A Low Transcriptional Level of *S^e-RNase* in the *S^e*-haplotype Confers Self-compatibility in Japanese Plum

Akiko Watari, Toshio Hanada, Hisayo Yamane, Tomoya Esumi, and Ryutaro Tao¹

Laboratory of Pomology, Graduate School of Agriculture, Kyoto University, Sakyo, Kyoto 606-8502, Japan

Hideaki Yaegaki and Masami Yamaguchi

National Institute of Fruit Tree Science, Tsukuba, Ibaraki 305-8605, Japan

Kenji Beppu and Ikuo Kataoka

Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-0795, Japan

ADDITIONAL INDEX WORDS. gametophytic self-incompatibility, pistil, pollen, *Prunus salicina*, Rosaceae, SFB

ABSTRACT. Most commercial cultivars of Japanese plum (*Prunus salicina* Lindl.) exhibit S-RNase-based gametophytic self-incompatibility (GSI), although some self-compatible (SC) cultivars exist. In this study, we characterized *S-RNase* and *SFB*, the pistil and pollen *S* determinants of the specificity of the GSI reaction, respectively, from four *S*-haplotypes, including a SC (*S^e*) and three SI (*S^a*, *S^b*, and *S^c*) *S*-haplotypes of Japanese plum. The genomic organization and structure of the SC *S^e*-haplotype appear intact, because the relative transcriptional orientation of its *S-RNase* and *SFB* and their intergenetic distance are similar to those of the other three SI *S*-haplotypes of Japanese plum and other *Prunus* L. species. Furthermore, there is no apparent defect in the DNA sequences of *S^e-RNase* and *SFB^e*. However, a series of transcriptional analyses, including real-time reverse transcriptase–polymerase chain reaction, showed that the *S^e-RNase* transcript levels in the pistil are significantly lower than those of the *S^a*-, *S^b*-, and *S^c*-*RNases*, although transcripts of *SFB^a*, *SFB^b*, *SFB^c*, and *SFB^e* are present at similar levels in pollen. Furthermore, no *S^e-RNase* spot was detected in two-dimensional polyacrylamide gel electrophoresis profiles of stylar extracts of the cultivars with the *S^e*-haplotype. We discuss the possible molecular basis of SC observed with the *S^e*-haplotype with special reference to the insufficient *S^e-RNase* accumulation incited by the very low transcriptional level of *S^e-RNase* in pistils.

Self-incompatibility (SI) is a widespread mechanism in flowering plants that prevents inbreeding and promotes outcrossing (de Nettancourt, 2001). In the Rosaceae, Scrophulariaceae, and Solanaceae plant families, SI is gametophytically controlled by the *S* locus, which contains the genes that control the pollen and pistil specificities. The pistil determinant of the three families is *S-RNase*, which encodes a basic glycoprotein with ribonuclease activity that is present in the extracellular matrix of the style-transmitting tract. The F-box genes named *SFB* or *SLF* were found to be good candidates for the pollen *S* component in *Prunus* (Rosaceae) (Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003b) and *Antirrhinum* L. (Scrophulariaceae) (Lai et al., 2002). A subsequent transformation experiment demonstrated that *SLF* is the pollen *S* component in *Petunia inflata* R.E. Fries (Solanaceae) (Sijacic et al., 2004).

SI in *Prunus* interests pomologists and has been extensively studied because most *Prunus* fruit tree species are SI and unable to bear fruit parthenocarpically. The pistil *S* gene, *S-RNase*, has been characterized at the molecular level in most SI *Prunus* fruit tree species, including almond [*P. dulcis* (Mill.) D.A. Webb. (Ushijima et al., 1998)], sweet cherry [*P. avium* L. (Tao et al., 1999)], sour cherry [*P. cerasus* L. (Yamane et al., 2001)], Japanese apricot [*P. mume* Siebold & Zucc. (Yaegaki et al.,

2001)], and apricot [*P. armeniaca* L. (Romero et al., 2004)]. Recently, pollen *S* component *SFB* (*S* haplotype-specific F-box protein gene) has also been characterized in almond (Ushijima et al., 2003), sweet and sour cherries (Yamane et al., 2003b), Japanese apricot (Entani et al., 2003; Yamane et al., 2003c), and apricot (Romero et al., 2004). The results from these studies have led to the development of not only molecular typing methods for *S*-haplotypes (Tao et al., 1999), but also molecular markers for self-compatibility (SC) in *Prunus* fruit tree species (Ikeda et al., 2004b; Tao et al., 2000, 2002a, 2002b).

There have been several reports on the characterization of Japanese plum *S-RNase* and *SFB*. Yamane et al. (1999) first isolated a Japanese plum *S-RNase* from the SI cultivar ‘Sordum’. Based on the *S-RNase* DNA sequence, Beppu et al. (2002, 2003) developed a polymerase chain reaction (PCR)-based *S*-haplotype typing system and identified 14 different Japanese plum *S-RNases* corresponding to the *S^a*- to *Sⁿ*-haplotypes. Later, Beppu et al. (2005) reported that one of the 14 *S*-haplotypes, the *S^e*-haplotype, could be responsible for SC in some of the cultivars based on pollination and genetic studies. Tao et al. (2007) and Zhang et al. (2007) isolated *SFB* from several *S*-haplotypes. Because only partial sequences are available for *S-RNase* and *SFB* from all of the *S*-haplotypes of Japanese plum, except for the *S^a*-haplotype (Tao et al., 2007), the nature of the SC conferred by the *S^e*-haplotype is unclear.

In the present study, we thoroughly characterized the pistil and pollen *S* determinants of the specificity of the gametophytic self-incompatibility (GSI) reaction, *S-RNase*

Received for publication 31 Oct. 2006. Accepted for publication 12 Mar. 2007. Ms. Watari and Mr. Hanada contributed equally.

¹Corresponding author. E-mail: rtao@kais.kyoto-u.ac.jp.

and *SFB*, respectively, from four *S*-haplotypes, including a SC (*S^c*) and three SI (*S^a*, *S^b*, and *S^c*) *S*-haplotypes of Japanese plum, to clarify the molecular basis of the SC observed in the

S^c-haplotype. Elucidation of the molecular basis of SC observed with *S^c*-haplotype could lead to the development of not only the molecular markers for SC, but also the novel SC breeding strategies in Japanese plum. A series of molecular analyses that were conducted in this study suggest that the SC in the *S^c*-haplotype is incited by the accumulation of insufficient levels of *S^c*-RNase incited by a very low *S^c*-RNase transcription level in the pistil.

Materials and Methods

PLANT MATERIAL. Twelve Japanese plum cultivars were used in this study (Table 1). Young leaves, pollen grains, and styles with stigmas at the balloon stage of development were collected and stored at -80 °C until use.

