Proteins implicated in mediating self-incompatibility-induced alterations to the actin cytoskeleton of *Papaver* pollen

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Background and aims Sexual reproduction in angiosperms involves a network of signalling and interactions between pollen and pistil. To promote out-breeding, an additional layer of interactions, involving self-incompatibility (SI), is used to prevent self-fertilization. SI is generally controlled by the S-locus, and comprises allelic pollen and pistil S-determinants. This provides the basis of recognition, and consequent rejection, of incompatible pollen. In *Papaver rhoeas*, SI involves interaction of pistil PrsS and pollen PrpS, triggering a Ca2+-dependent signalling network. This results in rapid and distinctive alterations to both the actin and microtubule cytoskeleton being triggered in ‘self’ pollen. Some of these alterations are implicated in mediating programmed cell death, involving activation of several caspase-like proteases.

Scope Here we review and discuss our current understanding of the cytoskeletal alterations induced in incompatible pollen during SI and their relationship with programmed cell death. We focus on data relating to the formation of F-actin punctate foci, which have, to date, not been well characterized. The identification of two actin-binding proteins that interact with these structures are reviewed. Using an approach that enriched for F-actin from SI-induced pollen tubes using affinity purification followed by mass spectrometry, further proteins were identified as putative interactors with the F-actin foci in an SI situation.

Key results Previously two important actin-binding proteins, CAP and ADF, had been identified whose localization altered with SI, both showing co-localization with the F-actin punctate foci based on immunolocalization studies. Further analysis has identified differences between proteins associated with F-actin from SI-induced pollen samples and those associated with F-actin in untreated pollen. This provides candidate proteins implicated in either the formation or stabilization of the punctate actin structures formed during SI.

Conclusions This review brings together for the first time, our current understanding of proteins and events involved in SI-induced signalling to the actin cytoskeleton in incompatible *Papaver* pollen.

Key words: Actin cytoskeleton, actin-binding proteins, mass spectrometry, *Papaver rhoeas*, pollen, self-incompatibility, signalling.

INTRODUCTION

Cellular responses require an integrated signal perception and signal transduction network. The self-incompatibility (SI) response in pollen of *Papaver rhoeas* (the field poppy), which prevents self-fertilization by rejecting incompatible pollen, provides a model system to study the components involved in cell–cell recognition and rejection. SI is an important genetically controlled system that has evolved independently several times, with different mechanisms used to regulate incompatibility in different plant species [see Franklin-Tong (2008) and other articles in this Special Issue].

In *Papaver*, the S-determinants comprise PrsS proteins, encoded by the pistil-expressed part of the S-locus, and PrpS, encoded by the pollen part of the S-locus. The PrsS gene was identified some time ago (Foote et al., 1994; Walker et al., 1996; Kurup et al., 1998); though it was recently re-named PrsS (for *Papaver rhoeas* stigma $S$ determinant) in order to provide a clearer nomenclature (Wheeler et al., 2009). The PrsS protein is a small (approx. 15 kDa) polymorphic secreted novel protein (Foote et al., 1994). Although when they were first identified PrsS proteins had no homologues in the databases, they were subsequently found to be members of the S-Protein Homologue (SPH) family (Ride et al., 1999), which has 84 members in arabidopsis. The pollen S-determinant was recently identified (Wheeler et al., 2009). PrpS is a novel gene with no immediately identifiable function, as extensive database searches failed to identify orthologues of PrpS genes. Sequence information indicates that PrpS encodes a highly hydrophobic protein with several predicted transmembrane passes, and experimental evidence has established that PrpS is a transmembrane protein, localized at the pollen tube plasma membrane (Wheeler et al., 2009). PrpS has a small predicted extracellular domain that has been shown to be involved in both binding to PrsS and to be involved in mediating SI (Wheeler et al., 2009).

