Recognition specificity of self-incompatibility in *Pyrus* and *Malus*

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Abstract *Pyrus* displays gametophytic self-incompatibility controlled by a single highly polymorphic gene complex termed *S* locus, which comprises a stylar-expressed gene (*S*-RNase) tighlty linked with a pollen expressed gene, that determines the specificity of the self-incompatibility locus. Deduced amino acid sequence of 'Meigetsu' S_8-RNase in *Pyrus pyrifolia* and 'Kuerlexiangli' S_{28}-RNase in *P. sinkiangensis* showed 100% identity. S_3-RNase in *Malus spectabilis* was also found to be similar to S_8-RNase in *P. pyrifolia* with 96.9% identity in the deduced amino acid sequence. The intron, which is generally highly polymorphic between alleles, was also remarkably well conserved within these allele pairs. The intron of PpS_8-RNase showed 95.3 and 91.9% identity with PsS_{28}-RNase and MsS_3-RNase, respectively. Pollen tube growth in styles, pollen tube length in artificial media containing different S-RNases and segregation of *S* haplotypes in F_1 plants revealed commonality of the recognition specificity between PpS_8-RNase and PsS_{28}-RNase and between PpS_8-RNase and MsS_3-RNase. Results suggested that PpS_8-RNase, PsS_{28}-RNase and MsS_3-RNase have maintained the same recognition specificity after the divergence of the two species and that amino acid substitutions found between PpS_8-RNase and MsS_3-RNase do not alter the recognition specificity.

Keywords *Pyrus* · *Malus* · Self-incompatibility (SI) · S-RNase · Recognition specificity · Trans-specific evolution

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Introduction

Self-incompatibility (SI) is a genetically determined system for recognition and rejection of self-pollen. The predominant form of SI is a gametophytic SI (GSI) system, in which pollen-tube growth is inhibited when an *S*-haplotype of pollen matches one of the *S*-haplotypes of the pistil. 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.

For most genes, new alleles can arise from the progressive accumulation of non-synonymous point mutations. Generation of new S-alleles is unlikely to be so straightforward, because of the co-adaptation of the two genes at the S-locus in S-RNase-based SI (Lewis 1949). Correct function of the S-locus depends upon interaction of stylar-S and pollen-S gene products in pollen tubes. Thus the generation of a new S-allele specificity requires complementary mutations in both stylar-S and pollen-S components. Mutation at only one or other component could breakdown the SI system, leading to self-compatibility, as demonstrated by the chimeric S-RNase experiments of Zurek et al. (1997).

Within plant families, S-RNases often show interspecific shared polymorphism, in which alleles from different species can be more similar than alleles of the same species (Ioerger et al. 1990). As a consequence of balancing selection, individual S-RNase lineages can persist for very long times in a population and in the subsequent species. Thus very similar S-haplotypes can be shared by relatively divergent species (Richman and Kohn 1996).

Exceptionally close identities between S-receptor kinase (SRK) and S-locus protein 11/S-locus cysteine-rich protein ($SP11/SCR$) alleles have been reported previously in Brassica oleracea and B. rapa (Kusaba et al. 1997, 2000; Kusaba and Nishio 1999; Kimura et al. 2002; Fujimoto et al. 2006) and the recognition specificity has been confirmed by cross pollination (Kimura et al. 2002; Sato et al. 2003). In the Rosaceae, S-RNase alleles of S_6 and S_{11} in Prunus dulcis showed 98 and 100% DNA sequence identity with MRSN-2 in Prunus mume and S_1-RNase in Prunus avium, respectively (Ortega et al. 2006). The S-RNase allele of S_8 in the dwarf almond Prunus tenella had 100% polypeptide identity with Prunus avium S_1-RNase and differed by one amino acid with S_{11}-RNase in Prunus dulcis (Surbanovski et al. 2007).

In the two plum species, six S-RNases have been identified with very high sequence identity to those of other Prunus spp., far higher than similarities typically observed in interspecific comparisons. High sequence identities of their corresponding pollen-S gene (S haplotype-specific F-box protein gene, SFB) have also been revealed by comparison with those in databases (Sutherland et al. 2008).

In this study, two pairs with high sequence similarities, S_8-RNase (accession no. AB104908) in Pyrus pyrifolia (PpS_8-RNase) with S_{28}-RNase (accession no. EF566872) in P. sinkiangensis (PsS_{28}-RNase); and S_8-RNase in P. pyrifolia with S_3-RNase (accession no. FJ943268) in Malus spectabilis (MsS_3-RNase), were identified. To elucidate whether PpS_8-RNase/PsS_{28}-RNase and PpS_8-RNase/MsS_3-RNase have the same recognition specificity in the pollen and stigma, we carried out interspecific pollination followed by microscopic observation of pollen-tube growth in styles, in vitro culture of pollen in liquid media containing different S-RNases, and segregation tests of S-haplotypes in F_1 plants to test recognition specificity.

