Chapter 23
Papaver rhoeas S-Determinants and the Signaling Networks They Trigger

Vernonica E. Franklin-Tong

Abstract Higher plants use specific interactions between pollen and pistil to achieve pollination. Self-incompatibility (SI) is an important mechanism used by many species to prevent inbreeding. It is controlled by a multi-allelic *S* locus. "Self" (incompatible) pollen is discriminated from "non-self" (compatible) pollen by interaction of pollen and pistil *S* locus components and is subsequently inhibited. Our studies of the SI system in *Papaver rhoeas* have revealed that the pistil *S* locus protein, PrsS, is a small novel secreted protein that interacts with the pollen *S* locus protein, PrpS, which is a small novel transmembrane protein. This interaction of PrsS with incompatible pollen induces a SI response, involving a Ca^{2+}-dependent signaling network, resulting in pollen inhibition and programmed cell death; this provides a neat way to destroy "self"-pollen. Several SI-induced events have been identified, including Ca^{2+} and K^+ influx, increases in cytosolic free Ca^{2+}, activation of a MAP kinase, alterations to the cytoskeleton, and phosphorylation of a soluble inorganic pyrophosphatase. Here we present an overview of our knowledge of the novel cell–cell recognition *S*-determinants and the signals, targets, and mechanisms triggered by an incompatible interaction. We hope this review is of interest to those involved in the origins and evolution of cell–cell recognition systems involved in discrimination between "self" and "non-self," which include histocompatibility systems in primitive chordates and vertebrates as well as plant self-incompatibility.

Keywords *Papaver rhoeas* • Pollen • Programmed cell death (PCD) • *S*-determinants • Self-incompatibility

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23.1 Introduction

The ability to discriminate between self and non-self (allorecognition) is important to most multicellular organisms. Self–non-self discrimination and other recognition systems, controlled by a highly polymorphic locus, are found in a wide range of organisms. Allorecognition is widespread and is integral to the animal immune response (Hughes 2002), fusion histocompatibility in lower animals (Scofield et al. 1982; De Tomaso et al. 2005; Nyholm et al. 2006), vegetative incompatibility in fungi (Glass et al. 2000), disease resistance in plants (Dangl and Jones 2001), and self-incompatibility (SI) systems found in many flowering plants (Takayama and Isogai 2005; Franklin-Tong 2008). Parallels between nonanalogous recognition systems were recognized, and their importance appreciated, many years ago (Burnet 1971), long before the molecular basis of these systems were elucidated. Allorecognition systems rely upon loci with multiple alleles with high levels of polymorphism. The nature of their polymorphism has intrigued population and evolutionary biologists for decades. Molecular and cellular studies are beginning to provide insights into these systems and provide interesting parallels and similarities. Here we provide an overview of our knowledge of the SI system in *Papaver rhoeas*, the field poppy.

Many plants are hermaphrodites, which provides a strategy that increases the chances of having progeny, but also the possibility of self-fertilization and concomitant problems with inbreeding depression. To avoid this problem, many plants utilize SI, controlled by the multi-allelic *S*- (Self-sterility or Self-incompatibility) locus, to prevent inbreeding (Takayama and Isogai 2005; Franklin-Tong 2008). The pollen and pistil *S* determinants must be physically linked to the *S* locus to maintain a functional SI system and are expected to have co-evolved. Other characteristics are high levels of allelic polymorphism and tissue-specific expression. Most importantly, they mediate pollen rejection by inhibition of some stage of the pollination process. Key aspects of understanding how SI systems operate are the identification and characterization of the pistil and pollen *S* determinants, coupled with establishing mechanisms involved in pollen inhibition.

Analysis of various pollen and pistil *S*-determinants gene sequences has shown that SI has evolved independently several times (Allen and Hiscock 2008). Well-characterized SI systems include the *Brassica* system, where the pistil *S*-determinant is a *S*-receptor kinase (SRK) and the pollen *S* determinant is SCR/SP11; and the *S*-RNase based SI systems with an S-RNase as the pistil *S*-determinant and F-box proteins SLF/SFB as the pollen *S*-determinant (Stein et al. 1991; Qiao et al. 2004; Sijacic et al. 2004; Takayama and Isogai 2005; McClure and Franklin-Tong 2006). The SI system in *Papaver* is distinct from these systems.

