A Mitogen-Activated Protein Kinase Signals to Programmed Cell Death Induced by Self-Incompatibility in Papaver Pollen

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Self-incompatibility (SI) in higher plants is an important mechanism to prevent inbreeding and involves specific rejection of incompatible (“self”) pollen. In field poppy (Papaver rhoeas), S proteins encoded by the sigma component of the S-locus interact with incompatible pollen, resulting in cessation of tip growth. This “self” interaction triggers a Ca\(^{2+}\)-dependent signaling network, involving programmed cell death (PCD). We previously identified p56, a mitogen-activated protein kinase (MAPK) that is activated during the SI response in incompatible pollen. Here, we show that p56 cross-reacts with AtMPK3, but not with AtMPK4 or salicylic acid-induced protein kinase antibodies. We provide good evidence that a MAPK is involved in initiation of SI-induced PCD in incompatible pollen. SI rapidly reduces pollen viability and the MAPK cascade inhibitor U0126, which prevents the SI-induced activation of p56 in incompatible pollen, “rescues” incompatible pollen, while its negative analog, U0124, does not. This strongly implicates the involvement of a MAPK in SI-mediated loss of pollen viability and cell death. SI also stimulates caspase-3-like (DEVDase) activity and later DNA fragmentation. Both these markers of PCD are significantly reduced by pretreatment with U0126, implicating the involvement of a MAPK in signaling during early PCD. As p56 appears to be the only MAPK activated by SI, our studies imply that p56 could be the MAPK involved in mediating SI-induced PCD.

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(Fischer et al., 2003). Various tetrapeptide inhibitors of caspases have aided the study of caspase-dependent apoptosis. Caspase-3 has the recognition sequence DxD, and the caspase-3 inhibitor DEVD is based on this. Inhibition of protease activity using DEVD implicates the involvement of a caspase-3-like activity. Caspases also cleave endogenous nuclease inhibitors, which allows activation of nucleases and leads to the fragmentation of nuclear DNA, which is a common marker for apoptosis and PCD.

PCD in plants is less studied compared with animals. However, PCD is well established to be triggered by biotic and abiotic stresses (Zhang and Klessig, 2001), as well as during development (Kuriyama and Fukuda, 2002). Although release of mitochondrial cytochrome c has been identified in plants (Thomas and Franklin-Tong, 2004; Yao et al., 2004) and biochemical evidence for caspase activation in plants during PCD is good (van Doorn and Woltering, 2005), many of the genes encoding components of the apoptotic machinery, including caspases, have either not yet been identified in plants or are simply not present (Woltering et al., 2002). Nevertheless, there is no doubt that PCD occurs in plants.

Self-incompatibility (SI) in Papaver involves interaction of pistil S-locus determinants (S proteins) with a pollen receptor. An incompatible (‘‘self’’) interaction triggers rapid, SI-specific increases in cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\); Franklin-Tong et al., 1993, 1997) and depolymerization of the F-actin cytoskeleton (Geitmann et al., 2000; Snowman et al., 2002), resulting in rapid arrest of incompatible pollen tube growth. The SI signaling cascade also triggers PCD involving a caspase-3-like activity (Thomas and Franklin-Tong, 2004), resulting in the specific destruction of ‘‘self’’ pollen. We recently established that changes in actin filament levels or dynamics play a functional role in initiating PCD in Papaver pollen, triggering a caspase-3-like activity (Thomas et al., 2006), providing evidence of a specific causative link between actin polymerization status and PCD in pollen. In incompatible pollen, SI also stimulates Ca\(^{2+}\)-dependent hyperphosphorylation of Pr-p26.1a/b, two soluble inorganic pyrophosphatases (sPPases), which reduces their sPPase activity (de Graaf et al., 2006). Since sPPases are important for biosynthesis, this provides a further mechanism for incompatible pollen inhibition.

Enhanced activation of a MAPK specifically in incompatible pollen originally identified p56 as being implicated in the SI-induced signaling cascade in Papaver pollen (Rudd et al., 2003). Convincing evidence that p56 is a MAPK included: myelin basic protein (MBP) in-gel kinase assays; immunoprecipitation with anti-phosphotyrosine antisera; immunoprecipitated proteins exhibiting MBP in-gel kinase activity; and sensitivity to the MAPK inhibitor apigenin (Rudd et al., 2003). However, the timing of its activation, peaking at 10 min after SI induction, which is after initial arrest of pollen tube inhibition, suggested that it might be involved in later events after initial arrest of pollen tube growth, which implicated a possible role in signaling to PCD. As MAPKs are known to be functionally involved in regulating PCD in plants (Ligterink et al., 1997; Yang et al., 2001; Zhang and Klessig, 2001; Ren et al., 2002), we investigated whether p56 might be involved in signaling to PCD in incompatible pollen. Here, we provide data indicating that a MAPK signals to PCD in incompatible pollen. This contributes to our understanding of the mechanisms involved in mediating SI and integration of the SI-induced PCD signaling network.

