/ha). Spirulina+OF and DAP+Urea applications provided 111% and 91% earliness when compared to the control. In terms of cultivars, 'Galia C8' was 38% earlier than 'Kirkagac 637'. In the 2013, early yield was 3 times greater (9.29 tonnes/ha) than the 2011. In total yield, plant activators, varieties and interaction of plant activators × variety × year were found to be statistically non-significant. The total yield in 2011 was 14.41 tonnes/ha while it was 32.02 tonnes/ha in 2013. Hence, yield was greater by 122% in 2013.

The mean values of fruit weight, TSS, fruit length, and fruit diameter are presented in Table 3 and 4. Except for TSS, plant activators × variety × year interactions were found to be statistically non-significant for all parameters. The average fruit weight of 'Galia C8' ranged from 1.49 kg (OSU 142+OF) to 1.86 kg (Endoroots+OF). The average fruit weight of 'Kirkagac 637' ranged from 1.15 kg (Control) to 2.03 kg (Spirulina) for 2011. TSS content of 'Galia C8' (10.3%) was higher than of 'Kirkagac 637' (8.3%). When applications were compared in terms of fruit length, the results were found to be statistically non-significant. However, according to varieties and years, average fruit length was found to be statistically significant at the 1% level. 'Kirkagac 637' (17.9 cm) has longer fruits than 'Galia C8' (13.3 cm). Fruit length was higher in 2013 (16.0 cm), when compared to 2011 (15.2 cm). In terms of fruit diameter, applications were statistically non-significant while variety and year were found to be statistically significant. The average fruit

Table 3. Fruit weight (kg) and total soluble solids content (%) in 'Galia C8' and 'Kirkagac 637'.

Applications	Fruit weight (kg)					Total soluble solid contents (%)				
	'Galia C8'		'Kirkagac 637'		mean	'Galia C8'		'Kirkagac 637'		mean
	2011	2013	2011	2013		2011	2013	2011	2013	
OSU 142	1.01	1.24	1.42	2.34	1.50	8.8	11.4	8.4	9.4	9.5
M3	1.07	1.35	1.64	2.65	1.68	10.3	10.2	8.6	8.8	9.5
SP245	0.93	1.49	1.45	2.46	1.58	9.4	10.5	8.4	8.3	9.2
Bioplin	1.13	1.48	1.62	2.64	1.72	8.9	10.5	9.3	7.2	9.0
Phosfert	1.14	1.35	1.50	2.55	1.64	10.4	10.4	7.9	7.5	9.0
Em1	1.03	1.52	1.91	2.47	1.73	9.4	11.3	8.3	8.2	9.3
Bio-One	1.25	1.30	1.67	2.70	1.73	10.7	10.1	8.1	9.6	9.6
Endoroots	1.26	1.68	1.70	2.63	1.82	9.5	11.3	8.1	8.9	9.5
Sim-Derma	1.04	1.26	1.85	2.59	1.68	9.1	10.4	8.0	8.8	9.1
Spirulina	1.05	1.66	2.03	2.45	1.80	8.4	11.1	8.7	8.3	9.1
OF	1.12	1.47	1.64	2.71	1.73	9.1	11.7	9.0	8.6	9.6
OSU142+OF	1.07	1.17	1.42	2.33	1.49	10.3	11.3	8.8	8.9	9.8
M3+OF	1.02	1.27	1.44	2.53	1.57	9.8	9.9	9.0	7.8	9.1
Sp245+OF	1.02	1.34	1.56	2.30	1.56	8.5	10.3	8.1	8.1	8.7
Bioplin+OF	1.10	1.42	1.52	2.51	1.64	10.2	10.0	7.9	6.7	8.7
Phosfert+OF	1.05	1.38	1.55	2.48	1.61	11.2	11.7	8.5	7.5	9.7
Em1+OF	1.04	1.36	1.70	2.91	1.75	9.7	11.1	8.3	8.0	9.3
Bio-One+OF	0.96	1.42	1.45	2.68	1.63	8.4	11.8	8.3	7.2	8.9
Endoroots+OF	1.30	1.72	1.62	2.82	1.86	9.3	11.0	8.2	8.4	9.2
Sim-Derma+OF	1.15	1.45	1.50	2.80	1.73	9.3	10.7	7.9	8.5	9.1
Spirulina+OF	1.22	1.45	1.40	2.60	1.67	9.1	11.3	8.4	7.5	9.1
DAP+Urea	1.06	1.42	1.55	2.69	1.68	11.4	12.1	7.4	7.9	9.7
Control	1.13	1.26	1.15	2.82	1.59	9.6	12.4	7.5	9.0	9.6
Variety mean	1.25 b		2.09 a			10.3 a		8.3 b		
Year mean	2011 = 1.34 b		2013 = 2.00 a			2011 = 9.0 b		2013 = 9.6 a		
D 1% (App.) = 0.33	D 5% (Var.) = ns		D 1% (Year)= 0.33			D 5% (App.) = ns		D 1% (Var.) = 2.1	D 1% (Year) = 2.1	
D 5% (Var. x Year) = ns	D 5% (App. x Year) = ns					D 1% (Var. x Year) = 0.3		D 1% (App. x Year) = 1		
D 5% (App. x Var.) = ns	D 5% (App. x Var. x Year) = ns					D 1% (App. x Var.) = 1.0		D 5% (App. x Var. x Year) = 0.3		

diameter of 'Kirkagac 637' (15.2 cm) was greater than that of 'Galia C8' (13.3 cm).

Discussion

It is noteworthy that conventional production provides early growth when compared to organic production. In terms of plant and fruit characteristics, 'Kirkagac 637' had higher values than 'Galia C8'. In our study, use of mycorrhiza increased fruit weight as compared with the control. Also, Vazquez-Hernandez et al. (2011) reported that *Glomus mosseae* increases fruit weight of papaya by 45.1%, while *Entrophospora colombia* increases it by 18.4%. Naidu et al. (2013) observed that the application of microbial-enriched compost tea to melons had positive effects on TSS, fruit weight, fruit length, and fruit flesh thickness. Karlidag et al. (2007) applied M3, OSU 142, FS01, and their combinations as plant activators, to examine their impact on the quality of 'Granny Smith' apples. They reported that plant activators had no statistically significant impact on fruit weight. For TSS, FS01 application gave the best result, 16.1%, while M3 (15.5%) and OSU 142 (15.5%) applications resulted in TSS lower than the control (15.8%). When they compared average the fruit diameter, all applications and interactions, excluding the FS01 (7.11 mm) application, were found to have lower values than the control (6.98 mm). Arikan et al. (2013) applied T8, OSU 142, and M3 to quince and reported that they had statistically significant effects on various fruit parameters, including fruit diameter, fruit length, and TSS. For fruit number, T8 application, for fruit weight, and fruit length, T8+OSU 142 application, for fruit diameter OSU 142 application, and for TSS control application provided the highest values.

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Table 4. Fruit length and diameter (cm) in 'Galia C8' and 'Kirkagac 637'.

Applications	Fruit length (cm)					Fruit diameter (cm)				
	'Galia C8'		'Kirkagac 637'		mean	'Galia C8'		'Kirkagac 637'		mean
	2011	2013	2011	2013		2011	2013	2011	2013	
OSU 142	12.2	14.1	18.1	17.9	15.6	12.8	13.2	13.7	16.1	14.0
M3	12.8	13.3	16.8	19.0	15.5	12.1	13.7	14.5	16.3	14.2
SP245	11.8	13.4	16.6	18.2	15.0	12.4	14.1	13.8	15.9	14.0
Bioplin	12.9	13.9	17.6	19.0	15.9	12.3	14.5	13.6	16.7	14.3
Phosfert	13.5	13.4	17.9	18.9	15.9	13.2	13.5	13.9	16.1	14.2
Em1	12.7	13.7	17.6	18.3	15.6	12.2	14.3	14.8	16.4	14.4
Bio-One	14.0	13.2	17.8	18.8	15.9	13.5	12.0	15.1	16.9	14.4
Endoroots	13.5	14.1	17.2	17.5	15.6	13.6	14.8	10.9	16.4	14.0
Sim-Derma	13.3	13.1	17.8	18.4	15.6	12.8	12.6	14.9	15.4	13.9
Spirulina	13.0	13.5	20.2	17.6	16.1	12.7	13.5	16.8	16.2	14.8
OF	13.4	12.0	18.0	18.1	15.4	12.9	13.1	14.2	16.7	14.2
OSU142+OF	13.0	13.6	16.9	17.7	15.3	12.7	14.2	13.3	16.0	14.1
M3+OF	12.8	13.5	17.0	17.9	15.3	12.6	13.9	12.9	16.3	14.0
Sp245+OF	12.9	13.4	17.5	18.7	15.6	12.5	13.4	14.3	16.2	14.1
Bioplin+OF	13.3	13.3	17.9	19.0	15.9	12.7	13.9	14.7	16.3	14.4
Phosfert+OF	12.9	13.3	16.9	18.3	15.3	12.9	13.6	13.5	16.8	14.2
Em1+OF	12.8	13.2	17.6	19.2	15.7	12.6	13.8	14.4	17.4	14.5
Bio-One+OF	12.8	14.2	16.2	18.5	15.4	12.3	14.5	13.6	16.7	14.3
Endoroots+OF	13.9	14.6	18.3	18.9	16.4	13.5	14.6	14.2	16.1	14.6
Sim-Derma+OF	13.4	13.7	16.2	19.2	15.6	12.9	14.1	14.2	16.2	14.3
Spirulina+OF	13.5	14.0	16.7	18.4	15.6	12.6	14.1	13.6	16.8	14.3
DAP+Urea	13.2	14.0	17.5	18.6	16.1	12.8	14.1	13.9	17.7	14.6
Control	13.2	13.5	14.7	18.7	15.0	13.0	13.9	13.2	17.2	14.3
Variety mean	13.3 b		17.9 a			13.3 b		15.2 a		
Year mean	2011 = 15.2 b		2013 = 16.0 a			2011 = 13.4 b		2013 = 15.1 a		
D 5% (App.) = ns	D 1% (Var.) = 0.27		D1% (Year) = 0.27			D 5% (App.) = ns	D 1% (Var.) = 1.97		D 1% (Year) = 1.97	
D 1% (Var. x Year) = 0.38	D 5% (App. x Year) = ns					D 1% (Var. x Year) = 0.37	D 5% (App. x Year) = 1.26			
D 5% (App. x Var.) = ns	D 5% (App. x Var. x Year) = ns					D 5% (App. x Var.) = ns	D 5% (App. x Var. x Year) = ns			

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Effects of Cadmium Stress on Growth, Reactive Oxygen Metabolism, and Photosynthesis of Melon Seedlings

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Abstract. An experiment was conducted with 'Hamilü' and 'Xiulü' melon seedlings grown in climate-controlled chambers, in order to investigate the effects of cadmium stress on growth, reactive oxygen metabolism, and photosynthesis, and to explore the possible mechanism of cadmium tolerance of melon. The results showed that, with the increasing cadmium concentration, seedling growth, net photosynthetic rate (P_n), stomatal conductance (G_s), transpiration rate (T_r), and stomatal limitation (L_s) decreased; meanwhile, intercellular carbon dioxide concentration (C_i) increased significantly. The plant growth and photosynthetic apparatus of 'Hamilü' were less affected than those of 'Xiulü'. Antioxidant enzyme activities and soluble protein content first increased, then decreased with increasing concentrations of cadmium. The proline content of 'Hamilü' increased significantly, meanwhile, the proline content of 'Xiulü' at first increased then decreased. With increasing cadmium concentrations, MDA content and $O_2^{\cdot-}$ producing rate increased, and 'Hamilü' seedlings were less affected than those of 'Xiulü'. These findings suggest that there are significant genotypic differences in melon response to cadmium toxicity.

Keywords: Cadmium, melon, reactive oxygen metabolism, photosynthesis

Introduction

Cadmium (Cd), one of the most highly toxic metals, is easily absorbed and accumulated by plants. It can adversely affect normal plant growth and development, and its bioaccumulation in the food chain can be highly dangerous (Li et al. 2004, Sato et al. 2010, Yu et al. 2012). It has been shown that Cd can impinge on photosynthesis, causing oxidative stress and membrane damage in plants, and change the activity of oxygen metabolism-related enzymes, such as the activity of anti-oxidases, and eventually lead to metabolic disorders that destroy the plant (Milone et al. 2003, Zhou et al. 2008). In recent years, investigators have conducted a lot of tests on the effects of Cd on wheat (Li et al. 2005), rice (Shi et al. 2013), and soybean (Huang et al. 2006) growth and development, physiology, and biochemistry. Ren et al. (2009) reported that 10 to 100 $\mu mol \cdot L^{-1}$ Cd^{2+} had obvious inhibitory effects on lettuce seedling growth, and the degree of inhibition increased with increasing concentration. At present, there is but little reported regarding effects of Cd on growth and physiology of cucurbits. Therefore, employing melon, *Cucumis melo* L., we herein report the effects of different concentrations of Cd on plant growth, anti-oxidase activity, and photosynthesis. We tested two melon cultivars in order to explore the possibility of the existence of relative tolerance in melon to Cd damage, for breeding resistant cultivars, and early diagnosis of Cd contamination in agricultural production.

Materials & Methods

Seeds of the melons 'Hamilü' and 'Xiulü' were provided by the Horticultural Research Institute, Shanghai Academy of Agricultural Sciences. Seeds were rinsed thoroughly with distilled water and germinated in an incubator at 30 °C. The uniformly germinated seeds were sown on plastic plates, 10 cm × 10 cm, filled with vermiculite and placed in a growth chamber at 30/20±1 °C (12-h photoperiod), irradiance of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and a relative humidity of 80%. The seedlings were watered with five concentrations: (1) nutrient solution (CK); (2) nutrient solution + 10 $mg \cdot L^{-1}$ Cd (Cd-10), (3) nutrient solution + 50 $mg \cdot L^{-1}$ Cd (Cd-50); (4) nutrient solution + 100 $mg \cdot L^{-1}$ Cd (Cd-100); (5) nutrient solution + 200 $mg \cdot L^{-1}$ Cd (Cd-200). There were 10 seedlings per treatment with three replicates, randomly selected. Measurements were taken 10 days after treatment.

The activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) were measured according to the method of Li (2000), the soluble protein content was measured with the method of Coomassie brilliant blue G250; the proline content was determined by acid ninhydrin colormetric method, and the malondialdehyde (MDA) content by the thiobarbituric acid method (Li 2000). The determination of the superoxide anion ($O_2^{\cdot-}$) production rate was measured by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{\cdot-}$ (Wang and Luo 1990). Net photosynthetic rate (Pn), transpiration rate (Tr), stomatal conduction degree (Gs), and intercellular CO_2 concentration (Ci) were observed and recorded on the third fully expanded leaf using a gas analyzer (Li-6400, Li-COR, Lincoln, NE,USA) between 9:00

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Table 1. The effects of cadmium stress on melon seedling growth.

Cultivar	Cd treatment ($mg \cdot L^{-1}$)	Plant heigh (cm)	Stem diameter (mm)	Leaf area (cm^2)	Shoot fresh weight (g)	Root fresh weight (g)
Hamilü	CK	14.33±0.91a	4.10±0.36a	38.38±3.48a	5.37±0.28a	0.65±0.04a
	Cd-10	14.03±0.40a	3.93±0.21a	38.75±1.88a	5.37±0.46a	0.63±0.05a
	Cd-50	13.30±0.70a	3.70±0.35a	32.11±0.52b	4.40±0.31b	0.57±0.04a
	Cd-100	10.50±0.90b	3.10±0.26b	31.03±2.74b	3.72±0.33b	0.41±0.03b
	Cd-200	9.07±0.42c	3.03±0.12b	25.29±1.01c	2.74±0.20c	0.36±0.03b
Xiulü	CK	16.30±1.05a	3.90±0.30a	36.75±2.66a	4.47±0.29a	0.49±0.04a
	Cd-10	15.63±0.46a	3.97±0.12a	37.95±3.51a	4.20±0.16a	0.50±0.04a
	Cd-50	13.23±1.21b	3.23±0.32b	29.31±1.21b	2.46±0.22b	0.45±0.03a
	Cd-100	11.37±0.74c	2.87±0.15b	27.44±1.09b	2.42±0.28b	0.29±0.03b
	Cd-200	9.33±0.13c	2.83±0.21b	23.54±2.14b	1.81±0.08c	0.28±0.02b

Note: the values in the table are means ± standard errors based on 3 measurements. Different letters in the same variety in the same column indicated defferences at the level of 5% of significance.

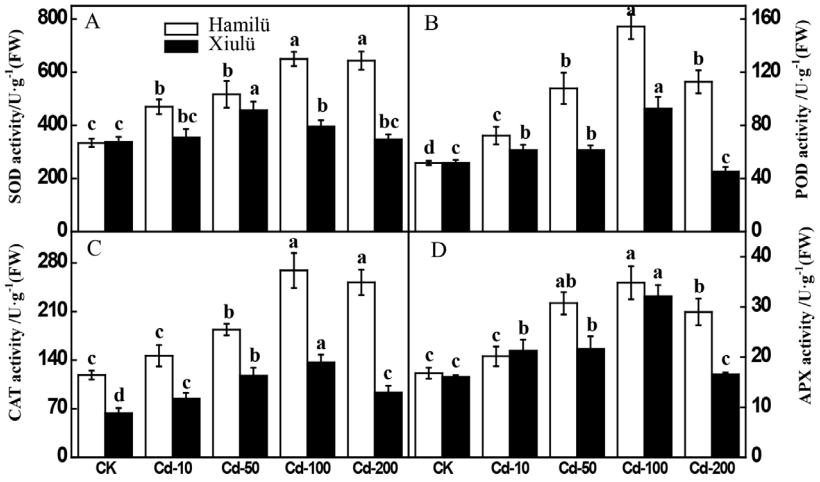

Figure 1. The effects of cadmium stress on antioxidant enzyme activities of melon seedlings. Different letters above bars for the same variety indicated differences at the level of 5% of significance.

and 11:00 am. For these measurements, the light intensity was about 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$, temperature 30±1 °C, concentration of CO_2 400±10 μmol/mol, stomatal limitation value (Ls) was 1-Ci/Co (Co means the CO_2 concentration of the air outside the leaves), and water use efficiency (WUE) was Pn/Tr (Li et al. 2012). After treatment, measurements were taken of plant height, stem diameter, shoot fresh weight and root fresh weight. Leaf area was calculated as maximum leaf length × maximum leaf width * 0.66 (a correction factor) (Wang et al. 2009). Each measurement had three replicates.

Results

Growth

As shown in Table 1, the growth of the seedlings of the two cultivars were different under different concentrations of Cd. Low concentrations (Cd-10 and Cd-50) to 'Hamilü' showed no significant differences (except Cd-50 leaf area and shoot fresh weight) from the control. With increase of Cd concentration, seedling growth of 'Xiulü' gradually but significantly decreased. At Cd concentration 200 $mg \cdot L^{-1}$ (Cd-200), the plants were wilted, and some died. Compared with the control, under the same Cd concentration treatment, the growth of 'Xiulü' declined much more than 'Hamilü'. The results indicate that the impact of Cd stress on 'Hamilü' was less than on 'Xiulü'.

Antioxidant enzymes

The activities of key antioxidant enzymes (SOD, POD, CAT, and APX) in the melon seedlings under Cd stress were estimated (Figure 1). The changing tendency of anti-oxidase enzyme activities of both cultivars were roughly the same, increased first and then decreased according to increasing Cd concentration. At Cd concentration 100 $mg \cdot L^{-1}$ (Cd-100), the activities reached their maxima (except 'Xiulü' SOD). Compared with the control, SOD, POD, CAT, and APX activities of 'Hamilü' increased by 95%, 199%, 127%, and 108%, respectively; for 'Xiulü', activities increased, respectively, by 17%, 78%, 115%, and 100%.

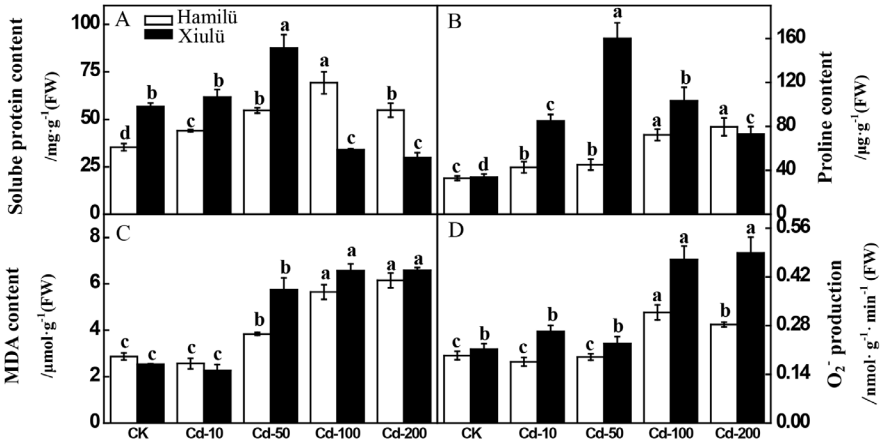

Figure 2. The effects of cadmium stress on soluble protein, proline, MDA and $O_2^{\cdot-}$ producing rate of melon seedlings. Different letters above bars indicated differences at the level of 5% of significance.

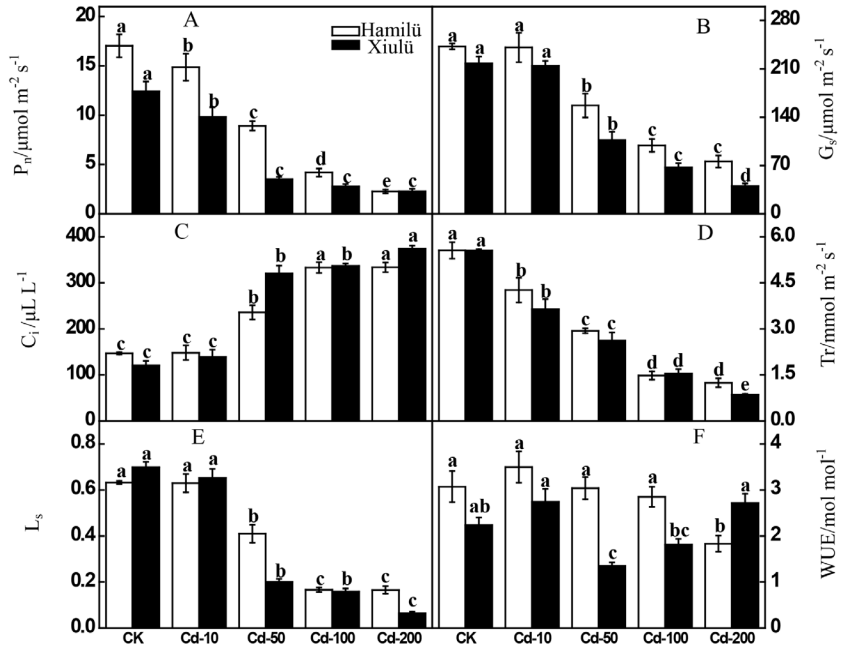

Figure 3. The effects of cadmium stress on photosynthesis parameters of melon seedlings. Different letters above bars indicated differences at the level of 5% of significance.

The increases in 'Hamilü' were more significant than those of 'Xiulü'.

Lipid peroxidation, $O_2^{\cdot-}$ production, soluble protein, and proline

As shown in Figure 2, soluble protein content in 'Hamilü' was increased first and then decreased with increasing of Cd concentrations; but compared with the control, soluble protein content were higher, and the differences were significant. At the Cd concentration of 100 mg·L^{-1}, the contents reached the maximum level, and were almost doubled compared with the control; at the Cd concentration of 50 mg·L^{-1}, the soluble protein of 'Xiulü' reached the maximum level, and was increased by over half compared with the control. When the Cd concentration reached 100 mg·L^{-1} and 200 mg·L^{-1}, the contents were lower than the controls, and decreased respectively by 40% and 47%.

The proline content in 'Hamilü' increased with increasing Cd concentration, and the differences were significant compared with the control. The proline content in 'Xiulü' increased first and then decreased with increases in Cd concentration. For both cultivars, proline was higher than that in the control with significant differences; when the Cd concentration was 50 mg·L^{-1}, the content reached the maximum level, almost quadrupling that of the control (Figure 2).

MDA contents of seedling leaves changed under differing Cd concentrations (Figure 2), but not significantly under the low

concentration Cd treatment (Cd-10). Content increased with the increase of Cd concentration; compared with the control, the differences in both cultivars were significant, but the increases of 'Hamilü' were smaller than those of 'Xiulü'.

As shown in Figure 2, compared with the control, $O_2^{\cdot -}$ production under Cd treatment of low concentrations was not significantly changed (Cd-10 and Cd-50). $O_2^{\cdot -}$ production increased with increasing of cadmium concentration, and the increase of 'Xiulü' was much higher that of 'Hamilü'.

Gas exchange

As seen in Figure 3, the net photosynthetic rate (Pn) under Cd treatment of different concentrations was significantly lower than the control, and the changes in both cultivars were basically the same. Compared with the control, Pn of 'Hamilü' under treatment with Cd-10, Cd-50, Cd-100, and Cd-200 decreased progressively with increasing Cd concentration. Pn of 'Xiulü' also decreased progressively, but by markedly greater margins at the lower concentrations.

The stomatal conductance (Gs), transpiration rate (Tr), and stomatal limitation (Ls) were consistent with the Pn trends (Figure 3). Compared with the control, Gs, Tr, and Ls decreased, and the effects were more obvious as the Cd concentration increased. 'Xiulü' showed larger decreases than 'Hamilü'.

Differences of intercellular CO_2 concentration were small between the control and Cd treatments of low concentration (Figure 3). However, these differences were apparent at the higher Cd concentrations. Trends in water use efficiency (WUE) were less consistent. In 'Hamilü' under Cd-200 treatment, WUE decreased significantly compared with the control, but the difference among other combinations were not significant. As compared with the control, WUE in the Cd-10 treatment of 'Xiulü' increased, in the Cd-50 treatment decreased, and at higher concentrations increased.

Discussion

Li et al. (2014) reported that a low concentration of Cd (≤ 50 mg·L^{-1}) can promote the growth of perennial ryegrass, but higher concentrations had an inhibitory effect. Increasing concentrations of Cd adversely affected the growth of rice seedlings, to a greater or lesser extent depending on the cultivar (Zhang et al. 2005). Likewise, in melon seedlings, low Cd treatment had little effect but as the concentration increased, the inhibitory effects became significant and the effects were greater or lesser depending on the cultivar (Figures 1,2,3). When the Cd concentration reached 200 mg·L^{-1}, after 10 days, 'Xiulü' seedlings were wilted and some died, but plants of 'Hamilü' were less adversely affected.

As the concentration of cadmium increased, four antioxidant enzyme activities of both melon cultivars increased first and then decreased, showing that there is a threshold value of cadmium concentration in the antioxidant enzyme activities. The increasing SOD and POD activities indicate that the protective capability was induced and enhanced. It is such protective effects in plants against stress that reduce the degree of membrane peroxidation, removing free radicals and preventing further damage (Wang et al. 2008). But when cadmium reached a certain high concentration, the activities began to decline. Excessive cadmium absorption affects the synthesis of SOD and POD, damages the membrane system, and may also inhibit the synthesis of antioxidant enzymes and their activities, and plant growth (Fu 2009, Deng et al. 2015). At high cadmium concentrations, the activities of CAT and APX increased, showing that the active oxygen produced by cadmium stress can play an effective role in removing activity. But the high concentration of cadmium had inhibited CAT and APX activities. This could be the result of high cadmium stress, the cadmium interfering with molecular structure or changes, or the accumulation speed of $O_2^{\cdot -}$ production and H_2O_2 in the plant might exceed that of CAT and APX removing speed, so the balance between ROS production and removal has been upset. This could cause decreased elimination capability of $O_2^{\cdot -}$ production and H_2O_2 in the plant, the continued accumulation of active oxygen, and exacerbated membrane peroxidation, as seen in the results of Fu (2009) and Ren et al. (2009). Furthermore, it would seem that under Cd stress, active oxygen species formation in the plant is increased and anti-oxidative enzyme system activity is increased, and one of the important reasons may be related to dysfunction of the respiratory system caused by cadmium stress.

The proline and soluble protein, as the intracellular important osmotic adjustment substances, can regulate the cellular osmotic balance and enhance the stability of the cellular structure and block the production of superoxide free radicals, in close relationship to the removal of reactive oxygen free radicals and reduction of membrane lipid peroxidation (Peng et al. 2002, Jiang et al. 2013). Under Cd treatment, the proline and soluble protein of the two cultivars of melon seedlings appeared different (Figure 2). This is not consistent with the results for proline content in rice under cadmium stress (Zhang et al. 2005). The mechanism of osmotic stress may be different, and needs further study.

The results show that higher concentrations of Cd stress can result in $O_2^{\cdot -}$ production in melon seedlings. $O_2^{\cdot -}$ and H_2O_2 can react to generate hydroxyl radicals, transforming fatty acids to toxic peroxides, destroying membranes and resulting in MDA accumulation (Zhang et al. 2006). The increased level of MDA was less in 'Hamilü' than in 'Xiulü'. Cadmium can have a profound effect on the peroxidation of the membrane lipids. The different reactions among cultivars are similar to those reported by He et al. (2008) on rice genotypes, who observed that MDA accumulation in the cadmium-tolerant genotypes was less than that in the cadmium-sensitive genotypes.

Decreases in the rate of photosynthesis can be caused by stomatal factors and non-stomatal factors. Stomatal factors are the main reason that can cause decreases in Ci (intercellular CO_2 concentration) and Gs (stomatal conductance). Increased Ci and decreased Gs can be caused by a decline of photosynthetic activity of mesophyll cells, and in this case the non-stomatal factors are the main factors (Farquhar and Sharkey 1982). Presently, in melon seedlings, Pn, Tr, Gs, and other photosynthetic indicators decreased under Cd treatment, the same as in rice (Zhang et al. 2005). The photosynthesis of rice subjected to Cd treatments (Cd 1.0 μmol·L^{-1} and 5.0 μmol·L^{-1}) was decreased. Under the conditions of the present study, while the Gs decreased, Ci increased and Ls decreased. This indicates that the decreasing photosynthetic rate is affected by mesophyll photosynthetic capacity. Transpiration rate was also affected by stomatal conductance and mesophyll cells. In a study of tomato, photosynthesis was decreased by cadmium stress, mainly due to stomatal

limitation (Hasan et al. 2011). This phenomenon, therefore, may be species-dependent and also dependent on the duration of cadmium stress. Cadmium is toxic to plants in many ways, but toxicity manifests differently among different crops and among the various physiological indicators.

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Construction of a High-Density DArT-seq SNP-Based Genetic Map in a Population Derived from a Cross Between a Feral and a Cultivated Watermelon

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Abstract. Watermelon is an economically important vegetable crop grown extensively worldwide. To facilitate the identification of horticulturally important traits and provide new information for genetic and genomic research on this species, a high-density genetic linkage map of watermelon was constructed using an F_2 population derived from a cross between an elite cultivated watermelon, K3, and a wild watermelon, PI 189225. Based on a sliding window approach, a total of 1,161 bin-markers representing 3,465 SNP markers were mapped onto 11 linkage groups corresponding to the chromosome pair number of watermelon. The total length of the genetic map is 1099.2 centimorgans, with an average distance between bins of 1.0 cM. The number of markers in each chromosome varies from 62 in chromosome 7 to 160 in chromosome 5. The length of individual chromosomes ranged between 61.8 cM for chromosome 7 to 140.2 cM for chromosome 5. Recombination rates varied greatly among each chromosome, from 2.0 to 4.2 centimorgans per megabase ($cM \cdot Mb^{-1}$). An inconsistency was found between the genetic and physical positions on the map for a segment on chromosome 11. The high-density genetic map described in the present study will facilitate fine mapping of quantitative trait loci, the identification of candidate genes, map-based cloning, as well as marker-assisted selection (MAS) in watermelon breeding programs.

Keywords: Watermelon, linkage map, SNP, high-density, DAr-Tseq

Introduction

Watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai (2n = 2x = 22), is an important vegetable crop with production of more than 100 million tonnes accounting for about 7% of total vegetable production worldwide (FAO Stat; http://www.fao.org/). Watermelon has an estimated genome size of 425 Mb (Arumuganathan and Earle 1991). The development of genetic linkage maps can facilitate the identification of biologically and horticulturally important trait loci and be deployed in crop improvement. High-quality and high-density genetic maps can also enable further functional genetics and genomics studies in plant species (Jones et al. 2009), leading to a better understanding of the genetic architecture of complex traits.

Recently, much progress has been made in genetic and genomic research for watermelon, such as construction of expressed sequence tag (EST) libraries (Levi et al. 2006a, Wechter et al. 2008, Guo et al. 2011), development of bacterial artificial chromosome libraries (Joobeur et al. 2006), and genome sequencing (Guo et al. 2013). In the last 20 years, several genetic linkage maps have been developed for watermelon using various molecular marker systems including randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), sequence tagged sites (STS), and sequence-related amplified polymorphism (SRAP) (Hashizume et al. 1996, 2003, Levi et al. 2001a, 2006b). Ren et al. (2012a) published a high resolution genetic map for watermelon that was constructed using 698 simple sequence repeats (SSR), 219 insertion-deletions (InDel) and 36 structure variations (SV) based on the genome sequences of the elite Chinese accession 97103 and the wild accession PI 296341-FR, and the map spanned on a total of 800 cM with an average distance of 0.8 cM between markers. Sandlin et al. (2012) constructed a single-nucleotide polymorphism (SNP)-based genetic map through comparative mapping of three separate populations developed from crosses of 'Klondike Black Seeded' (PI 635609) with 'New Hampshire Midget' (PI 635617), Strain II (PI 279461) with Egusi (PI 560023), and

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ZWRM50 (PI 593359) with *Citroides* (PI 244019). This map was comprised of 378 SNP markers with an average marker spacing of 5.1 cM. In order to create a more saturated map for watermelon, Ren et al. (2014) constructed an integrated map based on four populations containing 1,339 (698 SSR, 386 SNP, 219 InDel, and 36 SV) markers. This integrated map provided improved resolution, spanning 798 cM and bringing the average distance between markers down to 0.6 cM. However, marker density still remains far from saturation and the number of markers is fewer than needed to allow marker-assisted selection (MAS) and cloning of genes of interest in watermelon.

Recently, through the use of next generation sequencing (NGS) technologies, a rapid SNP discovery method, known as DAr-Tseq™, was developed utilizing the DArT marker platform in combination with next generation sequencing platforms (Sansaloni et al. 2011, Kilian et al. 2012, Courtois et al. 2013, Cruz et al. 2013, Raman et al. 2014). DAr-Tseq™ represents a new implementation of sequencing of complexity-reduced representations (Altshuler et al. 2000), and more recent applications of this concept have been on the next generation sequencing platforms (Baird et al. 2008, Elshire et al. 2011). DAr-Tseq™ has already been successfully applied in genetic diversity assessment studies (Cruz et al. 2013).

Thus, in order to increase marker density and expand marker resources for watermelon, here we demonstrate high-throughput SNP discovery using the DArT-seq™ method, resulting in a high-density genetic linkage map for watermelon based on corresponding SNP markers.

Materials & Methods

Plant material

The F_2 population of 144 plants derived from the cross between an elite watermelon accession K3 (female) and a wild watermelon PI 189225 (male) was used to generate the linkage map. Genomic DNA was extracted from freeze-dried leaves of each F_2 plant and parent using the CTAB (hexadecyl trimethyl-ammonium bromide) method as modified by Ren et al. (2012b).

High-throughput genotyping using the DArT-Seq™ method

A high-throughput genotyping method using DArT-Seq™ technology (Sansaloni et al. 2011) was employed to genotype the F_2 population. Restriction enzymes *Pst*I and *Mse*I were selected as the enzyme combination for genomic complexity reduction. DNA samples were processed in digestion/ligation reactions principally as per Kilian et al. (2012).

Bin map construction

Prior to the bin map construction, the SNP data that were not homozygous in both parents, as well as the proportion of missing data greater than 70%, were discarded. Due to potential sources of sequence errors and restrictions on the maximum number of markers that can be analyzed in JoinMap software, the SNP data were analyzed for genotype calling recombinations using a sliding window approach (Huang et al. 2009).

Genetic linkage map construction

Resulting bins on each chromosome were treated as genetic markers and analyzed for linkage using JoinMap version 4.1 (Van Ooijen 2011). Linkage groups (LG) were established and the marker order within each linkage group was determined using the maximum likelihood (ML) mapping algorithm (Jansen et al. 2001). The Kosambi mapping function was used to convert the recombination frequency into cM values (Kosambi 1943). The LG maps of each chromosome were drawn and aligned using MapChart v2.2 (Voorrips 2002).

Relationship between genetic position and physical position

In addition to the genetic map position, the physical positions of the bin markers were also determined based on the alignment with the watermelon reference genome sequence of accession

Table 1. Summary of chromosome assignment, number of SNP markers, number of bin markers, map length, number of gaps > 5.0 centimorgans, and marker density of the SNP bin genetic map in F_2 population derived from a cross between the elite watermelon K3 and the wild watermelon PI 189225.

Chromosome	No. SNP markers	No. bins	Map length (cM)	Bin intervals in cM		No. gaps >5.0 cM	Physical length (Mb)*	Recombination rates (cM/Mb)
				maximum	average			
1	367	120	119.9	9.9	1.0	2	34.1	3.5
2	348	128	119.9	4.8	0.9	0	34.4	3.5
3	281	93	99.3	6.9	1.1	2	28.9	3.4
4	189	70	77.3	4.4	1.1	0	24.3	3.2
5	440	160	140.2	1.1	0.9	0	33.7	4.2
6	300	84	79.3	4.6	1.0	0	27.0	2.9
7	272	62	61.8	4.0	1.0	0	31.5	2.0
8	275	95	82.8	3.1	0.9	0	26.1	3.2
9	365	131	114.5	3.1	0.9	0	35.0	3.3
10	331	105	104.0	5.4	1.0	1	28.4	3.7
11	297	113	100.3	10.4	0.9	1	27.1	3.7
Total	3465	1161	1099.2	--	--	6	330.6	--
Average	315	105	99.9	--	1.0	--	30.0	3.3

*Physical length is the distance between the first and last SNP on each chromosome.

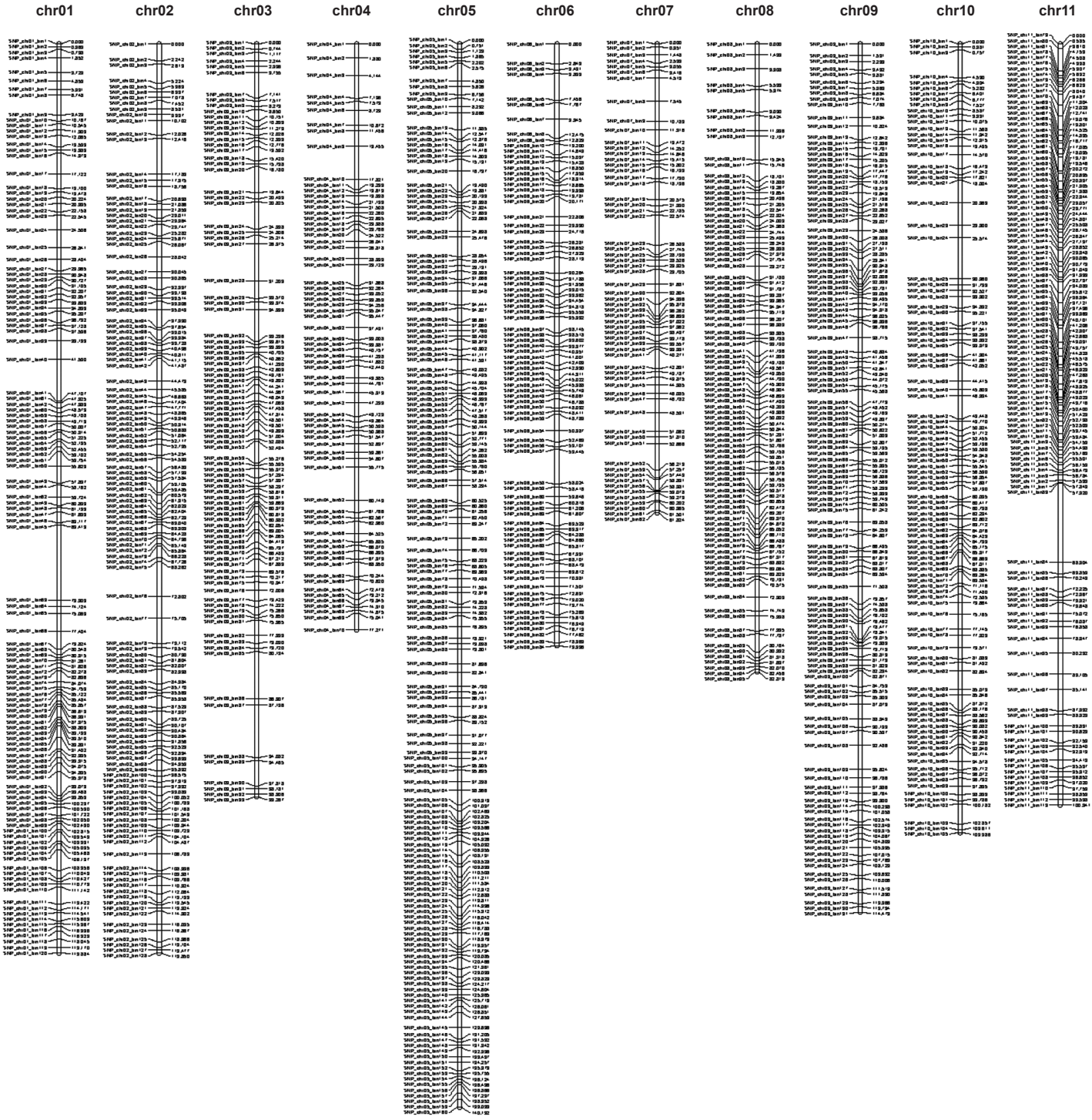

Figure 1. The SNP-based genetic linkage map for watermelon using the K3 × PI 189225 population. Bin marker names and map distances are shown on the left side and right side of each linkage group, respectively.

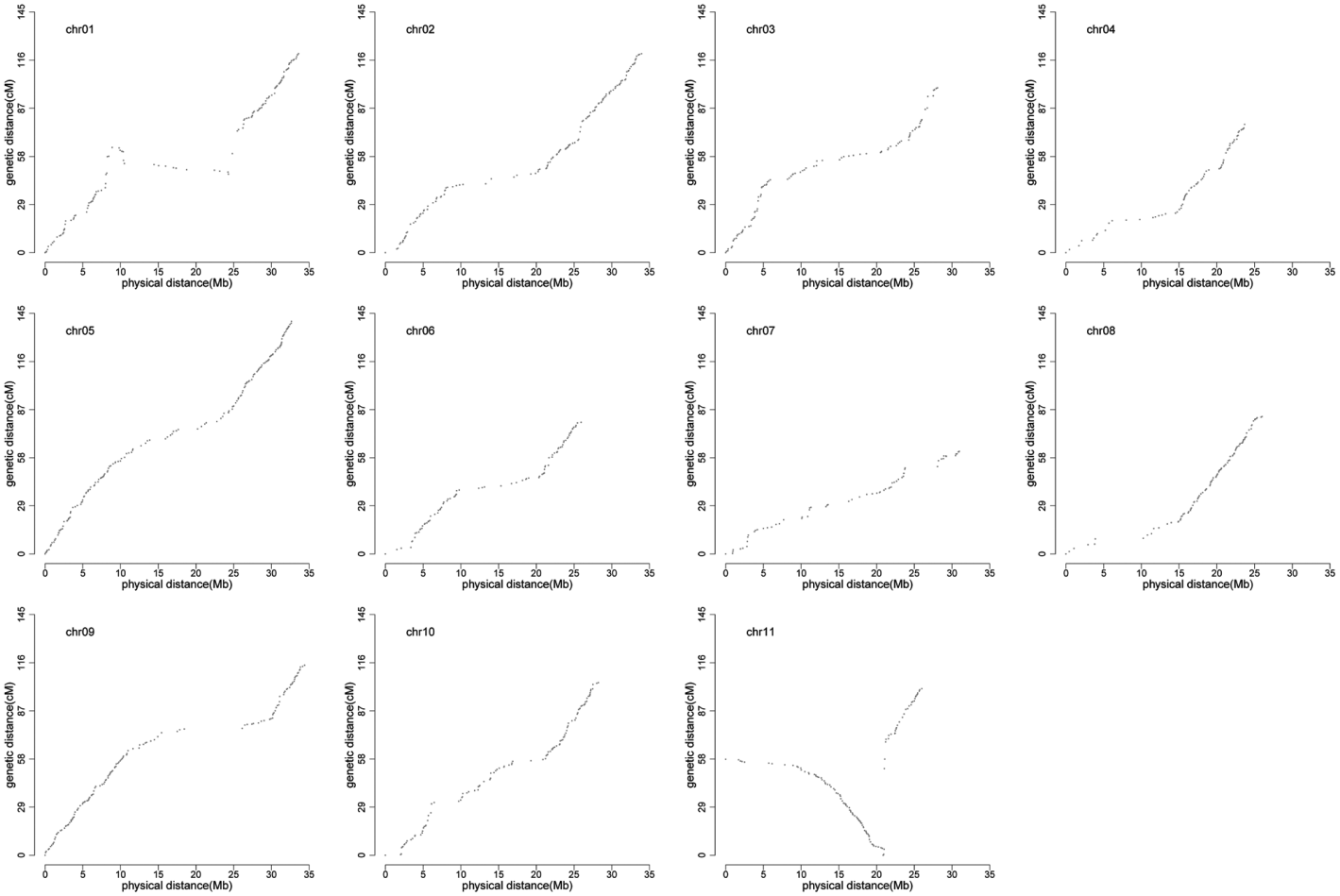

Figure 2. Relationship between genetic and physical positions of the bin markers within each chromosome. In each plot, the x-axis represents the physical positions in megabases (Mb) based on the 97103 reference v2 sequence. The y-axis represents the genetic marker positions in centimorgans.

97103. Colinearity between genetic and physical positions was determined by plotting genetic marker positions (in centimorgans) against their physical positions (in megabases) using the R statistical analysis package (Team 2012).

Results

Characterization of the DArTseq-based SNP marker and the SNP bin marker

A total of 4,808 SNPs were identified from the DArTseq-based genotyping systems. The average call rate of the markers was 91.3% and their PIC values ranged from 0.022 to 0.500, with an average of 0.454.

