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REVIEW: PART OF A SPECIAL ISSUE ON SEXUAL PLANT REPRODUCTION

Compatibility and incompatibility in S-RNase-based systems

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• *Background* S-RNase-based self-incompatibility (SI) occurs in the Solanaceae, Rosaceae and Plantaginaceae. In all three families, compatibility is controlled by a polymorphic *S*-locus encoding at least two genes. S-RNases determine the specificity of pollen rejection in the pistil, and *S*-locus F-box proteins fulfill this function in pollen. S-RNases are thought to function as *S*-specific cytotoxins as well as recognition proteins. Thus, incompatibility results from the cytotoxic activity of S-RNase, while compatible pollen tubes evade S-RNase cytotoxicity.
• *Scope* The *S*-specificity determinants are known, but many questions remain. In this review, the genetics of SI are introduced and the characteristics of S-RNases and pollen F-box proteins are briefly described. A variety of modifier genes also required for SI are also reviewed. Mutations affecting compatibility in pollen are especially important for defining models of compatibility and incompatibility. In Solanaceae, pollen-side mutations causing breakdown in SI have been attributed to the heteroallelic pollen effect, but a mutation in *Solanum chacoense* may be an exception. This has been interpreted to mean that pollen incompatibility is the default condition unless the *S*-locus F-box protein confers resistance to S-RNase. In *Prunus*, however, *S-locus F-box protein* gene mutations clearly cause compatibility.
• *Conclusions* Two alternative mechanisms have been proposed to explain compatibility and incompatibility: compatibility is explained either as a result of either degradation of non-self S-RNase or by its compartmentalization so that it does not have access to the pollen tube cytoplasm. These models are not necessarily mutually exclusive, but each makes different predictions about whether pollen compatibility or incompatibility is the default. As more factors required for SI are identified and characterized, it will be possible to determine the role each process plays in S-RNase-based SI.

Key words: S-RNase, self-incompatibility, Solanaceae, Rosaceae, *Nicotiana*, *Prunus*.

GENETICS OF SELF-INCOMPATIBILITY (SI)

Angiosperms display diverse reproductive strategies that have very different consequences for generating and maintaining genetic diversity. Plants that reproduce clonally are at one end of a spectrum and show little or no variability. Sexual reproduction generates new combinations of genes and alleles by crossing over, meiosis and gamete fusion. Clearly, so long as the cross is not so wide that fitness is reduced (as in interspecific crosses), crosses between less-related individuals favour diversity. Thus, although many plants reproduce by selfing, many others have evolved special mechanisms to enhance outcrossing. SI systems are genetically controlled mechanisms that inhibit fertilization by self-pollen or pollen from closely related plants (de Nettancourt, 2001). Outcrossing is enhanced in SI species by effectively dividing the population into compatibility groups, or mating types, where within-group crosses are sterile but crosses between groups are fertile (Darwin, 1877).

SI appears to have evolved independently in different angiosperm lineages. Three distinct mechanisms have been studied at the molecular level. SI plants in the Brassicaceae display a system in which proteins deposited by the tapetum onto the pollen coat interact with receptors in the stigmatic papillae to control whether pollen is accepted (Takayama and Isogai, 2005). In SI *Papaver*, low molecular-weight proteins secreted onto the stigma surface cause self-pollen to cease growth immediately and eventually die (Wheeler *et al.*, 2010). SI species in the Solanaceae, Rosaceae and Plantaginaceae employ ribonucleases for recognition and rejection of self-pollen. Further mechanisms will likely be discovered through studies of SI in other lineages. However, at this point, systems employing ribonucleases appear to be the most phylogentically widespread (Igic and Kohn, 2001).

SI fascinated botanists in the early part of the twentieth century when the foundations of genetics were being forged. In reviewing early work, East and Park (1917) highlight instances where the presence of SI, or 'self-sterility,' showed signs of Mendelian inheritance. They observed important regularities in crosses among SI *Nicotiana* species, but the correct genetic model for cross compatibility was only described later by East and Mangelsdorf (1925). After determining the inheritance of compatibility groups, these authors concluded that compatibility is controlled by a single locus, the *S*-locus, and that compatibility in individual crosses depends on whether the *S*-allele, or 'allelomorph', in the pollen is also present in

the pistil. As they described it, a plant would only provide stimulus for growth of pollen with an *S*-genotype not found in the pistil. This is now called gametophytic SI. Although these studies used *Nicotiana* species as the experimental system, other SI species in the Solanaceae, Rosaceae and Plantaginaceae display gametophytic control as well. *Papaver rhoeas* also shows gametophytic control of compatibility; although, the underlying mechanism is now known to be different (Wheeler *et al.*, 2009, 2010). SI in the Brassicaceae is referred to as sporophytic SI since compatibility determinants are produced in the tapetum, a sporophytic tissue, and deposited on the pollen coat (Takayama and Isogai, 2005).

The fact that compatibility is controlled by an interaction between the pollen and the pistil is foundational to all modern studies of SI. It is now known that separate, but closely linked, genes determine *S*-specificity in the pistil and pollen. Thus, the current convention is to use the term *S*-haplotype to describe *S*-locus variants. Framed in this way, in gametophytic SI pollen is rejected when there is a match between the single *S*-haplotype in the haploid pollen and either of the two *S*-haplotypes in the diploid pistil. Although early workers appreciated that this genetic interaction is mediated by the 'constituents' of the pollen and pistil (Darwin, 1877), the relationship between these constituents and the genetic interaction revealed by progeny analysis was unknown.

S-SPECIFICITY DETERMINANTS IN S-RNASE-BASED SI

Linking the genetics of SI with the physiology of compatibility requires identification of the genes and gene products that determine *S*-specific pollen rejection. Candidate genes must meet three criteria: (1) linkage to the *S*-locus; (2) sequence variability in allelic genes from different *S*-haplotypes; and (3) expression in the pistil or pollen as appropriate. Since SI *S*-specific pollen rejection is the defining feature of SI, candidate genes must be tested in a genetic experiment that directly addresses specificity.