23.2 Self-Incompatibility Pistil and Pollen *S*-Locus Determinants in *Papaver*

Cell–cell communication is often controlled by the interactions of secreted protein ligands with cell-surface receptors. The *Papaver* pollen and pistil *S*-determinants are proposed to act in this manner as extracts from stigmas inhibited incompatible, but not compatible, pollen tubes grown in vitro (Franklin-Tong et al. 1988).

23.2.1 The Pistil S*-Locus Determinants*

The *Papaver* pistil *S*-locus determinants, PrsS (*Papaver rhoeas* stigmatic S), are expressed in low abundance, secreted specifically by the stigma. The *PrsS* genes are single copy and encode small, secreted, hydrophilic proteins of ~15 kDa. They have no obvious close homologues (Foote et al. 1994), although subsequent analysis of the *Arabidopsis* genome has revealed a large gene family (*S Protein Homologues*, *SPHs*) with similar predicted secondary structures, despite very low homology. The primary amino-acid sequence of the PrsS proteins is highly polymorphic, with as much as 40–46 % divergence between alleles (Walker et al. 1996). However, their predicted secondary structures are very similar; they all have a highly conserved predicted secondary structure. Site-directed mutagenesis has identified variable and conserved amino acids in a hydrophilic predicted surface loop that play a role in recognition and inhibition of incompatible pollen (Kakeda et al. 1998; Jordan et al. 1999).

Demonstration that PrsS was the pistil *S*-locus determinant was achieved using a pollen SI bioassay. Addition of recombinant PrsS protein to pollen growing in vitro exhibited the expected *S*-haplotype-specific pollen inhibitory activity, (Foote et al. 1994). Because PrsS encodes a novel protein, their function was unknown. However, studies investigated whether they might be possible candidates for signaling ligands, as they were novel, small secreted proteins, and this was demonstrated using Ca^{2+} imaging studies (see later).

23.2.2 The Pollen S*-Locus Determinants*

Three alleles of the *Papaver* pollen *S*-determinant, *PrpS*, have been identified and cloned (Wheeler et al. 2009). *PrpS* is a single-copy gene linked to the pistil *S*-determinant, *PrsS*, and displays extensive polymorphism. *PrpS* encodes a novel, small highly hydrophobic protein with a predicted M_r ~20 kDa. Examination of the *PrpS* sequences for nonsynonymous to synonymous (*Ka*/*Ks*) substitutions revealed no significant difference between substitution rates in *PrpS* and *PrsS* genes (Wheeler et al. 2009). This observation suggests that the pollen and pistil *S* alleles coevolved, and are likely to be equally ancient, which is an expected characteristic of *S*-determinants. Analysis of the three *PrpS* allelic sequences indicates that they share a similar topology, with three or four predicted transmembrane domains and a small predicted extracellular loop. Extensive database searches failed to identify orthologues of *PrpS* genes, so *PrpS* is a novel gene. PrpS has been shown to be expressed at the plasma membrane, and the predicted extracellular loop of PrpS interacts with PrsS. Use of *PrpS* antisense oligonucleotides in the pollen in vitro SI bioassay allowed demonstration that PrpS is involved in *S*-specific inhibition of incompatible pollen, providing evidence that it has the biological function expected (Wheeler et al. 2009).

So, one big question is “What is PrpS?” As the PrpS amino-acid sequence contains no known catalytic domains, can PrpS be defined as a “receptor?” It clearly is not a “classic” defined/identified receptor. However, PrpS appears to act as a novel class of “receptor” that interacts with PrsS in a very specific manner, triggering an intracellular signaling network and resulting in a highly specific biological response.

As PrpS is novel, we have no real idea of how it functions, which presents a problem in moving forward in our understanding of how it interacts with PrsS to mediate SI in incompatible pollen. An intriguing possibility has emerged. A novel *Drosophila* protein, Flower, which functions in presynaptic vesicle endocytosis, has recently been characterized (Yao et al. 2009). Both Flower and PrpS are novel proteins with no obvious homologues and have very little primary sequence homology. However, they have similar topological predictions, so are "topological homologues." The Flower protein functions as a Ca^{2+}-permeable channel (Yao et al. 2009). Voltage-gated calcium channels have acidic amino acids (either glutamic acid or aspartic acid) in the transmembrane domains that form a pore. Flower has a single glutamic acid residue in a proposed transmembrane domain, and it has been shown to make a homo-multimeric complex that could form this pore (Yao et al. 2009). Excitingly, examination of PrpS sequences reveal that it has three aspartic acids and three glutamic acids conserved across all three PrpS proteins; several are close to the edges of putative predicted transmembrane domains. These are good candidates for a pore/channel selectivity generating amino-acid residue, and this provides a basis for a testable model for PrpS function.

