Characterization and cDNA Cloning for $S_r$-RNase, a Molecular Marker for Self-compatibility, in Japanese Apricot (Prunus mume)

Ryutaro Ta*, Tsuyoshi Habu, Hisayo Yamane and Akira Sugiura
Laboratory of Pomology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502

Summary
Self-compatible cultivars of Japanese apricot (Prunus mume Sieb. et Zucc.) have a common $S$-RNase ($S_r$-RNase) gene that can be used as a molecular marker for self-compatibility. In this study, we further characterized $S_r$-RNase by comparing $S$-RNases of self-compatible 'Kensaki' ($S_rS_r$) and self-incompatible 'Nankou' ($S_rS_r$). cDNA libraries from the styles with stigmas of these two cultivars were constructed and cDNAs encoding $S_r$, $S_r^{-}$, and $S_r$-RNases were cloned. Deduced amino acid sequences from these cDNAs all contained two active domains of the T2/S type RNase family and five conserved regions of the rosaceous $S$-RNase. RNA blot analysis showed that the $S_r$-, $S_r^{-}$, and $S_r$-RNase genes were transcribed in the pistil but not in the leaf as with other $S$-RNase genes of Prunus. Furthermore, 2D-PAGE analysis revealed that $S_r$-RNase has molecular mass, isoelectric point, and immunological characteristics similar to other $S$-RNase. These results indicate that the pollen-$S$ gene that is supposedly tightly linked to the $S_r$-RNase gene may be responsible for the self-compatibility observed in the $S_r$-haplotype.

Key Words: gametophytic self-incompatibility, pollen-$S$, self-compatibility, $S$-haplotype, $S$-RNase

Introduction
Japanese apricot (Prunus mume Sieb. et Zucc.) exhibits the RNase-based gametophytic self-incompatibility system (Tao et al., 2000; Yaegaki et al., 2001), as do other Prunus fruit tree species (Tao et al., 1997, 1999; Usijima et al., 1998; Yamane et al. 1999). Although both self-incompatible and self-compatible cultivars are grown commercially in Japan, self-compatible cultivars have a horticultural advantage over self-incompatible cultivars because the former requires no cross-pollinizer. Consequently, one of the major breeding goals for Japanese apricot is to produce self-compatible cultivars of horticultural importance.

Recently, we found that $S$-RNase genes are present in the genomes of both self-compatible and self-incompatible cultivars of Japanese apricot, and that the self-compatible cultivars have a common $S$-RNase gene, namely $S_r$-RNase gene (Tao et al., 2000). Furthermore, cosegregation of the $S_r$-RNase gene and self-compatibility was confirmed by using progenies and selections from controlled crosses (Tao et al., 2002). To establish self-compatibility by pollination and pollen tube growth tests requires several years, as trees must outgrow its juvenile stage and flower. Hence, molecular analysis of $S_r$-RNase gene as a molecular marker would be an efficient method to hasten the breeding of self-compatible Japanese apricots.

A recent molecular analysis revealed that the $S$-locus of the Rosaceae is bipartite, with different genes encoding the stylar component ($S$-RNase) and the pollen component (pollen-$S$) (Sassa et al., 1997). These genes are very tightly linked to each other so that they behave as if they were a single gene. On the basis of this finding, the term "haplotype" has been adopted to denote variants of the locus while the term "allele" is assigned to variants of a given polymorphic gene at the $S$-locus (McCubbin and Kao, 2000). Thus, it is possible that the $S_r$-RNase gene itself or pollen-$S$ that is tightly linked to the $S_r$-RNase gene is dysfunctional so that the cultivars with the $S_r$-RNase gene become self-compatible.

Our goal is to elucidate the nature of the dysfunction of the $S_r$-haplotype and to utilize the knowledge obtained to breed self-compatible Japanese apricot cultivars. As a first step towards this goal, we characterize and compare the $S$-RNases of self-incompatible and self-compatible cultivars by cDNA cloning, RNA gel blot, and two-dimensional gel electrophoresis (2D-PAGE) analyses.