Pistil determinant

Analysis of pistil extracts allowed identification of proteins meeting the three basic criteria for *S*-specificity determinants. Using isoelectric focusing, Bredimeijer and Blaas (1981) showed that abundant polypeptides expressed in the pistil cosegregate with *S*-haplotype in *Nicotiana alata*. Anderson *et al.* (1986) obtained N-terminal sequence information and cloned the protein coded by the *N. alata* S_2-haplotype. This breakthrough allowed many important facts about the structure and expression of the protein to be established. The S_2-protein contains a secretion signal, and the gene is expressed in the stigma, the style transmitting tract, and the epidermis of the placenta (Anderson *et al.*, 1986; Cornish *et al.*, 1988). Proteins from different *S*-haplotypes are distinct in their chromatographic behaviour and glycosylation patterns (Jahnen *et al.*, 1989; Woodward *et al.*, 1989). Comparing sequences from different *S*-haplotypes revealed conserved and variable domains (Ioerger *et al.*, 1991). These characteristics fit the important requirements of protein that functions in pollen recognition: it is secreted into the extracellular matrix (ECM) that forms the path from the stigma to the ovary and a distinct protein is expressed from each functional *S*-haplotype. These results are supported by identification of similar proteins in *Petunia* and *Solanum* (Broothaerts *et al.*, 1989, 1991; Clark *et al.*, 1990; Xu *et al.*, 1990; Singh *et al.*, 1991; Saba-El-Leil *et al.*, 1994) as well as other solanaceous genera. Similar proteins have also been identified in *Pyrus*, *Malus* and *Prunus* (Broothaerts *et al.*, 1995; Boskovic and Tobutt, 1996; Sassa *et al.*, 1996) in the Rosaceae and in *Antirrhinum* in the Plantaginaceae (Xue *et al.*, 1996). Interestingly, *Prunus S-RNase* genes contain two introns, while Maloideae and Solanaceae *S-RNases* contain only one (Igic and Kohn, 2001).

The ribonuclease activity of pistil S-proteins provided clues to the mechanism of self-pollen rejection. Studies of the active site residues in RNase T2 from *Aspergillus oryzae* revealed similarity to the *N. alata* S_2-glycoprotein (Kawata *et al.*, 1988; McClure *et al.*, 1989). Direct enzyme assays showed that each S-protein from *N. alata* copurifies with a major ribonuclease activity in pistil extracts, and the proteins are now referred to as S-RNases (McClure *et al.*, 1989). Ribonuclease activity suggested a potential link between the genetic function of the *S*-locus in pollen recognition and a plausible physiological mechanism for pollen rejection. Experiments following the fate of ^{32}P-labelled pollen RNA in compatible and incompatible pollination tested this hypothesis. The results showed that pollen RNA is stable in compatible pollinations and degraded in incompatible pollinations (McClure *et al.*, 1990). Further experiments showed that, as expected, S-RNase effectively inhibits translation. Moreover, experiments using ^{3}H-labelled S-RNase showed that the protein enters pollen tubes intact and, thus, retains its potentially cytotoxic enzyme activity (Gray *et al.*, 1991). Finally, Huang *et al.* (1994) showed that S-RNase ribonuclease activity is required for pollen rejection. Together, these results form the basis for the cytotoxic model for SI in the Solanaceae, Rosaceae and Plantaginaceae. In this model, S-RNases have dual functions, acting as recognition proteins as well as directly inhibiting growth of incompatible pollen.

The recognition function of S-RNase was confirmed using plant transformation and analysis of self-compatible mutants. Murfett *et al.* (1994) and Lee *et al.* (1994) showed that transforming an *S-RNase* gene into a new background causes a gain-of-function change that allows rejection of pollen expressing the corresponding *S*-genotype. Likewise, suppressing expression of a specific *S-RNase* causes loss of the ability to reject a specific pollen *S*-genotype (Lee *et al.*, 1994; Murfett *et al.*, 1994). Sassa *et al.* (1997) showed that S-RNase also determines *S*-specificity in the pistil in *Pyrus serotina*. These genetic results clearly demonstrate that S-RNases are the determinants of *S*-specificity in the pistil. Although structural differences exist between *S-RNase* genes in *Pyrus* and those in other taxa, the evidence suggests that the genes are derived from a common ancestor and that S-RNase-based SI may have emerged in the common ancestor to these diverse lineages (Igic and Kohn, 2001).

The most detailed sequence analysis of solanaceous sequences identified five conserved regions, C1 to C5, that account for about 40 of the residues in a typical S-RNase (Ioerger *et al.*, 1991). Regions C2 and C3 contain histidine

residues implicated in catalysis, and the others contribute to the hydrophobic core of the molecule (Ishimizu *et al.*, 1995; Kawata *et al.*, 1988). Although all other regions are variable, Ioerger *et al.* (1991) identified two areas with especially high sequence variability, HVa and HVb. Similar approaches were used to identify a single 'hypervariable' region in *S-RNases* from the Rosaceae (Ishimizu *et al.*, 1998). The role of these variable regions and other S-RNase sequences in pollen recognition was tested in domain-swap experiments in transformable solanaceous species. Working in *Nicotiana*, Zurek *et al.* (1997) showed that all regions contribute to *S*-specific recognition and that swapping any region destroyed recognition. Kao and McCubbin (1996) swapped two regions between petunia *S-RNase* genes and also found that both were required for recognition. In contrast, Matton *et al.* (1997) found that swapping just four residues between very closely related S-RNase proteins in potato could switch the *S*-specificity of the target protein. Thus, while a small number of residues may distinguish a particular pair of S-RNases, it is not possible to conclude that HVa and HVb are sufficient to determine *S*-specificity. Indeed, a pair of S-RNase sequences has been identified in *Prunus* that are identical in the regions of the molecule usually described as the most variable (Zisovich *et al.*, 2004).

Pollen determinant

Sequencing genomic regions surrounding *S-RNase* genes identified the pollen determinant of *S*-specificity. Since all genes located near *S-RNase* fulfill the criterion of linkage to the *S*-locus, the challenge was to identify the correct gene against a background that may include many genes showing sequence variation. Lai *et al.* (2002) identified an F-box protein gene (*SLF*, *S-locus F-box*) just 9 kb downstream of S-RNase in *Antirrhinum*. However, this gene, $AhSLF_2$, was not immediately judged to be a good pollen *S* candidate because it was not as polymorphic as expected. Similar analyses of *Prunus* species with compact genomes also revealed F-box protein genes (Entani *et al.*, 2003; Ushijima *et al.*, 2003). In *Prunus dulcis*, allelic *SLF* genes have sufficient sequence variation that probes have shown *S*-specific hybridization patterns on genomic DNA. This reflects much greater sequence variability than seen in *Antirrhinum* (Lai *et al.*, 2002) and is reminiscent of results with *S-RNase* genes (Anderson *et al.*, 1989; Ioerger *et al.*, 1991). The *P. dulcis SLF* genes are also expressed in pollen and, thus, appeared to be excellent candidates for *S*-specificity-determining genes: they are linked to S-RNase, show appropriate sequence variability, and are expressed in pollen (Ushijima *et al.*, 2003). Similar results were reported in *P. mume* (Entani *et al.*, 2003).

