

ASSESSMENT OF GERBERA PLANTS GENETICALLY MODIFIED WITH TSWV NUCLEOCAPSID GENE

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A B S T R A C T

Plants of four gerbera cultivars transformed with nucleocapsid N-gene of *Tomato Spotted Wilt Virus* were evaluated in terms of resistance to the virus and several phenotypical traits. Sixteen out of 33 transformed genotypes (with transgenic plant status confirmed by PCR with specific primers for N and *npt II* genes) survived when transferred to the greenhouse. After mechanical inoculation with TSWV, typical symptoms of viral infection appeared in the control plants after two to four weeks. No disease symptoms were observed at that time in any of the infected transgenic plants. Assessment of other phenotypical traits of gerbera confirmed lack of significant differences between transformed and control plants. Except for one genotype of 'Prince' and one genotype of 'Zuzanna', all of the transformed plants can be potentially good breeding material.

Key words: gerbera, transformation, resistance, TSWV, quality

INTRODUCTION

Tomato spotted wilt virus (TSWV) is a pathogen that infests many different plant species, causing great yield losses (Goldbach and Peters, 1994). In the 1990s, TSWV was identified as the causal agent of severe diseases of plants belonging to over 800 species, including many vegetables and ornamentals cultivated both in the field and in greenhouse (Bellardi and Vicchi, 1990; Berling et al., 1990; Kaminska and Korbin, 1991; 1994; Goldbach and Peters, 1994; Pottorff and Newman, 1999).

Most of affected plants lack natural resistance to TSWV (Smith and Gardner, 1951; Boiteux et al., 1993; Stevens et al., 1994). Therefore, genetic engineering has been used with some success to breed resistant cultivars, and

showed that this could be a very promising breeding strategy (Gielen et al., 1991; Ultzen et al., 1995, De Haan et al., 1996). However, only some of the recipient plants can serve as donors of the desirable traits after the introduction of new genetic material. This relatively low level of engineered and also classical breeding success is due to random positioning of the introduced genes and the interaction between the new genes and host genome, what can stimulate insufficient expression of the introduced genes or even induction of unexpected changes in the function of the host gene (Napoli et al., 1990; Van der Krol et al., 1990).

The aim of this study was to evaluate gerbera genotypes obtained by plant transformation with construct containing TSWV nucleocapsid gene (Korbin et al., 2002). To estimate the usefulness of this construct and to identify the best genetically modified plants to use as sources of resistance to TSWV, the phenotypic value of the transformants was compared to that of the maternal cultivars.

MATERIAL AND METHODS

Plants

The experiment was conducted on plants of four gerbera cultivars 'Prince', 'Paul', 'Alaska' and 'Zuzanna', which were kindly supplied by Mr. Petos, owner of a private breeding farm in central Poland. Plants were transformed with the construct pBIN19-pROK-N (Korbin et al., 2002). Transformed as well as untransformed control plants were propagated *in vitro* in accordance with standard procedures (Soczek and Hempel, 1988; Tymoszuk, 1988). After adaptation to *in vivo* conditions, ten to fifteen plants of each genotype were cultivated in the greenhouse under standard conditions (20-25°C). The plants were treated to control pests and pathogens. Transformed plants were autoclaved after the experiment.

Tests and assessment of plants

Plants were tested for the presence of transgenes by PCR on the template of genomic DNA isolated from fresh leaves (Doyle and Doyle, 1990). Primers specific for the N and *npt II* genes were utilized (Korbin et al., 2002; Yang et al., 1999). The following thermal profile of amplification (30 cycles) was used: 30s at 94°C (2 minutes in the first cycle), 30s at 55°C, and 60s at 72°C (10 minutes in the last cycle). Each test was repeated twice. The size of the PCR products was compared with the size of commercial DNA markers after electrophoresis in 1% agarose gel.

