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ORIGINAL PAPER

Two Different *Prunus SFB* Alleles Have the Same Function in the Self-incompatibility Reaction

C. Gu · J. Wu · Y.-H. Du · Y.-N. Yang · S.-L. Zhang

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Abstract Many species in the families of Rosaceae, Solanaceae, and Scrophulariaceae exhibit gametophytic self-incompatibility, a phenomenon controlled by two polymorphic genes at the *S*-locus, style-*S* (*S-RNase*) and pollen-*S* (*SFB*). Sequences of both genes show high levels of diversity, characteristic of genes involved in recognition of self-incompatibility systems in plants. In this study, S_{24}-*RNase* and SFB_{24} alleles were cloned from *Prunus armeniaca* cv. Chuanzhihong (Chinese apricot). Sequence comparisons of deduced amino acid sequences revealed that the *P. armeniaca* S_{24}-haplotype has different *SFB* alleles, but shares a single *S-RNase* allele with *P. armeniaca* S_4-haplotype. Moreover, *P. armeniaca* S_{24}-*RNase* haplotype has a single and three different alleles with S_1-*RNase* of *P. tenella* (dwarf almond) and S_1-*RNase* of *P. mira* (smooth pit peach), respectively. The functionalities of SFB_{24} and SFB_4 have been evaluated by pollen tube growth and controlled field tests of *P. tenella* and *P. mira*. Genetic analysis of the two intercrosses showed that progenies segregated 1:1 into two *S*-genotype classes, which is consistent with the expected ratio for semi-compatibility. These findings imply that the allelic function of the S_{24}-haplotype is identical to that of the S_4-haplotype in a self-incompatibility reaction. Thus, these two *Prunus S*-haplotypes are in fact two neutral variants of the same *S*-haplotype. The evolution of the *S*-allele is also discussed in terms of both functions and differences between S_{24}- and S_4-haplotypes in *Prunus*.

Keywords *Prunus armeniaca* · *S*-allele evolution · Self-incompatibility · *SFB* · *S-RNase*

Introduction

Gametophytic self-incompatibility (GSI), which prevents inbreeding and promotes out-crossing (De Nettancourt 2001), is a widespread mechanism in flowering plants that is often controlled by a single multiallelic locus, termed the *S*-locus. A pollen grain is rejected if its *S*-allele matches one of those in the style (Crane and Lawrence 1929). The *S*-locus is known to be a multigene complex, termed the *S*-haplotype, which contains a minimum of two polymorphic genes, called the *S*-determinant genes; one gene (stylar-*S*) controls stylar specificity and the other gene (pollen-*S*) controls pollen specificity of the self-incompatibility (SI) reaction.

In the Solanaceae, Plantaginaceae, and Rosaceae, the stylar-*S* gene encodes a ribonuclease (S-RNase) (McClure et al. 1989; Sassa et al. 1992; Xue et al. 1996), which is expressed in the pistil and specifically degrades the RNA of incompatible pollen (McClure et al. 1990). *S-RNase* alleles, which have been identified and characterised in many *Prunus* species (Gu et al. 2010; Halász et al. 2007; Heng et al. 2008; Romero et al. 2004; Tao et al. 1999; Wu et al.

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2009; Zhang et al. 2008b), contain five conserved regions (C1, C2, C3, RC4 and C5) and one hypervariable domain (RHV). The first introns of *S-RNase* are located between the mature protein and the C1 region, which has high sequence polymorphism but low length polymorphism. The second introns of *S-RNase* are in RHV and are located between the C2 and C3 regions. RHV is assumed to correspond to the HVa domain and to mediate pollen recognition in Solanaceae (Matton et al. 1997). In addition, RHV is a putative positively selected region where insertions, deletions, or nucleotide substitutions are suggested to occur more frequently (Ishimizu et al. 1998). The other variable region is located upstream of the C5 region (Gu et al. 2010; Verica et al. 1998).

The pollen-*S* determinant is encoded by multiple *SLF* genes in the Solanaceae (Kubo et al. 2010) and *Pyrus* (Kakui et al. 2011) and a single *SFB* gene in *Prunus* (Ikeda et al. 2004; Ushijima et al. 2003; Zhang et al. 2007). *Prunus SFBs* has high sequence polymorphism, is tightly linked with the corresponding *S-RNase* alleles and is expressed specifically in pollen grains (Ikeda et al. 2005; Wu et al. 2009; Zhang et al. 2007). Insertion, deletion and default in coding sequences of *Prunus SFBs* would break down the SI (Marchese et al. 2007; Sonneveld et al. 2005; Ushijima et al. 2004; Vilanova et al. 2006). The *SFB* alleles contain one F-box motif, two variable regions (V1 and V2), and two hypervariable regions (HVa and HVb). The F-box motif is located downstream of the start codon. Two hypervariable regions (HVa and HVb), located just upstream of the stop codon, are thought to be involved in the allelic specificity of the GSI reaction (Ikeda et al. 2004; Ushijima et al. 2003). The introns of *SFBs*, located upstream of the start codon, have low length polymorphism and high sequence polymorphism (Vaughan et al. 2006; Wu et al. 2009).

