Chapter 9
Molecular Biology of S-RNase-Based Self-Incompatibility

Y. Zhang and Y. Xue

Abstract Many flowering plants have developed self-incompatibility (SI) systems to avoid inbreeding and to promote out-crossing. Among the various SI systems, gametophytic SI (GSI) in the Solanaceae, Plantaginaceae and Rosaceae is believed to be the most common type, in which the specificity of SI response is controlled by a single polymorphic locus, termed the self-incompatibility *S*-locus. It has been shown that this locus is organised in a haplotype fashion carrying at least two genes determining the self and non-self pollen recognition specificity: *S-RNase* (*S-Ribonucleases*) expressed in pistil (pistil *S*) and *SLF* (*S*-Locus F-box)/*SFB*(*S*-haplotype-specific F-box) genes in pollen (pollen *S*). In this chapter, we present and discuss the current knowledge about molecular biology of S-RNase-based self-incompatibility.

Abbreviations

120K	120 kDa glycoprotein
AhSLF	*Antirrhinum hispanicum SLF*
C1 to C5	Conserved regions in the S-RNase sequence
CUL	Cullin
ECM	Extracellular Matrix
F-box	A protein motif; often components of SCF ubiquitin–ligase complexes
GSI	Gametophytic self-incompatibility
HT-B	H-Top Band; a small, novel asparagine-rich protein
HVa and HVb	Hypervariable regions in the S-RNase sequence

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PhSBP1	*Petunia hybrida S-RNase binding protein1*
PiSLF	*Petunia inflata SLF*
PPMs	Pollen-part mutants
RNAi	RNA interference
rRNA	Ribosomal ribonucleic acid
SC	Self-compatible
SCF	Skp1-cullin-F-box complex
SFB/SLF	*S-Locus F-box*/*S-haplotype-specific F-box* (the pollen *S*-determinant in many GSI systems; *SLF* in *Antirrhinum* and *Petunia*; *SFB* in *Prunus*)
SI	Self-incompatibility
S-locus	Self-incompatibility locus
S-RNase	*S*-locus ribonuclease (the pistil *S*-determinant in many GSI systems)
SSK	SLF-*interacting SKP1-like*
TAC	Transformation-competent artificial chromosome

9.1 Introduction

Self-incompatibility (SI) is one of the most important systems adopted by many flowering plants to prevent self-fertilisation and thereby to generate and maintain genetic diversity within a species. Classic genetic studies established that SI in most species is controlled by a single polymorphic locus, the self-incompatibility *S*-locus. It is now aware that this locus contains at least two separate genes: one controlling the self and non-self pollen recognition specificity on the pistil side (pistil *S*), and the other on the pollen side (pollen *S*), thus the term *haplotype* is used to describe variants of the *S*-locus. Pollen inhibition occurs when the same *S*-haplotype is expressed by both pollen and pistil (de Nettancourt 2001).

The molecular nature of the *S*-locus has been extensively studied in several species with different genetic features, and accordingly, the SI systems have been classified into several types. Generally, we can define these types of SI as the Brassicaceae-type, Solanaceae-type and Papaveraceae-type SI, because the species from the three families were used first for molecular studies and subsequent findings revealed that they represent three different molecular types of the *S*-locus (McCubbin and Kao 2000; Sims 2005; Takayama and Isogai 2005). The Solanaceae-type SI appears to be the most phylogenetically widespread form of SI found in angiosperms and is shared by two more families, the Plantaginaceae (formerly placed in the Scrophulariaceae) (Xue et al. 1996; Olmstead et al. 2001) and Rosaceae (Sassa et al. 1996; Ishimizu et al. 1998). This type of SI is genetically determined as a single-locus gametophytic SI (GSI) system, of which the recognition specificity is determined by the haplotypes of the polymorphic *S*-locus: pollen tube growth is

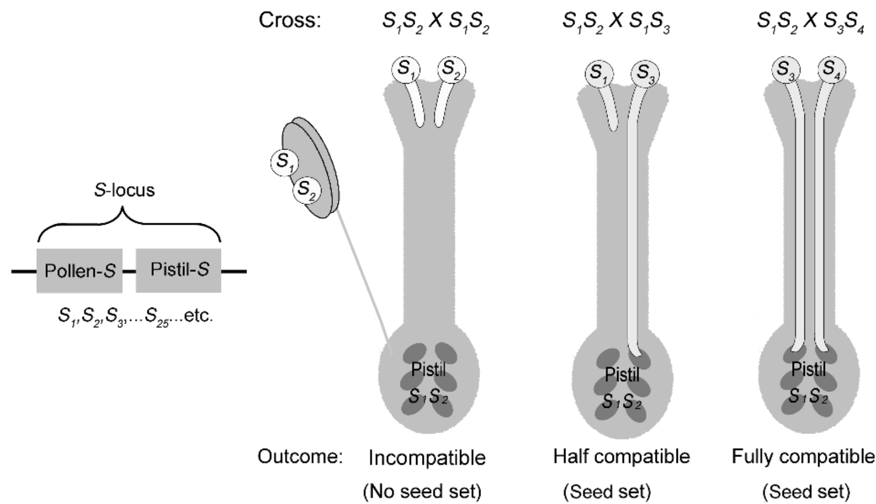


Fig. 9.1 Genetic control of gametophytic SI (GSI). Self-incompatibility is usually determined by a single polymorphic *S*-locus that is organised in a haplotype fashion carrying at least two genes: one controlling the self and non-self pollen recognition specificity on the pistil side (pistil *S*), and the other on the pollen side (pollen *S*). In GSI, the pollen SI phenotype is gametophytically controlled. Thus, half the pollen from an S_1S_2 plant is phenotypically S_1 and another half S_2. Pollen inhibition occurs on a 'like-matches-like' basis. When there is a match between the pollen *S*-haplotype and either of the two haplotypes present in the pistil, an incompatible reaction results and inhibition of the 'self' pollen occurs. This type of SI gives three possible classes of reaction: incompatible (all pollen is inhibited), half-compatible (50% inhibited, 50% grows normally) or compatible (all pollen grows normally) (modified from McClure and Franklin-Tong 2006, with kind permission from Springer)

