Molecular Markers for Self-compatibility in Japanese Apricot (*Prunus mume*)

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Abstract. Self-compatible cultivars of Japanese apricot (*Prunus mume* Sieb. et Zucc.) have a horticultural advantage over self-incompatible ones because no pollinizer is required. Self-incompatibility is gametophytic, as in other *Prunus* species. We searched for molecular markers to identify self-compatible cultivars based on the information about *S*-ribonucleases (*S*-RNases) of other *Prunus* species. Total DNA isolated from five self-incompatible and six self-compatible cultivars were PCR-amplified by oligonucleotide primers designed from conserved regions of *Prunus S*-RNases. Self-compatible cultivars exhibited a common band of ≈1.5 kbp. Self-compatible cultivars also showed a common band of ≈12.1 kbp when genomic DNA digested with *Hin*dIII was probed with the cDNA encoding S^2-RNase of sweet cherry (*Prunus avium* L.). These results suggest that self-compatible cultivars of Japanese apricot have a common *S*-RNase allele that can be used as a molecular marker for self-compatibility.

Japanese apricot, a species closely related to apricot (*Prunus armeniaca* L.) and Japanese plum (*P. salicina* Lindl.), exhibits gametophytic self-incompatibility, as do other self-incompatible crops in the Rosaceae (Janick and Moore, 1975; Miyake et al., 1995). Although both self-incompatible and self-compatible Japanese apricots are grown commercially in Japan, self-compatible cultivars have a horticultural advantage because no cross-pollinizer is required. Because Japanese apricot blooms very early in the spring, pollinating insects are not very active. In cooler areas of Japan, spring temperature and wind conditions are often very unfavorable to insects and only self-compatible cultivars can be grown commercially. These cultivars set fruit in the absence of insect-mediated pollen transfer. Consequently, one of the major breeding goals for Japanese apricot is to produce self-compatible cultivars.

Assessment of self-incompatibility, as determined by pollination and pollen tube growth tests, requires several years after the tree reaches the flowering age. The results obtained can be ambiguous because they are affected by environmental and cultural conditions. Molecular markers, such as restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR)-based markers for self-incompatibility, would enable identification of self-compatibility of juvenile seedlings and reduce the labor and time required for breeding.

Recently, *S*-RNase has been shown to be involved in the gametophytic self-incompatiblity of *Prunus* species such as almond [*P. dulcis* (Mill.) D.A. Webb] (Tao et al., 1997; Ushijima et al., 1998), sweet cherry (Tao et al., 1999a, 1999b), and Japanese plum (Yamane et al., 1999), and has also been reported in *Malus* and *Pyrus* (Sassa et al., 1992, 1996, 1997). For these fruit tree species, cDNAs encoding *S*-RNases have been cloned and molecular typing systems for *S*-alleles established (Ishimizu et al., 1999; Jansens et al., 1995; Tamura et al., 2000; Tao et al., 1999a, 1999b). Furthermore, possibilities exist for marker-assisted selection of self-compatible offspring of Japanese pear (*Pyrus serotina* Rehd.) (Sassa et al., 1997), sweet cherry (Tao et al., 1999b), and almond (Boskovic et al., 1999), based on the molecular and biochemical markers for *S*-alleles. In this study, using the information obtained about *S*-RNases of other *Prunus* species, we searched for molecular markers which identify self-compatible cultivars of Japanese apricot.

Materials and Methods

DNA isolation. Total DNA was isolated from young leaves by the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) using five self-incompatible ('Kotsubu-nanko', 'Oushuku', 'Nanko', 'Baigo', and 'Kairyo-uchida') and six self-compatible ('Orihime', 'Kensaki', 'Jizo-ume', 'Hachiro', 'Benisashi', and 'Ryukyo-koume') cultivars of Japanese apricot. After purifying the isolated DNA with polyethlylene glycol (PEG) precipitation (Mak and Ho, 1993), DNA was used for PCR and genomic DNA blot analyses.

