

RAPD AND ISSR MARKERS OF BLACK AND GREEN COLOUR OF BLACKCURRANT (*Ribes nigrum*) FRUITS

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A B S T R A C T

In 2002, studies on the identification of putative DNA markers of fruit color in the blackcurrant were initiated at the Research Institute of Pomology and Floriculture in Skierniewice. The aim of this study was to elaborate a simple technique to precisely select genotypes with black or green fruits at an early stage of plant development. This study was conducted on genotypes belonging to the cultivars 'Bona', 'Titania', 'Verti', Clone 27/75-6 and their selected progeny (F1 and F2). One hundred and twenty eight informative DNA fragments were amplified using RAPD and ISSR with 29 primers on template DNA from single genotypes and pooled genotype samples. Two PCR products were obtained. RAPD with primer OPA-01 amplified a specific product about 700 bp long only in plants bearing the dominant black allele. ISSR with primer 818 generated a DNA fragment about 1.2 kbp long only in plants bearing the recessive green allele.

Key words: blackcurrant, green fruits, early selection, genotype, RAPD, ISSR, PCR

INTRODUCTION

Blackcurrants with green fruits were first described in the 1800s (Anonymous, 1938). Breeders were interested in them because of their potential industrial uses. Several green-fruited cultivars were released (Keep and Knight, 1970; Iunnila and Hiirsalmi, 1987; Gwozdecki, 2002). Field studies on the inheritance of fruit color in the blackcurrant showed that this trait is controlled by a single dominant-recessive gene (Keep and Knight, 1970). Experiments on open-pollinated blackcurrants confirmed that those with the genotypes **BB** or **Bb** bore black fruits, and those with the genotype **bb** bore green fruits (Gwozdecki, 2002).

Fruit species with dark fruits such as blueberries, blackberries, red grapes and blackcurrants have been found to contain health-promoting vitamins and anti-oxidants, including anthocyanins (Frank et al., 2003; Viberg et al., 1997; Moreno-Alvarez et al., 2002). Interest in green-fruited blackcurrants has therefore decreased. However, recognizing the DNA fragments correlated with this trait is still useful in breeding programs and in targeted progeny selection. A quick and early DNA-based method for selecting blackcurrant genotypes with green or black fruits also seems to be an interesting tool for studies on pollen spread. This can provide valuable information on the safe co-existence of genetically modified and non-modified crops.

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MATERIAL AND METHODS

Plant material

Blackcurrant plants were cultivated in the experimental field of the Research Institute of Pomology and Floriculture. Randomly selected plants from the following genotypes were used as a source of material for molecular studies. Fruit color had previously been determined in field experiments (Gwozdecki, 2002).

- black fruits: 'Titania', 'Bona',
- green fruits: 'Verti', Clone 27/75-6,
- black fruits: progeny of 'Titania' x Clone 27/75-6 (F1),
- selected genotypes (F2) from open pollination of F1 ('Titania' x Clone 27/75-6) and Clone 27/75-6.

DNA isolation

DNA was isolated according to the procedure of Doyle and Doyle (1990). Nucleic acids were extracted directly from 2 grams of young leaf tissue from single genotypes as well as from pooled genotype samples. DNA concentration was determined by electrophoresis on 1% agarose gel using commercial dilutions of λ DNA (Gibco).

The following DNA templates were used:

- mix of ten genotypes of 'Titania' (BB) and 'Bona' (BB),
- mix of ten randomly selected progeny of 'Titania' x Clone 27/75-6 (Bb),
- mix of ten green progeny (F2) originating from open pollination of 'Titania' x Clone 27/75-6 (F1) and Clone 27/75-6 (bb),

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- individuals 10b, 6b and 7b from cross of 'Titania' x Clone 27/75-6 (BB or Bb), individual 9b (F2) from cross of 'Titania' x 'Bona' (BB) and 'Verti' (bb),
- individual of Clone 27/75-6 (bb), and
- green individual 9g from open pollination (bb).