Materials and methods

Plant material

Pyrus sinkiangensis cv. 'Kuerlexiangli', P. pyrifolia cvs. 'Meigetsu', 'Imamuraaki', and 'Hosui', and Malus spectabilis cvs. 'Haitang No.1', 'Haitang No.3' and 'Haitang No.7' were used for nucleotide sequence analysis and investigation of recognition specificity. Of 'Kuerlexiangli' × 'Meigetsu', 44 F_1 progenies and 45 F_1 progenies of 'Meigetsu' × 'Kuerlexiangli' were used for genetic analysis. All materials were collected from the Research Institute of Pomology, Chinese Academy of Agricultural Sciences, Xingcheng, Liaoning, China.

DNA and RNA extraction

Total genomic DNAs were isolated from young leaves using the CTAB method (Doyle and Doyle 1987) and treated with RNase (TaKaRa, Kyoto, Japan). Total RNAs from 'Kuerlexiangli', 'Meigetsu' and 'Haitang No.3' were isolated from young frozen pistil tissue collected from flower buds at the 'balloon stage' according to Ikoma et al. (1996). mRNAs were isolated from total RNA using the Micro-FastTrack™ 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

PCR amplification of the intron of S-alleles and sequence analysis of genomic S-RNases

PCR to identify the intron of S-RNase alleles in Pyrus and Malus was carried out using the consensus primer

pair PF and PR (Ishimizu et al. 1999), which have sequences of C1 and a region downstream of C2 of S-RNase, respectively (Supplementary table 1). PCR mixture (25 μL) contained 2.5 μL 10× reaction buffer (100 mM Tris–HCl pH 8.3, 500 mM KCl and 2.5 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 μM forward and reverse primers, 1.0 units *Taq* polymerase (TaKaRa), and 25–50 ng genomic DNA. Amplification was conducted in a thermal cycler PTC-200 (Bio-Rad, Foster, CA, USA) with the following cycle parameters: 3 min at 94°C for pre-denaturation; followed by 10 cycles of 30 s at 94°C, 30 s at 50°C and 45 s at 70°C; followed by 25 cycles of 30 s at 94°C, 30 s at 48°C and 45 s at 70°C; with a final extension of 7 min at 70°C. The amplified products were separated by electrophoresis on 1.5% (w/v) agarose gel.

PCR products were cloned into pGEM-T (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cloned PCR products were sequenced with a 3730 sequencer (Invitrogen). Nucleotide sequences were determined using DNASTAR software (Madison, WI, USA), and homology searches used the BLASTN program at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

3′- and 5′-RACE cloning

mRNAs were reverse-transcribed for 1 h at 42°C to synthesize first-strand cDNA with the adapter primer *Not*I-$(dT)_{18}$ (Takasaki et al. 2004). The 3′-region of cDNAs encoding S-RNase was amplified with *Not* I and PpS_8-, PsS_{28}- and MsS_3-specific primers designed from the region between exons C2 and C3 of the PpS_8-, PsS_{28}- and MsS_3-RNase genes, respectively (Supplementary table 1). The 5′-ends of the cDNAs were amplified using 5′-RACE System 2.0 (Invitrogen, Carlsbad, CA, USA) with reverse primers specific to PpS_8-, PsS_{28}- and MsS_3-RNase, respectively (Supplementary table 1).

Estimation of S-RNase concentration in the style

Style S-RNase from each cultivar was extracted and partially purified according to Hiratsuka et al. (2001) with some modifications. All procedures were carried out at 4°C. Of styles, about 4 g were ground in liquid nitrogen with a mortar and pestle. Then 170 ml of 50 mM MES/NaOH extraction buffer (pH 6.5)

containing 5 mM Na_2EDTA, 1.5% sodium ascorbate, and 3% polyvinylpolypyrrolidone (Polyclar-AT, Sigma Chemical, St. Louis, Mo, USA) were added immediately, the whole stirred for 30 min and then centrifuged at 16,000*g* for 10 min. The supernatant was collected for further purification. The pellet and beaker used in the experiment were washed again with 40 ml of the same buffer, centrifuged, and the supernatant combined with the S-RNase sample collected before. The sample was filtered with a Millipore membrane (0.45 μm, Millipore, Mass, USA) and then passed through a carboxymethyl (CM) Sepharose column (20 × 1.6 cm) to adsorb S-RNase. The column was washed with 50 ml of 50 mM MES/NaOH buffer to elute the acid S-RNase and the adsorbed alkalescent S-RNases were eluted with 100 ml of 350 mM NaCl dissolved in 50 mM MES/NaOH buffer. The eluate was saturated with $(NH_4)_2SO_4$ and then kept stable for 30 min, precipitated by centrifugation at 20,000*g* for 10 min. The sediment was washed with 1 ml of 50 mM MES/NaOH buffer and transferred into a 1.5-ml Eppendorf tube, centrifuged at 20,000*g* for 15 min. The sediment was redissolved with 50 μL of 50 mM MES/NaOH buffer and dialyzed overnight in the same buffer to remove $(NH_4)_2SO_4$. This S-RNase sample was stored in Eppendorf tubes at −80°C until use. S-RNase concentration was determined by the method of Bradford (1976).

Pollination tests and pollen-tube growth tests

For the 'Kuerlexiangli' × 'Meigetsu' and 'Meigetsu' × 'Kuerlexiangli' crosses, approximately 100 flowers at the balloon stage (1–2 days before anthesis) were emasculated, pollinated with pollen of tester cultivars, and covered with paper bags to avoid contamination. Subsequent fruits were collected about 3 months later.

