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Identification and cDNA Cloning for *S*-RNases in Self-incompatible Japanese Plum (*Prunus salicina* Lindl. cv. Sordum)

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Abstract

This report demonstrates the presence of *S*-RNases in style of self-incompatible Japanese plum (*Prunus salicina* Lindl.) and presents information about cDNA sequences encoding the S-RNases. Two stylar glycoprotein spots that were found in highly basic zone of 2D-PAGE gel with *Mr* of about 30 kDa shared similarity in N-terminal amino acid sequence and immunological characteristics with other rosaceous *S*-RNases and appeared to be *S*-RNases of Japanese plum. Deduced amino acid sequences from the cDNA clones encoding these glycoproteins contained two active sites of T2/*S* type RNases and five regions conserved among other rosaceous *S*-RNases. Genomic DNA blot and RNA blot analyses using the cDNA clones as probes and phylogenetic analysis based on the deduced amino acid sequences from the cDNAs all indicated that the cDNA clones encoded *S*-RNase corresponding to the stylar glycoproteins detected on 2D-PAGE gel. Phylogenetic analysis based on the deduced amino acid sequences from the cDNAs supported the hypothesis that the *S*-RNases of Rosaceae have diverged after the divergence of subfamilies but before the divergence of species.

1. Introduction

Self-incompatibility is a widespread mechanism in flowering plants which prevents self-fertilization and promotes out-crossing (de Nettancourt, 1977). Most rosaceous fruit crops exhibit gametophytic self-incompatibility that is controlled by a single locus (*S*-locus) with multiple alleles (Crane and Brown, 1937; Crane and Lewrence, 1929; de Nettancourt, 1977). In this type of self-incompatibility, growth of pollen tubes bearing either one of the two *S*-alleles carried by the recipient pistil is arrested in the style. The molecular mechanism of gametophytic self-incompatibility in Rosaceae has been studied most extensively with apple (*Malus* × *domestica* Borkh) and pear (*Pyrus* spp.) (Ishimizu *et al.*, 1996; Sassa *et al.*, 1992,1993,1994; Tomimoto *et al.*, 1996). As is the case with Solanaceae and Scrophulariaceae (for reviews, Kao and McCubbin, 1996; McCubbin and Kao, 1999), *S*-gene products in pistil of apple and pear were shown to be *S*-RNases (Ishimizu *et al.*, 1996; Sassa *et al.*, 1992,1993,1994; Tomimoto *et al.*, 1996).

Most of the fruit tree species of *Prunus*, one of the rosaceous genera, show also gametophytic self-incompatibility. Recently, *S*-RNases of sweet cherry (*Prunus avium* L.) and almond [*Prunus dulcis* (Mill.) D.A. Webb] have been identified, and cDNAs encoding them have been cloned (Tao *et al.*, 1997, 1999; Ushijima *et al.*, 1998). Based on the cDNA sequences, molecular typing system for *S*-alleles has been developed for sweet cherry (Tao *et al.*, 1999).

As with other fruit tree species of *Prunus*, most commercial cultivars of Japanese plum (*Prunus salicina* Lindl.) are self-incompatible (Mori, 1937), and cross-compatible cultivars that bloom simultaneously are required as pollinizers to ensure fruit set. In recent breeding programs a small number of particular excellent cultivars tend to be used as parents, which will lead to cross-incompatibility between cultivars. However, *S*-genotypes or even cross-incompatibility groups have not been described for Japanese plum cultivars.

As Japanese plum is one of the *Prunus* species, *S*-RNase-based self-incompatibility mechanism is likely to operate in this fruit tree. However, *S*-RNase of Japanese plum has not been reported although Burgos *et al.* (1998) characterized stylar RNases that linked to *S*-alleles of apricot (*Prunus armeniaca* L.), a very closely related species to Japanese plum. Information about DNA sequence of the *S*-alleles should be useful to develop molecular typing systems in Japanese plum. In this report, we characterized the stylar *S*-RNases in Japanese plum and cloned cDNAs encoding them. DNA sequence

information obtained from the cDNAs was used to evaluate the phylogenetic relationship among rosaceous *S*-RNases.

