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ORIGINAL PAPER

Identification and Characterisation of *SFBs* in *Prunus mume*

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Abstract The gametophytic self-incompatibility system in Rosaceae is controlled by a single highly polymorphic gene complex, termed the *S* locus, which is comprised of tightly linked stylar-expressed gene, *S*-RNase, with a pollen-expressed gene. In this study, seven novel *S* haplotype-specific F-box protein genes, $PmSFB_{10}$ to $PmSFB_{16}$ in *Prunus mume*, have been identified and characterised. Similarities amongst the deduced amino acid sequences of these *SFBs* ranged from 73.2% to 90.9%. Comparisons of two trans-specific pairs of haplotypes, PmS_{11} with $PspS_{3\text{-}1}$ and PmS_{13}/$ParS_9$ with $PspS_8$, indicated high degrees of polypeptide sequence identity. The deduced amino acid sequences of $PmSFB_{11}$ and $PspSFB_{3\text{-}1}$ showed similarity scores of 99.1%. In addition, the deduced amino acid sequences of the corresponding *S*-RNases, PmS_{11}-RNase and $PspS_{3\text{-}1}$-RNase exhibited a high similarity score of 99.0%. The similarity scores of the sequences physically positioned between *S*-RNase and *SFB* in the PmS_{11} haplotype and $PspS_{3\text{-}1}$ were 94.6%. The deduced amino acid sequence of $PmSFB_{13}$ showed high similarity scores with those of $PspSFB_8$ and $ParSFB_9$ at 99.3% and 97.9%, respectively. At the deduced amino acid level, the similarity scores of the PmS_{13}-RNase with the $PspS_8$-RNase or the $ParS_9$-RNase were 99.0% or 99.1%, respectively. Moreover, the similarity score of the sequence separating *S*-RNase and *SFB* in PmS_{13} was 96.1% compared with that of the $PspS_8$ haplotype and 99.0% compared with that of the $ParS_9$ haplotype. Our results suggest that the *S* haplotypes in *P. mume*, *P. armeniaca* and *P. spinosa* may have a common ancestor.

Keywords *Prunus mume* · Self-incompatibility · *SFB* · *S*-RNase

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Introduction

Prunus mume, which originated in China, is a flowering tree and an important commercial fruiting tree due to its flavourful fruit (Hou et al. 2011). *P. mume* belongs to the Amygdaloideae subfamily and exhibits gametophytic self-incompatibility (GSI) that is controlled by a single *S* locus with multiple alleles (de Nettancourt 2001). The specificity of the SI response is determined by the haplotypes of the polymorphic *S* locus, which contains at least two genes, i.e., one for the pistil determinant and one for the pollen determinant. Pollen is rejected when its *S* haplotype is the same as either of the *S* haplotypes in the diploid pistil.

The determinants of *S*-specificity in the pistil are ribonucleases (*S*-RNases) (Lee et al. 1994; Murfett et al. 1994). Based on the comparison of the cDNAs of several *S*-RNases in the Japanese pear (Norioka et al. 1996; Ishimizu et al. 1998; Castillo et al. 2002; Takasaki et al. 2004) and apple (Broothaerts et al. 1995; Li et al. 2011), the structural features of rosaceous *S*-RNases have been characterised. All *S*-RNases share five conserved domains (C1, C2, C3, RC4, and C5), and one hypervariable (HV) region is involved in allelic specificity in GSI reactions (Ushijima et al. 1998).

