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Identification of *S*-genotypes in 18 pear accessions and exploration of the breakdown of self-incompatibility in the pear cultivar Xinxue

Su-Li Shi, Hai-Yan Cheng, Lei Wu, Zhi-Hua Xie, Chao Gu*, Shao-Ling Zhang*

Centre of Pear Engineering Technology Research, State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China

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ABSTRACT

Self-incompatibility is a genetic mechanism in flowering plants that promotes outcrossing and prevents inbreeding. In this study, the reported 15 *S-RNase* alleles were isolated from 18 pear accessions that contained 14 different *S*-genotypes. The *S-RNase* alleles were only expressed in the style, not in the root, stem, leaf, fruit, sepal, and pollen. Pollen tube grew into self-styles and self-pollinated fruit set involved the breakdown of self-incompatibility in cv. Xinxue. However, the two *S-RNase* alleles in cv. Xinxue had identical amino acid sequences to those self-incompatible cultivars, and S_5- and S_6-*RNase* were normally expressed in style, indicating that the loss of self-incompatibility in cv. Xinxue likely resulted from pollen-part mutation. Further, S_5S_5 and S_6S_6 genotyped individuals were identified in self-pollinated progeny, indicating that S_5 and S_6 genotyped pollen were compatible with the self-styles of cv. Xinxue. A genetic analysis showed that the segregation ratio of S_5S_5, S_5S_6, and S_6S_6 was approximately 0:2:1 ($\chi^2 = 3.505 < \chi^2_{0.05,\,2} = 5.99$), which did not fit the theoretical ratio of 1:2:1 ($\chi^2 = 13.340 > \chi^2_{0.05,\,2} = 5.99$), suggesting that the breakdown of self-incompatibility could be caused by modified factor(s) located outside of the *S*-locus. These results are useful for parental assignment and understanding self-incompatibility reaction.

1. Introduction

Pear is a typical gametophytic self-incompatible (GSI) species, which is determined by a single multi-allelic locus (*S*-locus/*S*-haplotype) containing at least two genes that control stylar and pollen specificities, respectively (Okada et al., 2011; Ushijima et al., 2003). In pear, the stylar determinant is determined as an *S-RNase* gene (Sassa et al., 1992, 1997), while the pollen determinant may be controlled by multiple *F-box* genes (Kakui et al., 2011). Since the original cloning of *S-RNase* alleles by PCR with gene-specific primer pairs (Ishimizu et al., 1999), increasing numbers of *S-RNase* alleles has been isolated from *Pyrus* species (Heng et al., 2008; Wu et al., 2013a; Zuccherelli et al., 2002), and the identified *S*-genotypes have served as pollinator assignment to avoid a reduction in fruit production owning to self-incompatibility (SI).

Presently, a larger number (over 100) of *Pyrus S-RNase* alleles have been submitted into National Center for Biotechnology Information (NCBI) database, and the designation is disordered. For instance, the *S-RNase* alleles isolated from *P. communis* are numbered by English letters and Arabic numerals (Moriya et al., 2007; Sanzol et al., 2006), while they are independently numbered by English letters within *P. pyrifolia*, *P. bretschneideri*, *P. ussuriensis*, and *P. sinkiangensis* (Heng et al., 2008; Ishimizu et al., 1999). The disordered designation was first rearranged in *P. communis* (Goldway et al., 2009), and recently, the integration of *S-RNase* alleles occurred in *P. pyrifolia*, *P. bretschneideri*, *P. ussuriensis*, and *P. sinkiangensis* (Wang et al., 2017). The renumbered *S-RNase* alleles are more convenient for correct parental assignments.

Although approximately 462 pear accessions have been *S*-genotyped in the past two decades (Wang et al., 2017), what are the *S*-genotypes of new varieties and landraces is unclear. To select the suitable pollinator for new varieties and acceptors for landraces, first, 18 pear accession were collected, and then the *S-RNase* alleles were identified using PCR-based method. Second, based on allelic information, the breakdown of SI in cv. Xinxue was explored by observing pollen tube grew into self-style, self-pollinated fruit set, and the inheritance of *S* alleles in the self-pollinated progeny. Finally, the reason for self-compatibility (SC) in cv. Xinxue was discussed. These results will be useful for both parental assignment and understanding the SI reaction.

