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Identification of Stylar RNases Associated with Gametophytic Self-Incompatibility in Almond (*Prunus dulcis*)

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Stylar proteins of 13 almond (*Prunus dulcis*) cultivars with known *S*-genotypes were surveyed by IEF and 2D-PAGE combined with immunoblot and N-terminal amino acid sequence analyses to identify *S*-RNases associated with gametophytic self-incompatibility (SI) in this plant species. RNase activities corresponding to S_a and S_b, two of the four *S*-alleles tested, were identified by IEF and RNase activity staining. The S_a-RNase band reacted with the anti-S_f-serum prepared from Japanese pear (*Pyrus serotina*); no reaction with the antiserum was observed with the S_b-RNase band. When the S_a-RNase band was excised from an IEF gel stained for RNase activity, subjected to SDS-PAGE, and detected by immunoblotting, it appeared that this band consisted of a single protein that reacted with the anti-S_f-serum with M_r of about 28 kDa. With 2D-PAGE and silver staining of the stylar extracts, all four *S*-proteins could be successfully distinguished from each other in the highly basic zone of the gel. Although S_b-, S_c-, and S_d-proteins had roughly the same M_r of about 30 kDa, the S_c-protein seemed to be slightly smaller than the S_b-protein and slightly larger than the S_d-protein. In 2D-PAGE profiles as well, the S_a-protein had M_r of about 28 kDa, apparently smaller than the other three proteins. A bud sport, in which one of the two *S*-alleles of the original cultivar is impaired, was visualized as a loss of S_c-protein, which is consistent with the previous pollination study. All four *S*-proteins reacted with the anti-S_f-serum, probably because of the differing conformations of these *S*-proteins in the IEF and 2D-PAGE gels. The S_a-protein in 2D-PAGE appeared to be identical to S_a-RNase in IEF; both had the same M_r and were reactive with the anti-S_f-serum. N-terminal amino acid sequence analysis of the four *S*-proteins revealed that they were highly homologous to each other and similar to the *S*-RNases of *Malus, Pyrus*, Scrophulariaceae, and Solanaceae. Taken together, RNases in the style are strongly suggested to be associated with the gametophytic SI of almond. This is the first report identifying and characterizing *S*-RNase in almond.

Key words: Almond — Gametophytic self incompatibility — Isoelectric focusing — *Prunus dulcis* — *S*-RNase — Two-dimensional gel electrophoresis.

SI is a widespread mechanism in flowering plants which prevents self-fertilization and promotes out-crossing (de Nettancourt 1977). The predominant class of SI is the gametophytic SI system that is controlled by a single locus with multiple alleles (de Nettancourt 1977, Kao and Huang 1994, Newbigin et al. 1993). With this system, growth of pollen tube bearing either one of the two *S*-alleles carried by the recipient pistil is arrested in the style. The physiology and mechanisms of gametophytic SI have been studied in solanaceous plant species. cDNAs associated with *S*-locus of gametophytic SI were first cloned from *Nicotiana alata*, a Solanaceae (Anderson et al. 1986, 1989). The deduced amino acid sequence strongly implicated stylar RNase involvement in the recognition and rejection reaction within the style (McClure et al. 1989). From these and other studies, it has been shown that the *S*-gene products in pistils of Solanaceae are highly basic glycoproteins containing sequence motifs characteristic of the active site of the fungal RNases T2 (Kawata et al. 1988) and Rh (Horiuchi et al. 1988). Recent results with transgenic *Petunia inflata* (Lee et al. 1994, Huang et al. 1994) and *Nicotiana* (Murfett et al. 1994, 1995) have clearly demonstrated that *S*-RNases alone are sufficient for determining pollen rejection response in the gametophytic SI system of Solanaceae. Very recently, Xue et al. (1996) cloned cDNAs encoding polypeptides homologous to *S*-RNase from the Scrophulariaceae (*Antirrhinum*), a family closely related to the Solanaceae (Brewbaker 1967, Chase et al. 1993), and clearly showed that these cDNA clones are encoded by genes at the *S*-locus. cDNAs associated with the *S*-locus have also been cloned from Papaveraceae (*Papaver*) (Foote et al. 1994) and Poaceae (*Phalaris*) (Li et al. 1994). The deduced amino acid sequences of *S*-proteins in these families are quite different from each other and contain no RNase catalytic

Abbreviations: CIG, cross incompatibility group; 2D-PAGE, two dimensional polyacrylamide gel electrophoresis; IEF, isoelectric focusing; NEPHGE, nonequilibrium pH gradient electrophoresis; M_r, relative molecular weight; SC, self-compatibility; SI, self-incompatibility.

