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REVIEW PAPER

'A life or death decision' for pollen tubes in S-RNase-based self-incompatibility

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Abstract

Mate choice is an essential process during sexual plant reproduction, in which self-incompatibility (SI) is widely adopted as an intraspecific reproductive barrier to inhibit self-fertilization by many flowering plants. Genetic studies show that a single polymorphic *S*-locus, encoding at least two components from both the pollen and pistil sides, controls the discrimination of self and non-self pollen. In the Solanaceae, Plantaginaceae, and Rosaceae, an S-RNase-based SI mechanism is involved in such a discrimination process. Recent studies have provided some important clues to how a decision is made to accept cross pollen or specifically to reject self pollen. In this review, the molecular features of the pistil and pollen *S*-specificity factors are briefly summarized and then our current knowledge of the molecular control of cross-pollen compatibility (CPC) and self-pollen incompatibility (SPI) responses, respectively, is presented. The possible biochemical mechanisms of the specificity determinant between the pistil and pollen S factors are discussed and a hypothetical S-RNase endosome sorting model is proposed to illustrate the distinct destinies of pollen tubes following compatible and incompatible pollination.

Key words: Endosome sorting, pollen tube growth, programmed cell death, self-incompatibility, SLF, S-RNase, ubiquitin–proteasome system.

Introduction

Recognition and discrimination of self and non-self gametes is a fundamental and widespread mechanism in the plant kingdom. In many flowering plants, self-incompatibility (SI) serves as one such mechanism (de Nettancourt, 2001; Franklin-Tong, 2008). Being a genetically controlled intraspecific reproductive barrier, SI is essential for the acceptance of cross pollen and the rejection of self pollen. Genetic studies in several SI species demonstrate that the pollen–pistil recognition is controlled by a single polymorphic locus known as the *S*-locus. The locus has been shown to encode components specifically expressed in pistil and pollen that together control SI. Thus, variants of the *S*-locus are defined as haplotypes and variants of the pollen or pistil components are termed alleles (Kao and Tsukamoto, 2004; Takayama and Isogai, 2005; Zhang *et al.*, 2009). In the SI response, genetically unrelated pollen is able to complete fertilization whereas self pollen is rejected; this is referred to as cross-pollen compatibility (CPC) and self-pollen incompatibility (SPI) responses, respectively. On the basis of the genetic control of pollen behaviour, SI is classified as gametophytic (GSI) and sporophytic (SSI) types. In several GSI families (Solanaceae, Plantaginaceae, Rosaceae, and Papaveraceae), pollen carrying an *S*-haplotype identical to one of the two *S*-haplotypes of the diploid pistil is recognized as self pollen and its tube growth arrested in the pistil, resulting in SPI, whereas pollen with an *S*-haplotype that is not present in the pistil, is recognized as non-self pollen and thus accepted by the pistil leading to CPC. For the SSI family (Brassicaceae), pollen acceptance or rejection is determined by the *S*-haplotypes of its diploid (sporophytic) parent.

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Recent studies have generated important insights into our understanding of the molecular mechanisms of SI in several model species (for recent reviews, see Takayama and Isogai, 2005; Franklin-Tong, 2008; Hua *et al.*, 2008; McClure, 2009; Zhang *et al.*, 2009). In this review, current progress in the making of a 'life or death' decision of pollen tubes in S-RNase-based SI found in the Solanaceae, Plantaginaceae, and Rosaceae is mainly described, and some emerging clues about how the pistil and pollen components interact with each other to arrive at this decision are discussed.

Known players in S-RNase-based SI response

During the last two decades, the molecular natures of the two determining components from both the pistil (pistil *S*) and the pollen (pollen *S*) sides have been successively revealed in several families (Takayama and Isogai, 2005; Wheeler *et al.*, 2009). In the Solanaceae, Plantaginaceae, and Rosaceae, *S*-ribonucleases (S-RNases), sharing amino acid sequence similarity to RNase T_2 of *Aspergillus oryzae* (McClure *et al.*, 1989), have been shown to be central to the pistil SI response through both gain-of-function and antisense suppression transgenic experiments (Lee *et al.*, 1994; Murfett *et al.*, 1994) (Table 1). This type of SI is thus often referred to as the S-RNase-based SI or the Solanaceae-type gametophytic SI because S-RNases were first detected in *Nicotiana alata*, a solanaceous species (Bredemeijer and Blaas, 1981; Anderson *et al.*, 1986).

Table 1. Factors and their interacting partners involved in S-RNase-based SI response

Proteins	Interaction partners[a]	References[b]
S-RNase	SLF [a, b, c]	Qiao *et al.*, 2004*a*; Hua and Kao, 2006
	SBP1 [a, b]	Hua and Kao, 2006; Hua and Kao, 2008
	Na120K [b, c]	Cruz-Garcia *et al.*, 2005
	NaTTS [b, c]	Cruz-Garcia *et al.*, 2005
	NaPELPIII [b, c]	Cruz-Garcia *et al.*, 2005
	PGPS/D3 [a]	Guo *et al.*, 2006
SLF	S-RNase [a, b, c]	Qiao *et al.*, 2004*a*; Hua and Kao, 2006
	SSK1 [a, b]	Huang *et al.*, 2006
	SBP1 [a, b]	Hua and Kao, 2006
SLFL	S-RNase [b]	Hua *et al.*, 2007
SSK1	SLF [a, b]	Huang *et al.*, 2006
	Cul1-like [b]	Huang *et al.*, 2006
SBP1	S-RNase [a, b]	Hua and Kao, 2006; Hua and Kao, 2008
	SLF [a, b]	Hua and Kao, 2006
	Cul1 [a, b]	Hua and Kao, 2006
	PhUBC1 (E2) [a, b]	Hua and Kao, 2006
	Na120K [b]	Lee *et al.*, 2008
	NaTTS [b]	Lee *et al.*, 2008
HT-B	None	None
Na120K	S-RNase [b, c]	Cruz-Garcia *et al.*, 2005
	SBP1 [b]	Lee *et al.*, 2008
	NaPCCP [b]	Lee *et al.*, 2008
NaTTS	S-RNase [b, c]	Cruz-Garcia *et al.*, 2005
	SBP1 [b]	Lee *et al.*, 2008
	NaPCCP [b]	Lee *et al.*, 2008
NaPCCP	Na120K [b]	Lee *et al.*, 2008
	NaTTS [b]	Lee *et al.*, 2008
NaPELPIII	S-RNase [b, c]	Cruz-Garcia *et al.*, 2005

