Invited Review

DNA Markers and the Molecular Mechanism of Self-(in)compatibility in Japanese Pear (*Pyrus pyrifolia* Nakai)

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Japanese pear (*Pyrus pyrifolia* Nakai) is an important Rosaceous fruit tree in Japan. This species exhibits gametophytic self-incompatibility (GSI), which is controlled by a single *S*-locus with multiple *S*-haplotypes. Although GSI is a genetic mechanism to prevent inbreeding and promote outcrossing to maintain genetic diversity, it can be problematic in fruit trees because it causes unstable fruit set. Therefore, orchardists interplant Japanese pear with other lines as pollinizers or conduct artificial pollination to ensure fruit set. To achieve stable fruit production and to reduce or eliminate the need for artificial pollination, research on the GSI of Japanese pear has been conducted by pollination experiments and by characterization of self-compatible (SC) mutants. Additionally, breeding programs have progressed to produce SC cultivars with high fruit quality. Recently, molecular analyses of GSI and SC mutants in Japanese pear have provided new information that is relevant to the stable fruit production and efficient breeding of Japanese pear. This review focuses on studies of the GSI of Japanese pear, and especially on the recent development of DNA markers for *S*-genotyping and marker-assisted selection of SC trees. In addition, the candidate genes controlling pollen *S*-specificity and a model of the molecular mechanism of GSI in Japanese pear are described.


Introduction

Flowering plants have evolved various genetic mechanisms to prevent inbreeding and promote outcrossing to maintain genetic diversity within a species. One such mechanism is self-incompatibility (SI), which allows the pistil to distinguish between self (genetically related) pollen and non-self (genetically unrelated) pollen. The self pollen is rejected, while the non-self pollen is accepted for fertilization. To date, SI has been recorded in more than 50% of flowering plant species (Kao and McCubbin, 1996). In many species, SI is controlled by a single polymorphic locus, the *S*-locus. This locus consists of at least two linked genes: the pistil *S* gene (female determinant gene) and the pollen *S* gene (male determinant gene), so variants of the gene complex are called *S*-haplotypes (Kao and McCubbin, 1996; Takayama and Isogai, 2005).

Among the Rosaceous fruit trees, *Malus* (apple), *Pyrus* (pear), and *Prunus* (almond, cherry, plum, and apricot) exhibit SI (Yamane and Tao, 2009). However, SI in fruit trees can be problematic because it causes unstable fruit set. Therefore, to ensure fruit set in orchards, cross-compatible cultivars are interplanted as pollinizers, or artificial pollination is conducted.

Japanese pear (*Pyrus pyrifolia* Nakai) is an important Rosaceous fruit tree in Japan, and its self- and cross-sterility have been studied for a long time. Kikuchi (1929) revealed that Japanese pear exhibited gametophytic SI (GSI) controlled by a single *S*-locus with multiple *S*-haplotypes. In GSI, the SI phenotype of pollen is determined by its own *S*-genotype. The growth of the pollen tube is inhibited at the style when its *S*-haplotype matches one of the *S*-haplotypes of the pistil. When two different cultivars have the same *S*-genotype, they exhibit cross-incompatibility. Therefore, *S*-genotype assignment is important to select compatible pollinizers in orchards and compatible parents in breeding programs. In contrast, self-compatible (SC) cultivars are useful because they save on artificial pollination labor costs and achieve stable fruit set. Therefore, an important objective of Japanese pear-breeding programs is to produce...
SC cultivars with high fruit quality. In Japanese pear, the first SC cultivar ‘Osanijisseiki’ was discovered as a bud mutation from ‘Nijisseiki’ (Furuta et al., 1980). ‘Osanijisseiki’ has been used as a parent for cross-breeding of SC cultivars in Japan and Korea, producing new SC cultivars such as ‘Akiiba’, ‘Akiikansen’, ‘Shinmizuki’, and ‘Shino’ (Kim et al., 2004; 1982; Ogaki, 1958).

‘Shinmizuki’, and ‘Shino’ (Kim et al., 2004; 1982; Ogaki, 1958).

However, it is time-consuming to select SC trees by pollination experiments, there is a need to develop DNA markers to select SC trees at the seedling stage. This review focuses on GSI research on Japanese pear, especially the recently developed DNA markers for S-genotyping and marker-assisted selection of SC trees. In addition, candidate genes controlling pollen S-specificity and a model for the molecular mechanism of GSI in Japanese pear are described.

1. Analyses of S-genotype

1) Determination of S-genotypes by pollination experiments

In early pollination experiments on Japanese pear, Kikuchi (1929) found that cross combinations of some cultivars exhibited cross-incompatibility. In addition, Kikuchi (1929) found that approximately half of the first filial generation (F1) plants in cross combinations of some cultivars exhibited cross-incompatibility with their pollen parent; this was named paterclinal incompatibility. On the basis of the patterns of cross-incompatibility and paterclinal incompatibility, Terami et al. (1946) identified seven S-haplotypes (S1 to S7) and 10 S-genotypes in 23 cultivars for the first time. Using these S-genotypes as first cross indicators, the S-genotypes of other cultivars were determined in pollination experiments; when a cultivar was cross-incompatible with the cross indicator, they had the same S-genotype (Hiratsuka et al., 1998; Machida et al., 1982; Ogaki, 1958).

The S-genotype of the SC cultivar ‘Osanijisseiki’ was assigned as follows: the cross-pollination of ‘Nijisseiki’ (S3S4) × ‘Osanijisseiki’ was cross-incompatible, whereas that of ‘Osanijisseiki’ × ‘Nijisseiki’ (S2S4) was cross-compatible. These results suggested that ‘Osanijisseiki’ was a stylar-part mutant (Sato, 1993). Most of the F1 plants from ‘Kosui’ (S3S4) × ‘Osanijisseiki’ were self-incompatible, suggesting that the mutation was in the S4 gene, and that the S-genotype of ‘Osanijisseiki’ was S3S4sm (where sm indicates stylar-part mutant) (Sato, 1993).

In this way, conventional pollination experiments have been used to assign the S-genotypes of approximately 40 Japanese pear cultivars. However, pollination tests can be affected by environmental conditions, and are time-consuming and labor-intensive. Additionally, the cross indicators currently available do not cover the 21 S-genotypes consisting of combinations of seven S-haplotypes.