inhibited when its *S*-haplotype is the same as either of the *S*-haplotypes of the diploid pistil; conversely, pollen with any *S*-haplotype not present in the pistil is compatible (Franklin-Tong et al. 2003; McClure and Franklin-Tong 2006) (Fig. 9.1). Much of the molecular information about this type of SI has been obtained from four genera of the Solanaceae (*Lycopersicon*, *Nicotiana*, *Petunia* and *Solanum*), three genera of the Rosaceae (*Malus*, *Prunus* and *Pyrus*) and one genus of the Plantaginaceae (*Antirrhinum*) (Kao and Tsukamoto 2004; McClure 2004, 2006). Since their female *S*-determinant (*S-RNase*) was the first gene identified in the *S*-locus, this type of SI is often referred to as the S-RNase-based SI. In this chapter, we present the current knowledge about the molecular biology of S-RNase-based SI. The readers are also referred to several excellent reviews about the earlier work in this area (McClure et al. 2000; Cruz-Garcia et al. 2003; Roalson and McCubbin 2003; Wang et al. 2003; Nasrallah 2005) and in this volume Chap. 10, which deals with mechanisms of S-RNase-based SI; Chaps. 4 and 6 deal with evolutionary aspects of S-RNase-based SI.

9.2 S-RNase Determines *S*-Specificity in Pistil

9.2.1 Isolation and Identification of S-RNase as the Pistil S

As the first step to understand the molecular mechanism underlying the SI response, it is imperative to identify the products of the *S*-locus. Putative specificity determinants must meet three criteria suggested by the biology and genetics of SI: linkage to the *S*-locus, polymorphism between different *S*-haplotypes and expression in the pollen or pistil. The searches for the pistil *S* and pollen *S* were essentially based on these three requirements.

Pistil *S*-allele products were first reported in *Nicotiana alata* as polymorphic basic glycoproteins that varied in molecular mass and isoelectric point and segregated with *S*-alleles (Bredemeijer and Blaas 1981). And a cDNA encoding a 32 kD stylar glycoprotein that segregated with the S_2-allele was isolated (Anderson et al. 1986). Similar stylar proteins and their cDNAs were also identified from members of Solanaceae, Rosaceae and Plantaginaceae (de Nettancourt 2001).

The biochemical nature of the pistil S-proteins remained unclear until their sequences were found to share similarity with RNase T_2 of *Aspergillus oryzae* (Kawata et al. 1988; McClure et al. 1989). Further studies confirmed the ribonuclease activity of the pistil S-proteins and thus they were referred to as S-RNases (McClure et al. 1990; Singh et al. 1991). S-RNases are primarily expressed in pistil and secreted into the extracellular matrix (ECM) of transmitting tract (Cornish et al. 1987; Anderson et al. 1989; Xue et al. 1996).

Although the *S-RNase* gene has satisfied the criteria expected for the pistil *S* determinant, direct proof that the S-RNases are necessary and sufficient for self-pollen rejection was obtained from transformation experiments using both loss- and gain-of-function approaches (Lee et al. 1994; Murfett et al. 1994). These studies demonstrated that introduction of a new *S-RNase* allele was sufficient to confer the transgenic plants the ability to reject pollen with the same haplotype as the *S-RNase* introduced. Conversely, suppression of expression of the *S-RNase* by antisense transformation abolished the ability of the transgenic plants to reject the pollen carrying the affected haplotype. Stylar-part mutations reported in Solanaceae (Royo et al. 1994) and Rosaceae (Sassa et al. 1997) reinforced the notion that the S-RNase is essential for expression of SI in pistil.

9.2.2 S-RNase Sequence Features and the Specificity Determinant

9.2.2.1 The Role of the Carbohydrate Moiety

Attempts have been made to identify where the *S*-specificity resides in the S-RNase sequences. Since S-RNases are secreted glycoproteins that vary in the number and position of N-linked glycan chains, the allelic specificity might lie in the

carbohydrate moiety (Woodward et al. 1989, 1992; Oxley et al., 1996). However, the non-glycosylated S-RNase of *Petunia inflata*, with the only potential N-glycosylation site Asn replaced with Asp, functions as well as the wild-type glycosylated S-RNase in rejecting pollen carrying the same *S*-haplotype (Karunanandaa et al. 1994). Thus, the encoding of *S*-specificity does not reside in the glycan side chains.

9.2.2.2 The Role of Amino Acid Sequence

S-RNases are highly divergent, with amino acid sequence identity ranging from 38 to 98% (McCubbin and Kao 2000). Despite this high sequence diversity, five small conserved regions, C1 to C5, accounting for about 40 of the approximately 230 amino acids in a typical S-RNase, have been identified (Ioerger et al. 1991; Tsai et al. 1992). Similar structural features have been observed in the Rosaceae and the Plantaginaceae, with the exception that the position of C4 is occupied by conserved regions that share no homology with C4 or with each other (Xue et al. 1996; Ushijima et al. 1998). Most notably, the C2 and C3 regions, which share a high degree of sequence similarity with the corresponding regions of RNase T_2, contain conserved catalytic His residues, indicating that the RNase activity could be involved in self pollen inhibition (Fig. 9.2). Phylogenetic analysis of S-RNases suggested that this type of SI possessed by the species in these three distantly related dicot families shares a common origin, and might be exhibited by the ancestor of ~75% of all dicots (Xue et al. 1996; Igic and Kohn 2001). See also Chap. 5 for a review of this topic.