2. Materials and methods

2.1 Protein assay

Styles with stigmas were dissected from flower buds of a self-incompatible cv. Sordum of Japanese plum at the balloon stage of development, immediately frozen in liquid N_2 and stored at − 80 °C until use. Acetone powder was prepared from the frozen samples as described previously (Tao *et al.*, 1997) and used for protein assay. Crude extracts from the acetone powder were subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) using nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and SDS-PAGE in the second dimension (Tao *et al.*, 1997). After electrophoresis, proteins in the gel were detected by silver staining using Sil-Best Stain for Protein/PAGE (Nacalai tesque, Kyoto, Japan). The proteins separated by 2D-PAGE were electroblotted onto a PVDF membrane as described previously (Tao *et al.*, 1997) and glycoproteins were detected with biotin-conjugated concanavalin A and horseradish peroxidase-conjugated streptavidin. Peroxidase activity on the membrane was visualized using 4-chloro-1-naphthol as a substrate. Immunodetection of proteins electroblotted onto PVDF membrane was also conducted using the mouse anti-S^4-serum prepared against purified S^4-RNase of Japanese pear (Sassa *et al.*, 1993, Tao *et al.*, 1997). In addition, after the proteins were blotted onto the PVDF membrane and detected by coomassie blue staining, portions of the PVDF membrane carrying the proteins of interest were cut out and subjected to a gas-phase protein sequencer (476A, Applied Biosystems, Tokyo, Japan) for N-terminal amino acid sequencing (Tao *et al.*, 1997).

2.2 PCR cloning of S-RNase cDNAs

Total RNA was isolated from styles with stigmas of 'Sordum' at the balloon stage of development using GLASSMAX™ RNA Microisolation Spin Cartridge System (Life Technologies, MD). One microgram of the total RNA was used for first strand cDNA synthesis by SuperScript II RT (Life Technologies, MD) with an Adapter-dT primer (5'-CGA CGT TGT AAA ACG ACG GCC AGT TTT TTT TTT TTT TTT-3') consisted of M13-20 sequence primer and oligo$(dT)_{16}$. Pru-T2 primer (5'-TST TST TGS TTT TGC TTT CTT C-3') (Tao *et al.*, 1999) derived from the DNA sequence corresponding to the signal peptide sequence of *S*-RNase of sweet cherry was used in 3' rapid amplification of cDNA ends (3'RACE) with M13-20 primer as the adapter primer. PCR was performed using a program of 30 cycles at 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 1 min with an initial denaturing of 94 °C for 3 min and a final extension of 72 °C for 7 min. The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 400 μM each of primers, the template cDNA equivalent to the amount synthesized from 0.1 μg of the total RNA, and 1 U of Ex Taq polymerase (TaKaRa Shuzo Co, Shiga, Japan) in a 50 μl reaction volume. The PCR products were subcloned into the T-A cloning vector (pGEM-T Easy Vector System; Promega, Madison, WI). Nucleotide sequences of several clones were determined with an automatic DNA sequencer (model 373A; Applied Biosystems, Tokyo, Japan).

2.3 Genomic DNA blot analysis

Total DNA was isolated from young leaves of 'Sordum' as described previously (Ushijima *et al.*, 1998). In brief, young leaves were ground into powder in liquid-N_2 using a mortar and a pestle, and homogenized with homogenization buffer (Zhang *et al.*, 1995) consisting of 10 mM Tris-HCl (pH 9.0), 10 mM EDTA (pH 8.0), 80 mM KCl, 0.5 M sucrose, 0.1 % PVP, 2 % 2-mercaptoethanol. The homogenate was centrifuged (8000 rpm at 4 °C for 20 min). After discarding the supernatant, the precipitate was resuspended in the homogenization buffer and centrifuged once again. Total DNA was isolated from the precipitate as described by Kim *et al.* (1997). Five micrograms of total DNA were digested by *Hin*dIII, run on a 0.8 % agarose gel and transferred to a nylon membrane (Hybond-N, Amersham, Tokyo, Japan). Two cDNAs encoding *S*-RNase of 'Sordum' obtained in this study were labeled by PCR with DIG-dUTP using the Pru-C2 and Pru-C5 as gene specific primers (Tao *et al.*, 1999) and used as probes for Southern hybridization. After high stringency washes of the membrane (2 × 5 min at room temperature with 2 × SSC and 0.1 % SDS followed by 2 × 15 min at 68 ℃ with 0.1 × SSC and 0.1 % SDS), hybridization signal was detected using the anti-DIG-alkaline phosphatase conjugate and the chemiluminescent substrate CSPD (Boehringer Mannheim). Chemiluminescence was documented on X-ray films.