For SI to function, S-locus determinants are multi-allelic, with cognate pollen and pistil pairs interacting to mediate
the response. In *Papaver*, over 60 alleles have been identified. *S*-locus proteins are highly polymorphic, and in *P. rhoes*, there is a very high-level (approx. 40–50 %) divergence between the predicted primary amino acid sequences of alleles (Wheeler et al., 2010). A long-standing model for the operation of SI in *Papaver* has proposed that the female *S*-determinant, PrsS, acts as a signalling ligand that interacts with the male *S*-determinant. Recent data have demonstrated that PrsS and PrpS interact in an *S*-allele-specific manner (Wheeler et al., 2009, 2010).

Programmed cell death (PCD) is a highly conserved process used to kill unwanted cells, which includes apoptosis. In SI, PCD provides a strategy to specifically target and destroy unwanted ‘self’ pollen. In animal cells this involves caspases, a family of cysteine proteases, that when activated, cleave target proteins, which sets in motion organized cellular degradation and death. Caspase-3 is the main executioner protease in animal cells. Although plants have no true caspase gene homologues (see Woltering et al., 2002; Sammartin et al., 2005), it is well established that plants utilize PCD and good biochemical evidence for several caspase-like protease activities involved in PCD exist (Bonneau et al., 2008). DEVDase/caspase-3-like activities have also been detected in plants through cleavage of the animal caspase-3-specific substrate, Ac-DEVD-AMC (Danon et al., 2004; Thomas et al., 2006) and use of DEVD-CHO tetrapeptide inhibitors (del Pozo and Lam, 1998; Richael et al., 2001; Thomas and Franklin-Tong, 2004). Recently RNAi plants with a defective proteasome subunit PBA1 were shown to have reduced DEVDase activity, suggesting that PBA1 may function as a caspase-3-like enzyme (Hatsugai et al., 2009). Other plant caspase-like activities have been identified, including a VEIDase (caspase-6-like) activity (Bozhkov et al., 2004; Boren et al., 2006).

With respect to SI, studies have established that DNA fragmentation was triggered in incompatible pollen tubes (Jordan et al., 2000) and that this involved a caspase-3/DEVDase activity (Thomas and Franklin-Tong, 2004). These data, together with evidence for cytochrome *c* leakage and poly(ADP-ribose) polymerase (PARP) cleavage activity (Thomas et al., 2006), and use of DEVD-CHO tetrapeptide inhibitors (del Pozo and Lam, 1998; Richael et al., 2001; Thomas and Franklin-Tong, 2004), provided evidence for SI-induced PCD in incompatible *Papaver* pollen. To investigate the nature of the caspase-like activities triggered by SI, tetrapeptide caspase substrates were used as a pre-treatment prior to SI induction, to monitor and measure the caspase-like activities triggered by SI using Ac-tetrapeptide AMC derivatives (Bosch and Franklin-Tong, 2007). There was no evidence for any metacaspase activity, neither was there evidence for ‘initiator’ (caspase 8 and 9) activities. However, SI triggered substantial DEVDase/caspase-3-like and VEIDase/caspase-6-like activities. Low LEVDase/caspase-4-like and YVADase/caspase-1-like activities were also detected but, although the LEVDase activity increased later (Bosch and Franklin-Tong, 2007), the YVADase activity did not substantially increase (N. S. Poulter, M. Bosch and V. E. Franklin-Tong, unpublished results), and there was evidence that the YVADase did not play a role in SI-mediated PCD (Thomas and Franklin-Tong, 2004). The SI-activated caspase activities were verified by their inhibition by tetrapeptide inhibitors; out of eight inhibitors tested, only DEVD-CHO, VEID-CHO and LEVD-CHO were effective. As expected, they were not inhibited by broad-spectrum protease inhibitors. Moreover, live-cell imaging, using a DEVD substrate, established that the caspase-3/LEVDase activity was localized to both the cytosol and the generative cell (GC) and vegetative nucleus (Bosch and Franklin-Tong, 2007). Thus, SI appears to trigger several caspase-like activities: DEVDase, VEIDase and LEVDase activities which are likely to mediate PCD in this system.