2) Identification of pistil S gene

To identify the pistil S gene of Japanese pear, researchers attempted to identify S-haplotype-specific proteins (S-proteins) in the style. Two-dimensional gel electrophoresis analyses of proteins in style extracts identified seven S-proteins corresponding to the S1- to S7-haplotypes. These S-proteins were identified as S-RNases, that is, basic glycoproteins with ribonuclease activity (Ishimizu et al., 1996; Sassa et al., 1993). S-RNases are developmentally expressed in the style during flower maturation (Ishimizu et al., 1996), but are not expressed in the leaf, pollen, or germinating pollen (Sassa et al., 1993).

The stylar-part SC mutant ‘Osanijisseiki’ (S2S4sm) was shown to produce S2-RNase, but not S4-RNase (Ishimizu et al., 1996). The cDNA encoding the S2-RNase was cloned from the stylar cDNA library, but that encoding the S4-RNase was not (Norioka et al., 1996). Finally, it was revealed that ‘Osanijisseiki’ lacked a region more than 4 kb long, including the full-length S2-RNase sequence (Sassa et al., 1997). These findings suggested that the S-RNase is involved in pistil SI, but not pollen SI, in Japanese pear.

3) Development of S-genotyping systems using the pistil S gene

The cDNAs encoding seven S-RNases (S1- to S7-RNases) were cloned from styles of Japanese pear (Ishimizu et al., 1998; Norioka et al., 1996). Alignment of the deduced amino acid sequences of these S-RNases revealed five conserved regions (C1, C2, C3, RC4, and C5) and one hypervariable (HV) region with an S-haplotype-specific sequence (Ishimizu et al., 1998; Ushijima et al., 1998) (Fig. 1A). The HV region was thought to interact with the pollen S gene product(s) to recognize self and non-self pollen (Matsuura et al., 2001). One intron located within the HV region also showed considerable diversification in terms of sequence and length among the S-RNases (Ishimizu et al., 1999).

On the basis of the variations in the HV region and the intron, the cleaved amplified polymorphic sequence (CAPS) (S1 to S7) system for discriminating S1- to S7-haplotypes was developed as a rapid method to identify the S-genotypes of Japanese pear cultivars (Ishimizu et al., 1999). In this system, S-RNase fragments including the intron are amplified from genomic DNA by polymerase chain reaction (PCR) with the consensus primers “FTQQYQ” (5’-TTTACGCAGCAATATCAG-3’) and “anti-IIWPNV” [5’-AC(A/G)TTCCGGCAAA TAATT-3’] (Fig. 1A). Electrophoresis of PCR products can discriminate the 1347 bp S2-RNase fragment, but not the S1-, S3- to S7-RNase fragments of ca. 350 bp (Fig. 1B, lanes 1–4). These ca. 350 bp fragments are subsequently discriminated by digestion with six S-haplotype-specific restriction endonucleases: SfcI (S1-specific), PpuMI (S3-, S6-specific), Ndel (S4-specific),
AlwNI (\(S_r\)-specific), HincII (\(S_s\)-specific), and AccII (\(S_s\), \(S_r\)-specific). Castillo et al. (2001b) confirmed that the assignment of \(S\)-genotypes by the CAPS (\(S_1\) to \(S_7\)) system coincided with that by pollination experiments. Thus, the CAPS (\(S_1\) to \(S_7\)) system was shown to be highly reliable for identifying the \(S\)-genotypes of cultivars with \(S_1\) to \(S_7\)-haplotypes. However, the \(S\)-genotypes of some cultivars could not be identified using this system. It was thought that these cultivars had other uncharacterized \(S\)-RNases.

Castillo et al. (2001b) amplified a new 436 bp \(S\)-RNase fragment from ‘Ichiharawase’ (Fig. 1B, lane 5), ‘Meigetsu’, and ‘Heiwa’ (‘Nijisseiki’ × ‘Ichiharawase’) using the CAPS (\(S_1\) to \(S_7\)) system. The length of this fragment and the deduced amino acid sequence in the HV region differed from those of \(S_1\) to \(S_7\)-RNases. The new \(S\)-RNase was designated as \(S_8\)-RNase, and the \(S\)-genotypes of ‘Ichiharawase’, ‘Meigetsu’, and ‘Heiwa’ were identified as \(S_1S_8\), \(S_1S_8\), and \(S_4S_8\), respectively. Castillo et al. (2002) determined the complete sequence of the \(S_8\)-RNase, and selected NruI as an \(S_8\)-haplotype-specific restriction endonuclease. This established the CAPS (\(S_1\) to \(S_8\)) system for discriminating \(S_1\)- to \(S_8\)-haplotypes.

In Japan, three main cultivars of Japanese pear accounted for 78% of the total growing area (11745 ha) in 2011: ‘Kosui’ (\(S_4S_5\)), ‘Hosui’ (\(S_3S_5\)), and ‘Nijisseiki’ (\(S_2S_4\)) and its bud mutation (Statistics Bureau of Japan, http://www.e-stat.go.jp/SG1/estat/List.do?lid=000001115710, February 16, 2014). The next most common cultivars were ‘Niitaka’, ‘Akizuki’ (\(S_3S_4\)), ‘Shinkou’, and ‘Nansui’, occupying 9.5%, 2.7%, 2.4%, and 2.1% of the total growing area in Japan, respectively. However, \(S\)-genotypes of ‘Niitaka’, ‘Shinkou’, and ‘Nansui’ had not been determined by pollination experiments or using a CAPS system (\(S_1\) to \(S_7\)) (Fig. 1B, lane 6). Sawamura et al. (2002a) amplified a new \(S\)-RNase fragment from ‘Shinkou’ and ‘Shinsei’ (‘Suisei’ × ‘Shinkou’) using the consensus primers “FTQQYQ” and “anti-F(I/R)(N/D)CP(H/R)”.