Based on markers with proportion of missing genotype greater than 70% as well as the markers whose parental genotypes were not homozygous or could not be determined for their chromosome location, 1,343 were removed from the total markers. The remaining 3,465 (72.1%) SNP markers were assembled in a total of 1,161 bin signatures representing the corresponding recombination events according to their physical positions and their genotypes (Table 1).

Genetic map construction

The final genetic linkage map consisted of 1,161 SNP bin markers, giving a total map length of 1099.2 cM, with individual linkage groups ranging from 61.8 cM for chromosome 7 to 140.2 cM for chromosome 5 (Table 1). The number of markers per chromosome ranged from 62 for chromosome 7 to 160 for chromosome 5, with an average number of 105.5 bin markers. In general, the SNP bin markers were well-distributed across the genome and most of the spaces between any two bin markers on all 11 chromosomes were smaller than 5.0 cM; some exceptions were observed gaps of 5.5 cM and 9.9 cM on chromosome 1, 6.2 cM and 6.9 cM on chromosome 3, 5.4 cM on chromosome 10, and 10.4 cM chromosome 11 (Table 1). The total physical length of the map is 330.6 Mb and the average physical length per chromosome was 30.0 Mb, ranging from 24.3 Mb for chromosome 4 to 35.0 Mb for chromosome 9 (Table 1, Figure 1).

Comparison of genetic and physical positions

With the availability of complete watermelon genome sequences, we also carried out the comparisons between genetic and physical positions of each chromosome. In the present study, the relationship between genetic and physical positions

was mostly linear for each chromosome, except for a large segment of approximately 21.2 Mb on chromosome 11, which is in opposite orientation between genetic and physical positions (Figure 2). The variation in the relationship between the genetic and physical positions for each chromosome can be directly reflected by the different slopes of the curves of each chromosome (Figure 2).

In order to compare the genetic and physical distances, we also calculated recombination rates for each chromosome by comparing the genetic distance (cM) to the physical distance (Mb). The within-chromosome recombination rate varied greatly among the chromosomes, ranging from 2.0 cM/Mb for chromosome 7 to 4.2 (cM/Mb) for chromosome 5, with a genome-wide average recombination rate of 3.3 cM/Mb (Table 1).

Discussion

Genetic linkage maps are highly valuable tools for the identification of novel genes/QTLs, map-based cloning, and in marker-assisted breeding programs. SNP markers have been used extensively over the past few years for genetic mapping of many crops, including wheat (*Triticum aestivum* L. em. Thell.) (Akhunov et al. 2009), maize (*Zea mays* L.) (Ganal et al. 2009), and rice (*Oryza sativa* L.) (Chen et al. 2014), as well as other cucurbits including melon (*Cucumis melo* L.) (Deleu et al. 2009, Harel-Beja et al. 2010, Garcia-Mas et al. 2012), and pumpkin and squash (*Cucurbita pepo* L.) (Zraidi et al. 2007, Esteras et al. 2012).

Cultivated watermelon has been reported as having narrow genetic diversity (Levi et al. 2001a, 2001b), resulting in low polymorphism of molecular markers between parents of mapping populations. In the present map, the mapping population was generated from a cross between a cultivated genotype and a wild genotype. The wide genetic diversity between the two parents provided a higher frequency of polymorphisms for map construction. Furthermore, the wild type PI 189225 possesses resistance to powdery mildew (Tetteh et al. 2010, 2013), anthracnose (Boyhan et al. 1994), and gummy stem blight (Gusmini et al. 2005). The K3 parent is an advanced breeding line with high fruit quality and has been used as a parental line for several commercial watermelon hybrids in Jiangsu province of China, however it is highly susceptible to the fungal diseases. Therefore, the present map and the markers can facilitate the identification of new genes/QTLs for disease resistance useful for watermelon breeding.

Presently, we constructed a genetic map of watermelon using 1,161 bin markers representing 3,465 SNP markers by sequencing DArT tags using Illumina short-read sequencing technology. The mapped SNP marker number was larger than recently reported watermelon genetic maps, containing 378 (Sandlin et al. 2012) and 386 SNP markers (Ren et al. 2014), respectively. The distribution of markers on the 11 chromosomes was generally uniform and despite the presence of a 9.9 cM and 10.4 cM gaps in chromosome 1 and 11, respectively, the greater number of SNP markers obtained in the present study will significantly expand marker pools in watermelon genetic and genomics studies. The whole genome average marker density between bins was 0.9 cM, which is comparable to that of 0.8 cM obtained by Ren et al. (2012a) and 0.6 cM obtained by Ren et al. (2014).

The completion of the watermelon genomic sequence facilitated the comparison of genetic and physical distances. In the present study, recombination rates in the population varied greatly among chromosomes, from 2.0 to 4.2 cM/Mb, corresponding to the actual physical length of the smallest and largest chromosomes, 7 and 5, respectively. The whole genome recombination rate was 3.3 cM/Mb, and this ratio is similar to that of cucumber (*Cucumis sativus* L.) (3.2 cM/Mb) (Huang et al. 2009, Ren et al. 2012a), but is larger than the average ratio of 2.3 cM/Mb for watermelon reported earlier (Ren et al. 2012a). Thus, there seems to be an inconsistency between the genetic positions and sequence-based physical positions for a segment on chromosome 11. This inconsistency could be due to either structural variations (such as an inversion for this part of the genome) or more likely incorrect assembly of the reference genome, but further studies are needed to elucidate this phenomenon.

In the present study, we constructed a genetic map in watermelon (*Citrullus lanatus*) from a F_2 population developed from a cross between feral and cultivated watermelons. The utilized map construction method produced a large number of SNPs leading to the development of a high density and accurate genetic linkage map. The developed high density and quality genetic map will facilitate further basic and applied research on the genome structure of watermelon and assist breeding efforts for the selection of horticulturally valuable traits in this crop.

Acknowledgements

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Effects of Low Night Temperature on Morphological Anatomy Structure of Grafted Watermelon Seedlings

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Abstract. Watermelon (*Citrullus lanatus*) is an important horticultural crop worldwide. Grafting is commonly used in watermelon production for improving tolerance to low temperatures, high salinity, drought, and soil-borne diseases. Low temperature stress is a main influencing factor for grafted watermelon seedlings in the early spring. However, little information is available regarding the effects of grafting on watermelon under low temperature stress. In the present study, we compared the morphological anatomy structures of the healing process of grafted watermelon seedlings under different night temperature treatments. The results showed that the concrescence speed of the grafted watermelon seedlings under the treatment of night temperature of 18 °C was the fastest. The vascular bridges were connected to vascular bundles at 5 days post grafting (dpg), while for the treatment of night temperatures of 15 °C and 12 °C, the vascular bridges were connected to vascular bundles at 7 dpg and 10 dpg, respectively. In general, as the night temperature decreased, the healing rate was slower. The low temperature delayed the differentiation of vascular tissue, which was not conducive to the connection of the vascular bundles. The wider gully between the rootstock and scion caused the formation of callus on both sides to have failed to link and the grafted seedlings died. These observations indicated a direction for improving the grafting technology and survival rate.

Keywords: Watermelon, grafted seedlings, low temperature, morphological anatomy, structure

Introduction

As the main fruit vegetable in summer, watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) plays an important role in the world horticulture industry. With the increasing consumer demand, the cultivation area of watermelon is also expanding. However, larger areas and a larger scale of production have been restricted by continuous cropping, soil-borne diseases, and soil salinization, which greatly affect sustainable development and facility of production.

Grafting is the preferred technology for obtaining continuous cropping through the avoidance of soil pathogens and an increased tolerance of low temperatures, high salinity, and drought (Davis et al. 2008, Wu et al. 2009, Hao et al. 2010). The grafted watermelon seedlings should be placed in high-temperature, high-humidity, and shaded conditions, because of their susceptibility to low temperature stress, which is the main limiting factor on the seedlings in the early spring.

Currently, anatomical studies of graft-union formation under low temperature stress have been conducted in different species, such as tomato, grape, and apple (Errea et al. 1994, Soumelidou et al. 1994, Ermel et al. 1997, Fernandez-Garcia et al. 2004, Zarrouk et al. 2010, Mayeul et al. 2012, Pina et al. 2012). However, very little information is available regarding the effects of grafting on watermelon under low-temperature stress. Thus the need for information on survival of grafted watermelon seedlings and anatomical analysis of grafted watermelon is essential to gain insights into the healing process after grafting. In the present study, we observed the effects of low night temperature stress on grafted watermelon seedlings, focusing on anatomical structures. We also examined some physiological characteristics of grafted watermelon seedlings.

Materials & Methods

Plant material and culture conditions

Watermelon, *C. lanatus* 'Su Mi 8', was grafted onto bottle gourd, *Lagenaria siceraria* (Molina) Standl. rootstock 'Hengxi No. 1', using the top approach grafting method when the first true leaf of the rootstock and two cotyledons of the scion had expanded. The grafted watermelon seedlings were divided into three groups and placed in artificial climate boxes with the night temperatures of 12 °C, 15 °C, or 18 °C (control), with a day temperature of 28 °C for 10 hours per day. The seedlings had been

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covered with plastic film with no light source for the first three days, and a constant humidity of 95 to 100%. They were then placed in growth chambers at an illuminance of 9600 lux and a humidity of 90%.

Observing anatomical structure

Observation of anatomical structure was carried out on samples from stems of individual plants at 3, 5, 7, 9, and 11 days after grafting (dpg) under light microscopy. The samples were cut into resin semi-thin sections and stained in 1% toluidine blue. The stem segment tissues of the grafted seedlings were also collected from six different time points and the vascular bundles were observed by freehand sections under light microscopy.

Results

Anatomical structure of the grafting union at 3 dpg

Rootstock-scion sections revealed a thin and deep staining layer, agglomerated by dead cells and protoplasm of disrupted parenchyma on the wound surface, named the isolation layer (Figure 1A3,B3,C3). At the forming of the isolation layers, the cells of isolation layers on both sides underwent dedifferentiation, resumed meristematic activity and induced callus. No callus was found near the isolation layers, under the treatment of 12 °C night temperature (Figure 1A3). Under the 15 °C treatment, the isolation layers of the stock side produced 1 or 2 layers of small volume callus cells (Figure 1B3). Under the treatment of night temperature of 18 °C, the stock sides produced 2 or 3 layers of small volume callus cells and the scion sides also induced 1 or 2 layers of small volume callus cells. With the higher night temperature, the cells on both sides of the isolation layers could restore the ability of division more quickly, and promote the production of the irregular callus. Comparing three different treatments for grafting union development processes, we found that the cells close to the isolation layer on the side of the rootstock recovered the ability to divide faster than the cells on the side of the scions.

Anatomical structure of the grafting union at 5 dpg

At 5 dpg, callus production was evident. At this time, callus cells kept on dividing and proliferating on both sides of the isolation layers, during which cell differentiation took place along with formation of a continuous cambial connection between rootstock and scion. On the other hand, after the isolation layer produced callus, the parenchyma cells and the callus cells could directly differentiate vessel elements, or form sieve tubes and companion cells after one or more divisions. After the original vascular bundles were cut off, residual cambial cells or parenchyma cells could differentiate new vessel elements and sieve tubes as well, to be the middle vessel elements and sieve tubes of the transport organizational systems connecting the rootstock and scion. In Figure 1A5, 1B5, and 1C5, the isolation layers can be seen clearly. At the night temperature of 12 °C, the stock-side cells had obvious enlargement in volume compared with the scion-side cells. The initial vascular bundles started to differentiate new vessel elements and sieve tubes (Figure 1A5). At 15 °C night temperature, the expansion of the callus cells on one side of the stock was irregular, the original vascular bundles in the scion incision differentiated into vessel elements and sieve tubes, and began to break through the isolation layers (Figure 1B5). With the night temperature of 18 °C, callus cells on the side of scions had almost the same volume as on the other side (Figure 1C5). Callus cells of isolation layers on both sides induced differentiation and proliferated a lot, and some had broken through the isolation layers to mutually contact. The vessel elements and sieve tubes differentiated from the initial vascular bundles in the scion incision connected with those differentiated from callus cells and parenchyma cells of the stocks.

Anatomical structure of the grafting union at 7 dpg

In general, the earlier the formation of the callus of rootstock-scion, the higher the survival rate of grafting. When the rootstock-scion callus connected, the isolation layers began to gradually disappear. At 7 dpg under 12 °C night temperature, cambial layers were noticeably activated, and callus cells continued to divide and increase. Vessel elements and sieve tubes that differentiated from some callus cells on the scion sides had formed active cells with a certain divisive direction, showing signs of vascular bundle formation, and had a tendency to link with the initial vascular bundles of the scions (Figure 1A7). Under the night temperature of 15 °C, direct vessel-element organogenesis of scion callus cells had extended to the initial scion vascular bundles. The initial vascular bundles of the stock were not as close to the graft interface, but the distance of the nearest, differentiated lots of vessel elements extended to the interface (Figure 1B7). Living cells from the graft interface divided and proliferated in most combinations, continuing to produce a large number of vessel elements and sieve tubes to interconnect the rootstock-scion transfusion tissue. Along with the formation of rootstock-scion callus, callus cells staggered growth and combined into a cohesive whole. The isolation layers attenuated further (Figure 1C7).

Anatomical structure of the grafting union at 9 to 11 dpg

The effects of mechanical pressure and absorption, which come from cell division and proliferation on both sides of the grafted surfaces, dispersed the remaining materials in the isolation layers, and these disappeared gradually (Figure 1B9,A11,C11). New vascular bundles formed in the healing part of the grafted bodies under different treatments, and split into new inside xylem and new outside phloem. As shown in Figure 1C11, the vascular bundles have been completely connected and the rootstock and the scion have converged into an integral whole.

Anatomical structure of the dead grafted seedlings

Death of grafted seedlings was evident at 5 dpg at night temperature of 18 °C. Paraffin sections were made of dead seedlings (Figure 1). On the straight-cut section of the grafting union, the scion existed obviously in a state of being separated from the rootstock. There was no adhesion between the two parts. Instead, there was a gap, which means that the caliber of the scion was smaller than that of the stock. The major morphological anatomy characteristic of the dead grafted seedlings was that the aperture of the stock did not match the caliber of the scion. Due to the difference among the diameters of the rootstock-scion, graft success could not occur, and the grafted seedling eventually died.

Histological difference of vascular bridge formation

During the healing period of grafting, the parenchyma cells and the callus cells differentiated tracheids and vessel elements, thereby forming the connection of rootstock and scion

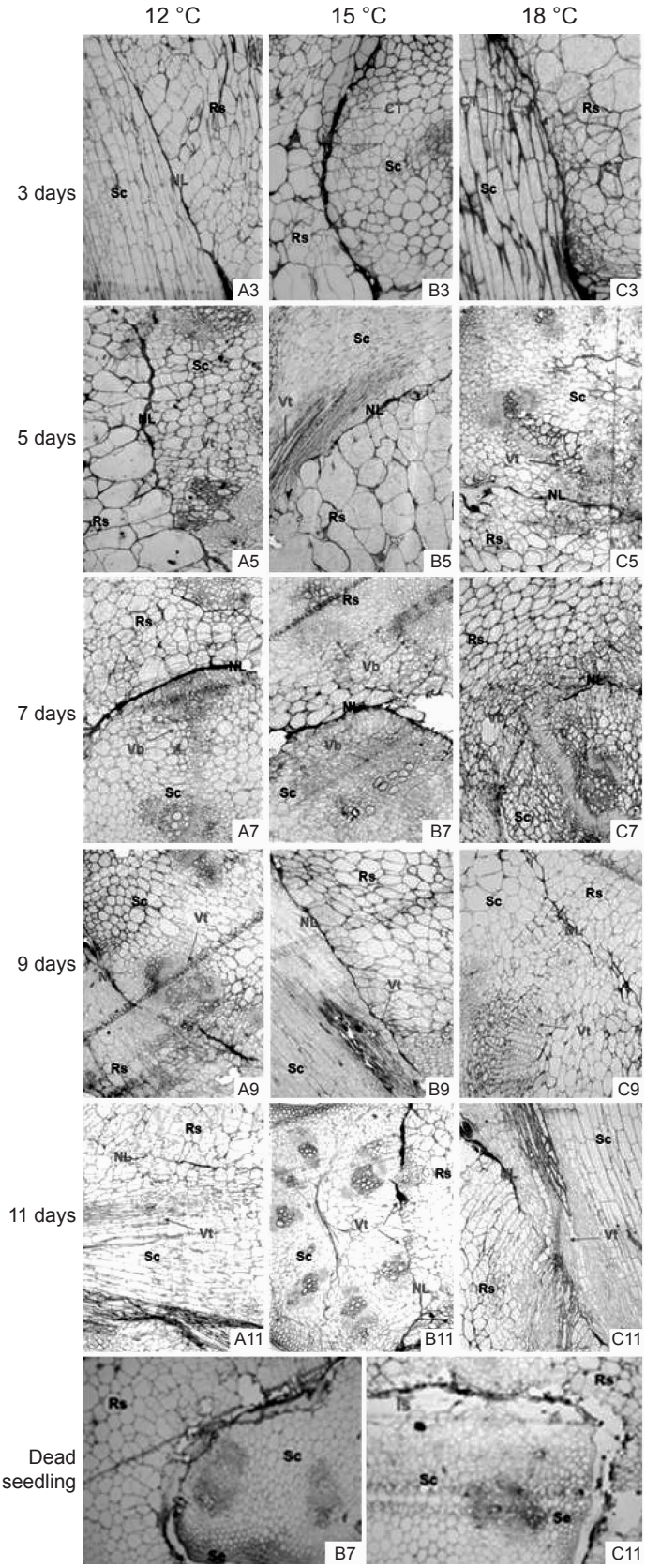

Figure 1. Graft union formation under 12 °C, 15 °C and 18 °C night temperature treatments at 3, 5, 7, 9, and 11 days post grafting (dpg). 'A', 'B' and 'C' refers to 12 °C, 15 °C, and 18 °C, respectively; Numbers of "1-11" represent the number of graft days; Example "A5" means at 5 dpg under the treatment of night temperature of 12 °C.

by vascular bridges. This process occurred only in the successful grafts. Tracheary elements, including vessel elements, tracheids, and fibers, are important members of the secondary xylem in vascular plants; they play an important role in the transport of intercellular substances, and mechanical support. Observation of the formation of vascular bundles in the healing surface, of seedlings after four days of treatment at 18 °C night temperature, revealed that tracheary elements differentiated first and formed vascular bridges on the fifth day (Figure 2c4). The grafted seedlings survived and the vascular bridge consisted of a large number of vascular molecules (Figure 2c5). Under the treatment of 15 °C night temperature, tracheary elements were observed on the sixth day (Figure 2b6), and vascular bundles formed by seven days (Figure 2b7). Under the treatment of the lowest night temperature, 12 °C, tracheary elements of graft unions were observed on the seventh day (Figure 2a7) and vascular bundles formed at 10 days (Figure 2a10). In conclusion, the lower the night temperature, the slower the vascular bridges were built and the lower the survival of the grafted seedlings.

Discussion

The development of grafted watermelon seedlings includes initial adhesion, production of callus, and formation of the secondary plasmodesmata and differentiation of vascular bundles. The formation of vascular bundles is one of the requirements for the survival of the graft (Hartmann et al. 1997). Soon after grafting, injured cells of rootstock and scion at the graft interface form isolation layers and separate from the outside world. This process is conducive to wound healing and resists the invasion of bacteria (Ermel et al. 1997). Vascular bundle cells can be formed by three ways of differentiation: the original residual vascular bundle cells close to the rootstock-scion incision, parenchyma and callus cells of the isolation layer, and the original vascular bundle cells of the rootstock-scion. The differentiation direction of these new tracheary elements turned towards the graft union surface, and the results were identical with histological and cytological research on graft union formation of grafted watermelon seedlings with different scion ages (Zhang et al. 2012).

Connectivity between the scion and rootstock of vascular tissue was an important sign in graft survival and development (Fernández-García et al. 2004). In this experiment, grafts under the 18 °C treatment at 4 dpg showed vessel elements. The vascular tissue of the scion and stock linked by 5 dpg and the healing process was basically over. The vessel elements under the treatment of night temperature of 15 °C at 6 dpg could be observed and the connections of stocks and scions had formed by 7 dpg. The vessel elements at the night temperature of 12 °C could be observed at 7 dpg and the rootstock-scion connections had been formed by 10 dpg. For all the temperature treatments at 11 dpg, the isolation layer basically disappeared and the vascular bundle bridge had been connected and the rootstock-scion had fully integrated into one union. It is suggested that temperature plays an important role in the survival of grafted seedlings. The higher the night temperature of grafted watermelon seedlings, the faster the cell division and proliferation, the earlier the vessel elements are produced, and callus and conducting tissue of the rootstock and scion is connected earlier.

In conclusion, the night temperature should not be less than 18 degrees. However, the grafting and healing process takes longer

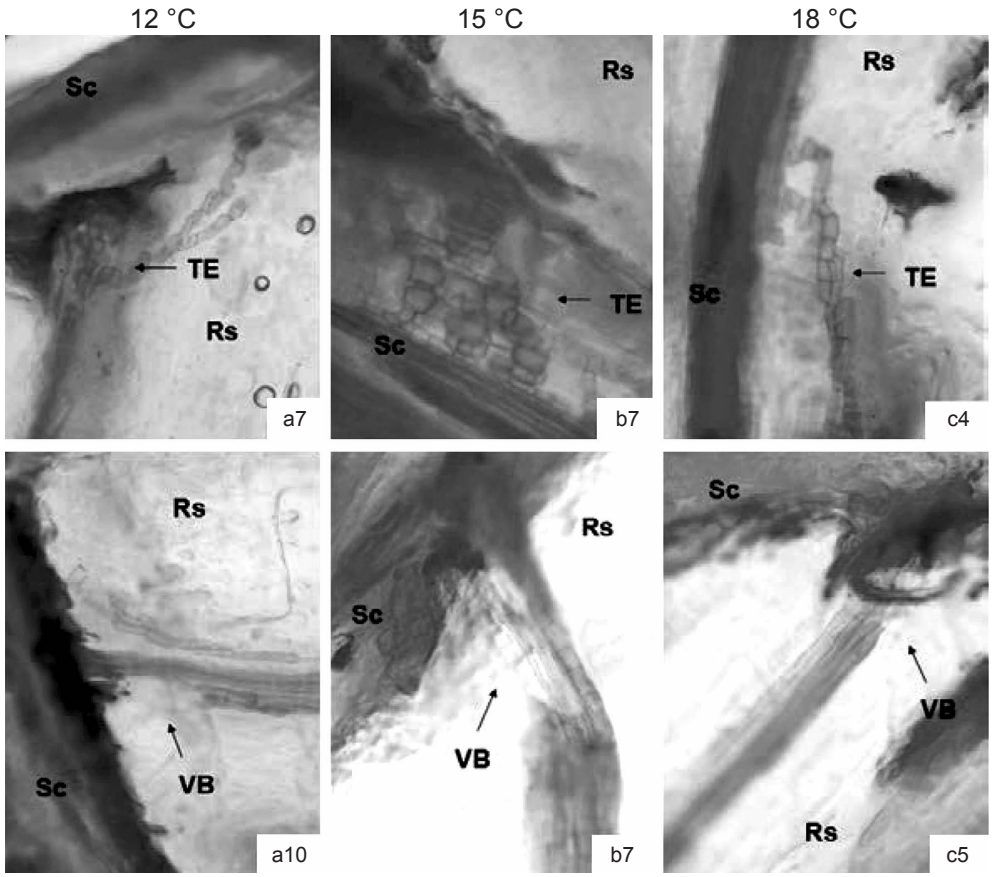

Figure 2. Formation of the vascular bridges of graft unions under different night temperature treatments. 'a', 'b' and 'c' respectively refer to 12 °C, 15 °C, and 18 °C; numbers of "1 to 10" represent the number of graft days; for example "a7" means at the 7th day of 12 °C night temperature treatment.

and has a higher cost in the early spring, often suffering from low temperature stress, so understanding the process of grafting in watermelon seedlings under low temperature conditions is crucial to post-grafting management. The present results provide direction for improving grafting techniques and instruction for reasonable and efficient management of grafted watermelon seedlings.

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Analysis of Fruit-Flesh Aroma Profile in a Melon Core Collection

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Abstract. Melon quality largely depends on fruit aroma, which is a complex trait that is highly genotype-dependent. With the aim of studying the variation in aroma profiles, we selected a set of 71 accessions from a core collection representing the extant variability of the species. Flesh volatile compounds were analyzed by gas chromatography-mass spectrometry. More than 200 compounds were detected. Cluster analysis revealed two main groups, aromatic genotypes having a predominance of esters and acetates, and non- or low aroma types, with a lower level of volatiles. The esters-rich-aromatic cluster mainly grouped genotypes belonging to the subspecies *melo*, mostly *cantalupensis* and *ameri* cultivars having high contents of propyl and butyl esters and apocarotenoids, associated with flesh color. Interestingly, this aromatic cluster included accessions of the exotic *conomon* and *dudaim* groups, the latter displaying the strongest and most singular aroma of the collection, with a particularly high content of g-dodecalactone, a compound typical to peach and strawberry. The low aroma cluster included two subclusters with different levels of lipid-derived compounds, some of them responsible for leafy odor. The less aromatic subcluster included (a) non-climacteric, non-sweet wild *agrestis*, *acidulus,* and *tibish* characterized by a strong leafy odor, (b) non-climacteric, sweet *inodorus* (including the main commercial market class of 'Piel de Sapo'), and (c) climacteric low sugar exotic *momordica* and *flexuosus-chate,* with moderately high contents of some esters and sulfur compounds. The second subcluster consisted of *inodorus* landraces with aroma profiles different from those of commercial *inodorus,* and more similar to exotic melons. The high volatile diversity found in the collection is reflective of the large amount of genetic variation still underexploited in melon breeding. This knowledge will encourage the improvement of melon aroma.

Keywords: Volatiles, aroma profile, melon, diversity, fruit-quality breeding

Introduction

Melon (*Cucumis melo* L.) is one of the most important crops worldwide. This species shows great variation in morphological, physiological, and organoleptic traits (Stepansky et al. 1999), and has been traditionally divided into two subspecies (*melo* and *agrestis*) (Kirkbride 1993) on which several intraspecific classifications are based. One of the most accepted of these is by Pitrat (2008), which consists of the following groups: *cantalupensis, reticulatus* (cantaloupe, muskmelon), *inodorus* (winter melon, casaba melon), *ameri, flexuosus* (snake melon), *chate* (adzhur melon), *dudaim* (pocket melon), and *tibish* within subsp. *melo* (though *tibish* was later reclassified into subsp. *agrestis* according to molecular studies by Esteras et al. (2013), and *flexuosus*, *chate*, and *dudaim* are often considered intermediate between the two subspecies); and *acidulus*, *conomon*, *makuwa*, *chinensis* (pickling melon), and *momordica* (snap melon) within subsp. *agrestis*.

Melon breeding programs nowadays are especially focused on fruit quality, including sweetness or aroma. Melon aroma is a complex trait which is dictated by the content of several volatile organic compounds (VOCs) (Aubert and Bourger 2004). Complex and finely regulated processes control the biosynthesis and emission of these VOCs during fruit ripening. Fruit volatiles are synthesized from different phytonutrient compounds (Granell and Rambla 2013). In melon, volatiles derived from amino acids seem to be the major contributors to aroma. In the aromatic-climacteric varieties, volatiles are mostly esterified (El-Sharkawy et al. 2005). These esters are compounds with low-odor thresholds that usually provide fruity notes to the aroma. In the non-aromatic varieties, volatiles occur mostly as aldehydes and alcohols.

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Sulfur-containing aroma compounds have also been reported in melons (Aubert and Pitrat 2006).

The aroma of melon flesh has been analyzed in several studies, employing genotypes of commercial interest like the climacteric Charentais-type (*cantalupensis*) or the non-climacteric honeydew melons (*inodorus*). Although the many intermediate forms between strict climacteric and non-climacteric types could be of great interest, only a few references exist that describe aroma profiles in other melon groups (Aubert and Pitrat 2006, Obando Ulloa et al. 2008, Escribano and Lázaro 2012, Pang et al. 2012, Vallone et al. 2013, Bernillon et al. 2013, Verzera et al. 2014). In addition, only a few aroma-related genes and QTLs have been studied and mapped (Diaz et al. 2015). One of the most recent studies regarding metabolic traits like VOCs has analyzed the fruit composition of a RIL population derived from two climacteric types and has performed a RNA-seq analysis finding interesting correlations between some metabolites, and the expression of certain genes in important pathways (Freilich et al. 2015).

In this context, the goal of this work was the characterization of the aroma profile in a melon core collection including not only commercial types but also landraces and wild types, to obtain a better knowledge of the variability extant in this crop and to encourage future studies of specific profiles in interesting types.

Materials & Methods

A core collection of 71 genotypes was assessed for VOCs in this study. These accessions were selected from the whole melon collection maintained by the Cucurbit Breeding Group in COMAV. This germplasm was previously molecularly characterized with AFLPs and SNP markers (Esteras et al. 2013, Leida et al. 2015), and represent the variability of *Cucumis melo*, including wild, cultivated, and feral types worldwide, belonging to both subspecies.

Fruits of each genotype were collected when mature, and the flesh juice was treated with saturated $CaCl_2$ solution and stored at -80 ºC until use. Three fruits per accession were analyzed. Flesh volatile compounds were analyzed by using gas chromatography-mass spectrometry (gas extraction by solid-phase microextraction, SPME, at 50 ºC).

Hierarchical Cluster Analysis (HCA) was used to analyze the volatile dataset. The ratio of the levels of each volatile in a sample to the average of all the genotypes analyzed was $\log_2$ transformed. Acuity 4.0 software (Axon Instruments) was used for HCA and to create the Heat map, with the distance metrics based on the Pearson correlation.

Results & Discussion

Among the melon genotypes in the diverse collection, a total of 210 compounds were detected. According to the volatile profiles seen in HCA, there appear to be two major clusters of genotypes, one grouping the most aromatic genotypes and the other the non-aromatic or low aroma accessions.

Aromatic genotypes

The first cluster, rich in esters, represents different volatile profiles. Most of the accessions are sweet aromatic melon cultivars of the *cantalupensis* group of the subspecies *melo*. This is the botanical group that includes many commercially important cultivars of different market classes. Our results indicate that there are cultivars with different aroma profiles within each *cantalupensis* market class. In fact, five groups of cantaloupes could be distinguished. This cluster also includes some Asian cultivars of the *ameri* group. Cultivars of the *cantalupensis* and *ameri* groups have orange or green fruit flesh, and are quite variable in the content of the apocarotenoid compounds related to flesh color, such as geranyl acetone or b-ionone, thus supporting the previous idea of carotenoid pigmentation of the flesh affecting volatile composition of fruits (Ibdah et al. 2006).

This cluster also includes a few accessions of the *conomon* group (subspecies *agrestis*) and of the *dudaim* group (considered intermediate between both subspecies). Accessions of both groups show volatile profiles similar to that of the commercial *cantalupensis*, which may reflect their role in the origin of these melon cultivars or be a consequence of introgressions due to modern breeding, as the *conomon* group is one of the most used to introgress disease resistances into the sweet melons (Pitrat 2008). Regarding the *dudaim* group, it exhibits a quite singular aroma profile, with a remarkably high content of some monoterpenes and g-dodecalactone, a compound that was also reported in *dudaim* types by Aubert and Pitrat (2006), and which is abundant in peach and strawberry. This variety is considered as having the strongest and most singular aroma of the melons, and is used as an ornamental.

Non-aromatic or low-aroma melon genotypes

The cluster with low ester content has two subclusters, that mostly differ in the amount of acetate esters and lipid-derived compounds, both including accessions belonging to the two melon subspecies.

Accessions of the first subcluster had a characteristic higher level of lipid-derived compounds and lower acetate contents. Three general volatile profiles could be distinguished: (a) high levels of lipid-derived compounds and low levels of esters and acetates are found in wild *agrestis* and the cultivated groups with cucumber-like uses, *acidulus* and *tibish,* of Indian and African, (b) high amounts of some lipid-derived compounds, but low amounts of some others, associated with a high content of a few specific esters and acetates, are found in non-climacteric sweet *inodorus*, including cultivars representing some important market classes, including the Spanish 'Piel de Sapo' and 'Amarillo Oro', and 'HoneyDew', which likely originated in France, (c) a lipid-derived profile similar to that of wild and exotic *agrestis* types, associated with a moderate to high content of ethyl and other esters that are more abundant in cantaloupes, is found in some Asian varieties of the *momordica*, *flexuosus*, *chate*, and *ameri* groups. This group of accessions is also characterized by a higher abundance of sulfur compounds, which are less frequent in wild types and other *agrestis* varieties, such as *acidulus* and *conomon.* The sulfurous aroma of one *momordica* accession has been studied in detail in Gonda et al. (2013), and the sulfurous profile also found in the aromatic *ameri* group probably suggests an early derivation of these melons from *momordica* types.

The second low-ester subcluster had, in general, less lipid-derived compounds and a higher acetate content, similar to that described in the *momordica* types, but with a higher content in some specific methyl esters, methyl acetates, and diacetates, but

they were still quite low in ethyl, propyl, and butyl esters. This includes a set of Spanish landraces that are not usually found in the commercial chain, thus confirming a high variability within this important group of melons, as well as a group of Far Eastern *conomon,* which also specifically exhibited high amounts of some alcohols and sesquiterpenes, such as a-farnesene.

As a conclusion, the use of this large collection of genotypes has allowed us the opportunity to identify volatiles that are responsible for the differences in aroma among the various horticultural groups, and will facilitate the association of specific compounds with specific characteristics of the varieties.

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Long-Lasting (2001 to 2009) Variation in Virulence Among Czech Cucurbit Powdery Mildew Populations Screened on Eleven *Cucumis melo* Differential Genotypes

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Abstract. The virulence structure of the cucurbit powdery mildews (CPM), *Golovinomyces orontii s.l.* (*Go*) and *Podosphaera xanthii* (*Px*), was studied on a set of 504 isolates (373 *Go* and 131 *Px*). CPM isolates originated from different Cucurbitaceae host species, sampled in various geographical areas of the Czech Republic in the years 2001 to 2009. They were screened on a set of 11 *Cucumis melo* differential genotypes (Iran H, Védrantais, PMR45, WMR29, Edisto 47, PI 414723, PMR5, PI 124112, MR-1, Nantais Oblong, Solartur). Since the year 2005, *C. melo* genotype PI 313970 has been also tested. Seven differentials and also PI 313970 exhibited a broad resistance variation to both CPM species when compared with the other four differentials (Iran H, Védrantais, Nantains Oblong, Solartur) which were highly susceptible. Altogether, 114 different reaction patterns in Czech CPM populations were observed using a differential set of 11 *C. melo* genotypes (old standard set; see Lebeda et al. 2011). There were also noted differential responses to PI 313970 among screened CPM isolates. Isolates virulent to line MR-1 but avirulent to Iran H were found in both CPM species. Differences in virulence variation were found between and within both CPM pathogens, as well as among the studied years and areas. There were substantial differences in the frequencies of low, medium, and highly virulent CPM strains. Medium and highly virulent strains prevailed mostly in Czech CPM populations, with the exception of 2006 when strains with low virulence occurred in more than 50% of the cases in both CPM species.

Keywords: Host-pathogen interaction, cucurbit powdery mildew, *Cucumis melo*, *Golovinomyces orontii*, *Podosphaera xanthii,* virulence variability

Introduction

Cucurbit powdery mildew (CPM) is one of the most intensively worldwide studied powdery mildews because of its economic impact (Jahn et al. 2002, Cohen et al. 2004, Pérez-García et al. 2009). Recently, two genera (*Golovinomyces, Podosphaera*) and three species (*Golovinomyces orontii, G. cucurbitacearum, Podosphaera xanthii*) are reported as causal agents on cucurbits across Central Europe (Braun and Cook 2012). *Golovinomyces* is probably a complex of two species (*G. orontii, G. cucurbitacearum*) that are very closely related and differ in some morphological features of anamorph stages (Braun and Cook 2012). In this manuscript we use the names *Golovinomyces orontii s.l.* (*Go*) and *Podosphaera xanthii* (*Px*) for the cucurbit powdery mildew (CPM) causal agents occurring in the Czech Republic (CR). These two species differ in many features, mainly by their host range, ecological requirements, geographical distribution (Křístková et al. 2009), pathogenicity and virulence (Lebeda et al. 2011), and by their variability in response to fungicides (Sedláková and Lebeda 2008, Lebeda et al. 2010, Sedláková et al. 2012).

Pathogenic specialization in CPM is well known. There is often a very clear expression of compatibility or incompatibility in host plant-powdery mildew interactions that allows for the classification of pathotypes and races based on the patterns of compatible and incompatible reactions on the differential host species or genotypes (Lebeda et al. 2008). Altogether, seven pathotypes (4 *Go*, 3 *Px*) were described internationally (Lebeda et al. 2011). Races of *Gc* and *Px* have, to date, been reported only on melon; two races of *Go* and about 31 races of *Px* have been identified on melons (Lebeda et al. 2011, McCreight et al. 2012). Recent results suggest that even more races exist (McCreight 2006, Lebeda et al. 2011, McCreight et al. 2012).

Preliminary studies of Czech CPM population virulence structure showed that Czech CPM populations are highly variable in their pathogenicity, and are unique and markedly different compared to those of some western and southern European countries and other parts of the world (Lebeda and Sedláková 2004, 2006, Lebeda et al. 2004). Various independent systems of CPM pathotype and race determinations and denominations have been used. Lebeda et al. (2008, 2011, 2016) critically reviewed the current state, gaps, and perspectives in our understanding of pathogenicity

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variation in these two CPM pathogens at the pathotype and race levels. They proposed two sets of differential cucurbit genotypes for the identification of CPM pathotypes and races, and an objective, efficient, uniform, and comprehensive coded system for meaningful, concise designation of CPM pathotypes (sextet code) and races (septet code). The preliminary study of a set of 18 CPM isolates using a new proposed race-differential set according to Lebeda et al. (2008) was presented by Lebeda et al. (2012) at Cucurbitaceae 2012 (Adana, Turkey). Also, an extensive study of 115 CPM isolates from different host species of Cucurbitaceae, at various locations in the Czech Republic, and over the years 2010 to 2012, was surveyed by Sedláková et al. (2014) at the subsequent meeting, Cucurbitaceae 2014 (Bay Harbor, Michigan, U.S.A.).

Here we are presenting a case study of virulence variation in Czech CPM populations from 2001 to 2009 using a large set of 504 Czech CPM isolates screened on 11 *Cucumis melo* L. differential genotypes and genotype PI 313970 from 2005.

Materials & Methods

Plant material

Seeds of differential genotypes of sets for race determination (11 differentials of *C. melo* and since the year 2005, also *C. melo* genotype PI 313970) were sown in perlite and grown in a growth chamber. Seedlings were transplanted at the cotyledon stage and grown in a CPM-free greenhouse (for details see Lebeda and Sedláková 2010).

Pathogen isolation, multiplication, and maintenance

CPM samples were microscopically examined before isolation; those determined to be a mixture of *Px* and *Go* were excluded. Conidia of pure cultures were transferred by tapping onto primary leaves of highly susceptible cucumber, *C. sativus* L. 'Stela F_1'. 504 Czech CPM isolates (373 *Go,*131 *Px*) originated from different host species, sampled in various geographical areas of the Czech Republic in the years 2001 to 2009 were used for the study. Isolates were cultured on leaf discs in plastic boxes (one isolate per box; 24/18 °C day/night; 12 h-day). Multiplication and maintenance of isolates were done as previously described (Lebeda and Sedláková 2010).

Determination of pathogenic variability

All CPM isolates described above were screened for pathogenic variability by a leaf-disc method (Bertrand et al. 1992, Lebeda and Sedláková 2010). There was used a set of 11 differential genotypes of *C. melo* proposed by Křístková and Lebeda (1999) for survey of pathogenicity variation in Czech CPM populations. Since the year 2005, *C. melo* genotype PI 313970 has been also tested. The differential set was comprised of nine of *C. melo* race-differentials (Iran H, Védrantais, PMR 45, WMR 29, Edisto 47, PI 414723, PMR 5, PI 124112, MR-1) proposed by Pitrat et al. (1998) and one *C. melo* genotype Nantais Oblong suggested by Bardin et al. (1999). *C. melo* differential Solartur was added by Křístková and Lebeda (1999). Each genotype was represented by 3 leaf discs (15 mm in diameter) in 3 replicates (one replicate per plant). Discs from true leaves (2 or 3-leaf stage) of cucumber plants were used for screening. Discs were inoculated by tapping a primary leaf of cucumber 'Stela F_1' covered with 3 or 4-day-old sporulating mycelium and incubated under the conditions described above. Evaluations were conducted 6 to 14 days after inoculation by using a 0 to 4 scale (Lebeda 1984). Data were used to calculate a degree of infection (DI) value for each genotype that was classified as resistant /R/ ($0 \leq DI \leq 1$), or susceptible /S/ ($1 < DI \leq 4$).

Results & Discussion

Differences in response to the majority of the 11 *C. melo* differentials and genotype PI 313970 were found within individual CPM species, between both CPM pathogens, as well as

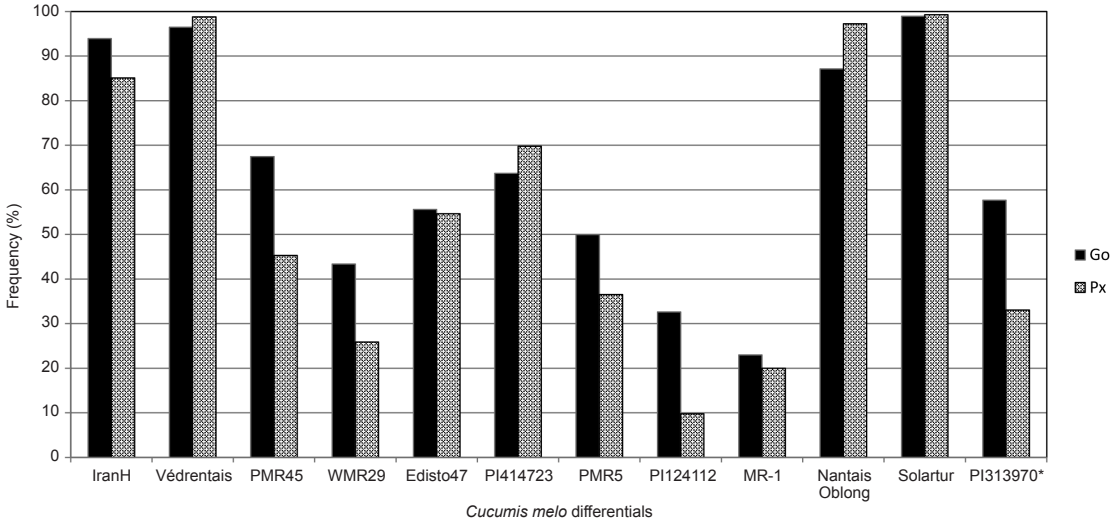

Figure 1. Frequency of occurrence of susceptible reactions of *Cucumis melo* differentials in reaction to the set of Czech CPM isolates (*G. orontii, P. xanthii*) originated from 2001 to 2009.
Go – *Golovinomyces orontii*, Px – *Podosphaera xanthii.*
*Response of Czech CPM populations to *C. melo* genotype PI 313970 has been screened since 2005.

among the studied years (Figure 1). Eight differentials (PMR 45, WMR 29, Edisto 47, PI 414723, PMR 5, PI 124112, MR-1, and PI 313970) exhibited a broad resistance variation to both CPM species, mainly among individual years (detailed data from individual years are not presented here). On the contrary, four differentials (Iran H, Védrantais, Nantains Oblong, Solartur) were highly susceptible, with frequency of susceptible reactions more than 66% in every year of study. Solartur was the most susceptible differential of all, with more than 93% compatible reactions with screened CPM populations over the nine year-period of study. For differential PI 124112, compatible interactions were the least frequent (10%). On differential MR-1, there was observed a similar reaction pattern of screened isolates of both CPM species, with a lower increase in frequency of susceptible reactions, 20 to 23%. Differences in reaction between both CPM species were observed with these five differentials: PMR 45, WMR 29, PMR 5, PI 124112, and PI 313970, and they ranged from 13% to 24%. In contrast, for the other six differentials, differences did not exceed 10%.

Altogether, 114 different reaction patterns in Czech CPM populations were observed using a set of 11 *C. melo* differentials (calculated without genotype PI 313970). Thirty-six reaction patterns were common for both pathogens whereas 57 of them were observed only in *Go* and 21 only in *Px*. Since 2005, when Czech CPM populations were screened also for reaction on PI 313970, there were noted different responses to this genotype. There were also observed differences in the frequency of occurrence of individual reaction patterns within individual CPM species as well as among the studied years (detailed data are not presented here). For both pathogens, we repeatedly observed a variety of reaction patterns (at least twice per studied period) during the nine-years period of study (36% of all reactions detected for individual pathogens). Isolates virulent to line MR-1 but avirulent to Iran H were found in both CPM species. Fifty-five of the isolates (51 *Go*, 4 *Px*) expressed profuse sporulation on all 11 *C. melo* differentials as well as on genotype PI 313970. Differences in virulence variation (number of virulent factors, VF) were found between and within both CPM pathogens, as well as among the studied years and areas. Substantial differences were observed in frequencies of low (number of VF: 1 to 4), medium (VF: 5 to 7) and highly virulent (VF: 9 to 11) CPM strains during the nine-years period of study. Medium and highly virulent strains prevailed mostly in Czech CPM populations, with the exception of 2006 when strains with low virulence occurred in more than 50% frequency. In contrary, in 2006 the highly virulent strains were found in less than 7% in populations of both CPM species.

The results presented here have verified our preliminary studies (Lebeda and Sedláková 2004, 2006, Lebeda et al. 2004) on a large set of 504 CPM isolates and also corresponded with early published results from CR by Křístková et al. (2004). A broad resistance variation of Czech CPM populations observed on genotype PI 313970 confirmed results published by Sedlářová et al. (2009) who studied histological aspects of *C. melo* PI 313970 resistance to *Px* and *Go*. The results presented here are interesting from these points of view: (1) they reveal substantial virulence variation in both CPM species and the occurrence of a huge number of races, (2) they demonstrate the existence of new (and until now unknown) virulence/avirulence patterns and susceptibility/resistance factors in pathogen populations as well as in differential host genotypes, (3) they show that Czech CPM populations are very heterogeneous and flexible in time and space in their virulence; also, they differ significantly from CPM populations in other countries, (4) they demonstrate that both CPM species have high evolutionary potential and according to the terminology of McDonald and Linde (2002) could be considered as "risky" pathogens, and (5) they substantially contribute to the development and verification of a new, unified system of determination and denomination of CPM races (Lebeda et al. 2016).