ISOLATION OF DNA. Total DNA was isolated from young leaves as described previously (Yamane et al., 1999). In brief, leaves were ground to a powder using a mortar and pestle and homogenized in washing buffer [0.1 M HEPES (pH 8.0), 0.1% PVP, 2% mercaptoethanol]. The homogenate was centrifuged (6500 g_n at 4 °C for 20 min) to collect the pellet, and the pellet was resuspended in washing buffer and centrifuged again. Next, the pellet was suspended in extraction buffer [0.2 M Tris-HCl

(pH 8.0), 0.25 M NaCl, 25 mM ethylenediaminetetraacetic acid, 6% PVP], and one-half volume of 7.5 M ammonium acetate was added. The mixture was incubated on ice for 30 min, after which one volume of chloroform was added. After centrifugation (2500 g_n at 4 °C for 15 min), the supernatant was transferred to a new tube and one volume of isopropanol was added. The mixture was centrifuged (8000 g_n at 4 °C for 20 min) to collect the pellet. The resulting DNA pellet was dissolved in 10 mM Tris-HCl + 1 mM EDTA buffer (pH 8.0) (TE). After RNase treatment, the DNA solution was further purified by phenol/chloroform extraction and the DNA was precipitated with ethanol, rinsed with 75% ethanol, and dissolved in TE.

CONSTRUCTION AND SCREENING OF A GENOMIC LIBRARY. A fosmid library was constructed from genomic DNA of 'Sordum' (*S^aS^b*) and 'Santa Rosa' (*S^cS^c*) using the CopyControl Fosmid Library Production Kit (Epicentre, Madison, Wis.). The library was screened with a DIG-dUTP-labeled (Roche, Basel, Switzerland) probe corresponding to a partial fragment of the coding region of almond *S^k*-RNase or *SFB^k* (Tao et al., 2007). Plasmid was extracted from positive colonies, and DNA sequences were determined with the BigDye Terminator version 3.1 Cycle Sequencing Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.). The deduced amino acid sequences of the *SFBs* and *S-RNases* were aligned using Clustal X (Thompson et al., 1997).

ANALYSIS OF THE PHYSICAL DISTANCES BETWEEN *S-RNASE* AND *SFB*. PCR reactions were performed to determine the physical distance between *S-RNase* and *SFB* in the

Table 1. *S*-haplotypes of 16 Japanese plum cultivars.

<i>S</i> -haplotype	Cultivar	Reference
<i>S^aS^b</i>	Sordum	Yamane et al., 1999
	Burmosa	Beppu et al., 2003
<i>S^aS^c</i>	Rio	Beppu et al., 2002
<i>S^aS^f</i>	Terada	Beppu et al., 2002
<i>S^aS^m</i>	Botan	Beppu et al., 2003
<i>S^bS^c</i>	Oishinakate	Beppu et al., 2002
	Taiyo	Beppu et al., 2002
<i>S^cS^c</i>	Santa Rosa	Beppu et al., 2002
	Beauty	Beppu et al., 2002
<i>S^bS^d</i>	Formasa	Beppu et al., 2002
<i>S^bS^f</i>	Frontier	Beppu et al., 2003
<i>S^bS^g</i>	Honey Rosa	Beppu et al., 2003
<i>S^bS^h</i>	Yonemomo	Beppu et al., 2002
<i>S^bSⁱ</i>	Bakemonosumomo	Beppu et al., 2002
<i>S^bS^k</i>	Harypickstone	Beppu et al., 2003
<i>S^bS^l</i>	Lantz	Beppu et al., 2003

Table 2. DNA sequences of oligonucleotide primers.

Primer	Sequence
ActF1	5'-ATGGTGAGGATATTCACCC-3'
ActR1	5'-CTCCTGTGGACAATGGATGG-3'
ccFos-F	5'-GGATGTGCTGCAAGGCGATTAAGTTGG-3'
ccFos-R	5'-CTCGTATGTTGTGTGGAATTGTGAGC-3'
PdS4-RNase-F4	5'-CCAGTAATTGCAATGGGTCG-3'
PpSFB1F1	5'-ATAGCACCAGATGCCATTTGC-3'
PpSFB2R2	5'-TTCGTCATATTTACGACGG-3'
Pru-C2	5'-CTATGGCCAAGTAATTATTCAAACC-3'
Pru-C4R	5'-GGATGTGGTACGATTGAAGCG-3'
PsSFBa-F1	5'-ATTACTGCTGCGAGGAAGAG-3'
PsSFBb-F3	5'-CGTCGTTCTGTTTCTTTGTA-3'
PsSFBb-R3	5'-TCTGCAATGCCCATATTGTA-3'
PS_SFBcR	5'-CGCCCTTATTGAGATCTCGTT-3'
PS_SFBcR	5'-GCAATCACACAAATGTCATCT-3'
qpActF	5'-CAACTGGTATTGTGCTGGATTCTG-3'
qpActR	5'-CAGCAAGGTCAAGACGAAGGAT-3'
rt_PS_SaF	5'-CGATGCAAACGTGATCCAGTAC-3'
rt_PS_Sa_RII	5'-TGTC AACGTTCTTCCAGCAATC-3'
rt_PS_SbF	5'-TGCAAAAATAACGTTGGCATCTC-3'
rt_PS_Sb_RII	5'-AATCATTCATTTTGTGTTTTATTACAGTAGTACCA-3'
rt_PS_ScF	5'-TTGCAAACGTGATCCAGCAC-3'
rt_PS_ScR	5'-TTCATATAACAACTGAGTCTTTGGCC-3'
rt_PS_SeF	5'-GGCGAACTCTCTGCTGTTACATG-3'
rt_PS_SeR	5'-CAGTCAATCAGCTTTAACGCATTATATC-3'
rt_PS_SFBaF	5'-TTATTTTCCATTGGATGAATGGAG-3'
rt_PS_SFBaR	5'-TTAATTCCTGTTTCAAGAACATCCTG-3'
rt_PS_SFBbF	5'-TTTTATTTTCTTTGGATGATTACTATTCTA-3'
rt_PS_SFBbR	5'-TGCCCATATTGTAATCTGTTCTTTG-3'
rt_PS_SFBcF	5'-TCCTTTTATTTATCCTCGGAGCTG-3'
rt_PS_SFBcR	5'-TTACAGAGATATAGATAAGCTACGCCCTTAT-3'
rt_PS_SFBcF	5'-GTGTCCTTTTATTTATCCTTCGGATTAT-3'
rt_PS_SFBcR	5'-CGCAATCACACAA ATGCATATCTAA-3'
SFB-C2R	5'-GGGTTCCATATKTGTATWGG-3'
SFB-C3R	5'-ACAGCCYTRTAGTCRTTAAC-3'
SFBC5F	5'-TACCAYWTMATTGAGAAAGGTCC-3'

Fig. 1. Alignment of deduced SFB amino acid sequences. The deduced amino acid sequences were aligned using the Clustal X program (Thompson et al., 1997). The F-box motif, the variable regions V1 and V2, and the hypervariable regions HVa and HVb, are boxed (Ikeda et al., 2004a). Gaps are marked by dashes. Sharps (#) indicate the positions that have a single, fully conserved residue. EMBL/GenBank/DBJ accession numbers are as follows: *SFB^a* (Ps_SFBa; AB252409), *SFB^b* (Ps_SFBb; AB252412), *SFB^c* (Ps_SFBc; AB280792), and *SFB^d* (Ps_SFBd; AB280794) of Japanese plum (*P. salicina*); *SFB³* (Pav_SFB3; AV096857) and *SFB⁶* (Pav_SFB6; AB096858) of sweet cherry (*P. avium*); *SFB¹* (Par_SFB1; AY587561) and *SFB²* (Par_SFB2; AY587562) of apricot (*P. armeniaca*); *SFB^a* (Pd_SFBa; AB092966) and *SFB^c* (Pd_SFBc; AB079776) of almond (*P. dulcis*); and *SFB¹* (Pm_SFB1; AB101440) and *SFB⁷* (Pm_SFB7; AB101441) of Japanese apricot (*Prunus mume*).