The *SLF* (*S* locus F-box) gene that controls specificity in the pollen determinant *SLF* genes were first reported in the

Plantaginaceae and are located approximately 9 kb downstream from the *S*-RNase (Lai et al. 2002). In the *Prunus* species, the putative pollen *S* gene encodes an F-box protein, which is termed the *S* haplotype-specific F-box protein gene (*SFB*) in sweet and sour cherries (Yamane et al. 2003a, b; Ikeda et al. 2004, 2005; Wunsch et al. 2010), almonds (*P. dulcis*) (Ushijima et al. 2003), Japanese plums (Zhang et al. 2007), peaches (Tao et al. 2007), and Japanese apricots (*P. mume*) (Entani et al. 2003; Yamane et al. 2003b). The primary structural features of *Prunus SFBs*, based on a comparison of 13 alleles, include the presence of one F-box motif, two variable regions (V1 and V2), and two hypervariable regions (HVa and HVb) in the amino acid sequence (Ikeda et al. 2004). The physical distance between *SFB* and *S*-RNase varied from 380 bp to 30 kb depending on the *S* haplotypes (Entani et al. 2003; Ushijima et al. 2003; Yamane et al. 2003a).

P. mume exhibits *S*-RNase-based gametophytic self-incompatibility similar to that of most other *Prunus* species. Based on the analyses of PCR and genomic DNA blots, a common *S*-RNase gene, which is designated as S^f-RNase, has been found in Japanese apricots (Tao et al. 2000, 2002). Three partial cDNA fragments (MSRN 1–3) (Yaegaki et al. 2001) and seven *S*-RNase genes (PMSRNA 1–7) have been identified using PCR methods. Subsequently, two *S*-RNase genes have been isolated and are designated as S_8-RNase (Tao et al. 2002) and S_9-RNase (Entani et al. 2003). The PmS_{10}–S_{16}-RNases have been identified in cultivars of *P. mume* (Heng et al. 2008). Based on the sequences of *S*-RNase, the corresponding *SFBs* and the physical distances between *S*-RNase and *SFB* in the *S* haplotypes of trans-specific were identified in the current study. Our results may help reveal the characteristics of *SFBs* and the patterns of trans-specific evolution in *Prunus*.

Materials and Methods

Plant Materials and Genomic DNA Extraction

The *P. mume* cultivars 'Xiaoyezhugan' ($S_{10}S_{14}$), 'Fubantiaozhi' ($S_{12}S_{13}$), 'Duoezhusha' (S_3S_4), 'Dantaofen' (S_4S_{12}), 'Longyanmei' ($S_{13}S_{14}$), 'Hongding' ($S_{15}S_{16}$), 'Xiyeqing' (S_3S_{15}) and 'Musashino' (S_5S_{11}) (Heng et al. 2008) as well as the *P. armeniaca* cultivars 'Jiguang' (S_8S_9) and 'Erhong' (S_9S_{11}) were used. Young leaves were collected in the spring and used for total DNA extraction (Bourguiba et al. 2010; Campoy et al. 2010).

PCR Amplification for *PmSFBs*

Consensus primers were designed from the conserved regions *SFB*-F1 and *SFB*-R1 (Ikeda et al. 2004) for amplifying $PmSFB_{10}$, $PmSFB_{12}$, $PmSFB_{13}$, $PmSFB_{14}$, $PmSFB_{15}$, $PmSFB_{16}$, and $ParSFB_9$, respectively. The PmS_{11}-RNase-specific primer designed between RC4 and C5 and the *PmSFB*-F1 primer were used to obtain the intergenic sequence that contains $PmSFB_{11}$. The PCR amplification was performed using *Ex*Taq polymerase (Takara, Kyoto, Japan) using a temperature profile of a cycle of 95°C for 3 min; 10 cycles of 94°C for 30 s, 58°C for 60 s, and 72°C for 1 min 30 s; 25 cycles of 95°C for 30 s, 55°C for 60 s, and 72°C for 1 min 30 s; and a final extension cycle at 72°C for 10 min in a Thermal Cycler (BioRad, PTC-200). The PCR products were purified from agarose gels using the QIAEX® kit (Qiagen, Hilden, Germany) and cloned into the pGEM-T vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. Approximately 20 plasmid clones were analysed using the restriction enzymes Csp6 I and EcoR I. Three clones with the same restriction patterns were sequenced to obtain a consensus sequence. If the same sequence was isolated from two cultivars possessing a common *S*-allele, the sequence was considered to represent the shared allele. Subsequent additional sequences were attributed to the remaining allele.

