PCR markers of apple resistance to scab (*Venturia inaequalis* CKE.) controlled by *Vf* gene in Czech apple breeding

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ABSTRACT

The varieties of apples resistant to apple scab (*Venturia inaequalis* CKE.) are among the latest outcomes of breeding this fruit species not only worldwide but also in the Czech Republic. A common donor of resistance to this disease is *Malus floribunda* Sieb., clone 821, which has conferred the qualitative resistance based on the dominant *Vf* gene to a range of varieties. The objective of this article is to characterise *Vf* gene in a collection of new Czech apple varieties. The published primers were used for PCR evaluation of the collection of twenty Czech varieties, seventeen varieties of world assortment and six new resistant selections of Czech breeding. All varieties possessing resistance based on *Vf* gene had the heterozygous constitution of *Vf* gene (*Vfvf*). Segregated F_1 progeny, which was already selected by infection tests in a greenhouse, was included in the same PCR test. In all progenies recessive homozygous genotypes (*vfvf*) were also found that were not selected by the infection test. The higher occurrence of recessive homozygous genotypes (*vfvf*) was detected above all in the progenies of such parental combinations where one of the parents was a donor of quantitative resistance to apple scab.

Keywords: apple trees; *Malus* × *domestica*; *Malus floribunda*; apple scab; *Venturia inaequalis*; resistance; *Vf* gene; DNA; PCR

Apples belong to the main fruit species and are the most important fruit in the temperate zone. Apples contain a lot of dietetically important substances, for example vitamins, saccharides, pectins, minerals and fibre and that is why they are a very important part of human nutrition. Apples are also an important raw material for many branches of processing industry (Dvořák et al. 1976). One of the main breeding objectives is to increase resistance to abiotic and biotic factors. Breeding for resistance to diseases is targeted at development of varieties resistant to scab and mildew.

Apple scab is one of the most widespread diseases of apple trees. This disease is caused by *Venturia inaequalis* (*Ascomycetes*) (Tenzer and Gessler 1997). Leaves attacked by scab have a lower assimilation rate. They are falling off earlier, fruits do not develop to normal size and do not have the full taste quality (Dvořák et al. 1976).

Breeding for resistance is one of the effective measures for a protection against this disease. Crosby et al. (1992) describe two basic types of resistance of apple trees to scab. The first is qualitative (monogenic) resistance that is typical of some species of the genus *Malus*. Segregation of susceptible and resistant progenies is guided by Mendel's laws. An important source of this resistance is *Malus floribunda* Sieb., clone 821. Prima was the first resistant variety that was bred with *Vf* gene (Dayton et al. 1970). The resistance of older apple varieties of Czech origin is usually quantitatively determined (Vondráček 1960). Simultaneously with the development of apple resistance to scab new races of *Venturia inaequalis* were also found (Parisi et al. 1993).

Besides traditional approaches, DNA markers are employed in breeding programmes. A majority of DNA markers of resistance to *V. inaequalis* is based on the polymerase chain reaction (PCR). Gardiner et al. (1995) developed RAPD (Random amplified polymorphic DNA) markers for detection of a resistance gene from *M. floribunda.* The marker is localised 28cM from *Vf* gene. Gianfranceschi et al. (1996) and Hemmat et al. (1998) found two RAPD markers. One of these markers detected the main gene, *Vf* gene. Tartarini et al. (1999) obtained co-dominant specific PCR for detection of dominant homozygous, heterozygous and recessive homozygous markers.

MATERIAL AND METHODS

Plant material

Two collections were investigated. A collection of twenty apple varieties of Czech origin (Table 1) was used. The presence of *Vf* gene was confirmed in ten varieties. Two varieties (Angold and Zuzana) had a high level of quantitatively determined resistance. The second collection of eighteen foreign varieties with known resistance to scab is presented in Table 2.

These results were obtained by support of these grants: MZe ČR QD1267, MZe ČR QD1049, MŠM 412100002, GA ČZU 232/10/32899 and GA ČZU 21190/1312/213151.