* Corresponding authors.
E-mail addresses: guchao@njau.edu.cn (C. Gu), slzhang@njau.edu.cn (S.-L. Zhang).

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2. Materials and methods

2.1. Plant materials

A total of 18 pear accessions, Xinxue, Guihuali, ShinKo, Hongxiangsu, Liuyuesu, Ruanzhiqing, LeConte, Beurre Giffard, Bayuehong, Zheli, Tienong No.1, Baihuaguang, Tiantang, Hongshan, Sumen, Qingsong, Shanxihongjinpin, and Jingzhoushanli, were maintained at Jiangpu orchard, Nanjing Agricultural University (Nanjing, China). Young leaf tissues of these accessions were collected in the spring, and the root, stem, sepal, pollen, and styles of cv. Xinxue were sampled from the pre-blooming flowers and fruits that were collected at 30 days after flower blooming. All of the collected samples were frozen by liquid nitrogen and stored at −80 ℃ until use.

2.2. Field pollination tests

Approximately 400 flowers were selected for self-pollination by bagging in translucent paper. After 96 h, the self-pollinated styles were collected and fixed in FAA buffer (formalin:acetic acid :70% ethanol = 1:1:18). The treatment and observation of the collected styles were identical to those performed in a previous study (Heng et al., 2008). Fruit set due to self-pollination was determined at 30 days after pollination, and the seeds were collected from the ripening fruits in the autumn. The collected seeds were treated with stratification at 4 ℃ and planted in nutritional pots at 25 ℃, and the leaves of each seedling were independently sampled for DNA extraction.

2.3. Nucleic acid extraction

Genomic DNA was extracted from the leaves of the 18 accessions and all self-pollinated progeny using the cetyl trimethyl ammonium bromide (CTAB)-based extraction method with few modifications (Gu et al., 2014). Total RNA was extracted from the root, stem, leaf, fruit, sepal, pollen, and styles of cv. Xinxue using an RNAprep Pure Plant Kit of Polysaccharides & Polyphenolics-rich (Tiangen, Beijing, China), and then the first-strand cDNA was synthesized using a TransScript One-Step gDNA Removal and cDNA synthesis Supermix (TransGen, Beijing, China) according to the manufacturer's instructions.

2.4. PCR amplification of S-RNase alleles

PCR was performed with the primers PF (5′-TTTACGCAGCAATATCAGC-3′) and PR (5′-AC(A/G)TTCGGCCAAATAATA-3′) to amplify the *S-RNase* alleles, and the reaction mixture and condition were identical to those in a previous study (Heng et al., 2008), using genomic DNA or cDNA. *PbTUB*, which is expressed in all tissues and amplified after 35 cycles using the TUB-F (5′- TGGGCTTTGCTCCTCTTAC-3′) and TUB-R (5′- TCAGTCGCCGCCGGCCTTTTG-3′ (Chen et al., 2015), primers served as the positive control for RT-PCR. The amplification products were separated by an 8% non-denaturing polyacrylamide gel in 1 × TBE buffer (Liu et al., 2015).

To produce full-length sequences of the *S-RNase* alleles, 5′ and 3′ rapid amplification of cDNA ends (5′- and 3′-RACE respectively) were performed using the GeneRacer™ Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The purification, cloning, and sequencing protocols of the amplification products were identical to those in a previous study (Heng et al., 2008). A sequence alignment was performed using DNAMAN software (Lynnon BioSoft, Vaudreuil, Quebec, Canada).