Table 3. Identities of the amino acid sequences of the *Prunus S* locus genes.^z

	<i>P. salicina</i>				<i>P. avium</i>		<i>P. armeniaca</i>		<i>P. dulcis</i>		<i>P. mume</i>	
	PsSa	PsSb	PsSc	PsSe	PavS3	PavS6	ParS1	ParS2	PdSa	PdSc	PmS1	PmS7
PsSa	—	77.9	76.0	74.9	76.9	77.9	78.2	76.8	66.2	75.4	78.7	75.8
PsSb	71.0	—	76.3	81.9	82.2	77.9	78.5	81.6	66.5	77.8	81.1	79.3
PsSc	68.6	59.7	—	74.4	74.7	75.2	75.7	75.5	67.2	76.5	76.0	78.4
PsSe	70.8	73.8	67.0	—	78.4	78.7	76.8	80.3	66.1	77.3	79.2	77.6
PavS3	69.5	74.7	66.4	74.7	—	79.5	77.1	77.6	66.2	77.3	82.1	77.7
PavS6	76.2	78.3	65.9	74.0	71.3	—	80.3	79.7	68.3	76.5	80.3	80.0
ParS1	74.8	76.0	69.3	82.5	71.5	75.8	—	79.5	66.6	75.4	80.8	78.0
ParS2	69.9	74.2	69.6	76.5	73.0	69.5	75.7	—	67.2	78.9	81.6	79.7
PdSa	52.7	56.1	51.1	52.9	52.4	52.0	54.2	49.1	—	69.3	68.8	68.7
PdSc	78.9	72.4	67.3	72.6	70.4	75.3	76.7	72.6	52.9	—	78.6	79.4
PmS1	66.5	71.0	64.3	77.7	74.6	72.6	75.9	68.3	54.0	69.5	—	80.3
PmS7	64.2	72.4	62.8	73.0	65.0	70.4	69.9	67.7	50.0	64.1	72.8	—

^zThe upper half provides amino acid sequence identities (%) between *Prunus* SFBs and the lower half shows identities between the S-RNases. EMBL/GenBank/DBJ accession numbers are as follows: *PsSFB^a* (AB252409), *PsSFB^b* (AB252412), *PsSFB^c* (AB280792), *PsSFB^e* (AB280794), *SaSFB³* (AV096857), *PaSFB⁶* (AB096858), *ParSFB¹* (AY587561), *ParSFB²* (AY587562), *PdSFB^a* (AB092966), *PdSFB^c* (AB079776), *PmSFB¹* (AB101440), *PmSFB⁷* (AB101441), *PsS^a-RNase* (AB252411), *PsS^b-RNase* (AB252413), *PsS^c-RNase* (AB280791), *PsS^e-RNase* (AB280793), *PaS³-RNase* (AB010306), *PaS⁶-RNase* (AB010305), *ParS¹-RNase* (AY587561), *ParS²-RNase* (AY587562), *PdS^a-RNase* (AB026836), *PdS^c-RNase* (AB011470), *PmS¹-RNase* (AB101438), and *PmS⁷-RNase* (AB101439).

S^b-, *S^c*-, and *S^e*-haplotypes of Japanese plum. For the *S^b*-haplotype, the primers PdS4-RNase-F4 and SFB-C2F (Table 2) were used along with *ExTaq* polymerase. The primers rtPS_ScF and rtPS_SFBcF (Table 2) were used with the Expand Long Template PCR System (Roche) for the *S^c*-haplotypes. Because it appeared that *SFB* and *S-RNase* are located in close proximity in the *S^b*- and *S^c*-haplotypes, the numbers of nucleotides flanked by the coding regions of the two genes were determined by sequencing. For the *S^e*-haplotype, the distance between *S-RNase* and *SFB* was determined by estimating the positions of the two genes. The primers ccFos-F and ccFos-R, which are specific to the flanking regions of the cloning site of the CopyControl pCC1FOS cloning vector (Epicentre), were used in combination with the primers rt_PS_SeF and rt_PS_SFBcF (Table 2). The distance between the flanking region of the cloning site and the respective genes was determined by long PCR with the Expand Long Template PCR System. The distance between *S-RNase* and *SFB* was calculated from sizes of the PCR fragments and the inserted genomic DNA fragment. The transcriptional orientations of the genes were determined by PCR amplification with various combinations of primers designed to complement the flanking regions of the vector cloning site and gene-specific primers for *SFB* or *S-RNase*. All PCR reactions contained 1X PCR buffer, 300 µM of each dNTP, 3.75 mM MgCl₂, 500 nM of each primer, 1 to 25 ng of template plasmid DNA, and 1 U *Taq* polymerase in a 15-µL reaction volume. Long PCR was performed using a program of an initial denaturation step at 94 °C for 2 min, 10 cycles of 94 °C for 10 s, 65 °C for 30 s, and 68 °C for 15 min, 20 cycles of 94 °C for 10 s, 65 °C for 30 s, and 68 °C for 15 min with an extension of an extra 20 s after each cycle and a final extension at 68 °C for 7 min. PCR products were separated on 1.5% or 0.6% agarose gels and visualized with ethidium bromide under ultraviolet light.

DNA GEL BLOT ANALYSIS. Six micrograms of genomic DNA were digested with *Hind*III, separated on a 0.6% agarose gel and transferred to a nylon membrane (Hybond-N; Amersham, Tokyo). The membrane was probed with a DIG-dUTP-labeled

DNA fragment and washed under high-stringency conditions (twice at room temperature in 2X SSC, 0.1% SDS for 5 min, and twice at 68 °C in 0.1X SSC, 0.1% SDS) or low-stringency condition (twice at room temperature in 2X SSC, 0.1% SDS, and twice at 60 °C in 0.1X SSC, 0.1% SDS). Hybridization signals were visualized as described (Tao et al., 1999). The DIG-dUTP-labeled probe was synthesized by PCR using the *S^a-RNase* cDNA with the primers, Pru-C2 and Pru-C4R (Tao et al., 1999). For *SFB* probes, genomic clones of *SFB^a*, *SFB^b*, *SFB^c*, and *SFB^e* were used as templates with the primer sets, a forward primer SFB-C5F and reverse primers SFB-AR2, SFBRR3-PS, PSSFBcR, and PSSFBcR, respectively (Table 2).

