Alleles controlling apple skin colour and incompatibility in new Czech apple varieties with different degrees of resistance against *Venturia inaequalis* CKE.

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ABSTRACT

The skin colour of 21 varieties was assessed on a molecular level. Four varieties had yellow skin colour with the genotype constitution a^1a^1, a^1a^2 or a^2a^2. Seventeen varieties had dominant red colour. Homozygous A^1A^1 constitution was present in 7 varieties, and heterozygous constitution A^1a^1 or A^1a^2 was found in the rest of the assessed varieties. The S-alleles controlled the incompatibility system of the pistil. Their detection is possible by means of the PCR method on the basis of allele specific primers. Six S-alleles (S1, S2, S3, S5, S7 and S9) were studied in the same collection of 21 diploid Czech varieties. This paper brings new findings on S-allele characterization, because the Czech varieties have not yet been studied on a molecular level. Both types of S-allele were found in 12 varieties. Only one type of S-allele was described in 9 varieties. Simultaneously, the presence of the *Vf* gene was screened in the collection of 21 Czech apple varieties. Ten varieties with a field resistance against the scab had a heterozygous constitution of the *Vf* gene. All 11 susceptible varieties were recessive homozygous *vfvf*.

Keywords: *Malus*; PCR; *Vf* gene; incompatibility; S-allele; skin colour; A-allele

The apple skin colour plays an important role in apple fruit trading, because customers prefer red coloured apple skin and thus the cost-effectiveness of red skin apples is greater. Molecular markers using enable to recognize the future fruit colour by creating a variety at the beginning of the breeding process. Fruit skin colour is determined by carothenoids, chlorophyll and anthocyanins (Lancaster 1992). The red colour is conditioned by anthocyanins, green and yellow colour is controlled by the quantity and the ratio of carothenoids and chlorophyll. There were three hypotheses about fruit skin colour controlling – single dominant gene control (Crane and Lawrence 1933), modifier genes overriding *Rf* locus (Schmidt 1988) and two complementary dominant genes (White and Lespinasse 1986). Cheng et al. (1996) confirmed that the skin colour of the apple (red/yellow dimorphism) is directed by a single gene localised in *Rf* locus and the presence of red anthocyanin pigmentation is dominant. By means of bulked segregant analysis and testing oligomer primer in crossing populations Cheng et al. (1996) detected four fragments associating with apple skin colour. Two of them – marked as A^1 (1160 bp) and A^2 (1180 bp) associate with red skin colour and a^1 (1230 bp) and a^2 (1320 bp) associate with yellow skin colour. These fragments were partly sequenced and there were found a high sequence of homologies confirming that fragments were generated from the same locus, *Rf* locus.

The incompatibility is the most widespread system preventing the pollination by its own or relative pollen. The incompatibility was studied in many plant families – *Solanaceae*, *Brassicacea*, *Rosaceae*. The single locus gametophytic incompatibility is typical for the *Malus* species. The incompatibility is directed by a group of S-alelles localized at multialellic locus – the S-locus in the pistil. The S-alleles encode high basic proteins – glycoproteins with ribonuclease activity (McClure et al. 1989), thus they are often called S-RNases. These RNases specifically interact with similar S-locus in the male partner (Golz et al. 2001) – the pollen – and the recognition of relative or unrelated S-alleles in pollen direct the pollination and the fertilization. The pollination and the pollen-tube growth are inhibited, when the pollen and the pistil have the same S-allele. An S-alleles

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understanding can be used in an orchards design, because only some varieties are good pollinators. A bad choice of pollinators can cause lower yields and production.

Another trend of apple breeding is obtaining of varieties with a resistance against the apple scab (*Venturia inaequalis* CKE.). The dominant *Vf* allele derived from *Malus floribunda* Sieb. clone 821 is mostly used in resistance breeding (Dayton et al. 1970). Tartarini et al. (1999) described the co-dominant PCR marker of *Vf* gene detection. Vejl et al. (2003) and Melounová et al. (2004) evaluated the Czech apple varieties collection by PCR method.