PCR

DNA was amplified using RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Specific Repeats). The reaction mixture contained: 10 ng of DNA template; 4.5 pmol primer; 10 x PCR buffer; 2mMol MgCl₂; 1mMol dNTP mixture; and 0.05 Units of Ampli Taq Gold Polymerase (Perkin Elmer).

In all, 29 primers were used. These included six random decamers from Operon Technologies: OPA-01, OPA-02, OPA-04, OPA-06, OPB-11, OPC-02. Also included were 23 SSR primers from UBC: 807-827, and 843-845. Reactions were carried out in a PTC-200 Thermocycler

using the following thermal profiles:

- RAPD: 49 cycles (45 s at 94°C, 60 s at 36°C, 2 min at 72°C),
- ISSR: 35 cycles (40s at 94°C, 50 s at 55°C, 1 min at 72°C).

Amplified DNA fragments were fractionated according to size by electrophoresis in 1.5% agarose gels and visualized under UV light after staining with ethidium bromide. Only reproducible PCR products were chosen for the further analysis.

Cloning of PCR products

Two PCR products were obtained after RAPD with primer OPA-01. One was 900 bp long, and the other was 700 bp long. They were purified from agarose gel using commercial NUCLEO SPIN columns (Marshall-Nagel). They were then cloned using PCR 2.1 TOPO Vector into competent *Escherichia coli* cells (TOPO TA Cloning Kit, Invitrogen).

After multiplication of *E. coli* in LB medium, plasmids with inserts were isolated using the alkaline lysis procedure. Sequences of inserts were determined with the CEQ Sequencing System (Beckman Coulter) using the universal primer M13. These sequences were then compared with available sequences in GenBank databases.

RESULTS AND DISCUSSION

One hundred and twenty eight informative DNA fragments were amplified in reactions with all 29 primers on template of DNA from single genotypes and pooled genotype samples. No polymorphism was observed with nine primers: 807, 809, 810, 824, 825, 843, OPA-02, OPA-04, OPA06.

Polymorphic DNA was observed in reactions with other primers, and permitted single genotypes and groups of genotypes to be distinguished from one another. Primers 816 and 845 generated a characteristic DNA pattern only with 'Bona'. Reaction with primer 816 distinguished between 'Bona' and 'Titania'. PCR with primers 813, 812 and OPC-02 allowed confirming that the DNA patterns of the black-fruited individuals 9b and 10b were similar to one another. Primer 808 distinguished between black-fruited individual 10b and green-fruited individual 9b. Primer 827 distinguished between all of the genotypes analyzed.

In PCR with primer OPA-01, a specific product about 700 bp long was amplified only in plants bearing the dominant black allele (Fig. 1). This band was lacking in green-fruited homozygotes what was confirmed in amplification conducted on the pooled DNA templates (Fig. 2). The 900 bp DNA fragment generated by primer OPA-01 was characteristic for all plants, both black-fruited and green-fruited. The sequence of this fragment was 75% homologous with non-annotated regions of the genome of *Arabidopsis thaliana*, located on chromosome 4 (Accession No. CO048680). No homology was found between the sequence of the 700 bp fragment and the sequences available in database.

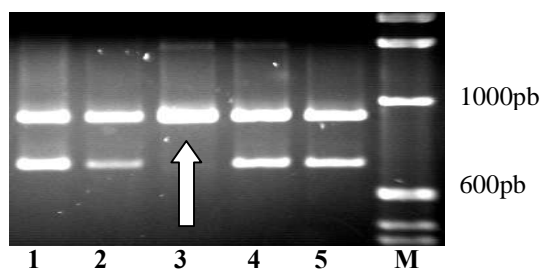


Figure 1. Electropherogram of products amplified in RAPD reaction with primer OPA-01 on the template of mixed DNA pools

1. progenies derived from cross 'Titania' x 'Titania' and 'Titania' x 'Bona': ten selected individuals (BB);
 2. progenies (F1) from 'Titania' x Clone 27/75-6: mix of ten selected individuals producing black fruits (Bb);
 3. Mix of 'Verti', Clone 27/75-6 and ten F2 individuals producing green fruits and derived from open pollination between genotypes 'Titania' x Clone 27/75-6 (Bb) and Clone 27/75-6 x Clone 27/75-6 (bb);
 4. F2 progeny from 'Titania' x Clone 27/75-6: ten selected individuals producing black fruits, derived from open pollination between genotypes 'Titania' x Clone 27/75-6 (Bb) and 'Titania' x 'Titania' (BB);
 5. 'Titania' and 'Bona', ten plants in all (BB);
- M.** Marker.

