ISSN 1392-3196
Žemdirbystė / Zemdirbyste / Agriculture, vol. 94, No. 4 (2007), p. 139–145
UDK [634.11+364.75]:631.527:631.526.32

SCREENING OF APPLE AND STRAWBERRY PLANTS CARRYING FUNGAL DISEASE RESISTANCE OLIGOGENES USING MOLECULAR MARKERS

Dalia GELVONAUSKIENĖ, Rytis RUGIENIUS, Tadeušas ŠIKŠNIANAS, Gražina STANIENĖ, Audrius SASNAUSKAS, Vidmantas STANYS

Lithuanian Institute of Horticulture
Babtai, Kaunas district
E-mail: D.Gelvonauskiene@lsdi.lt; R.Rugienius@lsdi.lt

Abstract

DNA analysis using of apple scab resistance gene *Vfa1* sequence specific marker was performed to identify the presence of this gene like sequences in apple cultivars. Our results showed that the cultivars, which are immune to 1–5 *Venturia ineaqualis* races, have a DNA fragment specific to the *Vfa1* gene. A SCAR marker was constructed based upon RAPD marker, linked to the strawberry red stele resistance *Rpf1* gene. Utilizing this marker strawberry cultivars and seedlings were screened for presence or absence of this gene. Evaluation of strawberry seedlings cross combinations red stele susceptible x resistant ('Selen' x 952002 and 'Honeoye' x 'Anapolis') in laboratory and field conditions showed resistant : susceptible segregation ratio 1:1.

Key words: *Malus domestica, Fragaria ananassa, Phytophthora fragariae, Venturia inaequalis,* marker assisted selection, breeding, cultivars, seedlings, SCAR, RAPD.

Introduction

Apple sensitivity to scab (*Venturia inaequalis* (Cooke) Aderh.), is a major problem for commercial fruit growing /Fischer, 2000; Kozlovskaya, 2001/. Advanced studies of apple (*Malus domestica* Borkh) development, physiology, and biochemistry have been hampered by the lack of appropriate genomics tools. One exception is the recent acquisition of extensive expressed sequence tag (EST) data. The entire available EST dataset for apple resulted from the efforts of at least 20 contributors and was derived from more than 70 cDNA libraries representing diverse transcriptional profiles from a variety of organs, fruit parts, developmental stages, biotic and abiotic stresses, and from at least nine cultivars, covering approximately one-half the expressed genes from apple /Park et al., 2006; Newcomb et al., 2006/. Many potential molecular markers are abundant in the apple transcripts. Molecular markers provide good potential for plant breeders. Several sources of apple scab resistance have been identified and used in breeding programs, but mainly resistance determined by *Vf* gene derived from *Malus floribunda* 821 was used /Williams et al., 1966; Ždanov, Sedov, 1991/. Particularly it is important for selecting trees that combine two or more genes for scab resistance, because traditional methods can be inefficient. A cluster of four resistance paralogs (*Vfa1, Vfa2, Vfa3* and *Vfa4*) was identified in the *Vf* locus homologous to the *Cladosporium fulvum*

resistance gene family of tomato. One of these genes, *Vf2* was used to transform the susceptible apple cultivar Gala /Belfanti et al., 2004/. *Vfa1* had no introns and is predicted to encode proteins characterized with extracellular leucine-rich repeats and transmembrane domains /Xu, Korban, 2002/.

The fungus *Phytophthora fragariae* C.J. Hickman, is able to cause red stele root rot in the strawberry. Symptoms of the disease is 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 /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. Bulked segregant analysis (BSA) was used to identify seven random amplified polymorphic DNA (RAPD) markers linked to the *Rpf1* 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/.

Molecular marker assisted selection allows to screen resistant plants in early developing stages despite biotic and abiotic conditions – without direct contact with pathogens.

The aim of our study was to select apple cultivars carrying scab resistance gene *Vf1* and cultivars of strawberry carrying red stele resistance gene *Rpf1*.

Material and methods

Apple (*Malus domestica*) cultivars: 'Noris', 'Papirovka','Antonovka', 'Katja', 'Tellissaare', 'Auksis', 'Prima', 'Štaris', 'Aldas' (Fig. 1), 'Skaistis', 'Rudenis'; strawberry (*Fragaria ananassa*) cultivars 'Anapolis', 'Senga Sengana', 'Venta', 'Kama', 'Selen', 'Honeoye', 'Elsanta', 'Tristar', 'Dangė' (Fig. 2), 'Elkat', seedling 940101 ('Guardian'x 'Pegasus'), seedlings of the combinations 'Selen' x 952002 ('Tristar' x *F. chiloensis* Del Norte) and Anapolis' x 'Honeoye' were used in the experiments.

