Invited Review

Distinct Self-recognition in the *Prunus* S-RNase-based Gametophytic Self-incompatibility System

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Prunus fruit tree species exhibit S-ribonuclease (S-RNase)-based gametophytic self-incompatibility (GSI). This system is also present in the families Plantaginaceae and Solanaceae and the tribe Maleae of the family Rosaceae. In S-RNase-based GSI, self/nonself-recognition between the pistil and pollen is controlled by the pistil *S* determinant S-ribonuclease gene (*S-RNase*) and the pollen *S* determinant F-box gene(s). Accumulated evidence indicates the *Prunus* pollen *S* locus contains a single F-box gene, while that of other plants consists of multiple F-box genes. The pollen *S* F-box genes are called *S* haplotype-specific F-box (SFB), *S*-locus F-box brothers (SFBB), and *S*-locus F-box (SLF) in *Prunus*, Maleae, and Solanaceae species, respectively. The consequences of pollen *S* gene mutations and heterodiallelic pollen production differ between *Prunus* species and other plants, suggesting there are different pollen *S* functions during self/nonself-recognition. The GSI systems of *Prunus* and other plants are believed to include the ubiquitin proteasome system for protein degradation. However, *Prunus* SFB is assumed to facilitate the S-RNase cytotoxic effects during self-recognition, while SLFs and SFBBs are thought to function collaboratively during nonself-recognition to avoid S-RNase cytotoxicity. This review summarizes the distinct features of the S-RNase-based GSI mechanism in *Prunus* species, with special references to the recent advances in our understanding of S-RNase-based GSI.

Key Words: general inhibitor, pollen *S* determinant, SCF complex, self/nonself-recognition.

Introduction

The genus *Prunus* L. is classified in the tribe Amygdalae within the subfamily Amygdaloideae in the family Rosaceae. It consists of over 200 species distributed across the northern hemisphere, including several economically important cultivated species, which are horticulturally often referred to as stone fruits (McNeill et al., 2012; Potter et al., 2007). Successful pollination, fertilization, and seed formation are indispensable to *Prunus* species fruit production because they do not bear fruit parthenocarpically. In addition to the absence of parthenocarpic ability, fruit set in *Prunus* species is hindered by a prezygotic reproductive barrier called the self-incompatibility (SI) system, which allows pistils to reject pollen from self and genetically related individu-

als (de Nettancourt, 2001; Tao and Iezzoni, 2010). The mode of *Prunus* SI is classified as gametophytic SI (GSI), where pollen specificity is determined based on its own haploid genome (Fig. 1). *Prunus* SI specificity is controlled by a single polymorphic *S* locus harboring pistil *S* and pollen *S* determinant genes, and variants of the *S* locus are referred to as *S* haplotypes. When the *S* haplotype of the pollen matches either of the two *S* haplotypes of the pistils, the pollen is recognized as self-pollen and the pollen tube stops growing in the style (de Nettancourt, 2001). In commercial orchards, simultaneously flowering cross-compatible cultivars belonging to different incompatibility groups are required to ensure sufficient fruit set.

During the late 1990s to early 2000s, *S*-ribonuclease (*S-RNase*) and F-box genes were identified as the pistil *S* and pollen *S* genes in *Prunus* species, respectively (Entani et al., 2003; Sonneveld et al., 2005; Tao et al., 1997, 1999; Ushijima et al., 1998, 2003, 2004; Yamane et al., 2003b). These findings led to the development of polymerase chain reaction-based *S* haplotyping, which is a fast, accurate, and stable method to determine the *S* haplotype. This procedure has been widely and success-

Received; February 11, 2016. Accepted; February 28, 2016.

First Published Online in J-STAGE on April 27, 2016.

This work was supported by Grant-in-Aid (nos. 20248004, 24248007 and 15H02431) for Scientific Research (A) from Japan Society for the Promotion of Science (JSPS) to R.T.

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fully used to re-evaluate *S* haplotypes of cultivars, and has recently been used in *Prunus* fruit tree breeding programs (Beppu et al., 2003; Habu et al., 2008; Ikeda et al., 2004b; Sonneveld et al., 2001, 2003; Sutherland et al., 2004; Tao et al., 1999; Yamane and Tao, 2009; Yamane et al., 2003c, 2009). Although these advances make it possible to circumvent some of the problems related to SI, further characterizing the underlying molecular mechanism is necessary to artificially control GSI in *Prunus* species.

Fig. 1. Genetic control of gametophytic self-incompatibility (GSI). (A) In GSI, the pollen is rejected when its *S* haplotype matches either of the pistil *S* haplotypes. The cross is incompatible when the *S* haplotypes of the male and female parents match completely ($S^1S^2 \times S^1S^2$), while the cross is compatible when a portion of the *S* haplotypes is shared by the male and female parents ($S^1S^2 \times S^1S^3$). (B) The GSI is generally controlled by a single *S* locus, which contains at least two genes, one for pollen specificity and another for pistil specificity.

The GSI system involving S-RNase as its pistil *S* determinant has been detected in the tribe Maleae of the family Rosaceae, as well as in the families Solanaceae, Plantaginaceae, and Rubiaceae (Anderson et al., 1986; Asquini et al., 2011; McClure et al., 1989; Nowak et al., 2011; Sassa et al., 1992, 1993, 1996; Xue et al., 1996). Phylogenetic analyses of S-RNase and its related sequences have revealed that the S-RNase-based GSI system has a single evolutionary origin, dating back about 120 million years (Igic and Kohn, 2001; Steinbachs and Holsinger, 2002; Vieira et al., 2008a). The F-box genes have been commonly identified as the pollen *S* determinants, except in the family Rubiaceae, which was recently observed to exhibit the S-RNase-based GSI with no identified pollen *S* determinant. Based on the similarities of the *S* determinant molecules, the molecular mechanism regulating *Prunus* GSI was assumed to be similar to that of the S-RNase-based GSI in plants from the family Solanaceae, as well as other plants. The features of the *Prunus* GSI molecular mechanism that distinguish it even from the corresponding systems in plants from the sister tribe Maleae have been identified, as the functional aspects of pollen *S* F-box proteins in different plant families have become characterized (Table 1, discussed below in detail; de Franceschi et al., 2012; McClure et al., 2011; Meng et al., 2010; Tao and Iezzoni, 2010). Currently, the *Prunus* pollen *S* F-box protein is assumed to function in self-recognition, while those in other plant families appear to function in nonself-recognition. Interestingly, recent genome-wide studies have suggested duplication events involving the *S* locus occurred in ancestral rosaceous species, and plants from the genus *Prunus* and tribe Maleae adopted different paralogs as specificity determinants (Aguiar et al., 2015; Morimoto et al., 2015). Although the *Prunus*-specific mechanism is still largely uncharacterized, an increasing number of studies have been conducted to clarify the molecular basis of *Prunus* GSI recognition.

