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Isolation and identification of new pollen-specific *SFB* genes in Japanese apricot (*Prunus mume*)

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ABSTRACT. *SFB*, a candidate gene for the pollen *S* gene, has been identified in several species of *Prunus* (Rosaceae). We isolated 5 new *SFB* alleles from 6 Japanese apricot (*Prunus mume*) lines using a specific *Prunus SFB* primer pair (SFB-C1F and Pm-Vb), which was designed from conserved regions of *Prunus SFB*. The nucleotide sequences of these *SFB* genes were submitted to the GenBank database. The 5 new *SFB* alleles share typical structural features with *SFB* alleles from other *Prunus* species and were found to be polymorphic, with 67.08 to 96.91% amino acid identity. These new *SFB* alleles were specifically expressed in the pollen. We conclude that the *PmSFB* alleles that we identified are the pollen *S* determinants of Japanese apricot; they have potential as a tool for studies of the mechanisms of pollen self-incompatibility.

Key words: Gametophytic self-incompatibility; Japanese apricot; *SFB*; *S-RNase*

INTRODUCTION

Most flowering plants have developed self-incompatibility (SI) systems to avoid inbreeding and to promote out-crossing (De Nettancourt, 2001). Among the various SI systems, the predominant form is gametophytic self-incompatibility (GSI), which has been found in 3 plant families, namely Solanaceae, Plantaginaceae, and Rosaceae (McCubbin and Kao, 2000). GSI is controlled by a single multiallelic locus, termed the *S* locus, which contains at least 2 closely linked genes: 1 pistil determinant *S*-ribonuclease gene (*S-RNase* gene) (Ushijima et al., 1998; Tao et al., 1997, 1999) and 1 pollen determinant *S*-haplotype-specific F-box gene/*S* locus F-box (*SFB/SLF*) gene (Entani et al., 2003; Yamane et al., 2003a; Ushijima et al., 2003, 2004). Thus, the term haplotype is used to describe variants of the *S* locus. Pollen inhibition occurs when the same *S*-haplotype is expressed by both pollen and pistil (De Nettancourt, 2001).

After the first identification of *S-RNase* in the Solanaceae (McClure et al., 1989), a very good candidate gene for the pollen *S* gene, an *SFB*, was recently found in several species of *Prunus* in the Rosaceae, including sweet cherry (*P. avium*; Yamane et al., 2003a; Ikeda et al., 2004; Sonneveld et al., 2005; Vaughan et al., 2006), almond (*P. dulcis*; Ushijima et al., 2003, 2004; Sutherland et al., 2008), Japanese apricot (*P. mume*; Entani et al., 2003; Yamane et al., 2003b), apricot (*P. armeniaca*; Romero et al., 2004; Vilanova et al., 2006; Wu et al., 2009), Japanese plum (*P. salicina*; Zhang et al., 2007), dwarf almond (*P. tenella*; Šurbanovski et al., 2007), and Chinese cherry (*P. pseudocerasus*; Huang et al., 2008; Gu et al., 2011).

Features of *SFB*, such as pollen-specific expression, the high level of allelic polymorphism, and the close physical distance to *S-RNase*, are appropriate characteristics for the pollen *S* gene (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003). *SFB* was first confirmed to be physically linked to *S-RNase* in 3 other *Prunus* species: sweet and sour cherries (*P. avium* and *P. cerasus*; Yamane et al., 2003a) and Japanese apricot (*P. mume*; Entani et al., 2003; Yamane et al., 2003b). Recently, the physical distance between *SFB* and *S-RNase* alleles has been determined in sweet cherry (Ikeda et al., 2005), Japanese plum (Zhang et al., 2007), apricot (Romero et al., 2004; Wu et al., 2009), and Chinese cherry (Gu et al., 2011). Furthermore, the location of *SFB* relative to *S-RNase* seems to be conserved as *SFB* is located downstream of *S-RNase* with the opposite direction of transcription in the *S* locus region, which is defined by the *S*-locus boundary markers 'NP79R' (SLFL1) and 'NP182F' in almond (Ushijima et al., 2001, 2003).