Genomic DNA was extracted from leaf material using the minipreparation method as described by Dellaporta et al. (1983).

For polymerase chain reaction (PCR) was used 1 unit of Taq DNA polymerase (MBI Fermentas, Lithuania), 1.5 mM $MgSO_4$, 0.2 mM dNTP and 1μM of each oligonucleotide primer. For apple the DNA denaturation was performed at 95 ºC for 4 min., further 35 cycles – 94 ºC 1.15 min, 47 ºC 1.15 min, 72 ºC 2 min. For strawberry respectively: 94 ºC for 4 min, further 35 cycles of 1.0 min at 94 ºC, 51 ºC for 1.0 min, 72 ºC for 1.30 min, 72 ºC for 7 min.

For apple the primers used were according to Xu and Korban (2002):

For: 5'-TCTATCTCAGTAGTTTCTATAATTCC-3',

Rev: 5'-GTAGTTACTCTCAAGATTAAGAACTT-3'

For strawberry:
RPFNF1: 5’ CGGTTCCCCAAAAGATAGTAGTTAC 3’,
RPFNR1: 5’ GTTCTACGCATTAAGATGCACTTGC 3’

Results and discussion

Apple scab DNA analysis using a *Vfa1* sequence specific marker was performed to identify the presence of *Vfa1*gene like sequences in apple cultivars and seedlings. Our results showed that the cultivars ‘Prima’, ‘Aldas’, ‘Skaistis’ and ‘Rudenis’, which are immune to 1–5 *Venturia ineaqualis* races, have a DNA fragment of 500bp specific to the *Vfa1* gene (Fig. 1).

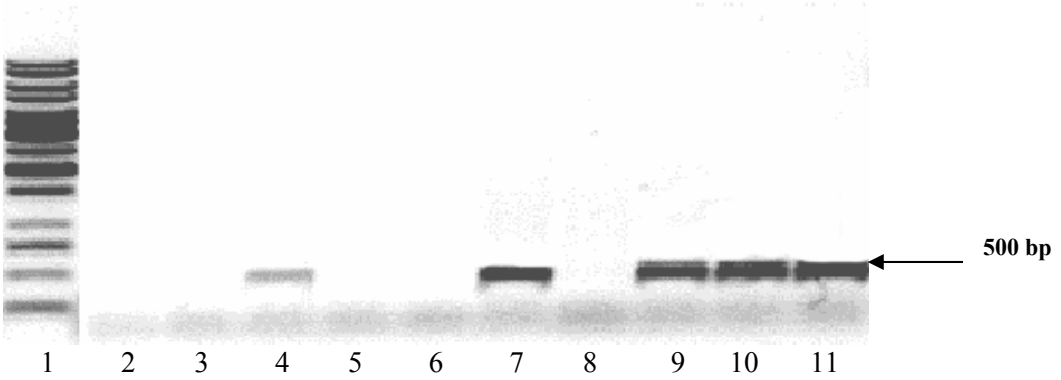

Figure 1. Apple DNA fragments amplified by PCR using *Vf1*a specific primer pair.
1 – GeneRuler^{TM} 1kb DNA Ladder; 2 – ‘Noris’; 3 – ‘Papirovka’; 4 – ‘Tellissare’;
5 – ‘Auksis’; 6 – ‘Katja’; 7 – ‘Prima’; 8 – ‘Štaris’; 9 – ‘Aldas’; 10 – ‘Skaistis’;
11 – ‘Rudenis’
1 paveikslas. *Obelų DNR fragmentai po PGR, naudojant Vfa1 genui specifinius pradmenis*

The same fragment was obtained in apple seedlings from crosses where one of parent form was scab immune variety ‘Prima’. However, cultivar ‘Štaris’ that is has not been injured by scab so far, has not *Vfa1* gene. Scab resistance in this cultivar probably is governed by an alternative genetic mechanism or combination of polygenes.

