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ORIGINAL PAPER

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The use of the *S* haplotype-specific F-box protein gene, *SFB*, as a molecular marker for *S*-haplotypes and self-compatibility in Japanese apricot (*Prunus mume*)

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Abstract Japanese apricot (*Prunus mume*) exhibits the S-RNase-based gametophytic self-incompatibility system as do other self-incompatible *Prunus* species. This report identifies the *S* haplotype-specific F-box protein gene (*SFB*), a candidate gene for pollen-*S*, of Japanese apricot, which leads to the development of a molecular typing system for *S*-haplotype in this fruit species. Both 5′- and 3′-RACE (rapid amplification of cDNA ends) were performed with *SFB* gene-specific oligonucleotide primers to clone *Pm-SFB*^{1} and *Pm-SFB*^{7} of 'Nanko (*S*^{1}*S*^{7})'. As in the case of *SFB* of other *Prunus* species, *Pm-SFB*^{1} and *Pm-SFB*^{7} showed a high level of *S*-haplotype-specific sequence polymorphism and their expression was specific to pollen. Genomic DNA-blot analyses of 11 Japanese apricot cultivars with the *Pm-SFB* probes under low stringency conditions yielded RFLP bands specific to the *S*^{1}- to *S*^{8}-haplotypes as well as a self-compatible *S*^{f}-haplotype. A practical usage of *SFB* as a molecular marker for *S*-haplotypes and self-compatibility in Japanese apricot is discussed.

Key words F-box motif · Gametophytic self-incompatibility · Pollen determinant · *Prunus mume* · SFB · S-RNase

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The nucleotide sequences reported in this paper have been submitted to the EMBL/GenBank/DDBJ database under accession numbers, AB101440 and AB101441, for *SFB*^{1} and *SFB*^{7}, respectively

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Introduction

Self-incompatibility (SI) is a widespread mechanism in flowering plants that prevents self-fertilization and promotes outcrossing (de Nettancourt 1977, 2001). The predominant form of SI is the gametophytic SI (GSI) system, in which the compatibility of a cross is determined by the haploid genome of the pollen and the diploid genome of the pistil (McCubbin and Kao 2000). The specificity of the SI response is determined by the haplotypes of the polymorphic *S*-locus, which presumably contains at least one gene for the pistil determinant and one for the pollen determinant. The pistil determinant of the SI reaction in three plant families, namely, Rosaceae (Sassa et al. 1992, 1997), Solanaceae (Anderson et al. 1986; McClure et al. 1989) and Scrophulariaceae (Xue et al. 1996), is known to be an S-RNase. Although the pollen determinant (pollen-*S*) in these families had long been unidentified since the first identification of S-RNase in the Solanaceae (McClure et al. 1989), a very good candidate gene for the pollen-*S*, an *S* haplotype-specific F-box protein gene (*SFB*), has been found recently in three species of *Prunus* in the Rosaceae, namely almond (*Prunus dulcis* (Mill.) D.A. Webb; Ushijima et al. 2003), and cherries (*Prunus avium* L. and *Prunus cerasus* L.; Yamane et al. 2003). The features of *SFB*, such as its pollen-specific expression, the tight linkage with the S-RNase gene, and the high level of allelic polymorphism, are appropriate characteristics for the male determinant in the S-RNase-based GSI system.

Most Japanese apricot (*Prunus mume*) cultivars exhibit the S-RNase-based GSI system (Tao et al. 2000, 2002a, b; Yaegaki et al. 2001), as do other fruit tree species of *Prunus*, such as almond (*P. dulcis*) and cherries (*P. avium and P. cerasus* L.) (Tao et al. 1997, 1999; Ushijima et al. 1998; Yamane et al. 2001). For fruit production in SI *Prunus*, fertilization is essential because they are unable to bear fruit parthenocarpically. In commercial orchards, cross-compatible cultivars that flower simultaneously and have different *S*-haplotypes are inter-planted, and beehives are introduced to ensure cross-pollination. Thus,

determination of correct *S*-haplotypes of cultivars is important.