Susceptibility to TSWV infection was tested by mechanically inoculating plants with TSWV-G isolate (Korbin, 1995). Plants were inoculated at the two-to-four-leaf stage in accordance with the procedure given by Gajos

(1972). Both inoculated and untreated plants were observed for three months. In the fifth and tenth weeks after treatment, all plants were serologically tested (Korbin, 1995). Infected tobacco (*Nicotiana benthamiana*) plants were used as a positive control.

Plants were evaluated in terms of growth rate, yield, and the presence of atypical traits such as flower malformation and discoloration. Growth rate was determined by measuring leaf length and width just before flowering. Flower shoot length and diameter were measured at harvest. Flowers were collected over a period of three months. Well-formed flowers which met the standards determined for each cultivar were considered as marketable yield.

Data were statistically elaborated using R.A Fisher analysis, followed by T-Duncan multiple-range t-tests.

RESULTS AND DISCUSSION

Sixteen out of 33 transformed genotypes survived after being transfer to the greenhouse. Their successful transformation was confirmed by PCR with specific primers for the N and *npt II* genes. In PCR on template of the genomic DNA of these transformed plants, products with expected size 800 bp for the N gene and 770 bp for the *npt II* gene were obtained. The other seventeen genotypes had regenerated from explants on medium containing kanamycin, but did not survive after being transferred to the greenhouse. These genotypes include those in which DNA was amplified with both set of primers in PCR as well as those in which the presence of only the N-gene (full N-sequence in direct PCR or N-gene fragment amplified in nested PCR) was confirmed. As expected, the absence of *npt II* gene was correlated with death of the young explants. One reason this gene might not have been detected in some regenerated plant which grew well *in vitro* is that the flanking sequences in the transgene had been destroyed. A similar phenomenon had been observed for the N gene in two 'Alaska' genotypes, in which the nucleocapsid gene sequence was detected only by nested PCR. On the other hand, thirteen genotypes reacted positively with both sets of primers, but did not survive after being transferred to the greenhouse. This strongly suggests that introduction of the kanamycin resistance gene does not guarantee growth *ex vivo*. The survival capacity of each genotype could depend on various interactions between the transgenes and the host genetic material (genetic background influence) (Peach and Velten, 1991).

After inoculation of both transgenic and control plants with TSWV, typical symptoms of viral infection were observed in the control plants after two to four weeks. No disease symptoms were observed at that time in any of the infected transgenic plants. Resistant transgenic plants included genotypes

containing either the full sequence of the N gene and only a fragment of this gene. This is compatible with the hypothesis that resistance to TSWV is mediated by RNA (Baulcombe, 1996; De Haan et al., 1992; Jan et al., 2000).

DAS-ELISA with antiserum against TSWV gave similar absorbance values for transgenic genotypes both before and after inoculation. The same values were also obtained for the untransformed healthy gerbera (0.1-0.2). Simultaneously, A₄₀₅ was five times higher for infected non-transgenic gerberas, and twenty times higher for tobacco (*N. benthamiana*), the positive control for the inoculation treatment. The fact that the translation product was below detectable level in transgenic plants is also compatible with the hypothesis that resistance to TSWV is RNA-mediated (Baulcombe, 1999). Our results agree with the results of experiments conducted in the Netherlands and Italy, in which nucleoprotein was not detected in plant resistant to TSWV, but only in susceptible, diseased tobacco genotypes (Gielen et al., 1991; Vaira et al., 1995). On the other hand, some of the transformed plants exhibited symptoms of flower malformation and discoloration that are similar to the symptoms of TSWV infection. This could suggest that the viral transgene was being expressed. However, the fact that the N-protein was not detected in gerbera tissues rather suggested that gene silencing was involved (Hobbs et al., 1993; English et al., 1996, Van der Krol et al., 1990; Yu et al., 1999; Przybecki, 2001). Generally, flower color is known to be an unstable trait in transgenic plants (Jain et al., 1998).