23.3 Mechanisms Involved in SI in the *Papaver* System

An area that has received considerable attention is the downstream signals and targets when incompatible PrsS and PrpS interact. The remainder of this review focuses on this important aspect.

23.3.1 Calcium Signaling Mediates Papaver *SI*

Ca^{2+}-dependent signaling networks are used to generate responses to a huge variety of stimuli in both animal and plant cells. Ca^{2+} imaging established that $[Ca^{2+}]_i$ acted as a second messenger in *Papaver* pollen, triggered specifically by interaction with incompatible PrsS proteins (Franklin-Tong et al. 1993, 1995). These studies formed the basis of a hypothesis proposing that only in an incompatible situation would PrsS-PrpS interaction occur, and this initiates a Ca^{2+}-dependent signaling cascade in incompatible pollen. Unchallenged pollen tubes have low basal $[Ca^{2+}]_i$ with high apical $[Ca^{2+}]_i$ (Fig. 23.1a). Incompatible pollen tubes rapidly exhibit increases in $[Ca^{2+}]_i$ with

Fig. 23.1 (continued) rapidly depolymerized (indicated by *dotted lines*). F-actin subsequently starts to form small foci. Phosphorylation of sPPases occurs within minutes, resulting in inhibition of activity and biosynthesis. At 5–10 min, a MAPK is activated. Increases in ROS (~1–5 min) are observed. (**c**) An incompatible pollen tube at ~5 h. Normal pollen tube cytosolic pH is ~7.0 (*green*). A drop in cytosolic pH (*yellow*) occurs several hours after initiation of SI. This acidification allows activation of several caspase-like activities, resulting in programmed cell death (PCD). Actin foci have become larger. Ultimately, DNA fragmentation and cellular dismantling occurs. (Adapted from Fig. 11.2, Chap. 11. Copyright: Franklin-Tong 2008)