**Fig. 1.** Analysis of \(S\)-RNase fragments using the CAPS systems in Japanese pear. (A) Structural features of \(S\)-RNase in Japanese pear. Boxes show exons and a bar shows an intron. Orange, blue, and red regions in boxes correspond to signal peptide (SP), conserved regions (C1, C2, C3, RC4, and C5), and hypervariable (HV) region, respectively. Arrows indicate primers “FTQQYQ”, “anti-IIWPNV”, and “anti-(I/T)IWPNV”. (B) \(S\)-RNase fragments amplified from genomic DNA of Japanese pear cultivars by PCR with primers “FTQQYQ” and “anti-IIWPNV” (Takasaki et al., 2004). (C) \(S\)-RNase fragments amplified from genomic DNA of Japanese pear cultivars by PCR with primers “FTQQYQ” and “anti-(I/T)IWPNV” (Takasaki et al., 2004). Fragments were subjected to 2% agarose gel electrophoresis. 1: ‘Imamuraaki’ (\(S_1S_6\)), 2: ‘Nijisseiki’ (\(S_2S_5\)), 3: ‘Hosui’ (\(S_3S_5\)), 4: ‘Okusankichi’ (\(S_5S_6\)), 5: ‘Ichiharawase’ (\(S_1S_8\)), 6: ‘Shinkou’ (\(S_4S_5\)).
The new \textit{S-RNase} was designated as \textit{S}_{	ext{fr}}-\textit{RNase}, and the \textit{S}-genotypes of ‘Shinkou’ and ‘Shinsei’ were identified as \textit{S}_S\text{S}_p. Takasaki et al. (2004) isolated the full-length cDNA encoding the \textit{S}_S-RNase from pistils of ‘Shinkou’ and ‘Shinsei’, and established the CAPS (\textit{S}_1 to \textit{S}_9) system for discriminating \textit{S}_1 to \textit{S}_9-haplotypes. In this system, the nine \textit{S-RNase} fragments are amplified from genomic DNA by PCR using the primer pair ‘FTQQYQ’ and ‘anti-(I/T)IWPNV’ [a mix of the primer ‘anti-IIWPNV’ and an \textit{S}_S-RNase-specific primer ‘anti-TIWPNV’ (\textit{S}‘ACGGTTGGCCAAATAGTT-3’) (Fig. 1A): 1347 bp (\textit{S}_S), 1307 bp (\textit{S}_p), 436 bp (\textit{S}_1), 367 bp (\textit{S}_2), 368 bp (\textit{S}_3), 376 bp (\textit{S}_4), 347 bp (\textit{S}_5), and 352 bp (\textit{S}_6)] (Fig. 1C). The \textit{S-RNase} fragments have similar lengths and are subsequently discriminated by digestion with \textit{S}-haplotype-specific restriction endonucleases (Table 1). The ca. 1.3 kb fragments are discriminated by digestion with \textbf{AflII} (\textit{S}_1-specific) and \textbf{BstBI} (\textit{S}_2-specific). The 436 bp fragment is discriminated by digestion with \textbf{NruI} (\textit{S}_3-specific). The ca. 350 bp fragments are digested with \textbf{SfcI} (\textit{S}_4-specific), \textbf{PpuMI} (\textit{S}_5-specific), \textbf{NdeI} (\textit{S}_6-specific), \textbf{AlwNI} (\textit{S}_7-specific), \textbf{HinII} (\textit{S}_8-specific), and \textbf{AccII} (\textit{S}_9-specific). Using this system, the \textit{S}-genotypes of ‘Niitaka’, ‘Nansui’, ‘Nangetsu’, ‘Amanogawa’, and ‘Ishiiwase’ were identified as \textit{S}_5\text{S}_9\text{S}_8\text{S}_1\text{S}_3, \textit{S}_3\text{S}_5\text{S}_9\text{S}_7\text{S}_1, \textit{S}_1\text{S}_9\text{S}_8\text{S}_5\text{S}_2, \textit{S}_9\text{S}_7\text{S}_8\text{S}_3\text{S}_1, and \textit{S}_5\text{S}_6\text{S}_8\text{S}_9\text{S}_2\text{S}_4\text{S}_3, respectively (Okada et al., 2004; Takasaki et al., 2004).

Moreover, these CAPS systems were used to reconsider the \textit{S}-genotypes of five cultivars for which there was some uncertainty. The \textit{S}-genotypes of ‘Akaho’, ‘Tanzawa’, ‘Kimizukawase’, ‘Choju’, and ‘Kumoi’ were previously determined by pollination experiments as \textit{S}_5\text{S}_7, \textit{S}_5\text{S}_6\text{S}_5\text{S}_6, \textit{S}_5\text{S}_5, \textit{S}_5\text{S}_5, and \textit{S}_5\text{S}_9\text{S}_6, respectively (Ogaki, 1958; Terami et al., 1946; Yasunobu et al., 1977). Using the CAPS systems, the \textit{S}-genotypes of ‘Akaho’, ‘Tanzawa’, ‘Kimizukawase’, ‘Choju’, and ‘Kumoi’ were reassigned as \textit{S}_5\text{S}_3, \textit{S}_5\text{S}_3, \textit{S}_5\text{S}_3, \textit{S}_5\text{S}_3, and \textit{S}_5\text{S}_3, respectively (Castillo et al., 2001b; Okada et al., 2004). The reassigned \textit{S}-genotypes of ‘Akaho’, ‘Tanzawa’, and ‘Kumoi’ were confirmed by pollination experiments (Castillo et al., 2001b; Okada et al., 2004). The reassigned \textit{S}-genotypes of ‘Kimizukawase’ and ‘Choju’ [‘Asahi’ (\textit{S}_5\text{S}_3) × ‘Kimizukawase’] were consistent with those according to the pedigree: ‘Shinsui’ (\textit{S}_5\text{S}_3) [‘Kikusui’ (\textit{S}_5\text{S}_3) × ‘Kimizukawase’], ‘Hayatama’ (\textit{S}_5\text{S}_3) [‘Gion’ (\textit{S}_5\text{S}_3) × ‘Kimizukawase’], and ‘Aikansui’ (\textit{S}_5\text{S}_3) [‘Choju’ × ‘Tama’ (\textit{S}_5\text{S}_3)] (Hiratsuka et al., 1998; Kajiura and Sato, 1990).

