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Genotyping Hungarian apricot cultivars for self-(in)compatibility by isoelectric focusing and PCR analysis

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Summary: Self-incompatibility (SI) in flowering plants is a widespread genetic system that promotes out-crossing. In Prunus species the SI is a gametophytic trait, which is controlled by a single multiallelic locus, termed *S*-locus. *S*-alleles codify stylar glycoproteins with ribonuclease activity (*S*-RNases). Our objective was to assess the *S*-genotype of some Hungarian apricot varieties by isoelectric focusing of stylar RNases as well as by PCR analysis using cherry consensus primers. Consensus primers amplified one or two bands of various sizes. Primers amplifying the 1st intron gained fragments the size of which ranged from 250 to 500 bp; while those amplifying the 2nd intron resulted in fragments of 800–2000 bp length. Our data demonstrated that the first intron of the apricot *S*-RNase gene is shorter than the second one, which coincides with the structure of cherry *S*-RNase alleles. 'Hargrand' (S_1S_2) and 'Harcot' (S_1S_4) possessed one common *S*-RNase isoenzyme. Hungarian 'Óriás' apricot cultivars showed different bands compared to the previous cultivars, but they shared completely identical patterns confirming that they possess the same *S*-genotype. 'Bergeron', 'Harmat' and 'Korai zamatos' are characterised by an evidently distinct *S*-RNase pattern. The self-compatible cultivar ('Bergeron') had one allele, which suggests its correspondence to the *Sc*. Primers for the 2nd intron was unsuccessful in gaining fragments, which indicates that the 2nd intron in the *Sc* allele is too long to get any amplification. On the basis of our data, identities and differences were revealed in the *S*-allele constitution of some economically important Hungarian apricot cultivars at protein and DNA levels.

Key words: apricot, isoelectric focusing, PCR, *Prunus armeniaca*, self-incompatibility, *S*-allele, *S*-RNase

Introduction

Species of the *Rosaceae* family show gametophytic self-incompatibility to promote outcrossing. Incompatible pollen tubes are degraded by pistil *S*-RNases, the products of a unique gene with several alleles (*S*-alleles) (Nettancourt, 1977).

Almost all the European apricot cultivars have been traditionally considered self-compatible *(Kostina,* 1977); nevertheless, more and more exceptions were found *(Schultz,* 1948; *Egea* et al., 1991, *Burgos* et al., 1997). Some of the North American and the majority of the Asian, African and Iranian-Caucasian varieties are self-incompatible. These are involved in genetic improvement programs due to their valuable properties, but self-incompatibility may also be inherited in the offsprings, which is of economical significance since pollen donators are required for sufficient yield. *Burgos* et al. (1998) presented the first report describing *S*-alleles encoding seven RNase izozymes in the North American and Spanish apricot genotypes: S_1–S_6 controls the self-incompatible trait and S_c is responsible for self-compatibility. Number of self-incompatibility alleles was later extended when S_7, a previously not the identified allele was revealed by non-equilibrium pH-gradient electrofocusing *(Alburquerque* et al., 2002).

In Hungary, *Horn* (1939) evaluated most cultivars as being self-fertile. *Brózik & Nyéki* (1975) revealed that cultivar 'Szegedi mammut' is self-sterile. Later it was established that 'Szegedi mammut' and all the cultivars ('Ceglédi óriás', 'Nagykőrösi óriás' and 'Ligeti óriás') within the Hungarian Óriás group show cross-incompatibility *(Nyújtó* et al., 1985). Nevertheless, no *S*-genotype was identified for the Hungarian apricot cultivars until the present day.

PCR based DNA fingerprinting is a recent and valuable technique to assess *S*-genotypes in a number of *Prunus* species *(Ushijima* et al., 1998; *Tao* et al., 2000; *Yaegaki* et al., 2001; *Wiersma* et al., 2001). *Sonneveld* et al. (2001) designed consensus primers from cherry cDNA sequences that amplify introns of the *Rosaceae S*-RNase gene variable in length more or less in an allele-specific manner. These proved to be useful to analyse *S*-allele composition in a wide

range of sweet cherry cultivars *(Sonneveld* et al., 2003; *Békefi* et al., 2003) as well as in wild cherry species *(Sonneveld* et al., 2003).

