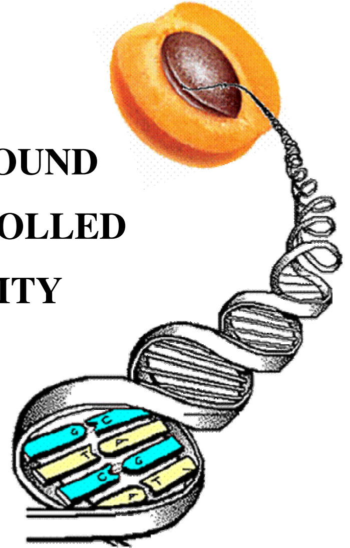
PhD Thesis



MOLECULAR BACKGROUND OF THE *S*-LOCUS CONTROLLED SELF-INCOMPATIBILITY IN APRICOT

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1. INTRODUCTION

Genetic variability caused by cross-pollination provides selective evolutionary advantage for plants. In the hermaphrodite flowers of angiosperms different mechanisms evolved to prevent self-pollination. One of the most efficient mechanisms is the so-called incompatibility, which results in the inhibition of self-pollen tube growth. Incompatibility is a genetically determined process.

Although cross-pollination is profitable for plants at an evolutionary scale, it is disadvantageous for growers because pollen donor trees must be provided in the orchards of self-incompatible cultivars. In addition, self-incompatible cultivars carrying the same *S*-genotype could not fertilize each other.

Fruit trees belonging to the *Rosaceae* family are characterized by gametophytic self-incompatibility based on ribonuclease enzymes. Most European apricot cultivars have been traditionally considered self-compatible, while the majority of Asian and North American ones were qualified as self-incompatible. In the last years many self-incompatible cultivars were released from breeding programs using Asian and American genotypes to introduce frost tolerance and virus resistance. Therefore, knowledge on the *S*-genotypes is necessary for planning the orchards. Open field experiments to determine compatibility relations give doubtful results because environmental and weather conditions can greatly influence fruit set ratios, and mature trees are required for these analyses. DNA-based analysis can support breeders' work by providing the possibility for the early selection of seedlings carrying the favourable trait.

Besides knowledge on the *S*-genotypes of self-incompatible cultivars presents useful information for the optimal cultivar association, elucidation of the molecular mechanisms controlling the fertility trait of fruit trees is also a very important task of basic research.

2. OBJECTIVES

The experimental design covered the followings:

1. To estimate the applicability of molecular techniques (NEpHGE, IEF and PCR) designed for other *Prunus* species and develop new methods optimized for apricot.
2. To determine and characterize as many as possible allelic variants of the *S*-locus by screening many cultivars of different origins.
3. To assign *S*-genotypes for economically important apricot cultivars and hybrids of great breeding value, and especially for the Hungarian apricot cultivars and to clarify *S*-genotype of cultivars with previously unknown or doubtful compatibility properties.
4. To establish a table demonstrating compatibility relationships of apricot cultivars by comparing our results with the data published previously. This table would be used as a genetic database by both growers and breeders.
5. To describe the molecular mechanism behind the self-compatibility trait in apricot and detect the original, functional version of the self-compatibility haplotype.
6. To provide an easy to apply, rapid and reliable tool for breeders that enables the early selection of seedlings for self-compatibility.
7. To discuss crop evolutionary history of apricot in function of the newly obtained molecular data on the *S*-locus.

3. MATERIALS AND METHODS

3.1. Plant material

74 apricot cultivars and hybrids of diverse type and origin were used in the experiments obtained from the orchard of the Corvinus University of Budapest, Department of Genetics and Plant Breeding in Szigetcsép, from the apricot germplasm collection of the Hungarian National Institute for Agricultural Quality Control in Tordas, from the orchard of the Fruit Culture Research Institute in Cegléd, from the orchard of the Kecskemét College in Kecskemét and from an orchard in Boldogkőváralja.

3.2. Pollination tests

Pollination tests were carried out in the germplasm of Corvinus University of Budapest, Szigetcsép, in 2005 and 2006. During compatibility analysis pollinated flowers were counted, branches were isolated, and fruit development was continuously monitored after 13, 21, 28, 34, 48 és 64 days.

