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Self-Incompatibility in *Papaver*: Advances in integrating the signalling network

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Abbreviations: I-V, current-voltage; $[Ca^{2+}]_i$, intracellular levels of Ca^{2+}; ADF, actin depolymerizing factor; CAP, cyclase associated protein; cPTIO, (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide); DPI, diphenyleneiodonium; F-actin, filamentous actin; MAPK, mitogen activated protein kinase; MYA, million years ago; NO, nitric oxide; NSCC, Non-Specific Cation Channel; $O_2^{\bullet -}$, superoxide anion radical; PCD, programmed cell death; PrABP80, *Papaver rhoeas* actin binding protein; PrpS, *Papaver rhoeas* pollen *S*; PrsS, *Papaver rhoeas* stigma *S*; ROS, reactive oxygen species; SI, self-incompatibility; sPPase, soluble inorganic pyrophosphatase; TEMPOL, 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl;

Abstract

Self-fertilization, which results in reduced fitness of offspring, is a common problem in hermaphrodite angiosperms. To prevent this, many plants utilize self-incompatibility (SI), which is determined by the multi-allelic *S* locus, that allows discrimination between self (incompatible) and non-self (compatible) pollen by the pistil. In poppy (*Papaver rhoeas*), the pistil *S*-determinant (PrsS) is a small secreted protein which interacts with the pollen *S*-determinant PrpS, a ~20 kDa novel transmembrane protein. Interaction of matching pollen and pistil *S*-determinants results in self-recognition, initiating a Ca^{2+}-dependent signalling network in incompatible pollen. This triggers several downstream events including alterations to the cytoskeleton, phosphorylation of soluble inorganic pyrophosphatases (sPPases) and a Mitogen Activated Protein Kinase (MAPK), increases in ROS and NO, and activation of several caspase-like activities. This results in the inhibition of pollen tube growth, prevention of self-fertilization and ultimately programmed cell death (PCD) in incompatible pollen. This review will focus on our current understanding of the integration of these signals with their targets in the SI-PCD network. We also discuss our recent functional expression of PrpS in *Arabidopsis thaliana* pollen.

Introduction

Successful sexual reproduction in many flowering plants involves a genetically controlled mechanism called self-incompatibility (SI). SI is genetically controlled by a multi-allelic *S*-locus that encodes linked pollen and pistil *S*-determinants that provide self-recognition. Interactions between pollen and pistil of the same haplotype initiate an SI response, which causes inhibition of "self" pollen tube growth, so fertilization is inhibited. Pollen carrying *S*-determinants that are different to those expressed on the surface of the recipient stigma are able to germinate, pollen tube growth proceeds and fertilization is achieved. The SI mechanism therefore acts to prevent inbreeding and increase genetic diversity. The SI systems that are well characterized at the molecular level to date are: the Brassicaceae, the Papaveraceae and the *S*-RNase-type which includes the Solanaceae, Plantaginaceae and Rosaceae. The reader is referred to other reviews for detailed information on the mechanisms of Brassicaceae [1] and *S*-RNase-type SI [2]. Here we focus on recent advances in our understanding of the Papaveraceae SI system in poppy (*Papaver rhoeas*).

The two *S*-determinants of the poppy *S*-locus have been well characterized. PrpS encodes a novel transmembrane ~20 kDa protein that is specifically expressed in pollen [3], while PrsS is a ~14 kDa protein secreted to the pistil surface [4]; see Poulter et al, 2010 for a recent review [5]. A robust *in vitro* SI bioassay that uses recombinant PrsS proteins to induce the *Papaver* SI response in germinating pollen has been developed in our laboratory, and this has enabled investigations at the molecular and cellular level, making it one of the best characterized SI systems at the mechanistic level. In brief, upon the cognate interaction of PrsS and PrpS, Ca^{2+} influx is triggered, which increases intracellular levels of Ca^{2+} ($[Ca^{2+}]_i$), and induces a signalling cascade, resulting in the pollen tube growth inhibition and ultimately, PCD of incompatible pollen. Ca^{2+}-dependent phosphorylation of sPPases has been observed in the initiation stage of this SI response. Both Ca^{2+} and phosphorylation reduce their activities, causing arrest of pollen tube growth [6, 7]. Another rapidly modified target is the actin cytoskeleton. SI induces rapid (within 1 min) alterations in F-actin organization, and a sustained depolymerization of actin filaments [8], followed by an accumulation of F-actin into "punctate foci" [9]. Between 90 s and 10 min post-SI induction, a MAPK, p56, is activated [10]. MAPKs are known to play a key role in mediating signals to PCD in the plant-

