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Characteristic of pollen tube that grew into self style in pear cultivar and parent assignment for cross-pollination

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ABSTRACT

Pear is one of the most important fruits in the world. In recent years, many new varieties have been bred. However, little is known about these new varieties, including pollen germination, self-(in)compatibility, and the best pollinator for the varieties. In this study, by surveying the length of the pollen tubes that grew into the self styles, pollen germination and self-(in)compatibility of the 127 pear cultivars were determined. All tested pollen germinated on the self stigmas, but the lengths of pollen tube growth into the self styles were different among the pear cultivars. Of these 127 cultivars, 56, 58, and 8 cultivars presented strong, intermediate, and weak self-incompatibility, respectively. Moreover, 5 cultivars presented self-compatibility because their pollen tubes grew to the bottom of the self styles and higher fruit sets with self-pollination were verified in the field. Furthermore, to assign the best pollinator for fruit production and/or new variety breeding, the *S-RNase* alleles that shared almost identical sequences were integrated together and then re-designated to discern different function-specificity. As a result, the original 68 *S-RNase* alleles in eastern pear were integrated into 48 re-designated function-specific *S-RNase* alleles. This integration is promising for assigning the best pollinator for the 36 main pear cultivars in China. Unless the integrated *S-RNase* alleles, in addition, the *S-RNase* alleles, which have extremely high identities in amino acid sequences, should be excluded to avoid cross-incompatibility between two parents, leading to poor fruit production and the loss of genetic information in this chromosome

1. Introduction

Pear has been widely cultivated around the world for at least 3, 000 years. Its cultivated area and fruit production are only less than orange and apple, making pear the third most important fruit tree. Pear presents typical gametophytic self-incompatibility (GSI) in the Rosaceae species, which is determined by a single multi-allelic locus named the *S*-locus (Nettancourt 1997; Kubo et al., 2015). It was determined that the *S*-locus contains one gene controlling stylar specificity, which has been identified as an *S-RNase* gene (Sassa et al., 1992, 1997; Ushijima et al., 2003). However, the gene(s) controlling pollen specificity have not be confirmed, and only a few positive results have suggested that a *F-box* gene cluster located within the *S*-locus is involved in the self-incompatibility reaction (Kakui et al., 2011; Sassa et al., 2007). In Rosaceae, the pollen-*S* has been recognized as a set of F-box protein(s), which has been documented with different terms, such as-locus F-box (SLF), *S*-haplotype-specific F-box (SFB), and/or *S*-haplotype-specific F-box brothers (SFBB), and functions as a component of the SCF ubiquitin-ligase complexes (Kubo et al., 2015). Therefore, *S*-genotypes can only be identified on the basis of *S-RNase* alleles in pear cultivars.

Self-incompatibility (SI) is a genetic mechanism in flowering plants. The identification of SI is usually examined by two main channels. One is to test fruit set from self-pollination (Hiratsuka and Zhang, 2002), and the other is to survey the length of the pollen tube that grows into the self style (Sanzol and Herrero, 2002). Fruit set is the best feedback for SI examination but is often a reflection of the surrounding environment; whereas, pollen tube growth surveys can be performed in the lab and the results are reliable and stable. If pollen tubes in a cultivar can grow to the bottom of the self style, then the cultivar is self-compatible (SC), but if pollen tubes can grow into but are stopped at the middle of the self style, the cultivar is self-incompatible. In general, pollen tubes stop growing at the position of before 2/3 length of the self style (Heng et al., 2008).

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Usually, pollen tube growth is arrested in a non-self style if the *S* allele in the pollen is identical to one of the two *S* alleles in the style. Therefore, two different cultivars sharing one *S*-genotype will likely present cross-incompatibility in pear. Cross-incompatibility is undesirable for pear breeders and farmers due to lower or no fruit set after cross-pollination. Clearly, cross-incompatibility should not occur in fruit production program and in new variety pear breeding. To avoid cross-incompatibility, a large number of pear cultivars and accessions were genotyped for *S-RNase* alleles in the last twenty years (Supplementary Table 1). The identified *S*-genotypes consist of at least 87 *S-RNase* alleles isolated from the *Pyrus* species (Supplementary Table 2). Of these isolated *S-RNase* alleles, 68 alleles in eastern pear cultivars were designated S_1 to S_{52} (Heng et al., 2008; Norioka et al., 1995), while 19 alleles in western pear cultivars were originally named S_a to S_t (Takasaki et al., 2006; Zuccherelli et al., 2002) and then re-named S_{101} to S_{109}, respectively (Goldway et al., 2009).

In recent years, few cultivars have been genotyped for *S-RNase* alleles in pear, but it is unknown if many cultivars are self-incompatible. In this study, a pollen tube growth assay was first performed to further determine the SI/SC of modern pear cultivars. Second, *Pyrus S-RNase* alleles were analyzed in homology to integrate the *S*-alleles that share one allelic number but have different allelic sequences and that have different allelic numbers but have identical allelic sequences. Third, *S*-genotypes in eastern pear cultivars were evaluated to determine the best parent assignment for cross-pollination. These results may be able to prevent fruit reduction and accelerate the process of new variety pear breeding.