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION. Total RNA was isolated from pollen grains, styles with stigmas at the balloon stage of development, and leaves of 'Sordum' (*S^aS^b*) and 'Santa Rosa' (*S^cS^e*) as described (Tao et al., 1999). One microgram of total RNA treated with DNase I (Invitrogen, Carlsbad, Calif.) was used for first-strand cDNA synthesis by SuperScript III RT (Invitrogen) with an adapter-dT primer. The primer combinations used to amplify the RT-PCR products from *SFB^a*, *SFB^b*, *SFB^c*, and *SFB^e* were PpSFB1F1 and PpSFB2R2, PsSFBb-F3 and PsSFBb-R3, rt_PS_SFBcF and rt_PS_SFBcR, and rt_PS_SFBcF and rt_PS_SFBcR, respectively (Table 2). The PCR mixture contained 1X *ExTaq* buffer, 200 µM of each dNTP, 400 nM of each primer, 20 ng of template cDNA, and 0.5 U of TaKaRa *ExTaq* polymerase (TaKaRa Bio, Ohtsu, Japan). PCR was performed using a program of 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s with an initial denaturation step at 94 °C for 2.5 min and a final extension step at 72 °C for 7 min. Expression of the actin gene was used as a control, using the ActF1 and ActR1 primers (Table 2). The control PCR conditions were identical to those of Yamane et al. (2003b).

CONSTRUCTION OF A PHYLOGENETIC TREE. The coding DNA sequences for 23 *S-RNases* were used to construct a phylogenetic tree. The *S-RNase* sequences encompassed 17 *Prunus S-RNases*, including three Japanese plum *S-RNases* that were identified in this study, three *P. inflata S-RNases*, and

Primer Express (version 2.0; Applied Biosystems). The primer combinations used to amplify real-time RT-PCR products from *SFB^a*, *SFB^b*, *SFB^c*, and *SFB^e* were rt_PS_SFBaF and rt_PS_SFBaR, rt_PS_SFBbF and rt_PS_SFBbR, rt_PS_SFBcF and rt_PS_SFBcR, and rt_PS_SFBeF and rt_PS_SFBeR, respectively (Table 2). The primer combinations used to amplify real-time PCR products from *S^a-RNase*, *S^b-RNase*, *S^c-RNase*, and *S^e-RNase* were rt_PS_SaF and rt_PS_SaR, rt_PS_SbF and rt_PS_SbR, rt_PS_ScF and rt_PS_ScR, and rt_PS_SeF and rt_PS_SeR, respectively (Table 2). As a control, *ACTIN* transcripts were quantified using two actin-specific primers designed for real-time RT-PCR analysis, qpActF and qpActR (Table 2). For real-time RT-PCR, 1 μ L of cDNA solution, equivalent to the amount synthesized from 10 ng of total RNA, was used. Reactions were carried out in 96-well plates in a 25- μ L reaction volume containing 12.5 μ L of 2X SYBR ExTaq Master Mix (TaKaRa Bio), 0.2 μ M each of forward and reverse primers, and 1 μ L of cDNA solution. Three reactions were performed. The threshold cycle value (Ct) of each sample was automatically measured using SDS software (version 2.1; Applied Biosystems). After the PCR reaction, a dissociation curve analysis was performed to confirm that the observed fluorescence resulted only from the gene-specific amplification. Based on the Ct values, the original cDNA quantities of *S-RNase* and *SFB* were normalized using the *ACTIN* cDNA values and compared as relative amounts of their transcripts.

TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS ANALYSIS OF PISTIL PROTEINS. Acetone powders were prepared from styles with stigmas from ‘Sordum’ (*S^aS^b*), ‘Taiyo’ (*S^bS^c*), ‘Santa Rosa’ (*S^cS^e*), and ‘Rio’ (*S^cS^e*) as described previously (Tao et al., 1997). Crude extracts from the acetone powders were subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) using nonequilibrium pH gradient electrophoresis in the first dimension and SDS-PAGE in the second dimension as described (Tao et al., 1997). After electrophoresis, the proteins in the gel were detected by silver staining using Sil-Best Stain for Protein/PAGE (Nacalai Tesque, Kyoto, Japan).

Results

ISOLATION AND PRIMARY STRUCTURES OF *SFB* AND *S-RNase* FROM FOUR *S*-HAPLOTYPES. The entire coding sequences for *SFB* and *S-RNase* of the *S^b*-haplotype of ‘Sordum’ (*S^aS^b*) and the *S^c*- and *S^e*-haplotypes of ‘Santa Rosa’ (*S^cS^e*) were determined using fosmid clones obtained with probes designed for the almond *S^k-RNase* or *SFB^k*. The deduced amino acid sequences of *SFB^b*, *SFB^c*, and *SFB^e* were aligned with those of *SFB^a* from Japanese plum (Tao et al., 2007), *SFB³* and *SFB⁶* from sweet cherry, *SFB¹* and *SFB²* from apricot, *SFB^a* and *SFB^c* from almond, and *SFB¹* and *SFB⁷* from Japanese apricot (Fig. 1). The amino acid identities among the 12 *SFB* alleles, including three Japanese plum alleles identified in this study, range from 66.1% to 82.2%, which is comparable to the amino acid identities between Japanese plum *S-RNases* and other *Prunus* *S-RNases* ranging from 49.1% to 78.9% (Table 3). All of the *S-RNases* from the four *S*-haplotypes, including the SC *S^c*-haplotype, contain the two active sites of the T2/S-type RNases, including the two histidine residues essential for activity and five regions conserved in the *Prunus* *S-RNases* (Fig. 2). Similarly, all four *SFBs* contain the F-box motif within the N-terminal region as do other *Prunus* *SFBs* (Fig. 1). Two hypervariable hydrophilic

regions that are under positive selection (Ikeda et al., 2004a) are also present in the C-terminal region (Fig. 1). No substantial difference was observed in the primary structures of the *S-RNase* and *SFB* from the SC *S^c*-haplotype as compared with other *S-RNases* and *SFBs* from SI *S*-haplotypes.

The relative transcriptional orientations of *SFB* and *S-RNase* and their physical distances in the *S^b*-, *S^c*-, and *S^e*-haplotypes were determined in this study (Fig. 3), and those of the *S^a*-haplotype had already been determined (Tao et al., 2007). In all four *S*-haplotypes, *SFB* is located downstream of *S-RNase* in a reverse transcriptional orientation, like in all of the other *S*-haplotypes of *Prunus* that have been investigated to date except for the *S²*-haplotype of *P. armeniaca* (Fig. 3; Ikeda et al., 2005; Romero et al., 2004; Tao et al., 2007; Ushijima et al., 2003). The estimated distances between *SFB* and *S-RNase* in the *S^b*-, *S^c*-, and *S^e*-haplotypes are 539 base pairs (bp), 756 bp, and 18 kilobp (kbp), respectively, whereas that for the *S^a*-haplotype was described previously (Tao et al., 2007) as $\approx$ 41 kbp (Fig. 3). These results show that the distance between the two genes in the *S^b*-, *S^c*-, and *S^e*-haplotypes is within the range of those in other SI *Prunus* *S*-haplotypes, and the *SFBs* are physically tightly linked to the *S-RNases* in Japanese plum like in other *Prunus* species.

