Cloning of the $S_8$-RNase ($S_8$-allele) of Japanese Pear (Pyrus pyrifolia Nakai)

Carlos CASTILLO1,5, Takeshi TAKASAKI2*, Toshihiro SAITO5, Shigemi NORIOKA4 and Tetsu NAKANISHI1,2

1 Graduate School of Science and Technology, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe, Hyogo 657-8501, Japan
2 Faculty of Agriculture, Kobe University, 1-1 Rokkodai, Nada-ku Kobe, Hyogo 657-8501, Japan
3 National Institute of Fruit Tree Science, 2-1 Fujimoto, Tsukuba, Ibaraki 305-8605, Japan
4 Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan
5 National Institute of Agrarian Research, Av. La Universidad s/n, Lima 12, Peru

*Corresponding author E-mail address: taka@kobe-u.ac.jp

Received 24 May 2001; accepted 11 September 2001

Abstract

The expression of $S_8$-RNase was confirmed in pistils of two Japanese pear cultivars, ‘Ichiharawase’ ($S_8S_8$) and ‘Heiwa’ ($S_8S_0$). The complete sequence of the $S_8$-RNase gene was determined connecting the nucleotide sequences of partial cDNA and 5′ terminal genomic DNA fragments amplified by RT-PCR and genomic PCR. The $S_8$-RNase has an open reading frame of 684 nucleotides encoding 228 amino acid residues. A hypervariable region (HV) of $S_8$-RNase, which is quite different from those of $S_7$- to $S_7$-RNases, includes an intron of 234 bp. The similarity of deduced amino acid sequences between $S_8$-RNase and the seven $S$-RNases of Japanese pear ranged from 56.7% ($S_7$-RNase) to 70.2% ($S_7$-RNase). Based on its nucleotide sequence, we selected NruI as $S_8$-RNase specific restriction endonuclease and established the PCR-RFLP system for discriminating $S_7$- to $S_8$-alleles.

Keywords: PCR - RFLP, Pyrus pyrifolia, Self-incompatibility, $S$-RNases.

Abbreviations

PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; PCR-RFLP, PCR-restriction fragment length polymorphism.

Introduction

Self-incompatibility (SI) is a widespread genetic mechanism to prevent inbreeding in plants (de Nettancourt, 1977). Japanese pear “Nashi” (Pyrus pyrifolia Nakai) exhibits gametophytic self-incompatibility (GSI) that is controlled by a single gene ($S$-locus) with multi-alleles (Kikuchi, 1929). Seven alleles ($S_1$ to $S_7$) were identified in 22 cultivars by pollen infection experiments (Terami et al., 1946). Since then, these $S$-genotype assignments have been used as cross indicators for identifying the genotype of other cultivars. Several pollen infection studies have been performed, and $S$-genotypes of around 40 cultivars have been described to date (Terami et al., 1946; Ogaki, 1958; Machida et al., 1982; Hiratsuka et al., 1998).

In Japanese pear, $S$-allele-associated stylar glycoproteins with RNase activity (termed $S$-RNases) have been identified by protein analysis (Sassa et al., 1992; Ishimizu et al., 1996a). $S_8$-RNase has been proved to be responsible for GSI in Japanese pear (Sassa et al., 1997). The cDNAs encoding the $S_8$ to $S_7$-RNases have been isolated and sequenced from four cultivars, ‘Imamurakaki’ ($S_8S_8$), ‘Nijisseiki’ ($S_8S_0$), ‘Hosui’ ($S_8S_2$) and ‘Okusankichi’ ($S_8S_7$) (Norioka et al., 1996; Ishimizu et al., 1998). The diversity of their deduced amino acid sequences has allowed us to appoint the hypervariable (HV) region, which is thought to be responsible for recognition of $S$-alleles (Ishimizu et al., 1998). The introns inserted in the HV regions have also been sequenced from genomic DNAs encoding the $S_7$ to $S_7$-RNases. Based on the nucleotide sequences within their HV region and intron, we previously proposed a PCR-RFLP system for identifying the seven $S$-alleles of Japanese pear (Ishimizu et al., 1999).

