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Initiation of Programmed Cell Death in Self-Incompatibility: Role for Cytoskeleton Modifications and Several Caspase-Like Activities

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ABSTRACT Programmed cell death (PCD) is an important and universal process regulating precise death of unwanted cells in eukaryotes. In plants, the existence of PCD has been firmly established for about a decade, and many components shown to be involved in apoptosis/PCD in mammalian systems are found in plant cells undergoing PCD. Here, we review work from our lab demonstrating the involvement of PCD in the self-incompatibility response in *Papaver rhoeas* pollen. This utilization of PCD as a consequence of a specific pollen–pistil interaction provides a very neat way to destroy unwanted 'self', but not 'non-self' pollen. We discuss recent data providing evidence for SI-induced activation of several caspase-like activities and suggest that an acidification of the cytosol may be a key turning point in the activation of caspase-like proteases executing PCD. We also review data showing the involvement of the actin and microtubule cytoskeletons as well as that of a MAPK in signalling to caspase-mediated PCD. Potential links between these various components in signalling to PCD are discussed. Together, this begins to build a picture of PCD in a single cell system, triggered by a receptor–ligand interaction.

INTRODUCTION

Programmed cell death (PCD) is a crucial process used by eukaryotic cells to remove unwanted cells, and there is good evidence that PCD/apoptosis has common origins. Many of the molecular components involved in PCD are highly conserved (Koonin and Aravind, 2002). Apoptosis, also referred to as type I PCD, is the best characterized form of PCD and exhibits characteristic morphological and biochemical features. Some of the genes that regulate apoptosis, such as Bax-1 inhibitor (*BI-1*) have homologues in plants (Kawai et al., 1999; Sanchez et al., 2000; Kawai-Yamada et al., 2004). Surprisingly, others, like the *Bcl*-family genes, such as *Bcl-2* or *BAX*, are not present in plants or yeast, though these genes have the expected survival/death function if expressed in plant cells (see, e.g. Kawai-Yamada et al., 2001). Mitochondria play a key role in the initiation of many apoptotic signalling cascades, preparing the cell for entry into the execution phase of PCD. An increase in the permeability of the mitochondrial outer membrane leads to the release of pro-apoptotic molecules, such as cytochrome c (cyt c), into the cytosol. Although cyt c release has been observed in plant cells (Balk et al., 1999; Yao et al., 2004), its role in PCD in plants is not well established. The production of reactive oxygen species (ROS) is also often a key component in activating signalling pathways that initiate apoptosis and there is evidence that these molecules are also involved in the initiation of plant PCD (see Van Breusegem and Dat, 2006). After the initiation phase, the execution phase is entered, in which the majority of proteolytic events take place that create the characteristic cellular changes known as apoptosis/PCD.

In mammalian cells, both the initiation and execution phases involve caspases, a specific family of cysteine proteases, which are key regulators of PCD. A cascade of caspases with different specificities is activated, whereby initiator caspases activate executioner caspases, and cleave target substrate proteins after an aspartate residue. Caspase-3, the main executioner caspase in animal cells (Fischer et al., 2003), has the general substrate recognition sequence DXXD and the tetrapeptide Ac-DEVD-CHO is based on this, acting as an inhibitor of caspase-3 activity. Similar tetra-peptide inhibitors designed against other caspase specificities exist. Tetrapeptides with a fluorophore attached to the substrate recognition sequence

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(e.g. Ac-DEVD-AMC) can be used to assay caspase-like activities. The activation of a caspase cascade elicits an organized degradation of many cellular proteins and accelerates the progression of cell death. Cleavage of nuclease inhibitors results in the eventual fragmentation of nuclear DNA, which is often used as a useful endpoint marker of apoptosis/PCD.

The evidence for the involvement of caspase-like enzymes in PCD in plants is good (Bonneau et al., 2008). Probably the most important example for a caspase-like protease identified in plants to date is the vacuolar processing enzyme (VPE), which has a YVADase (caspase-1-like) activity. VPEs are involved in pathogen-induced PCD and in developmental cell death triggered by vacuolar collapse and have been relatively well characterized (Hatsugai et al., 2004; Rojo et al., 2004). Other examples of plant caspase-like activities identified to date include a VEIDase (caspase-6-like) (Bozhkov et al., 2004; Boren et al., 2006), a TATDase (Chichkova et al., 2004), and serine aspartate-specific proteases ('saspases'; (Coffeen and Wolpert, 2004)). DEVDase/caspase-3-like activities have been identified in several plant systems by using Ac-DEVD tetra-peptide inhibitors that alleviate PCD (del Pozo and Lam, 1998; Richael et al., 2001; Thomas and Franklin-Tong, 2004), and also through cleavage of a caspase-3-specific substrate, Ac-DEVD-AMC (Danon et al., 2004; Thomas et al., 2006).

