S6-RNase Is a Marker for Self-compatibility in Loquat (*Eriobotrya japonica* Lindl.)

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Abstract. **Loquat (*Eriobotrya japonica* Lindl.), a member of the Rosaceae, carries the RNase-dependent gametophytic self-incompatibility fertilization system. Analysis of S-RNase-allele content in the commercial loquat cultivars Avri, Yehuda, and Akko 1 revealed that each of them contains one different S-RNase allele—S2, S3, and S4, respectively, and one that they all share, S6. Although all four S-alleles were isolated in this work, only S6 was found to be novel. Amino acid similarity between the partial sequence of S6-RNase and other known loquat RNases (S1 to S4) ranged between 62% and 65% with highest similarity (83%) to the S110-allele of European pear (*Pyrus communis*). Determination of S-RNase-allele content in progeny of 'Avri', 'Yehuda', and 'Akko 1', obtained in an open-pollinated, mixed-cultivar orchard, revealed that all of the progeny derived from self-fertilization contained the S6 haplotype, indicating that a mutation in the S6 locus is responsible for the self-fertilization. However, sequencing of most of the S6-RNase gene (from C1 to C5) did not reveal any mutation and the alignment of the deduced amino acid sequence showed that it has the expected S-RNase primary and tertiary structural organization. Nonetheless, because it is apparent that the S6-RNase allele is linked to the self-compatibility trait, it could serve as a marker for early selection of self-compatible loquat cultivars.**

Loquat (*Eriobotrya japonica* Lindl.) is a subtropical evergreen fruit tree of the family Rosaceae, subfamily Maloideae (Lin et al., 1999), which carries the RNase-dependent gametophytic self-incompatibility fertilization system. This system is controlled by a single multiallelic locus (*S*-locus) that contains at least two haplotype-specific genes: the *S-RNase* gene, which is expressed in the pistil, and a pollen *S* gene, expressed in the pollen tube (Kao and Tsukamoto, 2004; McCubbin and Kao, 2000). However, some loquat cultivars such as 'Akko 1', 'Mogi', 'Pale Yellow', 'Advance', and 'Tanaka' are self-fertile or partially self-fertile (Cuevas et al., 2003; Morton, 1987; Tous and Ferguson, 1996).

In the Maloideae subfamily, self-compatibility has been shown to occur as a result of mutation in the *S-RNase* gene: the self-compatible Japanese pear (*Pyrus pyrifolia*) cv. Osa-Nijisseiki carries a deletion in the *S4* locus that includes the *S-RNase* gene (Okada et al., 2008) and the European pears (*Pyrus communis*) 'Abugo' and 'Ceremeno' are self-compatible as a result of the absence of *S21°*-RNase protein in their styles (Sanzol, 2009).

From an agricultural point of view, self-compatibility provides an advantage in that an orchard can contain a single cultivar (solid block) as opposed to self-incompatible cultivars that depend on cross-pollination of a compatible cultivar with synchronous flowering. Nevertheless, in loquats, cross-pollination is generally considered to be beneficial in terms of productivity (Crane and Caldeira, 2006; McGregor, 1976; Morton, 1987; Thapa, 2006). For example, artificial cross-pollination of cv. Algerie resulted in more fruits of larger size with more seeds than artificial self-pollination (Cuevas et al., 2003), and cross-pollination of cv. Jie-fangzhong increased its fruit weight (Xu et al., 2007).

In Israel, 'Akko 1' and 'Yehuda' are the main commercial cultivars, whereas 'Avri' is a new cultivar that is widespread in new plantations. The three cultivars were bred from local chance seedlings. They are considered self-fertile and are usually planted in solid blocks. In this study, the *S*-genotype of the loquat cultivars Avri, Yehuda, and Akko 1 and their progeny was determined, indicating that the *S6-RNase* allele can be used as a marker for the identification and selection of self-compatible cultivars.