pathogen hypersensitive response and also have been described as universal signal transduction networks that connect many diverse signalling cascades [11], suggesting a potential role of the p56 MAPK in *Papaver* SI signalling pathway. Studies based primarily on the use of a MAPK cascade inhibitor in combination with PCD markers, implicated an important role for MAPKs in signalling to PCD in incompatible pollen [12]. Moreover SI resulted in a cytosolic acidification, providing the optimal pH for the SI-induced caspase-like activities detected in the pollen tube during SI [13]. The ultimate mechanism of poppy self-pollen rejection is through PCD [14]; evidence in support of this includes cytochrome *c* leakage, caspase-like activities and DNA fragmentation in incompatible pollen [13-15]. In recent years, further components have been identified as being involved in the SI signalling network, contributing to the pollen tube growth inhibition and PCD.

In this review, we will highlight some of the major intracellular events that are triggered during a *Papaver* SI response and focus on recent findings that contribute to our understanding of the integration of the signals and targets of this intricate SI-initiated PCD pathway. We will also describe the functional transferral of poppy SI system into self-compatible *Arabidopsis thaliana* and its implications. A model, based on our current understanding of the SI signalling cascade, is provided.

SI triggers membrane channel activation permeable for divalent and monovalent cations

The earliest identified physiological event triggered by SI recognition is the almost instantaneous increase in $[Ca^{2+}]_i$ in incompatible pollen tubes [16-18]. These studies formed the basis of a hypothesis, proposing that a receptor-ligand type of interaction, involving PrsS and PrpS of the same haplotype, triggers a Ca^{2+} dependent signalling cascade within incompatible pollen. More recently, studies using an electrophysiological approach, have demonstrated evidence not only for Ca^{2+} influx but also a large influx of K^+ in incompatible pollen [19] (**Figure 1**). Currents were identified by means of whole-cell patch clamping of pollen protoplasts; plasma membrane ion channel activation was measured during an *S*-allele-specific SI response in pollen protoplasts exposed to either Ca^{2+} or K^+. For the divalent cation Ca^{2+}, after exposure to recombinant incompatible PrsS protein, the current-voltage (I-V) relationship revealed a large increase in the current (**Figure 1**). A similar result was obtained for the monovalent cation K^+, where I-V relationship showed that SI stimulates a high conductance for K^+, with greater amplitude than that for Ca^{2+} (**Figure 1**). These studies suggest that the SI-induced conductance(s) is ligand-gated and may involve a Non-Specific Cation Channel (NSCC). Other studies have shown that the elevated $[Ca^{2+}]_i$ functions as a second messenger [16-18], initiating a complex set of events within incompatible pollen that result in the rapid inhibition of pollen tube growth and, ultimately, in the specific destruction of incompatible pollen by PCD.

Involvement of cytoskeleton in SI-mediated PCD

Integrity of the actin cytoskeleton is crucial for pollen tube growth as it is important for both regulating growth and modulating signal-response coupling [20, 21]. SI induction triggers several striking alterations to the actin cytoskeleton. The typical longitudinal F-actin bundles in normally growing pollen tubes (**Figure 2a**) are rapidly depolymerized in SI-induced pollen (**Figure 2b**), and subsequently, the F-actin aggregates into "punctate foci" (**Figure 2c**), which increase in size over time [8, 9]. An actin-binding protein, PrABP80, with properties of a gelsolin was identified as a candidate to mediate SI-induced actin depolymerization due to its potent Ca^{2+}-dependent severing activity, probably together with profilin [22]. Immunolocalization studies have shown that the punctate F-actin foci are associated with the

actin-binding proteins Actin Depolymerizing Factor (ADF/cofilin; **Figure 2d-e**) and Cyclase Associated Protein (CAP) [23]. Although the name ADF suggests its filament severing activity under normal cellular conditions, it has been reported that ADFs exhibit pH-sensitive activity, with actin depolymerization at an alkaline pH and binding to and stabilizing F-actin at an acidic pH [24]. Thus, in the context of SI, with an SI-induced acidification [13], ADF is likely to be playing a role in the formation and stabilization of the SI-induced actin foci.