Recently, using the PCR-RFLP system, we reconsidered the $S$-genotype assignments of six Japa-
nese pear cultivars, ‘Akaho’, ‘Tanzawa’, ‘Kimizukawase’, ‘Choju’, ‘Ichiharawase’ and ‘Meigetsu’ (Castillo et al., 2001). ‘Ichiharawase’ and ‘Meigetsu’ were both identified to have the S-genotype of S$_{1}S_{2}$ by pollination tests (Terami et al., 1946). From ‘Ichiharawase’ and ‘Meigetsu’, the S$_{1}$-RNase fragment (367 bp) and a new S-RNase fragment (436 bp) were amplified by PCR–RFLP analysis, but the S$_{2}$-RNase was not. The new S-RNase fragment presented a unique size and digestion pattern compared to the S$_{1}$- to S$_{2}$-RNases, and its partial deduced amino acid sequence included a quite different HV region. In addition, the intron inserted within the HV region had a different size. These distinctions led us to designate the new S-RNase fragment as the S$_{0}$-RNase, and as a result, the S-genotype of S$_{1}S_{0}$ was reassigned to ‘Ichiharawase’ and ‘Meigetsu’ (Castillo et al., 2001).

In this study, we confirmed the expression of S$_{0}$- RNase in pistils of two Japanese pear cultivars by RT-PCR analysis and determined the complete nucleotide sequence of S$_{0}$-RNase including the intron. Based on its nucleotide sequence, we selected an S$_{0}$-RNase specific restriction endonuclease and established the PCR–RFLP system for discriminating among S$_{1}$- to S$_{0}$-alleles.

Material and Methods

Plant Material and DNA

Young leaves of seven Japanese pear cultivars, ‘Ichiharawase’, ‘Meigetsu’, ‘Heiwa’, ‘Imamuraaki’, ‘Nijisseiki’, ‘Hosui’ and ‘Okusankichi’ were collected in spring at the National Institute of Fruit Tree Science, Ministry of Agriculture, Forestry and Fisheries of Japan in Tsukuba. The leaves were stored at -80°C. Genomic DNA was extracted from 0.1-0.2 g of leaves according to the method of Doyle and Doyle (1987). Flowers of ‘Ichiharawase’ and ‘Heiwa’ were collected at the white bud stage. Pistils were removed, frozen in liquid nitrogen and then stored at -80°C until use.

Isolation of mRNA

Total RNA was extracted from pistils of ‘Ichiharawase’ (S$_{1}S_{0}$) and ‘Heiwa’ (S$_{0}S_{0}$) according to the method described by Chomczynski and Sacchi (1987). Two hundred pistils were ground in liquid nitrogen and 5 ml of the extract buffer (5 M guanidine isothiocyanate, 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 30 mg ml$^{-1}$ Polyclar AT, 5% 2-mercaptoethanol and 0.5% sodium N-sauroyl sarsosinate) was added. After centrifugation, the supernatant was recovered and mixed well with 0.2 ml of 2 M NaOAc (pH 4.0), 2 ml of water-saturated phenol, and 1 ml of chloroform–isoamyl alcohol (24:1). The mixture was cooled on ice for 15 min. After centrifugation, the aqueous phase was mixed with 4.5 ml of isopropanol and held at -20°C for 2 h to precipitate RNA. The RNA pellet was washed with 75% ethanol, dried for 1 h at room temperature, and then it was dissolved in 0.8 ml of distilled water. mRNAs were isolated from total RNA using the Micro–FastTrack™ 2.0 mRNA Isolation Kit (Invitrogen) according to the manufacturer’s instructions.