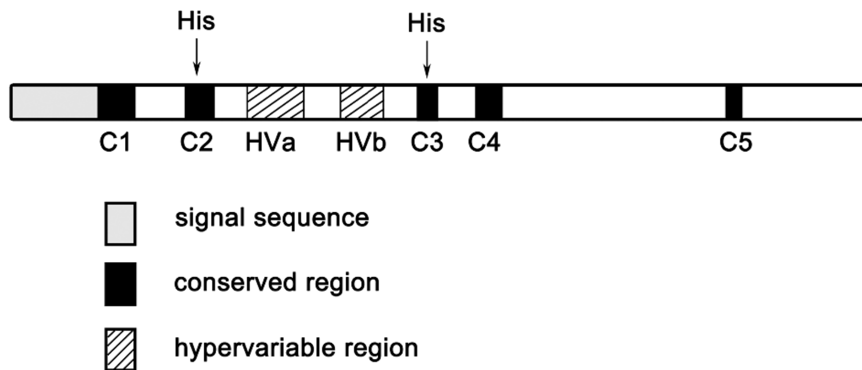


Fig. 9.2 Generalised features of S-RNases. A comparison of allelic S-RNases from several solanaceous species reveals a conserved structure among them. All contain a secretion signal sequence (*gray box*) and share five conserved domains (C1–C5; *black boxes*). Among them, C2 and C5 contain two histidine residues (His) that are required for ribonuclease activity as shown for related fungal RNases. Two hyper-variable regions (Hva and Hvb; *hatched boxes*) are also shown (modified form de Nettancourt 2001, with kind permission from Springer)

Sequence comparison of S-RNases from the Solanaceae also has revealed two hypervariable (HV) regions, HVa and HVb (Ioerger et al. 1991; Tsai et al. 1992), which were found subsequently to be the hypervariable regions of *Antirrhinum* S-RNases as well (Xue et al. 1996) and to correspond to two of the four regions of rosaceous S-RNases under positive selection (Ishimizu et al. 1998). The crystal structures of a solanaceous S-RNase and a rosaceous S-RNase show that HVa and HVb form a continuous solvent surface on one side of the protein (Ida et al. 2001; Matsuura et al. 2001), with a putative function to form a domain interacting with the pollen *S*-determinant. Therefore, HVa and HVb are considered the most likely candidates for the determinant of *S*-allele specificity. To assess this role, three sets of mosaic constructs among S-RNases had been utilised (Kao and McCubbin 1996; Zurek et al. 1997; Matton et al. 1997). However, these experiments led to different conclusions, and it remains possible that amino acids outside HVa and HVb are also involved in *S*-allele specificity (Verica et al. 1998). Nonetheless, it is clear that the HVa and HVb regions play a key role in the *S*-allele specificity determination.

9.2.3 The Role of S-RNase: A Cytotoxin Specifically Inhibits Self Pollen

9.2.3.1 S-RNase Cytotoxicity as the Underlying Cause of Pollen Tube Growth Inhibition

It has been shown that the two His residues important for ribonuclease activity are completely conserved in all S-RNases that are functional in pollen rejection (Ioerger et al. 1991), implicating that S-RNase enzymatic activity is involved in SI response. This has been supported by a series of experiments. A tracer experiment using ^{32}P-labelled pollen RNA showed that pollen rRNA are degraded after incompatible but not compatible pollination (McClure et al. 1990). In addition, catalytically inactive S-RNases due to the mutation of His residues lost the ability to reject self pollen (Huang et al. 1994; Royo et al., 1994). A cytotoxic model for S-RNase-based SI has been proposed accordingly (McClure et al. 1990). In this model, each *S*-haplotype encodes a unique S-RNase. In incompatible pollination, S-RNase gains access to the pollen tube cytoplasm where its ribonuclease activity causes a cytotoxic effect, whereas in compatible pollination, this cytotoxic effect is evaded.

Although rRNA degradation in incompatible pollen tubes is correlative with the expression of SI, it is difficult to discriminate whether it is the direct cause or secondary effect of incompatibility (McClure et al. 1990, 2000). And since no substrate specificity of S-RNase has been detected in vitro, it is still not clear how S-RNase activity could be restricted to self pollen tube (McClure et al. 1990). Moreover, incompatible pollen tubes could be ‘rescued’ if they were grafted onto compatible styles (i.e. they reverted to normal growth); thus the cytotoxic effect of S-RNase is not permanent. This seems inconsistent with the cytotoxic model, and had been interpreted that pollen tubes could synthesise rRNA continuously (Lush

and Clarke 1997). Nevertheless, it has been widely accepted that S-RNase functions as a cytotoxin to inhibit self pollen, no matter how this inhibition is achieved through the intrinsic RNase activity or other effects.

9.2.3.2 S-RNases Enter the Pollen Tubes in a Non-Haplotype-Specific Manner

How do the S-RNases specifically exert their cytotoxicity specifically in self pollen tubes? It was originally proposed that the membrane or cell wall-bound receptors of pollen tubes function as specific gatekeepers to allow the S-RNase of the same *S*-haplotype, but not S-RNases of different *S*-haplotypes to enter, and thereby the growth of self pollen tubes is selectively inhibited. Although intuitively this appeared to be a most efficient or likely mechanism, subsequent evidence strongly suggested that this model was incorrect. S-RNases have been shown by immunolocalisation to be present in the cytoplasm of both self and non-self pollen tubes growing in the style, strongly indicating that uptake of S-RNases into pollen tubes is not *S*-haplotype specific (Luu et al. 2000).

9.3 F-Box Proteins Determine *S*-Specificity in Pollen

9.3.1 Clues from Pollen-Part Self Compatible Mutants

Early attempts to isolate the pollen *S* were focused on the analysis of pollen-part mutants (PPMs) (de Nettancourt 2001). For species from Solanaceae, the most frequent types of lesions were either translocations or small ‘centric’ fragments (short extra chromosomes) that carried a duplicated copy of an *S*-allele. Breakdown of the pollen SI response in those plants has mainly been ascribed to a ‘competitive interaction’ that enabled pollen with two different *S*-haplotypes (but not two identical *S*-haplotypes) to grow through an incompatible style. Apart from PPMs, competitive interaction was also thought to be the reason why tetraploids that were derived from self-incompatible diploids in the Solanaceae were often self-fertile (de Nettancourt 2001). Consistently, molecular studies have lent support for competitive interaction (Thompson et al. 1991; Golz et al. 1999, 2001). Golz et al. (1999, 2001) performed a large scale gamma ray mutagenesis in *N. alata*, with the expectation to isolate PPMs with the pollen *S* deletion; however, all PPMs acquired were found to be associated with duplicated *S*-haplotypes. Similar results were obtained from a transposon-based mutagenesis conducted in *Antirrhinum* (our unpublished data).

