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Molecular characterization of *S*-RNase genes and *S*-genotypes in the Japanese apricot (*Prunus mume* Sieb. et Zucc.)

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Abstract Three partial *S*-RNase genes, MSRN-1, MSRN-2, and MSRN-3, in the Japanese apricot (*Prunus mume* Sieb. et Zucc.) were isolated from the three cultivars Nankou, Gyokuei, and Kairyouuchidaume, respectively. The structural characteristics revealed that *S*-RNase genes from the Japanese apricot were in the T2/SRNase-type *S*-RNase family with five conserved regions (C1, C2, C3, RC4, and C5) and one variable region (RHV) as reported in the other rosaceous plants. In the phylogenetic tree of T2/S SRNase-type RNases, three *S*-RNase genes of the Japanese apricot did not form a species-specific subgroup but the *Prunus* subfamily did. At least seven *S*-allelic genes were present in the Japanese apricot, and *S*-genotypes of six representative cultivars, including Nankou, Gyokuei, Kairyouuchidaume, Baigou, Kagajizou, and Oushuku were first established in this study as S_1S_7, S_2S_6, S_3S_4, S_3S_6, S_3S_6 and S_1S_5, respectively. An extended elucidation of the *S*-genotype would contribute to a more efficient breeding program of the Japanese apricot.

Keywords Gametophytic self-incompatibility · Japanese apricot · Molecular identification · *S*-genotype · *S*-RNase

Introduction

Major fruit trees cultivated in Japan, such as the apple (*Malus domestica* Borkh.), Japanese pear (*Pyrus pyrifolia* Nakai var. *culta* Nakai), and sweet cherry (*Prunus avium* L.), exhibit gametophytic self-incompatibility (GSI). This hampers economical cultivation of these fruits without pollination with suitably compatible pollen. Due to their importance, physiological, morphological, and genetic studies on self-incompatibility have been carried out extensively. The GSI is controlled by a single multiallelic gene, called the *S*-locus. The *S*-locus in the pistils produces basic glycoprotein with ribonuclease (*S*-RNase) activity in Rosaceae, Solanaceae, and Scrophulariaceae (Newbigin et al. 1993; Matton et al. 1994; Golz et al. 1995; Kao and McCubbin 1996), although it has been reported that the product of GSI is not *S*-RNase in *Papaver rhoeas* (Franklin-Tong et al.1991). The *S*-genotype of each cultivar needs to be determined in order to select a compatible pollinizer and to promote breeding. For these purposes, the *S*-genotypes of cultivars in the apple (Kobel et al. 1939; Komori et al. 1999), Japanese pear (Terami et al. 1946), almond (Kester et al. 1994), and sweet cherry (Knight 1969; Tehrani and Brown 1992) have been determined using pollination or pollen tube growth tests.

The Japanese apricot (*Prunus mume* Sieb. et Zucc.), which originated in southeastern China, is a major commercial fruit in Japan, and it exhibits GSI (Miyake et al. 1995; Yamane et al. 1998). However, it is difficult to apply the tests for controlled pollination or pollen tube growth that clearly determine the *S*-genotypes of the Japanese apricot because cold weather during flowering (February through March in Japan) disturbs reproducibility. Thus, the *S*-genotypes of the Japanese apricot remain uncertain. On the other hand, molecular analyses of self-incompatibility have been applied to the apple and Japanese pear, and the primal structures of *S*-RNase genes reported. The basic structural features of *S*-RNase genes in the apple and Japanese pear are similar to solanaceous *S*-RNases, which are called T2/SRNase-type *S*-RNases. The *S*-RNase genes of the apple and Japanese pear show a high homology, 62–69% at the amino acid level (Sassa et al. 1996). Recently, the *S*-RNase genes of almond and cherry were also isolated (Ushijima et al. 1998; Tao et al. 1999). They had the structural features of the

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T2/SRNase-type *S*-RNases in the active domain, but the arrangements of other regions were specific to the *Prunus* species, resulting in a low amino acid homology (25%–30%) when compared to the apple and Japanese pear in this region (Ushijima et al. 1998). Based on this sequence information, simple and convenient PCR-RFLP and PCR methods were developed to elucidate *S*-genotypes in apple (Janssens et al. 1995), Japanese pear (Ishimizu et al. 1999) and sweet cherry (Tao et al. 1999). More recently, Tao et al. (2000) reported a molecular marker linked to self-compatibility in the Japanese apricot. However, correlation between the *S*-RNase genes and *S*-genotypes of the Japanese apricot has not yet been established.