RT-PCR

RT-PCR was carried out using the Titan™ One Tube RT-PCR system (Roche Diagnostics) according to the manufacturer’s instructions. mRNA was reverse-transcribed for 30 min at 50°C to synthesize first-strand cDNA. PCR amplification was performed with a set of primers, ‘FTQQYQ’ (5’-TTTACGCAGAATATCG-3’) and adapter primer NotI-(dT)$_{6}$ (Amersham-Pharcmaica) for 10 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C and extension for 45 s at 68°C, followed by 25 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C and initial extension for 45 s at 68°C, adding 5 s for each cycle. A final extension step for 7 min at 68°C was performed. Nested PCR was carried out with a set of primers, ‘CNSNPT’ (5’-TGCAACTCTYAAWCGTACTC-3’) and NotI-dT (5’-AACTGGAAGAATTGCCGC-GCGAGAT T-’3’) for 10 cycles of denaturation for 15 s at 94°C, annealing for 30 s at 60°C and extension for 1 min at 70°C. Followed by 20 cycles of denaturation for 15 s at 94°C, annealing for 30 s at 60°C and extension for 1.5 min at 70°C. Then, a final extension step for 7 min at 70°C. After PCR-products were digested with EcoRI (which cleavages S$_{1}$- and S$_{0}$-RNases specifically), the undigested S$_{0}$-RNase fragment was directly sequenced.

Amplification of S-RNase fragment from genomic DNA

S-RNase fragments were amplified from genomic DNA by PCR with S-RNase specific primers, ‘5’-32 bp’ (5’-TGCTCCTCTTTGAACAAA-3’), ‘FTQQYQ’ and ‘anti-IFPQ’ (5’-AC(A/G)TTCCGGCCAATAATT-3’). Genomic DNA (50 ng) was mixed with 0.3 μM of each primer, 200 μM dNTP, 1x PCR-buffer, 1U Taq polymerase and distilled water up to final volume of 30 μl. PCR amplification was carried out for 10 cycles of denaturation for 15 s at 94°C, annealing for 30 s at 48°C and extension for 1 min at 70°C, followed by 20 cycles of denaturation for 15 s at 94°C, annealing for 30 s at 48°C and extension for 1.5 min at 70°C,
with a final extension for 7 min at 70 °C.

Sequencing analysis

PCR products were run on 2% agarose gels and each fragment was isolated using the GENECLEAN II Kit (Bio 101, Inc.). Nucleotide sequences were determined by the dye-deoxy-nucleotide chain termination method, with the primers described above, using an ABI PRISM™ 310 DNA capillary sequencer. All data were analyzed with DNASIS-Mac software (Hitachi Software Engineering Co.).

Restriction endonuclease digestion

*S*-allele specific restriction endonuclease digestion was carried out under conditions described previously (Castillo et al., 2001). *S*-RNase fragments were amplified from the seven allele set cultivars, ‘Imamuraaki’ (*S1S6*), ‘Nijisseiki’ (*S2S6*), ‘Hosui’ (*S3S6*) and ‘Okusankichi’ (*S4S6*), as well as from ‘Ichiharawase’ (*S5S6*), ‘Meigetsu’ (*S6S6*) and ‘Heiwa’ (*S5S5*) harboring *S5*-allele. The amplified *S*-RNase fragments were digested with *S*-allele specific restriction endonucleases (SfiI, PpuMI, NdeI, I.AhuNI, HincII, AccII and NruI) and digested fragments were electrophoresed on 2% agarose gels.

Results and Discussion

To investigate the expression of *S8*-RNase in pistils of ‘Ichiharawase’ and ‘Heiwa’, we amplified cDNA corresponding to *S8*-RNase by RT-PCR from mRNA of both cultivars. Two fragments of almost the same size (around 750 bp) were amplified with primers ‘FTQQYQ’ and NotI- (dT)₁₈ (Fig. 1A). RT-PCR products were then used as the template for nested PCR amplification with ‘CNSNPT’ and NotI- dT primers. The nested PCR yielded two *S*-RNase fragments of about 720 bp that corresponded with the expected sizes of partial *S*-RNase cDNAs. Because the amplicons from ‘Ichiharawase’ and ‘Heiwa’ also included the *S1*- and *S2*-RNase cDNA fragments, respectively, they were digested with EcoRI whose recognition site is present within the HV region of *S1*- and *S2*-RNases but not within that of *S8*-RNase. The digested *S*-RNase fragments were electrophoresed on 2% agarose gel, and then undigested *S8*-RNase fragments were extracted from the gel and sequenced. The partial *S8*-RNase cDNAs (558 bp) amplified from ‘Ichiharawase’ and ‘Heiwa’ were found to have the same nucleotide sequence. This indicates that *S8*-RNase is expressed in pistils of ‘Ichiharawase’ and ‘Heiwa’.

