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Characterization of the *S-RNase* genomic DNA allele sequence in *Prunus speciosa* and *P. pseudocerasus*

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ABSTRACT

In this study, eight *S-RNase* alleles were isolated from two *Prunus pseudocerasus* and two *Prunus speciosa* accessions by PCR amplification from genomic DNA. Analysis of the amino acid sequences revealed five novel and three published *S*-alleles. These *S-RNases* share typical structural features with *S-RNases* from other *Prunus* species exhibiting gametophytic self-incompatibility. The deduced amino acid identities ranged from 60.8 to 75.6% among four *S-RNase* alleles in *Prunus speciosa* and ranged from 73 to 81.4% among four *S-RNase* alleles in *Prunus pseudocerasus*. The size of the first introns ranged from 197 to 341 bp, and the size of second introns ranged from 81 to 1182 bp. Sequence analysis demonstrated that the deduced amino acid identities, by comparison with other *Prunus* species, were often higher than those of intraspecific identities. Moreover, exceptionally high identities were found between *Pspe-S_1* and *Pd-S_{28}*; between *Pspe-S_{31}* and *Pm-S_6*; among *Pspe-S_{51}*, *Pa-S_{29}* and *Pweb-S_7*; and among *Pps-S_{13}*, *Psim-S_4* and *Ps-S_b*, indicating that the *S-RNase* alleles evolved before *Prunus* species divergence. Interestingly, the similarities of the first and second introns were also high between the two *S-RNase* alleles, which range from 83.63 to 100% among the first introns and from 46.13 to 100% among the second introns. These information could not increase our knowledge on the *S*-alleles of *Prunus* species, but is available for molecular breeding of fruit trees, to avoid cross-incompatibility.

1. Introduction

Self-incompatibility (SI) is a widespread mechanism in flowering plants that prevents self-fertilization and the deleterious effects of inbreeding (De Nettancourt, 2001). Gametophytic self-incompatibility (GSI) is one such system that is controlled by a single multi-allelic locus, termed the *S*-locus (De Nettancourt, 1977). The *S*-locus is thought to contain at least two linked genes: one for the pistil determinant, and the other for the pollen determinant (Kao and Tsukamoto, 2004). Pollen-tube growth is arrested in the style when the haploid pollen *S*-allele matches either of the two *S*-alleles of the diploid pistil (Roalson and McCubbin, 2003).

S-RNase alleles have been determined as the stylar component of GSI in Rosaceae (Bŏsković and Tobutt, 1996; Sassa et al., 1996) and have been identified in many *Prunus* species including almond (*P. dulcis*; Ushijima et al., 1998; Tamura et al., 2000; Ortega et al., 2006), apricot (*P. armeniaca*; Romero et al., 2004; Vilanova et al., 2006; Wu et al., 2009), Japanese plum (*P. salicina*; Sapir et al., 2004; Zhang et al., 2008), Japanese apricot (*P. mume*; Yaegaki et al., 2001; Heng et al., 2008) and sweet cherry (*P. avium*; Tao et al., 1999; Sonneveld et al., 2001; Wünsh and Hormaza, 2004). The *S-RNases* are expressed in the pistil but not in the leaves and pollen and specifically degrade incompatible pollen RNAs (McClure et al., 1990). Recently, *S-RNase* alleles with exceptionally high identity had been found between different Rosaceae species, for example, *PtenS_8-RNase* have 99% identity with *PaS_1-RNase*, and *PtenS_1-RNase* have 99% identity with *ParS_4-RNase* (Šurbanovski et al., 2007). *PpyS_8-RNase* have 96.9% identity and the same recognition specificity with *MsS_3-RNase* (Heng et al., 2011). The phenomenon maybe makes against for molecular breeding of fruit trees.

