SFBs of Japanese Plum (*Prunus salicina*): Cloning Seven Alleles and Determining Their Linkage to the *S-RNase* Gene

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Additional index words. SFB, Japanese plum, *Prunus salicina*, self-incompatibility

Abstract. Japanese plum (*Prunus salicina* Lindl.), a species of the *Rosaceae* family, carries the *S-RNase*-mediated gametophytic self-incompatibility system. Self-incompatibility is manifested if the *S*-haplotype of the pollen is carried also by the pollinated flower. Thus, for fertilization to occur, the cultivars have to be genetically compatible. The haplotype is conferred by an *S*-locus, which contains the style-specific expressed *S-RNase* and the pollen-specific expressed F-box genes (SFB). Since both the *S-RNase* and the SFB genes are multiallelic and are characteristic of each of the *S*-haplotypes, they are ideal markers for molecular *S*-typing. In this work, seven SFBs, from eight Japanese plum cultivars, were cloned and sequenced. Five of the alleles were published recently and two SFB^a and SFB^b are new. The physical linkage of SFB^b and SFB^c to their adjacent *S-RNase* was determined; it is 544 base pairs (bp) and 404 bp for the *S*^b and *S*^c loci, respectively.

Self-incompatibility (SI) is the ability of a fertile hermaphrodite flowering plant to prevent self-fertilization by discriminating between self and nonself pollen. Japanese plum (*Prunus salicina* Lindl.), a species of the *Rosaceae* family, carries the *S-RNase*-mediated gametophytic self-incompatibility (GSI) system. This system was first identified in *Solanaceae* (Anderson et al., 1986) and later in *Rosaceae* (Sassa et al., 1992) and in *Scrophulariaceae* (Xue et al., 1996). Research in the three botanical families is brought together in an attempt to explore the *S-RNase*-mediated GSI system.

In GSI, the inhibition of a pollen grain is based on its haploid genotype (termed *S*-haplotype). SI is manifested if the *S*-haplotype of the pollen is carried also by the pollinated flower (McCubbin and Kao, 2000). The

haplotype is conferred by a *S*-locus, which contains, among others, the style-specific expressed *S-RNase* and the pollen-specific expressed F-box genes (McClure et al., 1989; Zhu et al., 2004). Both genes are heteroallelic and are suspected of being involved in determining the specific self-pollen rejection; however, the mechanism of the system is still not fully understood. Several reviews describing the current perception of the *S-RNase*-mediated GSI system have been published recently (Goldway et al., 2007; McClure and Franklin-Tong, 2006; Takayama and Isogai, 2005).

As a result of the SI, for obtaining satisfactory yield, it is essential that Japanese plum orchards contain at least two cultivars that serve as pollinators of each other. Before the molecular genetic era, compatibility was determined in field experiments by using natural and hand-pollination of cultivar couples. However, because agrotechnical and environmental factors affect fruit-set levels, the method is inaccurate. Although *S-RNase* alleles are well known and have been studied for more than a decade, the pollen F-Box gene was identified only recently, first in *Antirrhinum* (Lai et al., 2002) and then in

almond (*Prunus dulcis*) and Japanese apricot (*Prunus mume*) (Entani et al., 2003; Ushijima et al., 2003), both of the *Prunus* genus included in *Prunoideae*, a subfamily of *Rosaceae*, in *Petunia inflata* of the *Solanaceae* (Sijacic et al., 2004), and recently in apple (*Malus domestica*) and Japanese pear (*Pyrus pyrifolia*) (Cheng et al., 2006; Sassa et al., 2007), which are in *Maloideae*, another subfamily of *Rosaceae*. The gene was termed SLF (*S*-Locus F-box) by Entani et al. (2003) and also SFB (*S*-haplotype-specific F-Box protein) by Ushijima et al. (2003). In this article, we followed the latter, which has been already applied to other *Prunus* species.

Because both the *S-RNase* and the SFB genes are multiallelic and are characteristic of each of the *S*-haplotypes, they are ideal markers for molecular *S*-typing. To date, 14 *S-RNases* were cloned from Japanese plum (Beppu et al., 2002, 2003; Sapir et al., 2004).