PCR analysis. PCR analysis of *S*-alleles was performed with the *S*-RNase gene-specific oligonucleotide primers, Pru-C2 (5′-CTA TGG CCA AGT AAT TAT TCA AAC C-3′) and Pru-C4R (5′-GGA TGT GGT ACG ATT GAA GCG-3′). These primers were designed from the second and the fourth conserved regions, C2 and RC4, of the *Prunus S*-RNase, respectively (Tao et al., 1999a, 1999b; Ushijima et al., 1998). PCR was performed using a program of 35 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min with an initial denaturing of 94 °C for 3 min and a final extension of 72 °C for 7 min. The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 200 μM each of dNTPs, 400 nM each of primers, 50 ng of template DNA, and 1 unit of TaKaRa Ex Taq polymerase (Takara Shuzo Co., Shiga, Japan) in a 50-μL reaction volume. The PCR mixture was subjected to electrophoresis on a 1% agarose gel and DNA bands were visualized by ethidium bromide staining.

Genomic DNA blot analysis. Genomic DNA blot analysis was performed using PCR fragments from the cDNA encoding S^2-RNase of sweet cherry (Tao et al., 1999b). Five μg of total DNA was digested with *Hin*dIII and run on a 0.8% agarose gel. After electrophoresis, DNA in the gel was transferred to a nylon membrane (Hybond-N, Amersham, Tokyo), and probed with the C2-C5 fragment of the cDNA that was PCR-labeled by digoxigenin (DIG)-dUTP with the Pru-C2 and Pru-C5 primers (5′-TAC CAC TTC ATG TAA CAA CTG AG-3′). The Pru-C5 primer was designed from the fifth conserved region of *Prunus S*-RNase (Tao et al., 1999b; Ushijima et al., 1998). After low stringency washes (2 × 5 min at room temperature with 5 × SSC and 0.1% SDS followed by 2 × 15 min at 60 °C with 0.5 × SSC and 0.1% SDS), immunological detection of the hybridization was carried out using the anti-DIG-alkaline phosphatase conjugate and the chemiluminescent substrate CSPD™ (Boehringer Mannheim, Tokyo). Chemiluminescence was documented on X-ray film.

Results and Discussion

PCR analysis. As with *S*-RNases of other *Prunus* species (Tao et al., 1999a, 1999b; Ushijima et al., 1998; Yamane et al., 1999), the C2 and RC4 regions of the *S*-RNase of Japanese apricot seemed to be conserved, and all 11 cultivars tested yielded one or two bands

by PCR with the Pru-C2 and Pru-C4R primers (Fig. 1). Among the amplification products obtained, a band of 1.5 kbp is of particular interest. All six self-compatible cultivars yielded this band, but none of the five self-incompatible cultivars did so. Therefore, the band of 1.5 kbp could be a good molecular marker for self-compatibility in Japanese apricot. The shortest band obtained among the PCR products from the 11 cultivars was ≈360 bp which is longer than the expected size from the coding sequences of the S-RNase genes of other *Prunus* species, such as almond (Ushijima et al., 1998), sweet cherry (Tao et al., 1999a, 1999b), and Japanese plum (Yamane et al., 1999). This indicated the existence of some introns in the hypervariable (HV) region located between the Pru-C2 and Pru-C4R sequences of putative S-RNase genes. Since all the S-RNase genes reported so far for Rosaceae and Solanaceae contain an intron in the HV region (Chung et al., 1995; Matton et al., 1995; Tamura et al., 2000; Tao et al., 1999a), the insertion of introns in the HV region may be a common feature in S-RNase genes. As HV regions of S-RNases in Solanaceae are suspected to be responsible for the determination of the self-recognition specificity (Matton et al., 1995), the fact that a HV region includes the insertion of an intron that varies in size with different S-alleles is of interest.