In this work, as a first step in a comprehensive study of self-incompatibility in the Japanese apricot, we isolated partial cDNA fragments of *S*-RNase genes from the Japanese apricot by RT-PCR, characterized the primal structural features, and determined the *S*-genotypes of representative Japanese apricot cultivars using the PCR method.

Materials and methods

Plant materials

Three cultivars, Nankou, Gyokuei, and Kairyouuchidaume, of the Japanese apricot were used for isolation of *S*-RNase genes. For determination of *S*-genotypes, six commercially important cultivars were used: Nankou, Gyokuei, Kairyouuchidaume, Baigou, Kagajizou, and Oushuku. All plant materials were cultivated at the Chiyoda experimental farm of the National Institute of Fruit Tree Science (Chiyoda, Ibaraki, Japan).

Isolation of DNA and RNA

Total DNA was extracted from young frozen leaves by the method of Dellaporta et al. (1983). The total RNA was isolated from young frozen leaf, stem and pistil tissue of Nankou, according to Ikoma et al. (1996).

Cloning and sequencing of cDNAs encoding *S*-RNase

cDNA was synthesized using total RNA from the balloon stage pistil taken from Nankou, Gyokuei, and Kairyouuchidaume using the SuperScript preamplification system (Gibco, Rockville, Md., USA). RT-PCR was conducted using a primer set composed of Pru-T2 and Pru-C5 (Tao et al. 1999), which were designed from the signal peptide sequence of *S*-RNase of sweet cherry and the fifth conserved regions of solanaceous *S*-RNase, respectively. The amplified fragments were cloned using a TA cloning kit (Stratagene, La Jolla, Calif., USA) and were sequenced using an automatic ABI377 DNA sequencer (Perkin-Elmer, Foster City, Calif., USA). Phylogenetic trees were constructed with the UPGMA program (GENETYX, SDC, Tokyo, Japan).

RNA blot analyses

Ten micrograms of total RNA from Nankou was electrophoresed on a 1.2% agarose gel containing 8% formaldehyde. It was then blotted on Hybond-N nylon membrane (Amersham, Little Chalfont, UK). The loading of equal amounts of RNA was checked by gel staining with ethidium bromide. The membrane was hybridized with an MSRN-1 cDNA insert labeled with a Dig-11-dUTP system supplied by the manufacturer (Roche, Tokyo, Japan). Hybridization was done at 50°C in a hybridization buffer containing 50% formamide, 2×SSC, 0.1% SDS, 0.1% lauroylsarcosine, and 2% blocking reagent. After the membrane was washed twice for 5 min with 2×SSC and 0.1% SDS at room temperature, and then twice for 15 min with 0.1×SSC and 0.1% SDS at 55°C, it was exposed to X-ray film (Fuji, Kanagawa, Japan).

PCR amplification of *S*-alleles

PCR was conducted using primers of Pru-C2, designed from the second conserved regions of solanaceous *S*-RNase (Tao et al. 1999), and Pru-C5 for *S*-allele typing of six cultivars. PCR was performed with 35 cycles of 94°C for 1 min, 51°C for 2 min, and 72°C for 2 min with initial denaturing at 94°C for 3 min and a final extension of 72°C for 10 min. The reaction mixture consisted of 160 μM Tris-HCl (pH 8.0), 800 μM of KCl, 40 μM of $MgCl_2$, 5 μM each of dNTPs, 5 μM of each primer, 1 ng total DNA, and 1 U Ampli*Taq* (TaKaRa LA *Taq* polymerase, TaKaRa Shuzo, Shiga, Japan) in a 20 μl reaction volume. Amplified fragments were separated on 1% agarose gel and visualized by ethidium bromide staining. Southern blot analyses of the six cultivars were carried out using amplified fragments after PCR. The separated PCR amplified fragments were blotted on Hybond-N$^+$ nylon membrane (Amersham). The membrane was hybridized with an MSRN-1 cDNA insert that had been labeled with a Dig-11-dUTP system supplied by the manufacturer (Roche). Hybridization was at 42°C in a buffer containing 50% formamide, 5×SSC, 7% SDS, 0.1% lauroylsarcosine, and a 2% blocking reagent. Then, the membrane was washed twice for 5 min with 2×SSC and 0.1% SDS at room temperature and twice again for 15 min with 1×SSC and 0.1% SDS at 55°C. It was then exposed to X-ray film (Fuji).

