J. Biochem. 120, 335-345 (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[1]

Naoko Norioka,* Shigemi Norioka,*,[2] Yoshimi Ohnishi,* Takeshi Ishimizu,* Chitose Oneyama,* Tetsu Nakanishi,† and Fumio Sakiyama*

*Division of Protein Chemistry, Institute for Protein Research, Osaka University, Suita, Osaka 565; and †Department of Agriculture and Horticulture, Faculty of Agriculture, Kobe University, Kobe, Hyogo 657

Received for publication, March 21, 1996

The genes encoding three RNases were cloned from the style of a self-incompatible cultivar, Nijisseiki (S_2S_4), and its self-compatible mutant, Osa-Nijisseiki ($S_2S_4^{sm}$, sm means stylar part mutant), of Japanese pear. For Nijisseiki, cDNAs coding for two *S*-RNases (S_2-RNase and S_4-RNase) and an RNase unrelated to self-incompatibility (non-*S*-RNase) were cloned from the stylar cDNA library. The cDNAs coding for S_2-RNase, S_4-RNase, and non-*S*-RNase include 678-, 684-, and 681-bp open reading frames, respectively. Their deduced amino acid sequences were composed of signal peptides and mature RNases (201-203 residues) which were verified by partial amino acid sequencing. The primary structures of mature proteins revealed that these RNases are of the RNase T_2 type; only the two *S*-RNases have several potential *N*-glycosylation sites and 60% of their amino acid residues are identical, compared with 25% sequence identity with the non-*S*-RNase. Such a distinct difference in the primary structures between *S*-RNases and non-*S*-RNase has not previously been reported and may be a feature typical of *S*-RNases in the family Rosaceae. Similar experiments were performed for Osa-Nijisseiki. The cDNAs coding for S_2-RNase and non-*S*-RNase were similarly cloned from the stylar cDNA library. However, the cDNA coding for S_4-RNase was neither amplified by PCR nor cloned from the library, suggesting that the mutation of self-incompatible Nijisseiki to self-compatible Osa-Nijisseiki is due to a failure of expression of S_4-RNase. These results lead to the idea that Osa-Nijisseiki is a variant of Nijisseiki in which the S_4-allelic gene in the *S*-locus is exclusively mutated or deleted, causing severely impaired or suppressed expression of its gene product, S_4-RNase, at the style.

Key words: cloning, Japanese pear, nucleotide sequence, self-incompatibility, *S*-RNase.

Self-incompatibility is a system that prevents inbreeding and promotes outbreeding in higher plants and that is controlled by a single locus (*S*-locus) with multiple alleles (*1*). This system is classified into two types, gametophytic and sporophytic, on the timing of *S* gene expression in pollen. In gametophytic self-incompatibility, growth of the pollen tube having the same *S*-allele as one of the *S*-alleles in the pistil is inhibited in the style. The *S*-allele specific protein (*S*-glycoprotein) that is synthesized in the style prior to anthesis has been proposed to be responsible for segregation between self and nonself pollens and for arrest of pollen tube growth (*2-7*). We have predicted that *Nicotiana alata* (tobacco) S_2-glycoprotein is a member of the $RNaseT_2$ family (*8, 9*) and this has been confirmed experimentally (*10*). RNase activity has subsequently been found for other *S*-glycoproteins from tobacco (*11*), petunia (*12-16*), tomato (*17-21*), and potato (*22-25*). The *S*-glycoproteins showing this enzyme activity are referred to as *S*-RNase. Very recently, experiments using transgenic plants for petunia (*26, 27*) and tobacco (*28*) revealed that active RNase in the style is essential to the expression of self-incompatibility for these solanaceous plants. The involvement of *S*-RNase in the degradation of ribosomal RNA in the pollen tube upon self-pollination has been proposed, but the mechanism of access of the enzyme to the ribosome remains unclear (*29, 30*).

We are interested in the system of gametophytic self-incompatibility in Japanese pear of the family Rosaceae for several reasons. The first is to determine if the self-incompatibility system of rosaceous plants involves *S*-RNase. Seven *S*-genotypes have been genetically identified and assigned to available individual cultivars of Japanese pear (*31-33*). This indicates that, in most cases, the stylar *S*-RNase can be unambiguously assigned to each *S*-genotype by identifying a common *S*-RNase among two or more

[1] 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. The nucleotide sequences data reported in this paper will appear in the GSDB, DDBJ, EMBL, NCBI nucleotide sequence databases with the accession numbers D49527, D49528, and D49529 for S_2-, S_4-, and non-*S*-RNases, respectively.

[2] To whom correspondence should be addressed. Tel: +81-6-879-8618, Fax: +81-6-879-8619, E-mail: norioka@protein.osaka-u.ac.jp

Abbreviation: PTH, phenylthiohydantoin.

cultivars of which the *S*-genotypes are genetically identified. The second reason is based on the fact that a self-incompatible cultivar, Nijisseiki (S_2S_4), and its natural self-compatible mutant, Osa-Nijisseiki, have been found in Japanese pear. Based on the result of crossing experiments, Osa-Nijisseiki is identified as a cultivar in which the self-incompatibility gene (*S*-gene) in the style of Nijisseiki is exclusively mutated without affecting the *S*-genotype of its haploid pollen. This self-compatible mutant of Nijisseiki is referred to as $S_2S_4^{sm}$ (sm denotes stylar-part mutant) (*34*). Accordingly, it was expected that the comparative investigation of *S*-RNases in these two closely related cultivars would provide a deeper understanding of the molecular basis of the association between gametophytic self-incompatibility and *S*-RNase.

In this communication, we deal with the molecular cloning and nucleotide sequences of cDNAs encoding RNases from the style of Nijisseiki and Osa-Nijisseiki and discuss a possible mechanism for the mutation of the former self-incompatible cultivar to the latter self-compatible cultivar. We also describe the amino acid sequence deduced from nucleotide sequences, assignment of individual RNases to *S*-RNase or non-*S*-RNase, a self-incompatibility unlinked RNase, and the analysis of sequence information of the three RNases in the style of Nijisseiki.

MATERIALS AND METHODS

Materials—The flowers of Nijisseiki and Osa-Nijisseiki were collected at the Tottori Horticultural Experiment Station (Yura, Tottori) in 1994. The styles of each flower at the white bud stage were collected, rapidly frozen in liquid nitrogen and stored at −70°C until use. Fast Track™ mRNA isolation kits were purchased from Invitrogen. AmpliTaq DNA polymerase, cDNA synthesis kit and BcaBEST™ dideoxy sequencing kit were bought from Takara. *Eco*RI-*Not*I adaptor and Packaging INN were from Pharmacia and Nippon Gene, respectively. Hybond N^+ filter and α-^{32}P-dCTP were purchased from Amersham.

Isolation of Total RNA—Total RNA was extracted from the styles of Nijisseiki white buds based on the method of Chomczynski and Sacchi (*35*). The frozen styles from 500 Nijisseiki white buds were ground to a fine powder with a mortar and a pestle in liquid nitrogen. After the liquid nitrogen had evaporated, 10 ml of the extract buffer (5 M guanidine isothiocyanate, 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 30 mg/ml Polyclar AT, 5% 2-mercaptoethanol, and 0.5% sodium *N*-lauroyl sarcosinate) was added and the insoluble materials were removed by centrifugation. Subsequently, 2 M sodium acetate (pH 4.0) (800 μl), water-saturated phenol (8 ml), and chloroform-isoamyl alcohol mixture (24:1) (4 ml) were added to the supernatant (8 ml), and the solution was mixed thoroughly and cooled on ice for 15 min. After centrifugation, the aqueous phase (9 ml) was mixed with isopropanol (9 ml) and held at −20°C for 2 h to precipitate RNA. The RNA pellet collected by centrifugation was washed with 70% ethanol and dried *in vacuo*.

Isolation of mRNA—mRNAs were isolated from total RNA using the Fast Track™ mRNA isolation kit according to the manufacturer's instructions.