To obtain a complete nucleotide sequence of the *S8*-RNase gene, we amplified the 5’ terminal region of *S8*-RNase from genomic DNA of ‘Ichiharawase’, ‘Meigetsu’ and ‘Heiwa’ by PCR with a set of primers, ‘5’-32bp’ and ‘anti-IWPNV’. The ‘5’-32bp’ primer was designed based on conserved nucleotide sequences between the initiation codon (ATG) and the putative TATA box in the 5’ flanking region of Japanese pear *S2*, *S3*, *S4* and *S8*-RNase genes (Ushijima et al., 1998; Norioka et al., 2001). Only one fragment of 565 bp was amplified from ‘Ichiharawase’ and ‘Meigetsu’, while two fragments of 565 bp and 496 bp were amplified from ‘Heiwa’ (Fig. 1B). The 565 bp fragment corresponds to the 5’ terminal region of *S8*-RNase because the intron of *S8*-RNase is larger than that of *S2*-RNase (Castillo et al., 2001). The failure to amplify the *S1*-RNase from ‘Ichiharawase’ and ‘Meigetsu’ may be due to mismatches between the ‘5’-32bp’ primer and the 5’ flanking region of *S1*-RNase. The 5’ terminal fragments of *S8*-RNase were extracted from the gel and sequenced. The 5’ region of each fragment, except the upstream region of the initiation codon, was found to have the same 533 bp nucleotide sequence including the intron.

The complete nucleotide sequence of the *S8*-RNase was established by overlapping sequences between the partial cDNA fragment of 558 bp and 5’ terminal genomic DNA fragment of 533 bp. *S8*-RNase contains an open reading frame of 684 bp.

![Fig. 1](image_url)

**Fig. 1** *S*-RNase fragments amplified from mRNA and genomic DNA of Japanese pear cultivars. (A) The cDNA fragments of *S*-RNase amplified from ‘Ichiharawase’ by RT-PCR with ‘FTQQYQ’ and NotI-(dT)₁₈ primers (lane 1) and by nested PCR with ‘CNSNPT’ and NotI-dT primers (lane 2), and digested with EcoRI (lane 3). (B) The 5’ terminal *S*-RNase fragments amplified from genomic DNA of ‘Ichiharawase’ (lane 1), ‘Meigetsu’ (lane 2) and ‘Heiwa’ (lane 3) by PCR with ‘5’-32bp’ and ‘anti-IWPNV’ primers. *S*-RNase fragments were electrophoresed on 2% agarose gels.
encoding 228 amino acid residues. There is a single intron of 234 bp in \( S_8 \)-RNase located between amino acids 85 and 86 (Fig. 2). \( S_8 \)-RNase shows one HV region and five conserved regions (C1 through C5) described for seven \( S \)-RNase of Japanese pear (Norioka et al., 1996; Ishimizu et al., 1998). The \( S_8 \)-RNase shares 98 perfectly conserved amino acid residues with Japanese pear \( S \)-RNases.

The \( S_8 \)-RNase shows a high homology, ranging from 56.7% (\( S_5 \)-RNase) to 70.2% (\( S_7 \)-RNase). A putative signal peptide of \( S_8 \)-RNase is predicted by Neural Networks (Nielsen et al., 1997) and its most likely cleavage site is indicated between amino acids 26 and 27. The predicted mature \( S_8 \)-RNase protein has a calculated pI value of 9.19 (Skoog and Wichman, 1986) that agrees with the basic nature of \( S_7 \) to \( S_8 \)-RNases of Japanese pearl (Sassa et al., 1993; Ishimizu et al., 1996a). \( S_8 \)-RNase possessed the primary structural features of \( S \)-RNases of Japanese pearl, two histidine residues (His-61 and His-117) essential for T2/S type RNase activity (Kawata et al., 1989) and eight cysteine residues forming four disulfide bridges important for the formation or stabilization of their tertiary structure (Ishimizu et al., 1996b). The \( S_8 \)-RNase also possessed eight potential \( N \)-glycosylation sites with the consensus sequence Asn-X-Ser/Thr (X is not Pro and Asp), including Asn-145 the only conserved in rosaceous \( S \)-RNases and whose glycans may be important for the folding of the core structure (Ishimizu et al., 1998). The \( S_8 \)-RNase shows only 31.9% of homology with a non-\( S \)-RNase isolated from the pistils of ‘Nijisseiki’ by Norioka et al. (1996). All the features of \( S_8 \)-RNase, as well as the primer specificity, the cultivar specificity and its Mendelian inheritance described previously (Castillo et al., 2001), discard the possibility of \( S_8 \)-RNase being a non-\( S \)-RNase protein.