Results and discussion

RT-PCR was carried out to obtain the partial cDNA fragment of *S*-RNase from the Nankou, Gyokuei, and Kairyouuchidaume cultivars. Fragments of 549 bp (MSRN-1, accession number AB047100), 543 bp (MSRN-2, accession number AB047101) and 546 bp (MSRN-3, accession number AB047102) were obtained from Nankou, Gyokuei, and Kairyouuchidaume, respectively (Fig. 1). Nucleotide sequences of three cDNA clones indicated high homology (80%–90%) to the previously reported *S*-RNase genes of the almond (S_b, S_c, S_d; Ushijima et al. 1998) and sweet cherry (S_2, S_3, S_6; Tao et al. 1999). Alignment of the deduced amino acid sequence of three cDNA clones is shown along with the *S*-RNases from other fruit trees in Fig. 2. Although three cDNA clones lacked N- and C-terminal regions, they were in the T2/SRNase-type *S*-RNases with five conserved domains (C1, C2, C3, RC4, and C5) and a hypervariable region (RHV) common to *S*-RNase genes in Rosaceae (Ushijima et al. 1998). MSRN-1, MSRN-2, and MSRN-3 showed high identity (74%–85%) at the amino acid level. The amino acid identity of the RHV among the three clones ranged from 46% to 69%, indicating that the RHV was also highly variable in the Japanese apricot.

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              *************** * **** * *********     * ***************************************** ** ** * *** ***
MSRN-1      1 GGTTTTGCTTTCTTCGTGTGTTTCATTATGAGCACTGGCGATGGATCTTATGACTATTTTCAATTTGTGCAACAATGGCCACCAACTACCTGTAGA   96
MSRN-2      1 ...............T.T....A.G........-----.-.......................................G..C.A...C...   90
MSRN-3      1 ...............T.T....A.G........-----.-....................................G..G..C.A...C...   90
              _______________
              Pru - T2

              * *   * *        ***** ** ********* ******  ********************************* ************* ***
MSRN-1     97 G-TACGC-GG-G----AAATGCTCCAATCCCCGGCCAATACAAATCTTCACCATCCATGGCCTATGGCCAAGTAATTATTCAAACCCAACGACGCC  185
MSRN-2     91 .-.--T.--.A-A-CT..........A.........T......G..........................................T...  179
MSRN-3     91 .C.-...A..A.ACCT---.....T..A.........T......A...............................C............T...  182

              ***********  ***** ******     **** **  *  **** ***** *****  *  *** ******* * ******** ******** *
MSRN-1    186 CAGTAATTGCATTGGGTCGCAATTT-----AAGGAAAGCATGGTGTCCCCTCGATTGCG-ATCCAAACTGAAGAGATCTTGGCCCAACGTGGAAGG  275
MSRN-2    180 ...........A......A......GACGC....-..--.--..........A......A.-A.....................G........A.  269
MSRN-3    183 ...........AA.....A......GAGGC....-..--.--....A.....A.....-A......T.......T.G........G........A.  272

              * **** *******  *********** ************* **** ******* ********** **** **   ***** ************
MSRN-1    276 TAGCAATGATACAAGATTTTGGGAAGGTGAATGGAACAAACATGGTACATGTTCCCAACAGACACTTAACC-AATACCAATACTTCGAGCGATCCC  370
MSRN-2    270 .G....C.......A...........C.............C...........G.........C....-...T...................  364
MSRN-3    273 .G.............G..........C..................G.......G..............T..-TG.....T............T  367

              *  ** ****** *   **  * ********* * *************************** * * * ************* *** * ** ***
MSRN-1    371 A-CGAAATGTGGCACTTTC--ACAATATTACAAATATCCTTAAAAACGCTTCAATCGTACCACA-TCCGACACAAACATGGACCTACTCCGACATA  462
MSRN-2    365 .-A..C......-.-GG..GT...........G.G.........................-.G.G.T....................G..T...  456
MSRN-3    368 .TG..-.......-.-AA..CT.T.........G.G..............................-..................T...G.G......  459