SI also targets the microtubule cytoskeleton, triggering very rapid depolymerisation of cortical microtubules [25]. However, unlike actin, the microtubules do not reorganize into punctate foci. The use of specific drugs that alter the polymerization status of F-actin showed that actin depolymerization triggered the depolymerization of cortical microtubules. However, artificial depolymerization of microtubules did not affect actin, suggesting that there is one-way "cross-talk" from the actin to the tubulin cytoskeleton [25]. Both actin and microtubule alterations are implicated in mediating PCD. Several studies have shown that either actin depolymerization or stabilization can influence whether a eukaryotic cell goes through an apoptotic pathway [26, 27]. Investigations examining the effect of the actin-depolymerizing drug, latrunculin B and the actin-stabilizing/polymerizing drug, jasplakinolide in *Papaver* pollen revealed that both treatments stimulated high levels of DNA fragmentation which was mediated by a caspase-3 like/DEVDase activity [28]. This demonstrated that disturbance of actin polymer dynamics can trigger PCD in pollen. These data implicated both actin depolymerization and stabilization being functionally involved in the initiation of SI-induced PCD, and established a causal link between actin polymerization status and initiation of PCD. Thus, the rapid and substantial actin depolymerization triggered by SI signalling not only results in the rapid inhibition of incompatible pollen tip growth, but it also activates a caspase-3-like/DEVDase activity, triggering PCD. Therefore the formation of the F-actin foci and their association with ADF, appears to be an active process that is also involved in signalling towards PCD; they are also a reliable marker for SI-induced PCD in poppy pollen, and their formation is alleviated concomitantly with alleviation of PCD [29].

A role for ROS and NO signalling in SI-mediated PCD

In plants, ROS and NO have been shown to play a key role in a variety of cellular responses including PCD [30, 31]. Live cell imaging has recently been used to visualize ROS and NO in growing *Papaver* pollen tubes during SI. Around 5 min after SI-induction, rapid and transient increases in cytosolic ROS was observed in incompatible pollen tubes, and later, transient increases in NO [29]. Preventing ROS and NO increases in pollen tubes prior to SI-induction, using a pre-treatment with both the NADPH oxidase inhibitor, DPI (diphenyleneiodonium) and the NO scavenger cPTIO (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) substantially decreased the caspase-3-like/DEVDase activity (**Figure 3a**), which is a key feature of the poppy SI-PCD response. Neither of these scavengers alone could significantly reduce the amount of caspase-3-like/DEVDase activity (**Figure 3a**). This suggests that ROS and NO act either in concert or tandem to signal towards SI-induced PCD through activation of DEVDase activity. Evidence also suggested that ROS and NO increases during SI also contribute to increased numbers of pollen tubes containing punctate actin foci [29]. **Figure 3b** shows that untreated pollen tubes had negligible actin foci, SI induced pollen tubes had high levels, and when pollen was pre-treated with the ROS scavenger TEMPOL (4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) and/or c-PTIO prior to SI-induction, the number of actin foci was significantly reduced in all three cases [29]. As ROS and NO scavengers can alleviate the formation of punctate actin foci and also prevent the activation of caspase-3-like/DEVDase activity, this suggests that those ROS and NO

signals act upstream of these SI markers.