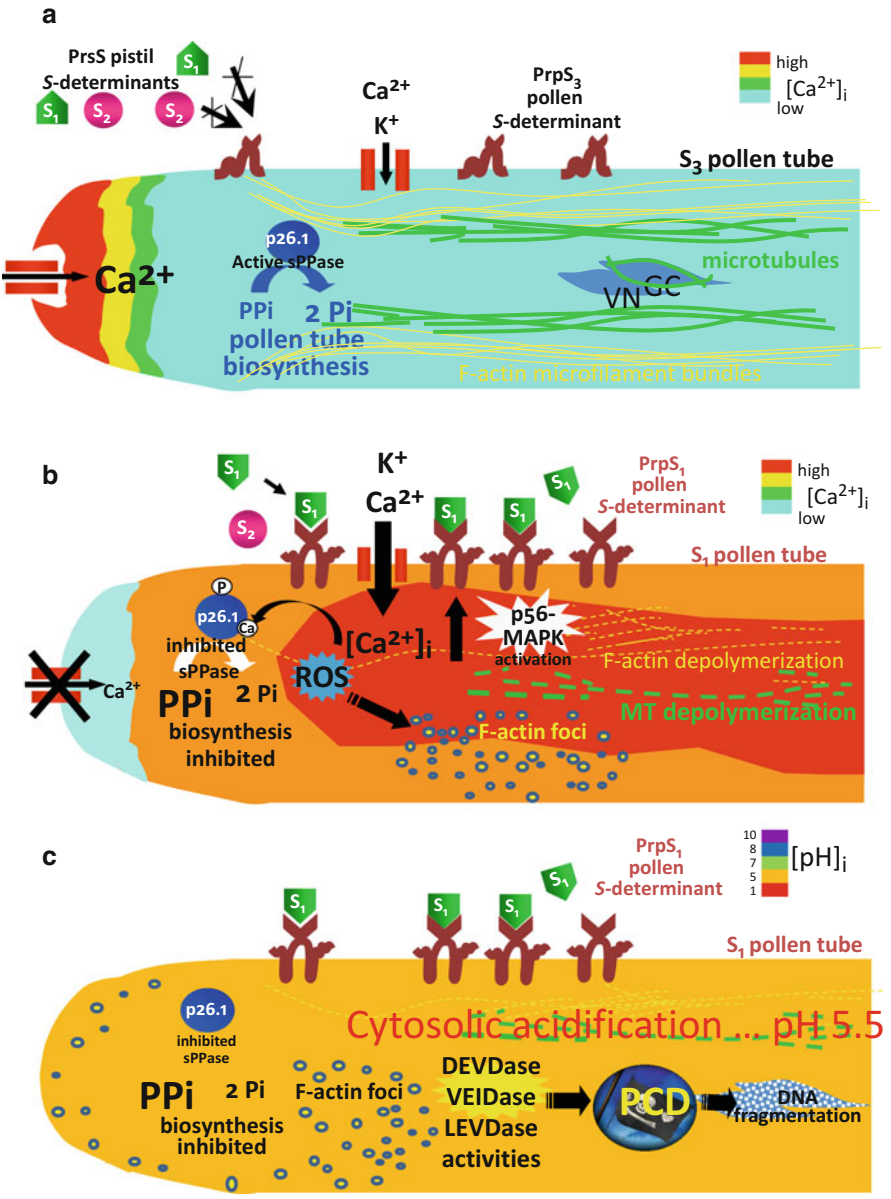

Fig. 23.1 Major changes in a pollen tube during different phases of self-incompatibility (SI). The pollen tubes are shown in pseudo-color to show $[Ca^{2+}]_i$ (a, b), with "hot" colors indicating high $[Ca^{2+}]_i$ and "cool" colors (*green*) indicating low, basal levels of $[Ca^{2+}]_i$. c Pseudo-color shows pH, with "cool" colors (*green*) indicating normal neutral levels of $[pH]_i$ and "hot" colors indicating low, acidic $[pH]_i$. (**a**) An untreated pollen tube, or a compatible situation, where pollen and pistil *S*-determinants do not match. Pistil *S*-determinants $PrsS_1$ and $PrsS_2$ do not interact with the pollen *S*-determinant PrsS, which has an S_3 specificity. Pollen germinates and grows, appearing indistinguishable from unchallenged pollen tubes. The pollen tube has a high apical $[Ca^{2+}]_i$ gradient and low, basal levels of $[Ca^{2+}]_i$ in the "shank" of the pollen tube; normal levels of sPPase activity enables biosynthesis; actin microfilaments enable delivery of vesicles to the tip allowing growth. (**b**) An incompatible pollen tube at ~1–10 min. The pistil *S*-determinant $PrsS_1$ matches and interacts with the pollen *S*-determinant, $PrpS_1$; this results in rapid Ca^{2+} influx and high $[Ca^{2+}]_i$ in the "shank" (indicated by *orange*/*red*), whereas $[Ca^{2+}]_i$ at the tip decreases. F-actin and microtubules are

loss of the apical gradient (Fig. 23.1b). Some of these increases in $[Ca^{2+}]_i$ were from extracellular sources (Franklin-Tong et al. 2002), implicating Ca^{2+} influx as being triggered by an incompatible SI response. More recent studies, using an electrophysiological approach, have demonstrated evidence for Ca^{2+} influx, and also K^+ influx triggered by PrsS interaction, specifically in incompatible pollen, has been obtained (Wu et al. 2011); see Fig. 23.1b. This rapid signaling implicates a receptor–ligand type of interaction being involved, leading to incompatible pollen tube inhibition.

23.3.2 Phosphorylation Events Identified in Papaver *SI*

Ca^{2+} signaling often causes altered protein kinase activity that results in posttranslational modification such as phosphorylation. Investigations revealed that alterations in protein phosphorylation were stimulated by SI in incompatible *Papaver* pollen with two major targets for SI-specific phosphorylation identified: a soluble inorganic pyrophosphatase (sPPase) and a mitogen-activated protein kinase (MAPK).