[a] Physical interactions are demonstrated by yeast two-hybrid ([a]), pull-down ([b]) and co-immunoprecipitation ([c]) assays, respectively.
[b] References are given for initial detection of the physical interactions.

More recently, *SLF* (*S*-locus F-box gene) in Solanaceae and Plantaginaceae and *SFB* (*S*-haplotype-specific F-box gene) in Rosaceae are shown to encode the pollen *S*-specificity determinant in S-RNase-based SI. *SLF* genes were first reported in Plantaginaceae (as represented in *Antirrhinum*) by sequencing an *S-RNase*-linked region (Lai *et al.*, 2002). Importantly, gain-of-function transformation experiments in Solanaceae and Plantaginaceae demonstrated that the *SLF* gene is the pollen *S* (Sijacic *et al.*, 2004; Qiao *et al.*, 2004*b*) (Table 1). Interestingly, Hua *et al.* (2007) excluded the function of *SLF-like* genes paralogous to *SLF* in SI because they showed no competitive interaction phenomenon where two heteroallelic pollen *S* produce pollen compatibility as *SLF*. For SLF and SFB proteins, several different features have been indicated, suggesting that they appear to function differently. First, competitive interaction appears not to be present in tetraploid sour cherry (Rosaceae) plants (Hauck *et al.*, 2006*b*), although competitive interaction of heteroallelic pollen is responsible for SI in many other species (Golz *et al.*, 1999, 2001; Xue *et al.*, 2009) including some from the Rosaceae (Huang *et al.*, 2008). Second, truncated or deleted *SFB* forms have been widely reported (Ushijima *et al.*, 2004; Sonneveld *et al.*, 2005; Hauck *et al.*, 2006*a*; Tsukamoto *et al.*, 2006; Vilanova *et al.*, 2006), whereas no *SLF* deletion mutants have been found in Solanaceae and Plantaginaceae as their deletion is considered to be gametophytic lethal (Golz *et al.*, 2001; Xue *et al.*, 2009). Third, the *SFB* genes characterized so far show more polymorphisms than that of the *SLF* genes (Newbigin *et al.*, 2008). Fourth, *SLF* and *SFB* apparently show a differential pattern of positive selection (Xue *et al.*, 2009).

Besides the genetically determined specificity factors respectively expressed in pollen and pistil, other factors have been shown to be required to aid the complex SI process and are known as non-*S*-specificity factors (Table 1). These factors, not encoded by the *S*-locus, have been identified in both sides and are considered to function during different stages of the SI reaction, ranging from the initiation, recognition to the final cross-pollen acceptance or to self-pollen growth inhibition. For example, from the pistil side, HT-B (H-Top Band) and 120K (120 kDa glycoprotein) proteins have been identified to enter pollen tubes and are involved in expressing the SI phenotype because their reduced expression led to compatibility, although their direct relations with the *S*-specificity factors are still unknown (McClure *et al.*, 1999; Hancock *et al.*, 2005; McClure, 2006). Whereas, SSK1 (SLF-interacting Skp1-like 1), a protein not only sharing a similar expression pattern with SLF as a pollen-specific protein but also interacting with several SLF proteins, appears to be involved in the CPC response (Huang *et al.*, 2006). Besides, a ubiquitously expressed

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protein, SBP1 (S-RNase Binding Protein 1), whose role has been characterized in pollen biochemically, is assumed to be associated with several factors taken up from the style transmitting tissue (Sims and Ordanic, 2001; Hua and Kao, 2006; Lee *et al.*, 2008). In general, it is believed that there must be more unidentified factors from both sides, although unrelated to the specificity determination, assisting S-RNase and SLF directly or indirectly for the final completion of CPC and SPI responses.

A multilayered restriction of S-RNase in cross-pollen compatibility

In Solanaceae-type SI plants, cross-pollen grains undergo the normal pollen hydration, germination, pollen tube penetration, and tip-growth steps, during which the most important one is the pollen tube penetration into the transmitting tissue of the style. After the entry into the style, pollen tubes will experience an autotropic-heterotropic transition for nutrient uptake (Stephenson *et al.*, 2003). These observations indicated that most of the materials needed for growth and final delivery of the sperm cell into the ovary were largely dependent on supply from the style extracellular matrix (ECM) (Stephenson *et al.*, 2003). Interestingly, the pistil S-RNase is expressed abundantly in the transmitting track of the style (Cornish *et al.*, 1987; Anderson *et al.*, 1989; Xue *et al.*, 1996), suggesting that the secreted S-RNases are taken up into the pollen tube along with other materials and this possibility has subsequently been demonstrated (Luu *et al.*, 2000; Goldraij *et al.*, 2006). However, the RNase activity could make it harmful to the pollen tubes and thus an adequate control of S-RNase activity would be essential for the CPC response. To maintain this tight control of S-RNase, the pollen tube must have evolved an intricate mechanism for its restriction. Recent findings appear to support two seemingly different ways of restricting S-RNase activity. One involves degradation of non-self S-RNase and another spatial sequestration by the pollen tube's endomembrane system (McClure, 2009; Zhang *et al.*, 2009). Most recently, an additional feedback regulation at the mRNA level of non-self S-RNase in the style has been revealed (Liu *et al.*, 2009). Therefore, it is essential for cross pollen actively to evade destruction by the incompatible system in the pistil and remain active until the double-fertilization.