Recently, a pistil *S*-allele, S-RNase, has been cloned and successfully used as a molecular marker for the *S*-haplotype in Japanese apricot (Yaegaki et al. 2001; Tao et al. 2002a). Furthermore, it was found that self-compatible (SC) cultivars of Japanese apricot have a common S-RNase gene, named the S^f-RNase (Tao et al. 2000). Since the S^f-RNase gene was cosegregated with SC (Tao et al. 2002a, b), it can be used as a molecular marker for SC. With the molecular markers for *S*-haplotypes and SC in Japanese apricot, the time required for the breeding could be considerably shortened because they can be used on vegetative materials, independently of age and season.

Since pistil and pollen *S*-alleles are tightly linked to each other as if they were a single gene, *SFB*, a possible candidate for the pollen *S*-allele in *Prunus*, should also be able to be utilized as a molecular marker for *S*-haplotypes and/or self-compatibility in Japanese apricot. The use of molecular markers for both pistil and pollen determinants would lead to a firm determination of *S*-haplotypes, especially when the S-RNase genes from different *S*-haplotypes gave the same PCR and RFLP bands (Tao et al. 1999; Hauck et al. 2001). In addition, availability of pollen-*S* markers would make it possible to identify pollen-part mutants such as $S^{4'}$ in sweet cherry (Lapins 1970; Boskovic et al. 2000). The S^f-haplotype of Japanese apricot has also been considered to be a pollen-part mutant since there found to be no substantial difference between the S^f-RNase gene of the S^f-haplotype and other S-RNase genes of the SI *S*-haplotypes (Tao et al. 2002a).

This report identifies the *S* haplotype-specific F-box protein gene, *SFB*, of Japanese apricot, which leads to the development of a novel molecular typing system for the *S*-haplotype in this fruit species. A practical usage of *SFB* as a molecular marker for *S*-haplotypes and SC in Japanese apricot is discussed.

Materials and Methods

Plant material

Five SI Japanese apricot cultivars (*Prunus mume* Sieb. et Zucc.), 'Gyokuei (S^2S^6)', 'Kairyouchida-ume (S^3S^4)', 'Nanko (S^1S^7)', 'Oushuku (S^1S^5)' and a selection TK (Tamaume × Koushusaisyo) (S^2S^8), and four SC cultivars, 'Benisashi (S^7S^f)', 'Hachiro(S^8S^f)', 'Kensaki (S^fS^f)' and 'Koshinoume (S^3S^f)' (Yaegaki et al. 2001), were used in this study.

DNA isolation and PCR

Total DNA was isolated from young leaves using the Nucleon Phytopure for the Plant DNA extraction kit (Amersham Biosciences), and used as a template for PCR. PCR was performed using *SFB* gene-specific primers, SFB-C1F and SFB-FB3 (Table 1; Yamane et al. 2003), to amplify homologous sequences of *SFB* of almond (Ushijima et al. 2003; hereafter *Pd-SFB*) and cherry (Yamane et al. 2003; heareafter *Pa-SFB*). These primers were designed from the previously identified conserved regions of Pd-SFB and Pa-SFB (Ushijima et al. 2003; Yamane et al. 2003). The PCR reaction mixture contained 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, 200 μM each of dNTPs, 400 nM each of primers, 50 ng of template DNA, and 1 U of TaKaRa Ex *Taq* polymerase (TaKaRa Shuzo Co., Japan) in a 50-μl reaction volume. PCR reactions were run with a program of 35 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min 30 s, with an initial denaturing at 94°C for 3 min and a final extension of 72°C for 7 min by TaKaRa PCR Thermal Cycler MP (Takara Shuzo Co.). The PCR products were subcloned into the T-A cloning vector (pGEM-T Easy Vector System; Promega). DNA sequences of the inserts of several clones were determined using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Table 1 Sequences of oligonucleotide primers used in this study

Primer	Sequence
SFB-C1F	*5′-RTTCGRTTTCTDTTTACRTG-3′*
SFB-FB3	*5′-CCCAAATTGGAGAGAAACATGG-3′*
SFB-C4R	*5′-CCAAKYGEATTTTAACCAAGG-3′*
PM-SFB-F1	*5′-TCRTGGAKKGATTTGATTGGC-3′*
PM-S7-C2F	*5′-ACATATGGAATCCATCGGTC-3′*
PM-S7-C3R	*5′-GACTATAGACCTCAACAGCC-3′*
ActF1	*5′-ATGGTGAGGATATTCAACCC-3′*
ActR1	*5′-CTTCCTGTGGACAATGGATGG-3′*