Table 1. Varieties of Czech origin used for analyses

Variety	Origin	Year of registration in the CR	Presence of resistance	Detected genotype
Angold	Sempra a.s.	1995	partial	*vfvf*
Denár	Sempra a.s.	1989	none	*vfvf*
Diadém	Sempra a.s.	1992	none	*vfvf*
Doris	Sempra a.s.	1988	none	*vfvf*
Jarka	Sempra a.s.	1995	none	*vfvf*
Jonalord	O. Louda	1993	none	*vfvf*
Julia	Sempra a.s.	1994	none	*vfvf*
Karmína	ÚEB ČAV	1995	yes	*Vfvf*
Klára	Sempra a.s.	1994	none	*vfvf*
Melodie	O. Louda	1991	yes	*Vfvf*
Nabella	Sempra a.s.	1994	none	*vfvf*
Otava	ÚEB ČAV	1997	yes	*Vfvf*
Produkta	Sempra a.s.	1998	none	*vfvf*
Rajka	ÚEB ČAV	1999	yes	*Vfvf*
Resista	Sempra a.s	1997	yes	*Vfvf*
Rosana	ÚEB ČAV	1994	yes	*Vfvf*
Rubinola	ÚEB ČAV	1997	yes	*Vfvf*
Topaz	ÚEB ČAV	1997	yes	*Vfvf*
Vanda	ÚEB ČAV	1994	yes	*Vfvf*
Zuzana	Sempra a.s.	1997	partial	*vfvf*

Progenies of six experimental crosses were also included: Resista × Angold, Resista × Topaz, Retina × Topaz, Resista × HL665 (Spartan × Antonovka), HL665 (Spartan × Antonovka) × Retina and HL665 (Spartan × Antonovka) × Topaz. Resista, Retina and Topaz were donors of dominant allele *Vf* while Angold and Antonovka were donors of quantitatively based resistance. These breeding progenies are characterised in Table 3.

Infection tests in greenhouse conditions according to Chevalier et al. (1991) were used for selection of resistant plants. Mixtures of isolates were used for plantlet inoculation. Seedlings were sprayed with a conidial suspension of *Venturia inaequalis* CKE. Seedlings were incubated for 48 hours at 18°C and 100% relative humidity. Disease symptoms were evaluated macroscopically after 21 days of cultivation in a greenhouse. Seedlings were divided into 5 classes. Plants in class 0 were without symptoms of infection. Plants of class 4 had lesions with full sporulation. For PCR analyses only pre-selected seedlings without symptoms of apple scab on the leaves – class 0 to 3 were used.

DNA isolation

DNA was isolated from leaves in all evaluated genotypes. Plants of variety collection were cultivated in field conditions and hybrids were grown in greenhouses. Leaves were immediately fixed in liquid nitrogen and used for extraction. The CTAB method according to Saghai-Maroof (1984) was used. DNAs were concurrently isolated by GenElute Plant Genomic DNA Kit (Sigma, SRN). Differences between extraction methods were evaluated by analysis of variance (Statgraphics Vers. 4.0).

PCR markers

The primers according to Tartarini et al. (1999) were used for detection of dominant allele *Vf*. For multi PCR two pairs of primers in single reaction were used. Primers A (5'TGAAAGAGAGATCCAGAAAGTG3') and B (5'CATCCCTCCACAAATGCC3') amplified a co-dominant marker. 466 bp fragment characterised dominant allele *Vf* and 724 bp fragment characterised recessive allele *vf*. The pair of primers C (5'CGTAGAACGGAATTTGACAGTG3') and D (5'GACAAAGGGCTTAAG TGCTCC 3') amplified a marker of dominant allele *Vf* (526 bp fragment) in the same reaction. The composition of 25 µl multi-PCR was: 25 ng DNA/25 µl, 0.2µM primer A, 0.2µM primer B, 0.1µM primer C, 0.1µM primer D, 1.5mM $MgCl_2$, 0.2mM dNTP, 0.8 U Taq/25 µl. The programme of amplification was: 1× (94°C – 150 s, 60°C – 60 s, 72°C – 120 s), 35× (94°C – 30 s, 60°C – 60 s, 72°C – 120 s) and 1× (72°C – 600 s).