Our experiments were carried out to acquire preliminary knowledge concerning the *S*-genotypes of Hungarian apricot cultivars by *S*-RNase detection and PCR analysis with cherry consensus primers.

Material and method

Plant material

Genotypes from the apricot collection of the Corvinus University of Budapest, Department of Genetics and Plant Breeding in Szigetcsép were used in the present study.

PCR amplification

The genomic DNA was isolated from fully expanded young leaves following the protocol of DNeasy Plant kit (QIAGEN). PCR amplification with consensus primers for the first and the second intron of *Rosaceae S*-RNase gene was carried out as described by Sonneveld at al (2003).

Sample preparation for isoelectric focusing

Flowers were emasculated and forty styles with stigmas uncontaminated with pollen were harvested just before or soon after anthesis. Extraction of stylar proteins was performed as described for cherry by *Bošković & Tobutt* (1996). The homogenate was centrifuged by a Hettich Zentrifugen Micro 22 R equipment (–4 °C, 35 min, 13500 rpm). Supernatant stored at –80 °C was used for further analyses. Leaf extracts from most of the assayed cultivars were also prepared. The ratio of leaf material to extraction buffer was approximately 1/5 (w/v).

Non-equilibrium pH gradient electro-focusing (NEpHGE)

The extracts were separated on vertical slab gels, 15 cm × 21 cm × 0.1 cm, consisting of 7.5% polyacrylamide with 10% saccharose and one or more ampholytes as described below. The first gels were prepared in general accordance with Burgos et al. (1998) and contained 4% Pharmalyte pH 5–8. As this revealed only limited polymorphism various other ampholytes and their combinations were tried. The main results presented here were obtained with 4% Pharmalyte pH 5–8 and 1.4% Ampholine pH 7–9. The catalyte and analyte used were 0.1 M sodium hydroxide and 0.04 M DL-glutamic acid, respectively. After a 30 min prefocusing at 100 V, the samples of 50 µl, were loaded at the anodal end, and the focusing run comprised 1 hour at 130 V, 2 hours at 260 V, 1 hour at 350 V, and 1 hour at 400 V. The temperature was maintained at 4 °C.

Isoelectric focusing (IEF)

IEF was carried out in a PhastSystem (LKB-Pharmacia, Sweden) electrophoresis unit under the parameters successfully used for taxonomic identification of a wide range of horticultural crops *(Stefanovits-Bányai* et al., 1999; Stefanovits-Bányai et al., 2002). In order to develop a pH gradient (pH 3–9) ready-made gels were prefocused by 2.5 mA at 10 °C for 75 Vh, and crude extracts of style samples were applied onto the acidic (pH 4.5) end of the gel and run by 2.5 mA at 10 °C for 700 Vh.

Ribonuclease detection in gels

Gels were stained for ribonuclease activity based on the procedures described by *Wilson* (1971) and modifications proposed by *Bošković & Tobutt* (1996) were taken into consideration. The interpretative diagrams show the main bands we regard as associated with incompatibility alleles as solid lines. The secondary bands marked by dotted lines refer to isoenzymes which participation in the SI reactions is doubtful.

Results

The RNase zymograms resulting NEPHGE *(Figure 1)* show that 'Hargrand' and 'Harcot' share one common allele, while both bands of 'Ceglédi óriás' and 'Ligeti óriás' seem to be identical. 'Bergeron', 'Harmat' and 'Korai zamatos' possess clearly distinct izozyme patterns. Leaf extracts ran under NEPHGE or isoelectric focusing did not show any RNase activity. Dashed lines correspond to secondary bands associated with ribonucleases not linked to incompatibility alleles.

In PCR analyses PaConsI-F and PaConsI-R primers gained fragments the size of which ranged from 250 to 500 bp *(Figure 2A)*, while PaConsII-F and PaConsII-R amplified fragments of 800–2000 bp length *(Figure 2B)*. 'Hargrand' and 'Harcot' showed a common band as a result of the analysis for the 1st and 2nd introns *(Figure 2A)*.

Completely identical patterns of the 2 cultivars belonging to the Óriás cultivar group could suggest that they possess the same *S*-genotype *(Figure 2A)*. The 2nd intron amplification of these cultivars performed only one of the two bands *(Figure 2B)*.