Construction of cDNA Library in λgt10—cDNAs were synthesized with cDNA synthesis kits according to the manufacturer's instructions. Following the addition of the *Eco*RI-*Not*I adaptors and the kination of their 5′-ends, the cDNAs were ligated to the *Eco*RI site of λgt10 and packaged *in vitro* with Packaging INN according to the manufacturer's instructions.

PCR Amplification of cDNA—Oligonucleotides #1 and #2 were synthesized based on the amino acid sequence of non-*S*-RNase and oligonucleotides #3 and #4 were synthesized based on the amino acid sequence of S_4-RNase. A pair of primers #1 and #2 (primer #1-#2) or primers #3 and #4 (primer #3-#4) was used for PCR amplification. The sequences of these oligonucleotides are as follows:

#1 5′ CA(C/T)ACITG(C/T)TG(C/T)TA(T/C)CC 3′
#2 5′ (C/T)TT(A/G)TT(A/G)CA(C/T)TGIATICC 3′
#3 5′ TTTACGCAGCAATATCAG 3′
#4 5′ G(C/T)GGGGGCA(A/G)T(T/C)TATGAA 3′

The reaction mixture for PCR amplification contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM each of dNTP, 2.5 U AmpliTaq DNA polymerase, 15 pmol each of two appropriate primers, and 30 ng of cDNA in a 50 μl reaction volume. The reaction was run for 30–35 cycles of 40 s at 94°C, 2 min at 40°C for primer #1-#2 or at 48°C for primer #3-#4, and 2 min at 70°C, with initial denaturation for 2 min at 94°C and final extension for 10 min at 70°C using a HYBAID™ Thermal Reactor (Hook & Tucker Instruments). The PCR products were applied to 6% polyacrylamide gels to check their sizes, or separated by 1.5% agarose gel electrophoresis to purify DNA fragments. DNA fragments were also purified from excised gel slices according to the method of Qian and Wilkinson (*36*), blunt-ended by T4 polymerase and ligated to the *Hinc*II site of the multiple cloning site of M13mp19 for DNA sequencing.

Screening of the cDNA Library—Plaques were grown at a density of 10,000–20,000/10×14 cm square plate at 37°C overnight. For the Nijisseiki cDNA library, 100,000 plaques were screened with PCR-fragments from primer #1-#2 and 80,000 plaques with those from primer #3-#4.

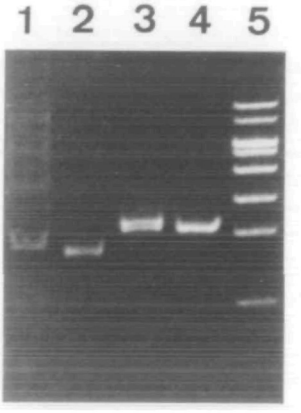

Fig. 1. **PCR-amplified fragments from Nijisseiki and Osa-Nijisseiki cDNAs.** PCR was carried out by the method described in "MATERIALS AND METHODS" and 5 μl of a 50 μl PCR mixture of each sample was subjected to 6% PAGE. Lane 1, amplified with primers #1 and #2 for Nijisseiki cDNA; lane 2, with primers #1 and #2 for Osa-Nijisseiki cDNA; lane 3, with primers #3 and #4 for Nijisseiki cDNA; lane 4, with primers #3 and #4 for Osa-Nijisseiki cDNA; lane 5, pHY molecular marker.

For the Osa-Nijisseiki cDNA library, 50,000 plaques were screened with those from primer #1-#2 or #3-#4. Plaques were transferred to a Hybond N^+ filter according to the manufacturer's instructions. Prehybridization of the filters was carried out for 1-4 h at 42°C in a hybridization buffer containing 50% formamide, 5×SSC, 5×Denhardt's solution, and 100 μg/ml of denatured salmon sperm DNA. The filters were then hybridized at 42°C overnight with the ^{32}P-labeled PCR-amplified fragments, washed three times with 0.1×SSC containing 0.1% SDS at 65°C for 30 min and autoradiographed for one day with an intensifying screen at −80°C.

DNA Sequencing Analysis—cDNA clones encoding RNases were subcloned into M13mp19 or M13mp18. The single strand DNA sequencing was performed by the dideoxynucleotide chain terminating method using Bca-BEST™ Dideoxy Sequencing Kits with a DNA sequencer DSQ-1 (Shimadzu).

FR1

```
CACACGTGCTGCTATCCCAAGTCAGGAAAGCCTACAGCAGATTTTGGCATTCATGGTCTATGGCCTAATTATAAGAATGGTGGCTACCCC  90
 H  T  C  C  Y  P  K  S  G  K  P  T  A  D  F  G  I  H  G  L  W  P  N  Y  K  N  G  G  Y  P

TCCAACTGTGATCCCGACAGCGTCTTCGACAAATCTCAGATCTCAGAGCTGTTGACCAGTCTTAATAAGAACTGGCCATCACTGAGCTGC 180
 S  N  C  D  P  D  S  V  F  D  K  S  Q  I  S  E  L  L  T  S  L  N  K  N  W  P  S  L  S  C

CCAAGTAGCAACGGTTACAGGTTCTGGTCACATGAATGGGAAAAGCACGGGACTTGCTCCGAGTCCGAGCTCGATCAGAAAGAGTACTTC 270
 P  S  S  N  G  Y  R  F  W  S  H  E  W  E  K  H  G  T  C  S  E  S  E  L  D  Q  K  E  Y  F

GAAGCAGCCCTCAAACTCAGAGAAAAAGTTAACCTTCTACAAATTCTAAAAAAATGCTGGAATCGTGCCAAATGATGAACTTTACAACCTA 360
 E  A  A  L  K  L  R  E  K  V  N  L  L  Q  I  L  K  N  A  G  I  V  P  N  D  E  L  Y  N  L

GAGAGCATAGTGGAAGCTATAAAAGTAGGTGTTGGGCACACCCCAGGCATCCAATGCAACAAG                          423
 E  S  I  V  E  A  I  K  V  G  V  G  H  T  P  G  I  Q  C  N  K
```

FR2

```
TTTACGCAGCAATATCAGCAGGCTTTCTGCAACTCTAATCCTACTCCTTGTAAGGATCCTCCTGACAAGTTGTTTACGGTTCACGGTTTG  90
 F  T  Q  Q  Y  Q  Q  A  F  C  N  S  N  P  T  P  C  K  D  P  P  D  K  L  F  T  V  H  G  L

TGGCCTTCAACCAAAGTAGGACGTGACCCAGAATATTGCAAGACAAAGAGATATCGGAAGATACAAAGACTCGAACCCCAGTTGGAAATT 180
 W  P  S  T  K  V  G  R  D  P  E  Y  C  K  T  K  R  Y  R  K  I  Q  R  L  E  P  Q  L  E  I

ATTTGGCCGAACGTATCCGATCGAAAAGCTAATCGAGGCTTCTGGCGTAAACAGTGGTACAAACATGGCTCCTGTGCGTCTCCCGCATTG 270
 I  W  P  N  V  S  D  R  K  A  N  R  G  F  W  R  K  Q  W  Y  K  H  G  S  C  A  S  P  A  L

CCGAACCAGAAGCATTACTTTGAAACAGTAATCAGAATGTTCTTAGCGGAGAAACAAAACGTCTCTAGAATCCTCTCAATGGCGACGATT 360
 P  N  Q  K  H  Y  F  E  T  V  I  R  M  F  L  A  E  K  Q  N  V  S  R  I  L  S  M  A  T  I

GAACCGGAGGGGAAAAACAGGACACTGTTGGAAATTCAAAATGCCATACGCGCTGGTACCAACAATATGATACCAAAACTCAAGTGCCAA 450
 E  P  E  G  K  N  R  T  L  L  E  I  Q  N  A  I  R  A  G  T  N  N  M  I  P  K  L  K  C  Q

AAGGTTAATGGGATGACTGAATTGGTTGAGGTCACTCTTTGCCACGATAGCAACTTAACGCAGTTCATAAATTGCCCCCGC          531
 K  V  N  G  M  T  E  L  V  E  V  T  L  C  H  D  S  N  L  T  Q  F  I  N  C  P  R
```