2.4 RNA blot analysis

Total RNA was isolated from six different tissues of 'Sordum', styles with stigmas, ovaries, petals, calyxes, stamens of flower buds at the balloon stage of development and young leaves. Ten micrograms of the total

RNA were run on a formaldehyde (1 %) agarose gel and blotted onto a nylon membrane (Hybond-N, Amersham, Tokyo, Japan). Hybridization was performed with the two labeled-cDNAs as probes and immunologically detected as described above.

2.5 Construction of a phylogenetic tree

To construct a phylogenetic tree, amino acid sequences deduced from the two cDNA clones of 'Sordum' were used with those of 20 S-RNases from other rosaceous species, six S-RNases from Solanaceae, two S-RNases from *Antirrhinum* (Scrophulariaceae) and four S-like RNases of angiosperms as an outgroup. The 32 amino acid sequences were aligned using Clustal X (Thompson *et al.*, 1997), as described by Richman *et al.* (1997). The neighbor-joining method (Saitou and Nei, 1987) was used for genealogical reconstruction.

3. Results and discussion

3.1 Protein assay

After 2D-PAGE and silver staining of stylar extracts from 'Sordum', we detected two major protein spots (referred as S^a- and S^b-RNases hereafter) that had many characteristics in common with S-RNase of other rosaceous species **(Fig. 1A)**. These proteins showed similar *Mr* and pI to those of other S-RNases in Rosaceae (Ishimizu *et al.*, 1996; Sassa *et al.*, 1993, 1994; Tao *et al.*, 1997, 1999). As with other rosaceous S-RNases, these stylar proteins were also shown to be glycoproteins that

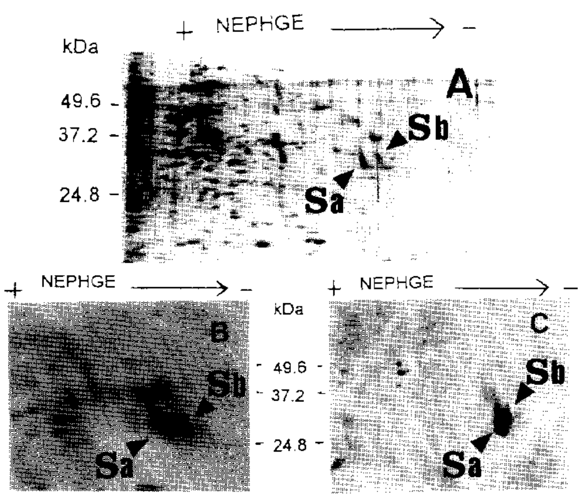

Fig. 1. Identification and characterization of S-RNases of Japanese plum cv. Sordum. Stylar proteins were separated by 2D-PAGE and detected by silver staining (A), glycoprotein staining with concanavalin A (B), and immunoblotting with the anti-S^4-serum prepared against purified S^4-RNase of Japanese pear (C). S-RNases are marked with *arrowheads*.

contained sugar chains reacting with concanavalin A **(Fig. 1B)**. Furthermore, these proteins exhibited an immuno-reaction with anti-S^4-serum raised against S^4-RNase of Japanese pear. This indicates that they are immunologically similar to S-RNase of Japanese pear **(Fig. 1C)**. The N-terminal amino acid sequence of S^a-protein was highly homologous to those of S-RNases of other *Prunus* spp., such as almond (Tao *et al.*, 1997) and sweet cherry (Tao *et al.*, 1999) **(Fig. 2)**. N-terminal sequence data of S^b-