Primers for the 1st intron 'Bergeron' amplified only one allele, while primers for the 2nd intron did not result in any fragments. 'Harmat' amplified a weaker and a stronger band in the analysis for the 1st intron *(Figure 2A)*, while for the 2nd intron it performed also two, well distinguishable bands, which were completely different from any of the previously described bands. In the case of 'Korai zamatos' only one *S*-allele representing band could be observed as a result of PCR reactions carried out by primers either amplifying the 1st or the 2nd intron.

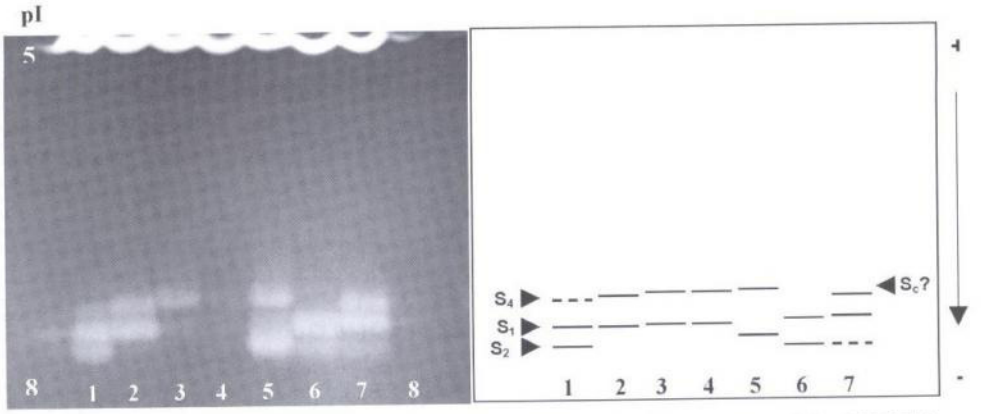

Figure 1. Zymogram and interpretative diagram of stylar ribonucleases separated by NEpHGE. 1. 'Hargrand', 2. 'Harcot', 3. 'Ceglédi óriás', 4. 'Ligeti óriás', 5. 'Bergeron', 6. 'Harmat', 7. 'Korai zamatos', 8. Leaf extract of 'Bergeron'.

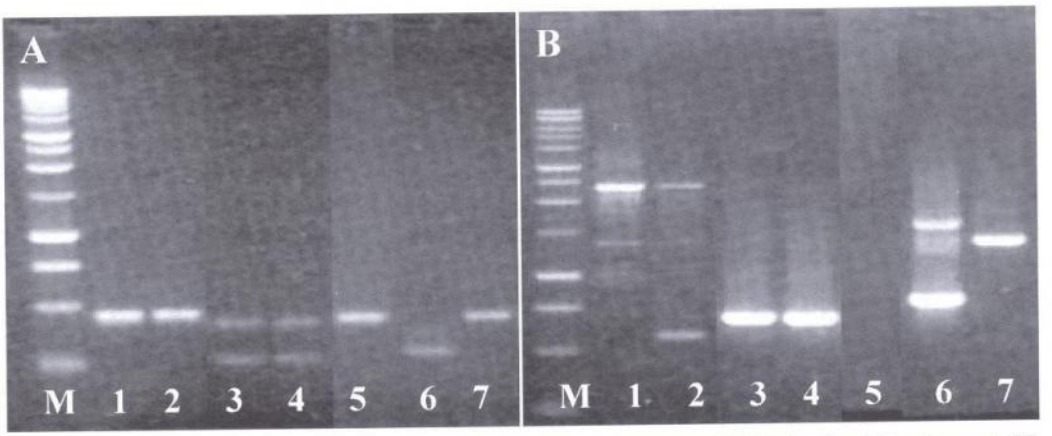

Figure 2. PCR analysis of apricot genotypes by cherry consensus primers amplifying the first (A) and second (B) introns of Prunus S-RNase gene. M Marker 1 kbp ladder, 1 'Hargrand', 2 'Harcot', 3 'Ceglédi óriás', 4 'Ligeti óriás', 5 'Bergeron' 6 'Harmat', 7 'Korai zamatos'

Discussion

The *S*-RNase zymograms represented three izozymes (S_1, S_2 and S_4) that were previously described by *Burgos* et al. (1998). Non-polymorphic bands, which were present in cherry stylar extracts *Boškovič & Tobutt*, 1996), did not occur in the tested pH range. Leaf extracts did not perform RNase activity, which also confirms that all izozymes detected in the gel mediate (in)compatibility reactions. Cultivars belonging to the Óriás cultivar group shared identical zymograms, elucidating the genetic background of cross-incompatibility among these cultivars, which has been well-known for a long time *(Nyujtó* et al., 1985; *Nyéki & Szabó,* 1995).