Histidine (H) and Threonine (T), which are completely conserved among \( S_7 \) to \( S_8 \)-RNases in Japanese pear were substituted for Gln-130 (Q) and

| Table 1: Analysis of \( S_7 \) to \( S_8 \)-RNase of Japanese pear by PCR - RFLP. |
|---------------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| Cultivar                        | \( S_7 \) | \( S_7 \) | \( S_7 \) | \( S_7 \) | \( S_7 \) | \( S_7 \) | \( S_7 \) |
| Imamuraaki                      |       |       |       |       |       |       |       |
| Nijisseiki                      |       |       |       |       |       |       |       |
| Hosui                           |       |       |       |       |       |       |       |
| Okusankichi                     |       |       |       |       |       |       |       |
| Ichiharawase                    |       |       |       |       |       |       |       |
| Meietsu                         |       |       |       |       |       |       |       |
| Heiwa                           |       |       |       |       |       |       |       |
| **O:S-**-RNase fragment amplified by PCR** | \( \text{++} \) | \( \text{++} \) | \( \text{++} \) | \( \text{++} \) | \( \text{++} \) | \( \text{++} \) | \( \text{++} \) |
| \( \text{++} \): Two \( S \)-RNase fragments were digested with restriction endonucleases | | | | | | | |
| \(+\): One of two \( S \)-RNase fragments was digested with restriction endonucleases | | | | | | | |
| \( \text{--} \): \( S \)-RNase fragments were not digested with restriction endonucleases | | | | | | | |
Cultivars with $S_8$-allele are compatible with all tester cultivars harboring any of the seven other alleles, thus it is difficult that $S$-genotype assignment of these cultivars be identified by pollination tests. The PCR–RFLP system has proved to be a reliable method for $S$-genotype typing (Ishimizu et al., 1999; Castillo et al., 2001). The PCR–RFLP system with ‘FTQQYQ’ and ‘anti-IWPNV’ primers amplified the $S_8$-RNase fragment, which was slightly different in size from $S_1$- to $S_7$-RNase fragments and was cleaved by Sfcl and AccII specific for $S_1$ and $S_5$, $S_7$-RNase, respectively, producing a distinct but complicated digestion pattern (Castillo et al., 2001). To obtain a clear discrimination of the $S_8$-RNase, we selected a new restriction endonuclease, Nrl1 that digests the $S_8$-RNase fragment producing 251 bp and 185 bp fragments. The PCR–RFLP system with the addition of Nrl1, was tested by determining $S$-genotypes of the seven allele set cultivars, ‘Imamuraaki’ ($S_5S_8$), ‘Nijisseiki’ ($S_2S_5$), ‘Hosui’ ($S_6S_5$), ‘Okusan-kichi’ ($S_6S_8$) and three cultivars, ‘Ichiharawase’ ($S_5S_9$), ‘Meigetsu’ ($S_5S_9$) and ‘Heiwa’ ($S_5S_9$) (Table 1). Nrl1 digested only the $S_8$-RNase fragment but not the $S_1$- to $S_7$-RNase fragments (Fig. 3).

In this study, we confirmed the expression of $S_8$-RNase in pistils of ‘Ichiharawase’ and ‘Heiwa’, and determined the complete nucleotide sequence of the $S_8$-RNase including its intron. Based on the nucleotide sequence, we selected an $S_8$-RNase specific restriction endonuclease, Nrl1, and established an PCR–RFLP system for identifying $S$-genotypes of Japanese pear cultivars harboring $S_1$ to $S_8$ alleles.

**Acknowledgement**

This work was supported in part by Grants-in-Aid for Special Research on Priority Areas (10660025 and 13760005) from the Ministry of Education, Science, Culture and Sports, Japan.

**References**