All samples were also evaluated by PCR test using the primers of pair E (5'GTAAAGCAAGCACTTCAACG') and F (5'GTAAAATAGATGTGTGGGTAGC') according to Gianfranceschi et al. (1996). This pair was able to amplify the 400 bp marker of dominant *Vf* allele. The composition of 25 µl reaction was: 10 ng DNA/25 µl, 0.3µM primer E, 0.3µM primer F, 2.5mM $MgCl_2$, 0.1mM dNTP, 0.7 U Taq/25 µl. Touchdown amplification steps according to Hemmat et al. (1998) were applied to amplify the PCR marker. The programme of amplification was: 1× (94°C – 120 s, 69°C – 120 s, 72°C – 120 s), 5× (94°C – 60 s, 68°C – 120 s, 72°C – 120 s), 5× (94°C – 60 s, 67°C – 120 s,

Table 2. Analysed foreign varieties

Variety	Country of origin	Presence of resistance	Detected genotype
Antonovka	Russia	partial	*vfvf*
Discovery	Great Britain	none	*vfvf*
Florina	France	yes	*Vfvf*
Gala	New Zealand	none	*vfvf*
Gloster	Germany	none	*vfvf*
Golden Delicious	USA	none	*vfvf*
Hrivna	Slovakia	none	*vfvf*
Idared	USA	none	*vfvf*
James Grieve Red	Germany	none	*vfvf*
Lord Lambourne	USA	none	*vfvf*
Melrose	USA	none	*vfvf*
Mio	Sweden	none	*vfvf*
Quinte	USA	none	*vfvf*
Retina	Germany	yes	*Vfvf*
Sir Prize	USA	yes	*Vfvf*
Spartan	Canada	none	*vfvf*
Wealthy Red	USA	none	*vfvf*

Table 3. Parental combinations of resistant progenies and their segregation after greenhouse tests

Parents	Donor of *Vf* allele	Results of infection test in greenhouse		
		number of analysed seedlings	frequency of resistant seedlings – classes 0–3 (%)	frequency of susceptible seedlings (%)
Resista × Angold	Resista	301	59	41
Resista × Topaz	Resista, Topaz	371	62	38
Retina × Topaz	Retina, Topaz	234	76	24
Resista × HL665 (Spartan × Antonovka)	Resista	411	74	26
HL665 (Spartan × Antonovka) × Retina	Retina	329	78	22
HL665 (Spartan × Antonovka) × Topaz	Topaz	319	73	27

72°C – 120 s), 5× (94°C – 60 s, 66°C – 120 s, 72°C – 120 s), 5× (94°C – 60 s, 65°C – 120 s, 72°C – 120 s), 5× (94°C – 60 s, 64°C – 120 s, 72°C – 120 s), 5× (94°C – 60 s, 63°C – 120 s, 72°C – 120 s), 5× (94°C – 60 s, 62°C – 120 s, 72°C – 120 s) and 1× (72°C – 480 s).

Amplified DNA fragments were visualised by ethidium bromide (Sambrook et al. 1989).

RESULTS

Infection test

Results of macroscopic evaluation of resistant and susceptible plants are presented in Table 3.

DNA isolation

Using CTAB method according to Saghai-Maroof et al. (1984) high-molecular DNA was obtained in all genotypes. Average quantity was 64.8 µg DNA/g fresh leaves. Coefficient of variation was 12.3%. Using GenElute Plant Genomic DNA Kit (Sigma, SRN) degraded DNA was obtained in 56% of genotypes. Average quantity was 17.6 µg DNA/g fresh leaves. Coefficient of variation was 16.1%. Differences between the two isolation methods in 20 Czech varieties were evaluated by three-way ANOVA (Statgraphics Vers. 4.0). Differences between the varieties and replications were not statistically significant. Differences in DNA quantity (µg DNA/g fresh leaves) between the extraction methods were significant on $P = 0.05$ probability level.