Transformation experiments offer the most straightforward approach to test the function of pollen *S* genes. Simple gain-of-function tests of the role of *SLF*, however, were deemed unlikely to succeed, and a more complex strategy was devised. In the solanaceous species where transformation experiments are practical, evidence suggested that the only pollen-side mutations that cause self-compatibility (SC) are due to the so-called heteroallelic pollen (HAP) effect. In these species, converting an SI diploid (e.g. S_1S_2) to a tetraploid causes SC (de Nettancourt, 1977), and the defect occurs only in the pollen; thus, $S_1S_1S_2S_2$ pistils reject S_1- and S_2-pollen normally, but diploid pollen is not rejected. Moreover, of the three types of diploid pollen produced by a tetraploid (i.e. S_1S_1, S_1S_2 and S_2S_2), SI breakdown only occurs in the HAP case, S_1S_2. However, noteworthy differences have been described between the behaviour of tetraploids in the Solanaceae compared with the Rosaceae, and there even appears to be variability within the latter family. Hauck *et al.* (2006) showed that SI only breaks down in tetraploid *P. cerasus* when at least two defective *S*-haplotypes are present. Thus, the HAP effect does not explain SC in this species. However, the HAP effect does seem to be effective in *P. pseudocerasus* and *Malus* (Lewis and Modlibowska, 1942; Huang *et al.*, 2008; Sassa *et al.*, 2009).

An influential series of modern mutagenesis experiments in *N. alata* nevertheless provided strong support for the idea that breakdown of SI in pollen occurs when two different pollen *S* genes are expressed together, at least in the Solanaceae. Golz *et al.* (1999, 2000) examined radiation-induced pollen-part mutants (PPMs) and concluded that all the mutants could be accounted for by duplications or translocations of the pollen *S* gene, effectively creating HAP (Golz *et al.*, 1999, 2001). The absence of other classes of mutants was interpreted to mean that pollen *S* functions to provide resistance to S-RNase. The implication for transgenic tests of candidate pollen *S* genes is that, since pollen *S* function is essential, knock-outs are lethal and the HAP effect is the best way to test function.

Pollen *S* candidate *SLF* genes were identified by genomic sequencing in *Petunia inflata*, a species where transformation experiments could be conducted (McCubbin *et al.*, 2000*a*, *b*; Wang *et al.*, 2003, 2004). In a classic experiment, Sijacic *et al.* (2004) transformed the $PiSLF_2$ gene into SI S_1S_1 and S_2S_3 plants. As expected from the HAP effect, expression of the transgene caused SC. In the transformed S_1S_1 plants, only pollen expressing $PiSLF_2$ shows breakdown in SI; consistent with the HAP effect, S_1-pollen without the transgene behaves normally. Thus, the effect of the $PiSLF_2$ transgene is clearly gametophytic. However, *S*-specificity *per se*, can only be tested by comparing effects on both self- and non-self *S*-haplotypes. This complication is inherent in the HAP effect. Thus, *S*-specificity was demonstrated in S_2S_3 plants transformed with the $PiSLF_2$ gene (Sijacic *et al.*, 2004). Here, breakdown of SI is only seen when the transgene is expressed in S_3-pollen. Since the $PiSLF_2$ transgene did not interfere with SI in S_2-pollen, the effect is *S*-specific. Qiao *et al.* (2004*a*) transformed the *Antirrhinum* $AhSLF_2$ gene into SI *Petunia hybrida* and also reported breakdown of SI in pollen, but this experimental design does not directly address *S*-specificity since only a single *S*-haplotype can be tested.

As noted, studies of *S*-linked genes in *Prunus* provided strong evidence for a role of F-box protein genes in SI. However, it is now clear that important differences exist between the ways these genes, often referred to as *SFB* (*S-locus F-box*) genes, behave in the Rosaceae and Solanaceae (Tao and Iezzoni, 2010). For example, the level of sequence polymorphism between *S*-linked F-box genes (*SLF* or *SFB*) from different *S*-haplotypes is much lower in the Solanaceae and Plantaginaceae than in the Rosaceae. The significance of this is not clear, but the difference is striking

(Wheeler and Newbigin, 2007). In another example, results in *Malus* and *Pyrus* unexpectedly identified multiple copies of pollen-expressed *S*-haplotype-specific F-box genes at the *S*-locus (*SFBB*, *S-locus F-box brothers*; Sassa *et al.*, 2007).

MUTATIONS AFFECTING POLLEN COMPATIBILITY

Pollen-side breakdown of SI in the Rosaceae also differs from breakdown in the Solanaceae. SC mutants have attracted the attention of fruit tree breeders for many years, and their collections are a valuable resource for SI studies. Self-compatibe PPMs have been identified and analysed in *P. avium* (Ushijima *et al.*, 2004; Sonneveld *et al.*, 2005; Marchese *et al.*, 2007), *P. armeniaca* (Vilanova *et al.*, 2006), *P. cerasus* (Hauck *et al.*, 2006), *P. mume* (Ushijima *et al.*, 2004) and *P. persica* (Tao *et al.*, 2007). In most cases, genomic analyses of *Prunus* PPMs reveal insertions or deletions in the *SFB* coding region, providing additional evidence that *SFB* functions as the pollen *S*-gene (Yamane and Tao, 2009). This is a sharp contrast to the situation just described in *N. alata*, where mutant studies suggest that loss of pollen *S* gene function is lethal (Golz *et al.*, 2001). The contrast is further highlighted by the discovery that a PPM in an SC *P. avium* accession is caused by an *SFB* gene deletion in the $S^{3'}$-haplotype (Sonneveld *et al.*, 2005). Together, these data clearly show that mutation or deletion of *Prunus SFB* genes leads to SC. These genes are, therefore, not essential for pollen tube growth *per se*.

The apparent differences between PPM behaviour in *Prunus* and in *N. alata* are difficult to reconcile. However, these differences speak directly to the role of the interaction between the genetically defined pollen and pistil *S*-specificity determinants. What is the default condition: compatibility or incompatibility? Does interaction of the pollen and pistil *S*-specificity determinants confer resistance to S-RNase, or does it initiate rejection? In *N. alata*, the absence of mutations other than those that can be explained by the HAP effect is interpreted as evidence that interaction between S-RNase and SLF confers resistance to the cytotoxic effects of S-RNase in compatible pollinations (Golz *et al.*, 2001). In this very reasonable view, pollen *S* acts as a genetic inhibitor of the action of S-RNase; the default, in the absence of pollen *S*, is rejection. The *Prunus* PPM results, however, support the opposite interpretation: mutation or deletion of pollen *S* leads to SC, and compatibility is the default condition.