MATERIAL AND METHODS

Plant material

Twenty-one Czech varieties were used for A-alleles, S-alleles and *Vf* gene analyses (Table 1). The following varieties were chosen as a reference:
Skin colour (Cheng et al. 1996): Empire (A^1A^1), Esopus Spitzenburg (A^1a^1), Gala (A^1a^2), and Golden Delicious (a^1a^2);
For S-alleles controlling incompatibility (Janssens et al. 1995): Ontario (S1S8), Golden Delicious (S2S3), Gala (S2S5), Idared (S3S7) and Jonathan (S7S9);
For resistance against apple scab (Vejl et al. 2003): Varieties Denár (*vfvf*), Doris (*vf vf*), Resista (*Vfvf*) and Topaz (*Vfvf*).

All of the analysed varieties were obtained from the gene source collection at the Research and Breeding Institute of Pomology in Holovousy.

DNA isolation

DNA isolation was realized from 100 mg of apple tissue by means of a DNA isolation kit (Qiagen, Germany). The quality and quantity of DNA was confirmed spectrophotometrically and electrophoretically.

PCR amplification of A-alleles controlling skin colour

The primers according to Cheng et al. (1996) (F-5′ GACAGGCTACGGTCCACTGCT 3′, R 5′ ACGTAAGGTCAAAGATTCAGATC 3′) were used

Table 1. Origin of analysed varieties

Variety	Breeder	Year of registration in the CR
Aneta	Institute of Experimental Botany AS CR, Střížovice, CR	1998
Angold	Research and Breeding Institute of Pomology, Holovousy, CR	1995
Biogolden	Institute of Experimental Botany AS CR, Střížovice, CR	2001
Goldstar	Institute of Experimental Botany AS CR, Střížovice, CR	1998
James Grieve Red	Research and Breeding Institute of Pomology, Holovousy, CR	1970
Jantar	Jaroslav Lepeška, CR	1993
Jonalord	Otto Louda, CR	1993
Julia	Research and Breeding Institute of Pomology, Holovousy, CR	1994
Karmína	Institute of Experimental Botany AS CR, Střížovice, CR	1995
Klára	Research and Breeding Institute of Pomology, Holovousy, CR	1994
Melodie	Otto Louda, CR	1991
Nabella	Research and Breeding Institute of Pomology, Holovousy, CR	1994
Nela	Institute of Experimental Botany AS CR, Střížovice, CR	2001
Otava	Institute of Experimental Botany AS CR, Střížovice, CR	1997
Rajka	Institute of Experimental Botany AS CR, Střížovice, CR	1999
Rosana	Institute of Experimental Botany AS CR, Střížovice, CR	1994
Šampion	Otto Louda, CR	1977
Selena	Research and Breeding Institute of Pomology, Holovousy, CR	1994
Sparjon	Jiří Červený, CR	2001
Vanda	Institute of Experimental Botany AS CR, Střížovice, CR	1994
Zuzana	Research and Breeding Institute of Pomology, Holovousy, CR	1997

Table 2. Primers and annealing temperatures used for PCR S-alleles detection

S-allele	Sequence of primers	Annealing temperature	Amplified fragment size
S1 F	5′ ATATTGTAAGGCACCGCCATATCAT 3′	60°C	530 bp
S1 R	5′ GGTTCTGTATTGGGGAAGACGCACAA 3′		
S2 F	5′ GTTCAAACGTGACTTATGCG 3′	60°C	449 bp
S2 R	5′ GGTTTGGTTCCTTACCATGG 3′		
S3 F	5′ CAAACGATAACAAATCTTAC 3′	49°C	375 bp
S3 R	5′ TATATGGAAATCACCATTCG 3′		
S5 F	5′ ATGAATTCTGCAAGGTCAAACCCACG 3′	58°C	1700 bp
S5 R	5′ ATGAATTCATATGGATAATGGTCAACCG 3′		
S7 F	5′ GCCTTCAGACTCGAATGGACA 3′	55°C	440 bp
S7 R	5′ TGGCATTTACAATATCTACC 3′		
S9 F	5′ CAGCCGGCTGTCTGCCACTT 3′	62°C	343 bp
S9 R	5′ CGGTTCGATCGAGTACGTTG 3′		
D F	5′ ATGACGGTTCTTATCCATCC 3′	59°C	236 bp
D R	5′ TGAGCCATTCCCGCTGGGGC 3′		

for the detection of A-alleles constitution. The composition of the 25 µl reaction was: 50 ng of genomic DNA, 0.32µM of each primers, 1.5mM of $MgCl_2$, 0.3mM of dNTP and 1 unit of TAQ polymerase (Fermentas, Lithuania). The amplification was performed in the T-Gradient thermocycler (Biometra, Germany). The programme of the amplification was 40 × (94°C – 30 s, 47°C – 60 s, 72°C – 60 s) and 1 × (72°C – 480 s). The co-dominant PCR markers were electrophoresed through a 2% agarose gel and visualised by ethidium bromide (Sambrook et al. 1989).