For most genes, new alleles arise from the accumulation of non-synonymous point mutations. The generation of new *S*-specificity could also occur in this manner, which would proceed by a series of sequential mutagenic steps that are difficult to monitor (Fisher 1961; Lewis 1951). The two genes at the *S*-locus were co-adapted in *S*-RNase-based SI (Lewis 1949). However, the correct function of the *S*-locus is based on the interaction of stylar-*S* and pollen-*S* gene products; therefore, for the generation of a new *S*-allele specificity, mutations would need to occur and be complementary in both stylar-*S* and pollen-*S* (Šurbanovski et al. 2007; Sutherland et al. 2008). Mutations could break down the SI system, resulting in self-compatibility in most cases, as described in *Nicotiana alata* (Zurek et al. 1997) and *P. avium* (Sonneveld et al. 2005); however, self-compatibility will not necessarily occur. In *Pyrus* and *Malus* species, *Pyrus pyrifolia* S_8*-RNase*, *Pyrus sinkiangensis* S_{28}*-RNase* and *Malus spectabilis* S_3*-RNase* have maintained the same recognition specificity (Heng et al. 2011). *Pyrus communis* S_{104}*-RNase* has two neutral variants, S_4*-RNase* and S_b*-RNase*, which have two different amino acid residues (Sanzol 2010). *Malus*×*domestica* S_{kb}*-RNase* shows 98 % identity to *Sorbus aucuparia* S_{19}*-RNase* and 97 % identity to *Crataegus monogyna* S_{17}*-RNase*, indicate that was a transgeneric *S-RNase* allele (Bokszczanin et al. 2009). In *Prunus species*, *P. persica* S_1*-RNase* was identical to *P. dulcis Sk-RNase*, *P. persica* S_2*-RNase* have 97.8 % identity with *P. salicina Sa*-haplotype (Tao et al. 2007). *P. tenella* S_8*-RNase* was identical to *P. avium* S_1*-RNase*, but has 12 amino acid replacements compared to *P. tenella* SFB_8 and *P. avium* SFB_1 (Šurbanovski et al. 2007). Moreover, Sutherland et al. (2008) found a few pairs of *S*-haplotypes, the identities of which were exceptionally high between two different *Prunus* species. Regrettably, they could not execute intercross-pollination of the two species.

Luckily, two *S*-haplotypes with exceptionally high identity has been found within a single *Prunus* species: the S_{24}-haplotype was derived from three Chinese apricot cultivars, 'Chaoxian', 'Chaoxian 1' and 'Zhanggongyuan' (Wu et al. 2009), and the S_4-haplotype was derived from two Northern American apricot cultivars, 'Harcot' (Burgos et al. 1998; Romero et al. 2004). Thus, their intercross-pollination could be executed in the field. In the present study, full-length *Prunus armeniaca* S_{24}*-RNase* (*Par-*S_{24}) and *P. armeniaca* SFB_{24} (*Par-*SFB_{24}) alleles were cloned from the other Chinese apricot cultivar 'Chuanzhihong', and full-length *P. armeniaca* S_4*-RNase* (*Par-*S_4) and *P. armeniaca* SFB_4 (*Par-*SFB_4) alleles were cloned from the Northern American apricot cultivar 'Harcot'. Understanding their functions, examined by pollen tube growth and intercrosses, would expand current knowledge regarding the relationships between non-synonymous substitutions and the SI response. Moreover, given the exceptionally high identity among *P. armeniaca* S_{24}-, *P. armeniaca* S_4-, *P. tenella* S_1- and *P. mira* S_1-haplotype alleles at the amino acid and nucleotide levels, as well as functionally, we focused on the discussion of the possible evolutionary process in *Prunus* species.

Materials and Methods

Plant Material

Young leaf tissue (spring) and flowers (before anthesis) of two apricot cultivars, 'Harcot' (S_1S_4) (Burgos et al. 1998) and 'Chuanzhihong' (formerly described as $S_{12}S_{14}$; Zhang et al. 2008a), were collected from the fruit tree genebank of the National germplasm repository for plums and apricots. Styles and pollen grains were detached, weighed, and stored in liquid nitrogen until being used. In total, 223 seedlings of intercross-pollinated progeny from two cultivars were used.

Total genomic DNA was extracted using the CTAB protocol with modifications (Doyle and Doyle 1987; Sonneveld et al. 2001), treated with RNase (TaKaRa, Kyoto, Japan), and incubated at 37 °C for 1 h. Total RNA was extracted from the styles, leaves and pollen grains of two apricot cultivars according to Tao et al. (1999) and then treated with DNaseI (Invitrogen, Carlsbad, CA). DNA and RNA integrity was assessed by electrophoresis, and the concentration of extracted DNA was determined by spectrophotometry. Total RNA (1 μg) from styles, leaves and pollen grains was used for first-strand cDNA synthesis by using an RNA PCR Kit Ver 2.1(TaKaRa), with an Adp-dT primer set which consists of the M13-20 sequence primer and oligo $(dT)_{16}$ (Tao et al. 1999).

PCR Amplification of *SFB* and *S-RNase* Alleles

Full-length *SFB* alleles were obtained from pollen grain cDNA by PCR with the forward primer F-BOX5′A and the reverse primer M13-20 (Table S1), and the introns of *SFB* alleles in *Prunus armeniaca* (*Par*) were amplified from genomic DNA by PCR with the forward primer F-BOX5′A and the reverse primer PsSFB-R1 (Table S1). The PCR reaction mixture contained 50 ng cDNA/DNA, 2.5 μl 10× PCR buffer (TaKaRa, Kyoto, Japan), 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.15 μM of each primer and 1 Unit *Ex Taq* DNA polymerase (TaKaRa). The PCR conditions were the same as described in Zhang et al. (2007), except that the annealing temperature was 52 °C.