Degradation of non-self S-RNase

When the first pollen *S* candidate, *AhSLF-S*$_2$, was identified to be an F-box gene, the hypothesis that it could function through the ubiquitin-mediated protein degradation pathway was proposed (Lai *et al.*, 2002), because an F-box protein usually serves as a member of an SCF (Skp1/Cullin1/F-box) ubiquitin ligase complex that often results in substrate ubiquitination and subsequent sorting and/or degradation (Smalle and Vierstra, 2004). In most cases, the F-box proteins often bind to the Skp1 adaptor by their loosely conserved F-box motifs and they also contain other distinct motifs for substrate binding (Lechner *et al.*, 2006). By contrast, in a substrate protein of an SCF complex, the lysine (K) residue(s) always serves as a site(s) specific for ubiquitination and degradation (Kerscher *et al.*, 2006).

Although it remains largely unclear how SLF functions biochemically, the available evidence indicates that it adopts an SCF^{SLF} ubiquitin ligase to interact with S-RNase for its ubiquitination and degradation. Qiao *et al.* (2004*a*) showed the first direct physical interaction between S-RNase and SLF proteins by both yeast two-hybrid and pull-down assays, suggesting that they could interact with each other for pollen–pistil recognition in pollen tubes albeit with no *S*-specificity. In addition, they used the proteasome-specific inhibitor, MG132, to reveal a potential role of the proteasome in S-RNase degradation during the CPC response, indicating that a specific degradation of non-self S-RNase might be a way of protecting non-self pollen tubes. Subsequently, Huang *et al.* (2006) identified the SSK1 protein in *Antirrhinum hispanicum* through a yeast two-hybrid assay using *AhSLF-S*$_2$ as the bait to screen a pollen cDNA library and further confirmed its interaction with SLF and Cullin1-like proteins in a pull-down assay, suggesting the presence of an AhSSK1-containing SCF^{SLF} complex. Such a typical SCF complex, recruited in the ubiquitin 26S-proteasome system (UPS), has been revealed in diverse pathways such as plant hormone signalling and defence responses (Vierstra, 2009). Meanwhile, the SBP1 protein (Sims and Ordanic, 2001) was reported to form a non-canonical SCF^{SLF} complex in *Petunia inflata*, with SBP1 replacing Skp1 and Rbx1 (RING HC finger protein) (Hua and Kao, 2006). SBP1 was first identified in *P. hybrida* (Sims and Ordanic, 2001), and later on PiSBP1 was isolated by screening against a pollen cDNA library using PiSLF$_2$ (CTD, C-terminal domain) as bait and found to have 98% amino acid identity to PhSBP1. PiSBP1, as a potential RING-HC protein, interacted with PiS-RNases, PiSLFs, PiCUL1, and an E2 ubiquitin-conjugating enzyme, strongly suggesting a role of the SBP1 protein in an SCF complex. Nonetheless, the *SBP1* gene is neither an *S*-haplotype-specific nor a pollen-specific gene, and is not encoded by the *S*-locus, indicating that SBP1 is less likely to be involved in SI (Hua and Kao, 2006). Intriguingly, however, SBP1 had self-ubiquitination activity and it alone could ubiquitinate S-RNases *in vitro* using bacterial-expressed UBA1 as E1, and PhUBC1 as E2, apparently making it a functional ligase (Hua and Kao, 2008). Moreover, a recent study showed that SBP1 in *Nicotiana alata* could interact with NaTTS (transmitting tract-specific glycoprotein) and 120K proteins through their conserved C-terminal domain (Lee *et al.*, 2008), suggesting that the SBP1 protein interacts with more potential partners and possibly acts as a general factor in pollen development. For AhSSK1 protein, its pollen-specific expression pattern, interaction with SLF and CUL1-like proteins, and a unique position in the phylogenetic tree of Skp1-like proteins, strongly support that SSK1 probably represents a Skp1-like adaptor in the SCF complex not required for pollen development *per se* except in the SI response (Huang *et al.*, 2006). Nevertheless, the

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possibility that two different ligases participate in one reaction has been reported with one ligase acting in the nucleus and another in the cytoplasm (Lee and Kay, 2008). If SBP1 and SSK1 proteins are differentially localized, their roles in SI might be not mutually exclusive. Thus, a possible explanation for the presence of SSK1- and SBP1-containing SCF complexes is that they could have different functions in the SI responses, which remains to be investigated, particularly their loss-of-function studies.