Curiously, although caspase-like activities have been identified, caspases have no true homologues in plants or yeast (see Woltering et al., 2002; Sanmartin et al., 2005). Metacaspases, which resemble animal caspases to some extent, have been identified in plants and yeast (Uren et al., 2000; Madeo et al., 2002). However, although they are involved in PCD (Madeo et al., 2002; Hoeberichts and Woltering, 2003; Suarez et al., 2004; Bozhkov et al., 2005), plant metacaspases do not cleave caspase-specific substrates, as they are arginine/lysine-specific cysteine proteases (Vercammen et al., 2004; Watanabe and Lam, 2005). VPEs, although they are proposed to share some structural properties with caspase-1, share very limited sequence identity with this caspase (Hatsugai et al., 2006). Thus, the identity of the caspase-like proteases in plant cells remains the subject of considerable debate.

Self-incompatibility (SI) provides an important genetically controlled mechanism that prevents inbreeding. We have been investigating the SI response in pollen of *Papaver rhoeas* L. (the field poppy) for a number of years, as it provides a model system to investigate cell–cell interactions and intracellular signalling in plant cells. SI uses the multi-allelic *S*-locus to prevent self-fertilization. It operates using specific recognition and consequent rejection of incompatible pollen. Discrimination occurs between different haplotypes, with incompatible pollen being inhibited when haplotypes (pollen *S*- and pistil *S*-) match. Distinct and diverse SI mechanisms have evolved in different species (see Takayama and Isogai, 2005; McClure and Franklin-Tong, 2006, for recent reviews). In *Papaver*, SI uses small ~15-kDa S-proteins, which are encoded by the pistil part of the *S*-locus (Foote et al., 1994). These interact with the pollen *S*-determinant in an *S*-haplotype-specific manner, so that when incompatible pollen interacts with a matching pistil S-protein, rapid inhibition of tip growth results. Our current working model is that the S-proteins act as signalling ligands by interacting in an *S*-specific manner with the pollen *S*-determinant, which is proposed to be a transmembrane receptor. We have preliminary data that the pollen *S*-determinant is a small transmembrane protein that could act in this manner (M.J. Wheeler, B.H.J. de Graaf, N. Hadjiosif, R.M. Perry, N.S. Poulter, K. Osman, F.C.H. Franklin, V.E. Franklin-Tong, unpublished data).

Most emphasis of our research has focused on the downstream events after S-proteins interact with incompatible pollen. An incompatible interaction triggers a Ca^{2+}-dependent signalling cascade in incompatible, but not compatible, pollen (Franklin-Tong et al., 1993). This results in rapid inhibition of pollen tube growth. At a cellular level, a number of SI-specific events are triggered in incompatible pollen, including rapid depolymerization of the F-actin cytoskeleton (Geitmann et al., 2000; Snowman et al., 2002); activation of a mitogen-activated protein kinase (MAPK), p56 (Rudd et al., 2003); and a rapid increase in phosphorylation of two cytosolic pollen proteins (Rudd et al., 1996), which were recently identified as soluble inorganic pyrophosphatases (sPPases), Pr-p26.1a and Pr-p26.1b (de Graaf et al., 2006). A major breakthrough in our understanding of mechanisms involved in mediating SI came with the finding that PCD, mediated by a caspase-3-like activity, is induced in incompatible pollen (Thomas and Franklin-Tong, 2004). The employment of PCD provides a very neat and precise mechanism for the destruction of 'self' pollen. We are currently investigating components involved in mediating PCD in incompatible pollen tubes. Here, we review our current knowledge of components that we have shown to be involved in mediating the SI response, primarily focusing on components interfacing with the PCD signalling cascade in incompatible pollen.