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Comparison of Anatomic Structure and Enzyme Activity of Fruit Rinds in Crack-Resistant and Crack-Prone Watermelons

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Abstract. Fruit cracking has been a topic of great concern for growers and researchers of watermelon (*Citrullus lanatus*), but the mechanism of fruit cracking is poorly understood. To compare fruit pericarp anatomic structure and enzyme activity in fruit cracking-resistant and cracking-prone mini-watermelons, two homozygous mini-watermelon lines were compared, the thick pericarp (~1 cm), crack-resistant K_2 and the thin pericarp (~0.6 cm) crack-susceptible L_1. The fruit pericarps were sampled 21, 24, 27, and 30 days after self-pollination and were observed by paraffin section. The stone-cells in the crack-resistant accession were smaller and rounder than those in the crack-susceptible accession. An obvious transition from the small cells to the big cells was observed in the crack-resistant. Variation in the degree of pericarp structure of the crack-resistant was more obvious than of the crack-susceptible late in fruit development. Fruit-cracking resistance was associated with the variation and arrangement of pericarp structure in the process of mini-watermelon maturity. At the same time, the fruit pericarps that were sampled 30 days after self-pollination were observed for enzyme activity. Pectinase activity, cellulose-enzyme activity, and activity of peroxidase (POD) in the thin pericarp, crack-susceptible line were higher than those in the thick pericarp crack-resistant line. Cracking tendency and pectinase activity in the pericarp were significantly positively correlated. Pericarp thickness and pectinase activity in the pericarp were significantly negatively correlated. In the pericarp, POD activity and SOD activity were significantly negatively correlated.

Keywords: Mini-watermelon, *Citrullus lanatus*, fruit cracking, anatomic structure, enzyme activity

Introduction

Fruit cracking is a major physiological disorder that can cause significant economic losses in a wide variety of fruit including tomato (*Solanum lycopersicum* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum. &Nakai), and melon (*Cucumis melo* L.) (Fernandez-Trujillo et al. 2013). Among vegetables, fruit cracking has been investigated extensively in tomato (Peet 1992, 1995, Moctezuma et al. 2003, Matas et al. 2004, Savvas et al. 2008). Certain environmental conditions, such as irregular rainfall or irrigation, high temperatures, rapid fruit growth, high humidity, thin-skinned cultivars, and wide day/night temperature differences seem to precede fruit cracking (Peet 1992). The anatomic structure of the fruit pericarp is associated with fruit cracking. Matas et al. (2004) have reported correlations between cracking in cherry tomatoes and membrane integrity, and presented data indicating that cell membrane thickness can be a useful indicator of crack susceptibility among cherry tomato cultivars. Continuous growth in ripening tomato fruit affects cuticle properties and cracking (Kou et al. 2001). Antisense suppression of a µ-galactosidase gene (*TBG*6) in tomato has also been shown to increase fruit cracking and reduce fruit firmness during early fruit development (Moctezuma et al. 2003).

Enzyme activities are associated with fruit cracking in the pericarp. Calcium affected the incidence of fruit cracking through its effect on the activities of cell wall enzymes in litchi pericarp (Chen et al. 2014). The activities of the wall-metabolic hydrolases and wall-bound POD and PPO in the pericarp were positively related to fruit cracking in litchi pericarp (Li et al. 2003). The cracking-resistant jujube 'Yuanling' has more capacity to concentrate calcium than the susceptible 'Jun' (Cao et al. 2013). The pectinase and cellulase activities in the pericarp of jujube fruit during late developmental stages affect the occurrence of cracking, the activities of POD and PPO in the susceptible cultivar were higher than in the crack-resistant cultivar (Cao et al. 2014).

Watermelon is one of the major horticultural crops. Total yield in 2009 was over 100 million tonnes, accounting for 10.7% of total vegetable production according to the 2009 FAO Statistics (http://www.fao.org/). Currently, China is the top watermelon producer with 68.2 million tonnes in 2009 which was 67.7% of the world production. Watermelon is more and more popular throughout the world on account of its economic

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and nutraceutical importance. Area and yield of early and mid-season varieties of watermelon is 56% and 59% in China, respectively. Production area of small, thin-skin watermelon is maintaining steady growth (Wang et al. 2013). Watermelon fruit cracking is becoming more prevalent in China, especially with mini-watermelons. Some researchers have studied aspects of physiological anatomy, cultivation techniques (Man and Zhang 2006, Jiang et al. 2009a) and genetics in watermelon (Sugiyama et al. 1999, Fan et al. 2000, Sugiyama 2001). Genotype effects on fruit cracking of watermelon have been studied (Jiang et al. 2009b). The correlation between watermelon fruit split rate and major fruit traits was investigated (Xu et al. 2013). However, the mechanism of cracking resistance is poorly understood. In the present study, two mini-watermelons, K_2 with high resistance to cracking and L_1 with cracking susceptibility, were used as plant materials. Fruit pericarp anatomic structure was observed by paraffin section these cracking-resistant and cracking-susceptible mini-watermelons. Pectinase activity, cellulase activity, SOD activity, and POD activity in fruit pericarp were measured. The results provide a basis for the mechanism of fruit cracking in watermelon.

Materials & Methods

Plant material

Two mini-watermelon inbred lines (*Citrullus lanatus*) were compared. K_2 has red flesh, oval fruit shape, and is highly resistant to cracking, and L_1 has canary yellow flesh, round fruit shape, and is susceptible to cracking. These two mini-watermelon inbred lines were maintained at the Qiqihar Vegetable Research Institute. The characteristics of the two inbred lines are summarized in Table 1 and Figure 1. In both, the period from pollination to fruit maturation is about 30 days.

Methods

K_2 and L_1 were sown in the spring of 2013 and seedlings were transplanted three weeks later to a plastic-covered house at Qiqihar University. A randomized complete block design with three replicates was used. The number of plants of K_2 and L_1 were 36 and 42, respectively, with 30 cm separation within and 50 cm between rows. All plants were regularly cultivated in vertical culture with single vine pruning, and drip irrigation was employed. Fruit pericarp from the equatorial region of the fruit (mid-way between the peduncle end and the stylar end) was sampled 21, 24, 27, and 30 days after pollination to observe the anatomic structure by paraffin section, according to the conventional paraffin section method (Jiang et al. 1994). The fruit pericarp, 30 days after pollination, was also sampled for pectinase activity (Zhang et al. 2004), cellulase activity (Bai and Wang 2012), SOD activity (Zhang et al. 2008), and POD activity (Zou 2000). There were three biological replicates. Fruit pericarps were collected immediately after the samples were transported back to the laboratory on ice. All pericarps were quickly frozen in liquid nitrogen and stored at -80 °C for analysis.

Fruit cracking was observed 30 days after pollination and cracking percentage was calculated.

Results

Characteristics of crack-resistant and crack-susceptible watermelon

The results show that fruit cracking percentage of L_1 was 36% and that of K_2 0% (Table 1). Fruit skin thickness of L_1 was approximately 5 mm and that of K_2 was significantly thicker, approximately 9 mm. Sample K_2 and L_1 fruits are shown as in Figure 1.

Comparison of pericarp anatomic structure of cracking-resistant and cracking-susceptible lines

The epidermis was thicker in K_2 than in L_1 (Table 2). The exocarp of K_2 was significantly thicker than that of L_1 (Table 2), and there were more cell layers in the exocarp (Figure 2). The stone cells of K_2 were smaller and there was a transition from disconnected bulk to connected bulk (Figure 2B1–B4). The stone cells in L_1 were larger with looser bulk and layers (Figure 2C1–C4).

There were smaller cells and an obvious transition from smaller cells to larger cells in the mesocarp of K_2. But there was no transition, and looser larger cells and no small and dense cells in L_1 (Figure 2C1–C4).

Comparison of enzyme activities in watermelon pericarp

As shown in Table 3, the pectinase, cellulase, and POD activities in L_1 were significantly higher than those in K_2. SOD activity in L_1 was significantly higher than that in K_2.

Correlation analysis of cracking fruit rate, pericarp thickness and enzyme activities

Fruit cracking percentage and pectinase activity of the pericarp were significantly positively correlated. Pericarp thickness and pectinase activity of the pericarp were significantly negatively correlated. POD activity of the pericarp and SOD activity of the pericarp were significantly negatively correlated (Table 4).

Discussion

We found that fruit cracking resistance was associated with the variation and arrangement of pericarp structure in the process of mini-watermelon maturity. The exocarp thickness of the crack-resistant K_2 was thicker than that of the crack-susceptible L_1. Size and shape of the stone cells in the crack-resistant

Table 1. Comparison of fruit cracking and pericarp thickness in the inbred lines.

Line	No. observed fruit	No. cracked fruit	Cracked fruit (%)	Pericarp thickness (cm)*
K_2	36	0	0.0	0.867±0.125A
L_1	42	15	35.7	0.467±0.047B

*Capital letters show the significant differences at $\alpha = 0.05$.

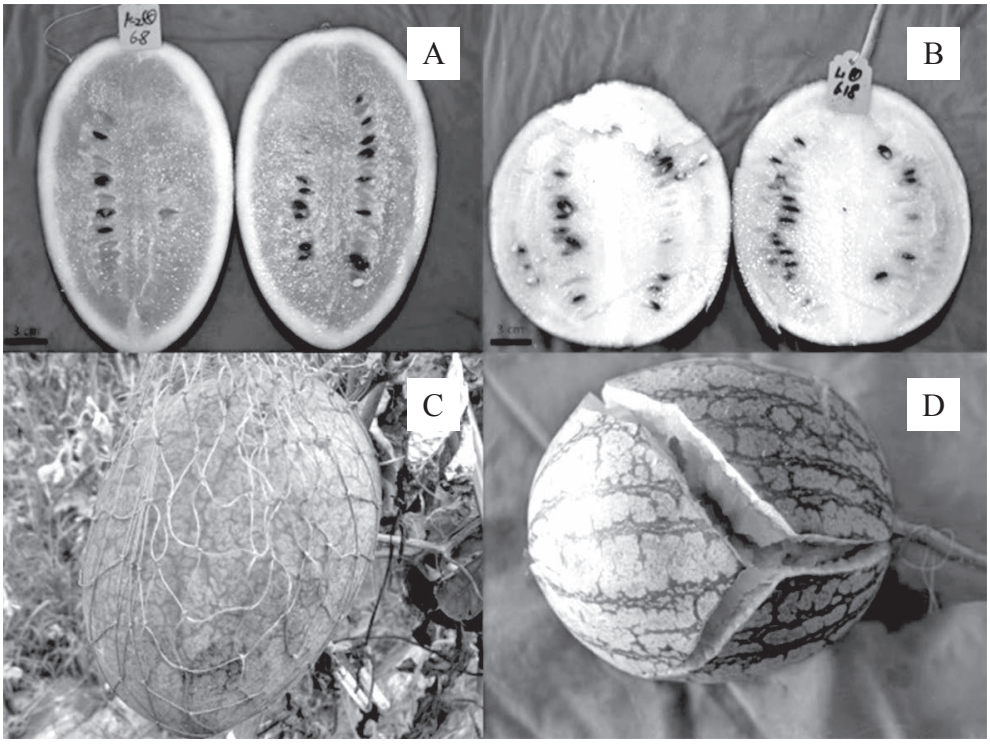

Figure 1. Comparison of two watermelon lines. Mini-watermelon K_2 (A). Mini-watermelon L_1 (B). Non-cracked fruit of K_2 (C) and cracked fruit of L_1 30 days after pollination (D).

Table 2. Comparison of fruit pericarp anatomic structure of K_2 and L_1 at different growth stages.

Lines	Days after pollination	Epidermis thickness (μm)	Exocarp thickness (μm)	Stone cells	Mesocarp	Pericarp thickness (mm)
K_2	21	14.68	118.73	Disconnected bulk, smaller and denser stone cells	Smaller cells shift to larger ones, smaller and denser cells	8.8
	24	16.13	151.77	Disconnected bulk, smaller and denser stone cells	Smaller cells shift to larger ones, smaller and denser cells	8.3
	27	14.80	129.35	Connected bulk, smaller and denser stone cells	Smaller cells shift to larger ones, smaller and denser cells	8.7
	30	18.78	129.48	Connected bulk, smaller and denser stone cells	Smaller cells shift to larger ones, smaller and denser cells	8.8
L_1	21	22.06	89.51	Loose bulk, larger stone cells	Loose larger cells, no small and dense cells	5.0
	24	17.86	59.55	Loose layer, larger stone cells	Loose larger cells, no small and dense cells	5.1
	27	18.22	69.09	Loose layer, larger stone cells	Loose larger cells, no small and dense cells	5.0
	30	15.37	67.17	Loose bulk, larger stone cells	Loose larger cells, no small and dense cells	4.9

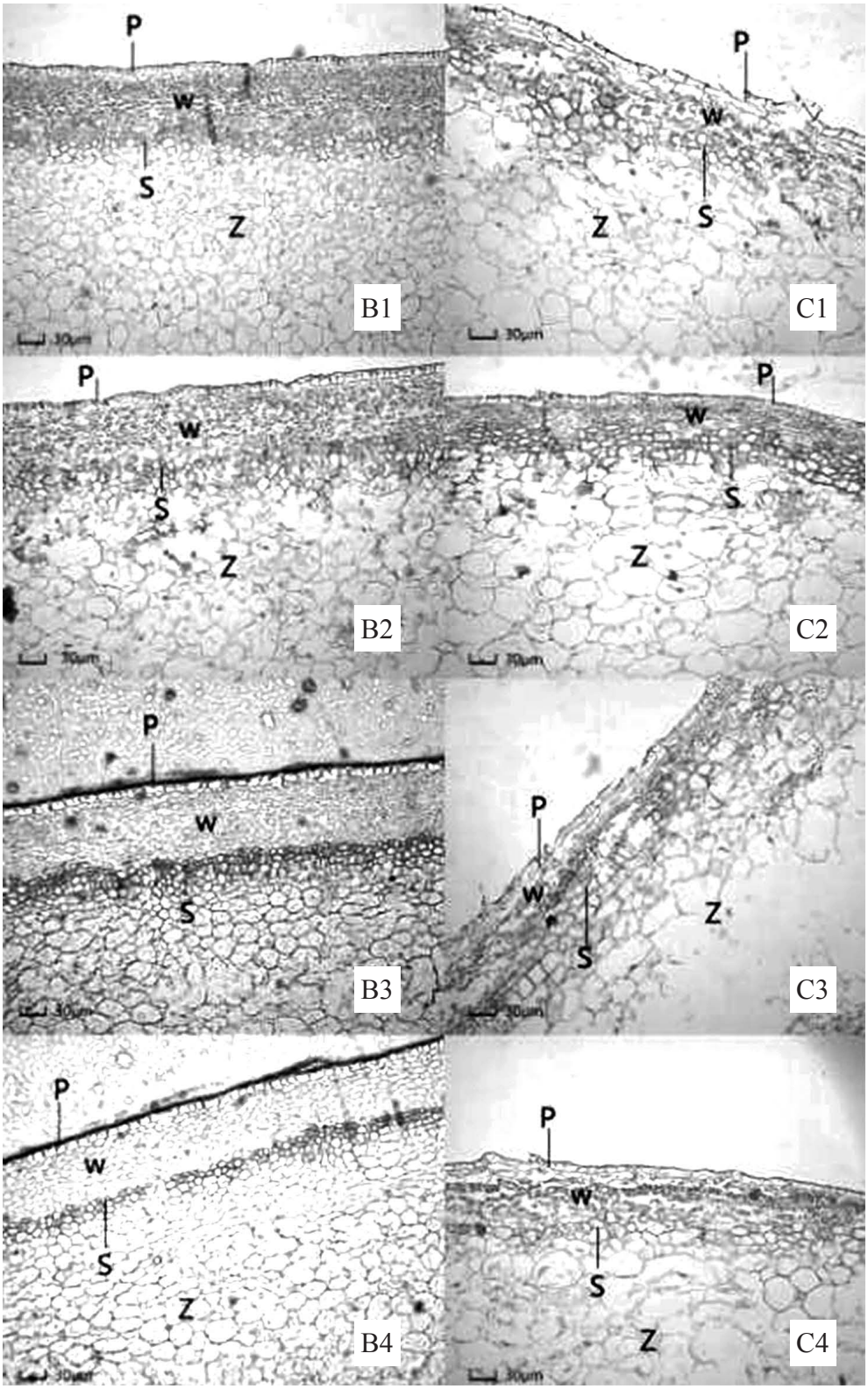

Figure 2. B1–B4: the pericarp structure of K_2 (21, 24, 27, 30 days after pollination). C1–C4: The pericarp structure of L_1 (21, 24, 27, 30 days after pollination) (Magnification: 10 × 40). P, Epidermis; W, Excocarp; S, Stone cell; Z, Mesocarp.

Table 3. Comparison of enzyme activity in watermelon pericarp (U/g).

Lines	Means±SE			
	Pectinase activity	Cellulase activity	SOD activity	POD activity
K_2	1035.594±65.129 bB	0.575±0.004 bB	44.500±0.496 aA	41.667±2.357 bB
L_1	2144.744±166.382 aA	0.592±0.003 aB	5.352±1.982 bB	192.500±7.500 aA

Lower case letters show the significant differences at the $\alpha = 0.05$ level, capital letters show the significant differences at the $\alpha = 0.01$ level.

Table 4. Correlation analysis of fruit cracking fruit rate, pericarp thickness and enzyme activities.

Activity	Fruit cracking percentage	Pericarp thickness	Pectinase activity	Cellulase activity	SOD activity
Pericarp thickness	- 0.948	--			
Pectinase activity	0.886	- 0.987*	--		
Cellulase activity	0.992*	- 0.902	0.823	--	
SOD activity	- 0.463	0.720	- 0.821	- 0.351	--
POD activity	0.402	0.671	0.780	0.286	- 0.998*

*represents significance at the $\alpha = 0.05$ level, no mark represents no significance of correlation.

line were smaller and rounder than in the crack-susceptible. An obvious transition from the small cells to the big cells was observed in the crack-resistant K_2. The variation degree of pericarp in the crack-resistant line structure was more obvious than in the crack-susceptible during the late growth stage. Man and Zhang (2006) found that the watermelon fruits of cultivars with good storage and transport quality had thick cuticles in the epidermal cells, more cell layers in the exocarp, bigger aggregation bodies of stone cells, and smaller and denser cells in mesocarp; the fruits of poor storage and transport quality cultivars had thin cuticles, fewer cell layers, smaller aggregation bodies of stone cells, and few small, dense cells. The resistance to fruit cracking in jujube is positively correlated the density and uniformity of fruit epidermal cells but not the density of pulp cells (Cao et al. 2013), consistent with our results.

We found that the activity of pectinase and cellulase was higher in L_1 pericarp (cracking-susceptible) than that in K_2 (cracking-resistant). Similar results were found in litchi pericarp (Li et al. 2003). It is reported that fruit cracking was accompanied by increased pectinase (PG) activity in wax apple (Michelle et al. 2013). Antisense inhibition of PE and PG activity in tomato can reduce fruit cracking (Brummell and Harpster 2001). Cracking in tomato was significantly reduced due to decreased PG and PE levels by transgenic antisense technology (Schuch et al. 1991). Five *LcPG*, one *LcEG*, and three *LcPE* genes were upregulated in cracking fruits (Li et al. 2014).

Fruit cracking is an undesireable characteristic for crop production and storage although it is a favourable trait for plant evolution in taking seeds further afield. In order to solve this problem, the most effective solution is breeding varieties with resistance to cracking (Fernandez-Trujillo et al. 2013). There are some encouraging results in tomato (Moctezuma et al. 2003, Matas et al. 2004, Savvas et al. 2008) and litchi (Li et al. 2014). But in watermelon, breeding varieties with resistance to cracking has just begun. In this study, the anatomic structure and enzyme activity of the fruit pericarp in crack-resistant and crack-susceptible mini-watermelon lines have been compared. The results provide a theoretical reference for mechanisms of cracking fruit in watermelon.

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Determination of Salt Tolerance Potential of Turkish Bottle Gourd (*Lagenaria siceraria*) Germplasm

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Abstract. Salinity is one of the global environmental problems negatively affecting plant productivity, because it causes reduced yield and quality, particularly in arid and semi-arid regions. Breeding salt-tolerant cultivars/ rootstocks and reclamation or advanced irrigation methods allowing use of low-quality water can make possible crop production under saline conditions. In this study, 160 Turkish bottle gourd accessions as well as introduced germplasm from international gene banks were screened at 10 dSm^{-1} salinity in a hydroponic system. Two commercial *Lagenaria* rootstocks, 'Macis' and 'Argentario', and two watermelon cultivars, 'Crisby' and 'Crimson Tide', were grown for comparison. Electrical conductivity of the solution was 1.5 dSm^{-1} in the control treatment and the experiment was continued for three weeks. All accessions were negatively affected by salt application and plant growth parameters were reduced at different levels. Reductions in shoot fresh weight varied from 6% to 93% as compared with the control. Decreases in leaf area compared with the control varied from 5% to 90%. Leaf number per plant under salinity stress ranged from 3 to 14 leaves/plant. Main stem length varied from 3.4 cm to 66.9 cm and the decrease in main stem length due to salinity ranged from 9% to 92% as compared with control plants. Bottle gourd accessions showed significant differences under salinity stress. Some promising accessions for use in rootstocks breeding programs against salinity stress were identified.

Keywords: Salinity, bottle gourd, rootstock, fresh weight, leaf area

Introduction

Soil salinity is the salt content in the soil; salinization is known as the process of accumulation of various salts in soil. Salinity in soil water is an important abiotic stress restricting plant growth and productivity that occurs in arid and semi-arid regions of the world (Arzani 2008). It was reported that over 800 million ha are affected by salinity and this area is 65% of total terrestrial area of the world (FAO 2009). The future of crop production in most agricultural regions of the world is under danger of progressive salinization (Flowers and Yeo 1986, Parida and Das 2005). The main reasons of soil salinization are high evaporation-to-rainfall rate (high evaporation, low rainfall), saline native rock, low quality irrigation water, and poor water management practices. Most crop plants are not able to survive or able to survive only with a reduced growth rate and lower yields and quality under high salinity stress conditions. The well-known impact of salinity is slower growth rate expressed as a reduction in number and size of leaves and a smaller and stunted plant canopy. The first and primary impact of salinity is due to its osmotic effects leading to low water potential of the rhizosphere. Other effects are toxicity of the ions, mainly Na^+ and Cl^-, and nutrient imbalance resulting from depression in plant nutrition uptake and/or transport (Jacoby 1994, Marschner 1995). Overcoming the salinity problems in soil will increase plant production by positively affecting plant growth and development. Salinity management through reclamation or advanced irrigation methods is often prohibitively expensive and provides only short-term solutions to solve salinity problems in arid and semi-arid areas where severe water shortage and high evapotranspiration occur (Singh and Singh 2000). Development of salt-tolerant cultivars or rootstocks by breeding is a more permanent solution to minimize deleterious effects of salinity (Foolad 1996). Several attempts have been made to improve crop salt tolerance through traditional breeding programs, but commercial success has been quite limited. Although salt tolerant species/genotypes are usually present in gene pool, breeding of improved cultivars with salt tolerance is tedious due to complex genetic mechanisms of salinity tolerance (Cuartero and Fernandez-Munoz 1999) and various effects of different levels of salinity. Recently, significant

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numbers of studies have been carried out to develop salt tolerant plants by transgenic plant technology. Although the improvement of salt tolerance was reported by transfer of a single gene (Rus et al. 2001), due to the polygenic nature of abiotic stress as such, salinity requires transfer of more genes for the improvement of salt-tolerance (Bonhert and Jensen 1996). Given the high grower demand for cultivars with high yield, quality, and marketing attributes, although they are not tolerant to salinity, alleviating negative effect of salt stress in plants has been still a significant research area. One possible way of reducing deleterious effects of salt stress on growth and development of high yielding cultivars would be to graft these elite genotypes onto rootstocks that are capable of growing under saline conditions and induce salt tolerance in the scion. Determining the effect of root characteristics, at least partially, on the salinity response of tomato was reported by Santa–Cruz et al. (2002), and they suggested grafting as a valid strategy for the alleviation of the deleterious effect of salt stress on the shoot growth. Similarly, the primary role of root characteristics on salt response was reported in melon, *Cucumis melo* L. (Romero et al. 1997). Interspecific grafting has been used for crop plants of the Cucurbitaceae and Solanaceae. This compatibility widens the opportunities of finding suitable rootstocks for related scion cultivars.

Grafting is an important technique for the sustainable production of some crops of the Cucurbitaceae and Solanaceae in Japan, Korea, and some other Asian and European countries, where intensive production is performed without plant rotation. Grafting in vegetables was first performed in Korea and Japan in the late 1920s by grafting watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, onto bottle or calabash gourd, *Lagenaria siceraria* (Molina) Standl. rootstocks (Ashita 1927, Lee 1994, Oda 1995). Some purposes of grafting in watermelons are to control Fusarium wilt, to increase low-temperature tolerance and yield and quality with increased water and nutrient uptake (Lee 1994, Oda 1995). For these purposes, watermelons have been grafted onto *Cucurbita moschata* Duchesne, *C. maxima* Duchesne, *Benincasa hispida* (Thunb.) Cogn., and *L. siceraria* (Lee 1994).

Bottle gourd is a monoecious annual, a vigorous climber, and there are five wild perennial dioecious *Lagenaria* species (Motimoto et al. 2005). *L. siceraria* has been utilized as a vegetable, musical instrument, decoration, tools, pipe, and containers, corresponding to characteristics of the young and mature fruits (Yetisir et al. 2008). Furthermore, as mentioned above, *L. siceraria* is used as rootstocks for watermelon against soil-borne diseases, particularly for Fusarium wilt and low soil temperature. *L. siceraria* shows high compatibility with watermelon (Lee 1994, Oda 1995, Yetisir and Sari 2003). Turkish bottle gourd germplasm was collected from 2003 to 2013, and seed multiplication and morphological characterization of 322 accessions were done. As some Turkish bottle gourds landraces showed superior plant growth and plant nutrition uptake as compared with watermelon in our previous studies (Uygur and Yetisir 2009, Yetisir and Uygur 2009), we aimed to screen Turkish bottle gourd germplasm against salt stress by comparing with watermelon regarding the plant growth parameters, and to prepare background information about accessions for further rootstock breeding programs.

Materials & Methods

The experiment was carried out in an unheated greenhouse at the Alata Horticultural Research Institution, Erdemli-Mersin, in April and May of 2013. The location is a Mediterranean coastal area (36°37' N; 34°20' E; altitude 5 m). One hundred sixty *L. siceraria* accessions selected based on morphological characteristics from Turkish bottle gourd germplasm, two commercial rootstocks, 'Argentario' (Arg, Syngenta) and 'Macis' (Nunhems), and two watermelons: 'Crimson Tide' (C.T. Syngenta) and 'Crisby (Nunhems) were used. Commercial rootstocks and watermelon cultivars were used for comparison. An aerated hydroponic system was used as growing system. The basic nutrient solution used in the experiments was a modified Hoagland formulation. All chemicals used were of analytical grade, and composition of the nutrient solution was: 2.0 mM $Ca(N0_3)_2$, 0.75 mM K_2SO_4, 0.65 mM $MgSO_4$, 0.5 mM KH_2PO_4, 50 µM NaCl, 10 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.4 µM $CuSO_4$, 0.4 µM $(NH_4)_6Mo_7O_{24}$, 0.5 µM $ZnSO_4$, and 80 µM Fe-EDDHA. The pH of the nutrient solution was 6.5 to 7.0. The two-to-three true-leaf stage seedlings, grown in a mixture of peat : perlite (2 : 1), were transplanted to foam-plugged holes (one plant per hole) in styrofoam sheets floating over l00 L of nutrient solution. The salt application was done by adding NaCl to the nutrient solution. The salt treatment was initialized 5 days after transplanting. The salt application (10 dSm^{-1}) was treated in an increasing manner (2.5 dSm^{-1} per day). In the control (1.5 dSm^{-1}) and salt treatments, the nutrient solution was changed once a week to ensure sufficient nutrients for plant growth and to keep the pH level in the solution close to the targeted level. The experimental design was a split block; main plots were salt treatment and sub-plots were genotypes. Each accession was replicated 3 times with 3 plants. After 21 days of salt application, main stem length, leaf number and area (LI 3100 C USA) per plant, and shoot and root fresh weight (g) of plants were determined. Reduction (%) in all measured parameters total, shoot and root due to salt stress were calculated and compared with the control by the following formula: Percent reduction = [(Control-Treatment)/Control]×100.

Results & Discussion

Plant growth was significantly affected by NaCl treatment and bottle gourd genotypes showed great variations. Reduction in plant growth parameters (%) were presented as frequency distribution of genotypes in figures (number of genotypes at each percent reduction level). Leaf number was significantly affected by salt application and reduction varied from 20 to 90%. About 28 bottle gourd accessions were less affected by salt application than commercial rootstocks and watermelon cultivars with regard to leaf number. While 'Argentario' and 'Macis' showed 50 and 60% reductions in leaf number, respectively, decrease in leaf number in both watermelon cultivars was 90% (Figure 1A). Leaf area was also significantly affected by salt application and genotypes presented significant differences. A large number of accessions (about 90) had larger leaves than commercial rootstocks and watermelon cultivars. Reductions in leaf area were 50% in 'Argentario' and 90% in 'Macis', while 'Crimson Tide' and 'Crisby' had 70 and 80% reductions in leaf area (Figure 1B).

Results for total plant fresh weight and main stem length are presented in Figure 2. Salt application caused a significant decrease in plant total fresh weight. Some accessions (about 15) showed better performance than the watermelon cultivars and commercial rootstocks. Reduction in total fresh weight was 30% in commercial rootstocks, while total fresh weight was decreased by salt stress in 'Crimson Tide' and 'Crisby' by 70 and 80%, respectively. Decrease in main stem length was not as marked as leaf area, leaf number and total fresh weight. Reduction in main stem length varied from 10 to 40% in a large number of accessions. While 30 to 40% shortening of the main stem was observed in 'Argentario' and 'Macis', respectively, reduction in main stem length of 'Crimson Tide' and 'Crisby' was 60 and 90%, respectively. Fifty-nine accessions showed 20% or less reduction in main stem length as compared with control plants.

Root fresh weight was significantly reduced by salt application in all accessions, commercial rootstocks and watermelon cultivars, to varying degrees (Figure 3). While watermelon cultivars showed a 90% decrease in root fresh weight, root fresh weight decreased 40% in commercial rootstocks. Among the bottle gourd accessions, about 12 genotypes showed superior performance over the commercial cultivars with regard to root fresh weight while reduction in root fresh weight was higher than commercial rootstocks in about 130 accessions (Figure 3A). Shoot fresh weight results were similar to those of plant total fresh weight. While 30 and 40% reductions in shoot fresh weight

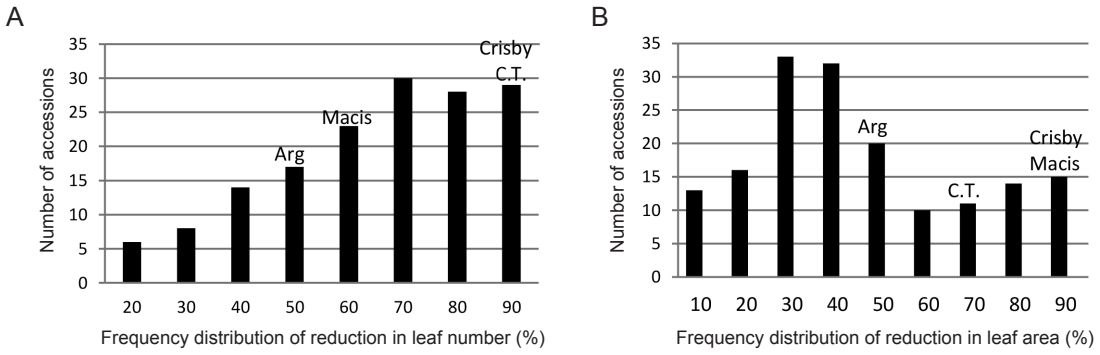

Figure 1. Frequency distribution of reduction in leaf number (A) and leaf area (B).

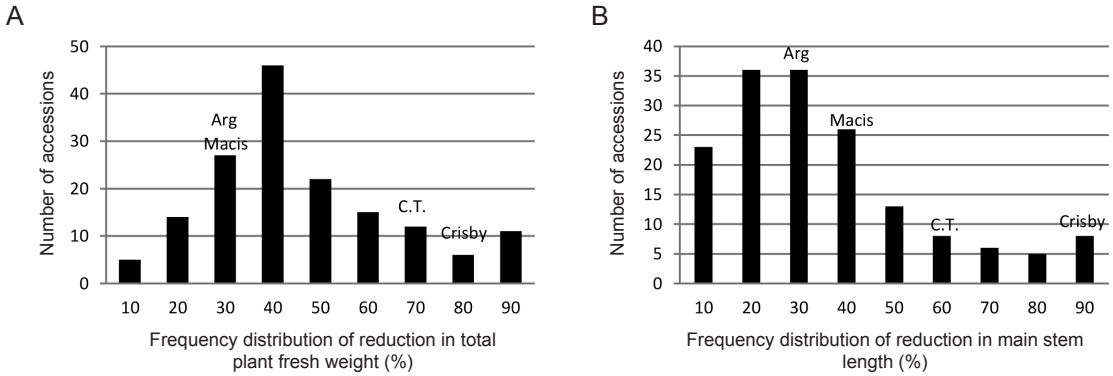

Figure 2. Frequency distribution of reduction in total plant fresh weight (A) and main stem length (B).

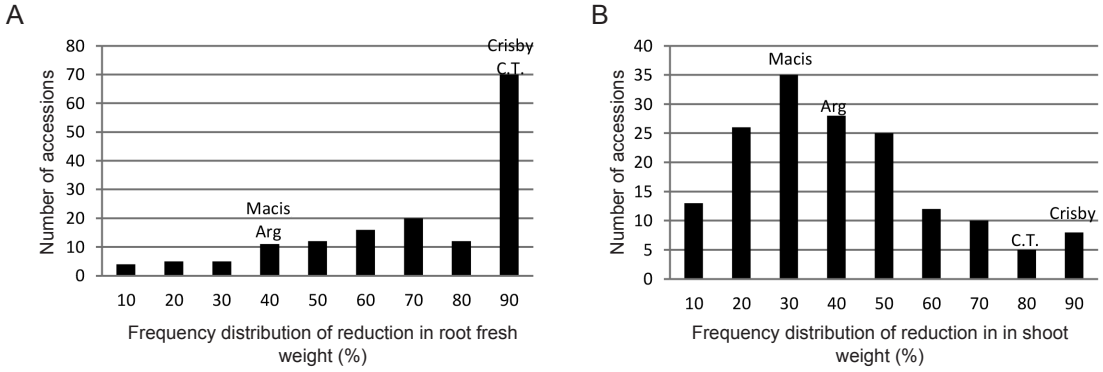

Figure 3. Frequency distribution of reduction in root (A) and shoot fresh weight (B).

were observed in 'Argentario' and 'Macis', respectively, 'Crimson Tide' and 'Crisby' showed 60 and 90% reductions in shoot fresh weight. About 60 bottle gourd genotypes had higher shoot fresh than 'Argentario' and 'Macis', and 49 and 74 genotypes produced lower shoot fresh weight respectively.

Plant genetic resources are unique sources for cultivar breeding with improved tolerance/resistance against biotic and abiotic stresses. Therefore, collection, preservation, characterization, and evaluation of plant genetic resources are invaluable for sustainable agriculture and food safety. Wide variation in salinity tolerance among plants has been reported. Among vegetable crops, asparagus and beets are the most tolerant, beans, carrots, and turnips are the most sensitive species. Pumpkin/squash, melon, and watermelon line in-between. Squash/pumpkins are more tolerant than watermelon, melon, and cucumber (Kotuby-Amacher et al. 2000). In our study, 160 bottle gourd genotypes from Turkey were screened for tolerance to salinity stress (10 dSm^{-1}). Significant variation in response to salinity stress was observed. The majority of the accessions showed better performance than watermelon cultivars with regard to plant growth parameters, and a significant number of bottle gourd genotypes even had superior performance over hybrid commercial rootstocks. In agreement with our results, tolerance of bottle gourd to salinity has been reported elsewhere. In a screening study, 65 bottle gourd genotypes were tested against salt stress (3 dSm^{-1}) at germination, and while two local accessions, 'Hapcheon' and 'Cheongdo' showed the highest germination rate, introduced genotypes from different sources did not germinate at all; in addition, 'Hapcheon' showed better rootstock performance under saline conditions (Chung et al. 2003). Taffouo et al. (2010) found bottle gourd to be more salt tolerant than watermelon and *C. moschata* at 50 mM salinity. Similarly, watermelons grafted onto bottle gourds were more tolerant to salinity and productive than ungrafted control plants (Huang et al. 2009). Zhu and Bie (2008) also reported that grafting cucumber, *Cucumis sativus* L., plants onto bottle gourd induced salt tolerance at 100 mM NaCl. In our previous studies, plant growth, plant nutrient uptake, and ion regulation (Uygur and Yetisir 2009, Yetisir and Uygur 2009) of bottle gourds under salinity stress were found better as compared with watermelon. Similarly, Yang et al. (2015) reported increased photosynthesis by the activation of stomatal and non-stomatal activities in watermelon grafted onto bottle gourd rootstocks under saline conditions.

Conclusions

The results of this study show that bottle gourd accessions possess significant variation with regard to plant growth parameters under saline conditions. The majority of the bottle gourd accessions were found to be more tolerant to salinity than two watermelon cultivars, 'Crisby' and 'Crimson Tide'. Furthermore, a significant number of accessions showed better plant growth than two leading commercial rootstocks, 'Argentario' and 'Macis'. Thus, Turkish *L. siceraria* germplasm is promising material as a good resource for rootstock/cultivar (as vegetables) breeding programs for tolerance to salinity stress.

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Open-Field Survey of Turkish Bottle Gourd Germplasm Reaction to Virus Diseases

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Abstract. Grafting of vegetables, particularly watermelon, cucumber, and melon, has become widespread in Turkey. *Zucchini yellow mosaic virus* (ZYMV) is one of the most damaging diseases of cucurbit crops. The possibility of seed-transmission of ZYMV makes it an even more dangerous virus. Therefore, identification of potential sources of ZYMV resistance in bottle gourd is important. Resistant germplasm could be used for developing inbred lines as rootstocks for watermelon. The aim of this study was to screen Turkish bottle gourd genotypes for ZYMV, *Watermelon mosaic virus* (WMV), and *Cucumber mosaic virus* (CMV) resistance under open field conditions. Of the 324 accessions that were screened, 188 (55%) were found to be infected with ZYMV, 71 genotypes (21%) with WMV, and 40 (12%) with CMV, in single or mixed infections, and the rest of the accessions were virus-free. Seeds were collected from ZYMV-infected plants and sown. The seedlings were tested serologically and with RT-PCR techniques for presence of ZYMV, WMV, and CMV. We observed that ZYMV was transmitted by 3.19% of the seeds from obviously infected fruits. As a result of these observations, we have become convinced that bottle gourd rootstock germplasm needs to be resistant to ZYMV.

Keywords: Bottle gourd, virus, rootstock, germplasm

Introduction

Viruses are the most important pathogens of the cultivated Cucurbitaceae. More than 30 viruses causing destructive symptoms and considerable economic losses have been reported in cucurbit crops (Zitter et al. 1996, Yuki et al. 2000, Ko et al. 2007). *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV), *Cucumber mosaic virus* (CMV), and *Papaya ring spot virus-W* (PRSV-W) are the most frequently encountered viruses in cucurbit crops in major cucurbit growing areas of the world (Unruean et al. 2013, Nontajak et al. unpublished). Their occurrence, spread, intensity of infection, and destructiveness depend on complex interrelations between the virus, its host plant, the vectors, and environmental conditions. It is usually not easy to find appropriate control measures to reduce the extent of destruction by viruses.

ZYMV, a member of the genus *Potyvirus* in the family Potyviridae, is one of the major viruses of cucurbits. Cultivars of *Cucurbita pepo* L., *Cucumis melo* L., and *Citrullus lanatus* (Thunb.) Matsum. & Nakai are particularly affected. However, other cucurbits, including bottle gourd, *Lagenaria siceraria* (Mol.) Standl., are also infected easily by ZYMV. Natural infection of bottle gourd by ZYMV has been observed in Hawaii (Ullman et al. 1991), India (Verma et al. 2004), Serbia (Dukić et al. 2006), U.S.A. (Provvidenti et al. 1984), and Turkey (Sertkaya et al. 2004, Fidan et al. 2013). In many cases, planting a disease-resistant cultivar is the best solution for controlling virus diseases in vegetable crops. Resistance to ZYMV in bottle gourd was reported in germplasm collected from several countries (Provvidenti 1977, 1995, Gerber 1978). However, only a limited number (18) of bottle gourd accessions have been tested for virus resistance (Provvidenti 1981).

The bottle gourd (*L. siceraria*) is one of the earliest domesticated plant species. Also known as calabash or white-flowered gourd, *L. siceraria* is an annual, a vigorous climber and monoecious. Geographic origin of this species is generally accepted as Africa (Whitaker 1971). Five perennial, dioecious, wild species of *Lagenaria*, *L. breviflora* (Benth.) Roberty, *L. abyssinica* (Hook. f.) C. Jeffrey, *L. rufa* (Gilg) C. Jeffrey, *L. sphaerica* E. Mey., and *L. guineensis* (G. Don) were reported from northern Africa (Decker-Walters et al. 2004). Two subspecies of *L. siceraria*, ssp. *asiatica* (Kob.) and ssp. *siceraria,* were described based on morphological characterization of vegetative

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and generative plant organs (Heiser 1979). Bottle gourd is known for its growing ability with limited horticultural requirements and high adaptation capacity to different environmental conditions (Mladonović et al. 2012).

Bottle gourd has become also an impressive plant species with different shapes and sizes of fruits, allowing use of mature dry fruit for sundry purposes. Reported fruit shapes for bottle gourds are oblate, spherical, ovoid, pyriform, scoop-shape, club-shape, bilobal (proximal lobe is smaller), cylindrical, and elongated (Morimoto et al. 2005).

Bottle gourd is grown as a vegetable, the immature young fruits are eaten after being cooked (boiling, frying, or stuffing), like *Cucurbita pepo*, in Africa and Asia. In the southern part of the Turkey, bottle gourd fresh fruit is also consumed as vegetable like summer squash. The genotypes with non-bitter fruits are generally consumed as a vegetable, while the wild bitter varieties are preferred for the medicinal use or other uses. However, the mature fruits with dried hard shell are used in making several types of containers for storing or transferring liquids and kitchen stuff (scoop, cup, plate, etc.), musical instruments, decorations, or fishing floats (Yetisir et al. 2008, Loukou et al. 2011). Vegetative part of plants (leaves, shoots, and tendrils) are also used as vegetables by cooking and mixing with other vegetables and the seeds are removed for oil extraction. Leaves can be dried and stored to be used out-of-season (Grubben and Denton 2004). In western Africa, bottle gourd seeds, rich in oil (45%) and protein (35%), are used for making sauce and oil extraction for human consumption (Loukou et al. 2011). Seeds, tendrils, and young leaves are also used for some medical purposes (Herklots 1972, Moerman 1998, Manandhar 2002). The high adaptability of bottle gourd to different soil and growth conditions and resistance to some soil-borne diseases has made bottle gourd a suitable rootstock for watermelon. Bottle gourd is used as a rootstock for watermelon against soil-borne diseases such as Fusarium wilt (Oda 1995, Yetisir et al. 2003), low soil temperature (Lee 1994), salinity (Colla et al. 2005, Yetisir and Uygur 2010), excessive water in soil (Yetisir et al. 2006), and high pH (Colla et al. 2010). In eastern Asia (China, Japan, and Korea), *L. siceraria* has been widely used as a rootstock, particularly for watermelon, for many years. Recently, significant interest has been given to bottle gourd as a rootstock in Europe and in the U.S.A., as an alternative way to control soil-borne diseases such as Fusarium wilt (Miguel et al. 2004, Cohen et al. 2007).

Use of resistant varieties is a commonly accepted solution to manage virus diseases in vegetable crops. Therefore, determination of the current status of Turkish bottle gourd germplasm for resistance to important cucurbit viruses has primary importance. The objective of this study was to determine which viruses cause disease in bottle gourds and find accessions possessing resistance (without symptoms) under open-field and natural infection conditions.

Material & Methods

Plant material and experimental field

In this study, Turkish bottle gourd germplasm (324 accessions) was surveyed for ZYMV, WMV, and CMV under open-field conditions and natural infection. The plants were grown in a plot at the Alata Horticultural Research Institution in Mersin, Turkey. The site is located in the coastal area of the Mediterranean Sea (36°37' N; 34°20' F; altitude 5 m). Seedlings with 2 to 3 true leaves were transplanted to the field at 3 m distances between rows and 0.5 m within rows. Ten plants from each of 324 accessions were grown. Plants were watered by drip irrigation, fertilization at 100 kg N/ha, 100 kg P_2O_5/ha and 100 kg K_2O/ha was applied with drip irrigation during the growing period.

In each plot, plants were inspected and samples from plants showing virus-like symptoms were collected. Each sample consisted of the youngest fully developed leaf from plants exhibiting symptoms such as mosaic, mottle, chlorosis, vein clearing or yellowing of the foliage, and fruit discoloration and deformation. All samples (299) were tested for the presence of ZYMV, WMV, CMV, PRSV-W, CYSDV, CVYV, SqMV, CGMMV, and MNSV. All collected samples were placed in plastic bags stored at 4 °C and tested within 1 to 3 days for virus infection either serologically [double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)] or reverse transcriptase polymerase chain reaction (RT-PCR) (Table 1).

Table 1. Surveyed viruses and testing methods.