3.3. Pollen tube growth

Pollen tube growth tests were carried out using UV fluorescence microscopy. Branches with at least 20 flower buds were transported to the laboratory and flowers were emasculated. Pistils were collected 72 h after pollination and fixed in the mixture of chlorophorm, 95% (v/v) ethanol and glacial acetic acid at a ratio of 1:3:1 for 24 h. Styles were stained with 0.1% (w/v) aniline blue in 33 mM K_3PO_4.

3.4. Assay of the *S*-ribonuclease isoenzymes

Developmentally controlled changes in *S*-RN-ase activity were demonstrated on agarose diffusion plates. Styles were placed on agarose gel containing *Torula* yeast RNA and incubated at 37 ^{o}C for 2 h. After removal of styles agarose plate was stained with 0.02 % toluidine blue.

About 50 styles were harvested 48 h before or soon after anthesis. Samples were homogenized in cold extraction buffer (100 mM sodium acetate pH 5.8, 100 μM phenylmethane-sulphonyl-fluorid, 1 % (v/v) 2-mercaptoethanol, 100 mM KCl) and centrifuged (2 ^{o}C, 25 min, 18,750 g). RNase activity assays were carried out spectrophotometrically at 260 nm. Absorbance was measured for 1 min at intervals of 10 s. Reaction mixture contained 100 mM sodium-acetate (pH 5.8), 25 μg/ml *Torula* yeast RNA, 1mM ditiothreitol and 100 mM potassium chloride. Protein quantity was determined with Bradford reagent using bovine serum albumin as standard.

3.4.3. Sample preparation for isoelectric focusing

40 styles before anthesis were collected and homogenized in 1 ml extraction buffer (30 % dimethyl-sulphoxide (v/v), 10 % (w/v) saccharose, 0.1 % (w/v) Na

metabisulphite, 0.2 % (v/v) Pharmalyte pH 3–10 és 0.5 % (v/v) 10 % Triton X-100) and centrifuged (–4 ^{o}C, 35 min, 18,750 g).

3.4.4. Isoelectric focusing (IEF) and non-equilibrium pH-gradient electrofocusing (NEpHGE)

Extracts were separated on vertical slab gels containing 7.5 % polyacrilamide, 10 % saccharose and various combinations of ampholytes (NEpHGE I-II: 4 % Pharmalyte pH 5–8 and 1.2 % Ampholine pH 7–9; NEpHGE III: 4 % Pharmalyte pH 3–10 and 1.2 % Ampholine pH 7–9). 40 µl of each sample was loaded at the anodal end. The focusing run comprised 1 h at 130 V, 2 h at 260 V, 1 h at 350 V and 1 h at 400 V (NEpHGE I); with additional 30 min at 450 V (NEpHGE II-III). The catalyte and analyte used were 0.1 M sodium hidroxide and 0.04 M DL-glutamic acid, respectively. The temperature was maintained at 4 ^{o}C.

Isoelectric focusing (IEF) was carried out on gels containing 4 % Pharmalyte pH 3–10 under the following conditions: 1 h at 150 V, 1 h at 300 V, 2 h at 450 V and 2 h at 550 V. To determine pI points, standard proteins with pI points from 5.9 to 9.3 (Sigma) were run alongside the cultivar samples. The standard proteins were stained with Coomassie Brilliant Blue R 250 and ribonuclease isoenzymes were detected using *Torula* yeast RNA and toluidine blue.

3.5. DNA based methods

Genomic DNA was extracted from fully expanded young leaves using the DNeasy Plant Mini Kit (Qiagen). Primer sequences amplifying *S*-RNase gene fragments are summarized in Table1 Approximately 20–80 ng of genomic DNA was used for PCR amplification in a 25-µl reaction volume, containing 1 × PCR buffer (Sigma) with final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.4 µM of the adequate primers and 0.625 U of *Taq* DNA polymerase (Sigma). PCR was carried out using protocols described for the given primer. PCR fragments were separated on 2% TAE agarose gels for 2 h at 100 V.

Degenerate primers, AprSFB-F1, AprSFB-F2 and AprSFB-R were designed to anneal within the conserved regions identified at the 5’ and 3’ ends of the apricot SFB gene. The amplification was carried out using a temperature profile with an initial denaturing of 94°C for 2 min, 35 cycles of 94°C for 30 s, 50°C for 1.5 min and 72°C for 2 min, and a final extension of 72°C for 5 min. PCR conditions were performed as described above.

