J. Biochem. 120, 326-334 (1996)

Identification and Partial Amino Acid Sequences of Seven *S*-RNases Associated with Self-Incompatibility of Japanese Pear, *Pyrus pyrifolia* Nakai[1]

Takeshi Ishimizu,* Yoshihiko Sato,† Toshihiro Saito,† Yumi Yoshimura,* Shigemi Norioka,*,[2] Tetsu Nakanishi,‡ and Fumio Sakiyama*

*Division of Protein Chemistry, Institute for Protein Research, Osaka University, Suita, Osaka 565; †Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki 305; and ‡Department of Agriculture and Horticulture, Faculty of Agriculture, Kobe University, Kobe, Hyogo 657

Received for publication, March 21, 1996

S-allele-specific proteins (S-proteins) were separated and identified by two-dimensional (2D) gel electrophoresis from the style extract of 14 cultivars of Japanese pear, *Pyrus pyrifolia* Nakai, which exhibits gametophytic self-incompatibility. These S-proteins were 30-32 kDa basic proteins with putative pIs of 9.6-10.1 and were distinct from the other proteins, which were common for all cultivars examined. Each S-protein was assigned to a given S-genotype based on electrophoretic mobility and the partial amino acid sequence. For S_1- to S_7-proteins, five different N-terminal amino acid sequences sharing the YFQFTQ-QY sequence were determined. Since the same N-terminal amino acid sequences were found for both S_1- and S_7-proteins, and for S_3- and S_5-proteins, the two S-proteins of each pair were distinguished based on their electrophoretic behavior. The internal amino acid sequences of S_2- and S_4-proteins, determined for *Achromobacter* protease I (API) digests, revealed that these proteins are S_2- and S_4-RNases, respectively. In the cultivar Nijisseiki, these two RNases were expressed from the white bud to mature flower stages when the cultivar acquires and enforces self-incompatibility. Osa-Nijisseiki, a self-compatible mutant of Nijisseiki, produced S_2-RNase, but did not produce S_4-RNase. The absence of S_4-RNase was also observed in self-compatible offsprings derived from Osa-Nijisseiki. These results suggest that Japanese pear in the family Rosaceae possesses a gametophytic self-incompatibility system involving an S-RNase, and that a reduction or lack of expression of S_4-RNase in the style is responsible for the self-compatibility of Osa-Nijisseiki.

Key words: Japanese pear, S-allele, self-incompatibility, S-RNase, two-dimensional gel electrophoresis.

Self-incompatibility is a system that enforces outbreeding in plants. In fruit trees, this system is poorly understood, especially as regards self-unfruitfulness (*1*). Japanese pear, *Pyrus pyrifolia* Nakai, is one of the fruit trees which need cross pollination to set fruit. Genetic analysis has disclosed that *P. pyrifolia* exhibits gametophytic self-incompatibility which is controlled by a single *S*-locus (*2*). When haploid pollen bearing a given *S*-allele is received by a style in which one of the two *S*-alleles matches that of the pollen, the self-incompatibility reaction takes place and the growth of the pollen tube within the female tissue is arrested. A total of seven *S*-alleles of *P. pyrifolia* have been identified by crossing experiments for diploid *S*-alleles among ~30 available cultivars (*3, 4*). From all genetic analyses of *P. pyrifolia* reported so far, Osa-Nijisseiki derived from self-incompatible Nijisseiki appears to be the sole detectable self-compatible mutant in this genus (*5*).

Gametophytic self-incompatibility has been studied mainly for the families, Solanaceae, Papaveraceae, and Rosaceae. In *Nicotiana alata* of the family Solanaceae, *S*-glycoproteins which are associated with *S*-alleles have been isolated and identified as RNases (*6, 7*), and these proteins are now known as *S*-RNases (*8*). Experiments using transgenic plants have demonstrated that RNase activity is necessary for *S*-RNase to serve for the self-incompatibility reaction in petunia (*9, 10*) and tobacco (*11*). However, an *S*-glycoprotein of *Papaver rhoeas* L. in the family Papaveraceae has neither RNase activity nor sequence homology to any of the *S*-RNases reported so far (*12, 13*).

The self-incompatibility system of the family Rosaceae has scarcely been studied, possibly due to the difficulty of protein and mRNA purification arising from the rapid browning of plant extracts. In cherry, some components

[1] Terminology recommended by the Nomenclature Committee of the International Union of Plant Biology. *Pyrus serotina* Rehd. is an alternative name of Japanese pear. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas to F.S. ("Genetic Dissection of Sexual Differentiation and Pollination Process in Higher Plants," No. 07281103) from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-879-8618, Fax: +81-879-8619, E-mail: norioka@protein.osaka-u.ac.jp

Abbreviations: 2D, two-dimensional; API, *Achromobacter* protease I (lysylendopeptidase from *Achromobacter lyticus*); MES, 2-(*N*-morpholino)ethanesulfonic acid; NEPHGE, non-equilibrium pH gradient electrophoresis; PVDF, polyvinylidene difluoride.

that are possibly associated with self-incompatibility have been isolated, but their characterization remains to be investigated (*14*). For Japanese pear, possible *S*-gene products in the style have been sought (*15-17*). (In this paper, the term style indicates the style with the stigma attached.) Three *S*-glycoproteins were separated by 2D gel electrophoresis and partially sequenced (*18*). Very recently, two cDNAs encoding for S_2- and S_3-RNases in the style of apple have been cloned and the similarity of their deduced amino acid sequences to those of *S*-RNases in the family Solanaceae has been reported (*19*). We have also cloned and sequenced cDNAs for S_2- and S_4-RNases in the style of Japanese pear and found a high degree of sequence identity between pear *S*-RNases of the RNase T_2-type and the apple counterparts (*20, 21*).

This investigation was designed to identify *S*-allelic products specific for all of the S_1- to S_7-alleles in the style of Japanese pear and to shed light on the mechanism by which Nijisseiki mutated to self-compatible Osa-Nijisseiki. We used 2D gel electrophoresis combined with N-terminal and partial amino acid sequence analyses, and seven *S*-allele specific proteins from 14 varieties, including typical cultivars and offsprings of which the *S*-genotypes were elucidated by conventional crossing tests, were separated and assigned to individual *S*-genotypes.