RESULTS

The p56 MAPK Is Activated in SI in an S-Specific Manner and Cross-Reacts with AtMPK3

Plant MAPKs have high homology to mammalian ERK1/2 MAPKs, and ERK1/2 antisera that recognize the dually phosphorylated forms of activated MAPKs can be used to monitor plant MAPK activity (Samuel et al., 2000; Lee et al., 2001; Kroj et al., 2003). We therefore used an anti-active MAPK pTEpY antibody (Cell Signaling Technology) that specifically recognizes activated MAPKs to confirm that p56 was phosphorylated on a pTEpY motif and was activated specifically in incompatible interactions when SI was induced. Figure 1A shows that p56 is activated during SI in incompatible, but not compatible or unchallenged Papaver pollen. Quantitation revealed that p56 activity was increased 7.7-±0.88-fold (n = 5) 10 min after SI challenge with incompatible S proteins, while compatible and biologically inactive S proteins had only 1.1-±0.09-fold and 1.2-±0.11-fold changes (n = 4) in MAPK activity, respectively. In addition to p56 activation, Figure 1A reveals that two other MAPKs are activated in hydrated and growing Papaver pollen. We have ascertained that there are at least three other MAPKs in addition to p56 in Papaver pollen, as we have cloned three MAPKs from Papaver pollen. Unfortunately, none of the cDNAs corresponds to p56 (K. Osman, F.C.H. Franklin, and V.E. Franklin-Tong, unpublished data; EMBL accessions AJ784995, AJ784996, and AJ784997). Two of these MAPKs can be seen to be activated in growing pollen (Fig. 1A), but their activity is clearly not stimulated by SI. The third MAPK, which we know to be present in Papaver pollen as we have obtained the cDNA, is apparently not activated in hydrated or normally growing pollen (Fig. 1A); we speculate that it may be activated, like many other MAPKs, during stress responses.

We also used antisera raised against known plant MAPKs in an attempt to provide further evidence that p56 is a MAPK, especially as it migrates as a rather high molecular mass band for a MAPK on SDS-PAGE. A peptide antibody, raised against the specific C-terminal part of AtMPK3 (which is not found in the other 19 known MAPKs in Arabidopsis), successfully identified p56. This antibody against AtMPK3 cross-reacts...
with a protein that migrates to the same position as p56 (Fig. 1B), thus further confirming that p56 is indeed a MAPK. This recognition of p56 by anti-AtMPK3 antibody in poppy (Papaver rhoes) extracts is specific, as we have found that p56 does not cross-react with tobacco (Nicotiana tabacum) salicylic acid-induced protein kinase (SIPK) antibody (Fig. 1C); SIPK is an ortholog of AtMPK6. p56 also is not recognized by a peptide antibody that specifically recognizes AtMPK4, an ortholog of AtMPK6. p56 also is not recognized by a protein kinase (SIPK) antibody (Fig. 1C); SIPK is an

The MAPK Inhibitor U0126 Inhibits Pollen Tube Growth and p56 Activity But Does Not Affect Pollen Viability

To establish whether a MAPK activity was involved in triggering SI-induced PCD, we used an inhibitor of MAPK cascades, U0126. This drug is a potent and specific inhibitor and has been shown to block the MAPK cascades in cell-based assays in both animal and plant cells (Lee et al., 2001). In tobacco, elicitor activation of SIPK was inhibited by 100 μM U0126 (Lee et al., 2001); MAPK phosphorylation in response to lipopolysaccharides was also inhibited by U0126 (Piater et al., 2004). However, while the use of this drug can serve as useful preliminary data suggestive of MAPK involvement, we cannot rule out the possibility that U0126 may have other targets.