Table 1. Nucleotide sequence of the tested primers.

primer	szekvencia 5’ -3’
PaConsI-F	MCT TGT TCT TGS TTT YGC TTT CTT C
PaConsI-R	CAT GRA TGG TGA ART WTT GTA ATG G
PaConsII-F	GGC CAA GTA ATT ATT CAA ACC
PaConsII-R	CAW AAC AAA RTA CCA CTT CAT GTA AC
EM-PC1consRD	GCC AYT GTT GMA CAA AYT GAA
EM-PC2consFD	TCA CMA TYC ATG GCC TAT GG
EM-PC3consRD	AWS TRC CRT GYT TGT TCC ATT C
EM-PC5consRD	CAA AAT ACC ACT TCA TGT AAC ARC
SRc-F	$_{JOE}$-CTC GCT TTC CTT GTT CTT GC
SRc-R	GGC CAT TGT TGC ACA AAT TG
AprSFB-F1	AAG AAW GAR AYY TTR RTC GAC AT
AprSFB-F2	TCY CTY RTT CGR TTT MTK TG
AprSFB-R	ATY GAG WAA AAC CAW RCT YTC

Total RNA was extracted from styles using an E.Z.N.A. Plant RNA Kit (Omega). The cDNAs were synthesized by First Strand cDNA Synthesis Kit (Fermentas) with oligo$(dT)_{18}$ primer. The obtained cDNAs were used as a template for PCR with primers SRc-F and EM-PC5consRD. PCRs were performed with *Taq* DNA polymerase (Sigma) using a program with initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 10 s, 55°C for 2 min and 72°C for 1 min and a final extension step of 72°C for 10 min. Electrophoresis was carried out on 1% TAE agarose gels as described above.

All genomic and cDNA fragments were extracted and purified from the agarose gels using the QIAquick Gel Extraction Kit (Qiagen) and cloned into a pGEM-T Easy plasmid vector (Promega). Plasmid DNA was isolated with the Rapid Plasmid DNA Daily Miniprep Kit (V-gene) and sequenced in the above described sequencer. For each fragment, the nucleotide sequences of five clones were determined in both directions. Analyses of DNA and deduced amino acid sequence data were performed using BLASTN at NCBI, the CLUSTAL W program with the Vector NTI 10.3.0 software (Invitrogen). Phylogenetic analyses were conducted using MEGA version 3.1.

5. RESULTS AND DISCUSSION

5.1. Determination of *S*-genotypes by isoelectric focusing

The first experiments aimed to elaborate the most appropriate isoelectric focusing methods for apricot. The most efficient separation was achieved with 1750–1975 Vh, which was not enough for proteins to reach their isoelectric points and thereby this technique should be regarded as non-equilibrium pH gradient electrofocusing (NEpHGE).

Variations in the NEpHGE protocols enabled us to detect four previously described (S_1, S_2, S_4 és S_C) and nine new (S_8–S_{16}) *S*-ribonuclease enzymes. Leaf extracts ran under NEpHGE did not show any RNase activity in the tested pH range, which proves that these alkaline ribonucleases are tissue-specific and take part in the fertilization process.

S-genotypes of cvs. 'Ceglédi óriás' and 'Ligeti óriás' that belong to the Hungarian Óriás (giant-fruited) cultivar group proved to be identical (S_8S_9). Our results elucidate the real background of cross-incompatibility between these cultivars and support their inclusion into the same incompatibility group.

NEpHGE detected only one band for the self-compatible cvs. 'Gönci magyarkajszi', 'Venus', 'Sulmona', 'Nikitskyi', 'Mari de Cenad' and 'Marculesti 5/5' while 'Bergeron', 'Mandulakajszi', 'Mamaia' and 'Konzervnyi Pozdnii' proved to be heterozygous.

'Harmat' ($S_{10}S_{11}$) and 'Korai zamatos' ($S_{12}S_{13}$) are early ripening cultivars, a feature that was introduced from the Central Asian genotypes. Allele S_{13} of 'Korai zamatos' was also present in 'Hybrid 8', 'Modesto' and 'Voski', accessions used in the same breeding program. 'Harmat' and 'Voski' have the S_{11}-allele in common, an allele not found in other accessions.