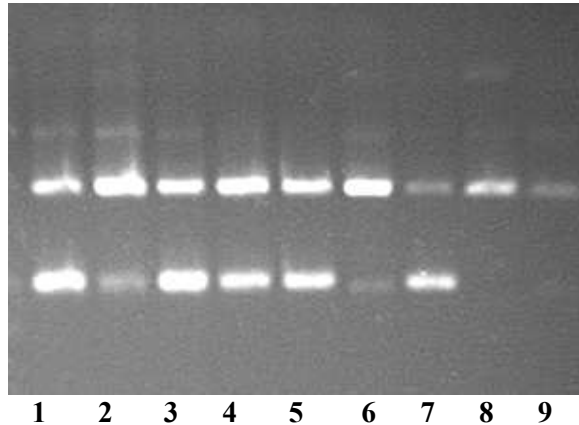


Figure 2. Electrophorogram of RAPD PCR products obtained with Primer OPA-01

1. 'Bona' (BB);
2. Individual producing black fruits, derived from cross 'Titania' x Clone 27/75-6 (Bb);
3. 'Titania' (BB);
4. Individual I (BB) producing black fruits, derived from cross 'Titania' x 'Titania';
5. Individual I (BB) producing black fruits, from cross 'Titania' x 'Bona';
6. Individual I (Bb) producing black fruits, derived from 'Titania' x Clone 27/75-6' (F1);
7. Individual II (Bb) producing black fruits, derived from 'Titania' x Clone 27/75-6';
- 8 and 9. Individuals (bb) with green fruits form open pollination between 'Titania' x Clone 27/75-6' (F1) and Clone 27/75-6 (F2).

ISSR with primer 818 generated a DNA fragment 1.2 kbp long only in plants bearing the recessive green allele (Fig. 3). This fragment was amplified on the DNA template derived from 'Verti', the green-fruited Clone 27/75-6, and the mix of DNA from crosses between black-fruited and green fruited plants.

Ever since the 1980s, molecular techniques have facilitated breeding and genetic research by allowing the identification of identifying interesting genome fragments at an early stage of fruit development independent of environmental conditions (Antonius-Klemola, 1999; Beadle et al., 1993; Godwin et al., 1997; Goodman et al., 1987; Klein-Lankhorst et al., 1991; Koller et al., 1993; Nybom, 1991). In our laboratory, color in blackcurrants has been studied using RAPD, ISSR, and BSA (Bulk Segregant Analysis) (Williams et al., 1990; Zientkiewicz et al., 1994; Michelmore et al., 1991). DNA pools were screened for specific genetic markers tightly linked with fruit color. Genetic and phenotypic pooling is a common tool for analyzing inheritance patterns (Williams et al., 1993). BSA has often been used in molecular testing, especially when searching for new markers or when

constructing genetic maps (Giovannoni et al., 1991; Powell et al., 1996). BSA also has practical applications in the early selection of near isogenic plant lines (Michelmore et al., 1991).