In Rosaceae, however, no SI breakdown mutants have been found to be associated with the presence of a centric fragment (de Nettancourt 2001), and the tetraploids can be either self-compatible or self incompatible (reviewed in Mable 2004). In fact, analyses of these mutants have provided foundation for the isolation and confirmation of the pollen *S*, and for the dissection of the molecular mechanisms underlying the SI response.

9.3.2 Isolation of the Pollen SI Determinant, SLF/SFB

During the past several years, extensive efforts have been made for the pollen *S* identification based on its predicted genetic features similar to the pistil determinant. This has included RNA differential display, subtractive hybridisation (Dowd et al. 2000; Li et al. 2000; McCubbin et al. 2000) and yeast two hybrid assay using HV domain of S-RNases as bait (Sims and Ordanic, 2001). However, no gene that segregated with *S-RNase* was identified through these routes. This was a real challenge since the pollen S protein appeared to be not as abundant as the S-RNase. Eventually, the pollen *S* gene candidates were successfully identified by a large-scale genomic DNA sequencing in the vicinity of *S-RNase* genes.

The first promising candidate was obtained from DNA sequence analysis of a ~64 kb region containing the S_2-*RNase* of *Antirrhinum hispanicum* (Lai et al. 2002). This region contained a novel F-box protein gene, *AhSLF*-S_2 (*A. hispanicum* *S*-locus F-box of S_2-haplotype), which was specifically expressed in pollen. More extensive searches of the adjacent regions of S_2-*RNase* revealed the existence of multiple paralogous genes that encoded similar F-box proteins. However, recombination analyses confirmed that *AhSLF*-S_2 was the most adjacent haplotype-specific gene among those F-box-encoding genes linked to the *S*-locus, although their allelic products had much lower sequence diversity (ca. 97% amino acid sequence identity) than those of S-RNases (Zhou et al. 2003).

Similar experiments have been conducted in species of Rosaceae (Entani et al. 2003; Ushijima et al. 2003) and Solanaceae (Sijacic et al. 2004). Entani et al. (2003) analysed the ~60 kb genomic region around the *S*-locus in self-incompatible *Prunus mume* (Japanese apricot) and found that the *S-RNase* gene was present in a region that contained at least four independent F-box genes. The F-box gene closest to the *S-RNase* gene was named *SLF*. It showed a high level of allelic diversity, with amino acid sequence identities of three alleles of *PmSLF* ranging from 78 to 81%, and was expressed exclusively in pollen.

At the same time, a ~70 kb segment of the *S*-locus of the rosaceous species *P. dulcis* (almond) was sequenced completely (Ushijima et al. 2003). This region was found to contain two pollen-expressed F-box genes. One of them, named *SFB* (*S*-haplotype-specific F-box), was expressed specifically in pollen and showed a high level of *S*-haplotype-specific sequence polymorphism. Another showed little allelic sequence polymorphism and was expressed also in pistil and named *PdSLF* (*P. dulcis* *S*-locus F-box). It should be noted that *PdSLF* was so named to emphasise that it shows approximately the same low level of allelic sequence diversity as detected among *AhSLF* rather than to indicate that *PdSLF* is an ortholog of *AhSLF*. In fact, both *SFB* and *PdSLF* are less than 25% identical to *AhSLF* at the deduced amino acid sequence level (Kao and Tsukamoto 2004).

In *P. inflata*, a thorough search of the pollen *S*-determinant in a large *S*-locus region (328 kb BAC contig of S_2-haplotype) identified a polymorphic F-box gene, named *PiSLF*, 161 kb downstream of the *S-RNase* gene. Although the genomic region outside this contig contained two more polymorphic F-box genes that were genetically linked to the *S*-locus, the PiSLF exhibited the highest sequence diversity

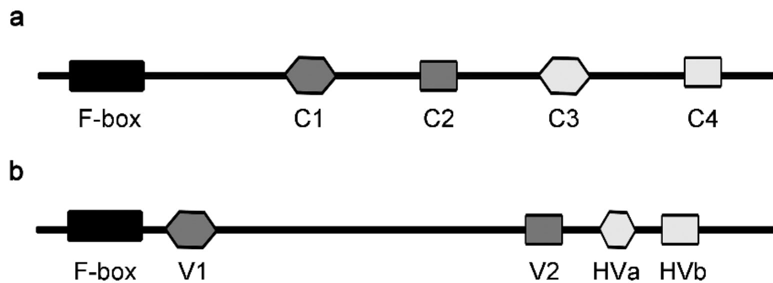


Fig. 9.3 Structural features of SLFs and SFBs. (**a**) Phylogenetic analyses of AhSLFs and their family members from *Arabidopsis* and several other plant species revealed that the *SLF* genes appeared to have had a monophyletic origin. Besides the F-box motif (*black box*), these SLFs contain four conserved domains (C1–C4; *gray boxes*) (Wang et al. 2004a). (**b**) Amino acid sequence alignment of *Prunus* SFBs reveals a conserved feature among them. All share an F-box domain (*black box*) and (hyper)variable domains (V1, V2, HV1 and HV2; *grey boxes*) (Ikeda et al. 2004)

(ca 90% in amino acid identity), but this was still much lower than that among *Prunus* SFBs (60–80% in amino acid identity) (Wang et al. 2004b).

9.3.3 Sequence Analysis of SLFs and SFBs

More alleles of *SLF* and *SFB* have been cloned by PCR, based on the conserved regions found among these genes (Ushijima et al. 2003; Yamane et al. 2003a, b; Zhou et al. 2003; Wang et al. 2004a; Tsukamoto et al. 2005; Cheng et al. 2006; Nunes et al. 2006; Vaughan et al. 2006). The phylogenetic analyses suggested that all of the SLF/SFBs from *Antirrhinum*, *Petunia* and *Prunus* belong to a monophyletic group sharing four conserved domains (Wang et al. 2004a) (Fig. 9.3a), implying that the common ancestor of many dicots possessed S-RNase/F-box-based GSI. This result provides further support on the monophyletic origin of the S-RNases (Xue et al. 1996; Igic and Kohn 2001). Nevertheless, all of the SFBs from the *Prunus* species formed a sub-lineage distinct from the functionally known SLF from *Antirrhinum* and *Petunia*, suggesting that they are likely diverged earlier during the S-RNase-based SI evolution (Ushijima et al. 2004; Wang et al. 2004a). See Chap. 5 for further discussion of the phylogeny of SLFs and SFBs. Based on amino acid sequence comparison, Ikeda et al. (2004) summarised the structural features for *Prunus* SFBs. All share an N terminal F-box domain, and four (hyper)variable regions, which were shown to be under positive selection (Fig. 9.3b).

