Investigating mechanisms involved in the self-incompatibility response in *Papaver rhoeas*

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Sexual reproduction in flowering plants is controlled by recognition mechanisms involving the male gametophyte (the pollen) and the female sporophyte (the pistil). Self-incompatibility (SI) involves the recognition and rejection of self- or incompatible pollen by the pistil. In *Papaver rhoeas*, SI uses a Ca^{2+} -based signalling cascade triggered by the *S*-protein, which is encoded by the stigmatic component of the *S*-locus. This results in the rapid inhibition of incompatible pollen tube growth. We have identified several targets of the SI signalling cascade, including protein kinases, the actin cytoskeleton and nuclear DNA. Here, we summarize progress made on currently funded projects in our laboratory investigating some of the components targeted by SI, comprising (i) the characterization of a pollen phosphoprotein (p26) that is rapidly phosphorylated upon an incompatible SI response; (ii) the identification and characterization of a pollen mitogen-activated protein kinase (p56), which exhibits enhanced activation during SI; (iii) characterizing components involved in the reorganization and depolymerization of the actin cytoskeleton during the SI response; and (iv) investigating whether the SI response involves a programmed cell death signalling cascade.

Keywords: self-incompatibility; pollen tube inhibition; calcium signalling; protein kinase; actin depolymerization; programmed cell death

1. INTRODUCTION

Self-incompatibility is an important recognition system used by many flowering plants to prevent self-fertilization. *Papaver rhoeas* employs a gametophytic system of SI control governed by a single, multi-allelic *S*-locus (Lawrence *et al.* 1978) with linked pollen and pistil components. This is described in detail in Franklin-Tong & Franklin (2003). The SI response of the poppy occurs on the stigma surface and involves a highly specific cell–cell recognition interaction resulting in the inhibition of pollen tube growth and ultimately its death. The pistil component of the system, the *S*-protein, has been identified and characterized (Foote *et al.* 1994). The *S*-proteins are *ca.* 15 kDa proteins, secreted by the stigmatic papillae cells, and share 51.3–63.7% homology (Kurup *et al.* 1998). All members of the *Papaver S*-protein family have a predicted conserved secondary structure comprising β -strand motifs and four conserved cysteine residues, linked by surface loops (Walker *et al.* 1996). Residues in loop six have been demonstrated to play an important part in the SI response (Kakeda *et al.* 1998). Although the pollen *S* gene has not yet been identified, a pollen membrane-bound protein, SBP, which binds to *S*-proteins, has been characterized (Hearn *et al.* 1996), and its importance in the SI response has been demonstrated (Jordan *et al.* 1999).

Considerable effort has been concentrated on elucidating the intracellular events downstream of the initial pollen–*S*-protein interaction. $[\text{Ca}^{2+}]_i$ has been shown to act as a second messenger (Franklin-Tong *et al.* 1993). Upon recognition of incompatible *S*-proteins, rapid increases in $[\text{Ca}^{2+}]_i$ are observed in the pollen tube shank. In pollen tubes, oscillating apical $[\text{Ca}^{2+}]_i$ gradients (see figure 1*a*) are critical for growth, although their role is unclear. During an incompatible SI reaction, the apical $[\text{Ca}^{2+}]_i$ gradient is lost and tube growth is inhibited (Franklin-Tong *et al.* 1993, 1997). Figure 1 illustrates some of the key changes in $[\text{Ca}^{2+}]_i$ dynamics stimulated in *P. rhoeas* pollen tubes during the SI response. More recently, SI-stimulated Ca^{2+} influx in the pollen tube shank has been demonstrated, together with the inhibition of Ca^{2+} influx at the tip (Franklin-Tong *et al.* 2002). It is thought, therefore, that the increases in the shank $[\text{Ca}^{2+}]_i$ are due, at least in part, to Ca^{2+} influx. During the SI response, several events that involve increases in $[\text{Ca}^{2+}]_i$ are triggered. These include the phosphorylation of an inorganic PPase (p26), reorganization and depolymerization of the actin cytoskeleton, and the activation of a MAPK (p56). Here, we summarize progress in our understanding of the SI signals and their targets.