Pollen-tube growth was observed after cross-pollinations between 'Kuerlexiangli', the female parent, and 'Kuerlexiangli', 'Meigetsu' and 'Hosui', as male parents. The cross-pollinations between 'Meigetsu', as female parent, and 'Meigetsu', 'Haitang No.3', 'Imamuraaki' and 'Hosui', were also performed. Pistils were harvested 72 h after self- or cross-pollination, and fixed in 1:1:18 (v/v/v) (formalin : acetic acid : 70% ethanol), washed thoroughly under running tap water, and incubated in 2 M NaOH for 3 h to soften the tissues.

The styles were then soaked overnight in 0.1% (w/v) aniline blue solution in 0.1 M K_3PO_4 at 60°C in darkness. Pollen tubes in washed and squashed styles were observed by ultraviolet fluorescent microscopy (BX60; Olympus, Tokyo, Japan).

Preparation of liquid medium and bioassay

The liquid media were prepared according to Hiratsuka et al. (2001) with the S-RNase concentration at 1.2 μg μl^{-1} for culturing pollen of 'Meigetsu'. Percentage germination and pollen-tube length were determined under a light transmission microscope. For germination, >100 pollen grains were observed; and the lengths of >10 pollen tubes measured for each treatment. Each treatment was repeated three times.

Results

Sequence analysis of the S_8-RNase in *P. pyrifolia* and S_{28}-RNase in *P. sinkiangensis*

Two DNA fragments were amplified from genomic DNA of *P. pyrifolia* cv. 'Meigetsu' and *P. sinkiangensis* cv. 'Kuerlexiangli' by PCR using a primer set, PF and PR, which have sequences of the first and second exons including the RHV region, respectively. Nucleotide sequences of the amplified DNA fragments were determined and, based on deduced amino acid

sequences, PpS_8-RNase and PsS_{28}-RNase were identified.

To determine the full-length cDNA sequences of PpS_8-RNase and PsS_{28}-RNase, 3′-RACE and 5′-RACE were carried out using mRNA extracted from pistils of 'Meigetsu' and 'Kuerlexiangli', respectively. Both PpS_8-RNase and PsS_{28}-RNase sequences contained an open reading frame (ORF) of 684 nucleotides encoding 228 amino acid residues (Fig. 1a). PpS_8-RNase was identical with PsS_{28}-RNase in both nucleotide and deduced amino acid sequences. PpS_8-RNase and PsS_{28}-RNase were predicted to have a putative signal peptide from amino acids 1–27 by SignalP V2.0 (Nielsen et al. 1999).

Comparison of the cDNA sequences with the genomic sequences revealed the size of the introns was 234 and 227 bp in PpS_8-RNase and PsS_{28}-RNase, respectively. The intron sequences showed high similarity (95.3%) with only 4 bp substitutions and 7 bp indels (insertions/deletions) (Fig. 2a).

In vivo and in vitro pollen tube growth of 'Meigetsu' inhibited by PsS_{28}-RNase

In self-pollinated pistils of 'Kuerlexiangli', the pollen germinated well (Fig. 3a), but small numbers of pollen tubes penetrated only one-third into the style (Fig. 3d). Pollen-tube growth was arrested within the upper two-thirds of the style (Fig. 3g). In a cross between 'Kuerlexiangli' (PsS_{22}/PsS_{28}) and 'Meigetsu' (PpS_1/PpS_8), 'Meigetsu' pollen tubes could grow
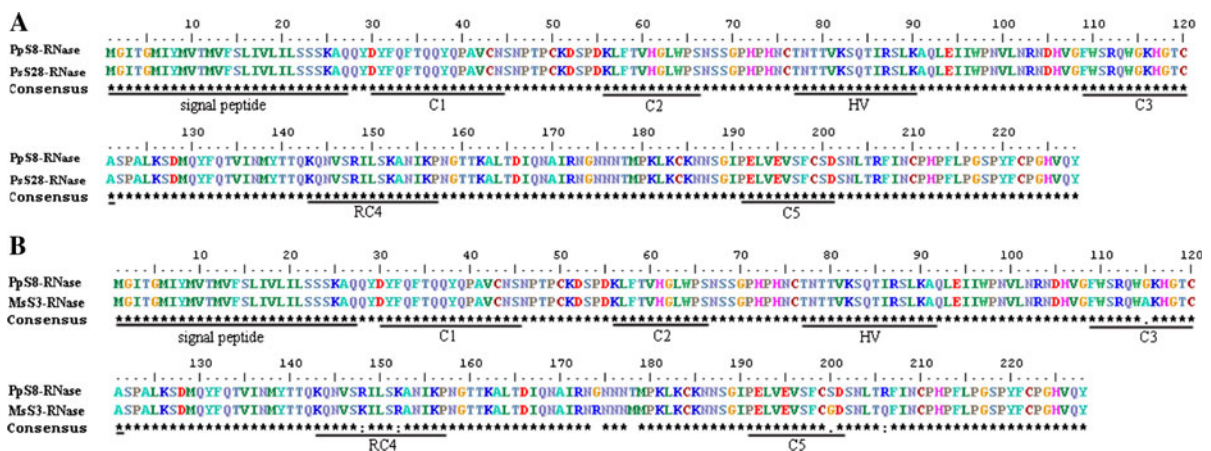

