Taking one for the team: self-recognition and cell suicide in pollen

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Abstract

Self-incompatibility (SI) is an important genetically controlled mechanism used by many angiosperms to prevent self-fertilization and inbreeding. A multiallelic S-locus allows discrimination between ‘self’ (incompatible) pollen from ‘nonself’ pollen at the pistil. Interaction of matching pollen and pistil S-determinants allows ‘self’ recognition and triggers rejection of incompatible pollen. The S-determinants for Papaver rhoeas (poppy) are PrsS and PrpS. PrsS is a small secreted protein that acts as a signalling ligand to interact with its cognate pollen S-determinant PrpS, a small novel transmembrane protein. Interaction of PrsS with incompatible pollen stimulates increases in cytosolic free Ca²⁺ and involves influx of Ca²⁺ and K⁺. Data implicate involvement of reactive oxygen species and nitric oxide signalling in the SI response. Downstream targets include the cytoskeleton, a soluble inorganic pyrophosphatase, Pr-p26.1, and a MAP kinase, PrMPK9-1. A major focus for SI-induced signalling is to initiate programmed cell death (PCD). In this review we provide an overview of our understanding of SI, with focus on how the signals and components are integrated, in particular, how reactive oxygen species, nitric oxide, and the actin cytoskeleton feed into a PCD network. We also discuss our recent functional expression of PrpS in Arabidopsis thaliana pollen in the context of understanding how PCD signalling systems may have evolved.

Key words: Actin cytoskeleton, caspase, nitric oxide (NO), oxidative stress, Papaver rhoeas, pollen, programmed cell death (PCD), reactive oxygen species (ROS), self-incompatibility (SI).

Introduction

Unwanted eukaryotic cells are often removed by programmed cell death (PCD). In recent years, plant PCD has been a topic of significant interest and this phenomenon has become accepted as a mainstream event, playing a critical role in most processes in plant development, abiotic stresses, and plant–pathogen interactions. Several categories of plant PCD have recently been defined according to morphological criteria (van Doorn et al., 2011), although biochemical criteria have yet to be dealt with in the same manner. However, not all PCD systems fit neatly into one category; a good example is the topic of this review, the Papaver self-incompatibility (SI) response, which exhibits hallmark features from several categories.

There are several markers for PCD that have been observed in both animal and plant cells. However, emerging data in both animals and plants suggest that many are also involved in normal development or necrosis, so attribution of characteristics to establish PCD is often difficult. In addition, it is clear that PCD in plants is quite different. Caspases play an important role in initiating and executing mammalian cell apoptosis by cleaving protein targets (Parrish et al., 2013). One of the key mammalian executioner caspases is caspase-3, which has a tetrapeptide recognition motif of DEVD, so is known as a DEVDase, due to its cleavage recognition specificity. Biochemical tools have been developed to identify caspase-like activities using caspase
specific substrates and inhibitors, based on specific caspase recognition motifs which allow the direct analysis of caspase activities. Despite the fact that plants do not have homologues of mammalian caspases, use of these mammalian tools has demonstrated the involvement of caspase-like activities in several plant PCD systems (Chichkova et al., 2004; Rojo et al., 2004; Bonneau et al., 2008).

Another important feature of many PCD networks in plants is an oxidative burst (Gechev et al., 2006; Mitter et al., 2011). Reactive oxygen species (ROS) play an important role in basal resistance and has been identified as being involved in the later stages of systemic acquired resistance and hypersensitive response, which leads to PCD (Torres et al., 2006). The role of the oxidative burst in plant PCD is the topic of several reviews in this special issue (Baxter et al., 2014; Demidchick, 2014), so will not be reviewed here.

The ability to discriminate between self and nonself (allorecognition) is usually controlled by a highly polymorphic locus and is an important feature of many organisms (Sawada et al., 2014). Many plants are hermaphrodites, with male and female organs in close proximity. This increases the chances of self-fertilization and also the possibility of undesirable inbreeding depression. Many plants utilize SI to prevent inbreeding, using a cell-cell recognition system in which pollen carrying the same haplotype as the pistil it lands on is rejected via the initiation of PCD. This process of self-rejection prevents inbreeding; for the ‘greater good’ of the species incompatible pollen to commit suicide. Here we review key events relating to SI signalling, stimulated by the S-specific interaction of recombinant pistil PrsS with pollen expressing PrpS. The Papaver SI system has recently been found to utilize ROS and nitric oxide (NO) to integrate signalling to PCD in incompatible pollen. This provides a good example of a system that straddles both these important topics that are the focus of this Special Issue. We therefore focus attention on the integration of these signalling components and their cellular targets.