This review summarizes the current information regarding the distinct molecular recognition mechanism

Table 1. Summary of the pollen *S* gene characteristics in different plant taxa exhibiting S-RNase based gametophytic self-incompatibility.

Plant family (subfamily)	Tribe	Representative SI genera	Pollen <i>S</i>		
			entity	gene name	proposed function
Rosaceae (Amygdaloideae)	Amygdalae	<i>Prunus</i>	Single gene	<i>SFB</i>	Self-recognition to trigger S-RNase cytotoxicity
	Maleae	<i>Malus</i> , <i>Pyrus</i>	Multiple paralogs	<i>SFBBx^z</i>	Nonself-recognition to inhibit S-RNase cytotoxicity
Solanaceae		<i>Petunia</i> , <i>Nicotiana</i> , <i>Solanum</i>	Multiple paralogs	<i>SLFx^z</i>	Nonself-recognition to inhibit S-RNase cytotoxicity
Plantaginaceae		<i>Antirrhinum</i>	Multiple paralogs (putative)	<i>SLF(x)^z</i>	Nonself-recognition to inhibit S-RNase cytotoxicity
Rubiaceae		<i>Coffea</i>	unknown	unidentified	—

^z x corresponds to a specific number that distinguishes paralogs

of the *Prunus* S-RNase-based GSI. We first describe the characteristics of the *S* determinants and the common S-RNase-based GSI molecular mechanism detected in non-*Prunus* taxa. We then introduce the *Prunus*-specific consequences of mutations in the pollen *S* locus and heteroallelic pollen (HAP) production. Based on these outcomes, we discuss the distinct molecular mechanism of *Prunus* S-RNase-based GSI, with special reference to the recent advances in our understanding of S-RNase-based GSI.

Identification and Characterization of S-RNase

S-RNase was first identified in *Nicotiana glauca* as a stylar basic glycoprotein (approximately 30 kDa) for which the gene co-segregated with its pistil *S* allele (Anderson et al., 1986). Later, it was revealed that the stylar glycoprotein exhibited RNase activity, and its sequence was homologous to that of *Aspergillus* RNase-T2 (McClure et al., 1989). Transformation experiments in *Nicotiana* and *Petunia* species confirmed that S-RNase loss-of-function leads to self-compatibility (SC) because of an inability to reject self-pollen. In contrast, S-RNase gain-of-function confers the ability to reject pollen with the newly introduced *S* haplotype. This indicates S-RNase is the sole pistil *S* gene of GSI in the family Solanaceae, and the encoded enzyme likely has cytotoxic effects against self-pollen (Lee et al., 1994; Murfett et al., 1994).

In the 1990s, following the identification of the pistil *S* locus in the family Solanaceae, S-RNase genes were cloned as the pistil *S* gene candidates in *Antirrhinum* species from the family Plantaginaceae, *Pyrus* and *Malus* species from the tribe Maleae of the family Rosaceae, and *Prunus* species from the family Rosaceae (Brootharerts et al., 1995; Sassa et al., 1992, 1993, 1996; Tao et al., 1997, 1999; Ushijima et al., 1998; Xue et al., 1996). The S-RNases of other genera in the tribe Maleae, including *Crataegus*, *Eriobotrya*, and *Sorbus*, and those of the family Rubiaceae were also isolated (Asquini et al., 2011; Carrera et al., 2009; Nowak et al., 2011; Raspé and Kohn, 2002). Transformation experiments in *Malus* species and analyses of the loss-of-function mutants in *Pyrus* and *Prunus* species indicated that Rosaceae S-RNase genes were the sole pistil *S* genes, and encoded enzymes that were assumed to be cytotoxic to self-pollen (Brootharerts et al., 2004; Hanada et al., 2009; Sanzol, 2009; Sassa et al., 1997; Watari et al., 2007; Yamane et al., 2003a).

The S-RNases from these families share many common features, although there is insufficient information regarding Rubiaceae S-RNases. All reported S-RNases are specifically expressed in the pistil, with high expression levels in the style (Anderson et al., 1986; Asquini et al., 2011; Nowak et al., 2011; Tao et al., 1999; Xue et al., 1996). *In situ* hybridization results indicated S-RNases are preferentially expressed in transmitting tissue tract cells of the style in the families

Solanaceae and Plantaginaceae (Anderson et al., 1989; Xue et al., 1996). An N-terminal secretion signal (i.e., signal peptide) is common in S-RNases, indicating they localize in the extracellular matrix of transmitting tissues, through which pollen tubes elongate, as observed in the family Solanaceae and tribe Maleae (Anderson et al., 1989; Certal et al., 1999). These features reflect the nature of the interaction between S-RNase and pollen.

S-RNase is a glycoprotein exhibiting RNase activity, which requires the presence of a well-conserved catalytic core motif consisting of two His residues. Transgenic experiments and mutant analyses in the family Solanaceae demonstrated that replacing the conserved His residues in S-RNase resulted in SC, indicating RNase activity is essential for S-RNase cytotoxicity during the SI reaction (Huang et al., 1994; Kowiyama et al., 1994). RNase activity in a Maleae SC haplotype is absent, possibly because of an amino acid insertion in the region facing the active site (Nyska et al., 2013). Although S-RNases exhibit diversity in the number and position of N-glycosylation sites depending on the alleles, the functions of N-linked glycans in self/nonself-recognition is still unclear. Analyses of *Petunia* and *Solanum* species using a recombinant non-glycosylated S-RNase indicated that carbohydrate moieties are not required to determine specificity or the SI reaction (Karunanandaa et al., 1994; Soulard et al., 2013). Soulard et al. (2013) also reported that recombinant S-RNase lacking its innate N-glycosylated sites, but with an artificially inserted N-glycosylation site in its hypervariable region, exhibited dual specificity. This enabled the recognition of an additional nonself *S* haplotype in addition to the original self *S* haplotype. It was suggested that a decrease in the number of S-RNase glycosylation sites may be related to a lower threshold for self-pollen rejection (Liu et al., 2008).