The 'Zard', 'Aurora' and 'Kech-pshar' showed one intense band under NEpHGE II, but our NEpHGE III protocol was successful in separation of these combined isoenzyme bands. Consequently, the genotype of 'Kech-pshar' was determined to be $S_{15}S_Z$, while 'Zard' was found to carry the S_{16}-allele. Only partial *S*-genotype could be determined for 'Aurora', with detecting allele S_X.

To determine isoelectric points of different *S*-RNases, 2450 Vh was used. Pairs of S_4 and S_{12}, S_{13} and S_X, and S_C and S_8 isoenzymes were characterized by the same isoelectric points.

The newly designed NEpHGE I-III protocols enabled us to determine the complete *S*-genotype for 12 cultivars and partial *S*-genotypes for 11 cultivars.

5.2. DNA-based *S*-genotyping: analysis of the *S*-RNase gene

The most important advantage of DNA-based *S*-genotyping is that vegetative tissue could be analysed, therefore it is applicable for the early selection of seedlings. *S*-RNase gene of *Prunus* species contains two introns. Determination of different alleles is based on size and sequence polymorphisms of the introns.

For the first time two pairs of sweet cherry consensus primers were used. Primers amplifying the first intron region revealed 10 alleles from 16, while primers amplifying the second intron region detected 11 alleles.

Another degenerate primer set designed from several available *Prunus* *S*-alleles, was applied to further improve the efficiency of *S*-allele detection (EM-PC2consFD and EM-PC3consRD). These primers could successfully amplify each of the alleles. Several amplification products were cloned and sequenced and found to be partial *S*-RNase sequences. Besides the 9 alleles identified by NEpHGE and cherry consensus primers (S_8–S_{16}) further 4 new alleles (S_{17}–S_{20}) were determined. EM-primers could detect S_C- and S_8-alleles containing the longest intron, where cherry primers did not give a successful amplification.

To analyze the smaller first intron, fluorescently labelled primers were used and precise length of PCR fragments was determined with an automated DNA sequencer. The length of the first intron in the ten new alleles unequivocally differed from that of the alleles described earlier, while the exact size of three alleles could not be determined. Since in some alleles lengths of the first or second intron regions are very similar, reliable *S*-genotyping should cover the analysis of both introns. This approach was successfully used to distinguish S_9-RNase from S_{20}-RNase; to assign precise *S*-genotypes to cvs. 'Korai piros' and 'Ceglédi Piroska'; and to revise the reported pedigree of the latter.

PCR typing of the progeny raised from the cross 'Bergeron' × 'Konservnyi Pozdnii' gave support for the parental S_CS_2 genotype. S_C-RNase was carried by all hybrids and the observed proportion (19:15) of the S_CS_C homozygote and S_CS_2 heterozygote seedlings fits well with the expected segregation ratio of 1:1 at $P<0.05$. This cross is also of breeding significance since the parents are late ripening cultivars yielding large and attractive fruits. In addition, all hybrids are SC, and half of them are homozygote at the *S*-locus. These are excellent crossing partners producing only SC offsprings. We have isolated a mutant version of the S_2-RNase from 'Mandulakajszi', which has a non-synonymous SNP in the middle of the RHV region. Further experiments are required to test its putatively altered function.

Since possessing the S_1S_2-genotype, the 'Ninfa' and 'Priboto' cultivars could be included in the I. incompatibility group encompassing 'Lambertin-1' 'Goldrich' and 'Hargrand'. 'Priboto' is a bud mutation of 'Goldrich', which explains their identical *S*-genotype (Table 2).

Besides the two groups identified previously, based on our results a new (III.) incompatibility group was established containing 'Antonio Errani' and 'Harcot' cultivars with the S_1S_4 genotype.

Table 2 Inter-incompatibility groups of apricot cultivars and universal pollen donors with self-compatible and unique self-incompatible genotypes. Only *S*-genotypes of underlined cultivars were known before our analysis.