GENOMIC DNA BLOT AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION ANALYSIS OF FOUR *SFBs*. Genomic DNA blot analysis showed that *SFB^a*, *SFB^b*, *SFB^c*, and *SFB^e* are cosegregated with the *S^a*-, *S^b*-, *S^c*-, and *S^e*-haplotypes of Japanese plum cultivars, respectively (Fig. 4). Under low-stringency washes at 60 °C with the *S^a-RNase* or *SFB^a* probe, all cultivars yielded *S^a*-haplotype-specific bands corresponding to their *S*-haplotypes (Fig. 4A, B). With high-stringency washes at 68 °C with the *SFB^a*, *SFB^b*, *SFB^c*, or *SFB^e* probes, only cultivars with the respective *S*-haplotypes yielded hybridization signals (Fig. 4C–F).

RT-PCR using primers specific for *SFB^a*, *SFB^b*, *SFB^c*, or *SFB^e* revealed that the transcription of these *SFBs* is specific to pollen (Fig. 5), like found with other *Prunus* *SFBs*.

Fig. 3. Schematic diagram illustrating the *S-RNase* and *SFB* gene organization in the *S* loci of four Japanese plum *S*-haplotypes. The illustration of the locus of the *S^a*-haplotype is from Tao et al. (2007). The nucleotide “A” of the start codon of *S-RNases* and *SFBs* is at position +1. Black boxes represent exons in the *S-RNases* and *SFBs*. Arrows denote the direction of transcription.

Fig. 4. Genomic DNA blot analyses for the S^a - and S^b -haplotypes of Japanese plum. Genomic DNAs from 14 Japanese plum cultivars were digested with *Hind*III, separated on a 0.6% agarose gel, and transferred to a nylon membrane. (A) Membrane hybridized with an S^a -RNase probe and washed under low-stringency conditions. (B) Membrane hybridized with an SFB^a probe and washed under low-stringency conditions. (C) Membrane hybridized with an SFB^b probe and washed under high-stringency conditions. (D) Membrane hybridized with an SFB^b probe and washed under high-stringency conditions. (E) Membrane was hybridized with an SFB^c probe and washed under high-stringency conditions. (F) Membrane was hybridized with an SFB^c probe and washed under high-stringency conditions. Lanes: A, 'Sordum' ($S^a S^b$); B, 'Burmosa' ($S^a S^b$); C, 'Rio' ($S^a S^c$); D, 'Terada' ($S^a S^c$); E, 'Botan' ($S^a S^m$); F, 'Oishinakate' ($S^b S^c$); G, 'Formasa' ($S^b S^c$); H, 'Frontier' ($S^b S^c$); I, 'Honey Rosa' ($S^b S^c$); J, 'Yonemomo' ($S^b S^c$); K, 'Bakemonosumomo' ($S^b S^c$); L, 'Harypickstone' ($S^b S^c$); M, 'Lantz' ($S^b S^c$); N, 'Santa Rosa' ($S^c S^c$).

PHYLOGENETIC ANALYSIS OF SFB s AND S -RNASES. To investigate the phylogenetic relationships of the S -RNases and SFB s of the Japanese plum S -haplotypes with other reported S -RNases or SFB /SLFs, we constructed phylogenetic trees for the S -RNase and SLF/ SFB from *Prunus* of the family Rosaceae, *A. hispanicum*, and *P. inflata*. All *Prunus* S -RNases fell into one clade, and the S -RNases from *A. hispanicum* and *P. inflata* were incorporated into the other two clades (Fig. 6). Similarly, all SFB s fell into one clade that was distinct from the clades of the

SLFs of *Prunus* and the SLFs of *A. hispanicum* and *P. inflata* (Fig. 7). As reported by Ushijima et al. (2004), the SLFs of *Prunus* were phylogenetically less similar to the SFB s of *Prunus* than to the SLFs of *Antirrhinum* and *Petunia* Juss. Like with the S -RNases, no species-specific subgroup of *Prunus* SFB s was formed. Both S -RNase and SFB from the SC S^c -haplotype fell into the clades of *Prunus* S -RNases and *Prunus* SFB s, respectively, confirming the finding that there are no substantial differences in the primary structures of S^c -RNase and SFB^c from other S -RNases and SFB s of SI S -haplotypes, respectively.

TRANSCRIPT ACCUMULATION OF SFB^c IN POLLEN AND S^c -RNASE IN STYLE. Beppu et al. (2005) reported the involvement of the S^c -haplotype in SC in Japanese plum based on segregation analysis. Because insufficient expression of either SFB or S -RNase leads to SC in *Prunus*, we performed real-time PCR to determine the amounts of the corresponding transcripts. The relative transcript amounts of S^c -RNase and those of the other three S -RNases, the S^a -, S^b - and S^c -RNases, in different lines, are shown in Figure 8A. The amount of S^c -RNase transcript in styles of 'Santa Rosa' was significantly lower (less than 1/10,000) than in those of S^c -RNase. Similarly, significantly fewer S^c -RNase transcripts accumulated than S^c - or S^a -RNase transcripts in the styles of 'Beauty' and 'Rio', respectively (Fig. 8A). Given that the amounts of S^a -RNase and S^b -RNase transcripts are comparable in 'Sordum' pistils, the significantly lower amount of S^c -RNase transcripts in styles could be responsible for the SC observed in the S^c -haplotype. The amounts of SFB^c transcript were equivalent to those of SFB^c in 'Santa Rosa' pollen, as was the

case with SFB^a and SFB^b in 'Sordum' pollen (Fig. 8B).

S -RNASE PROTEIN ASSAY. The 2D-PAGE profiles of 'Santa Rosa' ($S^c S^c$), 'Taiyo' ($S^b S^c$), and 'Rio' ($S^a S^c$) styles contained major protein spots with molecular weights and isoelectric points similar to those of the S -RNases of the Japanese plum 'Sordum' (Yamane et al., 1999) and other *Prunus* species, including almond (Tao et al., 1997), sweet cherry (Tao et al., 1999), and Japanese apricot (Tao et al., 2002b) (Fig. 9). Although two S -RNase spots corresponding to the S^b and S^c

Fig. 5. Expression analysis of the *SFBs* of four *S*-haplotypes of Japanese plum. RT-PCR for *SFBs* and *ACTIN* was performed with total RNA from pollen, styles, and leaves of 'Sordum' ($S^a S^b$) and 'Santa Rosa' ($S^c S^e$).

