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

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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^{g} *and* SFB^{k} 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' ($S^{k}S^{f}$), 'Black Diamond' ($S^{c}S^{h}$), 'Royal-Zee' ($S^{c}S^{c}$), '440' ($S^{b}S^{h}$), 'Songold' ($S^{h}S^{k}$), 'Methely' ($S^{b}S^{g}$), 'Shiro' ($S^{g}S^{f}$), 'Newyorker' ($S^{c}S^{k}$), and 'Golfrose' ($S^{b}S^{c}$) (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 ethylenediaminetetra acetic 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 pelted with ethanol and dissolved in DDW.

S-haplotype-specific F-Box protein poly merase chain reaction amplification. Polymerase chain reaction (PCR) was conducted using ≈20 ng of DNA, 5 µL of 10× PCR buffer containing 15 mM $MgCl_2$ (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 poly merase chain reaction products. Each PCR product was cloned into pGEM-T plasmid (Promega, Madison, WI). Each allele was

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sequenced from two different clones in both directions with automated sequencing using dye terminator cycle sequencing with fluorescent-labeled dye terminators on an ABI PRISM 377 DNA sequencer (PE Bio System, Foster City, CA).

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 μL of 10× PCR buffer containing 27.5 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), 0.5 mM dNTP mix, 1 μM of PRU-C2 primer, designed from *S*-RNase C2 conserved region (Tao et al., 1999), 1 μM of SFBc-F primer, designed from the F-BOX motif of SFB (Romero et al., 2004), and 1 μL of Expand Long Template PCR System (5 unit/μL; Roche Diagnostics) in a 50-μL reaction mixture. The PCR conditions were as follows: initial cycle of 2 min at 94 °C followed by 10 cycles of 10 s at 94 °C, 30 s at 54 °C, and 10 min at 68 °C followed by 25 cycles of 15 s at 94 °C, 30 s at 54 °C, and 10 min + 20 s for each successive cycle at 68 °C. The final cycle was 7 min at 68 °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 Lasergene 6 (Madison, WI) software.

Results and Discussion

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^f from 'Wickson', SFB^e SFB^h from 'Black Diamond', SFB^c SFB^e from 'Royal Zee', SFB^b SFB^h from '440', SFB^h SFB^k from 'Songold', SFB^b SFB^g from 'Methely', SFB^g SFB^f from 'Shiro', SFB^b SFB^c from 'Golfrose', and SFB^e SFB^k from 'Newyorker'.

DNA alignment revealed that all seven japanese plum *SFB* alleles carry the previously described features of the *SFB* gene: a single F-box domain, four (hyper) variable regions (V1, V2, HVa, and HVb), and no introns (Fig. 1). The comparison of their putative amino acid showed they were highly polymorphic, ranging from 73.3% to 82.5% (Table 1). These levels of polymorphism resemble those of other *Prunus* SFBs. For example, in almond (*Prunus dulcis*), it ranges from 68.4% to 76.4% (Ushijima et al., 2003), in japanese apricot (*Prunus mume*) from 74.9% to 80.2% (Entani et al., 2003), in sweet cherry (*Prunus avium*) from 75.3% to 81.1% (Ikeda et al., 2004), and in european apricot (*Prunus armeniaca*) from 67% to 83% (Romero et al., 2004). Furthermore, this SFB polymorphism is similar to the japanese plum *S*-RNases polymorphism (63.8% to 84.1%) (Table 1).