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domains.

Recently, Sassa et al. (1992, 1993, 1994, 1996) and Broothaerts et al. (1995) have reported that *S*-RNases are also associated with the gametophytic SI of Japanese pear (*Pyrus serotina*) and apple (*Malus domestica*), which belong to the Rosaceae. It is interesting that the Rosaceae and Solanaceae share the same mechanisms of gametophytic SI, despite the fact that these two families are taxonomically remote (Brewbaker 1967, Chase et al. 1993). The N-terminal amino acid sequences of *S*-RNases of Japanese pear and apple are quite similar (Sassa et al. 1993, 1996). PCR clones from genomic DNA and cDNAs of *S*-RNases have been cloned for Japanese pear (Norioka et al. 1995, 1996, Sassa et al. 1996) and apple (Broothaerts et al. 1995, Sassa et al. 1996) and the sequence data obtained was used to deduce the amino acid sequences. Although these sequences contained two conserved domains characteristic of the T2/S-type RNases, differences were evident between rosaceous and solanaceous *S*-RNases (Sassa et al. 1996). Based on the DNA sequence data, a practical method was developed to type *S*-alleles using allele-specific PCR amplification and restriction enzyme digestion (Janssens et al. 1995).

Prunus, one of the genera in the Rosaceae, includes important commercial fruit and nut tree species (horticulturally often referred as stone fruits or drupes) such as almond (*Prunus dulcis*), apricot (*Prunus armeniaca*), European or common plum (*Prunus domestica*), Japanese plum (*Prunus salicina*), Japanese apricot (*Prunus mume*), peach (*Prunus persica*), sour cherry (*Prunus cerasus*), and sweet cherry (*Prunus avium*). Fertilization is a very important factor in stone fruit production; they are unable to bear fruits parthenocarpically. For almond production, fertilization is essential because the commercial part of the fruit is the seed. As most of the fruit tree species of *Prunus* are partially or fully SI, cross-compatible cultivars that flower simultaneously are inter-planted in commercial orchards and beehives are introduced to ensure cross-pollination. In Japan, hand-pollination is sometimes practiced although it is a very labor intensive and expensive operation. Thus, there has been recurring interest in SC cultivars in SI species of *Prunus* (Janick and Moore 1975, Kester et al. 1991, Socias i Company 1991, Tehrani and Brown 1992). Elucidating the physiological and molecular mechanisms of gametophytic SI in *Prunus* would help breeders to produce SC cultivars through molecular techniques such as the antisense-expression of cDNAs associated with SI in Solanaceae (Lee et al. 1994, Huang et al. 1994, Murfett et al. 1994, 1995).

Since *Prunus* is one of the Rosaceae genera, *S*-RNase may play a role in rejecting SI pollen tube growth similar to that in *Malus* and *Pyrus*. As expected, while we were preparing this manuscript, Boskovic and Tobutt (1996) reported the correlation of stylar RNase zymograms with incompatibility alleles in sweet cherry. In their study, however, the identical *S*-allele of different cultivars or genotypes sometimes yielded different RNase activity bands with different pIs on IEF gel and it is unclear whether the RNase activities they found are true *S*-allelic products. We chose to use California almond cultivars to address the question of whether RNase plays an important role in gametophytic SI of *Prunus*, because all the possible combinations of the four known *S*-alleles are available in California almond cultivars (Kester et al. 1994a); not all the combinations of the known six *S*-alleles are available in sweet cherry (Tehrani and Brown 1992).

Materials and Methods

Plant materials—Twelve almond cultivars with known *S*-genotypes (Kester et al. 1994a) and 'Jeffries' (Kester et al. 1994b), a mutant of 'Nonpareil', were used in this study (Table 1). Styles were dissected from the flower buds at the pink bud to balloon stage of development (Flint and Kobbe 1985), immediately frozen in liquid N_2, and stored at −80°C until use. Acetone powder was prepared from the styles using pre-chilled acetone (−20°C) containing 0.07% (v/v) 2-mercaptoethanol and used for protein extraction.