SI TRIGGERS PCD IN INCOMPATIBLE POLLEN

SI selectively prevents incompatible (but not compatible) pollen tube growth soon after germination. As it has been proposed that the SI response may have some analogies with the hypersensitive response, a form of PCD in plants, we decided to investigate whether this might be the case, by examining if PCD was triggered in incompatible pollen by the SI response. Initial studies found evidence for SI-specific DNA fragmentation in incompatible, but not compatible, pollen tubes (Jordan et al., 2000), which hinted that SI might induce PCD. We subsequently obtained data showing high levels of cytosolic cyt c within 1 h of SI induction in incompatible, but not compatible, *Papaver* pollen (Thomas and Franklin-Tong, 2004), implicating leakage of cyt c from the mitochondria. We also investigated whether a caspase-like activity was triggered by SI. Pre-treatment of incompatible pollen tubes with tetra-peptide inhibitors resulted in much lower levels

of DNA fragmentation (Thomas and Franklin-Tong, 2004). This was substantiated by data using poly (ADP-ribose) polymerase (PARP), a classic substrate for caspase-3. Extracts from SI-induced incompatible pollen added to bovine PARP in vitro generated a 24-kDa PARP cleavage product, with a corresponding decrease in the amount of uncleaved PARP. Addition of Ac-DEVD-CHO (and not Ac-YVAD-CHO) inhibited formation of the PARP-cleavage product. More recently, we have used the fluorescent caspase-3 substrate, Ac-DEVD-AMC, as a tool to quantify the SI-induced caspase-3-like/DEVDase activity (Bosch and Franklin-Tong, 2007; Li et al., 2007). Together, these data provide compelling evidence for an SI-induced caspase-3-like/DEVDase activity. The specific activation of PCD in incompatible pollen provides an effective way in which to ensure that this pollen does not achieve fertilization.

VISUALIZATION OF SI-INDUCED DEVDase ACTIVITIES IN POLLEN TUBES

We examined the localization of DEVDase activity in living pollen tubes, using the CR$(DEVD)_2$ substrate, which comprises the fluorophore cresyl violet (CR) coupled to two DEVD peptides. In the presence of a DEVDase activity, the cleavage of DEVD-CR releases the fluorescent CR marker, thereby allowing visualization of DEVDase activity in living cells. Unstimulated pollen tubes showed no fluorescence (Figure 1A and 1B), but, in SI-induced pollen tubes, CR fluorescence revealed DEVDase activity in the cytosol and the nuclei (Figure 1C and 1D). Interestingly, the generative cell (GC) exhibited high DEVDase activity, suggesting it is preferentially targeted. Further examination showed that cytosolic DEVDase activity was detected first (as early as 1–2 h in some pollen tubes) and that DEVDase activity in the vegetative nucleus (VN) and GC was detected later (Bosch and Franklin-Tong, 2007). To our knowledge, this represents the first visualization of an activated DEVDase in a living higher plant cell undergoing PCD. Although it has previously been reported by Korthout et al. (2000) that *Chara* (an alga) exhibits a cytosolic DEVDase activity, the authors admitted that this was likely to be a basal level of activity that was not involved in PCD, as no elevations in fluorescence were detected and the cells remained alive for a month. The nuclear localization is of considerable interest, as this has been observed in several animal cells; moreover, it is well established that activated caspase-3 translocates from the cytosol to the

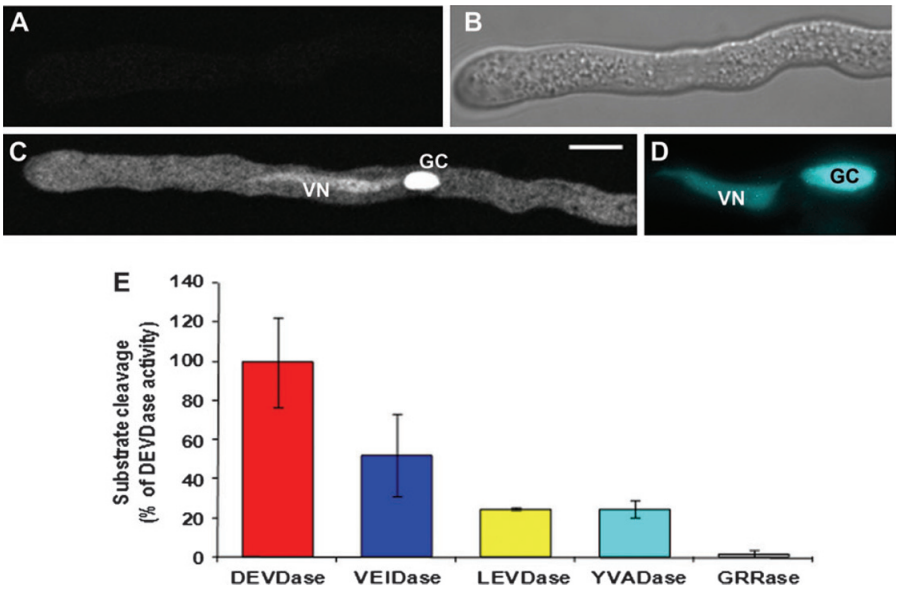

Figure 1. SI-Induced Caspase Activities in Pollen Tubes. CR$(DEVD)_2$ live-cell imaging of DEVDase activity in SI-induced pollen (A–D).