In the present work, seven Japanese plum SFBs were cloned from nine cultivars. Five SFBs were also described in a recent work of Zhang et al. (2007).

Materials and Methods

Plant materials. Nine Japanese plum cultivars (*Prunus salicina* Lindl.) were used in this study: 'Wickson' (*S*^a), 'Black Diamond' (*S*^b), 'Royal-Zee' (*S*^c), '440' (*S*^d), 'Songold' (*S*^e), 'Methely' (*S*^f), 'Shiro' (*S*^g), 'Newyorker' (*S*^h), and 'Gol-frose' (*S*ⁱ) (Sapir et al., 2004; unpublished data). Young leaves, ~1 month old, were collected during spring and stored at -80 °C until use.

DNA extraction. Extraction of DNA from the leaves was based on the method used by Doyle and Doyle (1987). Briefly, 700 µL extraction buffer (2% hexadecyltrimethylammonium bromide, 100 mM Tris pH 8, 20 mM ethylenediaminetetraacetic acid, pH 8, 1.4 M NaCl, 1% polyvinylpyrrolidone, MW 40000, 1% β-mercaptoethanol) was added to 100 to 200 mg of leaves, which were powdered by a pestle and mortar containing liquid nitrogen. This mixture was incubated for 30 min at 65 °C with occasional mixing. After cooling to room temperature, two extractions were performed with chloroform:octanol (24:1). The DNA was pelleted with ethanol and dissolved in DDW.

***S*-haplotype-specific F-Box protein polymerase chain reaction amplification.** Polymerase chain reaction (PCR) was conducted using ~20 ng of DNA, 5 µL of 10× PCR buffer containing 15 mM MgCl₂ (Sigma, St. Louis), 0.1 mM dNTP mix, 0.4 µM of each primer (FBOX5'A or SRc-F as forward primers and FBOX3'A as backward primer (Romero et al., 2004; Sonneveld et al., 2005), and 0.25 µL of Taq polymerase (5 unit/µL; Sigma) in a 50-µL reaction mixture. PCR conditions were as described in Sonneveld et al. (2005).

Cloning and DNA sequencing of polymerase chain reaction products. Each PCR product was cloned into pGEM-T plasmid (Promega, Madison, WI). Each allele was

Received for publication 28 Mar. 2007. Accepted for publication 19 June 2007.

Our research is supported by grants from the Israeli Ministry of Agriculture and Rural Development and by the JCA Charitable Foundation.

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Analysis of the physical distance between S-RNase and SFB in the cultivar 'Golfrose'. The physical distance between *S-RNase* and *SFB* in the cultivar 'Golfrose' was analyzed by PCR amplification using ≈ 20 ng of DNA, 5 μ L of 10 $\times$ PCR buffer containing 27.5 mM MgCl₂ (Roche Diagnostics, Mannheim, Germany), 0.5 mM dNTP mix, 1 μ M of PRU-C2 primer, designed from *S-RNase* C2 conserved region (Tao et al., 1999), 1 μ M of SFBc-F primer, designed from the F-BOX motif of SFB (Romero et al., 2004), and 1 μ L of Expand Long Template PCR System (5 unit/ μ L; Roche Diagnostics) in a 50- μ L reaction mixture. The PCR conditions were as follows: initial cycle of 2 min at 94 $^{\circ}$ C followed by 10 cycles of 10 s at 94 $^{\circ}$ C, 30 s at 54 $^{\circ}$ C, and 10 min at 68 $^{\circ}$ C followed by 25 cycles of 15 s at 94 $^{\circ}$ C, 30 s at 54 $^{\circ}$ C, and 10 min + 20 s for each successive cycle at 68 $^{\circ}$ C. The final cycle was 7 min at 68 $^{\circ}$ C.

DNA sequence and its putative protein analysis. Analysis of the consensus contigs of each SFB genomic DNA and deduced amino acid sequence data were performed by Laser-gene 6 (Madison, WI) software.