9.3.4 Identification of SLF as the Pollen S

Direct evidence that *SLF* encodes the pollen *S*-determinant was finally obtained from transformation experiments in *P. inflata* (Sijacic et al. 2004) and *P. hybrida*

(Qiao et al. 2004a). To ascertain whether *PiSLF* encodes the pollen *S*-determinant, the phenomenon of competitive interaction, which occurs in heteroallelic pollen was utilised (see Sect. 3.1). Consistent with this phenomenon, the transformation of S_1S_1, S_1S_2 and S_2S_3 plants with the S_2-allele of *PiSLF* (*PiSLF2*) caused breakdown of the pollen function in SI. Furthermore, molecular genetic analyses of the progeny from self pollination of S_1S_2/*PiSLF2* and S_2S_3/*PiSLF2* revealed that only S_1 and S_3 pollen carrying the *PiSLF2* transgene (corresponding to heteroallelic pollen), but not S_2 pollen carrying *PiSLF2* (corresponding to homoallelic pollen), became self-compatible (Sijacic et al. 2004). These results conclusively demonstrated that *SLF* is the long-sought after pollen *S*-determinant in S-RNase-based SI. Similar experiments were conducted with the *AhSLF* genes (Qiao et al. 2004a). Both a pollen-expressed *AhSLF*-S_2 gene constructs and a transformation-competent artificial chromosome (TAC) clone containing both S_2-*RNase* and *SLF*-S_2 were transformed into SI *P. hybrida*, and SI breakdown on the pollen side was subsequently observed. Moreover, introduction of other F-box proteins in the vicinity of the *S*-locus of *A. hispanicum* had no effect on the pollen SI behaviour (our unpublished results). These findings showed that the *AhSLF* and *PiSLF* are orthologs, representing the sole pollen *S*-determinant. Moreover, consistently, duplication of the *SLF* was associated with breakdown of pollen SI in a natural population of self-incompatible *P. axillaris* (Tsukamoto et al. 2005), lending further support to the proposal that *SLF* encodes the pollen *S*.

9.3.5 SFBs from Rosaceae Likely Represent Another Class of F-Box Genes

Gain-of-function or loss-of-function transgenic experiments in *Prunus* tree species are hindered by the lack of an efficient transformation system and a long juvenile period. Functional dissection of *SFB* mainly came from analysis of naturally occurring pollen-part self compatible mutants. Intriguingly, in contrast with the *SLF*, whose deletion could be lethal (Sect. 9.3.1), deletion of *SFB* appeared to be associated with SI breakdown on the pollen side in *Prunus* (Ushijima et al. 2004; Sonneveld et al. 2005; Hauck et al. 2006a; Tsukamoto et al. 2006; Vilanova et al. 2006). In addition, the heteroallelic pollen of a tetraploid *Prunus* was found to be self-incompatible (Hauck et al. 2006b), contrary to the competitive interaction phenomena observed in the Solanaceae and Plantaginaceae. These differences between *SLF* and *SFB* could possibly reflect a mechanistic diversity, which will be discussed later (Sect. 9.6).

Although *SFB* fulfills the genetic requirements for the pollen *S*-determinant: linkage to *S-RNase*, haplotype-polymorphism and expression in pollen, recent findings call for more careful attention about the nature of *SFB*. The S_8-RNase from SI *P. tenella* was found to be identical to S_1-RNase from *P. avium*, whereas the corresponding SFB alleles were different. This finding was interpreted as the pollen *S* specificity is more tolerant of non-conservative replacements and this

tolerance is important for SI evolution (Surbanovski et al. 2007). However, further functional dissection is needed to clarify whether these variants of *SFB* are the intermediate form of the pollen *S* during evolution or merely polymorphic genes linked to the *S*-locus rather than the pollen *S*. Furthermore, it is puzzling when the *S*-locus linked and pollen-expressed F-box genes from *Malus domestica* (apple), another species from Rosaceae, had been isolated by PCR and showed more sequence identity to the *SLFs* in the Solanaceae and Plantaginaceae rather than the *SFBs* in the Rosaceae (Cheng et al. 2006). What made the problem even more complicated was the finding that multiple pollen-specific F-box genes in apple (*M. domestica*) and Japanese pear (*Pyrus pyrifolia*), termed the *S*-locus F-box brothers (*SFBB*), display high homology to the *SLFs* isolated by Cheng et al. (2006), which also appear to meet the expected characteristics of the pollen *S* (Sassa et al. 2007). Although species from these three families (Solanaceae, Plantaginaceae and Rosaceae) use a similar molecule as the pistil *S*-determinant (S-RNase), the status of the pollen *S*-determinant in Rosaceae seems more complicated. It seems possible that there may be more than one gene product involved in S-RNase recognition (Sassa et al. 2007).

9.4 Other Genes That Modulate the SI Response

Although the *S*-locus encodes the determinants for *S*-haplotype specificity, there is evidence for the existence of other unlinked genes, termed modifier genes that are required for the SI response (reviewed in McClure et al. 2000, 2006; Cruz-Garcia et al. 2003; Kao and Tsukamoto 2004), which will be discussed here.

9.4.1 The Pistil Modifier Factors

9.4.1.1 HT-B

HT-B protein, a small asparagine-rich protein expressed late in style development, was originally identified by differential cDNA hybridisation to screen style genes that were expressed in self-incompatible species *N. alata* but not in *N. plumbaginifolia*, a closely related self-compatible species (McClure et al. 1999). Homologs of HT-B also have been identified in two other genera of the Solanaceae, *Lycopersicon* and *Solanum* (Kondo et al. 2002b; O'Brien et al. 2002). Down-regulation of *HT-B* expression by anti-sense transformation and RNAi deprived the transformants of the ability to reject self pollen, suggesting that it is essential for SI (McClure et al. 1999; O'Brien et al. 2002). In a comparative analysis of self-incompatible and self-compatible taxa of *Lycopersicon*, the expression of *HT-B* gene was not detected in all self-compatible taxa (Kondo et al. 2002a). Since no direct interaction between HT-B and S-RNases was detected, the exact role of HT-B for SI response was unclear until a recent immunolocalisation experiment (Goldraij et al. 2006).