	1 5 * ** 10 * 15 20
PS-Sa	DG**SY**D**YFQFVQQWPPT**N**C**RVR
PA-S2	DG**SY**D**YFQFVQQWPPT**N**C**RVR
PA-S3	DG**SY**V**YFQFVQQWPPT**T**C**RVQ
PA-S6	--**SY**V**YFQFVQQWPPT**N**C**RVR
PD-Sb	--**SY**V**YFQFVQQWPPT**N**C**R
PD-Sc	SG**SY**D**YFQFVQQWPPT**N**C**R
MD-Sc	---YDYFQFTQQYQPAVCH
MD-Sf	---FDYYQFTQQYQPAVCN
PC-S5	---YDYFQFTQQYQPAA
PP-S4	---FDYFQFTQQYQPAVCN
PP-S5	---YDYFQFTQQYQLAVCN
PU-S4	---FDYYQFTQQYQPAV
	C1

Fig. 2. Alignment of N-terminal amino acid sequences of S-RNase of Japanese plum cv. Sordum and other rosaceous S-RNases. Amino acid sequences corresponding to the C1, previously reported conserved domain of S-RNases in Solanaceae (Ioerger *et al.*, 1991) and Rosaceae (Norioka *et al.*, 1996; Ushijima *et al.*, 1998), are indicated at the bottom. Amino acid residues conserved in all *Prunus* S-RNases are indicated by bold face and those conserved in all rosaceous S-RNases are marked with *asterisks*. Sequence data for the S-RNases included are as follows: PS-Sa [S^a-RNase of *Prunus salicina* (Japanese plum) (this study)]; PA-S2, S3, and S6 [S^2, S^3, and S^6-RNases of *Prunus avium* (sweet cherry) (Tao *et al.*, 1999)]; PD-Sb and Sc [S^b and S^c-RNases of *Prunus dulcis* (almond) (Ushijima *et al.*, 1998)]; MD-Sc and Sf [S^c and S^f-RNases of *Malus x domestica* (apple) (Sassa *et al.*, 1996); PC-S5 [S^5-RNase of *Pyrus communis* (European pear) (Tomimoto *et al.*, 1996)]; PP-S4 and S5 [S^4 and S^5-RNases of *Pyrus pyrifolia* (Japanese pear) (Sassa *et al.*, 1996; Sassa and Hirano, 1997)]; PU-S4 [S^4-RNase of *Pyrus ussuriensis* (Chinese pear) (Tomimoto *et al.*, 1996)].

