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MAP Kinase PrMPK9-1 Contributes to the Self-Incompatibility Response^{1[CC-BY]}

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Mitogen-activated protein kinases (MAPKs) form important signaling modules for a variety of cellular responses in eukaryotic cells. In plants, MAPKs play key roles in growth and development as well as in immunity/stress responses. Pollen-pistil interactions are critical early events regulating pollination and fertilization and involve many signaling events. Self-incompatibility (SI) is an important mechanism to prevent self-fertilization and inbreeding in higher plants and also is known to utilize signaling to achieve incompatible pollen rejection. Although several pollen-expressed MAPKs exist, very little is known about their function. We previously identified a pollen-expressed MAPK (p56) from *Papaver rhoeas* that was rapidly activated during SI; several studies implicated its role in signaling to SI-induced programmed cell death involving a DEVDase. However, to date, the identity of the MAPK involved has been unknown. Here, we have identified and cloned a pollen-expressed *P. rhoeas* threonine-aspartate-tyrosine (TDY) MAPK, *PrMPK9-1*. Rather few data relating to the function of TDY MAPKs in plants currently exist. We provide evidence that *PrMPK9-1* has a cell type-specific function, with a distinct role from *AtMPK9*. To our knowledge, this is the first study implicating a function for a TDY MAPK in pollen. We show that *PrMPK9-1* corresponds to p56 and demonstrate that it is functionally involved in mediating SI in *P. rhoeas* pollen: *PrMPK9-1* is a key regulator for SI in pollen and acts upstream of programmed cell death involving actin and activation of a DEVDase. Our study provides an important advance in elucidating functional roles for this class of MAPKs.

Cellular responses require an integrated signal perception and signal transduction network. Mitogen-activated protein kinases (MAPKs) form highly conserved signaling networks that are common to all eukaryotic cells, including animals, plants, and fungi. They are commonly used to

transduce information from sensors to cellular responses. A MAPK cascade minimally consists of three kinases: a MAPKKK, a MAPKK, and a MAPK, which sequentially phosphorylate and activate each other. MAPKs are Ser/Thr kinases, and upon phosphorylation, which activates them, the MAPK can phosphorylate a range of target proteins, which include other kinases and transcription factors. This results, ultimately, in changes in gene expression, metabolism, cell division, and growth. In plants, they function in diverse signaling networks, including development, but perhaps the best characterized and well known involve responses to biotic stresses stimulated by interactions with pathogens, involving defense responses, and to a range of abiotic stresses, including wounding, drought, cold, heat, UV, touch, osmotic shock, and salt (Rodriguez et al., 2010; Meng and Zhang, 2013; Xu and Zhang, 2015). The involvement of MAPKs in signaling to the expression of defense genes, involving apoptosis and programmed cell death (PCD), also is well documented (Pitzschke et al., 2009; Mase et al., 2012; Bigeard et al., 2015).

Arabidopsis (*Arabidopsis thaliana*) has 20 MAPK genes, and there are a similar number in the sequenced genomes of other plant species. Plant MAPKs have two types of activation motif: either a Thr-Glu-Tyr (TEY) activation motif or a Thr-Asp-Tyr (TDY) activation motif, which is unique to plant MAPKs (Ichimura et al., 2002; Hamel et al., 2006). Plant MAPKs are highly conserved between species and can be divided into four clades (A–D) based on sequence similarities. The plant

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TEY MAPKs can be classified into three groups (A–C), while the TDY MAPKs belong to a more distant clade (D; Ichimura et al., 2002; Hamel et al., 2006). Group A MAPKs are well characterized; many are functionally involved in stress responses and include the Arabidopsis MPK3 and MPK6 and their orthologs in other species, such as the stress-activated MAPK from alfalfa (*Medicago sativa*), salt-induced MAPK, tobacco (*Nicotiana tabacum*) salicylic acid-induced protein kinase, and wounding-induced protein kinase. The group B MAPKs (Arabidopsis MPK4 and MPK11) also have functions implicated in pathogen defense and stress responses and also play an essential role in cell division (for review, see Meng and Zhang, 2013). The functions of MAPKs in groups C and D are currently less well established (Meng and Zhang, 2013; Zhang et al., 2014).

Plants use PCD for a wide range of functions, including disease resistance and development as well as reproduction (for review, see Van Hautegeem et al., 2015; Daneva et al., 2016; Van Durme and Nowack, 2016). Animal cells use a cascade of caspases (Cys proteases), which, when activated, cleave target proteins, resulting in cellular dismantling and, ultimately, death. Although there are no true caspase gene homologs in plants, there is good biochemical evidence for several caspase-like protease activities and their involvement in plant PCD (Bozhkov et al., 2004; Sanmartín et al., 2005). Caspase-like activities have been detected in plants through cleavage of the animal caspase-specific tetrapeptide substrates (e.g. Ac-DEVD-AMC for caspase-3; del Pozo and Lam, 1998; Richael et al., 2001; Danon et al., 2004; Thomas and Franklin-Tong, 2004; Thomas et al., 2006).

Pollination involves many interactions between the pistil and pollen grain/tube as it germinates and grows to achieve fertilization (for review, see Dresselhaus and Franklin-Tong, 2013). Many angiosperms face the possibility of self-fertilization and have evolved self-incompatibility (SI) to avoid this problem. This is an important genetically controlled system that has evolved independently several times; several different mechanisms regulate SI in different plant species (for review, see Takayama and Isogai, 2005; Franklin-Tong, 2008). SI is genetically controlled by a multiallelic *S*-locus that encodes linked pollen and pistil *S*-determinants. These allow recognition and rejection, so when male and female *S*-determinant allelic specificities match, self-(incompatible) pollen is recognized and rejected before fertilization can occur.

The SI system found in *Papaver rhoeas* (the field poppy) has provided a model system in which to study the components involved in cell-cell recognition and rejection, in particular the signaling network downstream of the interaction between the pistil *S*-determinant, PrsS, and the pollen *S*-determinant, PrpS. PrsS encodes a small secreted protein (Foote et al., 1994), and PrpS encodes a small, novel transmembrane protein (Wheeler et al., 2009). The major focus for SI-induced signaling is the initiation of PCD (Thomas and Franklin-Tong, 2004; Bosch and Franklin-Tong, 2007). A long-standing model

for SI in *P. rhoeas* is that PrsS acts as a signaling ligand, initiating a network of signaling events within incompatible pollen that results in the rapid inhibition of pollen tube growth and, ultimately, PCD. A key target for SI signals is the actin cytoskeleton. Within a few minutes of SI initiation, F-actin in incompatible pollen is depolymerized, and later, F-actin reorganizes into punctate foci (Snowman et al., 2002; Poultier et al., 2010). These large-scale alterations to the actin polymerization status of incompatible pollen activate a signaling network resulting in PCD (Thomas et al., 2006). The predominant caspase-like activity during *P. rhoeas* SI is a caspase-3-like/DEVDase activity (Thomas and Franklin-Tong, 2004; Bosch and Franklin-Tong, 2007). More recently, it has been demonstrated that reactive oxygen species (ROS) play a key role in signaling to alterations in actin and SI-PCD (Wilkins et al., 2011). How these events integrate to participate in SI-PCD is reviewed by Wilkins et al. (2014).

We previously showed that a MAPK named p56 was activated in SI-induced pollen (Rudd et al., 2003). This implicated MAPK signaling in *P. rhoeas* SI. Activation of the p56-MAPK peaked ~10 min after SI, so it occurred after the inhibition of pollen tube growth, suggesting a function in later events. A further study showed that the inhibition of SI-induced p56 activation resulted in the alleviation of SI-induced caspase-3-like activity. This provided evidence that the MAPK p56 participates in initiating the PCD signaling cascade (Li et al., 2007). Here, we have identified and cloned a pollen-expressed *P. rhoeas* MAPK, PrMPK9-1, that encodes the p56-MAPK. We provide evidence that it functions to participate in signaling to SI-mediated inhibition of growth and actin alterations that are key features of the SI response in *P. rhoeas* and in the activation of a DEVDase, which is required for SI-PCD. The identification of PrMPK9-1 provides an important step forward in identifying components involved in the SI signaling network. Moreover, our demonstration that this is a TDY-type MAPK and that it participates in signaling upstream of actin alterations and DEVDase activation in the PCD network provides an important example of the function of a cell-specific TDY MAPK, rather few of which have been characterized to date.