RACE

Total RNA was isolated from pollen grains of 'Nanko' by the cold phenol method, as described earlier (Tao et al. 1999). One microgram of total RNA was used for first-strand cDNA synthesis by SUPER SCRIPTII RT (Invitrogen). cDNA was then used to amplify the 5′- and 3′-ends of cDNA for *Pm-SFB*^{1} with a GeneRacer Kit (Invitrogen) and the forward and reverse gene-specific primers, PM-SFB-F1 and SFB-C4R (Table 1; Yamane et al. 2003), respectively. To clone the full-length cDNA for *Pm-SFB*^{7}, we used the forward and reverse gene-specific primers, PM-S7-C2F and PM-S7-C3R, respectively (Table 1). Deduced amino-acid sequences from the cDNAs obtained were aligned by Clustal X (Thompson et al. 1997).

RT-PCR

Total RNA was isolated from leaves of five SI and four SC cultivars of Japanese apricot that were described above, styles with stigmas at the balloon stage of development, and pollen grains from 'Nanko' as described elsewhere (Tao et al. 1999). One microgram of total RNA treated with DNase I (Invitrogen) was used for first-strand cDNA synthesis by SUPER SCRIPTII RT (Invitrogen) with an Adapter-dT primer that consisted of the M13-20 sequence primer attached to oligo$(dT)_{16}$, as described earlier (Tao et al. 1999). We performed PCR using the cDNA equivalent to the amount synthesized from 50 ng of total RNA with the *SFB*-specific primer set, PM-SFB-F1 and SFB-C4R. As references, we also performed RT-PCR with an actin gene-specific primer pair, ActF1 and ActRI (Table 1; Ushijima et al. 2003). The PCR condition was identical to that used by Yamane et al. (2003).

Genomic DNA blot analysis

Five micrograms of total DNA was digested by *Hin*dIII and run on a 0.9% agarose gel. After electrophoresis, DNA in the gel was transferred to a nylon membrane (Hybond-N, Amersham), and probed with the fragments of the *Pm-SFB*^{1} and *Pm-SFB*^{7} cDNAs that were PCR-labeled by DIG-dUTP (Roche) with SFB-C1F and SFB-C4R primers (Table 1). After hybridization and washing at 60°C or 65°C, hybridization signals were detected with alkaline phosphatase-conjugated antibodies against DIG and the chemilu-

minescent substrate CDP-star (New England Biolabs). Chemiluminescence was recorded on X-ray film, as described by Tao et al. (1999).

Results

Cloning and expression analysis of *Pm-SFB*^{1} and *Pm-SFB*^{7}

DNA sequencing of several different clones that were obtained by PCR of the genomic DNA of 'Nanko' (S^1S^7) with an SFB-C1F and SFB-FB3 primer set revealed two different kinds of clones. These clones encode partial sequences resembling *Pd-SFB* and *Pa-SFB* with about 85% DNA sequence identity (Ushijima et al. 2003; Yamane et al. 2003). We named these clones *Pm-SFB*^{1} and *Pm-SFB*^{7} based on their sequence specificities to haplotypes as described below. Based on the nucleotide sequences of these two clones, we performed 5′ and 3′RACE to obtain full-length cDNAs for *SFBs* from 'Nanko' pollen. Both 5′- and 3′-ends of the *Pm-SFB*^{1} fragment were obtained with RACE using PM-SFB-F1 and SFB-C4R primers, whereas those of *Pm-SFB*^{7} were obtained with PM-S7-C2F and PM-S7-C3R primers (Table 1).