Influence of DNA isolation methods on PCR amplification of *Vf* gene markers

Dominant or co-dominant PCR markers according to Tartarini et al. (1999) and Gianfranceschi et al. (1996) were specified only by using DNA that was isolated by CTAB method according to Saghai-Maroof et al. (1984). Template DNA isolated by GenElute Plant Genomic DNA Kit (Sigma, SRN) was typical of unspecific amplification of both markers. The products of amplification were not obtained in 80% of analysed genotypes. On the basis of this result only DNA that was isolated by the method according to Saghai-Maroof et al. (1984) was used in this experiment.

Detection of dominant and recessive allele of *Vf* gene

The specificity of optimised PCR co-dominant and dominant markers was confirmed in the collection of world varieties. All varieties with declared resistance always demonstrated the existence of dominant and co-dominant markers. PCR products with 526 bp and 466 bp size were typical of resistant varieties (Florina, Retina, Sir Prize). All susceptible varieties (Discovery, Gala, Gloster, Golden Delicious, Hrivna, Idared, James Grieve Red, Lord Lambourne, Melrose, Mio, Quinte, Spartan, Wealthy Red) had PCR markers only for recessive allele – genotype *vfvf*. The size of this marker was 724 bp. Partial resistance, polygenically determined, is typical of Antonovka variety. This variety had a recessive homozygous PCR marker. Figure 1 shows the result of amplification of multi-PCR (primers A, B, C and D in single reaction) in a sample of foreign assortment varieties.

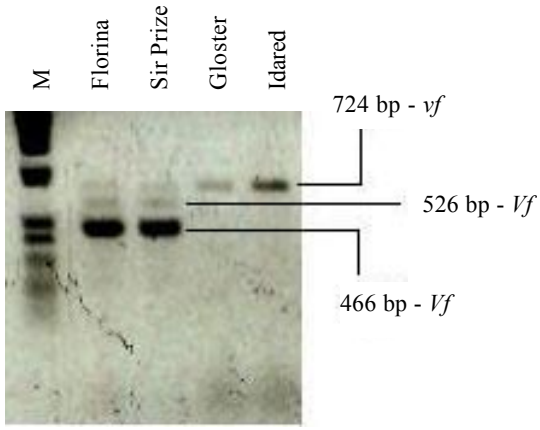

Figure 1. Multi-PCR amplification of co-dominant and dominant markers of *Vf* gene
M – marker λDNA/*Eco*47I(*Ava*II)

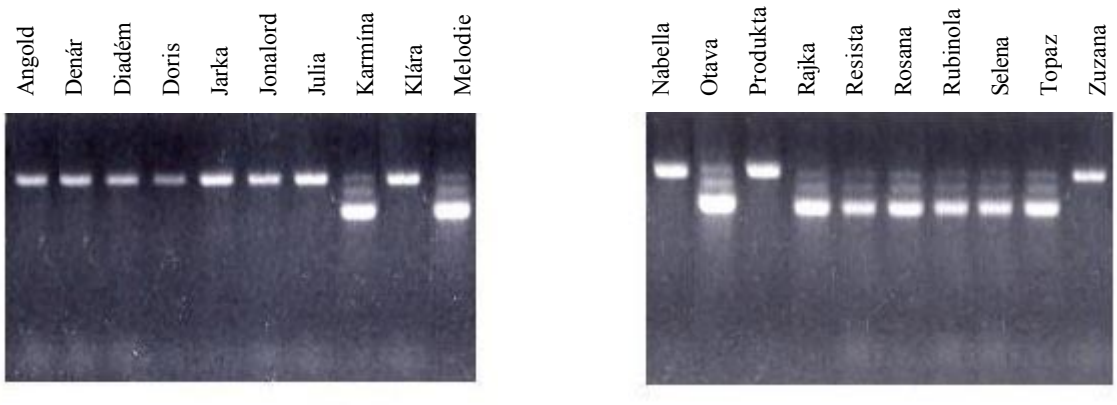

Figure 2. Multi-PCR (primers A, B, C, D) detection of dominant allele *Vf* in the collection of new Czech varieties of apple trees