The flowering cherry (*Prunus speciosa*) is self-incompatible and grows on Japanese islands, its *S-RNase* alleles have been surveyed and the distribution of *S*-alleles have been characterized among the populations on the Izu Peninsula and Izu Islands of Japan (Kato et al., 2007). The flowers of Chinese cherry (*P. pseudocerasus*) are

Abbreviations: Pspe, *Prunus speciosa*; Pps, *Prunus pseudocerasus*; Par, *Prunus armeniaca*; Pa, *Prunus avium*; Pd, *Prunus dulcis*; Pm, *Prunus mume*; Ps, *Prunus salicina*; Pten, *Prunus tenella*; Pweb, *Prunus webbii*; Psim, *Prunus simonii*; Pspi, *Prunus spinosa*; Pdom, *Prunus domestica*; Pbre, *Pyrus×bretschneideri*; Pcom, *Pyrus communis*; Ppy, *Pyrus pyrifolia*; Md, *Malus×domestica*; Ms, *Malus spectabilis*.

☆ Plant species from which each sequence is derived are represented by their initials in 'Abbreviations' section.

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self-compatible (Mizutani et al., 1995), and its *S-RNase* alleles have been recently identified. Notably, the deduced amino acid identities between the *S-RNase* alleles of Chinese cherry and flowering cherry are exceptionally high (Huang et al., 2008; Gu et al., 2010). To examine whether these two species are available for molecular breeding of fruit trees, two wild *P. pseudocerasus* and two *P. speciosa* accessions were studied. Eight *S-RNase* alleles were isolated and their deduced amino acid, coding region, and first and second intron sequences were characterized and compared with other *Prunus S-RNase* alleles. The results were then used to analyze why the *S-RNase* allele variable residues were less than that of the *SFB* [*S*-locus haplotype F-box] alleles, between the two *S*-haplotypes which have exceptionally high identities. The analysis of *Prunus S*-alleles would not further help to understanding the evolutionary process of *Prunus* species, but also could provide a reference for breeding of new varieties of fruit trees.

2. Materials and methods

2.1. Plant materials

This study used two wild *P. pseudocerasus* accessions, which were collected from the Xiong yue mountain area in Liaoning province, north of China, and two *P. speciosa* accessions that were collected from the Nanjing Agricultural University campus in Jiangsu province, south of China. Young leaf tissues were collected in the spring, frozen in liquid nitrogen and stored at −20 °C for DNA extraction.

2.2. Nucleic acid extraction

Total genomic DNA was extracted using the CTAB protocol with modifications (Doyle and Doyle, 1987; Sonneveld et al., 2001), treated with RNase (TaKaRa, Kyoto, Japan), and incubated at 37 °C for 1 h. The DNA integrity was confirmed by electrophoresis, and the extracted DNA concentration was examined by spectrophotometry.

2.3. PCR amplification of the second introns in the S-RNase alleles

The forward primer Pru-C2, which was designed using the conserved C2 regions, and the reverse primer Pa-C5R, which was designed using the conserved C5 regions (Table 1), were used to amplify the second introns of the putative *S-RNase* alleles in the *Prunus* species. The PCR reaction contained 50 ng of genomic DNA, 2.5 μl 10× PCR buffer (TaKaRa), 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.2 μM of each primer and 1 U of *Ex Taq* DNA polymerase (TaKaRa). The PCR reaction conditions were as follows: an initial denaturation at 94 °C for 5 min, then 40 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 2 min and a final 10 min extension at 72 °C. The PCR products were analyzed in 1.5% agarose gels in 1× TAE buffer and visualized by ethidium bromide staining under UV light.

2.4. PCR amplification of first introns of S-RNase alleles

The forward primer PMT2, which was designed using the 5′ *S-RNase* allele-flanking regions, and the reverse primer Pa-C3R, which was designed using the conserved C3 regions (Table 1), were used to amplify the first introns of the putative *S-RNase* alleles in the *Prunus* species. The PCR reaction mix and cycling conditions were similar to above except the extension time was 2 min.

For several first introns of *S-RNase* alleles that were unsuccessfully PCR amplified with the above primer pairs, specific reverse primers were designed: Pspe-S51R was designed for the $PspeS_{51}$-*RNase* allele (Table 1), and two specific reverse primers, Pps-S14R1 and Pps-S14R2, were designed for the $PpsS_{14}$-*RNase* allele (Table 1). The PCR reaction mix and cycling conditions were similar to above; however, the annealing temperature was 55 °C. The PCR products were analyzed in 1.5% agarose gels in 1× TAE buffer and visualized by ethidium bromide staining under UV light.