Group	Cultivar	*S*-genotype
I. incompatibility group	Goldrich, Hargrand, Lambertin-1 Ninfa, Priboto	S_1S_2
II incompatibility group	Ceglédi óriás Ligeti óriás	S_8S_9
III. incompatibility group	Harcot Antonio Errani	S_1S_4
0.group: universal pollen donors	Ananasniyi cjurpinszkii (S_CS_C); Borsi-féle kései rózsa (S_CS_C); Ceglédi kedves (S_CS_C); Currot (S_CS_C); Ginesta (S_CS_C); NJA-8 (S_CS_C); Nyujtó Ferenc emléke (S_CS_C), Palau (S_CS_C); Pannónia (S_CS_C); Pasinok (S_CS_C); Rózsakajszi C.1406 (S_CS_C); Sirena (S_CS_C); Sulmona (S_CS_C); Zaposdolye (S_CS_C) Mauricio (S_CS_1); Bayoto (S_CS_2); Bergeron (S_CS_2); Budapest (S_CS_2); Canino (S_CS_2); Konzervnyi Pozdnii (S_CS_2); Mamaia (S_CS_2); Mandulakajszi (S_CS_{2m}); Pepito (S_CS_2); Rakovszky (S_CS_2); Roxana (S_CS_2); Toyuda (S_CS_2) Colorao (S_CS_5)*; Rial Fino (S_CS_6); Beliana (S_CS_7) Andornaktályai magyarkajszi (S_CS_8); Cacansko zlato (S_CS_8); Crvena ungarska (S_CS_8); Darunec malahoyeva (S_CS_8); Effect (S_CS_8); Gönci magyarkajszi (S_CS_8); Kâsna ungarska (S_CS_8); Krimszkyi Amur (S_CS_8); Magyarkajszi C. 235 (S_CS_8); Marculesti 5/5 (S_CS_8); Nagygyümölcsű magyarkajszi (S_CS_8); Nikitskyi (S_CS_8); Paksi magyarkajszi (S_CS_8); Pisana (S_CS_8); Venus (S_CS_8) Ceglédi arany (S_CS_9); Ceglédi bíborkajszi (S_CS_9); Korai piros (S_CS_{20}); Mari de Cenad (S_CS_{19}); Modesto (S_CS_{13});	
	Ceglédi Piroska (S_8S_{20}), Harmat ($S_{10}S_{11}$); Kech-pshar ($S_{15}S_{18}$); Korai zamatos ($S_{12}S_{13}$); Moniquí (S_2S_6); Priana (S_2S_7); Sunglo (S_2S_3); T-8 ($S_{13}S_{14}$); Voski ($S_{11}S_{13}$)	

*'Colorao' was described as universal donor on the basis of its *S*-genotype, however, it is a male-sterile cultivar

In our experiments 13 new *S*-haplotypes were identified in Eastern European and Central Asian cultivars and hybrids, thus the number of known apricot alleles was increased to a total of 21. In addition, further 13 new alleles were detected in Chinese apricot accessions. The *S*-locus of apricot is more variable than previously believed. The development of frost tolerant and virus resistant hybrids, a process in which many Asian genotypes are used as parents greatly increased the variability of *S*-locus.

PCR analysis with EM-primers of the *S*-RNase gene first and second intron regions enabled us to determine the complete *S*-genotype of 22 cultivars and the partial *S*-genotype of 3 cultivars. Sequencing of PCR fragments in case of 5 cultivars facilitated the determination of genotypes. However, in many cases we could only identify genotypes with the previous knowledge on fertility properties since the S_8-RNase allele is characterized by the same fragment lengths as the S_C-RNase allele.

5.3. Molecular backround of self-compatibility

Isoenzyme analysis revealed that self-compatibility in apricot was not a consequence of activity loss of *S*-RNases. S_C- and S_8-RNases were not distinguishable with any methods applied.

To compare the coding regions of the two alleles, their cDNAs were cloned and sequenced. The identities between the deduced amino acid sequences of the S_8- and S_C-RNases and between those of the mature S_C-RNases from 'Pannónia' or 'Currot' were 99.5 and 98.2%, respectively. The deduced AA sequence of the RHV region of the S_8- and S_C-RNases from both cultivars was identical. All Spanish and Hungarian cultivars carrying the S_C-allele were characterized by self-compatibility, indicating that the 3 AA differences between the alleles may not affect their functional identity. The alignment of the S_C- and S_8-RNase cDNAs allowed us to determine the precise position and length (258 bp) of the 1st intron as well as the correct AA sequence within the neighbouring exon regions of the S_C-RNase allele. Previously, for lack of cDNA analyses the length of the 1st intron was assessed to be 260 bp and consequently the AA sequence was deduced mistakenly.