Although it has not been clearly defined yet which and/or both of the SBP1- and SSK1-containing SCF^{SLF} complex is employed in the SI response, the role of SCF^{SLF} in S-RNase degradation has been supported by several studies (Qiao *et al.*, 2004*a*; Hua and Kao, 2006; Huang *et al.*, 2006), suggesting that each allelic product of *SLF* specifically mediates non-self S-RNase ubiquitination and degradation. This specific interaction and recognition between SLF and S-RNase is assumed to occur as follows. All S-RNases are proposed to have two domains, a specificity domain (SD) and a catalytic domain (CD). The SD is described to be the recognition portion by the corresponding SLF protein; whereas, the CD is in charge of triggering the next step of the SI response. Similarly, SLF proteins are also supposed to adopt two domains, a specificity domain, used for specific interaction with cognate S-RNase, and an inhibitor domain (ID), for preventing non-self S-RNase catalytic activity (Kao and Tsukamoto, 2004). Thus, after non-self S-RNase enters the pollen tube, its catalytic domain interacts with the inhibitor domain, resulting in the formation of a functional SCF complex that targets the S-RNase for ubiquitination and degradation through the UPS pathway and the pollen tube continues its normal growth. In this model, SLF could protect self-S-RNase during the SPI response through its differential interaction with S-RNase (Zhang and Xue, 2008). Interestingly, SFB has been suggested to play such a role (Luu *et al.*, 2001; Ushijima *et al.*, 2004; Sonneveld *et al.*, 2005; Hauck *et al.*, 2006*a*). Nevertheless, it remains unknown how the proposed dual role of SLF could be achieved during the CPC and SPI responses.

However, it is still unclear how S-RNase degradation occurs during compatible pollen tube growth. So far, most evidence that supports a role for ubiquitination in S-RNase discrimination was obtained from *in vitro* experiments. Further *in vivo* experiments are essential to determine the fate of S-RNase during the CPC response. First, unequivocal identification of the potential lysine residue(s) for S-RNase ubiquitination is central to revealing the role of ubiquitination in the CPC response, although Hua and Kao (2008) provided some initial evidence for this *in vitro*. Second, careful examination of the S-RNase ubiquitination type, if any, could be another revealing issue since it is known that distinct ubiquitination forms are required for different biochemical and cellular events. For example, for polyubiquitination, seven different linkages between ubiquitins are known with diverse functions (Mukhopadhyay and Riezman, 2007). Two that are well characterized are Lys^{48} and Lys^{63} linkages. Lys^{48} (i.e. Ub^{K48}) is probably used for targeting substrate protein to the proteasome, whereas Lys^{63} (Ub^{K63}) plays key roles in the endocytic pathway (Pickart and Fushman, 2004). Previous studies have indicated that the 26S proteasome-dependent polyubiquitination is involved in S-RNase degradation, suggesting that the Lys^{48} linkage is more likely to be related to the S-RNase ubiquitination. However, whether other linkage types of ubiquitination are associated with the SI responses remains unknown. Third, to confirm when and how the recruitment of the SCF complex is accomplished will probably provide new evidence about the CPC response. Until now, we have no idea whether the SCF complex is formed at the beginning of their synthesis or upon some signal of CPC and/or SPI responses. If the latter is the case, what is an efficient way for homeostasis maintenance of pollen tube growth, what kind of and where is the signal released from? Is there any difference between the CPC and SPI responses in forming the SCF complex? Taken together, identification of the ubiquitination site (s) and the ubiquitination type, as well as the way of recruitment of the SCF complex, represent three important questions to be answered for further dissection of S-RNase fate within the growing pollen tubes.

In addition, besides the main working mode of the formation of a typical SCF complex, other functions of F-box protein have been reported recently. For example, F-box protein has been shown to participate in the protein turnover process in a proteasome-independent manner in the regulation of mitochondrial fusion (Escobar-Henriques *et al.*, 2006). Moreover, the Skp1 and F-box proteins could also form a non-SCF complex to regulate protein recycling in yeast (Galan *et al.*, 2001). However, despite its recognized role in S-RNase degradation in the CPC response, it is worth studying whether there are other working modes of the SLF protein in the SI response.

Spatially sequestration of S-RNase

Evidence obtained from the S-RNase–SLF interaction has shed some light on the fate determination of S-RNase. However, more questions are arising. For example, how does S-RNase traffic in the pollen tubes? Where do S-RNase and SLF interact with each other; is it cytoplasm or endosomal membrane associated? New insights into the mechanism for S-RNase protein endocytosis and endosome trafficking are emerging.

Recently, Goldraij *et al.* (2006) usd an immunolocalization assay to show that S-RNase was sorted into the pollen vacuole after its uptake from stylar ECM, suggesting that S-RNase is probably compartmentalized in the pollen tube. However, a previous study concerning S-RNase uptake showed that no obvious S-RNase was detected in a compartment (Luu *et al.*, 2000), which might be due to a different tissue fixation time and the experimental conditions used. Interestingly, a time-course observation of non-self S-RNase in the pollen tube was performed, showing that, in cross-pollen tubes, most compartments remained intact to restrict the S-RNase (Goldraij *et al.*, 2006). Therefore, a spatial sequestration of non-self S-RNase by the endomembrane

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system of pollen tubes represents another new way for restricting S-RNase.

However, it remains largely unclear how S-RNase enters pollen tubes, which is the first step for S-RNase to function and is fundamental in understanding the whole process of S-RNase trafficking within pollen tubes. As a small (~20–30 kDa) glycoprotein, abundant pistil S-RNases are taken up by pollen tubes mainly through a specialized tip growth mechanism (Cornish *et al.*, 1987; Anderson *et al.*, 1989). Nonetheless, the exact manner remains elusive. Recently, it was found that S-RNases gain access to pollen tubes via endocytosis (Y Zhang, Y Xue, unpublished data), and if this is so, it will be consistent with the consequent fate of S-RNase, that has been shown to be associated with the pollen tube endomembrane system. Taken together, the available evidence shows that, during the CPC response, S-RNase appears to be restricted from its entry to its subsequent trafficking process.