In the case of resistant varieties (Florina, Sir Prize) the result of PCR was three amplified fragments. The pair of primers A and B allowed the amplification of two fragments of co-dominant marker. 466 bp fragment corresponded to dominant allele *Vf* and 724 bp fragment detected the presence of recessive allele *vf*. The pair of primers C and D in the same reaction confirmed the result of amplification. In susceptible varieties (Idared, Gloster) the 724 bp fragment corresponded to recessive allele *vf*.

The results of the method applied to all genotypes are shown in Tables 1 and 2. Figure 2 shows the results of PCR detection of the studied gene in a collection of novel Czech varieties.

Tables 1 and 2 and Figure 2 document that all resistant varieties derived in interspecific hybridisation with *Malus floribunda* have the heterozygous constitution of its *Vfvf* genotypes.

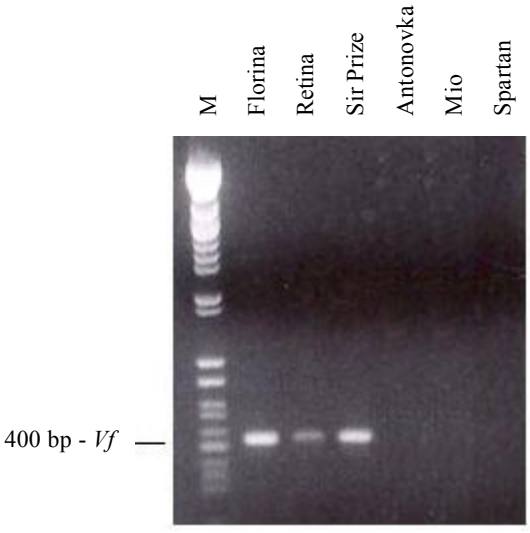

Figure 3. Dominant PCR marker (400 bp) for detection of dominant allele *Vf*
M – marker λDNA/*Eco*47I(*Ava*II)

Confirmation of results by means of an independent marker

Another dominant PCR marker (primers E and F) was used to confirm amplification reliability. Specificity of amplification was confirmed in all studied genotypes. 400 bp PCR fragment was obtained only in those genotypes that were identified as *VfVf* or *Vfvf* in previous analyses. An example of this possibility of dominant allele detection is shown in Figure 3.

Analyses of progenies of selected parental combinations

The same multi-PCR and PCR markers were used to evaluate the occurrence of dominant allele *Vf* in progenies and also in the studied collections of varieties. Figure 4 demonstrates the occurrence of allelic combination of *Vf* in segregated progenies. The obtained genotype segregations did not correspond with Mendel's laws because pre-selection was applied before DNA analysis.

DISCUSSION

The success of PCR detection of *Vf* was confirmed to depend on the quality and quantity of isolated DNA during the experiments. Gardiner et al. (1995), Yang and Korban (1996) and Guilford et al. (1997) applied a similar method of isolation in CTAB buffer. They considered this method as suitable for PCR analyses in the genus *Malus*. Gianfranceschi et al. (1996) used the method according to Dellaporta's protocol for DNA isolation in apple varieties (Dellaporta et al. 1983). Gianfranceschi et al. (1996) considered this DNA isolation method as suitable for PCR detection of *Vf* gene in apples. Some authors who applied PCR markers for *Vf* gene detection used the DNA isolation method with GenElute Plant Genomic DNA Kit (Sigma, SRN) .

