

SINTEZĂ PRIVIND APLICAȚIILE MARKERILOR MOLECULARI ÎN PROGRAMELE DE AMELIORARE DERULATE LA ICDP PITESTI, ROMANIA

APPLICATIONS OF MOLECULAR MARKERS IN FRUIT BREEDING PROGRAMS DEVELOPMENT AT ICDP PITEȘTI, ROMANIA – A REVIEW

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Abstract

Molecular markers are considered as efficient tools for details DNA based characterization of fruit crops and provide new directions to the efforts of breeders in taxonomy, gene mapping, phylogenetic analysis, and the assessment of disease resistance. Compared with traditional breeding programs, molecular markers can increase the efficiency and effectiveness of fruit breeding programs. This study looks at various molecular markers, including RAPD, SCAR, STS, SSRs, QTL analyses and how they contribute to our understanding of disease resistance, genetic diversity, and evolutionary of Romanian cultivars.

Cuvinte cheie: gene de rezistență, rapănul mărului, făinare, mana coletului, antracnoza căpșunului, phytoplasme, polimorfism allelic.

Key words: resistance genes, apple scab, powdery mildew, Phytophthora crown rot, anthracnose of strawberry, phytoplasma, allelic polymorphism.

1. Introduction

Developing and releasing new fruit cultivars take a long time and involve many steps due to the biology of fruit plants (large trees, long juvenile periods, intensive agricultural practice). For example, apples trees take up to 8-10 years to fully mature, even than using early-fruited initial forms. Although vegetative propagation allows for the production of a significant number of clonal replicates for the evaluation of environmental effects and genotype × environment interactions, the spaces required for plant cultivation and the intensity of work necessary for phenotypic surveys slow down the work of researchers (Madenova et al., 2024).

The translation of trait loci and whole-genome sequences into diagnostic genetic markers that are effective and affordable for use by breeders, who must choose genetically superior parents and subsequently choose genetically superior individuals among their progeny, are one of the most difficult tasks still facing tree fruit geneticists (Gloria and Cipriani, 2023).

Marker-assisted selection makes it possible to indirectly identify the presence of the genetic determinant or determinants of the traits of interest in the progeny obtained by crossing the appropriate parents. The success of the MAS depends on several factors, including the complexity of the trait considered and the proximity of the marker to the trait to be selected. MAS is gaining considerable importance as it can improve the efficiency of plant breeding through the precise transfer of genomic regions of interest and acceleration of the recovery of the section of the genome that contains the trait of interest (Gloria and Cipriani, 2023).

One way to achieve durable resistance is to pyramid multiple scab resistance genes in a cultivar, and it is desirable to combine several genetic factors that control immunity in one genotype (Bus et al. 2011).

2. Material and methods

Different fruit cultivars and rootstocks which belong to the experimental field of the Research Institute for Fruit Growing (ICDP) Pitesti, Romania were used in this study. The institute is located in an area with solar radiation 114-128 Kcal/cm², mean temperatures between 9.8-11.2°C, 3,400-4,100 degrees over 0°C, 2,800 - 3,500 degrees over 10°C, mean precipitations 450-700 mm, in dry years, from April until October. The experimental plots were located on poor loam-clay soil with over 30% clay.

Young leaves from each cultivar were collected direct in plastic bags and stored at -80° in the freezer. Total DNA was extracted using the protocol recommended for the 2% CTAB by the Isolate II Plant DNA Kit. The amplification reactions were performed using the kit 2x MyTaq™ Red Mix from Meridian Bioscience and specific primers for each study. Amplifications were performed under the following conditions: initial denaturation step at 95°C for 2.45 min., followed by 40 cycles of 55 sec. at 94°C, 55 sec. at (58°C - 60°C), 1.39 min. at 72°C, and a final extension of 10 min. at 72°C for the markers used in molecular screening for resistance to fire blight, and for two markers for resistance to scab (OPL19 and AD13); initial denaturation step at 94°C for 3 min., followed by 40 cycles of 1 min. at 94°C, 55 sec. at (Tm°C), 2 min. at 72°C, and a final extension of 10 min. at 72°C for the markers K08 (Tm=65°C), OPB18 (Tm=55°C), OPU02(Tm=60°C) and OPB12 (Tm=58°C); initial denaturation step at 94°C for 5 min., followed by 40 cycles of 2 min. at 94°C, 2 min. at 55°C, 2 min. at 72°C, and a final extension of 10 min. at 72°C for the EM M01 marker.