Virus name	Testing methods	Results
Cucumber mosaic virus (CMV)	ELISA	+
Zucchini yellow mosaic virus (ZYMV)	ELISA/RT-PCR	+
Watermelon mosaic virus (WMV)	ELISA	+
Squash mosaic virus (SqMV)	ELISA	-
Papaya ringspot virus (PRSV)	ELISA	-
Cucumber vein yellowing virus (CVYV)	RT-PCR	-
Cucumber green mottle mosaic virus (CGMMV)*	RT-PCR	-
Cucurbit aphid-borne yellows virus (CABYV)	RT-PCR	-
Cucurbit yellow stunting disorder virus (CYSDV)	RT-PCR	-
Melon necrotic spot virus (MNSV)	RT-PCR	-

*Not reported on cucurbit crops in Turkey.

Table 2. Primers used in RT-PCR and predicted amplicon size for major cucurbit virus diseases.

Primer	Sequence	Amplicon size bp
ZYMVF	ATGCTCCAATCAGGCACYC	791
ZYMVR	GTGTGCCGTTCAGTGTCTTC	
CVYVF	AGCTAGCGCGTATGGGGTGAC	450
CVYVR	GCGCCGCAAGTGCAAATAAAT	
CGMMVF*	TTGCGTTTAGTGCTTCTTATGT	440
CGMMVR*	GAGGTGGTAGCCTCTGACCAGA	
CABYVF	GAATACGGTCGCGGCTAGAAATC	600
CABYVR	CTATTTCGGGTTCTGGACCTGGC	
CYSDVF	AGTGACATGCCTAACTGTTACTT	364
CYSDVR	ATAGCTGCTGCAGATGGTTC	
MNSVF	CTCCATAAGCGCCAAGCAACC	485
MNSVR	AGCGGGGGAAAACAGAAGAA	

*Not reported on cucurbit crops in Turkey.

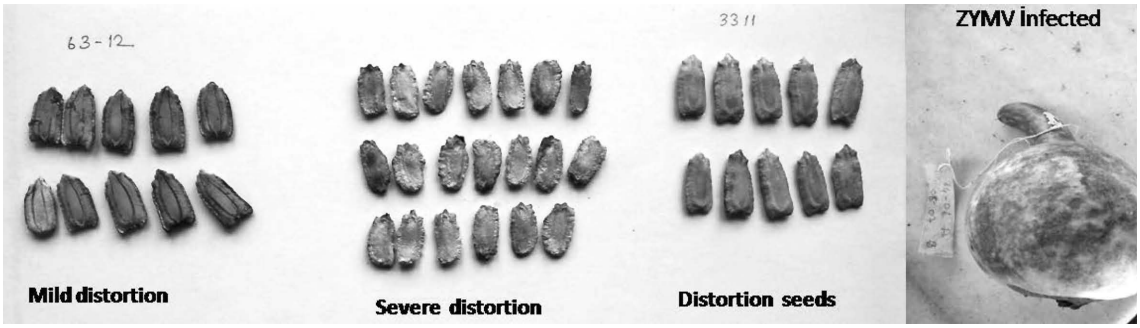

Figure 1. Distorted seeds harvested from infected bottle gourd plants.

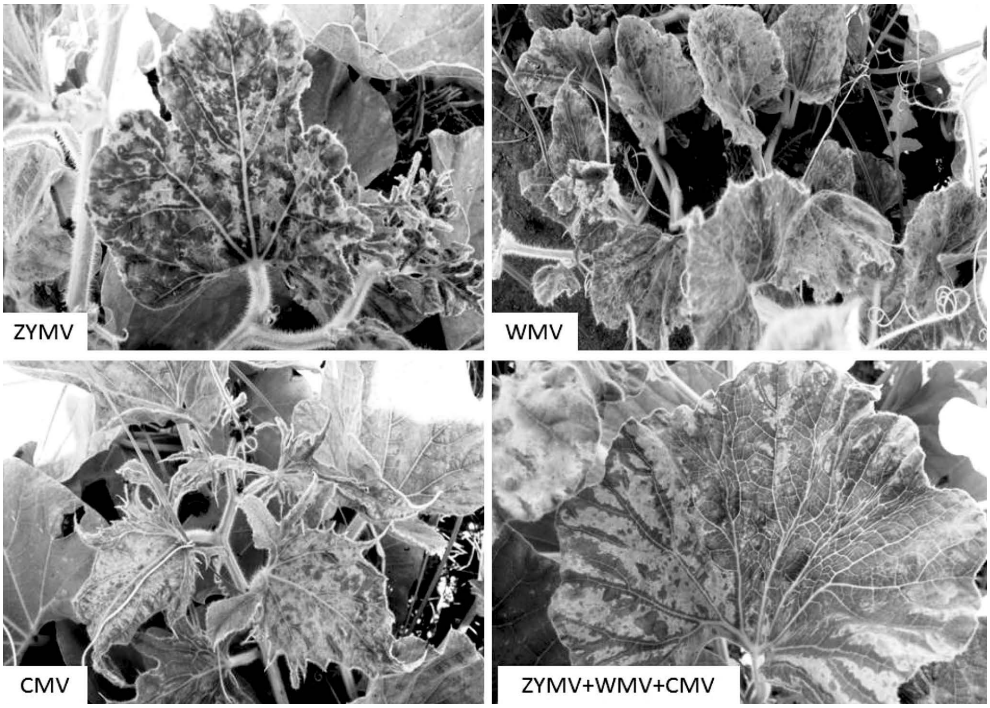

Figure 2. Leaf distortion, green mosaic (ZYMV), mosaic (WMV), and shoestring (CMV) symptoms in *Lagenaria siceraria* leaves caused by single or mixed infections by viruses.

Serological test

Serological assays were carried out using DAS-ELISA with polyclonal antibodies. Tests involved detection of viruses in infected young leaves and carried out according to Clark and Adams (1977). Polystyrene microtiter plates were coated with 1 : 1000 dilution of gamma globulin. The leaves of infected young plants were ground in the extraction buffer (phosphate buffered saline, PBS, pH 7.0). Plant samples were applied at a dilution of 1 : 5 (Wff) in PBS (pH 7.0), containing 0.05 volume Tween-20, 0.2% polyvinyl pyrolidone (PVP-40), and 2% bovine serum albumin (BSA). Plates were incubated at 4 °C overnight and washed four times. PBST Tween 20 buffer IgG-conjugate was applied at the concentration of 1 µl/1000 µl (Bioreba). Alkaline phosphatase conjugate was used at a 1/1000 dilution.

Results were acquired spectrophotometrically at 405 nm using a Medispec ESR 200 ELISA microplate reader. Also, against negative controls (healthy samples), twice the mean value and above, was considered positive. Positive controls of all viruses were supplied in lyophilized form with the kits and were resuspended in the sample buffer as recommended by the manufacturers (BIOREBA AG. Switzerland).

Total RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA extracts (Thermo Scientific GeneJETPlant RNA Purification Mini Kit) of virus-infected plants and seeds were used in a RT-PCR with sense and antisense primers. Primers used in this study and the expected length of each amplicon are presented in Table 2. First and second strand cDNAs were synthesized from RNA using Thermo Scientific Verso 1-Step RT-PCR Hot-Start Kit according to manufacturer's protocol. Amplification for each virus proceeded through a cycle of denaturation at 95 °C (45 s), annealing at 55 °C (1 min) and extension at 72 °C (1 min) for a total of 35 cycles in Techne Genius thermal cycler.

Table 3. Viruses found in open field survey and tested seeds from infected plants.

Virus name	Tested by	Infected plants	Infected seeds
Zucchini yellow mosaic virus (ZYMV)	ELISA/ RT-PCR	188 (55%)	8 (3.19%)
Watermelon mosaic virus (WMV)	ELISA	71 (21%)	--
Cucumber mosaic virus (CMV)	ELISA	40 (12%)	--
Papaya ring spot virus Type W (PRSV-W)	ELISA	0	--
Squash mosaic virus (SqMV)	ELISA	0	--
Cucurbit aphid-borne yellows virus (CABYV)	RT-PCR	0	--
Cucurbit yellow stunting disorder virus (CYSDV)	RT-PCR	0	--
Cucumber vein yellowing virus (CVYV)	RT-PCR	0	--
Cucumber green mottle mosaic virus (CGMMV)	RT-PCR	0	--
ZYMV+WMV	ELISA	23	--
ZYMV+CMV	ELISA	14	--
ZYMV+CMV+WMV	ELISA	11	--

Analysis of RT-PCR products

Ten µl of the PCR product was combined with gel loading buffer and analyzed on a 2% agarose gel containing 0.5 mg·ml^{-1} of ethidium bromide and photographed (Sambrook et al. 1989); 100 bp DNA ladder (Thermo) was used on each gel to determine the length of the amplified product.

Seed material and experimental conditions

Seeds were extracted from naturally ZYMV-infected fruits showing mild to severe symptoms and, after drying, were stored at 4 °C. The seeds were sown in an insect-proof greenhouse and the plants were visually observed. The plants with abnormal growth or virus symptoms were classified as severely distorted, distorted, and mildly distorted, and tested by ELISA techniques (Figure 1). In ELISA serological tests, Zucchini Yellow Mosaic Virus (Bioreba), Watermelon Mosaic Virus 2 (Bioreba), and Cucumber Mosaic Virus (Bioreba) kits were used. Others viruses were tested by the RT-PCR.

Results

A total of 299 *L. siceraria* leaf samples showing virus symptoms and seed samples harvested from infected plants were tested by ELISA and RT-PCR. Symptomological distinction of the viruses (ZYMV, WMV, PRSV, and CMV) is not easy (Figure 2). Therefore, we used ELISA and RT-PCR methods to determine which virus diseases were in the symptomatic plants.

The results showed that 188 genotypes (55%) were infected with ZYMV, 71 genotypes (21%) with WMV, 40 (12%) with CMV single or mixed infection and the rest (25 accessions) were virus-free (Table 3). Then the seeds were harvested from ZYMV-infected plants. Seeds were sown and seedlings were tested serologically using RT-PCR techniques for ZYMV, WMV, and CMV. The tests showed that ZYMV was transmissible by seeds at a frequencyof 3.19% in infected genotypes.

Discussion

The Cucurbitaceae are highly sensitive to virus infection. They are infected by more than 30 viruses, the most important being: CMV, WMV, ZYMV, PRSV, and *Squash mosaic comovirus* (SqMV) (Zitter et al. 1996). *Cucumber green mottle mosaic virus* (CGMMV) has not been reported on cucurbit crops in Turkey. Some of the bottle gourd accessions appear to be tolerant/resistant to ZYMV (Table 3). CYDSV, CABYV, PRSV, and SqMV have been reported in watermelon, melon, cucumber, and squash production fields in Turkey (Köklü and Yımaz 2006, Kaya and Erkan 2011).

Our results confirm the presence of ZYMV, WMV, and CMV in bottle gourd in our country. These viruses had been reported here previously at other localities (Sertkaya et al. 2004). ZYMV and WMV can be considered to be widespread. The most frequent virus, ZYMV, was present in 55% of the samples. This virus occurred in a relatively large number of samples (23) in combination with WMV. Compared with the other two viruses, CMV was detected only sporadically. As the symptoms caused by these three viruses in different bottle gourd accessions varied, it was not possible to establish a high correlation between the type of symptom and the virus. Therefore, field symptoms cannot be used as reliable indicators, even in the case when infection is caused by a single virus, due to complexity of the disease symptoms caused by many of the viruses. Also, in contrast with the results of other experiments with ZYMV that did not show transmission via seed, our study indicated seed transmission in bottle gourd. Seed-transmitted diseases, viral, bacterial, and fungal, are most significant in grafted transplant production. Because one single contaminated seedling (rootstock or scion) can easily contaminate several others during the grafting process and eventually the entire production field. Hence, identification of virus-resistant genotypes of bottle gourd will be important for further watermelon rootstock breeding programs.

Conclusion

Infection of bottle gourd with ZYMV, WMV, and CMV was observed in bottle gourd accessions. While the majority of bottle gourd accessions were infected by a single virus or mixture of viruses, some accessions were symptom-free. ZYMV was observed to be transmitted in bottle gourd seeds at the level of 2.5%. As grafting of watermelon on bottle gourd becomes more common, breeding virus-resistant bottle gourd rootstocks will become extremely important. Future research needs to continue to focus on the evaluation of bottle gourd germplasm for cucurbit virus resistance, particularly the seed-transmittable viruses.

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Reaction of Some Melon (*Cucumis melo*) Genotypes to Drought Stress

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Abstract. Plants encounter many stress factors which affect their growth and development throughout their life-cycles. These stress conditions, biotic and abiotic, can adversely affect the quantity and quality of the product, and lead to physiological and biochemical damage in crops. Plants have molecular response mechanisms for protecting and reducing negative effects of stress factors. The necessity of improved varieties resistant to biotic and abiotic stresses is becoming more and more appreciated. As the chemicals used against pests and diseases have adverse effects on health and are costly and often ineffective, and given the even higher cost of "organic" pesticides, breeding crop plants for stress tolerance has been initiated. The high local adaptation of indigenous genetic resources could increase the value of developed varieties. Drought stress was applied to 177 melon (*Cucumis melo*) genotypes, and the first branch, first male flower, number of flower buds, number of male flower buds, flower width, canopy diameter, length of main stem, root width, fresh root weight and dry root weight, condition of dehydration, leaf temperature, length of root, and growing status are parameters that were observed. Of the 177 genotypes, 43 showed some resistance to drought stress.

Keywords: Melon, drought stress, genetic resources, abiotic stress

Introduction

Temperature and drought are two important environmental parameters that can limit melon production. Abiotic stress can be defined as "stress caused by the inanimate components of the environment associated with climatic, edaphic, and physiographic factors that substantially limit plant growth and survival" (Jenks and Hasegawa 2005). Studying resistance to abiotic stresses can strengthen the breeding of new cultivars. Most agricultural practices are designated to optimize crop growth by avoiding or reducing abiotic stress (by irrigation, fertilization, etc.). Melon (*Cucumis melo* L.) is an important horticultural crop, often cultivated in arid and semi-arid regions of the world, where drought begins to be a threat, or has already been a problem. In general, melon is known to be moderately resistant to salinity and drought. It has been shown that these stresses cause several types of damage such as growth inhibition (Mendlinger 1994, Franco et al. 1997, Dasgan and Koc 2009, Kusvuran 2010), metabolic disturbances (Mavrogianopoulos et al. 1999), and yield and quality losses (del Amor et al. 1999). Our aim was to observe the effects of drought stress on 177 melon accessions collected from different regions of Turkey and determine which ones are the most drought tolerant. In our region, local accessions are used because of their relatively high drought tolerance. This project's target would be of interest in entire large regions, arid and semi-arid, which have high temperatures and high evaporation. At the end of this project, selected accessions will be used as parents for creation of new cultivars.

Material & Methods

This research was conducted at the Koruklu Research Station of the GAP Agriculture Research Institute (GAPTAEM), Turkish Ministry of Agriculture and Rural Affairs in Sanlıurfa (Turkey). The experiment was carried out on a clay textured soil (Vertic Calciorthid aridisol) during the 2012 production season. The altitude, latitude, and longitude of the experimental site were 410 m, 36°42'N, and 38°58'E, respectively. Average field capacity, permanent wilting point, dry bulk density, and pH of the site for 90 cm soil depth were 32.21%, 21.61%, 1.41 g/cm^3, and 7.6, respectively. Water quality at the site was good enough for irrigation with EC = 0.52 dS/m and pH = 7.3. Weather conditions at the site were hot, and dry from May to September with air temperatures up to 46 °C and relative humidity averaging about 34%. Annual average rainfall is about 380 mm (Dinc et al. 1988, Almaca and Gok 1997).

Plant material

There is a large collection of over 400 melon accessions held at Cukurova University, Faculty of Agriculture, Department of Horticulture. These accessions were collected from different regions of Turkey and some of them were imported from foreign countries. Of them, 177 were selected as the plant material for this project.

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Methods

Seeds were sown 2 m × 1 m between- and within-row spaces with 12 plants per plot. The first and last plants of each accession on a row were used as a buffer and the middle 10 plants were used for harvesting and measurements. Two irrigation levels were imposed to determine potential drought resistance of accessions. All plots in the beginning were irrigated with enough water for healthy seed germination. The plants received enough irrigation until the stage of true leaves. Irrigation treatments were initiated at the beginning of the true leaves. While S1 treatment received full irrigation based on depleted soil moisture in the root zone, S2 treatment received 50% of S1. Irrigation water was delivered by a pump from an irrigation channel near the experimental site. The control unit consisted of a pump, screen filter with 10 $L \cdot s^{-1}$ capacity, flow meter, control valves, fertilizer tank, and pressure gauges. Pressure compensating drippers were used to supply uniform water distribution. Each plot had a separate flow meter to monitor water input. A single drip irrigation tube for each row with 4 $L \cdot h^{-1}$ was placed over the soil surface. The operating pressure of the drip irrigation system was constant during the experiment at 100 kPa. Soil moisture content at 90 cm soil depth was measured gravimetrically before each irrigation event. The following formula was used to determine the amount of water applied to the treatments (Gungor et al. 2002):

$$d = \frac{FC - PM}{100} \gamma_t D P$$

In this formula, FC is the field capacity, PM is the present moisture at sampling, D is the effective plant root depth, P is the percentage of canopy cover, γ_t is the dry bulk density, and d is the amount of water applied for each irrigation event. A basic fertilizer application of 200 kg/ha P_2O_5 as triple superphosphate was incorporated in the soil before transplanting.

Fourteen traits were measured: first branch, condition of dehydration, leaf temperature, length of root, first male flower, number of flower buds, number of male flower buds, flower width, canopy diameter, length of main stem, root width, fresh root weight, growing status, and dry root weight. The drought tolerance was estimated by the ratio of the value of a trait under the S1 irrigation level and the value of this trait under the S2 irrigation level.

This ratio was transformed according to the following criteria. For the 10 parameters, first branch, first male flower, number of flower buds, number of male flower buds, flower width, canopy diameter, length of main stem, root width, fresh root weight, and dry root weight, the ratio was multiplied by 5 to obtain a value between 1 and 5. For the three parameters, condition of dehydration, leaf temperature, and length of root, the ratio was transformed to obtain a value between 1 to 10. For the growing status, the ratio was transformed to obtain a value between 1 to 20. The sum of the transformed values for the 14 traits was the drought tolerance index (DT) for an accession. A value of DT close 100 indicates a high tolerance to drought.

Results

The 43 accessions with a DT equal to or higher than 80 were classified as drought tolerant (Table 1). The accessions nos. 41, 106, 183, and 248 had the highest drought rating, 100. The next most tolerant accessions were nos. 188, 210, 228, 242, and 257, with ratings of 99. The 51 accessions with a DT lower than 65 but higher than or equal to 50 were classified as tolerant/intolerant accessions. The 79 accessions with a DT of lower than 50 were classified as intolerant accessions. The lowest rate was 10 and it came from accession no. 28, which was collected from Manisa (Aegean region of Turkey).

Discussion

Stem length is a morphological feature. Reducing amount of irrigation water caused decreased stem length. Shorter stem length was observed on grape vine (Eris et al. 1989), apple (Anju et al. 1994), and mandarin (Castel 1994). High stress condition increases root/stem ratio (Kang et al. 1998). Karipcin et al. (2008, 2010) determined that drought stress reduces diameter and length of the stem in watermelon. According to Zhang et al. (2004), supplemental irrigation actually optimizes water-use efficiency by increasing yield of watermelon. Also irrigation caused a change of biomass in watermelon (Xie et al. 2006).

Drought stress significantly decreased shoot dry weight of the melon genotypes in comparison to the control plants. Resistant genotypes could adjust growth performance under drought stress. While the tolerant genotypes had high shoot dry weights, the intolerant genotypes had low shoot dry weights (Table 1).

The drought tolerant melon genotypes showed a higher temperature drought stress than in the control treatment (Table 1). Generally, drought stresses had big effects on melon genotypes. According to the results on shoot fresh and dry weights, melon plant growth is restricted by drought stress. In intolerant genotypes, plant growth was significantly decreased in comparison with the tolerant genotypes. During drought, leaves are subjected to both heat and water-deficiency stresses (Clarke et al. 1993). Leaf temperature increases in all melon genotypes but intolerant melon genotypes had higher leaf temperatures than tolerant genotypes. All of the melon genotypes had increased leaf temperature under drought stress. Mohammadian et al. (2001) suggested that, under drought conditions, sugar beet leaves wilt in response to water deficiency and tend to lay flat on the soil and thus increase the effective area exposed to the sun, therefore, reducing transpiration rates of such leaves, and leaf temperature increases.

Conclusion

By analyzing 14 drought-related features, we attempted to determine the most tolerant melon germplasm in a high-temperature, open field area. We considered 43 of the accessions to be tolerant and 79 intolerant. The 55 other genotypes were classified as tolerant/intolerant.

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Table 1. Accessions used in this study, each with its drought tolerance index (DT).

Tolerant				Moderate				Intolerant			
accession number	points	accession number	points	accession number	points	accession number	points	accession number	points	accession number	points
41	100	8	79	80	64	233	51	24	49	269	33
106	100	12	79	185	64	240	51	37	49	4	32
183	100	51	79	195	64	261	51	64	49	13	32
248	100	53	79	10	63	267	51	90	48	26	32
188	99	72	79	96	62	6	50	149	44	48	32
210	99	84	79	124	62	11	50	262	44	67	32
228	99	177	79	220	62	19	50	191	43	107	32
242	99	250	79	237	62	33	50	223	42	209	32
257	99	254	79	36	61	252	50	42	41	221	32
1	98	170	78	52	61			125	41	244	32
29	98	175	78	55	61			281	38	275	32
69	98	192	78	62	61			77	37	16	31
215	98	225	78	97	61			263	37	60	31
253	98	40	77	184	61			25	36	79	31
284	98	249	77	190	61			43	36	105	31
22	97	171	75	194	61			15	35	127	31
187	97	214	75	216	61			32	35	247	31
7	95	286	75	219	61			35	35	260	31
270	94	231	65	236	61			54	35	266	31
287	94	245	65	239	61			83	35	47	30
264	84	282	65	102	60			88	35	57	30
3	82			130	60			131	35	169	30
23	81			229	60			2	34	255	30
39	81			268	60			21	34	256	29
46	81			45	59			38	34	272	29
68	81			66	59			44	34	274	29
238	81			246	56			85	34	276	29
273	81			213	54			180	34	277	19
289	81			50	53			197	34	27	17
294	81			157	53			204	34	30	17
61	80			9	52			211	34	174	15
173	80			18	52			212	34	198	15
186	80			243	52			234	34	20	13
217	80			285	52			65	33	70	12
226	80			290	52			159	33	63	11
232	80			56	51			178	33	28	10
265	80			59	51			179	33		
271	80			73	51			208	33		
278	80			83-1	51			218	33		
279	80			99	51			230	33		
283	80			116	51			235	33		
288	80			138	51			241	33		

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Evaluation of Genetic Relationships Among Hungarian and Turkish Watermelon (*Citrullus lanatus* var. *lanatus*) Accessions by SSR Markers

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Abstract. Molecular diversity and genetic relationships among Hungarian and Turkish watermelon accessions were evaluated using SSR markers. Thirty watermelon accessions of which 19 were from Hungary and 11 from Turkey were assessed by 11 SSR primers. SSR primers generated a total of 29 alleles of which 28 were polymorphic. The sizes of the bands ranged from 102 (CMCTN19) to 230 (Cgb5009) bp. The genetic similarity ranged from 0.32 to 1.00. Accessions G 34 and G 41 (*Citrullus lanatus* var. *citroides* (Bailey) Mansf.) showed the lowest similarity rate (0.32) while some accessions could not be separated from each other even though they had remarkable phenotypic differences. A clustering dendrogram based on the genetic similarity matrix was produced by NTSYS-PC. The investigated accessions were divided into two main groups (I and II) and an unclustered additional single accession (Kar 334). Within group I, three subclusters (SC) were defined. Group II included two citron (*C. lanatus* var. *citroides*) accessions. Despite the quite different morphological characteristics of accessions G 14 and Kar 216, they appear to be the most closely related (95% similarity) accessions in considering genetic relationships of the two countries.

Keywords: Genetic diversity, molecular markers, SSR, Cucurbitaceae

Introduction

Watermelon (*Citrullus lanatus* var. *lanatus*) is a member of the Cucurbitaceae and belongs to the genus *Citrullus*. It is considered as a popular vegetable crop having economic value and grown in many areas of the world.

Genetic diversity analysis facilitates utilization of genetic resources for conservation of germplasm, association genetics, and development of breeding programmes (Hu et al. 2015). Molecular markers have significantly contributed to understanding the genetic diversity and relatedness in many crops (Nantoume et al. 2013). They are not affected by environmental factors and developmental stages of the plant (Esposito et al. 2007, Wang et al. 2015).

Several studies indicate the ability of molecular markers to detect polymorphisms across diverse genotypes of watermelons. However, these studies revealed low levels of DNA polymorphism among cultivated watermelons, but high genetic diversity among the *Citrullus* subspecies (Zhang et al. 2012, Levi et al. 2013). Simple sequence repeats (SSRs) have proven to be the most useful marker system in diversity studies (Aka Kacar et al. 2012, Sheng et al. 2012, Zhang et al. 2012, Nantoume et al. 2013, Wang et al. 2015).

Although Turkey is not the origin of watermelon, a wide range of diversity has been observed among its watermelon genetic resources (Sari et al. 2007, Solmaz and Sari 2009). Based on the old Hungarian references about melon and watermelon production, it is believed that during the times of the Ottoman Empire the Turks carried watermelon cultivars into Hungary (Szamosi et al. 2008).

The aim of the present study was to (1) characterize and compare the molecular diversity of the most typical Hungarian and Turkish watermelon accessions and (2) to evaluate whether there is a close genetic relationship among the landraces of the two countries.

Materials & Methods

This study was carried out at the Department of Horticulture, Faculty of Agriculture, University of Cukurova. A total of 30 watermelon accessions were studied. The majority of the Hungarian seed materials (19 watermelon accessions) were collected

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from different regions of Hungary or obtained from the Gene Bank of Corvinus University of Budapest, Department of Genetics and Breeding. Eight watermelon accessions were mainly collected from various geographical regions of Turkey, seeds of 'Charleston Grey' and 'Calhoun Grey' were obtained from Seminis (USA), and *Praecitrullus fistulosus* seeds were provided by the USDA (USA). The origins of the investigated accessions are presented in Table 1.

Seeds of the measured accessions were sown in plastic multipots consisting of 45 pots (4 × 4 × 4 cm), containing perlite and peat mixture (1 : 2 v/v) under glasshouse conditions. Young leaves were collected at the second or third true leaf stage from nine seedlings of each genotype and were stored immediately frozen in liquid nitrogen and stored at -80 °C. Total genomic DNA was extracted from leaf tissue by modifying the procedure of Edwards at al. (1991). DNA concentrations were assessed by Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) and gel electrophoresis. DNA was diluted in water to a final concentration of 50 ng/µl and stored at -20 °C.

Eleven SSR primers (Cgb4765, Cgb4767, ASUW2, ASUW13, ASUW19, Cgb5009, C.I. 1-06, C.I. 1-20, C.I. 2-23, C.I. 2-140, CMCTN19; Table 2) were used in this study based on their successful amplification ability reported in previous studies (Katzir et al. 1996, Levi et al. 2006). PCR amplifications were carried out in 20 µl final reaction volumes each containing 5 µl (5 ng) DNA, 8 µl 2X PCR Master Mix (Fermentas), 5 µl ddH_2O, 0.5 µl $MgCl_2$ (Fermentas), 0.05 µl Taq polymerase (Fermentas), 0.5 µl M13 primer, and 1 µl forward and reverse primers. The DNA amplifications were carried out in a thermocycler (Eppendorf Mastercyler Gradient). The mixture was initially denatured at 94 °C for 5 min; followed by 35 cycles at 94 °C for 1 min; 55 to 60 °C for 30 s; 72 °C for 1 min; and final extension step at 72 °C for 4 min. PCR products were stored at 4 °C before analysis.

After amplification, 1 to 25 µl of loading buffer were added to each reaction tube containing 95% formamide, 10 mM EDTA (pH 8.0), 0.025% of xylene cyanol, and 0.025% of bromophenol blue. The samples were heat denatured for 5 min in 95 °C and quickly cooled on ice. After loading 1.0 µl of each sample, PCR products were separated in a 25 cm, 8% denaturing polyacrylamide gel (Long Ranger, FMC Biozym, Hessisch Oldendorf, Germany) that had been preheated for 30 min. Electrophoresis

Table 1. Sources of seeds and flesh colour of the investigated watermelon accessions.

Code	Accession	Origin	Flesh colour
G 1	Sándor Pál	Hungary	red
G 2	Marsowszky	Hungary	red
G 3	Hevesi feketemagvú	Hungary	red
G 4	Korai Kincs	Hungary	red
G 5	Kecskeméti Vöröshúsú	Hungary	red
G 7	Szász Zoltán B	Hungary	red
G 8	Nyírbátori A	Hungary	red
G 12	Gyulavári	Hungary	orange
G 13	Szentkirályi Óriás	Hungary	yellow
G 14	Téli görög	Hungary	orange
G 15	Szőkehajú	Hungary	yellow
G 16	Kömörői	Hungary	red
G 29	Unknown1	Hungary	yellow
G 31	Unknown2	Hungary	yellow
G 34	Unknown3	Hungary	yellow
G 36	Királyhalmi	Hungary	yellow
G 37	Barátmagvú	Hungary	yellow
G 39	RCAT036099	Hungary	cream
G 41	RCAT055816 (Újszilvási)	Hungary	cream
KAR 23	Diyarbakır Tat Karpuzu	Turkey (Urfa)	red
KAR 37	Halep Karası	Turkey (Adana)	red
KAR 178	Komando karpuzu	Turkey (Manisa)	red
KAR 216	Kore karpuzu	Turkey (Çanakkale)	red
KAR 229	Unknown	Turkey	red
KAR 233	Calhoun grey	USA	red
KAR 235	Charleston grey	USA	red
KAR 242	Unknown	Turkey (Hatay)	red
KAR 247	Ala çeşidi	Turkey (Aksaray)	red
KAR 249	Unknown	Turkey (Şereflikoçhisar)	red
KAR 334	*Praecitrullus fistulosus*	USDA	white

was conducted in 1.0 Long Ranger TBE buffer at 1500 V, 50 W, 35 mA, and 48 °C using a Li-Cor DNA Analyzer 4300 (LiCor Biosciences, Bad Homburg, Germany). A 50 to 350 bp DNA ladder mix (MWG Biotech AG, Ebersberg, Germany) was run alongside the amplified PCR products to determine DNA sizes.

Reproducible DNA bands were scored in a binary mode with 1 indicating the presence and 0 indicating the absence of a band. The unweighted pair-group method using the arithmetic average clustering procedure (UPGMA) was employed to construct the clustering dendrogram based on the genetic distance matrix using NTSYS-PC program (version 2.02i) (Rohlf 1998). The representativeness of dendrogram was evaluated by estimating cophenetic correlation for the dendrogram and comparing it with the similarity matrix, using Mantel's matrix correspondence test (Mantel 1967). The result of this test is a cophenetic correlation coefficient, r, indicating how well dendrogram represents similarity data.

Results & Discussion

Eleven SSR primers produced a total of 29 bands across the 30 watermelon accessions, of which 28 were polymorphic. The polymorphism rate was found to be 96.6%. The sizes of the allele ranged from 102 (CMCTN19) to 230 (Cgb5009) bp. The primers code, their sequences, size-range of the amplified products, and their polymorphism rates are presented in Table 2. Primers Cgb4767 and C.I. 1-20 gave the biggest number (4) of polymorphic alleles. The pair-wise genetic distances (GD) for the 30 watermelon accessions, based on 11 SSR markers that gave amplification, ranged from 0.32 to 1.00. Accessions G34 and G41 (*C. lanatus* var. *citroides* (Bailey) Mansf.) showed the biggest genetic distance (0.32). Contrary to the remarkable phenotypic differences, we were not able to separate some accessions (G1-G31, G2-G3, G4-G15-G37, and G29-G36) from each other with the used SSR primers.

The clustering dendrogram showing the genetic relationships among the 30 investigated watermelon accessions, based on the SSR markers, is presented in Figure 1. The Mantel test's cophenetic correlation coefficient indicated that the dendrogram was a very good (r = 0.94) representation of the similarity matrix.

The investigated accessions were divided into two main groups (I and II) and an unclustered additional single genotype (Kar334). This separate clustering of the *P. fistulosus* genotype is comparable to the previous report on phylogenetic relationships of *P. fistulosus* and *C. lanatus* var. *lanatus* based on RAPD and ISSR markers (Levi et al. 2005).

Table 2. SSR primers used for the watermelon accessions, their sequences, size-range of the amplified products and their polymorphism.

SSR Primers	Primer Sequence	Allele size range (bp)	Total allele number	Polymorphic allele number	Polymorphism (%)
Cgb4765	F: TTCTCTTCATTCCCCCAAAATC R: RACGGGTGAGGGAAAACGAG	175-200	3	3	100
Cgb4767	F: GAGAGGGAAAGAAAAGAGGAGAG R: AACGGAGGATGATGATTTGGTA	180-204	4	4	100
ASUW2	F: GCTTCGTTGTTGCTGCCGTTG R: GCATAAAATCACACTCAAAC	185-190	2	2	100
ASUW13	F: CTAGAGAAACCCCATC R: CTCCACTCACATACACAG	125	1	0	0
ASUW19	F: GTGTGTTTTTGCGTGTG R: GGGCAAATCCAATAATCCAG	170-190	3	3	100
Cgb5009	F: CAGTGGCACCGTCATCTAAAG R: AGTGGGGGATTCTCTTCCTAAG	195-230	3	3	100
CI. 1-06	F: CACCCTCCTCCAGTTGTCATTCG R: AAGGTCAGCAAAGCGGCATAGG	135-145	2	2	100
C.I. 1-20	F: CGCGCGTGAGGACCCTATA R: AGCAATTGATTGAGGCGGTTCT	163-190	4	4	100
C.I. 2-23	F: GAGGCGGAGGAGTTGAGAG R: ACAAAACAACGAAACCCATAGC	204-212	3	3	100
C.I. 2-140	F: CTTTTTCTTCTGATTTGACTGG R: ACTGTTTATCCCGACTTCACTA	202-215	2	2	100
CMCTN19	F: GAATGATTGGAGCAACCAGT R: GCTTTTTGAATTTGTGCAGGG	102-110	2	2	100
TOTAL			29	28	96.6

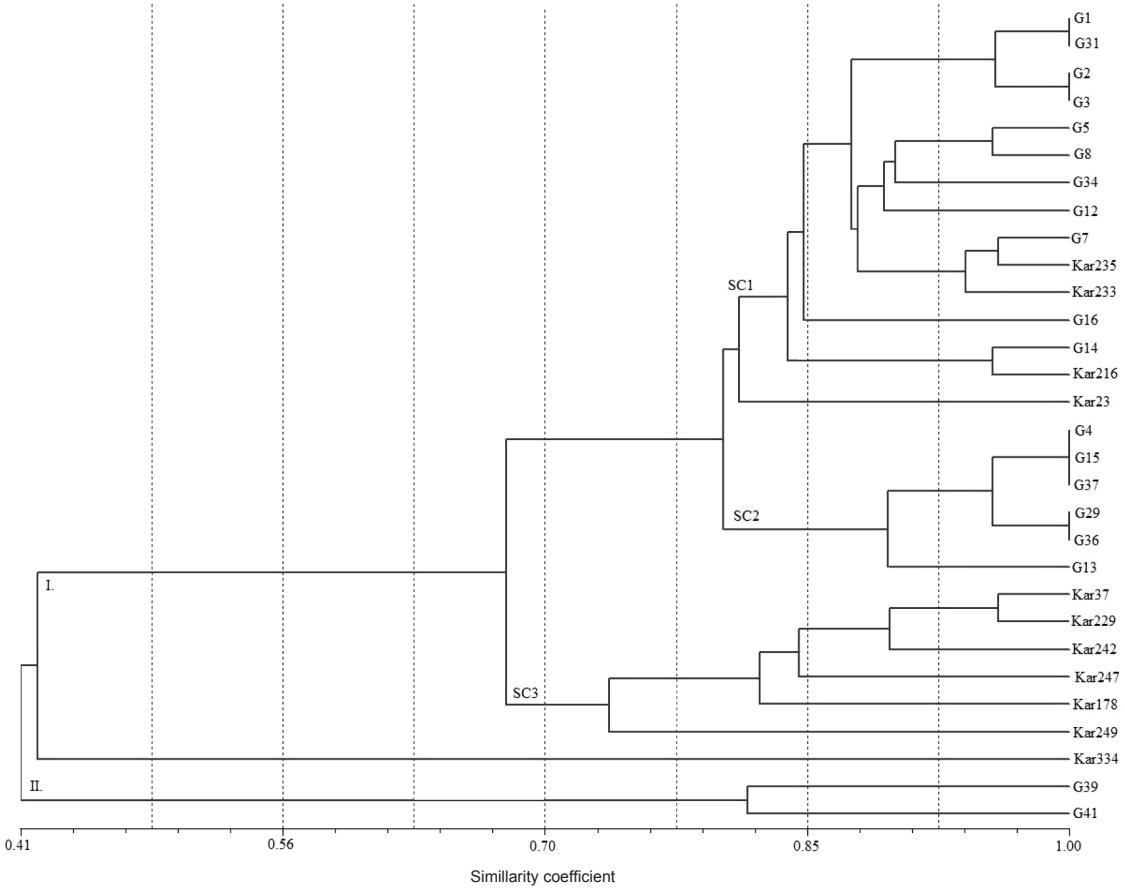

Figure 1. UPGMA dendrogram showing the genetic relationships among the 30 investigated watermelon accessions, based on the SSR markers.

Within group I, three sub-clusters (SC) were defined. SC1 was formed mainly by Hungarian accessions. However this is the only sub-cluster that includes Turkish or American reference genotypes as well. Accession G7 showed 96% similarity to Kar235. Considering the close similarities in shape and other morphological parameters, we can state that G7 is a slightly modified genotype of 'Charleston Gray'. Due to the open-pollinated nature of watermelon, different genotypes may occur during seed regeneration or cultivation. The findings of Levi et al. (2001) are comparable with our results. They compared the RAPD patterns of 'Charleston Gray' plants from two different seed sources, and the obtained patterns proved that the two types were similar but not identical.

Despite the quite different morphological characteristics of accessions G14 and Kar216, they appear to be the most closely related (95% similarity) genotypes considering genetic relationships of the two countries. Kar23 also showed similarity with the Hungarian accessions. Until the middle of the 16th century, only yellow-fleshed watermelons were grown in Hungary. Red-fleshed genotypes started to spread within the country from the middle of the century (Takáts 1917) during the time of the Ottoman Empire. Therefore it is interesting that both Turkish accessions (Kar216 and Kar23) which showed genetic similarities to the Hungarian watermelon genotypes are red-fleshed.

The second sub-cluster (SC2) consisted of only Hungarian accessions, while SC3 included exclusively Turkish watermelon accessions. The generally separate clustering results of the accessions of the two countries also supported the genetic relationship among the above-mentioned genotypes clustered together in SC1.

Group II includes the two preserving melon (*C. lanatus* var. *citroides*) genotypes. Although preserving melons were represented only with two accessions (G39 and G42), the separate clustering with high genetic distance from *C. lanatus* genotypes is consistent with the results obtained in previous studies (Jarret et al. 1997, Levi et al. 2001).

The data show that the investigated germplasm contains high genetic variability. The dendrogram based on the genetic distance matrix produced by the NTSYS-PC program is reflective of genetic relationships among the watermelon accessions of the two countries. Turkish accessions (Kar216 and Kar23) that showed close genetic similarities to the Hungarian watermelon genotypes are red-fleshed, which fits with the historical record that until the middle of the 16th century only yellow-fleshed watermelons were grown in Hungary and red-fleshed genotypes started to spread within the country during the time of the Ottoman Empire.

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Genome-Wide Association Mapping of Free Glutamic Acid Content Responsible for Umami Taste in Bottle Gourd

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Abstract. Bottle gourd or calabash (*Lagenaria siceraria*) (2n = 2x = 22), also known as opo squash or long melon or white-flowered gourd, is an important cucurbit crop worldwide. Bottle gourd is commonly used as a vegetable in many regions of Asia and Africa. Umami taste plays an important role in the flavor of bottle gourd fruits, usually determining consumers' choice and acceptance. However, the compound(s) responsible for umami taste in bottle gourd are barely known and the genes conferring umami taste remain unidentified. In the current study, free amino acid (FAA) concentrations were determined by ion chromatography among 139 bottle gourd accessions. By comparing the FAA concentrations among four accessions with high umami content and five accessions with low umami content, we found that free glutamic acid (Glu) was most likely the key compound determining umami taste in bottle gourd. The 139 accessions were then genotyped with the restriction site-associated DNA sequencing (RAD-seq) technology. Genome-wide association applied to free glutamic acid content was performed with 19,226 single nucleotide polymorphism markers (SNPs), leading to the identification of nine significant SNPs that were reproducible over two years. These SNPs were distributed on four chromosomes of the bottle gourd genome and each accounted for 6% to 14% of the pheotypic variation. These results advance our knowledge on the determining factors of umami taste in bottle gourd and will facilitate molecular breeding of flavor-improved bottle gourd cultivars.

Keywords: Bottle gourd, free amino acid, umami taste, genome-wide association mapping

Introduction

Umami is the meaty, mouth-filling, rich taste found in various types of food. It is the fifth basic taste quality that humans can detect, in addition to sweet, salt, bitter, and sour. Since Ikeda first discovered monosodium glutamate (MSG) as the prototypic umami stimulus (Ikeda 1908), a set of small molecules that can elicit umami taste have been identified, including amino acids (glutamate and aspartate), nucleotides (monophosphates of inosinate or guanylate, inosine 5'-monophosphate, and guanosine-5'-monophosphate), short peptides, and organic acids (Kuninaka 1960, Kurihara 2009). Among these umami compounds, glutamic acid is well recognized to cause umami taste. Most foods with high free glutamate content such as cheese, tomato, and mushrooms, have been found showing a strong umami taste in cooking. Thus to a certain extent, the free glutamate content could provide the characteristic umami taste of the food.

Bottle gourd or calabash [*Lagenaria siceraria* (Mol.) Standl.] (2n = 2x = 22), also known as opo squash or long melon or white-flowered gourd, is a diploid species of the Cucurbitaceae (Beevy and Kuriachan 1996). As one of the first domesticated plant species, bottle gourd is believed to have originated from Africa, or could have been independently domesticated in Africa and Asia, and is cultivated today all over the tropics (Heiser 1979, 1989, Erickson et al. 2005). Fresh young fruits of bottle gourd are a common vegetable in many regions of Asia and Africa (Morimoto and Mvere 2004); mature dry fruits can be used as containers, pipes, floats, music instruments, for medicine, artistic endeavors, or even clothes accessories (Heiser 1979). Bottle gourd is also widely used as a rootstock for watermelon to defend against soil-borne diseases and low soil temperatures (Lee 1994, Yetisir and Sari 2003). China has a long history of cultivating bottle gourd and it has been one of the most common and popular gourd vegetables for the people of southern China. Umami taste of bottle gourd is one of the most important criteria determining consumer choice and acceptance. Thus a key goal in the breeding program of bottle gourd is increasing the umami taste intensity of cultivars.

Unlike horticultural traits such as flowering time and yield, umami taste is difficult to measure and the results given by different tasters vary highly. Fortunately, molecular-marker-assisted breeding approaches that are based on the tight linkage between molecular markers and target traits have been successfully implemented in the selection of complex traits whose phenotype

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is difficult to evaluate. In the current study, we aim to use the whole genome-wide association to identify the genomic regions or markers associated with the free amino acid concentrations which elicit umami taste, focusing on free glutamic acid. And the detected markers will be used for umami-taste breeding in bottle gourd.

Materials & Methods

Plant materials

A population consisting of 135 bottle gourd accessions from across China and four accessions from the U.S.D.A./A.R.S. (United States Department of Agriculture, Agricultural Research Service) were used in the current study. All the accessions were inbred lines through multi-generation self-pollination.

Umami taste testing

Nine accessions including landraces and cultivars from China were tasted and compared for umami flavor. Marketable fruits, 8 to 12 days after pollination, were harvested, fried or cooked. Ten tasters, 20 to 60 years of age, male and female, were requested to taste the cooked food flavor. The umami taste was recorded on a scale of 1 to 10, and each taster scored independently.

RAD sequencing and SNP calling

Genomic DNA of the 139 bottle gourd accessions was extracted from young leaves of two-week-old seedlings using a DNA extraction kit (TIANGEN Co. Ltd, Beijing), following the manufacturer's instructions. The RAD library was constructed according to the protocol described by Baird et al. (2008), *Eco*RI and *NIa*III were used to cut the DNA. Sequencing was carried out using the Illumina NGS platform HiSeq2000.

Raw sequence reads without MID barcode sequences were trimmed to 85 nucleotides from the 3' end to ensure more than 90% of the nucleotides have a quality value above Q30 (equals 0.1% sequencing error) and more than 99% above Q20 (equals 1% sequencing error). Reads of low quality, including reads with < 85 bp after trimming or with ambiguous barcodes, were discarded. For SNPs calling, the trimmed reads were clustered into RAD-tags based on sequence similarity using Stacks under default parameters (Catchen et al. 2011). Clustered RAD-tags with very high read depth (> 500) were excluded. SNPs were identified in alignment results, and regarded as true polymorphisms when each allele was observed at least three times.

Sample preparation and free amino acid analysis

The accessions were evaluated in the field-houses of the Haining Experiment Station (30 °N, 120 °E) in the autumn of 2014 and 2015. Ten individuals of each accession were grown in 30-m rows spaced 0.5 m apart. When the fruits were 8 to 12 days after pollination, the prime stage for marketing and eating, three gourds from each accession were harvested. The intermediate sections of the three fruits (about 50 to 80 g for each gourd) were cut and blended into a homogenate. The prepared samples were stored at -80 °C for further analysis.

Free amino acids were extracted from 1.5 g of the homogenates with 3% sulfosalicylic acid in a constant volume to 8 ml by vigorous shaking for 1 h. The suspension was then centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was filtered with 0.45 μm syringe filters and then used to measure the free amino acid concentrations on an automatic amino acid analyzer (L-8900; Hitachi, Tokyo, Japan).

Association mapping

The SNP genotypes of each accession were used for association mapping. A mixed linear model (MLM) module implemented in TASSEL 5.2.19 with the effects of principle component analysis (PCA) and kinship (K) both taken into account was used for genome-wide association mapping of free amino acid concentrations responsible for umami taste.