FR3

```
TTTACGCAGCAATATCAGCCGGCCGTATGCAACTCTAATCCTACTCCTTGTAACGATCCTACTGACAAGTTGTTTACGGTTCACGGTTTG  90
 F  T  Q  Q  Y  Q  P  A  V  C  N  S  N  P  T  P  C  N  D  P  T  D  K  L  F  T  V  H  G  L

TGGCCTTCAAACAGGAATGGACCTGACCCAGAAAAATGCAAGACTACAACCATGAATTCTCAGAAGATAGGAAATATGACAGCCCAGTTG 180
 W  P  S  N  R  N  G  P  D  P  E  K  C  K  T  T  T  M  N  S  Q  K  I  G  N  M  T  A  Q  L

GAAATTATTTGGCCGAACGTTCTCAATCGAAGCGATCATGTAGGCTTCTGGGAAAGAGAGTGGCTCAAACATGGCACCTGTGGGTATCCC 270
 E  I  I  W  P  N  V  L  N  R  S  D  H  V  G  F  W  E  R  E  W  L  K  H  G  T  C  G  Y  P

ACAATAAAGGACGACATGCATTATTTAAAAACAGTAATCAAAATGTACATAACCCAGAAACAAAACGTCTCTGCAATCCTCTCAAAGGCG 360
 T  I  K  D  D  M  H  Y  L  K  T  V  I  K  M  Y  I  T  Q  K  Q  N  V  S  A  I  L  S  K  A

ACGATTCAACCGAACGGGAATAACAGGTCACTGGTGGATATTGAAAATGCCATACGCAGTGGTAACAACAATACGAAACCAAAATTCAAG 450
 T  I  Q  P  N  G  N  N  R  S  L  V  D  I  E  N  A  I  R  S  G  N  N  N  T  K  P  K  F  K

TGCCAAAAGAATACTAGGACGACGACTGAATTGGTTGAGGTCACTCTTTGCAGTAATAGAGACTTGACTAAGTTCATAAATTGCCCCCAC 540
 C  Q  K  N  T  R  T  T  T  E  L  V  E  V  T  L  C  S  N  R  D  L  T  K  F  I  N  C  P  H
```

Fig. 2. **Nucleotide sequences of the fragments amplified with primers #1 and #2 (FR1) and with primers #3 and #4 (FR2 and FR3) for Nijisseiki cDNA library.** The regions corresponding to primers #1, #2, #3, and #4 are underlined.

RESULTS

Construction of cDNA Library and PCR-Amplified Fragments—The cDNA library was constructed with highly pure mRNA that was carefully prepared from styles collected at the white bud stage of Nijisseiki or Osa-Nijisseiki. Since two and more RNases have been detected in the style of either cultivar, primers for PCR amplification using cDNA were selected based on partial amino acid sequences determined for non-*S*-RNase and S_4-RNase separated chromatographically (manuscript in preparation). Primers #1 and #2 were designed according to two amino acid sequences, HTCCYP (residues 21-26) and GIECNK (residues 156-161), for non-S-RNase, respectively. Similarly, primers #3 and #4 were prepared according to the amino acid sequences of FTQQYQ (residues 6-11) and FINCPH (residues 180-185), respectively, in S_4-RNase. For Nijisseiki, a 450-bp fragment was amplified with a set of primers #1 and #2 and two closely migrating 520-bp fragments were amplified with a pair of primers #3 and #4 (lanes 1 and 3 in Fig. 1). For Osa-Nijisseiki, the 450-bp fragment was similarly amplified, but the 520-bp fragment was detected as a single band (lanes 2 and 4 in Fig. 1). Then, amplified 450- and 520-bp DNAs were purified on 1.5% SeaPlaque^R GTG^R agarose gels and separately ligated into the *Hin*cII site of M13mp19. Although the two Nijisseiki 520-bp DNAs were separated on 6% PAGE (lane 3 in Fig. 1), they were not isolable individually on the 1.5% agarose gel and were ligated into M13 phage without further separation.

Nucleotide Sequences of PCR Fragments—Nucleotide sequence analyses revealed that the 450-bp fragment from either Nijisseiki or Osa-Nijisseiki was composed of 423 bp

(a)