The behaviour of a PPM in *Solanum chacoense* offers a potentially different view from the one supported by the *N. alata* results and suggests that compatibility may also be the default condition for pollen in the Solanaceae. The *S. chacoense S-locus inhibitor* (*Sli*) gene has been characterized in numerous inter- and intra-specific crosses and has been followed through several generations (Hosaka and Hanneman, 1998*a*, *b*; Phumichai *et al.*, 2005; Phumichai and Hosaka, 2006). Hosaka and Hanneman (1998*a*, *b*) interpret the behaviour of *Sli* as a single dominant factor that displays sporophytic inhibition of SI. Figure 1 illustrates the intriguing behaviour of *Sli*. In cross #1, SI *S. phureja* (S_2S_3) pollinates the inbred (i.e. seventh selfed generation) SC *S. chacoense* source of *Sli* (S_1S_1). In this compatible cross, the progeny segregate

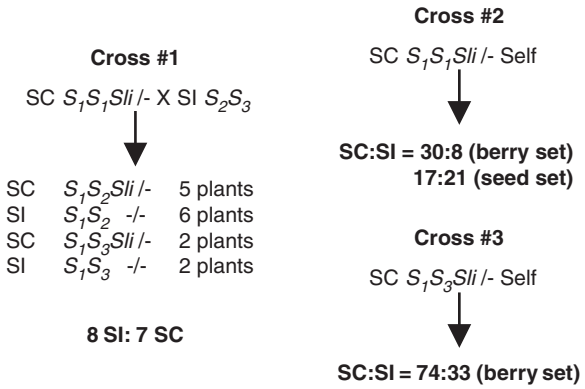

FIG. 1. Genetic behaviour of *S-locus inhibitor* (*Sli*) from *Solanum chacoense*. Cross #1, a cross between the inbred *S. chacoense* S_1S_1 *Sli* source plant and an SI *S. phureja* plant designated S_2S_3. The *S. chacoense* parent is heterozygous for *Sli* and behaves as a dominant factor that inhibits SI in half the progeny. SC is unlikely to be due to a defective *S*-locus since half the progeny are SI. Crosses #2 and #3, selfing the *S. chacoense* parent or one of the SC progeny of cross #1 also results in SI progeny. Thus, functional *S*-haplotypes are transmitted through the pollen even when *Sli* is not.

about 1 : 1 for SC, indicating that the defect is heterozygous. Importantly, SC is not due to a defective *S*-locus; both S_1S_2 and S_1S_3 SI progeny are obtained. Also, reciprocal sib-crosses (e.g. SI S_1S_2 × SC S_1S_2 and vice versa, not shown) indicate that pistil function is normal; thus, *Sli* only affects pollen function. The novelty of the *Sli* defect is evident from crosses #2 and #3, showing results from selfing the SC S_1S_1 parent in cross #1 and one of the SC S_1S_3 progeny from cross #1. Progeny from these self-pollinations also segregate for SC and SI. The existence of SI progeny shows that fully functional *S*-haplotypes are transmitted through pollen even when the *Sli* factor is not. Thus, *Sli* appears to act as a sporophytic factor that suppresses pollen function in a gametophytic SI system.

This surprising behaviour of *Sli* contrasts dramatically with the behaviour of pollen SI defects induced by radiation or transgenesis (Golz *et al.*, 1999, 2001; Sijacic *et al.*, 2004). As just described, these are explained in terms of the HAP effect: pollen expressing two different pollen *S*-alleles is not rejected and plants that display this effect are, therefore, SC. Crucially, the HAP effect is strictly gametophytic (Golz *et al.*, 2000). SC caused by the HAP effect can be due to changes at the *S*-locus or elsewhere. For instance, an *Sc*-haplotype with a translocation closely linked to the *S*-locus such that pollen *S* genes from two haplotypes, S_1 and S_2, segregate together would cause SC (Golz *et al.*, 2001). Since the effect is expressed gametophytically, selfing an S_xS_c plant would yield only SC progeny (i.e. S_xS_c and S_cS_c but not S_xS_x). An unlinked pollen *S* gene would have a similar gametophytic effect. For example, when the HAP effect is used to induce SC by transforming *Petunia inflata* with the pollen *S* $PiSLF_2$ gene, all self progeny are SC because pollen not expressing the transgene is rejected (Sijacic *et al.*, 2004). Thus, SC of *Sli*-containing plants cannot be easily explained by a duplication or translocation of a pollen *S* gene.

The behaviour of *Sli* has potentially important implications for understanding the mechanism of SI. Incompatibility is the default condition in one model (Hua *et al.*, 2008), while

compatibility is the default condition in the other (McClure, 2008). The behaviour of *Sli* may favour the latter because the mutant causes SC. Furthermore, the difference in PPM behaviour between Solanaceae and Rosaceae, along with differences in S-RNase gene structure and other observations, has been interpreted as evidence that S-RNase-based SI in the two families is fundamentally different (Tao and Iezzoni, 2010). *Sli* could, in principle, inhibit SI at several stages (Fig. 2): S-RNase uptake, the interaction of S-RNase and SLF, a later event necessary for pollen rejection, or through the action on a non-*S*-specific factor such. Information about the molecular nature of *Sli* is clearly needed to distinguish between these possibilities. Nevertheless, *Sli* represents an under-appreciated type of mutation that allows pollen to overcome the cytotoxic activity of S-RNase independent of its pollen *S*-genotype. Further studies of *Sli* should, therefore, reveal new insights into the mechanism of SI.

NON-*S*-SPECIFIC FACTORS

Although the simple genetics of S-RNase-based SI demonstrate that a single locus determines the specificity of pollen rejection, other genes are also required. Candidates for these modifier genes have been identified through genetic studies of breakdown of pollen- and pistil-side functions in SI as well as through biochemical studies. The functions of modifier genes can be grouped into genes directly required for expression of *S*-specificity determinants (*S-RNase*, *SLF*), genes required for SI but not for pollination *per se*, and genes required for SI and other pollen–pistil interactions (McClure *et al.*, 2000). Modifier genes with a unique function in SI are of special interest, as they offer insights not available through studies of *S*-specificity determinants alone.

Pollen factors

Biochemical and genetic studies have identified several potential modifier genes expressed in pollen. Although further studies are needed, the *Sli* factor already discussed has the characteristics of a pollen gene required for SI but not pollination *per se*. Vilanova *et al.* (2006) described a PPM in SC *Prunus armeniaca* 'Canino' that behaves somewhat similarly although it acts gametophytically: the gene is unlinked to the *S*-locus and does not affect expression of

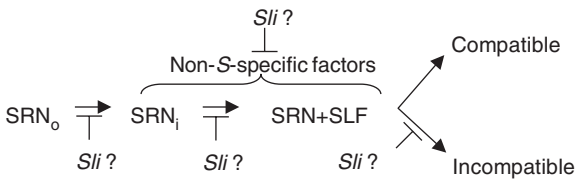

FIG. 2. Possible functions of *Sli*. In S-RNase-based SI, S-RNase is secreted into the pistil extracellular matrix (SRN_o). It is taken up into the pollen tube (SRN_i), where it interacts with SLF (SRN + SLF) to determine whether a pollination is compatible or incompatible. *Sli* may prevent S-RNase uptake, its interaction with SLF, or a downstream step needed for incompatibility. It may also prevent the action of non-S-RNase factors needed for pollen rejection. It is not, however, required for pollen tube growth *per se* and does not appear to interfere with the compatible pathway.