Recruitment of signalling for SI events in other distantly related species

Recent studies have begun to explore the possibility for functional transfer of the *Papaver* SI determinants into other species. The *Papaver rhoeas* pollen *S*-determinant, PrpS, was expressed as a GFP-fusion protein and demonstrated to be functional in self-compatible *A. thaliana* pollen [32]. When *A. thaliana* pollen expressing PrpS-GFP was treated with recombinant PrsS protein, pollen tube growth was inhibited and its viability decreased in an *S*-specific manner, demonstrating functional "SI" in *A. thaliana* (**Figure 4a**). This *S*-specific decrease in pollen viability could be rescued by pre-treatment with the caspase-3 inhibitor, Ac-DEVD-CHO (**Figure 4a**), demonstrating the involvement of PCD [32]. Recombinant PrsS protein treatment also stimulated *S*-specific actin foci formation in *At*-PrpS-GFP pollen in an *S*-specific manner (**Figure 4b-c)**, demonstrating that a similar signalling cascade to that in *Papaver* SI response was triggered in the transgenic *A. thaliana* pollen.

Successful SI trait transferral between species was first obtained nearly a decade ago by the Nasrallah laboratory [33, 34]. They demonstrated that *S*-haplotype pairs from *A. lyrata* and *Capsella grandiflora* (which are diverged from *A. thaliana* around ~5 [35], and ~6.2–9.8 million years ago (MYA) [36], respectively), confer self-incompatibility in *A. thaliana*. However, as these are closely related species that share a mechanistically common SI ancestor, this does not provide major insights into the evolution of SI signalling across angiosperm families. Our findings provide a breakthrough in this area, as functional transferral of the *Papaver* pollen *S*-determinant into *A. thaliana* is obtained between highly diverged species with an evolutionary distance ~144 MYA [37], and which has no orthologues of the *Papaver S*-determinants. This represents the first demonstration that an SI system can be functionally transferred into a very distantly related species with a different ancestral SI system.

Our data indicate that the *Papaver* SI system works in *A. thaliana* due to the ability to execute and co-ordinate more than one endogenous component at the same time and can act in signalling networks that they do not normally operate in. The *Papaver* SI signalling networks (e.g. Ca^{2+}) and targets (e.g. the actin cytoskeleton) appear to be universal and may be present in most of the angiosperms from the very early times. If these "common" cellular elements can be recruited to operate under the control of a newly introduced system (as we have shown with introduction of PrpS), it appears that a novel and functional signalling pathway can be initiated that results in a specific, predictable physiological outcome (PCD in this case). This could be similar to situations where gene redundancy and plasticity operate. MAPK cascade components are classic examples of signalling components that can participate in more than one signalling network in certain situations. For example, in *Saccharomyces cerevisiae* (yeast), many components can play a role in more than one pathway; (see [38-40] for further discussion of this phenomenon). Examples of dual functioning or 'multi-tasking' have been cited in the context of innate immune signalling pathways [41]. We think this is a likely explanation of why PrpS functions in *A. thaliana* pollen. Our data provides good evidence that *A. thaliana* possesses proteins that can be recruited to form a new signalling network for SI response which does not normally operate in *A. thaliana*. This also suggests that the *Papaver* SI system uses ubiquitous cellular targets (such as the cytoskeleton and sPPases), and that endogenous signalling components (such as $Ca^{2+,}$ ROS, NO, MAPKs) in the host species can somehow be recruited to elicit an appropriate "SI" response in a species that has most likely never had this type of SI system.

This has potentially important implications. Crop F_1 hybrid seed production currently relies on artificial emasculations, which are time- and labour-consuming. Transferral of *S*-determinants from poppy into crop plants to make them self-incompatible provides a novel and efficient way to obtain hybrid seeds naturally. Our findings also are of interest from a scientific viewpoint. Studies of the evolution of self-/non-self- recognition systems have generally focused on the receptor-ligand recognition, rather than the downstream signalling networks triggered by their interaction. Our findings suggest either conservation of an ancient signalling system or recruitment of signalling components to mediate the downstream responses for SI recognition. Our data suggest that the postulated parallels between SI and plant-pathogen resistance, with the idea that SI may utilize some of these signalling networks, may not be as unlikely as it initially seems.