Analyses of the primary structure of solanaceous S-RNases identified five conserved domains (C1–C5) and two hypervariable (HV) regions (HV_a and HV_b), both of which are located between C2 and C3 (Fig. 2; Ioerger et al., 1991). The conserved domains are assumed to be important for maintaining the basic three dimensional structure of a functioning S-RNase. In fact, domains C2 and C3 each contain one of the two His residues required for enzymatic activity. An investigation of the enzyme crystal structure revealed C1, C2, and C5 comprised the RNase active site (Ida et al., 2001). Additionally, the conserved N-glycosylation site is located in the C2 domain. Although the role of the C4 region remains unknown, transgenic experiments involving amino acid substitutions indicated that none of the C4 amino acid residues are important for the SI reaction (Qin et al., 2005). The HV_a and HV_b regions exhibit high sequence variability and are located on the surface of the crystal structure, which make them prime candidates for the *S* specificity-determining regions (Ida

Fig. 2. S-RNase protein structures. Triangles indicate intron locations. *S-RNase* genes of all plant families that exhibit S-RNase-based gametophytic self-incompatibility have an intron located in hypervariable region a (HVa) or the rosaceous hypervariable region (RHV). The intron located between the signal peptide and the C1 region is present only in *Prunus S-RNase* genes. SP: signal peptide; C1–C5: conserved regions; HVb: hypervariable region b; RC4: rosaceous conserved region; NT: amino-terminus; CT: carboxyl-terminus.

et al., 2001; Ioerger et al., 1991). However, a series of experiments in which chimeric S-RNases were expressed in transgenic plants led to inconsistent conclusions about the roles of HV regions, suggesting they had necessary, but insufficient, roles in determining specificity (Kao and McCubbin, 1996; Matton et al., 1997; Zurek et al., 1997).

The S-RNases of species from families other than Solanaceae contain structural features similar to those of solanaceous S-RNases. For example, plantaginaceous S-RNases have five conserved regions and two hypervariable regions. However, there is little sequence homology between the C4 regions of Plantaginaceae and Solanaceae S-RNases (Xue et al., 1996). Rosaceous S-RNases differ from solanaceous S-RNases more than plantaginaceous S-RNases (Fig. 2). Although rosaceous S-RNases have structural characteristics similar to those of S-RNases from other plants, their C4 and HV regions are distinct (Ushijima et al., 1998). Rosaceous S-RNases possess a fourth conserved domain, called RC4, which differs from the C4 region of solanaceous and plantaginaceous S-RNases regarding position and amino acid sequence. Furthermore, only a single rosaceous HV (RHV) region has been detected in the position corresponding to that of HVa (Ishimizu et al., 1998; Ushijima et al., 1998). Interestingly, the amino acid sites under positive selection in solanaceous S-RNases, where non-synonymous substitutions are preferred to synonymous substitutions, are located mainly in the HVa and HVb regions. In contrast, sites under positive selection in rosaceous S-RNases are distributed over their entire sequence, except for the region encoding the N-terminal secretion signal (Ishimizu et al., 1998; Ortega et al., 2006; Vieira et al., 2007, 2010). These results suggest that the RHV region may not be the only one with a significant role in determining specificity in rosaceous S-RNases. This possibility is supported by the fact that the same RHV sequence is present in different *S-RNase* alleles that exhibit diverse specificities (Matsumoto et al., 2010; Ortega et al., 2006; Zisovich

et al., 2004). Furthermore, the conserved N-glycosylation site of rosaceous S-RNases is located in RC4, whereas it is located in C2 in the S-RNases of all other plants.

Phylogenetic analyses of S-RNases from different families have revealed their shared origin, as well as lineage-specific clustering (Igic and Kohn, 2001; Nowak et al., 2011; Steinbachs and Holsinger, 2002; Vieira et al., 2008a). Their intron structures also reflected lineage specificity. A single intron is present in the coding sequences of Solanaceae, Plantaginaceae, and Maleae *S-RNase* genes. There is an additional intron in the coding sequence located downstream of the secretion signal in *Prunus S-RNase* genes (Fig. 2; Tao et al., 1999). S-RNases acquired different features as they evolved, even if they retained their cytotoxicity to self-pollen.

Identification of F-Box Genes as the Pollen *S* Gene Candidate

The pollen *S* locus involved in S-RNase-based GSI has been extensively studied in terms of its expected characteristics, such as its complete linkage to *S-RNase*, allele-specific sequence polymorphisms, and pollen-specific expression. Map-based cloning and chromosome walking approaches to study the Solanaceae, Plantaginaceae, and Rosaceae *S* loci have commonly identified multiple pollen-expressed F-box genes flanking the *S-RNase* gene (Fig. 3; de Franceschi et al., 2011; Entani et al., 2003; Lai et al., 2002; Minamikawa et al., 2010; Okada et al., 2008, 2010, 2013; Sassa et al., 2007; Ushijima et al., 2001, 2004; Wang et al., 2003, 2004; Wheeler and Newbigin, 2007; Zhou et al., 2003). Compared with that of the genus *Prunus*, the recombination-suppressed region around the *S* locus is much larger and more highly heterochromatic in the tribe Maleae and the families Solanaceae and Plantaginaceae, making it difficult to isolate the true pollen *S* gene in these three plant taxa (Entani et al., 1999a; Wang et al., 2012; Yang et al., 2007). This

Fig. 3. Schematic diagram of the *S* locus controlling S-RNase-based gametophytic self-incompatibility. Multiple F-box genes expressed in pollen are located around the *S-RNase* gene in all plant families. In the tribe Maleae and the families Solanaceae and Plantaginaceae, the *S* loci can be more than several Mb and contain multiple F-box genes, each of which acts as a pollen *S* gene. In contrast, the *Prunus* *S* locus is as small as approximately 70 kb, and contains a single pollen *S* F-box gene, *SFB*. Other pollen-expressed F-box genes (e.g., *SLFL1–3*) flank the *S* locus. The *Prunus* *SLFL1–3* genes are not involved in determining pollen specificity.

region was estimated to be 4.4 Mb in *Petunia* species, at least 1 Mb in *Malus* species, and could be as small as about 70 kb in *Prunus* species (Entani et al., 2003; Ushijima et al., 2001, 2003; Wang et al., 2003, 2012).

The pollen *S* candidate F-box genes (except for those in *Prunus* species) exhibit a lower level of allelic sequence polymorphism than expected based on the allelic sequence diversity of *S-RNases*. The Solanaceae and Plantaginaceae F-box genes are referred to as *S-locus F-box* (*SLF*) or *SLF-like* (*SLFL*) genes. In the tribe Maleae, the pollen *S* candidate F-box genes are named *S-locus F-box brothers* (*SFBBs*). The pollen *S* candidate F-box genes of the families Solanaceae and Plantaginaceae and the tribe Maleae exhibit high allelic amino acid sequence identities of 86.4%–100%, 90%–98.6%, and 83.2%–99%, respectively, which are much higher than the sequence identities of their corresponding *S-RNases* (i.e., 40.1%–79.4%, 40%–55%, and 58.0%–92.5%, respectively) (Kakui et al., 2011; Kubo et al., 2010; Xue et al., 1996; Zhou et al., 2003). In contrast, a single *Prunus* F-box gene located in the *S* locus had high allelic sequence polymorphisms (66%–82.5% amino acid identities), which were equivalent to the allelic sequence polymorphisms in *S-RNase* (54.6%–82.5%). The *Prunus* F-box gene was identified as a pollen *S* gene candidate, and two research groups gave it different names, *SLF* and *S haplotype-specific F-box* (*SFB*) (Entani et al., 2003; Ikeda et al., 2004a; Ushijima et al., 2003, 2004; Yamane et al., 2003b). In this review, the term *SFB* is used to reflect its features that differ from the pollen *S* gene of other plants with S-RNase-based GSI.