23.3.2.1 Identification of sPPases as Targets for Phosphorylation

Use of a phospho-proteomic-type approach revealed rapid *S*-specific phosphorylation of a 26-kDa cytosolic protein named Pr-p26.1, which was phosphorylated in a Ca^{2+}-dependent manner in incompatible pollen (Rudd et al. 1996). Analysis and cloning identified *Pr-p26.1a/b* as soluble inorganic pyrophosphatases, sPPases (de Graaf et al. 2006). sPPases are important enzymes involved in hydrolysis of inorganic pyrophosphate (PPi). During biopolymer synthesis PPi is generated, and sPPase hydrolysis of PPi generates inorganic phosphate (Pi), providing a thermodynamic pull, driving biosynthesis (Kornberg 1962).

The *Papaver* pollen sPPases have classic sPPase activities, which are Mg^{2+} dependent. Although it is well known that sPPases are inhibited by Ca^{2+}, it had not been previously established that phosphorylation could modify sPPase activity (de Graaf et al. 2006). Thus, identification of these sPPases as an early target of SI-induced Ca^{2+}-dependent signaling and kinase-dependent phosphorylation revealed mechanisms for regulating sPPase activity. It also suggested a possible mechanism to inhibit incompatible pollen tube growth. As sPPases are key enzymes that regulate biosynthesis, in principle, high sPPase activity results in faster biosynthesis (Fig. 23.1a), and low sPPase activity will result in a decrease in biosynthesis. SI would be expected to inhibit sPPase activity, which would therefore be predicted to inhibit pollen tube growth (Fig. 23.1b), which requires extensive biosynthesis of membrane and cell wall components. Use of antisense oligonucleotides based on *Pr-p26.1a/b* sequences to downregulate Pr-p26.1 resulted in significantly inhibited pollen tubes, suggesting that sPPases are necessary for pollen tube growth. SI-induced incompatible pollen tubes also had increased [PPi], which is predicted if sPPase activity is inhibited (de Graaf et al. 2006).

Together, these studies identified sPPases as novel, early targets for the Ca^{2+}-dependent SI signals, provided evidence for SI-stimulated modification of the Pr-p26.1a/b sPPase activity by Ca^{2+} and phosphorylation, and suggested that these sPPases play a role in regulating *Papaver* pollen tube growth. These findings provide an important part of the jigsaw puzzle of how SI operates.

23.3.2.2 A MAPK Is Activated by SI

MAPK cascades are involved in triggering numerous signaling networks. They are activated by dual phosphorylation of threonine and tyrosine residues in a TXY motif via a MAPKKK cascade. Investigations of SI-induced phosphorylation revealed the activation of a MAPK, p56, specifically in incompatible, but not compatible, pollen (Rudd et al. 2003; Li et al. 2007) (see Fig. 23.1b). Data suggested that the p56-MAPK plays a role in integrating SI signals. However, as its activation peaked 10 min after SI induction, it could not be involved in the rapid arrest of pollen tube inhibition. Studies have provided evidence that this MAPK is involved in signaling to PCD (see later).

23.3.3 Papaver *SI Triggers Alterations to the Cytoskeleton*

The actin cytoskeleton is both a major target and effector of signaling networks, and $[Ca^{2+}]_i$ is known to have a key role in mediating these responses (Staiger 2000). It has been found to be a target for SI signals, which involves both Ca^{2+} signaling and inhibition of pollen tube growth. The major findings are summarized here.

23.3.3.1 SI-Induced Alterations to F-Actin

Dramatic alterations in F-actin organization, specifically in incompatible pollen tubes, have been observed (Geitmann et al. 2000) (see Fig. 23.1a,b. Depolymerization of the actin filament bundles occurs within a few minutes of SI induction (Snowman et al. 2002), which provides a highly effective mechanism to rapidly inhibit incompatible pollen tube growth. Two Ca^{2+}-dependent actin-binding proteins (ABPs) that could potentially act synergistically in SI-induced depolymerization are profilin and PrABP80 (a putative gelsolin), which has potent Ca^{2+}-dependent severing activity (Huang et al. 2004). Subsequently, and within 30 min after SI induction, F-actin begins to aggregate to form highly stable punctate actin foci. Two ABPS, actin depolymerizing factor (ADF/cofilin) and cyclase-associated protein (CAP), have been identified as being involved in punctate actin foci formation, as they rapidly colocalize to the actin foci (Poulter et al. 2010). It is thought that these unusually stable actin structures play a role in signaling to programmed cell death (PCD) (see later).