We examined whether U0126 inhibited p56 activity using anti-phospho-MAPK immunoblotting. We pretreated pollen with 100 μM of either U0126 or its negative analog, U0124, and then induced SI. The p56 activity was stimulated by SI and strongly inhibited by U0126, while the U0124 pretreatment did not affect p56 activity (Fig. 2). Although p56 was not obviously activated in normally growing pollen tubes, we found that U0126 inhibited normal pollen tube growth, while U0124, which is a negative analog of U0126 (Favata et al., 1998), did not (Fig. 3). These data (assuming that the specificity of U0126 is as expected) suggest that MAPK activity is required for normal pollen tube growth, which to our knowledge has not previously been demonstrated. We have identified three other MAPK sequences in Papaver pollen (K. Osman, F.C.H. Franklin, and V.E. Franklin-Tong, unpublished data) that do not correspond to p56; two of these MAPKs are activated in hydrated and normally growing pollen, but are not specifically activated by SI (Fig. 1A). This suggests that these MAPKs may play a functional role in signaling to pollen tube growth. Examination of the viability of pollen treated with U0126 and U0124, using fluorescein diacetate (FDA), revealed that viabilities for untreated pollen and pollen treated with U0124 or U0126 for 8 h were 95.3% ± 0.88%, 94.0% ± 2.65% (P = 0.830, N.S., n = 3), and 94.7% ± 1.2% (P = 0.658, N.S., n = 3), respectively (Table I). These data established that U0126 does not affect pollen viability and is a suitable inhibitor to investigate the effects of inhibiting p56 activity.

![Figure 2](image-url) The MAPK inhibitor U0126 inhibits p56 activity. p56 activity was detected using a pTEpY antibody. No p56 activity was detected in controls: GM, DMSO, U0124, or U0126 treatment alone. Activity was detected in incompatible SI-induced samples (Inc) and SI-induced samples pretreated with DMSO (Inc/DMSO). The p56 activity was inhibited in extracts from SI-induced pollen pretreated with 100 μM U0126 (Inc/U0126), but not inhibited in extracts from pollen pretreated with 100 μM U0124 (Inc/U0124). p56 activity was not detected in compatible SI-induced samples (Comp).
The MAPK Inhibitor U0126 Inhibits DNA Fragmentation

To investigate if p56 was involved in signaling to SI-induced PCD, we used one of the hallmark features of PCD, DNA fragmentation. We previously demonstrated that this is triggered by SI in an S-specific manner and involves a caspase-3-like (DEVDase) activity (Thomas and Franklin-Tong, 2004). We pretreated growing pollen with 100 μM U0126 or U0124 for 1 h and then induced SI, and subsequently measured DNA fragmentation after 8 h as a marker for PCD. In cells that had undergone PCD, TUNEL labeling was detected coincident with nuclear DNA. Levels of DNA fragmentation in untreated and control pollen tubes were low (9.99% ± 0.64%, n = 6), as were levels in pollen tubes that had undergone a compatible SI interaction (7.52% ± 0.68%, n = 3). Pollen tubes treated with 100 μM U0126 or U0124 had low levels of DNA fragmentation (13.25% ± 1.2% and 7.18% ± 0.82%, respectively, n = 3), with no significant difference in levels of DNA fragmentation compared to untreated controls (P = 0.102 and 0.054, respectively). SI induced high levels of DNA fragmentation in incompatible pollen tubes (76.42% ± 2.49%, n = 6; Fig. 4). Importantly, pollen tubes pretreated with U0126 and then SI induced had reduced levels of DNA fragmentation (41.64% ± 0.82%, n = 3), significantly different from SI induced alone (P < 0.001, **). In contrast, the negative analog U0124 did not inhibit the SI-induced DNA fragmentation, and levels were 75.91% ± 3.71% and not significantly different from SI treatments alone (Fig. 4; P = 0.915, N.S.). These data indicate that inhibiting MAPK activation prevents DNA fragmentation.

Since U0126 prevented both p56 activation and DNA fragmentation normally induced by SI, our data implicate the functional involvement of a MAPK in initiating the signaling cascade leading to SI-induced DNA fragmentation. Assuming that the specificity of U0126 is as expected, our data implicate MAPK signaling involvement in SI-mediated PCD. Although U0126 will not target p56 activity specifically, as p56 appears to be the only MAPK activated by SI, our studies imply that p56 could be the MAPK involved in mediating SI-induced PCD.

### Table 1. SI stimulates loss of viability of pollen tubes and pretreatment with U0126 “rescues” pollen tube viability after SI Challenge