Fig. 1 Comparison of the deduced amino sequences PpS_8-RNase with PsS_{28}-RNase (**a**) and PpS_8-RNase with MsS_3-RNase (**b**), respectively. The putative signal peptide region, a hypervariable (*HV*) region, and five conserved regions (*C1*, *C2*, *C3*, *RC4* and *C5*) described for the Rosaceae (Ushijima et al. 1998) are *underlined*
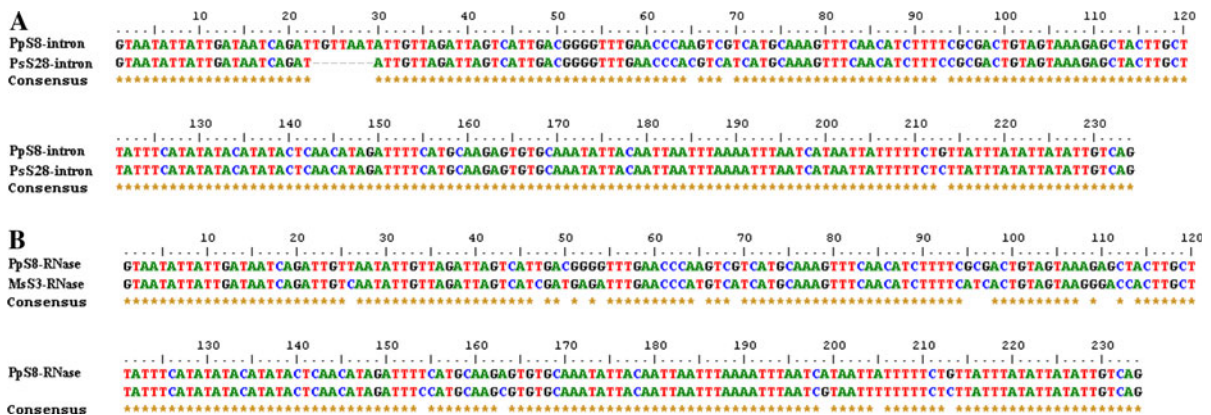
A

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              10        20        30        40        50        60        70        80        90       100       110       120
PpS8-intron   GTAATATTATTGATAATCAGATTGTTAATATTGTTAGATTAGTCATTGACGGGGTTTGAACCCAAGTCGTCATGCAAAGTTTCAACATCTTTTCGCGACTGTAGTAAAGAGCTACTTGCT
PsS28-intron  GTAATATTATTGATAATCAGAT------ATTGTTAGATTAGTCATTGACGGGGTTTGAACCCACGTCATCATGCAAAGTTTCAACATCTTTTCGCGACTGTAGTAAAGAGCTACTTGCT
Consensus     *******************         ************************************ *** ***************************** ***************

              130       140       150       160       170       180       190       200       210       220       230
PpS8-intron   TATTTCATATATACATATACTCAACATAGATTTTCATGCAAGAGTGTGCAAATATTACAATTAATTTAAAATTTAATCATAATTATTTTTCTGTTATTTATATTATATTGTCAG
PsS28-intron  TATTTCATATATACATATACTCAACATAGATTTTCATGCAAGAGTGTGCAAATATTACAATTAATTTAAAATTTAATCATAATTATTTTTCTCTTATTTATATTATATTGTCAG
Consensus     ********************************************************************************************** *****************
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B

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              10        20        30        40        50        60        70        80        90       100       110       120
PpS8-RNase    GTAATATTATTGATAATCAGATTGTTAATATTGTTAGATTAGTCATTGACGGGGTTTGAACCCAAGTCGTCATGCAAAGTTTCAACATCTTTTCGCGACTGTAGTAAAGAGCTACTTGCT
MsS3-RNase    GTAATATTATTGATAATCAGATTGTCAATATTGTTAGATTAGTCATCGATGAGAGTTTGAACCCATGTCATCATGCAAAGTTTCAACATCTTTTCATCACTGTAGTAAGGGGACCACTTGCT
Consensus     ************************* ********************* ** * ******** *** ****************** ***** * *

              130       140       150       160       170       180       190       200       210       220       230
PpS8-RNase    TATTTCATATATACATATACTCAACATAGATTTTCATGCAAGAGTGTGCAAATATTACAATTAATTTAAAATTTAATCATAATTATTTTTCTGTTATTTATATTATATTGTCAG
              TATTTCATATATACATATACTCAACATAGATTTCCATGCAAGCGTGTGCAAATATTACAATTAATTTAAAATTTAATCGTAATTTTTTTTCTCTTATTTATATTATATTGTCAG
Consensus     ********************************* ********* *********************************** ***** ******* *****************
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Fig. 2 Comparison of the intron sequences PpS_8-RNase with PsS_{28}-RNase (**a**) and PpS_8-RNase with MsS_3-RNase (**b**), respectively

the full length of 'Kuerlexiangli' style (Fig. 3h), but about half were arrested in the middle of the style (Fig. 3e). In hybridization of 'Kuerlexiangli' and 'Hosui' (PpS_3/PpS_5), most 'Hosui' pollen tubes reached the base of the 'Kuerlexiangli' style (Fig. 3i).