Collaboration of Multiple F-Box Genes to Determine Specificity in Taxa Other than *Prunus*

After pollen *S* gene candidates were identified, transgenic and mutant analyses were used to identify the true

Fig. 4. Function of the pollen *S* locus during the inhibition of nonself *S-RNases* in the tribe Maleae and families Solanaceae and Plantaginaceae. (A) Tetraploid plants exhibit self-compatibility, which is conferred by heteroallelic pollen. This self-compatibility results from the inhibition of all *S-RNases* through the complementary effects of two pollen *S* genes. (B) Nonself recognition by multiple pollen *S* F-box proteins, each of which recognizes and inhibits a subset of nonself *S-RNases*. Additionally, a complete set of pollen *S* F-box proteins function against everything but self *S-RNases*.

pollen *S* gene. Early mutagenesis studies in the family Solanaceae revealed that a single pollen grain containing two different pollen *S* alleles (i.e., HAP) became self- and cross-compatible, indicating that the pollen *S* gene functions as an inhibitor of nonself *S-RNase* (Fig. 4A; Entani et al., 1999b; Golz et al., 1999, 2001). This phenomenon, called competitive interaction, can be explained by assuming the pollen *S* gene product inhibits everything but self *S-RNase*. Competitive interactions have also been observed in the family Plantaginaceae and the tribe Maleae (Adachi et al., 2009; Lewis and Modilbowska, 1942; Mase et al., 2014; Qi et al., 2011; Sassa et al., 2009; Xue et al., 2009). Transgenic studies determined that the integra-

tion of an additional F-box allele from a different *S* haplotype resulted in SC by inducing the competitive interaction (Qiao et al., 2004b; Sijacic et al., 2004). Based on these studies, it was suggested that a single F-box gene corresponded to the pollen *S* gene in these plant families, and the single F-box gene was named *SLF* to indicate it is a true pollen *S* gene, while the other F-box genes in the *S* locus were referred to as *SLFL*.

However, recent studies revealed there are elements in addition to the pollen *S* gene in the family Solanaceae. Kubo et al. (2010) reported that two *SLFL* genes (renamed as *SLF2* and *SLF3*; see below) could also induce competitive interactions in pollen grains of certain *S* haplotypes. This indicated *SLF* and *SLFL* genes function together as the pollen *S* gene. The *SLF* and *SLFL* genes cloned from pollen were classified into six subgroups, with the *SLF* gene renamed as *type-1 SLF* (*SLF1*) and the *SLFL* genes renamed as *type-2–6 SLF* (*SLF2–6*). To date, 16–20 *SLF* genes have been identified in each *S* haplotype, and are classified into 18 types in *Petunia* species (Kubo et al., 2015; Williams et al., 2014a, b). Of these, *SLF1–6*, *-8*, and *-9* were confirmed to cause competitive interactions in certain *S* haplotypes. Each *SLF* can inhibit a subset of S-RNases, and a set of all *SLFs* in a given *S* haplotype is assumed to inhibit all nonself S-RNases. Several *SLFs* appear to target the same S-RNase, as indicated by the fact silencing of one *SLF* does not necessarily result in cross-incompatibility (Sun and Kao, 2013).

The presence of multiple pollen *S* factors is also consistent with observations in the tribe Maleae. Mutant analyses indicated *SFBB1* of the *Pyrus pyrifolia* *S*⁴ haplotype is required for pollen tube growth in a pistil producing *S*¹-RNase, indicating multiple *SFBB* genes, including *SFBB1*, confer cross-compatibility (Kakui et al., 2011; Okada et al., 2008; Saito et al., 2012). Although only two or three types of *SFBB* were reported when they were initially discovered, at least 8, 11, and 16 types have subsequently been identified in the genera *Pyrus*, *Malus*, and *Sorbus*, respectively (Aguiar et al., 2013; Kakui et al., 2011; Okada et al., 2013; Sassa et al., 2007). Furthermore, in contrast to the low sequence polymorphism observed among alleles of the same type of *SFBB*, synonymous and non-synonymous substitution rates among different types of *SFBB* were comparable to those among different *S*-RNase alleles (Kakui et al., 2011). This suggests the diversification of SI specificity was due to the duplication and differentiation of the *SFBB* loci as well as the diversification of *S*-RNase alleles. Aguiar et al. (2013) reported that the most closely related *Sorbus* *SFBB* genes diverged 8.27 million years ago (MYA). The most distantly related *SFBB* genes diverged 23.96 MYA, which is similar to the age of the oldest Maleae S-RNase specificity lineage (approximately 23 million years old; Vieira et al., 2010).

Considered together, several lines of recent evidence

indicate the Solanaceae and Maleae pollen *S* locus consists of multiple *SLF* and *SFBB* F-box genes, respectively, and each F-box protein inhibits a subset of nonself S-RNases (Fig. 4B). Although there has been no report describing the multiplicity of the plantaginaceous pollen *S*, a very low level of *SLF* allelic sequence polymorphism (i.e., 97%–99% amino acid identities) may indicate the existence of multiple F-box genes at the pollen *S* locus.

Molecular Mechanism of Self/Nonself Discrimination by Multiple F-Box Genes

A protein degradation model for the self/nonself discrimination mechanism was developed based on the expected biochemical functions of an F-box protein (Meng et al., 2010; Ushijima et al., 2004). An F-box protein forms part of the SCF complex, which consists of Skp1, Cull1, and Rbx1 (Deshaies and Joazeiro, 2009). The SCF complex is one of the RING-type E3 ubiquitin ligases, which polyubiquitinates substrate proteins to be degraded by the ubiquitin proteasome system (UPS). In the SCF complex, the F-box protein determines substrate specificity, Skp1 serves as an adaptor to connect variable F-box proteins to Cull1, and Cull1 forms a core catalytic scaffold with Rbx1 (Zheng et al., 2002). The Rbx1 component recruits the ubiquitin-charged E2 enzyme with its zinc finger domain, and the ubiquitin moiety is transferred from E2 to the substrate. The protein degradation model proposed that the SCF complex with the pollen *S* gene product, which was once assumed to be a single element, recognize and polyubiquitinate all nonself S-RNases (but not self S-RNases) for degradation (Fig. 5). This nonself-recognition is considered to be completed through the collaborative activities of multiple pollen *S* F-box proteins (Iwano and Takayama, 2012; Kubo et al., 2010; Wang and Kao, 2012). A series of biochemical experiments have provided data supporting this nonself S-RNase degradation model.