Japanese apricot (*P. mume* Sieb. et Zucc.) originated in China, exhibits GSI, as do other self-incompatible crops in the Rosaceae (Miyake et al., 1995; Yaegaki et al., 2001). Japanese apricot blooms very early in spring, during which there are many constraints on pollination (e.g., weather, available insects, and pollinizers). Thus, determination of correct *S* haplotypes of cultivars is very important. In Japanese apricot, many *S-RNase* alleles have been identified (Yaegaki et al., 2001; Habu et al., 2008; Xu et al., 2010). As for *SFB* alleles, Yamane et al. (2003b) cloned *PmSFB1* and *PmSFB7* of 'Nanko (S_1S_7)' with *SFB* gene-specific oligonucleotide primers. *PmSFB10* to *PmSFB16* have been identified and characterized by Heng et al. (2012). In this study, we identified 10 *SFB* alleles from 6 Japanese apricot cultivars. In addition, we compared the deduced amino acid sequences of these alleles with other *SFB* alleles previously identified in *Prunus*.

MATERIAL AND METHODS

Plant materials

Six Japanese apricot cultivars, 'Dalizhong', 'Daqiandi', 'Daqingmei', 'Yeliqing', 'Huangdou', and 'Xiaomei', from the National Field Genebank for Japanese apricot located at Nanjing Agricultural University were used. Young leaves, pollen grains, and styles with stigmas at the balloon stage of development were collected, frozen in liquid nitrogen, and stored at -70°C.

Extraction of nucleic acids

Genomic DNA was extracted from 0.2 g frozen young leaves of 6 Japanese apricot cultivars by the modified cetyltrimethylammonium bromide (CTAB) method (Wang et al., 2006), treated with RNase (TaKaRa, Kyoto, Japan), and incubated at 37°C for 1 h. DNA integrity was verified by electrophoresis and the concentration of extracted DNA was determined with a Biophotometer (Eppendorf, Hamburg, Germany).

Total RNA was extracted from pollen grains, styles, and leaves of 'Dalizhong' according to Tao et al. (1999) and then treated with gDNA Eraser (TaKaRa). RNA integrity was checked by electrophoresis and the concentration was determined with a Biophotometer.

PCR amplification of *SFB* alleles

PCR amplification was performed using the primer pair SFB-C1F (5'-RTTCGRTTTC TDTTTACRTG-3'; Yamane et al., 2003b) and Pm-Vb (5'-ATCCAAGCAAGTTCTTGAAAC A-3'; Gao et al., 2013). PCR was performed in a 25-μL reaction volume containing 70 ng genomic DNA, 2.0 μL 10X PCR buffer (TaKaRa), 1.5 mM $MgCl_2$, 0.15 mM dNTPs, 0.1 μM each primer, and 1 U Taq DNA polymerase (TaKaRa) in a PTC-100 thermal cycler (MJ Research, Cambridge, MA, USA). PCRs were run with a program of 35 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min 30 s, with an initial denaturation at 94°C for 3 min and a final extension of 72°C for 10 min. The PCR products were separated by 1.2% agarose gel electrophoresis in 1X TAE buffer and observed using a ultraviolet light system (FR-200, Peiqing, China).

RT-PCR

First-strand cDNAs were synthesized from 1 μg total RNA using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) and served as templates for RT-PCR amplification. Primers for PCR amplification were SFB-C1F and Pm-Vb as above. As references, we also performed RT-PCR with an actin gene-specific primer pair, ActF1 (5'-ATGGTGAGGATATTCA ACCC-3') and ActRI (5'-CTTCCTGTGGACAATGGATGG-3') (Ushijima et al., 2003). The PCR mixture and conditions were the same as above. PCR products were separated by 1.2% agarose gel electrophoresis in 1X TAE buffer and observed using a ultraviolet light system.

Sequencing of PCR products

PCR products were extracted from 1.2% agarose gels and purified using an Agarose

Gel DNA Purification kit (TaKaRa). The purified products were cloned into the PMD19-T vector (TaKaRa) following manufacturer instructions and transformed into *Escherichia coli* DH5α cell. Target clones were sequenced on an ABI Prism 3700 DNA analyzer (ABI, 3730, USA) using the M13 primer and the Big Dye Terminator Version 3.1.

Sequence and phylogenetic analysis

Homology searches were performed using the BLASTN program from the National Center for Biotechnology Information (NCBI; Altschul et al., 1997). Amino acid sequence alignments were performed using DNAMAN (version 5.2; Lynnon Biosoft) with the dynamic alignment method (Thompson et al., 1994). A phylogenetic tree was constructed based on the deduced amino acid sequences of the *S*-locus F-box genes in *Prunus* using the MEGA software version 4.0 by the P-distance and neighbor-joining methods with 1000 bootstrap replication tests (Tamura et al., 2007).