**Mechanisms involved in SI in the Papaver system**

**Calcium signalling mediates Papaver SI**

Ca\(^{2+}\) is an important second messenger, and Ca\(^{2+}\)-dependent signalling networks are used to generate responses to many stimuli in both animal and plant cells. The Papaver SI response is thought to be initiated by an almost instantaneous increases in cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)) forming a ‘wave’ in the shank of the pollen tube (Franklin-Tong et al., 1993, 1995, 1997). These studies formed the basis of a hypothesis, proposing that a receptor-ligand type of interaction, involving PrsS and PrpS, triggers a Ca\(^{2+}\)-dependent signalling cascade in incompatible pollen. More recent studies, using an electrophysiological approach, have provided evidence for Ca\(^{2+}\) influx and also revealed a large influx of K\(^{+}\) in incompatible pollen (Wu et al., 2011). These studies suggested that the SI-induced conductances may involve nonspecific cation channel(s). Several signalling targets downstream of the SI-specific increases in cytosolic Ca\(^{2+}\) in Papaver pollen tubes have been identified. Ca\(^{2+}\) signalling often results in altered protein kinase activity, resulting in post-translational modification such as phosphorylation.

**Phosphorylation events identified in Papaver SI**

MAPK cascades trigger numerous signalling networks. They are activated by dual phosphorylation of threonine and tyrosine residues in a TXY motif via a MAPKKK cascade. During the SI response, a MAPK, p56, is activated specifically in incompatible, but not compatible pollen (Rudd and Franklin-Tong, 1996; Li et al., 2007). p56 activation, which peaks 10min after SI induction, is temporally too late to be involved in the inhibition of pollen tube growth; however, studies have provided evidence that this MAPK is involved in signalling to PCD (as will be discussed later).
SI also triggers the rapid phosphorylation of Pr-p26.1a/b, two pollen-expressed 26-kDa soluble inorganic pyrophosphatases (sPPases) (Rudd et al., 1996; de Graaf et al., 2006). sPPases are important enzymes involved in hydrolysis of inorganic pyrophosphate (PPI) (Kornberg, 1962) and it makes good sense that their activity is associated with driving biosynthesis in these rapidly growing cells. sPPases are well documented to have their activity inhibited by Ca\(^{2+}\), so this provides a neat and potentially rapid mechanism to inhibit incompatible pollen tube growth (de Graaf et al., 2006). Phosphorylation of sPPases is a novel event and it may provide an additional inhibitory mechanism. However, whether these sPPases somehow feed into the PCD network is not known.

**Papaver SI triggers alterations to the cytoskeleton**

The actin cytoskeleton is a major target and effector of signalling networks (Staiger, 2000) and an intact F-actin cytoskeleton is required for pollen tube growth (Gibbon et al., 1999). With these two pieces of information, the actin cytoskeleton was investigated as a possible early target for SI signals, which involve both Ca\(^{2+}\) signalling and inhibition of pollen tube growth.

The normal actin cytoskeleton of *Papaver* pollen tubes comprises longitudinal filament bundles of actin in the shank, with a typical cone-shaped configuration just behind the tip (Fig. 1A). Dramatic alterations in F-actin organization are triggered specifically in incompatible pollen tubes by SI induction, which involves F-actin depolymerization (Fig. 1B) (Geitmann et al., 2000; Snowman et al., 2002). This is predicted to result in the rapid inhibition of pollen tube growth. Two Ca\(^{2+}\)-dependent actin-binding proteins (ABP), profilin and PrABP80 (a putative gelsolin), which have strong Ca\(^{2+}\)-dependent severing activity (Huang, 2004), are implicated in the SI-induced depolymerization. The actin then begins to aggregate to form punctate F-actin foci and they increase in size over time (Fig. 1C, D). Two ABPs, actin depolymerizing factor (ADF/cofilin) and cyclase-associated protein (CAP), rapidly colocalize to the actin foci (Poulter et al., 2010). It is thought that both the depolymerization and the formation of these highly stable actin structures play a role in signalling to PCD (as will be discussed further). Compatible interactions have no effect on the cytoskeleton (Fig. 1E). The pollen tube microtubule cytoskeleton is also a target for the SI signals. Cortical microtubules are rapidly depolymerized and there is evidence that this, too, plays a role in the PCD network (Poulter et al., 2008; see also further discussion).