Table 2 Identities of the amino-acid sequences of SFBs (upper half) and S-RNases (lower half) of various *S*-haplotypes. Pm refers to *P. mume* (Japanese apricot), Pa refers to *P. avium* (sweet cherry) and Pd refers to *P. dulcis* (almond). The accession numbers for the respective sequences are as follows: S^1-RNase, AB101438; S^7-RNase, AB101439; S^3-RNase, AB010306; S^6-RNase, AB010305; S^c-RNase, AB011470; S^d-RNase, AB011471; SFB^1, AB101440; SFB^7, AB101441; SFB^3, AB096857; SFB^6, AB096858; SFB^c, AB079776; and SFB^d, AB081648

Type	*Pm-S*^{1}	*Pm-S*^{7}	*Pa-S*^{3}	*Pa-S*^{6}	*Pd-S*^{c}	*Pd-S*^{d}
Pm-S^1	–	80	77	80	79	80
Pm-S^7	76	–	82	79	79	76
Pa-S^3	75	69	–	79	78	76
Pa-S^6	76	75	75	–	76	77
Pd-S^c	73	69	74	78	–	75
Pd-S^d	67	72	72	72	72	–

The deduced amino-acid sequences from the cDNAs for *Pm-SFB*^{1} and *Pm-SFB*^{7} contained the F-box motif at the N-terminal region to those of almond and cherry SFBs (Fig. 1; Ushijima et al. 2003; Yamane et al. 2003). The amino-acid identity among Pm-SFB^1 and Pm-SFB^7, and SFBs of almond and cherry, ranged from 71% to 82%, which is comparable to the identity observed among S-RNases (Table 2).