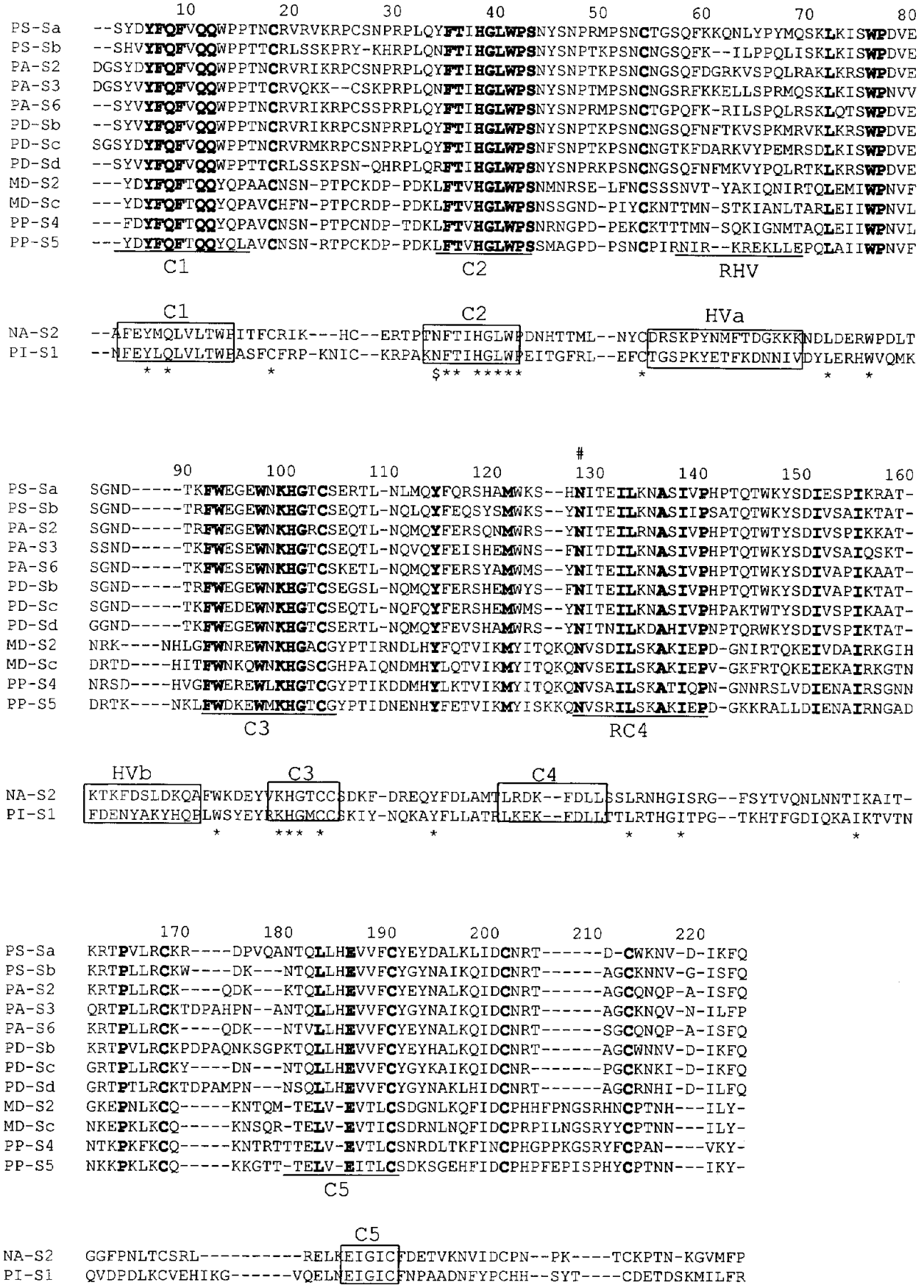

Fig. 3. Amino acid sequence alignment of *S*-RNases of Japanese plum and other species. The alignment was generated by CLUSTAL X (Thompson *et al.*, 1997). Gaps are marked by *dashes*. The five conserved regions, C1, C2, C3, RC4 and C5 (Ushijima *et al.*, 1998), and one hypervariable region, RHV (Ushijima *et al.*, 1998), reported in the rosaceous *S*-RNases are shown under the alignment of the rosaceous *S*-RNases and underlined. The five conserved regions, C1 to C5 (Ioerger *et al.*, 1991), and two hypervariable region, HVa and HVb (Ioerger *et al.*, 1991),

Fig. 3. (continued) reported in the solanaceous *S*-RNases are shown over the alignment of NA-S2 [S^2-RNase of *Nicotiana alata* (DDBJ/EMBL/GenBank Accession No. U08860)] and PI-S1 [S^1-RNase of *Petunia inflata* (Ai *et al.*, 1990)] and *boxed*. Conserved residues in rosaceous and all *S*-RNases are indicated by bold face and asterisks, respectively. Potential *N*-glycosylation sites conserved among the *S*-RNases of Rosaceae and Solanaceae are marked with # over the alignment of rosaceous *S*-RNases and $ under the alignment of solanaceous *S*-RNases, respectively. The amino acid sequences of the *S*-RNases included are as follows: PS-Sa and Sb [S^a and S^b-RNases of *Prunus salicina* (Japanese plum) (Acc. Nos. AB026981, AB026982) (this study)]; PA-S2, S3, and S6 [S^2, S^3, and S^6-RNases of *Prunus avium* (sweet cherry) (Acc. Nos. AB010304, AB010305, AB010306) (Tao *et al.*, 1999)]; PD-Sb, Sc, and Sd [S^b, S^c and S^d-RNases of *Prunus dulcis* (almond) (Acc. Nos. AB011469, AB011470, AB011471) (Ushijima *et al.*, 1998)]; MD-S2 and Sc [S^2 and S^c-RNases of *Malus x domestica* (apple) (Acc. Nos. U12199, D50836) (Broothearts *et al.*, 1995; Sassa *et al.*, 1996)]; PP-S4 and S5 [S^4 and S^5-RNases of *Pyrus pyrifolia*, syn. *Pyrus serotina*) (Japanese pear) (Sassa *et al.*, 1996; Sassa and Hirano, 1997)].