2. Materials and methods

2.1. Plant materials

A total of 127 pear cultivars sampled from different *Pyrus* species at the Pukou District pomology farm of Nanjing Agricultural University were used in this study. Of these pear cultivars, 64, 17, 10, 5, and 3 cultivars were collected from *P. pyrifolia* (*Pp*), *P. bretschneideri* (*Pb*), *P. ussuriensis* (*Pu*), *P. sinkiangensis* (*Ps*), and *P. communis* (*Pc*), respectively. Moreover, 27 cultivars were inter-specific hybridizations derived from cross-pollinations between at least two different *Pyrus* species. The flowers of all the tested pear cultivars were emasculated before flowering and then bagged until pollination.

2.2. Acquirement of nucleotide sequences of other Pyrus S-RNase genes

According to the accession numbers reported in other research, most *Pyrus S-RNase* genes were found in NCBI (http://www.ncbi.nlm.nih.gov), and their nucleotide sequences were downloaded from the GenBank/EMBL/DDBJ database. For each downloaded gene sequence, BLAST was executed to search for sequences that were as long as possible. The longest nucleotide sequences for each gene were used for homology analysis among *Pyrus S-RNase* genes in Vector NTI Version 11 (http://www.invitrogen.com) with CLUSTAL W program.

2.3. Phylogenetic analysis of Pyrus S-RNase alleles

Together with the acquired nucleotide sequences, the deduced amino acid sequences of *Pyrus S-RNase* alleles were also downloaded. These amino acid sequences were then aligned using BioEdit software version 7.1.11 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) with CLUSTAL W and CLUSTAL X programs. The aligned amino acid sequences were used to construct phylogenetic trees from a distance matrix implemented in MEGA version 6 (Tamura et al., 2013) with the neighbor-joining method based on multiple alignments and the Kimura two-parameter correction (Kimura, 1980). One thousand replicates were conducted to calculate bootstrap values and to assay the statistical significance of the phylogenetic tree.

2.4. PCR amplification and cloning of Pyrus S-RNase alleles

To acquire more sequence information of several partial *S-RNase* alleles, two primer pairs (PMT/Pyr-C3R and Pyr-C2F/Pyr-C5R) were used. Of these four primers, the two forward primers PMT (5′-TAATCTGCCTCGCTCTTGAAC-3′) and Pyr-C2F (5′-GTTGTTTACGGTTCACGGTTTG-3′) were designed for the upstream start codon and the conserved C2 region, respectively, and the two reverse primers Pyr-C3R (5′-GAGACGTTTTGTTTCTGGGTTATG-3′) and Pyr-C5R (5′-GCTGCAAATAGTGACCTCAACCAAT-3′) were designed for the conserved C3 and C5 regions, respectively. The 50 μLPCR mixtures contained 100 ng genomic DNA, 5 μL 10× KOD PCR buffer (ToYoBo, Kyoto, Japan), 0.2 mM dNTPs, 0.1 μM of each primer and 1 Unit KOD plus DNA polymerase (ToYoBo). The PCR reaction conditions were as follows: Initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 68 °C for 2 min, with a final extension at 68 °C for 10 min. The objective PCR fragments were purified using the Qiagen II GelExtraction Kit (Qiagen, Valencia, CA, USA) and then ligated using pEASY®-Blunt Zero Cloning Vector (TransGen, Beijing, China). The constructed products were transformed into *Escherichia coli* DH5a, and the positive colonies were selected and examined using PCR with universal primers of the vector. Finally, the extracted plasmids containing the target fragment were sequenced at least three times using independent colonies.

2.5. Field pollination and pollen tube growth

To check the self-incompatibility in Chinese pear, 127 pear cultivars distributed in five *Pyrus* species and inter-specific hybridizations were selected for a pollen tube growth assay of self-pollinated styles. In brief, the self-pollinated styles were first collected at 72 h after pollination, and then fixed using FAA mixture. Second, the fixed styles were incubated in 4N NaOH to soften the tissues and then soaked in 0.1% aniline blue solution overnight at 65 °C in darkness to dye the pollen tubes. Finally, the dyed pollen tubes were washed and observed by ultraviolet fluorescence microscopy (BX60, Olympus, Tokyo, Japan) (Gu et al., 2010). Moreover, to confirm the accuracy of the pollen tube growth assay, self-pollinations of a few representative pear cultivars were performed and their fruit sets were counted at 20 and 30 days after flowering (DAF). The number of self-pollinated flowers of cvs. Meigetsu, Xinxue, Zhenghedaxueli, Kuerlexiangli, Hululi, Cuiyu, Daxiangshui, Hongtaiyang, and Osa-Nijisseiki were 300, 192, 282, 201, 155, 288, 312, 192, and 306, respectively. Before anthesis, two to three flowers per inflorescence were left. The emasculated flowers were self-pollinated and re-bagged at full bloom. Fruit set greater than 20% in a cultivar was considered self-compatible (Komori et al., 1999).

3. Results

3.1. Identification of self-incompatibility by surveying pollen tube growth

According to previous research, *S*-genotypes have been identified in 462 pear cultivars and accessions in the *Pyrus* species, including 118 *P. pyrifolia*, 65 *P. bretschneideri*, 50 *P. ussuriensis*, 39 *P. sinkiangensis*, 133 *P. communis*, 42 inter-specific hybridization cultivars (*Ph*), and 15 landrace (Supplementary Table 1). To test the