Genomic DNA blot analysis. Hybridization signals with the cDNA encoding S^2-RNase of sweet cherry were observed with all 11 cultivars tested (Fig. 2). The number of bands hybridized with the cDNA varied from one to three depending on the cultivar. As with the PCR analysis, self-compatible cultivars produced a common band. Since the hybridization signal at 12.1 kbp only was observed with self-compatible cultivars, it can be used as a molecular marker for self-compatibility. Although there was a strong correlation between the PCR band of 1.5 kbp and the hybridization signal of 12.1 kbp, there was only a weak correspondence between other PCR bands and hybridization signals. For example, a PCR band of 360 bp was observed only with two cultivars Oushuku and Nanko, and seemed to correspond to a hybridization signal of ≈3.5 kbp; another cultivar, 'Benisashi', produced the same hybridization signal of 3.5 kbp. Similarly, a PCR band of ≈1.6 kbp observed in 'Hachiro' and 'Ryukyo-koume' seemed to correspond to the hybridization signal of 4.2 kbp, another cultivar, 'Kairyo-uchida', produced the same signal. Although flanking regions of S-RNase genes were reported to be highly divergent in Solanaceae (Chung et al., 1995; Matton et al., 1995), different S-alleles of sweet cherry produced the same hybridization signals (Tao et al., 1999b) as in this study for Japanese apricot. Perhaps the S-locus of *Prunus* is not as divergent in the flanking regions of the S-RNase gene as in those of the Solanaceae.

Molecular markers for self-incompatibility. As described above, with both the PCR and genomic DNA blot analyses, all self-compatible cultivars of Japanese apricot showed a distinct band that can be used as a molecular
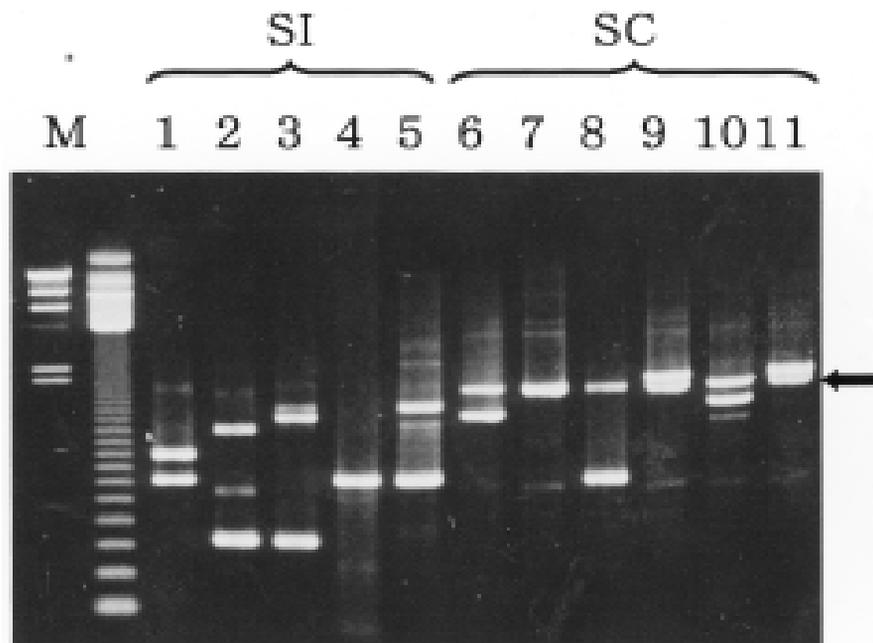