PCR products of *SFBs* were identified from cultivars with a previously determined *S*-RNase genotype (Sapir et al., 2004; unpublished data). For preventing misidentification, each *SFB* allele was cloned and sequenced at least twice from two or three different cultivars (Fig. 1). A total of seven alleles were cloned from the following nine cultivars as follows: *SFB^k SFB'* from 'Wickson', *SFB^e SFB^h* from 'Black Diamond', *SFB^c SFB^e* from 'Royal Zee', *SFB^b SFB^h* from '440', *SFB^b SFB^k* from 'Songold', *SFB^b SFB^g* from 'Methely', *SFB^g SFB'* from 'Shiro', *SFB^b SFB^c* from 'Golfrose', and *SFB^e SFB^k* from 'Newvorker'.

F-BOX

PS-SFBc	MTTTPRQXK ILIDI LARLPAX SLVRFLCTCK SWSIDLIGSSSFVSTHLRMTKMAHVYLL	60
PS-SFBb	MTTRLHKEE ILIDI LVRLPAK SLVRFLCTCK SWSIDLIGSSSFVSTHLRMTKMAHVYLL	60
PS-SFBb	-----1LVRLPAK SLVRFLCTCK SWSIDLIGSSSFVSTHLRMTKMAHVYLL	47
PS-SFBk	-----1LVRLPAK SLVRFLCTCK SWSIDLIGSSSFVSTHLRMTKMAHALL	47
PS-SFBf	-----1LVRLPAK SLRFLCTCK SWSIDLIGSSSFVSTHLRMTKMAHVYLL	47
PS-SFBg	-----1LVRLPAK SLVRFLCTCK SWSIDLIGSSSFVSTHLRMTKMAHVYLL	47
PS-SFBc	-----1LVRLPAK SLVRFLCTCK SWSIDLIGSSSFVSTHLRMTI MAHVYLL	47
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PS-SFBc	CLHHPMFERQMDTDDPDYDI EELQWSLFS NETTFEQFS KL SHPLC STEHYVYVGS SMLGVC	120
PS-SFBb	CLHHPMFERQMDTDDPDYDI EELQWSLFS NETTFEQFS EL SPPLMCKEYVYVGS SMLGVC	120
PS-SFBb	CLHHPMFERQMDTDDPDYDI EELQWSLFS NETTFEQFS KL SHPLMCKEYVYVGS SMLGVC	107
PS-SFBk	CLHHPMFERQMDTDDPDYDI EELQWSLFS NETTFEQFS KL SHPLESTEYVYVGS SMLGVC	107
PS-SFBf	CLHHPFSERLUMRDPYVYKKEFQWSLFS NETTFEYVYVGS SMLGVC	107
PS-SFBg	CLHHSMPERQADPDYVYKKEFQWSLFS NETTFEYVYVGS SMLGVC	107
PS-SFBc	CLHP SNEFAVDPDYVYKKEFQWSLFS NETTFEYVYVGS SMLGVC	107
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V1