Materials and Methods
Plant materials
Styles with stigmas at the balloon stage of development and young leaves of two Japanese apricot cultivars, self-incompatible 'Nankou' and self-compat-

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*Corresponding author (E-mail: rtao@kais.kyoto-u.ac.jp).
ible ‘Kensaki’, were used in this study. The S- haplo-
types of ‘Nankou’ and ‘Kensaki’ have been determined
to be S_S (Yaeagaki et al., 2001) and S_S (Tao et al.,
2002), respectively. Plant materials of ‘Nankou’ were
obtained from the Horticulture Experiment Center of
Wakayama Research Center of Agriculture, Forestry and
Fisheries, and those of ‘Kensaki’ were obtained from the
Fuku Prefectural Horticultural Experiment Station.
They were collected, frozen with liquid N₂ immediately,
and stored at -80 °C until use.

Cloning of PCR-amplified fragments

Genomic DNAs of ‘Nankou’ and ‘Kensaki’ were
isolated, purified and used as a template for PCR with
the Pru-C2 and Pru-C4R primers. Sequences for the
Pru-C2 and Pru-C4R primers were designed based on
DNA sequences of the conserved regions C2 and C4 of
the Prunus S- RNases, respectively (Tao et al., 1999).
The C2-C4R fragments that correspond to S - and S -
RNase genes of ‘Nankou’ and S - RNase genes of
‘Kensaki’ were cloned to the TA cloning vector (pgEM
-T Easy Vector System; Promega, Madison, WI) and
sequenced by the ABI PRISM 310 Genetic Analyzer
(Applied Biosystem, Tokyo, Japan).

cDNA cloning

Total RNA was isolated from styles with stigmas at
the balloon stage of development by the cold-phenol
method as described by McClure et al. (1990) with
several minor modifications. Poly(A) RNA was puri-
fied from the total RNA by using Oligotex dT30 (Takara
Shuzo, Shiga, Japan). cDNA was synthesized from the
Poly(A) RNA, cloned into Uni-ZAP XR vector, and
packaged in vitro as described previously (Tao et al.,
1999). A portion of cDNA for S - RNase of sweet
cherry (P. avium) was PCR - labeled by digoxigenin
(DIG) -dUTP (Roche, Tokyo, Japan) with the Pru-C2 and
Pru-C5 primer set and used as a probe to screen the
cDNA library. The sequence for the Pru-C5 primer was

designed based on the DNA sequence of the conserved
region C5 of the Prunus S- RNases (Tao et al., 1999).
Selected clones from the cDNA library were converted
to pBluescript and DNA -sequenced by ABI PRISM 310
Genetic Analyzer (Applied Biosystems, Tokyo, Japan).
Deduced amino acid alignment was generated by CLUS-
TAL X (Thomson et al., 1997).

RNA gel blot analysis

Total RNA was isolated from the styles with stigmas
and young leaves of ‘Nankou’ and ‘Kensaki’ by the cold
-phenol method as described above. Ten µg of total
RNA was run on formaldehyde (1%) agarose gel and
blotted onto a nylon membrane (Hybond-N, Amersham
Pharmacia, Tokyo, Japan). Hybridization was performed
with the DIG - labeled cDNA fragment for S - RNase of
sweet cherry that was prepared as described above. After
high stringency washes of the membrane (2 x 5 min at
room temperature with 2 x SSC and 0.1% SDS followed
by 2 x 15 min at 68 °C with 0.1 x SSC and 0.1% SDS),
hybridization signals were detected by using the anti-
DIG-alkaline phosphatase conjugate and the chemilu-
minescent substrate CSPD (Roche, Tokyo, Japan).
Chemiluminescence was documented on X-ray films.

Protein assay

Acetone powder was prepared from the styles with
stigmas and crude extracts were prepared from the
acetone powder as previously described (Tao et al.,
1997). The crude extracts were subjected to 2D-PAGE
by using nonequilibrium pH gradient electrophoresis
(NEPHGE) for the first dimension and SDS-PAGE for
the second dimension as previously reported (Tao et al.,
1997). After electrophoresis, proteins in the gel were
detected by silver staining. In addition, proteins sepa-
rated by 2D-PAGE were electroblotted onto a PVDF
membrane as previously described (Tao et al., 1997).
Glycoprotein and S- RNase were detected with conca-
avalin A and the rabbit antiserum prepared against
purified S - RNase of almond (P. dulcis), respectively
(Tao et al., 1999; Ushijima et al., 2001). Furthermore,
portions of the PVDF membrane carrying the protein of
interest were cut out and subjected to a gas-phase
protein sequencer (476A, Applied Biosystem, Tokyo,
Japan) for N-terminal amino acid sequencing.

