

Use of RAPD and SCAR markers for identification of strawberry genotypes carrying red stele (*Phytophthora fragariae*) resistance gene *Rpfl*

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Abstract. *Phytophthora fragariae* is responsible for strawberry red stele disease that spreads in almost all areas where strawberries are grown. RAPD markers that were developed previously for the strawberry *Rpfl* gene are difficult to reproduce and preferentially, therefore, should be converted into sequence characterized amplified region (SCAR) markers. The aim of our study was to develop new SCAR markers, associated with *Rpfl*, to make a comparative study of RAPD and SCAR markers, and to select strawberry varieties and seedlings carrying the *Rpfl* gene. A SCAR marker was constructed based upon RAPD marker OPO-16C, linked to the susceptibility allele of the *Rpfl* gene. Following the cloning and sequencing of this marker we were able to design SCAR primers specific to this gene. Utilizing newly developed SCAR markers it was shown that resistant varieties like 'Anapolis', 'Redgauntlet' contain *Rpfl* region. Common varieties 'Elsanta', 'Kama', 'Venta', 'Senga Sengana' did not exhibit this gene. The segregation rate of seedling populations (susceptible x resistant) by this marker was approximately 1: 1.

Key words: *Fragaria ananassa* Duch., marker-assisted selection, strawberry varieties, seedlings, wilt disease

INTRODUCTION

The fungus *Phytophthora fragariae* is able to cause red stele root rot in the strawberry. Symptoms of the disease are discoloration of the stele of the roots, rotting away of the infected roots, dwarfism, wilting, and finally plant death (Maas, 1998; Santos et al, 2003). Resistance to *P. fragariae* has long been assumed to be polygenically inherited (Stembridge & Scott 1959; Scott et al., 1984) but Van de Weg (1989;1997) found evidence that red stele resistance in strawberry and corresponding avirulence in *P. fragariae* interact according to a gene-for-gene system. At least five race-specific plant resistance genes and corresponding avirulence genes are believed to exist (Van de Weg 1997).

The ability to identify resistance genes and accurately screen them is laborious, expensive, and at times problematic due to epistatic interactions between resistance genes. Therefore using indirect selection with molecular markers is promising. Bulk segregant analysis (BSA) was used to identify seven random amplified polymorphic DNA (RAPD) markers linked to the *Rpfl* gene (Haymes et al. 1997). However RAPD markers are difficult to reproduce and therefore, they preferentially should be

converted into sequence characterized amplified region (SCAR) markers. An advantage of SCAR markers is their potential for quick and robust assessment (Guerin et al. 2003). The aim of our study was to develop new SCAR markers, associated with *Rpf1*, to make a comparative study of RAPD and SCAR markers, and select strawberry varieties and seedlings carrying the *Rpf1* gene.

MATERIALS AND METHODS

Strawberry (*Fragaria ananassa* Duch) cultivars 'Anapolis', 'Senga Sengana', 'Venta', 'Kama', 'Selen', 'Honeoye', 'Elsanta', 'Tristar', 'Dange', 'Elkat' and seedling 940101 ('Guardian' x 'Pegasus') seedlings of the combinations 'Selen' x 952002 ('Tristar' x *F. chiloensis* DeL Norte) and Anapolis' x 'Honeoye'. Total DNA was isolated from strawberry by cetyltrimethylammonium bromide (CTAB)-based method (Doyle and Doyle, 1990).

The amplification reaction (PCR) was conducted using ep gradient S cycler, a 20 µl final reaction volume that contained 1.25 units of *Taq* DNA polymerase, 1 ml 10x*Taq* buffer + (NH₄)₂SO₄, 1.5 mM MgCl₂, 1 µM of oligonucleotide primer OPO16 (5' TCGGCGGTT 3'), 0.2 mM dNTP and 20 ng/µl of total DNA. Conditions for the amplification were as follows: 94°C for 4 min; 35 cycles consisting of 1.0 min denaturation at 94°C; annealing at 35°C for 1.0 min; polymerization at 72°C for 1.30 min, and a final extension at 72°C for 7 min. Agarose gels with appropriate DNA fragments were photographed, saved and analysed using Easy Win32 computer software. GeneRuler™ 1 kb DNR ladder (MBI Fermentas, Lithuania) was used as a DNA size marker.