PS-SFBc	SDEI LMFDS PI HIOMPSISIKFRTPPMSTNINLKFAYVALQGFHPGVNDYKAVMMRTMK	180
PS-SFBb	SDEI LMFDS PI HIOMPSVUKFRTPPI STNM- IKFSYVALQGFHPGVNDYKAVMMRTMK	179
PS-SFBb	SDEI LMFDS PI HIOMPSVUKFRTPPMSTNINMKFSHVALQGFHPGVNDYKAVMMRTMK	167
PS-SFBk	SDEI LMFDS PI HIOMPSVUKFRTPPMSTNINIKFSYVALQGFHPGVNDYKAVMMRTMK	167
PS-SFBf	SDEI LMFDS PI HIOMPSVUKFRTPPI SNNINIKFSYVALQGFHPGVNDYKAVMMRTMK	167
PS-SFBg	SDEI LMFDS PI HIOMPSVUKFRTPPI STNINIKFSHVALQGFHPGVNDYKAVMMRTMK	167
PS-SFBc	SDEI LMFDS PI HIOMPSIRKFRTPPMSTNINIKSYVALQGFHPGVNDYKAVMMRTMK	167
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PS-SFBc	DAFAVEVYS LQTDGKMKIEAI PPOLK CTQGHQGTFFNGVAYHIIKKGPIFSIMSFDSGS	240
PS-SFBb	DUFAVEVYS LRTNSGKMKIETI PPOLR CTQGHQGTFFNGVAYHIVKNGHIFSIMSFDSGS	239
PS-SFBb	GALAVEVYS LRTDSGKMKIEAI PPOLK CTQGHQGTFFNGVAYHIIKKGPIFSIMSFDSGS	227
PS-SFBk	GALAVEVYS LRTDSGKMKIETI PPOLI CTQGHQGTFFNGVAYHIVKNGHIFSIMSFDSGS	227
PS-SFBf	DAFAVEVYS LGTDCGKMKIEAI PPOLK CTQGHQGTFFNGVAYHIIKKGPIFSIMSFDSGS	227
PS-SFBg	NALAVEVYS LRADSGKMKIEAI PPOLK CTQGHQGTFFNGVAYHIIKKGPIFSIMSFDSGS	227
PS-SFBc	DAFTAVEVYS LGTDSGKMKIEAI PPOLK CTQGHQGTFFNGVAYHIIKKGPIFSIMSFDSGS	227
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PS-SFBc	EEFQEF IAPDA ICS SGLC IDVYKDEI CLLLMFYSCEEG-MEKIDLOVLQEKRWKQLCPT	299
PS-SFBb	EEFQEF IAPDA ICS SGLC IDVYKDEI CLLLMFYSCEEG-MEKIDLOVLQEKRWKQLCPT	299
PS-SFBb	EEFEF IAPDA ICS SGLC IDVYKDEI CLLLMFYSCEEG-MEKIDLOVLQEKRWKQLCPT	287
PS-SFBk	EEFEF IAPDA ICS SGLC IDVYKDEI CLLLMFYSCEEG-MEKIDLOVLQEKRWKQLCPT	287
PS-SFBf	EEFEF IAPDA ICS SGLC IDVYKDEI CLLLMFYSCEEG-MEKIDLOVLQEKRWKQLCPT	287
PS-SFBg	EEFEF IAPDA ICS SGLC IDVYKDEI CLLLMFYSCEEG-MEKIDLOVLQEKRWKQLCPT	287
PS-SFBc	EEFEF IAPDA ICS SGLC IDVYKDEI CLLLMFYSCEEG-MEKIDLOVLQEKRWKQLCPT	285
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V2

PS-SFBc	IF-PSDYVYR-TIF IS IDS KILALRADHIRTGLDMDL DCE SKKVLET GIKLATUKYGEI	357
PS-SFBb	TF-PLDYVYR-TIF IS IDMKLALRRMD ISG IADLHL DYE SK QVLET GIKLATUKYGEI	358
PS-SFBb	IF-PLDYVYR-TIF IS IDMKLALRRMD ISG IADLHL DYE SK QVLET GIKLATUKYGEI	345
PS-SFBk	IF-PLDYVYR-TIF IS IDMKLALRRMD ISG IADLHL DYE SK QVLET GIKLATUKYGEI	345
PS-SFBf	IF-PFDYVYR-TIF IS IDMKLALRRMD ISG IADLHL DYE SK QVLET GIKLATUKYGEI	344
PS-SFBg	IF-PLDYVYR-TIF IS IDMKLALRRMD ISG IADLHL DYE SK QVLET GIKLATUKYGEI	344
PS-SFBc	IF-PRS CYRI--IF IS IDMKLALRRMD ISG IADLHL DYE SK QVLET GIKLATUKYGEI	344
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	HVa	HVb
PS-SFBc	EFLFSITYIETLPLL--	373
PS-SFBb	EFLFSITYIETLPLL--	374
PS-SFBb	EFVFSITYIETLPLL--	363
PS-SFBk	EFLFAITYIETLPLL--	361
PS-SFBf	EFLFAITYIETLPLL--	360
PS-SFBg	EFLFSITYIETLPLL--	360
PS-SFBc	EFLFSITYIETLPLL--	359
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amino acid sequences was identical except for a single substitution: in SFB^b (gly₇₇ to ser). It is doubtful that those minor differences change the characteristics of the SFBs. *S-RNase* and *SFB* must segregate as one genetic entity. Therefore, it is expected that the two genes will be physically close to each other to prevent recombination. Hence, a

Table 1. Identities of the putative amino acid sequences of Japanese plum *S*-locus genes. The upper half represents amino acid sequence identities (%) between SFBs and the lower half between the *S*-RNases. SFBs GenBank accession numbers: *SFB^b* (DQ646488), *SFB^c* (DQ646489), *SFB^e* (DQ646490), *SFB^f* (DQ989578), *SFB^g* (DQ989579), *SFB^h* (DQ646491), and *SFB^k* (DQ992485). *S*-RNases GenBank accession numbers: *S*-RNase^b (AB08413), *S*-RNase^c (AB084147), *S*-RNase^e (AB084146), *S*-RNase^f (AB084147), *S*-RNase^g (AB093131), *S*-RNase^h (AB084148), and *S*-RNase^k (AB093133).