RESULTS

Evidence Suggests That p56-MAPK Is Encoded by PrMPK9-1, an Ortholog of AtMPK9

The strategy adopted to identify the gene encoding p56-MAPK was based on combining extant data on plant MAPKs, properties of the protein, and proteomic analysis. MAPKs comprise a large gene family with 20 members identified in the Arabidopsis genome. Although MAPKs are highly conserved, they fall into four groups: the TEY groups of MAPKs (A–C) are generally smaller (~42–45 kD) than the TDY group of MAPKs (group D), which are ~55 to 79 kD (Supplemental Fig. S1). This suggested that the poppy p56-MAPK was possibly a TDY group MAPK. Expression data from

Genevestigator indicated that four Arabidopsis TDY MAPKs, *AtMPK8*, *AtMPK9*, *AtMPK17*, and *AtMPK19*, are expressed at high levels in pollen (Supplemental Table S1). Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometry of p56 isolated by SDS-PAGE fractionation of pollen proteins (see “Materials and Methods”) identified five peptides present in *AtMPK9*, comprising three peptides that were unique to *AtMPK9*, one peptide unique to *AtMPK8*, and two peptide sequences that were present in *AtMPK8*, *AtMPK9*, and *AtMPK15* (Supplemental Table S2). Several other peptides corresponding to TDY MAPKs from *Brassica napus*, rice (*Oryza sativa*), and *Populus trichocarpa* also were identified (Supplemental Table S2). Taken together, these data suggested that the poppy p56-MAPK was likely encoded by an *AtMPK8/9* ortholog.

We next designed primers based on sequences from Arabidopsis TDY MAPKs with high expression in pollen (*AtMPK8*, *AtMPK9*, and *AtMPK17*). An ~950-bp fragment was cloned from poppy pollen, and sequencing of this revealed that it shared highest nucleotide identity with *AtMPK8* and *AtMPK9* (Supplemental Table S2). To obtain the full-length gene sequence, 5' RACE and 3' RACE were carried out on pollen cDNA (see “Materials and Methods”). An ~1.6-kb mRNA product was amplified, cloned, and sequenced. The pollen open reading frame was 1,479 bp long, predicted to encode a protein of 493 amino acids, with a molecular mass of 56.3 kD and a pI of 6.49. The full-length sequence showed highest homology to *AtMPK8* and *AtMPK9*, sharing 79.4% identity with *AtMPK9* and 65.5% identity to the *AtMPK8* gene at the amino acid level. Therefore, we designated the poppy gene *PrMPK9-1*. To determine if the *PrMPK9-1* sequence was the gene encoding the SI-activated p56-MAPK, we reanalyzed the FT-ICR mass spectrometry data using the predicted protein sequences obtained. The predicted *PrMPK9-1* protein had two peptide matches that were highly significant (KGSYGVVASAVDTHTEK and KVDPLALNLLERL; Supplemental Table S2).

We also cloned another pollen open reading frame, designated *PrMPK9-2* (1,818 bp, encoding a predicted protein of 606 amino acids), that also showed high homology to *AtMPK8* and *AtMPK9* and homology with *PrMPK9-1* (Supplemental Fig. S2). However, its predicted molecular mass of 68.95 kD was too large to be a good candidate for the gene encoding the SI-activated p56-MAPK. Comparing the *PrMPK9-2* predicted protein sequences with the p56 pollen extract, FT-ICR peptide data revealed no significant matches (Supplemental Table S2). This discounted *PrMPK9-2* as a candidate for the poppy p56-MAPK and confirmed that *PrMPK9-1* was the best candidate.

The expression of *PrMPK9-1* was examined using semiquantitative reverse transcription (RT)-PCR. Transcript was detected in mature pollen, with weak expression during late anther development; there was negligible expression in root, leaf, or stigma (Fig. 1A). This expression pattern is consistent with the known

function of the p56-MAPK involvement in the SI response in mature pollen. We examined whether *PrMPK9-1* was up-regulated after SI induction, as several MAPKs are up-regulated after stimulation, particularly stress treatments (Zhang et al., 2014). RT-PCR expression analysis of *PrMPK9-1* revealed increased expression 10 min after SI induction in incompatible pollen specifically; compatible pollen challenged with the same PrsS did not show increased expression, nor did other control pollen samples (Fig. 1B). Thus, *PrMPK9-1* also exhibits increased expression after SI induction.

PrMPK9-1 Encodes a TDY MAPK

Analysis of the predicted *PrMPK9-1* protein sequence reveals the expected features of a MAPK, including 11 characteristic kinase domains, the ATP-binding site, the substrate-binding site, and the kinase interaction motif. The presence of a TDY motif, rather than a TEY motif, is characteristic of a group D MAPK (for review, see Rodriguez et al., 2010). Consistent with this, *PrMPK9-1* possesses a long C-terminal extension of ~140 amino acids that is typical of group D MAPKs (Jonak et al., 2002). In addition, the protein lacks the C-terminal common-docking domain that is found in groups A, B, and C MAPKs but not in group D proteins. In addition, a phylogenetic tree (Supplemental Fig. S1) based on ClustalX analysis of the predicted *PrMPK9-1* and *PrMPK9-2* protein sequences with MAPK sequences from Arabidopsis, rice, *P. trichocarpa*, alfalfa, *Zea mays*, and *Homo sapiens* also placed the proteins in the large

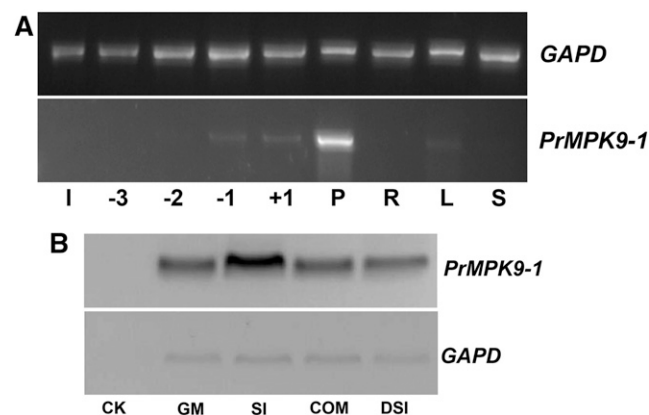


Figure 1. Expression analysis of *PrMPK9-1*. A, Semiquantitative RT-PCR expression analysis of *PrMPK9-1*. *PrMPK9-1* shows increasing expression during pollen development and low expression in other tissues. I, Immature anthers; -3/-2/-1/+1, stages of anther development (number of days from anthesis); P, hydrated pollen; R, root; L, leaf; S, stigma. GAPD shows control gene expression. B, RT-PCR expression analysis of *PrMPK9-1* shows increased expression 10 min after SI induction in incompatible (SI) and compatible (COM) pollen or heat-denatured PrsS (DSI). CK, Control using water as a template; GM, control pollen growing in growth medium (GM). GAPD shows control gene expression.

clade of group D MAPKs. The poppy proteins exhibited highest homology with AtMPK9. Gene duplication is a prominent feature within both the eudicot and monocot MPK gene families (Hamel et al., 2006; Rodriguez et al., 2010). For example, in *P. trichocarpa*, two paralogs of AtMPK16 have been identified (*PtMPK16-1* and *PtMPK16-2*). The high similarity between *PrMPK9-1* and *PrMPK9-2* suggests this to be the case for the poppy MPK9. Thus, *PrMPK9-1* and *PrMPK9-2* appear to be paralogs of AtMPK9, although the latter is present only as a single copy in the Arabidopsis genome.

Evidence of a Direct Link between *PrMPK9-1* and p56-MAPK

Although evidence indicated that p56-MAPK is not an ortholog of AtMPK3, we had shown previously that p56-MAPK cross-reacts with an anti-AtMPK3 antibody (Li et al., 2007). Therefore, we checked if the product of *PrMPK9-1* was detected by the anti-AtMPK3 antiserum. We expressed the full-length His-tagged *PrMPK9-1* protein in *Escherichia coli*. Western blotting confirmed that this recombinant protein could be detected with anti-His tag antiserum (Fig. 2A) and anti-AtMPK3 (Fig. 2B). Given this observation, we sought to obtain direct lines of evidence to demonstrate that *PrMPK9-1* encodes the p56-MAPK.