MATERIALS AND METHODS

Plant Materials—The flowers of Japanese pear were collected in 1992 to 1994 at the Tottori Horticultural Experiment Station in Daiei, Tottori and the Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries of Japan in Tsukuba, Ibaraki. The cultivars of Japanese pear used in this investigation are as follows: Imamura-aki (S_1S_6), Chojuro (S_2S_3), Nijisseiki (S_2S_4), Osa-Nijisseiki ($S_2S_4^{sm}$) (sm, stylar-part mutant), Seigyoku (S_3S_4), Kosui (S_4S_5), and Okusankichi (S_5S_7) at Tottori and Hayatama (S_1S_2), S_2S_2 homozygote, S_3S_3 homozygote and offsprings (267-4, 267-39, and 268-26) between Osa-Nijisseiki (female) and Kosui (male) at Tsukuba. The *S*-genotypes of these varieties have been assigned by crossing experiments (*3, 4, 22, 23, 33*). The styles of each flower were collected, rapidly frozen in liquid nitrogen, and stored at −170°C until required.

2D Gel Electrophoresis—Samples for electrophoresis were prepared as follows. The styles of each cultivar (from approximately 500 flowers) were ground in liquid nitrogen using a mortar and a pestle, extracted with lysis buffer (5 ml) (*24*) containing 3% Polyclar-AT (polyvinylpyrrolidone, GAF Chemicals) and 1.5% sodium ascorbate, and centrifuged at 25,000×*g* for 30 min at 4°C. The supernatant was stored at −80°C until use.

2D gel electrophoresis was performed by non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and SDS-PAGE in the second dimension according to the method of O'Farrell *et al.* (*25*) with slight modifications. The first-dimensional gel (2.5×130 mm) was composed of 9 M urea, 2% Nonidet P-40, 4.73% acrylamide, 0.27% *N,N'*-methylene-bisacrylamide, 2% Ampholine pH 3.5-10, 1% Pharmalyte pH 8-10.5. Samples corresponding to approximately 5 flowers were applied to the cathode end of the gel and electrophoresed for 2,100-2,900 V·h until the current in the gel was less than 100 μA. Then the gel was equilibrated with SDS sample buffer (*26*) for 1 h and applied to 12% SDS-polyacrylamide gel. After electrophoresis in the second dimension, proteins in the gel were detected by silver staining using 2D-Silver stain·II (Daiichi Pure Chemicals, Tokyo) or Coomassie Brilliant Blue R-250.

Preparation of S-RNases—The styles of Nijisseiki, Kosui, or Chojuro (approximately 1,000 flowers) were ground in liquid nitrogen as described previously and extracted with 50 mM MES/NaOH buffer, pH 6.5 (150 ml), containing 5 mM EDTA·2Na, 1.5% sodium ascorbate, and 3% Polyclar-AT, for 30 min on ice. After centrifugation at 16,000×*g* for 10 min at 4°C, the supernatant was collected and chromatographed on a CM-cellulose column (14×250 mm) equilibrated with 50 mM MES/NaOH buffer, pH 6.5, with a linear gradient of 0 to 0.5 M NaCl at 4°C. The *S*-RNase fraction bearing RNase activity was further purified by reverse-phase HPLC on a Vydac C_4 column (4.6×250 mm) with a gradient of 2-propanol/acetonitrile (7 : 3, v/v) (0-80%) in 0.1% trifluoroacetic acid.

Amino Acid Sequence Analyses—*S*-RNases separated by 2D gel electrophoresis were electroblotted onto polyvinylidene difluoride (PVDF) membranes as described by Hirano and Watanabe (*27*). Membrane-blotted *S*-RNase was excised, placed in a Blott cartridge (Perkin Elmer/Applied Biosystems) and sequenced with a gas-phase protein sequencer (470A Applied Biosystems). To analyze internal sequences, *S*-RNase was blotted on PVDF membrane, digested with API in 0.1 M Tris/HCl, pH 9.0, containing 10% acetonitrile and 1% reduced Triton X-100 (*28*) for 24 h at 37°C at a substrate/enzyme ratio of 50/1 (mol/mol), and eluted with 0.1% trifluoroacetic acid in 40% acetonitrile by sonication for 5 min. Fragmented peptides were separated on a YMC-Pack MB-ODS column (2.1×50 mm) with a linear gradient of acetonitrile (0-40%) in 0.1% trifluoroacetic acid at a flow rate of 200 μl/min. Each peptide fragment thus obtained was sequenced with the protein sequencer.

RESULTS

Separation of Seven Basic Style Proteins and Their Assignment to Individual S-Genotypes—Cultivars and

TABLE I. **Cultivars of Japanese pear and *S*-genotypes.**

Cultivar	*S*-genotype	Refs.
Hayatama	S_1S_2	(*3*)
Imamura-aki	S_1S_6	(*4*)
Chojuro	S_2S_3	(*4*)
Kikusui	S_2S_4	(*4*)
Nijisseiki	S_2S_4	(*4*)
Osa-Nijisseiki (self-compatible)	$S_2S_4^{sm}$	(*23*)
Seigyoku	S_3S_4	(*22*)
Kosui	S_4S_5	(*3*)
Okusankichi	S_5S_7	(*4*)
267-4 (self-compatible)	$S_4^{sm}S_5$	*
267-39	S_2S_5	*
268-26	S_3S_5	*
S_2S_2 homozygote	S_2S_2	(*33*)
S_3S_3 homozygote	S_3S_3	(*33*)

*Sato, unpublished data.

offsprings of Japanese pear used in this experiment are listed with their genetically established *S*-alleles in Table I. Stylar proteins were extracted with lysis buffer in the presence of polyvinylpyrrolidone and sodium ascorbate. The style extract from each cultivar was submitted to 2D gel electrophoresis in which NEPHGE, separating proteins of pI 4.5 to 10.5, and SDS-PAGE were used as the first and second dimensions, respectively. Figure 1 shows a typical pattern of 2D gel electrophoresis of the style extract of *P. pyrifolia*. Almost all proteins separated over the whole region of the gel were detected for all cultivars tested, but critical differences in the pattern were found in a particular region (hereafter called the *S*-protein zone) to which 30–32 kDa basic *S*-proteins migrated (Figs. 1 and 2). Positional comparison of these proteins for any of two cultivars sharing a given *S*-allele made it possible to assign tentatively each basic protein to one of the S_1- to S_5-alleles (Fig. 2). This assignment was then confirmed by analyzing the N-terminal amino acid sequence (Fig. 3). In this assignment, the S_6-protein of Imamura-aki (S_1S_6) and the S_7-protein of Okusankichi (S_5S_7) were assigned based on the position in the *S*-protein zone and the N-terminal amino acid sequence since S_6- or S_7-protein is known to exist only in one of these cultivars. Seven style *S*-proteins separated on a gel were eventually assigned to individual *S*-genotypes. A composite of the locations of these assigned proteins is presented in Fig. 4. Detailed procedures for the identification of *S*-proteins are described below.

a) S_1-protein: Imamura-aki (S_1S_6) gave two separate protein spots in the *S*-protein zone. One was a 30.5 kDa protein which migrated slower than the other one in NEPHGE. The same 30.5 kDa protein was detected for Hayatama (S_1S_2), and was assigned as S_1-protein. Its N-terminal amino acid sequence was determined as YDYFQFTQQYxPAVxN (x denotes an unidentified residue). The YDYFQFTQQYWPAV sequence has been reported for the N-terminus of S_1-RNase (*18*).