Electrophoresis DNA band of strawberry 'Elsanta' amplified using OPO16 primer and associated with *Rpf1* in repulsion phase with respect to this gene was chosen for developing the SCAR marker. About 430 bp in size-amplified DNA fragment of 'Elsanta' was purified from 1% agarose gel using Cyclo-Pure Gel Extraction Kit from Agarose Slides (Amresco). The DNA fragment was cloned into pTZ57R plasmid and Inst/Aclone™ PCR Product Cloning Kit (MBI Fermentas, Lithuania) according to producer-supplied methodology. The DNA fragment was sequenced in the Sequencing Centre of the Institute of Biotechnology (BTI).

According to the sequence specific forward (RPF1NF1 5' CGGTTCCCAAAAAGATAGTAGTTAC 3') and reverse (RPF1NR1 5' GTTCTACGCATTAAGATGCACTTGC 3') primers were developed. The PCR was conducted in the same conditions as described before, except 1 µM of oligonucleotide primer RPF1NF1 and RPF1NR1 were added instead of OPO16.

RESULTS AND DISCUSSION

DNA electrophoresis results show that several polymorphic bands could be obtained after PGR using primer OPO-16 (Fig 1). There was a quite clear band of about 430 bp in size and characteristic only for susceptible strawberry cultivars and seedlings. We decided that it is a match to marker OPO- 16C discovered by Haymes et al. (1997) existing in repulsion phase to *Rpf1*.

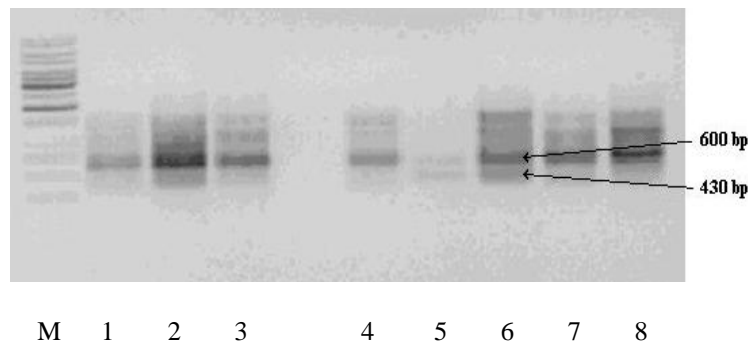


Fig. 1. Strawberry DNA electrophoregram, after PCR using primer OPO-16. M-DNA fragments size marker (GeneRuler™ 1kb DNA Ladder), 1- 005001, 2- 940101 ('Guardian' x 'Pegasus'), 3- 'Redgauntlet', 4- 'Anapolis'; 5- 'Selen', 6- 'Elsanta', - 7- 'Tristar', 8- 005002.

This marker band was characteristic to susceptible cultivars 'Selen', 'Elsanta' and seedling 940101 ('Guardian' x 'Pegasus'). The seedling and its parent forms are resistant to red stele disease despite the absence of *Rpf1*. Red stele disease resistance of this seedling is likely determined by other resistance genes such as *Rpf2* or *Rpf3*. In our experiment seedlings 005001 and 005002 ('Selen' x 'Tristar'), cultivars 'Anapolis', 'Redgauntlet', 'Tristar' lack 430 bp size DNA band characteristic to susceptible cultivars only. Plants of those 5 genotypes are resistant to *P. fragariae* in field conditions. Therefore it safe to say that our PCR data corresponds with data received in field conditions and allows a strong assumption that those 5 genotypes carry the *Rpf1* gene. According to suggestions of Van de Weg et al. (1989) received after analysis of cultivar race interactions with regard to a gene-for-gene model, and also pedigree data, cultivars 'Anapolis', 'Redgauntlet', 'Tristar' may carry the *Rpf1* gene. Our experiment data confirm those suggestions. Contrary to data of Haymes et al. (1997) we observed the exceptionally bright DNA band of about 600 bp in size (Fig. 1). This DNA band was monomorphic, characteristic of all tested varieties and did not coincide with OPO-16C marker. Discrimination of genotypes by the presence or absence of OPO-16C marker is quite problematic because there are not enough bright and clear bands. Sometimes it is quite difficult to distinguish an appropriate DNA band clearly from another band for unchallenged confirmation. Modifications of PCR conditions failed to help much. We decided to develop a SCAR marker which could assist positive confirmation of the presence or absence of the *Rpf1* gene. Such markers linked to the *Colletotrichum acutatum* resistance gene are being developed for strawberry (Guerin et al. 2003). A DNA fragment of 'Elsanta' – amplified about 400 bp in size- was purified from 1% agarose gel, cloned using pTZ57R plasmid and sequenced. According to sequence data, new specific primers were chosen. The PCR data using those primers are presented in Figure 2. According to our PCR data cultivars and seedlings 'Redgauntlet', 'Anapolis'; 'Tristar', 'Dange, 005001; 005002 carry the *Rpf1* gene, cultivars 'Honeye'; 'Elsanta', 'Venta', 'Kama'; 940101; 'Selen'; 'Elkat', 'Senga Sengana' – show no evidence of this gene. Such findings correspond with our previous experimental and field trial data. Without using RAPD primers, we observed only one band that clearly indicated the presence or absence of the *Rpf1* gene.