An RNase extraction method and a simple and rapid RNase activity assay were adopted to check whether sequence similarity/diversity is reflected by the enzyme activity levels of the proteins or not. RNase activity showed great differences according to the developmental stages and *S*-genotypes. RNase activity was higher at the balloon stage than before, reflecting the developmentally controlled *S*-RNase expression. Cultivars with the same *S*-genotype produced nearly equal activity values, while those expressing RNases encoded by different *S*-alleles showed significantly different activities. Although differences in the non-*S*-specific RNase activity of crude stylar extracts may also influence the results, a similar correlation could be recorded for apricot *S*-RNases. The identical RNase activity of the S_CS_9 and S_8S_9 pistils confirms that S_C-RNases are functional and suggests identical affinity of the S_C- and S_8-RNases for the substrate *Torula* yeast

RNA and coincides with their identical or very similar AA sequences, providing biochemical support for the identity of the S_C- and S_8-alleles.

The hypothesized pollen-part mutation within the S_C-haplotype was functionally verified by test crosses. The SC cultivar 'Ceglédi arany' (S_CS_9) shares the S_9 incompatibility allele with the SI cultivar 'Ceglédi óriás' (S_8S_9). When 'Ceglédi óriás' as pollen parent was crossed on to the 'Ceglédi arany' as seed parent, the combination has not resulted in fruit set or the percentage of the fruit set was negligible and 'Ceglédi óriás' pollen tubes showed typical incompatible phenotype in 'Ceglédi arany' styles. The reciprocal cross and the cross 'Gönci magyarkajszi' × 'Ceglédi óriás' set around 40% fruit confirming again the loss of pollen function and excluding the possibility that the failure of the cross 'Ceglédi arany' × 'Ceglédi óriás' may be due to the lack of viable pollens released by the pollen parent 'Ceglédi óriás'.

Degenerate primers were designed from *P. armeniaca* and *P. mume* sequences. PCR amplified one or two differently sized fragments from 'Ceglédi óriás', 'Ceglédi arany', and 'Pannónia'. Cloning and sequencing of the amplicons revealed that the nucleotide sequence of the SFB_8 and SFB_C showed almost 100% identity except for a 358 bp insertion in the latter, similarly to the results described by a Spanish research group in the same time. Alignment with the non-mutated, original version of the same allele (S_8) allowed us to verify the position of the inserted sequence.

The SFB_8 and the $OriSFB_C$ (without the insertion) differed in only one amino acid residue within the F-box region, and the 358 bp insertion in the 'Pannónia' and 'Currot' differed only in two nucleotides. Therefore, following the allele labelling system established for sweet cherry, S_8' may be a synonymic designation for the S_C. The premature stop codon located near the beginning of the inserted sequences resulted in a truncated F-box protein that lacks the HVa and HVb hypervariable regions essential for the allele-specific recognition.

The function of a general inactivation mechanism for non-self *S*-RNases in the pollen tubes was previously hypothesized with SFB conferring specificity by protecting self *S*-RNases from inactivation. Accordingly, the truncated SFB_C by not recognizing the S_8-RNase as self cannot protect it from being degraded in the 26S proteasomes and pollen rRNAs will remain intact.

5.4. SFB-based *S*-genotyping of cultivars of various origins

Primers specific for the S_C-haplotype are required for the early selection of self-compatible seedlings. A degenerate primer pair was designed from the available apricot and Japanese apricot SFB/SLF sequences. The new AprSFB-F1/R primer pair amplifies approximately the whole coding region of the SFB gene excluding 27 (18+9) nucleotides. Since mutated SFB_C allele contains a 358 bp insertion, size of the SFB_C fragment amplified by this primer pair is 1419 bp, while the original allele without insertion gives a 1061 bp long fragment. Our primer pair resulted successful amplification in case of all alleles and the *S*-genotypes showed

perfect correlation with compatibility phenotypes reported previously or analysed in the present study.

These consensus primers were not only useful in the detection of SC phenotype but as a co-dominant marker they also gave information on the heterozygote or homozygote state at the locus. These primers provide more reliable results than the allele-specific primers used for the detection of mutated SFB alleles in other *Prunus* species. Results of allele-specific primers can be burdened with false negatives, which can only be prevented by using internal PCR controls. Robustness of our primers was checked in an analysis of 35 cultivars, from which the precise *S*-genotype of 32 cultivars could only be identified with these primers.