In liquid media, both pollen germination and pollen-tube elongation of 'Meigetsu' were inhibited by addition of S-RNase of 'Meigetsu'. Pollen germination and pollen-tube elongation were 12.9% and 35.7 μm, respectively, while those of the controls without addition of S-RNase were 47.2% and 98.1 μm. The germination and tube elongation of 'Meigetsu' pollen in the media containing 'Kuerlexiangli' S-RNase were 25.2% and 68.4 μm, respectively, and those in media containing S-RNase of 'Imamuraaki'(PpS_1/PpS_6) were 21.3% and 60.6 μm. The germination and tube growth of 'Meigetsu' pollen were strongly inhibited by a mixture of S-RNases of 'Kuerlexiangli' and 'Imamuraaki', being 10.0% and 32.2 μm, respectively, comparable to those by S-RNase of 'Meigetsu'. In liquid media containing S-RNase of 'Hosui' (PpS_3/PpS_5), inhibition of germination and tube growth of 'Meigetsu' pollen were slight, 40.3% and 92.6 μm, respectively (Fig. 4).

Genetic analysis of F_1 progeny between 'Kuerlexiangli' and 'Meigetsu'

Since the S-genotypes of 'Kuerlexiangli' and 'Meigetsu' were PsS_{22}/PsS_{28} and PpS_1/PpS_8, respectively, the S-genotypes of F_1 progeny in the 'Kuerlexiangli' × 'Meigetsu' cross should segregate into four classes: PpS_1/PsS_{22}, PpS_1/PsS_{28}, PpS_8/PsS_{22} and PpS_8/PsS_{28}. However, S-genotypes of the F_1 progeny of 'Kuerlexiangli' × 'Meigetsu' were inconsistent with this hypothesis, and only PpS_1/PsS_{22} and PpS_1/PsS_{28} genotypes were observed with segregation ratio of 21:24, corresponding to 1:1 ($\chi^2 = 0.2$, $P < 0.05$). F_1 progeny of a reciprocal crossing, 'Meigetsu' × 'Kuerlexiangli', also produced two S-genotypes, PpS_1/PsS_{22} and PpS_8/PsS_{22}, with a segregation ratio of 22:22. These results showed that PpS_8 and PsS_{28} function as the same S-haplotype.

Sequence analysis of the PpS_8-RNase from *Pyrus* and MsS_3-RNase from *Malus*

Two different DNA fragments were amplified from genomic DNA of a *M. spectabilis* cv. 'Haitang No.3' (MsS_3/MsS_4) by PCR using the primer set, PF and PR. Based on sequence analysis, MsS_3-RNase was identified. To determine the full-length cDNA sequence encoding MsS_3-RNase, 3′-RACE and 5′-RACE were carried out using mRNA extracted from pistils of 'Haitang No.3'. The MsS_3-RNase cDNA sequence contained an ORF of 684 nucleotides encoding 228 amino acid residues. MsS_3-RNase showed 96.9% amino acid identity with PpS_8-RNase from the signal peptide region to the C-terminal region. Between MsS_3-RNase and PpS_8-RNase, there were seven different amino acid residues scattered throughout the sequences, three of which were one-conservative and two half-conservative replacements (Fig. 1b). The hypervariable regions, considered important for recognition specificity of S-RNase, were identical to each other.