S-locus genes or cause sterility. It is noteworthy that both mutations result in SC.

Sims and Ordanic (2001) used a yeast two-hybrid (Y2H) approach to identify *Ph*SBP1, a RING domain protein from *P. hybrida* pollen that binds to S-RNase. Proteins similar to SBP1 have been identified in *S. chacoense*, *P. inflata* and *N. alata* (O'Brien *et al.*, 2004; Hua and Kao, 2006; Lee *et al.*, 2008). *SBP1* is expressed in a variety of tissues. In addition to binding S-RNase, *SBP1* binds other proteins in Y2H assays, including the C-terminal domain of pistil arabinogalactan proteins (AGPs) such as the 120-kDa glycoprotein (120K), SLF, and certain transcription factors (Hua and Kao, 2006; Ben-Naim *et al.*, 2007; Lee *et al.*, 2008). Hua and Kao (2006) proposed that SBP1 forms a complex with SLF that allows degradation of non-self S-RNase in pollen and thus provides resistance to its cytotoxic effect. Although its broad expression pattern and variety of binding partners suggest that SBP1 functions in a process more general than SI *per se*, this does not exclude a role in degradation of S-RNase.

A number of additional putative pollen modifier genes encode proteins that form complexes with SLF. These SLF-binding proteins have been reviewed elsewhere (Hua *et al.*, 2008; Zhang *et al.*, 2009). Briefly, most F-box proteins are thought to function as adaptors that allow specific client proteins destined for degradation to enter one of several types of E3 ubiquitin ligase complexes. In some models for S-RNase-based SI, an SCF-like (Skp1-Cullin-F-box) E3 complex containing SLF provides for ubiquitylation of non-self S-RNase leading to its degradation, while self S-RNases fail to bind productively and thus escape degradation (Hua *et al.*, 2008; Zhang *et al.*, 2009). A canonical SCF E3 ubiquitin ligase complex includes a Skp1-like protein that links a cullin 1 scaffold protein to an F-box protein. Huang *et al.* (2006) used Y2H to identify *Ah*SSK1, a Skp1-like protein from *Antirrhinum* that binds to SLF. They also found evidence that *Ah*SSK1, in turn, binds a cullin 1-like protein and suggested that the ternary complex functions in SI. Although the F-box domain is generally thought to confer interaction with Skp1-like proteins, Hua and Kao (2006) reported that the three Skp1-like proteins expressed in *P. inflata* pollen do not bind SLF. Instead, as just described, these authors presented evidence that SLF binds the RING domain protein SBP1 and further suggested that it functions in a complex with a specific cullin 1 (Hua and Kao, 2008; Hua and Kao, 2006). Thus, several SLF-containing protein complexes are possible. Tests of whether the genes encoding these proteins truly behave as SI modifier genes have not been reported. However, if SLF provides for ubiquitylation and degradation of non-self S-RNase as a necessary step to overcome the cytotoxicity of S-RNase, then preventing expression of genes encoding other proteins in an SLF-containing complex would be lethal. On the other hand, the behaviour of *Sli* and PPMs in the Rosaceae suggest that suppressing pollen *S* function can lead to compatibility. Thus, direct functional tests to determine whether putative SLF-complex members are truly SI modifier genes should be considered.

Pistil factors

S-RNase is an abundant component of the pistil ECM. Other factors are also required for SI, albeit for functions other than

determining *S*-specificity. Modifier genes encoding putative pistil SI factors have been identified through biochemical and genetic studies.

120K

S-RNase binding studies identified some of these factors and revealed possible connections between SI and other pollen-pistil interactions. Four S-RNase-binding proteins were identified in pistil extracts using immobilized S-RNase (Cruz-García *et al.*, 2003, 2005). These include a small protein similar to chemocyanin, a pistil protein from *Lilium longiflorum* that displays pollen tube chemotactic activity *in vitro* (Kim *et al.*, 2003). Three other S-RNase-binding proteins from *N. alata* are abundant transmitting tract AGPs that share a homologous cysteine-rich C-terminal domain: 120K, the pistil extensin-like protein III (PELPIII) and the transmitting tract-specific glycoprotein (TTS) (Cheung *et al.*, 1993; Schultz *et al.*, 1997; de Graaf *et al.*, 2003). Given the extraordinary concentration of these proteins in the ECM, AGPs are likely to form complexes with S-RNase *in planta* (Cruz-Garcia *et al.*, 2003). The functional significance of such putative S-RNase complexes is uncertain. It is noteworthy that each of the S-RNase binding proteins has been reported to interact with pollen tubes (Lind *et al.*, 1996; Wu *et al.*, 1995; Kim *et al.*, 2003; de Graaf *et al.*, 2004).

The function of *120K* was tested using RNAi. Careful selection of the RNAi target region allowed production of plants with no detectable 120K protein yet near normal levels of TTS and PELP III proteins (Hancock *et al.*, 2005). These plants fail to show *S*-specific pollen rejection, suggesting a direct role for *120K*. Neither pollen tube growth nor uptake of *S*-RNase into pollen tubes is greatly affected in *120K*-silenced plants (Hancock *et al.*, 2005; Goldraij *et al.*, 2006). Thus, 120K is not essential for these processes. Interestingly, 120K is known to enter pollen tubes, but this has not been shown for the other S-RNase-binding AGPs (Wu *et al.*, 1995; Lind *et al.*, 1996; de Graaf *et al.*, 2004; Wolf *et al.*, 2004).

Immunolocalization studies in *N. alata*-compatible pollen tubes using antibodies to both S-RNase and 120K showed that 120K associates with the margin of large pollen-tube vacuoles far from the tube tip (Goldraij *et al.*, 2006) and that S-RNase is present in the lumen of these vacuoles. In *N. alata*, pollen rejection occurs only after a delay of several hours. Experiments comparing compatible and incompatible pollinations prior to rejection showed similar compartmentalization patterns for 120K and S-RNase. However, at later times (i.e. 36 h), the vacuolar localization of S-RNase and 120K in incompatible pollen tubes breaks down (Goldraij *et al.*, 2006). S-RNase compartmentalization could provide a mechanism for pollen tubes to evade rejection, and this concept is the basis for one model of S-RNase-based SI (McClure, 2008).

Little is known about how S-RNase, 120K and other pistil proteins are taken up and sorted in pollen tubes. Y2H or pull-down experiments to test for interactions between pollen and pistil proteins could contribute to these potentially important processes. Lee *et al.* (2009) used the C-terminal domain of 120K to identify a pollen protein, *Na*PCCP (Pollen C2 domain-containing protein from *N. alata*), that binds both 120K and phosphatidyl inositol-3-phosphate. Biochemical experiments, live imaging and immunolocalization experiments showed that *Na*PCCP associates with the pollen tube plasma membrane and internal compartments (Lee *et al.*, 2009). Since phosphatidyl inositol-3-phosphate is implicated in endocytosis and endomembrane transport, *Na*PCCP may contribute to sorting pistil proteins in pollen. However, there is no evidence that this function is restricted to proteins implicated in SI.