RNase were not obtained because S^b-RNase overlapped with other proteins in the 2D-PAGE profile.

3.2 cDNA cloning of S-RNases

The Pru-T2 primer designed from DNA sequences corresponding to the signal peptide sequence of the *S*-RNase in sweet cherry (Tao *et al.*, 1999) was successfully used to obtain cDNA clones encoding *S*-RNases of 'Sordum' in 3'RACE by PCR. Nine cDNA clones obtained were classified into two groups by PCR amplification of the insert DNA using the AS1 and the Pru-C5 primers that correspond to the N-terminal and the fifth conserved domain of S-RNases of sweet cherry, respectively (Tao *et al.*, 1999). The PCR-amplified fragments of seven of the nine cDNA clones had the same *Sty*I restriction site, while the other two had no *Sty*I site. Thus the seven clones seemed to be the same and the other two to be the same. From DNA sequencing conducted for two each of the clones with and without *Sty*I site, it was confirmed that the clones with *Sty*I site had exactly the same sequence and the clones with no *Sty*I site had the same DNA sequence. Comparisons of the deduced amino acid sequences from the two different cDNA clones and the N-terminal sequence of the S^a-RNase revealed that the cDNA clones with *Sty*I site encoded the S^a-RNase and the other cDNA clones with no *Sty*I site encoded the S^b-RNase. The deduced amino acid sequences from the S^a- and S^b-cDNA clones contained two active sites of T2/*S* type RNases (Kawata *et al.*, 1988), and five regions conserved among the rosaceous *S*-RNases (Ushijima *et al.*, 1998) (**Fig. 3**). In addition, seven cysteine residues conserved among other *S*-RNases and potential *N*-glycosylation site conserved among other rosaceous *S*-RNases were present in S^a- and S^b-RNases of Japanese plum.

3.3 Genomic DNA blot analysis

Two bands of 4.8 kb and 14.5 kb were observed with *Hin*dIII digestion (**Fig. 4**). Relative intensities of the bands obtained with *Hin*dIII, however, varied with the different probes. When the S^a-probe was used, the intensity of the 4.8 kb fragment was stronger than that of the 14.5 kb fragment, while when S^b-probe was used *vice versa*. Thus the 4.8 kb fragment seemed to be from the S^a-allele and the 14.5 kb fragment from the S^b-allele. Since Japanese plum is a diploid species two different *S*-alleles should be present as a single copy gene. Genomic DNA blot analysis supported that a single copy each of the S^a- and S^b-RNase genes exist in the genome of Japanese plum.

3.4 RNA blot analysis

Hybridization signal at about 900 bp, which coincided with the estimated size from the cDNAs, was detected only with the total RNA isolated from styles with stigmas, whereas the total RNAs from

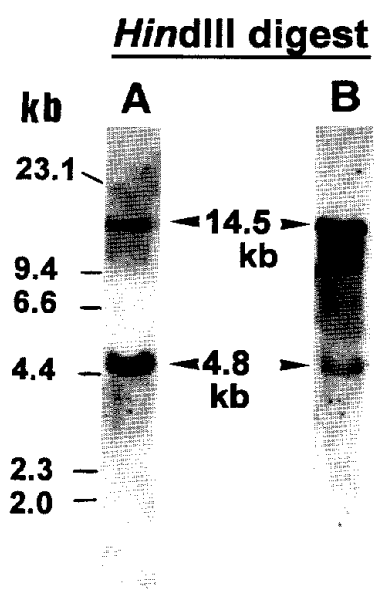

Fig. 4. Genomic DNA blot analysis of Japanese plum cv. Sordum. The blot was hybridized to the C2-C5 fragments (Tao *et al.*, 1999) of the cDNAs encoding S^a-RNase (A) and S^b-RNase (B).