(A) A normally growing untreated pollen tube (control) shows no background DEVDase activity.

(B) Bright-field image of the same pollen tube.

(C) Typical representative image of CR fluorescence 170 min after SI induction. DEVDase activity is cytosolic and also localizes to the generative cell (GC) and vegetative nucleus (VN).

(D) DAPI staining shows nuclear DNA in the GC and VN.

Images (A & C) are median plane confocal sections; all were captured using identical detection settings; (D) is an epifluorescence image. Scale bar: 10 μm.

(E) Substrate specificity of SI-induced caspase-like protease activities.

SI-induced pollen extracts were tested for substrate specificity using different caspase substrates: Ac-DEVD-AMC (DEVDase; red bar), Ac-VEID-AMC (VEIDase; blue), Ac-LEVD-AMC (LEVDase; yellow), Ac-YVAD-AMC (YVADase; cyan), Ac-GRR-MCA (GRRase; white). Relative fluorescence units were measured and are expressed as a percentage of the DEVDase activity. Error bars ± SEM (n = 3).

Part (E) taken from Bosch and Franklin-Tong (2007), © 2007 The National Academy of Sciences, USA.

nucleus during apoptosis (Zhivotosky et al., 1999; Faleiro and Lazebnik, 2000).

OTHER CASPASE-LIKE ACTIVITIES ACTIVATED BY SI

We used Ac-tetrapeptide-AMC derivatives to fully characterize the *Papaver* pollen caspase-like activities stimulated by SI. These specific peptide substrates have been used extensively to establish the substrate specificities of animal caspases (Thornberry et al., 1997). Caspase-3 has an optimal tetrapeptide recognition motif DEVD; caspase-6 has a VEID tetrapeptide recognition motif. These two caspases are the key 'executioner' caspases in animal cells. The initiator caspases-8 and -9 preferentially recognize (L/V)EXD motifs. Our studies, using these substrates to test for caspase-like activities activated in our SI-induced samples, revealed that SI activates not only significant levels of DEVDase activity, but also VEIDase and, to a lesser extent, LEVDase and YVADase activity; there was no evidence for metacaspase activity, as there was no increase in cleavage of the metacaspase substrate Ac-GRR-MCA (see Figure 1E; Bosch and Franklin-Tong, 2007). Moreover, we established that both the DEVDase and VEIDase are functionally involved in SI-mediated PCD. Examination of the temporal activation of the four SI-induced caspase-like activities revealed that increases in DEVDase activity could be detected 90 min after SI-induction, and that this activity significantly increased by 3 h, with activity peaking 5 h after SI induction. The temporal profile of the VEIDase activity was very similar. However, the LEVDase activity had a different profile; it increased less rapidly and its activity was still increasing at 8 h after the DEVDase and VEIDase activities had significantly decreased. The YVADase activity remained constant at a basal level (Bosch and Franklin-Tong, unpublished results). Thus, the DEVDase and VEIDase activities are stimulated early in SI-stimulated PCD, while the LEVDase activity peaks later. Imaging revealed that pollen tubes exhibiting DEVDase activity were rarely observed at 1 or 2 h after SI, but ~57% of pollen tubes had DEVDase activity 3 h after SI-induction, and ~89% exhibited DEVDase activity at 5 h.