RESULTS

Identification of the *SFB* alleles from Japanese apricot cultivars

The *Prunus SFB* primer pair, SFB-C1F and Pm-Vb, was designed from the conserved regions of *Prunus SFB*: the F-box motif and region downstream of variable HVb, respectively. Only one PCR amplification fragment of approximately 1000 bp was obtained from each cultivar (Figure 1). According to the DNA homologous sequence analysis using DNAMAN (version 5.2; Lynnon Biosoft), these sequences were classified into 10 different types. There were 5 new *SFB* alleles showing high homology with the *SFB* alleles identified in other *Prunus* species and were named pollen-specific *SFB*: *PmSFB*. The nucleotide sequences of 5 new *SFB* alleles were submitted to the GenBank database, and the accession numbers of *SFB* alleles were as follows: *PmSFB12* (JQ356586), *PmSFB40* (JQ356585), *PmSFB41* (JQ356593), *PmSFB42* (JQ356581), *PmSFB43* (JQ356578). The other *SFB* alleles were *PmSFB1* and *PmSFB7*, as previously reported. The *SFB* alleles of the 6 cultivars tested were different and the results are presented in Table 1.

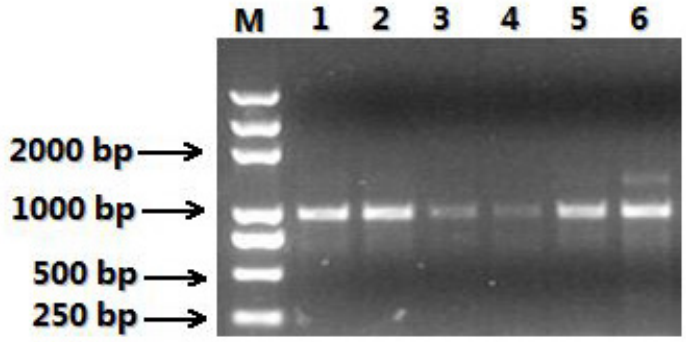

Figure 1. PCR amplification fragments of *SFB* alleles from 6 Japanese apricot cultivars. *Lane M* = 2000-bp ladder marker; *lane 1* = Dalizhong; *lane 2* = Daqingmei; *lane 3* = Daqiandi; *lane 4* = Yeliqing; *lane 5* = Huangdou; *lane 6* = Xiaomei.

Table 1. The *SFB* alleles of 6 Japanese apricot cultivars.

Cultivar	*SFB* alleles
Dalizhong	*PmSFB7*/*PmSFB14*
Daqiandi	*PmSFB2*/*PmSFB22*
Daqingmei	*PmSFB7*/*PmSFB12*
Yeliqing	*PmSFB7*/*PmSFB40*
Huangdou	*PmSFB41*/*PmSFB42*
Xiaomei	*PmSFB1*/*PmSFB43*

Sequence analysis and comparison of *SFB* alleles identified in Japanese apricot

Based on amino acid sequence comparison, Ikeda et al. (2004) first summarized the structural features for *Prunus SFB* alleles. In this study, the structures of the 5 new *SFB* alleles were similar to other *Prunus SFB* alleles. The structures were described as follows: the conserved F-box motif located in the N-terminal region and 2 hypervariable regions, HVa and HVb, located in the C-terminal region; variable regions, V1 and V2, located downstream of the F-box motif and upstream of the non-conserved HVa, respectively (Figure 2).