A multilayered restriction of S-RNase during the CPC response

Although there are still many unknowns associated with S-RNase fates in compatible pollen, it is likely that S-RNase degradation and compartmentalization are both involved, since the SLF protein has been shown to localize to the endomembrane system of pollen tubes (Wang and Xue, 2005). To address the relationship between these two mechanisms, a hypothetical S-RNase endosome sorting model has been proposed to explain the CPC and SPI responses (Fig. 1; Zhang *et al.*, 2009), in which recognition between S-RNase and SLF determines whether a functional SCF^{SLF} complex is formed and where the S-RNase is subsequently sorted to: the cytoplasm or vacuole. For the CPC response, non-self S-RNase is postulated to be recognized and activated by an activation domain within SLF to allow the formation of a functional SCF complex to ubiquitinate non-self S-RNase. Then the ubiquitinated non-self S-RNase is sorted through the endosome into the proteasome and/or vacuole for degradation (Piper and Katzmann, 2007) and thus, the pollen tube could normally grow until the double-fertilization. Therefore, it is likely that a multilayered restriction of S-RNase is involved in the CPC response because if, by any chance, S-RNase is out of control in the pollen tubes, it would be a disaster for successful sexual plant reproduction.

Control of self-pollen incompatibility

In contrast with cross pollen, the ultimate fate of self pollen is its growth inhibition. However, for self-pollen grains, there are no obvious cytological alterations observed before they enter the pistil (Herrero and Dickinson, 1981). Pollen tube growth inhibition was initiated after the penetration of self-pollen tubes into the transmitting tissue of style. Importantly, S-RNases have been shown to enter not only into compatible but also incompatible pollen tubes (Luu *et al.*, 2000; Goldraij *et al.*, 2006). The available evidence strongly suggests that S-RNase plays a role in rejecting self-pollen by acting as a cytotoxin (McClure *et al.*, 1990), although how this cytotoxicity is accomplished remains relatively unclear. Nonetheless, the pistil-secreted S-RNase acting as a determinant molecule to trigger the SI process comes from early cytological observations of pistil and pollen following compatible and incompatible pollinations.

Cytological changes of pistil and pollen during the SI response

Self pollen is known to germinate in an identical manner to cross pollen, but after penetration, self-pollen tubes elongate more slowly than compatible ones. Moreover, after a short period of growth in the transmitting tissue, incompatible tubes show thicker cell walls and cytoplasm packed with organelles and reserves and gradually stop growth (Herrero and Dickinson, 1981). On the pistil side, a major difference between compatible and incompatible pollination appeared to be nutrition mobilization when the pollen tubes gain access into the transmitting tissue. The incompatible tubes take much less of the stylar reserves, which coincides with their growth arrest (Herrero and Dickinson, 1979). However, most of the studies were performed with *in vitro* pollen growth assays. A detailed cellular description of pollen tube growth through the style is largely missing, due to technique difficulties associated with *in vivo* observation.

Role of self S-RNase in pollen tubes

Based on previous cytological studies, it seems that S-RNases enter self pollen tubes via a similar way to cross pollen. After entering the pollen tube, how self S-RNase controls the growth inhibition is a key issue in understanding SPI. Where does S-RNase play its role, staying within a compartment or getting out of it into the cytoplasm?

Recent results in SI and other fields have shed some light on the fate of S-RNase during SPI. A time-course observation of self S-RNase in pollen tubes showed that, compared with cross-pollen tubes, the vacuole-like endomembrane system for S-RNase appeared to break down in the late stage of pollen rejection in the SPI response (Goldraij *et al.*, 2006). Thus, self S-RNase could be specifically translocated into the cytoplasm of pollen tubes, providing an opportunity to trigger the downstream signalling cascade. Although it is unknown what triggers the breakdown of the vacuole in incompatible pollen tubes, it appears that a small asparagine-rich non-*S*-specific factor, HT-B, was involved because it remained intact in self-pollen tubes, but largely degraded at the late stage in compatible ones (Goldraij *et al.*, 2006). In particular, in *Nicotiana* and *Petunia*, a substantial knockdown of expression of *HT-B* led to an inability of the pistil to reject self-pollen tubes (McClure *et al.*, 1999; Puerta *et al.*, 2009). Therefore, HT-B appears to be required for the SPI response by controlling vacuolar membrane integrity, although by what mechanism is unknown. Recent progress in innate and adaptive immune responses have indicated that perforins and perforin-like proteins attack membrane structure through a pore-forming mechanism (Kafsack