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 h</th>
<th>1 h</th>
<th>3 h</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT</td>
<td>98.33 ± 0.88</td>
<td>97.33 ± 1.76</td>
<td>95.00 ± 2.65</td>
<td>94.0 ± 2.65</td>
</tr>
<tr>
<td>U0126</td>
<td>98.67 ± 0.33</td>
<td>97.67 ± 0.33</td>
<td>97.33 ± 0.67</td>
<td>94.67 ± 1.2</td>
</tr>
<tr>
<td>U0124</td>
<td>98.0 ± 0.58</td>
<td>98.0 ± 0.58</td>
<td>95.67 ± 0.33</td>
<td>95.33 ± 0.88</td>
</tr>
<tr>
<td>Comp SI</td>
<td>98.0 ± 1.15</td>
<td>97.0 ± 1.73</td>
<td>96.33 ± 1.45</td>
<td>93.33 ± 2.68</td>
</tr>
<tr>
<td>Inc SI</td>
<td>98.0 ± 1.0</td>
<td>58.33 ± 2.03</td>
<td>18.33 ± 0.88</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Inc SI + U0126</td>
<td>98.67 ± 0.33</td>
<td>98.37 ± 0.58</td>
<td>97.33 ± 0.67</td>
<td>94.67 ± 2.08</td>
</tr>
<tr>
<td>Inc SI + U0124</td>
<td>98.0 ± 1.0</td>
<td>55.0 ± 2.08</td>
<td>20.33 ± 0.33</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Pollen tube viability was measured using FDA at different time intervals (h) for different treatments. Inc SI, Incompatible pollen; Comp SI, compatible pollen; Inc SI + U0126, pollen pretreated with 100 μM U0126 prior to SI challenge; Inc SI + U0124, pollen pretreated with 100 μM U0124 prior to SI challenge. Controls comprised untreated pollen (UT) and pollen treated with drugs alone (U0126, U0124). One hundred pollen tubes were counted for each treatment and percentage viability determined from a total of three independent experiments.
tubes and pollen tubes treated with U0126 and U0124 at 8 h ($P = 0.830$ and $P = 0.658$, respectively). In contrast, SI-challenged incompatible pollen tubes showed significant loss of viability within 1 h ($P < 0.001$, *** and 0.001, ***) and had completely lost viability by 8 h. Notably, pretreatment with U0126 prior to SI induction prevented loss of viability; there was a highly significant difference between SI-induced samples and those pretreated with U0126 ($P < 0.001$, *** at 1 and 3 h, while pretreatment with U0124 did not have an effect on SI-induced death ($P = 0.315$ [N.S.] and 0.101 [N.S.], respectively; Table I). Thus, pretreatment with U0126 and prevention of p56 activation prior to SI “rescues” incompatible pollen from death. Together with the DNA fragmentation data, this clearly implicates the involvement of MAPK activation in playing a functional role in mediating PCD.

SI-Mediated Poly (ADP-Rib) Polymerase Cleavage Is Inhibited by U0126

SI-induced PCD in *Papaver* involves a caspase-3-like (DEVDase-like) activity (Thomas and Franklin-Tong, 2004). We previously demonstrated that SI-induced pollen protein extracts exhibited a caspase-3-like cleavage activity in vitro, using bovine poly (ADP-Rib) polymerase (PARP) as a substrate. The nuclear DNA repair protein PARP is a classic substrate for caspase-3 activity in animal cells (Lazebnik et al., 1994), and its cleavage is often used as evidence for apoptosis. We showed that incompatible SI-induced pollen extracts cleave PARP, producing a 24-kD PARP cleavage fragment, which was prevented by addition of the caspase-3 tetrapeptide inhibitor Ac-DEVD-CHO (Thomas and Franklin-Tong, 2004). To establish if a MAPK was implicated in signaling to caspase-3 activation, we examined if inhibition of MAPK activation by U0126 prevented the PARP cleavage activity exhibited by SI-induced pollen. SI-induced incompatible pollen protein extracts have a caspase-3-like activity that cleaves bovine PARP in vitro (Thomas and Franklin-Tong, 2004; Fig. 5). A doublet at approximately 24 kD was consistently detected only in PARP samples incubated with incompatible SI-induced pollen extracts ($n = 3$). The detection of a PARP cleavage fragment, coupled with a corresponding decrease in the amount of uncleaved PARP at approximately 116 kD, provides compelling evidence for a SI-induced PARP cleavage activity. The specificity of this PARP cleavage activity was demonstrated because SI-induced compatible pollen, untreated pollen, or pollen treated with the U0124 and U0126 drugs alone did not show a disappearance of the 116-kD intact PARP fragment or the appearance of a 24-kD PARP fragment (Fig. 5). Notably, in the samples pretreated with U0126 prior to SI induction, the 116-kD intact PARP fragment was still present and the 24-kD SI-specific doublet was much less intense in the U0126-pretreated samples (Fig. 5). The samples pretreated with U0124 prior to SI induction were indistinguishable from those without pretreatment, and

Figure 5. SI-mediated PARP cleavage is inhibited by U0126. Uncleaved PARP (PARP) comprises a major band at 116 kD (white arrow). Extracts from untreated pollen (UT), compatible SI-induced pollen (Comp), and pollen treated with 100 μM drugs alone (U0124, U0126) show nonspecific proteolysis of PARP. Extracts from incompatible SI-induced pollen (Inc) showed an S-specific cleavage pattern: disappearance of the 116-kD intact PARP fragment or the appearance of a 24-kD PARP fragment (Fig. 5). Notably, in the samples pretreated with U0126 prior to SI induction, the 116-kD intact PARP fragment was still present and the 24-kD SI-specific doublet was much less intense in the U0126-pretreated samples (Fig. 5). The samples pretreated with U0124 prior to SI induction were indistinguishable from those without pretreatment, and
showed loss of the 116-kD intact PARP fragment and a strong 24-kD SI-specific doublet (Fig. 5). The alleviation of SI-mediated PARP cleavage by U0126, but not U0124, suggests that inhibiting MAPK activity prevents activation of caspase-3-like activity. These data implicate MAPK signaling to PCD in incompatible pollen.