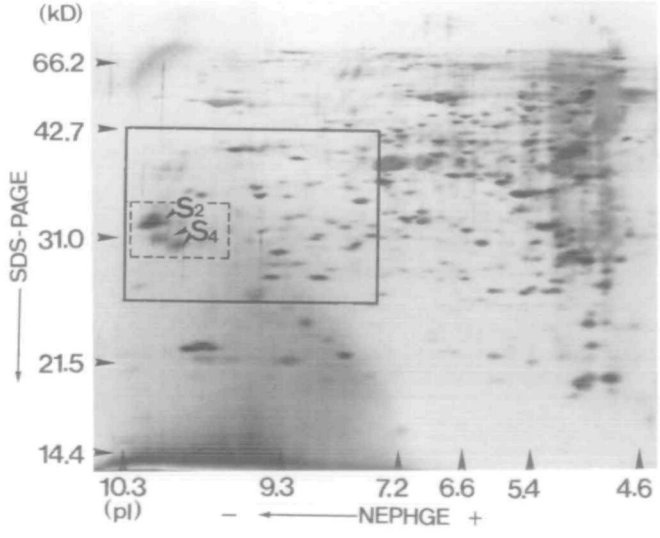

Fig. 1. **Pear stylar proteins separated by 2D gel electrophoresis.** Stylar proteins from Nijisseiki (S_2S_4) were separated by 2D gel electrophoresis (NEPHGE/SDS-PAGE) and detected by silver staining. Numbers in the vertical column indicate molecular masses of standard proteins and those in the horizontal column denote their pIs. The *S*-protein zone and the zone presented in Figs. 2, 4, 5, 7, and 8 are boxed by broken and solid lines, respectively.

b) S_2-protein: In the *S*-protein zone, Nijisseiki (S_2S_4) gave four protein spots which were divided into two groups, each composed of two proteins. One group with a molecular mass of 32 kDa was found for Hayatama (S_1S_2), Osa-Nijisseiki ($S_2S_4{}^{sm}$), an S_2S_2 homozygote and two offsprings [267-39 (S_2S_5) and 268-26 (S_2S_5)] derived from Osa-Nijisseiki, and was assigned as S_2-protein. The two components of S_2-protein were named S_2a- and S_2b-proteins (low and high mobility on SDS-PAGE, respectively). Only S_2b-protein was detected in other S_2-allelic cultivars such as Chojuro (S_2S_3) and Kikusui (S_2S_4). The N-terminal amino acid sequence analysis of PVDF membrane-blotted proteins revealed that S_2a- and S_2b-proteins from Nijisseiki and S_2b-protein from Hayatama shared the same sequence, ARYDYFQFTQQYQxAF. For most of the S_2-allelic cultivars tested, S_2b-protein was the major component.

c) S_3-protein: Chojuro (S_2S_3) gave three proteins in the *S*-protein zone. Of the three, the protein which migrated in the first dimension on gel electrophoresis had been assigned as S_2b-protein as described above. The remaining two proteins were also detected for Seigyoku (S_3S_4) and the S_3S_3 homozygote. The amounts of these two proteins estimated from the intensity of silver staining were roughly equal and they were named S_3a (32 kDa)- and S_3b (31 kDa)-proteins on the same basis as mentioned earlier. The N-terminal amino acid sequences of both S_3a- and S_3b-proteins were the same, being YDYFQFTQQYxLAVxN. The mobility of S_3b-protein on NEPHGE was the smallest among the seven *S*-proteins tested.

d) S_4-protein: For Nijisseiki (S_2S_4), two protein spots with similar intensities on silver staining migrated more slowly than S_2a- and S_2b-proteins in the first dimension. These two slowly migrating proteins, which were also found for Kikusui (S_2S_4), were assigned as S_4a (31 kDa)- and S_4b (30.5 kDa)-proteins. Seigyoku (S_3S_4) and Kosui (S_4S_5) gave S_4b-protein only. The N-terminal FDYFQFTQQYQP-AVxN sequence was found for either S_4a- or S_4b-protein from Nijisseiki. All residues except C-terminal Asn have been determined for S_4-RNase (*18*).

e) S_5-protein: In addition to S_4b-protein, two slower-migrating proteins were clearly detected by 2D-PAGE for Kosui (S_4S_5) and were assigned as S_5a (32 kDa)- and S_5b (31 kDa)-proteins. S_5a-protein was a major component of Kosui, and the same compositional feature was observed for offspring 267-39 (S_2S_5). Okusankichi (S_5S_7) shared S_5a-protein with Kosui and offspring 268-26 (S_2S_5) gave S_5b-protein as a major component. The N-terminal amino acid sequence, YDYFQFTQQYQLAVxN, was found for both S_5a- and S_5b-proteins from Kosui. All residues except the eleventh were identical with those of S_3-protein as described above or with those of S_5-RNase (*18*).

f) S_6-protein: S_6-protein, a 31 kDa protein, was detected as a single spot together with S_1-protein identified for Imamura-aki (S_1S_6) and had the highest mobility in the first direction among the seven *S*-proteins identified by 2D gel electrophoresis. The spot of S_6-protein on the gel was not superimposable on that of any of the *S*-proteins separated from other cultivars. The N-terminal amino acid sequence was found to be YNYFQFTQQYxPAVxN. The sequence contains all of the nine conserved residues (underlined), though Asn2 was distinct from the Asp counterpart ob-