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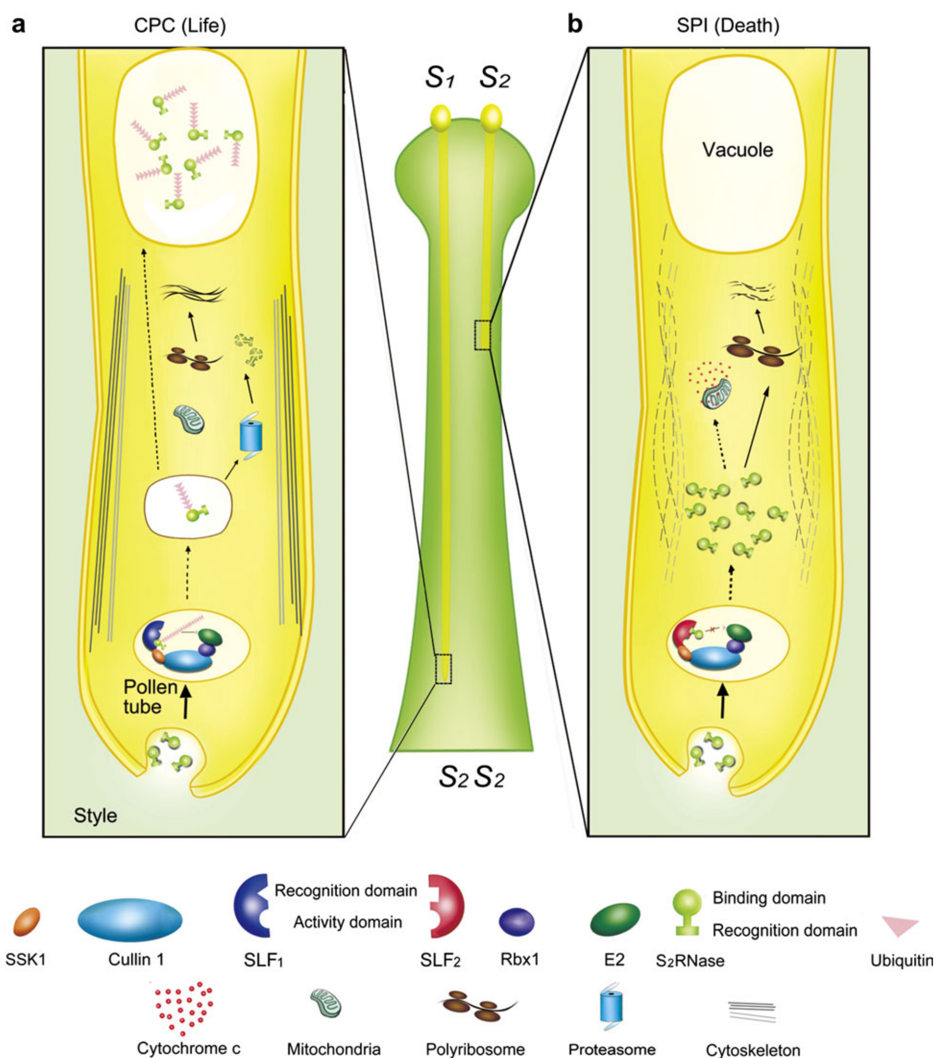

Fig. 1. A hypothetical S-RNase endosome sorting model of S-RNase-based self-incompatibility. (a) Cross-pollen compatibility response (CPC). Once cross pollen (S_1) lands on the stigma and germinates into a style (S_2S_2), non-self S-RNases are endocytosed into the pollen tubes and subsequently detected by a putative activity domain of the pollen S (i.e. SLF in Solanaceae and Plantaginaceae). Then, SLF_1 adopts an SCF^{SLF1} complex to tag the non-self S-RNases with polyubiquitins. Thus the marked non-self S-RNases are sorted into the proteasome and/or vacuole for degradation and the cross-pollen tubes remain active, leading to a 'life' decision of cross-pollen tubes. (b) Self-pollen incompatibility response (SPI). When self pollen (S_2) arrives at the stigma and penetrates into a style (S_2S_2), self S-RNases enter the pollen tube in a similar way to the non-self ones. Consequently, self S-RNases bind to a putative recognition domain but not the activity domain of the pollen S, resulting in dysfunction of an SCF^{SLF2} complex for polyubiquitination of self S-RNases. However, it is still possible that self S-RNases are tagged with ubiquitins in some different way if the SCF^{SLF2} complex is formed. Self S-RNases are not restricted but somehow released from the endosome to trigger subsequent pollen tube growth inhibition events, such as the direct degradation of ribosome RNA or the indirect (through an unknown way) destabilization of cytoskeletons and the release of cytochrome *c* from mitochondria, which eventually result in a 'death' decision of self pollen tubes. Solid arrows indicate the pathways with evidence and the dotted ones speculative pathways. SSK1, SLF-interacting SKP1-like1; SLF, *S*-locus F-box; Rbx1, RING Box1; E2, ubiquitin-conjugating enzyme.

et al., 2009). Although there is no significant sequence and structural similarity between HT-B and perforin-like proteins (G Chen, unpublished data), the mode of action of the perforin-like proteins might provide a suggestive role for HT-B. In addition, the precise role of HT-B protein still needs to be determined since neither S-RNase nor SLF protein appears to interact with it. So, how is the HT-B degradation regulated during compatible pollen-tube growth? Does this degradation result in the SI response or is it an inevitable consequence of self-pollen arrest? To address these questions, it would really be interesting to discover the interactive partners of the HT-B protein in style transmitting tissue and/or in pollen tubes. Also, the dynamic trafficking route of HT-B from the transmitting tissue to the inside of the pollen tube is an important future issue to investigate.

After self S-RNase is released from the spatial sequestration, the next issue is the target of S-RNase in the

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cytoplasm, in other words, the exact substrate it binds to or pathway it activates or inhibits. When the RNase property was first uncovered in the pistil S, a cytotoxic role of S-RNase was shown to be involved in the inhibition of self-pollen-tube growth by degrading pollen ribosome RNAs (rRNA) (McClure *et al.*, 1990; Huang *et al.*, 1994). Recently, however, additional functions of T_2-type RNases have been found. For example, Rny1p, which is a *Saccharomyces cerevisiae* secreted T_2-RNase, has a dual role in both the cleavage of tRNA and rRNA dependent on its RNase activity and the induction of cell death under oxidative stress conditions independent of its RNase activity (Thompson and Parker, 2009; Yamasaki *et al.*, 2009). Moreover, an *Aspergillus niger* extracellular T_2-RNase has been shown to possess an F-actin-binding activity and to inhibit pollen tube elongation *in vitro* (Roiz *et al.*, 2006). Indeed, the degradation of pollen rRNAs was a clear sign of arrest of pollen tube growth (McClure *et al.*, 1990), but the reversible grafting assay, by grafting incompatible pollen tubes onto compatible styles, in which the growth-arrest pollen reverted to normal growth, seemed to be inconsistent with the cytotoxic hypothesis (Lush and Clarke, 1997), indicating a continuous synthesis of rRNAs in pollen tubes. Thus, it would be difficult to discriminate whether the degradation of rRNAs was the consequence or the cause of the SPI response. To characterize the precise role of self S-RNase, the growth inhibition of self pollen tubes *per se* should be carefully examined.