The SI-Induced Caspase-3-Like (DEVDase) Activity Is Inhibited by Pretreatment with U0126

We also used the fluorescent caspase-3 substrate Ac-DEVD-AMC to measure the caspase-3-like (DEVDase) activity generated by SI and to test whether U0126 inhibited this activity. Extracts from untreated pollen, compatible SI-treated pollen, and those treated with U0126 and U0124 alone gave low levels of DEVDase activity (Fig. 6). SI induced high levels of DEVDase activity. The tetrapeptide Ac-DEVD-CHO (DEVD), a caspase-3 inhibitor, was used to provide evidence for a caspase-3 activity in apoptotic animal cells and in plant cells undergoing PCD (Garcia-Calvo et al., 1998; Richael et al., 2001; Danon et al., 2004; Thomas and Franklin-Tong, 2004). Addition of Ac-DEVD-CHO inhibited this activity, while addition of the Ac-YVAD-CHO caspase-1 inhibitor did not. Pretreatment of pollen with U0126 prior to SI induction resulted in a significant alleviation of the DEVDase activity ($P = 0.003$, **, $n = 3$), while pretreatment with U0124 did not ($P = 0.949$, N.S., $n = 3$).

These data clearly demonstrate that SI-mediated caspase-3-like (DEVDase) activity is inhibited by a MAPK cascade inhibitor. Assuming the specificity of U0126, this implicates that a MAPK is activated during early PCD and functions to signal to PCD. Since p56 is apparently the only MAPK activated by SI, our data suggest that p56 activation is necessary for progression of PCD in pollen. As sequence information is not yet available for p56, reverse genetic studies cannot currently be carried out to provide definitive evidence that it is p56 that is involved.

**p56 Activation Is Upstream of the SI-Induced DEVDase Stimulation**

We also investigated whether the caspase-3 inhibitor DEVD might inhibit p56 activity in order to establish if p56 activation is upstream of the SI-induced caspase-3-like activation. We pretreated pollen with DEVD for 1 h prior to the induction of SI and pollen extracts were examined for p56 activation. Pretreatment of pollen with DEVD prior to SI induction did not alter p56 activation (Fig. 7), indicating that the SI-induced MAPK activation is upstream of caspase-3-like activity triggered by SI.

In summary, we have shown that SI induces p56 activation in incompatible pollen and that the MAPK cascade inhibitor U0126 prevents its activation. SI rapidly reduces pollen viability and U0126 “rescues” incompatible pollen, while its negative analog does not. These data implicate the involvement of a MAPK in SI-mediated loss of pollen viability and cell death, though we cannot rule out the possibility that U0126 may also act on other targets. SI also stimulates DNA fragmentation, which is significantly alleviated by pretreatment with U0126, implicating the functional involvement of a MAPK in initiating signaling upstream of SI-induced DNA fragmentation. SI stimulates a caspase-3-like (DEVDase) activity, and this activity is inhibited by U0126. Our demonstration that the SI-mediated caspase-3-like (DEVDase) activity is inhibited by a MAPK cascade inhibitor strongly implicates a MAPK being involved in signaling to activate a caspase-3-like (DEVDase) activity involved in PCD.

**DISCUSSION**

We previously established that PCD is triggered in incompatible pollen (Thomas and Franklin-Tong, 2004), which will ensure that pollen tube growth does not resume and provides a neat way of disposing of unwanted incompatible pollen. Here, we provide evidence that MAPK activation is likely to be required for SI-induced PCD. Our data, assuming the lack of side effects of U0126, strongly implicate a MAPK in signaling to early events initiating PCD in poppy pollen. To our knowledge, this represents the first evidence for involvement of MAPK signaling in PCD...
triggered by SI. SI appears to stimulate a single MAPK activity: p56 (Rudd et al., 2003; this study). As activation of p56 peaks 10 min after SI induction, it cannot be involved in the rapid arrest of pollen tube growth. Here, we provide evidence implicating a MAPK as being involved in the initiation phase of PCD in incompatible pollen, which provides a significant step forward in our understanding of SI-induced events.