Previously, differentiation of S_C- and S_8-alleles could only be achieved with the addition of fruit set or pollen tube growth studies. Homo- (S_CS_C) or heterozygote (S_CS_8) genotypes of cultivars (e.g. the Rózsakajszi (S_CS_C) or Magyarkajszi (S_CS_8) cultivar groups, respectively) equally produced only one fragment of the same size in case of *S*-RNase based PCR.

Since all tested economically important self-compatible cultivars carry the insertion in their pollen component gene, the detection of S_C-haplotypes provides an easy to apply, rapid and reliable tool for breeders that allows for the early selection of self-compatible seedlings.

5.5. The structure of the *S*-RNase alleles and phylogenetic analysis

The structure of the S_C and 13 new (S_8–S_{20}) ribonuclease alleles were characterized by sequencing and fragment length analysis. The length of introns was determined by comparing with molecular weight markers or sized precisely with an automated sequencer. In case of S_8–S_{20} alleles variability of the second intron length was higher (118–2680 bp) as compared to the first intron length (110–320 bp), which is in agreement with the tendency characteristic for the S_1-S_7 alleles. In general, *S*-RNase alleles of *Prunus* species contain longer second intron except for the S_3-allele (175 bp and ~110 bp) and the S_{18}-allele (~230 bp and 118 bp).

Deduced amino acid sequences from cDNA of S_C; S_8; S_9; S_{11}; S_{13}; S_{15} and S_{16} *S*-ribonucleases were determined and used for phylogenetic analysis together with other RNase sequences.

Rhizopus T_2 type RNase formed an out-group in the dendrogram. The rosaceous *S*-RNase alleles formed two subfamily-specific clades, i.e. the *Maloideae* and *Prunoideae* groups.

Among the *Prunus* alleles an outgroup occurred encompassing the *S*-like RNases isolated from cherry and almond, which enzymes do not take part in the fertility processes. Their expression is not stylar specific, and aging, phosphate starvation and pathogen attacks increase the rate of expression.

Our phylogenetic analysis supports the trans-specific evolution in the *Rosaceae* family described earlier. Alleles from *Prunus* species do not form a

monophyletic group since interspecific sequence homology was found to be higher than intraspecific homology. Alleles of *P. armeniaca* show the highest homology with some sweet cherry, *P. pseudocerasus*, Japanese apricot or almond *S*-RNases. Consequently, alleles of apricot do not form a species specific subgroup. Apricot S_{11} and Japanese apricot S_f (100 %), apricot S_{15} and *P. pseudocerasus* S_1 (100 %), apricot S_1 and sweet cherry S_2-RNases (80 %) had the highest bootstrap support.

5.6. *S*-genotyping and crop evolutionary perspectives

The S_CS_2 genotype could be assigned to 8 cultivars on the basis of NEpHGE, PCR and DNA sequencing, 2 of which originated from North America, and 1 from Afghanistan.

The two American cultivars of S_CS_2 genotype analysed in this study originate from the breeding program conducted in Washington State. Pedigree of 'Bayoto' is unknown; while 'Toyuda' is considered to be a progeny of 'Goldrich' (S_1S_2) thus its S_2-allele can be transmitted by the cv. 'Goldrich'. Our work revealed the S_CS_2 genotype of cultivar 'Budapest' bred at the University of Horticulture. 'Budapest' originates from 'Nancy' pollinated with a pollen mixture of 'Akme', 'Magyarkajszi' and 'Kései rózsa'. Among pollen donors, genotype of 'Magyarkajszi' is supposed to be S_CS_8, while 'Kései rózsa' might be S_CS_C. Although 'Akme' was not available for our experiments, it seems likely that the S_C allele of 'Budapest' was inherited from the pollen donor. This supposition means that S_2 was carried by the cv. 'Nancy'. Several authors hypothesize that 'Nancy' can be traced back to cultivars grown in Hungary. Since 'Nancy' is involved in the pedigree of almost all important Western European cultivars ('Moor Park', 'Royal', 'Blenheim' etc.), it reflects the narrow genetic background of European cultivars to which Hungarian genotypes had also sizable contribution.