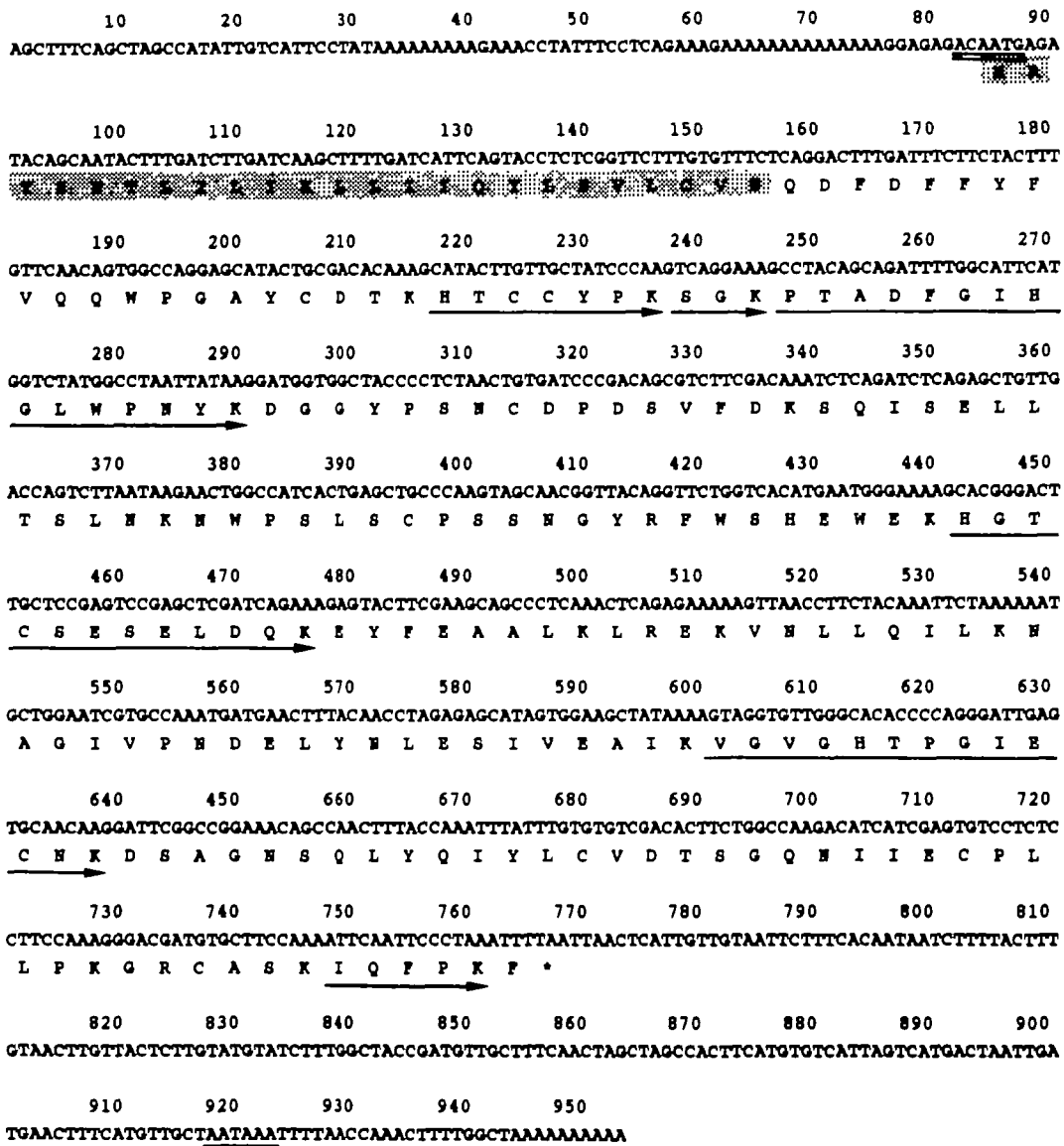

Fig. 3a

(FR1) and has the same nucleotide sequence (Fig. 2). The amino acid sequence deduced from the nucleotide sequence included all partial amino acid sequences determined by protein sequencing for non-S-RNase, FR1 being assigned as the PCR product of mRNA for non-*S*-RNase. Sequence analyses also revealed that the two 520-bp DNAs from Nijisseiki were actually two fragments composed of FR2 and FR3 (Fig. 2), because, of ten M13 plaques bearing the 520-bp DNA, four plaques held the FR2 fragment and the six others included the FR3 fragment. FR2 was composed of 531 bp and its deduced amino acid sequence completely matched all partial amino acid sequences determined for S_2-RNase, implying that FR2 is the PCR product of mRNA for S_2-RNase. FR2 could be amplified by PCR using the primers for the cloning of the S_4-RNase gene because of the extremely high sequence similarity (97%) of the primer regions between S_2-RNase and S_4-RNase. FR3 was a 540-bp fragment whose deduced amino acid sequence was identical with the partial amino acid sequences of S_4-RNase. These results are consistent with the fact that the *S*-genotype of Nijisseiki is S_2S_4 and that the 520-bp fragment was amplified from Nijisseiki as double bands, corresponding to the genes of S_2- and S_4-RNases, on the polyacrylamide gel. On the other hand, the nucleotide sequence analysis revealed that the 520-bp fragment from Osa-Nijisseiki was a single DNA composed of 531 bp and its nucleotide sequence was identical with that of FR2 from S_2-RNase.

Screening of cDNA Libraries and Nucleotide Sequences of Genes Encoding Non-S-RNase, S_2-RNase, and S_4-RNase—For the Nijisseiki style cDNA library, four, six, and six positive transformants were obtained by plaque hybridization using FR1, FR2, and FR3, respectively, as probes. The size of the inserted gene of each transformant was checked by PCR with λgt10 primers and insertion of the target gene was confirmed by PCR with primer #1-#2 or #3-#4. Two, five, and six positive clones were selected as genes encoding non-*S*-RNase, S_2-RNase, and S_4-RNase, respectively. Among these clones, the longest cDNA for each RNase (*ns-nons* for non-*S*-RNase, *ns-s*$_2$ for S_2-RNase,