Results & Discussion

Free glutamic acid content is the main component for umami taste in bottle gourd

Based on the flavor assessment of nine accessions from the ten tasters, four lines showed little umami taste and five lines showed strong umami taste. According to the free amino acid concentrations of the nine accessions, the free glutamic acid concentration was found to have a significant association with umami taste. These results indicated that the free glutamic acid concentration might play a key role in the umami taste of bottle gourd.

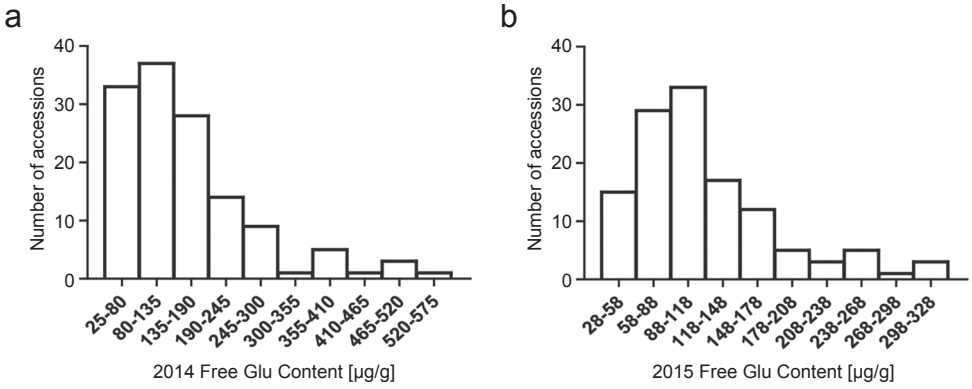

Figure 1. The free glutamic acid concentration distribution among bottle gourd accessions, assessed in 2014 (a) and 2015 (b).

Assessment of free glutamic acid concentration

Free glutamic acid concentration was determined over two growing seasons, including autumn 2014 and autumn 2015. The free glutamic acid concentration (μg/g) showed high variation (Figure 1), ranging from 25.14 to 582.63 with an average of 155.67 in 2014 and from 28.02 to 328.07 with an average of 117.39 in 2015. The free glutamic acid concentration for single accession also showed large fluctuations in different environments, and the correlation coefficient between the two seasons was low. This indicated that the free glutamic acid concentration was greatly affected by the environment.

Genome-wide marker-trait associations

Based on the RAD-Seq data, 19,226 SNPs were originally detected in the sample set. Of these, 442 SNPs showed an allele frequency value of 0 in all 139 accessions, and 8,360 SNPs were found to be monomorphic in the population, which were removed from further analyses. Then 10,424 polymorphic SNPs in the population were further filtered, and the filter criteria were: SNP with < 20% missing data, and the lower allele frequency value should be over 0.05. Finally, 8,180 SNPs were used for association mapping. Under the MLM model, nine loci were both detected in the two years of experiments as significantly ($P < 0.01$) associated with the free glutamic acid concentration. These SNPs were distributed on four chromosomes of the bottle gourd genome and each accounted for 6% to 14% of the phenotypic variation. These results revealed the genetic architecture of the free amino acid concentration responsible for umami taste in bottle gourd and the association markers will aid in the breeding of flavor-improved bottle gourd cultivars.

Acknowledgements

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Use of Wild *Cucumis* as Potential New Rootstocks for Melons

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Abstract. Grafting is a common practice in cucurbits. However, in melon, the use of common commercial rootstocks of the *Cucurbita* genus is hampered by the occurrence of negative impacts on fruit quality. The genus *Cucumis* is a variable resource of new rootstocks. However, wild *Cucumis* species are difficult to handle in grafting assays due to their thin hypocotyls. One way of increasing hypocotyl diameter is performing interspecific crosses. Interspecific hybrids have also the advantage of combining resistances coming from the two parents. There exist severe crossability barriers within the genus, but some hybrid combinations are viable. We have successfully obtained interspecific hybrids between *Cucumis ficifolius* and *C. anguria*. Their hybrid nature has been proven according to taxonomic traits. These hybrids are more vigorous and have higher germination rates in different conditions than their corresponding parents. These interspecific hybrids are being characterized for resistance to biotic stresses. Also, grafting compatibility of the hybrids has been assayed as a first step to evaluate their potential as new rootstocks for melon.

Keywords: Hybrids, rootstock, resistance, *Cucumis,* melon

Introduction

Grafting is a cultural practice used to avoid soil stress. Intensive agriculture and global warming are increasing soil biotic and abiotic problems, such as fungal diseases, nematodes, drought, salinity, and extreme temperatures. Grafting was used in cucurbits for the first time in Asia, but nowadays is a very common technique spread worldwide (King et al. 2008). It is routinely used for watermelon to increase yields even under Fusarium wilt pressure. This crop is grafted onto various rootstock species, but the most common are interspecific hybrids between *Cucurbita maxima* Duchesne and *C. moschata* Duchesne (Davis et al. 2008). These hybrids have two main shortcomings, they are not resistant to nematodes and often cause negative impacts on fruit quality (Cohen et al. 2014, Soteriou et al. 2014).

In melon (*Cucumis melo* L.), the use of grafting has been delayed, but is becoming necessary with the increase of soilborne problems. The *C. maxima* × *C. moschata* hybrids have been the most common rootstock choice, even when the negative impact of these hybrids on melon quality is more significant than the effect on watermelons (variable rind netting, reduced sugar content, altered flesh color, aroma, etc.) (Rouphael et al. 2010, Gisbert et al. 2016). The assessment of new germplasm that provides a wider range of resistances without decreasing quality is a new challenge for grafting technology of melon crops.

One of the best alternatives to the *Cucurbita* hybrids is the use of intrageneric or intraspecific variability. This strategy is being used in watermelon, using *Citrullus* germplasm (Cohen et al. 2014). Within *C. melo* L., there are few resources resistant to soil stress. However, useful resistances have been identified in other species within *Cucumis*. For example, *C. metuliferus* E. Mey. ex Naudin and *C. pustulatus* Naudin ex Hook.f. are resistant to nematodes (Siguenza et al. 2005, Liu et al. 2015). Also *C. metuliferus, C. ficifolius* A. Rich, and *C. anguria* L. were reported as resistant to Fusarium wilt and *Meloidogyne incognita* (Nisini et al. 2002, Matsumoto 2012, Liu et al. 2015).

These wild *Cucumis* spp. resources could be an alternative to *Cucurbita* hybrids as rootstocks for melon crops. However, the plants of wild *Cucumis* develop small seedlings with very thin hypocotyls. Size differences with the melon scions present a difficulty for the grafting process. Interspecific hybridization could be a strategy to improve hypocotyl vigor and diameter of wild *Cucumis* and at the same time could lead to combined resistances to soil stresses. Attempts to obtain *Cucumis* interspecific hybrids have been done, but strong reproductive barriers exist in most combinations, although they can be overcome in some specific crosses (Kho et al. 1980, Weng 2010, Matsumoto et al. 2012). We generated some interspecific crosses between selected accessions of wild *Cucumis* and started evaluating their possible efficacy as new rootstocks for melon.

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Materials & Methods

Interspecific crosses

The assays were conducted in greenhouses at COMAV-UPV (Universitat Politècnica de València). During the spring-summer season, five plants of each of the following wild *Cucumis* species were grown in the greenhouse: *C. metuliferus, C. ficifolius, C. myriocarpus* Naudin*, C. zeyhery* Sond.*,* and *C. anguria.* All possible cross combinations were attempted to produce interspecific hybrids, using each species as male and female parent. Fruit set was observed after pollination and the fruits were collected when mature. Seeds were extracted from the fruits and cleaned. Germination rate was registered for all genotypes.

The hybrid *C. ficifolius* × *C. anguria* (F × A) was the one for which we obtained the highest number of seeds and on which we focused phenotypic characterization, the study of grafting ability, and the evaluation of resistance to pathogens.

Phenotypic characterization of F_1 hybrids

Seeds of the F × A hybrid and their corresponding parents were germinated in Petri dishes and then sown in plastic pots (6 × 6 cm) with substrate and vermiculite (2 : 1) in the greenhouse. Hypocotyl length and diameter, plant length, and number of true leaves were measured in five plants per genotype. Also, botanical identification keys were used to confirm the hybrid nature of the characterized plants (Kirkbride 1993, Schaefer 2007). We focused on the most discriminant characters between both parents, such as the presence of aculei in the stems of *C. ficifolius*, while stems of *C. anguria* are only hispidulous, and the ratio between the length of the hyaline part of the aculei of the ovary and the length of the opaque part, which is less than one in *C. anguria* (especially for var. *longaculeatus* J.H. Kirkbride, to which the plants used here as parents belong), and more than one in *C. ficifolius*.

Grafting assay

'Charentais' melon (*C. melo* L. var. *cantalupensis* Naudin) was grafted onto the F × A hybrid to determine its grafting compatibility and value as a rootstock. Grafting was performed at two stages of development of scion and rootstocks: (a) early stage: using young rootstock seedlings with developed cotyledons and scion seedlings just after germination; (b) mature stage: using rootstock plants with five true leaves and scion seedlings with developed cotyledons. For both procedures, grafting was performed above the cotyledons using the cleft method.

Resistance to pathogens

F_1 hybrids (F × A) and parents were tested for resistance to Fusarium wilt (using a culture of *F. oxysporum* f. sp. *melonis* race 1, 2 for artificial inoculation). The resistance to the fungus was evaluated at 30 days after inoculation (DAI). Also, the level of resistance/susceptibility against important viruses was assessed through an artificial inoculation assay. *Watermelon mosaic virus* (WMV), *Zucchini mosaic virus* (ZYMV) and *Tomato leaf curl New Delhi virus* (ToLCNDV) were inoculated and visual evaluation was performed at 30 DAI (López et al. 2015).

Results & Discussion

Interspecific crosses

The use of wild *Cucumis* species in melon breeding has been hampered by the occurrence of severe reproductive barriers (Walters and Wehner 2002, Sebastian et al. 2010, Matsumoto et al. 2012). However, crosses between some of these wild species are viable (Chen and Adelberg 2000). We report fruit set from the following crosses: *C. ficifolius* × C. *anguria* (F × A)*, C. ficifolius* × *C. myriocarpus* (F × My), and *C. ficifolius* × *C. metuliferus* (F × M), always using *C. ficifolius* as the female parent. The germination rate was variable in the three different hybrids. High germination rates were obtained from seeds of F × A and F × My, and the parent species *C. ficifolius, C. myriocarpus, C. anguria*, whereas no germination was observed in F × M seeds (Caceres et al. 2016). These seeds were analyzed in detail and they presented no or poor embryo formation

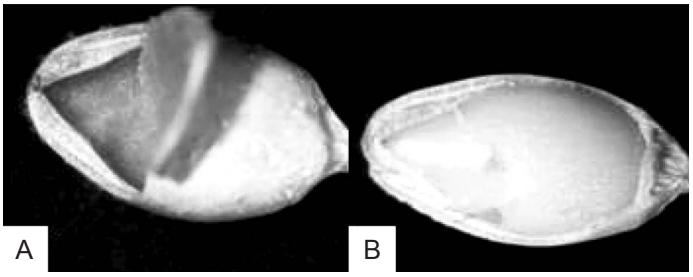

Figure 1. Empty seed from the hybrid *C. ficifolius* × *C. metuliferus* (A), viable embryo from the hybrid *C. ficifolius* × *C. anguria* (B).

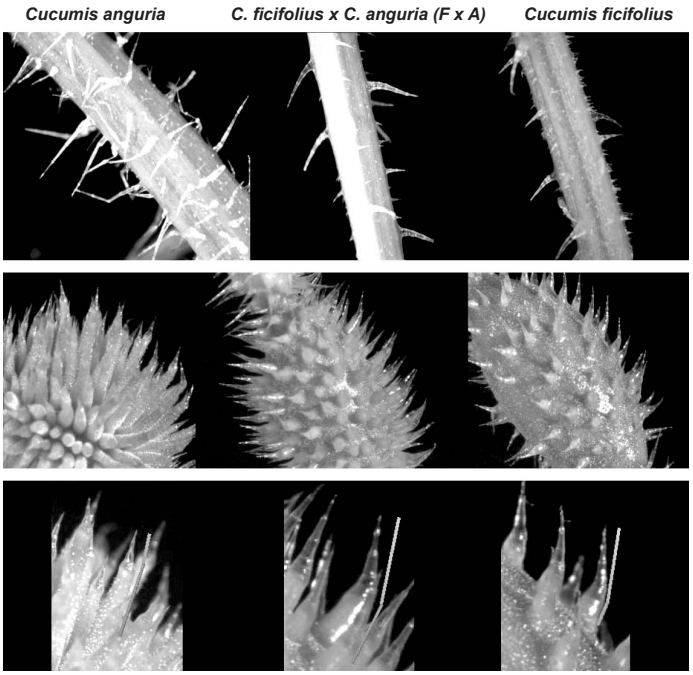

Figure 2. Taxonomic characters of *Cucumis anguria*, *C. ficifolius* and the hybrid *C. ficifolius* × *C. anguria* (F × A); Top: Presence of simple hairs on the stems of *C. anguria*, while F × A and *C. ficifolius* have aculei. Middle; Shape and presence of aculei on ovaries of both parents and the intermediate F × A; Bottom: detail of aculei. The blue bar represents the hyaline part and the red bar the opaque part.

(Figure 1). Singh and Yadava (1984) grouped *C. ficifolius*, *C. anguria,* and *C. myriocarpus* in the same compatibility group, whereas *C. metuliferus* was classified in a different group, less compatible with the others. Consistent with this classification and with our results, Kho et al. (1980) obtained seeded and unseeded fruits from *C. ficifolius* × *C. anguria* and *C. ficifolius* × *C. metuliferus*, respectively. These authors did not obtain fruit set in *C. ficifolius* × *C. myriocarpus.* The discrepancies with the results of the cross involving *C. myriocarpus* could be explained by the variation among different accessions.

Characterization of the hybrid C. ficifolius × C. anguria

The cross F × A was the most compatible, from which the highest number of seeds and the highest germination rate was observed, and then we focused on the characterization of this hybrid.

Taxonomic characterization of the F × A hybrid

Taxonomic traits, such as the presence of aculei in stems and petioles, the ratio between the hyaline and opaque parts of aculei of ovaries, and shape of ovaries were evaluated in the F × A hybrid and in their corresponding parents. Leaf size and shape were not evaluated as they appeared to be polymorphic, as was also indicated in the identification keys (Kirkbride 1993). This characterization showed that the F × A hybrids were morphologically intermediate in the most discriminant characters between parents, as shown in Figure 2. This supports the hybrid nature of these intermediate individuals.

Germination, seedling and plant growth

A good germination ratio is one required property for using the F × A hybrid as a rootstock. Few reports have studied germination in wild *Cucumis*, but some describe problems in germination efficiency and uniformity with *C. myriocarpus* and *C. metuliferus* (Benzioni et al. 1991, Mafeo 2014). In our study, the germination rate of the F × A hybrid was higher than that of the corresponding parentals, 96.3%*, versus* 89.4% and 93.8%, in F × A, *C. ficifolius,* and *C. anguria*, respectively (Cáceres et al. 2016). This effect is likely explained by genotype interactions and hybrid vigor.

Other traits, such as hypocotyl length and diameter, can affect grafting success. An adequate diameter facilitates the rootstock/scion union and a tall hypocotyl avoids the penetration of soil into the join union. Figure 3 shows the hypocotyl length and diameter of F × A and the corresponding parentals, measured at 20 days after germination. Significant differences are found among the three genotypes, but the most vigorous is the parent *C. anguria*, the hybrid having better characteristics than the *C. ficifolius* parent. This result was observed at the very early seedling stage, but the growth of the plants was followed until the adult stage. Figure 4 shows the number of leaves and plant length from 15 days until 60 days after transplanting. Despite *C. anguria* having a better early growth, late growth was more

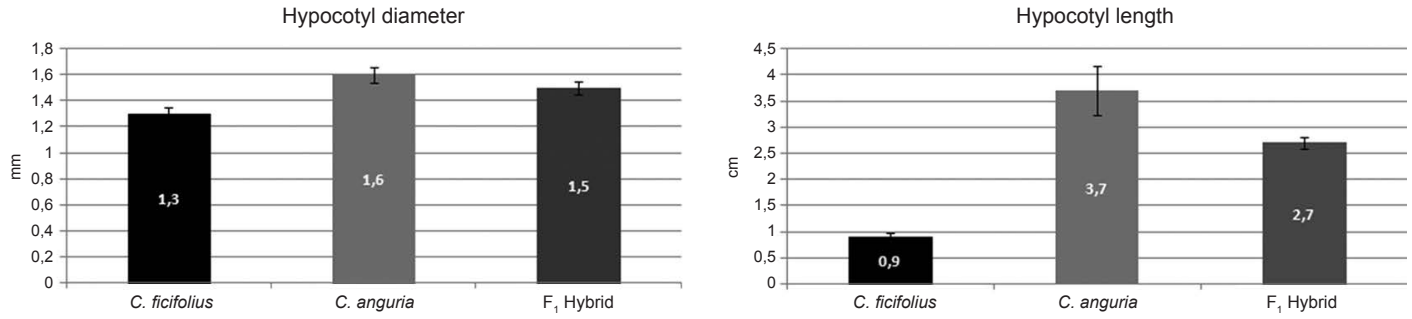

Figure 3. Hypocotyls diameter (left) and length (right), means and standard errors measured in *C. ficifolius, C. anguria*, and their corresponding F_1 hybrid (F × A), 20 days after germination.

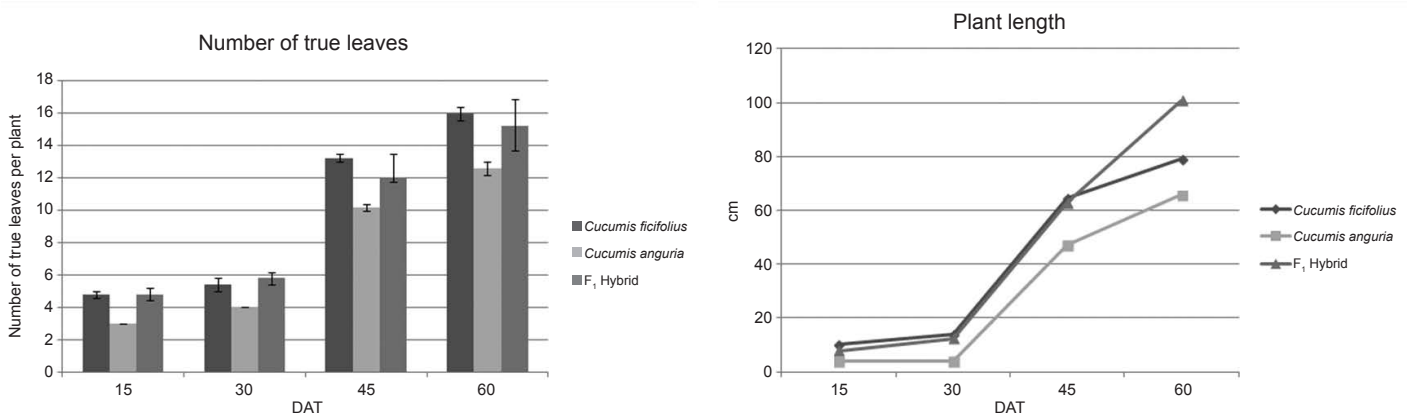

Figure 4. True leaves (left) and plant length (right) measured in *C. ficifolius, C. anguria,* and the F_1 hybrid (F × A) from 15 to 60 days after transplanting (DAT).

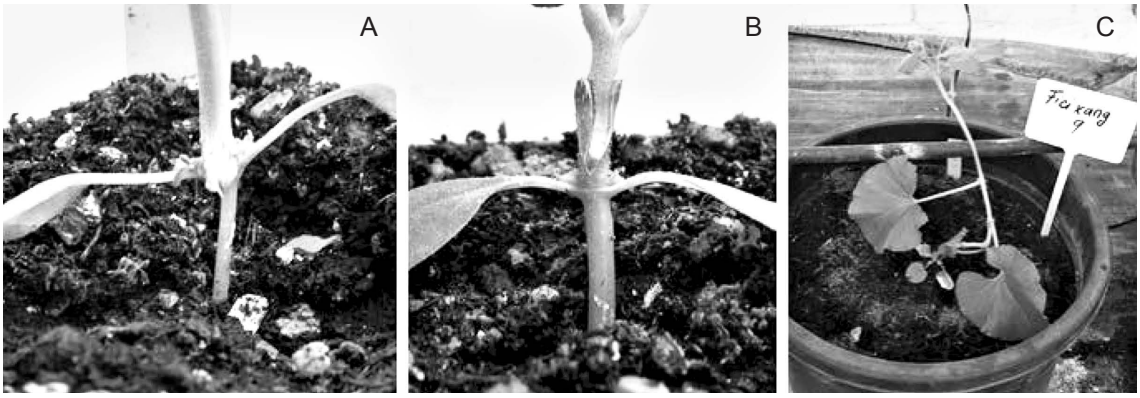

Figure 5. Plants of *C. melo* var. *cantalupensis* grafted onto F × A, 10 days after grafting by the cleft procedure using early grafting (A) or mature grafting (B). The second ones were transplanted for evaluation under greenhouse conditions (C).

vigorous in *C. ficifolius* and even more in the interspecific hybrid, mainly at the end of the assay.

Grafting melon onto the F × A hybrid

Good rootstock/scion union was obtained when melon seedlings were grafted onto F × A seedlings at the two developmental stages (early and mature), however only plants grafted at the mature stage developed properly. In the first procedure, the development of the scion (melon) was faster than that of the rootstock and the rootstock collapsed (Figure 5A). Plants grafted at the mature stage were transplanted to a greenhouse to study their horticultural behavior as compared with ungrafted and self-grafted plants (Figure 5B,C).

Resistance to pathogens

As reported previously, *C. ficifolius* has been observed to be resistant to *Fusarium oxysporum* f. sp. *melonis* race 1,2 (Matsumoto et al. 2011). In our assay, plants from *C. ficifolius*, *C. anguria,* and from the F × A F_1 hybrid did not show symptoms of Fusarium wilt at 30 DPI, whereas the melon control was completely dead by this time. All genotypes assayed, the parents and the F_1, showed mild symptoms of the viruses ZYMV, WMV, and ToLCNDV, whereas the melon control showed severe symptoms of the three viruses. Viral presence was confirmed by western blot and PCR in all the assayed genotypes (López et al. 2015).

In conclusion, we have obtained the hybrids *C. ficifolius* × *C. anguria* and *C. ficifolius* × *C. myriocarpus*, which might be useful as rootstocks for melon. These hybrids are unexploited materials that could be also useful in breeding programs. The characterization of these hybrids was initiated with F × A, confirming its hybrid nature. F × A showed an early growth better than *C. ficifolius* and a late growth better than *C. anguria*. Its high germination rates, good compatibility with melon after grafting, and resistance to pathogens make this hybrid a promising rootstock for melon.

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Evaluation of Organically Produced Melon Cultivars for Powdery and Downy Mildew Severity in Maryland, U.S.A.

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Abstract. The mid-Atlantic region of the U.S.A. has many small diversified organic vegetable farms that grow cucurbit species, including melon (*Cucumis melo*). These farms need information on performance of melon cultivars under organic production practices. A trial was conducted in 2015 at the University of Maryland's Lower Eastern Shore Research and Education Center Farm on organically managed land. Eight melon cultivars, 'Athena', 'Dulce Nectar', 'Escorial', 'Eden's Gem', 'Juane', 'Sivan', 'Snow Mass', and 'Sunbeam', were planted in a randomized complete block design with three replicates. The experiment was conducted under two different tillage systems; a mixed-species cover crop (*Vicia villosa* and *Trifolium incarnatum*) was planted in the fall and either tilled or mown (no-till) in the spring. Powdery mildew (*Podosphaera xanthii*), downy mildew (*Pseudoperonospora cubensis*), and anthracnose (*Colletotrichum orbiculare*) severities were rated on 26 July, 2 and 13 August (tilled section), and 12, 20, and 26 August (no-till section). 'Athena' and 'Dulce Nectar' had the lowest powdery mildew severities in both the tilled and the no-till section ($P = 0.0001$). 'Escorial' and 'Sivan' had the lowest downy mildew severities in both the tilled and no-till section ($P = 0.0001$). No melon cultivar sustained low levels of all diseases. Of the cultivars advertised as resistant to powdery mildew, only 'Athena' and 'Dulce Nectar' had consistently low severity ratings.

Keywords: Pathology, cover crops, no-till, anthracnose, *Colletotrichum orbiculare*

Introduction

Small specialty organic farms are increasing in the mid-Atlantic U.S.A., especially farms that specialize in vegetable production, which have increased 26% since 2008 (USDA 2015). Downy mildew (caused by *Pseudoperonospora cubensis*) and powdery mildew (caused by *Podosphaera xanthii*, syn. *Sphaerotheca fuliginea*) are two of the most economically important and widespread foliar cucurbit diseases (Lebeda and Cohen 2011, Ojiambo and Holmes 2011). The pathogens causing downy and powdery mildew are obligate biotrophs, and disease outbreaks occur annually throughout the mid-Atlantic states of Maryland and Delaware, resulting in defoliation, reduced yield, and reduced sugar content. Anthracnose, caused by *Colletotrichum orbiculare*, is also widespread in the region where it is endemic and causes vine, leaf, and fruit lesions, which reduce marketable yield.

Some disease management practices, such as use of synthetic fungicides, are prohibited in organic production. Host resistance, however, is an allowed management practice. This study evaluated eight melon cultivars of four types for severities of downy mildew, powdery mildew, and anthracnose under two organic production systems (till and no-till).

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Materials & Methods

The experiment was conducted at the University of Maryland's Lower Eastern Shore Research and Education Center, Salisbury. The certified organic field of Fort Mott and Rosedale loamy sand soil was seeded with a hairy vetch (*Vicia villosa*) and crimson clover (*Trifolium incarnatum*) cover crop mixture on October 2, 2014. The experiment was conducted as a randomized complete block design with three replicates on each of two production systems: green manure (till section) and mowed mulch (no-till section). Plots were split among eight melon cultivars: 'Athena' and 'Eden's Gem' (muskmelon types), 'Dulce Nectar' and 'Snow Mass' (honeydew types), 'Escorial' and 'Sivan' (Charentais types), and 'Juane', also known as 'Jaune des Canaries,' and 'Sunbeam' (canary types). Five of the cultivars ('Athena', 'Dulce Nectar', 'Escorial', 'Sivan', and 'Sunbeam') were advertised resistant to powdery mildew. Plots in both production systems consisted of single-row beds, 27 m long with 36 plants on 2.1-m centers and drip irrigation. In the till section, raised beds were covered with 1.25 mil (0.032 mm thick) black plastic mulch on May 27. In the no-till section, in-ground beds were left uncovered. Cultivars were transplanted into the field on 10 June; plants were 61 cm apart in the row. Weeds were managed by rototilling (till section only), mowing (no-till section only), and hand-weeding (both sections). Insects were managed with Entrust (420 $gm \cdot ha^{-1}$), which contains spinosad, applied on June 29, July 29, and August 24, and with

Pyganic (2.24 kg·ha^{-1}), which contains botanical pyrethrum, applied on July 9 and August 6. Melon foliage was evaluated three times in each production system, but evaluation dates were staggered due to differences in plant growth. Foliar data represent the mean disease severity of powdery mildew, downy mildew and anthracnose, for six plants per cultivar per replicate using a 0 to 4 scale, where 0 = no disease, 1 = less than 10% disease, 2 = 10 to 25% disease, 3 = 25 to 50% disease, and 4 = more than 50% disease. In the till section, disease severity was assessed on July 26, August 2, and August 13. In the no-till section, where plant growth was delayed, disease severity was assessed on August 12, 20, and 26. Mature fruit were counted and weighed throughout the season; yield varied significantly by cultivar, production system, and harvest date (data not shown). Disease severity was analyzed using JMP version 10, and means separated using Student's t-test.

Results

Rainfall in June, July, and August was 23, 11, and 10 cm, respectively. Cover crop system (till versus no-till) was a significant factor for all diseases evaluated (anthracnose $P = 0.008$; powdery mildew $P = 0.029$; and downy mildew $P = 0.004$). Melon in the till section had less anthracnose and powdery mildew (Table 1), while melon in the no-till section had less downy mildew (Table 2). Cultivar was also a significant factor for all diseases evaluated ($P \leq 0.001$), but performance varied by disease. 'Athena' and 'Dulce Nectar' had low levels of powdery mildew in both tillage systems (Tables 1,2). 'Escorial' and 'Sivan' had low levels of downy mildew in both tillage systems (Tables 1,2).

Table 1. Severity of anthracnose, powdery mildew, and downy mildew on eight melon cultivars transplanted into ground where a cover crop was planted in the fall and tilled in the spring.

Cultivar[y]	Foliar disease severity[z]								
	Anthracnose			Powdery mildew			Downy mildew		
	Jul 26	Aug 2	Aug 13	Jul 26	Aug 2	Aug 13	Jul 26	Aug 2	Aug 13
Athena	0.5	0.9	2.1 d[x]	0.1	0.1	0.3 e	0.5	1.7	4.0 a
Dulce Nectar	0.4	0.8	3.2 ab	0.1	0	1.1 de	0.1	0.4	3.9 a
Eden's Gem	1.4	2.1	--	1.9	4.0	--	0.7	1.7	--
Escorial	0.7	1.8	3.8 a	0	0.5	2.4 bc	0.1	0.1	0.6 c
Juane	0.4	1.2	3.8 a	1.3	3.2	3.7 a	0.2	0.9	3.1 b
Sivan	0.2	0.9	2.3 cd	0.2	0.4	2.8 ab	0	0	0.1 c
Snow Mass	0.1	0.8	3.0 b	0.1	0.8	3.0 ab	0.6	1.6	3.8 a
Sunbeam	0.9	1.9	2.9 bc	0.4	0.6	1.7 cd	0.3	1.6	3.9 a

[z]Data based on foliar ratings of six plants per cultivar per replicate using a 0 to 4 scale, where 0 = no disease, 1 = less than 10% disease, 2 = 10 to 25% disease, 3 = 25 to 50% disease, and 4 = more than 50% disease. Three replications.
[y]Cultivar was a significant factor for anthracnose ($P = 0.003$), powdery mildew ($P < 0.001$), and downy mildew ($P < 0.001$).
[x]Mean separation by Student's t-test ($P < 0.05$). No disease severity data for Eden's Gem, as too few living plants.

Table 2. Severity of anthracnose, powdery mildew, and downy mildew on eight melon cultivars transplanted into ground where a cover crop was planted in the fall and mowed (no-till) in the spring.

Cultivar[y]	Foliar disease severity[z]								
	Anthracnose			Powdery mildew			Downy mildew		
	Aug 12	Aug 20	Aug 26	Aug 12	Aug 20	Aug 26	Aug 12	Aug 20	Aug 26
Athena	0.9	3.0	3.4 a[x]	0.1	0.6	0.6 c	0	0.7	3.2 a
Dulce Nectar	0.9	1.7	1.4 c	0	0.4	0.8 c	0	0.2	2.4 ab
Eden's Gem	1.0	3.4	1.9 bc	2.3	2.1	2.4 b	0.4	1.6	3.0 a
Escorial	0.4	2.4	2.2 bc	0.1	1.1	2.2 b	0	0	1.2 cd
Juane	0.7	1.9	2.1 bc	1.8	3.1	3.5 a	0	0.5	1.5 bc
Sivan	0.3	1.3	2.1 bc	0.2	1.4	3.1 ab	0	0.1	0.3 d
Snow Mass	1.6	3.8	2.6 ab	0.4	1.9	2.3 b	1.3	0.6	2.9 a
Sunbeam	0.7	1.9	2.5 b	0.2	2.0	3.6 a	0	1.2	3.1 a

[z]Data based on foliar ratings of six plants per cultivar per replicate using a 0 to 4 scale, where 0 = no disease, 1 = less than 10% disease, 2 = 10 to 25% disease, 3 = 25 to 50% disease, and 4 = more than 50% disease. Three replications.
[y]Cultivar was a significant factor for all diseases evaluated ($P < 0.001$).
[x]Mean separation by Student's t-test ($P < 0.05$).

Discussion

No melon cultivar sustained low levels of all three diseases. Of the cultivars advertised as resistant to powdery mildew, only 'Athena' and 'Dulce Nectar' had consistently low foliar ratings, which were significantly lower than all other cultivars on 26 August in the no-till section. The only correlation between melon type and disease severity was that downy mildew severity on the Charentais types, 'Escorial' and 'Sivan,' was low in both tillage systems. Guan et al. (2013) also evaluated melon cultivars in Florida. They found that 'Honey Yellow' honeydew type, and 'Camposol' canary melon, had low defoliation severity in both an organic and conventional field. Both powdery and downy mildew were present in the Florida fields, however the diseases were not evaluated separately. Similar to our observations in the mid-Atlantic, their disease pressure was high. They observed that yields were lower in the organic production field (Guan et al. 2013).

No melon cultivar sustained low disease severity of anthracnose, powdery, and downy mildew in our trials. A good strategy for organic production in the mid-Atlantic region, given the differential reaction of cultivars and melon types to foliar disease, is to plant a diversity of cultivars.

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Association Analysis of Morphological Traits in Iranian Melon Accessions

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Abstract. Association analysis is a method for identifying informative markers associated with morphological traits. These markers can be applicable to breeding programs, especially in selecting elite plants and constructing genetic maps. Ten ISSR primers produced 88 polymorphic markers in 27 endemic melon accessions with ten replications. Stepwise regression analysis was applied to determine informative markers association with morphological traits as dependent variables and molecular independent data. The morphological traits included 15 quantitative characters: fruit length-to-width ratio, days to first mature fruit, flesh thickness, skin thickness, placenta diameter, cavity diameter, 100 seed weight, average number of fruits per plant, placenta width, total soluble solids, dry matter content, skin and flesh thickness, seed length, seed width, and seed diameter. Minimum and maximum numbers of markers were found for placenta diameter (7 markers) and seed diameter (27 markers), respectively. The associated markers explained a maximum of 32.2% (days to first mature fruit) to 72.1% (total soluble solids content) of the maximum adjusted coefficient of determination (adjusted R^2) for individual traits. Some markers were observed to have a close association with morphological traits but the relationship between primers and morphological traits is independent of marker index and polymorphism information content.

Keywords: Melon, association analysis, coefficient determination, cucurbits

Introduction

Melon, *Cucumis melo* L., has a basic chromosome number of $n = 12$ (Kerje and Grum 2000). Plant breeders use genetic markers (or simply markers) to study genomic organization and location of interesting genes, and to facilitate the plant breeding process (Acquaah 2009). The selection efficiency of desired traits has been remarkably improved through the use of markers. Inter simple sequence repeat (ISSR) markers are PCR-based molecular markers occurring in DNA segments between microsatellite repeats. These markers combine most of the benefits of AFLP and microsatellite analysis with the universality of RAPD (Reddy et al. 2002). As some markers and genes reside close together on the same chromosome, they tend to stay together during successive generations. When sites of markers on chromosomes are detected and it is ascertained how close they are to specific genes, a genetic linkage map can be created. Such genetic maps achieve several aims, including detailed analysis of associations between economically important traits and genes or quantitative trait loci (QTLs), and facilitating the introgression of desirable genes or QTLs through marker-assisted selection (Semagn et al. 2006).

Association analysis (AA), also known as association mapping or linkage disequilibrium mapping, is a method to study the relationship between phenotypic variation and molecular genetic polymorphisms. The analysis holds promise as a strategy for applying marker-assisted selection for quantitative traits into plant breeding programs (Breseghello and Sorrells 2006). So far, association analysis has been applied to only a few crops such as date palm (Marsafari et al. 2014) and wheat (Roy et al. 2006, Rashidi Monfared et al. 2008, Hu et al. 2015).The aim of our study was identification of informative ISSR markers related to morphological traits in 27 endemic Iranian melon accessions.

Materials & Methods

Plant materials

Seeds of 27 melon accessions were collected from all over Iran. The seeds were planted outside at the Agriculture Faculty of the Tarbiat Modares University during 2012 to 2013 (Table 1). Assessment of morphological characteristics was performed on ten fruits of each accession, including fruit length-to-width ratio, days to first mature fruit, flesh thickness, skin thickness, placenta

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diameter, cavity diameter, 100-seed weight, average fruit weight per plant, placenta width, total soluble solids content (TSS), dry matter, fruit skin, flesh hardness, seed length, seed width, and seed diameter.

Molecular markers

DNA from 10 individuals of each accession was separately extracted using the modified Cetyltrimethyl ammonium bromide (CTAB) method (Edwards et al. 1991). Ten primers which produced clear fragments (Table 2) were used for the polymerase chain reaction mixture, which contained: 20 ng DNA, 1.15 mM $MgCl_2$, 0.77 µM primer, 0.15 mM dNTPs, 1 × PCR buffer, 0.5 unit of Taq DNA polymerase (Smart taq DNA polymerase, Sinaclon™) in a total volume of 13 µL. The ISSR PCR was performed in a Thermocycler (C 1000TM Thermal Cycler, BIORAD), as follows: 3 min of denaturing at 95 °C followed by 1 min of denaturing at 95 °C, 1 min annealing at 30 to 55 °C (depending on primer), and 2 min of elongation at 72 °C for 34 cycles. A final cycle was 72 °C for 10 min. The PCR of ISSR was performed. The PCR products were analyzed on 10% polyacrylamide gels in 1 × TBE buffer running at 400 V for 1 h and eventually silver stained according to described protocol (Stift et al. 2004).

Data analysis

A binary data matrix (1 for presence and 0 for absence) was obtained from scoring polymorphic bands. Polymorphism information content (PIC) of ISSRs was calculated in an Excel file as $PIC=1-(\sum_{i=1}^{n} pi^2 + \sum_{i=1}^{n} qi^2)/n$, where the p*i* and q*i* are the frequency of the presence and absence of band i^{th} for an ISSR marker, respectively. Data analysis involved estimation of polymorphism content (PIC) and Marker Index, MI = PIC·N·β (PIC = average polymorphism information for each primer, N = number of bands for each primer, β = ratio polymorphism of each primer) (Dangi et al. 2004). PIC for codominant markers (such as SSR) ranged from 0 to 1 but for dominant markers (like ISSR) ranged from 0 to 0.5. PIC values are a criterion measuring the discriminatory power of each primer to detect polymorphism in populations (Anderson et al. 1993, De Riek et al. 2001).

The association analysis was performed using linear regression by stepwise method on 15 morphological traits and 10 ISSR primers in 27 endemic melon accessions by PASW Statistics 18

Table 1. Details of 27 cultivated melon accessions.

No.	Code	Local name	Origin city	Horticultural variety (group)	Latitude	Longitude
1	GER	Gergah	Gilan-e Gharb	Dudaim	34°10'N	46°00'E
2	MIKE	Mirpanj	Kermanshah	Inodorus	34°18'N	47°04'E
3	GES	Garmak	Esfahan	Reticulatus	32°38'N	51°39'E
4	SHA	Talebi Shahabadi	Esfahan	Reticulatus	32°38'N	51°39'E
5	GOR	Gorgab	Esfahan	Inodorus	32°38'N	51°39'E
6	KONA	Komboze	Najafabad	Inodorus	32°45'N	51°15'E
7	AHAR	Ahar	Ahar	Ameri	38°28'N	47°04'E
8	TSAV	Talebi Saveh	Saveh	Reticulatus	35°01'N	50°21'E
9	BANI	Barge Ney	Mahallat	Inodorus	33°54'N	50°27'E
10	TONI	Tozard	Mahallat	Inodorus	33°54'N	50°27'E
11	ZANI	Zard	Mahallat	Inodorus	33°54'N	50°27'E
12	BONI	Bodagh	Mahallat	Inodorus	33°54'N	50°27'E
13	AlSI	AlashaltaSijaval	Bandar Torkaman	ND*	36°54'N	54°04'E
14	KSI	Khiyari Sijaval	Bandar Torkaman	ND*	36°54'N	54°04'E
15	KHM	Khatoni	Neyshabur	Inodorus	36°12'N	58°47'E
16	NB	Nishbaba	Neyshabur	Ameri	36°12'N	58°47'E
17	SANI	Sabz	Neyshabur	Inodorus	36°12'N	58°47'E
18	GNI	Garmak	Neyshabur	Reticulatus	36°12'N	58°47'E
19	GB	Ghanat Boshroye	Neyshabur	Inodorus	36°12'N	58°47'E
20	TS	Tashkandi	Neyshabur	Inodorus	36°12'N	58°47'E
21	TISA	TilSabz	Neyshabur	Cantalupensis	36°12'N	58°47'E
22	TA	TilAtashi	Neyshabur	Cantalupensis	36°12'N	58°47'E
23	TM	TilMagasi	Neyshabur	Cantalupensis	36°12'N	58°47'E
24	KN	Khaghani	Neyshabur	Inodorus	36°12'N	58°47'E
25	AV	Eyvanaki	Garmsar	Inodorus	35°13'N	52°20'E
26	HM	Hajimoshalahi	Gonabad	Inodorus	34°21'N	58 °41'E
27	CP	Chahpaliz	Gonabad	Inodorus	34°21'N	58 °41'E

*ND = not determined.

Table 2. Polymorphism information content (PIC), marker index (MI), and polymorphism percentage (PP) for 10 primers that were studied on 27 Iranian melon accessions.

Name	PP (%)	PIC	MI	Sequence (5'-3')
$(TG)_8G$	50.0	0.20	2.03	TGTGTGTGTGTGTGTGG
$(AG)_8T$	73.08	0.36	6.76	AGAGAGAGAGAGAGAGT
$(GA)_8T$	70.0	0.25	3.46	GAGAGAGAGAGAGAGAT
$(GA)_8YC$	35.3	0.15	0.92	GAGAGAGAGAGAGAGAYC
$(AC)_8YG$	10.0	0.27	0.54	ACACACACACACACACYG
$(CA)_8A$	35.7	0.36	1.79	CACACACACACACACAA
$(AC)_8G$	50.0	0.15	0.76	ACACACACACACACACG
$(GAA)_6$	62.5	0.29	2.85	GAAGAAGAAGAAGAAGAA
$(GACA)_4$	84.6	0.30	3.30	GACAGACAGACAGACA
$(GA)_8A$	37.5	0.31	1.87	GAGAGAGAGAGAGAGAA
Mean	50.9	0.26	2.43	

Table 3. Analysis of association of 15 morphological traits and molecular data using stepwise regression.

Traits	N	R^2 (Max)%	R^2 (T) %	df		ISSR Max	df		ISSR Complete
				Y	X		Y	X	
Fruit length-width ratio	15	57.4	92.8	56	1	12.8***	13	44	1.6***
Days to first mature fruit	25	32.2	98.2	56	1	2932***	15	42	578***
Flesh thickness	13	54.1	90.8	56	1	663***	11	46	101***
Skin thickness	11	33.0	85.3	56	1	27.0***	11	46	6.34***
Placenta diameter	7	56.4	83.1	56	1	928***	7	50	197***
Cavity diameter	18	26.5	92.8	56	1	2542***	12	45	719***
100 - seed weight	28	49.5	99.7	56	1	49***	24	33	4.05***
Average fruit weight per bush	19	37.1	96.1	56	1	7211547***	15	42	1222204***
Placenta width	13	53.7	97.2	56	1	2346***	17	40	326***
Total solid soluble content	9	72.1	92.3	56	1	188***	9	48	27.0***
Dry matter	11	55.7	96.2	56	1	98.6***	11	46	15.4***
Fruit skin and flesh hardness	18	45.5	97.8	56	1	182***	12	45	12.7***
Seed length	23	68.7	99.5	56	1	79.4***	18	39	6.01***
Seed width	13	54.3	99.6	56	1	13.7***	11	46	2.21***
Seed diameter	27	34.5	95.2	56	1	0.53***	17	40	0.09***

*** Significant at the 0.001 level.
N - number of markers.
Df - degrees of freedom.
R^2 adjusted (Max) % - maximum adjusted coefficient of determination for a molecular marker related to morphological traits.
R^2 adjusted (T) % - total adjusted coefficient of determination for a molecular marker related to morphological traits.
ISSR Max - percentage of variations that is explained by first independent variables.
ISSR Complete - percentage of variations that is explained by total of independent variables.

(formerly SPSS statistics) software. For each analysis, one morphological trait was considered as a dependent variable (Y) and molecular markers as independent variables (X).

Results & Discussion

The ISSR primers produced 172 markers of which 88 were polymorphic (56.16%). Of the polymorphic markers, nine had a high determination coefficient (R^2), $(GAA)_6$ having numerous (four times) and the highest values for determination coefficient. The highest MI was from $(AG)_8T$ and the highest PIC was from $(AG)_8T$ and $(CA)_8A$ (Table 2). Yildiz et al. (2011), in an investigation on 63 Turkish melons and 19 melon accessions from around the world, detected 57.5 percent polymorphism for ISSR markers.

The proportion of the total variance in Y explained by X can be measured by R^2, also called the coefficient of determination. The determination coefficient ranges from 0 to 1 and represents the strength of the linear association between variables (X and Y) (Zuur et al. 2007). Results of linear regression are shown in Table 3. The associated markers each explained a maximum of 32.2% (days to first mature fruit) to 72.1% (TSS) of the maximum adjusted coefficient determination (adjusted R^2) for indi-

vidual traits. The highest and lowest numbers of markers were related to seed diameter (27 indicators) and placenta diameter (7 markers), respectively. The highest value of R^2 total (99.7%) was observed for 100 seed weight. Markers with a length of 1044 bp in the $(TG)_8G$ primer for total soluble solids content, a length of 536 bp in the $(TG)_8G$ primer for seed diameter and 100 seed weight, a length of 1063 bp in the $(AC)_8YG$ primer for fruit length-to-width ratio, a length of 3100 bp in the $(AC)_8G$ primer for seed width, a length of 2785 bp in the $(GAA)_6$ primer for days to first mature fruit, a length of 1044 bp in the $(GAA)_6$ primer for seed diameter, a length of 585 bp in the $(GAA)_6$ primer for average fruit weight per plant, a length of 543 bp in the $(GAA)_6$ primer for seed diameter and seed width, and a length of 2770 bp in the $(GA)_8A$ primer for cavity diameter, dry matter, fruit skin, and flesh hardness indicated the highest significant R^2 max at the 1% level of significance.

The results show that in the $(GAA)_6$ primer, while polymorphism information content and marker index was not included in the maximum amount, it had a much stronger correlation with morphological traits than the others. This probably indicates the presence of this primer sequence in the coding regions of morphological traits. Marsafari et al. (2014) identified RAPD and ISSR informative markers with 11 morphological traits in Iranian date palm. They concluded that some of the markers were associated with more than one trait, so correlation of these traits should be more significant. Rashidi Monfared et al. (2008) carried out association analysis on six agronomic traits with 74 SSAP markers in durum wheat. They concluded that if more primer combinations are used, more markers correlated with traits related to yield and its components can be expected.

