Discovery of a New Self-incompatibility Allele in Apple

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Additional index words. Malus ×domestica, Se-RNase, Se-allele, PCR

Abstract. A polymerase chain reaction (PCR)-based method for identifying the S-alleles in the Asian pear [Pyrus pyrifolia (Burn) Nak.] was applied to apple (Malus ×domestica Borkh.) cultivars. With minor modifications in one of the primers, the fragments from S-genes (S-RNAs) with introns were amplified from total DNA of apple cultivars possessing S2-, S3-, S5-, S7- (=Sd)-, S9- (=Sc-), Sf- and Sg-allele genotypes. S-alleles within S24- (=Sb-) and S26-alleles were also amplified. The PCR amplification step of this method appears to be useful for preliminary investigation of apple S-alleles, especially for species or cultivars of unknown origin or history. Using the primers, which are a part of a new S-allele, the Se-allele encoding Se-RNase with an intron in the Se-allele was amplified. We cloned the cDNA of Se-RNase, and developed a PCR-restriction fragment length polymorphism (RFLP) analysis method for Se-allele identification. S-allele genotypes of seven apple cultivars were investigated.

Apple has gametophytic self-incompatibility (GSI) controlled by a single multiallelic S-locus. In this system, fertilization is inhibited when the pollen S-allele matches one of the pistil S-alleles. In GSI, both pollen and pistil are normal, but flowers can reject their own pollen (de Nettancourt, 1977).

The S-locus in apple has S2- (=Sa-), S3- (=Sb-), S5-, S7- (=Sd-), S9- (=Sc-), Sf-, Sg-, S24- (=Sb-), S26- and S27-alleles. S-alleles encode S-RNAs, many of which have been cloned (Broothaerts et al., 1995; Janssens et al., 1996; Verdoodt et al., 1998). Based on the nucleotide sequences of the S-genes encoding S-RNAs, a PCR-RFLP analysis method for S-allele identification has been developed (Janssens et al., 1995; Kitahara et al., 1999, 2000; Sassa et al., 1996; Verdoordt et al., 1998). Based on the nucleotide sequences of the S-genes encoding S-RNAs, a PCR-RFLP analysis method for S-allele identification has been developed (Janssens et al., 1995; Kitahara et al., 2000; Matsumoto et al., 1999a, b; Verdoordt et al., 1998). The method consists of PCR amplification of each S-gene using specific primers for individual S-genes, followed by digestion of the fragments by specific restriction enzymes.

Previously, we identified the S-allele genotype of ‘Delicious’ apple as 39 using the PCR-RFLP method (Matsumoto et al., 1999a), although the S-allele genotype of ‘Delicious’ previously had been identified as Sc (=S9) Se by pollination and progeny analysis (Komori et al., 1996). Sc was represented as Sd by Katoh et al. (1997), but we used Sc in this study since Sc has priority over Sd. Because the existing PCR-RFLP technique (Matsumoto et al., 1999a) does not detect the Se-allele, a new or modified method is needed.

Recently, a PCR-based method for identification of S-allele genotypes of the Asian pear was developed by Ishimizu et al. (1999). The Asian pear also has GSI, and some sections of the S-RNAs are well conserved between the Asian pear and apple. The method uses primers from the conserved regions of the S-RNAs (Table 1), and the PCR amplification step within the method is useful not only for the Asian pear but also for apples that have S-alleles containing new S-RNAs.

In this study, we applied a method for Asian pears to apple cultivars, and determined the cDNA sequence encoding Se-RNase. We also developed a PCR-RFLP method for Se-allele identification, and investigated S-allele genotypes of seven apple cultivars.