The samples were migrates through the agarose gel, in a horizontal electrophoresis system.

Visualization of the results was done with a high-quality imaging system Uvitec Cambridge Essential and UVITec14D analysis software.

3. Results and discussions

Disease resistance genes

Apple. In many regions of Romania, apple scab, caused by the ascomycete fungus *Venturia inaequalis*, is the most serious disease of apple. For this reason, the apple breeding program develop at ICDP Pitesti is focus on durable disease resistance to apple scab and powdery mildew, as major goal. In this context, combining different resistance genes in the same genotype is considered a good way to achieve durable resistance. Presently, 20 scab resistance genes are mapped, but the only sources for durable resistance are *Rvi5* (*Vm*), *Rvi11* (*Vbj*), *Rvi12* (*Vb*), *Rvi14* and *Rvi15* (*Vr2*) (Patocchi et al., 2020, Höfer et al., 2021). In this study, for apple scab, screening was done with 6 SCAR markers (AL07, AM19, VfC, OPL19, AD13, K08), one STS marker (OPB12) and one SSR (Hi07h02).

Apple powdery mildew, caused by the obligate biotrophic ascomycete *Podosphaera leucotricha* (Ellis & Everh.), is another disease with a great impact on fungicide use. The genetic base for improving the resistance to powdery mildew is more limited. Only, few major resistance genes were found, like *PI-1* from *Malus robusta*, *PI-2* from *Malus zumi*, *PI-w* from ornamental crab apples, *White Angel*, *PI-d* from D12 clone, *PI-m* from MIS, and breeders have focused their efforts on introducing into bred cultivars. For apple powdery mildew, screening was done only with SCAR markers (EM M01, AT20, OPU02).

***Rvi2* (sin. *Vh2*).** The SCAR marker OPL19 was used to determinate presence of *Rvi2*, which amplified the allele linked to *Rvi2*, but also of recessive alleles of the gene in the following cultivars: 'Alex', 'Bistrițean', 'Cezar', 'Ciprian', 'Dany', 'Delicios de Voinești', 'Discoprim', 'Estival', 'Jonaprim', 'Luca', 'Pomona', 'Redix', 'Remar', 'Romus 2', 'Romus 3', 'Romus 4', 'Romus 5', 'Salva', 'Starkprim', 'Voinicel'; 'Voinea' is a cultivar that carries only the dominant allele of the *Rvi2* gene, no recessive alleles. The 'Delicios de Voinești' is a cultivar with a monogenic characteristic for scab resistance, having only *Rvi2* gene.

***Rvi4* (sin. *Vr1*, *Vh4*, *Vx*).** To confirm presence of *Rvi4* gene was used SCAR marker AD13 which amplified two fragments, 1100 bp and 1,200 bp, along with the dominant allele of 950 bp, in nine cultivars: 'Romus 3', 'Romus 5', 'Redix', 'Cezar', 'Pomona', 'Remar', 'Voinicel', 'Estival', 'Discoprim'. Recently, Peil and colab. demonstrate that *Rvi4* and *Rvi15* are the same resistance gene, and propose to use *Rvi4*, as this name was the first assigned to this resistance and because it is probably more well known in apple breeding than *Rvi15*.

***Rvi5* (sin. *Vm*),** located on LG-17 at the distal end, was shown to be responsible for resistance in *Malus micromalus* and *Malus atrosanguinea* 804. (Dayton and Williams, 1970). Recently, using the apple genome as a reference, a 228 kb region likely containing the *Rvi5* gene was identified (Bandara et al., 2015). With STS marker OPB12, an amplified fragment of 687 bp was identified in three cultivars: 'Nicol', 'Generos' and 'Irisem'.