This revealed that in self pollen tubes, HT-B was likely to facilitate S-RNase transport from an endomembrane compartment to the cytoplasm, where they could exert their cytotoxicity, leading to the arrest of pollen tube growth, while in compatible pollen tubes, HT-B level was significantly down regulated and the S-RNases were compartmentalised (Goldraij et al. 2006). See Chap. 10 for further details.

9.4.1.2 120K Glycoprotein

The 120 kDa glycoprotein (120K) is an abundant protein in the stylar ECM and taken up by the growing pollen tubes (Lind et al. 1994, 1996). The 120K protein binds to S-RNase in vitro and, like HT-B, suppression of its expression by RNAi prevented self pollen rejection (Cruz-Garcia et al. 2005; Hancock et al. 2005). In recent immunolocalisation experiments, antibodies to the 120 kDa glycoprotein were found to label the compartment membrane that surrounds the S-RNases inside the pollen tubes. However, since S-RNase uptake is normal in 120K protein defective plants, its precise role in SI is still not clear (Goldraij et al. 2006); see Chap. 10.

9.4.2 The Pollen Modifier Factors

9.4.2.1 SSK1

F-box proteins often serve as adaptors that bind specific substrate proteins to the SCF (Skp1-Cullin-F-box) E3 ubiquitin ligase complex (Petroski and Deshaies 2005). This raised the possibility of whether the SLF involved in the SI also participates in an SCF complex, mediating S-RNase ubiquitination. Identification of other components in such a putative complex is obviously necessary to address this question. *SSK1* (*SLF-interacting SKP1-like1*), a homolog of *SKP1*, was originally isolated in *A. hispanicum* through a yeast two-hybrid screening against a pollen cDNA library using *AhSLF-S*$_2$ as bait (Huang et al. 2006). Pull-down assays suggested that AhSSK1 could be an adaptor that connects SLF to CUL1 protein. Therefore, it is thought that SLF and SSK1 are likely to be recruited to a canonical SCF complex, which could be responsible for S-RNase ubiquitination (Fig. 9.4a); see also Chap. 10.

9.4.2.2 SBP1

In an attempt to isolate the pollen *S*, Sims and Ordanic (2001) screened a yeast two-hybrid library from mature pollen of *P. hybrida* using *P. hybrida* S$_1$-RNase as bait, and identified a gene named *PhSBP1* (*S-RNase Binding Protein1*). Its homolog in *S. chacoense* was obtained based on a similar approach (O'Brien et al. 2004). However,

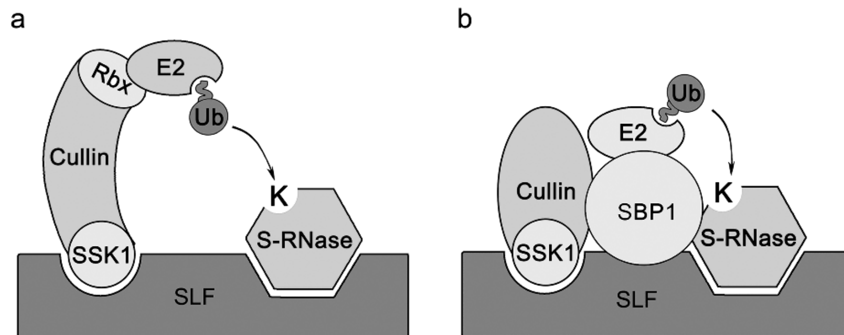


Fig. 9.4 Two proposed SCF^{SLF} recognition complexes in S-RNase-based self-incompatibility. Experiments described in the text demonstrate that the pollen *S* is an *S*-locus encoded F-box gene. F-box proteins are the recognition components of SCF E3 ubiquitin ligase complexes. AhSLF-S_2 from *A. hispanicum* has been shown to interact with S-RNases both in vitro and in vivo. AhSSK1, identified by yeast two-hybrid screening using AhSLF-S_2 as bait, is able to form a complex together with Cullin1 and S-RNase. These results suggest that SLF and SSK1 are components of a canonical SCF complex, which transfers ubiquitin from an E2 enzyme to S-RNase. PhSBP1, originally identified as a factor that binds to S-RNase in yeast two-hybrid assay, has been shown to be able to interact with PhSLF, Cullin1 and E2, with a possible role that replaces RBX1 as the RING protein of the complex. In either case, recognition of S-RNase by the SCF^{SLF} complex is predicted to lead to ubiquitination of the S-RNase protein

the *SBP1* gene displayed no haplotype polymorphism and was found to be expressed in almost all tissues. In addition, it was unlinked to the *S*-locus and therefore is unlikely to encode the pollen *S*-determinant. However, sequence analysis revealed that SBP1 contains a RING-finger domain, which is characteristic of E3 ubiquitin ligases (Kerscher et al. 2006), indicating a possible role for SBP1 in S-RNase ubiquitination and degradation (Sims and Ordanic 2001; O'Brien et al. 2004). Interestingly, *P. inflata* SBP1 (PiSBP1) has recently been shown to be able to interact with PiSLFs, Pi CUL1 and an ubiquitin-conjugating enzyme, and a novel E3 ligase complex has been suggested, with the possibility that PiSBP1 plays a combined role of SKP1 and RBX1 (Sims 2005; Hua and Kao 2006) (Fig. 9.4b); see also Chap. 10.

9.5 Molecular Mechanisms for S-RNase-based SI

9.5.1 Pollen S, the Positive or Negative Regulator of S-RNase? Clues from Genetic Evidence

It is now generally accepted that S-RNases exert their cytotoxicity inside the pollen tube. This raises the question of what the function of the pollen *S* product is. One possibility is that the pollen *S* product might promote or inhibit the activity of S-RNases. Genetic evidence has indicated possible distinct roles for *SLF* genes in

Solanaceae and Plantaginaceae and *SLF*/*SFB* genes in Rosaceae, and we briefly discuss this here.