Initially, we used western blotting to detect *PrMPK9-1*. As MAPKs share many conserved domains, we made an antibody raised against the divergent C-terminal region (103 amino acids) of *PrMPK9-1*, as the full-length protein was unlikely to be sufficiently specific. This C-terminal

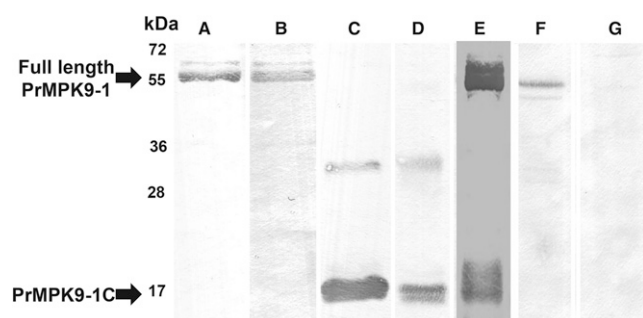


Figure 2. Western-blot analysis of *PrMPK9-1*. A, Expression of full-length *PrMPK9-1* recombinant protein was detected with anti-His tag antiserum (1:1,000). B, Anti-AtMPK3 antiserum (1:1,000) cross-reacted with recombinant full-length *PrMPK9-1*. C, C-terminal His-tagged *PrMPK9-1C* truncated recombinant protein (~16 kD) was detected with the anti-His tag antiserum; *PrMPK9-1C* antiserum was raised against this protein. D, *PrMPK9-1C* truncated recombinant protein cross-reacted with the *PrMPK9-1C* antiserum. E, *PrMPK9-1C* antiserum cross-reacted with the full-length *PrMPK9-1* recombinant protein. The smear at the bottom is a likely degradation product. F, *PrMPK9-1C* antiserum cross-reacted with a protein from *P. rhoeas* pollen extracts, migrating at 56 kD. G, Preimmune serum did not cross-react with the *PrMPK9-1C* recombinant protein. The arrows indicate the positions to which full-length *PrMPK9-1* and C-terminal truncated *PrMPK9-1C* migrate.

His-tagged *PrMPK9-1C* truncated recombinant protein (~16 kD) was detected with the anti-His tag antiserum (Fig. 2C). The *PrMPK9-1C* antiserum cross-reacted with the truncated *PrMPK9-1C* recombinant protein (Fig. 2D) and with full-length *PrMPK9-1* recombinant protein (Fig. 2E). We also ascertained that it detected a protein migrating at 56 kD in crude pollen extracts from *P. rhoeas* (Fig. 2F). Preimmune serum did not cross-react with the *PrMPK9-1C* recombinant protein (Fig. 2G).

We then examined pollen extracts that had been SI induced (Fig. 3A) to see if we could detect an ~56-kD MAPK protein that was activated by SI. Western blotting using anti-pTXpY to detect activated MAPKs showed minimal levels of phosphorylated p56-MAPK activity in untreated pollen and increased activity in SI-induced samples (Fig. 3B); several activated MAPKs also were detected, but their activity was approximately the same in both samples. When the membrane was reprobed with anti-*PrMPK9-1C* (Fig. 3C), this antibody detected a protein at the same molecular mass as the phosphorylated p56-MAPK, but, as expected, it did not distinguish between phosphorylated and unphosphorylated samples. This provides good evidence that *PrMPK9-1* corresponds with the p56-MAPK phosphoprotein. The top band at ~70 kD is a cross-reacting contaminant from the recombinant *PrsS* used for the SI induction (Supplemental Fig. S3).

We previously used calyculin A (a Ser/Thr protein phosphatase inhibitor) to obtain increased levels of phosphorylated MAPKs in pollen extracts and used a pTXpY antibody to detect the activated form of p56 (Rudd et al., 2003; Li et al., 2007). To determine that *PrMPK9-1* corresponded to this phosphoprotein, we treated pollen with calyculin A. Equal amounts of untreated- and calyculin A-treated pollen extracts (Fig. 3D) were immunoprecipitated using biotinylated *PrMPK9-1C* antiserum and probed with anti-pTXpY antibody (Fig. 3, E and F). This revealed an activated ~56-kD protein immunoprecipitated by anti-*PrMPK9-1C* in the calyculin-treated sample (Fig. 3E) but not in the untreated sample (Fig. 3F). Corresponding immunoprecipitates of untreated and SI-induced samples probed with anti-pTXpY antiserum (Fig. 3G) also gave an ~56-kD protein, present in the SI sample and not in the untreated sample. We believe the bottom band to be a degradation product. These data provide good evidence that *PrMPK9-1* corresponds with a pollen phosphoprotein that has the properties of the SI-activated p56-MAPK identified previously (Rudd et al., 2003; Li et al., 2007).

To unequivocally determine the identity of the protein in the calyculin A-treated pollen sample, a 56-kD band was excised from the sample immunoprecipitated by *PrMPK9-1C* antiserum (Fig. 3E) and analyzed using FT-ICR mass spectrometry. Importantly, this revealed that all the peptides present in this 56-kD band had highly significant matches with *PrMPK9-1* (Supplemental Table S2). Together, these data provide compelling evidence that *PrMPK9-1* encodes the p56-MAPK.

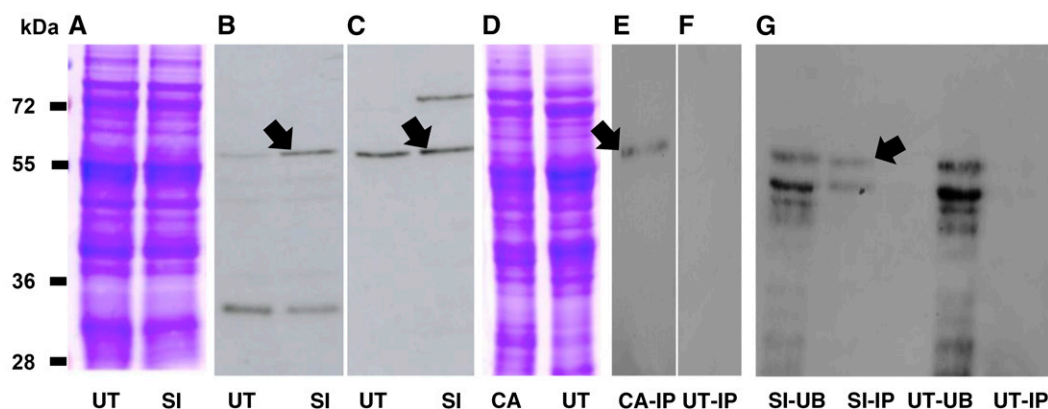


Figure 3. PrMPK9-1C antiserum cross-reacts with a 56-kD pollen phosphoprotein. A, Coomassie Blue staining of untreated (UT) and SI-induced (SI) pollen extracts. B, Western blotting of samples in A using anti-pTxpY antiserum revealed the detection of increased levels of a phosphorylated (activated) ~56-kD phosphoprotein in SI-induced samples (arrow) compared with untreated pollen (left). C, Reprobing this blot with PrMPK9-1C antibody revealed a 56-kD protein of approximately equal intensity in both untreated and SI-induced samples. For an explanation of the ~72-kD protein detected, see Supplemental Figure S3. D, Coomassie Blue staining of calyculin A-treated (CA) and untreated pollen samples. E and F, Immunoprecipitation of the samples in D using PrMPK9-1C antiserum followed by the detection of immunoprecipitated activated MAPKs using western blotting and probing with the anti-pTxpY antibody revealed that an ~56-kD protein was immunoprecipitated by PrMPK9-1C antibody specifically in the calyculin A-treated (CA-IP, arrow) sample (E) but not in the untreated (UT-IP) sample (F). G, Immunoprecipitation of SI-induced samples using PrMPK9-1C antiserum revealed an ~56-kD protein (SI-IP) detected by anti-pTxpY antiserum (arrow). Equivalent untreated samples did not have a detectable ~56-kD protein. UB, Unbound fractions.