Pollen *S* F-box proteins from the families Solanaceae and Plantaginaceae and the tribe Maleae, were confirmed to form SCF complexes with the Skp1-like protein SSK1 (Huang et al., 2006; Li et al., 2014; Minamikawa et al., 2014; Xu et al., 2013; Yuan et al., 2014; Zhao et al., 2010). In Solanaceae species, down-regulation of either SSK1 or Cull1 results in a loss of cross-compatibility, indicating the indispensable role of the SCF^{SLF} complex in compatible reactions, and the involvement of the UPS in S-RNase detoxification (Li and Chetlat, 2014; Zhao et al., 2010). S-RNase binding protein 1 (SBP1) was also identified as a component of an alternative E3 ligase complexed with the pollen *S* F-box protein (Hua and Kao, 2006). *In vitro* experiments revealed that the solanaceous SBP1 interacts with *SLF*, Cull1, and the E2 ubiquitin-conjugating enzyme. On the contrary, recombinant solanaceous *SLF* expressed in pollen grains did not appear to interact with SBP1, but

Fig. 5. The molecular basis of self/nonself discrimination in the tribe Maleae and families Solanaceae and Plantaginaceae involves a degradation model for the biochemical function of the pollen *S* gene. (A) Inhibition of nonself S-RNases with the ubiquitin proteasome system. The SLF/SFBBs form the SCF complex with the SLF/SFBB-interacting Skp1-like protein (SSK1), Cul1, and Rbx1. The SLF/SFBB in the complex recognizes nonself S-RNases as the substrate, and SCF^{SLF/SFBB} attaches ubiquitin molecules by recruiting the E2 enzyme carrying ubiquitin. The polyubiquitinated nonself S-RNases are degraded by the 26S proteasome. (B) Inability to inhibit self S-RNases. The SLF/SFBBs in the complex are unable to recognize self S-RNases as the substrate because of low affinity or a self-specific interaction. Therefore, self S-RNases remain intact and their cytotoxicity induces the self-incompatibility reaction.

mainly formed a canonical SCF complex with SSK1 (Entani et al., 2014; Hua and Kao, 2006; Li et al., 2014). Although SBP1 was also identified in the tribe Maleae, inconsistent results were obtained regarding the interaction between SBP1 and SFBB. Minamikawa et al. (2014) observed that SBP1 interacted with SFBB and Cul1, which contradicted the findings of Yuan et al. (2014). Nevertheless, the possible involvement of SBP1 in SI is still unclear because SBP1 by itself was reported to interact with S-RNase in the family Solanaceae and the tribe Maleae (Hua and Kao, 2006, 2008; Minamikawa et al., 2013; Sims and Ordanic, 2001). Furthermore, polyubiquitination of S-RNase by SBP1 was observed *in vitro* in Solanaceae species, while no such polyubiquitination was detected in Maleae species (Hua and Kao, 2006, 2008; Yuan et al., 2014).

Selective polyubiquitination of nonself S-RNases by pollen or recombinant SLF was observed *in vitro* in Solanaceae and Plantaginaceae species (Chen et al., 2012; Entani et al., 2014; Hua and Kao, 2006; Qiao et al., 2004a). Additionally, a recent *in vivo* study in a *Petunia* species confirmed that S-RNase is polyubiquitinated and degraded in compatible pollen tubes, but not in incompatible ones (Liu et al., 2014). Entani et al.

(2014) reported that *Petunia* *S*⁷-SLF2, which induces competitive interactions in *S*⁹ and *S*¹¹ pollen, but not in *S*⁵ or *S*⁷ pollen, polyubiquitinated S-RNases from *S*⁹ and *S*¹¹ haplotypes, but not from *S*⁵ and *S*⁷ haplotypes. These results suggest the polyubiquitination of S-RNases by the pollen *S* F-box protein is likely involved in regulating compatibility, and that all nonself S-RNases may be degraded by multiple pollen *S* F-box proteins in pollen tubes.

Specific self/nonself interactions between pollen *S* F-box proteins and S-RNases are considered the basis of selective polyubiquitination. However, it is unclear how self S-RNases evade polyubiquitination by pollen *S* F-box proteins. Several SLFs from the family Solanaceae and SFBBs from the tribe Maleae are known to interact with a subset of nonself S-RNases. Additionally, some SLFs from Solanaceae and Plantaginaceae species interact with self S-RNases (Hua and Kao, 2006; Hua et al., 2007; Kubo et al., 2010; Qiao et al., 2004a; Xu et al., 2013; Yuan et al., 2014). Hua and Kao (2006) revealed that *S*²-SLF1 has a greater affinity for *S*³-RNases than for self S-RNases, which suggests self-interactions may not be strong enough to induce polyubiquitination of self S-RNases. Interestingly, *S*²-SFBB1 can polyubiquitinate self *S*²-RNases *in vitro*, while the polyubiquitination of S-RNases by nonself SFBB was not tested (Yuan et al., 2014).

***Prunus* Pollen *S* F-Box Proteins Are the Sole Determinants Conferring Incompatibility Reaction Specificity**

Unlike other plants exhibiting S-RNase-based GSI, in which multiple pollen *S* F-box proteins function as the determining factor, in *Prunus* species, *SFB* is the sole pollen *S* determinant. Furthermore, it has been suggested there are distinct functions for the *Prunus* pollen *S* locus. As in other plants, multiple pollen-expressed F-box genes, *SFB*, and three *S*-locus F-box like genes (*SLFL1*–*3*), are located at the *S* locus and its flanking regions in *Prunus* species (Fig. 3; Entani et al., 2003; Ushijima et al., 2003, 2004). *SLFL1*, *SLFL2*, and *SLFL3* exhibit a much lower level of allelic sequence polymorphism (i.e., approximately 92.5%) than *SFB* (i.e., 66%–82.5%), and there are considerable differences in DNA sequences among the genes (Entani et al., 2003; Matsumoto et al., 2008; Ushijima et al., 2003, 2004). Genetic analyses revealed *SLFL1* was highly associated with the *S* locus, but had undergone some recombination events, while *SLFL2* and *SLFL3* were not associated with the *S* locus. This indicates *SFB* is the only F-box gene located in the *Prunus* *S* locus (Entani et al., 2003; Ushijima et al., 2001; Vieira et al., 2008b). The *S* haplotype with its *SLFL1* deleted was also reported to result in normal SI and cross-compatibility, which suggests *SFB* is the only F-box gene determining the specificity of the SI reaction in

Prunus species (Matsumoto et al., 2008).