Fig. 6. Phylogenetic tree of the *S-RNases* of *Prunus*, *Antirrhinum*, and *Petunia*. All phylogenetic reconstitution was conducted in PAUP* (Swoford, 2001) as described in the text. Numbers indicate the percentage of 100 bootstrap replicates in which a given group was found (values below 50% not shown). Sequence information for the *S-RNases* included in the tree is as follows: S^a -*RNase* (Ps Se; AB026981), S^b -*RNase* (Ps Sb; AB026982), S^c -*RNase* (Ps Sc; AB280791), and S^e -*RNase* (Ps Se; AB280793) of Japanese plum (*P. salicina*); S^1 -*RNase* (Pav S1; AB028153), S^3 -*RNase* (Pav S3; AB010306), S^4 -*RNase* (Pav S4; AB028154), S^5 -*RNase* (Pav S5; AJ298314), and S^6 -*RNase* (Pav S6; AB010305) of sweet cherry (*P. avium*); S^1 -*RNase* (Pm S1; AY587561), S^2 -*RNase* (Pm S2; AY587562), and S^4 -*RNase* (Pm S4; AY587564) of apricot (*P. armeniaca*); S^a -*RNase* (Pd Sa; AB026836), S^b -*RNase* (Pd Sb; AB011469), and S^c -*RNase* (Pd Sc; AB011470) of almond (*P. dulcis*); S^1 -*RNase* (Pm S1; AB101438) and S^7 -*RNase* (Pm S7; AB101439) of Japanese apricot (*P. mume*); S^2 -*RNase* (Ah S2; X96465), S^4 -*RNase* (Ah S4; X96466), and S^5 -*RNase* (Ah S5; X96464) of *Antirrhinum hispanicum*; S^1 -*RNase* (Pi S1; M67990), S^2 -*RNase* (Pi S2; AF301533), and S^3 -*RNase* (Pi S3; M67991) of *Petunia inflata*.

alleles were observed in 'Taiyo', only a single S-RNase spot was visible in the 2D-PAGE profiles of 'Santa Rosa' and 'Rio' (Fig. 9). Because the S-RNase spots of 'Santa Rosa' and 'Rio' had different molecular masses and isoelectric point (pI) values, and these are thought to correspond to S^c -RNase and S^a -RNase, respectively, we concluded that the S^c -RNase spot was missing in the 2D-PAGE profiles. This suggests that very

little S^e -RNase, if any, accumulates in the styles of cultivars with the S^c -haplotype.

Discussion

SI in *Prunus* is controlled by two genes, *S-RNase* and *SFB*, which are located in the *S* locus. The former controls pistil specificity and the latter controls the pollen specificity of the SI reaction. Although partial Japanese plum *S-RNase* sequences have been deduced through PCR and characterized at the molecular level (Beppu et al., 2002, 2003; Sapir et al., 2004; Yamane et al., 1999), *SFB* remained to be cloned until very recently (Tao et al., 2007; Zhang et al., 2007). Tao et al. (2007) isolated full-length *S-RNase* and *SFB* of the Japanese plum S^a -haplotype by screening a genomic library, and Zhang et al. (2007) isolated partial *SFB* sequences from eight different *S*-haplotypes by genomic PCR. The Japanese plum S^a -haplotype and the peach S^2 -haplotype appear to have the same origin (Tao et al., 2007). The former appears to be an intact SI *S*-haplotype and the latter to be a mutated SC *S*-haplotype from the same ancestral *S*-haplotype. As indicated by our phylogenetic analysis and those of others (Ikeda et al., 2004b; Ushijima et al., 1998), the diversification of *S*-haplotypes apparently occurred before speciation in *Prunus*.

In this study, we isolated the *S* locus region of the S^b -, S^c -, and S^e -haplotypes and characterized their full-length *S-RNase* and *SFB*. The molecular characteristics of the *S-RNase* and *SFB* of the three *S*-haplotypes support the idea that they encode factors that control the pistil and pollen specificity of the SI reaction in Japanese plum, like in S^a -haplotype (Tao et al., 2007). The full-length coding sequences for *S-RNase* and *SFB* of the four *S*-haplotypes, including the SC S^c -haplotype of Japanese plum, were used to elucidate the molecular basis of SC observed with the S^c -haplotype, because defects in either *S-RNase* or *SFB* often lead to SC in *Prunus* (Hauck et al., 2006a, 2006b; Tao et al., 2007; Tsukamoto et al., 2006; Ushijima et al., 2004; Yamane et al., 2003a). Although a series of molecular analyses, including a primary structural comparison, investigation of the gene organization in the *S* locus and a

Fig. 7. Phylogenetic tree of the *SFB/SLFs* of *Prunus*, *Antirrhinum*, and *Petunia*. All phylogenetic reconstitution was conducted in PAUP* (Swofford, 2001) as described in the text. Numbers indicate the percentage of 100 bootstrap replicates in which a given group was found (values below 50% not shown). Sequence information for the *SFBs* included in the tree is as follows: *SFB^a* (Ps SFBa; AB252409), *SFB^b* (Ps SFBb; AB252412), *SFB^c* (Ps SFBc; AB280792), and *SFB^d* (Ps SFBd; AB280794) of Japanese plum (*P. salicina*); *SFB¹* (Pav SFB1; AB111518), *SFB³* (Pav SFB3; AB096857), *SFB⁴* (Pav SFB4; AB111521), *SFB⁵* (Pav SFB5; AB111520), *SFB⁶* (Pav SFB6; AB096858), *SLFL1-S⁴* (Pav SLFL1S4; AB280953), *SLFL2-S⁴* (Pav SLFL1S4S4; AB280954), and *SLFL3-S⁴* (Pav SLFL3S4; AB280955) of sweet cherry (*P. avium*); *SFB¹* (Par SFB1; AY587563), *SFB²* (Par SFB2; AY587562), and *SFB⁴* (Par SFB4; AY587565) of apricot (*P. armeniaca*); *SFB^a* (Pd SFBa; AB092966), *SFB^b* (Pd SFBb; AB092967), and *SFB^c* (Pd SFBc; AB079776) of almond (*P. dulcis*); *SFB¹* (Pm SFB1; AB101440), *SFB⁷* (Pm SFB7; AB101441), *SLFL1-S¹* (Pm SLFL1S1; AB092623), *SLFL1-S⁷* (Pm SLFL1S7; AB092624), *SLFL2-S¹* (Pm SLFL2S1; AB092625), *SLFL2-S⁷* (Pm SLFL2S7; AB092626), and *SLFL3-S⁷* (Pm SLFL3S7; AB092627) of Japanese apricot (*P. mume*); *SLF-S²* (Ah SLF2; AJ297974), *SLF-S⁴* (Ah SLF4; AJ515534), and *SLF-S⁵* (Ah SLF5; AJ515536) of *Antirrhinum hispanicum*; *SLF¹* (Pi SLF1; AY500390), *SLF²* (Pi SLF2; AY500391), and *SLF³* (Pi SLF3; AY500392) of *Petunia inflata*.