Results and Discussion

Cloning of PCR-amplified fragments and cDNAs for
S-, S-, and S-haplotypes

Several independent clones were sequenced for the
PCR-amplified fragments that correspond to S- and S-
RNase genes of ‘Nankou’ and S- RNase gene of
‘Kensaki’ (Tao et al., 2000; Yaeagaki et al., 2001). A
single intron within the hypervariable region of Rosae-
ceae, RHV, located between the Pru-C2 and Pru-C4R
primer sequences was found in these clones as with
other Prunus S- RNase genes (Tamura et al., 2000;
Yamane et al., 2000). Deduced amino acid sequences of
the putative coding sequences of PCR amplified frag-
ments resembled other Prunus S- RNases. cDNA
clones, corresponding to the PCR-amplified fragments,
and, thus, encoding S- and S- RNase of ‘Nankou’ and
S- RNase of ‘Kensaki’ were obtained from their stylar
cDNA library. The cDNAs for S-, S- and S- RNases
encode 224, 226 and 221 amino acids, respectively (Fig.
1). All deduced amino acid sequences from cDNA
clones contained two active domains of the T2/S type
RNases and five conserved regions and one hyperva-
riable region, RHV, of rosaceous S- RNases (Ushijima
et al., 1998). In addition, eight cystein residues and an N-
glycosylation site conserved among other Japanese
apricot and rosaceous S- RNases were present in the
deduced amino acid sequences of S-, S- and S-
RNases of Japanese apricot. Identities of amino acid
sequences between the three S-RNases of Japanese apricot and other Prunus S-RNases were 66% to 79% and within the range of amino acid sequence identity observed among Prunus S-RNases (Table 1). Yaegaki et al. (2001) reported the partial sequences for three S-RNase genes from 'Nankou', 'Gyokuei', and 'Kairyouchidaume'. A comparison of the DNA sequence of MSRN-1, a clone derived from 'Nankou', (Yaegaki et al., 2001) with the cDNA sequences for S₁ and S₂-RNases revealed that MSRN-1 encoded the partial sequence of the S₂-RNase gene.

**RNA blot analysis**

Hybridization signals at about 900 bp, which coincided with the estimated size from the cDNAs, were detected with the total RNA isolated from styles with stigmas of ‘Nankou’ and ‘Kensaki’ (Fig. 2). There was no difference in the intensity of the hybridization signals between ‘Nankou’ and ‘Kensaki’. No signal was observed for total RNA from young leaves as were reported with the expression patterns of other S-RNase genes (Tao et al., 1999; Yamane et al., 2000).

**Protein assay**

Major protein spots (Fig. 3) that had molecular weights and isoelectric points similar to those of S-RNases of other Prunus species such as almond (Tao et al., 1997), sweet cherry (Tao et al., 1999), and Japanese plum (P. salicina) (Yamane et al., 1999) were found in the 2D-PAGE profiles of ‘Nankou’ and ‘Kensaki’. These spots were found to be glycoproteins that contained sugar chains that reacted with concanavalin A and exhibited an immunoreaction with antiserum raised against S₁-RNase of almond. The putative S-RNase spot from ‘Nankou’ was divided into regions, high and low molecular weight; their N-terminal sequences were determined because the spot seemed to consist of two different proteins, possibly S₁ and S₂-RNases. The N-terminal sequence of the lower molecular weight region contained deduced amino acid sequences of the C1 region, the N-terminal region of mature protein, from cDNA for S₁-RNase (Fig. 1). The N-terminal sequence for the higher molecular weight region resembled the deduced amino acid sequence of the C1 region from cDNA for S₁-RNase but the exact sequence could not be determined because the higher molecular weight region contained more than one protein. As S-RNases are glycoproteins and their spots often tail when electrophoresed, the higher molecular weight region could

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**Table 1.** Amino acid sequence identities between S-RNases of Japanese apricot and other Prunus S-RNases (%).

<table>
<thead>
<tr>
<th></th>
<th>PM-Sf</th>
<th>PM-S1</th>
<th>PM-S7</th>
<th>MSR N2</th>
<th>MSR N3</th>
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<td>71</td>
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<td>72</td>
<td>66</td>
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<tr>
<td>PM-S7</td>
<td>71</td>
<td>75</td>
<td>-</td>
<td>72</td>
<td>66</td>
<td>67</td>
<td>67</td>
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</tr>
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*Designation of S-RNases in as Fig. 1.