2. p26: AN INORGANIC PPASE

Pollen germination and pollen tube growth are almost instantly arrested by the interaction with stigmatic *S*-proteins during incompatible challenges. This observation shows the presence of very rapid SI signalling events in

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Figure 1. Alterations to $[Ca^{2+}]_i$ in *Papaver rhoeas* pollen during the SI response. Normally growing pollen tubes (a) have an oscillating apical gradient of $[Ca^{2+}]_i$, which is rapidly lost during an incompatible SI response (b). Images in (a) are at 10 s intervals from the top; images in (b) are at 0, 10, 60, 100 s after SI induction. In the 'shank' of the pollen tube $[Ca^{2+}]_i$ is normally low (top image in (c)), and this is rapidly increased during an incompatible response. Subsequent images in (c) are at 0, 20, 100 s after SI induction. The pseudocolour scale bar in (a) indicates $[Ca^{2+}]_i$ (red, high: ca. 1000 nM; blue, low: ca. 250 nM). Scale bars all indicate 10 μ m. (Images from (b) and (c) are taken from Franklin-Tong *et al.* (1997) and are reproduced with permission from Blackwell Publications.)

Papaver pollen. Besides a striking rise in $[Ca^{2+}]_i$ (Franklin-Tong *et al.* 1993, 1995, 2002), the interaction of S-proteins with incompatible pollen leads to SI-specific changes in the phosphorylation state of several pollen proteins (Rudd *et al.* 1996, 1997). One of these phosphoproteins is a soluble 26 kD protein (p26) that becomes phosphorylated within 90 s after incompatible challenges but not after compatible challenges. Phosphorylation of

p26 increases significantly in the next 400 s. Because the protein kinase activity responsible for p26 phosphorylation is dependent on Ca^{2+} and calmodulin, this suggests that, upon SI induction, p26 is the target for a Ca^{2+} - and calmodulin-dependent protein kinase (Rudd *et al.* 1996).

Recently, the p26 cDNA was obtained and its deduced amino acid sequence showed ca. 80% homology to soluble inorganic PPases. These enzymes are responsible for the conversion of PPi, formed during the many biosynthetic reactions that use ATP, into inorganic orthophosphate. Removal of PPi, which is toxic to the cell, also allows a shift of the equilibrium of the biosynthetic reactions towards biosynthesis. PPases are expressed in metabolically active tissues and their activity is dependent on the presence of divalent metal cations, usually Mg^{2+} . Biochemical analysis of a recombinant p26 protein produced in *Escherichia coli* confirmed that this protein is a true PPase and that its PPase activity is dependent on Mg^{2+} . Moreover, we were able to demonstrate that p26-PPase activity can be inhibited by phosphorylation (Rudd & Franklin-Tong 2003). The rapid inactivation of p26-PPase and the abrupt arrest of incompatible pollen upon SI challenge provides us with a putative link between SI signalling events and pollen tube physiology. We are currently studying the function of soluble organic PPases in pollen tube growth and the regulation of p26 activity by phosphorylation upon SI challenge.

3. THE ACTIN CYTOSKELETON

Pollen tubes grow by polarized tip growth via the localized deposition of cell wall and membrane components. The actin cytoskeleton plays a key part in the delivery and docking of vesicles at the growing tip. SI results in the cessation of pollen tube growth and the involvement of the actin cytoskeleton has been demonstrated. Studies using rhodamine-phalloidin staining showed that, in normally growing pollen tubes, the actin cytoskeleton is represented by thick axial bundles in the shank region and a 'basket-like' structure of fine actin bundles in the terminal 5–10 μ m of the tube, as illustrated in figure 2a. Because the cytoskeleton is a thought to be a major target for signals, we have studied the arrangements of F-actin during the SI response and have observed dramatic and rapid rearrangements (see figure 2b,c). Within 1–2 min of incompatible SI challenge, many F-actin bundles are lost, although cortical F-actin was retained. Later, punctate foci of actin in the cortex were observed to accumulate (Geitmann *et al.* 2000). Challenge with compatible S-proteins did not alter F-actin organization in pollen tubes, demonstrating that these rearrangements are S-specific. Quantitative analysis of F-actin in incompatible pollen tubes revealed an actin loss of ca. 30% within 1 min and ca. 75% after 1 h of SI challenge. This demonstrates that at least some of the alterations observed are due to actin depolymerization (Snowman *et al.* 2002).

It is interesting to note that drugs that increase $[Ca^{2+}]_i$ in pollen also stimulate actin reorganization and depolymerization (Geitmann *et al.* 2000; Snowman *et al.* 2002) that are similar to those observed during the SI response (see figure 2d,e). This indicates that the actin alterations are on the Ca^{2+} -mediated SI signalling cascade. We are currently investigating various actin-binding proteins to

Figure 2. Reorganization of the actin cytoskeleton during the SI response in *Papaver rhoeas* is similar to changes induced by increases in $[Ca^{2+}]_i$. Normally growing pollen tubes have a typical actin cytoskeleton configuration as in (a), which is altered during the SI response as in (b) and (c). (b) Alterations 10 min after SI challenge; (c) alterations after 60 min SI. The drug A23187, which raises $[Ca^{2+}]_i$ artificially in pollen tubes, also induces similar alterations to the actin cytoskeleton (d) and (e) as the SI response, suggesting that Ca^{2+} acts as a second messenger in both of these signalling pathways. Scale bar indicated in (a) is 10 μ m, and applies to all figure parts.

identify which of these proteins are involved in the rapid and dramatic reorganization and depolymerization of actin during the SI response.