Materials and Methods

The orchard. The experiment was conducted in a 0.3-ha loquat orchard planted in 2002 at 440 trees/ha spaced at 6 × 4 m. The nearest loquat orchard is 5 km away. The orchard is located north of the Sea of Galilee (long. 36° N, lat. 29.9° E, alt. 0 m) in a semiarid region with high temperatures (≈42 °C maximum). Winter precipitation (November to April) is ≈400 mm. The orchard contains six rows of 'Yehuda' as a single cultivar, apart from the fourth row in which 'Avri' and 'Akko 1' were planted between 'Yehuda' along the row in alternating fashion (i.e., 'Yehuda', 'Avri', 'Yehuda', 'Akko 1', 'Yehuda', 'Avri', 'Yehuda', 'Akko 1', and so on). The three cultivars flowered simultaneously during Oct. 2008. Fruit ripened early, between 15 Feb. and 5 Apr. 2009.

Plant material. For DNA analysis, young leaves were collected from the parental cultivars Avri, Yehuda, and Akko 1 and from 4-month-old seedlings of their progeny. Seedlings were obtained from seeds that were isolated at harvest, sown in boxes containing peat rooting medium, and left for germination in a nursery. The progeny *S-RNase* analysis was performed on one or two seedlings obtained from a single fruit. The leaves were stored at –70 °C until use.

DNA extraction. DNA was extracted from the leaves according to Doyle and Doyle (1987) with modifications. Briefly, 700 μL of extraction buffer (2% w/v hexadecyltrimethyl-lammonium bromide, 100 mᴍ Tris-HCl pH 8, 20 mᴍ ethylenediamine tetraacetic acid pH 8, 1.4 M NaCl, 1% w/v polyvinylpyrrolidone MW 40,000, 1% v/v β-mercaptoethanol) was added to 100 to 200 mg of leaves, which had been frozen in liquid nitrogen and pulverized with a mortar and pestle. This mixture was incubated for 30 min at 65 °C with occasional mixing. After the mixture cooled to room temperature, two extractions were performed with chloroform:octanol (24:1, v/v). The DNA was pelleted with ethanol and dissolved in double-distilled water. The DNA extract was kept at –20 °C pending use.

Polymerase chain reaction amplification. The polymerase chain reaction (PCR) was performed in a MyCycler apparatus (BioRad, Hercules, CA). The basic program was as follows: 1 cycle of 2 min at 94 °C, 30 cycles of 15 s at 94 °C, 30 s at the specific annealing temperature (Table 1), and 2 min at 70 °C; the final cycle was 7 min at 70 °C. Each PCR tube contained ≈20 ng DNA, 1× Ex *Taq* buffer (Takara Bio Inc., Shiga, Japan), 0.2 mᴍ dNTP mixture, 20 pmol of each primer, and

Table 1. Conditions and primers for polymerase chain reaction of loquat *S-RNase* genes.

RNase gene	Accession no.	Forward primer (5′→3′)	Reverse primer (5′→3′)	Fragment length (bp)	Annealing temp. (°C)
S		TTTACGCAGCAATATCAG[z]	GYGGGGGGCARTYTATGAA[z]		48
S2	GU384665	AATAAAACCATGAATTCCCTGGC (133–155)	CTGCAAAGASTRAYCTCAACC [*S2*- (627–647), *S4*- (654–674) and *S6*- (604–624)]	514	52
S3	GU384666	GGTAATTGCTAGATAGACTAA (274–294)[y]	As for *S2*	539[x]	56
S4	GU384667	GCATACCAGAGAGAAAAATTA (319–339)[y]	As for *S2*	355	56
S6	GQ202269	CAACCACAATACAGGACGACG (390–410)[y]	As for *S2*	234	58

[z]Universal primers of *Pyrus pyrifolia S-RNases* (Ishimizu et al., 1998).
[y]Part or all of the primer is located within the intron.
[x]Fragment length based on deduced gene sequence.

1.25 U of Ex *Taq* (Takara Bio Inc.) in 25 μL of reaction mixture. The universal primers were those used initially by Ishimizu et al. (1998) for the amplification of *P. pyrifolia S-RNases*. For amplification of each specific *S-RNase*, a unique forward primer was generated for each of the alleles and a degenerated reverse primer was generated for *S2, S3, S4,* and *S6-RNase* (Table 1).