![Fig. 1.](https://academic.oup.com/jxb/article-abstract/65/5/1331/2884868)

It might be argued that Ca\(^{2+}\) signalling, actin depolymerization, and inhibition of pollen tube growth, all of which occur very rapidly, might simply cause inhibition of tip growth and consequent cell death. However, studies showed that compatible pollen tubes that stop growing ‘naturally’ around 8 h, due to limitations in the artificial growth medium, did not exhibit DNA fragmentation (Jordan et al., 2000). This, coupled with the observation that ‘normal’ inhibition of pollen tube growth was not associated with the very distinctive Ca\(^{2+}\) signature observed in incompatible pollen, helped to rule out the idea that cell death is just a consequence of inhibited pollen tube growth. It is possible that the SI-induced Ca\(^{2+}\) signalling is primarily to stop the growth of the pollen tube, involving F-actin depolymerization. In support of this, low concentrations of LatB result in pollen-tube inhibition but not DNA fragmentation (Gibbon et al., 1999; Thomas et al., 2006). However, both the level of SI-induced [Ca\(^{2+}\)]\(_{cyt}\) and the amount of F-actin depolymerization are in huge excess of that required simply to stop growth. Caffeine, which inhibits tip growth but
does not affect the actin cytoskeleton of pollen tubes (Geitmann et al., 2000; Snowman et al., 2002), has been used to show that caffeine-inhibited pollen tubes have low DNA fragmentation levels, not significantly different from controls (Thomas et al., 2006). This study demonstrated that the SI-mediated DNA fragmentation is not merely a consequence of inhibition of growth, but is due to major changes in actin polymer levels or assembly dynamics. The evidence supports the idea that the SI Ca\(^{2+}\) signature is generated specifically to signal to trigger excess sustained F-actin depolymerization and then aggregation and stabilization of F-actin foci, which are highly distinctive and a feature of the SI-PCD (as will be discussed).

**SI-induced PCD is mediated by caspase-like activities**

Temporally, one of the latest SI events is DNA fragmentation. Jordan et al. (2000) showed that DNA fragmentation occurred in an S-specific manner. DNA fragmentation increased only in SI-induced pollen tubes, with maximal levels detected at 12–14 h after SI induction and not in compatible controls grown for the same length of time (Jordan et al., 2000; Thomas and Franklin-Tong, 2004). Subsequently it was shown that addition of the caspase-3 inhibitor, Ac-DEVD-CHO, prior to SI induction significantly reduced SI-specific pollen-tube inhibition as well as DNA fragmentation. In contrast, use of the caspase-1 inhibitor Ac-YVAD-CHO showed that a caspase-1-like/YVADase activity was not involved (Thomas and Franklin-Tong, 2004). This provided evidence that a DEVDase/caspase-like activity was involved in SI-mediated pollen-tube inhibition and DNA fragmentation. Subsequently, Bosch and Franklin-Tong (2007) used fluorescent AMC-based caspase substrates to verify and characterize the caspase-like activities involved in more detail. A caspase-3-like/DEVDDase and a caspase-6-like/VEIDase activity were identified in the pollen tube cytosol in the first 1–2 h after SI induction, peaking at 5 h, suggesting that they are involved in early PCD events (Bosch and Franklin-Tong, 2007). A caspase-4-like/LEVDDase activity was also identified in SI-induced pollen, but its activation was later, peaking at 8 h post SI induction, when DEVDDase and VEIDase activity was decreasing (Bosch and Franklin-Tong, 2007).

The identity of these ‘caspase-like’ proteins is not known; they are certainly not caspases, as plants do not possess these genes. Intriguingly, use of a DEVD-biotin pull-down with pollen extracts identified a vacuolar processing enzyme (VPE/legumain) from Papaver pollen extracts as interacting with the DEVD motif (Bosch et al., 2010). This is of interest, even though a YVADase was thought not to be required for the SI-induced PCD, as it hinted that a legumain/YVADase could interact with a DEVDDase-containing substrate. In tobacco, VPE is essential for a virus-induced hypersensitive response that involves PCD (Hatsugai, 2004). However, the significance of the VPE in the Papaver SI-PCD system is currently unknown. With respect to the identity of the Papaver DEVDDase, an intriguing possibility is raised by the identification of PBA1, a proteasome subunit exhibiting a caspase-3-like/DEVDDase activity during infection of avirulent bacterial strains in Arabidopsis thaliana which is implicated in disease resistance and PCD (Hatsugai et al., 2009). Proteasomal activities are critical not only during PCD but during many normal cellular processes, including pollen tube growth (Sheng et al., 2006). The Franklin-Tong group is currently exploring the possibility that the SI-activated DEVDDase may be due to proteasomal activity. Although data implicate involvement of the proteasome in modulating pollen tube growth, it is too early to speculate about the identity of the DEVDDase.