Table 1. Primers cited in this manuscript.

| Primer name | Primer sequence (5’–3’) | Reference |
|-------------|--------------------------|-----------|
| FTQQYQ      | TTTACCGCAAGAAATTCGAG     | Ishimizu et al., 1999 |
| anti-i/4-IWPVN | ACGTGGCAGCAAATCGATT    | This paper |
| PSNKNGP     | CTTTCAACAAAGAATGGGCC    | This paper |
| Sf-sense 2  | ATTAATCTGCCTCGCACTTG     | Matsumoto et al., 1999b |
| Se-sense 2  | TGTTCCTGCAATATCCGG      | This paper |
| Se-antisense| ATCGTGATCTGTTATGGT       | This paper |
| anti-IWPVN  | ACyTTCGGCCCAATAATT       | Ishimizu et al., 1999 |

Received for publication 3 Dec. 1999. Accepted for publication 17 Apr. 2000. We thank Junichi Soejima for supplying plant samples and Kaoji Sugishita for her assistance. This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (No. 11660025). The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

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Materials and Methods

Plant material. The Malus plants from collections at the Apple Research Center of the National Institute of Fruit Tree Science, Morioka, Japan, were used. Styles, together with the stigma, stamens, and petals of individual flowers of ‘Delicious’ were dissected at the popcorn stage, immediately frozen in liquid nitrogen, and stored at −80 °C. Young leaves were collected and stored at −80 °C until analysis.

PCR amplification. Total DNA was isolated from leaves of individual plants as described by Thomas et al. (1993), and PCR was conducted using the sense (‘FTQQYQ’) and antisense (‘anti-i/4-IWPVN’) primers (Table 1). Conditions for PCR were as described by Ishimizu et al. (1999). Amplified and digested fragments were separated on a 4% or 6% polyacrylamide gel in 40 mM Tris-acetate, 1 mM EDTA, pH 8.0 (TAE) at 50-70 V for 1-1.5 h.

Se-RNase cDNA cloning. Primers ‘FTQQYQ’ and ‘anti-i/4-IWPVN,’ amplified a 370 bp fragment in ‘Delicious’. This fragment was directly sequenced by dideoxy chain termination on an ABI PRISM™ 377 DNA sequencer (Perkin-Elmer Co., Beverly, MA) using dRhodamine Terminator Cycle Sequencing Kits (Perkin-Elmer Co.). A primer ‘PSNKNGP’ (nucleotides 209-228 in Fig. 3) from the 370 bp fragment (Table 1) was used as the gene-specific primer for rapid amplification of cDNA 3’ ends (3’ RACE) (Frohman et al., 1988).

Total RNAs from mature pistils of ‘Delicious’ were extracted essentially as described by Chang et al. (1993). Reverse transcription (RT) was performed in a total volume of 20 µL containing 1 µL (ca. 1.0 µg) of RNA, 0.125 µM Oligo dT-Adaptor Primer (Takara Shuzo Co., Kusatsu, Shiga, Japan), 0.25 U AMV Reverse Transcriptase XL (Takara Shuzo Co.), 20 U RNase Inhibitor (Takara Shuzo Co.), 1 mM dNTPs, 5 mM MgCl₂, and 1× RNA PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl). The analysis was programmed in a thermal cycler (GeneAmp 2400 apparatus; Perkin-Elmer Co.) and conducted under the following conditions: 60 min at 42 °C, 5 min at 99 °C, and cooling to 4 °C.

Primers ‘PSNKNGP’ and M13 Primer M4 (Takara Shuzo Co.), a primer complementary to the adaptor region of the oligo dT-Adaptor primer, were used for 3’ RACE. PCR was carried out in a total volume of 100 µL containing 20 µL of the RT reaction mixture,
0.2 μM ‘PSKNPG’ primer, 0.2 μM M13 Primer M4, 2.5 mM MgCl₂, 1x RNA PCR buffer, and 2.5 U Taq DNA polymerase (TaKaRa Taq™, Takara Shuzo Co.). The amplification program consisted of 2 min at 94 °C for initial denaturation, 45 cycles of 30 s at 94 °C, 30 s at 60 °C, 1.5 min at 72 °C, and a final extension of 7 min at 72 °C.

Three cDNA 3′ end clones named Se3′-2, Se3′-5 and Se3′-8 were obtained.