Full-length *S-RNase* alleles were obtained from stylar cDNA by PCR with the forward primer PMT2 and the reverse primer M13-20 (Table S1), and the first and second introns of putative *S-RNase* alleles in *P. armeniaca* were amplified from genomic DNA by PCR with the forward primer PMT2 and the reverse primer PCE-R (Table S1), The PCR reaction contained 50 ng cDNA/DNA, 2.5 μl 10×PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 μM of each primer and 1 Unit Ex *Taq* DNA polymerase (TaKaRa). PCR reaction conditions were the following: initial denaturation at 94 °C for 3 min; then 40 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 90 s; and a final 10 min extension at 72 °C.

Organ-Specific Expression

cDNA of styles of two apricot cultivars, ‘Chuanzhihong’ and ‘Harcot’, were used as templates for PCR amplification with the primers Pru-C2 and Amy-C5 (Table S1) for *S-RNase* alleles. PCR reaction mixtures and thermal cycling were the same as PCR amplification of the first and second introns of *S-RNase* alleles. cDNA of pollen grains of two apricot cultivars were used as templates for PCR amplification with the primers PsSFB-F1 and PsSFB-R1 for *SFB* alleles. PCR reaction mixtures and thermal cycling were the same as PCR amplification of the introns of *SFB* alleles. PCR analysis of *Actin* genes using the primers ActF and ActR (Table S1) was used as the internal control. Genomic DNA was used as an additional control.

Sequence Analysis of *S-RNase* and *SFB* Alleles

Specific PCR fragments were excised from 2 % agarose gels and purified using the Qiagen II Gel Extraction Kit (Qiagen, Valencia, CA). The purified products were cloned into the PMD19-T vector (TaKaRa) following the manufacturer’s instructions and transformed into *Escherichia coli* DH5α. Transformed colonies were selected, and plasmid purification was performed using the Plasmid Mini Kit (Qiagen). Plasmids with inserts of the expected size were examined using the same primer pairs as used for initial amplification by PCR. To obtain a consensus sequence, three positive clones from independent PCR reactions of each *S*-haplotype were sequenced by Invitrogen.

A consensus DNA sequence for each allele was obtained by assembling the data from all three replicates with DNAMAN (version 5.2; Lynnon Biosoft). Nucleotide sequences and deduced amino acid sequences of *S-RNases* were aligned using the CLUSTAL W (Thompson et al. 1994) and CLUSTAL X (Thompson et al. 1997) programs, respectively.

Field Pollination Tests and Pollen Tube Growth Tests

For self-pollination and cross-pollination, the numbers of flowers were the following: 503 for self-pollination of ‘Harcot’, 614 for self-pollination of ‘Chuanzhihong’, 1,302 for cross-pollination of ‘Harcot’×‘Chuanzhihong’ and 1,513 for cross-pollination of ‘Chuanzhihong’×‘Harcot’. The emasculated flowers were self-pollinated, counted and re-bagged at full bloom. The percentages of fruit sets were calculated prior to harvest; fruit sets ≥6 % were considered to be self- or cross-compatible, and <6 % were considered to be self- or cross-incompatible (Chen et al. 2006).

Pollen tube growth tests were performed for self- and cross-pollinations of the two cultivars. Styles were collected 72 h after self- or cross-pollination, fixed in FAA (37 %-formaldehyde: glacial-acetic-acid: 50 %-ethanol, 5:5:90), and stored at 4 °C until used. The styles were washed thoroughly under running tap water, incubated in 4 *N* NaOH for 2 h to soften the tissues, and then soaked in 0.1 % aniline blue solution with 0.1 % K_3PO_4 overnight at 65 °C in darkness. Pollen tubes in styles, which were washed and squashed, were observed by ultraviolet fluorescent microscopy (BX60, Olympus, Tokyo, Japan).

Genetic Segregation of *S*-Alleles

To study the segregation of *S*-alleles, the genotypes of seedlings were first detected by PCR amplification with primers Pru-C2 and PCE-R for *S-RNase*. The second intron in the S_1-*RNase* allele was too large to amplify successfully; thus, the specific primer S1-Intron-F was used in this study (Table S1). Then, the seedlings were analysed by PCR with the primers PsSFB-F1 and PsSFB-R1 for *SFB*. Amplification products were digested with *Csp*6I. Using this method, both *S-RNase* genotypes and *SFB* genotypes of seedlings from progenies were determined. The goodness-of-fit of segregation to Mendelian segregation ratios (1:1) was tested using χ^2 test.

Results

Sequence Analysis and Comparison of *S-RNase* and *SFB* Alleles

Sequence analysis revealed that deduced amino acid sequences of *P.armeniaca* SFB_{24} (*Par-*SFB_{24}) and *P.armeniaca* SFB_4 (*Par-*SFB_4) shared the F-box motif, two variable regions (V1 and V2), and two hypervariable regions (HVa and HVb; Fig. 1). DNA sequence comparisons of *Par-*SFB_{24} and *Par-*SFB_4 revealed two base substitutions: *Par-*SFB_{24} contains the bases 'G' and 'C' instead of two 'A' bases present in *Par-*SFB_4 (Fig. S1). This resulted in two non-synonymous amino acid substitutions of V→I and D→E, respectively, outside the above mentioned conserved and variable region (Fig. 1). Other regions of these alleles were identical, including the intron of *Par-*SFB_{24} (Fig. S2).