Characterization of the SI-activated caspase-1, 3, 6-like activities revealed that they exhibit activity within a very narrow pH range, with peak activity at pH 5 and virtually no activity at pH 6 (Bosch and Franklin-Tong, 2007). This suggested that, for these proteins to have activity in a biological context, the cytosol would need to become acidic. Investigations using the live-cell ratiometric pH probe, BCECF-AM, revealed that there was, indeed, a dramatic acidification of the pollen cytosol from pH 6.9 to pH 5.5, triggered in the first 1–4 h of SI (Bosch and Franklin-Tong, 2007; KA Wilkins, M Bosch, TH Haque, NT Teng, and VE Franklin-Tong, unpublished data). What is involved in this phenomenon is not yet clear, but it points to major alterations to the cytosol being triggered by SI. Thus, cytosolic acidification is likely to be an important decision-making step, as this will allow PCD to proceed by allowing caspase-like proteins to be activated. Surprisingly few measurements of cytosolic pH during PCD in any system exist, with the exception of Bosch and Franklin-Tong (2007) and another method, based on the extinction of YFP fluorescence at low pH, which has been used to monitor pH during PCD in onion cells, showing that loss of YFP fluorescence could be correlated with caspase-like activity (Young et al., 2010). More recent studies in the Papaver SI-PCD system have shown that dramatic SI-induced acidification of the pollen cytosol occurs much earlier, with substantial acidification occurring within 10 min (Wilkins et al., Submitted). We think that live-cell monitoring of cytosolic pH will be an important but rather neglected area to investigate more fully in the future to elucidate the early events of PCD in various systems.

**Integrating SI-triggered events that signal to PCD**

A major focus for the SI-induced signalling network is the activation of PCD. The interaction of the secreted pistil S-determinant, PrsS, with the ‘self’ pollen PrpS triggers Ca\(^{2+}\) influx and rapid increases in [Ca\(^{2+}\)]\(_{cyt}\). This kicks off several events which ultimately lead to PCD. SI-PCD hallmarks include: the cytoskeleton, the p56-MAPK, reactive oxygen species (ROS), and nitric oxide (NO) (Thomas et al., 2006; Li et al., 2007; Wilkins et al., 2011). The following discussion and Fig. 2 summarizes data relating to our understanding of how the signals and targets triggered by SI in incompatible pollen tubes are integrated to mediate cell death.

**Involvement of actin signalling in SI-mediated PCD**

As already described, both actin depolymerization and the formation of F-actin foci is a rapid event in Papaver SI
response. Furthermore, both actin and microtubule alterations are implicated in mediating PCD, with microtubule depolymerization being triggered by actin depolymerization (Thomas et al., 2006; Bosch et al., 2008; Poulter et al., 2008) (Fig. 2). Investigations showed that both actin depolymerization and stabilization treatments (using latrunculin B and jasplakinolide, respectively) stimulated DNA fragmentation in Papaver pollen, which was inhibited by the DEVD-CHO inhibitor but not the Ac-YVAD-CHO inhibitor (Thomas et al., 2006). This provided evidence that actin alterations were placed on a pathway involving a caspase-3-like/DEVDase activity (Fig. 2). Moreover, DNA fragmentation correlated with the duration and extent of actin depolymerization; 50% actin depolymerization for 10 min was sufficient to induce PCD (Thomas et al., 2006). The caspase-3 substrate Ac-DEVD-AMC was also used to establish that the DEVDase/caspase-3-like activity was directly; pollen tubes treated with 1 μM Latrunculin B for 6 h exhibited an increase in DEVDase activity similar to that induced by SI (Fig. 2). These data provided evidence that major disturbance of actin polymer dynamics could trigger a caspase-3-like/DEVDase activity in pollen that resulted in DNA fragmentation, establishing a causal link between actin polymerization status and initiation of PCD (Thomas et al., 2006). Furthermore, changes in cytoskeletal dynamics have been shown to influence whether a eukaryotic cell enters into a PCD pathway (see reviews by Franklin-Tong and Gourlay, 2008; Smertenko and Franklin-Tong, 2011). Thus, there is evidence that both the substantial actin depolymerization and the later aggregation into punctate foci triggered by SI signalling play a key role in triggering PCD, mediated by activation of a caspase-3-like/DEVDase activity (Thomas et al., 2006; see also Fig. 2).