9.5.1.1 SLF, the Inhibitor of S-RNase in Solanaceae and Plantaginaceae

It has been suggested that the pollen *S* product in Solanaceae and Plantaginaceae might be required for pollen tube growth in styles possessing functional S-RNases, possibly because its product acts as an RNase inhibitor (Sect. 9.3.1). A simple inhibitor model has been proposed in which the pollen component was assumed to be an RNase inhibitor inside the pollen tube, which was able to inhibit all S-RNases except the one of the corresponding *S*-haplotype (Dodds et al. 1996; Kao and McCubbin 1996; Luu et al. 2000). In this model, the pollen *S* encodes a protein that has two functions of both inhibiting non-self S-RNases and conferring specificity (Kao and Tsukamoto 2004) (Fig. 9.5a). The simple inhibitor model could readily explain the phenomenon of competitive interaction. When two different pollen *S*-alleles are expressed in the same pollen grain, their products could together inhibit the RNase activity of all S-RNases, thus resulting in the breakdown of SI (Kao and Tsukamoto 2004); also see Chap. 10.

The inhibitor effect of SLF could also provide an explanation for its low sequence divergence. Since SLF functions to inhibit all but self S-RNase, it would be fatal if the sequence varies to a higher degree. Moreover, relatively small amounts of sequence divergence can nevertheless result in allelic specificity, as is the case for the S_{11} and S_{13} alleles of *S. chacoense* (Saba-el-Leil et al. 1994). This possibility could be further tested by structural and functional studies on SLF.

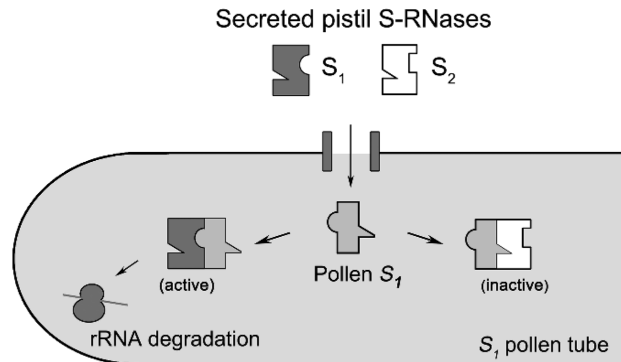
9.5.1.2 SFBs in Rosaceae Appear to Protect Self S-RNases

In Rosaceae, however, another scenario is emerging. The deletion of *SFB* appears to have resulted in pollen-part self-compatibility in *Prunus* (Sect. 9.3.5). Loss-of-function mutations strongly indicate the presence of a general inactivation mechanism, with SFB acting to confer specificity by protecting self S-RNases from inactivation (Ushijima et al. 2004; Sonneveld et al. 2005; Hauck et al. 2006a). Such a mechanism was originally proposed by Luu et al. (2001) as a modified inhibitor model to explain the SI behaviour of a dual-specificity chimeric S-RNase they had generated (Matton et al. 1999) (Fig. 9.5b); see also Chap. 10.

9.5.2 The Fate of S-RNases: S-RNase Restriction is Likely to Involve Ubiquitination

Current knowledge concerning the molecular mechanism of S-RNase-based SI has mainly been obtained from the Solanaceae and Plantaginaceae. Genetic evidence

a. Simple inhibitor model



b. General inhibitor model

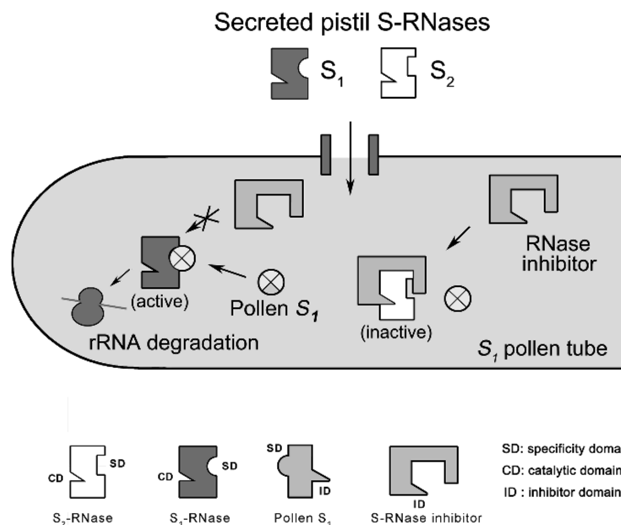


Fig. 9.5 Two models for the mechanism of S-RNase-based self-incompatibility. (**a**) Simple inhibitor model. Both S_1- and S_2-RNases are taken up by an S_1 pollen tube, but only S_1-RNase is active in degrading pollen RNA, because the pollen S_1-haplotype product specifically inhibits the RNase activity of S_2-RNase. Binding of S_1-RNase to the pollen S_1-haplotype product through their matching *S*-haplotype-specificity domains (SD) blocks binding of the inhibitor domain (ID) of the pollen S_1-haplotyoe product to the catalytic domain (CD) of S_1-RNase. Interaction between S_2-RNase and the pollen S_1-haplotype product is through the CD of the former and the ID of the latter, thus rendering S_2-RNase inactive. (**b**) General inhibitor model. This model differs from the simple inhibitor model in two major respects: the pollen *S*-haplotype products are homotetramers and only contain the SD and a general RNase inhibitor is assumed to be responsible for the inhibition of the RNase activity of S-RNases. The *S*-haplotype-specific inhibition of the RNase activity of S-RNases is achieved in a manner similar to that described for (**a**) (from Franklin-Tong and Franklin 2003, with kind permission from Elsevier)