Analysis of the Physical Distance Between *S*-RNase and *SFB* in *P. mume*

To determine the physical distance between the *S*-RNase and *SFB* in the *S* haplotype, the *S*-RNase gene-specific primer and the *SFB*-F2 primer, which were designed from the variable region of *S*-RNase and the conserved region of the 3′ *SFBs* in *Prunus* sequences, respectively, were used (Table 1). The PCR reactions, purification, cloning and sequencing were performed as described above. The DNA sequence data were analysed using the BioEdit ver. 7.0 software and the ClustalX program (Zhang et al. 2011).

Assessment of *SFB* Intron Polymorphism in *P. mume*

The *SFB*-5′A primer and the *SFB*-specific primer (Table 1), which were designed from the conserved region of the 5′ UTR and the variable region of *SFB*, respectively, were used to amplify the intron that was located in the 5′UTR of each isolated *SFB* allele. The amplification products were purified, cloned, and sequenced as described above.

Results

PCR Amplification for *PmSFBs*

Partial sequences of $PmSFB_{10}$, $PmSFB_{12}$, $PmSFB_{13}$, $PmSFB_{14}$, $PmSFB_{15}$, $PmSFB_{16}$, and $ParSFB_9$ were isolated successfully from the various cultivars as follows: $PmSFB_{10}$ from 'Xiaoyezhugan', $PmSFB_{12}$ from 'Dantaofen' and 'Fubantiaozhi', $PmSFB_{13}$ from 'Fubantiaozhi' and

Table 1 The primers for amplification of *SFB* genes in *P. mume* and *P. armeniaca*

Primer	Orientation (5′–3′)	Note
SFB-5′A	TTKSCHATTRYCAACCKCAAAAG	Vaughan et al. (2006)
SFB-F1	GAAAWCKTAATCGACATCCTMGTAAG	Ikeda et al. (2004)
PmSFB-F2	CCAAGCAAGTTCTTGANACAGG	This work
SFB-R1	CAMRAATTCGATTTCGYCATATTTC	Ikeda et al. (2004)
$PmSFB_{10}$-R	CTTTCTAAGGGATAGCTTAACTTGC	This work
$PmSFB_{11}$-R	TGCTCTGTGTTTTCTGAAGGATGGC	This work
$PmSFB_{13}$-R	GGGATGGCTTAACTTGGAGCACTCT	This work
$PmSFB_{14}$-R	GTTCTGTGCTCCCTAAAGGATGGCT	This work
$PmSFB_{16}$-R	CCGAAGGGATGACTTAACTTGGAGC	This work
$ParSFB_{9}$-R	GGGATGGCTTAACTTGGAGCACTCT	This work
PmS_{10}-*RNase*-F	GCAAACCTGATCCAGCAGCA	This work
PmS_{11}-*RNase*-F	GGGTCTCAATTTAACGAAAGTAG	This work
PmS_{12}-*RNase*-F	CAGAGTTCGCAATAAACCTTGCTC	This work
PmS_{13}-*RNase*-F	GGTCGCAATTTAAGGGAATATTG	This work
$ParS_{9}$-*RNase*-F	AAGGTTGTACTTTTTTCTTTGGCC	This work

'Longyanmei', $PmSFB_{14}$ from 'Xiaoyezhugan' and 'Longyanmei', $PmSFB_{15}$ from 'Hongding' and 'Xiyeqing', $PmSFB_{16}$ from 'Hongding', and $ParSFB_{9}$ from 'Jiguang' and 'Erhong'. However, $PmSFB_{11}$ was not confirmed because no other cultivar shared the same *PmSFB*. The PmS_{11}-RNase-specific primer that was designed between RC4 and C5 and the *PmSFB*-F1 primer were used to obtain the intergenic distance in this haplotype. Based on the DNA sequence analysis, open reading frames were identified that corresponded to PmS_{11}-RNase (partial) and $PmSFB_{11}$ (partial). In addition, the intergenic distance between PmS_{11}-RNase and $PmSFB_{11}$ was determined.