To obtain the 5′ ends of the cDNA 3′ end clones, PCR was conducted as described above using the primers ‘Se-sense 2’ (Matsumoto et al., 1999b) and M13 Primer M4. Three cDNA clones, named Se5′-1, Se5′-6 and Se5′-9, corresponding to the cDNA 5′ end clones, were obtained. PCR products were subeloned into the pCRII cloning vector. The nucleotide sequences of all the clones were determined as described above.

Se-allele specific PCR analysis. PCR was conducted using the ‘Se-sense’ (nucleotides 655–674 in Fig. 3) and ‘Se-antisense’ (nucleotides 448–467 in Fig. 3) primers (Table 1), for Se allele identification. Each 35 μL PCR mixture contained 1.0 μM of each primer, 200 μM deoxynucleotides, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 2.5 U Taq polymerase, and 50 ng template DNA. Amplification was performed in a GeneAmp 2400 thermal cycler under the following conditions: 1) 3 min at 94 °C for denaturation; 2) 1 min at 94 °C; 3) 1 min at 60 °C; and 4) 1 min at 72 °C for 30 cycles, followed by a final extension for 10 min at 72 °C. After PCR, the amplified fragments were digested by KpnI. Amplified and digested fragments were separated on a 4% polyacrylamide gel in TAE at 50–70 V for 1–1.5 h.

Results and Discussion

The PCR-based method for identifying the S-alleles in the Asian pear was developed by Ishimizu et al. (1999). This method can be used with cultivars having two S-alleles, including a new S-allele, because the amino acid sequences ‘FTQQYQ’ and ‘anti-IIWPNV’, which are conserved among rosaceous S-RNases, are used as the primers (Table 1). These sequences are well conserved not only in Asian pear but also in apple S-RNases. The nucleotide sequence of the S2-allele encoding S2-RNase corresponding to one of the oligonucleotide primers, ‘anti-IIWPNV,’ shows mismatches at 2 positions (ACGTTGACACATCAATT). We designed a new primer, ‘anti-1/2IWPNV’ (Table 1), with one mismatch or none for all apple S-alleles, including the S2-allele. From the length and sequence of apple S-alleles encoding S-RNases with introns, 349 bp for S2, ca. 1300 bp for S3 and S5, 320 bp for S7, 347 bp for S9, 538 bp for Sf and 512 bp for Sg, should be amplified by the primers FTQQYQ and ‘anti-1/2IWPNV’ (Table 1). As shown in Fig. 1A, bands were amplified from ‘Golden Delicious’ (S2S3), ‘Sansa’ (S3S7), ‘Fuji’ (S9S5) and ‘Meku 10’ (S3S7). Bands of ca. 350 bp and ca. 500–550 bp were amplified from ‘Baskatong’ (S26S27) and ‘Worcester Pearmain’ (S2S6), respectively (Fig. 1A). The length and sequence of the introns of the S-, S2- and S27-alleles are unknown. To confirm that the bands were from the S-alleles encoding S-RNases, they were digested by restriction enzymes. From the sequence data of coding regions of the S-alleles, a 55 bp fragment should be observed in Sf and S27, but not S2 and S26, by BamHI digestion. However, a 153 bp fragment should be observed at S26, but not S27, by NdeI digestion. A fragment close to 55 bp was detected from ‘Worcester Pearmain’ (S2S6), and a fragment close to 153 bp was detected from ‘Baskatong’ (S26S27), suggesting that the S- and S26-allele were amplified by the primers (Fig. 1B). From the lengths of the amplified fragments, nine S-alleles were divided into five groups; 347–350 bp of S2, S9 and S26, ca. 1300 bp of S3 and S5, ca. 320 bp of Sf, 538–540 bp of Sf and S27, and 512 bp of S9.