S-alleles PCR amplification

The PCR was performed according to Janssens et al. (1995) and Broothaerts (2003). Six S-alleles and one monomorphic fragment D were detected. The detection of S5, S7, S9 and D fragment was realized according to Janssens et al. (1995), and detection of S1, S2 and S3 according to Broothaerts (2003). The PCR composition was the same for all reactions; differences were only in the annealing temperature and the sequence of S-allele specific primers (Table 2). PCR amplification was performed with 25 µl reaction volume containing 100 ng of genomic DNA, 0.2µM of each primer, 1.5mM of $MgCl_2$, 0.2mM of dNTP and 1 unit of *Taq* polymerase (Fermentas, Lithuania). The annealing temperatures of the amplification profiles were optimised by means of thermocycler T-Gradient (Biometra, SRN). Table 2 shows the results of the optimisation. The programme of the amplification was: 1 × (94°C – 180 s), 30 × (94°C – 60 s, annealing temperature from Table 2 – 60 s, 72°C – 60 s), 1 × (72°C – 480 s).

Amplified PCR fragments were stained by ethidium bromide and electrophoresed through a 1.5% agarose gel.

Vf gene PCR amplification

Primer pair for co-dominant *Vf* gene marker described by Tartarini et al. (1999) was used for a resistance evaluation. The PCR protocol according to Vejl et al. (2003) and Melounová et al. (2004) was applied.

RESULTS

A-alleles controlling apple skin colour. The description of apple skin colour according to Sus et al. (2000) and Blažek (2001) shows Table 3. Cheng et al. (1996) used an explicit classification of green, yellow and red colour. The polymorphism of red/yellow skin colour is typical for many apple varieties. Red varieties are defined according to Cheng et al. (1996) as primarily (> 50%) red and yellow varieties as primarily (80–90%) yellow. The explicit classification of assessed Czech varieties is presented also in Table 3. PCR markers of al-

Table 3. Apple skin colour characteristic and detected constitutions of A-alleles

Variety	Skin colour	Classification of skin colour according to Cheng et al. (1996)	Detected genotype
Aneta	mainly dark red covering colour	red	A^1a^2
Angold	yellowish-green with little red blush	yellow	a^2a^2
Biogolden	yellow without covering colour	yellow	a^1a^2
Goldstar	yellow without covering colour	yellow	a^1a^1
James Grieve Red	mainly dark red covering colour	red	A^1A^1
Jantar	mainly scarlet red covering colour	red	A^1a^1
Jonalord	mainly dark red covering colour	red	A^1A^1
Julia	mainly dark red covering colour	red	A^1A^1
Karmína	scarlet red colour on the whole fruit	red	A^1A^1
Klára	mainly dark red covering colour	red	A^1a^2
Melodie	mainly purple red covering colour	red	A^1A^1
Nabella	mainly carmine red covering colour	red	A^1A^1
Nela	mainly dark red covering colour	red	A^1A^1
Otava	yellow with little orange blush	yellow	a^1a^2
Rajka	mainly fresh red covering colour	red	A^1a^1
Rosana	mainly carmine red covering colour	red	A^1a^1
Šampion	striped red colour	red	A^1a^2
Selena	mainly dark red covering colour	red	A^1a^1
Sparjon	mainly dark red covering colour	red	A^1a^1
Vanda	mainly fresh red covering colour	red	A^1a^1
Zuzana	mainly orange red covering colour	red	A^1a^1

leles A^1 (1160 bp), a^1 (1230 bp) and a^2 (1320 bp) were found in the analysed Czech varieties. The electrophoreogram of separated PCR markers is shown in Figure 1.

S-alleles controlling incompatibility. Nowadays, 25 types of S-alleles were described in worldwide varieties. S1–S11 are more common alleles, the rest of S-alleles occurred only in single cultivars (Broothaerts 2003). The S-allele constitution was described in a majority of important varieties (Janssens et al. 1995, Van Nerum et al. 2001, Broothaerts 2003) and the presence of S2, S3, S5, S7 and S9 alleles was the most common. The described S-alleles analysis was chosen for this reason and this fact was confirmed in our experiments. The exemplary electrophoreograms of S2, S3 and S7 allele detection are shown in Figures 2–4. A PCR product of monomorphic D fragment (the conservative part of S-alleles) was obtained in all of the analysed varieties. The S-allele constitution is described in Table 4.