According to the pedigree, recent commercial cultivars of Japanese pear (46 cultivars that occupied a growing area of ≥ 1 ha in 2011) are genetically derived from 10 local cultivars: ‘Chojuro’ (\textit{S}_5\text{S}_3), ‘Shinkozo’ (unknown), ‘Doitsu’ (\textit{S}_5\text{S}_3), ‘Taihaku’ (\textit{S}_5\text{S}_3), ‘Wasekouzou’ (\textit{S}_5\text{S}_3), ‘Nijisseiki’ (\textit{S}_5\text{S}_3), ‘Akaho’ (\textit{S}_5\text{S}_3), ‘Okusankichi’ (\textit{S}_5\text{S}_3), ‘Imamuraaki’ (\textit{S}_5\text{S}_3), and ‘Amanogawa’ (\textit{S}_5\text{S}_3) (Kajiura and Sato, 1990; Sawamura et al., 2004, 2008). These local cultivars were used as the first parents in the Japanese pear-breeding program initiated in 1915. The \textit{S}-genotype of ‘Shinkozo’ has not yet been identified, but ‘Shinkozo’ has only been used once as a parent, for ‘Kimizukawase’ (\textit{S}_5\text{S}_3) [‘Shinkozo’ × ‘Doitsu’ (\textit{S}_5\text{S}_3)] (Kajiura and Sato, 1990). Therefore, the \textit{S}-genotypes of recent commercial cultivars in Japanese pear comprise various pairs of only eight \textit{S}-haplotypes (\textit{S}_1 to \textit{S}_8) derived from 10 ancestral local cultivars, and can be identified using the CAPS (\textit{S}_1 to \textit{S}_9) system.

To date, the CAPS systems have been used to identify the \textit{S}-genotypes of 77 Japanese pear cultivars (Castillo et al., 2001a, b, 2002; Ishimizu et al., 1999; Okada et al., 2004; Sawamura et al., 2002b, 2008; Takasaki et al., 2004) (Table 2); 20 cultivars were identified as \textit{S}_5\text{S}_3 and 9 cultivars as \textit{S}_5\text{S}_6\text{S}_9. This result indicates that there are many cross-incompatible combinations among Japanese pear cultivars. The \textit{S}_5-\textit{S}_9- and \textit{S}_1-\textit{S}_9-haplotypes are distributed in 43, 34, and 21 cultivars, respectively. One reason for the imbalance of \textit{S}-haplotypes among cultivars is because ‘Nijisseiki’ (\textit{S}_3\text{S}_3), ‘Kosui’ (\textit{S}_5\text{S}_3), ‘Hosui’ (\textit{S}_5\text{S}_3), and their progeny have often been used in Japanese pear-breeding programs (Sawamura et al., 2002b). Recent breeding pro-

\begin{table}[h]
\centering
\caption{\textit{S}-genotype assignments of Japanese pear cultivars using CAPS (\textit{S}_1 to \textit{S}_9) system.}
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{Cultivar} & \textbf{ca. 1.3 kb} & \textbf{436 bp} & \textbf{ca. 350 bp} & \textbf{S-genotype} \\
\hline
\textbf{AflII} & \textbf{BstBI} & \textbf{NruI} & \textbf{SfcI} & \textbf{PpuMI} & \textbf{NdeI} & \textbf{AlwNI} & \textbf{HinII} & \textbf{AccII} & \textbf{S-genotype} \\
\hline
Imamuraaki & -- & -- & -- & + & -- & -- & -- & -- & + & \textit{S}_5\text{S}_9 \\
Nijisseiki & + & + & -- & -- & -- & -- & -- & -- & -- & \textit{S}_5\text{S}_3 \\
Hosui & -- & -- & -- & -- & -- & -- & ++ & -- & + & \textit{S}_5\text{S}_7 \\
Okusankichi & -- & -- & -- & -- & ++ & -- & -- & -- & -- & \textit{S}_5\text{S}_3 \\
Ichiharawase & -- & + & + & + & -- & -- & -- & -- & -- & \textit{S}_5\text{S}_3 \\
Shinkou & -- & + & -- & -- & -- & -- & ++ & -- & -- & \textit{S}_5\text{S}_9 \\
\hline
\end{tabular}
\begin{flushright}
++: Two \textit{S-RNase} fragments were digested with the restriction endonuclease. \\
+: One \textit{S-RNase} fragment was digested with the restriction endonuclease.
\end{flushright}
\end{table}
grams have tended to use a few excellent cultivars as parents. Consequently, the new cultivars show a narrow range of S-genotypes. Therefore, S-genotype assignment is becoming more and more important for stable fruit production and efficient breeding.

As mentioned above, use of the CAPS systems is a rapid and reliable method for identifying the S-genotypes of recent commercial cultivars and new cultivars selected from their progeny. However, the S-genotypes identified using these systems should be confirmed by polliination experiments to increase the reliability of the results. Accurate S-genotypes determined using both CAPS systems and pollination experiments make it possible to select compatible pollinizers for orchards and compatible parents in breeding programs.

4) S-genotype assignments of local cultivars

In Japanese pear, many local cultivars appear to have been selected from chance seedlings or bud mutations that arose during cultivation. To date, 1212 cultivars of Japanese pear have been reported (Kajiura and Sato, 1990). Although the recent commercial cultivars have only eight S-haplotypes (S₁ to S₇, and S₉), it is unclear whether this low number is because fewer genetic resources were used in the breeding program, or because there is a low diversity of S-haplotypes in Japanese pear. Kim et al. (2007) identified a new Sk-RNase in a local cultivar of Japanese pear, ‘Kinchaku’ (S₉S₉), which is an important breeding material for resistance to pear scab disease (Abe and Kotobuki, 1998). Okada et al. (2009) analyzed the S-haplotypes of three local Japanese pear cultivars, ‘Senryo’, ‘Kuroki’, and ‘Ryoko’, and identified three S₉-RNase of European pear in ‘Senryo’ (S₉S₉), the S₁₁-RNase of Chinese pear in ‘Kuroki’ (S₉S₁₁), and the S₁₀-RNase of Chinese pear and Sk-RNase in ‘Hogukyo’ (S₉S₁₀). These results revealed that Japanese pear has more S-haplotypes than the nine previously identified (S₁ to S₉). The number of S-haplotypes is expected to increase as more local cultivars are analyzed.

Table 2. S-genotypes of Japanese pear cultivars identified using the CAPS system.