Comparison of the cDNA sequence with the genomic sequence revealed the intron of 234 bp in the MsS_3-RNase allele. The intron sequence of the
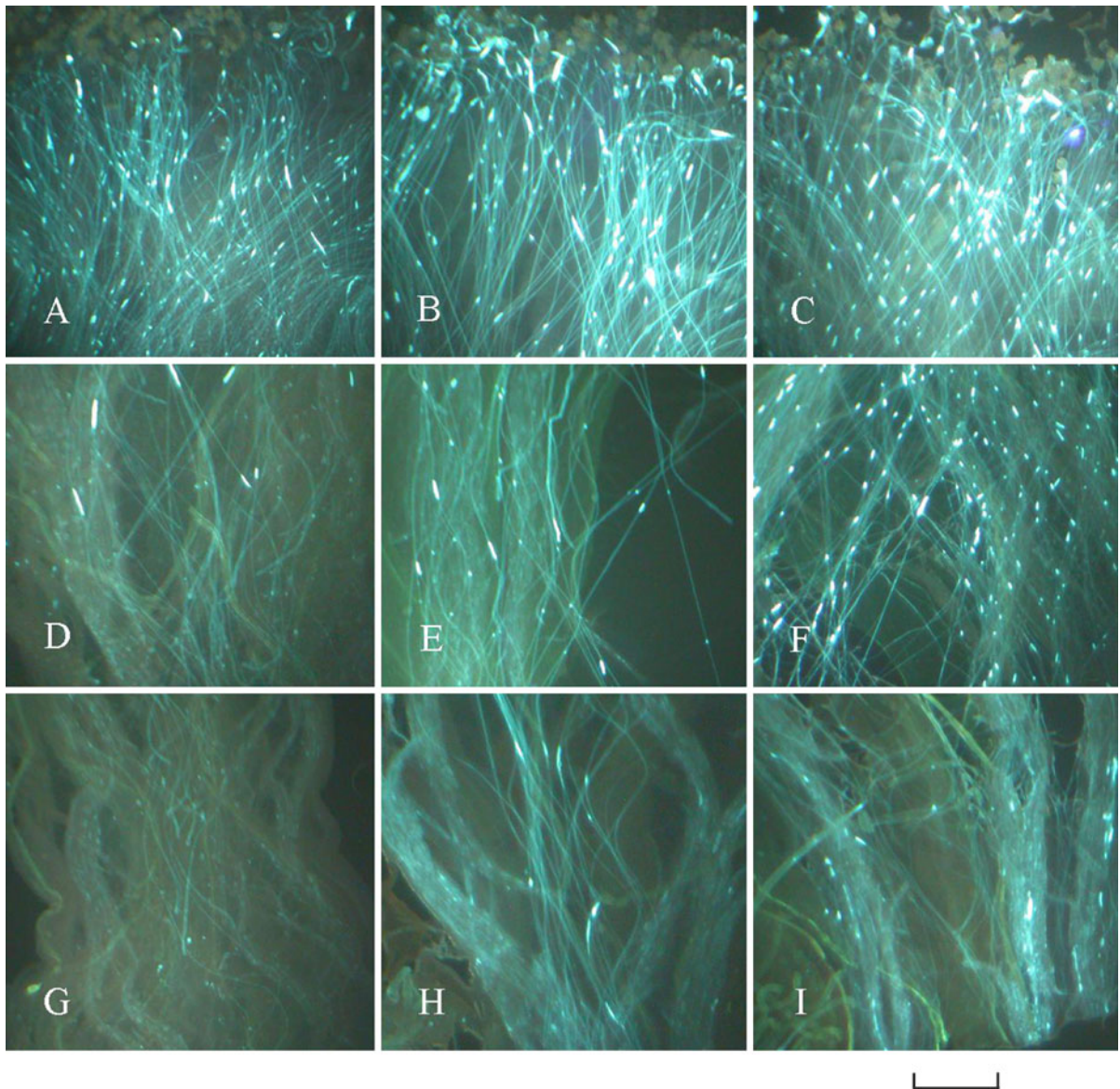
Fig. 3 Fluorescence microscopy of pollen-tube growth in styles of 'Kuerlexiangli': **a**, **d** and **g** self-pollination of 'Kuerlexiangli'; **b**, **e** and **h** cross-pollination of 'Kuerlexiangli' with pollen tubes from 'Meigetsu'; and **c**, **f** and **i** cross-pollination of 'Kuerlexiangli' with pollen tubes from 'Hosui'. *Scale bar* indicates 50 μm. **a**, **b** and **c** the upper part of the style; **d**, **e** and **f** the one-third of the style; and **g**, **h** and **i** the base of the style

MsS_3-RNase allele showed high similarity (91.9%) to the PpS_8-RNase allele with only 19 bp substitution (Fig. 2b).

Pollen tube growth of 'Meigetsu' inhibited by MsS_3-RNase

In self-pollinated pistils of 'Meigetsu', pollen-tube growth was arrested in the upper one-third of the style (Fig. 5f). In crosses with 'Haitang No.3' (MsS_3/MsS_4) and 'Imamuraaki' (PpS_1/PpS_6), both 'Haitang No.3' and 'Imamuraaki' pollen tubes could grow the full length of the 'Meigetsu' style (Fig. 5h, i), with about half of pollen tubes arrested in the upper one-third of styles (Fig. 5g, h). In the 'Meigetsu' × 'Hosui' cross, most 'Hosui' pollen tubes reached the base of the 'Meigetsu' style (Fig. 5n). In the 'Meigetsu' style hybridized with 'Haitang No.7' (MsS_2/MsS_5),
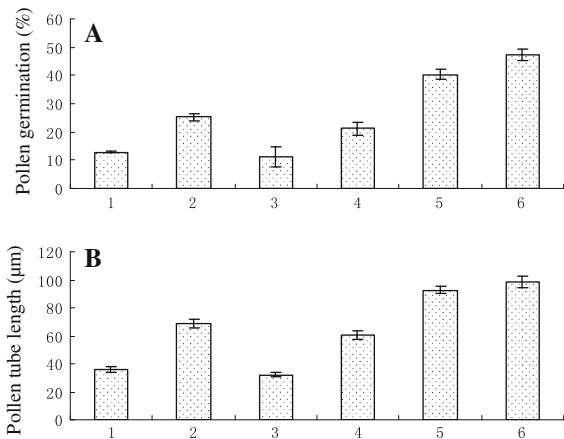
Fig. 4 Effect of different *S*-RNases on **a** pollen germination and **b** pollen-tube growth in vitro *S*-RNases added to the liquid media are of: *1* 'Meigetsu', *2* 'Kuerlexiangli', *3* 'Kuerlexiangli' and 'Imamuraaki', *4* 'Imamuraaki', *5* 'Hosui', and *6* Control

the pollen germination rate on the stigma was low because of low viability of 'Haitang No.7' pollen grains (Fig. 5e). Despite that, most pollen tubes penetrated the middle and reached the bottom of the 'Meigetsu' style, similar to that of 'Hosui' (Fig. 5j, o).