Na*StEP*

Comparisons between the pollen rejection activity associated with *S-RNase* transgenes in *N. alata* and SC *N. plumbaginifolia* provide clear evidence for pistil modifier genes required for SI. Murfett *et al.* (1996) showed that expressing S-RNase in *N. plumbaginifolia* does not cause *S*-specific pollen rejection. However, *S*-specific pollen rejection is fully restored by crossing in modifier genes from SC *N. alata* 'Breakthrough'. Diverse cDNA cloning strategies identified candidate modifier gene transcripts (McClure *et al.*, 1999; Juarez-Diaz *et al.*, 2006; Busot *et al.*, 2008). A cDNA clone corresponding to a stigma-expressed protein designated *Na*StEP shows a very strong differential signal (Busot *et al.*, 2008). The encoded *Na*StEP protein is acidic and homologous to Kunitz-type proteinase inhibitors. It is abundant in papillar cells on the stigma surface and accumulates after pollination. Low-level expression is also detectable in the style. *Na*StEP protein shows an intriguing change in localization in the stigma after pollination. A putative vacuolar targeting signal is present near the N-terminus; in unpollinated stigmas, *Na*StEP is present in papillar cell vacuoles. However, after pollination, *Na*StEP is released into the stigmatic exudate (Busot *et al.*, 2008), apparently through perforations in the papillar cell wall. Antibodies to *Na*StEP show cross-reacting pistil proteins in SI species, including *N. alata*, *N. forgetiana* and *N. bonariensis*, but not in SC *N. tabacum*, *N. plumbaginifolia*, *N. benthamiana*, *N. longiflora* and *N. glauca* (Busot *et al.*, 2008). Studies are currently underway to test whether *Na*StEP is required for SI and whether it is taken up by pollen tubes. Although it is not known whether *Na*StEP is an active proteinase inhibitor, an intriguing possibility is that it may modulate breakdown of pistil proteins inside the pollen tube.

Na*Trxh*

A cDNA-AFLP screen identified other pistil-expressed genes with higher expression in *N. alata* compared with *N. plumbaginifolia* (Juarez-Diaz *et al.*, 2006). One differentially expressed gene encodes *Na*Trxh, a protein belonging to thioredoxin *h* subgroup II (Fig. 3; Juarez-Diaz *et al.*, 2006). Participation of thioredoxin *h* proteins in pollen rejection has been demonstrated in *Brassica*, where down-regulation of *THL1* and *THL2* leads to a partial breakdown of SI (Haffani *et al.*, 2004). Other experiments suggest that THL1 prevents autophosphorylation of the *S*-receptor kinase in the absence of a ligand (Cabrillac *et al.*, 2001). The *Na*Trxh protein contains an N-terminal extension that may be important for targeting or activity but does not posses a canonical secretion signal. Immunolocalization experiments, nevertheless, showed that *Na*Trxh is secreted into the transmitting tract ECM where it

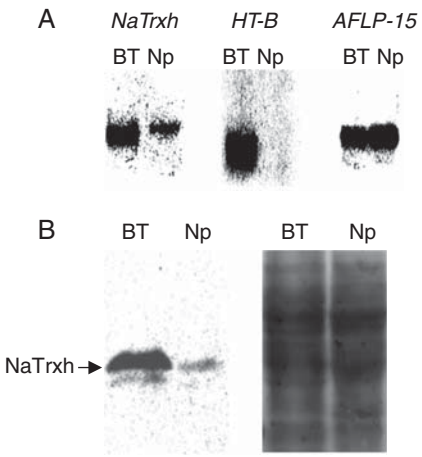

FIG. 3. Expression of *NaTrxh*. (A) RNA level expression: pistil RNA (10 μg) from *N. alata* 'Breakthrough' (BT) or *N. plumbaginifolia* hybridized with ^{32}P-labelled probes for *NaTrxh*, *HT-B* or a non-differential clone *AFLP-15*. (B) Protein blot analysis: left, total pistil protein (15 μg) probed with anti-*Na*Trxh; right, a similar gel stained with Coomassie Blue.

colocalizes with S-RNases and *Na*TTS. Moreover, the *Na*Trxh sequence is sufficient to confer secretion of green fluorescent protein into the extracellular space (Juarez-Diaz *et al.*, 2006). *In vitro* reduction assays show that *Na*Trxh reduces S-RNase in crude style extracts, suggesting that it is a potential extracellular substrate (Juarez-Diaz *et al.*, 2006). Although a functional test of *NaTrxh* in pollen rejection has not yet been performed, the results suggest it may function as a modifier gene in the SI response. It will be interesting to determine whether reduction of S-RNase by *Na*Trxh alters its three-dimensional structure or trafficking in pollen tubes. For example, *Na*Trxh may disrupt S-RNase–AGP complexes in pollen tube vacuoles or alter their structures and thus affect the destination in the pollen endomembrane system.

HT-B

HT-B was the first modifier gene cloned. Like *NaStEP*, it was identified as a differential cDNA expressed in *N. alata* but not in *N. plumbaginifolia* (McClure *et al.*, 1999). Antisense experiments in *Nicotiana* showed that suppressing *HT-B* expression in the pistil prevents *S*-specific pollen rejection (McClure *et al.*, 1999). In these experiments, an anti-HT-B antibody was used to detect expression in antisense-suppressed plants. Plants with reduced expression show partial breakdown in *S*-specific pollen rejection, and plants with no detectable HT-B protein allow many otherwise incompatible pollen tubes to penetrate to the base of the style. An *HT-B* gene was recently identified in SI *P. inflata*, and an RNAi construct tested its function in SI. Plants with extremely low *HT-B* expression show partial breakdown in SI. This observation supports a role in SI and also suggests that even small amounts of HT-B protein are sufficient (Puerta *et al.*, 2009). Plants in the genus *Solanum* (including relatives of potato and tomato) express two very similar genes, *HT-A* and *HT-B*. O'Brien *et al.* (2002) used RNAi to test the function of these genes in *S. chacoense*. Plants with strongly suppressed *HT-B* expression show partial breakdown in SI. Since these plants retain near normal levels of *HT-A* mRNA, the authors concluded that *HT-B* is implicated in SI but that *HT-A* is not. Kondo *et al.* (2002), who examined *HT-A* and *HT-B* genes in cultivated tomato and several of its wild relatives, noted that *HT-B* genes are disrupted or contain stop codons in SC species. However, a recent study of SI *S. habrochaites* showed that both SI and SC accessions have stop codons in *HT-B*. Protein-level studies similarly failed to detect HT-B protein in all accessions of *S. habrochaites* (Covey *et al.*, 2010). Since all indications are that even extremely low levels of HT-B protein are sufficient, it is worth reexamining the possibility that HT-A can function in *S*-specific pollen rejection. It is also possible, as Puerta *et al.* (2009) suggested, that *HT-B* has an indirect role in SI and strengthens the response. Such an effect could vary between different *S*-haplotypes.