***Vf* gene detection.** Heterozygous genotype was detected in all of the resistant varieties. Susceptible varieties or varieties with a partial polygenic tolerance against the apple scab possess homozygous constitution of *Vf* gene. The electrophoreogram of a co-dominant PCR marker is presented in Figure 5. Detected allelic constitutions of all varieties are shown in Table 5.

DISCUSSION

A-alleles controlling apple skin colour

The A-alleles constitution was evaluated in 21 new Czech apple varieties. Blažek (2001) and Sus et al. (2000) presented the full characterization of apple skin colour. Nongenetic variability of the red respectively yellow colour intensity is very often. The apple skin colour was evaluated according to Cheng et al. (1996) for this reason. Only

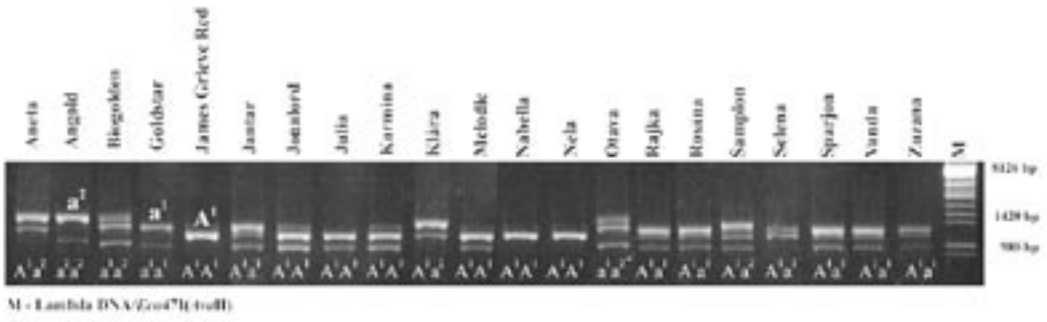

Figure 1. Codominant PCR marker of A alleles controlling the apple skin colour

four varieties from 21 evaluated apple varieties possessed yellow colour. Varieties Biogolden and Goldstar lacked the sharp covering colour or the blush. Varieties Angold and Otava had small red or orange blush. The absence of dominant A^1 allele was characteristic for all of yellow varieties. Cheng et