As all known S-alleles except the S27-allele in apple were amplified by the primers, we tried to amplify the unknown S-alleles encoding S-RNases within ‘Delicious’, ‘McIntosh’ and ‘Vista Bella’. The S-alleles in ‘McIntosh’ are unknown, and one of the S-alleles in ‘Vista Bella’ is ‘Delicious’ is known. Although no clear specific bands were obtained from ‘McIntosh’ and ‘Vista Bella’ except for a 540 bp fragment from the Sh allele in ‘Vista Bella’, a 370 bp fragment, which does not correspond to any apple S-alleles, was detected in ‘Delicious’ (Fig. 2), its progeny ‘Orei’ (‘Golden Delicious’ × ‘Delicious’), and its sport ‘Starking Delicious’, but was not detected in other S-genotype cultivars, suggesting that it was from an unknown S-allele within ‘Delicious’ (Fig. 1, 2). Previously, we identified the S-allele genotype of ‘Delicious’ as S9 by the PCR-RFLP method (Matsumoto et al., 1999a). Thus, its S-genotype appears to be S9S9 or a combination of S9 and an unknown S-allele. Since no S9-allele of ‘Orei’ was identified by S-allele-specific PCR-RFLP analysis (data not shown), the S-allele of ‘Delicious’ must be a combination of S9 and an unknown S-allele. As the S-allele genotype of ‘Delicious’ was identified as Scr=S9S as through pollination and progeny analyses.

Fig. 1. Analysis of PCR fragments from six S-genotype-known apple cultivars: (A) Golden Delicious (S2S3) (lane 1), Sansa (S3S7) (lane 2), Fuji (S9S5) (lane 3), Meku 10 (S3S7) (lane 4), Worcester Pearmain (S2S6) (lane 5), and Baskatong (S26S27) (lane 6). Analyses of the PCR fragment digests with restriction endonucleases: (B) BamHI digestion of the fragment of lane 5 in Fig. 1A (lane 7), BamHI digestion of the fragment of lane 6 in Fig. 1A (lane 8), and NdeI digestion of the fragment of lane 6 in Fig. 1A (lane 9).
Komori et al. (1996), we classified the unknown S-allele as Se-allele. To determine whether the 370 bp band is a part of a Se-RNase or not, we determined its sequence directly. The deduced amino acid sequence of the fragment shows 65.7% to 77.6% similarities with other apple S-alleles encoding S-RNases, and contains the C2 domain 'LFTVHGLWP' at a corresponding site. Moreover, it contains an intron (169 bp) deduced from the presence of plant 5' and 3' splice-site consensus sequences, and is comparable in location to those of apple S-alleles.

The Se-RNase cDNA clones were obtained and sequenced based on the sequence data of the genomic DNA (Fig. 3). Nucleotides 116–316 in Fig. 3 were completely matched with those of the 370 bp genomic fragment except for a deduced intron sequence. The nucleotide sequences of the cDNA clones were the same except for the site of the poly(A) addition. The cDNA insert (878 bp) contains 681 bp of coding region encoding 227 amino acids. In apple, S2-, S3-, Sd-, S9-, Sf-, Sg-, Sh-, S24-, S26-, and S27-RNase each code for 226–228 amino acids. The putative initiation codon ATG at position 20–22 is preceded by a stop codon TAA at position 8–10 in frame. The sequence surrounding the first ATG codon (ATTCAATG) was well-conserved in the cDNAs of the apple S-alleles. The terminat

Fig. 2. Analysis of PCR fragments from five apple cultivars possessing unknown S-RNases: 'Delicious' (lane 1), 'Starking Delicious' (lane 2), 'Orei' (lane 3), 'McIntosh' (lane 4), and 'Vista Bella' (lane 5).

Fig. 3. Nucleotide and deduced amino acid sequences of the Se-RNase cDNA from 'Delicious' apple. Asterisks indicate stop codon. The site of the intron is shown by a triangle, and the sites of poly (A) additions by diamonds. The sequence of the Se-RNase cDNA was deposited under the DDBJ accession number AB035273.