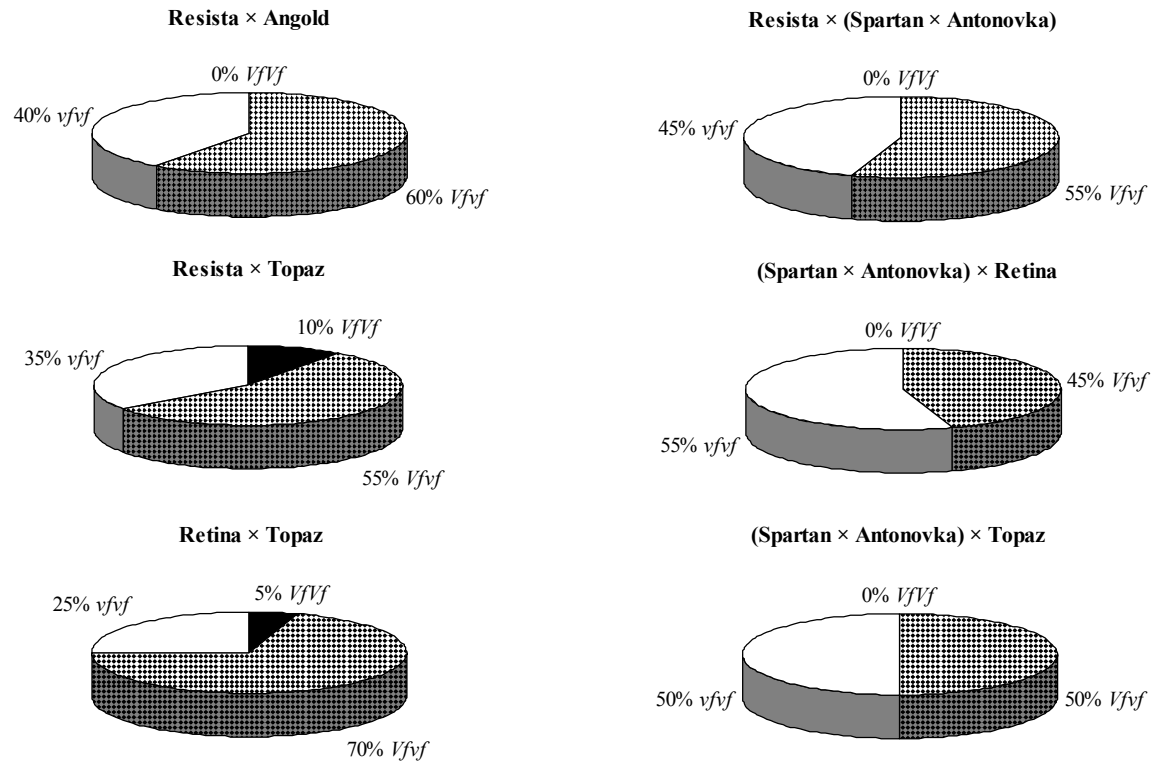

Figure 4. Occurrence of individual genotypic combinations of *Vf* in evaluated progenies

An objective of our experiments was also the verification of DNA isolated with GenElute Plant Genomic DNA Kit (Sigma, SRN). This method gave very different results of the quality and quantity of isolated DNA. The reason was difficult homogenisation of leaves in the initial steps of extraction. Therefore the isolation of genomic DNA by the method according to Saghai-Maroof et al. (1984) was carried out.

The same primers as those used by Tartarini et al. (1999) were employed for detection of *Vf* gene. The composition of PCR reaction was optimised: we used 0.8 U of *Taq* polymerase per 25 µl reaction against Tartarini et al. (1999), who used only 0.6 U *Taq* polymerase per 25 µl reaction. When only 0.6 U *Taq* polymerase was used, the intensity of amplification was very low. Unlike Gianfranceschi et al. (1996) the concentration of *Taq* polymerase was also increased from published 0.2 U to 0.7 U in 25 µl reaction.