As mentioned earlier, actin foci are associated with the ABP ADF/cofilin (Poulter et al., 2010). Although ADF’s name comes from its ability to depolymerize F-actin at normal cytosolic (~pH 7) in vitro (Carlier et al., 1997; Bamburg et al., 1999), it has been shown that at acidic pHs ADF does not exhibit its usual actin-filament-severing activity and instead binds to and stabilizes F-actin (Allwood, 2002). It therefore seems likely that SI-induced cytosolic acidification could play an important role in altering the activity of ADF from a depolymerizing activity to an actin-stabilizing one, which could result in the formation of the highly stable F-actin foci observed during SI in incompatible pollen (Fig. 1D). Thus, cytosolic acidification, coupled with the consequent binding of ADF to the actin foci, helps to explain the strong link between actin foci formation and PCD in incompatible pollen, and suggests that this can be used as a good hallmark feature of PCD, at least in the SI system (Fig. 2).

Involvement of MAPK signalling in SI-mediated PCD

MAPKs are well-known key players in plant signalling networks and are activated under both abiotic and biotic stress responses and during PCD (Colcombet and Hirt, 2008; Meng and Zhang, 2013). This places them as key mediators of PCD signalling networks. As aforementioned, SI activates the MAPK p56 (Li et al., 2007; Rudd et al., 2003). The MAPK cascade inhibitor U0126, which blocks MAPK activity in both animals and plants, prevented the activation of the SI-stimulated p56-MAPK and alleviated several SI-induced events, including DNA fragmentation, caspase-3-like/DEVDase activity, and loss of viability (Li et al., 2007); see Fig. 2. As p56 appears to be the only MAPK activated by SI, this provides evidence that p56 is implicated in signalling upstream of a caspase-3/DEVDase-like activity (Rudd et al., 2003; Li et al., 2007). The activation of the MAPK p56 peaked 10 min after the induction of SI (which is considered the initiation phase of SI, up to which time point any changes induced are reversible by washing out of PrsS; P Wright and VE Franklin-Tong, unpublished data). This potentially places p56, and other events around 10 min post SI, as key turning point events that may help form the decision to enter into irreversible PCD.

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

ROS and NO have been shown to often participate in PCD and various stresses in plant cells (see Baxter et al., 2014; Passaia et al., 2014, in this issue). The Franklin-Tong group has investigated whether ROS and NO play a role in mediating PCD in the SI response. Live-cell imaging of ROS and NO in growing Papaver pollen tubes, using chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate, respectively, showed that SI induces relatively rapid and transient increases in ROS and NO (Fig. 3A, B), with distinct temporal ‘signatures’ (Wilkins et al., 2011). SI-induced increases in ROS were documented as early at 2 min, lasting up to 15 min in some pollen (Fig. 3A) and increases in NO occurred ~15 min after SI induction, lasting for up to ~40 min (Fig. 3B). The integration of SI-PCD responses with these putative signals were investigated with the ROS and NO scavengers diphenylidionium, tempol, and c-PTIO. This revealed the alleviation of the formation of the distinctive SI-stimulated p56-MAPK and alleviated several SI-induced actin punctate foci and the activation of a DEVDase/caspase-3-like activity when using these drugs combined (Fig. 3; Wilkins et al., 2011). These data provided evidence that ROS and NO increases are not only upstream of these key SI markers, but contribute to signalling to activation of a DEVDase (Wilkins et al., 2011; see also Fig. 2). These data represent the first steps in understanding ROS/NO signalling, providing an important biological context and improving the understanding of how several key components of the SI response are integrated to bring about PCD in Papaver pollen tubes. A possible role of the p56-MAPK may be in stimulating NO. Although this link between MAPK activation and NO generation in SI is speculative at present, this link has been shown in plant pathogen signalling (Asai et al., 2008).