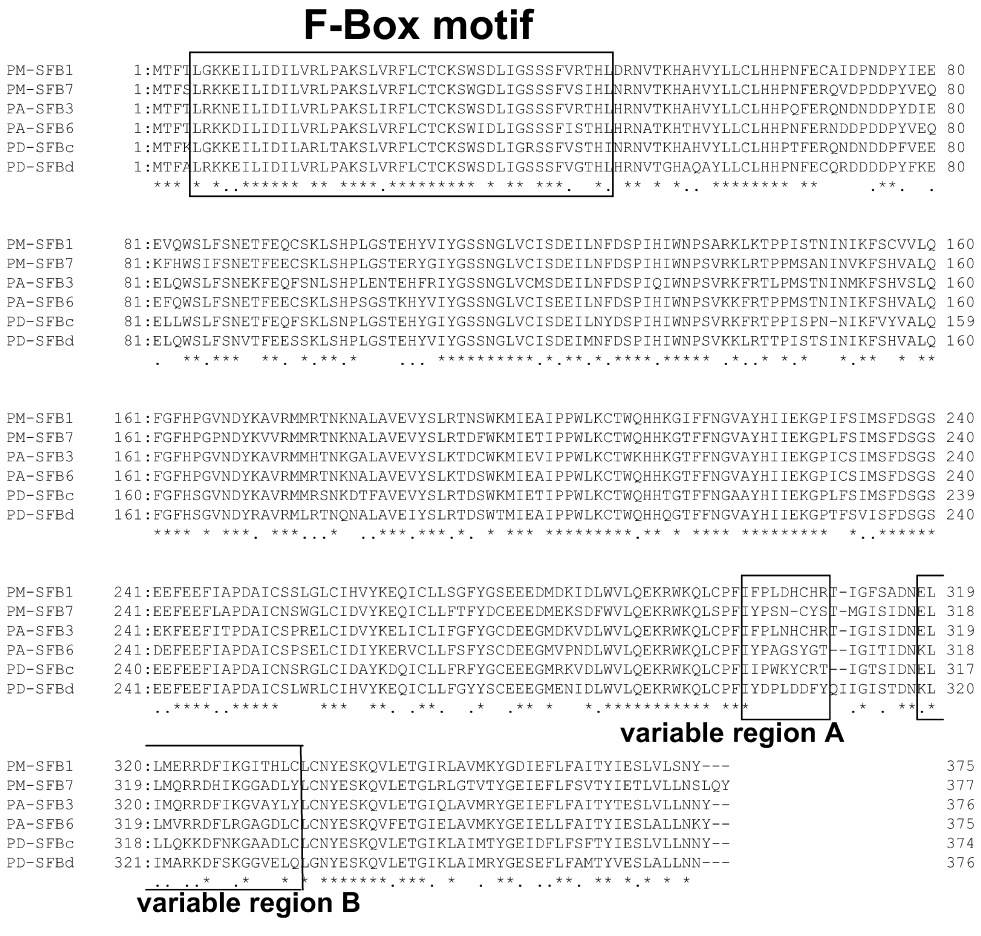

Fig. 1 Alignment of the deduced amino-acid sequences of *SFBs*. Deduced amino-acid sequences of *SFBs* were aligned by the Clustral X program (Thompson et al. 1997). The F-box motif and two variable regions (Ushijima et al. 2003; Yamane et al. 2003) are *boxed*. Among the marks underneath of sequences, '*' indicates the positions which have a single, fully conserved residue, and '.' indicates that the positions which have a conservative replacement in one of the following groups (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW) (Thompson et al. 1997). The EMBL/Genbank/DDBJ accession numbers of amino-acid sequences used were described in Table 2

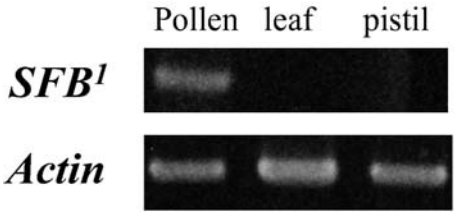

Fig. 2 Expression analysis for *Pm-SFB*^{1} and actin in 'Nanko (*S*^{1}*S*^{7})'. RT-PCR was performed with total RNA from pollen, leaves, and pistils of 'Nanko'. See text for details

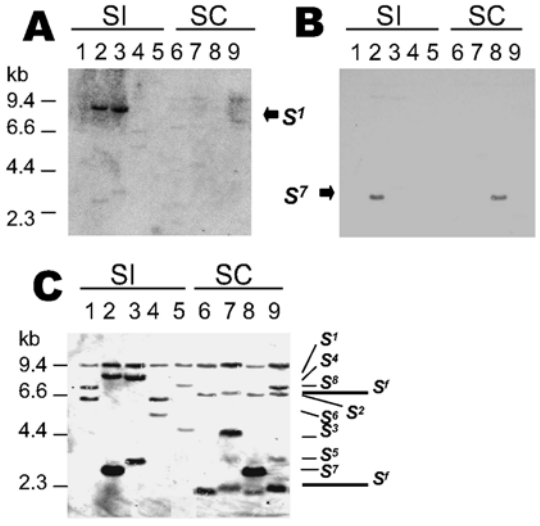

Fig. 3A–C Genomic DNA blot analysis with *Pm-SFB*^{1} (**A**) and *Pm-SFB*^{7} probes (**B** and **C**). Temperatures for pre-hybridization and hybridization were either 65°C for the photographs, **A** and **B**, or 60°C for **C**. *Lane* 1, a selection TK (Tamaume × Koshusaisyo) (*S*^{2}*S*^{8}); *lane* 2, 'Nanko (*S*^{1}*S*^{7})'; *lane* 3, 'Oushuku (*S*^{1}*S*^{5})'; *lane* 4, 'Gyokuei (*S*^{2}*S*^{6})'; *lane* 5, 'Kairyouchida-ume (*S*^{3}*S*^{4})'; *lane* 6, 'Kensaki (*S*^{f}*S*^{f})'; *lane* 7, 'Koshinoume (*S*^{3}*S*^{f})'; *lane* 8, 'Benisashi (*S*^{7}*S*^{f})'; and *lane* 9, 'Hachiro (*S*^{8}*S*^{f})'. SI, self-incompatible and SC, self-compatible

Expression analysis for *Pm-SFB*^{1} as investigated by RT-PCR, revealed the pollen-specific expression of *Pm-SFB* (Fig. 2) as in the case of *Pd-SFB* and *Pa-SFB* (Ushijima et al. 2003; Yamane et al. 2003).

Genomic DNA blot analysis with the *SFB* probe

Genomic DNA blot analysis showed that *Pm-SFB*^{1} and *Pm-SFB*^{7} were linked to the *S*^{1}- and *S*^{7}-haplotypes of Japanese apricot cultivars, respectively (Fig. 3A, B). Under high-stringency conditions at 65°C, only cultivars with *S*^{1}- and *S*^{7}-haplotypes yielded hybridization signals with the *Pm-SFB*^{1} and *Pm-SFB*^{7} probes, respectively (Fig. 3A, B). Under low-stringency conditions at 60°C, by contrast, all nine cultivars of the Japanese apricot tested yielded *S* haplotype-specific bands corresponding to eight SI haplotypes, *S*^{1} to *S*^{8}, as well as the SC haplotype, *S*^{f}, indicating the presence of *Pm-SFBs* that are linked to other *S*-haplotypes of Japanese apricot (Fig. 3C). In addition to the *S* haplotype-specific bands, all cultivars yielded a monomorphic band at 9.4 kb, indicating the presence of the homologous sequences to *SFB* in their genome (Fig. 3C).