Germination and tube elongation of 'Meigetsu' pollen in liquid media containing *S*-RNase of 'Haitang No.3' were 24.5% and 50.7 μm, respectively, and those in media containing 'Imamuraaki' *S*-RNase were 20.3% and 46.6 μm. In liquid media containing *S*-RNases of both 'Haitang No.3' and 'Imamuraaki', the germination and tube growth of 'Meigetsu' pollen were strongly inhibited, being 7.9% and 33.8 μm, respectively; comparable with those with *S*-RNase of 'Meigetsu'. *S*-RNases of 'Hosui' and 'Haitang No.1' (MsS_1/MsS_2) did not affect germination and tube growth of 'Meigetsu' pollen (Fig. 6).
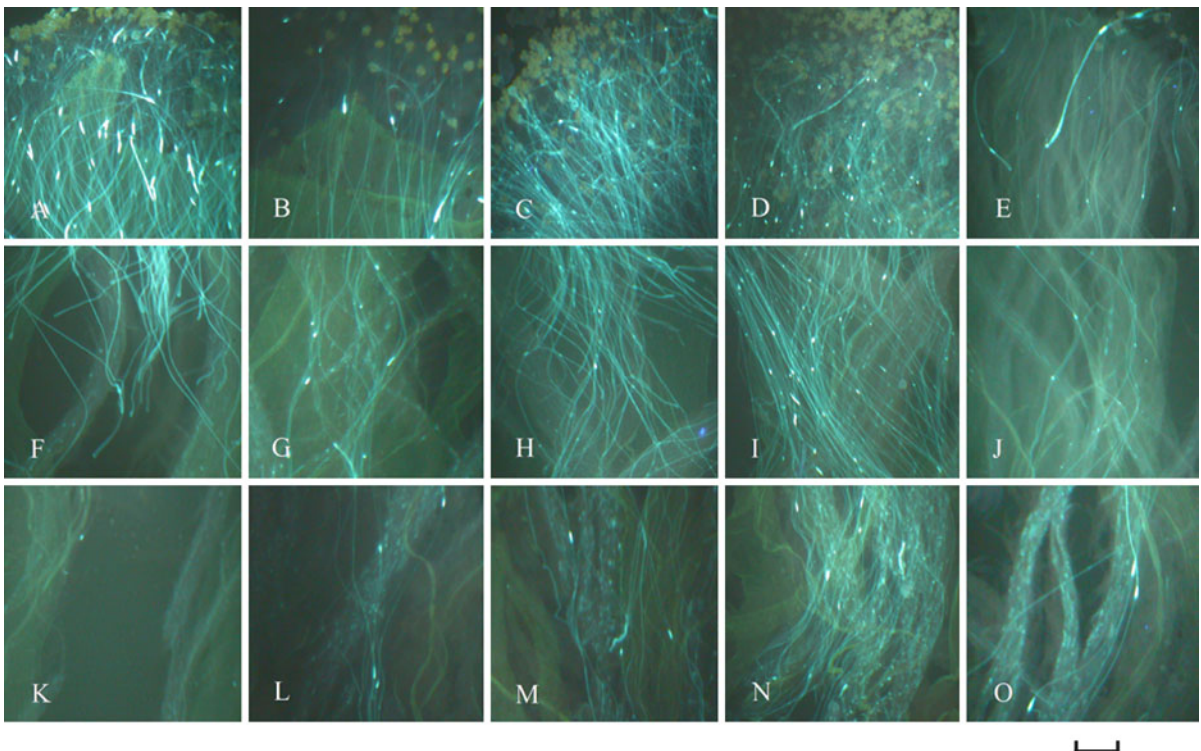

Fig. 5 Fluorescence microscopy of pollen-tube growth in styles of 'Meigetsu': **a**, **f** and **k** self-pollination of 'Meigetsu'; **b**, **g** and **l** cross-pollination of 'Meigetsu' with pollen tubes from 'Haitang No.3'; **c**, **h** and **m** cross-pollination of 'Meigetsu' with pollen tubes from 'Imamuraaki'; **d**, **i** and **n** cross-pollination of 'Meigetsu' with pollen tubes from 'Hosui'; and **e**, **j** and **o** cross-pollination of 'Meigetsu' with pollen tubes from 'Haitang No.7'. **a**, **b**, **c**, **d** and **e** the upper part of the style; **f**, **g**, **h**, **i** and **j** the one-third of the style; and **k**, **l**, **m**, **n** and **o** the base of the style. *Scale bar* indicates 50 μm
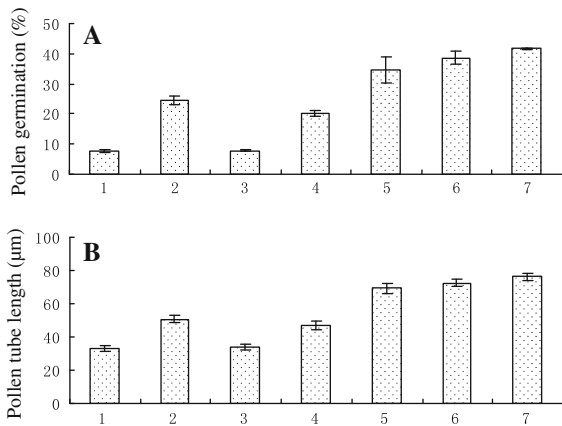
Fig. 6 Effect of different S-RNases on 'Meigetsu' **a** pollen germination and **b** pollen-tube growth in vitro. S-RNases added to the liquid media are of: *1* 'Meigetsu', *2* 'Haitang No.3', *3* 'Imamuraaki' and 'Haitang No.3', *4* 'Imamuraaki', *5* 'Hosui', *6* 'Haitang No.1', and *7* Control