Summary and future perspectives

Research on the *Papaver* SI system has shown that an *S*-specific interaction between the male and female *S*-determinants triggers an SI signalling network which integrates several cellular components in incompatible pollen (see **Figure 5**). These signalling events can be classified into two categories. First, signal initiation events which include Ca^{2+} and K^+ influx, inhibition of sPPase activity, depolymerization of F-actin and inhibition of incompatible pollen tube growth. Second, later suicide signalling events, are involved in commitment to PCD, through the activation of caspase-3-like/DEVDase activities. These include activation of the p56 MAPK, increases in ROS, and the formation of F-actin foci. Collectively, these signal to the "gateway" which incompatible pollen must pass to become irreversibly inhibited, by setting up self pollen "suicide". Once cytosolic acidification and caspase activation occur, the incompatible pollen is programmed to die and disassemble. Together, these mechanisms coordinate the prevention of self-fertilization. Although key components have been identified in the signalling network of the *Papaver* SI system, many unanswered questions remain, and further efforts are required to fully elucidate the mechanisms involved in the poppy SI response. Moreover, the demonstration of wide transgenera functionality of the *Papaver* SI system in *A. thaliana*, suggests that the transfer of the *Papaver* SI system to unrelated crop species, is potentially feasible. Transfer of this SI system could be used as a tractable SI system and heralds the possibility, at least in principle, of using this as an application to increase efficiency in the production of F_1 hybrids in crop plants.

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Figure legends

Figure 1. Conductance stimulated in pollen under SI conditions. Representative ramp-induced, whole cell currents obtained by patch-clamping pollen protoplasts bathed in Ca^{2+}-containing saline: untreated (UT, cyan line) and after SI induction, by addition of incompatible PrsS, shows an increase in Ca^{2+} current (SI, black line), and after SI induction in K^+-containing saline, shows an even larger increase in K^+ current (SI, red line). The dashed line indicates zero current.

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Figure 2: Alteration of F-actin organization during SI
(a) F-actin organization in an untreated pollen tube showing prominent actin filament cables in the shank of the tube and a subapical actin filament collar.
(b) 10 min post-SI many of the F-actin bundles have disappeared, but F-actin remains at the cortex and tip.
(c) 3 h post-SI induction, large F-actin foci are visible at the cortex throughout the length of the pollen tube.
(d) In untreated (UT) pollen tubes, ADF (green) was cytosolic and did not co-localize with F-actin (red).
(e) After 3 h of SI induction, a significant amount of ADF co-localized to the punctate F-actin foci (yellow).
All the images were obtained using confocal microscopy and are single optical sections. Images (a-c) show F-actin stained with rhodamine phalloidin; images (d-e) show F-actin stained with rhodamine phalloidin (red) and immunolocalization of ADF (green), as a merged images, with overlap shown as yellow. Scale bar = 10 μm.

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Figure 3. Analysis of the effect of ROS and NO on SI markers.
(a) The effect of ROS and NO on DEVDase activity in *Papaver* pollen extracts was measured using Ac-DEVD-AMC. The levels of DEVDase activity in untreated (UT) samples (white bar), samples pre-treated with inhibitors (dotted bars) and SI induced pollen (SI, black bar) were compared to activity in SI-induced pollen pre-treated with the NO scavenger cPTIO, the NADPH inhibitor DPI, and both inhibitors combined (cross-hatched bars). DEVDase activities are shown as a percentage of the untreated activity (100%). Data are means ± SE of five independent experiments.
(b) Effect of ROS and NO on actin configuration. The percentage of pollen exhibiting actin foci configuration (black bar) was low in untreated (UT) sample and high in SI induced sample. Pre-treatment with Tempol, cPTIO and both together did not alter the number of tubes pollen tubes containing foci. Pre-treatment of pollen with TEMPOL, cPTIO, and both together prior to SI-induction reduced actin foci formation. Actin configuration was assessed using fluorescence microscopy and rhodamine-phalloidin staining. Actin configuration was categorised as normal, punctate foci, or intermediate. 70 pollen tubes were scored for each treatment, and expressed as a percentage. Error bars indicate SE of three independent experiments.