PrMPK9-1 Is Cytosolic and Does Not Alter Its Localization after SI Induction

As some MAPKs translocate to the nucleus after activation (Cheong et al., 2003), we investigated the intracellular localization of PrMPK9-1 in untreated pollen tubes and after SI induction using immunolocalization. Fixed pollen tubes were probed with the purified anti-PrMPK9-1C antiserum and visualized using anti-rabbit IgG-fluorescein isothiocyanate (FITC) secondary antibody (Fig. 4). This revealed that PrMPK9-1C appeared to have a largely cytosolic localization throughout untreated pollen tubes (Fig. 4, A–C). Examining localization after SI induction revealed that PrMPK9-1C retained its mostly cytosolic localization (Fig. 4, D–F). Even several hours after SI induction, PrMPK9-1C localization remained similar to the observed localization in normally growing pollen tubes. We conclude that, unlike some other MAPKs that have been shown to undergo rapid translocation to the nucleus (e.g. PcMPK3a/b and PcMPK6 during the innate immunity response in parsley [*Petroselinum crispum*] cells; Lee et al., 2004), PrMPK9-1 does not alter its localization after activation.

Antisense Oligonucleotides for PrMPK9-1 Block the SI Response in Pollen Tube Growth

Having obtained compelling evidence that PrMPK9-1 is the poppy p56-MAPK, we wished to establish that PrMPK9-1 was involved in the SI response. Although we

had speculated earlier that p56 was unlikely to be involved directly in pollen inhibition, as its peak activation was 10 min after SI induction, this did not rule out a possible role in decision-making after the rapid tip inhibition. In order to establish whether the p56-MAPK was functionally involved in the early steps of the SI response, we used *in vitro* SI bioassays using antisense oligonucleotides. This approach has been used previously to demonstrate a functional role for a pollen-soluble inorganic pyrophosphatase (de Graaf et al., 2006) and for the PrpS pollen S-determinant (Wheeler et al., 2009) in SI, as they block the inhibition response. We hypothesized that if the PrMPK9-1 MAPK is functionally involved in signaling to targets/mechanisms involved in SI, knockdown of its expression should result in the alleviation of pollen tube inhibition in an S-specific manner. Therefore, we induced SI *in vitro* in the presence of either antisense (as-ODNs) or sense (s-ODNs) oligonucleotides for PrMPK9-1 to test this hypothesis.

SI induction in pollen grown *in vitro*, stimulated by the addition of recombinant PrsS proteins, resulted in highly significantly inhibited pollen tube growth (a reduction of 75.25% in length; $P = 2.55 \times 10^{-45}$, $n = 150$). Compatible pollen in combination with the same PrsS proteins did not result in any significant difference in lengths compared with compatible untreated pollen tubes ($P = 0.062$, not significant). When SI was induced in pollen pretreated with antisense MPK9-1 oligonucleotides (as-ODNs), we observed a significant alleviation of the SI-specific inhibition (Fig. 5). Addition of the

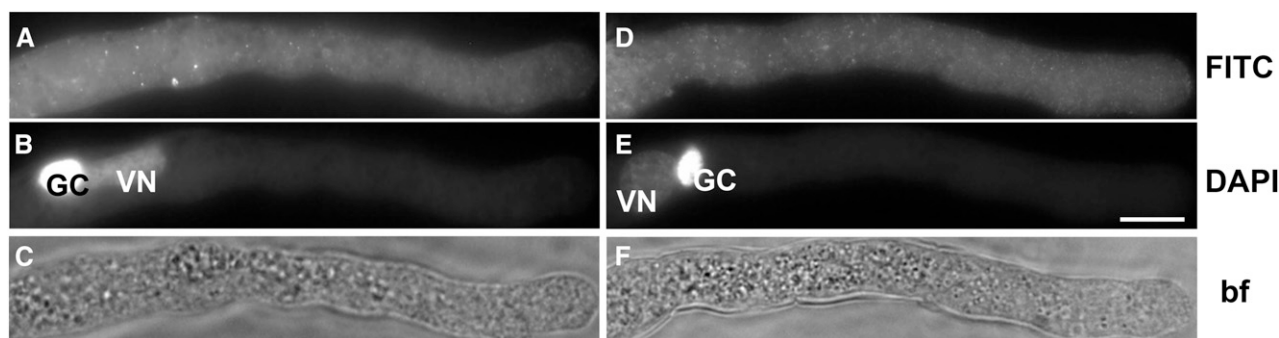


Figure 4. Immunolocalization of PrMPK9-1 in poppy pollen. A to C, Representative examples of the localization of PrMPK9-1 in a typical normally growing pollen tube. A, PrMPK9-1 localization using PrMPK9-1C antiserum and anti-rabbit IgG-FITC secondary antibody using fluorescence microscopy with an FITC filter. PrMPK9-1 appears to be a largely cytosolic protein localized throughout the pollen tube. B, 4',6-Diamino-phenylindole (DAPI) staining showing the pollen tube generative cell (GC) and vegetative nucleus (VN). C, Bright-field (bf) image. D to F, Representative examples of the localization of PrMPK9-1 at 30 min after SI induction. D, PrMPK9-1 localization using PrMPK9-1C antiserum and anti-rabbit IgG-FITC secondary antibody using fluorescence microscopy with an FITC filter. PrMPK9-1 retains its largely cytosolic localization. E, 4',6-Diamino-phenylindole staining showing the pollen tube generative cell and vegetative nucleus. F, Bright-field image. Bar = 10 μm .

as-ODNs resulted in a highly significant recovery of SI-induced tubes (333.4% longer pollen tube length compared with SI-induced tubes; $P = 2.4 \times 10^{-44}$, $n = 150$). The sense version of this oligonucleotide (s-ODN) did not alleviate SI-induced inhibition, and SI-induced pollen tubes in the presence of s-ODNs were significantly shorter than untreated pollen tubes ($P = 2.3 \times 10^{-36}$, $n = 150$). The as-ODN and s-ODNs alone had no effect on normal pollen tube growth ($P = 0.856$, not significant, and $P = 0.181$, not significant, respectively; Fig. 5). Together, these data demonstrate that PrMPK9-1 has a crucial role in the signaling network resulting in the SI-induced S-specific irreversible pollen tube inhibition.

Antisense Oligonucleotides for *PrMPK9-1* Block the Formation of SI-Induced Actin Punctate Foci

As we had shown previously that alterations in actin polymerization status can trigger PCD in pollen (Thomas et al., 2006) and that the formation of punctate F-actin foci is a key feature of SI induction (Geitmann et al., 2000), we wished to establish whether PrMPK9-1 was functionally involved in mediating the formation of these distinctive actin foci formed specifically in incompatible pollen. To examine this, we took a similar approach, inducing SI in vitro in the presence of either *PrMPK9-1* antisense (as-ODNs) or sense (s-ODNs) oligonucleotides and imaging F-actin in pollen tubes after 2 h. SI triggered the formation of the F-actin foci (Fig. 6A), while untreated pollen tubes had normal F-actin configurations (Fig. 6B); as-ODNs in combination with SI induction rescued the pollen F-actin, which showed a normal configuration (Fig. 6C), while s-ODNs in combination with SI induction had no effect (Fig. 6D); control treatment with as-ODNs (Fig. 6E) or s-ODNs alone had no effect. Quantification of these data (Fig.

6F) revealed that pollen tubes pretreated with as-ODNs prior to SI induction had significantly fewer actin foci compared with SI alone ($P = 1.14 \times 10^{-10}$, $n = 200$), while s-ODN pretreatment prior to SI was significantly

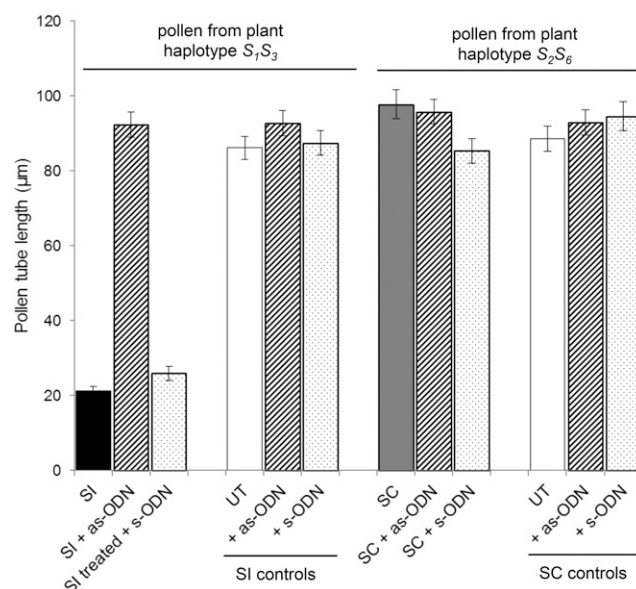


Figure 5. *PrMPK9-1* antisense oligonucleotides attenuate SI specifically in incompatible pollen tubes. Mean pollen tube length is shown for incompatible (black bar) and compatible (gray bar) pollen after treatment with antisense (as-ODN; diagonals) or sense (s-ODN; dotted) oligonucleotides prior to SI treatment. With SI treatment, incompatible (SI) pollen tubes are inhibited (black bar); inhibition of growth is alleviated by the *PrMPK9-1* as-ODNs, while compatible (SC) pollen treated with the same recombinant PrsS proteins (PrsS₁ and PrsS₃) grow normally (gray bar). Untreated controls (UT; white bars) are unaffected by *PrMPK9-1* ODNs. Four independent data sets were used, totaling $n = 200$ for each treatment; error bars indicate $\pm \text{SE}$.