The plant cytoskeleton comprises highly dynamic and responsive arrays of actin microfilaments (F-actin) and tubulin microtubules. By responding to signals, they play an important role in mediating signalling networks, acting as both a target and effector in response to various signals in plant cells, playing a key role in determining cell shape and growth (Wasteney and Galway, 2003; Erhardt and Shaw, 2006; Hussey et al., 2006; Staiger and Blanchon, 2006; Thomas et al., 2009). These responses are achieved through their interaction with various actin-binding proteins (ABPs) and microtubule-associated proteins (MAPs). Many of the signalling intermediates that regulate actin and microtubule dynamics are well-defined in animal cells and yeast (see, for example, Erhardt and Shaw, 2006; Iden and Collard, 2008; Thomas et al., 2009). Although considerably less is known for plants, several monomer-binding proteins, including profilin, actin-depolymerizing factor (ADF) and cyclase-associated protein (CAP), are known to function synergistically to stimulate actin turnover in *vitro* (Chaudhry et al., 2007; Staiger et al., 2010). Plant profilin binds G-actin with high affinity, acting to prevent spontaneous nucleation of filaments and also shuttles monomers onto the ends of growing actin filaments. However, unlike other profilins, plant profilin is unable to catalyse nucleotide exchange (ADP to ATP) on monomers (Staiger and Blanchon, 2006). ADF is also a key regulator of actin dynamics. It is able to bind to both G- and F-actin, depolymerizes F-actin by severing and is involved in F-actin treadmilling (Andriantoandro and Pollard, 2006; Dos Remedios et al., 2003). CAP (Srv2p is the yeast homologue) is a subunit of adenylly cyclase. It’s C-terminus binds ADP-actin monomers and is involved in actin treadmilling in yeast. There is evidence that arabidopsis CAP1 has a similar function (Barrero et al., 2002; Deeks et al., 2007). However, AtCAP1 has been shown to directly enhance nucleotide exchange on actin, replacing the role of profilin in other organisms (Chaudhry et al., 2007). Other ABPs act to bundle and cross-link actin filaments to form higher-order structures (for a recent review, see Staiger et al., 2010). One example of an ABP with this function in plants is fimbrin (Kovar et al., 2000b; Thomas et al., 2009). The arabidopsis fimbrin, AtFim1 binds and cross-links F-actin, and has been shown to associate with F-actin in *vivo* (Kovar et al., 2000b).

As the cytoskeleton is an important integrator of signalling events into physiological responses, we review our recent findings on SI-induced alterations to the actin and tubulin cytoskeletons in incompatible pollen, and discuss data suggesting their involvement in PCD. We also present new data identifying further candidates that may play a role in the formation of the SI-induced punctate actin structures. Together these data enrich our rather incomplete picture of how SI-induced signalling to the actin cytoskeleton is achieved in incompatible *Papaver* pollen.
SI TRIGGERS ALTERATIONS TO THE ACTIN AND MICROTUBULE CYTOSKELETON

The SI response in *Papaver* stimulates rapid and dramatic alterations of the actin cytoskeleton. Use of the F-actin-binding probe, rhodamine–phalloidin, has allowed examination of the F-actin cytoskeleton organization at different time-points after stimulation of SI (Fig. 1). Within a few minutes the typical longitudinal F-actin bundles observed in normally growing pollen tubes (Fig. 1A) had largely disappeared (Fig. 1B) and by 30 min the F-actin had a fine, speckled appearance (Fig. 1C). This established that the actin cytoskeleton was a very early target for the SI signals in incompatible *Papaver* pollen. Quantitative studies established that SI induces very rapid, large-scale depolymerization of F-actin in incompatible pollen (Snowman et al., 2002). The actin then forms ‘punctate foci’ (Geitmann et al., 2000; Snowman et al., 2002; Fig. 1D). More recent studies have started to characterize the formation of these punctate actin foci, which are unusually stable, being resistant to treatments with 1 μM of the actin-depolymerizing drug latrunculin B. Detailed quantitative analysis has established that the size of the punctate actin foci increases with time (Poulter et al., 2010).