Fig. 8. Relative amounts of *S-RNase* and *SFB* transcripts. (A) Relative amounts of *S-RNase* transcripts in styles of the Japanese plum cultivars 'Sordum' (S^aS^b), 'Santa Rosa' (S^cS^e), 'Beauty' (S^cS^e), and 'Rio' (S^aS^e) as analyzed by real-time RT-PCR. (B) Relative amounts of *SFB* transcripts in pollen grains of the Japanese plum cultivars 'Sordum' (S^aS^b) and 'Santa Rosa' (S^cS^e) as revealed by real-time RT-PCR. See text for details. Vertical bars indicate standard deviation.

phylogenetic reconstruction indicated that both *S-RNase* and *SFB* are intact in the S^e-haplotype, real-time RT-PCR analysis showed that the transcriptional level of S^e-*RNase* is much lower than those of the *S-RNases* of the other three Japanese plum SI S-haplotypes investigated. Although no difference was observed previously in the transcriptional levels of the S^e-*RNase* and the other *S-RNases* attributable to the use of nonquantitative RT-PCR in our earlier study (Beppu et al., 2005), the use of real-time RT-PCR in this study made it possible to demonstrate the low S^e-*RNase* transcription level. Furthermore, the 2D-PAGE analysis clearly showed that the low level of transcription leads to a very low level of S^e-*RNase* in the style.

In the Rosaceae, the molecular bases of four stylar-part mutants have been reported. The S^d-*RNase* of the S^{dsm}-haplotype of Japanese pear is completely deleted, whereas the respective *S-RNases* of the S^{6m}-, S^{6m2}-, and S^{13m}-haplotypes of sour cherry have defects that affect their transcription or translation. The molecular defect in the S^{6m}-haplotype was determined to be an insertion of a Mu-like element upstream of the S⁶-*RNase* that blocks *S-RNase* transcription (Yamane et al., 2003a). The S^{6m2}- and S^{13m}-haplotypes are disrupted by different defects: deletions of 1 bp and 23 bp in S^{6m2}-*RNase* and S^{13m}-*RNase*, respectively, leading to a premature stop codon, although transcripts of each are still present (Tsukamoto et al., 2006). Although the nature of the low level of S^e-*RNase* transcription remains to be characterized, it results in a low level of S^e-*RNase* accumulation in the style, possibly causing the SC observed in the S^e-haplotype.

Qin et al. (2006) estimated the threshold amount of S¹²-*RNase* of *Solanum chacoense* Bitt. that is required for pollen

Fig. 9. S-RNases of the Japanese plum cultivars (A) 'Sordum' ($S^a S^b$), (B) 'Taiyo' ($S^b S^c$), (C) 'Santa Rosa' ($S^c S^e$) and (D) 'Rio' ($S^a S^e$). Styler proteins were separated by 2D-PAGE and visualized by silver staining. S-RNases are indicated by arrows. The spatial distribution and relative intensities of the S-RNases in 2D-PAGE profiles are shown in E.

rejection. They showed that 68 ng of S^{12} -RNase per style did not confer SI, whereas 160 ng S^{12} -RNase per style led to SI. This indicates that approximately half of the threshold amount of S-RNase required for a successful SI reaction failed to confer SI in *S. chacoense*. Because the amount of S^e -RNase transcript was at least 10,000-fold lower than those of the SI *S-RNases* of Japanese plum and no S^e -RNase spot was found in the 2D-PAGE profile of the styler extracts, the SC conferred by the S^e -haplotype could be attributable to an insufficient level of S^e -RNase transcript. Because *SFB^e* is transcribed normally, the S^e -haplotype may contain a functional pollen *S* allele and a nonfunctional styler *S* allele, which would make the S^e -haplotype a styler-part mutant SC *S*-haplotype. However,

because the original functional version of S^e -haplotype has yet to be found, there is a possibility that *SFB^e* is also nonfunctional.

In conclusion, we isolated and characterized complete coding sequences for *S-RNase* and *SFB* from three *S*-haplotypes of Japanese plum, S^b , S^c , and S^e , and deduced a possible molecular basis for the SC observed in the S^e -haplotype. In addition to the previously characterized S^a -haplotype (Tao et al., 2007), full-length coding sequences for the *SFBs* and *S-RNases* of four *S*-haplotypes of Japanese plum are now available. Furthermore, partial sequence information is available for the *SFBs* and *S-RNases* of several other *S*-haplotypes (Zhang et al., 2007). Because SC mechanisms in addition to that related to the S^e -haplotype are thought to exist in Japanese plum (Beppu et al., 2002, 2003), the gene sequence information could provide further insight into the molecular mechanisms of SC in this fruit species, which, in turn, would provide valuable information for SC breeding.

Literature Cited

- Beppu, K., N. Komatsu, H. Yamane, H. Yaegaki, M. Yamaguchi, R. Tao, and I. Kataoka. 2005. S^e -haplotype confers self-compatibility in Japanese plum (*Prunus salicina* Lindl.). *J. Hort. Sci. Biotechnol.* 80:760–764.
- Beppu, K., Y. Takemoto, H. Yamane, H. Yaegaki, M. Yamaguchi, I. Kataoka, and R. Tao. 2003. Determination of *S*-haplotypes of Japanese plum (*Prunus salicina* Lindl.) cultivars by PCR and cross-pollination tests. *J. Hort. Sci. Biotechnol.* 78:315–318.
- Beppu, K., H. Yamane, H. Yaegaki, M. Yamaguchi, I. Kataoka, and R. Tao. 2002. Diversity of *S*-RNase genes and *S*-haplotypes in Japanese plum (*Prunus salicina* Lindl.). *J. Hort. Sci. Biotechnol.* 77:658–664.
- de Nettancourt, D. 2001. Incompatibility and incongruity in wild and cultivated plants. Springer-Verlag, Berlin.
- Entani, T., M. Iwano, H. Shiba, S. Takayama, K. Fukui, and A. Isogaandi. 2003. Comparative analysis of the self-incompatibility (*S*-) locus region of *Prunus mume*: Identification of a pollen-expressed F-box gene with allelic diversity. *Genes Cells* 8:203–213.
- Hauck, N.R., K. Ikeda, R. Tao, and A.F. Iezzoni. 2006a. The mutated S_I -haplotype in sour cherry has an altered *S*-haplotype-specific F-box protein gene. *J. Hered.* 97:514–520.
- Hauck, N.R., H. Yamane, R. Tao, and A.F. Iezzoni. 2006b. Accumulation of nonfunctional *S*-haplotypes results in the breakdown of gametophytic self-incompatibility in tetraploid *Prunus*. *Genetics* 172:1191–1198.