IEF—Acetone powder (1 mg) was homogenized with 40 μl of ice-cold extraction buffer consisting of 9% (v/v) glycerol, 2% (v/v) Ampholine pH 3.5–10 (Pharmacia Biotech, Tokyo, Japan), and 0.01% (v/v) 2-mercaptoethanol. After centrifugation at 10,000 × *g* for 10 min, 15 μl of supernatant was subjected to IEF slab gel consisting of 7.5% (w/v) acrylamide, 0.3% (w/v) bis-acrylamide, 0.5% (v/v) glycerol, 3.5% (v/v) Ampholine pH 3.5–10, and 1.5% (v/v) Pharmalyte pH 8–10.5 (Pharmacia Biotech, Tokyo, Japan). The acidic and basic electrode solutions were 0.01 M H_3PO_4 and 0.02 M NaOH, respectively. The samples were loaded onto the acidic end of the gel for separation of basic proteins. IEF was performed in a refrigerator (4°C) at 100 V for 30 min, at 200 V for 2 h, at 300 V for 1 h, and finally at 400 V for 30 min. After electrophoresis, the gel was subjected to either RNase activity staining (Sassa et al. 1992, Wilson 1971) or electroblotting onto a PVDF membrane for immunodetection of *S*-proteins. After IEF, the gel was equilibrated with SDS-sample buffer, which consisted of 62.5 mM Tris-HCl (pH 6.8) containing 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol, and electroblotted onto a PVDF membrane according to the method of Hirano and Watanabe (1990). For RNase activity staining, the gel

Table 1 Almond cultivars used and their *S*-genotypes

CIG	Genotype	Cultivar
I	S_cS_d	IXL, Nonpareil, Tardy Nonpareil
II	S_aS_b	Languedoc, Mission
III	S_aS_c	Sauret no. 2, Wood Colony
IV	S_bS_c	Merced, Rosetta
V	S_aS_d	Carmel, Sauret no. 1
VI	S_bS_d	Monterey
None	$S_c^mS_d$	Jeffries

was immersed in 0.1 M sodium acetate buffer (pH 5.4) containing 0.1 M KCl for 30 min after IEF. It was then incubated in the same fresh buffer with added 0.4% (w/v) yeast RNA for 20 min. The gel was washed with 0.1 M sodium acetate buffer (pH 5.4) for 3 min, stained with 0.2% (w/v) toluidine blue in 0.5% (v/v) acetic acid for 1 min, and washed several times with distilled water. After RNase activity staining, RNase bands were excised, equilibrated with the SDS-sample buffer, subjected to 15% SDS-PAGE, and electroblotted onto a PVDF membrane (Hirano and Watanabe 1990) for immunodetection.

2D-PAGE—Acetone powder (1 mg) was homogenized with 80 μl of lysis buffer (O'Farrell 1975) consisting of 9.5 M urea, 2% (v/v) Nonidet P-40, 2% (v/v) Ampholine pH 3.5–10, 5% (v/v) 2-mercaptoethanol, and 5% (w/v) polyvinylpyrrolidone K30. After incubation at 60°C for 10 min, the supernatant was isolated by centrifugation at 10,000 × *g* for 10 min, and 50 μl aliquots was subjected to NEPHGE in the first dimension and SDS-PAGE in the second dimension, as described by O'Farrell et al. (1977) with slight modifications (Sassa et al. 1993). The samples were applied to the acidic end of the first dimensional gels consisting of 4% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide, 8 M urea, 2% (v/v) Nonidet P-40, 2% (v/v) Ampholine pH 3.5–10, and 4% (v/v) Pharmalyte pH 8–10.5. NEPHGE was conducted at 200 V for 20 min, at 300 V for 20 min, at 400 V for 2 h, and finally at 800 V for 30 min. After electrophoresis, the gels were equilibrated with the SDS-sample buffer and applied to 15% SDS-PAGE for the second dimensional electrophoresis. Proteins in the gel were detected by silver staining using the Sil-Best Stain for Protein/PAGE (Nacalai tesque, Kyoto, Japan) or electroblotted onto a PVDF membrane (Hirano and Watanabe 1990) for immunodetection of *S*-proteins and N-terminal amino acid sequence analysis.

Immunodetection—*S*-proteins were detected using anti-S_4-serum prepared against purified S_4-glycoprotein of Japanese pear (Sassa et al. 1993). The primary antibodies were bound with biotin-conjugated goat IgG raised against mouse IgG and then with peroxidase-conjugated avidin D. Finally, chemiluminescence was documented on X-ray films using ECL system according to the manufacturer's instructions (Amersham International, Buckinghamshire, England).

N-terminal amino acid sequence analysis—Proteins electroblotted onto a PVDF membrane were detected by Coomassie Blue staining (Hirano et al. 1991). Portions of the PVDF membrane carrying the *S*-proteins were cut out and subjected to a gas-phase protein sequencer (476A, Applied Biosystems, CA) for N-terminal amino acid sequencing. Edman degradation was performed according to the standard program obtained from Applied Biosystems (Foster, CA).