Cloning and DNA sequencing of polymerase chain reaction products. PCR products were cloned into pGEMT (Promega, Madison, WI) and sequenced in both directions. At least two different clones were sequenced for each *S-RNase*. The sequencing was performed with an ABI 3730 XL automated DNA sequencer (PE Bio System, Foster, CA) using dye terminator cycle sequencing with fluorescently labeled dye terminators.

DNA and putative protein sequence analyses. Analyses of the DNA sequence data and of the predicted protein sequences were performed with BLAST of NCBI, Clustal W and the phylogenetic tree at EMBL BoxShade server (http://www.ebi.ac.uk/clustalw/index.html), and the DNAstar Lasergene 6 "SeqManII" software (Madison, WI).

S-RNase *structure modeling and alignment.* Models were created with the Swiss Model Workspace (Arnold et al., 2006) and aligned with the PyMOL Molecular Graphics System (2002) (DeLano Scientific, San Carlos, CA).

Results

The S-genotype of cvs. Avri, Yehuda, and Akko 1. Each of the *S-RNase* alleles from 'Avri', 'Yehuda', and 'Akko 1' was cloned and sequenced independently. Alignment of the clones to each other and to GenBank revealed that 'Avri' is *S2-S6* (Accession Nos. GU384665 and GQ202269, respectively), 'Yehuda' is *S3-S6* (Accession Nos. GU384666 and GQ202269, respectively), and 'Akko 1' is *S4-S6* (Accession Nos. GU384667 and GQ202269, respectively). Thus, they all share the *S6-RNase* allele. *S2, S3,* and *S4-RNase* have also been recently cloned by Carrera et al. (2009) (Accession Nos. EU442286, EU442287, and EU442289, respectively), whereas *S6* is a novel allele. Nevertheless, the sequences of *S2, S3,* and *S4-RNases* were deposited in GenBank, because they were 24 to 94 bases longer than those of Carrera et al. (2009).

The loquat S6-RNase. Because *S6*-RNase is carried by 'Avri', 'Yehuda', and 'Akko 1', three cultivars that are assumed to be self-compatible, we hypothesized that self-fertilization might be the result of mutation in the *S6-RNase* gene.

The segment cloned from the *S6-RNase* allele contained ≈80% of the gene's open reading frame. *S-RNase* genes carry an intron within the hypervariable (HV) region. The intron of loquat *S6-RNase* was localized to this native position and was 124 bases long. The deduced amino acid sequence of the two exons exhibited highest similarity (83%) with European pear (*Pyrus communis*) *S110*-RNase (also designated *S10* or *Sg*, Accession Nos. AB258360 and EF418041, respectively; Goldway et al., 2009). Amino acid identity between the *S6*-RNase and loquat *S1-S4* RNases ranged between 62% and 65% (Fig. 1).

The putative *S6*-RNase amino acid sequence possessed the primary structural features of all known *S-RNases*. *S6*-RNase was cloned from the border of C1 to the border of C5 conserved regions and contained the three other conserved regions, C2, C3, and RC4. It also included the HV region and the five conserved cysteine and two conserved histidine residues (Fig. 1), which have been shown to be essential for RNase activity in the Solanaceae (Royo et al., 1994).

The computerized protein structures of *S2*- and *S6*-RNases from loquat were generated and compared revealing a similar structure, except for the HV region (Fig. 2).