Fig. 1. PCR analysis of self-compatible (SC) and self-incompatible (SI) cultivars of Japanese apricot. Lane M (**left**), λ/*Hin*dIII marker; lane M (**right**), 123 bp ladder; lane 1, 'Kotsubu-nanko'; lane 2, 'Oushuku'; lane 3, 'Nanko'; lane 4, 'Baigo'; lane 5, 'Kairyo-uchida'; lane 6, 'Orihime'; lane 7, 'Kensaki'; lane 8, 'Jizo-ume'; lane 9, 'Hachiro'; lane 10, 'Benisashi'; and lane 11, 'Ryukyo-koume'. Arrow indicates a common band of 1.5 kbp observed in self-compatible cultivars.
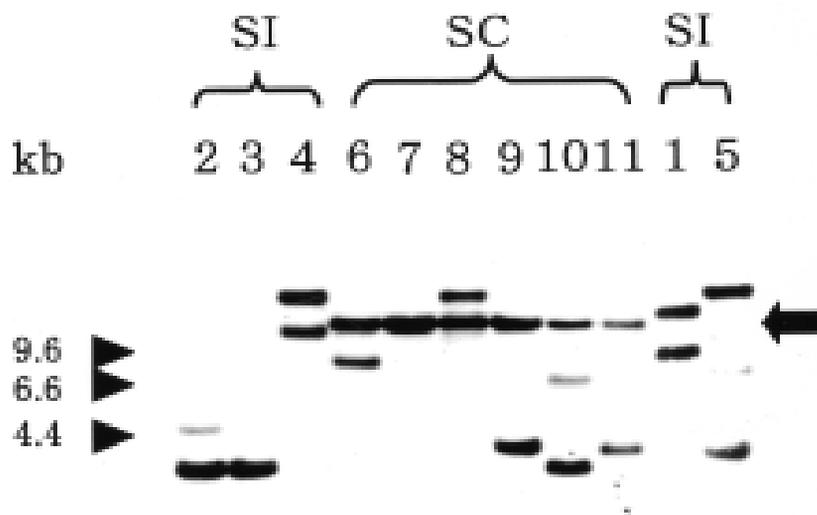

Fig. 2. Genomic DNA blot analysis of self-compatible (SC) and self-incompatible (SI) Japanese apricot cultivars. Total DNA was digested with *Hin*dIII and probed with the cDNA encoding S^2-RNase of sweet cherry. Lane numbers correspond to those in the legend for Fig. 1. Lane 1, 'Kotsubu-nanko'; lane 2, 'Oushuku'; lane 3, 'Nanko'; lane 4, 'Baigo'; lane 5, 'Kairyo-uchida'; lane 6, 'Orihime'; lane 7, 'Kensaki'; lane 8, 'Jizo-ume'; lane 9, 'Hachiro'; lane 10, 'Benisashi'; and lane 11, 'Ryukyo-koume'. Arrow indicates a common hybridization band observed in self-compatible cultivars.

marker for self-compatibility. We suggest that the common bands are from a mutated S-allele that confers self-compatibility. The mutated S-allele seems to be transmitted to the progenies because 'Hachiro' is from open-pollination of 'Jizo-ume' and both have the bands linked to the mutated S-allele. There are several explanations for the mode of mutation. First, a mutated S-RNase gene may code for inactive S-RNase with no RNase activity or the gene is not expressed at all. Kowyama et al. (1994) reported that the self-compatibility allele, S^c, of *Lycopersicon peruvianum* (L.) Mill encodes a defective protein that has no RNase activity. Also, a self-compatibility allele, S^c or S^f, of almond has codes for S-RNase with no RNase activity (Boskovic et al., 1999). The second explanation is that mutation may have occurred in a pollen component of S-allele that is tightly linked to the S-RNase gene. Golz et

al. (1999) suggested that the *S*-locus is bipartite, with different genes encoding the pollen component (pollen-*S*) and the style component (*S*-RNase). In *Nicotiana alata* Link et Otto, mutants with supposedly mutated pollen-*S* could be identified based on the *S*-RNase gene linked to the mutated pollen-*S* (Golz et al., 1999).

Although further studies are necessary to elucidate the nature of the mutation, clearly we have identified unique PCR and hybridization bands that may be linked to a mutated *S*-allele conferring self-compatibility in Japanese apricot. This finding should be useful in evaluating self-compatibility in this species.

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