Results and Discussion

Several bands of RNase activity were observed in the highly basic zone of the IEF gel (Fig. 1). From the IEF profiles, we could detect RNase activities corresponding to S_a and S_b, two of the four *S*-alleles tested. The most basic band could be identified as S_b-RNase because all the cultivars with this allele, Mission (S_aS_b), Languedoc (S_aS_b), Merced (S_bS_c), Rosetta (S_bS_c), and Monterey (S_bS_d), showed the band; no other cultivar tested yielded the band. The second basic band appeared to correspond to S_a-RNase because it was only detected with the cultivars possessing S_a allele, Mission (S_aS_b), Languedoc (S_aS_b), Sauret no. 2 (S_aS_c), Wood Colony (S_aS_c), Carmel (S_aS_d), and Sauret no. 1 (S_aS_d). All of the other less basic bands were observed with all the cultivars tested and seemed to be not associated with SI. The fact that we could not identify S_c- and S_d-RNase activities might be explained by comigration of S_c- and S_d-RNases with the non-specific RNases or low expression level of S_c and S_d, as is the case with Japanese pear S_2-protein (Sassa et al. 1992).

When 2D-PAGE was used to separate the crude preparation of the stylar extracts of almond, all four *S*-proteins could be successfully distinguished from each other (Fig. 2). 2D-PAGE profiles of all the cultivars tested were consistent with the *S*-genotypes obtained from the pollination study (Kester et al. 1994a). A schematic diagram of

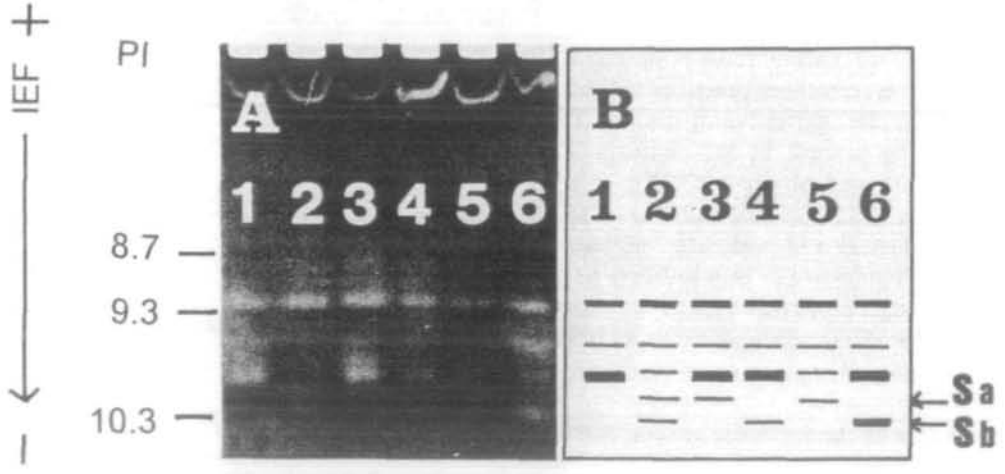

Fig. 1 Photograph (A) and interpretive drawing (B) of RNase activities in IEF gel of style proteins of almond cultivars. 1, Nonpareil (S_cS_d); 2, Mission (S_aS_b); 3, Sauret no. 2 (S_aS_c); 4, Merced (S_bS_c); 5, Carmel (S_aS_d); and 6, Monterey (S_bS_d). *S*-RNases are marked with *arrows*.