(b)

```
        10         20         30         40         50         60         70         80         90
AGTAATTAATTTGCCTCGCTCTTGAACAAATATTATTCAATGAGGATTACGGGGATGATATATATTTTTACGATGGTATTTTCATTAAAT

       100        110        120        130        140        150        160        170        180
GTATTAATATTGTCTTCGTCCGCGGCGAGATACGATTATTTTCAATTTACGCAGCAATATCAGCAGGCTTTCTGCAACTCTAATCCTACT
                                A  R  Y  D  Y  F  Q  F  T  Q  Q  Y  Q  Q  A  F  C  N  S  N  P  T

       190        200        210        220        230        240        250        260        270
CCTTGTAAGGATCCTCCTGACAAGTTGTTTACGGTTCACGGTTTGTGGCCTTCAACCAAAGTAGGACGTGACCCAGAATATTGCAAGACA
 P  C  K  D  P  P  D  K  L  F  T  V  H  G  L  W  P  S  T  K  V  G  R  D  P  E  Y  C  K  T

       280        290        300        310        320        330        340        350        360
AAGAGATATCGGAAGATACAAAGACTCGAACCCCAGTTGGAAATTATTTGGCCGAACGTATCCGATCGAAAAGCTAATCGAGGCTTCTGG
 K  R  Y  R  K  I  Q  R  L  E  P  Q  L  E  I  I  W  P  N  V  S  D  R  K  A  N  R  G  F  W

       370        380        390        400        410        420        430        440        450
CGTAAACAGTGGTACAAACATGGCTCCTGTGCGTCTCCCGCATTGCCGAACCAGAAGCATTACTTTGAAACAGTAATCAGAATGTTCTTA
 R  K  Q  W  Y  K  H  G  S  C  A  S  P  A  L  P  N  Q  K  H  Y  F  E  T  V  I  R  M  F  L

       460        470        480        490        500        510        520        530        540
GCGGAGAAACAAAACGTCTCTAGAATCCTCTCAATGGCGACGATTGAACCGGAGGGGAAAAACAGGACACTGTTGGAAATTCAAAATGCC
 A  E  K  Q  N  V  S  R  I  L  S  M  A  T  I  E  P  E  G  K  N  R  T  L  L  E  I  Q  N  A

       550        560        570        580        590        600        610        620        630
ATACGCGCTGGTACCAACAATATGATACCAAAACTCAAGTGCCAAAAGGTTAATGGGATGACTGAATTGGTTGAGGTCACTCTTTGCCAC
 I  R  A  G  T  N  N  M  I  P  K  L  K  C  Q  K  V  N  G  M  T  E  L  V  E  V  T  L  C  H

       640        650        660        670        680        690        700        710        720
GATAGCAACTTAACGCAGTTCATAAATTGCCCCCGCCCATTGCCACAAGCATCACCATATTTCTGCCCCATTGATGATATTCAGTATTAA
 D  S  N  L  T  Q  F  I  N  C  P  R  P  L  P  Q  A  S  P  Y  F  C  P  I  D  D  I  Q  Y  *

       730        740        750        760        770        780        790        800        810
GAGCTAGTATATATATAAGAAGTTGGCCTAATACGGATGGCGATTTCCATATATAGGACGAGGAATGCAGTTATGTGATTGTACTTCTAA

       820        830        840        850        860        870
TGAATAAATTGGCTTAATCAATTAAAACTCTTGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 3b

and *ns-s*$_4$ for S_4-RNase) was subcloned into the M13 vector and sequenced. The same experiment was performed for the Osa-Nijisseiki style cDNA library. Seven and fifteen positive transformants were obtained by plaque hybridization using FR1 and FR2, respectively, and cDNA clones positive to both λgt10 primers and primer #1-#2 or primer #3-#4 were selected. The number of positive clones thus selected was 6 for FR1 and 14 for FR2. The longest cDNA among these clones detected with FR1 (*os-nons*) and that detected with FR2 (*os-s*$_2$) were individually subcloned into M13 and sequenced. However, no positive DNA clone was detected by plaque hybridization using FR3, suggesting that the style cDNA library of Osa-Nijisseiki lacks the cDNA corresponding to the S_4-RNase gene.

The nucleotide sequence analysis revealed that *ns-nons* (853 bp) and *os-nons* (953 bp) shared the identical 853 nucleotide sequence. *Os-nons* sequence included an open reading frame of 681 nucleotides which encoded 227 amino

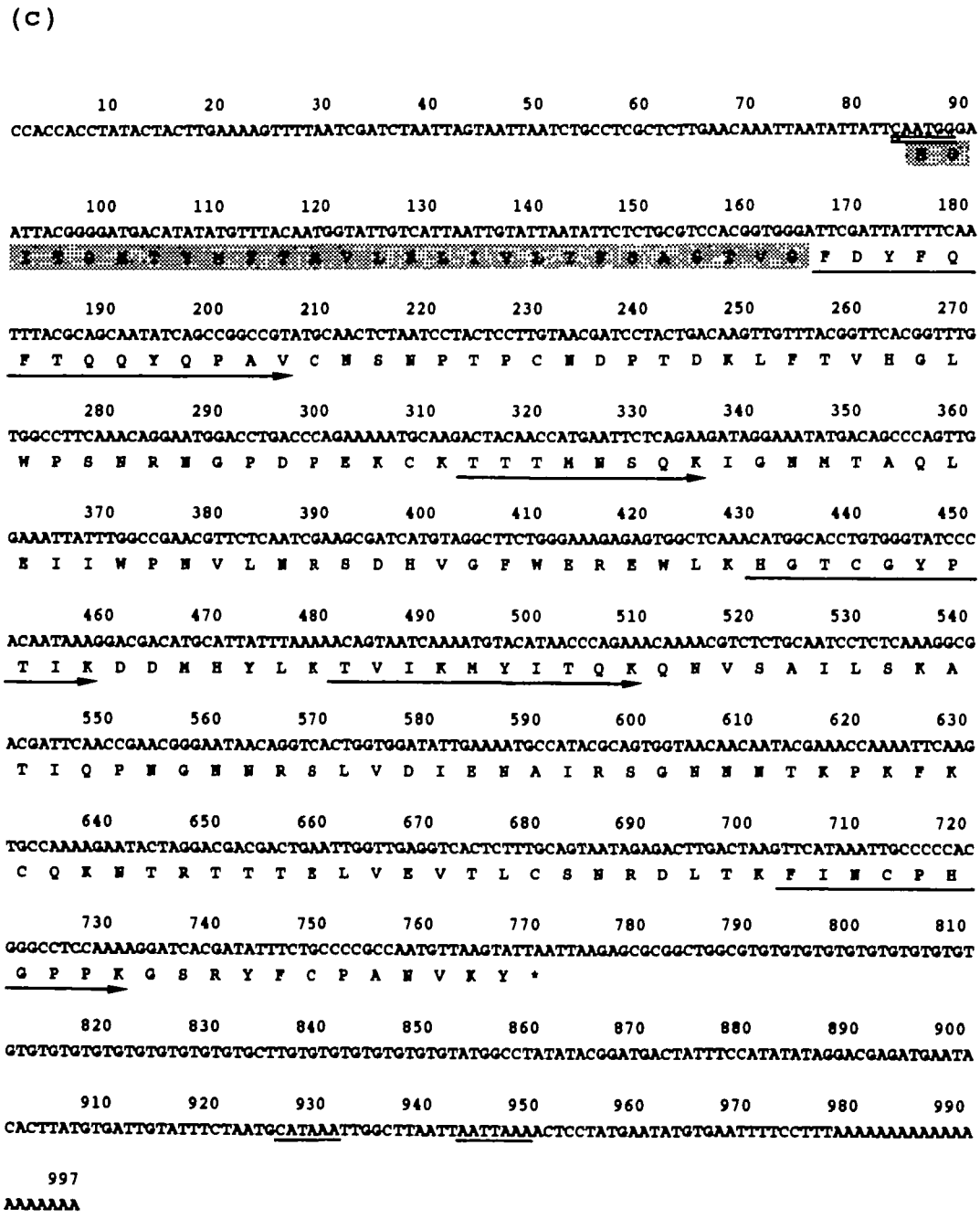

Fig. 3. **Nucleotide and deduced amino acid sequences of cDNAs encoding stylar RNases.** The consensus sequences encompassing a translation initiation codon in a plant gene are doubly underlined. Underlines and asterisks indicate a putative polyadenylation signal and stop codon, respectively. The putative signal peptides are shadowed. The amino acid sequences determined by protein sequence analysis are underlined with arrows. A dashed line denotes the unidentified PTH-amino acid. (a) *os-nons* coding for non-*S*-RNase; (b) *ns-s*$_2$ coding for S_2-RNase; (c) *ns-s*$_4$ coding for S_4-RNase.

acid residues (Fig. 3a). A putative polyadenylation signal was found at positions 918–923. The amino acid sequence deduced from the nucleotide sequence for mature protein was identical with the sequences of peptides from non-*S*-RNase as determined by protein sequencing (manuscript in preparation). A typical signal peptide sequence composed of initiator methionine and a cluster of hydrophobic residues rich in leucine and isoleucine was found in the N-terminal 24 residues. But the cleavage site with signal peptidase remains to be determined because the N-terminus of mature non-*S*-RNase is blocked (unpublished data).

Similarly, the nucleotide sequence was analyzed for *ns-s*$_2$, *os-s*$_2$, and *ns-s*$_4$. *Ns-s*$_2$ was 875 bp long and included an open reading frame of 678 nucleotides (positions 40–717 in Fig. 3b). Comparison of the amino acid sequence deduced from the nucleotide sequence and the sequences determined for S_2-RNase showed that *ns-s*$_2$ encoded the precursor of S_2-RNase, composed of a 201-residue mature protein and a 25-residue signal peptide. *Ns-s*$_2$ had a putative polyadenylation signal at positions 813-818. *Os-s*$_2$ was approximately 250 bp longer than *ns-s*$_2$. Elongation of the nucleotide chain of *os-s*$_2$ was due to a long polyA tail, starting at position 846, attached to the 3′-end. However, the size and the nucleotide sequence of an open reading frame and the sequence and location of a putative polyA addition signal were identical with those of *ns-s*$_2$. *Ns-s*$_4$ was composed of 997 bp including a 684-bp open reading frame at positions 85–768 (Fig. 3c). There were two candidates for the putative polyadenylation signal at positions 925–931 and 943–950. The amino acid sequence translated from the nucleotide sequence was 228 residues long, which comprised a 27-residue signal peptide and a 201-residue mature protein. Partial amino acid sequences including N-terminal and several internal sequences were verified by amino acid sequencing (underlined with arrows in Fig. 3c), suggesting that *ns-s*$_4$ encodes the precursor of S_4-RNase.

DISCUSSION