**Fig. 2.** RNA blot analysis of total RNA from leaf (Lf) and style with stigma (St) from flower buds of Japanese apricot 'Nankou' and 'Kensaki' at the balloon stage of development. The blot was hybridized with the C2-C5 fragment of the cDNA for S₁-RNase of sweet cherry (A). The RNA gel before blotting was stained with ethidium bromide (B).
contain both S_7- and S_7- RNases. The N-terminal sequence of the putative S-RNase spot of ‘Kensaki’ consisted of a single protein and contained the deduced amino acid sequence from the C1 region of S_7- RNase.

Possible cause of self-compatibility observed with Sf-RNase

In deduced amino acid sequences, transcription and translation levels revealed by RNA blot and 2D-PAGE analyses, there was no distinct difference between the S_7- RNase of self-compatible ‘Kensaki’ and S_7- and S_7- RNases of self-incompatible ‘Nankou’. Although the RNase activity of S_7- RNase remains to be investigated, S_7- RNase has two active sites of T2/S type RNases and supposedly RNase activity. Recent molecular analysis revealed that the S-locus region of Rosaceae contains at least two different genes that encode the stylar component (S-RNase) and the pollen component (pollen-S) (Sassa et al., 1997). Deletion or mutation of either the style or pollen component could result in the breakdown of self-recognition system. An example of stylar part mutation is observed in ‘Osa-Nijisseiki’ of Japanese pear (Pyrus pyrifolia Nakai, synonym of Pyrus serotina Rehd.) (Sassa et al., 1997), whereas that of pollen part mutation is present in the sweet cherry ‘Stella’ (Boskovic et al., 2000; Tao et al., 1999). Since we could not find any distinct difference between S_7- RNase and S_7- and S_7- RNases that are involved in a self-incompatible reaction, it is most likely that the pollen-S gene, tightly linked to the S_7- RNase gene, may be responsible for the self-compatibility. The S_7- haplotype of Japanese apricot could be useful for identifying the pollen component of the S-RNase-based gametophytic self-incompatibility system.

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Tao, R., T. Habu, H. Yamane, A. Sugiuira and K. Iwamoto.
ウメ（Prunus mume）の自家和合性分子マーカーS-RNaseの特性調査とcDNAクローニング

田尾龍太郎・羽生 剛・山根久代・杉浦 明

京都大学大学院農学研究科 606-8502 京都市左京区北白川追分町

摘 要

ウメ（Prunus mume Sieb. et Zucc.）の多くの栽培品種は、他の多くのバラ科果樹類と同様にS-RNaseの関与する配偶体型の自家不和合性を示す。しかしながら、中には自家和合性の品種も存在し、これらの品種はいくつかの点で自家不和合性品種に比べて優れている。我々は、以前の研究で、ウメの自家和合性形質の分子マーカーとして利用可能なS-RNase遺伝子（S-RNase遺伝子）を見つけ出しました。今回は、自家和合性品種である「剣先（S₁S₃）」と自家不和合性品種の「南高（S₂S₄）」のS-RNaseを比較検討することでS₁-RNaseの性質を明らかにしようとして、以下の実験を行った。

「剣先（S₁S₃）」と「南高（S₂S₄）」の花柱からcDNAライブラリを構築し、S₁- S₄-およびS₂-RNaseをコードするcDNAをクローニングした。これらcDNAより推定されるS₁- S₄-およびS₂-RNaseのアミノ酸配列にはT2/S型RNase特有の2つの活性中心とバラ科植物のS-RNaseに共通してみられる5種類の保存領域が存在した。RNAプロットを行ったところ、ウメのS-RNase遺伝子は、他のPrunus属のS-RNase遺伝子と同様に葉では転写されておらず、花柱のみで転写されていることが明らかになった。また、「剣先のS₁-RNase遺伝子と「南高のS₂-RNase遺伝子の転写産物量の差異は見いだされなかった。花柱抽出汁の2次元電気泳動を行ったところ、S₁-RNaseは他のS-RNaseと同様の分子量と等電点をもつ。そして免疫学的特性を持つことが示された。本研究の結果は、ウメのS₁-RNaseの個体の示す自家和合性はS₁-RNase遺伝子に強く関連したpollen-S遺伝子の作用によって生じている可能性を示唆するものであろう。