<table>
<thead>
<tr>
<th>S-genotype</th>
<th>Cultivar</th>
</tr>
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<tbody>
<tr>
<td>S₁S₂</td>
<td>Doitsu°, Echigoniishiki°, Hatsuaki°, Hayatama°</td>
</tr>
<tr>
<td>S₁S₃</td>
<td>Kumo°, Sekaichi°</td>
</tr>
<tr>
<td>S₁S₄</td>
<td>Higashino°, Suisi°, Yakumo°</td>
</tr>
<tr>
<td>S₁S₅</td>
<td>Akiakari°, Chouju°, Kimizukawase°, Shusui°</td>
</tr>
<tr>
<td>S₁S₆</td>
<td>Imamuraaki°</td>
</tr>
<tr>
<td>S₁S₇</td>
<td>Hogetsu°, Seiryuu°</td>
</tr>
<tr>
<td>S₁S₈</td>
<td>Ichiharawase°, Meigetsu°</td>
</tr>
<tr>
<td>S₂S₉</td>
<td>Amanogawa°</td>
</tr>
<tr>
<td>S₂S₁₀</td>
<td>Choujuro°, Hokkan°, Yoshikai°</td>
</tr>
<tr>
<td>S₂S₁₁</td>
<td>Gion°, Gold Nijisseiki°, Hokusin°, Kikusuri°, Nijisseiki°</td>
</tr>
<tr>
<td>S₃S₄</td>
<td>Wasekouzou°, Yanaga°, Yasato°</td>
</tr>
<tr>
<td>S₃S₅</td>
<td>Aikansui°, Ishiiwase°, Kumdai°</td>
</tr>
<tr>
<td>S₃S₆</td>
<td>Naitaki°, Natsumi°, Natsuhakari°, Ohtgon-nashi°, Seigyoku°, Shinseiki°, Shuurei°, Wakahikari°</td>
</tr>
<tr>
<td>S₃S₇</td>
<td>Akahoro°, Akemizu°, Hosu°</td>
</tr>
<tr>
<td>S₃S₈</td>
<td>Akitaka°, Echigo°, Ishiwasae°, Naitaka°</td>
</tr>
<tr>
<td>S₃S₉</td>
<td>Aikensui°, Asahi°, Hakkou°, Kisu°, Kiyomiki°, Kogiku°, Kosui°, Kotobukishinsui°, Kunitomi°, Momoechaki°, Oushuu°, Sagami°, Sankou°, Shinsui°, Shugyoku°, Taikou°, Tama°, Tanzawa°, Torikou°, Waseka°</td>
</tr>
<tr>
<td>S₄S₅</td>
<td>Akibae°</td>
</tr>
<tr>
<td>S₄S₆</td>
<td>Heiwa°</td>
</tr>
<tr>
<td>S₄S₇</td>
<td>Nangetsu°, Nansui°, Shinkou°, Shinsei°, Yachiyo°</td>
</tr>
<tr>
<td>S₄S₈</td>
<td>Shinsetsu°</td>
</tr>
<tr>
<td>S₄S₉</td>
<td>Okusankichi°</td>
</tr>
<tr>
<td>S₅S₁₀</td>
<td>Nikkori°</td>
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</tbody>
</table>

° Castillo et al. (2001b) |
°° Sawamura et al. (2002b) |
° Okada et al. (2004) |
°° Sawamura et al. (2008) |
° Ishimizu et al. (1999) |
° Castillo et al. (2002) |
° Takasaki et al. (2004) |
° Castillo et al. (2001a)
5) Development of an S-genotyping system using a pollen S candidate

Although the details of the pollen S gene are described later in section 3, multiple pollen S candidates, S-locus F-box brothers (SFBB) genes, have been identified in Japanese pear. Kakui et al. (2007) and Sassa et al. (2007) isolated 10 SFBBγ genes (SFBB1γ to SFBB8γ, and SFBB9γ) from Sγ to Sα, and Sβ-haplotypes. On the basis of the sequence polymorphisms of the SFBBγ genes, Kakui et al. (2007) developed a CAPS/derived cleaved amplified polymorphic sequence (dCAPS) system for S-genotyping of Japanese pear harboring Sα-genome. These results revealed that the Sα-haplotype lacks a sequence of > 4 kb including the full-length Sγ-RNase is missing from this haplotype (Sassa et al., 1997). However, the absence of an S-RNase fragment may be due to experimental errors, or could be because the cultivar harbors novel S-RNase alleles that were not amplified by the consensus primers. Therefore, the development of an Sβ-haplotype-specific DNA marker was necessary to confirm the Sβ-haplotype.

To identify the deleted region in the Sβ-haplotype, Okada et al. (2008) prepared a bacterial artificial chromosome (BAC) library from an Sβ-homozygote (SβSβ) in Japanese pear, and constructed a 570 kb BAC contig around the Sβ-RNase. Using primers designed from the contig sequence, the deleted region in the Sβ-haplotype was analyzed by PCR using the genomic DNA from Sγ and Sβ-homozygotes (SβSβ as the template, and by DNA sequencing of the BAC contig. These results revealed that the Sβ-haplotype lacks a 236 kb sequence spanning from 48 kb upstream to 188 kb downstream of the Sγ-RNase (Fig. 2A). To develop a DNA marker specific for the Sβ-haplotype, Okada et al. (2008) designed a new primer pair, “SM-F” and “SM-R”, from the regions outside both of the deletion breakpoints in the Sβ-haplotype (Fig. 2A). PCR using “SM-F” and “SM-R” primers amplified a