In an alignment of the *SFBs* of *P. mume*, 170 out of 377 sites were conserved, and an additional 62 sites had only conservative replacements, which were scattered throughout the *SFB*. Many residues were conserved at the N-terminal region of the F-box motif and at most of the variable sites, which were located at the C-terminal region of *SFB*, which also contained two hypervariable regions, HVa and HVb (Fig. 1) as previously reported (Ushijima et al. 2003; Ikeda et al. 2004). The identities amongst these *PmSFBs* ranged from 73.2 to 90.9% at the amino acid level.

Physical Distance Between *SFB* and *S*-RNase

S-RNase gene-specific primers and the *SFB*-F2 primer were used to investigate the PmS_{10}–PmS_{16} haplotypes. However, only four out of seven haplotypes were confirmed. The intergenic distances for PmS_{10}, PmS_{11}, PmS_{12} and PmS_{13} haplotypes were 339 bp, 974 bp, 1,710 bp and 623 bp, respectively; were lowly polymorphic with similarities that ranged from 31.9% to 41.9%; and displayed C+G content ranging from 32.91% to 40.29% at the nucleotide level. The intergenic region between the *S*-RNase and the *SFB* genes in $ParS_{9}$ haplotypes was also successfully obtained using $ParS_{9}$-RNase-F, which was a forward *S*-RNase-specific primer, and the *SFB*2 primer, which was a reverse primer that was designed from the 3′ region of *Prunus SFB* (Table 1). The size of the intergenic region was 619 bp, suggesting that the two genes were in opposite transcriptional orientations. *SFB* was located downstream of the *S*-RNase and in the reverse transcriptional orientation. The relative order and transcriptional orientation of *S*-RNase and *SFB* genes were conserved amongst the four *P. mume S* haplotypes.

Discrimination of the Introns of New *SFB* Alleles

The putative introns in *PmSFBs* were observed in the 5′UTRs showing small length polymorphisms. The intron lengths of $PmSFB_{10}$, $PmSFB_{11}$, $PmSFB_{13}$, $PmSFB_{14}$, $PmSFB_{16}$ and $PmSFB_{f}$ varied from 89 bp to 114 bp. The sequence polymorphism of introns varied from 69.3% ($PmSFB_{10}$ with $PmSFB_{11}$) to 94.4% ($PmSFB_{14}$ with $PmSFB_{16}$), and GC content ranging from 28.1% to 30.7% at the nucleotide level. The introns of *SFBs* in *P. mume* exhibited high sequence polymorphisms with those of other species in *Prunus*, which similarity scores ranged from 59.1% ($PmSFB_{10}$ with $PaSFB_{5}$) to 98.9% ($PmSFB_{13}$ with $ParSFB_{9}$) at the nucleotide level (Fig. 2). Similar to reports regarding the sweet cherry, the conserved motif at the 3′-intron border 'TDCAG' (except for $PaSFB_{16}$) and the conserved motif at the 5′-intron border 'TAAGT' were noted in *Prunus SFBs* (Yamane et al. 2003a).

Comparison of PmS_{11} with $PspS_{3\text{-}1}$ Haplotype and PmS_{13}, $PspS_{8}$ with $ParS_{9}$ Haplotype

The identity between $PmSFB_{11}$ and $PspSFB_{3\text{-}1}$ was exceptionally high (Nunes et al. 2006). The alignment of these