INTRACELLULAR ACIDIFICATION OCCURS DURING SI

Examination of the SI-induced caspase-like activities revealed that the activities detected had remarkably acidic pH optima. The DEVDase and VEIDase exhibited peak activity at pH 5.0, and the LEVDase had peak activity between pH 4 and 6 (Bosch and Franklin-Tong, 2007). This was surprising, as the physiological pH of the cytosol is usually around pH 7. However, mild cytosolic acidification (corresponding to ~0.3–0.4 pH units), mediated by the mitochondria, is known to commonly occur during the early stages of apoptosis in mammalian systems (Matsuyama et al., 2000; Matsuyama and Reed, 2000), though its exact role remains debated (Lagadic-Gossmann et al., 2004). As cytosolic acidification might provide a possible explanation for the unusually acidic pH optima of the SI-induced caspase-like activities, we investigated whether SI induction resulted in changes in intracellular cytosolic pH ($[pH]_i$). Ratio imaging with a pH-sensitive fluorophore revealed that SI-induced pollen tubes had a $[pH]_i$ of 5.5, which represents a dramatic drop of 1.4 pH units from that observed in normally growing pollen tubes (Bosch and Franklin-Tong, 2007). Notably, this SI-induced cytosolic pH fits the pH optima for the SI-induced caspase activities very closely. Thus, it appears likely that acidification is triggered prior to caspase activation, thereby allowing caspases to exhibit their optimal activity in the cytosol. Interestingly, vacuolar rupture occurs during PCD involving xylem development (Obara et al., 2001; Kuriyama and Fukuda, 2004), and VPE-mediated PCD presumably involves vacuolar collapse (Hatsugai et al., 2004), which is expected to result in a pH drop. We are currently investigating this, and have preliminary evidence to suggest that vacuolar integrity is lost early in the SI response (M. Bosch, R.M. Perry and V.E. Franklin-Tong, unpublished data).

SI TARGETS THE POLLEN ACTIN CYTOSKELETON

Actin microfilaments and tubulin microtubules, comprising the cytoskeleton, are highly dynamic (Erhardt and Shaw, 2006; Hussey et al., 2006) and the plant actin cytoskeleton has been shown to play a key role in modulating signal–response coupling (Staiger, 2000). As the actin cytoskeleton plays a critical role in regulating pollen tube growth (Gibbon et al., 1999), and pollen tube inhibition is stimulated by SI, we investigated whether SI induces alterations to the actin cytoskeleton of incompatible pollen tubes. These studies showed that SI triggers very striking *S*-specific alterations to the pollen tube actin cytoskeleton specifically in incompatible pollen tubes (Geitmann et al., 2000; Snowman et al., 2002). The alterations were surprisingly rapid, with substantial alterations within 1 min of SI induction, and were not observed in pollen tubes that stopped growing due to some non-specific trigger. There are at least two phases to the alterations. The first comprises disappearance of the longitudinal F-actin bundles, and F-actin conformation alters to give a fine, speckled appearance; the second phase involves F-actin accumulating into 'punctate foci' (Geitmann et al., 2000; Snowman et al., 2002) (Figure 2A, 2C, and 2E). We established that, in incompatible pollen, SI induces very rapid, massive, and sustained depolymerization of actin filaments (Snowman et al., 2002) and identified a calcium-sensitive actin-binding protein (ABP), PrABP80, which has the properties of a gelsolin and is a good candidate for being involved in this event, as it exhibits potent Ca^{2+}-dependent severing activity (Huang et al., 2004). The actin cytoskeleton is crucial for the delivery of secretory vesicles to the tip, which sustains pollen tube growth. Actin depolymerization would therefore almost certainly result in rapid inhibition of pollen tube growth. However, the level of F-actin depolymerization was far more than that required to inhibit tip growth.

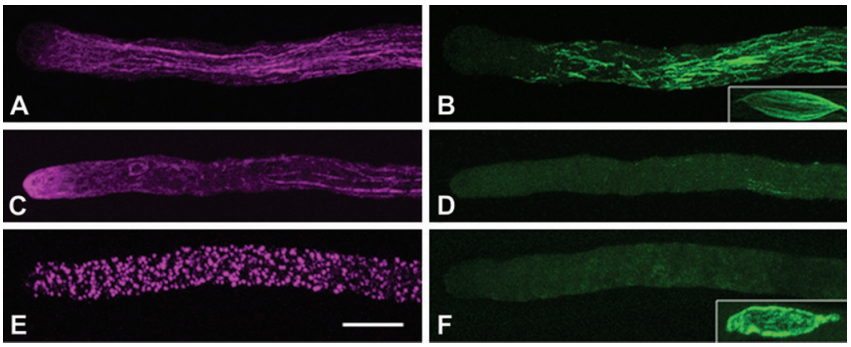

Figure 2. SI Stimulates Alterations to Both the Actin and Microtubule Cytoskeletons. F-actin images (left-hand side) are shown in magenta pseudocolour; F-actin was detected using rhodamine-phalloidin. Microtubules (right-hand side) are shown in green pseudocolour and were detected using immunolocalization with anti-α-tubulin.