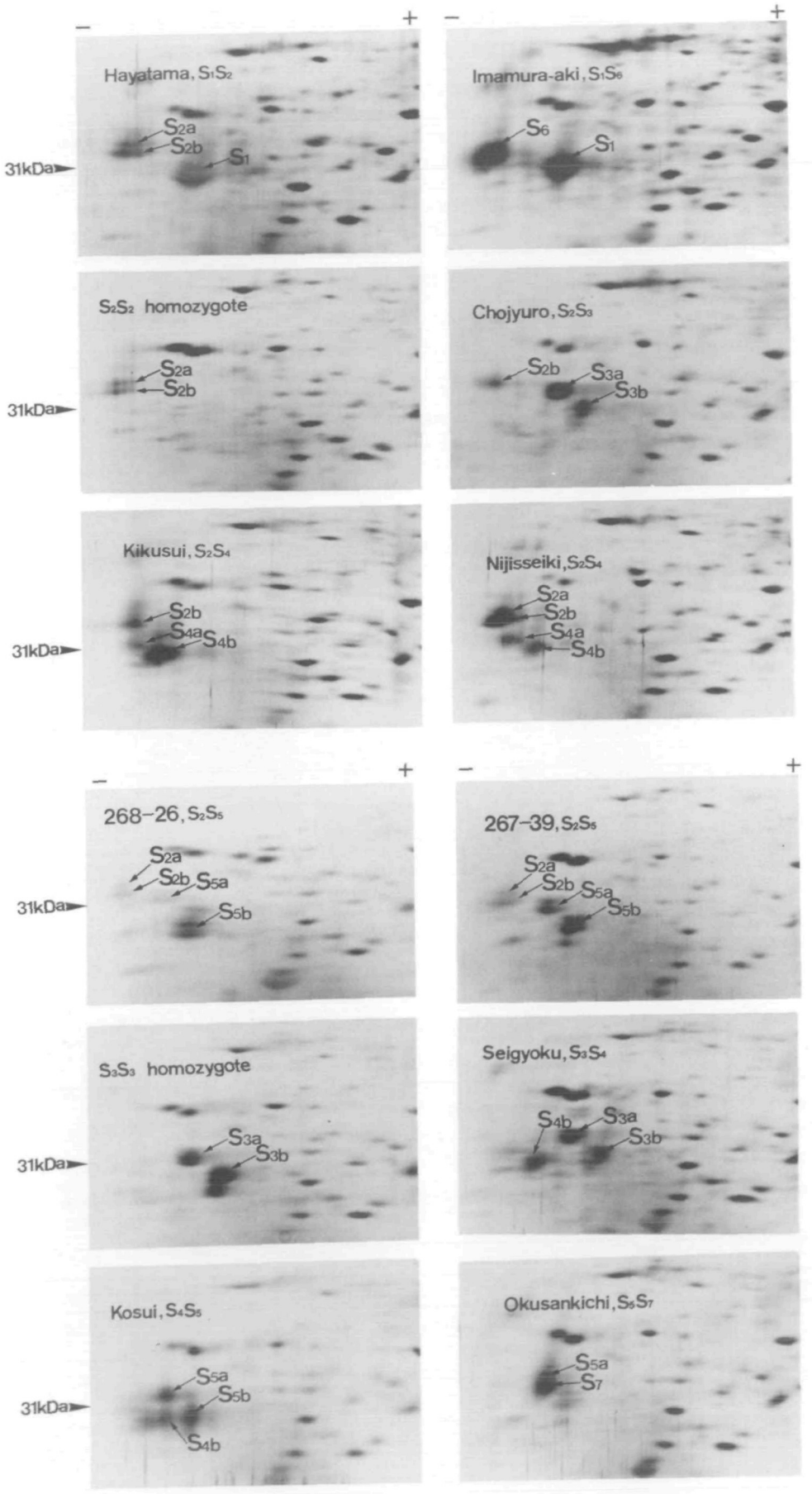

Fig. 2. Separation of *S*-proteins from 12 varieties. Each panel shows the separation of proteins in the *S*-protein zone. Assigned *S*-protein spots are indicated by arrows with *S*-alleles.

served for S_1- to S_5-proteins and S_7-protein. From these results, the 31 kDa protein was assigned as S_6-protein.

g) S_7-protein: Okusankichi (S_5S_7) gave a protein spot with a slightly faster mobility than S_5a-protein in the *S*-protein zone on SDS-PAGE. This spot was distinct from all other *S*-proteins that have ever been identified, and was assigned as S_7-protein. The sequence YDYFQFTQQYx-PAV was found for its N-terminus, which is the same as the N-terminal sequence of S_1-protein.

S-Proteins Are S-RNases—Very recently, *S*-RNases have been successfully purified by a series of chromatographic steps from the style of *P. pyrifolia* in our laboratory (manuscript in preparation). To see whether S_4-protein is active S_4-RNase, chromatographically purified S_4-RNase from Nijisseiki or Kosui was mixed with the style extract of the former cultivar and submitted to 2D gel electrophoresis. The S_4-RNase, though stained as a diffuse spot, co-migrated with S_4a- and S_4b-proteins on the gel, thus identifying the S_4-protein as S_4-RNase (Fig. 5B). When S_3-RNase isolated from Chojuro was electrophoresed by the same procedure, the enzyme appeared as a sole additional protein at the position to which the S_3-protein migrates on the gel, leading to the assignment of S_3-protein as S_3-RNase (Fig. 5C).

To acquire crucial evidence for the assignment of S_2- and S_4-proteins as S_2- and S_4-RNases, internal amino acid sequences of S_2b- and S_4b-proteins from Nijisseiki were analyzed. Briefly, the protein electroblotted onto PVDF membrane after 2D gel electrophoretic separation was digested with API on the membrane. Fragment peptides were separated by reverse-phase HPLC and sequenced. As a result, 81 and 127 residues, all comprising independent peptides, were sequenced for S_2- and S_4-RNases, respectively. All these sequences agreed completely with the amino acid sequences deduced from the nucleotide se-

```
                 1    5     10    15
S1  P.p.     YDYFQFTQQYxPAVxN
S2  P.p.  ARYDYFQFTQQYQxAF
S3  P.p.     YDYFQFTQQYxLAVxN
S4  P.p.     FDYFQFTQQYQPAVxN
S5  P.p.     YDYFQFTQQYQLAVxN
S6  P.p.     YNYFQFTQQYxPAVxN
S7  P.p.     YDYFQFTQQYxPAV

S2  M.d.     YDYFQFTQQYQPAVCN
S3  M.d.     YDYFQFTQQYQPAVCS

S6  N.a.   AFEYMQLVLQWPTAFCH
S1  P.i.   NFEYLQLVLTWPASFCF
S2  S.t.   DFDYMQLVLTWPRSFCY
```

Fig. 3. **Comparison of N-terminal amino acid sequences of *S*-RNases from pear, apple, and the solanaceous plant.** S_1 to S_7 *P.p.*, S_2 and S_3 *M.d.*, S_6 *N.a.*, S_1 *P.i.*, and S_2 *S.t.* denote S_1- to S_7-RNases from *Pyrus pyrifolia*, S_2- and S_3-RNases from *Malus* × *domestica* (*19*), S_6-RNase from *Nicotiana alata* (*34*), S_1-RNase from *Petunia inflata* (*35*), and S_2-RNase from *Solanum tuberosum* (*36*), respectively. Residues conserved in pear *S*-RNases are shadowed. x indicates an unidentified residue.