Although HT-B's exact function is not known, it is clear that it is not required for S-RNase uptake. Goldraij *et al.* (2006) observed normal S-RNase uptake and compartmentalization in *HT-B*-suppressed plants that are incapable of *S*-specific pollen rejection. Thus, S-RNase uptake is not sufficient to cause pollen rejection, and S-RNase sequestered in the vacuole also is not necessarily detrimental to pollen-tube growth, at least in the absence of HT-B protein. Therefore, it is very intriguing that HT-B protein appears to be degraded after compatible pollination. Immunolocalization experiments in *Nicotiana* show little or no anti-HT-B reactive protein in compatible pollen tubes but substantial amounts in incompatible pollen tubes (Goldraij *et al.*, 2006). This observation is consistent with HT-B protein being taken up and degraded in pollen tubes. Moreover, analysis of style extracts showed that, after compatible pollination, HT-B levels drop to 3–25 % of unpollinated controls. HT-B levels also drop after incompatible pollination but only by about 2-fold (Goldraij *et al.*, 2006).

It is now clear that several HT-like proteins are expressed in the pistil. Kondo and McClure (2008) noted similarities between HT-proteins and certain glycine-rich proteins, including nodulin-24. Interestingly, the putative secretion signals are among the most conserved features of the HT/NOD-24 family. It is followed by a variable core sequence of about 50 amino acid residues. All family members contain one or more cysteine motifs, including CXXCXC, CXXXCC or CXXCC. HT-A and HT-B proteins are distinguishable by a characteristic stretch of about 20 asparagine and aspartic acid residues (ND domain) that is flanked by the cysteine motifs CXXCXC and CXXXCC. Sassa and Hirano (2006) identified the *PiHTL-A* and *PiHTl-B* transcripts in *P. inflata* as products of alternative splicing of a single gene. The encoded proteins contain a CXXCXCCXXXCXXXC motif but lack an ND domain. *PiHTL-A* and *PiHTl-B* do not appear to be required for SI. Kondo and McClure (2008) identified a family of small HT-family proteins in *Nicotiana* designated HT-M. Like the *P. inflata* HTL-A and -B proteins, HT-M proteins lack an ND domain and contain a single cysteine motif. Like HT-B, HT-M protein levels decrease after pollination. Unlike HT-B, the response to compatible and incompatible pollen is similar.

MODELS

Figure 4 shows two models used to explain S-RNase-based SI: the S-RNase degradation model (left) and the

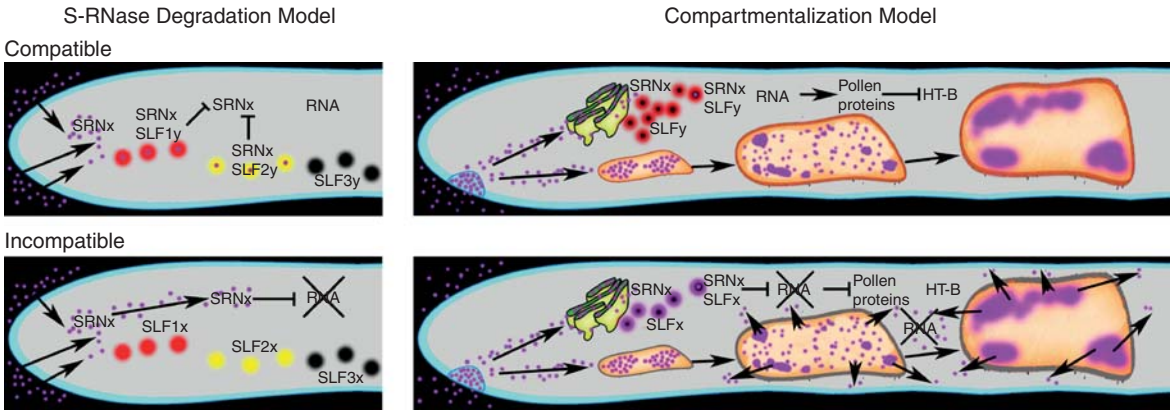

FIG. 4. Models for *S*-specific pollen rejection. Pollen tubes are shown in the pistil ECM containing a single S-RNase (SRN_x, purple); although, in a typical *S*-heterozygote two S-RNases would be present. Compatible (top, S_y-pollen tube in a pistil expressing S_x-RNase) and incompatible (bottom, S_x-pollen tube) pollinations are shown. Left: an S-RNase degradation model that implicates multiple SLF proteins (SLF1, red; SLF2, yellow; SLF3, black regardless of whether they are derived from the S_x- or S_y-haplotype) collaborating to cause S-RNase degradation. These models do not specify a route from the ECM to the pollen cytoplasm where the S-RNase-SLF interaction occurs. Right: the compartmentalization model shows most S-RNase taken up by endocytosis and trafficking by default to progressively larger vacuoles in more mature regions of the pollen tube. S-RNase must exit the endomembrane system to interact with SLF; a single SLF (red, SLF_x; blue SLF_y) is shown. Both models show degradation of pollen RNA (cross) in incompatible pollen tubes, a process that do not occur in compatible pollen tubes (no cross). In S-RNase degradation models, compatibility is attributed to wholesale degradation of S-RNase. The compartmentalization model, in contrast, emphasizes S-RNase isolation from the cytoplasm.

compartmentalization model (right). In both, interaction between S-RNase and SLF determines whether pollination is compatible or incompatible, as shown in Fig. 2. Both models entail degradation of pollen RNA after incompatible pollination (RNA, crossed out), which leads to a general inhibition of pollen protein synthesis needed for continued growth. The latter is explicitly shown only in the compartmentalization model, which emphasizes homeostatic mechanisms that maintain the integrity of the endomembrane system and facilitate degradation of HT-B from the pistil. The explanations for how compatible pollen evades S-RNase cytotoxicity are different in the two models.