S ^k	S ^h	S ^g	S ^f	S ^e	S ^c	S ^b
82.0	81.4	76.7	77.0	82.5	77.0	S ^b
76.7	73.3	76.9	74.7	76.1	74.1	S ^c
80.6	82.5	76.7	78.6		77.1	S ^e
78.1	76.9	78.3		75.8	71.0	S ^f
79.4	74.7		63.8	78.8	70.0	S ^g
80.3		73.8	65.2	78.0	69.1	S ^h
	74.4	83.8	72.0	84.1	74.7	S ^k

PCR was carried out with primers complementary with the F-Box motif of the *SFB* and the C2 conserved region of *S*-RNase (see "Materials and Methods"). PCR applied to the 'Golfrose' (S^bS^c) cultivar resulted in two bands of ≈ 3 and ≈ 3.5 Kb (Fig. 2A). The fragments were cloned and sequenced. Analyzing the sequences revealed that the distances between *S^b-RNase* and *SFB^b*, and between *S^c-RNase* and *SFB^c*, was 544 base pairs (bp) and 404 bp, respectively, with *SFB* and *S*-RNase positioned in an inverse orientation (Fig. 2B). An attempt to clone the

intergenic regions from a few of the other *S*-loci was unsuccessful, possibly because of their large size, which prevented regular PCR amplification. The *S^b* and *S^c* intergenic regions were aligned and analyzed and no significant identity or any motif was identified. Zhang et al. (2007) also sequenced the *S^c* intergenic regions and reported that it was 410 bp, six bases longer than in our study. This difference could be because cloning was performed in two different cultivars. The proximity between *S*-RNase and *SFB* in other *Prunus* species varies; for instance, in the *S6* locus of *Prunus avium*, it is 380 bp (Yamane et al., 2003a), whereas in the *S4* locus of *Prunus avium*, it is 38 Kb (Ikeda et al., 2005). It seems that a few tens of kilobytes are sufficient for the tight genetic linkage between *S*-RNase and *SFB*. In contrast, in *Solanaceae*, the distance between the two genes is much larger and the *S*-locus may reach the size of a megabase (Entani et al., 1999; Wang et al., 2003). This is because in *Solanaceae*, the *S*-locus is close to centromer, a region with suppressed recombination and therefore distantly located genes can remain genetically linked (Robbins et al., 2000), whereas in *Rosaceae*, the locus is distant from the centromer and consequently is only ≈ 70 Kb long (Entani et al., 2003; Lai et al., 2002; Ushijima et al., 2001).

The objective of this study was to clone *SFB* genes from Japanese plum to study their phylogeny, to obtain the potential for the identification of mutations that can lead to

autocompatibility, as observed in *Prunus mume* and *Prunus avium* (Ushijima et al., 2004; Yamane et al., 2003b), and to improve *S*-haplotyping possibilities of plums. *S*-typing is a key characteristic also in view of the reduced yield observed among semicompatible versus fully compatible apples, pears, and plums, which are cultivated in nonoptimal condition for growth and pollination (Schneider et al., 2005; Zisovich et al., 2005; unpublished data).

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Fig. 2. (A) PCR amplification of 'Golfrose' DNA with primer pair SFBc-F and Pru-C2. PCR products (left) and Fermentas (Ontario, CA) GeneRuler 1 Kb (right) were run on a 0.7% agarose gel, stained with ethidium bromide. (B) A scheme illustrating the distances between the *S*-RNase and *SFB* genes for *S^b* and *S^c* Japanese plum haplotypes. Full Arrows represent the transcriptional direction of the genes, solid line represent intergenic region.

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