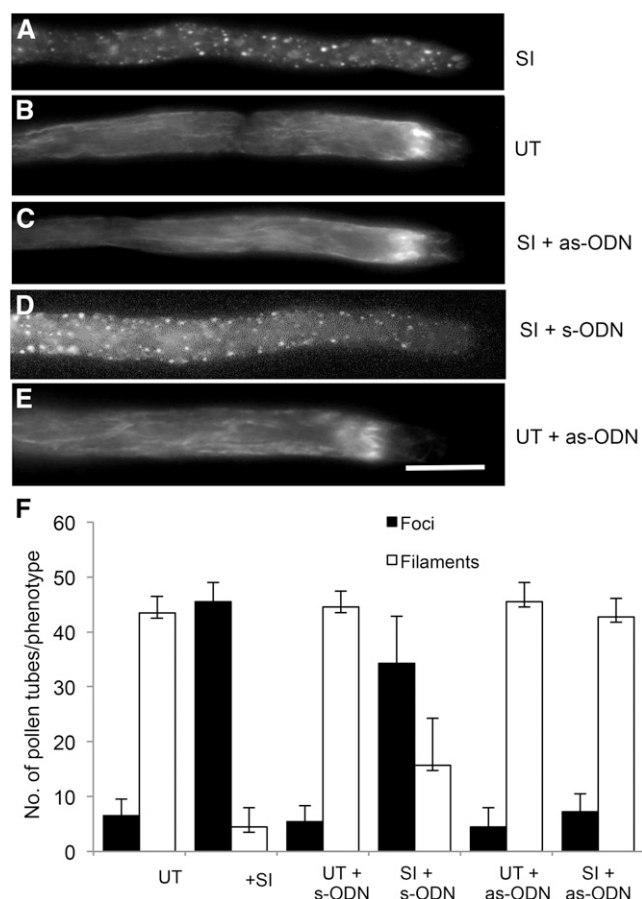


Figure 6. *PrMPK9-1* antisense oligonucleotides prevent the formation of SI-induced actin punctate foci. Pollen tubes were pretreated for 45 min with antisense (as-ODN) or sense (s-ODN) oligonucleotides, and SI was induced for 2 h. Typical representative images of actin configuration in pollen tubes are shown. A, Two hours after SI treatment, the pollen tube exhibits punctate actin foci (scored as phenotype foci in F). B, An untreated (UT) pollen tube control (scored as phenotype filaments in F). C, Actin configuration in an as-ODN-treated SI pollen tube showing normal actin filament organization. D, Actin configuration in an s-ODN-treated SI pollen tube showing punctate actin foci. E, Actin configuration in an as-ODN control untreated pollen tube showing filaments. Bar = 10 μ m. F, Quantification of the effect of pretreatment of pollen with antisense (as-ODN) or sense (s-ODN) oligonucleotides: untreated or SI treated. Four independent experiments scored 50 pollen tubes each for actin phenotype (foci or filaments). Error bars indicate SE.

different from the as-ODN pretreatment with SI ($P = 0.0005$). The as-ODN and s-ODNs alone had no effect ($P = 0.21$, not significant, and $P = 0.2814$, not significant, respectively). Together, these data demonstrate that *PrMPK9-1* is upstream of SI signaling to the formation of punctate actin foci.

Antisense Oligonucleotides for *PrMPK9-1* Block SI-Induced PCD in Incompatible Pollen

We previously obtained data implicating a MAPK as being functionally involved in mediating PCD in

incompatible pollen (Li et al., 2007). However, as this involved a pharmacological approach, which would inhibit all pollen MAPKs, and as the identity of p56 at that time was unknown, we could not link p56 to being functionally involved in mediating SI-induced PCD. Here, having cloned p56, we used the fluorogenic caspase-3/7 live-cell substrate, Image iT LIVE Green Caspase-3 and Green Caspase-7 Detection Kit (see "Materials and Methods"), in combination with the antisense *PrMPK9-1* oligonucleotides to examine whether *PrMPK9-1* was involved in mediating SI-induced PCD in incompatible pollen. We induced SI in vitro in pollen tubes that had been pretreated with either antisense (as-ODNs) or sense (s-ODNs) oligonucleotides and imaged caspase-3-like activity in pollen tubes after 4 to 5 h, when peak activity was expected (Bosch and Franklin-Tong 2007), to test this hypothesis. Untreated pollen tubes showed no FITC fluorescence indicating caspase-3-like activity (Fig. 7, A and B), while SI-treated pollen tubes had increased levels of fluorescence (Fig. 7, C and D); pollen tubes treated with as-ODNs prior to SI had background levels of fluorescence similar to untreated controls (Fig. 7, E and F), while the control treatment with s-ODNs prior to SI had no effect, with DEVDase activity still detected (Fig. 7, G and H). Quantification of these data (Fig. 7I) revealed that the as-ODNs resulted in a highly significant recovery of SI-induced tubes compared with SI-induced treatment alone ($P = 8.790 \times 10^{-10}$, $n = 250$), and s-ODN pretreatment prior to SI had a significantly different effect compared with that of the as-ODNs (3.60×10^{-8}). The as-ODN and s-ODNs alone had no effect ($P = 0.250$, not significant, and $P = 0.409$, not significant, respectively). Together, these data demonstrate that *PrMPK9-1* plays a key role in the signaling network resulting in caspase-3-like activation, which will induce PCD in incompatible pollen tubes.

DISCUSSION

MAPKs have been shown to be key players in regulating many signaling networks in plants (Pitzschke et al., 2009; Meng and Zhang, 2013; Xu and Zhang, 2015). Despite a good knowledge of the MPK gene families for some time now, the function of only a handful of MPK genes have been identified. Here, we have identified the pollen-specifically expressed *PrMPK9-1*, a developmentally expressed TDY MAPK, with high expression in mature pollen, as being involved in the SI response. We took an unusual backward approach, as we had a well-characterized biological phenomenon (SI) that we had shown previously to involve a MAPK activity (Rudd et al., 2003), but we did not know which of the 20 MAPKs was involved. To our knowledge, our studies are the first implicating a function for a MAPK that is expressed in pollen. Moreover, *PrMPK9-1* falls in the (so far) rather poorly characterized group of TDY class (group D,