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Figure 4: PrpS functions in *A. thaliana*.
"SI" was induced in *A. thaliana* pollen expressing PrpS by addition of cognate recombinant PrsS protein, and scored for two key features of the poppy SI response: DEVDase activity (a) and punctate actin foci formation (b-c).
(a) Recombinant PrsS protein treatment triggered *S*-specific PCD in *At-PrpS* pollen. Untreated *At*-PrpS pollen (white bar) had high viability and is significantly reduced in an *S*-specific manner by addition of cognate recombinant PrsS protein (black bars). Addition of caspase-3/DEVDase inhibitor Ac-DEVD-CHO alone did not affect pollen viability (cross-hatched bars), but pre-treatment with Ac-DEVD-CHO before SI resulted in a significantly increase of viability (diagonal cross bars). Error bars indicate results ±SEM.
(b-c) *S*-specific actin foci were stimulated by treating *A. thaliana* pollen expressing PrsS with cognate recombinant PrsS protein. *At*-$PrpS_1$ pollen treated with recombinant $PrsS_1$ protein showed punctate actin foci

(b). *At*-PrpS$_1$ pollen treated with recombinant PrsS$_3$ protein showed the normal actin filamentous organization (c).

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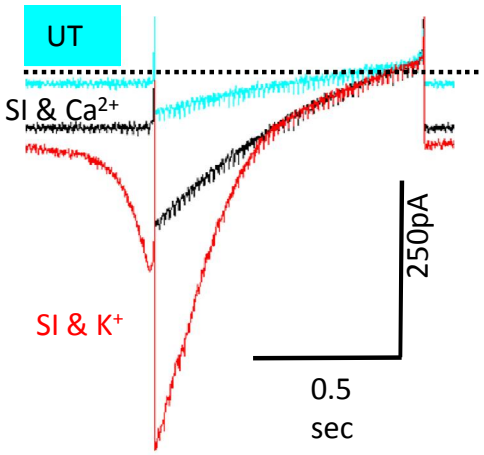
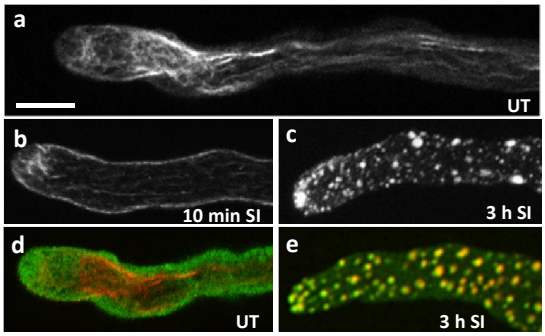
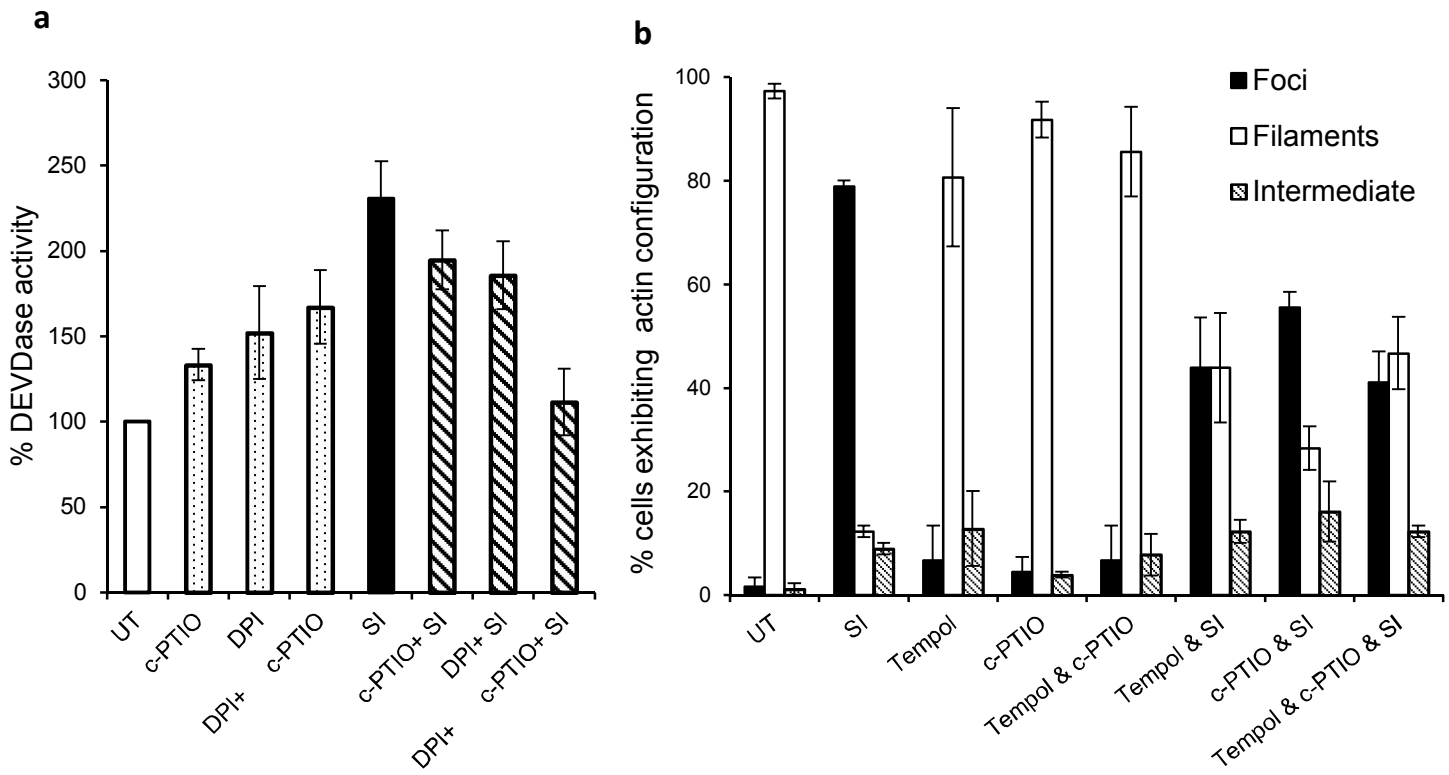
Figure 5: A model showing the integration of the signals and targets of the *Papaver* SI signalling network.