***Rvi6* (sin. *Vf*)** was the first scab resistance gene identified from a wild relative (*Malus floribunda*) of apples. Markers AL07, AM19 and VfC are indicators for *Rvi6* gene. The SCAR marker AL07 is located 0.2cM from de gene (Xu and Korban, 2000). AM19 was found closer to a resistance gene than AL07 and was used for chromosome walking of the BAC library of 'Florina'. The resistance conferred by this gene is influenced by the gene environment (Chizzali et al., 2016). In this study, using three SCAR marker (AL07, AM19 and VfC), alleles linked to *Rvi6* were reported in 32 Romanian bred cultivars: 'Aura', 'Rustic', 'Valery', 'Brumar', 'Revidar', 'Doina', 'Iris', 'Real', 'Inedit', 'Luca', 'Salva', 'Jonaprim', 'Starkprim', 'Romus 4', 'Voinea', 'Rebra', 'Dacian', 'Colmar', 'Colonade', 'Romus 5', 'Voinicel', 'Discoprim', 'Cezar', 'Pomona', 'Remar', 'Pionier', 'Bistrițean', 'Dany', 'Alex', 'Ciprian', 'Romus 3', 'Redix'.

Rvi8 (sin. Vh8). Bus et al., in 2005, reported an allele size of 799 bp linked to *Rvi8* for SCAR marker OPB18. In the present study, using marker OPL19 SCAR, a fragment of 430 bp was detected, that it is assigned the presence of *Rvi8* gene, after confirming the presence of the fragment of 799 bp, amplified with the OPB18 marker.

Rvi11 (sin. Vh). The *Rvi11* gene was identified in *Malus baccata* by Dayton and Williams (1968) and was mapped to LG-2 at about 0.6CM (Gygax et al., 2004). With SCAR marker K08, we showed that 22 Romanian apple cultivars ('Productiv de Cluj', 'Precoce de Ardeal', 'Remus', 'Rebra', 'Dacian', 'Colmar', 'Colonade', 'Romus 2', 'Irisem', 'Generos', 'Nicol', 'Pionier', 'Estival', 'Bistrițean', 'Dany', 'Alex', 'Ciprian', 'Romus 3', 'Redix', 'Pomona', 'Revidar', 'Remar') carried this gene.

PI-w. This gene for powdery mildew resistance has been mapped on LG 8 and been less used in apple breeding programs (Bus et al., 2011). The SCAR marker EM M01 amplified on 88bp fragment in Romanian three cultivars: 'Irisem', 'Generos', and 'Nicol' (compact cultivar). AT20 and OPU02 were used as SCAR markers for **PI1** and **PI2**, respectively, and not data about the alleles linked to these genes are available in Romanian cultivars studied.

The apple scab and powdery mildew screening of 43 Romanian cultivars bred in different research units shows different gene accumulation, unfortunately, unenough to obtain an immune response to these diseases (table 2).

Strawberry. In Romania, strawberry plants are usually affected by a number of pathogens including *Mycosphaerella fragariae* (Tul.) Lindau (leaf spot), *Diplocarpon earliana* (Ell. & Ev.) Wolf (leaf scorch) and recently *Colletotrichum acutatum* Simmonds (anthracnose) and *Phytophthora fragariae* Hickman (red stele). One of the most useful applications of genetic markers, as tools for indirect selection of hybrid plants in breeding program development at ICDP Pitesti, are usage of markers linked to these diseases resistance genes. Using SCAR marker R1A linked to *Rpf1* and STS marker *Rca2_240* linked to *Rca2*, were screened the Romanian cultivars and hybrids obtained from different hybridization for the presence or absence of the markers linked to these genes. The primer for STS-*Rca2_240* marker associated with the resistance allele *Rca2* to strawberry anthracnose was detected in three cultivars ('Floral', 'Real' and 'Sarom') out of the seven Romanian cultivars and the marker SCAR-R1A is dominant for the *Rpf1* resistance gene and it is present in 'Real' cultivar. With RAPD marker OPO-16C_438, an allele size of 438 bp linked to *rpf1* (susceptible allele for susceptibility to *Phytophthora fragariae*) were reported for a large number of strawberry hybrids.