Polymerase chain reaction analysis of S-RNase *alleles in the progeny.* To characterize the fertilization and pollination types of 'Avri', 'Yehuda', and 'Akko 1' in a mixed-cultivar commercial orchard, *S-RNase* allele distribution
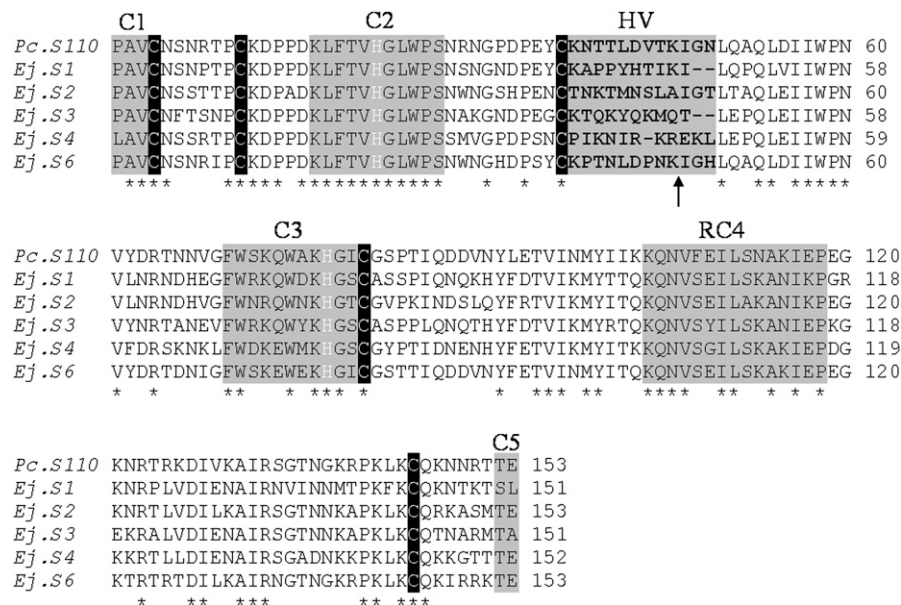

Fig. 1. Amino acid alignment of loquat *S-RNases*. The alignment was generated by Clustal W 1.8. Similarities are shown by asterisks and gaps are marked by dashes. The conserved regions (C1 to C5) are shaded. The hypervariable region (HV) is shaded with characters in bold type. The conserved cysteines (white characters on black background) and histidines (white characters on gray background) are indicated. *S6*-RNase intron position is marked with an arrow. The aligned *S-RNase* sequences are from *S1, S2, S3, S4,* and *S6* alleles of loquat (*Eriobotrya japonica, Ej*) (Accession Nos. ACC66149, ADC92282, ADC92283, ADC92284, and ACS94938, respectively) and from the *S110*-allele of European pear (*Pyrus communis, Pc*) (also designated *S10* or *Sg*, Accession Nos. ABR08577 and BAF35959, respectively).

was determined in their open-pollinated progeny. Fruits were collected from adjacent 'Avri', 'Yehuda', and 'Akko 1' trees and PCR analysis for *S-RNase* alleles was performed on leaves of seedlings raised from the seeds of the collected fruits. For the analysis, specific primer pairs were generated for loquat *S2*, *S3*, *S4,* and *S6-RNase* (Table 1; Fig. 3).

Because we assumed that self-compatibility is the result of a malfunction in the *S6* locus, we expected that the progeny derived from self-fertilization would all carry the *S6-RNase*, half of them in the homozygous state and the other half in the heterozygous state, with the second allele being the other parental *S-RNase*. The analysis revealed that all of the self-fertile progeny contained the *S6* haplotype (Table 2). Some of them were obtained by self-pollination and some by cross-pollination, because all of the pollen donors in the orchard contained the
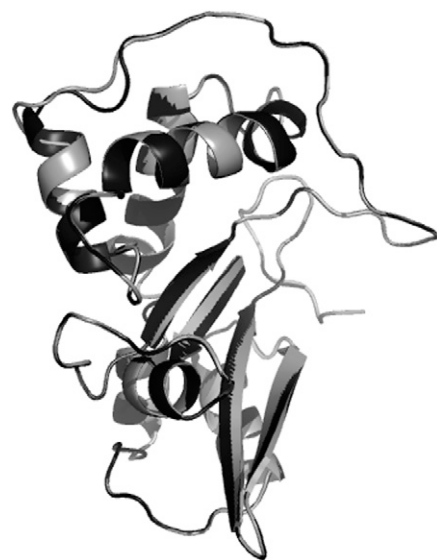

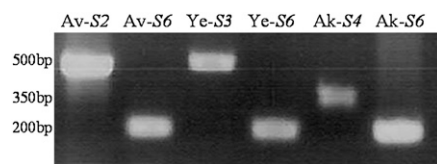Fig. 2. Stereo ribbon diagram of *S2*- and *S6*-RNases from loquat (black and gray, respectively).