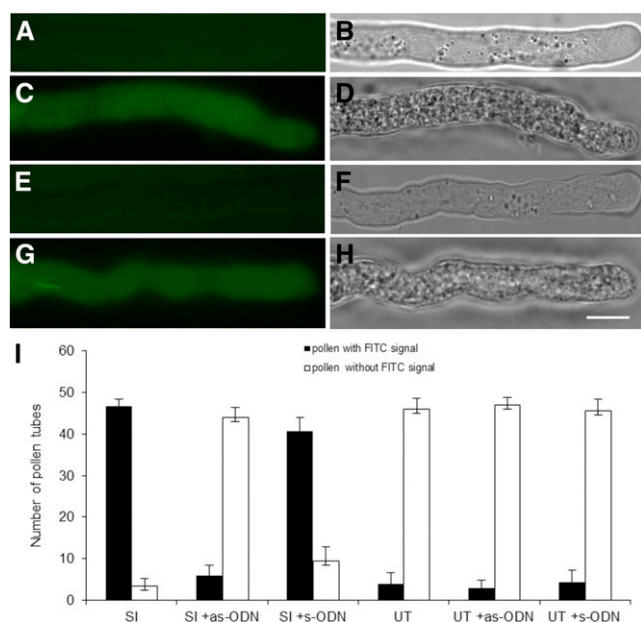


Figure 7. Antisense oligonucleotides block the SI-induced DEVDase/caspase-3-like activity. A, Representative image of an untreated (UT) pollen tube with no DEVDase/caspase 3-like activity. B, Corresponding bright-field image. C, Representative image of an SI-induced pollen tube after 4 h, using live-cell caspase 3/7 activity probe and an FITC filter set, showing fluorescence. D, Corresponding bright-field image. E, Representative image of an SI-induced pollen tube with antisense *PrMPK9-1* oligonucleotide pretreatment (SI + as-ODN) after 4 h, using live-cell caspase 3/7 activity probe and an FITC filter set, with no detectable FITC signal. F, Corresponding bright-field image. G, Representative image of an SI-induced pollen tube with sense *PrMPK9-1* oligonucleotide pretreatment (SI + s-ODN) after 4 h, using live-cell caspase 3/7 activity probe and an FITC filter set, with fluorescence detected. H, Corresponding bright-field image. Bar = 10 μ m. I, Quantification of the effect of oligonucleotide pretreatment of pollen tubes (as-ODN or s-ODN) on DEVDase activity. Five independent experiments scored 50 pollen tubes in total for each treatment, and the percentage of pollen tubes in each category is indicated. Error bars indicate SE.

family V) MAPKs. Below, we discuss the implications of our findings.

Identity of PrMPK9-1 as a TDY MAPK

The *P. rhoeas* *MPK9-1* gene belongs to the large MPK clade D, which has a TDY motif instead of TEY and an extended C-terminal region relative to the other MAPKs (Ichimura et al., 2002). The group D MAPKs are unique, owing to their TDY motif in the activation loop and long C-terminal common-docking domain (Rodriguez et al., 2010). Although the existence of the TDY-type clade D MAPKs is well established and the fact that all plants have MAPKs in this group indicates their functional importance, relatively little is known about the function of MAPK members of clade D. Moreover, functional redundancy between MAPKs makes it difficult to employ a genetic approach to

determine their functions. Thus, rather little is known about the function of TDY MAPKs in plants. A wealth of information relating to TEY MAPKs exists, and most of what we know about MAPK function relates to the TEY MAPKs, with studies largely focused on a relatively small subset of MAPKs, primarily MPK3/4/6, which are all in the A and B subgroups of TEY-type MAPKs. Early research on plant MAPKs focused on their functions in immunity and stress responses, as many of the TEY MAPKs were found to function in these (for review, see Meng and Zhang, 2013). More recent studies have revealed that many TEY MAPKs also play key roles in plant growth and development (for review, see Xu and Zhang, 2015).

Only a few members of subfamily D have been functionally characterized to date, so insights into functions for TDY MAPKs are limited. However, all four of those that have been characterized to date are phylogenetically closely clustered together in a group. The alfalfa MAPK, *TDY1*, was one of the first of the TDY-clade MAPKs to be characterized. High expression in root nodules and root and shoot apices suggested a possible role in tip growth initiation/development, and *TDY1* promoter studies revealed induction by mechanical and pathogen wounding (Schoenbeck et al., 1999). In rice, the TDY MAPK *BWMK1* (blast- and wound-induced MAPK) was shown to be induced by infection by both infection of the rice blast fungus *Magnaporthe grisea* and mechanical wounding (He et al., 1999). More recently, it was shown that *BWMK1* is activated by fungal elicitors, hydrogen peroxide, salicylic acid, and jasmonic acid (Cheong et al., 2003). Other studies in cotton (*Gossypium raimondii*) showed that *MPK9* (together with *MPK13* and *MPK25*) confer resistance to *Verticillium dahlia*, which causes defoliation (Zhang et al., 2014). These studies provide evidence that these TDY MAPKs play a role in signaling to defense signaling. In contrast, a landmark study in *Arabidopsis* revealed that *MPK9* was preferentially and highly expressed in stomatal guard cells and has a completely separate function. Studies revealed that *MPK9* is functionally redundant with *MPK12* (a TEY MAPK) and that together they acted as positive regulators of ROS-mediated abscisic acid-induced stomatal guard cell closure (Jammes et al., 2009). Although most of this study focused on *MPK12*, it clearly showed that *MPK9* and *MPK12* function together in regulating transpiration. More recently, it has been shown that these two MPKs also function in methyl jasmonate signaling to induce stomatal closure (Khokon et al., 2015). Data indicate that *MPK9* and *MPK12* function upstream of S-type anion channel activation and downstream of ROS production, cytosolic alkalization, and $[Ca^{2+}]_{cyt}$ elevation in guard cells (Khokon et al., 2015). A further *Arabidopsis* TDY MAPK has been functionally characterized recently: *Arabidopsis* *MPK8* is activated by mechanical wounding, and it has been shown that the *MPK8* pathway negatively regulates ROS accumulation by controlling the expression of

the *RbohD* gene (Takahashi et al., 2011). Here, we have shown that PrMPK9-1, which is closely related to these TDY MAPKs, functions in signaling to SI in incompatible pollen. We speculate that different functions have evolved for these MAPKs in different cell types.

PrMPK9-1 Functions in Pollen in the SI Signaling Network

To our knowledge, to date, no known function for *AtMPK9* in pollen has been found, although the Arabidopsis Affymetrix chip data indicate that *AtMPK9* is expressed in pollen. This suggests that this MAPK has evolved a specific function within the context of SI. *PrMPK9-1* exhibits increased expression after SI induction, and the protein is activated by phosphorylation after SI induction. Here, using an antisense oligonucleotide approach, we have demonstrated that PrMPK9-1 has a crucial role in the signaling network leading to the SI-induced S-specific pollen tube inhibition and PCD. Our study identifies a key role for PrMPK9-1 in signaling to characteristic SI-mediated actin cytoskeleton reorganization (Geitmann et al., 2000; Snowman et al., 2002; Poulter et al., 2010) and activation of a DEVDase activity that is required for PCD in incompatible pollen (Thomas and Franklin-Tong, 2004; Bosch and Franklin-Tong, 2007). Moreover, our previous studies on SI have shown that actin alterations are required for PCD (Thomas et al., 2006). Our data place PrMPK9-1 as a key player upstream of actin alterations and PCD. MAPK signaling to the actin cytoskeleton is well established (Samaj et al., 2004). Integrating our existing knowledge of the SI signaling network with our new data on PrMPK9-1, we can place PrMPK9-1 activation, which peaks at 10 min after SI induction (Rudd et al., 2003), downstream of the almost instantaneous increases in $[Ca^{2+}]_{cyt}$ (Franklin-Tong et al., 1993, 1997), downstream of increases in ROS that peak at 5 min post SI (Wilkins et al., 2011), and upstream of the later formation of punctate actin foci and DEVDase activation. Thus, PrMPK9-1 occupies a critical position within the SI-PCD signaling network.

Close Relatives *AtMPK8* and *AtMPK9* May Function in Similar Signaling Networks

It is of considerable interest that two of the few Arabidopsis MAPKs belonging to the TDY group that have been functionally characterized so far, *MPK8* and *MPK9*, are the closest relatives to the *P. rhoeas* PrMPK9-1 and have been shown to be involved in regulating ROS in different signaling networks (Jammes et al., 2009; Takahashi et al., 2011; Khokon et al., 2015). Thus, together, current data suggest that several TDY MAPKs, *AtMPK8*, *AtMPK9*, and *PrMPK9-1*, play a key role in regulating ROS homeostasis. As all three MAPKs are close relatives, this may not be a coincidence. It will be interesting to see if future studies show that further TDY MAPKs in this cluster generally share this

phenomenon. Moreover, a study by Menges et al. (2008), using Gene Ontology overrepresentation analysis to analyze biological processes potentially involved in unknown MAPK functions, identified *MPK9* as being potentially involved in cytoskeleton organization. Here, we have provided direct experimental evidence that the *P. rhoeas* PrMPK9-1 is upstream of and required for regulating the highly distinctive SI-induced actin reorganization into punctate foci. Indeed, it appears that the signaling network found in *P. rhoeas* pollen undergoing SI-PCD is rather similar (at least superficially) to that for *MPK8* expressed in various tissues (Khokon et al., 2015). While the methyl jasmonate pathway in guard cells shares several signaling components with the abscisic acid pathway (ROS, $[Ca^{2+}]_{cyt}$ alterations, and cytosolic alkalization), the SI-PCD network also involves ROS and $[Ca^{2+}]_{cyt}$ increases and alterations in cytosolic pH, but a dramatic acidification is triggered (Wilkins et al., 2015). Future studies should explore whether other TDY MAPKs in this cluster generally share these key signaling components.