Fig. 2. DNA marker specific for Sβ-haplotype in Japanese pear. (A) Schematic genomic structures around Sγ-RNase in Sγ- and Sβ-haplotypes. Arrowhead indicates Sγ-RNase. Sβ-haplotype lacks 236 kb spanning from 48 kb upstream to 188 kb downstream of Sγ-RNase. Arrows indicate Sβ-haplotype-specific primers “SM-F” and “SM-R”. (B) Sβ-haplotype-specific DNA marker amplified from genomic DNA of Japanese pear by PCR with primers “SM-F” and “SM-R” (Okada et al., 2008). Fragments were subjected to 2% agarose gel electrophoresis. SC and SI indicate self-compatibility and self-incompatibility, respectively. 1: Sβ-homozygote, 2: Sγ-homozygote, 3: ‘Imamuraaki’ (SγSγ), 4: ‘Nijisseiki’ (SγSγ), 5: ‘Hosui’ (SγSα), 6: ‘Okusankichi’ (SγSβ), 7: ‘Ichiharawase’ (SβSα), 8: ‘Shinkou’ (SγSα).
666 bp fragment from genomic DNA of the $S_{f}^{sm}$ homoygote, but not from that of the $S_{r}$-homozygote or six SI cultivars harboring nine $S$-haplotypes ($S_{f}$ to $S_{9}$) (Fig. 2B). This result indicated that the 666 bp fragment can be used as an $S_{f}^{sm}$-haplotype-specific marker. Its reliability was confirmed by the selection of SC trees from the cross seedlings of ‘Osanjisseeiki’ ($S_{S_{f}^{sm}}$) × ‘Nansui’ ($S_{S_{r}}$) (Okada et al., 2008).

However, trees with the $S_{f}^{sm}$-haplotype are not always SC. The $S_{f}^{sm}$ pollen was accepted by the pistils of ‘Osanjisseeiki’ ($S_{S_{f}^{sm}}$), but rejected by those of ‘Nijissseeiki’ ($S_{S_{r}}$) and ‘Doitsu’ ($S_{S_{r}}$). This observation suggested that the $S_{f}^{sm}$ pollen is rejected by pistils harboring $S_{r}$- or $S_{f}$-haplotypes (Saito et al., 2012; Sato, 1993). (The details of this phenomenon are described later in section 3.) That is, $S_{S_{r}}S_{r}$ and $S_{S_{f}}S_{f}$ trees exhibit SI. To select SC trees efficiently, therefore, $S$-genotypes of cross seedlings must first be predicted based on the $S$-genotypes of both parents assigned using the CAPS system, and by the $S_{f}^{sm}$-haplotype-specific marker. If the predicted $S$-genotypes are not $S_{S_{f}}S_{f}$ or $S_{S_{r}}S_{r}$, SC trees can be selected from the cross seedlings using only the $S_{f}^{sm}$-haplotype-specific marker. If the predicted $S$-genotypes contain $S_{S_{f}}S_{f}$ or $S_{S_{r}}S_{r}$, SC trees can be selected by eliminating seedlings assigned to $S_{f}$- or $S_{r}$-haplotypes using the CAPS system from those identified using the $S_{f}^{sm}$-haplotype-specific marker. The combination of the $S_{f}^{sm}$-haplotype-specific marker and the CAPS system provides an early and reliable system to select SC Japanese pear, and will facilitate the breeding of SC cultivars with the $S_{f}^{sm}$-haplotype (Okada et al., 2008).

3. Analysis of the pollen $S$ gene and model for the molecular mechanism of GSI

The GSI of Japanese pear is controlled by two genetically linked genes: the pistil $S$ gene and the pollen $S$ gene at the $S$-locus. As mentioned above, the pistil $S$ gene of Japanese pear is the $S$-RNase. In Japanese pear, Liu et al. (2007) showed that self S-RNases inhibited pollen tube growth in vitro, but non-self S-RNase did not. In the Solanaceae family, which also exhibits S-RNase-based GSI, both self and non-self S-RNases enter pollen tubes growing in the style (Luu et al., 2000). In the Solanaceae, the RNase activity of S-RNases is essential for the rejection of self pollen, and pollen rRNAs are degraded after self-pollination (Huang et al., 1994; McClure et al., 1990). On the basis of these results in the Solanaceae, it has been speculated that the self S-RNase of Japanese pear inhibits the growth of the self pollen tubes in the style by degrading pollen rRNAs, but the RNase activity of all non-self S-RNases is inhibited by the pollen $S$ gene product. Additionally, recent studies have shown that, in vitro, the S-RNase of Japanese pear induces alterations in mitochondria (membrane potential collapse, cytochrome $c$ leakage, and swelling), disruption of tip-localized reactive oxygen species (ROS; which are required for tip growth), depolymerization of the actin cytoskeleton, and degradation of the nuclear DNA of incompatible pollen tubes. These findings suggested that programmed cell death (PCD) may specifically occur in incompatible pollen tubes (Liu et al., 2007; Wang et al., 2009, 2010).

In contrast to the pistil $S$ gene ($S$-RNase), the pollen $S$ gene of Japanese pear remained unknown for a long time. To identify the pollen $S$ gene in apple ($Malus × domestica$ Borkh.) and Japanese pear, Sassa et al. (2007) analyzed the $S$-locus in apple, and identified two homologous F-box genes from the $S_{r}$-haplotype and two from the $S_{f}$-haplotype. These multiple and related F-box genes were named $SFBB$ of $M. domestica$: $MdSFBB^{a,b}d_1$ and $MdSFBB^{a,b}d_2$ from the $S_{r}$-haplotype, and $MdSFBB^{3-a}$ and $MdSFBB^{3-b}$ from the $S_{f}$-haplotype. Using primers designed from the $MdSFBB$ sequences, six cDNAs were isolated by RT-PCR from pollen of the Japanese pear cultivar ‘Kosui’ ($S_{S_{r}}$): $PpSFBB^{a,b}$, $PpSFBB^{a,b}d_1$, and $PpSFBB^{a,b}d_2$ from the $S_{r}$-haplotype, and $PpSFBB^{a,b}d_1$, $PpSFBB^{a,b}d_2$, $PpSFBB^{a,b}d_3$, and $PpSFBB^{a,b}d_4$ from the $S_{f}$-haplotype. Because these SFBBs showed linkage to the $S$-RNase, $S$-haplotype-specific sequence divergence, and pollen-specific expression, they seemed to be a good candidate for the pollen $S$ gene in apple and Japanese pear (Sassa et al., 2007). However, a later linkage analysis of $PpSFBB^{a,b}$ and $S_{r}$-RNase in Japanese pear detected two recombinants among 59 segregants, suggesting that $PpSFBB^{a,b}$ ($PpSFBB6-S_{r}$) was not involved in pollen $S$ specificity (Kakui et al., 2011). Generally, F-box proteins function as one of the four major subunits (CUL1, SKP1, RBX1/ROC1, and an F-box protein) of the SCF complex that catalyzes the attachment of polyubiquitin chains to target proteins for their subsequent degradation by the 26S proteasome (Lechner et al., 2006). Therefore, SFBBs are speculated to mediate ubiquitination and degradation of all non-self S-RNases through the ubiquitin/26S proteasome system.