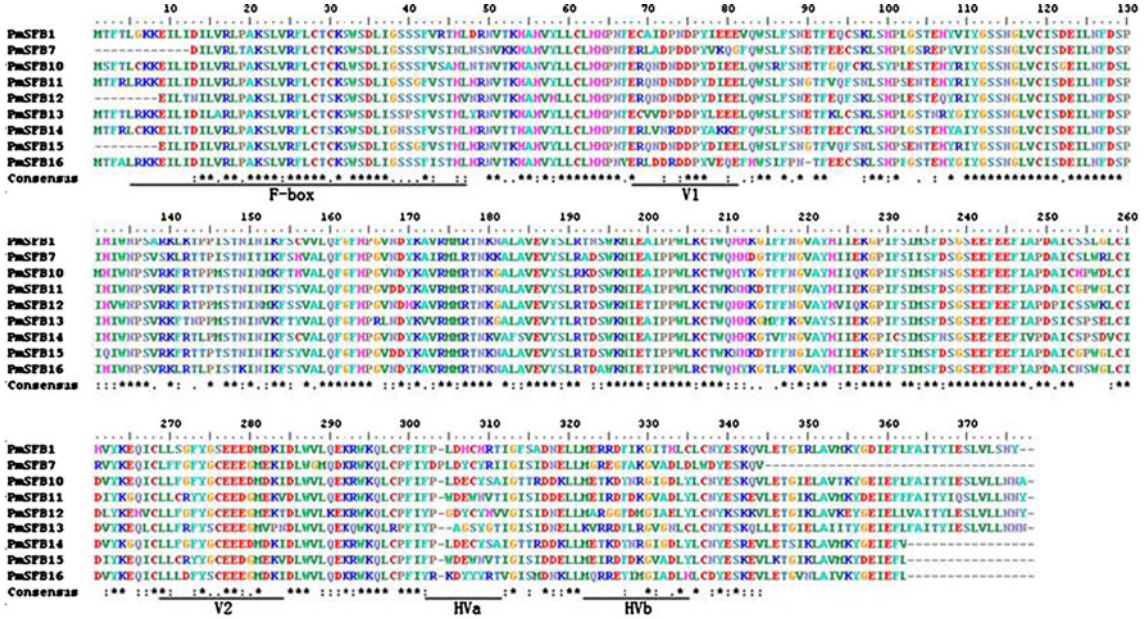

Fig. 1 Alignment of the deduced amino acid sequences of the *P. mume SFB*, S_1, S_7 and S_{10}–S_{16} by the ClustalX method using BioEdit software. The *F-box* motif and the hypervariable regions V1, V2, HVa and HVb are *underlined*. *Asterisks* indicate identical residues, *colons* indicate conserved substitutions, *dots* indicate semi-conserved substitutions and *dashes* within the sequences correspond to gaps

two *SFBs* showed that only 8 bp were different in the coding sequences with 98.8% similarity, and four residues differed from each other yielding 99.1% similarity at the amino acid level (Fig. 3a). Another alignment showed that the identity between the PmS_{11}-RNase (Heng et al. 2008) and the $PspS_{3\text{-}1}$-RNase was 99.2% at the nucleotide level with differences in 5 bp, and 99% at the amino acid level with differences in two residues (Fig. 3b). Moreover, sequence comparisons between the two introns in *S*-RNase indicated that the identity at the nucleotide level was 99.2% between the first intron sequences and 96.4% between the second intron sequences. The distance between the $PspS_{3\text{-}1}$-RNase and $PspSFB_{3\text{-}1}$ was 1,343 bp greater than that in the PmS_{11} haplotype. Surprisingly, the identity between the intergenic sequences of these two haplotypes was 94.6%.

The identities amongst $PmSFB_{13}$, $PspSFB_8$ and $ParSFB_9$ were also exceptionally high. The alignment showed that seven residues were different between $PmSFB_{13}$ and $PspSFB_8$ and five residues were different between $PmSFB_{13}$ and $ParSFB_9$ (Fig. 4a). PmS_{13}-RNase (Heng et al. 2008) was similar to $PspS_8$-RNase with 99.1% identity at the protein level and 98.3% at the nucleotide level, and it was similar to $ParS_9$-RNase with 99.1% identity at the protein level and 99.3% at the nucleotide level (Fig. 4b). Sequence comparison showed that the identity of the two intron sequences of PmS_{13}-RNase was high compared with $PspS_8$-RNase or the $ParS_9$-RNase. The identities between PmS_{13}-RNase and $PspS_8$-RNase were 96.9% at the first intron sequences and 97.4% at the second intron sequences. The identities between PmS_{13}-RNase and $ParS_9$-RNase were 98.8% at the first intron sequences and 98.3% at the second intron sequences. Moreover, the intergenic sequence comparison showed that the similarity of PmS_{13} was 96.1% to the $PspS_8$ haplotype and was 99.0% to the $ParS_9$ haplotype at the nucleotide level.