Fig. 3. Identification of *S-RNase*-allele content in loquat cvs. Avri (Av), Yehuda (Ye), and Akko 1 (Ak) by polymerase chain reaction with specific primers (primers are described in "Materials and Methods"; Table 1).

S6 locus. The distribution between the *S6* homozygous and heterozygous (with the other parental *S*-allele) states was, respectively, 71% and 29% in 'Avri', 67% and 33% in 'Yehuda', and 52% and 48% in 'Akko 1' (Table 2).

Cross-pollination levels were determined by the non-parental *S-RNase* allele (Tables 2 and 3). For example, the seven 'Avri' progeny that were the outcome of non-*S6* pollen fertilization (*S6-S3* and *S2-S3*) were identified by the *S3*-allele of 'Yehuda' (Table 2). If the same number of 'Avri' progeny was obtained by self-fertilization with 'Yehuda' *S6*-pollen grain, then the total cross-pollination level of 'Avri' open-pollinated progeny, obtained in the mixed-cultivar orchard, was 46% (14 of 31 progeny). By using this same method, the total cross-pollination rates of 'Yehuda' and 'Akko 1' were found to be 10% and 32%, respectively (Table 3).

Discussion

The *S-RNase*-allele content of three commercial loquat cultivars that are considered to be self-compatible was determined. It was found that 'Avri', 'Yehuda', and 'Akko 1' contain one unique *S-RNase*: *S2*, *S3,* and *S4*, respectively, and that the other allele, *S6*, is present in all of them. Although all four *S*-alleles were isolated in this work, only *S6* was novel. The *S6*-RNase exhibited the highest amino acid identity to the European pear (*P. communis*) *S110* allele (83%), whereas it showed only 62% to 65% identity to the loquat *S*-RNases. Thus, similar to many other cases (Ishimizu et al., 1998; Zisovich et al., 2004), including another study on loquat *S*-RNases (Carrera et al., 2009), the *S6*-RNase exhibits a transspecific pattern, suggesting that some of the polymorphism at the *S*-locus existed before divergence of the Maloideae species.

Self-fertilization in an open-pollinated, mixed-cultivar orchard containing 'Avri', 'Yehuda', and 'Akko 1' was determined by PCR analysis of progeny *S-RNase* alleles (concluded by the presence of the parental *S-RNase* alleles). Because all of the progeny derived from self-fertilization carried the *S6-RNase* allele, it was concluded that the *S6* locus is responsible for this self-fertilization: 77% to 95% of the three cultivars' progeny were the outcome of self-fertilization with the *S6* pollen grain, indicating dysfunction of a main component in the fertilization system.

No insertions or deletions were detected in the *S6*-RNase sequence from the border of C1 to the border of C5 (covering ≈80% of the

gene). Alignment of the deduced amino acid sequence and a computer-generated protein structure showed that it has the expected *S*-RNase primary, secondary, and tertiary structural organization (Ishimizu et al., 1998; Matsuura et al., 2001). Nevertheless, because the 5′ and 3′ ends of *S6-RNase* were not sequenced and its expression and function were not analyzed, this RNase cannot be conclusively determined as the reason for the breakdown of the fertilization system. Another possibility that would enable self-fertilization is dysfunction of the pollen *S*-gene. The *SFB* protein, a pollen *S*-haplotype-specific F-box protein gene, has been shown to confer self-compatibility in sweet cherry (*Prunus avium*), Japanese apricot (*Prunus mume*), peach (*Prunus persica*), and apricot (*Prunus armeniaca*) (Sonneveld et al., 2005; Tao et al., 2007; Ushijima et al., 2004; Vilanova et al., 2006). However, all of these fruits belong to the Amygdaloideae, whereas loquats are from the subfamily Maloideae. According to Sassa et al. (2007), there are two (in apple) or three (in pear) F-box brother (SFBB) genes, which are located in the *S*-locus and expressed in the pollen, and they are candidates for this role in the Maloideae subfamily. However, to date, there has been no report of a mutation in an SFBB conferring self-fertilization at all.