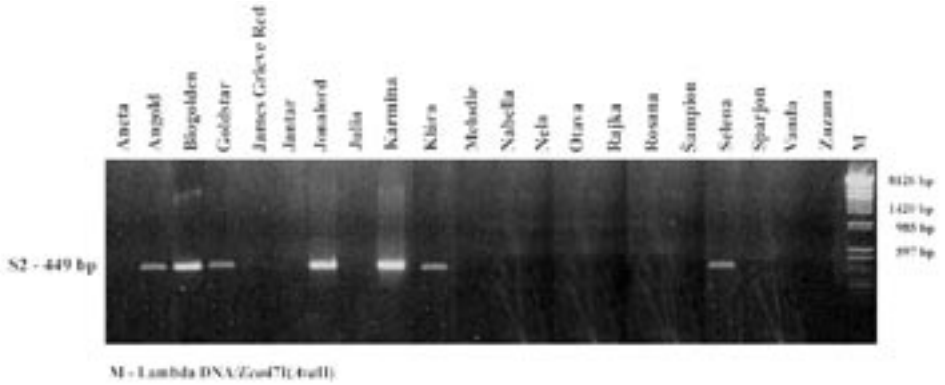

Figure 2. PCR marker of S2 allele controlling the apple incompatibility

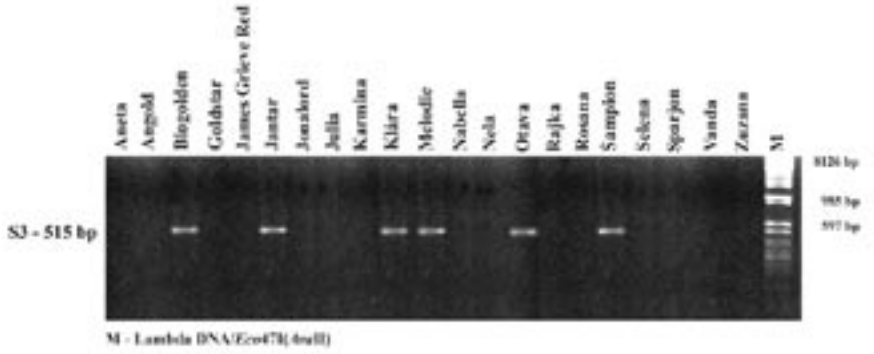

Figure 3. PCR marker of S3 allele controlling the apple incompatibility

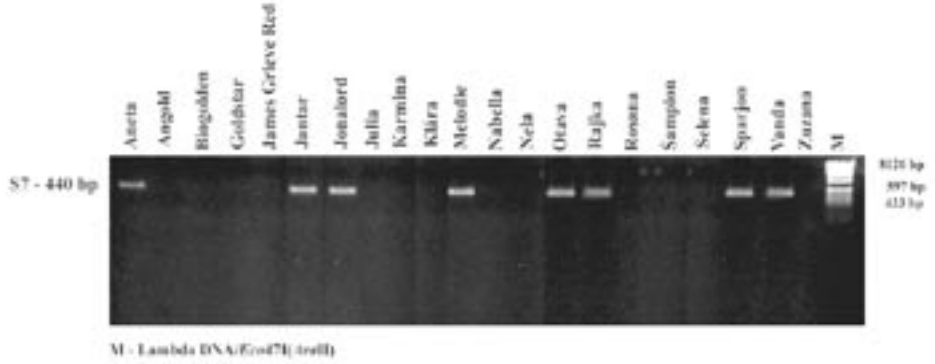

Figure 4. PCR marker of S7 allele controlling the apple incompatibility

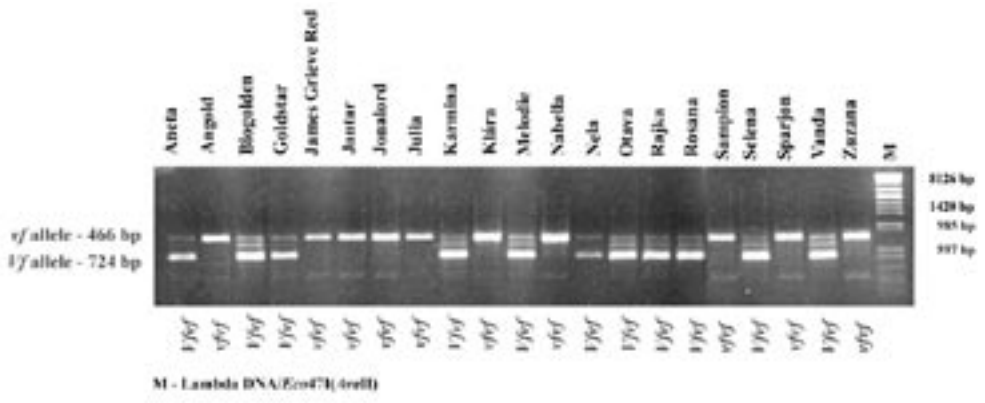

Figure 5. Codominant PCR marker of *Vf* alleles controlling the resistance against the apple scab

al. (1996) also described that varieties with a sharp yellow colour (Golden Delicious) do not have the dominant allele A^1. Variety Honeygold with a red blush has according to Cheng et al. (1996) mainly a yellow fruit skin and an absence of A^1 allele. Similar results were obtained in varieties Angold (little red blush, genotype a^2a^2) and Otava (little orange blush, genotype a^1a^2). Lancaster (1992)