Analyses of naturally occurring and artificially produced SC pollen-part mutant (PPM) *S* haplotypes indicate *SFB* is the sole pollen *S* gene in *Prunus* species (Tao and Iezzoni, 2010). The first PPM *S* haplotypes that were characterized at the molecular level were sweet cherry *S*^{4'} and Japanese apricot *S*^f, and both were reported to encode a truncated SFB caused by a frame-shift mutation (Ushijima et al., 2004). A complete deletion of *SFB* was later observed in the PPM *S*^{3'} haplotype of the sweet cherry (Sonneveld et al., 2005). These findings contradict the predicted pollen *S* gene function in other plants with S-RNase-based GSI, in which the pollen *S* locus is involved in degrading and detoxifying cytotoxic S-RNases. However, these results may indicate *SFB* is indispensable for the *Prunus* SI reaction. Subsequent studies on other SC PPM *S* haplotypes have confirmed the indispensable role of SFB in SI, and it is now generally accepted that SFB is not necessary for compatible reactions in *Prunus* species (Hauck et al., 2006a, b; Marchese et al., 2007; Tao et al., 2007; Tsukamoto et al., 2006, 2010; Vilanova et al., 2006; Yamane and Tao, 2009; Yamane et al., 2009). Given that the pollen *S* gene specifically inhibits everything except self S-RNase, the loss of the pollen *S* locus should result in self- and cross-incompatibility. Supporting this hypothesis, an SC PPM *S* haplotype conferred by the loss-of-function mutation of the pollen *S* locus has never been detected in non-*Prunus* plant taxa that show S-RNase-based GSI (de Franceschi et al., 2012; Golz et al., 2001; McClure et al., 2011; Meng et al., 2010; Sassa et al., 2009).

A distinct function for SFB has been revealed based on differences between *Prunus* species and other plants regarding HAP. The tetraploid sour cherry (*Prunus cerasus*) includes SI and SC individuals. A series of genetic and molecular studies revealed the genotype-dependent loss of SI, which requires the accumulation of non-functional *S* haplotypes (Hauck et al., 2006a, b; Tsukamoto et al., 2006, 2008a, b, 2010). Sour cherry produces diploid pollen, including HAP, and its SI behavior appears to be determined by the one-allele-match model (Fig. 6A; Hauck et al., 2006b). In this model, diploid pollen is rejected when it carries a functional pollen *S* allele matching its cognate functional S-RNase allele in the style. This finding indicates that SFB functions exclusively during incompatible but not compatible reactions, and that the competitive interaction is absent in *Prunus* species. The possible existence of competitive interactions in *Prunus* species was recently reported for SC Chinese cherry cultivars (*Prunus pseudocerasus* L.) (Gu et al., 2013, 2014; Huang et al., 2008). However, these results should be carefully verified because SC in Chinese cherry might be explained by mutations of factors unrelated to the *S* locus or the accumulation of non-functional *S* haplotypes (Tao and Iezzoni, 2010; Yu et al., 2010; Zhang et al., 2010).

The distinct SI characteristics of *Prunus* species are the result of the distinct molecular functions of SFB (Fig. 6B; Tao and Iezzoni, 2010). Phylogenetic analyses of the pollen *S* F-box genes indicated *SFB* possesses unique features (Aguiar et al., 2015; Vieira et al., 2009). *Prunus* *SFB* has been classified in a separate clade from that containing the Solanaceae, Plantaginaceae, and Maleae pollen *S* F-box genes (Fig. 7). Greater allelic polymorphisms observed in *SFB* enabled the characterization of its primary structural features. There are three variable regions in SFB (i.e., Vn, V1, and V2), and two hypervariable regions (i.e., HVa and HVb; Fig. 6C; Ikeda et al., 2004a; Nunes et al., 2006). Four of the five variable and hypervariable regions are located in the C-terminal region, and may influence allele-specific substrate recognition.

Working Model for Self/Nonspecific Discrimination in *Prunus* Species

Based on what is currently known, the *Prunus* pollen *S* F-box protein (i.e., SFB) has been hypothesized to help release the cytotoxic effects of self S-RNases and induce incompatible reactions. In contrast, the pollen *S* F-box proteins (i.e., SLFs and SFBs) of other species recognize and detoxify nonspecific S-RNases to avoid incompatible reactions (Figs. 4B and 6B). To explain the detoxification of *Prunus* S-RNases, researchers predicted the existence of a hypothetical general inhibitor (GI) that detoxifies S-RNases in compatible pollen tubes (Luu et al., 2001; Tao and Iezzoni, 2010). To characterize the distinct self-recognition molecular mechanism in *Prunus* species, the biochemical activities of SFB will need to be clarified, and the hypothetical GI should also be identified.

The SFB protein is considered to be part of the SCF complex because the F-box motif is conserved at the N-terminal, and was observed to be under purifying selection (Ikeda et al., 2004a; Nunes et al., 2006). This has been supported by the identification of the functional Skp1-like protein that interacts with SFB (i.e., SSK1). Additionally, the UPS may help regulate SI in *Prunus* species (Matsumoto et al., 2012). Interestingly, *Prunus* SSK1 is orthologous to the SSK1 of Solanaceae, Plantaginaceae, and Maleae species, while its pollen *S* F-box protein partner is phylogenetically remote (Matsumoto et al., 2012; Xu et al., 2013). These facts may indicate that the *Prunus* pollen *S* locus has gained different functions in SI recognition from the pollen *S* loci of other plants during its evolution, despite the shared basal SI molecular components. It is apparent that cytotoxic S-RNase is not the substrate of SCF^{SFB} in *Prunus* species.

Based on the knowledge obtained from other taxa with S-RNase-based GSI, it is possible that E3 ligases that contain a protein orthologous to SLF, SFB, or SBP1 may be involved in degrading S-RNases. Therefore, *Prunus* *SLFL* genes are the best candidates be-

cause phylogenetic studies have highlighted their close relationship with *SFBB* genes (Fig. 7; Aguiar et al., 2015; de Franceschi et al., 2012; Matsumoto et al., 2008; Morimoto et al., 2015; Sassa et al., 2007, 2009). The SLFLs may help degrade S-RNases in a manner similar to the hypothesized activity of SFBBs. As expected, SLFLs can interact with SSK1, indicating they function as E3 ligases (Matsumoto et al., 2012). Although phylogenetic studies have suggested it is possible, it remains unknown whether any SLFLs function as a GI. The detection of a disrupted *SLFL1* gene in a functional self-incompatible *S* haplotype indicates that *SLFL1* is not the only factor necessary for cross compatibility. The most likely GI candidate is *SLFL3* because of the *SLFL* genes, only *SLFL3* exhibits up-regulated expression during compatible pollination (Habu and Tao, 2013). The *Prunus* homolog of SBP1 is another candidate for GI. However, the *Prunus* SBP1 homolog does not appear to interact with S-RNases *in vitro* (Matsumoto and Tao, 2012a).