Inhibitors of MAPK cascades have been extremely useful for identifying some of the physiological roles of the cell signaling pathways that they inhibit. U0126 is recognized as a highly specific inhibitor of MAPK cascades (Davies et al., 2000). There is good evidence for U0126 preventing MAPK activation in plants. For example, pathogen response gene expression in tobacco cells was inhibited by U0126 at similar concentrations to those we used (Lee et al., 2001), providing what was considered good evidence of a functional involvement of a MAPK in plant defense activation. Our data therefore strongly suggest the involvement for a MAPK(s) playing a role in PCD. We cannot definitively say that p56 is involved, as we have not obtained the cDNA corresponding to it, so cannot currently use an antisense approach to explore this. However, because p56 appears to be the only MAPK stimulated by SI, our data strongly implicate the involvement of p56 in initiating PCD. Furthermore, we provide evidence that the SI-activated MAPK signals to activate a caspase-3-like (DEVDase) activity. This is confirmed by our finding that although the tetrapeptide inhibitor Ac-DEVD-CHO inhibits PCD, it does not inhibit p56 activation, suggesting that the caspase-3-like (DEVDase) activity is downstream of p56 in the signaling cascade. Together, our data provide important information about the integration of the SI signaling network, indicating that we have a complex network of events to stop pollen tube growth.

MAPK cascades have emerged as key players in some of the most essential roles in eukaryotic signaling networks. Plant MAPKS have been shown to be activated by a variety of stresses (Tena et al., 2001; Zhang and Klessig, 2001), and the involvement of MAPKS in activation of defense responses resulting in PCD (Ligterink et al., 1997; Yang et al., 2001; Kroj et al., 2003) and resistance to pathogens (Zhang and Klessig, 2001; Asai et al., 2002) is well established in plants. Surprisingly, although MAPKs have been demonstrated to play a role in PCD in plants, apart from an involvement in pollen hydration (Wilson et al., 1993), virtually nothing is known about MAPK signaling in mature pollen, though they are known to be implicated in pollen development (Prestamo et al., 1999).

Furthermore, since U0126 inhibits pollen tube growth, it implicates MAPKs as playing a role in regulating pollen tube growth. Interestingly, it has recently been demonstrated that SIMK (stress-induced MAPK) is involved in regulating root hair tip growth (Samaj et al., 2002). Our findings, therefore, not only provide a significant advance in our understanding of mechanisms involved in mediating the SI response in *Papaver*, but also shed some light on the role of MAPKs in pollen. Thus, to our knowledge, these data provide the first evidence for MAPK signaling in growing pollen tubes.

SI involves modification of several cellular components in incompatible pollen. Figure 8A outlines a model of the current state of play in our understanding of this signaling network. Figure 8B shows the new data described here, implicating MAPK involvement. PCD involves release of cytochrome c into the cytosol and later DNA fragmentation, which is mediated by a caspase-3-like (DEVDase) activity (Thomas and Franklin-Tong, 2004). One of the first SI-induced events is rapid Ca$^{2+}$ influx (Franklin-Tong et al., 2002), accompanied by SI-specific increases in [Ca$^{2+}$]i (Franklin-Tong et al., 1993, 1997; Fig. 8A); this appears to initiate the SI signaling network, causing rapid inhibition of pollen tube growth. We have evidence that the [Ca$^{2+}$]i increases are required for PCD (Thomas and Franklin-Tong, 2004). SI also triggers depolymerization of the F-actin cytoskeleton (Geitmann et al., 2000; Snowman et al., 2002), resulting in rapid arrest of incompatible pollen tube growth. Evidence that artificially raising [Ca$^{2+}$]i, also results in actin depolymerization suggests that this SI-induced event is on the same signaling pathway (Fig. 8A). We have recently shown that actin depolymerization is involved in initiating PCD (Thomas et al., 2006), providing the first piece of evidence integrating some of the SI-stimulated components.