The information obtained by research on the sites of quantitative traits needs to become practically suitable for plant selections. Association analysis can be an effective tool for overcoming the gap between QTL analysis and marker assisted selection (MAS) (Breseghello and Sorrells 2006). Moreover, informative markers with high R^2 can be detected and isolated from the gel and, after sequencing and sequence alignment in databases, detect candidate genome regions of interest with the highest similarity. Also, desired sequences can be utilized to design SCAR primers for recognition of target traits for use of MAS in breeding programs (Rashidi Monfared et al. 2008). Sometimes the accessibility and time-consuming creation of mapping populations, together with the lack of linkage between traits and markers, are great limitations to recognizing molecular markers for specific traits. To overcome these limitations, and as an alternative to planned populations, molecular markers for traits of interest have to be identified through association studies conducted on germplasm collections (Roy et al. 2006).

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Identification of Informative ISSR Markers for Morphological Characteristics of Iranian Dudaim Melons (*Cucumis melo* var. *dudaim*)

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Abstract. Association analysis of germplasm can be used for identification of informative markers for traits of interest which could be useful for future breeding programs. In this study, relationships between 28 morphological traits (as dependent variables) and molecular markers of 10 ISSR primers (as independent variables) in 14 accessions of Iranian dudaim melons (*Cucumis melo* var. *dudaim*) were investigated using stepwise regression analysis. The average number of polymorphic markers per primer was 15.5. Also, 155 polymorphic markers were detected with average polymorphism of 82.51%. The $(AG)_8T$ primer showed the highest polymorphism information content (PIC 0.44) and the $(AC)_8G$ primer showed the highest marker index (MI 8.2). The minimum and maximum number of markers were related to earliness of male flowers (4 markers) and male flower petal length/width ratio (27 markers), respectively. The maximum adjusted coefficient of determination (R^2) ranged from 27.2% (female flower petals length/width ratio) to 94% (days to 50% flowering). Maximum associated markers were related to the $(GA)_8T$, $(GAA)_6$, and $(GA)_8A$ primers (a total of 19 traits). Total adjusted coefficients of determination (R^2) for all traits were approximately 100%. These results showed that all of the markers explained significantly the variation of traits in this experiment.

Keywords: Coefficient determination, dudaim melons, informative markers, morphological traits

Introduction

Melon, which is native to tropical and subtropical regions, is one of the most important horticultural crops in Iran (Raghami et al. 2013). The genus *Cucumis* includes 32 species many of which, including melon, *C. melo*, have a chromosome number of $2n = 2x = 24$ (Parvathaneni et al. 2011). Dudaim melon (called "Dastanbu" in Persian), *C. melo* var. *dudaim*, is native to Iran and is used as a decorative and aromatic fruit. The plant is andromonoecious. Dudaim fruit is small and has an orange skin with maroon or brown stripes (Nesom 2011).

One of the important applications of molecular markers is to breeding programs, especially selection for a special trait. In fact, any technique that helps to select early in plant development for a particular trait contributes toward increased efficiency and shorter breeding cycles. This is especially true for cases in which the development of traits of interest takes a long time to be expressed or when the plant has been crossed and the traits of interest develop after crossing. In this way, linkage between the gene and a molecular marker can be useful for selection (Bagheri et al. 2007). Populations segregating for desired qualitative or quantitative traits are useful for identification of such markers (Naghavi et al. 2007), but this is costly and time-consuming. If markers with suitable relationships to desirable traits could be identified using regression analysis, they could be used in breeding programs as informative markers (Ranjbar et al. 2009). Informative markers can also be relevant for selection of parents to produce a population for mapping. Also, selection of superior genotypes can be useful especially when basic genetic information, such as their placement on a genetic map, is not available (Mohammadzedeh et al. 2014). Regression analysis between molecular markers and morphological traits could be used to construct maps that include genes of interest, with a high degree of confidence, and also enable the identification of markers that are otherwise not traceable (Roy et al. 2006). In the present study, we tried to identify informative markers for morphological traits in 14 genotypes of native Iranian dudaim melons. This information could be useful for selection in breeding programs.

Materials & Methods

Fourteen accessions of dudaim melon were collected from different locations in Iran (Table 1). The research was done in May 2012 at the Faculty of Agriculture, Tarbiat Modares Univer-

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Table 1. The geographic coordinates and origins of 14 accessions of Iranian dudaim melon (*Cucumis melo* var. *dudaim*) accessions.

Code	City	Geographic coordinates		Code	City	Geographic coordinates	
		Northern	Eastern			Northern	Eastern
BLE	Bandaer Lenge	26.56	55.03	CHA	Ramsar/ Chalakrood	36.90	50.68
ESF	Esfahan	32.60	51.60	RAZ	Ramsar/ Razimahalle	36.90	50.68
GHO	Ghome	34.64	50.89	CHAP	Ramsar/ Chaparsar	36.90	50.68
DAM	Damaghan	36.34	53.17	DAR	Ramsar/ Daryaposhteh	36.90	50.68
ELA	Ilam	33.28	46.52	SOR	Sorkh kalateh	36.91	54.59
SHI1	Ilam/ Shiravan 1	33.86	33.18	MAR	Maragheh	37.30	46.12
SHI2	Ilam/ Shiravan 2	33.86	33.18	KERS	Kermanshah	37.23	46.52

sity. The experiment was conducted in a randomized complete block design with 3 replications. The seeds were planted in the field, 5 to 6 cm deep, with 65 cm distance between plants within the row, and 2 meters between rows. Fruit characteristics at full maturity, traits of leaves, branches, flowers at the stage 50% of flowering and seed traits after drying the seeds, were measured.

A total of 28 morphological traits were studied: 100-seed weight (g) (SW), earliness of male flower (days) (MF), days to first flowering (FF), days to 50% flowering (FPF), days to first mature fruit (FAF), maturation period (days) (MP), total fruit weight (KG) (TFW), fruit acidity (DAM), flesh thickness (mm) (FS), dry matter percent (DM), placenta diameter (mm) (PD), cavity diameter (mm) (CD), leaf petiole length (mm) (LP), stem diameter (mm) (SS), male flower stem length/width ratio (mm) (LWMS), female flower stem length/width ratio (mm) (LWFS), ovary length/width ratio (mm) (LWO), female flower petal length/width ratio (mm) (LWFP), male flower petal length/width ratio (mm) (LWMP), fruit length/width ratio (mm) (LWF), leaf length/width ratio (mm) (LWL), pH of fruit juice (pH), hardness of skin with flesh (SFF), seed length (mm) (LS), seed weight (mm) (WS), seed diameter (mm) (DS), number of fruit per plant (NF), and total soluble solids content (TSS).

For identification of genetic diversity by ISSR the genomic DNA of 10 individuals from each accession was extracted using a modified Cetyltrimethyl ammonium bromide (CTAB) method (Rashidi Monfared et al. 2008). PCR was done in a thermocycler (BIORAD, C 1000™) in a final volume of 13 µl through three steps: denaturation at 94 °C for 3 min, 34 cycles of denaturation at 95 °C for 1 min followed by annealing at 30 °C for 1 min and extension at 72 °C for 2 min, with final extension for 10 min at 72 °C. PCR products were loaded on a 10% polyacrylamide gel, and bands were revealed with silver nitrate staining (Bassam et al. 1991). Molecular data were evaluated based on presence (as 1) or absence (as 0) of bands for each primer.

For the denotation of the markers, polymorphic information content (PIC) was calculated by Excel software using of the formula $PIC=1-(\Sigma_{i=1}^{n} pi^2 + \Sigma_{i=1}^{n} qi^2)/n$ in which p*i* and q*i* are the frequency of the presence and absence of band i^{th} in an ISSR marker (Anderson et al. 1993). The Marker Index (MI) was calculated using the formula of $MI = PIC \cdot N \cdot \beta$ for all primers (PIC = average polymorphism information for each primer, N = number of bands for each primer, β = ratio polymorphism of each primer) (Dangi et al. 2004).

Association analysis of 28 morphological traits and 10 ISSR markers on 14 accessions of Iranian dudaim melon using stepwise regression was performed by SPSS 18 software (IBM). In all analyses, one of the morphological traits was the dependent variable (Y) and all of the molecular markers were independent variables (X). Markers that authenticated a high percentage of variance were revealed.

Results & Discussion

The relationships between quantitative traits were calculated using Pearson correlation coefficient with Minitab 17.1.0 (Table 2). The pH of fruit juices had the highest negative significant correlation with fruit acidity (r = -0.92) and the highest positive correlation was observed between the two traits seed width and 100-seed weight (r = 0.91). There were significant negative correlations between the days to first mature fruit with days to 50% flowering, fruit acidity with days to first flowering, and seed width with days to 50% flowering. Seed length with 100-seed weight showed the highest negative correlation. Days to the first mature fruit, flesh thickness, placenta diameter, seed length, seed width, and seed diameter had positive correlations with days to 50% flowering. Cavity diameter was positively correlated with placenta diameter. Seed diameter had a significantly positive correlation with 100-seed weight. Taha et al. (2003) showed significant positive correlations between the number of fruits with the number of primary branches, netting development with number of primary branches, netting development with total soluble solids, number of primary branches with number of secondary branches, fruit weight with plant length, earliness with flavor, and netting development with flesh thickness. Earliness and netting development, total soluble solids and earliness, and the number of primary branches and stem length were found to be negatively associated.

Mohammadi et al. (2014), in a study on 49 cantaloupe melon accessions, reported a positive correlation between average fruit weight and fruit flesh thickness, but these traits lacked significant relationship with skin diameter and cavity diameter.

From a total of 10 ISSR primers, 186 clear bands and 155 polymorphic bands were scored. Six markers out of the 155 polymorphic bands had the maximum R^2 adjusted coefficient (R^2 = 94 %) with morphological traits. Of the six markers, the

Table 2. The Pearson correlation matrix through different traits in 14 Iranian native dudaim melon accessions.

Traits	SW	MF	FF	FPF	FAF	MP	TFW	FA	FS	DM	PD	CD	LP	SS	PH	LWFS	LWO	LWFP	LWMP	LWF	LWL	LWMS	SFF	LS	WS	DS	NF	TSS
SW	1																											
MF	-0.11	1																										
FF	0.17	-0.52	1																									
FPF	0.96	0.22	-0.57*	1																								
FAF	0.78	0.01	-0.66**	-0.73**	1																							
MP	0.64*	0.05	-0.56*	-0.66**	0.89**	1																						
TFW	0.22	0.25	0.00	-0.11	0.38	0.28	1																					
FA	-0.03	0.08	-0.64*	-0.59*	0.21	0.09	0.02	1																				
FS	0.64*	0.04	0.27	-0.43	0.75**	0.72**	0.72**	0.00	1																			
DM	-0.35	0.21	0.37	0.44	-0.18	-0.10	-0.45	-0.35	-0.50	1																		
PD	0.54*	-0.21	-0.56*	-0.66**	0.78**	0.84	0.36	0.23	0.69**	-0.33	1																	
CD	0.71**	-0.17	-0.69**	-073**	0.82**	0.79**	0.34	0.26	0.68**	-0.47	0.89**	1																
LP	0.35	0.03	-0.18	-0.20	0.43	0.27	0.03	-0.06	0.06	0.42	-0.00	0.01	1															
SS	0.60*	0.35	-0.45	-0.48	0.64*	0.57*	0.07	0.21	0.40	0.03	0.24	0.39	0.61	1														
pH	-0.13	-0.04	0.64*	0.61*	-0.34	-0.14	-0.11	-0.92**	-0.14	0.44	-0.32	-0.42	0.00	-0.31	1													
LWFS	0.01	-0.24	-0.38	-0.11	-0.09	-0.10	0.03	-0.03	-0.06	-0.06	-0.15	-0.13	0.28	0.00	0.07	1												
LWO	0.11	0.49	0.14	0.08	0.06	0.15	0.26	-0.04	0.36	-0.06	-0.01	-0.12	-0.22	0.13	0.20	-0.42	1											
LWFP	-0.28	-0.14	-0.27	-0.26	-0.33	-0.27	-0.06	0.37	-0.31	-0.26	-0.26	-0.24	-0.02	-0.04	-0.17	-0.21	-0.08	1										
LWMP	-0.03	0.85	0.27	0.26	0.05	0.18	0.24	0.02	0.17	0.12	-0.03	-0.10	0.01	0.29	0.02	-0.36	0.48	-0.25	1									
LWF	0.59*	-0.57*	-0.41	-0.48	0.45	0.28	0.10	0.00	0.35	-0.44	0.38	0.56*	0.26	0.20	-0.25	0.40	-0.55*	-0.10	-0.49	1								
LWL	0.02	-0.02	-0.01	0.00	-0.05	0.07	0.01	-0.11	0.02	-0.17	0.34	0.29	0.59*	0.28	0.04	0.47	0.07	-0.14	-0.20	-0.16	1							
LWMS	-0.24	0.11	0.58*	0.65*	-0.59*	-0.50	-0.23	-0.57*	-0.46	0.23	-0.57	-0.41	-0.04	-0.13	0.50	0.09	0.30	0.04	0.12	-0.05	0.03	1						
SFF	0.00	-0.07	-0.09	-0.11	0.15	0.11	0.01	-0.21	-0.12	0.48	-0.14	-0.25	0.71**	0.14	0.35	0.27	-0.04	0.28	-0.11	-0.07	-0.42	-0.11	1					
LS	-0.75**	-0.17	-0.55*	-0.59*	0.71**	0.39	0.38	0.18	0.60*	-0.36	0.32	0.50	0.49	0.56*	-0.33	0.59	0.56	-0.04	-0.33	0.54*	-0.28	-0.44	0.23	1				
WS	0.91**	-0.01	0.52	-0.57*	0.77**	0.60	0.42	0.08	0.71**	0.42	0.55*	0.75**	0.32	0.62*	-0.27	0.20	0.10	-0.23	-0.15	0.62*	-0.03	0.23	-0.09	0.76**	1			
DS	0.89**	-0.02	-0.53	-0.52	0.71**	0.47	0.26	0.15	0.55*	-0.29	0.41	0.58*	0.38	0.58*	-0.24	0.01	0.25	-0.18	0.09-	0.39	-0.09	-0.34	0.05	0.80**	0.87**	1		
NF	-0.44	-0.26	0.13	0.16	-0.66*	-0.60*	-0.17	0.04	-0.53	-0.08	-0.50	-0.45	-0.12	-0.33	0.10	0.58*	-0.19	0.79*	-0.34	-0.05	0.13	0.42	0.12	-0.28	-0.31	-0.33	1	
TSS	0.02	0.44	0.27	0.29	-0.11	-0.03	-0.29	-0.06	-0.21	0.45	-0.26	-0.27	0.37	0.53*	0.07	-0.16	0.12	0.06	0.53*	0.33	-0.16	0.41	0.00	-0.17	-0.04	0.07	-0.03	1

*and ** show significant differences at significance levels of 5% and 1%, respectively.

$(GA)_8T$ primers had the highest R^2. The highest PIC was related to the $(AG)_8T$ primer (Table 3).

Zhang et al. (2012) obtained a PIC and a percentage of polymorphic bands of 0.30 and 83.9, respectively, from 12 ISSR primers, a range comparable with our results. The results of Raghami et al. (2013) with SSR markers of melons, including some dudaims, showed an average PIC = 0.49, which is close to the results of our study. The MI coefficient indicated that the $(AC)_8G$ primer had the highest segregation among individual accessions. The average MI was 5.57, which was high compared with 3.74 from Fabriki Ourang et al. (2010). So in this assay, the MI showed greater differentiation among populations (Table 3). The results of stepwise regression analysis of morphology and molecular markers are shown in Table 4. The highest and lowest numbers of markers were related to male petals length/weight ratio (27 markers) and male flower earliness (4 markers), respectively. The highest R^2 coefficient ($R^2 = 94$) was for days to 50% flowering.

In the stepwise regression method, morphological characteristics and molecular markers were considered as the dependent and independent variables, respectively. Markers that associate with phenotypic changes were identified and introduced as informative markers (Sabouri et al. 2013). The traits of days to first flowering, cavity diameter, and seed diameter, had similar markers, but days to first flowering had negative correlations with cavity diameter and seed diameter, and cavity diameter with seed length and fruit weight had positive and significant correlations ($P < 0.05$). However, total fruit weight and fruit flesh thickness, and fruit juice pH and acidity had common markers, but these had no correlation. Seed length and fruit length/width ratio also had a common marker and a positive and significant correlation ($P < 0.05$) that indicates linkage between these traits. Similarly to the results of Marsafari et al. (2014), our data indicated that due to good distribution of polymorphic DNA markers in the genome of date and markers with high association to horticultural characters, linkages can be established for important traits. However, linkages between the markers and horticultural traits need to be tested in segregating populations for constructing linkage maps (Naghavi et al. 2007). Based on our results, the $(GA)_8T$ primer had more markers with the highest R^2. However, PIC and MI were not maximum, and this primer had a strong correlation with the studied morphological traits. Marsafari et al. (2014) analyzed 11 morphological traits and 122 RAPD markers on 15 date cultivars. The R^2 associated with seed diameter was 26% and the highest R^2 adjusted was for fruit shape (67%). They concluded that some of the markers have been associated with more than one trait; therefore these traits have high correlation with one another. Improvement in plant breeding depends on genetic diversity. Then the management and identification of this diversity is necessary for breeding programs. In addition, knowledge of genetic diversity needs to be supplemented with conservation management of plant germplasm. Although mapping based of quantitative traits is suitable for gene detection, it is time-consuming and laborious work (Rakshit et al. 2010). To overcome these limitations, identifying the markers associated with traits has been facilitated through regression methods (Gomez and Gomez 1984).

The results of this study show that Iranian dudaim melons have high morphological and molecular diversity. Molecular markers had high correlations with some morphological traits. The results also show that selection based on molecular markers could facilitate breeding programs by saving time in selection of superior genotypes (Sarayloo et al. 2015).

The primers could facilitate identification of informative markers for breeding programs, markers that are highly correlated with important horticultural traits (Marsafari et al. 2014). Also, informative markers associated with agronomic traits, especially markers in known linkage groups, can be useful in producing lines by chromosome substitution (Liu et al. 2002). The identified informative markers in this study could be helpful for selection of superior genotypes even without background information on the genotypes. Also, these markers could be used to select suitable parents to produce populations for mapping (Mohammadzadeh et al. 2014).

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Table 3. The sequence of 10 ISSR primers and data of molecular analysis related to these primers.

Primer code	Primers	Sequence (5'-3')	Numbers of bands total	Numbers of polymorphic bands	Percents of polymorphic bands	PIC	MI
A	$(TG)_8G$	TGTGTGTGTGTGTGTGG	16	15	93.75	0.26	3.9
B	$(AG)_8T$	AGAGAGAGAGAGAGAGT	16	13	81.25	0.44	5.72
C	$(GA)_8T$	GAGAGAGAGAGAGAGAT	18	16	88.88	0.33	5.28
D	$(GA)_8YC$	GAGAGAGAGAGAGAGAYC	18	14	77.77	0.27	3.78
E	$(AC)_8YG$	ACACACACACACACACYG	17	14	82.35	0.38	5.32
F	$(CA)_8A$	CACACACACACACACAA	12	7	58.23	0.40	2.8
G	$(AC)_8G$	ACACACACACACACACG	21	20	95.33	0.41	8.2
H	$(GAA)_6$	GAAGAAGAAGAAGAAGAA	26	21	80.76	0.35	7.35
K	$(GACA)_4$	GACAGACAGACAGACA	22	18	81.81	0.41	7.38
L	$(GA)_8A$	GAGAGAGAGAGAGAGAA	20	17	85.00	0.39	7.8
Average	-	-	186	155	82.51	0.36	5.75

Table 4. Data analysis of 28 morphological traits and molecular data using the stepwise regression method in Iranian dudaim melons.

Traits	Number of primers	R^2 (Max) %	R^2 (T) %	df of variance source		ISSR Max	df of variance source		ISSR Comlete	Marker
				X	Y		X	Y		
SW	9	54	100	1	22	7.14***	9	14	1.41***	L8
MF	4	35.6	100	1	22	18.97***	2	21	26.66**	D6
FF	6	92	100	1	22	126.92***	4	19	37.50**	C7
FPF	20	94	100	1	22	455.6***	20	3	25.7**	C4
FAF	21	79.5	100	1	22	557.1***	21	2	33.4**	K2
MP	19	88.7	100	1	22	496.9***	13	10	43***	B10
TFW	24	84.5	100	1	22	5.52***	20	3	0.38***	H10
FA	16	75	100	1	22	0.3***	14	9	0.03***	H20
FS	22	65.3	100	1	22	315.9***	18	5	26.87***	H10
DM	11	45.9	99.9	1	22	19.7***	17	40	3.72***	A10
PD	16	70.4	100	1	22	417.74***	16	7	37.07***	B10
CD	13	64.4	100	1	22	2558.05***	13	10	305.59***	C7
LP	17	66.9	100	1	22	5874.18***	13	10	675.63***	L10
SS	14	50.6	100	1	22	6.49***	12	11	1.02***	B2
pH	15	88.8	100	1	22	174.94***	15	8	0.55***	H20
LWFS	11	57	100	1	22	75.30***	11	12	11.46***	K13
LWO	17	45.1	100	1	22	0.31***	13	10	0.05***	H17
LWFP	20	27.2	100	1	22	7583.778***	20	3	1250.56***	H3
LWMP	27	4.03	100	1	22	0.39***	17	6	0.05**	L16
LWF	13	80	99.9	1	22	0.44***	13	10	0.04***	C9
LWL	22	43.1	100	1	22	0.01***	20	3	0.001***	L1
LWMS	17	74.7	100	1	22	143.71***	15	8	12.82***	C4
SFF	7	45.8	98.3	1	22	59.2***	7	16	18.16***	L2
LS	20	53.3	100	1	22	44.60***	20	3	4.18***	C9
WS	20	57.6	100	1	22	1.31***	20	3	0.11***	L11
DS	7	46.9	98.9	1	22	0.261**	7	16	0.08***	C7
NF	11	75.5	100	1	22	1365.44***	11	12	164.34***	K7
TSS	20	44.9	100	1	22	6.66**	20	3	0.74***	F2

*** significant in level of 0.001.
N - number of markers.
Df - degree of freedom.
R^2 adjusted (Max) % - maximum adjusted coefficient related to one marker for morphological traits.
R^2 adjusted (T) % - total of adjusted coefficient for informative markers of morphological traits.
ISSR Max - percent of changes that are justified by first dependent variables.
ISSR Complete - percent of changes that are justified by independent variables.

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Identification of ISSR Markers Linked to Morphological Traits in Iranian Snake Melons

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Abstract. Association between genotype and phenotype provides an opportunity to identify and map genes/QTLs with a high degree of confidence. We used 10 ISSR primers to identify markers linked to morphological characters in Iranian snake melons, *Cucumis melo* var. *flexuosus*. Across 120 individuals, a total of 93 markers were produced of which 82 were polymorphic. The average number of polymorphic markers, the average polymorphic information contents (PIC), and marker indices (MI) were 8.2, 0.34, and 2.58, respectively. Stepwise regression analysis was used to evaluate possible correlations between 21 morphological traits (as dependent variables) and 82 markers (independent variables). The lowest and highest numbers of markers were significantly associated with placenta width (6 markers) and petiole length (19 markers), respectively. Among all primers, $(AC)_8$YG had the greatest proportion in explaining the adjusted R^2 values for traits (8 traits). Maximum adjusted determination coefficient (adjusted R^2 maximum) ranged from 8.8% for placenta diameter to 53.8% for fruit length. The highest and lowest values for total R^2 were 88.7% for petiole length and 43.6% for placenta diameter, respectively.

Keywords: Association analysis, informative markers, *Cucumis melo* var. *flexuosus*, PIC, MI

Introduction

Melon (*Cucumis melo* L.) is an important horticultural crop worldwide in temperate and tropical regions. A large diversity of melons occurs throughout Iran, Afghanistan, India, and China. Wild melons and melon relatives are distributed from Egypt to Iran and northwest India (Robinson and Decker-Walters 1997). *Cucumis melo* var. *flexuosus* is a diploid, $2n = 2x = 24$, and is resistant to the heat of the summer growing season. Due to its high economic importance and easy cultivation, the melon plant can be used as a model for genetic and physiological studies within *Cucumis*. The plant can be used to identify genes associated with resistance to several abiotic (heat, salinity, and drought) and biotic (pests, fungal, and bacterial pathogens) stresses (Jaberizade et al. 2008). Molecular markers provide rapid tools to identify genomic regions correlated with various traits. Inter Simple Sequence Repeat (ISSR) markers can be applied to any species having a sufficient number of simple sequence repeats and for species for which genomic data are not available in databases. ISSR markers are dominant and to determine polymorphism among individuals, laboratory techniques can be conducted quickly, simply, and efficiently, with a relatively high reproducibility (Reddy et al. 2002). Large numbers of DNA fragments are amplified per reaction, encompassing multiple loci representing different regions of the genome (Doveri et al. 2007). Determining associations between genetic markers and the traits of interest allows to map quantitative trait loci (QTLs) (Neale and Savolainen 2004). The aim of this study was to evaluate association between ISSR markers and morphological traits in snake melon following a stepwise regression analysis.

Materials & Methods

Plant material

Seeds of 12 snake melon accessions were field collected from different regions of Iran or provided by the Gene Bank of the Seed and Plant Improvement Institute, Karaj, Iran (Table 1). This research was carried out at the Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran.

Measuring morphological traits

A randomized block design was used to measure 21 quantitative traits listed by the International Plant Genetic Resources Institute (IPGRI). These traits were: seed length, seed width, seed diameter, 100-seed weight, leaf length, leaf width, petiole length, internode length, number of branches, fruit weight, fruit length, fruit diameter, peduncle length, flesh thickness, placenta width, placenta diameter, cavity diameter, fruit skin hardness, total soluble solids, juice acidity, and flesh dry matter.

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DNA extraction

Genomic DNA was extracted from young leaves using a modified Cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thomson 1980). The quality and quantity of DNA were determined by using a spectrophotometer (Epoch™, BioTek), and the diluted DNA (10 ng/µl) was stored at -20 °C. Ten ISSR primers were used in this research (Table 2). Each PCR contained 25 ng genomic DNA, 1 × PCR buffer, 1.15 mM $MgCl_2$, 0.15 mM dNTPs, 0.76 µM primer, and 0.5 unit Taq polymerase in a total volume of 13 µl. PCR thermoprofile was: initial denaturating at 94 °C for 5 min, 34 cycles at 94 °C for 1 min, 30 to 55 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. Amplification reactions were done by using a ThermoCycler (Bio-Rad C1000). After amplification, the PCR products were run in a 10% polyacrylamide gel at a constant voltage (400 V) by using vertical gel electrophoresis for 3 h. DNA bands were visualized by silver staining (Bassam et al. 1991). Gels were dried by a gel dryer (Bio-Rad Model 583) for 50 min at 80 °C.

Data analysis

ISSR bands were scored as 1 for presence and 0 for absence. Polymorphism information content (PIC) was estimated as $PIC=1-(\Sigma_{i=1_m}^{n} pi^2 + \Sigma_{i=1}^{n} qi^2)/n$, where the p_i and q_i are the frequency of the presence and absence of band i^{th}, respectively (Anderson et al. 1993). Marker index (MI) was also calculated as MI = PIC·N·β for all markers (PIC = Polymorphism information content, N= total number of bands, β = ratio polymorphism of each primer) (Dangi et al. 2004). The association analysis was performed on 21 morphological traits and 10 ISSR primers in 12 endemic snake melon accessions using linear regression by stepwise method with PASW Statistics 18 (formerly SPSS statistics) software. In each analysis, one morphological trait was considered as a dependent variable (Y) and molecular markers as independent variables (X). The fragment sizes of the molecular markers that explained a higher proportion of variations were determined on the polyacrylamide gel.

Results & Discussion

Eighty-two out of the 93 identified bands across 12 snake melon (*Cucumis melo* var. *flexuosus*) accessions were polymorphic, with an average of 8.2 polymorphic bands per primer. The average percentage of polymorphic bands was 82.95% for ISSR markers, while using RAPD markers on Indian snake melon

Table 1. List and geographic origin of Iranian snake melon (*Cucumis melo* var. *flexuosus*) accessions.

Accession	Origin	Geographic coordinates	Accession	Origin	Geographic coordinates
BA	Kordestan (Baneh)	35˚98′N45˚89′E	NA	Isfahan (Najaf Abad)	32˚40′N51˚15′E
AH1	Khuzestan (Ahvaz 1)	31˚20′N48˚40′E	TH	Khorasan Razavi (TorbatHeidarieh)	35˚27′N59˚21′E
AH2	Khuzestan (Ahvaz 2)	31˚20′N48˚40′E	SHU	Khuzestan (Shushtar)	32˚04′N48˚85′E
BEH	Khuzestan (Behbahan)	30˚30′N50˚15′E	TA	Southern Khorasan (Tabas)	33˚35′N56˚05′E
FA3	Fars 3	29˚61′N52˚53′E	FA1	Fars 1	29˚61′N52˚53′E
ISF	Isfahan	32˚50′N51˚50′E	MA	Markazi (Mahallat)	33˚55′N55˚30′E

Table 2. Genetic polymorphism detected for 10 ISSR primers in 12 snake melon (*Cucumis melo* var. *flexuosus*) accessions.

Primer No.	Primer sequence (5´to 3´)	Nb	Np	Ppb	PIC	MI
1	$(TG)_8G$	5	2	40	0.33	0.66
2	$(AG)_8T$	18	18	100	0.15	2.70
3	$(GA)_8T$	4	3	75	0.40	1.20
4	$(GA)_8YC$	12	11	91.67	0.32	3.52
5	$(AC)_8YG$	14	13	92.86	0.37	4.81
6	$(CA)_8A$	5	4	80	0.31	1.24
7	$(AC)_8G$	11	11	100	0.36	3.96
8	$(GAA)_6$	8	7	87.5	0.31	2.17
9	$(GACA)_4$	8	7	87.5	0.40	2.80
10	$(GA)_8A$	8	6	75	0.46	2.76
Average		9.3	8.2	82.95	0.34	2.58

Note: Nb, Np, Ppb, PIC, and MI indicate total number of bands amplified, number of polymorphic bands, percentage of polymorphic bands, polymorphism information content, and marker index, respectively.

Table 3. Analysis of association of 21 morphological traits and molecular data using stepwise regression.

Traits	N	R^2 (Max) %	R^2 (T) %	df		ISSR Max	df		ISSR Complete
				X	Y		X	Y	
Seed length	16	23.4	75.9	1	73	15.56***	16	58	3.67***
Seed width	14	35.7	78.5	1	73	4.48***	14	60	0.72***
Seed diameter	12	19.4	65.1	1	73	0.68***	12	62	0.20***
100-seed width	14	23	68.9	1	73	23.16***	14	60	5.94***
Leaf length	13	23.5	64.8	1	73	5.79***	13	61	1.50***
Leaf width	7	17.9	48.7	1	73	3.48***	7	67	1.40***
Petiole length	19	29.3	87.7	1	73	220.68*	19	55	39.19***
Internode length	8	16.3	51.6	1	73	5.54***	8	66	2.25***
Number of secondary stems	7	13.2	46.7	1	73	3.99***	7	67	2.05***
Fruit weight	14	11.2	66.3	1	73	702250.42***	14	60	338547.70***
Fruit length	12	53.8	96.2	1	73	6023.70***	12	62	819.83***
Fruit diameter	10	38	68.4	1	73	14.67***	10	64	2.74***
Peduncle length	11	43.1	80.8	1	73	248.30***	11	63	43.06***
Flesh thickness	16	32.1	82.9	1	73	110.14***	16	58	20.54***
Placenta width	6	19.8	51.6	1	73	78.55***	6	68	34.89***
Placenta diameter	7	8.8	43.6	1	73	19.15***	7	67	13.37***
Cavity diameter	11	17.6	51.3	1	73	169.95***	11	63	57.69***
Skin hardness	12	22.5	69.8	1	73	5.96***	12	62	1.87***
Total soluble solid	10	21.5	69.4	1	73	15.70***	10	64	5.12***
Fruit acidity	16	18.3	74.9	1	73	0.08***	16	58	0.03***
Dry matter	8	30.4	66.2	1	73	16.87***	8	66	4.81***

*** significant at the 0.001 level.
N - number of markers linked to the traits.
df - degrees of freedom.
R^2 (Max) % - percentage of maximum adjusted coefficient of determination for a molecular marker associated with morphological traits.
R^2 (T) % - percentage of total adjusted coefficient of determination for a molecular marker associated with morphological traits.
ISSR Max - percentage of variation that is explained by first independent variables.
ISSR Complete - percentage of variations that is explained by the total of independent variables.

accessions was 86.6% (Dhillon et al. 2006). The average of polymorphic information contents (PIC) and marker index (MI) was 0.34 and 2.58, respectively. The highest MI was observed with $(AC)_8$YG and the highest PIC was found with $(GA)_8$A, $(GA)_8$T and $(GACA)_4$ (Table 2).

Among all of the polymorphic markers, $(AC)_8$YG with a length of 875 bp had the highest coefficient of determination (R^2). The relationship between molecular markers and morpho logical traits estimated with linear regression is shown in Table 3. The highest (n = 19) and lowest (n = 6) numbers of markers were associated with petiole length and placenta diameter, respectively. Petiole length also exhibited the highest value of total R^2 (88.7%). From the $(AC)_8$YG primer, a 1050 bp marker expressed a significant R^2 Max for number of secondary stems. From the $(GAA)_6$ primer, a 208 bp marker with significant R^2 Max was found for leaf width; with a length of 500 bp from the $(GAA)_6$ primer for leaf length, placenta width and placenta diameter traits; with a length of 720 bp from the $(CA)_8$A primer for fruit acidity; with a length of 180 bp from the $(GA)_8$A primer for petiole length and total soluble solid traits; with a length of 287 bp from the $(GA)_8$YC primer for seed diameter and internode length traits; with a length of 243 bp from the $(GA)_8$YC primer for fruit cavity diameter; with a length of 487 bp from the $(GA)_8$YC primer for seed length; and with a length of 580 bp from the $(GA)_8$T primer for fruit dry weight showed the highest and significant R^2 Max.

Primer $(AC)_8$YG, while its polymorphism information content was not included in the maximum amount, its marker index was included in the highest amount and it indicated a much stronger correlation with morphological traits than others. Marsafari et al. (2014) identified RAPD and ISSR markers associated with 11 morphological traits in Iranian date palm. They concluded that some of the markers were associated with more than one trait. Basaki et al. (2011) assessed association analysis for morphological traits in 202 pomegranate accessions representing 22 provinces of Iran using 30 microsatellite markers. The results showed that there are significant and positive correlations among these traits. Moustafa et al. (2014) identified TRAP and SRAP markers in wheat that are linked with yield components under drought stress. The results revealed that 10 of 14 TRAP and nine of 19 SRAP markers are linked to agronomic traits and can be introduced as new markers.

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Effect of Melon Rootstocks with Multiple Disease Resistance on Yield and Quality of Melon (*Cucumis melo* L.)

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Abstract. Soil-borne diseases in continuous cropping areas cause severe losses of yield and quality of melons. Melons are mostly produced in protected cultivation in Korea. Two major soil-borne pathogens are *Fusarium oxysporum* f. sp. *melonis* (Fom) and *Monosporascus cannonballus*, and they cause sudden plant wilting just prior to harvest. Rural Development Administration (RDA) has developed F_1 hybrids of melon with resistance to both pathogens, for use as melon rootstocks. The objective of this study was to investigate the effect of the developed F_1 hybrids as melon rootstocks on seven farms located in two different areas, Namwon and Gumi. In the Namwon area, leaf yellowing symptoms presumed to be caused by CABYV infection were observed on three farms. On one farm, the melon crop failed due to severe symptoms and on another farm the marketable fruits were only 31.3% of the total in non-grafted cultivation but 61.9% in grafted cultivation (by commercial and RDA rootstocks). On another farm, 74.2% of the fruits were marketable in non-grafting and 75.3% in grafting. Therefore, it seems that grafting reduces damage caused by leaf yellowing. Grafted plants had a higher yield, by 101% to 198%, than non-grafted plants on the various farms. On all six farms in Namwon, the percentage of big fruits, about 2.0 kg, increased, and the percentage of small fruits, below 1.6 kg, decreased. Wilting did not occur in grafted plants on some farms which had mild wilting in non-grafted plants during the hot season. In the Gumi area, there were no leaf yellowing symptoms and no wilting on the farms. There were no significant differences in fruit size, fruit weight, fruit skin thickness, sugar contents, and net formation between grafted and non-grafted plants. Accordingly, the use of melon rootstocks with multiple disease resistance for melon crops can increase yield and improve quality.

Keywords: Cucurbitaceae, grafting, grafting affinity, soil-borne diseases

Introduction

Melon (*Cucumis melo* L.) is a promising crop for which cultivation area and export quantity are steadily increasing in Korea. However, serious losses, including sudden plant wilting immediately prior to harvest, frequently occurs in the main production area of continuous cropping. Plant wilting causes reduced sugar content and deteriorated net formation of fruits, threatenening stable quality production. In the soil on which melons show wilting, in main production areas, *Fusarium oxysporum* f. sp. *melonis* (Fom) and *Monosporascus cannonballus* have been isolated. Melon seedlings grafted on pumpkin rootstocks have been used by some growers to prevent plant wilting. *Cucurbita* rootstocks have strong resistance to soil-borne diseases and vigorous root systems but often adversely affect melon fruit quality. So Rural Development Administration (RDA) has developed F_1 hybrids of melon resistant to both *Fusarium oxysporum* f. sp. *melonis* (Fom) and *Monosporascus cannonballus*. This study was conducted to investigate the effect of these melon hybrids as rootstocks on yield and quality of melons grown in protected culture.

Materials & Methods

Plant material

Three melon F_1 hybrids developed by RDA were used as rootstocks, 'Hanmaeum', 'Green Power', and 'PS'. 'Summer Ace' muskmelon was used as the scion. Non-grafted 'Summer Ace' and 'Hero' were used as controls.

Methods

On-site tests were conducted in two regions, Namwon, Jeollabuk-do and Gumi, Gyeongsangbuk-do. In Namwon, six farms participated, using 'Hanmaeum', 'Green Power', or 'PS' as the rootstock. In Gumi, one farm participated and used 'Hanmaeum' as the rootstock. On each farm, 1,200 grafted seedlings with one of the melon rootstocks and 1,200 non-grafted seedlings

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were planted in July and harvested in late September. Yield and quality of melons were observed and recorded.

Results & Discussion

In the Namwon region, leaf yellowing symptoms presumed to be caused by CABYV infection were observed on three farms (Table 1). On one of them, Kim's farm, the melon crop failed completely due to severe damage and on Rho's farm, only 31.3% of the harvested fruits were marketable in non-grafted cultivation but 61.9% were marketable in grafted cultivation (by commercial and RDA rootstocks). On Park's farm, 74.2% of the fruits were marketable from non-grafted and 75.3% from grafted plants. The root functions of plants affected by leaf yellowing symptoms was 0.28 $mg \cdot g^{-1}$, about half that measured for the normal unaffected plants (0.48 $mg \cdot g^{-1}$) (Lee et al. 2015). Therefore, it seems that increase of root function by grafting reduces damage caused by leaf

Table 1. Yield of grafted and non-grafted melon in Namwon region.

Farm	Grafting	Rootstock	Yield		Marketable fruit (%)	Remarks
			fruit No./box (8 kg) - No. of boxes	kg		
Park	Grafted	'Hanmaeum'	4 fruits/box - 34 5 fruits/box - 84 6 fruits/box - 58	904	75.3	Leaf yellowing symptoms
	Non-grafted	-	4 fruits/box - 20 5 fruits/box - 102 6 fruits/box - 50	890	74.2	
Ha	Grafted	'Hanmaeum'	3 fruits/box - 25 4 fruits/box - 232 5 fruits/box - 4	1,023	85.3	
	Non-grafted	-	4 fruits/box - 23 5 fruits/box - 161	897	74.7	
Ahn	Grafted	'Green Power'	4 fruits/box - 280 5 fruits/box - 2	1,130	94.2	
	Non-grafted	-	4 fruits/box - 57 5 fruits/box - 157	1,013	84.4	
Rho	Grafted	'Green Power'	4 fruits/box - 82 5 fruits/box - 87	743	61.9	Leaf yellowing symptoms
	Non-grafted	-	5 fruits/box - 75	375	31.3	
Jo	Grafted	PS	4 fruits/box - 230 5 fruits/box - 18	1,010	84.2	
	Non-grafted	-	4 fruits/box - 155 5 fruits/box - 42 6 fruits/box - 8	878	73.2	
Kim	Grafted	PS	-	-	0	Leaf yellowing symptoms
	Non-grafted	-	-	-	0	

Non-grafted plants Grafted plants

Figure 1. Growth and development of grafted and non-grafted plants in Namwon.

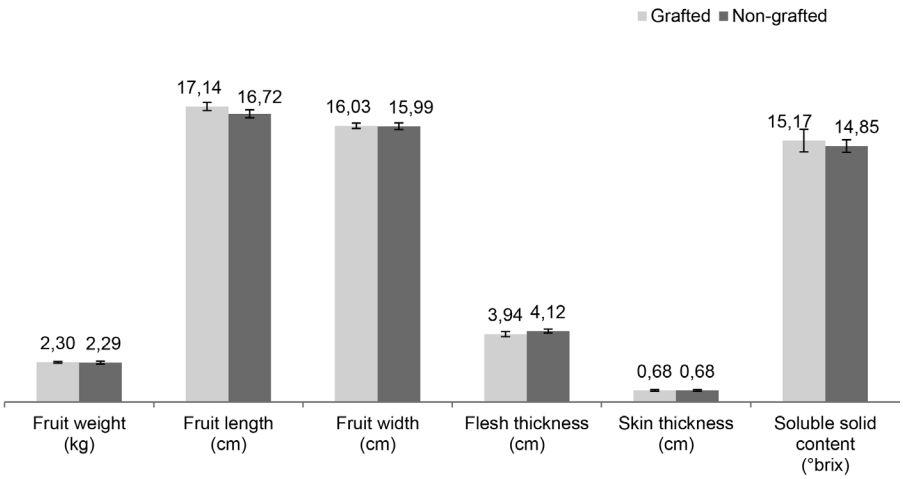

Figure 2. Fruit characteristics and fruit quality of grafted and non-grafted melons in the Gumi region.

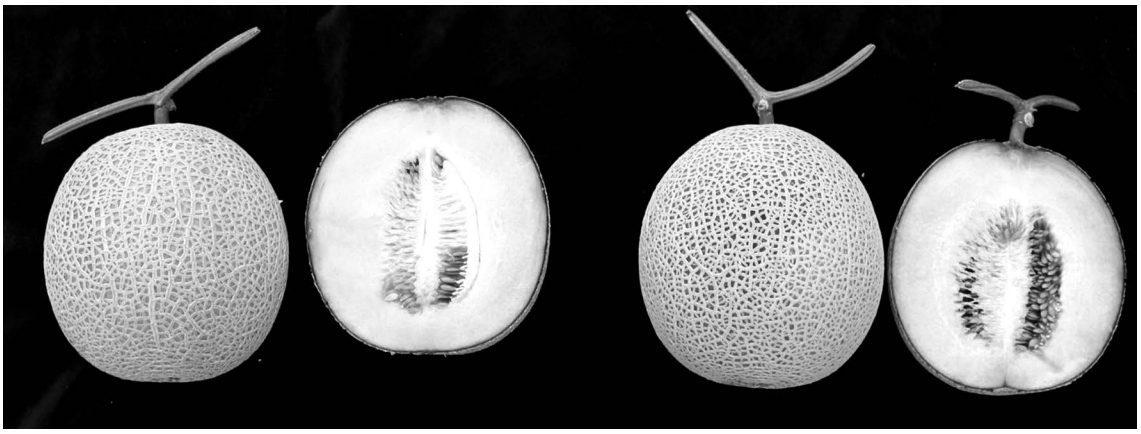

Figure 3. Fruits of non-grafted and grafted melon in the Gumi region.

yellowing. Grafted plants had increased yields by 101% to 198% over non-grafted plants, according to the farm. On all six farms in Namwon, the percentage of big fruits, about 2.0 kg, increased, and the percentage of small fruits, below 1.6 kg, decreased. Wilting did not occur in grafted plants on the farms which had mild wilting in non-grafted plants during the hot season (Figure 1). Sugar contents of fruits did not show differences between non-grafted and grafted plants. However, uniformity of net formation slightly decreased in grafted melons as compared with non-grafted melons. It seemed to be improved by setting of fruits at lower nodes.

In the Gumi region, one melon rootstock was tested on one farm. There were no leaf yellowing symptoms or wilting symptoms in both grafted and non-grafted plants. When the fruit quality of melon was compared, there was no significant differences in fruit size, fruit weight, fruit skin thickness, sugar contents, and net formation between grafted and non-grafted (Figures 2,3). When *Cucurbita* seedlings were used as rootstocks for melon cultivation, the fruit yield and sugar contents were differentially affected by different rootstock cultivars (Lee et al. 2009). Accordingly, the use of multiple disease-resistant melon rootstocks for melon production can increase yield and improve fruit quality.

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Breeding of F_1 Hybrid Melon 'Greenpower' with Multiple Disease Resistance as Rootstock for Melon (*Cucumis melo* L.)

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Abstract. The objective of this study was to develop F_1 hybrid melons resistant to Fusarium wilt and Monosporascus root rot (MRR). Plants of 292 melon accessions were collected and evaluated in soil artificially infested with *Fusarium oxysporum* f. sp. *melonis* (*Fom*) race 1 which was isolated from diseased melon plants grown in Suwon, Korea. Based on germplasm screening procedures, 18 genotypes were found to be resistant to *Fom* race 1. Screening of melon genotypes with resistance to *Monosporascus cannonballus* was conducted in an infested greenhouse. Twenty-seven melon accessions were compared in selecting for resistance to *M. cannonballus*. Eight genotypes exhibited high or moderate resistance against *M. cannonballus.* The resistant plants from the selected accessions were put in an advance trial from S_2 to S_4 generations on the basis of resistance to *Fom* and *M. cannonballus* from each generation. Ten inbreds resistant to both Fusarium wilt and *M. cannonballus* were used as parental lines to produce F_1 hybrid rootstocks for melon. An F_1 hybrid, named 'Greenpower', was developed from the cross of inbreds K153007 and K225290. Yield and quality of fruits harvested from 'Earlselite' (Muskmelon, Reticulatus Group) and 'Homerunstar' (Honeydew type, Inodorus Group) grafted onto 'Greenpower' rootstock were found to be comparable to or better than those of 'Shintozwa' (*Cucurbita* spp.) rootstock and non-grafted melons. However, no fruit fermentation was observed when 'Homerunstar' was grafted onto 'Greenpower' rootstock, unlike when it was grafted onto 'Shintozwa' rootstock where fruit fermentation was observed. The study confirmed the possibility of producing high-quality melons by using multiple disease-resistant melon rootstocks.