Seventeen varieties of the world assortment were characterised by means of PCR. Heterozygous constitution of *Vf* was confirmed in three varieties. The cause of heterozygous genotypes in the studied *Vf* gene is probably a high degree of open pollination in the genus *Malus*. Homozygous constitution was unequivocally detected in the remaining fifteen varieties with different level of resistance. It is very advantageous to use multi-PCR with all four primers (A, B, C, D) according to Tartarini et al. (1999) in single reaction. This advantage makes it possible to obtain co-dominant markers and to confirm the results of dominant marker amplification.

The main part of presented results is dedicated to PCR evaluation of varieties and unknown genotypes. Several research institutes in the Czech Republic are interested in breeding of apple trees with resistance to scab on the basis of *Vf* or *Vm* gene or quantitatively determined resistance. All Czech analysed varieties of apples where resistance to apple scab was decelerated by breeders had the heterozygous gene *Vf*. This result is confirmed using PCR co-dominant markers according to Tartarini et al. (1999). This result also shows that the Czech apple breeders intensively use donor resistance to apple scab with major genes *Vf*.

Only seedlings without apple scab symptoms were evaluated by means of PCR markers of *Vf* gene according to Tartarini et al. (1999) and Gianfranceschi et al. (1996). The crosses Resista × Topaz and Retina × Topaz are the products of crossing of two heterozygotes from the genetic aspect. In both cases dominant homozygotes were also detected by PCR test. These results are shown in Figure 4. The frequency of recessive homozygotes in both crosses was 25 and 35%, respectively. This fact confirms that single mass infection tests under certain circumstances are not able to disclose all undesirable genotypes. It could be caused by an inexpressive reaction of the host to the presence of pathogen, failure of inoculation or subjective view of the evaluator. 40% of recessive homozygotes were detected in the crossing Resista × Angold. This higher frequency of unselected recessive homozygotes is possible to explain by Angold variety. Similarly, a high frequency of recessive homozygotes (45–55%) was detected in all more complicated crosses where Antonovka was the donor of quantitative resistance.

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Received on February 20, 2003

ABSTRAKT

Hodnocení českých odrůd a novošlechtění jabloní pomocí PCR markeru genu *Vf* řídícího rezistenci vůči strupovitosti (*Venturia inaequalis* CKE.)

Odrůdy jabloní rezistentní vůči strupovitosti (*Venturia inaequalis* CKE.) jsou v současné době jedním z aktuálních výstupů šlechtění této ovocné dřeviny nejen ve světě, ale i v ČR. Častým donorem rezistence vůči této chorobě je botanický druh *Malus floribunda* Sieb. – klon 821, který předal odrůdám jabloní monogenní rezistenci řízenou dominantní alelou *Vf*. Cílem příspěvku je charakterizovat gen *Vf* v kolekci nových odrůd jabloní vyšlechtěných v ČR. Pro hodnocení kolekce 20 českých odrůd, 17 odrůd světového sortimentu a šesti štěpících generací českého rezistentního šlechtění byly použity publikované dominantní a kodominantní PCR markery. U všech českých i zahraničních odrůd, u kterých byla deklarována rezistence vůči strupovitosti řízená majorgenem *Vf*, byla zjištěna heterozygotní sestava *Vfvf*. Stejné analýze byla podrobena segregující F_1 generace, která byla již podrobena selekci na základě infekčních skleníkových testů. Ve všech potomstvech byl zjištěn výskyt recesivních homozygotů *vfvf*, které nebyly vyselektovány infekčním testem. Vyšší výskyt recesivních homozygotů *vfvf* byl zjištěn u potomstev takových rodičovských komponentů, u nichž jeden z rodičů byl donorem rovněž polygenně determinované rezistence vůči strupovitosti.

Klíčová slova: jabloně; *Malus* × *domestica*; *Malus floribunda*; strupovitost; *Venturia inaequalis*; rezistence; *Vf* gen; DNA; PCR

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