Analysis of 160 *in vitro* propagated plants showed that there were only slight phenotypical differences between transformed and untransformed genotypes of the same cultivars (Tab. 1). In 'Alaska', the mean leaf length was 28.5 cm in the control plants, and ranged from 26.8 cm to 32.3 cm in transformed plants. Leaf width was 14.1 cm in the control plants, and ranged from 13.6 to 15.7 cm in transformed genotypes (Tab. 1a). In 'Prince', however, the leaves of the transformed plants were substantially shorter and narrower than those of the control plants (Tab. 1c). In 'Zuzanna', leaf width was 13.7 cm in the control plants, and about the same in the transformed genotypes Z5, Z45, Z46. However, in the transformed Z2, Z42 and Z43, leaf width was much lower, ranging from 10.0 to 10.7 (Tab.1d). In 'Alaska' and 'Paul', flower diameter was somewhat larger in the transformed than in the control-plants. In 'Prince', it was the other way around: flower diameter was 13.5 cm in the control plants and 12.8 cm in the GM-plants (Tab. 1a,b). In 'Zuzanna', flower diameter was 13.7 cm in the control, and ranged from 12.9 to 14.6 cm in the transformed plants (Tab. 1d).

Flower malformation and discoloration were observed in both control and transgenic plants. However, flower morphology was similar to that seen in TSWV infection in two genotypes (Pr5 and Z42). This was probably caused during transgenesis and these plants were removed from further study.

Table. 1 Phenotypic traits in control and transformed genotypes of four gerbera cultivars. Means followed by the same letters do not differ significantly according to Duncan's multiple-range t-test at P 0.05. NS - not significance; ***significantly different at P 0.001; **significantly different at P 0.01; *significantly different at P 0.05

a) cultivar 'Alaska'

Genotype	TRAITS						
	leaf length [cm]	leaf width [cm]	flower shoot length [cm]	flower diameter [cm]	number of flowers per plant	market yield per plant	Normal flowers [%]
Control	28.49b	14.07bc	65.30a	12.49a	5.30a	5.10a	96.00
Alaska 4	27.24a	14.17c	68.13b	12.59a	6.20abc	5.90abc	96.10
Alaska 8	26.82a	13.61a	68.86b	12.51a	6.90c	6.50cd	94.60
Alaska 15	32.33d	15.76e	65.68a	13.17b	6.90c	6.60cd	96.10
Alaska 18	26.81a	14.96b	68.87b	13.25b	5.50ab	5.50ab	0.00
Alaska 23	26.87a	13.71ab	64.97a	12.50a	6.50c	6.40cd	98.90
Alaska 24	28.29b	15.37de	68.95b	13.06b	6.90c	6.90d	0.00
Alaska 25	30.14c	14.03c	68.70b	12.60a	6.40bc	6.30bcd	98.60
Significance	**	***	***	***	**	**	NS

b) cultivar 'Paul'

Genotype	TRAITS						
	leaf length [cm]	leaf width [cm]	flower shoot length [cm]	flower diameter [cm]	number of flowers per plant	market yield per plant	normal flowers [%]
Control	29.58	13.53b	54.48a	14.53a	7.40a	7.40a	86.70
Paul 41	30.31	12.58a	56.84b	15.12b	10.90b	10.90b	86.60
Significance	NS	***	**	*	***	***	NS

c) cultivar 'Prince'

Genotype	TRAITS						
	leaf length [cm]	leaf width [cm]	flower shoot length [cm]	flower diameter [cm]	no of flowers per plant	market yield per plant	normal flowers [%]
Control	32.63b	14.69b	58.10a	13.51b	7.30b	6.00	84.40
Prince 3	28.03a	13.52a	61.79c	12.61a	7.60b	6.10	81.20
Prince 5	29.00a	13.92a	59.59b	12.90a	6.10a	5.70	92.91
Significance	***	**	***	***	*	NS	NS

d) cultivar 'Zuzanna'