Historical and linguistic evidences indicate that a major entry of apricot from Turkey occurred during and after the time of Turkish occupation in Hungary. The theory that Hungarian apricots were carried further west and formed the northern line of the European apricots is supported by the results of our study. Nine cultivars of the SC Magyarkajszi group from 5 different countries had the same S_CS_8 genotype. It indicates that the ancestor of Magyarkajszi may have the S_8S_i genotype (S_i is an unidentified incompatibility allele), and a pollen-part mutation within the SFB_8 made the mutated S_8 (S_8'=S_C) pollen able to achieve self-fertilization. The presently grown Magyarkajszi cultivars of S_CS_8 genotype could develop from this mating. The non-mutated S_8-allele could be detected in Hungarian, Italian and Ukrainian accessions.

Geographic distribution of alleles found in 74 apricot cultivars showed that in Eurasia the allelic diversity gradually increases from West to East and its highest rate occurs in Central Asia and China.

In the 41 Eastern European cultivars and in the 15 Western European cultivars six or five alleles were identified, respectively. This fact indicates that the distribution of self-compatible genotypes from Eastern Europe to Western Europe

was more intensive since growing of these genotypes was more advantageous than that of the SI genotypes.

In the tested 13 American cultivars 7 alleles were detected, more, than from the 15 Western European cultivars belonging to the same eco-geographical group. Its reason is that in New Jersey (USA) where the NJA hybrids were released, cultivars and selections (e.g. 'Zard') from the Uzbek VIR Institution were used. However, most of these new cultivars (i.a. 'Aurora', 'Orange red') are self-incompatible and they carry previously unknown incompatibility alleles (e.g. S_{13}, S_{17}).

Similar tendency can be observed in Europe where in breeding programs (e.g. CUB Department of Genetics and Plant Breeding) Armenian cultivars were used to increase the genetic variability, but self-incompatible genotypes and inter-incompatibility groups might also be established. All these data highlight the importance of my PhD work.

Chinese and Central Asian cultivars are predominantly SI, while 60% of the Turkish and nearly 20% of the traditional Hungarian cultivars were shown to be SI, and SI is very rare among the Western European cultivars suggesting that the mutation leading to SC must have been evolved somewhere on the road from China to Turkey. It seems to be confirmed by the fact that the S_C-allele could also be detected in 'Roxana', a Central Asian genotype. Further investigations in the Eastern European gene pool may reveal valuable information in terms of the genetic background of the cultivated apricots.

Besides its direct economical importance, determination of *S*-genotypes can supply valuable and interesting information on the crop evolutionary process of apricot.

6. NEW SCIENTIFIC RESULTS

1. Protocols of non equilibrium pH gradient electrofocusing and different PCR based methods were adapted and developed for molecular characterization of fertility properties in apricot cultivars.
2. In 74 apricot cultivars and hybrids originated from 14 different countries and 4 eco-geographical groups, 13 previously unknown *S*-haplotypes (S_8–S_{20}) were identified, and intron lengths of their ribonuclease gene were determined.
3. Complete *S*-genotype of 51 economically important apricot cultivars and hybrids of great breeding value was determined, including Magyarkajszi (S_CS_8) and Óriás (S_8S_9) cultivar groups. Using molecular analyses and fruit set evaluation, the self-(in)compatibility phenotype of 21 cultivars with unknown or doubtful compatibility properties was clarified.
4. By comparing our results with data published previously, we established a table demonstrating compatibility relationships of apricot cultivars. Sixty-seven cultivars were assigned to 3 inter-incompatibility groups and a universal pollen donor group. One of the most important results of our work is that the first incompatibility group was extended with two cultivars and a new incompatibility group (III.) containing two cultivars was established. The information supplied by this table can give direct help for planning orchards with self-incompatible cultivars and selecting parental lines in breeding programs.
5. Determining fruit set and monitoring pollen tube growth after testcrosses and using molecular methods, it was unequivocally proved that self-compatibility in apricot is attributable to an insertion mutation within the pollen component SFB_C gene. The original version of the SFB_C was isolated and labelled as SFB_8 and found to be a frequent allele in Hungarian cultivars.

6. A degenerate primer pair was designed to amplify the pollen component SFB gene. These primers provide an easy to apply, rapid and reliable tool for breeders that allows for the early selection of self-compatible seedlings and as a co-dominant marker detects both homo- and heterozygote genotypes.
7. Our phylogenetic analysis of newly identified apricot and other *Prunus S*-RNase alleles confirmed their trans-specific evolution within the *Prunoideae* subfamily.

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