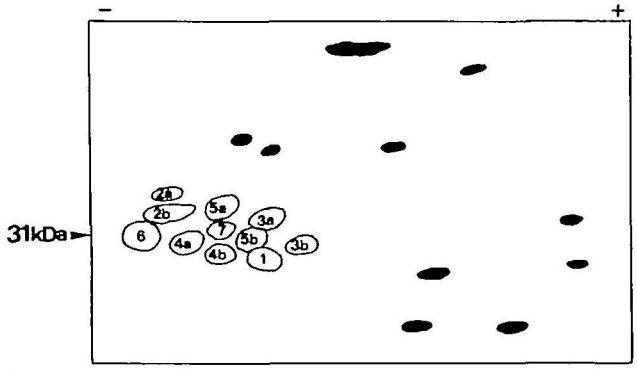

Fig. 4. **A composite for the location of seven *S*-RNases in the *S*-protein zone.** A composite panel was prepared by superimposing all panels shown in Fig. 2. Major proteins are shown by solid black. Open circles and numbers show the locations of *S*-proteins and their identified *S*-alleles, respectively.

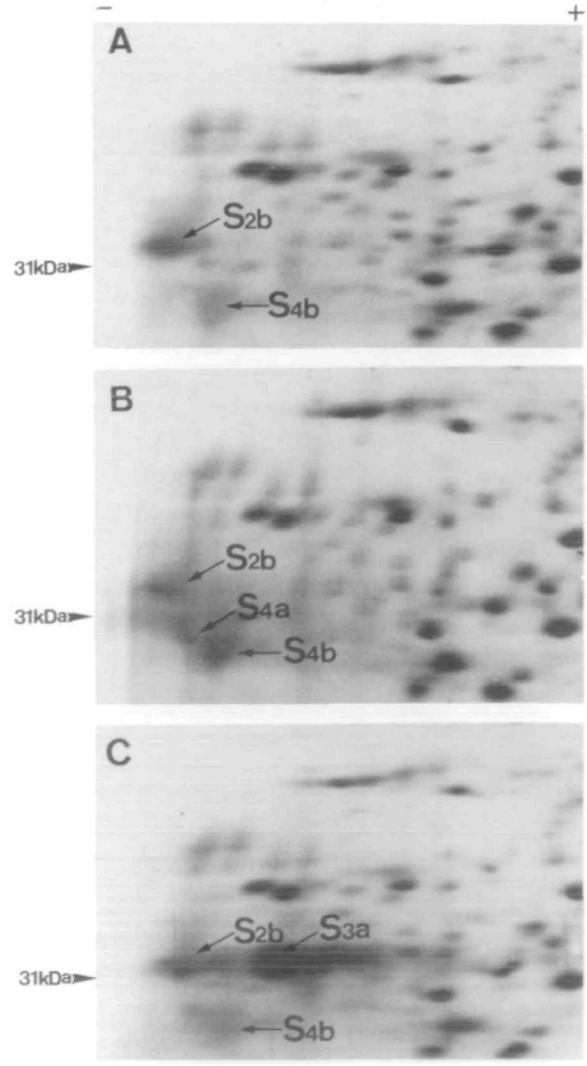

Fig. 5. **Co-migration of purified S_4- and S_3-RNases in the style extract of Nijisseiki to the *S*-protein zone.** (A), S_2- and S_4-RNases from the style extract of Nijisseiki (S_2S_4); (B), (A)+purified S_4-RNase; (C), (A)+purified S_3-RNase.

S_2	*P.p.*	FTVHGLWPS	YKHGSCA
S_4	*P.p.*	FTVHGLWPS	LKHGTCG
S_2	*M.d.*	FTVHGLWPS	NKHGACG
S_3	*M.d.*	FTVHGLWPS	RKHGTCG
S_6	*N.a.*	FTIHGLWPD	IKHGTCC
S_1	*P.i.*	FTIHGLWPE	RKHGMCC
S_2	*S.t.*	FTIHGLWPD	KKHGTCC
T_2	*A.o.*	WTIHGLWPD	NKHGTCI

Fig. 6. **Amino acid sequences of pear S_2- and S_4-RNases in the putative active site region.** The putative active site region of pear S_2- and S_4-RNases was assigned by comparing the amino acid sequences with those of solanaceous S-RNases and RNase T_2. Totally conserved sequences for the family of RNase T_2 are shadowed. Abbreviations: S_2 and S_4 *P. p., P. pyrifolia* S_2- and S_4-RNases; S_2 and S_3 *M.d., Malus* × *domestica* S_2- and S_3-RNases (*19*); S_6 *N.a., N. alata* S_6-RNase (*34*); S_1 *P.i., P. inflata* S_1-RNase (*35*); S_2 *S.t., S. tuberosum* S_2-RNase (*36*); and T_2 *A.o., Aspergillus oryzae* RNase T_2 (*37*).

quences of cDNA encoding S_2- and S_4-RNases (*20, 21*). In the sequences determined by these analyses, two short stretches of the peptide chains including counterparts corresponding to two essential histidine residues of RNase T_2 (*6*) were found (Fig. 6). From this and the previous results, it was concluded that S_2- and S_4-proteins purified by 2D gel electrophoresis are members of the RNase T_2 family and they were renamed S_2- and S_4-RNases, respectively. Thereafter, S-proteins separated by the same method as that used for these two S-RNases will be called S-RNases.

S_4-RNase Is Not Expressed in Self-Compatible Osa-Nijisseiki—When the style extract of Osa-Nijisseiki ($S_2S_4^{sm}$) was analyzed by 2D gel electrophoresis, S_2-RNase was clearly detected as S_2a- and S_2b-proteins. However, no proteins corresponding to S_4a- and S_4b-proteins were detected in the S-protein zone (Fig. 7), leading to the notion that no detectable S_4-RNase exists in the style of Osa-Nijisseiki. The absence of S_4-RNase was also observed for a self-compatible offspring, 267-4 ($S_4^{sm}S_5$), derived from crossing Osa-Nijisseiki (female) and Kosui (male). From this result, a close relationship of self-compatibility of Osa-Nijisseiki with failure of the synthesis of S_4-RNase in the style is suggested.

S-RNases Are Developmentally Expressed—The appearance of S-RNases in the style of Nijisseiki was followed by 2D gel electrophoresis during flower development. Figure 8 depicts the developmental appearance of S_2- and S_4-RNases in the S-protein zone at five stages from green bud, pink bud, white bud, balloon, and mature flower. The two RNases were hardly detectable at the green bud and pink bud stages and were clearly detected at subsequent stages up to anthesis, indicating that these S-RNases are developmentally expressed during the process of flower maturation.