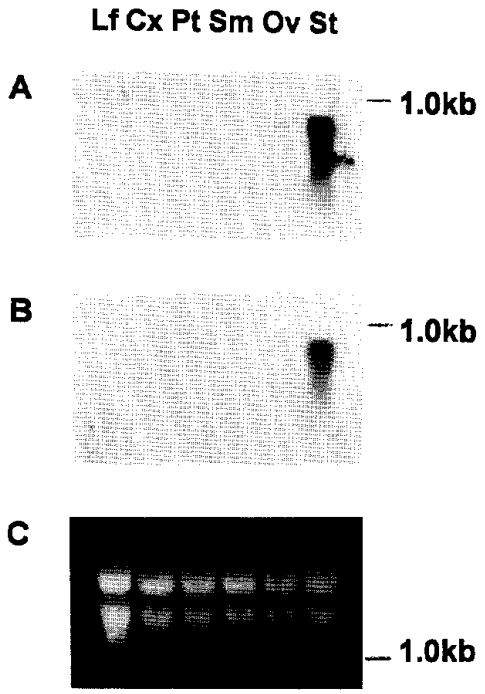

Fig. 5. RNA blot analysis of total RNA from leaf (Lf), calyx (Cx), petal (Pt), stamen (Sm), ovary (Ov) and style with stigma (St) from flower buds of Japanese plum cv. Sordum at the balloon stage of development. The blot was hybridized to the C2-C5 fragments of the cDNAs encoding S^a-RNase (A) and S^b-RNase (B). The RNA gel before blotting was stained by ethidium bromide (C).

petals, ovaries, calyxes, stamens, and young leaves gave no hybridization signal (**Fig. 5**). There was no difference in size of the band obtained using two different probes. The expression pattern of the genes corresponding to S^a- and S^b-cDNAs of Japanese plum is consistent with that of other rosaceous *S*-RNases (Sassa *et al.*, 1996; Tao *et al.*, 1999).

3.5 Sequence similarity and phylogenetical analysis

As shown in **Table 1**, similarities of amino acid sequences between the two *S*-RNases of Japanese plum and other *Prunus S*-RNases were high (71 to 82%). However, similarities between the *S*-RNases of Japanese plum and other rosaceous, solanaceous, and scrophulariaceous *S*-RNases were low (24 to 31%). Phylogenetic analysis further confirmed that S^a- and S^b-RNases belong to the *S*-RNase family (**Fig. 6**). Three distinct groups, *S*-like RNases, *S*-RNases of Asteridae and *S*-RNases of Rosaceae, were detected, and the *S*-RNases of Rosaceae were further divided into two subgroups, *S*-RNases of Prunoideae and Maloideae. The subgroup of Prunoideae included the *S*-RNases of Japanese plum but species-specific subgroup was not found in the subgroup of Prunoideae, which supports the hypothesis that the *S*-RNases of Rosaceae have diverged after the divergence of subfamilies but before the divergence of species (Ushijima *et al.*, 1998). In *Prunus*, many important commercial fruit tree species are included. If the rosaceous *S*-RNases evolved trans-specifically as expected, most of *Prunus* species should have *S*-RNases in the style. It is interesting to see whether self-compatible species of *Prunus*, such as peach (*P. persica*) and European plum (*P. domestica*), express a high level of *S*-RNases in the style.

Although no direct evidence for the linkage of S^a- and S^b-cDNAs to the *S*-locus of 'Sordum' is presented in this study, all the experimental evidence from the protein, DNA, and phylogenetic analyses indicate that the cDNAs indeed encode *S*-RNases of Japanese plum. The knowledge obtained here should lead to the further understanding of the

Table 1. Sequence similarities among *S*-RNases* (%).

	PS-Sb	PA-S2	PA-S3	PA-S6	PD-Sb	PP-S5	MD-S2	NA-S2	LP-S3	AH-S4
PS-Sa	72	74	71	76	82	28	27	24	31	26
PS-Sb		79	77	78	76	29	29	25	30	25
PA-S2			73	83	85	29	29	25	29	26
PA-S3				74	74	28	27	24	29	24
PA-S6					80	29	29	23	30	26
PD-Sb						27	27	24	29	25
PP-S5							64	20	21	19
MD-S2								20	18	20
NA-S2									43	31
LP-S3										30