Generally, in the SPI response, the ultimate growth inhibition of self-pollen tubes appears to be a kind of plant cell death since their inhibition is closely associated with swollen cells or cell death (Herrero and Dickinson, 1981). Interestingly, after biotic or abiotic stresses, plant cells usually undergo programmed cell death (Greenberg, 1996; Love *et al.*, 2008). The commonality of those programmed cell death responses (PCD) is that they are all under tight genetic control. In the S-RNase-based SI, the pollen rejection or death is specific to self pollen, indicating that such a rejection or death process should also be finely tuned and never happen randomly. However, what directly triggers this process in the pollen tube is still not well understood, except that self S-RNase must have a role in this process. What is the direct effect of RNase activity in S-RNase, as it has been shown to be essential for the SI response (Huang *et al.*, 1994)?

Recent studies in poppy (*Papaver rhoeas*) as well as a recent discovery in *Pyrus pyrifolia* threw some light on self-pollen inhibition with a close association with PCD. In poppy SI, which does not involve S-RNase, PCD is revealed to be specifically associated with the SPI response because the cytological evidence of self-pollen rejection is coincident with most of the characteristics of PCD, such as Ca^{2+} influx, MAPK-cascade activation, filamentous actin depolymerization, cytochrome *c* release, and DNA fragmentation (Thomas and Franklin-Tong, 2004; Bosch and Franklin-Tong, 2008; Li *et al.*, 2007). Moreover, in pear (*P. pyrifolia*), a rosaceous species that also possesses an S-RNase-based SI, the pollen tube PCD was also specifically detected for the SPI response (Wang *et al.*, 2009). Wang *et al.* (2009) found that the mitochondrial membrane potential ($\Delta\Psi_{mit}$) was comparatively collapsed in incompatible pollen tubes under S-RNase challenge *in vitro*. Also, the leakage of cytochrome *c* into the cytosol was detected as a result of $\Delta\Psi_{mit}$ collapse, and the degradation of nuclear DNA occurred in self-incompatible pollen tubes. These results provided another possibility that S-RNase could lead to the growth arrest of self pollen by triggering a PCD response.

However, a clear relationship between S-RNase and self-pollen-specific PCD is still fragmentary and it is unclear whether the mechanism of PCD during the SPI response has been adopted by Solanaceae and Plantaginaceae plants. In fact, the specific PCD occurrence in self-pollen tubes might not be mutually exclusive with a previous indication of specific degradation of rRNAs, as the degradation of rRNAs could be a result of PCD or these two events are independent of each other as has been shown in some cases (Smirnoff *et al.*, 2006; Thompson and Parker, 2009; Yamasaki *et al.*, 2009). Nevertheless, the role of S-RNase for degrading rRNA is challenged, from the reversible pollen growth by the grafting experiment (Lush and Clarke, 1997), to the recent discovery of PCD specifically participating in the SPI response of S-RNase-based SI (Wang *et al.*, 2009). Is S-RNase an early trigger of the PCD signalling pathway or a direct PCD initiator? In particular, does PCD, triggered by S-RNase, occurs *in vitro* and *in vivo* in a similar way? Future work including the transformation of fluorescent-tagged S-RNase may be helpful in revealing its dynamics *in vivo* during the SPI response. Furthermore, it would be interesting to find out whether S-RNase directly or indirectly triggers the alteration of the pollen tube cytoskeleton to induce the self-pollen-tube growth arrest. Further identification of other partners of S-RNase in pollen would be another way to test this possibility. In addition, although the role of self S-RNase have been assumed to occur in the cytoplasm, it could be possible that it functions in other places. For example, the human RNASET2 protein was detected in a perinuclear localization and found to be transported along the secretory pathway (Acquati *et al.*, 2005).

In conclusion, although the new idea of S-RNase triggering PCD has emerged recently, relatively little is known about the SPI mechanism (Fig. 1) in S-RNase-based SI compared with the CPC control. Whatever their mechanisms, an important control step in SI goes to the specificity determination of S-RNase and SLF, representing a life or death decision-making process for pollen tubes during both the CPC and the SPI responses.

How do S-RNase and SLF interact to determine allelic specificity?

Recognition of S-RNase and SLF proteins in pollen tubes to discriminate between self and non-self is one of the most intriguing processes, but it is still unclear how determinants of allelic specificity are biochemically controlled between S-RNase and SLF. Understanding of this control requires the

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molecular dissection of amino acids involved in their specific interaction.

Structural domains in S-RNase and SLF

S-RNases are highly polymorphic with amino acid sequence identity ranging from 38% to 98% and divided into five conserved domains (C1–C5) as well as two hypervariable domains (HVa and HVb) (Ioerger *et al.*, 1991; Tsai *et al.*, 1992). Although the hypervariable regions have been shown to be involved in specificity discrimination (Matton *et al.*, 1997), further studies have revealed that the hypervariable regions alone might not be sufficient to confer the overall recognition interface (Verica *et al.*, 1998; Wang *et al.*, 2001). Thus, the putative specificity domain (SD) of S-RNase, as predicted in the S-RNase degradation model (Kao and Tsukamoto, 2004; Zhang and Xue, 2008), would function at a hierarchical three-dimensional (3D) structural level in order to be recognized by SLF proteins. This might be similar to a recently proposed interacting configuration module between SCR-SRK in *Brassica* SI (Boggs *et al.*, 2009).