              *** ** ******** ***  *** *** ********* ********** * *  * ******* ***********************
MSRN-1    463 GTATCAACCATTAAAGCAGTAACTCAAACAACACCCCTTCTTCGTTGCA-A-ACAGCATAAGAAGACTCAGTTGTTACATGAAGTGGTA       549
MSRN-2    457 ...G..C........A...CT...A...G.........C..........A.T.-C.-...............................       543
MSRN-3    460 ......C........A...C....A...G.........C..........G.C.-G.-.......T.......................       546
                                                                                     _______________________
                                                                                     Pru - C5
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Fig. 1 Nucleotide sequence of MSRN-1, MSRN-2, and MSRN-3 from Nankou, Gyokuei, and Kairyouuchidaume cultivars, respectively. Gaps are marked by *dashes*. *Dots* indicate nucleotides identical to MSRN-1. *Asterisks* indicate conserved nucleotides. Primer sequences are *underlined*

A phylogenetic tree was constructed using the UPGMA method based on the amino acid alignment of rosaceous *S*-RNase genes, using S_2, S_3, and S_6 of tobacco (*Nicotiana alata*) as an outgroup (Fig. 3). Rosaceous *S*-RNase genes were comparatively diverged and classified into two subfamilies: the apple and Japanese pear subfamily and the *Prunus* subfamily. S_2, S_3, and S_6 of tobacco were in a different location than in the Rosaceous *S*-RNase group. MSRN-1, MSRN-2, and MSRN-3 also belonged to the *Prunus* subfamily, but they did not cluster in the same branch; instead, they clustered with the *S*-RNase genes of the almond and sweet cherry: MSRN-1 was placed next to cherry S_3, MSRN-2 next to cherry S_2, and MSRN-3 also close to cherry S_2. Ushijima et al. (1998) reported that while rosaceous *S*-RNase genes did not form species-specific subgroups, they did form subfamily-specific subgroups. Therefore, they speculated that *S*-RNase genes of Rosaceae diverged soon after the divergence of subfamilies and before the divergence of species. The hypothesis on their proposal was observed in the case of the Japanese apricot.

Expression analysis of MSRN-1 showed spatial expression only in the pistil tissue, and not in the leaf or stem by northern blot analysis (data not shown). It has been demonstrated that mRNA corresponding to the *S*-RNase gene accumulates in an organ-specific manner, where it functions only in pistils and not in other tissues of the apple (Broothaerts et al. 1995), Japanese pear (Sassa et al. 1993) and sweet cherry (Tao et al. 1999). The expression pattern of MSRN-1 coincided with that of the other T2/SRNase-type *S*-RNase in Rosaceae, indicating that the expression of *S*-RNase genes was highly regulated.

It has been reported that genotypes of *S*-RNase alleles in cultivars of sweet cherry can be determined easily by the PCR method using the primers of Pru-C2 and Pru-C4R designed from the fourth conserved region of *Prunus S*-RNase (Tao et al. 1999). Recently, Tao et al. (2000) showed the molecular marker for the discrimination of the Japanese apricot self-compatibility using Pru-C2 and Pru-C4R primers. However, they could not elucidate *S*-genotypes of the Japanese apricot because of the confusing PCR banding patterns on the agarose gel and the lack of information about the controlled pollina-