One possible explanation for how S-RNase cytotoxicity is controlled by SFBs and GIs to induce SI involves the degradation of the GI by SFB in a self-recognition-specific manner (i.e., GI degradation model; Fig. 8; Tao and Matsumoto, 2012). All S-

Fig. 7. Neighbor-joining tree constructed using aligned amino acid sequences corresponding to the *S* locus-linked F-box genes from different taxa exhibiting S-RNase-based gametophytic self-incompatibility. The *S* locus-linked F-box genes from well characterized *S* haplotypes in each plant species were chosen to construct the tree. MEGA ver. 6.0 (Tamura et al., 2013) was used to align the amino acid sequences and generate a phylogenetic tree with 1000 bootstrap replicates. *Prunus* SLFLs clustered with the Maleae SFBBs.

RNases are assumed to be recognized and inhibited by a GI unless they are affected by the cognate SFB. The SCF^{SFB} recognizes the complex consisting of self S-RNase and the GI, and polyubiquitinates the GI for degradation to release cytotoxic self S-RNases. In a compatible reaction, the SCF^{SFB} would not recognize

complexes containing nonself S-RNase, which would then be inhibited by a GI. This model is consistent with the *Prunus* SI characteristics described to date (i.e., self-compatibility conferred by a loss of SFB function, and the absence of competitive interactions in HAP) (Hauck et al., 2006b; Sonneveld et al., 2005; Tsukamoto et al., 2010; Ushijima et al., 2004; Yamane et al., 2003a). However, this hypothesis needs to be carefully verified, which means the GI must be identified.

Self-incompatibility Events That Arrest Self-pollen Tube Growth after Self/Nonself Discrimination

The molecular mechanism of self/nonself discrimination controlled by specificity determinants is likely different between *Prunus* species and other plants with S-RNase-based GSI. However, self S-RNases commonly avoid being degraded in pollen tubes. The degradation of RNA in incompatible pollen tubes is commonly observed in *Nicotiana* and *Solanum* species of the family Solanaceae and in the tribe Maleae of the Rosaceae (Hiratsuka et al., 2007; Liu et al., 2009, 2012; McClure et al., 1990). It remains unclear if an SI reaction cascade caused by self S-RNases varies among different plant taxa.

S-RNase behavior in pollen tubes has been extensively studied in Solanaceae species. It is believed that extracellular S-RNases are non-selectively endocytosed and invade the pollen cytosol similar to other protein toxins or viruses. This is because self/nonself discrimination is assumed to occur in the cytosol of pollen tubes where pollen S F-box proteins are localized (Liu et al., 2014; McClure, 2011; Wang and Xue, 2005). In fact, self and nonself S-RNases are taken in by compatible and incompatible pollen tubes in Solanaceae species (Boivin et al., 2014; Goldraij et al., 2006; Liu et al., 2014; Luu et al., 2000). However, based on microscopic observations, two different models have been proposed to explain how self-discrimination induces SI reactions (Figs. 9 and 10).

In *Petunia* and *Solanum* species, S-RNases are distributed in the cytoplasm of compatible and incompatible pollen tubes (Boivin et al., 2014; Liu et al., 2014; Luu et al., 2000). However, investigations involving immunoelectron microscopy revealed that S-RNase abundance decreases in the cytoplasm of compatible pollen tubes, and accumulates in the cytosol of incompatible pollen tubes, 18–24 h after pollination in *Solanum* species (Boivin et al., 2014). Additionally, higher GFP expression levels during compatible pollination than during incompatible pollination was observed from 24 h after pollination in transgenic *Solanum* plants (Liu et al., 2012). Furthermore, S-RNase uptake by *in vitro* cultured pollen tubes decreased in *Petunia* compatible pollen tubes (Liu et al., 2014). Based on these results, we suggest the detoxifi-

cation of S-RNases that have non-selectively entered the cytoplasm depends on the degradative activities of pollen S F-box proteins, and the SI reaction is caused by the cytotoxicity of non-degraded and accumulated self S-RNases (i.e., protein degradation model; Fig. 9).

A different scenario has been proposed following a study involving a *Nicotiana* species (Goldraij et al., 2006). Confocal microscopic analyses identified differences in the localization of S-RNases between compatible and incompatible pollen tubes (Goldraij et al., 2006). First, S-RNases taken in by pollen tubes were sequestered in vacuolar compartments. Later the vacuoles were degraded only in incompatible pollen tubes, which enabled S-RNases to enter the cytoplasm. It was assumed that some of the sequestered S-RNases avoid compartmentalization using an unknown route to participate in self/nonself discrimination. The non-degraded self S-RNases in the cytosol induce the vacuolar breakdown during incompatible pollination. In this scenario, it was assumed that the cytotoxic effects of S-RNases against pollen tubes relied on the compartmentalization controlled by self/nonself discrimination. The SI reaction would be induced by some of the self S-RNases present in the cytosol, and completed by the cytotoxicity of the S-RNases released from vacuoles (i.e., compartmentalization model; Fig. 10). This model is supported by observations of pollinated pistils lacking the stylar-part SI modifier, such as HT-B, 120K, and the unidentified 4936-factor (Goldraij et al., 2006; Jiménez-Durán et al., 2013; McClure et al., 2011). Down-regulation or mutation of the stylar-part modifiers results in continued vacuolar compartmentalization, which causes SC. Interestingly, the accumulation of sequestered HT-B in pollen vacuoles induces such disruption. The accumulation of HT-B in vacuoles continued in incompatible pollen tubes, while HT-B abundance decreased in the vacuoles of compatible pollen tubes (Goldraij et al., 2006). Another stylar-part SI modifier, StEP, which is a Kunitz-type proteinase inhibitor produced in the stigma and taken up by pollen tubes, is involved in SI by stabilizing HT-B in incompatible pollen tubes (Jiménez-Durán et al., 2013). Vacuolar breakdown is believed to be induced by the inhibition of HT-B degradation, which is supposed to be controlled by self-recognition mediated by S-RNases and F-box proteins in the pollen tube cytosol. However, it is unclear whether the SI reaction varies even among Solanaceae species. Interestingly, the suppression of HT-B leads to SC not only in *Nicotiana* species, but also in *Petunia* and *Solanum* species. This suggests there is a common HT-B role during the SI reaction in Solanaceae species, which share the same mechanism (O'Brien et al., 2002; Puerta et al., 2009).