'Bergeron', 'Harmat' and 'Korai zamatos' are characterised by evidently distinct *S*-RNase pattern. One allele of 'Bergeron' as a self-compatible cultivar must be assigned as S_c allele which is responsible for the self-compatibility trait in a dominant manner *(Burgos* et al., 1997). On the basis of the data presented by *Burgos* et al. (1998), it can be supposed that S_c-RNase may be the one focused in the less alkaline part of the gel. Nevertheless, some questions remained unanswered: whether less alkaline *S*-RNase izozyme of the cv. 'Harmat' is identical or not with the more alkaline isoenzyme of the Óriás group and how these are related to the S_1 self-incompatible allele present in 'Hargrand' and 'Harcot'?

PCR analysis with consensus primers may offer an efficient way to clarify this. *S*-RNase sequences are highly conserved *(Igic & Kohn,* 2001), especially within the *Prunoideae* subfamily *(Ma & Oliveira,* 2002), which makes the application of these primers possible in other *Prunus* species, as it was verified by *Sonneveld* et al. (2003) in the case of wild cherry species.

Introns that are inserted in the *Rosaceae S*-RNase genes are variable in length more or less specifically to the different alleles. Two pairs of consensus primers designed from conserved coding regions flanking the two introns of the *Prunus avium S*-RNase sequences yielded one or two bands in the case of the most diploid apricot accessions, which indicated that sweet cherry consensus primers also recognise *S*-RNase sequences in apricot.

Our data demonstrated that the first intron of the apricot *S*-RNase gene is shorter than the second one, which coincides with the structure of cherry *S*-RNase alleles

(Sonneveld et al., 2003). The common band of 'Hargrand' (S_1S_2) and 'Harcot' (S_1S_4) corresponds to the allele S_1. The smaller band occurring in case of the 2nd intron analysis for cv. 'Harcot' seemed to be S_4 *(Burgos* et al., 1998).

Completely identical *S*-genotypes of cross-incompatible Óriás cultivars, which were previously suggested, due to stylar RNase analyses were clearly validated and further confirmed by PCR. The self-compatible cultivar 'Bergeron' amplified one band, which was also present in all other self-compatible genotypes (data not shown), which suggests its correspondance to the S_c allele. Primers for the 2nd intron were unsuccessful in gaining fragments, which indicates that the 2nd intron of the S_c allele may be rather long. This fact may be exploited to find self-compatible apricot genotypes which are homozygous for the S_c allele. These genotypes would be rather valuable in a breeding programme, since all their progenies will surely be self-compatible *(Alburquerque* et al., 2002). 'Harmat' and 'Korai zamatos' gave distinct patterns, which may serve as an efficient tool to determine their *S*-genotypes and their fertility properties not exactly clarified at the moment.

The *S*-RNase isoenzyme pattern showed a good correlation with the data gained by cherry consensus primers. Proper *S*-genotyping of cultivars may be achieved by comparing our results with those obtained in the case of the Mediterranean and American apricot cultivars *(Burgos* et al., 1998) and by sequencing PCR products to approve or disapprove new *S*-alleles in apricot.

From a theoretical point of view, our results provided further support to the hypothesis that *S*-RNases do not form species-specific clusters in a phylogenetic tree *(Igic & Kohn,* 2001), and showed that besides wild cherries *(Sonneveld* et al., 2003) apricot also belongs to the *Prunus* species in which sweet cherry primers were efficient to evaluate compatibility properties.

On the basis of our preliminary data, possible identities and differences were revealed in the *S*-allele constitution of some economically important Hungarian apricot cultivars. It elucidates the genetic background of the fertility properties of cultivars with known fruit setting behaviour *(Brózik & Nyéki,* 1975; *Nyújtó* et al., 1985), while in other cultivars and genotypes with not currently known compatibility properties it may be ascertained in a safe and convenient way. Besides, correct *S*-genotyping of cultivars may be helpful for producers to establish more proper orchard structures.

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