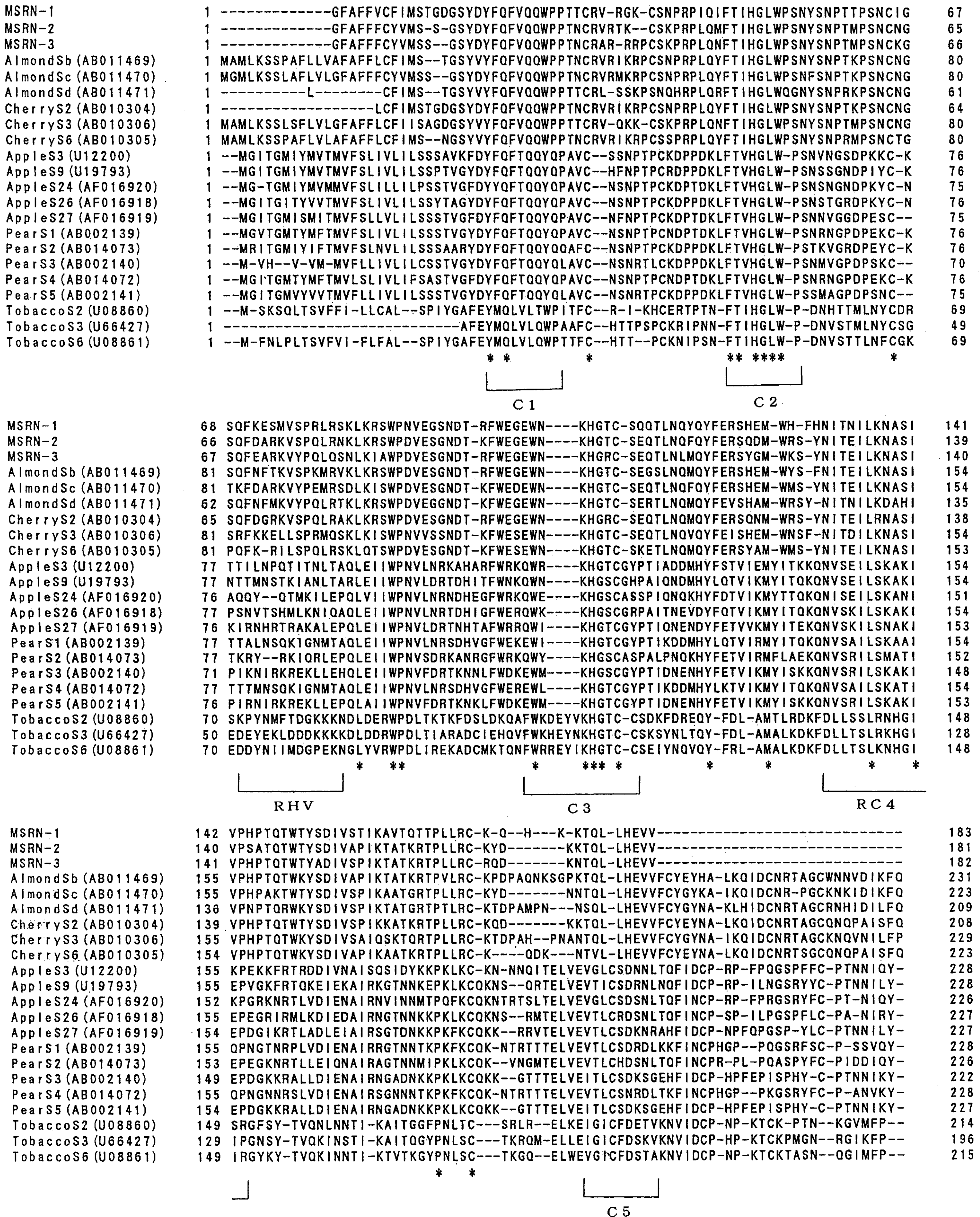

Fig. 2 Comparison of amino acid sequence alignments of *S*-RNases from Japanese apricot (MSRN-1, MSRN-2, and MSRN-3) and *S*-RNases from other fruit trees. Gaps are marked by *dashes*. *Asterisks* indicate identical polypeptides. Letters in *parentheses* show the accession number in DDBJ