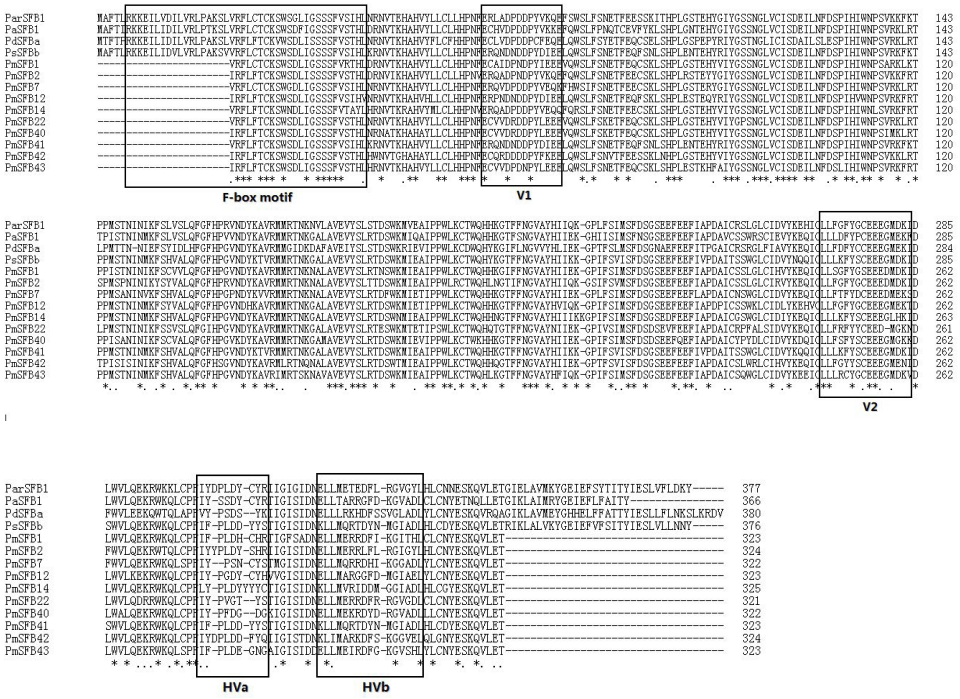

Figure 2. Alignment of the deduced amino acid sequences of *SFB* alleles from *Prunus armeniaca* (Par), *P. avium* (Pa), *P. dulcis* (Pd), *P. salicina* (Ps), and *P. mume* (Pm). Asterisks, dots, and dashes indicate conserved amino acid residues, conservative substitutions, and gaps, respectively. F-box motif, variable (V1 and V2) and hypervariable (HVa and HVb) regions are boxed. GenBank accession Nos.: *ParSFB1* (AY587563), *PaSFB1* (AB111518), *PdSFBa* (AB092966), *PsSFBb* (AB252412). *PmSFB1*, *PmSFB2*, *PmSFB7*, *PmSFB12*, *PmSFB14*, *PmSFB22*, *PmSFB40*, *PmSFB41*, *PmSFB42*, and *PmSFB43* were detected in this study.

The deduced amino acid identities ranged from 75.93 (*PmSFB22*/*PmSFB42*) to 84.83% (*PmSFB12*/*PmSFB41*) among the 10 *PmSFB* alleles (Table 2), which were different from the identities (from 66.85 to 89.73%) among *PmS-RNase* alleles (data not shown). The deduced amino acid identities compared with other *Prunus* species ranged from 67.08 to 96.91% for *PmSFB* alleles (data not shown), and the identities of the *PmSFB42* had a very high identity percentage (96.91%) with the *PdSFBd*.

Table 2. Identities (%) of the derived amino acid sequences of the *PmSFB* alleles.

SFB alleles	*PmSFB1*	*PmSFB2*	*PmSFB7*	*PmSFB12*	*PmSFB14*	*PmSFB22*	*PmSFB40*	*PmSFB41*	*PmSFB42*	*PmSFB43*
PmSFB1	100									
PmSFB2	82.41	100								
PmSFB7	82.04	79.94	100							
PmSFB12	80.19	79.32	81.11	100						
PmSFB14	80.00	79.08	81.54	79.69	100					
PmSFB22	82.04	77.16	80.43	78.33	77.54	100				
PmSFB40	82.35	77.16	80.43	79.88	78.15	84.78	100			
PmSFB41	82.35	79.32	81.73	84.83	84.31	78.33	79.88	100		
PmSFB42	79.94	78.09	77.16	77.78	76.62	75.93	78.70	79.63	100	
PmSFB43	83.28	79.63	80.19	82.04	79.38	79.88	82.04	82.35	78.09	100

Expression analysis of *PmSFB* alleles in pollen

We performed RT-PCR analysis with pollen grain cDNA and style and leaf cDNA of 'Dalizhong' using SFB-C1F and Pm-Vb to investigate the expression patterns of *PmSFB* genes. RT-PCR analysis of the actin gene was performed using ActF1 and ActRI as the positive control (Figure 3). Figure 3 (*SFB*) shows that RT-PCR of pollen grain cDNA produced DNA fragment(s) of the same size as genomic DNA, but no fragment was amplified from stylar cDNA, leaf cDNA, and the negative control. In Figure 3 (actin), amplification fragments were obtained from genomic DNA, pollen grain cDNA, stylar cDNA and leaf cDNA, but not from the negative control. The size of the fragment from genomic DNA was different compared to the fragments from pollen grain cDNA, stylar cDNA, and leaf cDNA, which indicated that the RNA preparations were free from genomic DNA. The above results showed that the extracted RNA used in this analysis was free from genomic DNA and that *PmSFB* alleles were specifically expressed in pollen as in the case of other *Prunus SFB* alleles. The sequencing results and comparison of sequences revealed that the amplification fragment of the *SFB* gene from pollen was identified as 2 *SFB* genes, *PmSFB7*, and *PmSFB14*.