Since the discovery of RNase activity in *S*-glycoprotein from *Nicotiana alata* (*10*), the mechanism of the involvement of the enzyme activity in the self-incompatibility reaction has been a major issue. To address the question of why the self-incompatible Nijisseiki had mutated to self-compatible Osa-Nijisseiki, we must establish the role of *S*-RNase in the reaction of Japanese pear. Based on genetic analysis using crossing experiments, it has been proposed that acquisition of self-compatibility by Osa-Nijisseiki is due to mutation only at the S_4-gene in the style (*34*). In the present investigation, emphasis was placed on both characterization of the genes coding for the style RNases from Nijisseiki and Osa-Nijisseiki and their structural comparison. It has been shown that except for one point, the fertilization by accepting self pollen upon pollination, Osa-Nijisseiki retains the same entities as Nijisseiki. In fact, the apparent features of fruit, flower, tree form and many other properties are identical for Nijisseiki and Osa-Nijisseiki. Accordingly, it is thought that the difference in the structure and function of style *S*-RNases between these two closely related self-incompatible and self-compatible cultivars must be biochemically subtle, but physiologically critical.

The present investigation revealed that the style of heterozygous Nijisseiki produces three RNases. These three RNases were unambiguously assigned as non-*S*-RNase, S_2-RNase, and S_4-RNase by a comparison of their deduced amino acid sequences with the amino acid sequences determined at the protein level, since these enzymes were separately isolated from style extract either by two-dimensional gel electrophoresis (*37*) or by a series of chromatographic steps (manuscript in preparation). The presence of non-*S*-RNase in the style is not surprising since

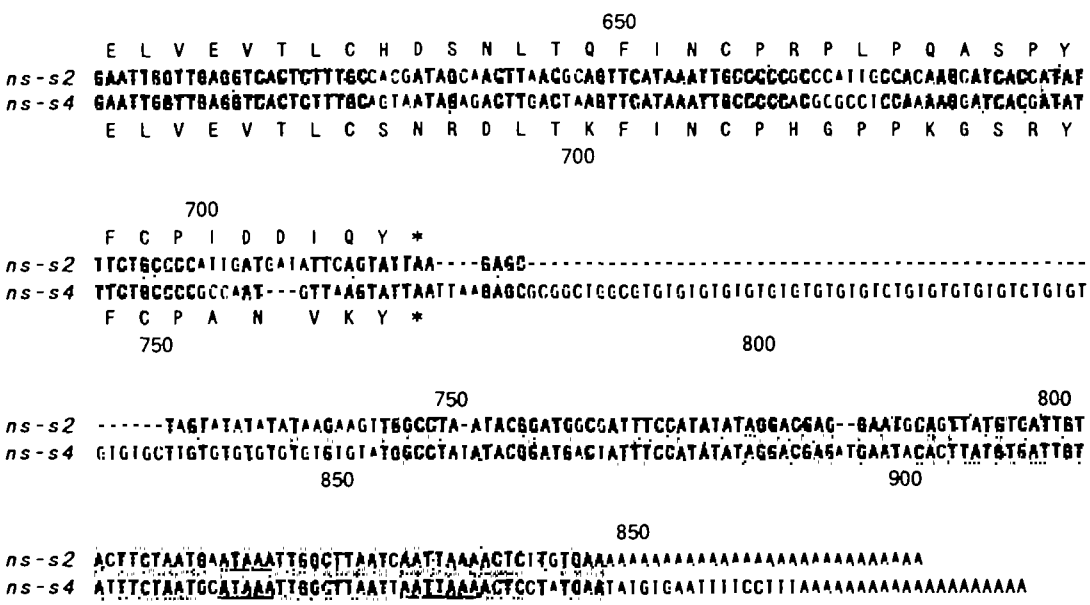

Fig. 4. **Nucleotide sequence alignment between *ns-s*$_2$ and *ns-s*$_4$.** Conserved nucleotides between the two genes are shadowed. Underlines and asterisks indicate a putative polyadenylation signal and stop codon, respectively. The numberings shown above and below the alignment are based on *ns-s*$_2$ and *ns-s*$_4$, respectively.

at least one non-*S*-RNase exists in the style of self-incompatible *Nicotiana alata* (*38*) and *Petunia inflata* (*39*).

Three cDNA clones encoding style RNases were obtained from Nijisseiki. However, the cDNA coding for S_4-RNase was not detected for Osa-Nijisseiki. This difference is critical for these two cultivars, leading to the notion that the absence of S_4-RNase mRNA in the style is responsible for self-compatibility in Osa-Nijisseiki. This is consistent with the finding that Osa-Nijisseiki is a stylar-part mutant of Nijisseiki (*34*) and that the former cultivar retains S_2-RNase, but lacks S_4-RNase in the style (manuscript in preparation). Two reasons could be considered for the absence of S_4-RNase mRNA in the style of Osa-Nijisseiki. One is to assume deletion of the S_4-RNase gene at the *S*-locus in the pear genome and the other is to assume no or extensively suppressed transcription of the *S*-RNase gene in the style of the self-compatible mutant. Interestingly, the acquisition of self-compatibility by Osa-Nijisseiki is a substantial but independent event that induces no or only minor effects on the other inherent characters of Nijisseiki. It is tempting to speculate that the stylar-part mutation represented by S_4^{sm} in Osa-Nijisseiki may have occurred by deletion of the S_4-RNase gene, one of multiple alleles at the *S*-locus, from the genome. Further studies of genomic DNA will be needed to verify this interesting hypothesis.

It is informative to analyze the sequences of *S*-RNases and non-*S*-RNase(s) with respect to their structures and functions at the DNA and protein levels. At the DNA level, several interesting features are apparent. Firstly, the sequence CAATG is consistently found at the putative initiator Met codon in three cloned cDNAs, *ns-nons*, *ns-s*$_2$, and *ns-s*$_4$. This structure is homologous to the AACAATGGC consensus sequence encompassing a translation initiation codon in plants (*40*) and serves to assign the initiator methionine. In particular, for *ns-s*$_2$, three sites (positions 40, 55, and 73) were detected as candidates for the translation initiation site. Eventually, position 40 was proposed as the initiation site because its neighboring sequence, TTCAATGAC, is very similar to the afore-mentioned consensus sequence. Similar structures, GACAATGAG and TTCAATGGG, were found around the codon for the initiator methionine in *ns-nons* and *ns-s*$_4$, respectively. Position 55 (the 20-residue signal peptide) in *ns-s*$_2$ and position 100 (the 22-residue signal peptide) in *ns-s*$_4$ have been assigned to translation initiation sites (*41*), since the solanaceous *S*-RNases have about a 22-residue signal peptide (*15, 16*). But, position 40 in *ns-s*$_2$ and position 85 in *ns-s*$_4$ are more likely to be translation initiation sites because of their CAATG consensus sequences.

Identity of the nucleotide sequences of *ns-s*$_2$ and *ns-s*$_4$ was 74% overall and 80% for the coding region. In contrast, the identity between *ns-nons* and either *ns-s*$_2$ or *ns-s*$_4$ was low and smaller than 30% even for the coding region. These results indicate that the two *S*-RNase genes, *ns-s*$_2$/*os-s*$_2$ and *ns-s*$_4$, are *S*-locus-associated and structurally distinct from the non-*S*-RNase gene. In spite of the high nucleotide sequence similarity between *ns-s*$_2$ and *ns-s*$_4$, there is a great structural difference between the two genes. Most important is the presence of GT repeats in *ns-s*$_4$; 30 consecutive GT sequences comprise the nucleotide chain from positions 789 to 852, though they are interrupted by a GCTT sequence at position 833 upstream from the putative polyadenylation signal (Fig. 3c). Except for the region from positions 780 to 834 involving part of the GT repeat in *ns-s*$_4$, the nucleotide sequences of *ns-s*$_2$ and *ns-s*$_4$ are highly homologous to each other (Fig. 4). The GT repeat, classified as a microsatellite, was first detected for the *S*-RNase gene. In general, satellite sequences, characterized by specifically repeated short nucleotide sequences, are ubiquitously present in eukaryotic genomes, but their biological function is not known (*42*). Whether the microsatellite of *ns-s*$_4$ has any connection to the occurrence of deletion or mutation of the S_4-RNase gene in the style of Nijisseiki remains to be investigated. In this regard, it is noteworthy that replication errors often occur in the microsatellite region (*43*).