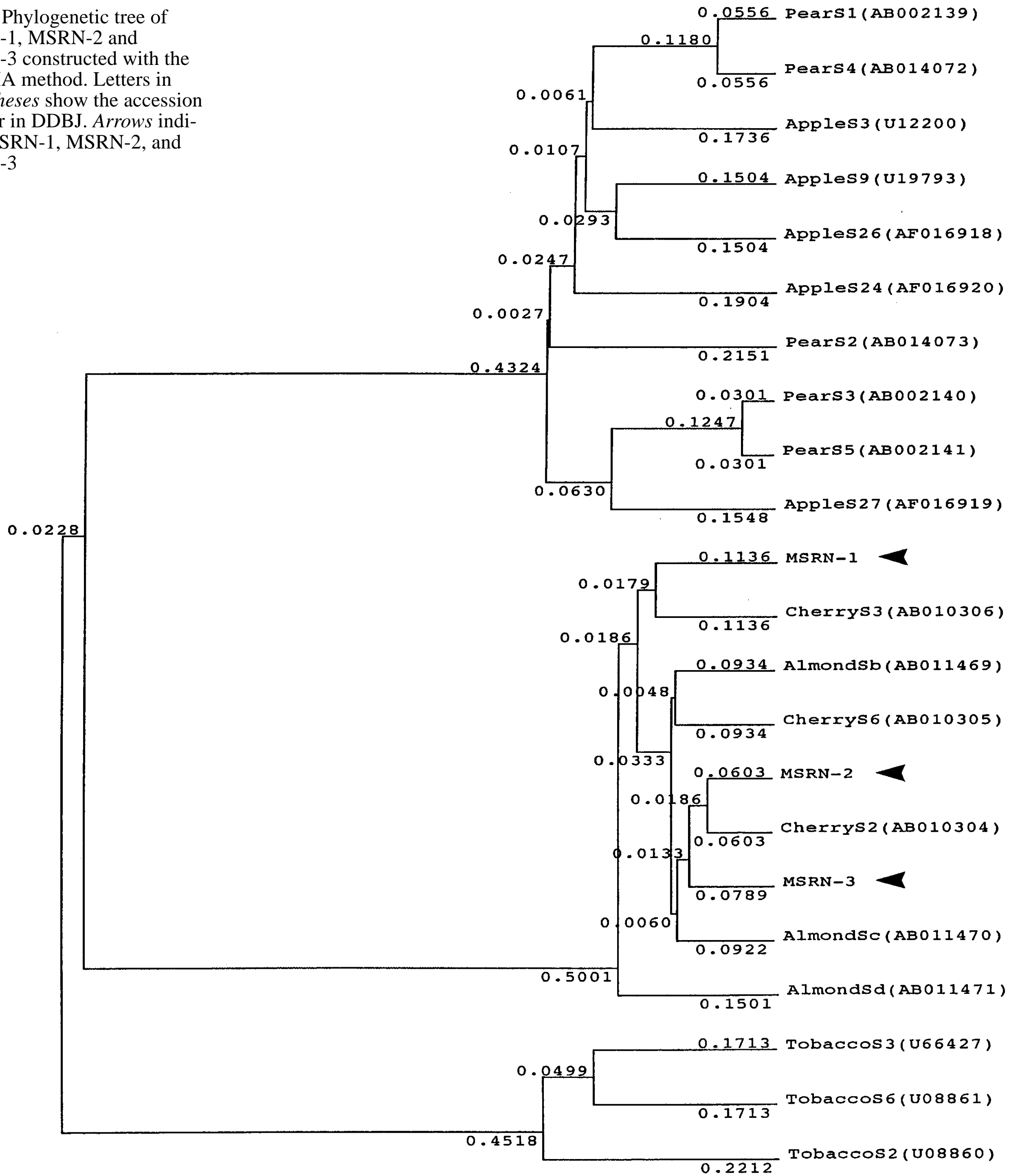

Fig. 3 Phylogenetic tree of MSRN-1, MSRN-2 and MSRN-3 constructed with the UPGMA method. Letters in *parentheses* show the accession number in DDBJ. *Arrows* indicate MSRN-1, MSRN-2, and MSRN-3

tion test. So, we also applied Pru-C2 and Pru-C4R primers to elucidate genotypes of *S*-allele in the representative six cultivars (Nankou, Gyokuei, Kairyouuchidaume, Baigou, Kagajizou, and Oushuku) of the Japanese apricot. We, too, were unable to detect a second fragment in Gyokuei, Baigou, or Kagajizou with these primers. However, when we applied Pru-C2 and Pru-C5 primers, two fragments were observed in each cultivar, and some of them showed polymorphism. As a result, seven differently sized fragments were obtained from the six cultivars (Fig. 4). As a result of the Southern blot analysis after PCR using MSRN-1 as a probe, all seven fragments hybridized with MSRN-1 (date not shown), indicating that they encoded *S*-RNase genes. Although we could not discriminate three cDNA clones (MSRN-1, MSRN-2, and MSRN-3) by their sizes (543 bp–549 bp), the polymorphism length of the *S*-alleles was obtained successfully by PCR. This was probably due to the intron insertion. The seven fragments obtained in Southern blot analysis after PCR were tentatively designated as S_1-S_7 according to their molecular weight: that is, S_1 was a 0.4-kbp fragment, S_2 (0.7 kbp), S_3 (0.9 kbp), S_4 (1.0 kbp), S_5 (1.3 kbp), S_6 (1.4 kbp), and S_7 (1.5 kbp). The established *S*-alleles enabled us to determine the ge-