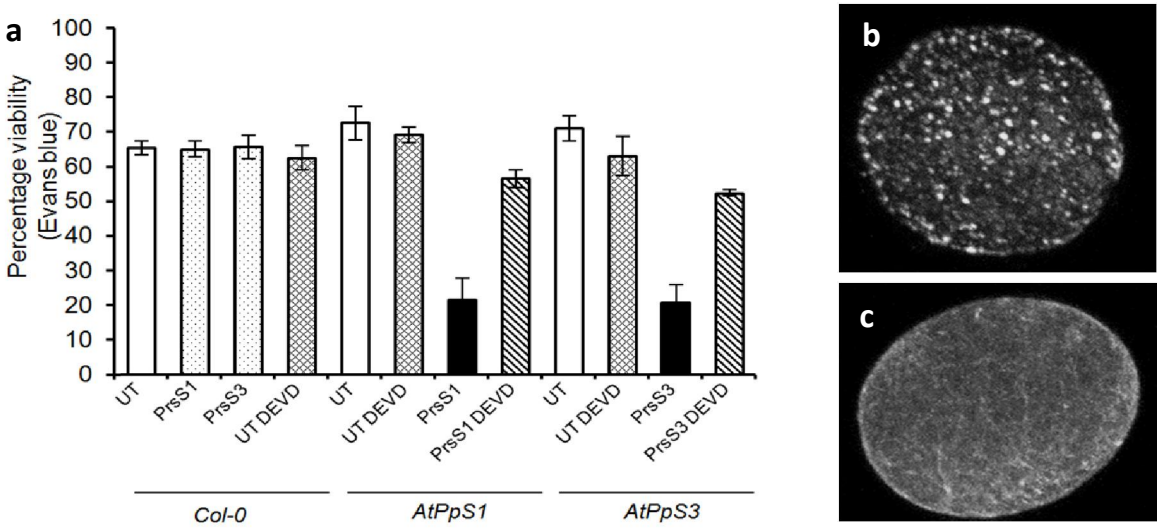
PrsS-proteins secreted by the stigma interact with the pollen *S*-determinant PrpS in an *S*-specific manner. This triggers both K^+ and Ca^{2+} influx. The increase of cytosolic Ca^{2+} triggers a signalling network which targets several downstream components, and results in the pollen tube growth inhibition and programmed cell death (PCD) of incompatible pollen. Soluble inorganic pyrophosphatases, Pr-p26.1a/b, are phosphorylated; this and increased Ca^{2+}, results in their inhibition and a results in a reduction in biosynthetic capability. The F-actin cytoskeleton is rapidly depolymerized, which also contributes to inhibition of pollen tube growth. Later in the SI response, F-actin forms large punctate foci. The microtubule cytoskeleton is also rapidly depolymerized. These changes to the cytoskeleton signal to PCD, involving caspase-like activities, as does a MAPK, p56, which is activated by phosphorylation. ROS and NO increases are also involved in signalling to PCD, acting upstream of actin foci formation and caspase-3-like activities. Together these events ensure that fertilization cannot occur in an incompatible situation.