The detection of self S-RNase may induce unknown cytotoxic effects that stimulate early intracellular changes before wide-scale pollen RNA degradation. Additionally, actin organization may be affected by S-

Fig. 8. The molecular basis of *Prunus* self/nonself discrimination involves the general inhibitor (GI) degradation model, which is based on *Prunus* pollen *S* biochemical functions. (A) Nonself S-RNases are thought to be recognized and inhibited by the unidentified GI. The inhibition of nonself S-RNases is believed to be unaffected by SFB. (B) The SFB is assumed to recognize the self S-RNase-GI complex and polyubiquitinate GI. Degradation of the polyubiquitinated GI by the ubiquitin proteasome system leads to the release of active self S-RNases, which enables the incompatibility reaction.

Fig. 9. Degradation model for the incompatibility reaction in pollen tubes. S-RNases non-selectively enter the cytoplasm of pollen tubes growing through the style. Left panel: compatible reaction. S-RNases taken up from nonself *S* haplotypes are polyubiquitinated by SCF^{SLF} and degraded by a proteasome. Right panel: incompatible reaction. Self S-RNases remain active in the pollen cytosol and degrade pollen RNA to elicit cytotoxicity.

Fig. 10. Compartmentalization model for the incompatibility reaction in pollen tubes. The HT-B and S-RNases are endocytosed by pollen tubes, and sequestered in vacuoles. Some S-RNases enter the cytoplasm from the endosome to participate in self/nonself discrimination with SLFs. Left panel: compatible reaction. Vacuolar compartmentalization is retained in compatible pollen tubes to circumvent the cytotoxic effects of S-RNases. This compartmentalization is maintained by an unknown HT-B degradation mechanism. Escaped S-RNases are also degraded by SLFs. Right panel: incompatible reaction. Escaped self S-RNases avoid being degraded by SLFs, and are assumed to inhibit HT-B degradation. The accumulation of HT-B disrupts vacuolar compartmentalization to release self and nonself S-RNases, which degrade pollen RNA to elicit cytotoxicity.

RNase cytotoxicity. Incompatible pollen tube growth is inhibited before vacuolar disruption, and F-actin disorganization occurs at the initiation of pollen tube growth inhibition during *in vitro* and *in vivo* experiments in *Nicotiana* species (Roldán et al., 2012, 2015). Delayed *Solanum* incompatible pollen tube growth occurs within 12 h of pollination. This is well before the considerable decrease in the synthesis of mRNA and protein, which has been observed once vacuoles are degraded 24 h after pollination in incompatible pollen tubes (Liu et al., 2012, 2014). Although there has been no report on the relationship between actin disruption and delayed incompatible pollen tube growth in *Solanum* species, S-RNases were observed to interact with actin through eEF1A, and to be co-localized with actin in incompatible pollen tubes (Soullard et al., 2014).

Our understanding of the SI reaction in the tribe Maleae has recently increased. S-RNase was observed to be non-selectively taken up by *in vitro* cultured apple pollen tubes. The uptake appeared to be mediated by the cytoskeleton and an ABC transporter family protein (MdABCF) localized in the pollen tube membrane (Meng et al., 2014a). This ABCF-mediated S-RNase-uptake pathway was also required for SI in *Petunia* species. In pollen tubes, S-RNases were initially localized

in vesicles, but then migrated into the cytoplasm to induce cytoskeletal disorganization, possibly through interactions with the specific apple Rho-like GTPase homolog (MdROP8; Meng et al., 2014b, c). The cytoskeletal disorganization may contribute to the delayed self-pollen tube growth during the early stages. Programmed cell death eventually inhibited self-pollen tube growth (Liu et al., 2007; Wang et al., 2009, 2010). Disturbance in generation of reactive oxygen species in incompatible pollen tubes is expected to lead to cytoskeletal disruption, mitochondrial collapse, and DNA fragmentation. Higher transglutaminase activity in incompatibly pollinated apple styles, in which proteins, including cytoskeletal components, may be modified by the crosslinking of polyamines to form high molecular weight aggregates, may lead to programmed cell death (del Duca et al., 2010).

Only a few studies have examined the intracellular SI events in *Prunus* species. In the early electron microscopy analyses by Uwate and Lin (1980), no obvious structural differences were observed between compatible and incompatible pollen tubes 6 h after pollination. Cytoskeletal changes may be involved in the *Prunus* SI reaction because the artificially reduced S-RNases (in a reducing intracellular environment) interact with actin *in vitro* (Matsumoto and Tao, 2012b). Habu and Tao (2013) recently identified thousands of genes differentially regulated between self- and cross-pollinated pistils using next-generation sequencing technologies. Interestingly, no homologs of the solanaceous pistil factors, such as HT-B or I20K, were detected, indicating there is a distinct *Prunus* SI reaction. A pollen-part non-S determinant (i.e., pollen modifier) may be important for elucidating some aspects of the *Prunus* SI reaction (Vilanova et al., 2006; Wünsch and Hormaza, 2004; Wünsch et al., 2010). In several sweet cherry and apricot cultivars, a mutated pollen modifier conferred SC without affecting *SFB* expression. Interestingly, the modifier loci of sweet cherry and apricot cultivars were located at the distal end of linkage group 3 (*M* locus), which is the syntenic region of the Maleae *S* locus (Cachi and Wünsch, 2011, 2014; Zuriaga et al., 2012, 2013). However, none of genes reported to be involved in SI are located in this region (Habu and Tao, 2013; Zuriaga et al., 2012). Identifying the pollen modifier gene will provide new insights into the *Prunus*-specific SI mechanism. Undoubtedly, further research is required to fully elucidate *Prunus* self/nonself-recognition, with a particular need for a histochemical analysis of the *Prunus* SI system.

Conclusion and Perspectives

Studies during the last decade have suggested the recognition mechanisms of the S-RNase-based GSI mechanism in *Prunus* are different from those of other taxa, including the families Solanaceae and Plantaginaceae and the tribe Maleae from the family

Rosaceae. There have been no findings that contradict the hypothesis that *Prunus* S-RNases have cytotoxic effects similar to the expected activities of S-RNases in other plants that exhibit S-RNase-based GSI. However, there is considerable evidence indicating the *Prunus* pollen *S* F-box protein may function differently than the corresponding proteins in other plants. *Prunus* SFB is believed to specifically recognize self S-RNases to induce their cytotoxicity, while the pollen *S* F-box proteins of other plants detoxify nonself S-RNases. In *Prunus* species, the GI is believed to detoxify all S-RNases. Recent biochemical and phylogenetic studies on SFB revealed several GI candidates, and provide important information regarding the distinct self-recognition mechanism in *Prunus* species. We expect that the molecular mechanism controlling *Prunus* SI will be fully elucidated in the near future, enabling the regulation of *Prunus* SI.

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