Table 4. Incompatibility and detected constitutions of S-alleles

Variety	Detected S-allele constitution	Parental combination	Parental S-allele constitution
Aneta	S7*S?*	Šampion × ÚEB 1200/1	*S?S?* × *S?S?*
Angold	S2*S?*	HL A28/38 × Golden Delicious	*S?S?* × S2S3
Biogolden	S2S3	Golden Delicious × ÚEB 1200/1	S2S3 × *S?S?*
Goldstar	S2*S?*	Rubin × Vanda	*S?S?* × S5S7
James Grieve Red	S5*S?*	mutation of James Grieve	*S?S?*
Jantar	S3S7	Golden Delicious × Jonathan	S2S3 × S7S9
Jonalord	S2S7	Jonathan × Lord Lambourne	S7S9 × *S?S?*
Julia	S9*S?*	Quinte × Discovery	*S?S?* × S10S25
Karmina	S2S5	Karmen × ÚEB 725/6	*S?S?* × *S?S?*
Klára	S2S3	Hvězdnatá × Hájkova reneta	*S?S?* × *S?S?*
Melodie	S3S7	Šampion × OR 38 T 16	S3S5 × *S?S?*
Nabella	S5S9	Nonnetit × Starking Delicious	*S?S?* × S9S19
Nela	S1*S?*	Prima × ÚEB 1200/1	*S?S?* × *S?S?*
Otava	S3S7	Šampion × Jolana	S3S5 × *S?S?*
Rajka	S5S7	Šampion × ÚEB 1200/1	S3S5 × *S?S?*
Rosana	S5*S?*	Jolana × Lord Lambourne	*S?S?* × *S?S?*
Selena	S2*S?*	Britemac × Prima	*S?S?* × S2S10
Sparjon	S7S9	Spartan × Jonared	*S?S?* × S7S9
Šampion	S3S5	Golden Delicious × Cox Orange Pipin	S2S3 × S5S9
Vanda	S5S7	Jolana × Lord Lambourne	*S?S?* × *S?S?*
Zuzana	S5*S?*	Glockenapfel × James Grieve	*S?S?* × *S?S?*

S? – unknown S-allele

Table 5. Resistance against the apple scab and detected allelic constitutions of *Vf* gene

Variety	Presence of resistance	Donor of resistance	Detected genotype
Aneta	monogenic vertical resistance	ÚEB 1200/1	*Vfvf*
Angold	partial polygenic tolerance	HL A28/38	*vfvf*
Biogolden	monogenic vertical resistance	ÚEB 1200/1	*Vfvf*
Goldstar	monogenic vertical resistance	Vanda	*Vfvf*
James Grieve Red	susceptible	none	*vfvf*
Jantar	susceptible	none	*vfvf*
Jonalord	susceptible	none	*vfvf*
Julia	susceptible	none	*vfvf*
Karmína	monogenic vertical resistance	ÚEB 725/6	*Vfvf*
Klára	susceptible	none	*vfvf*
Melodie	monogenic vertical resistance	OR 38 T 16	*Vfvf*
Nabella	less susceptible	none	*vfvf*
Nela	monogenic vertical resistance	Prima and ÚEB 1200/1	*Vfvf*
Otava	monogenic vertical resistance	Jolana	*Vfvf*
Rajka	monogenic vertical resistance	ÚEB 1200/1	*Vfvf*
Rosana	monogenic vertical resistance	Jolana	*Vfvf*
Šampion	susceptible	none	*vfvf*
Selena	monogenic vertical resistance	Prima	*Vfvf*
Sparjon	susceptible	none	*vfvf*
Vanda	monogenic vertical resistance	Jolana	*Vfvf*
Zuzana	less susceptible	none	*vfvf*

indicated that many yellow varieties display a distinctive blush of anthocynin pigmentation. Different genes in the anthocyanin biosynthetic pathway can activate a red blush formation.

The rest of the 17 varieties possessed a different intensity of red apple skin. The presence of a dominant A^1 allele was specific for all of the red coloured apple varieties. These results are fully congruous with the study of Cheng et al. (1996). Dominantly homozygous constitution A^1A^1 is characteristic in varieties with darkly red fruits (for example Karmína, Julia, Jonalord, Nela, Melodie, Nabella). Cheng et al. (1996) did not describe a relationship between dominantly homozygous A^1A^1 constitution and dark red colour of fruits. Opposite of this fact, Cheng et al. (1996) found that the constitution A^1A^1 is typical for dark red, respectively violet red apple varieties (for example Empire, Jerseymac, Jonathan, Macoun, Monroe).

Cheng et al. (1996) discovered the occurrence of a dominant A^2 allele controlling also the red skin colour. The donor of this A^2 allele is the red crabapple variety White Angel. White Angel's pedigree contains Asian apple genotypes (Simon and Weeden 1991). A PCR marker of allele A^2 (1180 bp) was not found in any analysed Czech varieties. This result corresponds to the fact that Asia genotypes are not often used in Czech apple breeding.