Adapted from Bosch & Franklin-Tong (2008), Journal of Experimental Botany, ***59****, 481-490, by kind permission of © Oxford University Press*


UT
SI & Ca2+
SI & K+
250pA
0.5
sec


a
UT
b
10 min SI
c
3 h SI
d
UT
e
3 h SI


a
% DEVDase activity
300
250
200
150
100
50
0
UT
c-PTIO
DPI
DPI+ c-PTIO
SI
c-PTIO+ SI
DPI+ SI
DPI+ c-PTIO+ SI
b
% cells exhibiting actin configuration
100
80
60
40
20
0
Foci
Filaments
Intermediate
UT
SI
Tempol
c-PTIO
Tempol & c-PTIO
Tempol & SI
c-PTIO & SI
Tempol & c-PTIO & SI


a
b
c
Percentage viability (Evans blue)
100
90
80
70
60
50
40
30
20
10
0
UT
PrsS1
PrsS3
UT DEVD
UT
UT DEVD
PrsS1
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UT
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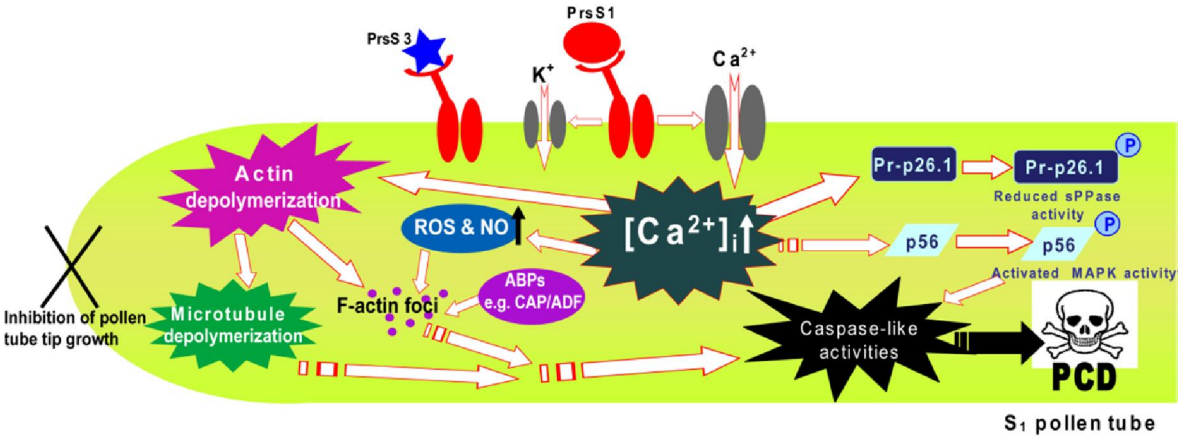
PrsS3 DEVD
Col-0
AtPpS1
AtPpS3


PrsS 3
PrsS1
K+
Ca2+
Actin depolymerization
ROS & NO
[Ca2+]i
Pr-p26.1
Pr-p26.1
Reduced sPPase activity
p56
p56
Activated MAPK activity
Inhibition of pollen tube tip growth
Microtubule depolymerization
F-actin foci
ABPs e.g. CAP/ADF
Caspase-like activities
PCD
S1 pollen tube