Here, we have provided important evidence linking a second SI-stimulated component, activation of a MAPK, to this PCD network (Fig. 8B). We previously showed that p56 activation is inhibited by La$^{3+}$ (Rudd et al., 2003), a Ca$^{2+}$ channel blocker, indicating that the SI-induced MAPK activation is downstream of the initiating Ca$^{2+}$ signals. Since p56 activation is 10 min after SI induction, this event must be downstream of actin depolymerization. Although we previously showed that altering actin dynamics using latrunculin B and jasplakinolide can trigger PCD in pollen tubes (Thomas et al., 2006), preliminary data suggest that...
Figure 8. A model of events triggered by SI in incompatible pollen. A. Current overall understanding of events involved. Incompatible stigmatic S proteins trigger a number of events in incompatible pollen. This starts with influx of Ca\(^{2+}\) and increases in [Ca\(^{2+}\)], (assumed to be triggered by the interaction of S proteins with their pollen receptors). This results in phosphorylation of two sPPases, Pr-p26.1a and Pr-p26.1b; their sPPase activity is inhibited by phosphorylation. Large-scale actin depolymerization occurs, which plays a role in triggering a caspase-3-like activity and PCD. p56-MAPK activation is also triggered by SI; here, we show that it is likely to be involved in mediating PCD (see B). B. Data described here that contributes to the model. SI triggers activation of a MAPK, p56. This activity is inhibited by U0126. Viability tests show that pretreatment with U0126 can “rescue” incompatible pollen that would normally rapidly die. Both SI-induced caspase-3-like activity and DNA fragmentation are dramatically reduced by pretreatment with U0126. Together, these data implicate the involvement of MAPK in regulating SI-induced PCD.

In conclusion, this is the first evidence (to our knowledge) suggesting a specific causal link between MAPK activation and initiation of PCD in SI. Our findings implicate a fundamentally important role for MAPK signaling in SI, showing that it most likely participates in initiating the early PCD signaling cascade, committing the pollen tube to die. Although these data fall short of providing definitive evidence, they nevertheless provide a significant advance in our understanding of SI and signaling to regulate incompatible pollen tube growth. We propose that this represents the “gateway” through which incompatible pollen must pass to become irreversibly inhibited.

MATERIALS AND METHODS

SI Induction and Pollen Treatments

Pollen of Papaver rhoas, the field poppy, was hydrated at 25°C for 45 min, then germinated and grown in vitro in liquid germination medium [GM; 0.01% H\(_3\)BO\(_3\), 0.01% KNO\(_3\), 0.01% Mg\((NO_3)\)_2, 6H\(_2\)O, 0.036% Ca\(_2\)Cl\(_2\)-2H\(_2\)O, 13.5% Suc], as described by Snowman et al. (2002), at 25°C for 45 min before any treatments were applied.

For SI treatments, recombinant proteins were produced by cloning the nucleotide sequences specifying the mature peptide of the S\(_5\), S\(_6\), and S\(_8\) alleles of the S gene (pFRS901, pFRS902, and pFRS800) into the expression vector pMS119 as described previously (Foote et al., 1994). Expression and purification of the proteins were as described (Kakeda et al., 1998). SI was induced by adding recombinant S proteins; S\(_5\), S\(_6\), and S\(_8\) (final concentration 10 \(\mu\)g mL\(^{-1}\)), to pollen from S\(_5\)S\(_6\) (incompatible) and S\(_8\)S\(_8\) (compatible) plants, growing in vitro, as described (Snowman et al., 2002). Heat-denatured S proteins boiled for 30 min prior to use were used as additional controls.

The inhibitor U0126 [1,4-diamino-2,3-dicyano-1,4-bis(methythio)butadiene; Calbiochem] was used at a concentration of 100 \(\mu\)M to inhibit P56 activity. Its inactive analog, U0124 [1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; Calbiochem], was used as a control and did not inhibit MAPK cascades even at concentrations of 100 \(\mu\)M. The drugs were added to pollen growing in vitro as a pretreatment for 1 h prior to experiments. Controls comprised addition of dimethyl sulfoxide (DMSO), which was used to solubilize the inhibitors, at a final concentration of 0.1% (v/v).

MAPK Activity Assays and Immunoblots

Cytosolic proteins were extracted from pollen according to Rudd et al. (1996). Pollen was collected by centrifugation at 2,000 rpm for 5 min and then extracted into IP buffer (Rudd et al., 2003) in a ground-glass homogenizer. Protein concentration was assayed using a Bio-Rad protein assay kit. Pollen protein extracts were separated using SDS-PAGE and then western blotted. Blots were probed with anti-ATMPK kinase polyclonal Ab (p56\(^{E1}\); Cell Signaling Technology), which recognizes activated MAPKs. For testing the cross-reaction of p56 with AtMPK3, immunoblots were probed with a 1:10,000 dilution of the polyclonal anti-AtMPK3 antibody raised against the specific peptide corresponding to amino acids 359 to 370 of AtMPK3 (Arabidopsis thaliana) PMK3 (M8318; Sigma). This sequence is specific to AtMPK3 because it is not found in the other 19 known MAPKs in Arabidopsis.

Subsequently, blots were washed and incubated with an anti-rabbit horse-radish peroxidase secondary antibody (A-1854; Sigma) and detected using an ECL kit (RPN2209; Amersham). As controls, tobacco (Nicotiana tabacum) SIPK antibody (generous gift from Shuqun Zhang, University of Missouri), which is an ortholog of Arabidopsis MPK6, and AtMPK4 antibody (A6979; Sigma) raised against synthetic peptide corresponding to the C terminus of Arabidopsis MPK4 (amino acids 363–376; this sequence is specific to AtMPK4 and it is not found in the other 19 known Arabidopsis MAPKs) were also used.