The alignment showed that the *P.armeniaca* S_{24} (*Par-*S_{24}) and *P.armeniaca* S_4 (*Par-*S_4) share identical sequences at the amino acid level (Fig. 2a); but the sequences differ at the nucleotide level (Fig. S3). The only difference between the *Par-*S_{24} and *Par-*S_4 alleles in the coding region was a single base substitution; *Par-*S_{24} contains the base 'A' while *Par-*S_4 contains the base 'G' (Fig. S3). Differences were detected in the two introns (compared with *Par-*S_4); 'TAGAGAAATACTGTGT' was inserted in the first intron of *Par-*S_{24} (Fig. 2b) and, in the second intron of *Par-*S_{24}, 'AT' was inverted and 'CCAA' was inserted (Fig. 2c). Moreover, blast analysis in GenBank showed that *Par-*S_{24} allele shares exceptionally high identity with *P. tenella* S_1-*RNase* (*Pte-*S_1) and *P. mira* S_1-*RNase* (*Pmi-*S_1) alleles. Sequence comparisons revealed that one base substitution resulted in one non-synonymous amino acid substitutions of K→R (Fig.2a, S3); and there have 2 base substitutions in the first intron (Fig. 2b) and 12 base substitutions in the second intron between *Par-*S_{24} and *Pte-*S_1 (Fig. 2c). Eight base substitutions resulted in three non-synonymous amino acid substitutions of I→V, L→F and S→R (Fig. 2a, S3); and there have three base substitutions in the first intron (Fig. 2b) and eight base substitutions in the second intron between *Par-*S_{24} and *Pmi-*S_1 (Fig. 2c).

Specific Expression of *S-RNase* and *SFB* Alleles

For total RNAs from leaf, style and pollen grain samples in 'Harcot' and 'Chuangzhihong', RT-PCR with the primers ActF and ActR yielded amplified fragments of the same size, which were shorter than the fragments amplified from genomic DNA due to the lack of introns in the RNA (Fig. 3a, b). The results confirmed the successful synthesis of the first-strand cDNA and the absence of genomic DNA. When using the gene-specific primers Pru-C2 and Amy-C5 for *S-RNase*, only RT-PCR performed with stylar cDNA yielded fragments shorter than those produced by PCR using the same primers with a genomic DNA template (Fig. 3c, d). Length polymorphism could not be revealed because of lack of the second intron, giving additional evidence of no genomic DNA contamination in these six RNAs. Meanwhile, when the gene-

Fig. 1 Alignment of deduced amino acid sequences of *Par-*SFB_{24} and *Par-*SFB_4. *Asterisks* Conserved amino acid residues, *dots* conservative substitutions. F-box, variable (V1 and V2) and hypervariable (HVa and HVb) regions are *boxed*. Accession numbers: *Par-*SFB_{24} (HQ615603) and *Par-*SFB_4 (AY587565)