23.3.3.2 SI-Induced Alterations to Microtubules

The pollen tube microtubule cytoskeleton is also a target for the SI signals. Rapid apparent depolymerization of cortical microtubules, within 1 min of SI induction, is observed in incompatible pollen tubes (Poulter et al. 2008) (see Fig. 23.1a,b). Interestingly, the distinctive spindle-shaped microtubules around the generative cell remained relatively intact until much later. Artificially depolymerizing actin (using the drug LatB) resulted in microtubule depolymerization, but stabilizing actin before SI prevented total microtubule depolymerization. This result suggested that the SI signals stimulating actin depolymerization trigger microtubule depolymerization (Poulter et al. 2008). Similar to actin, the cortical microtubules have also been shown to have another role, relating to signaling to PCD (see later).

23.3.4 SI Triggers Programmed Cell Death

Unwanted eukaryotic cells are often removed by apoptosis or PCD. Perhaps one of the most important findings relating to events triggered by SI in *Papaver* pollen was the demonstration of the involvement of PCD, which provides a highly effective way to prevent fertilization by incompatible pollen.

23.3.4.1 PCD Involving Caspase-Like Activities Is Triggered by SI

Animal cells utilize caspases to initiate and mediate apoptosis or PCD. Their activation is a classic feature often used to diagnose apoptosis/PCD, using tetrapeptide caspase-specific substrates and inhibitors. Caspase-3, a key executioner caspase in animal cells, has a tetrapeptide recognition motif DEVD. It is often called a DEVDase; its activity is inhibited by DEVD inhibitors, and substrates that act as fluorogenic indicators for caspase activities allow a direct analysis of caspase-like activities. Using these approaches, it has been shown that caspase-like activities are activated in plants. However, the nature of most plant caspases is a mystery, as there are no known caspase homologues (Lam and del Pozo 2000; Woltering et al. 2002; Woltering 2004).

PCD has been shown to be a key event in the *Papaver* SI response. Evidence includes leakage of cytochrome *c* into the cytosol, and SI-induced DNA fragmentation, which is inhibited by the caspase-3 inhibitor Ac-DEVD-CHO but not the caspase-1 inhibitor Ac-YVAD-CHO (Thomas and Franklin-Tong 2004); this implicated a caspase-3-like/DEVDase activity involved in mediating the SI-induced DNA fragmentation. Moreover, extracts from SI-induced incompatible pollen can cleave the classic caspase substrate, poly(ADP-ribose) polymerase (PARP) (Thomas and Franklin-Tong 2004). Use of a fluorescent caspase-3 substrate, Ac-DEVD-AMC, that acts as an indicator for DEVDase activity has provided more direct evidence for SI-induced DEVDase activity (Bosch and Franklin-Tong 2007; Li et al. 2007) and

allowed detailed characterization. Live-cell imaging of the DEVDase activity has revealed that this caspase-like activity first appears in the cytosol around 1–2 h after SI induction and increases over time (Bosch and Franklin-Tong 2007). Other probes have revealed the presence of SI-stimulated VEIDase (caspase-6) and LEVDase (caspase-4) activities in incompatible pollen tubes (Bosch and Franklin-Tong 2007) (see Fig. 23.1c). DEVDase and VEIDase had similar temporal activation profiles, with activity peaking at 5 h post-SI, suggesting that they are involved in early PCD. LEVDase activity, in contrast, was activated rather more slowly and later, with activity still increasing at 8 h, when the DEVDase and VEIDase activities had significantly decreased (Bosch and Franklin-Tong 2007).

Surprisingly, all three of the SI-activated caspase-like activities exhibit peak activity at pH 5, which hinted that a possible early SI-induced event was acidification of the cytosol. Investigation demonstrated that there was, indeed, a dramatic and rapid acidification of the pollen cytosol triggered in the first 1–2 h of SI (Bosch and Franklin-Tong 2007). What is involved in this phenomenon is not yet clear, but it points to major alterations to the nature of the cytosol being elicited by SI. Current studies are investigating this aspect further (Wilkins and Franklin-Tong, unpublished data).