It was expected that among the self-fertilized progeny, 50% would be homozygous for *S6*, and the other 50% would be heterozygous for *S6* and the *S-RNase* allele of the maternal *S*-haplotype as has been found for progeny of the partly self-compatible Japanese pear (*Pyrus serotina*) cv. Osa Nijisseiki (*S2S4^{sm}*) (Hiratsuka et al., 1995) and the partly self-compatible Japanese apricot (*Prunus mume*) cv. Hachiro (*S8Sf*) (Tao et al., 2002). The distribution among 'Akko 1' progeny was as expected; however, in 'Avri' and 'Yehuda', there was a bias of 71% and 67%, respectively, for *S6*-homozygous progeny. The reason for this imbalanced preference is not clear to us; it could be coincidental or the result of biological reasons such as an advantage for fertilization of *S6* haplotype ovules by *S6* haplotype pollen or a higher probability of seedling formation by seeds that are homozygous for *S6*. Regardless, the results clearly indicate that the *S6*-RNase is linked to the self-compatibility trait in loquats and that it can be applied as a marker for the identification and selection of self-compatible cultivars.

Cross-pollination level of 'Avri', 'Yehuda', and 'Akko 1' was 46%, 10%, and 32%, respectively. The lower rate of cross-pollination in 'Yehuda' could be because it was the main cultivar in the orchard and/or as a result of its flower properties, which were not examined in this study. Accordingly, all 'Avri' and 'Akko 1' cross-fertile progeny were the outcome of fertilization with 'Yehuda' *S3* pollen. However, the high rate of cross-pollination in 'Avri' and 'Akko 1', obtained in a mixed-cultivar orchard, implies honeybees' and other insects' contribution to loquat fruit set, as reported by others (Crane and Caldeira, 2006; Cuevas et al., 2003; McGregor, 1976; Morton, 1987; Thapa, 2006). According to Cuevas et al.

Table 2. *S*-allele distribution in 'Avri', 'Yehuda', and 'Akko 1' progeny obtained from open pollination in a mixed-cultivar orchard in 2008–2009.

Cultivar progeny	Progeny with parental *S* alleles (self-fertilization)			Progeny with a non-parental *S* allele (cross-fertilization)				No. progeny
Avri (*S6-S2*)	*S6-S6*	*S2-S2*	*S6-S2*	*S6-S3*	*S6-S4*	*S2-S3*	*S2-S4*	
	17	0	7	3	0	4	0	31
Yehuda (*S6-S3*)	*S6-S6*	*S3-S3*	*S6-S3*	*S6-S2*	*S6-S4*	*S3-S2*	*S3-S4*	
	24	0	12	0	2	0	0	38
Akko 1 (*S6-S4*)	*S6-S6*	*S4-S4*	*S6-S4*	*S6-S2*	*S6-S3*	*S4-S2*	*S4-S3*	
	16	0	15	0	2	0	4	37

Table 3. Cross-pollination levels in open-pollinated 'Avri', 'Yehuda', and 'Akko 1' progeny obtained in a mixed-cultivar orchard in 2008–2009.

Cultivar progeny	Cross-pollination with non-$S6$ pollen grains (%)[z]	Assumed cross-pollination with $S6$ pollen grains (%)	Total cross-pollination with $S6$ and non-$S6$ pollen grains (%)
Avri ($S6$-$S2$)	23% (7/31)	23% (7/31)	46% (14/31)
Yehuda ($S6$-$S3$)	5% (2/38)	5% (2/38)	10% (4/38)
Akko 1 ($S6$-$S4$)	16% (6/37)	16% (6/37)	32% (12/37)

[z]According to cross-fertilization results presented in Table 2.

(2003) and Xu et al. (2007), cross-pollination in loquat improves fruit set, seed set, and fruit size. Our results show for the first time that cross-pollination is significant in a mixed-cultivar loquat orchard. Therefore, despite the existence of self-compatibility in many loquat cultivars, this strategy could be applied to improve the productivity of loquat orchards.

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