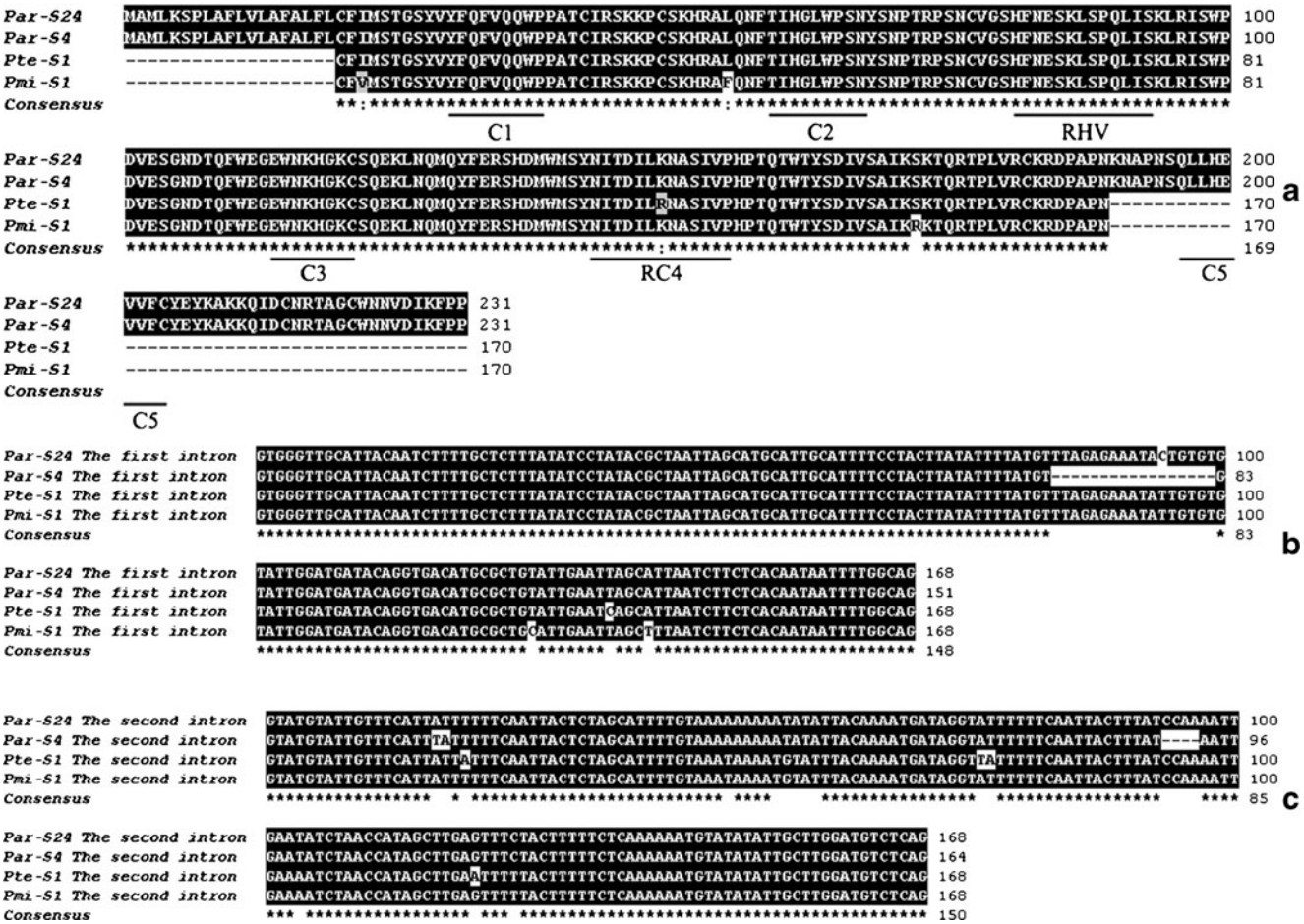

Fig. 2 Alignment of the deduced amino acid and the nucleotide sequences of *Par-S*$_{24}$, *Par-S*$_4$, *Pte-S*$_1$ and *Pmi-S*$_1$. *Asterisks* Conserved bases, *dashes* gaps. Accession numbers: *Par-S*$_{24}$ (HQ615602), *Par-S*$_4$ (AY587564), *Pte-S*$_1$ (DQ983373) and *Pmi-S*$_1$ (AB597200). **a** Alignment of the deduced amino acid sequences of *Par-S*$_{24}$, *Par-S*$_4$, *Pte-S*$_1$ and *Pmi-S*$_1$. **b** Alignment of the nucleotide sequences of the first intron of *Par-S*$_{24}$, *Par-S*$_4$, *Pte-S*$_1$ and *Pmi-S*$_1$. **c** Alignment of the nucleotide sequences of the second intron of *Par-S*$_{24}$, *Par-S*$_4$, *Pte-S*$_1$ and *Pmi-S*$_1$

specific primer set PsSFB-F1/PsSFB-R1 was used to amplify *SFB*s, only RT-PCR from pollen RNAs yielded fragment(s) of the same size as those produced by PCR from genomic DNA (Fig. 3e, f). These results indicate that the *S-RNase* and *SFB* alleles identified in the two cultivars were expressed specifically in the style and pollen, respectively.

Controlled Pollination Test and Pollen Tube Growth Assay

To check the self- and intercross-compatibility of the two apricot cultivars, fruit set data were analyzed. As described by Chen et al. (2006), the criterion used to define a cultivar as compatible in self- or cross-pollination was a fruit set of ≥6 %. The self-pollinated fruit sets of ‘Harcot’ and ‘Chuangzhihong’ were 0.57 % and 0 %, respectively. The intercross-pollinated fruit sets were 11.29 % and 22.87 %, respectively (Table 1). Therefore, based on these data, ‘Harcot’ and ‘Chuangzhihong’ are self-incompatible, but intercrosses are compatible.

Pollen tube growth was also monitored by fluorescence microscopy after 72 h of pollination (Fig. 4). Pollen tubes were arrested completely in self-pollination of the two cultivars (Fig. 4a, b). Pollen tube growth was arrested partially in intercross-pollination; several pollen tubes grew at the bottom of styles (Fig. 4c, d). Pollen tube growth was observed down to the bottom of styles in open-pollination of the two cultivars (Fig. 4e, f). Thus, these data confirmed that the cultivars ‘Harcot’ and ‘Chuangzhihong’ were completely incompatible, their intercrosses were semi-compatible, and natural pollinations were fully compatible.

Genetic Analysis of Cross-Pollinated Progenies

The pollination tests indicated that ‘Harcot’ was self-incompatible and few fruits could be generated by their self-pollination. The *S*-genotyping of intercross-pollinated progenies of ‘Harcot’ (S_1S_4)×‘Chuanzhihong’ (S_8S_{24}) showed that the individuals consisted of two *S*-genotype