It was demonstrated that the presence of *Vfa1* did not warrant scab immunity by itself. This fragment was found in cultivar ‘Tellissaare’ with polygenic scab resistance mechanism. Infection of leaves and fruit of this cultivar are estimated in 3 and 1 score points respectively /Gelvonauskienė, Bandaravičius, 1998/. In this case seemingly altered regulation of gene expression takes place. It could be influenced by small changes in gene sequence, caused by strong and steady horizontal selective pressures by the fungal pathogen *V. inaequalis*, and divergent selection on somatic variations /Xu, Korban, 2004/. It is possible also that scab immunity is not really monogenic but depends on a few close located or functionally related genetic factors.

The above-mentioned DNA fragment was not obtained for the susceptible cultivars ‘Noris’ and ‘Papirovka’ and the cultivars ‘Katja’ and ‘Auksis’ with polygenic scab resistance.

Strawberry red stele *Phytopthora fragariae*

Our results show that several polymorphic bands could be obtained in PCR (polymerase chain reaction) using primer OPO-16 (Fig 2). There was quite clear band of about 430 bp in size characteristic only of 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 *Rpf 1*.

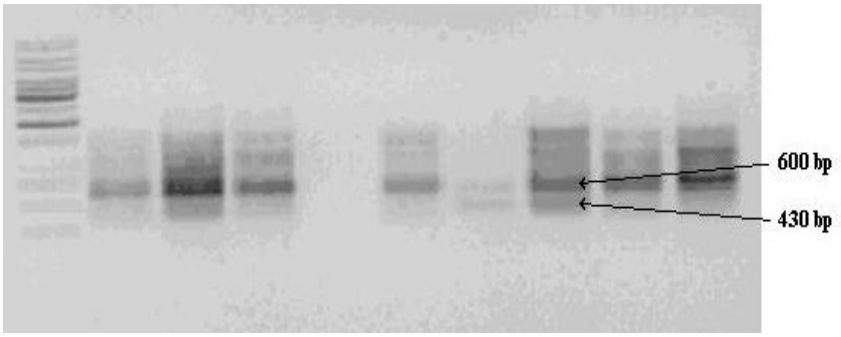

Figure 2. Strawberry DNA fingerprint, obtained by primer OPO-16. M-DNA fragments size marker (GeneRuler^{TM} 1kb DNA Ladder) (DNR fragmento dydžio žymuo),
1 – 005001, 2 – 940101 (‘Guardian’ x ‘Pegasus’), 3 – ‘Redgauntlet’, 4 – ‘Anapolis’;
5 – ‘Selen’, 6 – ‘Elsanta’, 7 – ‘Tristar’, 8 – 005002
2 paveikslas. *Braškių DNR fragmentai, gauti PGR, naudojant OPO-16 pradmenį*

This marker band was characteristic of 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 resistance genes such *Rpf2 or Rpf3*. In our experiment seedlings 005001 and 005001 (‘Selen’ x ‘Tristar’), cultivars ’Anapolis’, ‘Redgauntlet’, ‘Tristar’ lack 430 bp size DNA band characteristic to susceptible cultivars. Plants of those 5 genotypes were resistant to *P. fragariae* in the field conditions. Therefore it safe to say that our PCR data correspond to the data received in field conditions and allows strong assumption that those 5 genotypes carry *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 *Rpf1* gene. Our experimental data confirm those suggestions. Contrary to the data of Haymes et al. (1997) we observed the exceptionally bright DNA band of size about 600 bp (Fig. 2). 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 due to insufficiently bright and clear bands. Sometimes it is quite difficult to distinguish appropriate DNA band clearly from other bands. Modifications of PCR conditions failed to help a lot. We decided to develop a SCAR marker which could help undoubtedly confirm presence or absence of the *Rpf1* gene. Such markers linked to *Colletotrichum acutatum* resistance gene are being developed for strawberry /Guerin et al., 2003/. About 400bp in size amplified DNA fragment of 'Elsanta' 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 3. According to our PCR data cultivars and seedlings 'Redgauntlet', 'Anapolis'; 'Tristar', 'Dange', 005001; 005002 carry the *Rpf1* gene, cultivars 'Honeoye'; 'Elsanta', Venta', 'Kama'; 940101; 'Selen'; 'Elkat', 'Senga Sengana' – show lack of this gene. Such findings correspond to our previous experiment and field trial data. Differently from using RAPD primers we observed only one band that clearly shows presence or absence of the *Rpf1* gene.