Fig. 2 Comparison of *SFB* introns amongst the species in *Prunus*

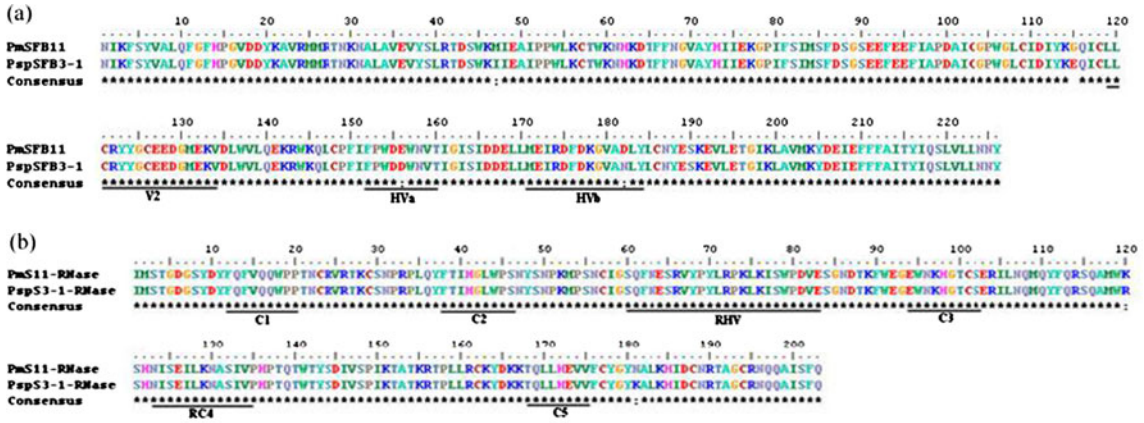

Fig. 3 **a** Comparison of the amino acid sequences of $PmSFB_{11}$ and $PspSFB_{3\text{-}1}$. The hypervariable regions V2, HVa and HVb are *underlined*. *Asterisks* indicate identical residues, *colons* indicate conserved substitution, *dots* indicated semi-conserved substitution and *dashes* correspond to gaps. **b** The amino acid sequences were compared between the PmS_{11}-RNase and the $PspS_{3\text{-}1}$-RNase. The conserved regions (C1, C2, C3, RC4 and C5) and the hypervariable region (RHV) are *underlined*. *Asterisks* indicate identical residues, *colons* indicate conserved substitutions, *dots* indicated semi-conserved substitution and *dashes* correspond to gaps

Discussion

Sequence Analysis and Comparison of *SFBs*

Similar to other *Prunus SFBs*, *PmSFBs* were found to have one F-box domain, V1, V2, HVa and HVb, which are involved in the allelic specificity of the GSI reaction (Ushijima et al. 2003). The identities amongst the *PmSFBs* ranged from 73.2 to 90.9% at the amino acid level. Interestingly, the high identities amongst the *SFBs* occurred frequently across *Prunus* species. For example, the identity between $PmSFB_{11}$ and $PspSFB_{3\text{-}1}$ was 94.6%, and the identity of $PmSFB_{13}$ was 98.7% with $PspSFB_8$ and 99.3% with $ParSFB_9$ at the amino acid level. These high identities indicated that the *Prunus S* haplotypes might have a common ancestor.