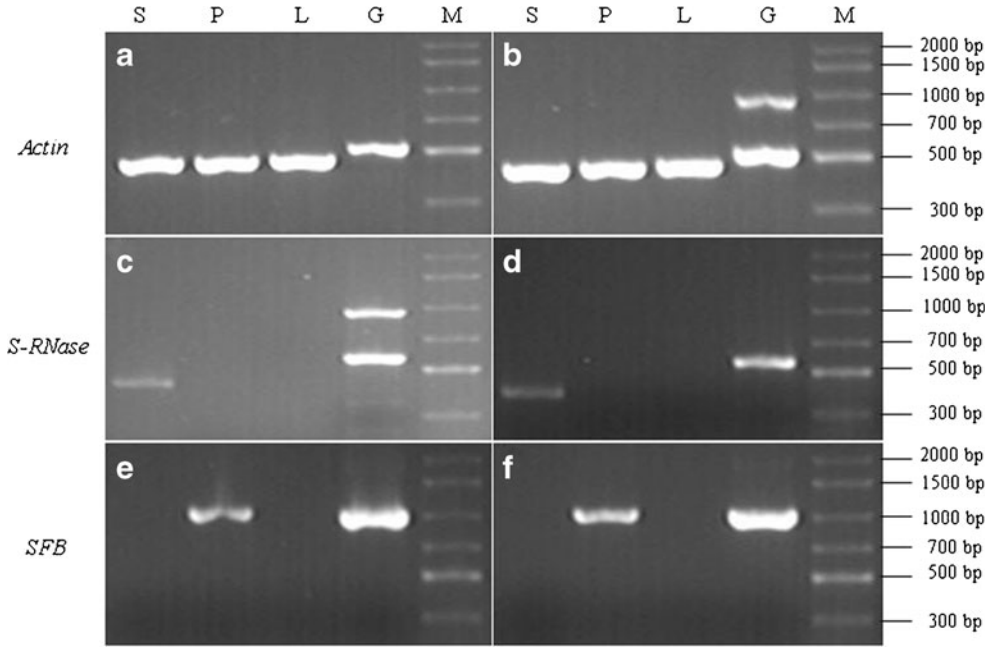

Fig. 3 Expression patterns of *S-RNase* and *SFB* genes in two apricot cultivars, Harcot and Chuanzhihong. Lanes: *M* Marker, *S* cDNA of style, *P* cDNA of pollen, *L* cDNA of leaf, *G* genomic DNA. **a** Expression pattern of *Actin* in 'Chuanzhihong': PCR with primer set ActF/ActR. **b** Expression pattern of *Actin* in 'Harcot': PCR with primer set ActF/ActR. **c** Expression patterns of *S-RNase* in 'Chuanzhihong': PCR with primer set Pru-C2/Amy-C5. **d** Expression patterns of *S-RNase* in 'Harcot': PCR with primer set Pru-C2/Amy-C5. **e** Expression patterns of *SFB* in 'Chuanzhihong': PCR with primer set PsSFB-F1/PsSFB-R1. **f** Expression patterns of *SFB* in 'Harcot': PCR with primer set PsSFB-F1/PsSFB-R1

classes, and the S_8-haplotype was detected in all progenies. The segregation ratio was S_1S_8:S_4S_8=42:54 (≈1:1, $\chi^2 = 1.26 < \chi^2_{0.05,\,1} = 3.84$), which fitted with the expected ratio (Table 2; Fig. S4). These results revealed that *P. armeniaca* S_4-RNase could cognise *Par*-SFB_{24}. Moreover, the pollination test indicated that 'Chuanzhihong' was also self-incompatible, and no fruits could be generated by self-pollination. The *S*-genotyping of intercross-pollinated progenies of 'Chuanzhihong'×'Harcot' showed that the progenies consisted of two *S*-genotype classes, and the S_1-haplotype was detected in all progenies. The segregation ratio was S_1S_8:S_1S_{24}=67:60 (≈1:1, $\chi^2 = 0.283 < \chi^2_{0.05,1} = 3.84$), which was also the same as the expected ratio (Table 2; Fig. S5). These results revealed that *P. armeniaca* S_{24}-RNase could cognise *Par*-SFB_4. Thus, the function of *Par*-SFB_4 was equivalent to *Par*-SFB_{24}.

In addition, the segregation results of *Par-SFB*$_{24}$ and *Par-SFB*$_4$ alleles were consistent with their cognate *S-RNase* alleles in progenies of 'Harcot'×'Chuanzhihong' and 'Chuanzhihong'×'Harcot', respectively (Fig. . S4, S5). Thus, both *Par-SFB*$_{24}$ and *Par-SFB*$_4$ alleles were co-segregated with their cognate *S-RNase* alleles.

Discussion

The *S*-genotype of 'Chuanzhihong' is identified as S_8S_{24}, which is different from a previous result that identified it as $S_{12}S_{14}$ (Zhang et al. 2008a). The reason may be that two different cultivars were coupled with one nomination or nominated incorrectly when the cultivar was introduced from one place to another. The two

Table 1 Frequency of fruit set in self- or cross-pollination of three apricot cultivars

Selfed or cross-pollinated	Number of flowers pollinated	Number of fruit set	Frequency of fruit set (%)
"Harcot" (S_1S_4) selfed	503	3	0.57
"Chuangzhihong" (S_8S_{24}) selfed	614	0	0
"Harcot" (S_1S_4)×"Chuangzhihong" (S_8S_{24})	1,302	147	11.29
"Chuangzhihong" (S_8S_{24})×Harcot (S_1S_4)	1,513	346	22.87