Phytoplasma identification. In order to maintain mother plants free from harmful organisms, primers which amplified products from all phytoplasma-infected plants were used as a diagnostic tool necessary for epidemiological studies, analysis of vector relationships and elaboration of disease control strategies. Using specific phytoplasmic primers, a PCR product containing the entire 16S rRNA gene as well as the distance region of 16S/23S rRNA was amplified, some rootstock plants ('Adaptabil', 'Baroc' and 'Mirodad 1') were tested (table 3). A PCR product, of approximately 1800 bp, was amplified on 11 from 19 tested samples (figure 1).

Analysis of genetic diversity. Molecular analysis with RAPD markers OPAC-11, OPBD-04, OPBC-04, OPBB-05 and OPBD-01 showed a moderate informative polymorphism and a medium to high genetic diversity for the apple cultivars grown and released in Romania, in different research centers. For plum species, markers OPAC-11, OPBD-04, OPBD-01 and OPBA-20 also indicated moderate polymorphism and medium to high genetic diversity, while markers OPBC-04 and OPBB-05 expressed a high genetic diversity and moderate polymorphism (Iancu and Chivu, 2021).

QTLs for fire blight resistance. The existence of different levels of susceptibility to fire blight (*Erwinia amylovora*) in European pear (*Pyrus communis* L.) cultivars suggests that it is possible to identify QTLs related to resistance in pear germplasm. Using 3 SSR molecular markers (CH05a03, CH02c02b, TsuEN01) associated with certain QTL for resistance to fire blight, 31 Romanian pear cultivars were screening. Significant values of the Pearson coefficient were obtained for 15 genotypes, for two markers (CH05a03 and TsuEN01), which means the association between major QTL, located on HS2, and resistance to the attack of the pathogen, *Erwinia amylovora*. Some of pear varieties tested ('Seckel' and 'Kieffer Seedling') have a moderate resistance (Bell, 1990). Another Romanian local varieties, such as: 'Decana Krier', 'Galbene', 'Pere de iarnă', 'Pere gutui', 'Tămâioase de Călimănești' are recognized as potential sources of genes for resistance to fire blight (Cociu et al., 1999; van der Zwet, 1982, Thibault, 1982; Bell et Itai, 2011; Mareși et al., 2023).

4. Conclusions

Maintaining a moderate to high level of genetic diversity remains a challenge for genetic breeding programs. Therefore, the study of some traits (organoleptic qualities, resistance to biotic and abiotic stress) by evaluating allelic polymorphism and genetic diversity will help breeding programs in the selection process. The molecular markers used to evaluate genetic diversity offers a clearly image

regarding the level of Romanian genetic diversity and extend previous work regarding the evaluation for main disease resistance of fruit cultivars bred in different research centers.

A combination of marker alleles linked to the fourth scab resistance genes, *Rvi₂*, *Rvi₄*, *Rvi₆* and *Rvi₁₁*, were detected in four cultivars, 'Romus 3', 'Redix', 'Pomona' and 'Remar'. The cultivars 'Bistrițean', 'Dany', 'Alex' and 'Ciprian' amplified marker alleles linked to *Rvi₂*, *Rvi₆* and *Rvi₁₁*. Alleles linked for three scab resistance genes, *Rvi₂*, *Rvi₄*, and *Rvi₁₁* was detected in summer cultivar 'Estival', and the combination *Rvi₆*, *Rvi₈*, and *Rvi₁₁* in old bred cultivar 'Pionier'. Marker alleles linked to powdery mildew resistance gene, *Plw*, and two scab resistance genes, *Rvi₅* and *Rvi₁₁*, were found only in three Romanian cultivars: 'Irisem', 'Generos' and 'Nicol'.