In summary, here, we show that *PrMPK9-1* is a TDY MAPK that is expressed in a distinct cell type (pollen) and provide evidence that it has a cell type-specific function for *PrMPK9-1*, with a distinct role from *AtMPK9*. PrMPK9 is a key regulator for SI in pollen and acts upstream of PCD involving alterations in actin organization and activation of a DEVDase/caspase-3-like enzyme. Thus, the role of *MPK9-1* expressed in pollen plays a role in a very different biological phenomenon to the other TDY MAPKs analyzed at this level to date (ROS, $[Ca^{2+}]_{cyt}$ alterations, and cytosolic alkalization). However, the signaling network in which it is placed appears (at least superficially) similar to other closely related MAPKs that have been characterized to date. It will be interesting in the future to see what functions and signaling networks further cell type-specific MPKs are involved in.

MATERIALS AND METHODS

Cloning of *Papaver rhoeas* MPK9-1: Sequence Analyses and Affymetrix Analysis

cDNA was synthesized using the Invitrogen SuperScript II Reverse Transcriptase kit using total RNA from *P. rhoeas* pollen (RNAeasy Plant Mini Kit; Qiagen). Initially, gene-specific primers based on Arabidopsis (*Arabidopsis thaliana*) MAPKs expressed in pollen (*AtMPK8*, *AtMPK9*, and *AtMPK17*) were used to obtain partial pollen-expressed *P. rhoeas* MPK cDNAs. An 880-bp cDNA fragment, amplified using primers *MPK17-5'4* and *MPK17-3'5*, was cloned and sequenced and shared highest identity with *AtMPK8* and *AtMPK9*. Full-length cDNAs of *PrMPK9-1* were obtained using 3' and 5' RACE PCR. 3' RACE amplification from *P. rhoeas* pollen cDNA used 3' RACE (3'RACE 17AP) and 5' gene-specific (*PrMPK8-3R1*) primers. The product was PCR purified and reamplified with 3' RACE primer (3'RACE UAP) and nested gene-specific 5' primer (*PrMPK8-3R2*). For 5' RACE, cDNA was synthesized using a primer specific to the 3' end of the gene (*PrMPK8-5R1*) instead of the oligo(dT) primer. cDNA was cleaned using the QIAquick PCR Purification Kit (Qiagen). A poly(C) tail was added at the 5' end. PCR amplification employed a 5' RACE abridged primer (5'RACE AAP) and a second gene-specific 3' primer (*PrMPK8-5R2*). This amplification product was reamplified with a 5' RACE unabridged primer (5'RACE UAP) and a third nested gene-specific 3' primer (*PrMPK8-5R3*). Full-length sequence was confirmed by

sequencing the mRNA product amplified using primers *PrMPK91gen5'* and *PrMPK8gen3'* (Table I).

DNA sequences were analyzed using Chromas software (Technelysium). Homology searches were prepared from the National Center for Biotechnology Information Web site utilizing the Entrez and BLAST (Altschul et al., 1997) functions. Alignments were made using ClustalW 1.7 and ClustalX2 (www.ebi.ac.uk/Tools/msa/clustalw2/). Arabidopsis MAPK gene expression was analyzed using data from Affymetrix gene chip experiments available via Genevestigator (https://www.genevestigator.com). Sequence alignments were prepared by ClustalW, and phylogenetic distributions were prepared by ClustalX on PHYLIP version 3.69.

Proteomic and Bioinformatic Analyses

For mass spectrometry analysis of the p56 protein, total pollen proteins were separated by SDS-PAGE and stained with Coomassie Blue. A band corresponding to 56 kD was excised and digested with Trypsin Gold (Promega). Peptides were identified by FT-ICR mass spectrometry searching NCBI Green Plant database using the SEQUEST algorithm.

RT-PCR

Standard RT-PCR techniques were used for expression analysis. Total RNA was extracted from various tissues from *P. rhoeas* plants (anthers at various stages of development, mature pollen, stigma, leaf, and root; RNeasy Plant Mini Kit; Qiagen) and cDNA was synthesized (Omniscript RT Kit; Qiagen). Gene-specific primers were used to amplify *PrMPK9-1* transcripts during RT-PCR (*PrMPK9-1RT-F/PrMPK9-1RT-R* and *PrMPK92gen5'/PrMPK92gen3'*) for semiquantitative experiments; primers for the *P. rhoeas* glyceraldehyde-3-phosphate dehydrogenase gene (*PrGAPD5'* and *PrGAPD3'*) acted as controls (Table II).

In Vitro Kinase Activity Assays

Kinase assays using pollen protein were performed according to Rudd et al. (1996). Briefly, pollen was homogenized on ice in 50 mM Tris-HCl, pH 7.5, and 0.1% Triton X-100 containing protease inhibitor cocktail, cell debris was removed by centrifugation, and supernatants were stored at -20°C until use. Recombinant His-tagged PrMPK9-1 was incubated with crude pollen extract in 50 mM Tris-HCl, pH 7.5, 1 mM ATP, 1 mM MgCl_2 , and 0.25 mM calyculin A for 15 min at 30°C . The His-tagged protein was isolated using an Ni-NTA Spin Column (Qiagen). Incorporation of ^{32}P into recombinant His-tagged proteins was measured after 10 min. Phosphorylated MAPK proteins were analyzed using SDS-PAGE followed by autoradiography. Phosphoproteins were enriched using the PhosphoProtein Purification Kit (Qiagen).

Pollen Tube Growth and SI Induction

Pollen was grown and SI was induced as described (Wilkins et al., 2011, 2015). Briefly, recombinant PrS proteins were expressed using nucleotide sequences specifying the mature peptide of the *PrS₁* and *PrS₃* alleles of the *PrS* gene (*pPRS100* and *pPRS300*) as described (Foote et al., 1994; Kakeda et al.,

Table II. Primers for RT-PCR

Name	Sequence 5'→3'
<i>PrMPK9-1RT-F</i>	GGAGTATCATCCCCAAATGCTTCAGG
<i>PrMPK9-1RT-R</i>	GCTTTTGTTGGGCTCTGAAGGG
<i>PrMPK92gen5'</i>	CTGCCATTAATTCATGTGTGGGG
<i>PrMPK92gen3'</i>	GAAAAACCCCTGAAACTATCAGTGG
<i>PrGAPD5'</i>	GAAGGGTGGTGCCAAGAAGGTTATC
<i>PrGAPD3'</i>	GCCTTCTTCTCAAGTCTCACAGTAAG

1998). SI was induced by adding recombinant PrS (to 10 mg mL^{-1}) to pollen of the appropriate S-haplotype to generate an incompatible combination (e.g. *PrS₁* and *PrS₃* with pollen from plants of haplotype *S₁S₃*). For calyculin A treatment, growing pollen was treated with 0.25 mM calyculin A (Sigma-Aldrich) for 10 min.

Pollen and Leaf Protein Extracts

Pollen proteins were prepared according to Rudd et al. (1996). Briefly, pollen was homogenized on ice in 50 mM Tris-HCl, pH 7.5, and 0.1% Triton X-100 containing protease inhibitor cocktail, cell debris was removed by centrifugation, and supernatants were stored at -20°C . Leaf proteins were extracted in a similar way, after grinding with liquid N_2 . Protein concentration was estimated using the Bio-Rad assay.