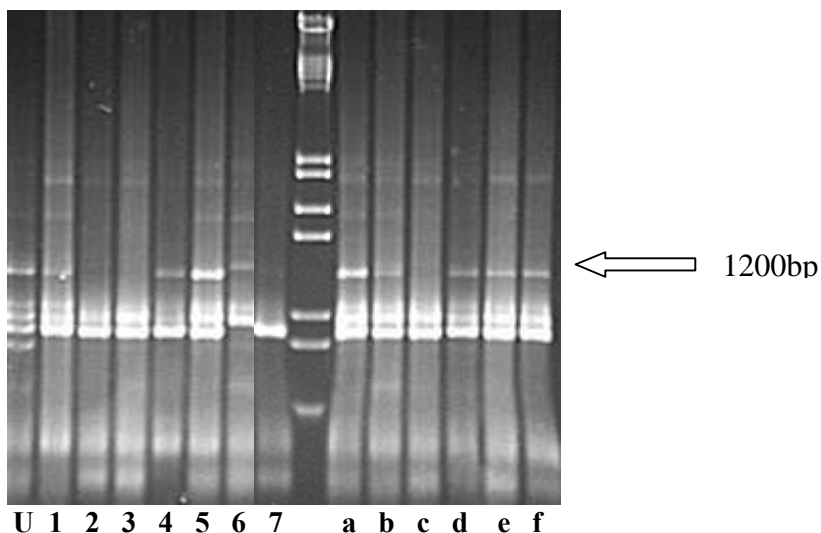


Figure 3. Electrophorogram of PCR products obtained with 818 ISSR primer for single genotypes and DNA pools

U. Clone 27/75-6 (bb);

1. 'Titania' x Clone 27/75-6 (Bb);

2. 'Titania' (BB);

3. Individual 9 (BB);

4. Individual 9 (bb);

5. Individual 10 (Bb);

6. Individual 10 (bb);

7. Individual 6 (Bb);

a. Mix of ten genotypes from 'Titania' x Clone 27/75-6, (Bb);

b. Mix of black genotypes: 'Titania' x Clone 27/75-6 (Bb), Individual 10 (Bb), Individual 6 (Bb) and Individual 7 (Bb);

c. Mix of black genotypes: 'Titania' (BB) and Individual 9 (BB);

d. Mix of genotypes: 'Verti' (bb), Clone 27/75-6 (bb) and Individual 10 (bb);

e. Mix of black genotypes: 'Titania' (BB), Individual 9 (BB), Individual 9 (Bb), Individual 10 (Bb), Individual 6 (Bb) and Individual 7 (Bb);

f. Mix of black and green genotypes: Individual 9 (Bb), Individual 10 (Bb), Individual 6 (Bb), Individual 7 (Bb), 'Verti' (bb) and Clone 27/75-6 (bb).

Our results confirm that this is a useful strategy for generating molecular markers of fruit color in the blackcurrant. The two markers proposed, one 700 bp long and the other 1.2 kpb long, distinguished between black-fruited and green-fruited genotypes and allowed for the selection of potential carriers of the green allele at a very early stage of plant development.

Blackcurrant plants with green fruits have not recently been popular on the European market, but they are still useful for breeding programs as donors of other desirable traits. They can also be used as an experimental model for studies on pollen spread (valuable information on the safe co-existence of genetically modified and non-modified crops). Our results seem to be suitable in this kind of analysis.