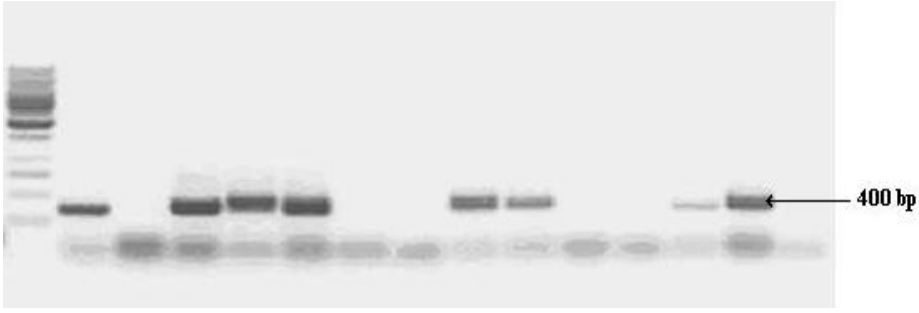

Figure 3. Strawberry DNA fingerprint, obtained by SCAR primers RPFNF1 and RPFNR1. M – DNA fragments size marker (GeneRuler^{TM} 1kb DNA Ladder) (DNR fragmentų dydžio žymuo), 1 – 'Honeoye'; 2 – 'Redgauntlet', 3 – 'Elsanta', 4 – 'Venta', 5 – 'Kama'; 6 – 'Anapolis'; 7 – 'Tristar'; 8 – 940101; 9 – 'Selen'; 10 – 005001; 11 – 005002; 12 – 'Elkat', 13 – 'Senga Sengana'; 14 – 'Dangė'
3 paveikslas. *Braškių DNR fragmentai po PGR naudojant SCAR pradmenis RPFNF1 ir RPFNR1*

It can be observed that brightness and position of DNA bands are not always exact. Anyway, 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 those genes of such genotypes.

Two years summarized evaluation data of seedlings from crosses 'Selen' x 952002 ('Tristar' x *F. chiloensis* DeL Norte) and 'Anapolis' x 'Honeoye' in a field condition showed resistant : susceptible segregation rate 1:1. The same segregation rate

was obtained in PCR experiments and it confirms that *Rpf1* is dominant and present in heterozygous state.

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

Conclusions

1. Apple DNA fragments from PCR using *Vf1a* specific primer pair show, that cultivars 'Prima', 'Aldas', 'Skaistis' and 'Rudenis' which are immune to 1–5 *Venturia ineaqualis* races, have *Vfa1* gene.

2. Strawberry DNA fragments obtained by PCR using SCAR primers demonstrate that strawberry seedlings of cross combinations red stele susceptible x resistant ('Selen' x 952002 and 'Honeoye' x 'Anapolis') in laboratory and field condition showed resistant: susceptible segregation ratio 1:1.

Received 07 08 2007
Accepted 02 10 2007

REFERENCES

1. Belfanti E., Silfverberg-Dilworth E., Tartarini S. et al. The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated mariety // Proceedings of the National Acccademy of Sciences. – 2004, vol. 101, iss. 3, p. 886–890

2. Dellaporta S. L., Wood J., Hicks J. B. A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter. – 1983, vol. 1, p. 19−21

3. Doyle J. J., Doyle J. L. Isolation of plant DNA from fresh tissue // Focus. – 1990, vol. 12, iss. 1, p. 13–15

4. Fischer C. Multiple resistant apple cultivars and consequences for apple breeding in the future // Acta Horticulturae. – 2000, vol. 538, iss. 1, p. 229–234

5. Gelvonauskienė D., Bandaravičius A. Obelų atsparumas rauplėms ir filostiktozei // Sodininkystė ir daržininkystė: mokslo darbai. – 1998, t. 17 (1), p. 30–38

6. Guérin G., Lerceteau-Kohler E., Laigret F. et al. Development of SCAR marker linked to dominant gene conferring resistance *Colletotrichum acutatum* in strawberry // Acta Horticulturae. – 2003, vol. 626, p. 85–91

7. Haymes K. M., Henken B., Davis T. M., van de Weg W. E. Identification of RAPD markers linked to a *Phytophthora fragariae* resistance gene (*Rpf1*) in the cultivated strawberry // Theoretical and Applied Genetics. – 1997, vol. 94, p. 1097–1101