Table 1 Percentage of fruit set by controlled pollination test between six cultivars

	♀ Predicted S-genotype	Oushuku S_1S_5	Nankou S_1S_7	Gyokuei S_2S_6	Kairyouuchidaume S_3S_4	Baigou S_3S_6	Kagajizou S_3S_6
♂	Predicted S-genotype						
Oushuku	S_1S_5	0	45.1	–[b]	–	–	–
Nankou	S_1S_7	46.9	0	21.0	–	15.0	38.0
Gyokuei[a]	S_2S_6	m-s	m-s	m-s	m-s	m-s	m-s
Kairyouuchidaume	S_3S_4	–	–	48.3	0	–	27.8
Baigou	S_3S_6	19.5	–	13.0	–	0	1.6
Kagajizou[a]	S_3S_6	m-s	m-s	m-s	m-s	m-s	m-s

[a] Both cultivars show male-sterility (m-s) and cannot be used as pollen parent
[b] Not carried out

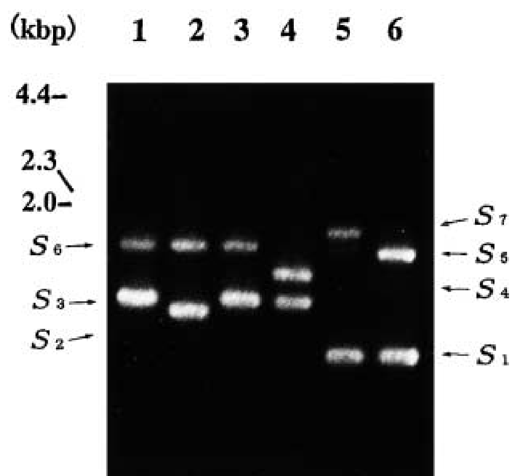

Fig. 4 PCR using Pru-C2 and Pru-C5 primers. PCR products were stained by ethidium bromide and tentatively designated as S_1-S_7 (*arrows*) according to their molecular weight. Size markers are indicated on the *left side*. Lane *1*: Baigou (S_3S_6), Lane *2*: Gyokuei (S_2S_6), Lane *3*: Kagajizou (S_3S_6), Lane *4*: Kairyouuchidaume (S_3S_4), Lane *5*: Nankou (S_1S_7), and Lane *6*: Oushuku (S_1S_5)

notypes of the six cultivars. The expected genotypes of each cultivar are: Nankou (S_1S_7), Gyokuei (S_2S_6), Kairyouuchidaume (S_3S_4), Baigou (S_3S_6), Kagajizou (S_3S_6), and Oushuku (S_1S_5).

Elucidation of the *S*-genotypes was confirmed by a pollination test using the six cultivars shown in Table 1. Combinations resulting in 5% or less seed set have been considered incompatible. (Since Gyokuei and Kagajizou show male-sterility, these cultivars cannot be used as pollen parents.) In this controlled pollination test, it would be expected that combinations between self- and the same *S*-genotype (in this paper, Kagajizou×Baigou) show a low percentage of fruit set. Indeed, all self-combinations showed 0% fruit set and the combination Kagajizou×Baigou had only 1.6% fruit set. Other combinations analyzed showed 13.0% to 48.3% fruit set; therefore, we determined that they were compatible. Thus, cross-incompatibility was confirmed on the basis of pollination tests as well as by examinations at the molecular level.

In conclusion, we isolated the partial *S*-RNase genes in the Japanese apricot and showed that at least seven alleles of the *S*-gene were present in the Japanese apricot genome. We described the *S*-genotypes of six representative Japanese apricot cultivars using the PCR method. Elucidation of the *S*-genotype is expected to contribute to improved efficiency in the breeding program of the Japanese apricot.

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