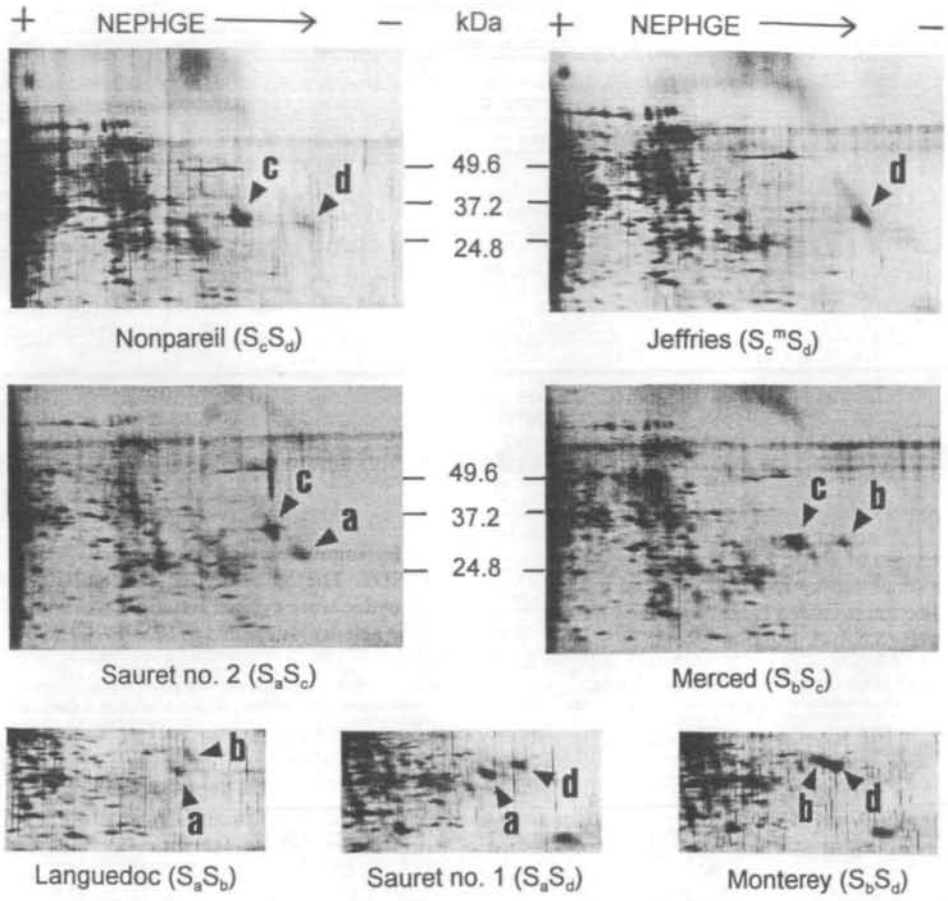

Fig. 2 2D-PAGE profiles of style proteins of almond cultivars. *S*-proteins are marked with *arrowheads*.

2D-PAGE profile of *S*-proteins is illustrated in Fig. 3. Although S_b-, S_c-, and S_d-proteins had roughly the same M_r of about 30 kDa, the S_c-protein seemed to be slightly smaller than the S_b-protein and slightly larger than the S_d-protein. S_a-protein had M_r of about 28 kDa and is apparently smaller than the other three proteins. Thus S_b-, S_c-, S_d-, and S_a-proteins could be aligned by their M_r from largest to smallest in that order. S_c-, S_a-, S_b-, and S_d-proteins could also be aligned by their pI values from least to most basic in that order. Taking comparison of the 2D-PAGE profiles (Fig. 2) into consideration, we conclude that no S_d-RNase activity could be detected on the gel; the S_c-RNase activity band might have overlapped with the third or less basic bands on IEF gel or, perhaps, no S_c-RNase activity could be detected as is the case with S_d-RNase. Since the S_d-spot after silver staining on 2D-PAGE gel was weaker than S_c-protein but as intense as S_a- and S_b-proteins (Fig. 2), the S_d-protein itself may have weaker RNase activity than the other three *S*-RNases.

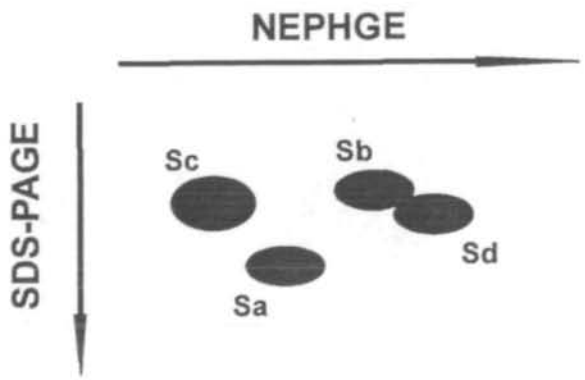

Fig. 3 Spacial distribution and relative intensities of almond *S*-proteins in 2D-PAGE profiles.

By comparing 2D-PAGE profiles for 'Jeffries' and its parent 'Nonpareil', we could further identify S_c- and S_d-proteins on the gel (Fig. 2) and confirm the previous results obtained from pollination studies (Kester et al 1994a, b). 'Jeffries' was discovered as a high yielding limb in a 'Nonpareil' orchard in which no other cultivar had been provided for cross-pollination. Except for its high productivity, the cultivar is identical to 'Nonpareil' in tree and nut characteristics (Kester et al. 1994b). 'Jeffries' is not SC but pro-