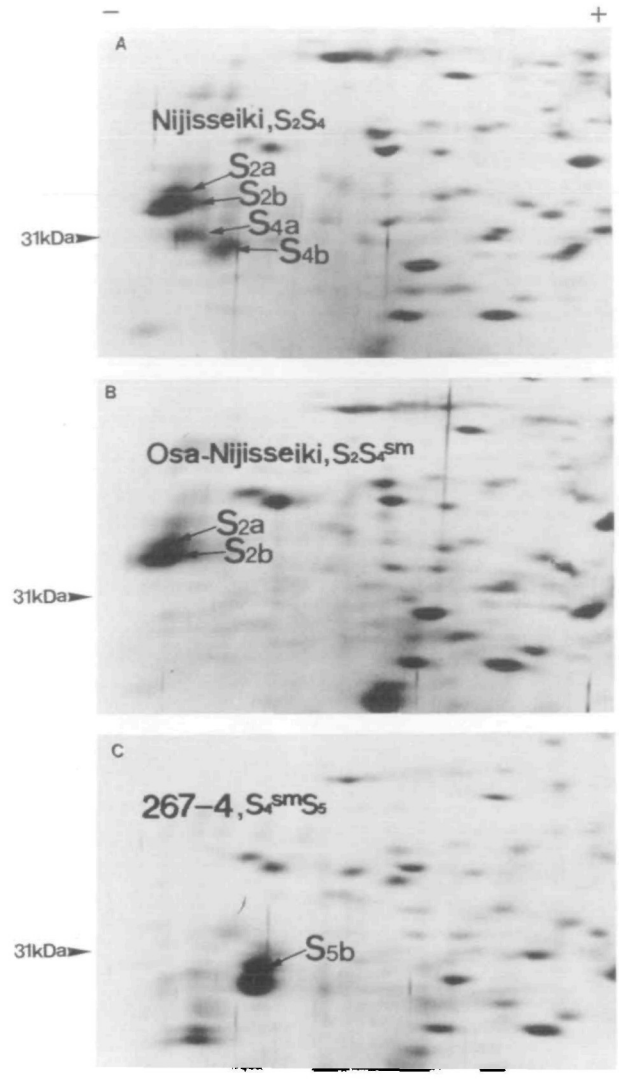

Fig. 7. **The S-protein zone of Osa-Nijisseiki and its self-compatible offspring.** (A) Nijisseiki (S_2S_4), (B) Osa-Nijisseiki ($S_2S_4^{sm}$), and (C) an offspring, 267-4 ($S_4^{sm}S_5$).

DISCUSSION

The present 2D gel electrophoresis results showed that seven S-allele specific proteins (S-RNases) of Japanese pear are separated without superimposition (Fig. 4). These S-RNases were assigned to individual S-alleles by positional comparison with those from cultivars bearing common S-genotypes and in most cases by comparison of their N-terminal amino acid sequences. A similar experiment has been reported by Sassa *et al.* (*18*), who analyzed the N-terminal amino acid sequences of three S-glycoproteins, which were identified as S_1-, S_4-, and S_5-RNases. However, for some reason S_2-RNase has never been detected by 2D gel electrophoresis in cultivars such as Nijisseiki (S_2S_4), Hayatama (S_1S_2), Doitsu (S_1S_2), and Osa-Nijisseiki ($S_2S_4^{sm}$). Since our procedures and conditions for gel electrophoresis are not much different from those used for the earlier experiments, our successful detection of S_2-RNase, a structurally distinct S-RNase molecule among pear S-RNases, on the gel is probably due to direct extraction with lysis buffer of the S-RNases from the pear style. In any event, the present electrophoretical separation of seven S-RNases was successful, since all detectable

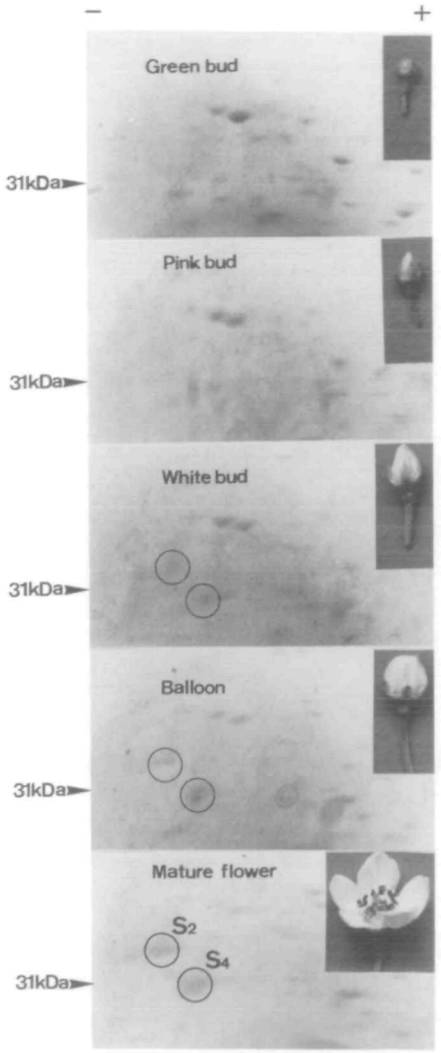

Fig. 8. **The *S*-protein zone during flower development of Nijisseiki.** Stylar proteins from each stage of Nijisseiki were separated by 2D gel electrophoresis and detected by staining with Coomassie Brilliant Blue R-250. The *S*-protein zone and the picture of the flower at each stage are shown. The spots of S_2- and S_4-RNases are marked with a circle.

S-RNases exclusively migrated to the *S*-protein zone. This method should also be applicable for the determination of *S*-genotypes of new cultivars which would be derived by crossing from known cultivars of Japanese pear.

It is important that the present method apparently separates subcomponents of *S*-RNases, which are often detected as a double spot. In these cases, determination of the identical N-terminal amino acid sequence for the two proteins is necessary but not sufficient to establish the identity of their amino acid sequences, since S_3-RNase and S_5-RNase electrophoretically behave differently but have the same sequence. Eventually, we compared the peptide map of the API digest of each subcomponent on a reverse-phase column under acidic conditions. For instance, the peptide maps of S_3a-RNase and S_3b-RNase were indistinguishable (data not shown). Since *N*-glycosylated peptides generally have very similar retention volumes on a reverse-phase column under acidic conditions, this observation supports the idea that the two subcomponent proteins which migrated at different positions have the same amino acid sequence. In fact, S_4-RNase, a mixture of S_4a- and S_4b-RNases, was detected as a single peak on a reverse-phase column, but two or more molecular ions were actually detected by mass spectrometry for the S_4-RNase purified chromatographically (data not shown). It is, therefore, likely that microheterogeneity of sugar moieties in an *S*-RNase is responsible for the occurrence of subcomponents separable by 2D electrophoresis. The presence of a double spot for a given *S*-RNase of potato has also been reported (*29*).