For further exploration of the pollen $S$ gene, Okada et al. (2008, 2011) constructed two BAC contigs around the $S_{r}$- and $S_{f}$-RNases of Japanese pear. A 649 kb region around the $S_{r}$-RNase and a 378 kb region around the $S_{f}$-RNase were sequenced, based on the prediction that the pollen $S$ gene was tightly linked to the $S$-RNase. As a result, six new pollen-specific F-box genes [$PpSFBB^{a,b}d_1$, $PpSFBB^{a,b}d_2$, $PpSFBB^{a,b}d_3$, $PpSFBB^{a,b}d_4$, $PpSFBB^{a,b}d_5$, and $PpSFBB^{a,b}d_6$] were identified named $S_{r}$-box0; Okada et al., 2008), and $PpSFBB^{d-2}$ were identified around the $S_{r}$-RNase, and 10 new pollen-specific F-box genes ($PpSFBB^{a,b}d_1$, $PpSFBB^{a,b}d_2$, $PpSFBB^{a,b}d_3$, $PpSFBB^{a,b}d_4$, $PpSFBB^{a,b}d_5$, $PpSFBB^{a,b}d_6$, $PpSFBB^{a,b}d_7$, $PpSFBB^{a,b}d_8$, and $PpSFBB^{a,b}d_9$) were found around the $S_{f}$-RNase (Fig. 3). $PpSFBB^{a,b}$, $PpSFBB^{a,b}d_1$, and $PpSFBB^{a,b}d_2$ were not within the sequenced region, and were located much further away from the $S_{f}$-RNase.
Moreover, Kakui et al. (2007, 2011) isolated 31 new PpSFBBs from different haplotypes of Japanese pear by PCR or RT-PCR. These studies on the pollen S gene (Kakui et al., 2007, 2011; Okada et al., 2008, 2011; Sassa et al., 2007) revealed that multiple PpSFBBs are clustered around the S-RNase of Japanese pear: 7 in the S1-haplotype, 11 in the S2-haplotype, 8 in the S3-haplotype, and 7 in the S4-haplotype. Interestingly, the deduced amino acid sequence identities of some PpSFBBs were higher between different S-haplotypes than within each S-haplotype (Okada et al., 2011). For example, there were high (>90%) pairwise deduced amino acid sequence identities between nine PpSFBB4 genes (PpSFBB4-a1–a4, PpSFBB4-d1–d2, and PpSFBB4-e–g) and 11 PpSFBB2 genes (PpSFBB2-a1–a5, PpSFBB2-d1–d5, and PpSFBB2-e–g); 92.1% identity between PpSFBB4-a2 and PpSFBB2-a1, 93.1% identity between PpSFBB4-a2 and PpSFBB2-a2, 94.9% identity between PpSFBB4-a2 and PpSFBB2-a4, and 99.0% identity between PpSFBB4-a2 and PpSFBB2-a4. Conversely, the deduced amino acid sequence identities ranged from 62.3% to 86.2% among the nine PpSFBB4 genes, and from 63.0% to 86.0% among the 11 PpSFBB2 genes. In a phylogenetic tree, 42 PpSFBBs isolated from S1- to S4-haplotypes clustered into eight types (SFBB1–SFBB8), and each type contained similar PpSFBBs isolated from different S-haplotypes (Kakui et al., 2011).

Similarly, 16–20 S-locus F-box (SLF) genes were identified in each S-haplotype in Petunia, a member of the Solanaceae family that exhibits the same S-RNase-based GSI as Japanese pear (Hua et al., 2007; Kubo et al., 2010, 2015; McCubbin et al., 2000; Wang et al., 2003, 2004; Williams et al., 2014a). These SLFs were classified into 18 types (SLF1–SLF18) based on their phylogenetic relationships (Kubo et al., 2015). Transformation experiments showed that at least eight of the SLFs (SLF1–SLF6, SLF8, and SLF9) were involved in pollen specificity (Kubo et al., 2010, 2015; Sijacic et al., 2004; Williams et al., 2014b). On the basis of these findings, a collaborative non-self recognition model was proposed. In this model, the pollen S gene in Petunia comprises multiple types of F-box genes. Each type of F-box protein recognizes and degrades only some of the non-self S-RNases, and multiple types of F-box proteins collaboratively inactivate all of the non-self S-RNases (Kubo et al., 2010).

As pollen S candidates, PpSFBBs in Japanese pear show characteristics that fit well with the collaborative non-self recognition model reported for Petunia, suggesting that this system may also exist in Japanese pear. This idea was supported by the results of a recent study on Japanese pear. For a long time, it was believed that S1 pollen and S4sm pollen in Japanese pear have the same function in pollen S specificity. Interestingly, however, pollination experiments showed that S4sm pollen was rejected by the pistils harboring not only the S1- but also the S2-haplotype, although S1 pollen was accepted by the pistils harboring the S1-haplotype (Saito et al., 2012). Moreover, the progeny of ‘Kimizukawase’ (S1S1) × ‘Osanijisseiki’ (S4S4sm) did not inherit the S4sm-haplotype, but the progeny of ‘Hosui’ (S3S3) × ‘Osanijisseiki’ (S4S4sm) did, indicating that S4sm pollen was accepted by the pistils harboring the S3- and S4-haplotypes but rejected by those harboring the S1-haplotype. On the other hand, the progeny of ‘Kimizukawase’ (S1S1) × ‘Nijisseiki’ (S2S2) inherited the S1-haplotype, showing that S1 pollen was accepted by the pistils harboring the S1-haplotype (Kakui et al., 2011). These results suggested that the S1sm-haplotype also has a mutation in the pollen S1 gene. As the S1sm-haplotype lacks not only the S1-RNase but also PpSFBB4-d1 (S-F-box0) (Okada et al., 2008) (Fig. 3), the collaborative non-self recognition model would predict that PpSFBB4-d1 is an element of the pollen S gene in Japanese pear that specifically recognizes the S1-RNase (Kubo et al., 2010) (Fig. 4).