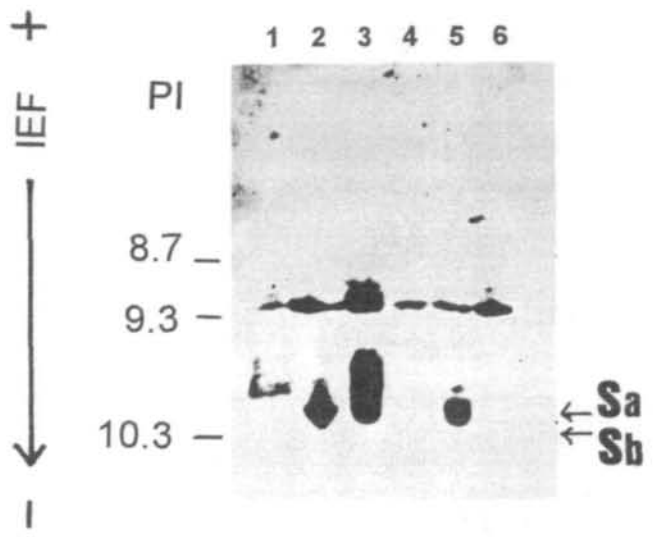

Fig. 4 Immunodetection of almond *S*-RNases separated by IEF. Stylar proteins were separated by IEF and detected by immunoblotting with anti-S_4-serum prepared from Japanese pear (Sassa et al. 1993). 1, Nonpareil (S_cS_d); 2, Mission (S_aS_b); 3, Sauret no. 2 (S_aS_c); 4, Merced (S_bS_c); 5, Carmel (S_aS_d); and 6, Monterey (S_bS_d). The positions where S_a- and S_b-RNase activity bands were located on the same gel after RNase activity staining are marked with *arrows*.

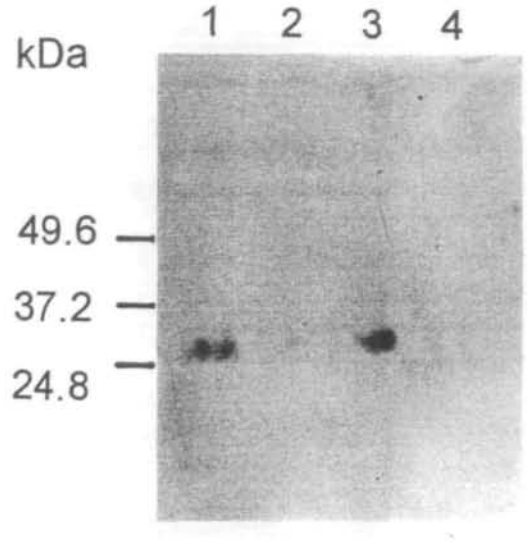

Fig. 5 Immunodetection of almond *S*-RNases separated by IEF and SDS. The S_a- and S_b-RNase activity bands of Mission and Languedoc were excised from IEF gel which had been stained for RNase activity, subjected to SDS-PAGE, and detected by immunoblotting with anti-S_4-serum prepared from Japanese pear (Sassa et al. 1993). 1, S_a of Mission; 2, S_b of Mission; 3, S_a of Languedoc; and 4, S_b of Languedoc.

duced good set when pollinated by 12 almond cultivars representing the entire range of CIGs (Kester et al. 1994a, b). Pollination studies revealed that 'Jeffries' expressed a loss of S_c allele, one of the two alleles (S_cS_d) of the parent 'Nonpareil', and behaved like S_dS_d (Kester et al. 1994a, b). Our results obtained from 2D-PAGE were consistent with these data and clearly demonstrated that S_c-protein was missing in the stylar extracts of 'Jeffries' (Fig. 2). Since 'Jeffries' is a rare type of mutation in the gametophytic SI system, comparisons of 'Jeffries' with its parent 'Nonpareil' might provide further understanding of the physiology of gametophytic SI.

When proteins separated by IEF were immunologically detected with the anti-S_4-serum prepared from Japanese pear, a band corresponding to S_a was found among the protein bands reacted with the anti-S_4-serum (Fig. 4). The S_a-corresponding protein band had the same pI value as the S_a-RNase on IEF gel; no reaction was observed in the area of PVDF membrane where S_b-RNase was located, and neither an S_c- nor S_d-corresponding reaction was found. Since a non-specific reaction with some almond stylar proteins and no reaction with S_b-RNase were observed with the anti-S_4-serum, the S_a- and S_b-RNase activity bands of Mission (S_aS_b) and Languedoc (S_aS_b) were cut out from the IEF gel after RNase activity staining, subjected to SDS-PAGE, and immuno-