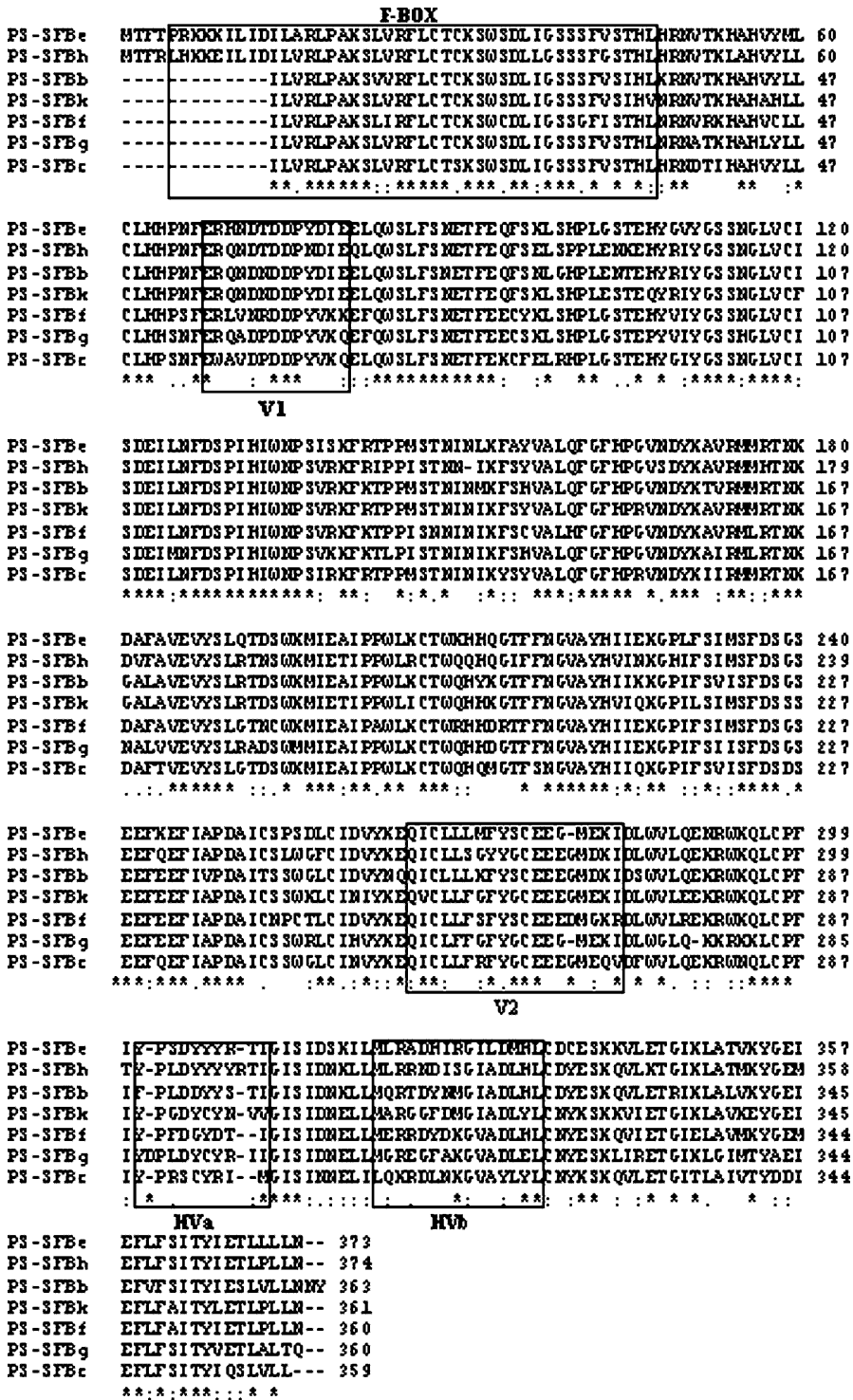

Fig. 1. Amino acid sequence alignment of the seven japanese plum SFBs. The amino acid sequences of SFBs were aligned using Clustal X (Thompson *et al.,* 1997). Dashes—gaps, asterisks—conserved sites, dots—conservative replacements [amino acid groups defined in Dayhoff *et al.,* (1979): C, STAPG, MILV, HRK, NDEQ, and FYW]. F-box and (hyper) variable regions, V1, V2, HVa and HVb are boxed. Aligned SFBs GenBank accession numbers: SFB^b (DQ646488), SFB^c (DQ646489), SFB^e (DQ646490), SFB^f (DQ989578), SFB^g (DQ989579), SFB^h (DQ646491), SFB^k (DQ992485).

While writing this paper, Zhang et al. (2007) published the sequence of eight SFBs, five of which are also described here: SFB^c, SFB^e, SFB^b, SFB^f and SFB^h. Those SFB clones are from different cultivars than ours. A comparison between the analogous SFBs exhibited minor differences between the DNA sequences and the putative deduced amino acid sequences was identical except for a single substitution: in SFB^b (gly_{77} 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} (ABO93131), *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	77.7	S^{e}
78.1	76.9	78.3		75.8	71.0	73.1	S^{f}
79.4	74.7		63.8	78.8	70.0	78.2	S^{g}
80.3		73.8	65.2	78.0	69.1	71.9	S^{h}
	74.4	83.8	72.0	84.1	74.7	76.6	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' ($S^{b}S^{c}$) 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 *S*6 locus of *Prunus avium*, it is 380 bp (Yamane et al., 2003a), whereas in the *S*4 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).

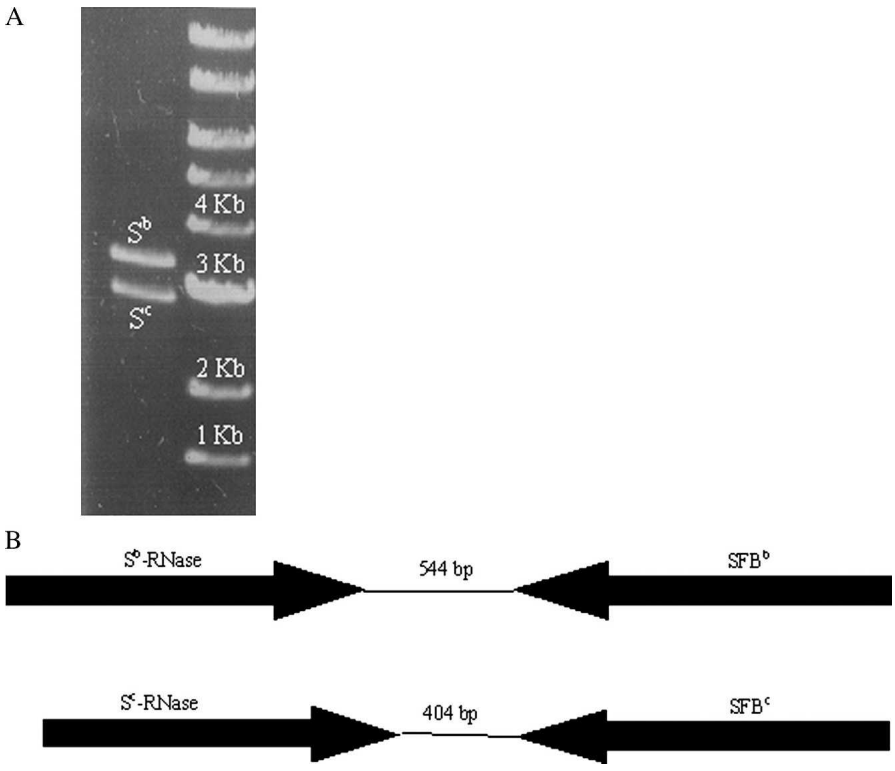

Fig. 2. (**A**) PCR amplification of 'Golfrose' DNA with primer pair SFBc-F and Pru-C2. PCR products (left) and Fermentas (Ontario, CA) GeneRuler 1 Kb (right) were run on a 0.7% agarose gel, stained with ethidium bromide. (**B**) A scheme illustrating the distances between the *S-RNase* and *SFB* genes 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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