Typical representative images of **(A)** F-actin in an untreated pollen tube, **(B)** cortical microtubules in the same pollen tube, inset: GC microtubules. At 2 min after SI induction, many of the F-actin bundles are depolymerized **(C)** and the microtubules are also apparently depolymerized **(D)**. At 30 min after SI induction, the F-actin has formed punctate foci **(E)**, and cortical microtubules remain depolymerized and undetectable **(F)** and the GC microtubules have started to degrade (inset).

Images are full projections of confocal sections. Scale bar: 10 μm.

ACTIN POLYMER DYNAMICS SIGNAL TO PCD IN INCOMPATIBLE POLLEN

There is evidence suggesting that changes to the actin cytoskeleton can initiate apoptosis (see Franklin-Tong and Gourlay, 2008, for a recent review). As the amount of F-actin depolymerization seemed excessive and the pattern of sustained (rather than transient) depolymerization was unusual, we investigated whether the actin depolymerization might be involved in mediating PCD.

We manipulated the F-actin polymerization status in pollen tubes, using either latrunculin B (LatB) to artificially stimulate F-actin depolymerization or jasplakinolide (Jasp) to stimulate stabilization of actin filaments, and then measured levels of PCD using DNA fragmentation as a marker. These studies established that PCD can be triggered in *Papaver* pollen by altering actin dynamics (assuming lack of side effects of these drugs), thereby suggesting that this is sufficient to induce PCD in these cells (Thomas et al., 2006). Briefly, both LatB and Jasp stimulated DNA fragmentation. Use of LatB and Jasp in combination with tetrapeptide caspase inhibitors established that the actin-mediated DNA fragmentation involved a caspase-3-like/DEVDase activity. Thus, these data implicated both actin depolymerization and stabilization in stimulating activation of a caspase-3-like protease-mediated PCD. Moreover, we established that reductions in F-actin to levels similar to what is usually achieved during SI were sufficient for this irreversible 'decision-making' step into PCD to be made (Thomas et al., 2006).

We also used a treatment of Jasp to counteract both LatB- and SI-mediated PCD. Experiments showed that Jasp significantly reduced the SI-induced DNA fragmentation, showing that it can 'rescue' SI-induced pollen from PCD, presumably by counteracting actin depolymerization. Other data showed that Jasp could also counteract the effect of LatB-induced depolymerization and 'rescue' pollen from entry into PCD (Thomas et al., 2006). These experiments clearly demonstrate that major changes to actin polymerization status can trigger caspase-like activity in pollen tubes, resulting in PCD. Moreover, with respect to SI, they demonstrate that the SI-mediated actin depolymerization operates to activate a PCD signalling network as well as being involved in regulating the inhibition of pollen tube growth.

SI TARGETS THE POLLEN MICROTUBULE (MT) CYTOSKELETON

We recently established that SI also targets the microtubule (MT) cytoskeleton, triggering very rapid apparent depolymerization of cortical microtubules (Poulter et al., 2008). Examining the MT cytoskeleton using immunolocalization at various time points after SI induction revealed that cortical microtubules were rapidly altered; within 1 min, the cortical MT bundles were virtually undetectable in incompatible pollen tubes. The characteristic generative cell spindle-shaped microtubules remained relatively intact at this time point, but were disintegrating by 30 min (Figure 2B, 2D, and 2F). Unlike actin, MTs did not reorganize into punctuate foci later, but remained apparently depolymerized (Poulter et al., 2008), so the two cytoskeletal components show quite distinct responses. As SI stimulated rapid actin depolymerization (Snowman et al., 2002), we wondered whether this might be responsible for alterations to the MTs. We used LatB to stimulate F-actin depolymerization and examined both pollen tube actin and microtubules using immunolocalization. This established that actin depolymerization results in apparent depolymerization of cortical MTs, providing evidence for signalling between these two cytoskeletal components (Poulter et al., 2008). However, use of oryzalin to artificially depolymerize microtubules

did not affect actin, suggesting that there is one-way 'cross-talk' from actin to tubulin cytoskeleton, but not *vice versa* (Poulter et al., 2008).