Discussion

There were no major barriers to hybridization between 'Meigetsu' and 'Haitang No.3'. *Pyrus* probably originated in the foothills of the Tian Shen mountains in Xinjiang in western China, with isolation and adaptation after spreading eastward and westward, leading to speciation. By current consensus the genus is classified into 21–26 primary species, including *P. bretschneideri*, *P. pyrifolia*, *P. sinkiangensis*, *P. ussuriensis* and *P. communis*. Reciprocal crosses between *Pyrus* spp. have been successful (Challice and Westwood 1973; Bell and Hough 1986). Intergeneric hybridization is evidence of the close relationship between *Pyrus* and *Malus* (Crane and Marks 1952), as is pollen-tube elongation of 'Haitang No.3' and 'Haitang No.7' in the styles of 'Meigetsu' in the present study.

The PpS_8 haplotype in *P. pyrifolia* 'Meigetsu' was shown to have the same recognition specificity as the PsS_{28} haplotype in *P. sinkiangensis* 'Kuerlexiangli' and the MsS_3 haplotype in *M. spectabilis* 'Haitang No.3', respectively. This was demonstrated by the methods of interspecific hybridization and cultivation of pollen in vitro. It is considered that the pairs of PpS_8PsS_{28} and PpS_8MsS_3 have maintained the same recognition specificity since the divergence of species and genera, and that the amino acid substitutions have no influence on recognition specificity. In *Prunus*, *Prunus tenella* S_8-RNase has 100% identity with

Prunus avium S_1-RNase, while 12 residues differ in *SFB* sequences at the protein level. Pollination tests have demonstrated that the SI function of *Prunus tenella* S_8 and *Prunus avium* S_1 haplotypes was not disturbed (Surbanovski et al. 2007). In several interspecific pairs of S-haplotypes identified between *Brassica oleracea* and *B. rapa* showing high similarities (<100%) identities in *SRK* and *SP11/SCR* sequences, the same recognition specificities have been demonstrated by interspecific hybridization, pollination tests with transgenic plants and bioassay using SP11 proteins (Kimura et al. 2002; Sato et al. 2003). These results indicated some flexibility of amino acid sequences in the specific recognition and that the different residues did not play a role in defining allelic specificities of the S-haplotypes.

The three S-haplotypes (i.e., PpS_8, PsS_{28} and MsS_3) exhibiting high sequence similarity of S-RNase and the same recognition specificity are considered to be derived from the same ancestral S-haplotype. There was higher sequence similarity between PpS_8-RNase and PsS_{28}-RNase than between PpS_8-RNase and MsS_3-RNase, indicating that diversification of PpS_8 and PsS_{28} haplotypes occurred after divergence of the MsS_3 haplotype, corresponding with divergence of species in *Pyrus* and *Malus* (Ishimizu et al. 1998; Takasaki et al. 2004). There are similar relationships of S-haplotypes in *Brassica* spp. and *Raphanus* spp., although the sequence similarities between these S-haplotypes are lower than those in *Pyrus* spp. and *Malus* spp. (Sato et al. 2004).

Uyenoyama et al. (2001) proposed that the evolution of S-specificities allows self-compatible intermediates, while the model proposed by Matton et al. (1999) predicted dual-specificity intermediates that are self-incompatible. Point mutation, recombination and gene conversion played an important role in the generation of new alleles. In this study, PpS_8-RNase, PsS_{28}-RNase or MsS_3-RNase might be intermediate steps to a new S-haplotype, without loss of SI. In fact, an S-haplotype could tolerate a small number of amino acid changes in the S-RNase or *SFB* and minor changes might broaden specificity. These incremental changes could in time give rise to a completely new specificity.

Alteration of SI strength might have occurred in evolution of S-haplotypes (Sato et al. 2004). In self-pollinated pistils of 'Kuerlexiangli', the pollen tubes could penetrate through the middle of style, reaching the bottom, but not be fertilized; while pollen tubes

were arrested at one-third down the style in self-pollinated pistils of 'Meigetsu'. The results suggested low SI strength in 'Kuerlexiangli' than in 'Meigetsu', possibly caused by changes in two S-haplotypes.

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