Keywords: Soil-borne diseases, *Fusarium oxysporum* f. sp. *melonis, Monosporascus cannonballus*, grafting, fruit quality

Introduction

Melon has been produced mostly in greenhouses for stable production and quality improvement purposes in Korea (Lee et al. 2007, Lee et al. 2009). However, the continuous and intensive cultivation in the same greenhouse causes the increase of pathogen density and salt accumulation in soil, resulting in the incidence of soil-borne diseases and crop yield reduction (Jang et al. 2014). Soil-borne diseases aggravated by continuous cropping cause severe yield loss of melon production in the major melon-growing areas of Korea. *Fusarium oxysporum* f. sp. *melonis* (*Fom*) and *Monosporascus cannonballus* were reported to induce sudden wilting in melon. *Fom* is believed to cause the most destructive disease of melon (Lee 1994, Oda 1995). *Fom* attacks melon at any growth stage, even before sprouting, but mainly when the fruit is ripe. This causes either slow wilting accompanied by progressive yellowing, or a sudden wilting without prior yellowing or other related symptoms (Mas et al. 1981). Based on the host resistance genes associated with variants of this pathogen, *Fom* isolates were classified into four physiological races, designated 0, 1, 2, and 1,2 (Risser et al. 1976).

M. cannonballus was reported to induce sudden wilting in melon which also has become a major production problem worldwide (Martyn and Miller 1996, Heo et al. 2001). Root infection and damage occurs at all developmental stages, but the increase in water demand of the plant during fruit development and maturation can lead to its collapse due to loss of water-uptake capacity, resulting in poor quality melons with small size, and lower sugar content and soluble solids (Martyn and Miller 1996, Martyn 2007).

Grafting has been widely used in order to reduce continuous cropping injuries, together with crop rotation, the use of resistant cultivars, and soil disinfestation. Cucurbit production using grafted seedlings is being safely adapted for organic as well as environment-friendly crop production to minimize uptake of undesirable agrochemical residues (Oda 1995, Lee et al. 2010, Jang et al. 2014). Grafting in cucurbit cultivation makes it possible to increase soil-borne disease resistance and tolerance to environmental stresses such as soil salinity and low temperature. The available rootstock species for melon grafting are melon,

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pumpkin (*Cucurbita* spp.), and wax gourd (*Benincasa hispida* (Thunb.) Cogn.) (Traka-Mavrona et al. 2000). When melons were grafted onto *Cucurbita* rootstocks, the wilt incidence on grafted melon was significantly lower than on non-grafted plants (Cohen et al. 2000, Davis et al. 2008). However, the fruit quality of grafted melons is influenced by the combination of scion and rootstock. When melons were grafted onto the vigorous interspecific hybrid 'Shintozwa' (*Cucurbita maxima* Duchesne × *C. moschata* Duchesne), preharvest internal decay or breakdown and abnormal fermentation occurred in the fruit (Park and Chung 1989, Park et al. 2013). *Cucurbita* rootstocks also reportedly cause decrease of sugar content and the increase of vitrescence (a physiological disorder in which the fruit flesh appears water-soaked) (Davis et al. 2008, Park et al. 2013). Melons grafted onto melon rootstocks are expected to have less horticultural problems related to scion-rootstock compatibility (King et al. 2010). Accordingly, grafting onto resistant melon rootstocks may improve plant resistance without reducing fruit quality and yield. Therefore, this study aimed at developing F_1 hybrid melons resistant to Fusarium wilt and Monosporascus root rot (MRR) for use as rootstocks in melon production.

Materials and Methods

The experiment was conducted at the National Institute of Horticultural & Herbal Science in Suwon, Korea (37°18'23''N, 126°58'40''E).

Selection of melon accessions and F_1 hybrids with multiple disease resistance

Plants of 292 melon accessions were evaluated in soil artificially infested with *Fom* race 1 which was isolated from diseased melon plants grown in Suwon, Korea. Isolates of *Fom* 1 were grown on potato dextrose agar (Difco) in plastic petri dishes of 5.5 cm in diameter at 25 °C for 8 days. Sterile distilled water (5 mL) was added per petri dish, and colonies were scraped off using a sterilized glass rod. The suspension was filtered through two layers of sterile cheesecloth and conidia were adjusted to 10^6 conidia·mL^{-1} using a hemacytometer. The roots of the 20-day-old plants were dipped into suspension of 10^6 conidia·mL^{-1} for one minute and later transplanted to pots. Disease ratings were recorded three weeks after inoculation.

Screening of melon germplasm for resistance to MRR was conducted in an infested greenhouse. Twenty-seven accessions were used in the study. Melon plants were transplanted in a randomized complete block design with three replications per accession. Each bed was 190 cm wide with an intra-row spacing of 45 cm.

The resistant plants from the selected accessions were put in an advanced trial of S_2 to S_4 generations for the purpose of selecting plants highly resistant to *Fom* and *M. cannonballus* from each generation.

Fruit quality and yield of grafted melons in greenhouse cultivation

Three experiments were conducted in unheated polyethylene greenhouses. In all experiments, tolerance to MRR, fruit quality and yield of 'Homerunstar' (Syngenta; Inodorus Group of *Cucumis melo*) and 'Earlselite' (Syngenta; Reticulatus Group) grafted onto 'Greenpower' (a F_1 hybrid melon rootstock with multiple disease resistance), were evaluated. Plants grafted onto the interspecific *Cucurbita* hybrid 'Shintozwa' (Nongwoo Bio), non-grafted 'Homerunstar', and non-grafted 'Earlselite' served as controls. 'Homerunstar' and 'Earlselite' are the most common cultivars being harvested between June and September in Korea. The former is a honeydew type melon with creamy-white color and oblong shape, while the latter is a muskmelon with round shape.

In the first experiment, both the rootstock and the scion were sown at the same time, on March 27, 2013, for synchronization of grafting. Grafting was done on April 6, 2013, at the cotyledon

Table 1. Disease severity of melon genotypes, F_1 hybrid and 'Shintoza' against *Fusarium oxysporum* f. sp. *melonis* race 1 and *Monosporascus cannonballus*.

Accession	Disease severity[z]		Accession	Disease severity	
	FOM[y]	*M. cannonballus*		*FOM*	*M. cannonballus*
'Akbal'	HR	S	PI 414723	HR	HR
'Daejeong'	HR	SR	'Oltin Tepa'	HR	MR
'Hasanbey'	HR	S	'Saxovot'	HR	MR
IT138001	HR	S	'Tuyona'	HR	MR
IT138010	HR	S	'Wondae'	HR	SR
IT190255	HR	S	'Charantais T'	S	S
IT190256	HR	S	'Charantais Fom-1'	S	S
IT190325	HR	S	'Charantais Fom-2'	HR	S
K134068	HR	HR	'Greenpower'	HR	HR
K134069	HR	HR	'Homerunstar'	S	S
K153007	HR	HR	'Earlselite'	SR	S
K225290	HR	MR	'Shintozwa'	HR	HR

[z]Disease severity: HR = highly resistant, MR = moderately resistant, SR = slightly resistant, S = susceptible.
[y]*Fusarium oxysporum* f. sp. *melonis* race 1.

stage. The splice-grafting technique was used in which eight-day-old watermelon seedlings were used for grafting (Lee et al. 2010). The grafted seedlings were transferred into a mist room maintained at a relative humidity of 95% for seven days, and the relative humidity was gradually decreased, for acclimatization, from one week prior to transplanting. 'Homerunstar' and 'Earlselite' seedlings, 37-days-old, grafted and non-grafted, were transplanted on May 3, 2013. Mature fruits were harvested from 12 to 20 July 2013. In the second experiment, the rootstock and scion were sown on March 27, 2014. Grafting work was done on April 15, 2014 at the cotyledon stage. 'Homerunstar' seedlings, 37-days-old grafted and non-grafted, were transplanted on May 14, 2014. Mature fruits were harvested from 25 to 30 July 2014. In the third experiment, the rootstock and scion were sown on July 19, 2014. Grafting work was done on July 28, 2014 at the cotyledon stage. 'Earlselite' seedlings, 26-days-old grafted and non-grafted, were transplanted on August 13, 2014. Mature fruits were harvested from November 5, 2014.

Melon seedlings were transplanted to a greenhouse, in rows with black polyethylene mulching films. Three double-rows (1.5 × 28 m, rows were 0.5 m apart in double-row) were made. The experiment was arranged in a randomized complete block design with three replicates. The melon plants were trained vertically by staking. Manual pollination was conducted for fruit set. One fruit per plant was set on lateral vines between the 10^{th} and 13^{th} nodes of the main vine, with removal of other lateral vines. After fruit set, the apical bud of the main vine was removed at the 10^{th} node above the fruit-bearing node. Cultivation practices were otherwise as described by the Rural Development Administration (RDA) (2005).

The data were statistically analyzed by analysis of variance using SAS version 9.1 software (SAS Institute Inc., Cary, NC, U.S.A.). Duncan's Multiple Range Test was performed at $\alpha = 0.05$ on each of the variables tested.

Results & Discussion

Selection of melon accessions and F_1 hybrids with multiple disease resistance

Fusarium wilt and MRR were reported to induce sudden wilting in melon which has become a major production problem worldwide (Martyn and Miller 1996, Bruton 1998). The incidence of severe foliage wilting at later stages by *Fom* and *M. cannonballus* has markedly increased in some major melon producing areas in Korea (Park et al. 2013).

Plants of 292 melon accessions were collected and evaluated in soil artificially infested with *Fom* race 1 which was isolated from diseased melon plants grown in Suwon, Korea. Based on germplasm screening procedures, 18 genotypes were found to be resistant to *Fom* race 1. Twenty-seven melon accessions were screened for resistance to *M. cannonballus*, of which eight genotypes exhibited high or moderate resistance. The resistant plants from the selected accessions were put in an advanced trial from S_2 to S_4 generations and plants highly resistant to *Fom* and *M. cannonballus* were selected in each generation. As a result, 10 inbred lines were observed to have resistance to both *Fom* and *M. cannonballus* and were used as parental lines to produce F_1 hybrid rootstocks for melon (Table 1). Of these, 'Greenpower' was selected, the result of the cross K153007 × K225290 (Figure 1).

Characteristics related to the fruit of 'Greenpower' are shown in Table 2. Its fruit shape is cylindrical while the color of the mature fruit skin is orange. Soluble solids content of the fruit fresh is about 5.0 °Brix.

Fruit quality and yield of grafted melons in greenhouse cultivation

Wilting symptoms, yield and quality of fruits harvested from 'Earlselite' (Muskmelon, Reticulatus Group) and 'Homerunstar' (Honeydew type, Inodorus Group) grafted onto 'Greenpower' melon rootstocks were recorded in a greenhouse infested with *M. cannonballus*.

Wilting by *M. cannonballus* occurred in non-grafted melons 45 days after transplanting. Non-grafted 'Homerunstar' plants showed high wilting symptoms in spring cultivation of 2013 and 2014, whereas plants grafted onto 'Greenpower' and 'Shintozwa' rootstocks showed lower wilting symptoms. Similarly, non-grafted plants of 'Earlselite' showed high wilting symptoms in spring cultivation of 2013 and in autumn cultivation of 2014, whereas plants grafted onto 'Greenpower' and 'Shintozwa' rootstocks showed lower wilting symptoms (Table 3).

The plants of 'Homerunstar' and 'Earlselite' grafted onto 'Greenpower' and 'Shintozwa' rootstocks exhibited less severe wilting symptoms in spring and autumn cultivation. The low wilting symptoms of 'Greenpower' and 'Shintozwa' rootstocks were attributed to their disease resistance and vigorous root system, respectively (Cohen et al. 2000).

'Homerunstar' grafted onto 'Greenpower' rootstock did not exhibit fruit fermentation and vitrescence, unlike when it was grafted onto 'Shintozwa' rootstock (Table 3). Rouphael et al. (2010) reported that increased vitrescence and the incidence of physiological disorders in grafting of melons would be closely associated with the rootstocks. Likewise, vigorous rootstocks such as *Cucurbita* interspecific hybrids tend to absorb more nitrogen and less calcium into the fruits, thus resulting in premature internal decay in 'Homerunstar' melons (Jang et al. 2014). In this study, fruit fermentation and vitrescence incidences in fruits of 'Homerunstar' grafted onto 'Shintozwa' mainly appeared in spring cultivation. It was considered that the mineral component, particularly the absorption of nitrogen and calcium, were imbalanced due to its vigorous root system.

Table 4 shows the yield and fruit quality of 'Homerunstar' melon influenced by the rootstock in spring cultivation of 2013 and 2014. 'Homerunstar' melons grafted onto 'Greenpower' and 'Shintozwa' yielded from 23 to 55% and from 19 to 47%, respectively, more than non-grafted melons. The 'Homerunstar' melon grafted onto 'Greenpower' rootstock had higher percentages of marketable fruit, compared to grafted onto 'Shintozwa' and non-grafted melons fruits. The 'Homerunstar' melons grafted onto 'Shintozwa' in the spring cultivation of 2013 had the highest soluble solid contents (SSC). However, the SSC of 'Homerunstar' melons grafted onto 'Greenpower' and 'Shintozwa' were higher than that of non-grafted melons in the spring cultivation of 2014.

The yield and fruit quality of 'Earlselite' melon influenced by the rootstock in spring cultivation of 2013 and in autumn cultivation of 2014 are shown in Table 5. The 'Earlselite' melons grafted onto 'Greenpower' and 'Shintozwa' yielded from 27 to 56% and from 38 to 79%, respectively, more than non-grafted melons. The percentages of marketable fruits of 'Earlselite'

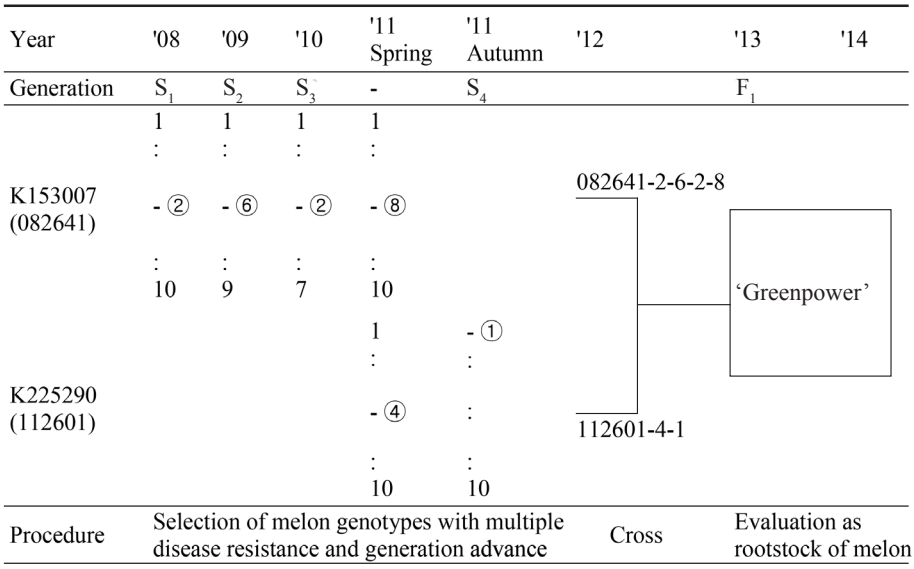

Figure 1. Pedigree diagram of F_1 hybrid melon 'Greenpower'.

Table 2. Characteristics related to fruit of 'Greenpower' (new cultivar) and 'Homerunstar' (control cultivar).

Cultivar	Fruit weight (kg·ea^{-1})	Fruit shape	Ground color of fruit skin		Width of fruit fresh (cm)	Soluble solid contents (°Brix)
			immature	mature		
'Greenpower'	4.0±0.25^{y}	cylindrical	green	orange	3.6±0.15	5.0±0.29
'Homerunstar'	1.8±0.14	round	light green	white	4.1±0.20	12.2±0.55

^{y}Standard error.

Table 3. Wilting symptoms and fruit fermentation of 'Homerunstar' and 'Earlselite' melon grafted onto 'Greenpower' and 'Shintozwa' rootstocks grown under greenhouse cultivation in summer of 2013 and autumn of 2014.

Rootstock	'Homerunstar'				'Earlselite'			
	wilting (%)		fruit fermentation (%)		wilting (%)		fruit fermentation (%)	
	2013	2014	2013	2014	2013	2014	2013	2014
'Greenpower'	3.3	6.7	0.0	0.0	3.3	0.0	0.0	0.0
'Shintozwa'	0.0	3.3	23.3	16.7	3.3	0.0	0.0	3.3
Non-grafted	33.3	50.0	10.0	6.7	46.7	30.0	0.0	0.0

Table 4. Fruit yield and quality of 'Homerunstar' grafted onto 'Greenpower'and 'Shintozwa' in spring cultivation of 2013 and autumn 2014.

Rootstock	Total yield (t·ha^{-1})		Marketable yield (%)		Fruit weight (kg·ea^{-1})		Width of fruit flesh (cm)		Soluble solid contents (°Brix)	
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
'Greenpower'	26.5 a^{z}	26.8 a	86.7	90.0	1.9 a	2.0 a	4.0 ns	4.4 ns	10.2 b	14.2 a
'Shintozwa'	25.6 a	25.4 a	70.0	73.3	1.9 a	1.9 a	4.0	4.4	11.3 a	13.2 a
'Homerunstar' (Non-grafted)	21.5 b	17.3 b	76.7	73.3	1.6 b	1.3 b	3.9	4.3	10.1 b	12.3 b

^{z}Mean separation within columns by Duncan's Multiple Range Test ($\alpha = 0.05$).

Table 5. Fruit yield and quality of 'Earlselite' grafted onto 'Greenpower' and 'Shintozwa' in spring cultivation of 2013 and in autumn of 2014.

Rootstock	Total yield ($t \cdot ha^{-1}$)		Marketable yield (%)		Fruit weight ($kg \cdot ea^{-1}$)		Width of fruit fresh (cm)		Soluble solid contents (°Brix)	
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
'Greenpower'	19.2 a[z]	14.2 a	96.7	93.3	1.5 a	1.1 a	4.0 a	3.2 a	11.7 ns	13.2 a
'Shintozwa'	20.8 a	16.3 a	93.3	90.0	1.6 a	1.2 a	3.8 ab	3.4 a	11.7	13.5 a
'Earlselite' (Non-grafted)	15.1 b	9.1 b	73.3	60.0	1.1 b	0.7 b	3.5 b	2.7 b	12.4	10.6 b

[z]Mean separation within columns by Duncan's Multiple Range Test ($\alpha = 0.05$).

melon grafted onto 'Greenpower' and 'Shintozwa' rootstocks was higher than that of non-grafted melons in both seasons. The SSC of fruits harvested from the 'Earlselite' melons grafted onto 'Greenpower' rootstocks was comparable to or better than that of those grown on 'Shintozwa' rootstocks and non-grafted melons.

The reduction of marketable fruits in 'Homerunstar' melons grafted onto 'Shintozwa' was caused by the fruit fermentation and vitrescence incidence. The reduction of total yield and marketable fruits of two non-grafted melon cultivars was caused by wilting, disease incidence, and small fruits, below 0.7 kg, or fruit cracking.

The SSC of melons grafted onto the *Cucurbita* interspecific hybrid rootstocks was also reported to be reduced or similar to non-grafted melons (Crino et al. 2007, Zhao et al. 2011). However, in this study, the SSC of fruits harvested from the two melon cultivars grafted onto 'Greenpower' and 'Shintozwa' rootstock was higher or comparable to those harvested from non-grafted melons. Rouphael et al. (2010) reported that decreased photosynthesis may be related to low quality. Although the photosynthesis for the two melon cultivars was not examined in this study, the lower SSC of melon fruits grown from the non-grafted plants could be attributed to lower photosynthesis.

In this study, yield and quality of fruits harvested from 'Earlselite' (Muskmelon, Reticulatus Group) and 'Homerunstar' (Honeydew type, Inodorus Group) grafted onto the 'Greenpower' rootstock were found to be comparable or better than those grafted onto the 'Shintozwa' rootstock, and non-grafted melons. Melons grafted onto the 'Greenpower' melon rootstock also had no reduction of fruit quality, expressed as SSC, fruit fermentation, and vitrescence. This study confirms the possibility of producing high-quality melons and increasing marketable yield by using multiple disease-resistant melon rootstocks.

Further breeding for combining desirable characteristics, such as disease resistance, cold tolerance, and salt tolerance could address additional needs for new, improved melon rootstocks for enhancing successful melon production under greenhouse conditions.

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Comparison of Some Cucumber Cultivars for Open-Field Production Present on the Polish National List

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Abstract. Cucumber is one of the most important field crops in Poland. In 2015, there were 78 cultivars of cucumber suitable for open-field production on the Polish National List. The cultivars were arranged into three groups: appropriate for gherkins, pickling, and brining (dill) pickles. Within each group, the yield, its quality, earliness, and susceptibility to two common diseases were evaluated.

Keywords: Cucumber, yield, tolerance, disease

Introduction

Cucumber, *Cucumis sativus* L., is one of the most important vegetable crops in Poland. It is grown on 15,300 ha (2014). The fruits can be harvested as gherkins (3 to 6 cm in length), for pickling (6 to 10 cm), and for brining (8 to 15 cm). There are 78 cultivars of cucumber grown for open field production on the Polish National List (2015). The newest cultivars and some well-known, as standards, were tested and compared.

Materials & Methods

The data consist of cucumber field trials performed from 2013 to 2015. The field trials were conducted in randomized complete block design, with four replicates. For each plot, several measurements were taken. Altogether, 18 cultivars (Table 1) were tested at six experimental stations belonging to COBORU. The cultivars were arranged in three groups: appropriate for gherkins, pickling, and brining (dill) pickles. Seeds were sown in the middle of May on beds containing two rows each with 60 cm spacing; the distance between beds was 120 cm. The tests were conducted according to the best current commercial practices. The harvest started from the first days of July; fruits were picked every 2 to 3 days until the end of August. Early yield refers to the first four harvests from the beginning of the harvest period of the earliest variety. Each year, during vegetative growth, the plants were naturally infected by downy mildew and angular leaf spot. For this reason, an evaluation of plant susceptibility was made. Additionally, after harvest for brining, the fruits were brined and then evaluated after 3 and 6 moths.

Statistical analysis of the results was made using REML (Restricted Maximum Likehood, from statistical package of GenStat). Least significant difference (LSD) among cultivars at the 5% was calculated.

Results & Discussion

Table 2 shows means obtained for yield, yield quality, and susceptibility to downy mildew and angular leaf spot of the gherkins cultivars. Table 3 shows the results for the same parameters of the pickling cultivars and Table 4 for the brining cultivars.

The REML showed significant differences for yield among cultivars in all groups.

Among the gherkins, 'Kajtek' had the highest yield (Table 2). 'Grot' and 'Rufus' had a large proportion of small fruits (3 to 4 cm in length). The most tolerant to downy mildew and angular leaf spot was 'Rodos'. Also, in earlier tests, 'Rodos' was the least susceptible to these diseases (Bartoszak and Frankowska 2011).

Among the cultivars for pickling, 'Traper', 'Kajtek' and 'Szeryf" produced the highest yield (Table 3). 'Ataman' was the earliest one. In some earlier tests, 'Śremski' was also one of the earliest (Bartoszak and Frankowska 2011). 'Traper' had the highest percentage of pickle-size fruits of the total yield, 'Ataman' showed a tendency to bear deformed fruits, and 'Kajtek' bore fruits that were too thick. 'Szeryf" was the most tolerant both to downy mildew and to angular leaf spot.

Among the cultivars for brining, 'Szeryf" produced the highest dill-size yield and 'Ataman' was the earliest but had a higher percentage of deformed and too thick fruits (Table 4). 'Julian' had a large proportion of fruits for brining-size pickles. 'Szeryf" and 'Julian' were the most tolerant to downy mildew and angular leaf spot.

Brining pickles were sensory evaluated after three and six months after start of the natural fermentation (Table 5). As in some earlier tests, 'Śremski' got the best ratings after both, three and six months (Bartoszak and Frankowska 2011).

Most of newer varieties had higher total yields with good quality of fruits and better tolerance to diseases, but were not as early. Some of the tested varieties were suitable for different types of use.

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Table 1. List of tested cucumber F_1 hybrids.

Cultivar	Year listed	Company or institution
Aladyn	1993	Instytut Ogrodnictwa, Skierniewice
Ataman	2012	Przedsiębiorstwo Nasienne „SELECTA" Anna Stolińska
Avatar	2012	SPÓJNIA Hodowla i Nasiennictwo Ogrodnicze, Nochowo
Boztom	2004	Golden Valley Seed
Grot	2005	Krakowska Hodowla i Nasiennictwo Ogrodnicze POLAN, Kraków
Izyd	2000	Instytut Ogrodnictwa, Skierniewice Przedsiębiorstwo Nasiennictwa Ogrodniczego i Szkółkarstwa w Ożarowie Mazowieckim
Julian	1996	Krakowska Hodowla i Nasiennictwo Ogrodnicze POLAN, Kraków
Kajtek	2012	PlantiCo Hodowla i Nasiennictwo Ogrodnicze, Zielonki
Lasso	2008	Hortag Seed
Markus	2005	Krakowska Hodowla i Nasiennictwo Ogrodnicze POLAN, Kraków
Pickling	2002	Kees Broersen Zaden
Rodos	2004	Instytut Ogrodnictwa, Skierniewice Przedsiębiorstwo Nasiennictwa Ogrodniczego i Szkółkarstwa, Ożarów Mazowiecki
Rufus	1997	Krakowska Hodowla i Nasiennictwo Ogrodnicze POLAN, Kraków
Słowiański	2012	SPÓJNIA Hodowla i Nasiennictwo Ogrodnicze, Nochowo
Szeryf	2010	Przedsiębiorstwo Nasiennictwa Ogrodniczego i Szkółkarstwa, Ożarów Mazowiecki
Śremski	1988	SPÓJNIA Hodowla i Nasiennictwo Ogrodnicze, Nochowo
Traper	2012	Szkoła Główna Gospodarstwa Wiejskiego, Warszawa
Yorker	2008	Hortag Seed

Table 2. Results for gherkins F_1 hybrids.

Cultivar	Yield (dt/ha)			Yield quality (% of total yield)				Susceptibility to*	
	total	gherkins	early	3 to 4 cm in length	4 to 6 cm in length	too thick	deformed	DM	AL
Grot	212	180	30	35.1	49.4	5.7	9.8	5.9	5.8
Kajtek	243	203	28	33.4	48.5	8.5	9.6	6.1	6.2
Pickling	204	167	17	32.4	49.4	7.1	11.1	5.9	6.1
Rodos	205	173	10	34.1	49.9	7.8	8.2	6.8	6.6
Rufus	224	186	25	34.7	47.1	9.3	8.9	5.8	6.1
Traper	227	194	27	33.2	52.0	6.9	7.9	5.6	5.8
$LSD_{0.05}$	25	22	4						

*DM = Downy Mildew, AL = Angular Leaf Spot.
9-point scale: 9 = not susceptible, 1 = highly susceptible.

Table 3. Results for pickling F_1 hybrids.

Cultivar	Yield (dt/ha)			Yield quality (% of total yield)				Susceptibility to*	
	total	pickling	early	pickling	too long	too thick	deformed	DM	AL
Aladyn	347	220	21	76.1	2.0	4.4	17.5	6.3	6.5
Ataman	355	241	71	69.1	2.1	7.6	20.9	5.2	5.5
Avatar	305	225	27	75.4	2.2	5.0	17.4	5.9	6.0
Izyd	342	253	24	75.0	1.8	6.5	16.7	6.4	6.3
Kajtek	386	271	37	70.5	1.4	9.4	18.7	6.3	6.3
Markus	318	240	24	76.2	2.9	4.9	16.0	6.2	6.2
Słowiański	321	239	29	75.3	2.2	5.2	17.3	6.1	6.2
Szeryf	359	263	17	75.0	4.2	3.9	16.9	7.0	6.7
Śremski	343	242	35	72.9	3.2	7.7	16.2	5.2	5.6
Traper	372	290	46	79.2	1.6	5.6	13.6	5.6	6.0
Yorker	294	215	29	74.4	3.7	4.9	16.9	5.4	6.0
$LSD_{0.05}$	47	34	8						

*DM = Downy Mildew, AL = Angular Leaf Spot.
9-point scale: 9 = not susceptible, 1 = highly susceptible.

Table 4. Results for brining F_1 hybrids.

Cultivar	Yield (dt/ha)			Yield quality (% of total yield)				Susceptibility to*	
	total	brined (dill) pickles	early	brined pickles	too long	too thick	deformed	DM	AL
Ataman	428	290	128	69.4	0.3	7.5	21.4	4.9	5.6
Boztom	358	270	75	76.6	0.6	3.5	19.3	5.7	6.2
Julian	381	291	42	78.0	0.4	4.8	16.8	6.6	6.9
Lasso	331	244	57	75.3	0.2	5.8	18.5	5.8	6.1
Słowiański	379	277	68	74.0	0.2	5.4	20.4	5.7	6.1
Szeryf	411	305	45	75.9	0.3	3.5	20.3	6.7	6.8
Śremski	399	293	72	74.6	0.3	5.8	19.3	5.2	5.5
$LSD_{0.05}$	55	49	11						

*DM = Downy Mildew, AL = Angular Leaf Spot.
9-point scale: 9 = not susceptible, 1 = highly susceptible.

Table 5. Sensory evaluation of for brining F_1 hybrids.

Cultivar	Sensory evaluation*	
	3 months	6 months
Ataman	4.3	4.4
Boztom	4.1	4.1
Julian	4.3	4.3
Lasso	4.3	4.4
Słowiański	4.4	4.5
Szeryf	4.1	4.0
Śremski	4.5	4.5

*5-point scale: 1 = very bad quality, 5 = the highest quality.

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Detection of Two QTLs Associated with Resistance to *Cucurbit yellow stunting disorder virus* in Melon Line TGR 1551

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Abstract. *Cucurbit yellow stunting disorder virus* (CYSDV), efficiently transmitted by the whitefly *Bemisia tabaci*, is a devastating disease reducing melon (*Cucumis melo*) fruit quality and yield in many areas around the world. Host plant resistance of melon to CYSDV and its vector are high priorities for sustainable melon production in affected production areas. The melon accession TGR 1551, from Zimbabwe, has exhibited a high-level resistance to CYSDV. Resistance to the virus has been evaluated in a RIL population (F_7/F_8) obtained from a cross between TGR 1551 and the Spanish yellow-rinded 'Bola de Oro'. Controlled inoculations using the whitefly as vector were carried out. Four replications (Spring 1, Spring 2, Spring 3, and Summer) have been observed, and yellowing symptoms were scored in all of them; in Summer, virus content was also recorded by RT-PCR. By using a genetic map generated by GBS from 125 RILs, two main QTLs have been identified in Linkage Group V to be associated with CYSDV resistance.

Keywords: *Cucumis melo*, resistance, susceptibility, single nucleotide polymorphisms, recombinant inbred lines

Introduction

Cucurbit yellow stunting disorder virus (CSYDV) greatly reduces quality and fruit yield in melon crops (*Cucumis melo* L.) in almost all areas where melons are cultivated. CYSDV is efficiently transmitted by the cotton whitefly, *Bemisia tabaci*. So far, the main strategy to limit the incidence of virus-infected plants has been the application of insecticides to reduce vector populations, aided to some extent by the use of selected integrated pest management cultural practices. However, due to concerns about the effect of insecticides on pollinators, consumer demand for reduced pesticide use, and the ability of the whitefly vectors to develop insecticide resistance, there is a growing need to develop and deploy strategies that do not rely on insecticides. The reduction in pesticide use will greatly increase the need for genetic resistance to the virus. Resistance combined with selected strategies could become a viable means to increase yields in crops produced in open fields despite the presence of viruses (Lapidot et al. 2014).

Resistance of melon to CYSDV is of high priority for sustainable melon production. There are currently two independent sources of resistance to CYSDV in melon germplasm, line TGR 1551 from Zimbabwe (López-Sesé and Gómez-Guillamón 2000) and accession PI 313970 from India (McCreight and Wintermantel 2008). In both of them, the resistance has a recessive control (McCreight and Wintermartel 2011, McCreight et al. 2016). There are no commercial cultivars resistant to CYSDV probably because the use of this exotic germplasm makes the introgression of resistance difficult. On the other hand, the selection of virus-resistant genotypes in a breeding program is a difficult task because viral symptoms take time to be expressed and susceptible plants can escape infection, a situation often caused by environmental conditions (Mohan et al. 1997, Collard et al. 2005, Shi et al. 2009). To avoid these problems, marker assisted selection (MAS) has been widely used to introduce virus resistance into major crops, including potato (Hämäläinen et al. 1997), soybean (Saghai Maroof et al. 2008, Shi et al. 2009), barley (Jefferies et al. 2003), rice (Sugiura et al. 2004), and tomato (Zamir et al. 1994). Although the choice of marker will obviously depend on the targeted use, microsatellites, single-nuceotide polymorphisms (SNPs), and genotyping by sequencing (GBS) largely fulfill most of the user requirements (Grover and Sharma 2016). However, application requires the previous identification

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of genetic markers tightly linked to the targeted genes. Regarding the resistance to CYSDV, neither mapping nor identification of markers linked to the genes conferring resistance in melon has so far been accomplished. Because the resistance found in TGR 1551 is recessive, which implies greater complexity in the introgression program, the use of molecular markers linked to the gene(s) conferring resistance would facilitate selection of resistant genotypes.

The objective of this work was to perform a quantitative trait loci (QTL) analysis to better depict the genetic architecture of the CYSDV resistance derived from TGR 1551 and to identify molecular markers useful for a MAS program directed to introgress this resistance into elite cultivars.

To attain this objective, a population (F_7/F_8) of recombinant inbred lines (RILs) derived from crossing TGR 1551 with the highly susceptible Spanish yellow-rinded 'Bola de Oro' has been evaluated for CYSDV resistance.

Materials & Methods

A RIL population was developed by the Single Seed Descent (SSD) method. A single F_1 plant derived from a cross between the Zimbabwean genotype TGR 1551 (CYSDV resistant) and the Spanish 'Bola de Oro' (CYSDV susceptible) was used to generate F_2 individuals, which were self-pollinated until the F_7–F_8 generations.

This RIL population, together with the parental genotypes and their F_1, has been evaluated for CYSDV resistance using three or four plants per genotype in four different environments: Spring 1 (86 RILs), in which virus inoculation was carried out using the clip-cage method as described in López-Sesé and Gómez-Guillamón (2000), plants were grown in pots (500 cm^3) filled with soil substrate and maintained in a glasshouse; Spring 2 (101 RILs), in this case the virus was introduced by massive inoculation with viruliferous whiteflies following a modification of the method described by de Ruiter et al. (2008); after the inoculation, plants were sprayed with imidacloprid and transplanted to a plastic greenhouse with sandy soil; in Spring 3 (69 RILs), virus inoculation was carried out using the clip-cage method and then the plants were transferred to a plastic greenhouse with sandy soil; in Summer (89 RILs), the lines were distributed randomly in a plastic greenhouse with bare soil, surrounded by 'Bola de Oro' virus-infected plants. Also, one week after transplanting, six viruliferous colonies of whiteflies were released in the plot to increase the opportunity of the virus to be transmitted. In this case, no spraying against whiteflies was done over the course of the experiment.

Plants were pruned to one branch and the presence of virus symptoms was visually assessed twice a week over a one-month period (Spring 1, Spring 2, and Spring 3) or over a two-month period (Summer). The symptom scoring in Spring 1 and Spring 2 was based on a visual scale of the number of leaves with viral symptoms, ranging from 0 = no symptoms to 9 = leaves with clear virus symptoms. In Spring 3 and Summer the score was based on a visual scale of virus symptoms, ranging from 0 = no symptoms to 5 = almost the entire plant with clear symptoms. Symptom score at 4 (Spring 1, Spring 2, and Spring 3) or 8 weeks (Summer) after inoculations was considered the phenotypic value of the line. Four weeks after the release of viruliferous whiteflies in the Summer experiment, the third leaf from the plant apex of each RIL was taken for RT-PCR analysis. Analysis of variance (ANOVA) was performed to determine the effects of the variable RIL genotypes and environments on the resistance. Comparisons of all pairs of means were performed using Tukey's test with $\alpha = 0.05$. Correlations between pairs of environments were estimated by using the Pearson correlation coefficient. Statistical analyses were conducted using SPSS Statistics software v.21.

Genomic DNAs from parental lines, F_1, and RIL plants were extracted from fresh young leaf tissue using the Plant DNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The quality and quantity of the DNA were examined using a NanoDrop ND-1000 (Thermo Fisher Scientific, U.S.A.), and used for GBS library preparation for SNP discovery by the Institute for Genomic Diversity (Cornell University, Ithaca, NY). SNP-filtered and genetic linkage map construction were performed by the Institute for the Conservation and Breeding of Agricultural Biodiversity (Universitat Politècnica de València, Valencia, Spain).

QTLs for CYSDV resistance were analyzed by composite interval mapping with Windows QTL Cartographer 2.5 (Voorrips 2002) using the developed genetic map. The LOD threshold for a Type I error, $\alpha = 0.05$ value, was calculated by a permutation test with 1,000 permutations independently for each environment. Additive QTL effect (a) and the proportion of phenotypic variance explained by QTL (R^2) were estimated at the highest peaks depicted by the QTL analysis.

Results & Discussion

All the inoculated plants of 'Bola de Oro' were susceptible to CYSDV and showed typical symptoms of yellowing although their intensity was variable depending on evaluations. The TGR 1551 plants showed a resistant response, without any symptoms in most of the cases although a dispersed vein clearing in several leaves could be observed in a few plants. Plants of the F_1 were susceptible, although they showed symptoms later than 'Bola de Oro', and only after four weeks of observation (Summer), which confirmed the recessive inheritance of the character (McCreight et al. 2016).

Normal frequency distribution was observed for Spring 1, Spring 2, and Summer. Spring 3 distribution was slightly deviated from normal distribution, since the number of RILs showing CYSDV symptoms was lower than in other evaluations. In all cases, the symptom scores for the RIL genotypes segregated widely between the parental genotypes and significant differences among environments and RIL genotypes were observed (Table 1).

Virus symptom score averages for Spring 1 (3.85/9) and Spring 3 (1.42/5) were significantly lower than for Spring 2 (4.26/9) and Summer (2.98/5). Pearson's correlation test among the four environments showed positive significant correlations for all of them, ranging from $r = 0.35$ to $r = 0.68$. Spring 2 and Summer evaluations were carried out by massive infestations with viruliferous whiteflies and plants were maintained in a plastic greenhouse; unexpectedly, massive infestation seems to have been a more suitable method than the use of leaf cages to successfully transmit CYSDV. The presence of transgressive genotypes

Table 1. Analysis of variance for resistance to CYSDV based on symptoms in an RIL population derived from the cross TGR 1551 × 'Bola de Oro' and assayed in four different growing conditions: Spring 1, Spring 2, Spring 3 and Summer.

Source of variation	df	Mean Square	F
Environment	3	364.4	242.1***
RILs	121	5.7	3.8***
Error	227	1.5	

df, degrees of freedom; *** Significant differences with $P < 0.001$.

was also noticed with some RIL genotypes showing lower values of disease than TGR 1551 and the presence of genotypes more susceptible than 'Bola de Oro'.

In the Summer experiment, there was the opportunity to compare virus presence with yellowing symptoms in plants. Usually, plants with virus detected by RT-PCR developed typical symptoms of yellowing; however, there were lines in which the virus was detected but no symptoms of yellowing were observed; and, on the contrary, some plants without virus developed yellow spots that could be misevaluated as susceptible because of those symptoms. In general, there was a good correspondence between the symptoms showed by plants and the virus detection by RT-PCR, the correlation of both pairs of values being 0.78. It is possible that the observed overall low level of CYSDV multiplication in TGR 1551 and several RILs is not enough to trigger the cascade of events associated with symptom induction (Maule et al. 2000). In fact, lack of symptom expression has been shown to be accompanied by reduced levels of virus multiplication in other cases (Fraser 1990). As Marco et al. (2003) revealed, the TGR 1551 resistance to CYSDV could restrict movement of the virus in the vascular system of the plants. Alternatively, it may be that the virus still has difficulty replicating even after it moves, such that it accumulates to low levels.

The GBS data of the RILs was utilized for generating the linkage map. The genetic map was constructed using a set of 1,593 SNPs spanning 1,477 cM that were distributed across 12 linkage groups (LGs). For identification of QTLs, the genotyping data were integrated with the phenotyping data of the four environments and of the RT-PCR analysis. QTL analysis indicated the presence of two major QTLs (*cysdvq5.1* and *cysdvq5.2*) mapped on the middle region of LGV (Table 2). One of them (*cysdvq5.1*) has been detected repeatedly in all of the evaluations when symptoms have been scored, and explains different percentages of phenotypic variance depending on evaluation; these percentages ranged between 9.20 to 61.7%, values highly significant. A second QTL (*cysdvq5.2*) was found in the Summer evaluation to be associated with virus titer in plants, which explained 29.8% of the phenotypic variance observed. It seems that there are two genomic regions associated with CYSDV resistance; one of them would be responsible for the resistance to CYSDV, impeding or making the virus multiplication in the plant difficult, and the other one that could be associated with the capability of the virus to spread into the plant and produce the typical symptoms of yellowing. Positive values of additive effects indicated that the TGR 1551 alleles increase the CYSDV resistance (Table 2).

Conclusions

Two QTL regions associated with CYSDV resistance are reported and mapped for the first time. The high value of LOD scores, the high percentage of variability explained, and the stable chromosomal localization of the discovered *cysdvq5.1* in the different environments make this QTL suitable for dissecting the CYSDV resistance. The second QTL, *cysdvq5.2*, associated with the virus titer in plants should be further confirmed using additional phenotypic replications. Further works focused on identification of putative candidate genes and QTL validation using additional populations should be done. This information will be essential for the utility of these QTLs for marker assisted selection in future breeding programs.

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Table 2. Quantitative trait loci (QTLs) associated with the CYSDV resistance, LOD score, additive effects and percentage of variance explained.

QTL name	Evaluation	Linkage group	Interval (cM)	LOD	Additive effect	Variance Explained (%)
cysdvq5.1	Spring 1	5	29.6–33.1	6.57	1.08	28.51
	Spring 2	5	33.5–35.2	20.07	1.99	61.68
	Spring 3	5	33.2–35.0	22.77	1.18	60.46
	Summer[a]	5	31.1–35.2	11.39	1.04	31.00
	Summer[b]	5	29.8–35.2	5.27	1.69	9.20
cysdvq5.2	Summer[a]	5	56.6–59.7	4.49	0.58	9.83
	Summer[b]	5	54.8–63.3	13.47	2.95	29.81

[a]Virus symptom score assessed visually in Summer.
[b] Virus content detected by RT-PCR.

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Mutation in a Conserved Position of 1-Aminocyclopropane-1-Carboxylate Synthase Leads to Andromonoecy in Watermelon

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Abstract. Although it has been reported previously that ethylene plays a critical role in sex determination in cucurbit species, how the andromonoecy that carries both the male and hermaphroditic flowers is determined in watermelon is still unknown. Here we show that the watermelon gene *1-aminocyclopropane-1-carboxylate synthase 4* (*CitACS4*), expressed specifically in carpel primordia, confers andromonoecy in watermelon. Among four SNPs and one InDel identified in the coding region of *CitACS4*, the C364W mutation located in the conserved box 6 co-segregated with andromonoecy. Enzymatic analyses showed that the C364W mutation caused a reduced activity in CitACS4. We believe that the reduced CitACS4 activity may hamper the programmed cell death of stamen primordia, leading to the formation of hermaphroditic flowers.

Keywords: 1-aminocyclopropane-1-carboxylic acid synthase, *Citrullus lanatus*, sex determination

Introduction

1-aminocyclopropane-1-carboxylic synthase (ACS), a key enzyme for ethylene biosynthesis, is important for sex determination in Cucurbitaceae (Boualem et al. 2008, 2009). In cucumber (*Cucumis sativus* L.) and melon (*C. melo* L.), mutations that cause reduced ACS activities lead to andromonoecy (Boualem et al. 2008, 2009). Watermelons, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, have been found in six sex forms, monoecious, andromonoecious, gynoecious, trimonoecious, gynomonocious, and hermaphrodite (Ji et al. 2015). The most common sex form of modern watermelons is monoecious (male and female flowers on the same plant). The next most common is andromonoecious (male and hermaphroditic flowers on the same plant). Recently, a gynoecious (carrying female flowers only) mutant was described (Ji et al. 2015). The recessive gene *a* confers andromonoecy and recessive gene *gy* confers gynoecy. In this study, we identified the *a* gene as *CitACS4* in watermelon, and did a preliminary analysis of CitACS4 function.

Materials & Methods

Plant material

The andromonoecious accession AKKZW (*C. lanatus* subsp. *mucosospermus* Fursa), genotype *a*/*a Gy*/*Gy*, was crossed with monoecious accession XHB (*C. lanatus* subsp. *vulgaris* Schrad.), genotype *A*/*A Gy*/*Gy*, to obtain an F_2 population for testing segregation at the *a* locus. Sex form of the plants was according to the method described by Ji et al. (2015).

Plant genotyping

A 163-bp fragment of *CitACS4* was amplified with primers, and the restriction enzyme *Fsp*BI was used. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used in this experiment are listed in Table 1.

Quantitative RT-PCR

Quantitative RT-PCR conditions were as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s. PCR specificity was verified using a dissociation curve (55 °C to 95 °C). The primers used in this experiment are listed in Table 1.

Enzyme activity assays

Three constructs, *His6-CitACS4, His6-CitACS4*^{C364W}, and *His6-CmACS-7*, were made and transformed into *Escherichia coli*. Fusion proteins were affinity-purified using Ni columns. The ACS enzymatic activity was measured using a buffer

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Table 1. Primers used in this study.