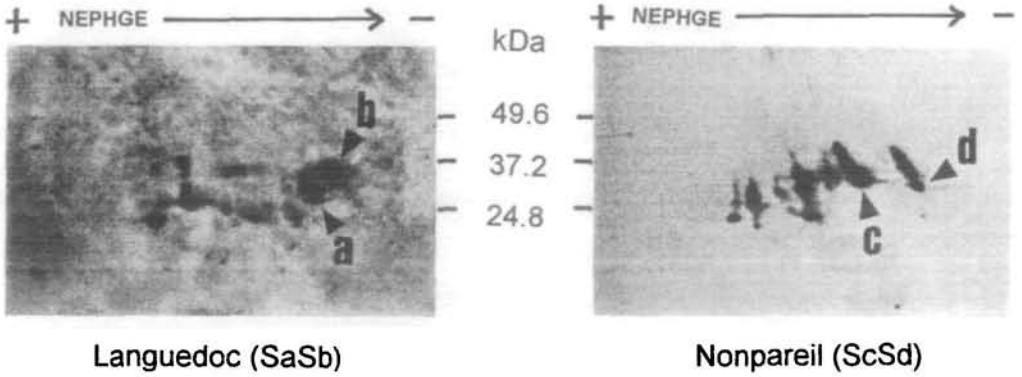

Fig. 6 Immunodetection of almond *S*-proteins separated by 2D-PAGE. Stylar proteins were separated by 2D-PAGE and detected by immunoblotting with anti-S_4-serum prepared from Japanese pear (Sassa et al. 1993). *S*-RNases are marked with *arrowheads*.

logically examined. The S_a-RNase appeared to consist of a single protein that reacted with the anti-S_4-serum and had the same M_r as that of S_a-protein separated by 2D-PAGE; no reaction was again observed with S_b-RNase activity band (Fig. 2, Fig. 5).

Immunodetection of the proteins separated by 2D-PAGE gave different results. All four S-proteins reacted with the anti-S_4-serum although some proteins unrelated to S-allele were also detected (Fig. 6). The S_a-protein in 2D-PAGE appeared to be identical to S_a-RNase in IEF; both had the same M_r and were reactive with the anti-S_4-serum. It is unclear why only the S_a-RNase band in IEF reacted with the anti-S_4-serum. One possible explanation is that urea and/or Nonidet P-40 used for 2D-PAGE affected the conformation of the S-proteins, so that the reactivity of the S-proteins with the anti-S_4-serum changed.

Using Languedoc (S_aS_b) and Tardy Nonpareil (S_cS_d), N-terminal amino acid sequences of all the four S-proteins were determined. N-terminal amino acid sequences of S-proteins of almonds were highly homologous to each other and similar to those of S-RNases of *Malus, Pyrus*, Scrophulariaceae, and Solanaceae. The identity of C1, which corresponds to the previously identified conserved domains among S-RNases of solanaceous species (Tsai et al. 1992), is about 60%, 60%, 50%, and 50–70%, respectively (Fig. 7). The 16th amino acid residue from the N-terminus (the 18th from N-terminal in the case of S_c) of almond S-proteins could not be determined by our procedure. It is quite possible that this residue is a cysteine, as cysteines are conserved in the corresponding amino acid position in all S-RNases reported so far. Phenylthiohydantoin (PTH) cysteine obtained from Edman degradation is very unstable and rarely recovered by HPLC. It is suggested, therefore, that the 16th amino acid from the N-terminal is also cysteine in almond. As all the four almond S-proteins are similar in terms of M_r, pI, reactivity with the anti-S_4-serum from Japanese pear, and N-terminal amino acid sequence to other S-RNases, we have termed the S-proteins observed in 2D-PAGE as S-RNases, although we have only experimentally shown that S_a-protein has RNase activity. It is interesting that some of the conserved amino acid residues in almond S-RNases, which differ from those of *Malus* and *Pyrus* S-RNases, are common to those conserved residues of scrophulariaceous and solanaceous S-RNases. For example, the amino acid residues marked with open circles in almond S-RNases in Fig. 7 are common to the corresponding conserved residues of scrophulariaceous and solanaceous S-RNases; in *Malus* and *Pyrus*, different amino acid residues are conserved. Ishimizu et al. (1996) and Norioka et al. (1996) reported that the C1 domain of rosaceous S-RNase is structurally distinct from solanaceous S-RNase and speculated that the N-terminal is Rosaceae-specific. However, our results with S-RNase of almond do not support their interpretation. It is possible that the C1 domain is only *Malus*- and *Pyrus*-specific but not Rosaceae-specific. In terms of morphological and genetic characteristics, *Malus* and *Pyrus* are more closely related to