The marker STS-Rca₂_240 allowed presence of the *Rca₂* gene in three Romanian cultivars ('Floral', 'Real' and 'Sarom') and their resistance to anthracnose. The strawberry cultivar 'Real' can be successfully used in future breeding program for resistance to the *Phytophthora fragariae* and *Colletotrichum acutatum* diseases, because it has both resistances genes.

Regarding resistance to fire blight, the markers CH05a03, TSUEN0 and CH02c02b can be successfully used for the screening pear progenies.

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Tables and Figures

Table 1. Molecular markers use for different genetic analyses

Type/name	Utilization / identified genes	References
SCAR	Disease resistance genes	
AL07	<i>Rvi6</i> (sin. <i>Vf</i>) – apple scab	Khajuria et al., 2014
AM19		Tartarini et al., 1999
VfC		Afunian et al., 2004
OPL19	<i>Rvi2</i> or <i>Rvi8</i> – apple scab	Bus et al., 2005a
OPB18	<i>Rvi8</i> – apple scab	Bus et al., 2005a
AD13	<i>Rvi4</i> (sin. <i>Vr1</i> , <i>Vh4</i> , <i>Vx</i>) – apple scab	Boudichevskaia et al., 2006
R1A	<i>Rpf1</i> – <i>Phytophthora</i> crown rot of strawberry	Haymes et al., 2000
K08	<i>Rvi11</i> – apple scab	Gygax et al., 2004
EM M01	<i>Pl-w</i> – powdery mildew	Evans and James, 2003
AT20	<i>Pl1</i> – powdery mildew	Markussen et al., 1995
OPU02	<i>Pl2</i> – powdery mildew	Liebhard et al., 2002 Gardiner et al., 2003
STS	Disease resistance genes	
OPB12	<i>Rvi5</i> (sin. <i>Vm</i>) – apple scab	Cheng et al., 1998
Rca2_240	<i>Rca2</i> – anthracnose of strawberry	Lerceteau-Kohler et al., 2005
SSR	Disease resistance genes	
Hi07h02	<i>Rvi5</i> (sin. <i>Vm</i>) – apple scab	
	Identification of phytoplasma	
P1, P7	16S rRNA and 23S rRNA	Smart et al., 1996; Deng and Hiruki, 1991
P1A, P7A	16S rRNA and 23S rRNA	Lee et al., 2004
RAPD	Disease susceptible genes	
OPO-16C_438	<i>Rpf1</i> (recessive allele for susceptibility to <i>Phytophthora</i> crown rot of strawberry)	Haymes et al., 1997
	Allelic polymorphism and genetic diversity	
OPAC-11	Apple and plum	Mehdi et al., 2012 Melid et al., 2015 Shahin et al., 2012
OPBD-04		
OPBC-04		
OPBB-05		
OPBD-01		
OPBA-20	Plum	Nikoumanesh et al., 2011

Table 2. Apple gene accumulation in Romanian bred cultivars

Disease resistance genes	Cultivar
Apple	
-	Frumos de Voinești, Auriu de Cluj
<i>Rvi2</i>	Delicios de Voinești
<i>Rvi6</i>	Aura, Rustic, Valery, Brumar, Revidar, Doina, Iris, Real, Inedit
<i>Rvi11</i>	Productiv de Cluj, Precoce de Ardeal, Remus
<i>Rvi2</i> + <i>Rvi6</i>	Luca, Salva, Jonaprim, Starkprim, Romus 4, Voinea
<i>Rvi6</i> + <i>Rvi11</i>	Rebra, Revidar, Dacian, Colmar, Colonade
<i>Rvi2</i> + <i>Rvi11</i>	Romus 2
<i>Rvi2</i> + <i>Rvi4</i> + <i>Rvi6</i>	Romus 5, Voinicel, Discoprim, Cezar
<i>Rvi5</i> + <i>Rvi11</i> + <i>Plw</i>	Irisem, Generos, Nicol
<i>Rvi6</i> + <i>Rvi8</i> + <i>Rvi11</i>	Pionier
<i>Rvi2</i> + <i>Rvi4</i> + <i>Rvi11</i>	Estival
<i>Rvi2</i> + <i>Rvi6</i> + <i>Rvi11</i>	Bistrițean, Dany, Alex, Ciprian
<i>Rvi2</i> + <i>Rvi4</i> + <i>Rvi6</i> + <i>Rvi11</i>	Romus 3, Redix, Pomona, Remar
Strawberry	
<i>Rca2</i>	Floral, Sarom
<i>Rca2</i> + <i>Rpf1</i>	Real