MTs ARE ALSO INVOLVED IN SIGNALLING TO PCD IN INCOMPATIBLE POLLEN

As alterations to actin polymerization status can trigger PCD in pollen tubes (Thomas et al., 2006), we examined whether microtubule depolymerization might also signal to PCD. We used oryzalin to depolymerize, or taxol to stabilize, pollen tube microtubules, and pollen tube extracts were tested for caspase-3-like activity using Ac-DEVD-AMC. This showed that MT stability did not affect entry into PCD in *Papaver* pollen. However, as microtubule depolymerization accompanies actin depolymerization, we wondered whether tubulin depolymerization might be required in conjunction with actin depolymerization to allow progression into SI-induced PCD. Use of taxol to stabilize MTs prior to SI induction showed that pollen tubes pre-treated with taxol prior to SI-induction significantly reduced the level of SI-induced DEVDase activity (Poulter et al., 2008). This alleviation of PCD by taxol provided evidence that microtubule depolymerization is required for SI-induced PCD to progress. Together, these studies showed that SI targets the MT cytoskeleton and that, through cross-talk with actin, they contribute to events mediating initiation of PCD in incompatible pollen tubes.

A MAPK SIGNALS TO PCD IN INCOMPATIBLE POLLEN

Mitogen activated protein kinase (MAPK) cascades act as universal signal transduction networks that connect many diverse signalling cascades (Chang and Karin, 2001). These kinases are activated by dual phosphorylation of a TXY motif on threonine and tyrosine residues. In plants, MAPKs are key players in stress responses, being activated by a variety of stresses, and have been shown to play a role in PCD (Zhang and Liu, 2001; Zhang and Klessig, 2001; Ren et al., 2002); *Arabidopsis* has 20 MAPK genes (Ichimura et al., 2002).

We investigated whether MAPKs might be involved in SI, and found activation of a MAPK, named p56, as it migrates at 56 kD on SDS–PAGE, specifically in SI-induced incompatible pollen and not in pollen challenged with compatible S-proteins (Rudd et al., 2003). Although at least two other MAPK activities are detected in pollen, only p56 was activated during SI-induction; the others remained at basal levels. Several pieces of data provide evidence that p56 is a *bona fide* MAPK. This included detection of MAPK activity using myelin basic protein as a kinase substrate in in-gel kinase assays (Rudd et al., 1996) and by using pTEpY antibodies, which detect phosphorylation of the TEY motif in activated MAPKs (Li et al., 2007); anti-phospho-tyrosine antibodies immunoprecipitated p56 in SI-induced pollen; and activated p56 was sensitive to the MAPK inhibitor, apigenin (Rudd et al., 2003). Thus, these data implicated MAPK signalling in *Papaver* SI. However, since activation of the p56-MAPK peaked 10 min after SI induction, which occurred after pollen tube inhibition of growth, this suggested to us that this MAPK may perhaps be involved in PCD, as PCD is triggered temporally later.

As MAPKs are known to signal to PCD in plants, we investigated whether there was any evidence for the p56-MAPK in signalling to PCD in incompatible pollen. We used an inhibitor approach as a first route to examine whether this might be the case. U0126 is a highly selective inhibitor of MAPK cascades, and blocks MAPK signalling in plant cells. For example, in tobacco, 100 μM U0126 inhibited activation of the salicylic acid-induced protein kinase (SIPK) MAPK (Lee et al., 2001).

Although growing pollen tubes only contain very low levels of activated p56, we found that U0126 inhibited normal pollen tube growth, while the inactive analogue, U0124, did not (Li et al., 2007), suggesting that MAPK activity is required for pollen tube growth. This appears to be the first evidence for the importance of MAPK signalling in growing pollen tubes, though MAPK involvement in root hair tip growth has previously been shown (Samaj et al., 2002). U0126 but not U0124 inhibited SI-induced p56 activation (Figure 3A); moreover, U0126 substantially reduced SI-induced DNA fragmentation and loss of pollen tube viability (Li et al., 2007). Pre-treatment with U0126 also alleviated SI-induced caspase-3-like activity, as measured using both a PARP cleavage assay and the fluorescent caspase-3 probe, Ac-DEVD-AMC (Figure 3B). Together, these data provide good evidence that inhibiting MAPK activity prevents activation of caspase-3-like (DEVDase) activity, and suggest that a MAPK, most likely p56, participates in initiating the PCD signalling cascade, committing the pollen tube to die.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Our knowledge of the components involved in SI in *Papaver* has increased significantly over the last few years, and there is now considerable evidence for PCD in playing a key role in SI. We propose that initiation of the PCD signalling cascade represents a 'point of no return' for incompatible pollen. Several caspase-like activities have been identified and it will be important to elucidate if (and how) these activities integrate to form a caspase-like signalling network/cascade. For instance, there is no information on whether there are, for example, initiator and activator caspase-like activities involved in plant PCD that are similar to mammalian systems. Moreover, current data point to a central role for cytosolic acidification during early PCD, which we are currently investigating. Actin depolymerization results in rapid inhibition of growth and also initiates PCD. There is a second phase of actin alterations that involves the formation of actin 'punctate foci'. This appears to be an active process, but whether this is also involved in PCD is not yet established. Cross-talk from actin to microtubules appears to play a role in mediating PCD, perhaps