The sizes of the introns varied from 89 to 114 bp, and the similarity amongst them ranged from 69.3% to 94.4%. Compared with the first and second introns of the *S*-RNases, the introns of *SFBs* exhibited lower length

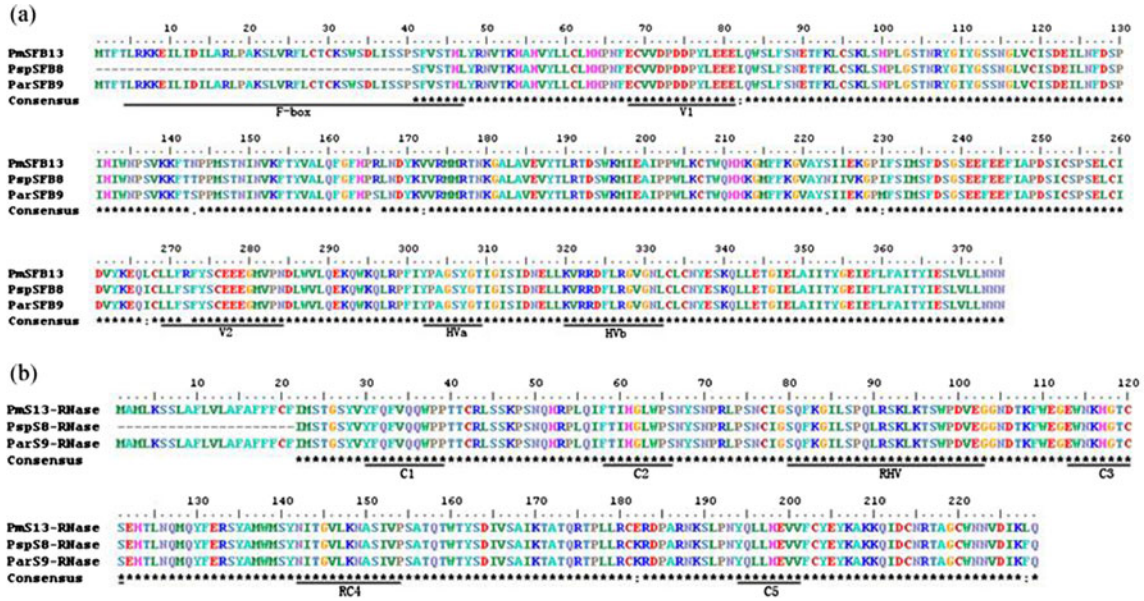

Fig. 4 **a** Comparison of the amino acid sequences of $PmSFB_{13}$, $PspSFB_8$ and $ParSFB_9$. The *F-box* motif and the hypervariable regions V1, V2, HVa, and HVb are *underlined*. *Asterisks* indicate identical residues, *colons* indicate conserved substitutions, *dots* indicate semi-conserved substitutions and *dashes* within the sequences correspond to gaps. **b** The amino acid sequences were compared amongst the PmS_{13}-RNase, the $PspS_8$-RNase and the $ParS_9$-RNase. Five conserved regions (C1, C2, C3, RC4 and C5) and the hypervariable (RHV) region are *underlined*. *Asterisks* indicate identical residues, *colons* indicate conserved substitutions and *dashes* correspond to gaps

polymorphism, which may be constrained due to a specific regulatory role for the 5′UTR intron in gene expression (Chung et al. 2006). In addition, 50 bp was the minimum requirement for effective splicing (Deutsch and Long 1999). Moreover, the introns of three pairs, $PmSFB_{13}$ with $ParSFB_9$, $PmSFB_{14}$ with *PdSFB-c*, and $PaSFB_6$ with $ParSFB_1$, showed high similarities, whereas the coding sequences of the *SFBs*, with the exception of $PmSFB_{13}$ with $ParSFB_9$, did not. These results indicate that the evolution of *SFB* coding and non-coding sequences may not occur simultaneously.