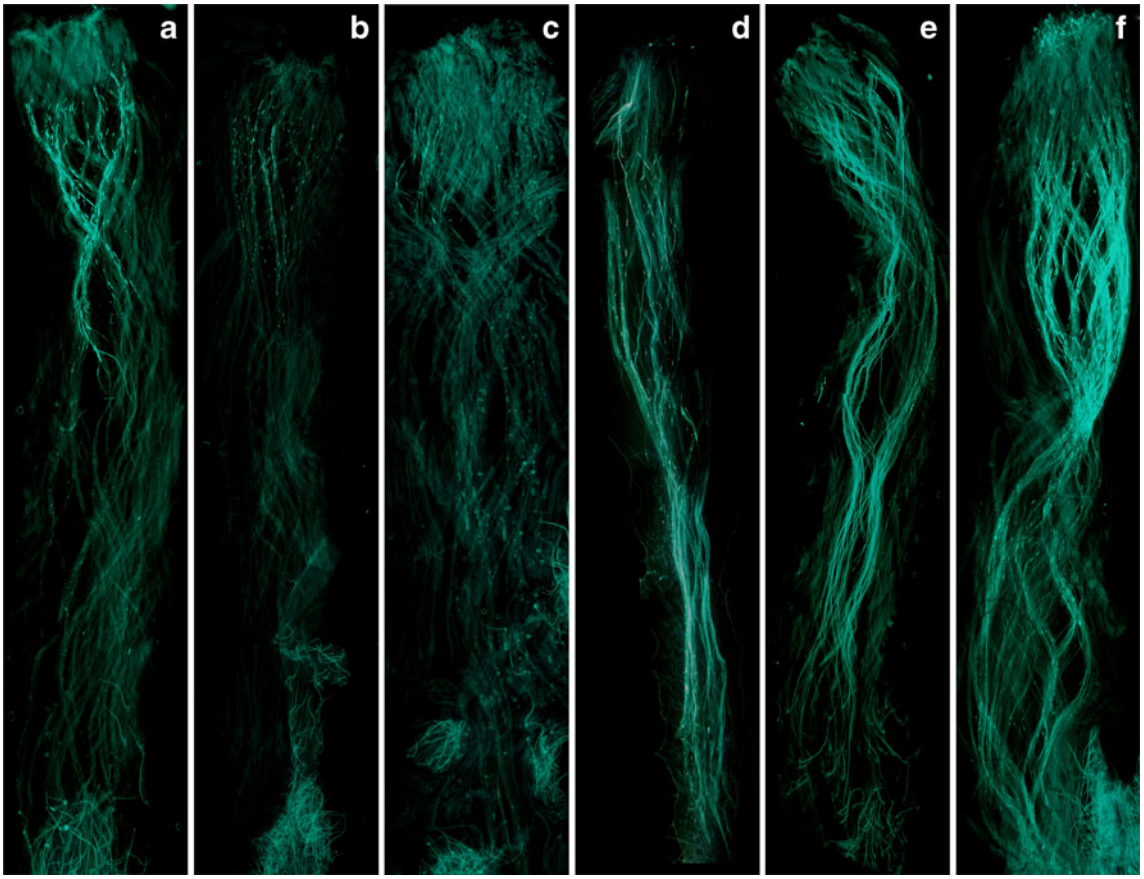

Fig. 4 Pollen tube growth 72 h after self-, cross-, and open-pollination. **a** Self-pollination of 'Chuanzhihong'. **b** Self-pollination of 'Harcot'. **c** Cross-pollination of 'Chuanzhihong' × 'Harcot'. **d** Cross-pollination of 'Harcot' × 'Chuanzhihong'. **e** Natural pollination of 'Chuanzhihong'. **f** Natural pollination of 'Harcot'

genes in *P. armeniaca* S_{24}-haplotypes were identified and isolated initially from genomic DNA of 'Chaoxian', 'Chaoxian 1' and 'Zhanggongyuan' (Wu et al. 2009), The full-length alleles were obtained by PCR with the primers PMT2 and M13-20 from stylar and pollen cDNA of 'Chuanzhihong', respectively. Surprisingly, *Par-SFB*$_{24}$ differed in only two amino acids from *Par-SFB*$_4$, but their cognate *S-RNase* alleles were identical at the amino acid level. The two genes in *P. armeniaca* S_{24}- and S_4-haplotypes were transmitted to the two cross-pollinated progenies (Table 2), and specifically expressed in style and pollen, respectively (Fig. 3).

In order to test the function of *Par-SFB*$_{24}$ and *Par-SFB*$_4$, pollen tube growth assays and fruit sets of self-pollination showed that 'Chuanzhihong' and 'Harcot' were self-incompatible (Fig. 4a, b; Table 1), which elucidate that the *P. armeniaca* S_{24}- and S_4-haplotypes confer self-incompatibility. Pollen tube growth assay of intercross-pollinations, which indicated that the two cultivars were semi-compatible with each other (Fig. 4c, d); the

Table 2 Segregation of *S*-genotypes in progenies of different crosses

Crosses	*S*-genotypes observed in progeny		Total	Segregation ratio	χ^2	$\chi^2_{0.05,\lambda}$
'Harcot' (S_1S_4) × 'Chuangzhihong' (S_8S_{24})	S_1S_8	S_4S_8		1:1[a]	1.260	3.84
	42	54	96	1:1[b]		
'Chuangzhihong' (S_8S_{24}) × 'Harcot' (S_1S_4)	S_1S_8	S_1S_{24}		1:1[a]	0.283	3.84
	67	60	127	1:1[b]		

[a] Theoretical ratios

[b] Expected ratios

[c] Degrees of freedom

precondition was that self-pollination of 'Chuanzhihong' and 'Harcot' were incompatible (Fig. 4a, b), and natural pollination were fully compatible (Fig. 4e, f). Moreover, genetic analysis of intercrosses showed that the *S*-genotype of the progenies segregated into two classes (1:1), and fitting the expected ratio (Table 2; Fig. S4, S5). Thus, *Par*-SFB_{24} had the same function as *Par*-SFB_4. Pollen grains of *P. armeniaca* S_{24} and S_4 were not transmitted to the two intercross-pollinated progenies also indicated that *Par*-S_{24} and *Par*-S_4 alleles have the same function too. Based on these data, we determined that the function of the S_{24}-haplotype of *P. armeniaca* was identical to that of the S_4-haplotype of *P. armeniaca*. Thus, these two *S*-haplotypes were neutral variants of the same *S*-haplotype.