- Ikeda, K., B. Igic, K. Ushijima, H. Yamane, N.R. Hauck, R. Nakano, H. Sassa, A.F. Iezzoni, J.R. Kohn, and R. Tao. 2004a. Primary structure features of the *S* haplotype-specific F-box protein, *SFB*, in *Prunus*. *Sex. Plant Reprod.* 16:235–243.
- Ikeda, K., K. Ushijima, H. Yamane, R. Tao, N.R. Hauck, A.M. Sebolt, and A.M. Iezzoni. 2005. Linkage and physical distance between the *S*-haplotype *S-RNase* and *SFB* genes in sweet cherry. *Sex. Plant Reprod.* 17:289–296.
- Ikeda, K., A. Watari, K. Ushijima, H. Yamane, N.R. Hauck, A.F. Iezzoni, and R. Tao. 2004b. Molecular markers for the self-compatible *S'*-haplotype, a pollen part mutant in sweet cherry (*Prunus avium* L.). *J. Amer. Soc. Hort. Sci.* 129:724–728.
- Lai, Z., W. Ma, B. Han, L. Liang, Y. Zhang, G. Hong, and Y. Xue. 2002. An F-box gene linked to the self-incompatibility (*S*) locus of *Antirrhinum* is expressed specifically in pollen and tapetum. *Plant Mol. Biol.* 50:29–42.
- Posada, D. and K.A. Crandall. 1998. MODELTEST: Testing the model of DNA substitution. *Bioinformatics* 14:817–818.
- Qin, X., B. Liu, J. Souillard, D. Morse, and M. Cappadocia. 2006. Style-by-style analysis of two sporadic self-compatible *Solanum chacoense* lines supports a primary role for S-RNases in determining pollen rejection thresholds. *J. Expt. Bot.* 57:2001–2013.
- Romero, C., S. Vilanova, L. Burgos, J. Martínez-Calvo, M. Vicente, G. Llácer, and M.L. Badenes. 2004. Analysis of the *S*-locus structure in *Prunus armeniaca* L. Identification of *S*-haplotype specific *S-RNase* and *F-box* genes. *Plant Mol. Biol.* 56:145–157.
- Sapir, G., R.A. Stern, D. Eisikowitch, and M. Goldway. 2004. Cloning of four new Japanese plum *S*-alleles and determination of the compatibility between cultivars by PCR analysis. *J. Hort. Sci. Biotechnol.* 79:223–227.
- Sijacic, P., X. Wang, A.L. Skirpan, Y. Wang, P.E. Dowd, A.G. McCubbin, S. Huang, and T. Kao. 2004. Identification of the pollen determinant of S-RNase-mediated self-incompatibility. *Nature* 429:302–305.
- Swofford, D.L. 2001. PAUP*. Phylogenetic analysis using parsimony (and other methods), version 4. Sinauer Associates, Sunderland, Mass.
- Tao, R., T. Habu, A. Namba, H. Yamane, F. Fuyuhiko, K. Iwamoto, and A. Sugiura. 2002a. Inheritance of *S'*-RNase in Japanese apricot (*Prunus mume*) and its relation to self-incompatibility. *Theor. Appl. Genet.* 105:222–228.
- Tao, R., T. Habu, H. Yamane, and A. Sugiura. 2000. Molecular markers for self-compatibility in Japanese apricot (*Prunus mume*). *HortScience* 35:1121–1123.
- Tao, R., T. Habu, H. Yamane, and A. Sugiura. 2002b. Characterization and cDNA cloning for *S'*-RNase, a molecular marker for self-compatibility, in Japanese apricot (*Prunus mume*). *J. Jpn. Soc. Hort. Sci.* 71:595–600.
- Tao, R., H. Yamane, H. Sassa, H. Mori, T.M. Gradziel, A.M. Dandekar, and A. Sugiura. 1997. Identification of stylar RNases associated with gametophytic self-incompatibility in almond (*Prunus dulcis*). *Plant Cell Physiol.* 38:304–311.
- Tao, R., H. Yamane, A. Sugiura, H. Murayama, H. Sassa, and H. Mori. 1999. Molecular typing of *S* alleles through identification, characterization and cDNA cloning for S-RNases in sweet cherry. *J. Amer. Soc. Hort. Sci.* 124:224–233.
- Tao, R., A. Watari, T. Hanada, T. Habu, H. Yaegaki, M. Yamaguchi, and H. Yamane. 2007. Self-compatible peach (*Prunus persica*) has mutant versions of the *S* haplotypes found in self-incompatible *Prunus* species. *Plant Mol. Biol.* 63:109–123.
- Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876–4882.
- Tsukamoto, T., N.R. Hauck, R. Tao, N. Jiang, and A.F. Iezzoni. 2006. Molecular characterization of three non-functional *S*-haplotypes in sour cherry (*Prunus cerasus*). *Plant Mol. Biol.* 62:371–383.
- Ushijima, K., H. Sassa, A.M. Dandekar, T.M. Gradziel, R. Tao, and H. Hirano. 2003. Structural and transcriptional analysis of the self-incompatibility locus of almond: Identification of a pollen-expressed F-box gene with haplotype-specific polymorphism. *Plant Cell* 15:771–781.
- Ushijima, K., H. Sassa, R. Tao, H. Yamane, A.M. Dandekar, T.M. Gradziel, and H. Hirano. 1998. Cloning and characterization of cDNAs encoding S-RNases from almond (*Prunus dulcis*): Primary structural features and sequence diversity of the S-RNases in Rosaceae. *Mol. Gen. Genet.* 260:261–268.
- Ushijima, K., H. Yamane, A. Watari, E. Kakehi, K. Ikeda, N.R. Hauck, A.F. Iezzoni, and R. Tao. 2004. The *S* haplotype-specific F-box protein gene, *SFB*, is defective in self-compatible haplotypes of *Prunus avium* and *P. mume*. *Plant J.* 39:573–586.
- Yaegaki, H., T. Shimada, T. Moriguchi, H. Hayama, T. Haji, and M. Yamaguchi. 2001. Molecular characterization of S-RNase genes and *S*-genotypes in the Japanese apricot (*Prunus mume* Sieb. et Zucc.). *Sex. Plant Reprod.* 13:251–257.
- Yamane, H., K. Ikeda, N.R. Hauck, A.F. Iezzoni, and R. Tao. 2003a. Self-incompatibility (*S*) locus region of the mutated *S₆*-haplotypes of sour cherry (*Prunus cerasus*) contains a functional pollen *S*-allele and a non-functional pistil *S* allele. *J. Expt. Bot.* 54:2431–2434.
- Yamane, H., K. Ikeda, K. Ushijima, H. Sassa, and R. Tao. 2003b. A pollen-expressed gene for a novel protein with an F-box motif that is very tightly linked to a gene for *S-RNase* in two species of cherry, *Prunus cerasus* and *P. avium*. *Plant Cell Physiol.* 44:764–769.
- Yamane, H., R. Tao, and A. Sugiura. 1999. Identification and cDNA cloning for S-RNases in self-incompatible Japanese plum (*Prunus salicina* Lindl. cv. Sordum). *Plant Biotechnol.* 16:389–396.
- Yamane, H., R. Tao, A. Sugiura, N.R. Hauck, and A.F. Iezzoni. 2001. Identification and characterization of S-RNases in tetraploid sour cherry (*Prunus cerasus*). *J. Amer. Soc. Hort. Sci.* 126:661–667.
- Yamane, H., K. Ushijima, H. Sassa, and R. Tao. 2003c. The use of the *S* haplotype-specific F-box protein gene, *SFB*, as a molecular marker for *S*-haplotypes and self-compatibility in Japanese apricot (*Prunus mume*). *Theor. Appl. Genet.* 107:1357–1361.
- Zhang, S.-L., S.-X. Huang, H. Kitashiba, and T. Nishio. 2007. Identification of *S*-haplotype-specific F-box gene in Japanese plum (*Prunus salicina* Lindl.). *Sex. Plant Reprod.* 20:1–8.