S-alleles controlling incompatibility

Table 4 shows the origins of the studied varieties. Variety Jantar was derived from a cross of Golden Delicious (S2S3) × Jonathan (S7S9). The PCR analysis confirmed that Golden Delicious was the donor of S3 allele and S7 allele is originated from the Jonathan in the variety Jantar. The origins of both S-alleles were confirmed also in the variety Šampion (S3S5), where the donor of S3 allele was the Golden Delicious and the donor of S5 allele was the Cox Orange Pipin. Also it was found that the donor of S2 allele in varieties Angold and Biogolden was the Golden Delicious, the donor of S7 allele in

variety the Jonalord was the Jonathan, the donor of S3 allele in varieties Melodie and Otava was the Šampion, the donor of S9 allele in variety Nabella was the Starking Delicious, the donor of S5 allele in variety Rajka was the Šampion, the donor of S2 allele in variety Selena was the Prima and the donor of S9 allele in variety Sparjon was the Jonared. Blažek (2001) described Vanda and Rosana varieties as reciprocally very bad pollinators. This result was confirmed on a molecular level. Both varieties contain the S5 allele.

S1 allele was detected only in the variety Nela. Broothaerts (2003) also indicated the low frequency of S1 allele in a world apple variety collection. Varieties with unusual S-alleles can be good pollinators for varieties with frequent S-alleles.

Vf gene detection

Varieties Aneta, Biogolden, Goldstar, James Grieve Red, Jantar, Nela, Šampion and Selena have not been evaluated by means of *Vf* gene PCR marker yet. Varieties Aneta, Biogolden, Goldstar, Nela and Selena show the field resistance against the apple scab. Each of the resistant varieties contains botanic species *Malus floribunda* in their pedigree. Heterozygous constitution *Vfvf* was found in all of the resistant genotypes. Dominant homozygous constitution *VfVf* was not described in any Czech apple variety (Vejl et al. 1993, Melounová et al. 2004). Varieties James Grieve Red, Jantar, Šampion and Sparjon are susceptible and show recessive homozygous constitution of *Vf* gene. The result of PCR markering in varieties Angold, Jonalord, Julia, Karmína, Klára, Melodie, Nabella, Otava, Rajka, Rosana, Vanda and Zuzana corresponds with the evaluation according to Vejl et al. (1993). The *Vm* gene is another possibility of breeding apple varieties with monogenic vertical resistance against the apple scab (Cheng et al. 1998, Melounová et al. 2004). The important donor of *Vm* gene (OR 45 T 132) was not found in any of the assessed new Czech varieties. Czech resistance breeding based on *Vf* gene corresponds to the fact that the resistance of genotype *Vfvf* is not overcome by new races of *Venturia inaequialis* CKE.

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ABSTRAKT

Alely řídící barvu slupky a inkompatibilitu u nových českých odrůd jabloní s odlišným stupněm odolnosti vůči *Venturia inaequalis* CKE.

Na molekulárně genetické úrovni byla hodnocena kolekce 21 vybraných českých odrůd. U čtyř odrůd byla detekována genotypová sestava a^1a^1, a^1a^2 a a^2a^2 korespondující se žlutým zbarvením plodů, u sedmnácti odrůd byla detekována dominantní červená barva. Dominantně homozygotní sestava A^1A^1 byla přítomna u sedmi odrůd, heterozygotní sestava A^1a^1 nebo A^1a^2 byla nalezena u zbytku testovaných odrůd. S-alely kontrolují inkompatibilní systém pestíku. Jejich detekce je možná pomocí PCR na základě specifických primerů. Šest S-alel (S1, S2, S3, S5, S7 a S9) bylo studováno u stejné kolekce odrůd jabloní. Tento příspěvek přináší nové poznatky v charakterizaci S-alel, neboť české odrůdy nebyly dosud na molekulární úrovni studovány. Obě S-alely byly popsány u dvanácti odrůd, u devíti odrůd byla nalezena pouze jedna S-alela. Současně v téže kolekci odrůd byla sledována přítomnost genu *Vf*. Deset odrůd vykazujících polní odolnost vůči strupovitosti mělo heterozygotní sestavu *Vf* genu. Všech jedenáct senzitivních odrůd mělo homozygotní sestavu *vfvf*.

Klíčová slova: *Malus*; PCR; *Vf* gen; inkompatibilita; S-alely; barva slupky; A-alely

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