The two key amino acid sequences, HGLWP and KHGTC, for the putative active site were detected in the sequences deduced from the nucleotide sequences of *ns-nons*, *ns-s*$_2$, and *ns-s*$_4$, suggesting that all three enzymes are of the RNaseT$_2$ type. Sequence comparison indicated that S_2-RNase and S_4-RNase share 60% identical residues, while the sequence identity between either *S*-RNase and non-*S*-RNase was only 25%. Such a large sequence difference between *S*-RNase and non-*S*-RNase has not previously been reported; no difference was observed in the degree of sequence identity between non-*S*-RNase and *S*-RNases of *Nicotiana alata* and *Petunia inflata* in the family Solanaceae (*38, 39*). The absence of a potential *N*-glycosylation site was an additional feature characteristic of pear non-*S*-RNase. S_2-RNase and S_4-RNase have five and six potential *N*-glycosylation sites, respectively.

Sequence comparison of *S*-RNases between pear and apple was very interesting (Table I). The sequence identity between pear S_2-RNase and an apple *S*-RNase and between pear S_4-RNase and two apple *S*-RNases (*44*) was a little higher than that between the two pear *S*-RNases. Such a close similarity of the *S*-RNase sequences from these two different genera in the same family may be related to the fact that certain pear cultivars accept apple pollens, producing an intergeneric hybrid of Japanese pear and apple (K. Banno, personal communication). Alignment of two pear *S*-RNases sequenced here and two apple *S*-RNases led us to assign seven conserved regions specific for the family Rosaceae and two hypervariable regions (Fig. 5). In particular, two conserved regions at the N-terminus (C1 and C2) are thought to be Rosaceae family-specific as discussed later.

The alignment of four rosaceous *S*-RNases and nine typical solanaceous *S*-RNases provided important information on the similarity and differences in their primary

TABLE I. **Percentage sequence identity among stylar ribonucleases from Rosaceae and Solanaceae families.** *PP*, Japanese pear (*Pyrus pyrifolia*); *MD*, apple (*Malus* × *domestica*); *NA*, tobacco (*Nicotiana alata*); *PI*, petunia (*Petunia inflata*); *SC*, potato (*Solanum chacoense*).

	Rosaceae					Solanaceae		
PPS$_2$	25							
PPS$_4$	26	60						
MDS$_2$	25	59	64					
MDS$_3$	25	62	67	65				
NAS$_2$	19	17	19	18	19			
NAS$_6$	20	21	20	22	23	57		
PIS$_1$	17	19	19	20	19	43	37	
SCS$_2$	22	21	22	21	22	46	43	39
	non*S*	*PPS*$_2$	*PPS*$_4$	*MDS*$_2$	*MDS*$_3$	*NAS*$_2$	*NAS*$_6$	*PIS*$_1$

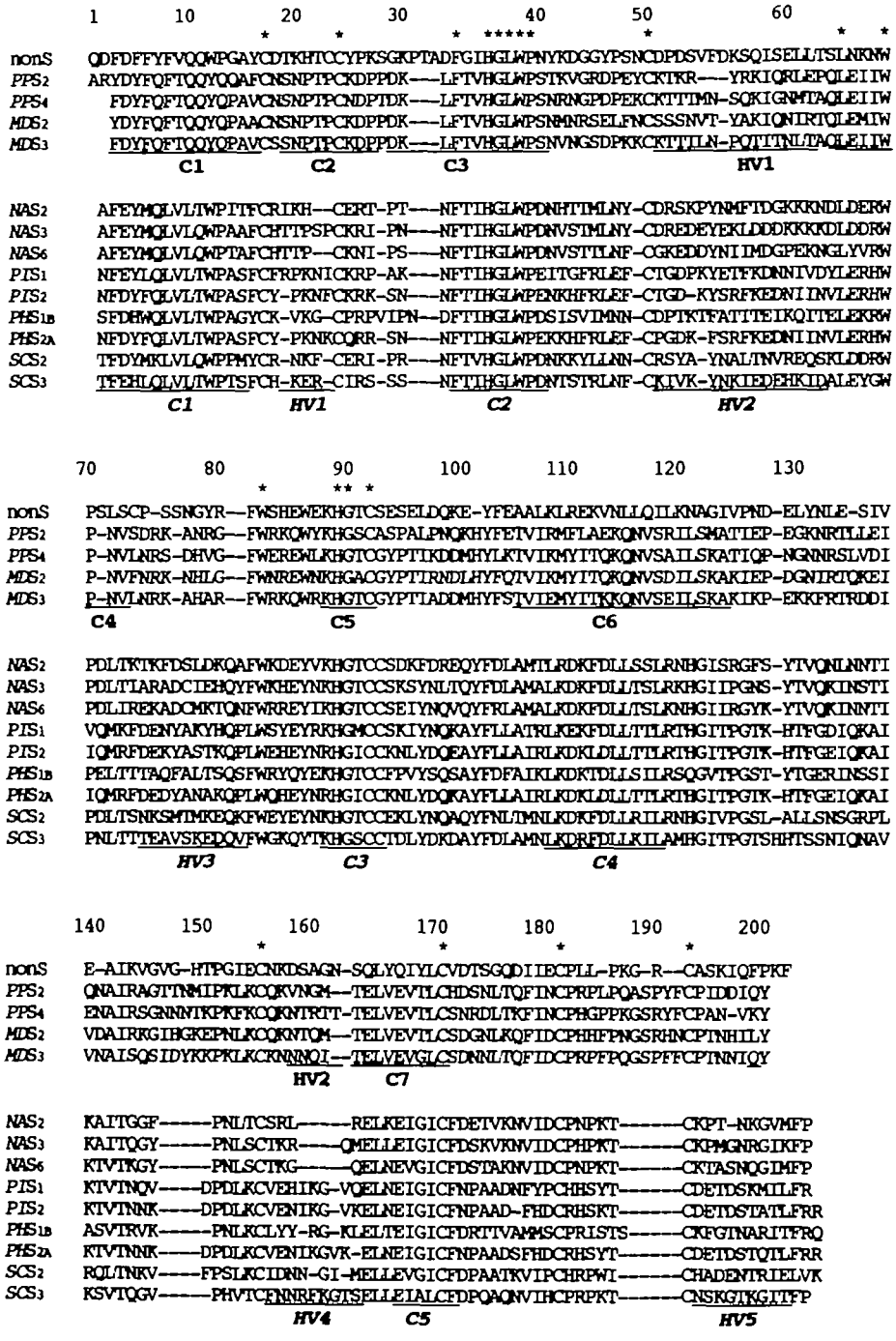

Fig. 5. **Amino acid sequence alignment of non-*S*-RNase, Rosaceae *S*-RNases, and Solanaceae *S*-RNases.** The numbering shown above the alignment is based on that of mature S_2-RNase. Asterisks show the amino acid conserved in all RNases listed. NonS, non-*S*-RNase from Japanese pear; PPS_2 and PPS_4, S_2- and S_4-RNases from Japanese pear; MDS_2 and MDS_3, S_2- and S_3-RNases from apple (*Malus* × *domestica*) (*44*); NAS_2, NAS_3, and NAS_6, S_2-, S_3-, and S_6-RNases from tobacco (*Nicotiana alata*) (*10, 11*); PIS_1 and PIS_2, S_1- and S_2-RNases from *Petunia inflata* (*12*); PHS_{1B} and PHS_{2A}, S_{1B}- and S_{2A}-RNases from *Petunia hybrida* (*16*); SCS_2 and SCS_3, S_2- and S_3-RNases from potato (*Solanum chacoense*) (*25*).

structures (Fig. 5). In this alignment, eight half-cystine residues were conserved and two short stretches of conserved active-site residues are placed at definite positions for all *S*-RNases. Four gaps ranging from one to six residues are observed between rosaceous and solanaceous *S*-RNases and the family-specific structural motif may be integrated into these regions. As for conserved and hypervariable regions, the Rosaceae-specific regions roughly overlapped with those in solanaceous *S*-RNases. But several critical differences in their numbers and locations were found by a comparison between rosaceous and solanaceous *S*-RNases. Rosaceous *S*-RNases have two more conserved regions with the loss of hypervariable regions *1, 3,* and *5* (C2 in place of *HV1*) and lack the thiol group at residue 95, which is readily modified by iodoacetic acid, leading to the inactivation of *Nicotiana alata* S_6-RNase (*45*). However, the eight conserved half-cystine residues are likely to form four disulfide linkages by analogy to the location of disulfides of the S_6-RNase (manuscript in preparation).

In conclusion, the present investigation has determined that Osa-Nijisseiki gains self-compatibility through severely impaired or extensively suppressed expression of the product of the S_4-RNase gene in the style. This is compatible with the finding that the S_4-RNase of this cultivar was not detectable at the *S*-protein zone on 2D gel electrophoresis, as described in the preceding paper (*37*).

The involvement of *S*-RNase in the self-incompatibility reaction has clearly been demonstrated by anti-sense targeted suppression of the expression of *S*-RNase (*26*), *S*-RNase activity-loss events by mutation (*27*), and *S*-RNase activity-gain experiments (*26, 28*). Osa-Nijisseiki is a case of a native activity-loss event for Nijisseiki. Its consequence is quite clear; Osa-Nijisseiki is self-compatible because of the absence of S_4-RNase, one of the two proteins responsible for self-incompatibility in heterozygous Nijisseiki. Knowing why and how Nijisseiki lost the ability to express S_4-RNase in the style will help us to understand the mechanism of gametophytic self-incompatibility involving *S*-RNase.

We thank Kohsuke Inoue (Tottori Horticultural Experiment Station), Yoshihiko Sato (Nagasaki Prefectural Fruit Tree Experiment Station), and Toshihiro Saito and Kazuo Kotobuki (Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries) for collecting Japanese pear flowers. We are grateful to Yoshiko Yagi and Yumi Yoshimura for amino acid and sequence analyses. Thanks are also due to Dr. Kiyoshi Banno of Shinsyu University, Japan for informing us of unpublished results. We also thank Dr. Ettore Appella of the National Institutes of Health for advice regarding the manuscript.

REFERENCES

1. de Nettancourt, D. (1977) *Incompatibility in Angiosperms*, Springer-Verlag, Berlin
2. Singh, A. and Kao, T.-h. (1992) Gametophytic self-incompatibility: Biochemical, molecular genetic and evolutionary aspects. *Int. Rev. Cytol.* **140**, 449-483
3. Clarke, A.E. and Newbigin, E. (1993) Molecular aspects of self-incompatibility in flowering plants. *Annu. Rev. Genet.* **27**, 257-279
4. Newbigin, E., Anderson, M.A., and Clarke, A.E. (1993) Gametophytic self-incompatibility systems. *Plant Cell* **5**, 1315-1324
5. Sims, T.L. (1993) Genetic regulation of self-incompatibility. *Crit. Rev. Plant Sci.* **12**, 129-167
6. Kao, T.-h. and Huang, S. (1994) Gametophytic self-incompatibility: A mechanism for self/nonself discrimination during sexual reproduction. *Plant Physiol.* **105**, 461-466