Fig. 4. Se-allele specific PCR-RFLP analysis. PCR products obtained by using 'Delicious' (lane 1), 'Golden Delicious' (S2S3) (lane 3), 'Sansa' (S5S7) (lane 4), 'Fuji' (S9Sf) (lane 5), 'Meku 10' (S3Sh) (lane 6), 'Worcester Pearmain' (S2Sh) (lane 7) and 'Baskatong' (S26S27) (lane 8) genomic DNA as templates, and 'Se-sense' and 'Se-antisense' primers. The product of lane 1 was digested by KpnI (lane 2). We carried out PCR amplification, using 'Delicious' as a template. A PCR product very close to 227 bp was obtained (Fig. 4, lane 1). The fragment was specific for 'Delicious' and was not obtained from other S-genotype cultivars (Fig. 4, lane 3–8). In addition, the fragment was digested by KpnI (227 bp into 91 bp and 136 bp), which is a specific endonuclease for the PCR product of the Se-allele (Fig. 4, lane 2). Using this method, the Se-alleles of 'Holly,' 'Jonadel,' 'Melrose,' 'Orei,' 'Sekihikari' and 'Starking Delicious' were identified. We also identified the S2- and S9-allele of 'Holly,' 'Jonadel,' 'Melrose,' 'Orei' and 'Starking Delicious' using the S-allele specific PCR-RFLP analysis method described by Matsumoto et al. (1999a, b) (Table 2). S-genotypes of seven apple cultivars possessing the Se-allele were identified (Table 2). All of the S-genotypes except for 'Sekihikari' are the expected ones based on their parental makeup.

Literature Cited
Broothaerts, W., A.G.A. Janssens, P. Proost, and W.F. Broekaert. 1995. cDNA cloning and molecular analysis of two self-incompatibility alleles from apple. Plant Mol. Biol. 27:499–511.
Chang, S.J., P. Puryear, and J. Cairney. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rpt. 11:113–116.
Table 2. S-allele genotype of apple cultivars.

| Cultivar     | Parentage                        | S-allele genotype |
|--------------|----------------------------------|-------------------|
| Delicious    | Jonathan (S7S9) × Delicious       | S9S9e             |
| Holly        | Jonathan (S7S9) × Delicious       | S9S9e             |
| Jonadel      | Jonathan (S7S9) × Delicious       | S9S9e             |
| Melrose      | Jonathan (S5S5) × Delicious       | S5S5e             |
| Orei         | Golden Delicious (S2S3) × Delicious| S2S3e             |
| Sekihikari   | Indo (S7Sg) × Richared Delicious  | Sg                |
| Starking Delicious | sport of Delicious | S9S9e             |

Katoh, N., K. Yamada, A. Kasai, M. Senda, K. Miyairi, and T. Okuno. 1997. cDNA cloning of apple S-RNase. Jpn. Soc. Biosci. Biotech. Agrochem. 71 (Suppl.):236.

Kitahara, K., H. Fukui, J. Soejima, and S. Matsumoto. 1999. Cloning and sequencing of a new S-gene ‘Sg-RNase’ (Accession No. AB019184) from Malus ×domestica Borkh. ‘Indo’. (PGR99-046) Plant Physiol. 119:1567.

Kitahara, K., J. Soejima, H. Komatsu, H. Fukui, and S. Matsumoto. 2000. Complete sequences of the S-genes, ‘Sd-’ and ‘Sh-RNase’ cDNA in apple. HortScience 35:712–715.

Sassa, H., T. Nishio, Y. Kowyama, H. Hirano, T. Koba, and H. Beaheshi. 1996. Self-incompatibility (S) alleles of the Rosaceae encode members of a distinct class of the T2/S ribonuclease superfamily. Mol. Gen. Genet. 250:547–557.

Thomas, M., S. Matsumoto, P. Cain, and N.S. Scott. 1993. Repetitive DNA of grapevine: Class present and sequences suitable for cultivar identification. Theor. Appl. Genet. 86:173–180.

Verdoodt, L., A. Van Haute, I.J. Goderis, K. De Witte, J. Keulemans, and W. Broothaerts. 1998. Use of the multi-allelic self-incompatibility gene in apple to assess homozygocity in shoots obtained through haploid induction. Theor. Appl. Genet. 96:294–300.