SFBs are Tightly Linked with *S*-RNases

The tight linkage between the *SFB* and *S*-RNase alleles is one of the mechanisms that are used to suppress recombination between these two genes (Zhang et al. 2007). In the current study, four intergenic sequences at the *S* locus were obtained from *P. mume* cultivars using allele-specific primers and *SFB*-F2. We demonstrated that the sizes of these sequences ranged from 339 to 1,710 bp and that the two intergenic sequences at the *S* locus were obtained from *P. armeniaca* cultivars. The intergenic regions in the Pm_{14}–Pm_{16} *S* haplotypes were not obtained successfully due to their great length as was also found for the *P. avium* S_4 haplotype (Ikeda et al. 2005). Moreover, the sequence order at the two ends of the intergenic region showed that *SFB* was located downstream of the *S*-RNase and in the reverse transcriptional orientation.

Encoding the Sequences of *S*-RNase and *SFB* Gene

SFBs that were recovered from the pairs of $PmS_{11}/PspS_{3\text{-}1}$ and $PmS_{13}/ParS_9/PspS_8$ showed a low degree of variation. However, this variation was greater than that amongst the *S*-RNases. There were 1–7 amino acid substitutions in the corresponding regions. Variant residues did not cluster in or near the four HV regions, which included the hypervariable residues that were identified by Ikeda et al. (2004) but were distributed uniformly throughout the polypeptide chain. Likewise, the alignment of the amino acid sequences of two pairs indicated differences that were distributed throughout the sequence, most of which were non-synonymous substitutions. In the pairs $PmS_{11}/PspS_{3\text{-}1}$ and $PmS_{13}/ParS_9$, there were two residues from C1 to the stop codon region. Interestingly, the pairs $PmS_{11}/PspS_{3\text{-}1}$ and $PmS_{13}/ParS_9/PspS_8$ shared identical RHV regions, which may encode specificity, and changes at these sites are more likely to generate new specificities (Ushijima et al. 1998). Given that *S*-RNases and *SFB* polypeptides interact in a specific way, we expect that the identity and polypeptide structure are highly conserved.

S-allele Evolution

Prunus S-RNase alleles have one small, variable-length intron immediately upstream of the C1 region and the other introns, which are located within the RHV region, have notable characteristics such as their lengths and sequence polymorphisms (Tao et al. 1999; Igic and Kohn 2001). The presence or absence of introns acts as a taxonomic marker between closely related species. The absence of introns from Maloideae *S*-RNases agrees with the marked divergence between the Maloideae and Prunoideae *S*-RNases, which has been noted by Ushijima et al. (1998). As non-coding regions, the introns of the *S*-RNase alleles have accumulated random mutations and show significant differences amongst the *S* haplotypes. The similar scores of the second introns, which ranged from 96.4% to 98.3%, between the PmS_{11}-RNase and the $PspS_{3\text{-}1}$-RNase as well as the PmS_{13}-RNase and $ParS_9$-RNase or the $PspS_8$-RNase may indicate that a relatively recent divergence of these alleles or a recent introgression event occurred after the divergence of the species but prior to their geographical spread.

Šurbanovski et al. (2007) have found a reproductive *Prunus* species, which had a different *SFB* allele that was coupled with the *S*-RNase allele. In addition, Sutherland et al. (2008) has reported six pairs of *S*-loci alleles that exhibited exceptionally high identities in *Prunus* species. In the current study, we found two pairs of *S* haplotypes that exhibited exceptionally high identities. All of the pairs of these *S* haplotypes may arise from introgressive hybridisation with each other or from a common ancestor that existed before the separation of these species. After species divergence, the *S* haplotypes may have retained the same protein sequences in different lineages. Moreover, two pairs of intergenic sequences at the *S* locus were exceptionally high. These haplotypes may be formed by trans-specific pairs (Sutherland et al. 2008). Minor divergences are evident in the *Prunus S-RNase* and the *SFB* sequences of the trans-specific pairs, and it is still unclear whether these divergences are sufficient to produce a new *S*-allele specificity. Ideally, the function and specificity of the trans-specific *S* haplotypes in *Prunus* that are reported here should be examined in controlled crosses.

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