8. Kozlovskaya Z. Apple genetic resources and their potential for breeding in Belarus // Sodininkystė ir daržininkystė. – 2001, vol. 20, iss. 3, p. 43–49

9. Maas J. L. (ed.). Compendium of Strawberry Diseases, 2nd edition. - APS Press, St. Paul, Minnesota, 1998. – 128 p.

10. Newcomb R. D., Crowhurst R. N., Gleave A. P. et al. Analyses of Expressed Sequence Tags from Apple // Plant Physiology. – 2006, vol. 141, p. 147–166

11. Park S., Sugimoto N., Larson M. D. et al. Identification of Genes with Potential Roles in Apple Fruit Development and Biochemistry through Large-Scale Statistical Analysis of Expressed Sequence Tags // Plant Physiology. – 2006, vol. 141, p. 811–824

12. Santos B., Barrau C., Romero F. Strawberry fungal diseases // Food, Agriculture & Environment. – 2003, vol. 1, iss. 3&4, p. 129–132

13. Scott D. H., Draper A. D., Galletta G. J. Breeding strawberries for red stele resistance // Plant Breeding Reviews. – 1984, vol. 2, p. 195–214

14. Stembridge G. E., Scott D. H. Inheritance of resistance of strawberry to the common race of the red stele root rot fungus // Plant Disease Report. – 1959, vol. 43, p. 1091–1094

15. Van de Weg W. E. Cultivar-race interactions of the strawberry *Phytophthora fragariae* system with regard to a gene-for-gene model // Acta Horticulturae. – 1989, vol. 265, p. 203–206

16. Van de Weg W. E. A gene-for-gene model to explain interactions between cultivars of strawberry and races of *Phytophthora fragariae* var. *fragariae* // Theoretical Applied Genetics. – 1997, vol. 94, p. 445–451

17. Williams E. B., Dayton D. F., Shay J. R. Allelic genes in *Malus* for resistance to *Venturia inaequalis* // Proceedings of the National Academy of Sciences. – 1966, vol. 88, p. 52–56

18. Xu M., Korban S. S. Somatic variation plays a key role in the evolution of the *Vf* gene family residing in the *Vf* locus that confers resistance to apple scab disease // Molecular Phylogenetics and Evolution. – 2004, vol. 32, iss. 1, p. 57–65

19. Xu M., Korban S. S. A cluster of four receptor-like genes reside in the Vf locus that confers resistance to apple scab disease // Genetics. – 2002, vol. 162, p. 1995–2006

20. Ždanov V. V., Sedov E. N. Selekcija jabloni na ustojčivost' k parše. – Tula, 1991. – 207 s.

ISSN 1392-3196
Žemdirbystė, t. 94, Nr. 4 (2007), p. 139–145
UDK [634.11+364.75]:631.527:631.526.32

OLIGOGENAIS DETERMINUOTŲ ATSPARIŲ GRYBINĖMS LIGOMS OBELŲ IR BRAŠKIŲ ATRANKA, NAUDOJANT MOLEKULINIUS ŽYMENIS

D. Gelvonauskienė, R. Rugienius, T. Šikšnianas, G. Stanienė, A. Sasnauskas, V. Stanys

Santrauka

Naudojant obelų atsparumo rauplėms geno *Vfa1* specifinius DNR žymenis, įvertintas šio geno buvimas skirtingose obelų veislėse. Tyrimo rezultatai rodo, kad veislės, kurios yra imunios 1–5 *Venturia inequalis* rasėms, turi specifinį *Vfa1* genui DNR fragmentą. Pagal RAPD žymens DNR seką sukurtas jai specifinis SCAR žymuo, susijęs su braškių atsparumo fitoftorozei (*Phytophthora fragariae*) *Rpf1* genu. Naudojant šį žymenį įvertintas geno *Rpf1* buvimas skirtingose braškių veislėse ir sėjinukuose. Kryžminimo kombinacijų 'Selen' x 952002 ('Tristar' x *F. chiloensis* DeL Norte) ir 'Anapolis' x 'Honeoye' braškių sėjinukų laboratoriniai ir lauko tyrimai rodo, kad atsparūs ir neatsparūs fitoftorozei braškių sėjinukai pasiskirsto santykiu 1:1.

Reikšminiai žodžiai: *Malus domestica, Fragaria ananassa, Phytophthora fragariae, Venturia inaequalis,* selekcija, molekuliniai žymenys, veislės, sėjinukai, SCAR, RAPD.