Table 3. Primers used for phytoplasma detection

Gene	Marker type	Product size (bp)	Oligonucleotide sequence (5'→3')	References
16S rRNA /23S rRNA	P1; P7	1756 1784	F: CGTCCTTCATCGGCTCTT R: AAGAGTTTGATCCTGGCTCAGGATT	Smart et al., 1996; Deng & Hiruki, 1991
16S rRNA /23S rDNA	P1A; P7A	1759	F: CCTTCATCGGCTCTTAGTGC R: ACGCTGGCGGCGCGCCTAATAC	Lee et al., 2004

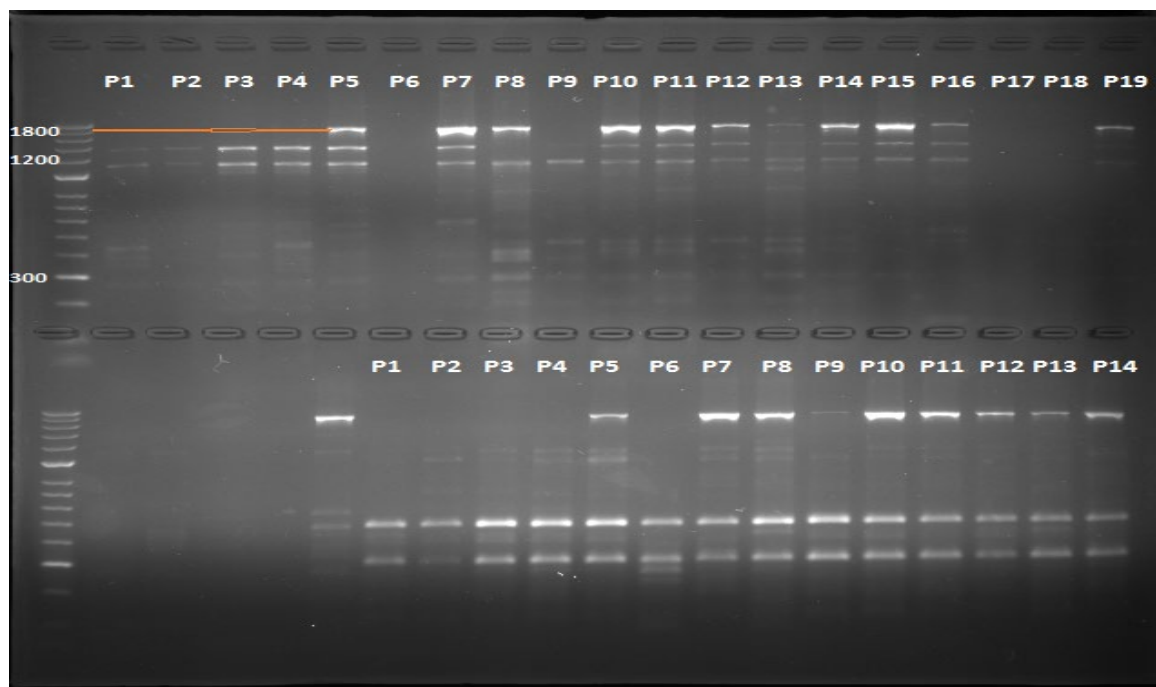


Fig. 1. Electrophoretic profile for P1/P7 (up) and P1A/P7A (down)