The N-terminal amino acid sequence of *S*-RNases has a structural motif characteristic of pear enzymes; $^{Y}/_{F}{}^{D}/_{N}$-YFQFTQQYxxA$^{V}/_{F}$ is conserved for all seven *S*-RNases identified (Fig. 3). It is noteworthy that the N-terminal sequence of apple *S*-RNases is YDYFQFTQQYQPAV (*19*), which is highly homologous to that of pear *S*-RNases. These motifs are structurally distinct from those of solanaceous *S*-RNases. In addition, pear S_2-RNase is unique in that the Ala-Arg sequence is attached to the N-terminus of the above motif. The very recent nucleotide sequence analysis of cDNA encoding S_2-RNase revealed that the protein is synthesized as a precursor bearing a signal peptide in which the Ala-Ala-Arg sequence is followed by Tyr1 (*20, 21*). This indicates that a signal peptidase in the pear style can cleave the Ala-Ala bond but can not cleave the Arg-Tyr bond in pro-S_2-RNase. The fact that the Gly-Phe and the Gly-Tyr bonds of the precursors of pear S_4-RNase (*20, 21*) and apple S_2-RNase (*19*), respectively, are sensitive to signal peptidase indicates that this peptidase favors non-polar side chains at the site of action in the propeptide-mature protein junction. These sites conform to the (−3, −1)-rule (*30*). This leads to the notion that, as in the case of pear S_2-RNase, the N-terminus of apple mature S_3-RNase is Val-Lys-Phe and is not Phe since Ala-Val-Lys-Phe is located at positions −3 to +1 (*19*).

Expression of two *S*-RNases in the style of Nijisseiki increased significantly between the pink bud stage and the balloon stage. These stages appear 1 to 3 days prior to anthesis and roughly correspond to the time of acquiring and enforcing self-incompatibility (*31*). Since, under the present conditions, no other detectable protein seems to be synchronized with acquisition of self-incompatibility, our observation supports the idea that *S*-RNase is the key protein in the system segregating self pollen from non-self pollens in the pear style.

According to the results of genetic analyses, Osa-Nijisseiki, a self-compatible mutant of Nijisseiki, is a heterozygous stylar-part mutant ($S_2S_4{}^{sm}$) in which the S_4 gene of the style is exclusively mutated (*23*). Throughout our experiments, neither S_4a-protein nor S_4b-protein was detected in the style, in contrast to S_2-RNase which is always detectable in both Nijisseiki and Osa-Nijisseiki. No exception has been found in any examined style sample collected from any of the trees of Osa-Nijisseiki at different years. S_4-RNase was also absent in the self-compatible offspring derived from Osa-Nijisseiki (female) and Kosui (male), suggesting that the potency for accepting self-pollen of the former cultivar is genetically transferred to its offsprings. Although there is as yet no information about the S_4-RNase gene in the *S*-locus, it is interesting to consider possible reasons why Nijisseiki has gained self-compatibility by

mutation. The first possibility is that the S_4-RNase gene is partially or completely deleted in Osa-Nijisseiki. The second possibility is the complete, or extensive, suppression of transcription or translation of mRNA of S_4-RNase. To examine possibilities, we first performed the cloning of cDNA encoding S_4-RNase for Nijisseiki and Osa-Nijisseiki. The result of this investigation will be reported in the accompanying paper (*21*).

Pear *S*-RNases exclusively migrated to the *S*-protein zone, to which *ca.* 30 kDa basic proteins move. Solanaceous *S*-RNases also migrated to the same zone (*32*). Both *S*-RNases are of the RNase T_2 type. Moreover, as revealed by the present investigation, the absence of style S_4-RNase is associated with self-compatibility in Osa-Nijisseiki. This result is consistent with the fact that the anti-sense targeted suppression of the expression of a given *S*-RNase abolishes the appearance of self-incompatibility in petunia (*9*). These results support the idea that *S*-RNase is associated with gametophytic self-incompatibility in rosaceaeous plants as well as in solanaceous plants.

We are grateful to the Tottori Horticultural Experiment Station and the Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries of Japan, for the supply of many cultivars and varieties of Japanese pear. We are indebted to members of the Department of Biotechnology, Faculty of Engineering, Tottori University and members of this Division for collecting an enormous number of pear flowers. We are also indebted to Mr. Akihiro Iwamatsu of Kirin Brewery Co. for valuable suggestions about the digestion of proteins blotted onto PVDF membranes. Thanks are also due to Dr. Ettore Appella of the National Institutes of Health for advice regarding the manuscript.