Recombinant PrMPK9-1 Protein Expression

The full-length coding region of *PrMPK9-1* was amplified using primers *PrMPK9-1_5'NdeI* and *PrMPK9-1_3'XhoI*, captured in cloning vector pDrive (Qiagen), excised, and ligated into expression vector pET21-b(+) (Novagen). The expression of recombinant PrMPK9-1 with a C-terminal 6× His tag was induced in *Escherichia coli* BL21 using 1 mM isopropylthio- β -D-galactosidase. Recombinant PrMPK9-1 was isolated using Ni-NTA agarose (Qiagen) and eluted with 100 mM imidazole. To produce the C-terminal region of PrMPK9-1 (residues 391–493; PrMPK9-1C), a partial coding region of *PrMPK9-1* was amplified using primers *PrMPK9-1C_5'NdeI* and *PrMPK9-1_3'XhoI* and cloned into pET21-b(+). The biotin-tagged version of PrMPK9-1C (used for antibody purification) was cloned using primers *PrMPK9-1PP5'HindIII* and *PrMPK9-1PP3'NotI* in the PinPoint Xa Protein Purification System (Promega; Table III).

Production and Purification of Recombinant PrMAPK9-1 Antisera

Polyclonal antibodies were raised in rabbits against full-length recombinant PrMPK9-1 (Biogenes) and also the C-terminal portion of PrMPK9-1 (residues 391–493; PrMPK9-1C) to obtain an antibody specific to PrMPK9 (Biogenes). The anti-PrMPK9-1C antibody was affinity purified using the biotinylated version of PrMPK9-1C (PrMPK9-1CPP) and the PinPoint Xa Protein Purification System (Promega). Antibody captured by the PrMPK9-1CPP-TetraLink Tetrameric Avidin Resin (Pierce) was eluted using 100 mM citric acid (pH 3).

Biotinylation of anti-PrMPK9-1C Antibody and Immunoprecipitation

Purified anti-PrMPK9-1C antibody was biotin labeled using the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce), following the manufacturer's instructions for calculations (<http://www.piercenet.com/haba/habacalc.cfm>).

Table I. Primers for cloning of PrMPK9-1

Name	Sequence 5'→3'
<i>MPK17-5'4</i>	GTGGAGATCAAGCAYATYATGC
<i>MPK17-3'5</i>	CGAGTCGGGCAAACTSTYTTTG
<i>3'RACE17AP</i>	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT
<i>PrMPK8-3R1</i>	GATGTACGAGAATTAATTTACCG
<i>3'RACEUAP</i>	GACTCGAGTCGACATCGA
<i>PrMPK8-3R2</i>	GGAGTATCATCCCCAAATGCTTC
<i>PrMPK8-5R1</i>	GTTCAGGAGTAAGATCATCATTTGCC
<i>5'RACE AAP</i>	GGCCACGCGTCGACTAGTACGGG55GGG55GGG55G
<i>PrMPK8-5R2</i>	GATCTGATTCCATCAACTCAAATACG
<i>5'RACEUAP</i>	GGCCACGCGTCGACTAGT
<i>PrMPK8-5R3</i>	AAATATCTTTAAATCTCTCCTAGATC
<i>PrMPK91gen5'</i>	GAATCAAGGAACAAGAGAAGAAGAAG
<i>PrMPK8gen3</i>	GTAGTTATCTGCCGACTTTCTGG

Table III. Primers for amplification of PrMPK9-1

Name	Sequence 5'→3'
<i>PrMPK9-1_5'NdeI</i>	ATTCATATGGGCGAGACGGAATTTTCAC
<i>PrMPK9-1_3'XhoI</i>	AATCTCGAGGGAACCTAGTTGAGAAATCTTC
<i>PrMPK9-1C_5'NdeI</i>	ATTCATATGGGTGAGAAAAGCACTCCTCC
<i>PrMPK9-1PP5'HindIII</i>	ATTAAGCTTGGTGAGAAAAGCACTCCTCC
<i>PrMPK9-1PP3'NotI</i>	AATGCGGCCGCTCAGGAACCTAGTTGAGAAATCTT

Biotin incorporation was confirmed using the Cuvette Format. The biotinylated PrMPK9-1C antibody was linked to TetraLink Tetrameric Avidin Resin (Pierce). Biotinylated anti-PrMPK9-1C antibody-avidin resin was added to pollen extracts, with immunoprecipitation buffer (PBS + 1 mM PMSF), and incubated overnight at 4°C. The resin was washed and recovered by centrifugation, the supernatant was removed and incubated in 100 mM citric acid, pH 3, and proteins were eluted into Tris buffer, pH 8.

SDS-PAGE and Western Blotting

Proteins were separated using SDS-PAGE and electroblotted (Bio-Rad). Immunoblots were probed with primary antibodies (rabbit anti-His tag [1:1,000; Sigma-Aldrich], rabbit anti-AtMPK3 antibody [1:1,000; Sigma-Aldrich], rabbit anti-full-length PrMPK9-1 antibody [1:1,000], rabbit anti-PrMPK9-1C antibody [1:1,000], and anti-ACTIVE MAPK polyclonal antibody [pTxpY; 1:2,000; Cell Signaling Technology]) in blocking solution (1× TBS and 5% milk powder). Blots were washed and then incubated with secondary antibody (goat anti-rabbit IgG-alkaline phosphatase; 1:5,000; Sigma-Aldrich). Proteins were detected using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium alkaline phosphatase detection (Promega) or ECL Detection Reagent (Promega) with an AGFA CURIX 60 processor. To reprobe, blots were stripped in 0.05 M sodium phosphate (pH 6.5), 10 M urea, and 0.1 M β -mercaptoethanol for 30 min at 60°C.

Immunolocalization

Pollen tubes were prefixed using 3-maleimodobenzoic acid *N*-hydroxy-succinimide ester (400 μ M; Pierce), then fixed in 2% formaldehyde, washed in actin-stabilizing buffer, and permeabilized, as described (Thomas et al., 2006). Samples were incubated with PinPoint purified anti-PrMPK9-1 antibody (1:1,000), washed in TBS, then incubated with anti-rabbit FITC antibody (1:300). Confocal images were collected using a Zeiss LSM 710 microscope with ZEN10 software. Images were analyzed using ImageJ and archived as TIF files.

Antisense Oligonucleotide Perturbation of PrMPK9-1 Function in Pollen Tubes

Phosphorothioated gene-specific antisense oligodeoxynucleotides (as-ODN) and their sense controls (s-ODN), corresponding to the 1,303- to 1,326-bp region of the gene that had unique sequences to allow discrimination among MAPKs, were designed. Sequences were as follows: *PrMPK9-1* as-ODN (ttgTTGGGCTCTGAAGgt) and *PrMPK9-1* s-ODN (accctTCAGAGCCCA-Caaaa). Pollen was grown in vitro and pretreated with as-ODNs and s-ODNs as described (de Graaf et al., 2006; Wheeler et al., 2009) for 45 min prior to induction of SI by the addition of recombinant PrS as described (Snowman et al., 2002; Wilkins et al., 2015) and then left to grow.

For assays testing the effect of as-ODNs on the formation of SI-induced F-actin foci, pollen tubes were grown for 2 h after treatment and then fixed and treated as described (Poulter et al., 2010; Wilkins et al., 2011), staining with rhodamine-phalloidin (Invitrogen). The fluorogenic caspase-3/7 live-cell substrates, Image iT LIVE Green Caspase-3 and Green Caspase-7 Detection Kit (Thermo-Fisher Scientific), were used to measure the effect of as-ODNs on DEVDase/caspase-3-like activity in individual pollen tubes after 5 h. Pollen tubes were scored for a high level of GFP fluorescence (indicating caspase-3-like activity). Images were collected using a Nikon Eclipse TE300 fluorescence microscope (Nikon 60× or 100× Plan Apo oil-immersion objective, 1.4 NA) and a FITC filter block (excitation, 492 nm; Chromas) with Nikon NIS-Elements software. For quantification, 50 pollen tubes were assessed for the formation of F-actin foci in four independent experiments (total of 200 pollen tubes per treatment). For DEVDase assays, 50 pollen tubes were assessed in five independent experiments (total of 250 pollen tubes per treatment). Means were calculated \pm SE. *P* values were calculated using a Student's *t* test.

Accession Numbers

Sequence data from this article can be found in the EMBL data libraries under accession numbers LT634154 and LT634155.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Protein sequence alignment of PrMPK9-1, PrMPK9-2, and other plant MAPKs.

Supplemental Figure S2. Phylogenetic tree of MAPKs.

Supplemental Figure S3. Explanation for ~72kDa band in SI-induced samples with PrMPK9-1C.

Supplemental Table S1. Expression of MAPKs in Arabidopsis pollen.

Supplemental Table S2. Peptides identified from the ~56-kD protein band using FT-ICR mass spectrometry.

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