*Designation of *S*-RNases is as Fig. 6

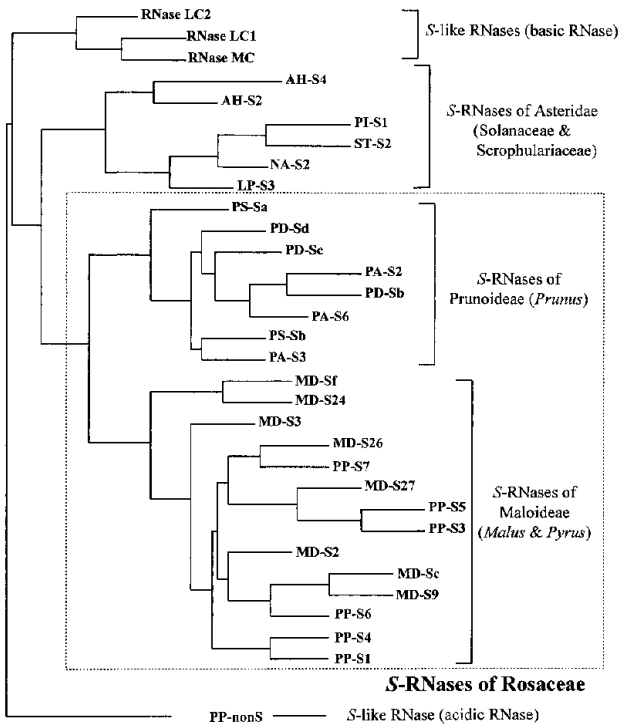

Fig. 6. Phylogenetic tree of the *S*-RNases. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) based on an alignment of 32 T2/*S* type RNases. Sequence data for the *S*-RNases included are as follows: PS-Sa and Sb [S^a and S^b-RNases of *Prunus salicina* (Japanese plum) (DDBJ/EMBL/GenBank Accession Nos. AB026981, AB026982) (this study)]; PA-S2, S3, and S6 [S^2, S^3, and S^6-RNases of *Prunus avium* (sweet cherry) (Acc. Nos. AB010304, AB010305, AB010306) (Tao *et al.*, 1999)]; PD-Sb, Sc, and Sd [S^b, S^c, and S^d-RNases of *Prunus dulcis* (almond) (Acc. Nos. AB011469, AB011470, AB011471) (Ushijima *et al.*, 1998)]; MD-S2 and S3 [S^2 and S^3-RNases of *Malus x domestica* (apple) (Acc. Nos. U12199, U12200) (Broothearts *et al.*, 1995)]; MD-Sc and Sf [S^c and S^f-RNases of apple (Acc. Nos. D50836, D50837) (Sassa *et al.*, 1996)]; MD-S9, S24, S26, and S27 [S^9, S^{24}, S^{26} and S^{27}-RNases of apple (Acc. Nos. U19793, AF016920, AF016918, AF016919)]; PP-S4 [S^4-RNase of *Pyrus pyrifolia* (syn. *Pyrus serotina*) (Japanese pear) (Sassa *et al.*, 1996)]; PP-S5 [S^5-RNase of Japanese pear (Acc. No. D88282) (Sassa and Hirano, 1997)]; PP-S1, S3, S6, and S7 [S^1, S^3, S^6, and S^7-RNases of Japanese pear (Acc. Nos. AB002139, AB002140, AB002142, AB002143) (Ishimizu *et al.*, 1998)]; AH-S2 and S4 [S^2 and S^4-RNases of *Antirrhinum hispanicum* (Acc. Nos. X96465, X96466) (Xue *et al.*, 1996)]; LP-S3 [S^3-RNase of *Lycopersicon peruvianum* (Acc. No. X76065) (Royo *et al.*, 1994)]; NA-S2 [S^2-RNase of *Nicotiana alata* (Acc. No. X76065)]; PI-S1 [S^1-RNase of *Petunia inflata* (Ai *et al.*, 1990)]; ST-S2 [S^2-RNase of *Solanum tuberosum* (Acc. No. X62727) (Kaufmann *et al.*, 1991)]. Sequence data for the *S*-like RNases included are as follows: RNase LC1 and LC2 from *Luffa cylindrica* (Acc. Nos. D64011, D64012); RNase MC from *Momordica charantia* (Acc. No. P23540); PP-nonS [non-*S*-RNase of Japanese pear (Acc. No. D49529) (Norioka *et al.*, 1996)].

molecular mechanisms underlying self-incompatibility in Japanese plum and facilitate the breeding of self-compatible cultivars through antisense RNA technology.

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