REFERENCES

1. Crane, M.B. (1925) Self-sterility and cross incompatibility in plums and cherries. *J. Genet.* **15**, 301-322
2. Kikuchi, A. (1929) Investigations in 1927 and 1928. 1. Paterclinical incompatibility in the Japanese pear (in Japanese). *J. Okitsu Hort. Soc.* **24**, 1-6
3. Machida, Y. (1972) Morphological and physiological characteristics of Japanese and Chinese pear in *Dictionary of Pomology* (Sato, K., Mori, H., Matsui, O., Kitajima, H., and Chiba, T., eds.) pp. 527-529, Yokendo, Tokyo
4. Terami, H., Torikata, H., and Shimazu, Y. (1946) Analysis of the sterility-factors existing in varieties of the Japanese pear (*Pyrus serotina* Rehd. *var. culta* Rehd.) (in Japanese). *Studies Hort. Inst. Kyoto Imp. Univ.* **3**, 267-271
5. Furuta, O., Imai, T., Miyoshi, T., Yatsumi, N., Ueki, S., Hayashi, S., and Hiragi, S. (1980) Properties of Japanese pear 'Osa-Nijisseiki.' (in Japanese). *Abst. Jpn. Soc. Hort. Sci. Autumn Meet.*, pp. 70-71
6. Kawata, Y., Sakiyama, F., Hayashi, F., and Kyogoku, Y. (1990) Identification of two essential histidine residues of ribonuclease T_2 from *Aspergillus oryzae*. *Eur. J. Biochem.* **187**, 255-262
7. McClure, B.A., Haring, V., Ebert, P.R., Anderson, M.A., Simpson, R.J., Sakiyama, F., and Clarke, A.E. (1989) Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. *Nature* **342**, 955-957
8. McClure, B.A., Gray, J.E., Anderson, M.A., and Clarke, A.E. (1990) Self-incompatibility in *Nicotiana alata* involves degradation of pollen rRNA. *Nature* **347**, 757-760
9. Lee, H.S., Huang, S., and Kao, T.-h. (1994) *S* proteins control rejection of incompatible pollen in *Petunia inflata*. *Nature* **367**, 560-563
10. Huang, S., Lee, H.S., Karunanaadaa, B., and Kao, T.-h. (1994) Ribonuclease activity of *Petunia inflata S* proteins is essential for rejection of self-pollen. *Plant Cell* **6**, 1021-1028
11. Murfett, J., Atherton, T.L., Beiquan, M., Gasser, C.S., and McClure, B.A. (1994) *S*-RNase expressed in transgenic *Nicotiana* causes *S*-allele-specific pollen rejection. *Nature* **367**, 563-566
12. Franklin-Tong, V.E., Atwal, K.K., Howell, E.C., Lawrence, M.J., and Franklin, F.C.H. (1991) Self-incompatibility in *Papaver rhoeas*: There is no evidence for the involvement of stigmatic ribonuclease activity. *Plant Cell Environ.* **14**, 423-429
13. Foote, H.C.C., Ride, J.P., Franklin-Tong, V.E., Walker, E.A., Lawrence, M.J., and Franklin, F.C.H. (1994) Cloning and expression of a distinctive class of self-incompatibility (*S*) gene from *Papaver rhoeas* L. *Proc. Natl. Acad. Sci. USA* **91**, 2265-2269
14. Mau, S.-L., Raff, J., and Clarke, A.E. (1982) Isolation and partial characterization of components of *Prunus avium* L. styles, including an antigenic glycoprotein associated with a self-incompatibility genotype. *Planta* **156**, 505-516
15. Hiratsuka, S. (1992) Detection and inheritance of a stylar protein associated with a self-incompatibility genotype of Japanese pear. *Euphytica* **61**, 55-59
16. Nakanishi, T., Yamazaki, T., Funadera, K., Tomonaga, H., Ozaki, T., Kawai, Y., Ichii, T., Satoh, Y., and Kurihara, A. (1992) Isoelectric focusing analysis of stylar proteins associated with self-incompatibility alleles in Japanese pear. *J. Jpn. Soc. Hort. Sci.* **61**, 239-248
17. Sassa, H., Hirano, H., and Ikehashi, H. (1992) Self-incompatibility-related RNases in styles of Japanese pear (*Pyrus serotina* Rehd.). *Plant Cell Physiol.* **33**, 811-814
18. Sassa, H., Hirano, H., and Ikehashi, H. (1993) Identification and characterization of stylar glycoproteins associated with self-incompatibility genes of Japanese pear, *Pyrus serotina* Rehd. *Mol. Gen. Genet.* **241**, 17-25
19. Broothaerts, W., Janssems, G.A., Proost, P., and Broekaert, W.F. (1995) cDNA cloning and molecular analysis of two self-incompatibility alleles from apple. *Plant Mol. Biol.* **27**, 499-511
20. Norioka, N., Ohnishi, Y., Norioka, S., Ishimizu, T., Nakanishi, T., and Sakiyama, F. (1995) Nucleotide sequences of cDNAs encoding S_2- and S_4-RNases (D49527 and D49528 for EMBL) from Japanese pear (*Pyrus pyrifolia* Nakai). *Plant Physiol.* **108**, 1343
21. Norioka, N., Norioka, S., Ohnishi, Y., Ishimizu, T., Oneyama, C., Nakanishi, T., and Sakiyama, F. (1996) Molecular cloning and nucleotide sequences of cDNAs encoding *S*-allele specific stylar RNases in a self-incompatible cultivar and its self-compatible mutant of Japanese pear, *Pyrus pyrifolia* Nakai. *J. Biochem.* **120**, 335-345
22. Sato, Y. (1992) Breeding of self-compatible Japanese pear (in Japanese). *Abst. Symp. Jpn. Soc. Hort. Sci.*, pp. 12-22
23. Sato, Y. (1993) Breeding of self-compatible Japanese pear in *Techniques on Gene Diagnosis and Breeding in Fruit Trees* (Hayashi, T. *et al.*, eds.) pp. 241-247, FTRS/Japan, Tokyo
24. O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007-4021
25. O'Farrell, P.Z., Goodman, H.M., and O'Farrell, P.H. (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**, 1133-1142
26. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
27. Hirano, H. and Watanabe, T. (1990) Microsequencing of proteins electrotransferred onto immobilizing matrices from polyacrylamide gel electrophoresis. *Electrophoresis* **11**, 573-580
28. Fernandez, J., Andrews, L., and Mische, S.M. (1994) An improved procedure for enzymatic digestion of polyvinylidene difluoride-bound proteins for internal sequence analysis. *Anal. Biochem.* **218**, 112-117
29. Thompson, R.D., Uhrig, H., Hermsen, J.G., Salamini, F., and Kaufmann, H. (1991) Investigation of a self-compatible mutation in *Solanum tuberosum* clones inhibiting *S*-allele activity in pollen differentially. *Mol. Gen. Genet.* **226**, 283-288
30. von Heijne, G. (1986) A new method for predicting signal sequence cleavage site. *Nucleic Acids Res.* **14**, 4683-4690
31. Hiratsuka, S., Hirata, N., Tezuka, T., and Yamamoto, Y. (1985) Self-incompatibility reaction of Japanese pear in various stages

of floral development. *J. Jpn. Soc. Hort. Sci.* **54**, 9-14
32. Mau, S.-L., Williams, E.G., Atkinson, M.A., Cornish, E.C., Grego, B., Simpson, R.J., Kheyr-Pour, A., and Clarke, A.E. (1986) Style proteins of a wild tomato (*Lycopersicon peruvianum*) associated with expression of self-incompatibility. *Planta* **169**, 184-191
33. Terai, O., Sato, Y., Abe, K., Saito, T., and Kotobuki, K. (1995) *S*-gene homozygote: Useful tool for *S*-genotype analysis in Japanese pear (in Japanese). *Abst. Jpn. Soc. Hort. Sci. Autumn Meet.*, pp. 150-151
34. Anderson, M.A., McFadden, G.I., Bernatzky, R., Atkinson, A., Orpin, T., Dedman, H., Tregear, G., Fernley, R., and Clarke, A.E. (1989) Sequence variability of three alleles of the self-incompatibility gene of *Nicotiana alata*. *Plant Cell* **1**, 483-491
35. Ai, Y., Singh, A., Coleman, C.E., Ioerger, T.R., Kheyr-Pour, A., and Kao, T.-h. (1990) Self-incompatibility in *Petunia inflata*: Isolation and characterization of cDNAs encoding three *S*-allele-associated proteins. *Sex. Plant Reprod.* **3**, 130-138
36. Kirch, H.H., Uhrig, H., Lottspeich, F., Salamini, F., and Thompson, R.D. (1989) Characterization of proteins associated with self-incompatibility in *Solanum tuberosum*. *Theor. Appl. Genet.* **78**, 581-588
37. Kawata, Y., Sakiyama, F., and Tamaoki, H. (1988) Amino-acid sequence of ribonuclease T_2 from *Aspergillus oryzae*. *Eur. J. Biochem.* **176**, 683-697