In S-RNase degradation models, interaction between S-RNase and SLF leads to wholesale destruction of S-RNase, thereby providing resistance to its cytotoxic effect (Hua *et al.*, 2008; Zhang *et al.*, 2009). In the version of the S-RNase-degradation model shown in Fig. 4, multiple SLF proteins (SLF1, red; SLF2, yellow; SLF3, black) collaborate to provide resistance to S_x-RNase (Kubo *et al.*, 2010). Different *S*-haplotypes express a unique array of allelic *SLF* genes (e.g. $SLF1_x$ and $SLF1_y$). Individual SLF proteins may or may not bind a given S-RNase. In the hypothetical compatible pollination shown (top, left), only $SLF1_y$ and $SLF2_y$ bind S_x-RNase, preventing RNA degradation. In contrast, in an incompatible pollination, none of the SLF proteins productively bind self S-RNase ($SLF1_x$, $SLF2_x$ and $SLF3_x$ are shown without bound S_x-RNase; bottom, left), pollen RNA is degraded, and pollen tube growth is inhibited. Thus, the collaborative S-RNase degradation model rationalizes the finding that multiple *SLF* genes are linked to a given *S-RNase* gene as well as results showing variable binding between individual S-RNase and SLF proteins (Kubo *et al.*, 2010). These features, however, make definitive tests difficult. Since the number of *SLF* genes in a given *S*-haplotype is unknown and since S-RNase binding for a given SLF protein cannot be predicted, almost any binding result or transgenic test of *S*-specificity can be accommodated.

Ubiquitylation and subsequent proteasomal degradation of non-self S-RNase is taken to be the fundamental process responsible for compatibility in S-RNase degradation mechanisms. Thus, the identification of SLF-containing complexes provides support for this model (Qiao *et al.*, 2004*a*, *b*; Hua and Kao, 2006; Huang *et al.*, 2006; Hua *et al.*, 2007). *S*-specific interactions between S-RNase and SLF have been reported *in vitro*, but degradation of S-RNase in pollen extracts is not *S*-specific (Hua and Kao, 2006). Although non-self S-RNase degradation is a plausible mechanism, a direct connection between S-RNase/SLF interactions and S-RNase degradation has not been established.

The compartmentalization model (Fig. 4, right) explains resistance to non-self S-RNase by its sequestration from the cytoplasm in compatible pollen tubes. Immunolocalization experiments show large amounts of S-RNase sequestered in vacuoles of compatible pollen tubes and compartmentalization breakdown in incompatible pollen tubes (Goldraij *et al.*, 2006). The compartmentalization model emphasizes the homeostatic effects of normal pollen gene expression (pollen proteins, Fig. 4) in relation to maintenance of the endomembrane system and elimination of HT-B protein. As previously discussed, degradation of HT-B occurs in compatible pollen tubes (Fig. 4; top, right) and is assumed to rely on normal pollen gene expression. Much S-RNase traffics to vacuoles, presumably, through another default process. Nevertheless, since SLF is a cytoplasmic protein, some S-RNase must exit the luminal compartment, possibly after retrograde transport to the endoplasmic reticulum, as has been demonstrated for other cytotoxins (McClure, 2006). In the compartmentalization model, the non-self S-RNase/SLF interaction does not cause wholesale degradation of S-RNase, which is largely inaccessible. This feature of the model rationalizes the

immunolocalization experiments showing large amounts of S-RNase in compatible pollen tubes (Goldraij *et al.*, 2006), but it does not address the question of the direct function of SLF-containing ubiquitin ligase complexes. For convenience, Fig. 4 shows alternate complexes for both compatible and incompatible interactions (i.e. SRN_x/SLF_y, top; SRN_x/SLFx, bottom), but the model places no specific restraint on the biochemical nature of these interactions. The model only predicts that, whatever the function of the SLF-complex, a non-self S-RNase does not cause RNA degradation, a normal spectrum of pollen proteins are produced, and growth proceeds. Incompatibility, however, is seen as an active process. One speculation is that self S-RNase activates the SLF-complex to target a pollen protein whose function is to degrade HT-B, which promotes the ability of S-RNase to cause pollen rejection. Regardless of the actual target of the SLF complex, it is clear that in an incompatible pollination HT-B is stabilized, pollen RNA is degraded, the endomembrane system loses its integrity and ever more S-RNase is released. Thus, incompatibility is seen as self-reinforcing.

The compartmentalization and S-RNase degradation models are not mutually exclusive. For example, the presence of large amounts of S-RNase in pollen tube vacuoles does not exclude degradation of smaller amounts in the cytoplasm, and vice versa. However, if non-self S-RNase degradation is the dominant mechanism for S-RNase resistance, then breakdown of SI in pollen should lead to rejection. Mutant studies in *N. alata*, the HAP effect, and the behaviour of *SLF* transgenes in *P. inflata* are interpreted as consistent with this prediction (Golz *et al.*, 2001; Hua *et al.*, 2008). On the other hand, *SBF* mutations in several *Prunus* species clearly result in pollen compatibility, not incompatibility (Ushijima *et al.*, 2004; Sonneveld *et al.*, 2005; Vilanova *et al.*, 2006; Marchese *et al.*, 2007; Tao *et al.*, 2007). Tao and Izzeoni (2010) suggest this latter observation is due to a fundamental difference between the mechanism of S-RNase-based SI in the Solanaceae and Rosaceae. Differences between the models may exist, but the major similarities (i.e. S-RNase determining *S*-specificity in the pistil and F-box proteins fulfilling this function in pollen) are more striking than the differences. The behaviour of *Sli* in *Solanum* may be important as well. Although no information is available about its identity or molecular mechanism, the genetic data are not consistent with gametophytic action. Therefore, *Sli* cannot be attributed to the well-documented HAP effect, which is strictly gametophtic. Yet, similar to the pollen-side mutations in *Prunus*, when *Sli* causes breakdown of SI in pollen, compatibility, not incompatibility, is the result. These pollen-side defects in *Prunus* and in *Solanum* are consistent with pollen rejection resulting from an active process in pollen: in the absence of recognition, pollen is compatible. Of note, this is also the situation in *Papaver* SI (Wheeler *et al.*, 2010). Although the molecular mechanism in *Papaver* is completely different, SI signalling clearly induces a series of active processes in pollen that result in incompatibility. In this regard, it is also important that pistil-modifier genes are required for S-RNase to function in S-RNase-based SI. Some modifier genes have been identified, and the activities of additional candidates are being tested. Nevertheless, none of those tested so far affect S-RNase uptake (Goldraij *et al.*, 2006). In the absence of 'activation' by modifier genes, S-RNase remains harmlessly sequestered in pollen tube vacuoles.

PROSPECTS

More questions than answers about S-RNase-based SI remain. Progress is needed at all levels. At the biochemical level, it would be helpful to establish clearly the target of the SLF complex. Is S-RNase a target or a modifier of its activity toward other proteins? At the genetic level, it would be helpful to identify all of the genes required for SI to function on both the pollen and pistil sides. *Sli* is an especially intriguing factor acting in pollen. Many additional factors are likely to contribute to SI in both the pollen and the pistil. Finally, at the cell biological level, it would be helpful to characterize the S-RNase trafficking pathway from the ECM to the vacuole and understand how it gains access to the cytoplasm.

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