23.3.5 Attempting to Integrate SI-Triggered Events That Signal to PCD

The interaction of the secreted pistil PrsS with the "self"-pollen PrpS causes Ca^{2+} influx and rapid increases in $[Ca^{2+}]_i$ and inactivation of the p26 sPPase. A major focus for the SI-induced signaling network is the activation of PCD. Alterations to the cytoskeleton are implicated in mediating PCD. The SI-induced acidification of the pollen cytosol (Fig. 23.1c) is also likely to be an important decision-making step, as this will allow PCD to proceed by allowing caspase-like proteins to be activated. Next, we summarize data relating to integration of the signals and targets triggered by this interaction. Figure 23.1 shows a cartoon of key events in pollen in three phases: (a) unchallenged or compatible, (b) early and rapid signaling events triggered by SI, and (c) late SI, constituting commitment to PCD.

23.3.5.1 Involvement of Actin Signaling in SI-Mediated PCD

Both actin and microtubule alterations are implicated in mediating PCD (Thomas et al. 2006; Poulter et al. 2008). A number of studies have shown that either actin depolymerization or stabilization can influence whether a eukaryotic cell enters into an apoptotic pathway; see (Franklin-Tong and Gourlay 2008; Smertenko and Franklin-Tong 2011). Investigations examining the effect of the actin-depolymerizing drug, latrunculin B (LatB), and the actin-stabilizing/polymerizing drug, jasplakinolide (Jasp), in *Papaver* pollen revealed that both treatments stimulated high levels of DNA fragmentation,

which was mediated by a caspase-3 like/DEVDase activity (Thomas et al. 2006). This finding suggested that disturbance of actin polymer dynamics could trigger PCD in pollen. Data implicated actin depolymerization being functionally involved in the initiation of SI-induced PCD and established a causal link between actin polymerization status and initiation of PCD in plant cells (Thomas et al. 2006). Thus, the rapid and substantial actin depolymerization triggered by SI signaling not only results in the rapid inhibition of incompatible pollen tip growth, but it also activates a caspase-3-like/DEVDase activity, triggering PCD (see Fig. 23.1b,c).

It seems likely that SI-induced cytosolic acidification plays an important role in the formation of the highly stable actin foci (Fig. 23.1c), as the actin-binding protein ADF/cofilin was found to be associated with the F-actin foci (Poulter et al. 2010). It has been shown that when F-actin is decorated with ADF, it does not exhibit its usual filament severing activity; this may explain why the foci are unusually stable. It is proposed that the formation of the actin foci and their association with ADF is an active process that is also involved in signaling to PCD (Fig. 23.1c), especially as they appear to be a marker for SI-induced PCD that can be alleviated concomitantly with alleviation of PCD.

23.3.5.2 Involvement of MAPK Signaling in SI-Mediated PCD

MAPKs are known to play a key role in mediating signaling to PCD in the plant–pathogen hypersensitive response. Studies based primarily on use of the MAPK cascade inhibitor U0126 in combination with PCD markers implicated a key role for MAPKs in signaling to PCD in incompatible pollen (Li et al. 2007) (see Fig. 23.1b). U0126 prevented the SI-induced activation of p56 in incompatible pollen and "rescued" incompatible pollen and also significantly reduced caspase-3-like (DEVDase) activity and later DNA fragmentation (Li et al. 2007). As p56 appears to be the only MAPK activated by SI, this suggests that p56 could involved in mediating SI-induced PCD and implicated involvement of a MAPK in signaling to PCD.

23.3.5.3 A Role for ROS and NO Signaling in SI-Mediated PCD

It has recently been shown, using live-cell imaging to visualize reactive oxygen species (ROS) and nitric oxide (NO) in growing *Papaver* pollen tubes, that SI induces relatively rapid and transient increases in ROS and NO, which have distinct temporal "signatures" in incompatible pollen tubes (Wilkins et al. 2011). Investigating how these signals integrate with the SI responses, using ROS/NO scavengers, revealed the alleviation of both the formation of SI-induced actin punctate foci and the activation of a DEVDase/caspase-3-like activity, providing evidence that ROS and NO act upstream of these key SI markers and suggesting they signal to these SI events (Wilkins et al. 2011) (see Fig. 23.1b). As the actin foci appear to be an integral part of the PCD response, this suggests that actin stabilization also plays a role in mediating PCD (Fig. 23.1c). These data represent the first steps in understanding ROS/NO signaling triggered by SI in pollen tubes.