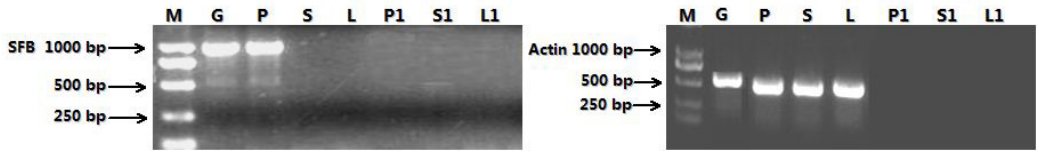

Figure 3. Expression analysis for *PmSFB* genes and actin in pollen (P), styles (S), and leaves (L) RNA of 'Dalizhong'. RT-PCR was performed using total RNA with gene-specific primers to investigate the expression patterns of the *SFB* and Actin genes. *Lanes P1*, *S1*, and *L1* = negative control of pollen, style, and leaf RNA without reverse transcription, respectively; *lane G* = genomic DNA; *lane M* = 1000-bp ladder marker. SFB: PCR with the primer pair SFB-C1F and Pm-Vb; Actin: PCR with primer pair ActF1 and ActR1.

Phylogenetic analysis of *SFB* and *SLFL* genes in *Prunus*

A phylogenic tree was constructed using the predicted amino acid sequences of *S*-locus F-box genes in *Prunus* by the neighbor-joining method. Figure 4 shows that the tree was divided into 2 major classes, the *SFB* clade and the *SLFL* clade, which were clustered together. Therefore, *S*-locus F-box genes in *Prunus* shared a high similarity. All new *SFB* alleles in *P. mume* were placed within the *SFB* clade and showed high similarity with other *SFB* alleles, and even the homology of *SFB* alleles between *P. mume* and other species was higher than the homology of *SFB* alleles between *P. mume*. From the phylogenic tree, *PmSFB41* and *PmSFB42* had a high similarity with *PsSFBb* and *PdSFBd*, respectively. These results suggested that new *SFB* alleles are orthologs of the *SFB* alleles in different *Prunus* species.