Measurement of Pollen Tube Lengths

Pollen tubes were treated as described above, but incubated at 25°C for 1 h, and then fixed in 4% paraformaldehyde and mounted in Tris-buffered saline, pH 7.6. Pollen tubes were imaged and pollen tube lengths measured using a Quips PathVysion image analysis system (Applied Imaging International) and with IPlab software. One hundred pollen tubes were measured for each treatment and mean length determined from a total of three independent experiments.

Viability Assays

Pollen viability was assessed using the fluorochromatic test procedure as described (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison et al., 1984). Pollen was incubated in 5 \(\mu\)g mL\(^{-1}\) FDA in GM for 5 min at
room temperature. FDA is an indicator of cellular metabolic activity; viable pollen can cleave FDA, so is fluorescent; unviable pollen is not. Images were captured with a Nikon T300 fluorescence microscope, using a Quips PathVysion image analysis system. One hundred pollen tubes were counted for each treatment and percentage viability determined from a total of three independent experiments.

DNA Fragmentation Assays

Pollen was treated and grown for a total of 8 h, after which it was fixed in 2% paraformaldehyde and then assayed for DNA fragmentation. For “untreated” controls, pollen was grown for 1 h, an aliquot of liquid GM (Rudd et al., 1996) was added as a “mock treatment,” and pollen was incubated for a further 8 h. Pollen tubes were then fixed in 4% paraformaldehyde (Jordan et al., 2000). Fixed pollen tubes were labeled with a DeadEnd fluorometric TUNEL kit (Promega). Fifty pollen tubes were scored for DNA fragmentation for each of at least three independent experiments using a Nikon T300 fluorescence microscope, with a Nikon 60× Plan-Apo, 1.4-NA oil objective. Capture and analysis of images was made at 20°C using a Photometrics SenSys camera (model KAF1400-G2) and a Quips PathVysion image analysis system (Applied Imaging International).

PARP Cleavage Assays

Pollen was grown in liquid GM (Rudd et al., 1996) and SI induced or treated with 100 μM U0126/U0124 inhibitors prior to SI induction; as described above. Pollen tubes were collected by centrifugation and HEPES buffer (50 mM HEPES, pH 7.4, 10 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol) added. Samples were snap-frozen in liquid N2, and pollen protein extracts were prepared by grinding, centrifuged (14,000 rpm, 20 min, 4°C), and the supernatant tested for PARP cleavage activity, as described by Thomas and Franklin-Tong (2004). Thirty micrograms of pollen cytosolic protein was incubated with 250 ng of bovine PARP (BioMol) at 37°C for 15 min. SDS final sample buffer was added and samples were analyzed using SDS-PAGE and western blotting with PARP antisera (clone F1-23; BioMol), which detects the PARP 24-kD fragment. Controls comprised bovine PARP incubated with HEPES buffer or untreated pollen extracts. At least three independent experiments were carried out.

Caspase-3-Like Activity Measurements

We used the fluorogenic caspase-3 substrate Ac-DEVD-AMC (Calbiochem) to establish the caspase-3-like activity directly. Protein extracts were made from treated pollen tubes by grinding in extraction buffer (50 mM sodium acetate, 10 mM l-Cys, pH 6.0). Ten micrograms of total protein was incubated with 50 μM Ac-DEVD-AMC at 27°C, and fluorescence was monitored at 460 nm using a FLUOstar Optima 413-1183 time-resolved fluorescence plate reader (BMG-LabTech; 380 excitation, 460 emission) as described by others (Thomas et al., 2006). Fluorescence at 480 nm indicated cleavage of the substrate by a DEVDAse activity. Relative fluorescence units were expressed as percentage increase relative to the control after 4 h. At least three independent experiments were carried out.

Caspase Inhibitor Treatments

To confirm that the caspase-3-like activity was induced by SI, 100 μM Ac-DEVD-CHO or Ac-YVAD-CHO (Calbiochem) was added to pollen extracts in caspase substrate experiments using Ac-DEVD-AMC. To test if MAPK activation was upstream of caspase activation, pollen was grown as described and pretreated by addition of 100 μM Ac-DEVD-CHO or Ac-YVAD-CHO (Calbiochem) for 1 h; SI was then induced. Extracts for MAPK activity were made at 10 min and western blotted with anti-ACTIVE MAP kinase polyclonal Ab (pTEpY; Promega; see above). At least three independent experiments were carried out.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ784995, AJ784996, and AJ784997.

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