It has also been established that the pollen tube microtubule cytoskeleton is a target for SI signals. SI triggered very rapid apparent depolymerization of cortical microtubules, but these microtubule alterations are quite different from the actin alterations. The organization of the microtubule cytoskeleton in normally growing *Papaver rhoeas* pollen tubes is very similar to that described previously for pollen tubes. The tip region is relatively microtubule-free; behind this region are arrays of short, longitudinally organized microtubule bundles, behind which are longer, more regularly organized longitudinal microtubule bundles in the ‘shank’ region (Fig. 1E). The GC has a distinctive population of spindle-shaped GC microtubules associated with it (Fig. 1E, inset). As the pollen tube actin cytoskeleton is a target for the SI signals, being rapidly depolymerized, we investigated if the microtubules might also be affected. Using immunolocalization with α-tubulin antisera at various time points after SI induction, it was found that the microtubule cytoskeleton was very rapidly depolymerized (Poulter et al., 2008). Within approx. 1 min of SI induction the cortical microtubule bundles were virtually undetectable in incompatible pollen tubes (Fig. 1F), but the distinctive GC population of microtubules remained (Fig 1F, inset). At 30-min post-SI (when actin was starting to aggregate), the cortical microtubules remained depolymerized (Fig. 1G), while the GC spindle-shaped microtubules were disintegrating, but still evident (Fig. 1G, inset). No further microtubule reorganization was seen at 1-h post-SI-induction but the GC microtubules continued to degrade (Fig. 1H) Thus, SI induces rapid alterations to the cortical microtubule cytoskeleton of incompatible pollen tubes, but the spindle-shaped microtubules associated with the GC were much more stable (Poulter et al., 2008). When the overall levels of α-tubulin were examined in SI-induced pollen tubes using western blotting, the amount of α-tubulin in the pollen tubes remained virtually constant for at least 60 min after SI was induced. This suggests that the cortical microtubule

**Fig. 1.** Actin and microtubule alterations stimulated by SI in incompatible pollen. F-actin was visualized in fixed cells using rhodamine–phalloidin; tubulin was visualized using monoclonal anti-α-tubulin clone B 5-1-2 (Sigma). (A) Typical longitudinal F-actin bundles observed in normally growing pollen tubes. (B) The F-actin bundles had largely disappeared 2 min after SI induction. (C) At 30 min, actin has formed small ‘punctate foci’. (D) At 3 h, actin has formed large ‘punctate foci’ that are very stable. (E) Microtubule organization in normally growing pollen tubes shows a relatively microtubule-free apical region, then a region with shorter microtubule bundles; behind this are longer arrays of longitudinally arranged cortical microtubule bundles. Inset: the generative cell (GC) has a distinctive population of spindle-shaped microtubules. (F) Cortical microtubules are apparently virtually completely depolymerized 1 min after SI-induction. Inset: GC microtubules are more or less intact. (G) 30 min after SI-induction, microtubules remain depolymerized whilst the GC microtubules (inset) begin to degrade. (H) No further reorganization of the microtubules is seen at 1 h SI, GC microtubules (inset) continue to degrade. (A–D), taken from Poulter et al. (2010); (E–H), adapted from Poulter et al. (2008).
disappearance is due to depolymerization of microtubules, rather than tubulin degradation. Importantly, these comparisons between SI-induced microtubule and microfilament responses show that, although both respond very rapidly, they have quite distinct characteristics.

**ALTERATIONS TO ACTIN DYNAMICS IS SUFFICIENT TO TRIGGER PCD IN POLLEN TUBES**

A number of studies in animal cells and in yeast has shown that either actin stabilization or depolymerization can influence whether a cell enters into an apoptotic pathway (see Franklin-Tong and Gourlay, 2008). As it had been established that SI triggered PCD (Thomas and Franklin-Tong, 2004), this raised the question of whether the SI-induced alterations in actin dynamics might play a role in this. A pharmacological approach was taken to manipulate actin dynamics in pollen tubes, and it was subsequently assessed if levels of PCD were altered. The cytoskeleton drugs used were: latrunculin B (LatB), which binds to monomeric actin, inhibiting polymerization and results in the preferential disassembly of actin filaments that are undergoing rapid turnover; or jasplakinolide (Jasp), which stabilizes actin filaments and stimulates polymerization. It was found that both LatB and Jasp stimulated DNA fragmentation (a marker for PCD), suggesting, somewhat surprisingly, that both actin depolymerization and stabilization can stimulate PCD (Thomas et al., 2006). Pre-treatment with the caspase tetrapeptide inhibitor DEVD-CHO prior to addition of LatB or Jasp resulted in significantly reduced levels of the LatB- or Jasp-induced DNA fragmentation (Thomas et al., 2006). These studies demonstrated that changes in actin filament dynamics are sufficient to initiate PCD in *Papaver* pollen, and that a DEVDase/caspase-3 like activity is involved. These data imply a key functional role for actin dynamics (both depolymerization and stabilization) in initiating PCD in *Papaver* pollen.