Because diphenylidionium, which is a NADPH oxidase inhibitor, resulted in inhibition of SI-induced increases in ROS, this suggests that a respiratory burst oxidase homologue (RBOH) might be responsible for some of these increases in ROS. RBOHs, which are present on the plasma membrane, are key contributors of the oxidative burst in stress responses (Torres et al., 2005). Imaging of
Fig. 2. Cartoon showing a model of the integrated self-incompatibility (SI) programmed cell death (PCD) signalling network in Papaver rhoeas pollen. Interaction of a pistil S-determinant PrsS with its cognate pollen S-determinant, PrpS, in a S haplotype-specific manner (e.g. PrsS1 with PrpS1) rapidly triggers the SI signalling network, resulting in the rapid inhibition of pollen tube tip growth and culminating with DNA fragmentation and death of incompatible pollen. Different coloured boxes indicate key events triggered by SI; grey arrow indicates approximate timings; yellow arrows show SI-induced events; red arrows indicate agonist drugs used to stimulate events; black symbols show drugs used to inhibit events; pink vertical line represents the cell wall; double green line represents the plasma membrane. One of the earliest events include rapid influx of Ca$^{2+}$ and K$^+$, and almost instantaneous increases in cytosolic free Ca$^{2+}$ ([Ca$^{2+}]_{cyt}$) increases are required for many SI events, including phosphorylation and inactivation of soluble inorganic pyrophosphatases (sPPases) Pr-26.1a/b, actin depolymerization, and transient increases in reactive oxygen species (ROS) and nitric oxide (NO). Moreover, mastoparan or the Ca$^{2+}$ ionophore A23187 (red arrows) can mimic SI-induced increases in [Ca$^{2+}]_{cyt}$ as a key mediator of SI signalling to death. This places Ca$^{2+}$ signalling as one of the first SI events, upstream of many of the targets. The end focus of the SI-induced network appears to be DNA fragmentation and cell death (red box), which can be prevented by pretreatment with the tetrapeptide inhibitor Ac-DEVD-CHO, but not Ac-YVAD-CHO. The inferred DEVDase activity has been more directly measured using the tetrapeptide substrate, Ac-DEVD-AMC, which is cleaved by SI-induced pollen extracts. This approach revealed three activities (green box): DEVDase and VEIDase increase between 1–5h post SI and LEVDase increases later (still increasing at 8h). DEVDase activity peaks at 5h and has been shown to be required for DNA fragmentation, which can be prevented by the DEVDase inhibitor Ac-DEVD-CHO. These activities are optimal at pH 5. The caspase-like activities, which do not have any activity at normal cytosolic pH, need the pollen cytosol to acidify; this has been shown to occur in the first few hours of SI (rainbow box). Several components appear to be integrated in signalling to SI-mediated cell death. Central to this is the cytoskeleton (yellow boxes). SI-stimulates actin depolymerization, which causes inhibition of pollen tube growth. Low concentrations of Latrunculin B (Lat B) also inhibits pollen tube tip growth (orange box). SI triggers much higher levels of depolymerization than that required to inhibit pollen tube growth and higher concentrations of LatB that mimic this triggers activation of a DEVDase activity (measured with the Ac-DEVD-AMC substrate) and DNA fragmentation that is prevented by pretreatment with Ac-DEVD-CHO and not Ac-YVAD-CHO. Moreover, low concentrations of actin stabilizing Jasplakinolide (Jasp) can counteract and alleviate SI- or Lat B-induced DNA fragmentation, presumably by lowering the level of actin depolymerization. Subsequently, in SI, F-actin aggregates form highly stable punctate F-actin foci that are resistant to depolymerization and are associated with the actin-binding proteins (ABPs) actin-depolymerizing factor (ADF), and cyclase-associated protein (CAP; yellow box). High concentrations of Jasp can also trigger DNA depolymerization which is alleviated by pretreatment with Ac-DEVD-CHO and not Ac-YVAD-CHO. The formation of stable F-actin foci may be analogous to the stabilization using Jasp. Caffeine, which inhibits pollen tube growth without affecting the cytoskeleton, does not trigger DNA fragmentation. Together, these data implicate actin dynamics in modulating the DEVDase activity. SI also triggers microtubule depolymerization. This can be mimicked by use of the microtubule depolymerizer, oryzalin. Actin depolymerization using LatB triggers microtubule depolymerization but not vice versa, suggesting that SI-induced F-actin depolymerization signals to microtubule depolymerization. The tubulin-stabilizing drug, taxol, alleviates SI-induced Ac-DEVD-AMC cleavage, implicating a requirement for tubulin depolymerization for the DEVDase activation. Disrupting microtubule dynamics alone using oryzalin does not trigger increased cleavage of the DEVDase substrate Ac-DEVD-AMC; this suggests that both actin and microtubule depolymerization are required for DEVDase-mediated SI-induced PCD. Evidence for involvement of a SI-activated...
the SI-induced ROS signatures showed that the increases in ROS as a wave in the shank of the pollen tube and that the source of the ROS appeared to be intracellular organelles, as ‘hot spots’ (Fig. 3A). This suggests that at least some of the SI-induced ROS may originate from organelles. Thus, there might be several sources of SI-induced ROS. There is good evidence that the intracellular sites of ROS production influence its signalling role (Laloi et al., 2004; Foyer and Noctor, 2005). SI results in inhibition of tip growth (and presumably tip-ROS is lost during SI, although this has not been directly ascertained) and stimulates increases in ROS in the ‘shank’ of the pollen tube. This is a very different response to that observed in root hairs in response to NOD factors that occur at the tip (Cárdenas et al., 2008).