Very recently, another SC mutant 415-1 was produced by crossing ‘Kosui’ (S1S1) with pollen from gamma-irradiated ‘Kosui’. The S-genotype of 415-1

Fig. 3. Schematic genomic structures and locations of PpSFBBs around S1-RNase and S2-RNase in Japanese pear. Arrows indicate directions of transcription of S-RNases and PpSFBBs.
was identified as $S_4S_5$ using the CAPS system. Cross-pollinations of $415-1$ ($S_4S_5$) $\times$ ‘Shugyoku’ ($S_4S_5$) and $415-1$ ($S_4S_5$) $\times$ ‘Oushuu’ ($S_4S_5$) were cross-incompatible, whereas cross-pollinations of ‘Shugyoku’ ($S_4S_5$) $\times$ $415-1$ ($S_4S_5$) and ‘Oushuu’ ($S_4S_5$) $\times$ $415-1$ ($S_4S_5$) were cross-compatible. These results suggested that $415-1$ is a pollen-part mutant (Sawamura et al., 2013). A ploidy analysis, and the segregation in the progeny of $S_4$-haplotypes and simple sequence repeat (SSR) markers around the $S_4$-locus, indicated that the pollen-part self-compatibility of $415-1$ was not caused by a loss of function of pollen $S$ genes in the $S_4$- or $S_3$-haplotype, but by segmental duplication of the chromosome containing the $S_4$-haplotype. Consequently, $415-1$ was able to produce $S_4S_5$ pollen with two different $S$-haplotypes ($S$-heteroallelic pollen). This pollen is capable of breaking down SI by competitive interaction between the two different $S$-haplotypes in a single pollen grain (Mase et al., 2014). Because the pollen of a pollen-part SC mutant would be cross-compatible with cultivars of any $S$-genotype, $415-1$ can serve as additional useful breeding material to produce new SC cultivars with high fruit quality.

Matsumoto et al. (2006) reported that two tetraploids arising as a bud mutation from ‘Shinkou’ ($S_4S_9$) exhibited self-compatibility. The cross-pollination of the tetraploid $\times$ ‘Shinkou’ ($S_4S_9$) was cross-incompatible, whereas that of ‘Shinkou’ ($S_4S_9$) $\times$ the tetraploid was cross-compatible. These results suggested that the tetraploids are also pollen-part mutants.

According to the collaborative non-self recognition model, mutations that disrupt the function of the pollen $S$ gene, such as the deletion of $PpSFBB^{4-d1}$ in the $S_4$-haplotype (Okada et al., 2008), cause pollen to be incompatible with more $S$-haplotypes because decreasing the repertoire of the pollen $S$ gene eliminates the ability to recognize and degrade its target $S$-RNase(s). Conversely, mutations to increase the repertoire of the pollen $S$ gene, such as segmental duplication of the chromosome containing the $S$-locus in $415-1$ (Mase et al., 2014) or tetraploidization (Matsumoto et al., 2006), allow pollen to recognize and degrade more $S$-RNases. Therefore, inducing mutations to increase the repertoire of the pollen $S$ gene would be one effective method to create new pollen-part SC mutants of Japanese pear.
4. Conclusions

Japanese pear is an important fruit tree with a long history of cultivation in Japan. Since almost all cultivars of Japanese pear exhibit SI, S-genotype assignment is essential for stable fruit production and efficient breeding. Studies on the pistil S gene have led to the establishment of the CAPS system for S-genotyping based on sequence polymorphisms of the S-RNase allele (Castillo et al., 2002; Ishimizu et al., 1999; Takasaki et al., 2004). Similarly, studies on the pollen S gene have led to the development of the CAPS/dCAPS system for S-genotyping based on sequence polymorphisms of the PpSFBB\(^7\) allele (Kakui et al., 2007). The use of the CAPS systems has allowed more S-genotypes to be determined quickly and accurately. Consequently, the S-genotypes of the main commercial cultivars and important breeding materials have been revealed. This information has already been used to select compatible pollinizers for orchards and compatible parents in breeding programs. In addition, analyses of the SC mutant ‘Osanijisseiki’ have led to the development of a DNA marker specific for the S\(^{100}\)-haplotype (Okada et al., 2008). This marker has allowed the early and accurate selection of SC trees, and it has already been used in breeding programs to develop new SC cultivars with high fruit quality. Thus, studies on the GSI of Japanese pear have contributed to the development of new techniques to identify S-genotypes and to select SC trees efficiently.

In addition to its importance as a fruit tree, Japanese pear is an important model plant for elucidating the molecular mechanism of S-RNase-based GSI in the Rosaceae. The recent discovery of PpSFBBs as a candidate for the pollen S gene suggested that Japanese pear may use the collaborative non-self recognition system that has been identified in Petunia of the Solanaceae. On the other hand, surprisingly, recent studies have reported that the features of the GSI differ between the tribe Pyreae (Prunus and Malus) and the tribe Amygdaleae (Prunus) in the Rosaceae (for reviews, see De Franceschi et al., 2012; Meng et al., 2011; Sassa et al., 2010; Yamane and Tao, 2009). These findings suggested that the role of the pollen S gene may differ between the tribe Pyreae (Prunus and Malus) and the tribe Amygdaleae (Prunus) in the Rosaceae, although Prunus also has an S-RNase as the pistil S gene and an F-box gene [called SFB (S-haplotype-specific F-box) or SLF (S-locus F-box)] as the pollen S gene (Entani et al., 2003; Usui et al., 2003). In Japanese pear, however, pollen S candidates have only been found recently, and further experiments are required to confirm that multiple PpSFBBs are the pollen S gene in the GSI. Future research on PpSFBBs should determine how many PpSFBBs exist in each S-haplotype; which PpSFBB recognizes each S-RNase; how PpSFBBs degrade non-self S-RNases; and how a single S-RNase and multiple PpSFBBs coevolved to maintain GSI. Further research is also required to determine the mechanism of self pollen tube growth arrest. Resolving these issues will provide a better understanding of the mechanism and the evolution of GSI, and will contribute to the development of new techniques for stable fruit production and efficient breeding of Japanese pear.

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