has suggested that SLF in these two families functions to inhibit the S-RNase activity; therefore, the key questions now facing us are how the compatible pollen tube evades the S-RNase cytotoxicity and what the molecular function of pollen *S* product is during this process? It has been shown that SLF and SSK1, the components of an SCF complex, function to inhibit the S-RNase activity (Sect. 9.5.2.1). Since SCF is well known to act in conjunction with the E2 enzymes to ubiquitinate target proteins (Kerscher et al. 2006), it is assumed that the SCF^{SLF} could recognise and ubiquitinate S-RNase. This speculation has been tested and confirmed by two separate experiments in *A. hispanicum* and *P. inflata*, respectively, although no *S*-haplotype-specificity was observed in both cases (Qiao et al. 2004b; Hua and Kao 2006). Furthermore, compatible pollinations were specifically blocked after the treatments of the proteasomal inhibitors MG115 and MG132, which had little effect on incompatible pollination, indicating that ubiquitination/26S proteasomal activity is involved in compatible pollination processes. Based on these findings, the S-RNase degradation model was proposed, in which S-RNases are ubiquitinated by non-self SLF followed by proteasome-mediated degradation after compatible pollination, while the S-RNases remain active in self pollen tube due to an ineffective interaction of SLF and self S-RNase. This latter situation would be predicted to result in pollen tube growth arrest (Qiao et al. 2004b; Hua and Kao 2006) (Fig. 9.6).

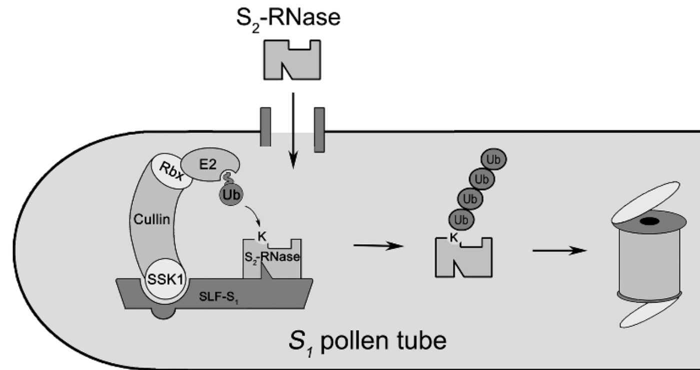
There are still some questions remaining with respect to this model. For example, how is specificity attained? Why does the quantity of S-RNases show no significant decrease during compatible pollination though there is no detectable S-RNase activity within the pollen tube? Nevertheless, consistent with the genetic evidence and with an explanation for the competitive interaction, the inhibitor model has been widely accepted to interpret the molecular mechanism of SI response in the Solanaceae and Plantaginaceae; see also Chap. 10.

However, a recent study at a cytological level has provided a new vision. It has been suggested that the compartmentalisation of S-RNases in compatible pollen tube is the reason for the restriction of the S-RNase cytotoxicity, and the role of HT-B during this process has been discussed (Goldraij et al. 2006; McClure 2006). These two proposed mechanisms are not necessarily mutually exclusive. Immunolocalisation studies have suggested that the distribution of AhSLF appears associated with the membrane system, indicating that the SLF probably has a function in membrane trafficking (Wang and Xue 2005). Thus, it is possible that these two mechanisms could work together to inhibit non-self S-RNase activity during compatible pollination. A closer inspection of the molecular function of the pollen S protein will be helpful for a better understanding of the fate of S-RNases. See also Chap. 10 for further discussion of this perplexing problem.

9.5.3 Future Perspectives

During the last two decades, extensive efforts have been made to elucidate the molecular mechanism of S-RNase-based SI response, and the most breathtaking

a. Compatible pollination



b. Incompatible pollination

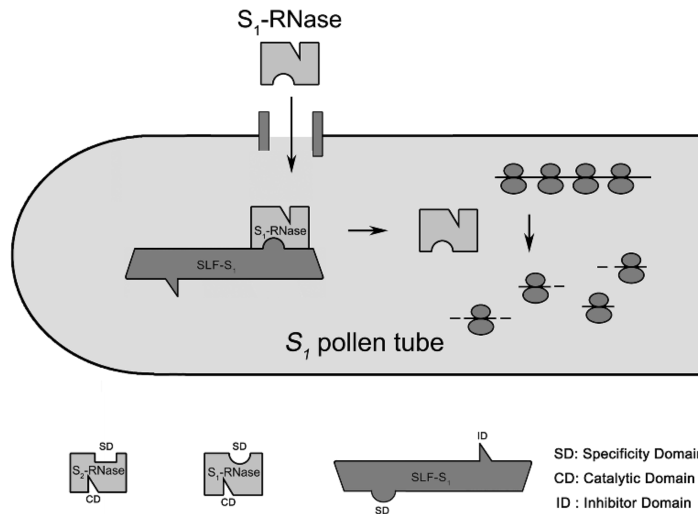


Fig. 9.6 S-RNase degradation model. After non-self S-RNase enters the pollen tube, its catalytic domain (CD) interacts with the inhibitor domain (ID), leading to formation of a functional SCF complex that targets the S-RNase for ubiquitination and degradation by the 26S proteasome, thus the pollen tube growth continues normally (**a**). By contrast, after self S-RNase enters the pollen tube, the specificity domains (SD) of S-RNase and SLF interact with each other, resulting in conformational change in SCF^{SLF} and thus the S-RNase is not ubiquitinated and degraded. S-RNase cytotoxicity results in RNA degradation and consequent pollen tube growth inhibition (**b**). However, it is unknown how the *S*-haplotype specificity is attained

advances were the molecular identification of the pistil and pollen *S*-determinants. Although several attractive working models have been proposed to explain how they interact to elicit the SI signalling, we are still some distance from understanding the nature of the molecular and cell biology of the interaction and how exactly this GSI system operates mechanistically. Several outstanding questions clearly need immediate attention. For example, we are still ignorant of the molecular function of SFBs in Rosaceae. Could these SFBs also interact and ubiquitinate S-RNases like the SLFs in the Solanaceae and Plantaginaceae do, or is there a distinct mechanism for non-self S-RNase inhibition in the Rosaceae? It is also unclear why so many F-box genes reside in the vicinity of the *S*-locus. No interaction between these F-box proteins and S-RNase or SSK1 were detected (Qiao et al. 2004b; Huang et al. 2006) and they did not appear to have a direct role for S-RNase inhibition. What roles, if any, do they have? Further experiments, with the integration of the genetic, biochemical and cytological techniques, will help us to get a better understanding of this unique cell recognition and rejection system in flowering plants.

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