23.4 Recruitment of Signaling for SI Events in Other Species

Recent studies, exploring the possibility for transfer of the SI determinants into other species, have demonstrated that the pollen male determinant, PrpS, can be expressed in *Arabidopsis thaliana* pollen. The PrpS-GFP-expressing transgenic pollen exhibits key features of the *Papaver* pollen SI response, including inhibition, formation of actin foci, and increases in caspase-3-like activity when cognate recombinant *Papaver* PrsS is added to it (de Graaf Barend et al. 2012). This observation represents the first demonstration that a SI system can be transferred into a distantly related species and function to trigger appropriate responses. Until now, it was thought that transfer of SI would be limited to close relatives, as so far the only movement of SI systems has been within species or closely related species, *Arabidopsis lyrata* and *Capsella grandiflora* into *A. thaliana* (Nasrallah et al. 2002; Boggs et al. 2009). Although these are important demonstrations, they diverged only ~5 million (Koch et al. 2000), and ~6.2–9.8 million years ago (Acarkan et al. 2000), respectively, and so do not provide major insights into the evolution of SI signaling across angiosperm families because of their close relationship and their possession of a mechanistically common SI system. Our findings provide a breakthrough in this area, as transferral of the *Papaver* pollen *S*-determinant into *A. thaliana* is between highly diverged species with ~144 million years separating them (Bell et al. 2010), and they do not share a common SI system.

Our data provide good evidence that *A. thaliana* possesses proteins that can be recruited to form new signaling networks and targets that are used for a function that does not normally operate in this species. These data suggest that the *Papaver* SI system uses common cellular targets, and that endogenous signaling components can be recruited to elicit a response that most likely never operated in this species, which has potentially important implications. Studies of the evolution of self/non-self recognition systems have generally focused on the receptors and ligands involved in recognition, rather than the downstream signaling networks triggered by their interaction. Our findings suggest either conservation of an ancient signaling system or recruitment of signaling components to mediate the downstream responses for SI recognition. Also, our data suggest that the postulated parallels between SI and plant–pathogen resistance (Hodgkin et al. 1988; Sanabria et al. 2008), with the idea that SI may utilize some of these signaling networks, may not be as unlikely as it initially seems. It appears that the *Papaver* SI system works in *A. thaliana* as a result of "multi-tasking" of endogenous components that can "plug and play" to act in signaling networks in which they do not normally operate. The signaling networks (e.g., Ca^{2+}) and targets for *Papaver* SI (e.g., the actin cytoskeleton) appear to be "universal," unspecialized, and ancient and may be present in all angiosperm cells. If these "common" cellular elements can be recruited to operate under the control of a newly introduced system (as we have shown with PrpS), it appears that a novel and functional signaling network can be set up that results in a specific, predictable physiological outcome; this could be similar to situations where gene redundancy and plasticity operate. MAPK cascade components are classic examples of signaling components that can participate in more than one signaling

network in certain situations. For example, in *Saccharomyces cerevisiae* (yeast), components can play a role in more than one pathway; see Widmann et al. (1999), Asai et al. (2002), and Eckardt (2002) for further discussion of this phenomenon. Examples of dual functioning or "multi-tasking" have been cited in the context of innate immune signaling pathways (Ausubel 2005). We think this is a likely explanation of why PrpS functions in *A. thaliana* pollen.

23.5 Summary

Research into the *Papaver* SI system, as outlined in this review, has shown that several major cellular components are targets for SI signals in pollen, which are triggered when the male and female *S*-determinants interact (see Fig. 23.1a for a cartoon of the situation in an unstimulated pollen tube). These events contribute, first, to initiation of signaling events (Fig. 23.1b) and the inhibition of incompatible pollen tube growth, and second, to key mechanisms involved in steps to commitment to PCD (Fig. 23.1c). Together, these mechanisms ensure that self-fertilization does not occur. *Papaver* clearly has a complex network of signaling events that are integrated to contribute to SI-mediated inhibition and death of incompatible pollen tubes. However, there still remain many unanswered questions about mechanisms to be investigated. For the future, the demonstration of wide transgenera functionality of the *Papaver* SI system opens up the possibility that this may, in the longer term, provide a tractable SI system to transfer to crop plants to make F_1 hybrids more efficiently. Demonstration that interaction of PrsS with *A. thaliana* PrpS-GFP pollen to elicit a "SI" response suggests there is scope for transfer of the *Papaver* SI system to completely unrelated crop species. This possibility will be a major challenge for the future.

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