Analysis	Primer name	Sequence (5' to 3')	Fragment size (bp)
Sequence analysis	wma_Cla011230_F	CCAATTTCTATCAAATATGGGCAATG	1720
	wma_Cla011230_R	TCTCTGGATCTTAAAACCCGATT	
Plant genotyping	dCAPs_FspBI_F	CAATAACGGGCTTAAATTCATCCG	163
	dCAPs_FspBI_R	CATGTTGTCGAACCGGAAGTTTAC	
Quantitative RT-PCR	wma_qPCR_F	AACGCCGAGCGAGTCCACAT	180
	wma_qPCR_R	CCGGTTCGACAACATGGACGCTAA	
	ClActin_F	CCTACAACTCAATTATGAAGTGTG	250
	ClActin_R	GAAATCCACATCTGCTGGAAGGTG	
in situ experiment	wma_in suit_F	GATTTAGGTGACACTATAGAATGCTATAGAGTTGTTTTAACTGCTGGTGC	955
	wma_in suit_R	TGTAATACGACTCACTATAGGGATCCTCTCCATGAAGCAATGTATTC	

containing 10 μM pyridoxal 5′-phosphate (PLP) and 200 μM *S*-adenosyl methionine (SAM) (Boualem et al. 2008, 2009).

In situ *hybridization*

CitACS4 in situ hybridization was performed using the method described by Liu et al. (2013). The primers used in this experiment are listed in Table 1.

Results & Discussion

Eight *ACS* genes, *Cla014652* (*CitACS1*), *Cla014057* (*CitACS2*), *Cla006634* (*CitACS3*), *Cla011230* (*CitACS4*), *Cla000483* (*CitACS9*), *Cla011522* (*CitACS10*), *Cla022653* (*CitACS11*), and *Cla006245* (*CitACS12*), are present in the watermelon genome (Guo et al. 2015). Four ACS family members (*CitACS1-4*) are associated with sex determination (Salman-Minkov et al. 2008, Prothro et al. 2013, Guo et al. 2015). CitACS4 shared 94% sequence identity with CmACS-7 in melon at the protein level (Figure 1B). The 1,720 bp genomic region of *CitACS4*, containing two introns and three exons (Figure 1A), encodes a polypeptide with 444 amino acids (Figure 1B). Therefore, it has been proposed that *CitACS4* is the ortholog of *CmACS-7*, so we also suggested that *CitACS4* is the candidate gene for the *a* locus in watermelon (Prothro et al. 2013). To address this possibility, we used polymerase chain reaction (PCR) to amplify the genomic sequences of *CitACS4* from 25 watermelon accessions with different sex forms: 19 monoecious, 1 gynoecious, and 5 andromonoecious (Figure 1A). Among five polymorphic sites with either single nucleotide polymorphisms (SNPs) or insertion-deletions (InDels) in *CitACS4*, only the SNP of G1477C co-segregated with andromonoecy (Figure 1A). The mutation caused a cysteine (C) to tryptophan (W) substitution at residue 364 (named C364W; Figure 1B). The genotypic analysis of 440 F_2-population plants showed that all 107 andromonoecious plants co-segregated with the *a/a* genotype of dCAPs_FspBI, while none of the remaining 333 plants showed andromonoecy, suggesting a tight link of the C364W substitution with the *a* locus (Figure 1C).

C364W is located in box 6 (Figure 2A), of a total of 12 conserved boxes of ACS protein (Rottmann et al. 1991). It has been shown previously that the G33C, P209S, and S399L mutations in cucumber ACS and the A57V mutation in melon all reduced ACS enzymatic activities, and plants carrying these mutations showed andromonoecious sex expression (Boualem et al. 2008, 2009). To determine if the C364W mutation had compromised the enzymatic activity of ACS, three constructs, *His6-CitACS4,* *His6-CitACS4*^{C364W}, and *His6-CmACS-7*, were made to analyze the activity of ACS. The results showed that the ACS activity of *His6-CitACS4* was similar to that of *His6-CmACS-7*, but the ACS activity of *His6-CitACS4*^{C364W} was significantly reduced, suggesting that the C364W mutation compromised the enzymatic activity of CitACS4 (Figure 2B). Referred to as the LeACS8 in tomato (Huai et al. 2001), the 3-dimensional modeling of CitACS4 showed that the C364 residue is located in the α-carboxylate backbone (Figure 2C,D). The cysteine residue possesses a mercapto group that can potentially form a disulfide bond to maintain protein stability in this model (Figure 2D). The C364W substitution may have disrupted the stability of CitACS4, and subsequently damaged the activity of the enzyme.

Total RNA from different parts of the watermelon plant was extracted to examine the expression pattern of *CitACS4*. Quantitative real-time PCR assays were performed and the results showed that *CitACS4* was expressed specifically in female and hermaphroditic flowers (Figure 2E–G). According to the stages defined for flower development in cucumber (Bai et al. 2004), *CitACS4* was expressed primarily in stage 6 female and hermaphroditic flower buds, and lower levels of expression were detected in flowers of stages 10 and 14 (Figure 2E,F). RNA *in situ* hybridization analysis revealed that *CitACS4* was expressed specifically in the carpel primordia of female and hermaphroditic flower buds at stages 5 and 6, while no expression was detected in male flower buds. The *CitACS4* expression pattern is similar to that of *CsACS2* in cucumber and *CmACS-7* in melon (Boualem et al. 2008, 2009).

The production of ethylene in carpel primordia of female flowers, mediated by the ACS activity, triggers programmed cell death (PCD) in stamen primordia (Bai et al. 2004). In this

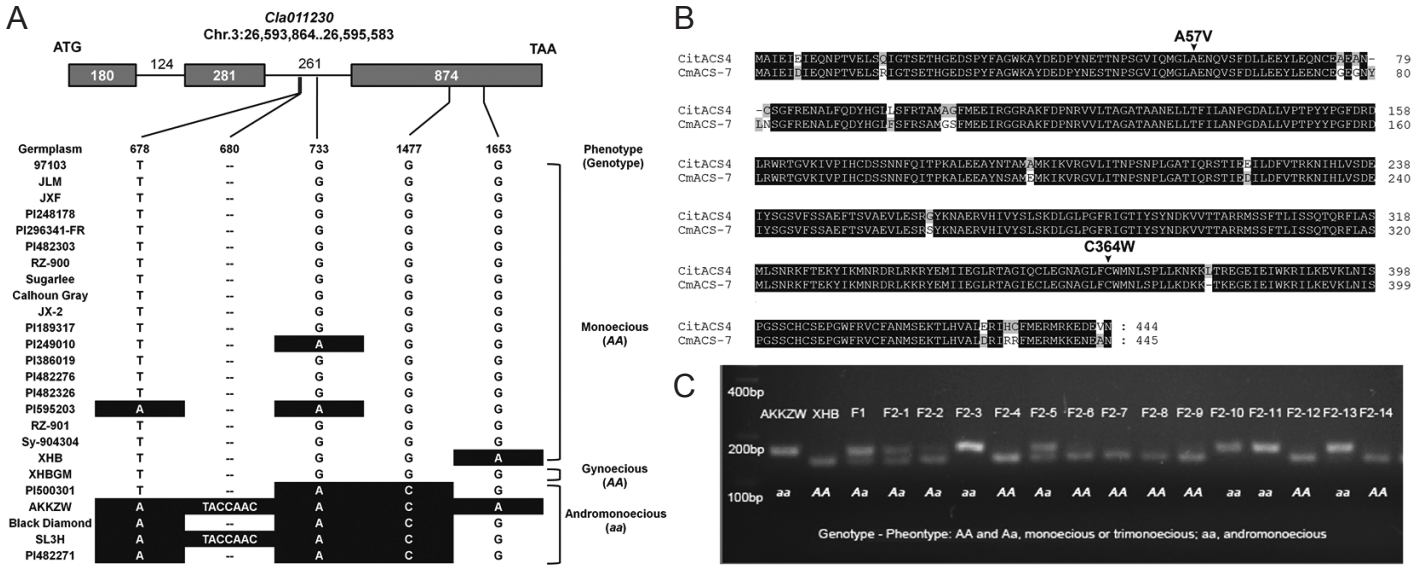

Figure 1. The identified analysis for *CitACS4*. (A) Sequence analysis of *CitACS4*. (B) Alignment of CmACS-7 and CitACS4 proteins. A57V and C364W indicate the amino acid changes associated with the andromonoecious genotype in melon and watermelon, respectively. (C) Analysis of the F_2 population using the dCAPs_FspBI marker.

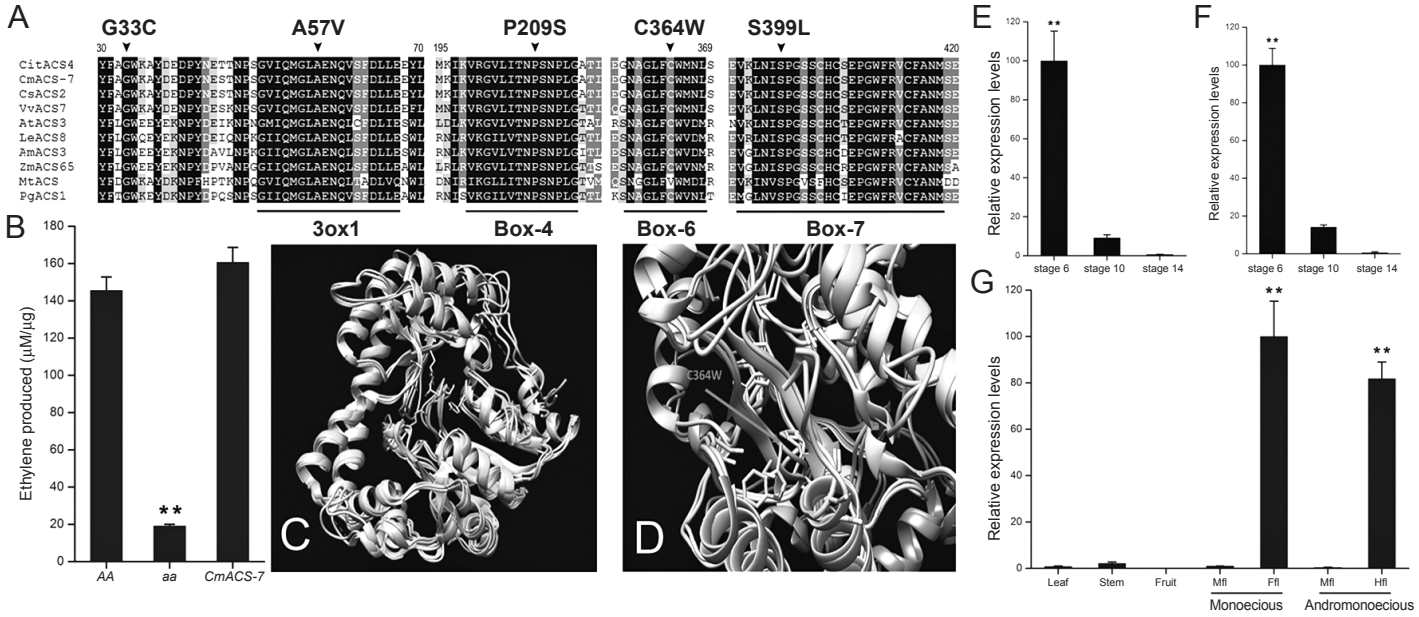

Figure 2. Function analysis of *ClWIP1*. (A) Alignments of CitACS4, CmACS-7, CsACS2 and homologus proteins from AmACS3 (*Antirrhinum majus*, AAC70353), AtACS3 (*Arabidopsis thaliana*, AF322390), LeACS8 (*Lycopersicon esculentum*, AF179247), MtACS (*Medicago truncatula*, AAL35745), PgACS1 (*Picea glauca*, ABM60747), VvACS7 (*Vitis vinifera*, CAN66901), and ZmACS65 (*Zea mays*, AAR25560). G33C, P209S, and S399L are mutations in CsACS2, A57V is the mutation in CmACS-7, and C364W is the mutation in CitACS4. All these mutations lead to andromonoecy. (B) Enzyme activities of His6-CitACS4, His6-CitACS4^{C364W}, and His6-CmACS-7 produced in *E. coli*. Note that amounts of ethylene produced by CitACS4^{C364W} is significantly lower (indicated with**) than those produced by His6-CitACS4 and His6-CmACS-7. AA: His6-CitACS4; aa: His6-CitACS4^{C364W}; CmACS-7: His6-CmACS-7. (C) Superposition of the ACS structure determined using x-ray crystallography in tomato and indicated in white, and the three 3D models of CitACS4, indicated in blue, green, and purple. (D) Magnification of the ACS active site. C^{364} form is indicated in white, and W^{364} form is indicated in blue. Expressiom analysis of *CitACS4* in female (E) or hermaphroditic (F) flowers. In stage 6 flower buds, *CitACS4* expressions in female (E) and hermaphroditic (F) flowers are both significantly higher (indicated with **) than in stage 10 and stage 14 buds. (G) Expression analyses of *CitACS4* in different organs. Note that, in stage 6 floral buds, *CitACS4* expressions in female (Ffl) and hermaphroditic (Hfl) flower buds are significantly higher (indicated with **) than those in male flower buds (Mfl) and any other organs tested.

study, we showed that andromonoecy in watermelon is caused by a recessive mutation in *CitACS4*. The compromised enzymatic activity of CitACS4 may cause reduced ethylene production in carpel primordia, leading to the formation of flowers with both male and female organs. This finding may potentially be used in breeding and genetic improvement of watermelon in the future.

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Breeding of New Watermelon Hybrid 'Shenmi-968' with Disease Resistance and High Quality

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Abstract. The objective of this study was to break the linkage drag between disease resistance and poor quality and improve both disease resistance and quality traits for watermelons adapted to protected cultivation. Both seedling testing in the greenhouse and adult plant testing in the field disease nursery were employed to identify resistance to *Fusarium oxysporum* f. sp. *niveum* (Fusarium wilt). Multiple parents and four-way crosses were used to develop inbred lines with the pedigree method. Then, inbred lines were test-crossed to develop hybrids with strong resistance to Fusarium wilt and good quality. A hybrid with high quality and resistance to Fusarium wilt, 'Shenmi-968', was developed and released in 2014. In multiple regional tests, 'Shenmi-968' has shown strong resistance to multiple diseases and broad adaptation. In the continuous cultivation field, its incidence of Fusarium wilt was less than 5%. It is medium-early, ripening about 33 days after flowering. It has a high fruit setting ability under cool and weak light conditions in the spring in southern of China. Its fruit shape is round-oval sphere, size ranges from 5.0 to 8.0 kg. It has a high yield potential, exceeding 49 $t \cdot ha^{-1}$. It has light green skin with dark green stripes and dark pink flesh. The fruit flesh is very tasty, juicy, and delicate with over 12.5% soluble solids content in the center and over 8.5% near the rind. This new hybrid variety is suitable for protected cultivation in spring, summer, or fall in China. 'Shenmi-968' has good quality, high yield, strong resistance to Fusarium wilt, and broad adaptation. It has been planted on over 1,000 ha in several provinces in China, including Shanghai, Jiangsu, Zhejiang, Anhui, and Yunnan.

Keywords: Watermelon, hybrid variety, disease resistance, Fusarium wilt, fruit quality

Introduction

For the past 20 years, crop rotation in China has become very difficult because more and more hectares of protected cultivation are devoted to watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai. Continuous cropping of watermelon has caused epidemics of Fusarium wilt (*Fusarium oxysporum* Schlecht. f. sp. *niveum*), gummy stem blight (*Didymella bryoniae*), and anthracnose (*Colletotrichum orbiculare*). Most recently, Fusarium wilt has caused great economic losses and has become the most severe disease in watermelon production. Host resistance is the most economic and effective way to control diseases and reduce chemical applications.

F. oxysporum f. sp. *niveum* (Fom) is a soil-borne fungus. It causes great yield and economic losses. Since Orton started breeding for Fusarium wilt resistance over a century ago, there have been numerous great achievements in the U.S.A., where many watermelon varieties with Fusarium wilt resistance and good horticultural traits have been developed. Some of those U.S. varieties have been used as parental lines in breeding programs in other countries (Norton et al. 1993, 1995, Crall et al. 1994, Wang et al. 1996). The research on Fusarium wilt in China started in the 1980s. Since then, studies on pathogen identification, race classification, and resistance source screening have been conducted (Xu and Yang 1992, Gu et al. 1994, Wang et al. 2003). Using U.S. sources of resistance, several wilt-resistant varieties have been developed in China, including 'XiNong 8', 'ZheKang 2', 'KangBinSuMi', 'JinKang 1', and 'JinKang 2'. These varieties have made great contributions to watermelon production in China (Huang and Zhang 1993, Wang 1993, Zhou 1993, Xu et al. 1995, Dai et al. 1998, Yu and Na 2001, Gu et al. 2006). However, these Chinese varieties have late maturity, low fruit-setting percentage, poor quality, and are not well-adapted to protected cultivation. The current market requires new varieties with early maturity, good quality, and disease resistance. Previous studies showed linkage drags between good quality and disease resistance, which is why most disease-resistant varieties have inferior quality (Zhou and Kang 1996a, 1996b, Zhang et al. 1999). The objective of this

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work was to develop new hybrid varieties combining disease resistance and good quality. For this purpose, crosses with multiple parental lines were designed, selections were made using the pedigree method, disease resistance was screened at both seedling and adult plant stages, and newly developed lines were tested at multiple locations and over several years.

Materials & Methods

Identification and selection of disease-resistant watermelon germplasm

From 1988 to 1993, 11 *Fom* strains were collected. Through inoculation tests, a strain referred to as Js-3 was identified as the most pathogenic. Subsequently, Js-3 was used to test 176 watermelon accessions using a standard procedure of root-soaking inoculation at the seedling stage (Crall et al. 1994). This test had three replications, each replication containing 30 inoculated seedlings, and using 'Calhoun Gray' as a resistant control and 'Sugar Baby' as a susceptible control. The inoculum concentration was 5.5×10^5 spores per ml. Wilted seedlings were counted from the 14th day after inoculation, and continually thereafter, every other day, ending on the 30th day after inoculation. The accessions were scored as highly resistant (< 20% of the plants wilted), moderately resistant (21 to 50% of the plants wilted), moderately susceptible (51 to 80% of the plants wilted), and susceptible (81 to 100% of the plants wilted). Based on the seedling test, 'Dixilee', 'Sugarlee', 'AU-Producer', and 'All-Sweet Scarlet' were identified as highly resistant. The resistant seedlings of these four cultivars were transplanted to the field disease nursery for adult plant testing. The surviving plants were self-pollinated and seeds were harvested. After three generations of testing and selection, four re-selected lines having good disease resistance were developed, including 'Dixilee-1' with Fusarium wilt resistance and large fruit, 'Sugarlee-3' with Fusarium wilt resistance and high sugar content, 'AU-Producer-7' with Fusarium wilt resistance and large fruit, and 'All-Sweet Scarlet-4' with Fusarium wilt resistance, high sugar content and intense red flesh.

Breeding new watermelon inbred lines with disease resistance and high quality

Using 'Sugarlee-3', 'Dixilee-1', 'AU-Producer-7', and 'All-Sweet Scarlet-4' as core disease-resistant parental lines, a multi-parent crossing method was used to combine their disease resistances. The resulting F_1s were selfed for several generations and progenies were tested for disease resistance as seedlings and as adult plants. New lines were selected combining disease resistance with good quality (fruit center sugar content > 10%, moderate fiber content) and high yield. Then these newly developed lines were used as disease-resistant parents to cross with other parental lines that had good quality, early maturity, stress tolerance (tolerance to cool and weak light conditions, or heat), and high fruit-setting rate to further improve their horticultural traits. Self-pollination and pedigree selection were employed to develop inbred lines with multiple-disease resistance, good quality, and high yield. The inbred lines were then test-crossed to identify hybrids with strong resistance to Fusarium wilt and good fruit quality. After 17 years of breeding, a new hybrid, designated 'Shenmi-968', with high quality and Fusarium wilt resistance, was developed. 'Shenmi-968'was released in 2014.

Results

The breeding process of 'Shenmi-968'

Breeding of new watermelon inbred lines with multiple disease resistance

A four-way cross of 'Sugarlee-3' × 'Dixilee-1' × 'AU-Producer-7' × 'All-Sweet Scarlet-4' was made and then selfed for six generations. The screening for disease resistance started in the F_2 generation with both seedling and adult plant testing. An independent culling selection approach was used to select traits, with the order of disease resistance, good quality, and high yield. Two new inbred lines, designated V13-1 and V13-7, were developed with high resistance to Fusarium wilt, moderate resistance to gummy stem blight and anthracnose, good quality (fruit center sugar content > 10%), and high yields (single fruit weight > 6 kg). However, both V13-1 and V13-7 have undesirable traits, including late maturity (growing period > 115 d), low fruit-setting percentage (76% and 89%, respectively), low fruit center sugar content (< 11%), and more fiber in the fruit flesh, and thus are in need of further improvement.

Improvement of inbred V13-7

Inbred V13-7 was used as a male parent to cross with 'Hongbaoshi', introduced from Japan, which has early maturity, good quality, tolerance to cool and poor light, and high fruit-setting percentage. Self-pollinations for several generations resulted in the development of inbred lines that combined Fusarium wilt resistance, good quality, high yield, and high fruit-setting. Both seedling and adult-plant testing were used to screen for Fusarium wilt resistance. Progenies were selected with an independent culling method for horticultural traits in the order of good quality, early maturity, high yield, and disease resistance. A new $F_{2:7}$ inbred line designated W6-9-1 was developed. W6-9-1 has early maturity, good quality (tender, juicy and refreshing flesh with fruit center sugar content > 12.8%), high fruit-setting (1.2 fruits per plant), and moderate resistance to Fusarium wilt and gummy stem blight.

Improvement of inbred V13-1

Inbred V13-1 was crossed as a female parent with 'Sugarlee-3', which has good resistance to Fusarium wilt and high sugar content. The F_1 was top crossed with accession WJ9-2, which has mid-season maturity, high sugar content, high fruit-setting rate, and tolerance to heat. The progeny was self-pollinated for seven generations. Both seedling testing and adult-plant testing were used to identify the resistance to Fusarium wilt, and an independent culling approach was used to select horticultural traits with the order of disease resistance, good quality, and high yield. A new inbred line, designated V13-9-3, was developed with strong resistance to Fusarium wilt, moderate resistance to gummy stem blight and anthracnose, good quality (fruit center sugar content was 11.5%), high fruit-setting rate (more than one fruit per plant), tough rind, and medium-late maturity (growing period > 105 d).

Development and evaluation of a new hybrid

A new hybrid, designated WF29-9, was obtained by crossing inbred W6-9-1 as a female parent with inbred V13-9-3 as a male parent, in the spring of 2009. The performance tests for this hybrid started in 2010. In the tests, this hybrid showed strong resistance

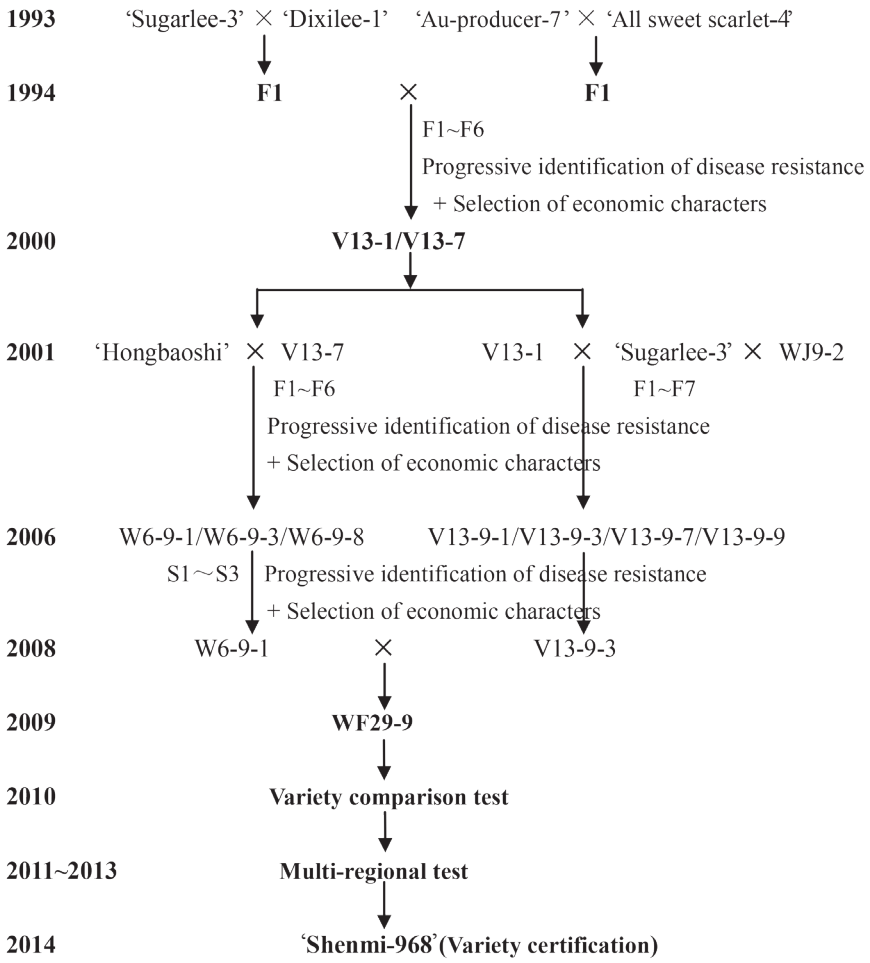

Figure 1. Breeding process for new watermelon variety 'Shenmi-968'.

to Fusarium wilt, moderate resistance to gummy stem blight, high fruit-setting rate (1.12 fruits per plant), and large fruits (single fruit weight of 5 to 8 kg). Its fruit shape is oval-spherical and the rind is green with dark green stripes. Its flesh is dark pink, with sugar content in the center > 12.5%. Its flesh flavor is very tender and crispy. Its rind is tough and suitable for storage and transportation. Multi-regional tests of hybrid WF29-9 were conducted in 2011 and 2012, and the multi-production tests were conducted in 2013 and 2014. All of the test results showed that this new hybrid has good horticultural traits such as disease resistance, abiotic stress tolerance (tolerance to cool and poor light), high yielding, and good quality. This new hybrid was approved for release by the Shanghai Crop Variety Release Committee and named as 'Shenmi-968' in 2014. The breeding process of 'Shenmi-968' was shown in Figure 1.

Multi-regional test of 'Shenmi-968'

Growth period and fruit quality of 'Shenmi-968'

When planted in the spring season, the growth period of 'Shenmi-968' is about 102 d, and the fruit development period is about 32 d. Fruit shape is very slightly longer than broad, thus can be described as "high-round sphere". Its rind is tough with a thickness of about 1.06 cm. The flesh color is dark pink, and is very delicate and crispy due to its low fiber content and high soluble solids content (13.2% in the center, and 8.8% just under the rind).

Performance of disease resistance

The resistance to Fusarium wilt of 'Shenmi-968' and 'Zaojia' was evaluated with seedling tests in 2011 and 2012 (Table 2). The percentages of wilted plants for 'Shenmi-968' were 7.5% and 10.6%, which allows classifying the line as highly resistant. But for the check variety 'Zaojia', the percentages of wilted plants were 69.2% and 73.2%, based on which data this line classifies as susceptible to Fusarium wilt. In the multi-regional tests of 2011 and 2012, 'Shenmi-968' had an average disease index of 1.2 and 3.6 for gummy stem blight, and 1.7 and 2.2 for anthracnose, indicative of moderate resistance to both diseases.

Yield performance of 'Shenmi-968'

The regional test results in 2011 and 2012 are shown in Table 3. The average fruit number per plant for 'Shenmi-968' was 1.18, and average fruit weight was 5.41 kg, which was heavier than 'Zaojia' by 1.1 kg. Its average yield was 49.57 $t \cdot ha^{-1}$, which was 23.4% more than 'Zaojia'.

Table 1. Growth period and quality for 'Shenmi-968' and control variety 'Zaojia'.

Cultivar	Year	Growth period (d)	Fruit development period (d)	Fruit shape index	Rind thickness (cm)	Flesh color	Soluble sugar content (%)		Flavor	Fruit cracking rate (%)
							side	center		
	2011	102.2	32.3	1.06	1.06	pink	8.5	12.9	delicate, crispy	0
'Shenmi-968'	2012	101.9	31.9	1.05	1.05	pink	9.0	13.3	delicate, crispy	0
	average	102.1	32.1	1.06	1.06	pink	8.8	13.2	delicate, crispy	0
	2011	100.1	30.7	1.00	0.95	pink	8.5	12.5	crispy tender	17.7
'Zaojia'	2012	98.7	29.6	0.95	0.85	pink	8.5	12.8	crispy, tender	20.6
	average	99.4	30.2	0.98	0.90	pink	8.5	12.6	crispy, tender	19.2

Table 2. Disease resistance rating** in regional tests, Shanghai City, China.

Cultivar	Year	Fusarium wilt		Gummy stem blight		Anthracnose	
		wilt rate (%)	resistance grade	disease index (DI)	resistance grade	disease index (DI)	resistance grade
'Shenmi-968'	2011	7.5	HR	1.2	HR	1.7	MR
	2012	10.6	HR	3.6	MR	2.2	MR
	Average	9.1	HR	2.4	MR	1.9	MR
'Zaojia' (control)	2011	69.2	HS	5.1	S	3.7	S
	2012	73.2	HS	7.5	S	5.8	S
	Average	71.2	HS	6.3	S	4.7	S

**Disease rate (DR) of Fusarium wilt was identified using 0 to 100%. HR = means with a wilting rate lower than 20%; MR = means with a wilting rate between 21 and 50%; S = means with a wilting rate between 51 and 80%; HS = means with a wilting rate between 81 and 100%.

Disease index (DI) of gummy stem blight was identified using a 0 to 9 scale: 0 = immune; 1 = leaves infected, but stem immune; 3 = leaves infected severely, but stem immune; 5 = stem infected lightly; 7 = stem infected severely; 9 = plant died. DI = 0, 1, and 2 mean highly resistant (HR), DI = 3, 4, and 5 mean moderately resistant (MR), and the others mean susceptible.

Disease index (DI) of anthracnose was identified using a 0 to 5 scale: 0 = immune; 1 = necrosis of the leaf area 0 to 20%; 2 = necrosis of the leaf area 21 to 40%; 3 = necrosis of the leaf area 41 to 60%; 4 = necrosis of the leaf area 61 to 80%; 5 = necrosis of the leaf area 81 to 100%. DI = 0 or 1 means high resistance to anthracnose, DI = 2 or 3 means moderate resistance to anthracnose, and others mean susceptible.

Discussion

Watermelon is an important horticultural crop with a high commercial value. Consumers focus on quality while producers lay emphasis on the yield. The most effective way to satisfy both consumers and producers is to develop new watermelon varieties with high yield, disease resistance, and good quality. The prerequisite for development of these new varieties is to obtain watermelon germplasm with high yield, disease resistance, and good quality. Fusarium wilt in watermelon is conferred by a single dominant gene (Netzer and Weintall 1980, Zhou and Kang 1996a, 1996b, Xiao et al. 1998, 2000, Zhang et al. 1999), therefore, it should be relatively easy to combine Fusarium wilt resistance with other resistances by means of multi-parent crossing, backcross, and pedigree methods.

In this project, multi-parent crossing, and backcross and pedigree methods, were used to develop new disease-resistant germplasm, and also to simultaneously improve quality traits and yield. Through several generations of self-pollination and selection with the pedigree method, the undesirable association between disease-resistance and poor quality was broken and two new watermelon inbred lines, W6-9-1 and V13-9-3, were developed. A new hybrid, 'Shenmi-968', was created from crossing between W6-9-1 and V13-9-3. This hybrid has improved and multi-disease resistance, and good quality. It has been planted on more than 1,000 ha in Shanghai, Jiangsu, Zhejiang, and Anhui in China. Due to the limitations in modern molecular plant breeding techniques, in order to improve watermelon germplasm, traditional breeding techniques including multi-parent crossing, backcrossing pedigree breeding are still the most practical and effective ways.

Table 3. Results from five localities in regional tests conducted in 2011 and 2012 in Shanghai City, China.

Cultivar	Year	Mean fruit weight (kg)	Fruit no. per plant	Mean yield ($t \cdot ha^{-1}$)	Increase rate for yield over control (±%)
'Shenmi-968'	2011	5.34	1.21	50.37	+ 23.4**
	2012	5.47	1.16	48.76	
	Average	5.41	1.18	49.57	
'Zaojia' (control)	2011	4.25	1.32	40.09	
	2012	4.36	1.22	40.26	
	Average	4.31	1.27	40.18	

**$P < 0.01$

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A New Type of Semi-Bush Habit Processing Cucumber (*Cucumis sativus*) for Simplified Fruit Harvest

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Abstract. For many years, attempts have been made in the Department of Plant Genetics, Breeding, and Biotechnology, Warsaw Life Sciences University, to obtain a new type of cucumber, fit for mechanical field harvest. The main objective was to develop strongly vigorous plants with shorter internodes, allowing for an increased plant density and a higher concentration of fruiting. Four new hybrids (nos. 26, 28, 29, and 30) were obtained by crossing a female bush habit accession, R39, with four monoecious sublines selected from accession W-19. These were then compared, over two years, with two Polish widely grown commercial hybrids, 'Szeryf' and 'Śremski'. Plants were harvested at a low frequency, every three to four days. Hybrid no. 26 had significantly higher commercial yields, 528 kg/100 m^2 (2014) and 442 kg/100 m^2 (2015), as compared with 'Śremski', 428 kg/100m^2 (2014) and 336 kg/100m^2 (2015). As compared with 'Śremski', the hybrid plants had shorter main stems (by 33%, 111 cm) and branches (by 43%, 454 cm), fewer leaves (by 40%, 93), and shortened leaf petioles (by 33%, 15 cm). These high-yielding hybrids can be planted at higher densities than standard commercial hybrids.

Keywords: Field cucumber, semi-bush-type growth, plant architecture

Introduction

Cucumber has one of the highest consumption levels of fresh vegetables in Poland. In 2014, it was estimated that 13% of the total consumption of fresh vegetables and mushrooms (yearly 6.24 kg per capita) were cucumbers, second only to tomato (Dudkowska and Strelczuk 2015).

Cucumber cultivation largely relies on manual harvest. The estimated cost of manual harvesting amounts to as much as 70% of the direct cost of cucumber production and up to 50% of the total cost of the field cucumber production in Poland (Dudkowska and Strelczuk 2015). In view of the growing labour cost, it seems necessary to reduce its input at harvest. For many years now, the Department of Plant Genetics, Breeding, and Biotechnology, Warsaw Life Sciences University, has endeavored to obtain cucumber plants suitable for simplified field harvesting.

In this setting, the aim of the present work was to evaluate important traits of four semi-bush habit experimental hybrids in comparison with two typical commercial Polish cucumber hybrids. One of the commercial hybrids, 'Śremski', has the typical viney plant habit and 'Szeryf' has a semi-bush plant habit that is more pronounced than in the new experimental hybrids.

Materials & Methods

In 2014 and 2015, trials were conducted at the Department of Plant Genetics, Breeding, and Biotechnology Experimental Field at Wolica. Tested were four new hybrids (nos. 26, 28, 29, and 30), obtained from crossing a female vigorous bush accession of unknown origin, designated R39, with each of four monoecious accessions of the W-19 type. Two Polish commercial hybrids, 'Śremski' (a typical vine, early, vigorous, processing form) and 'Szeryf' (pronounced semi-bush habit, more so than in the new hybrids) served as the standards. The trials were designed as randomized blocks in three replications. Seeds were sown directly into the soil on May 19 at 135 × 25 cm spacing, and the plot area was 10.1 m^2. Cultural practices were in accordance with recommended procedures. The harvesting frequency was limited to twice a week, every 3 to 4 days. The harvest period was from July 14 to August 24 (a total of 14 harvests). The fruits were sorted by fractions as recommended by the Research Centre for Cultivar Testing (COBORU). Data were calculated as the total, commercial, and early commercial yield (up to August 1).

At the end of the harvest period, the growth habit of the forms tested was examined by measuring 8 plants per replication.

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The number of lateral branches, internodes, and leaves per plant were counted. The length of the main stem, lateral branches, and their internodes were measured. Length and width of the leaf laminae and petioles were measured, using the 20th leaf from the apical meristem. The commercial fruit shape coefficient was calculated, based on the maximal length and width of 30 fruits for each of the tested varieties. Statistical analysis was conducted using the Statistica 12 programme and statistical differences were evaluated by Tukey's test ($\alpha = 0.05$).

Results

Fruit yield

In both years, the highest yield was produced by hybrid no. 26 528.7 kg/100 m^2 (2014) and 441.9 kg/100 m^2 (2015) (Table 1). The commercial hybrid 'Śremski' had significantly less, 427.7 kg/100 m^2 (2014) and 335.9 kg/100 m^2 (2015). The early commercial yield of hybrid no. 26 was also high (224 kg/100 m^2 in 2014 and 215 kg/100 m^2 in 2015), more than that produced by the early-producing 'Śremski' (207 kg/100 m^2 in 2014 and 164 kg/100 m^2 in 2015). Hybrid no. 26 also had a remarkable marketable yield percentage (67.2–67.5%), exceeding that of 'Śremski' (48.1 to 57.7%) (Table 1).

In order to measure marketable fruit yield, it was necessary to determine the fruit shape coefficient. In the consumers' opinion, the most preferable fruit shape coefficient is 3.00 (Table 3). Such fruits are also the best for processing (Grabowska et al. 2015). The most favourable results were obtained in the hybrids nos. 26 and 30, which had coefficients 2.92 and 3.15, respectively. No major differences in the average commercial fruit weight were noted among the hybrids tested (Table 3).

Plant growth habit

The new hybrids nos. 26, 29, and 30 had a main stem length ranging from 104 cm to 130 cm, significantly shorter than the standard 'Śremski' (166 cm) and the new hybrid no. 28 (147 cm). Another characteristic feature was variable length of internodes on the main stem, from the shortest for hybrid no. 26 (4.3 cm) to the longest for 'Śremski' (5.1 cm) and hybrid no. 28 (5.4 cm).

The new hybrids were also characterized by a lower number of lateral branches, namely, 7.8 (hybrid no. 26), 9.0 (hybrid no. 30), and 9.5 (hybrid no. 28), as compared to 15.8 of 'Śremski'. Moreover, the total length of all the lateral branches of the new hybrids was low, hybrid no. 29 having the shortest, 282 cm, and 'Śremski' having the longest, 607 cm.

The internode length of the lateral branches ranged from 4.4 to 5.2 cm, but the differences were statistically non-significant (Table 2). It was typical of the new hybrid plants that all of their shoots were shortened, by 40% in no. 30, 41% in no. 29, and 50% in no. 26, as compared with 'Śremski', which had a total length of all the shoots equal to 773 cm. The shortest internodes on the main stem were noted in hybrids nos. 26 and 29 (4.3 cm and 4.5 cm, respectively), whereas the standard variety 'Śremski F_1' had 5.1 cm.

Leaf blades and petioles have an impact on harvesting. The smaller the blades and shorter the petioles, the easier the access to the fruits, facilitating manual harvesting. The leaves with the shortest petioles (14.5 cm), the smallest length (11.3 cm) and width (14.2 cm) of the leaf blades were observed in 'Szeryf' (Table 3). 'Szeryf' also had the shortest petioles (14.5 cm). Similar petiole length was noted for hybrids nos. 26 (15.3 cm) and 29 (15.9 cm), however, their leaf blades were broader (17.6 to 17.7 cm) and vertical longer (12.6 to 13.1 cm). The longest petioles were observed in 'Śremski' (20.3 cm) and hybrid no. 28 (20.1 cm), and their leaf laminae were also found to be the largest (13.1 to 13.6 cm 16.9 to 18.3 cm).

Discussion

Since the 1960 s, many research centers have been testing cucumber germplasm which might provide improved suitability for once-over mechanical fruit harvesting (Robinson and Mishanec 1965, George 1970, Denna 1971, Biegert and Pike 1976, Tkachenko 1976, Pike and Biegert 1978, Miller and George

Table 1. Fruit yield characteristics in the new hybrids of the semi-bush habit field cucumber, Wolica, 2014 and 2015.

Hybrid	Year	Fruit yield kg/100m^2			
		total	commercial	early commercial	percent early commercial of total
No. 26	2014	782.3 bc	528.4 a	223.7 a	67.5
	2015	657.2 a	441.9 a	214.6 a	67.2
No. 28	2014	729.0 d	332.0 c	172.0 b	45.5
	2015	617.7 ab	402.9 ab	173.8 ab	65.2
No. 29	2014	680.7 e	345.0 c	117.7 d	50.7
	2015	532.2 b	398.5 ab	192.6 ab	74.9
No. 30	2014	754.0 c	456.0 b	145.0 c	60.5
	2015	623.7 ab	433.9 a	184.6 ab	69.6
'Szeryf' (standard)	2014	788.3 b	444.7 b	126.7 cd	56.4
	2015	680.3 a	382.2 ab	183.6 ab	56.2
'Śremski' (standard)	2014	889.0 a	427.7 b	207.0 a	48.1
	2015	582.4 ab	335.9 b	163.8 b	57.7

Means followed by the same letter within each column are not significantly different to $\alpha = 0.05$.

1979, Franken 1981, Edwards and Lower 1982, Polyanska 1985, Kubicki et al. 1986a,b, Sołtysiak et al. 1986, Bolotskikh 1988, Gashkowa 1989). The dwarf, determinant, compact, and bushy plants that were tested seemed to be fit for simplified harvesting. However, in spite of the optimistic results of experiments (Kauffman and Lower 1976) with determinant and compact habit forms, they were not applied in the development of new varieties (Sołtysiak et al. 1986) fit for field cultivation with once-over mechanical harvesting.

As a result of research launched in the 1970s by B. Kubicki, the W-19 bush-type mutant was derived from 'Borszczagowski', through the use of ethylene imine (Kubicki 1983, Kubicki et al. 1986a). The most typical trait of this mutant was the shortening of internodes of all branches by approximately half. The mutant plants produced a similar number of lateral branches and, in spite of a significant reduction of the total length of shoots, they produced more internodes and leaves than normal plants. In the field, the mutant plants occupied smaller areas and thus could be planted at a higher density than normal plants. An additional important trait of the mutants was their vigor and viability, at the same level as normal plants.

The bush-type growth of W-19, conferred by a single recessive gene, *bu*, was strongly linked (5 to 6 cM) in repulsion phase to the recessive gene for femaleness, *gy*. In bush-type plants, weak female expression was obvious. As a result of crossing, self-pollination, and selection, stronger female expression, lack of bitterness, white prickles, and partial tolerance to downy mildew were introgressed into W-19. The greatest obstacle was encountered when trying to combine bush habit, *bu*, with *gy*. No purely female lines with the *bu* gene were developed. A number of original, highly vigorous lines containing the *bu* gene were obtained, but they were monoecious though more strongly female than the original mutant.

In 1993, one of the bush accessions was registered in the Polish National List of Original Varieties as 'Dar'. It is still cultivated, but mainly by amateur gardeners because of its lower yield than that of the other varieties.

The new hybrids were developed in an attempt to obtain a type of cucumber that would facilitate hand harvesting and simultaneously have relatively high yields. The hybrids have modified plant architecture. Analysis of the new hybrids (nos. 26, 29, and 30) has shown that they have foliar traits indicative of them possessing the desirable plant habit.

Outstanding was hybrid no. 26, which had a significantly higher marketable yield in both years of the experiment than even the high-yielding commercial leader 'Śremski'. The earliness of hybrid no. 26 is remarkable considering the fact that 42% of its marketable fruits in 2014 and 48% in 2015 were harvested within

Table 2. Lengths of shoots and internodes of the new experimental semi-bush hybrids and commercial hybrids, 2015.

Hybrid	Main stem		Lateral branches			Whole plant stems (cm)	Average internode length (cm)
	length (cm)	internode length (cm)	number	all lateral branches (cm)	internode length (cm)		
No. 26	111.3 ab	4.3 a	7.8	342.5 a	5.21	453.7 a	4.9 ab
No. 28	147.4 bc	5.4 c	9.5	416.9 ab	5.42	564.3 ab	5.4 b
No. 29	104.4 a	4.5 ab	10.3	282.1 a	4.38	386.5 a	4.4 a
No. 30	120.5 ab	4.6 abc	9.0	342.6 a	4.92	463.1 a	4.8 ab
'Szeryf' (standard)	137.2 ab	4.7 abc	9.8	408.9 ab	4.78	546.0 ab	4.8 ab
'Śremski' (standard)	166.3 c	5.1 bc	15.8	606.9 b	4.88	773.2 b	4.9 ab

Means followed by the same letter within each column are not significantly different at $\alpha = 0.05$.

Table 3. Foliar and fruit characteristics of new, experimental semi-bush hybrids and commercial hybrids, 2015.

Hybrid	Leaf				Commercial fruit	
	leaf blade		petiole length (cm)	no of leaves per plant	fruit weight (g)	fruit shape coefficient
	vertical diameter	horizontal diameter				
No. 26	13.1 bc	17.6 bc	15.3 a	92.7 a	61.8	2.92
No. 28	13.6 c	18.3 c	20.1 b	102.8 ab	59.7	3.60
No. 29	12.6 b	17.7 bc	15.9 a	90.0 a	61.5	2.63
No. 30	13.5 c	18.2 c	18.3 b	99.7 a	63.1	3.15
'Szeryf' (standard)	11.3 a	14.2 a	14.5 a	117.2 ab	61.1	3.37
'Śremski' (standard)	13.1 bc	16.9 b	20.3 b	156.5 b	64.6	3.28

Means followed by the same letter within each column are not significantly different at $\alpha = 0.05$.

the first 2 weeks (first 5 harvests, by August 1). This is similar to 'Śremski', the leader among the early-bearing Polish varieties (48% of the commercial yield of this hybrid is the early yield).

Cucumber cultivation still relies on multiple manual harvesting taking up to two months and requiring a high labour input. The new hybrids of the semi-bush growth type, evaluated in the two-year experiment (nos. 26, 29, and 30), give hope for partial simplification of harvesting, reduced to twice a week. Besides, a smaller plant size allows for a higher density of plants in the field, which can be expected to result in higher yields. Further research is needed to find out the relationship between the yield and plant spacing. It is possible that the hybrids will be suitable for planting at a density which will enable once-over harvesting or the use of a combine after several manual harvests.

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