The evolution of *S*-allelic diversity is still debated by many geneticists. A new *S*-allele specificity may have arisen initially through mutations in the pollen-*S* gene, and then corresponding mutations occurred in the stylar-*S* gene to maintain SI (Uyenoyama and Newbigin 2000). This viewpoint was supported by Chookajorn et al. (2004) where they found that pollen-*S* specificity could tolerate a variety of induced amino acid changes and domain swapping without the loss of a functional SI response; Matton et al. (1999; 2000) also predicted that a new *S*-allele came about from a series of intermediate steps and without the loss of SI, such as dual *S*-specificity. Although a dual *S*-specificity-haplotype has not been found until now, that two *S*-haplotypes shared one *S-RNase* allele, and different *SFB* alleles have been detected in *Prunus* species (Tao et al. 2007). This evidence supported the inference that *SFB* alleles are more plastic, and can tolerate a greater degree of variation without breaking down the SI (Sutherland et al. 2008). However, as two different *Prunus* species can not intercross, the function of the two genes in different *S*-haplotypes with exceptionally high sequence identities could not be tested by intercross pollination (Šurbanovski et al. 2007; Sutherland et al. 2008; Tao et al. 2007).

In the present study, the two neutral variants of the same *S*-haplotype segregated from *P. armeniaca*, which have two changes between two *SFBs* (Fig. 1) and share one *S-RNase* allele (Fig. S1), at the amino acid level. This is an example for plastic *Prunus SFB* alleles. However, when comparing the two genes in *P. armeniaca* S_{24}- and S_4-haplotypes at the nucleotide acid level, the differences between the two *S-RNase* alleles were greater than between the two *SFB* alleles. Two point mutations and two indels found in the two introns (Ka=0.0655; Fig. 2b, c) and one synonymous substitution occurred in the coding region of the two *S-RNase* alleles (Ka=0, Ks=0.001; Fig. S3), while only two non-synonymous substitutions occurred in the two *SFB* alleles (Ka=0.0016, Ks=0; Fig. S1). Similar results were also discovered in the *Prunus S*-haplotype, which shares one *S-RNase* allele and has different *SFB* alleles (Šurbanovski et al. 2007; Sutherland et al. 2008; Tao et al. 2007). The reason is that the two introns of *Prunus S-RNase* alleles could incorporate numerous mutations that then reduce the number of non-synonymous substitutions in coding regions, while the single intron of *SFB* alleles, which is located upstream of the start codon, incorporated longer insertions with lower efficiency (Vaughan et al. 2006); thus the number of non-synonymous substitutions in the coding region was larger than in the *S-RNase* alleles. This inference could be used to explain why the different *Prunus SFB* alleles have the chance to match the same *S-RNase* in the GSI reaction, and thus, this function of pollen-*S* would allow the formation of new *S*-specificity without the loss of GSI (Šurbanovski et al. 2007; Sutherland et al. 2008).

Matton et al. (1997, 1999) revealed that *Solanum chacoense* S_{13}*-RNase* allele could maintain SI when either six or seven residues are changed. Moreover, the S_{11}*-RNase* allele of *S. chacoense* could also maintain SI when three residues located within hypervariable regions were changed. Sanzol (2010) described two neutral variants from two self-incompatible European pears, which have two changes between the two *S-RNase* alleles, at the amino acid level. These results suggest that *S-RNase* alleles are dynamic, and can tolerate degrees of variation without breaking down SI. In *Prunus* species, two *S-RNase* alleles, with a maximum of seven different residues, have been discovered in many plants (Šurbanovski et al. 2007; Sutherland et al. 2008; Tao et al. 2007). *Par*-S_{24} and *Par*-S_4 has a single different residue with *Pte*-S_1 and three different residues with *Pmi*-S_1 (Fig. 2a). It is likely that these four *S-RNase* alleles have the same function in a GSI reaction. At the nucleotide level, 15 point mutations have been detected in the two introns (Ka=0.0446; Fig. 2b, c). Among those, a single synonymous and three non-synonymous substitutions have been found in the coding region of *Par*-S_{24} and *Pte*-S_1 (Ka=0.002, Ks=0.0059; Fig. S3); 11 point mutations have been detected in the two introns (Ka=0.0327; Fig. 2b, c), and three synonymous and eight non-synonymous substitutions must have occurred in the coding regions of *Par*-S_{24} and *Pmi*-S_1 (Ka=0.0157, Ks=0.0059; Fig. S3). Thus, the inference that the two introns of *S-RNase* alleles could incorporate many mutations thus reducing the number of non-synonymous substitutions in coding regions is tenable in *Prunus* species. However, *Pte*-SFB_1 and *Pmi*-SFB_1 have not been found in GenBank, which is inconvenient as they cannot be used to analyze the process of *Prunus S*-allele evolution. If the models of Uyenoyama and Newbigin (2000) and Matton et al. (1999, 2000) fit with *Prunus S*-alleles, the *Par*-SFB_{24} or *Par*-SFB_4 would share at least two and three different residues, respectively, with *Pte*-SFB_1 and *Pmi*-SFB_1.

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