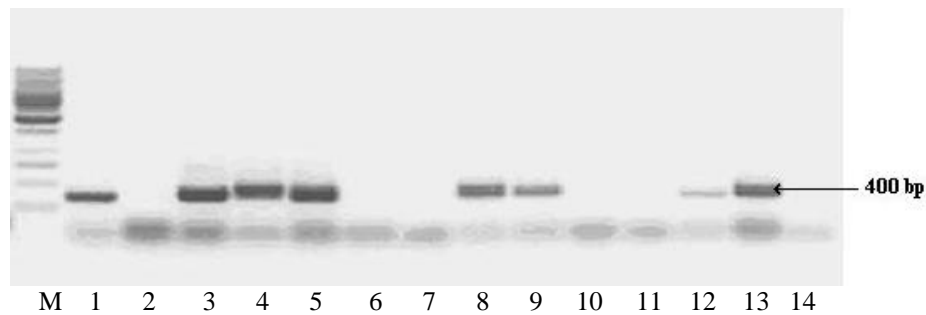


Fig. 2. Strawberry DNA electrophoregram, after PCR using primers RPFNF1 and RPFNR1. M- DNA fragments size marker (GeneRuler™ 1kb DNA Ladder), 1- 'Honeoye'; 2- 'Redgauntlet', 3- 'Elsanta', 4- 'Venta', 5- 'Kama'; 6- 'Anapolis'; 7- 'Tristar'; 8- 940101; 9- 'Selen'; 10- 005001; 11- 005002; 12- 'Elkat', 13- 'Senga Sengana'; 14- 'Dange'.

It can be observed that brightness and position of DNA bands are not always exact. It could be explained by DNA molecular variation in different genotypes and heterogeneity or heterozygosity. In any case, the advantages of SCAR markers are evident.

SCAR markers could be used for identification of genetic background of resistance to red stele. Seedlings 005001, 005002 received from interspecific crosses *F. ananassa* x *F. chiloensis* D.N. potentially contain 4 resistance genes *Rpf1*, *Rpf2*, *Rpf3*, *Rpf4*. Development of SCAR markers for each of these genes is required for identification of genetic constitution of such genotypes.

Evaluation of seedlings from crosses 'Selen' x 952002 ('Tristar' x *F. chiloensis* DeL Norte) and 'Anapolis' x 'Honeoye' in a field condition showed resistant x susceptible segregation rate 1: 1. The same segregation rate was achieved in PCR experiments, confirming that *Rpf1* is dominant and present in a heterozygous state.

Genetic investigation of disease resistance is time consuming, difficult to manage in field conditions and depends on both pathogen and plant state, therefore the use of DNA markers can save time and expense and prove the presence or absence of appropriate genetic factors at the DNA level.

CONCLUSIONS

1. Our results show that RAPD and SCAR markers are suitable for selection of strawberry genotypes carrying the red stele resistance gene *Rpf1*.
2. SCAR markers are more convenient than RAPD for quick and robust assessment of the genetic background of strawberry red stele resistance.

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