REFERENCES

- Anonymous 1938. Pomology department. REP. JOHN INNES HORT. INSTN FOR 1937: 12-15.
- Antonius-Klemola K. 1999. Molecular markers in *Rubus* (*Rosaceae*) research and breeding. J. HORT. SCI. BIOTECH. 74: 149-160.
- Beadle D.J., Bishop D.H.L., Copping L.G., Dixon G.K., Hollomon D.W. 1993. Opportunities for molecular biology in crop production. BRITISH CROP PROTECTION COUNCIL 55: 121-274.
- Doyle J.J., Doyle J.L. 1990. Isolation of plant DNA from fresh tissue. FOCUS 12: 13.
- Frank I., Kamal-Eldin A., Lundh T., Maatt K., Torrnén R., Vessby B. 2003. Effects of dietary anthocyanins on tocopherols and lipids in rats. J AGRIC FOOD CHEM. 51: 3196.
- Goodman F.L., Hauptli H., Crossway E., Knauf V.C. 1987. Gene transfer in crop improvement. SCIENCE 236: 48-54.
- Godwin I.D., Atken E.A., Smith L.W. 1997. Application of inter simple sequence repeat (ISSR) markers to plant genetics. ELECTROPHORESIS 18: 1524-1528.
- Giovannoni J., Wing R.A., Ganai M.W., Tanksley S.D. 1991. Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations". NUC. ACIDS RES. 19: 6553-6558.
- Gwozdecki J. 2002. Cross-fertilization on blackcurrant (*Ribes nigrum* L.) plantation. J. FRUIT ORNAM. PLANT RES. 10: 27-30.
- Iunnila S., Hiirsalmi H. 1987. Genetic background of green fruit colour in black currant. ANNAL. AGRIC. FENNIAE 26: 275-278.
- Keep E., Knight R.L. 1970. Inheritance of fruit colour in currants and gooseberries. REP. E. MALLING RES: 139-142.
- Klein-Lankhorst R.M., Vermunt A., Weide R., Liharska T., Zabel P. 1991. Isolation of molecular markers for tomato (*L. esculentum*) using Random Amplified Polymorphic DNA (RAPD). THEOR. APPL. GENET 83: 108-114.
- Koller B., Lehmann A., Mc Dermott J.M., Gessler C. 1993. Identification of apple cultivars using RAPD markers. THEOR. APPL. GENET 85: 911-914.
- Michelmore R.W., Paran I, Kesseli R.V. 1991. Identification of markers linked to disease-resistant genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. GENETICS 88: 9828-9832.
- Moreno-Alvarez M.J., Viloriamatos A., Lopez E., Belen D. 2002. Stability of anthocyanins in pasteurized juice of blackberry (*Rubus glaucus* Benth.). ARCH LATINOAM. NUTR. 52(2): 181-186.
- Nybohm H. 1991. Application of DNA-fingerprinting in plant breeding. PROC. INTERN. SYMP. DNA FINGERPRINTING: pp. 294-311.

- Powell W., Morgante M., Andre C., Hanafey M., Vogel J., Tingey S., Rafalski A. 1996. The comparison of RAPD, AFLP and SSR (microsatelite) markers for germplasm analysis. MOLEC. BREED 2: 225-238.
- Williams J.K.G., Kubelik A.R., Livak K.J., Rafalski J.A., Tingey S.V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. NUCL. ACIDS RES. 18: 6531-6535.
- Williams J.G.K., Reiter R.S., Young R.M., Scolnik P.A. 1993. Genetic mapping of mutations using phenotypic pools and mapped RAPD markers”, NUCL. ACIDS RES. 21: 2697-2702.
- Viberg U., Ekstram G., Fredlund K., Oste R.E., Sjöholm J. 1997. A study of some important vitamins and antioxidants in a blackcurrant jam with low sugar content and without additives. INT. J. FOOD SCI. NUTR. 48: 57-66.
- Zientkiewicz E., Rafalski A., Labuda D. 1994. Genome fingerprinting by simple sequence repeat (SSR) – anchored polymerase chain reaction amplification. GENOMICS 20: 176-183.

MARKERY RAPD i ISSR DLA WCZESNEJ IDENTYFIKACJI ROŚLIN CZARNEJ PORZECZKI (*Ribes nigrum*) O CZARNYCH I ZIELONYCH OWOCACH

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

Dwa markery molekularne związane z czarną i zieloną barwą owoców czarnej porzeczki (*Ribes nigrum*) wygenerowano technikami PCR (RAPD i ISSR). Badania prowadzono na roślinach odmian: ‘Bona’, ‘Titania’, ‘Verti’, klonu No. 27/75-6 oraz wybranych genotypach pochodzących z wyżej wymienionego krzyżowania (pokolenie F1 i F2). Analizom poddano materiał genetyczny pochodzący zarówno z pojedynczych genotypów, jak i z prób mieszanych. W reakcjach z 6 starterami arbitralnymi (RAPD) i 23 starterami mikrosatelitarnymi (SSR) uzyskano ogółem 128 informatywnych produktów. W reakcji ze starterem OPA-01 uzyskano specyficzny produkt, wielkości około 700bp, umożliwiający odróżnienie genotypów czarno (BB i Bb) i zielono owocujących (bb). Natomiast w reakcji ze starterem 818 uzyskano produkt 1,2kb, specyficzny dla genotypów będących donorem recesywnego allelu, warunkującego zieloną barwę owoców (Bb i bb).

Słowa kluczowe: porzeczka czarna, zielone owoce, wczesna selekcja, RAPD, ISSR, PCR