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Rosaceae          * **o *oo   ↓
PD-Sa          SYQYFQFVQQWPPTTxA
PD-Sb          SYQYFQFVQQWPPTNxA
PD-Sc        SGSYDYFQFVQQWPPTNxR
PD-Sd          SYVYFQFVQQWPPTxxR
MD-Sc           YDYFQFTQQYQPAVCH
MD-Sf           FDYYQFTQQYQPAVCN
PC-S5           YDYFQFTQQYQPAA
PC-S6         AKYDYLQFTQQYQPA
PS-S1           YDYFQFTQQYWPAV
PS-S4           FDYFQFTQQYQPAVCN
PS-S5           YDYFQFTQQYQLAVCN
PU-S2           YDYFQFTLQYQPAV
PU-S4           FDYYQFTQQYQPAV

Scrophulariaceae
AH-S2           FDYFKLVLQWPNSYCS
AH-S4           CDYLKLVLQWPKSFCL
AH-S5           FEILKLVLQWPNSYCS

Solanaceae
LP-S3           FDYLQLVLQWPRSFCK
LP-S5           FDYMQLVLTWPRSFCY
NA-S1           FEYMQLVLTWPTAFCN
NA-S2           FEYMQLVLTWPITFCR
PH-S1b          FDHWQLVLTWPAGYCK
PH-S3a          FDYFQLVLTWPASFCY
PI-S1           FEYLQLVLTWPASFCF
PI-S2           FDYFQLVLTWPASFCY
SC-S2           FDYMKLVLQWPPMYCR
SC-S3           FEHLQLVLTWPTSFCH
ST-S1           FELLELVSTWPATFCY
ST-S2           FDYMQLVLTWPRSFCY
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                     C1
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Fig. 7 N-terminal amino acid sequence alignment of the S-RNases in rosaceous plants. Amino acid sequences corresponding to C1, previously reported conserved domain of Solanaceae S-RNases (Tsai et al. 1992), and the following amino acid residues of scrophulariaceous and solanaceous S-RNases are also presented. Amino acid residues conserved in all almond S-RNases are indicated by boldface. Amino acid residues conserved in all rosaceous S-RNases are marked with *asterisks* and those conserved in all almond, scrophulariaceous, and solanaceous S-RNases are indicated by open circles. The amino acid residue conserved in all S-RNases is marked with an *arrow*. Plant species from which each sequence is derived are denoted by the initials of their scientific names, i.e., PD means *Prunus dulcis*. Sequence data for the S-RNases included are as follows: S_a, S_b, S_c, and S_d of *Prunus dulcis* (almond) (this study); S_c and S_f of *Malus* × *domestica* (apple) (Sassa et al. 1996); S_5 and S_6 of *Pyrus communis* (European pear) (Tomimoto et al. 1996); S_1, S_4 and S_5 of *P. serotina* (Japanese pear) (Sassa et al. 1993, 1996); S_2 and S_4 of *P. ussuriensis* (Chinese pear) (Tomimoto et al. in press); S_2, S_4, and S_5 of *Antirrhinum hispanicum* (Xue et al. 1996); S_3 and S_5 of *Lycopersicon peruvianum* (Royo et al. 1994, Tsai et al. 1992); S_1 and S_2 of *Nicotiana alata* (Anderson et al. 1986, Kheyr-Pour et al. 1990); S_{1b} and S_{3a} of *Petunia hybrida* (Clark et al. 1990); S_1 and S_2 of *P. inflata* (Ai et al. 1990); S_2 and S_3 of *Solanum chacoense* (Xu et al. 1990); S_1 and S_2 of *S. tuberosum* (Kaufmann et al. 1991).

each other than to *Prunus* (Janick and Moore 1975, Westwood 1993), which, in turn, shares more similarity in the N-terminal amino acid sequence with Scrophulariaceae and Solanaceae than with *Malus* and *Pyrus*. It is necessary to survey more *S*-RNases in different genera in Rosaceae before firm conclusions can be made.

This study demonstrated that RNases in the style are strongly suggested to be associated with the gametophytic SI of almond. In terms of M_r, pI values, immunological characteristics, and N-terminal amino acid sequences, *S*-RNases of almond appeared to share the same characteristics with those of *Malus, Pyrus*, Scrophulariaceae, and Solanaceae. This is the first report identifying and characterizing *S*-RNases in almond and the knowledge obtained from this study should open the door for further understanding of the molecular mechanisms underlying SI in this fruit species, which in turn will lead to development of molecular methods for *S*-allele typing and facilitate the breeding of SC cultivars of almond.

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