Discussion

In this study, two *Pm-SFBs* in *S*^{1}- and *S*^{7}-haplotypes were identified and cloned from the SI Japanese apricot cultivar 'Nanko (*S*^{1}*S*^{7})'. As in the case of *SFB* of other *Prunus* species, *Pm-SFB*^{1} and *Pm-SFB*^{7} showed a high level of *S* haplotype-specific sequence polymorphism (Fig. 3) and their expression was specific to pollen (Fig. 2). Ushijima et al. (2003) found two variable hydrophilic regions, variable regions A and B, that are supposed to be responsible for the discrimination between self- and non-self S-RNases in Pd-SFBs. The two variable regions were also present in the Pm-SFB sequences. Furthermore, amino-acid sequence comparisons among eight SFBs from three different *Prunus* species, *P. dulcis* (Ushijima et al. 2003), *P. avium* (Yamane et al. 2003) and *P. mume* (this study), revealed that the region A could be more variable than the region B. All these data support the idea that *SFB* could be a very good candidate gene for the pollen determinant of the S-RNase-based GSI system in *Prunus*.

One of the most important outcomes of this study was the demonstration of molecular typing of the *S*-haplotype and the SC of Japanese apricot cultivars through genomic DNA blot analyses with the *SFB* probe (Fig. 3C). Under low stringency conditions, all cultivars yielded bands that correspond to their respective *S*-haplotypes as well as a single monomorphic band (Fig. 3C). Since *S* haplotype-specific bands have been also obtained with the genomic DNA blot analysis for sweet cherry cultivars with the *Pa-SFB* probe under low stringency conditions (Yamane et al. 2003), it is possible that *S*-haplotype-typing with the *SFB* probe could be used not only for Japanese apricot but also for other *Prunus* spp. The use of molecular markers for both pistil and pollen determinants would lead to a firm determination of *S*-haplotypes, especially when S-RNase genes from different *S*-haplotypes gave the same PCR and RFLP bands (Tao et al. 1999; Hauck et al. 2001). In addition, availability of the molecular marker for the pollen-determinant would be advantageous in that we could have a means to identify the pollen-part mutant such as *S*$^{4'}$ in sweet cherry (Lapins 1970; Boskovic et al. 2000).

It appeared that the *S*^{f}-haplotype corresponded to two bands because 'Kensaki', homogenous for *S*^{f}, yielded the monomorphic band and the two bands that were found in SC cultivars in common. This may indicate the presence of two different genes for SFB in the *S*^{f}-haplotype, which may involve the SC observed with the *S*^{f}-haplotype that has been considered to be a pollen-part mutant (Tao et al. 2002a). Cloning and further insight into *SFB* of the *S*^{f}-haplotype would be intriguing.

A single monomorphic band that was found in all Japanese apricot cultivars tested in this study (Fig. 3C) indicated the existence of homologous sequences to *SFB* in their genome. The sweet cherry genome also seemed to contain the homologous sequence to *SFB* because genomic DNA blot analysis of sweet cherry cultivars with the *Pa-SFB* probe under low stringency conditions yielded a single common monomorphic band (Yamane et al. 2003). Since the sequence is unlikely to control the specificity of the SI reaction, care should be taken to avoid the mis-typing of *S*-haplotypes by wrongly scoring the monomorphic band.

In conclusion, this study identified *Pm-SFB*, a candidate gene for pollen-*S*, in Japanese apricot. As do *Pa-SFB* and *Pd-SFB*, *Pm-SFB* encodes the F-Box protein with *S* haplotype-specific polymorphism. Based on the genomic DNA blot with the *Pm-SFB* probe, a molecular typing system of *S*-haplotypes has been developed, which would enable us to identify the pollen-part mutant in the future breeding programs.

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