By contrast, SLF are highly conserved proteins with small allelic variations. In *P. inflata*, SLF are separated into three domains: FD1 (Functional Domain1), FD2, and FD3, according to their different properties of interaction with S-RNase *in vitro*. Significantly, FD2 was shown to be a conserved region that contributed to strong interactions between SLF and non-self S-RNase, whereas FD1 and FD3 regions were dedicated to a specific interaction of SLF with S-RNase possibly through negative modules since together they could bind self S-RNase to a greater extent than non-self S-RNases (Hua *et al.*, 2007). However, besides the available *in vitro* data in *P. inflata*, a three-dimensional structure of SLF is necessary for the further dissection of the relationship between S-RNase and SLF, instead of the random separation of proteins from their primary amino acid sequences.

Low polymorphism of SLF appears sufficient for recognition

The lower polymorphism in SLF protein compared with its interacting partner S-RNase is still a puzzling issue (Newbigin *et al.*, 2008). The SLF proteins are expected to exhibit the same properties of a long-term negative frequency-dependent selection as found in S-RNases (Newbigin *et al.*, 2008) so that they could co-evolve and interact with each other with allelic specificity as found in studies of poppy and *Brassica* (Boggs *et al.*, 2009; Wheeler *et al.*, 2009). However, in the case of *Antirrhinum*, four allelic SLF proteins show about 97% identity in the amino acid sequences (Zhou *et al.*, 2003). Although S-RNase-based SI is the most common form adopted by many eudicots (de Nettancourt, 2001), the origin for the *SLF* alleles seems to be much younger (Newbigin *et al.*, 2008) than their cognate ancient *S-RNase* alleles (Igic and Kohn, 2001; Steinbachs and Holsinger, 2002). How could this happen? There could be several possibilities. First, S-RNases in different families sharing a common ancestor does not mean that they were born as an initial key factor in the SI response. It is possible that *S-RNase* did not server as an SI factor until the recruitment of *SLF* by chance. S-RNase could be assumed to act as an 'attacker' so it needs to be polymorphic in order to evade the restriction by SLF, whereas SLF would confer recognition and subsequent manipulation of S-RNase as a 'defence' factor so that several, or even only a one-site alteration, would be sufficient for its role. A similar scenario has recently been found in the *Brassica* study where *in vivo* studies revealed that only a few amino acid residues are required for the specificity determination, whereas other abundant polymorphism and/or positively selected sites might not be involved in the recognition between specificity factors (Boggs *et al.*, 2009). Second, the limited polymorphic sites, along with the conserved ones, may form a specific interface pattern sufficient for recognition. Third, SLF-like proteins may also play a role in SI, either directly or indirectly co-ordinating with SLF protein to realize an overall specificity. Actually, the function of *SLF-like* genes in the vicinity of the *S*-locus is intriguing, although Hua *et al.* (2007) used the competitive interaction rule to exclude a role of SLF-like protein in the CPC response. Do SLF-like proteins somehow participate in the SPI response? If not, why are they maintained during SI evolution especially for those *SLF-like* genes with a pollen-specific expression pattern? Or is there a supporting role of SLF-like protein in the pollen elongation process? Apparently, the roles of SLF-like proteins are still far from clear.

Nevertheless, it might not be too surprising when we think about the highly conserved *SLF* genes acting as the pollen *S* because any gene expressed in a haploid cell like a pollen grain would experience a stronger selection against deleterious mutations than that expressed in a diploid cell, especially if it is of importance to the reproductive fitness of pollen (Wheeler and Newbigin, 2007). Therefore, such an evolutionary selection in the haploid cell might shape the SLF as a protein product with a limited variation to maintain its 'defensive' function. Thus, the low polymorphism of SLF proteins should not serve as an obstruction for its recognition function, and it is expected that new pieces of evidence for their precise role in allelic specificity will come from a specific *in vivo* assay for those proposed allelic specificity amino acids and regions.

In vitro *evidence for specificity determination and beyond*

To date, the most impressive and intriguing experiment concerning specificity discrimination was accomplished by an *in vitro* detection of affinity differences between allelic S-RNase and SLF proteins based on a protein pull-down assay, suggesting that the physical interaction between S-RNase and non-self SLF could be stronger than that between S-RNase and self SLF (Hua and Kao, 2006). Nonetheless, *in vitro* ubiquitination of S-RNase did not show any obvious difference in self-incompatible and self-

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compatible pollen tube extracts (Hua and Kao, 2006). This provided the first biochemical clue to explain the specific preferences between self and non-self pollen–pistil recognition. However, those *in vitro* observations need further *in vivo* verification before we get a step closer to the elucidation of the nature of the specific interaction of S-RNase-SLF proteins. The available evidence suggests that the biochemical control of allelic specificity of S-RNase and SLF seemed a complicated process and several amino acids and regions were identified as being involved, thus a high-resolution crystal structure of the SLF protein in both its S-RNase-bound and unbound forms will be helpful for dissecting allelic specificity.

Conclusions and perspectives

Our understanding towards the molecular mechanisms of S-RNase-based SI has progressed significantly in recent years. Molecular identification of the pistil and pollen components encoded by the *S*-locus represents two milestones in our research of this type of SI over the past two decades. Important new clues to the biochemical and cellular mechanisms of S-RNase and SLF action are emerging, indicating that these proteins must have a finely tuned interaction in order to make the life or death decision for pollen tubes during the CPC and SPI responses. Further work on S-RNase traffic, its interaction with SLF, and the three-dimensional structural determination of SLF will be among the immediate experiments to address the molecular control of the S-RNase-based SI. Those studies are expected to provide an unprecedented opportunity to dissect and manipulate this important intra-specific reproductive barrier for its biology and application in crop breeding.

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