4. MAPK ACTIVATION DURING SI

MAPK phosphorylation cascades are highly conserved signal-transduction mechanisms. In higher plants, MAPKs have been implicated in a wide range of stress responses (see Tena *et al.* 2001). As the response of pollen to signals from an incompatible stigma may be considered a biological stress response, we have investigated the possible involvement of MAPKs in the pollen SI response. Analysing pollen MAPK activity, using an in-gel assay with MBP as a substrate, revealed an increase in the MBPK activity of a 56 kDa protein (p56). The earliest detectable increase in activity occurred 5 min into the SI response. Peak activity was at *ca.* 10 min, representing a three- to fourfold increase over basal levels. Activity remained significantly higher than basal levels for at least

30 min. Compatible and heat-denatured incompatible S-proteins failed to stimulate activity appreciably demonstrating S-specificity. Pretreatment of growing pollen with lanthanum, a known blocker of plasma membrane calcium channels, prevented SI-induced stimulation of Ca^{2+} influx (Franklin-Tong *et al.* 2002) and also p56 activity, implying that Ca^{2+} influx is critical for p56 activation events.

A diagnostic feature of MAPKs is their activation via phosphorylation on tyrosine and threonine residues. Activated p56 was immunoprecipitated using phosphotyrosine specific antiserum, lending support to the view that it is a MAPK. Further evidence comes from using the specific MAPK inhibitor, apigenin. When this was included in the in-gel kinase assay, p56 activity was abolished. The timing of p56 activation, after pollen tube inhibition has occurred, suggests that it may be involved in signalling to the irreversible inhibition of pollen tube growth. Current research is directed towards cloning and characterizing p56 to further our understanding of the highly complex SI response.

5. PCD IN THE SI RESPONSE

Apparent similarities between the SI-induced events in *Papaver* and the HR to pathogens have been noted previously (Wheeler *et al.* 1999; Rudd & Franklin-Tong 2003). In HR, activated host plant cells ultimately die due to a controlled PCD pathway. A key marker for PCD is the nicking of nuclear DNA and its degradation to oligonucleosomal fragments. Using both fragment end labelling and TdT-mediated dUTP nick-end labelling reactions for DNA fragmentation, we have found evidence for DNA fragmentation triggered in an S-specific manner (Jordan *et al.* 2000). Fragmentation was first detected 4 h after S-protein challenge and increased to *ca.* 80% of pollen tubes affected 14 h after challenge. DNA fragmentation was also observed in pollen tubes treated with mastoparan, which increases $[Ca^{2+}]_i$, therefore implicating Ca^{2+} in this signalling cascade as well.

Studies of PCD in animal systems have implicated cysteine-aspartate proteases (caspases) in initiating cellular changes associated with PCD. Although no caspase homologues have been identified in plants, evidence suggests that caspase-like activity is activated in both HR and the *Papaver* SI response. Current data indicate that inhibitors of caspase-3 (Ac-DEVD-CHO) alleviate the inhibitory effects of S-proteins on pollen tube growth and significantly reduce S-induced DNA fragmentation. Furthermore, we have preliminary evidence that at least some of the SI-stimulated actin alterations can also be alleviated by these inhibitors. This suggests a link between the actin and PCD signalling cascades. We are currently working on obtaining unequivocal evidence that PCD is operating in this SI system. To do this, we are investigating other markers for PCD that could be involved. This includes examining whether fragmentation of a caspase-3 substrate, poly-ADP ribose polymerase, is triggered. We also intend to investigate whether cytochrome *c* leakage from mitochondria might occur.

6. CONCLUSIONS

Here, we have briefly outlined current data and ongoing work on investigating the signalling cascades and targets for the SI response in *P. rhoeas*. For a model of current thought on the contribution of these components to the inhibition of incompatible pollen, see Franklin-Tong & Franklin (2003). From these emerging data, it is clear that quite a complex system has evolved to ensure that self-pollen does not achieve fertilization. An ongoing challenge is understanding how these components interrelate, and what level of cross talk is used by this system.

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GLOSSARY

- $[\text{Ca}^{2+}]_i$: cytosolic free Ca^{2+}
 HR: hypersensitive response
 MAPK: mitogen-activated protein kinase
 MBP: myelin basic protein
 PCD: programmed cell death
 PPase: pyrophosphatase
 PPI: pyrophosphate
 SI: self-incompatibility