2.5. Genetic segregation of S-RNase alleles

Because of the sizes of amplification products, the two *S-RNase* alleles were indistinguishable in cv. Xinxue, therefore, four specific primer pairs were designed based on the sequence polymorphism between the two *S-RNase* alleles. Of the four primer pairs, two pairs, S5F1 (5′-CGATAAGGAACATTCGGAAGAGAG-3′)/S5R1 (5′-CTATGAAATGTTCTCCGCTTCTGTC-3′) and S5F2 (5′-AAGTTGTTTACGGTTCACGGTTTG-3′)/S5R2 (5′-AAATGTTCTCCGCTTTTGTCACTGC-3′), were used to identify S_5-*RNase* alleles, while the other two pairs, S6F1 (5′- TCTGGAATAGACAGTGGAACAAAC-3′)/S6R1 (5′-AATACAGAATATGATTGGTGGGGC-3′) and S6F2 (5′-ATAGACAGTGGAACAAACATGGC AGC-3′)/S6R2 (5′-ACAGAATAT GATTGGTGGGGCAGTG-3′), were used to identify S_6-*RNase* alleles. The chi squared ($\chi 2$) goodness-of-fit was applied to the genotyping data to test for deviations from theoretical Mendelian segregation ratios.

2.6. Quantitative PCR (qPCR)

Using the DNA isolated from *S*-genotyped homozygous (S_5S_5 and S_6S_6) and heterozygous (S_5S_6) individuals as material, quantitative PCR (qPCR) was conducted in a LightCycler 480® II/96 Thermal Cycler (Roche Diagostics, Rotkreuz, Swizerland). The primer pairs, S5YG-F (5′-GGACGGGAAAAAAAGAGCACTGTT-3′)/S5YG-R (5′-GGGCAATCTATGAAATGTTCTCCG-3′) and S6YG-F (5′-ACTGTATTGGTCGGGAGCACTAT-3′)/S6TG-R (5′-TCGTCCTATGTATGGAAATGGTC-3′), were used in the qPCR to test the expression levels of S_5- and S_6-*RNase* alleles, respectively. The 20 μl reaction volume contained 10 μl of LightCycler 480 SYBR GREEN I Master (Roche, Diagostics, Rotkreuz, Switzerland), 0.5 μM of each primer, and 10 ng genomic DNA. A melting curve analysis was performed at the end of 40 cycles to ensure specificity of the amplified fragments. Moreover, *PbTUB* was used as a constitutive control.

3. Results

3.1. Identification of S-RNase alleles in 18 pear accessions

Besides single fragment was detected in the accessions Tienong No.1, Zheli, Liuyuesu, and Xinxue, two fragments of different lengths were amplified from the remaining 14 pear accession (Fig. 1). Sequenced result of PCR products revealed that a total of 15 *S-RNase* alleles were isolated from the 18 pear accessions and each accession had two different *S-RNase* alleles. The single amplified fragment in the accessions Tienong No.1, Zheli, Liuyuesu, and Xinxue resulted from almost identical fragment sizes between the detected two *S-RNase* alleles (Table 1). Moreover, to detect the expression levels of *S-RNase* alleles in the tissues, the total RNA was extracted from root, stem, leaf, fruit, pollen, and style of cv. Xinxue and used in the synthesis of the first-strand cDNA for PCR amplification. As seen in Fig. 2, the fragment of *PbTUB* gene was amplified from all tissues, confirming that the first-strand cDNAs of all tissues were successfully synthesized and had uniform concentrations. However, no *S-RNase* allele amplification products were detected in the root, stem, leaf, fruit, and pollen, while only fragment was found in style. Thus, *S-RNase* alleles were exclusively expressed in style.

3.2. Evidences of cv. Xinxue presenting self-compatibility

To test the SI/SC of cv. Xinxue, the lengths of pollen tubes in self-pollinated styles was observed. The pollen tubes were not only found in the top 1/3rd of the style, but were also visualized at the bottom (Fig. 3A). Moreover, the fruit set after self-pollination was 22.9%, which was above 20%, which is the threshold of self-compatibility (Wang et al., 2017). Therefore, cv. Xinxue appears to be a self-compatible cultivar (Wang et al., 2017).

3.3. Sequence analysis of S_5- and S_6-RNase alleles in cv. Xinxue

Full-lengths of S_5- *and* S_6-*RNase* alleles were acquired from cv. Xinxue using 5′- and 3′-RACE. Alignments showed that the two alleles