Genotype	TRAITS						
	leaf length [cm]	leaf width [cm]	flowers shoot length [cm]	flower diameter [cm]	no of flowers per plant	market yield per plant	normal flowers [%]
Control	35.61d	13.69b	68.77b	13.69b	7.80c	6.00c	77.20
Zuz-2	30.51b	10.56a	68.12b	14.49c	7.20c	5.40bc	75.20
Zuz-5	36.97e	13.89b	67.18b	12.94a	6.60bc	5.20abc	79.40
Zuz-42	32.66c	10.67a	58.22a	13.00a	5.60ab	4.30a	79.10
Zuz-43	28.84a	10.03a	69.98b	14.57c	4.80a	4.40a	87.00
Zuz-45	35.77d	13.45b	69.06b	13.70b	5.30ab	4.60ab	90.50
Zuz-46	34.59d	13.73b	66.03b	13.75b	5.60ab	4.70ab	86.60
Significance	***	***	***	***	***	**	NS



Figure 1. Transformed (T) and control (C) plants just before flowering

Transformed plants started flowering two weeks later than the controls (Fig. 1). Transformation did not have a negative impact on yield. In 'Alaska', 'Paul' and 'Prince', yield was actually higher in the transformed plants than in the control plants. In 'Zuzanna', the number of flowers per plant was 6.9 in the control plants, and ranged from 4.3 to 5.4 in the transformed plants. This confirms that potential in a given transformed plant may depend not only on genotype, but also on interactions between transgenes, and the host genome.

Phenotypical assessment of gerbera plants confirmed that these traits may vary not only from cultivar to cultivar, but also from genotype to genotype within the same cultivar. Generally, however, no significant differences between transformed and untransformed plants were observed. Except for two genotypes (Pr5, Z42), all of the transformed plants are potentially good material for breeding purposes, especially since preliminary testing has confirmed the stable integration of the N transgene (Gielen et al., 1991). The genes used for this experiment seem to be environmentally safe (Powell et al., 1990; Green and Allison, 1994). Furthermore, growing the gerberas in the greenhouse and propagating them by vegetative methods provided additional security against unintentional gene spread.

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OCENA ROŚLIN GERBERY ZMODYFIKOWANEJ GENEM NUKLEOKAPSYDU WIRUSA BRĄZOWEJ PLAMISTOŚCI POMIDORA (TSWV)

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S T R E S Z C Z E N I E

Cechy szesnastu genotypów gerbera transformowanej konstrukcją binarną z genem N nukleokapsydu wirusa brązowej plamistości pomidora (TSWV) oceniano podczas ich wzrostu w warunkach szklarniowych. Status genotypów jako roślin transgenicznych potwierdzono w testach PCR ze starterami specyficznymi dla genów N i *npt II*. Rośliny transgeniczne charakteryzowały się odpornością na TSWV i nie wykazywały żadnych objawów chorobowych po potraktowaniu inokulum z preparatem wirusa, podczas gdy na nietransformowanych roślinach kontrolnych symptomy porażenia przez TSWV były widoczne po 2-4 tygodniach od inokulacji.

Analiza cech użytkowych takich jak, wielkość i jakość kwiatostanu, pokrój rośliny, długość szypułki kwiatowej oraz wysokość i jakość plonu, wykazała brak istotnych różnic między roślinami zmodyfikowanymi a roślinami kontrolnymi tej samej odmiany. Termin kwitnienia roślin transgenicznych był o dwa tygodnie opóźniony w stosunku do roślin kontrolnych. Poza dwoma genotypami 'Prince' i 'Zuzanna', które ze względu na powtarzające się przebarwienia i deformacje kwiatostanu usunięto z badań, pozostałe uzyskane rośliny transgeniczne mogą stanowić dobrej jakości materiał wyjściowy dla hodowli odpornościowej gerbera.

Słowa kluczowe: gerbera, cechy jakościowe, transformacja genetyczna, odporność