This supports the concept that distinct spatial-temporal ‘signatures’ can generate signal specificity and (Foyer and Noctor, 2005) implicates different sources for ROS and NO, as well as different enzymes for PCD and tip growth inhibition. This provides an interesting area to pursue in the future, which would help achieve a better understanding of how the growth ROS/NO signals and the SI-induced ROS/NO signals involved in PCD achieve their very different biological outputs in a single cell.

How is the pollen tube cellular disassembly achieved?

One area that has been neglected to date is the question of how the pollen tube is disassembled during SI-induced death. Electron microscopy studies have shown that organelles are reorganized within 1 h of SI induction (Geitmann et al., 2004). In contrast to control pollen grains (Fig. 5A), SI-induced pollen grains had striking curved endoplasmic reticulum cisternae that were wrapped around empty structures that may be the vacuole (Fig. 5B) and mitochondria.
were greatly altered (Fig. 5C). By 3–4 h, the organelles were almost indistinguishable but there did not seem to be wholesale lysis (Fig. 5D; Geitmann et al., 2004), even though cytosolic pH was 5.5 by this time. Subsequent studies showed that, within 30 min of SI induction, the endoplasmic reticulum configuration had altered and it appeared to be wrapped around other organelles and vacuoles. Organelles appeared to be engulfed in what appeared to be vacuolar compartments within 3 h of SI induction (NS Poulter and VE Franklin-Tong, unpublished data; Fig. 5D). These studies hint at the possibility of autophagy being involved.

Recruitment of signalling for SI events in other species

Recent studies have involved introduction of PrpS, the Papaver pollen S-determinant into self-compatible A. thaliana, a model plant. Exposing transgenic A. thaliana pollen expressing PrpS-GFP to recombinant Papaver PrsS protein resulted in the hallmark features of incompatible Papaver pollen being triggered, including inhibition of pollen tube growth, formation of F-actin foci, and increases in caspase-3-like/DEVDase activity after addition of recombinant PrsS (de Graaf et al., 2012). This demonstrated PrpS functions as an S-determinant and is capable of triggering PCD when transferred into a self-compatible species from a distantly related genus. It is well established that these two SI systems evolved independently (Allen and Hiscock, 2008) and all the available evidence indicates that Arabidopsis does not possess the P. rhoes S-determinants, so it is unlikely that A. thaliana possesses genes that have operated in an ancestral form of Papaver-like SI, although formally this cannot be discounted. This finding is notable because the evolutionary distance between the Ranunculales and Brassicales is very large, estimated at ~140 million years (Bell et al., 2010), and because Papaver and Arabidopsis lack a common SI system. Despite this, transgenic A. thaliana pollen was not only rejected, but also displayed hallmark features of the Papaver PCD responses in incompatible pollen (de Graaf et al., 2012). Our data provide good evidence that A. thaliana possesses proteins that can be recruited to form new PCD signalling networks and targets that are used for a function that does not normally operate in this species. Indeed, as the features of a ‘Papaver-like’ SI-induced PCD response, which have not been observed in the Brassica-type SI response, this suggests that the PrpS–PrsS interaction is sufficient to specify a particular downstream signalling network to obtain this PCD outcome.

Summary and future perspectives

ROS and NO signalling form an integral part of SI-PCD (see this issue). Here, we have described evidence that ROS and NO signalling is central to SI-induced alterations in the actin cytoskeleton and the activation of a caspase-3-like/DEVDase activity, which suggests that they play a key role in SI-induced PCD. We have begun to tease out the relationship between ROS and NO and their role in triggering
and executing PCD in incompatible pollen. The finding that Papaver SI can be induced by Papaver PrsS-PrpS interaction in transgenic A. thaliana carrying PrpS suggests that the Papaver SI system either can recruit ‘common’ endogenous signalling components in A. thaliana pollen and utilize them to signal using a Papaver-like PCD response, via actin and caspase-3-like/DEVDase activities, or that it activates an existing PCD pathway in A. thaliana. The latter is supported by the fact that different cell death processes are known to share signalling pathways (van Doorn et al., 2011). However, it is surprising that it produces a Papaver-like PCD response that has not (so far) been described for any Arabidopsis PCD response. We believe that this may work because the signals (e.g. Ca\textsuperscript{2+}, phosphorylation, ROS, NO) and targets for Papaver SI (e.g. the actin cytoskeleton) are ‘universal’, ancient, and perhaps common to all eudicots. This has implications for our perspective of evolution of signalling networks both between different SI systems and, more generally, potentially in PCD and other signalling responses.