7. Matton, D.P., Nass, N., Clarke, A.E., and Newbigin, E. (1994) Self-incompatibility: How plants avoid illegitimate offspring. *Proc. Natl. Acad. Sci. USA* **91**, 1992-1997
8. 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
9. Kawata, Y., Sakiyama, F., and Tamaoki, H. (1988) Amino-acid sequence of ribonuclease T_2 from *Aspergillus oryzae*. *Eur. J. Biochem.* **176**, 683-697
10. 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
11. Anderson, M.A., Cornish, E.C., Mau, S.-L., Williams, E.G., Hoggart, R., Atkinson, A., Bonig, I., Grego, B., Simpson, R.J., Roche, P.J., Haley, J.D., Penshow, J.D., Niall, H.D., Tregear, G. W., Crawford, J.P., and Clarke, A.E. (1986) Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. *Nature* **321**, 38-44
12. 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
13. Coleman, C.E. and Kao, T.-h. (1992) The flanking regions of two *Petunia inflata S* allele are heterogeneous and contain repetitive sequence. *Plant Mol. Biol.* **18**, 725-737
14. Broothaerts, W.J., van Laere, A., Witters, R., Preaux, G., Decock, B., van Damme, J., and Vendrig, J.C. (1989) Purification and N-terminal sequencing of style glycoproteins associated with self-incompatibility in *Petunia hybrida*. *Plant Mol. Biol.* **14**, 93-102
15. Clark, K.R., Okuley, J.J., Collins, P.D., and Sims, T.L. (1990) Sequence variability and developmental expression of *S*-alleles in self-incompatible and pseudo-self-compatible petunia. *Plant Cell* **2**, 815-826
16. Ai, Y., Tsai, D.-S., and Kao, T.-h. (1992) Self-incompatibility in *Petunia inflata*: Isolation and characterization of cDNAs encoding three *S*-allele associated proteins. *Plant Mol. Biol.* **19**, 523-528
17. 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
18. Rivers, B.A., Bernatzky, R., Robinson, S.J., and Jahnen-Dechent, W. (1993) Molecular diversity at the self-incompatibility locus is a salient feature in natural populations of wild tomato (*Lycopersicon peruvianum*). *Mol. Gen. Genet.* **238**, 419-427
19. Chung, I.-K., Nakata, K., Tanaka, H., Ito, T., Horiuchi, H., Ohta, A., and Takagi, M. (1993) Identification of cDNA clones coding for the style specific S_{11a}-glycoprotein gene associated with gametophytic self-incompatibility in tomato (*Lycopersicon peruvianum*). *Biosci. Biotech. Biochem.* **57**, 1172-1176
20. Royo, J., Kowyama, Y., and Clarke, A.E. (1994) Cloning and nucleotide sequence of two *S*-RNases from *Lycopersicon peruvianum* (L.) Mill. *Plant Physiol.* **105**, 751-752
21. Royo, J., Kunz, C., Kowyama, Y., Anderson, M., Clarke, A.E., and Newbigin, E. (1994) Loss of a histidine residue at the active site of *S*-locus ribonuclease is associated with self-compatibility in *Lycopersicon peruvianum*. *Proc. Natl. Acad. Sci. USA* **91**, 6511-6514
22. Kaufmann, H., Salamini, F., and Thompson, R.D. (1991) Sequence variability and gene structure at the self-incompatibility locus of *Solanum tuberosum*. *Mol. Gen. Genet.* **226**, 457-466
23. 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
24. Xu, B., Grun, P., Kheyr-Pour, A., and Kao, T.-h. (1990) Identification of pistil-specific proteins associated with three self-incompatibility alleles in *Solanum chacoense*. *Sex. Plant Reprod.* **3**, 54-60
25. Xu, B., Mu, J., Nevins, D.L., Grun, P., and Kao, T.-h. (1990) Cloning and sequencing of cDNA encoding two self-incompatibility associated proteins in *Solanum chacoense*. *Mol. Gen. Genet.* **224**, 341-346
26. Lee, H.-S., Huang, S., and Kao, T.-h. (1994) *S* proteins control rejection of incompatible pollen in *Petunia inflata*. *Nature* **367**, 560-563
27. Huang, S., Lee, H.-S., Karunanandaa, 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
28. Murfett, J., Atherton, T.L., Mou, B., Gasser, C.S., and McClure, B.A. (1994) *S*-RNase expressed in transgenic *Nicotiana* causes *S*-allele-specific pollen rejection. *Nature* **367**, 563-566
29. 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
30. Gray, J.E., McClure, B.A., Boning, I., Anderson, M.A., and Clarke, A.E. (1991) Action of the style product of the self-incompatibility gene of *Nicotiana alata*. *Plant Cell* **3**, 271-283
31. 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.). *Studies Hort. Inst. Kyoto Imp. Univ.* **3**, 267-271
32. Ogaki, C. (1958) The sterility factors in new varieties of the Japanese pear (*Pyrus serotina* Rehd. *var. culta* Rehd.). *Res. Rep. Kanagawa Agri. Inst. Hort.* **5**, 23-26
33. Machida, Y. (1972) Morphological and physiological characteristics of Japanese and Chinese pear in *Dictionary of Pomology*

(Satoh, K., Mori, H., Matsui, O., Kitajima, H., and Chiba, T., eds.) pp. 527-529, Yokendo, Tokyo
34. 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
35. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
36. Qian, L. and Wilkinson, M. (1991) DNA fragment purification: Removal of agarose 10 minutes after electrophoresis. *Biotechniques* **10**, 736-738
37. Ishimizu, T., Sato, T., Saito, Y., Yoshimura, Y., Norioka, S., Nakanishi, T., and Sakiyama, F. (1996) Identification and partial amino acid sequences of seven *S*-RNases associated with self-incompatibility of Japanese pear, *Pyrus pyrifolia* Nakai. *J. Biochem.* **120**, 326-334
38. Kuroda, S., Norioka, S., Mitta, M., Kato, I., and Sakiyama, F. (1994) Primary structure of a novel stylar RNase unassociated with self-incompatibility in tobacco plant, *Nicotiana alata*. *J. Protein Chem.* **13**, 438-439
39. Lee, H.-S., Singh, A., and Kao, T.-h. (1992) RNase X2, a pistile-specific ribonuclease from *Petunia inflata*, shares sequence similarity with solanaceous *S* proteins. *Plant Mol. Biol.* **20**, 1131-1141
40. Lutcke, H.A., Chow, K.C., Mickel, F.S., Moss, K.A., Kern, H.F., and Scheele, G.A. (1987) Selection of AUG initiation codons differs in plants and animals. *EMBO J.* **6**, 43-48
41. 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
42. Beckmann, J.S. and Soller, M. (1990) Toward a unified approach to genetic mapping of eukaryotes based on sequence tagged microsatellite sites. *Biotechnology* **8**, 930-932
43. Eshleman, J.R. and Markowitz, S.D. (1995) Microsatellite instability in inherited and sporadic neoplasms. *Curr. Opin. Oncol.* **7**, 83-89
44. Broothaerts, W., Janssens, 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
45. Ishimizu, T., Miyagi, M., Norioka, S., Liu, Y.-H., Clarke, A.E., and Sakiyama, F. (1995) Identification of histidine 31 and cysteine 95 in the active site of self-incompatibility associated S_6-RNase in *Nicotiana alata*. *J. Biochem.* **108**, 1007-1013