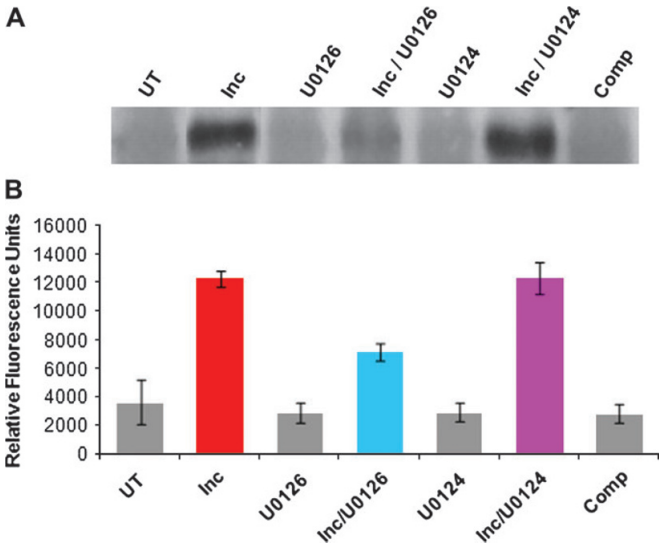

Figure 3. Evidence for Involvement of a MAPK in SI-Mediated PCD.
(A) The MAPK inhibitor U0126 inhibits p56 activity. MAPK activity was detected using a pTEpY antibody; the only MAPK significantly activated after SI challenge (Inc) was that identified as p56, shown here. Activity was detected in incompatible SI-induced samples (Inc) and basal, low levels of p56 activity were detected in controls comprising untreated pollen (UT), U0124, or U0126 treatment alone, or compatible SI-induced samples (Comp). Extracts from SI-induced pollen pre-treated with 100 μM U0126 (Inc/U0126) exhibited no p56 activity; p56 activity was not inhibited in extracts from pollen pre-treated with 100 μM U0124 (Inc/U0124).
(B) The SI-induced caspase-3-like (DEVDase) activity is inhibited by pre-treatment with U0126. Controls comprising untreated pollen (UT), compatible pollen (Comp), and pollen treated with 100 μM U0126 or U0124 alone (gray bars) gave low levels of caspase-3-like activity. Incompatible SI-induced pollen (Inc; red bar) had high levels of caspase-3-like activity. Incompatible SI-induced pollen pre-treated with 100 μM U0126 (Inc-U0126; blue bar) had reduced levels of caspase-3-like activity, while incompatible SI-induced pollen pre-treated with 100 μM U0124 (Inc-U0124; magenta bar) did not. Caspase-3-like activity was measured using Ac-DEVD-AMC, and data are means ± SEM of three independent experiments.
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explaining the rapid depolymerization of microtubules, but the exact roles of the MTs is not known, nor are the downstream components with which they interact. The involvement of the p56-MAPK is perhaps not surprising, given their role in PCD in other systems. However, we do not yet know which components involved in PCD it signals to. There are indications that MAPKs are involved in the dynamic organization of the actin cytoskeleton in both animals and plants (Samaj et al., 2004). Thus, it is relevant to examine if whether there is cross-talk between the SI-induced actin alterations and the activation of the p56-MAPK. As the timing of p56-MAPK peak activation occurs after actin depolymerization, it is more likely to be involved in the later events involving formation of punctate actin foci. Cloning of p56 will open the door to many further investigations into the functional role of the p56-MAPK. Finally, recent preliminary data implicate a role for ROS and NO signalling in the SI response (J.M. Bancroft, M.B. and V.E.F.-T., unpublished data). As they are known to be involved in signalling to PCD in plants, future research will focus on establishing whether this is the case in the SI response too. There are clearly many aspects of the SI-PCD signalling network still to investigate; the current ongoing challenge is to establish how all these components are integrated and how they participate in SI-induced PCD.

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