The finding that Papaver SI-PCD can be reproduced by stimulating interaction of PrpS-GFP in transgenic A. thaliana pollen expressing PrpS-GFP opens up new approaches for the future. Use of the Arabidopsis system expressing PrpS-GFP may allow use of the toolbox of analytical tools and resources available for Arabidopsis, including genetic approaches, with the availability of knockout lines, to analysing how Papaver SI-induced PCD is integrated. Postulated parallels between SI and other well-characterized PCD responses, such as plant–pathogen resistance (Hodgkin et al., 1988; Sanabria et al., 2008), which also use ROS in an oxidative burst, have been made. Like the SI response, hypersensitive response PCD has been associated with increases in [Ca\textsuperscript{2+}]\textsubscript{cyt}, ROS, and microtubule depolymerization (Levine et al., 1997; Lecourieux et al., 2002; Rentel et al., 2004) and use of Arabidopsis knockout lines could provide an approach to analysing this possibility.

Another area to investigate in the future is to understand how the source and location of intracellular ROS influences its signalling role (Laloi et al., 2004; Foyer and Noctor, 2005) and establish the differences between ROS signals in normal cells and during PCD. This could help to tease out the role of specific ROS ‘signatures’. At present, the source of ROS during SI-PCD is unknown, although both NADPH oxidase and an organelle-based source has been implicated. To mediate downstream responses, ROS and NO must presumably alter target proteins; these types of studies in plant cells are in their infancy. However, it is known that NO-mediated S-nitrosylation of proteins, particularly cysteine residues of redox-sensitive proteins (Wang et al., 2006; Moreau et al., 2010), can affect protein activity and so has the potential to play a key role in regulating cellular events (Lindermayr et al., 2005). A possible target of interest is actin, as this is a major target of SI-PCD signalling which utilizes NO/ROS signals (Wilkins et al., 2011). Although there are virtually no data on NO signalling to actin in plant cells, actin has been identified as being both S-nitrosylated and Y-nitrated in Arabidopsis (Lindermayr et al., 2005; Lozano-Juste et al., 2011). Moreover, nitrosylation of a single cysteine residue on mammalian actin can alter actin dynamics (Dalle-Donne et al., 2000) and this can affect the outcome of NO-induced apoptosis in animal cells. Despite the known sensitivity of the actin cytoskeleton to ROS and NO, how they alter the dynamics and organization of F-actin is far from clear, even in animal cell systems. Preliminary investigations aimed at identifying pollen proteins that are modified by S-nitrosylation, nitration, and oxidation as a result of SI signalling to PCD are currently being undertaken.

Looking further into the future, in a translational context, transfer of the Papaver PrpS and PrsS S-determinants could possibly, in the longer term, perhaps provide a tractable SI system for some crop plants, which has long been the goal of plant breeders. Many self-compatible crop plants could benefit from improved yields that could be obtained from generating F1 hybrid plants (which are generally superior, but expensive to produce). If SI could be harnessed, these could potentially be made more easily and cheaply. To date, focus has been on SI relatives of crop plants or species closely related to crops, as it was thought that transfer of SI would be limited to close relatives. So far, the only movement of SI systems has been within species or closely related...
species in the same family that share an ancestral SI system. Our demonstration that interaction of PrpS with *A. thaliana* PrpS-GFP pollen to elicit a ‘S’ response suggests there is scope for transfer of the *Papaver* SI system to completely unrelated crop species. This has implications for helping to solve food security issues, by allowing breeding of superior F1 hybrid plants more easily and cheaply. We are currently exploring whether PrpS-GFP functions in a self-compatible monocot crop, barley, as a case study to see if transfer of this SI-PCD system can operate in even more distantly related angiosperms. Furthermore, assuming that PrpS can be readily functionally expressed in other cell types, our findings open up the possibility of developing an inducible PCD system in a variety of cell types. If these were tissue-specifically expressed, this might open up the possibility to be able to control the death of particular cell types. We are starting to explore this possibility by seeing if PrpS-GFP can operate a PCD response in *A. thaliana* leaf protoplasts, as a test system to investigate if this SI-PCD system might work in cells other than pollen and we hope to see how far we can extend this system. This approach may also provide some answers relating to evolutionary aspects of how this PCD system might have evolved.

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