2.5. Thermal asymmetric interlaced PCR (TAIL-PCR) for stop codon

Three specific primers, Pa-C3F1, Pa-C3F2 and Pa-C4F, were designed using the *S-RNase* allele conserved sequences, and four arbitrary degenerate primers, AD1, AD2, AD3 and AD4, were used for TAIL-PCR amplification (Li et al., 2007). The first PCR was performed using genomic DNA as template, Pa-C3F1 as the forward primer, the four AD primers for reverse primers, and the reaction mixtures were as follows: 2.5 μl 10× LA PCR buffer II (TaKaRa), 0.6 mM dNTP, 0.4 μM of one of the four arbitrary degenerate primers, 0.4 μM outer specific primer, 2.5 U LA Taq DNA polymerase (TaKaRa), and ddH_2O was added to bring the total volume to 25 μl. The cycling conditions were as follows: 94 °C for 2 min; 95 °C for 2 min; 1 cycle of 94 °C for 30 s, 65 °C for 1 min, 72 °C for 2 min; 1 cycle of 94 °C for 30 s, 25 °C for 1 min, 72 °C for 1 min; 15 cycles of 94 °C for 30 s, 63 °C for 1 min, 72 °C for 1 min, 94 °C for 30 s, 63 °C for 1 min, 72 °C for 1 min, 94 °C for 30 s, 44 °C for 1 min, 72 °C for 1 min and a final 10 min extension at 72 °C. The product of the first TAIL-PCR was diluted one hundred fold and used as template for the second PCR along with Pa-C3F2 as the forward primer and the four AD primers as the reverse primer, respectively. The cycling conditions were the same as above. The third PCR was performed using the diluted product from the second PCR as template, Pa-C4F as the forward primer, and the four AD primers as the reverse primer, respectively. The cycling conditions were also the same as above. The PCR products were analyzed in 1.5% agarose gels in 1× TAE buffer and visualized by ethidium bromide staining under UV light.

2.6. Cloning and sequencing

The PCR bands were excised from 2% agarose gels and purified using the Qiagen II Gel Extraction Kit (Qiagen, Valencia, CA, USA). The purified PCR products were cloned into the PMD19-T Vector (TaKaRa) following the instructions of the manufacturer and transformed into DH5α *E. coli*. Transformed colonies were selected and amplified, and plasmid purification was performed using the Plasmid Mini Kit (Qiagen). Plasmids with inserts of the expected size were examined using the same primer pairs used for their initial PCR amplification. To obtain a consensus sequence and to avoid errors caused by PCR, each allele was sequenced a minimum of three times using independent colonies.

2.7. Sequence and phylogenetic analysis

Homology searches were performed using BLASTX and BLASTN (Altschul et al., 1990, 1997) in the NCBI database. A DNA consensus sequence for each allele was obtained by assembling the data from all three replicates using DNAMAN (version 5.2; Lynnon Biosoft). Nucleotide sequences or deduced amino acid sequences of *S-RNase*s and *SFB*s were aligned using the CLUSTAL W (Thompson et al., 1994) and CLUSTAL X (Thompson et al., 1997) programs, respectively. Phylogenetic trees were constructed from a distance matrix implemented in MEGA (version 3.1; Kumar et al., 2004) by the neighbor-joining method based on multiple alignments, which was applied with Kimura two-parameter correction (Kimura, 1980). One thousand bootstrap analyses replicates were conducted to provide confidence estimates for phylogenetic tree topologies.

Sequence data for the *S-RNases* used in this study are as follows: $Par\text{-}S_9$ (AY864826), $Par\text{-}S_2$ (AY587562), $Par\text{-}S_4$ (AY587564), $Par\text{-}S_{17}$ (EU516388), $Pa\text{-}S_5$ (AJ298314), $Pa\text{-}S_{23}$ (AY259114),