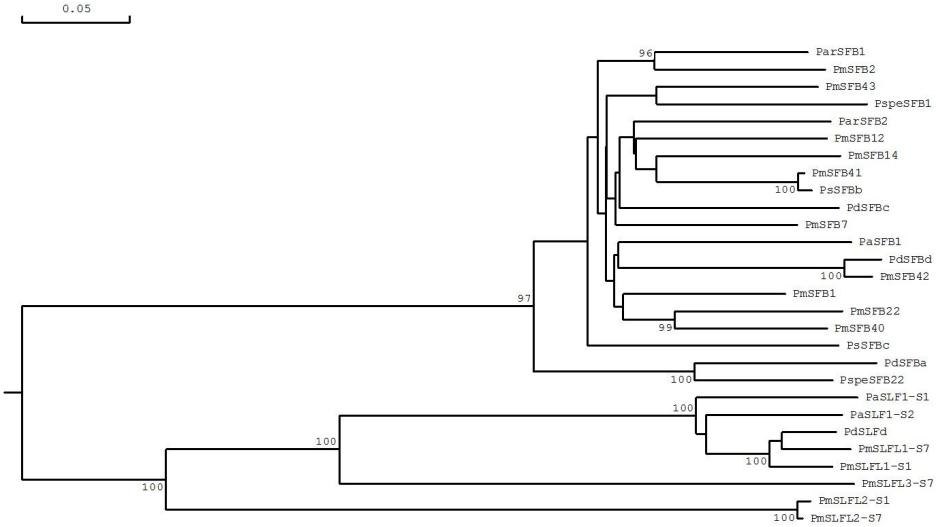

Figure 4. Phylogenetic tree constructed base on aligned amino acid sequences of *Prunus SFB* and *SLFL* genes, including 28 *S*-locus F-box genes, from *P. armeniaca* (Par), *P. avium* (Pa), *P. dulcis* (Pd), *P. salicina* (Ps), *P. speciosa* (Pspe), and *P. mume* (Pm). The scale bar represents 0.05 substitutions per amino acid site. The numbers at each branch indicate the percentage of 1000 bootstrap, which supports the grouping at that node. GenBank accession Nos.: *ParSFB1* (AY587563), *ParSFB2* (AY587562), *PaSFB1* (AY805048), *PdSFBa* (AB092966), *PdSFBd* (AB081648), *PdSFBc* (AB079776), *PmSLFL1-S1* (AB092623), *PmSLFL1-S7* (AB092624), *PmSLFL2-S1* (AB092625), *PmSLFL2-S7* (AB092626), *PmSLFL3-S7* (AB092627), *PdSLFd* (AB101660), *PaSLF1-S1* (AB360339), *PaSLF1-S2* (AB360340), *PsSFBb* (AB252412), *PsSFBc* (AB280792), *PspeSFB1* (HM347508), *PspeSFB22* (HM347509); the accession Nos. of *PmSFB* alleles were detailed in the text.

DISCUSSION

Identification of *PmSFB* alleles

Arumuganathan and Early (1991) and Zhang (2008) reported that apricot, Japanese apricot, and their varieties are diploid. The 6 Japanese apricot cultivars used in this study were diploid. In theory, these cultivars should result in 2 amplified bands of *SFB* alleles. In fact, the

6 Japanese apricot cultivars possessed 2 different sequences using the *Prunus SFB* primer pair, SFB-C1F and Pm-Vb, which proved the correctness of this primer pair.

The presence of F-box genes (*AhSLF*) physically linked to *S-RNase* and exhibiting pollen-specific expression was first reported in *Antirrhinum* (Lai et al., 2002). Yamane et al. (2003b) demonstrated a method for the molecular typing of *P. mume SFB* genes to determine the *S* haplotype using genomic DNA blots and a *PmSFB* probe. Vaughan et al. (2006) established a rapid method based on the PCR-amplified region to determine the *S* genotypes of sweet cherry cultivars. Zhang et al. (2007) designed allele-specific primers according to the hypervariable regions of *PsSFB* to determine the *SFB* alleles of Japanese plum cultivars. Gu et al. (2011) discriminated different *SFB* alleles by CAPS markers in *P. pseudocerasus* and *P. speciosa*. In this study, we successfully determined the *SFB* alleles of Japanese apricot cultivars by the method based on PCR-amplified region, which was simple, less demanding with regard to instrumentation, and more conducive to practical application.

Sequence analysis of *SFB* alleles

The deduced amino acid sequences of the 5 new *PmSFB* alleles contained the F-box motif, 2 variable regions (V1 and V2), and 2 hypervariable regions (HVa and HVb), the same as other *Prunus SFB* alleles. Previous research has suggested that the ubiquitin/26S proteasome proteolytic pathway plays a central role in self/non-self pollen discrimination in *S-RNase*-based GSI (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003). However, the F-box protein functions as a receptor to incorporate proteins targeted for polyubiquitination into the SCF complex (Ushijima et al., 2003). Thus, the F-box motif is essential for forming the SCF complex for protein degradation and important for self/non-self pollen discrimination. The 4 variable regions (V1, V2, HVa, and HVb) may play an important role in the haplotype-specific interaction with *S-RNase* for the discrimination of self and non-self *S-RNases* (Ushijima et al., 2004).

As for *PmSFB* alleles, interspecies amino acid identities by comparison with other *Prunus SFBs* were often higher than intraspecies identities. The deduced amino acid identities of *PmSFB42* had a very high percentage (96.91%) with *PdSFBd*, but a low percentage with the other *PmSFB* alleles. The phenomenon indicated that the evolution of *SFB* genes was before the formation of the species. Besides, according to pairwise comparisons of deduced amino acid sequences, pollen *SFB* genes showed a higher conservativeness than the *S-RNase* genes. About one-half of the total residues of the *SFB* alleles were conserved or conservative replacements, which may be important for the structure and function of *SFB* genes (Ikeda et al., 2004).

In conclusion, we identified 5 new *PmSFB* alleles from 6 Japanese apricot cultivars. The 5 *PmSFB* alleles had the same typical feature as *SFB* alleles from other *Prunus* species. The results of this study will enrich the information available on *SFB* alleles of *Prunus*.

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