Normally cells respond to stimuli using very rapid, transient alterations in actin dynamics. To explore how extreme a change might be required to ‘tip pollen tubes over the edge’ and induce PCD, limited treatment times were carried out by performing ‘washouts’ to remove the drugs. These data established that relatively transient changes in actin filament dynamics (e.g. reducing F-actin levels to approx. 50% for 10 min) could initiate high levels of DNA fragmentation, even though F-actin levels were returned to normal after treatments (Thomas et al., 2006). Moreover, pre-treatment with Jasp, in an attempt to counteract and reduce both Lat-B- and SI-induced depolymerization, significantly reduced both the Lat-B- and SI-induced DNA fragmentation. This ‘rescue’ of LatB- or SI-induced pollen from PCD added further strength to the idea that actin polymerization status plays a key role in the initiation of PCD in pollen. Together, these data implicate the actin cytoskeleton as a sensor of cellular stress.

Both actin depolymerization and stabilization/aggregation are triggered by SI, and the experiments by Thomas et al. (2006) suggest that both of these phases may potentially be involved in mediating PCD, triggering death of incompatible pollen through activation of caspase-3-like activities. We discuss possible candidate ABPs that may be involved in mediating the depolymerization later. How the actin depolymerization and stabilization/aggregation relate to each other and are regulated is something that needs to be explored further, as does establishing which signalling components are implicated and how the cytoskeleton interacts and integrates with signalling networks.

**ALTERATIONS TO MICROTUBULE DYNAMICS CAN ALSO TRIGGER PCD IN POLLEN TUBES**

Since our data indicated that tubulin depolymerization, rather than degradation is involved in SI, we investigated whether there was cross-talk between actin and tubulin networks. Using a 1 μM LatB treatment to mimic the SI-induced effect as closely as possible, we showed that actin depolymerization stimulated rapid and virtually complete apparent microtubule depolymerization, while actin stabilization using Jasp alleviated or delayed SI-induced apparent microtubule depolymerization. Moreover, altering microtubule dynamics through addition of oryzalin to artificially depolymerize, or taxol to stabilize the microtubules, did not result in any detectable effect on the actin cytoskeleton (Poulter et al., 2008). This implicated actin depolymerization as being upstream of microtubule depolymerization. We also investigated the possibility that the pollen tube microtubule cytoskeleton might play a role in mediating PCD, using a similar approach to that used for the actin studies, using pre-treatments with the microtubule drugs, oryzalin and taxol. We found that neither microtubule depolymerization nor stabilization was sufficient to trigger PCD. However, prevention of total microtubule depolymerization using a pre-treatment with taxol prior to SI induction, significantly reduced the level of caspase-3-like/DEVDase activity (Poulter et al., 2008). This suggests that, while tubulin depolymerization on its own is insufficient to trigger PCD, this step does play an important role in allowing full activation of PCD, perhaps mediating progression. Thus, SI targets the microtubule cytoskeleton and its depolymerization appears to play a functional role. We discuss possible candidate MAPs that may be involved in the microtubule depolymerization step later.

**INVESTIGATIONS INTO THE NATURE OF THE PUNCTATE ACTIN FOCI**

Next our attention was turned to initiate analysis of the SI-mediated punctate actin foci, which are a very characteristic feature of the SI response. Until recently we had very little information on the punctate actin foci, except that they appear to be large aggregates of filamentous (F-)actin, based on phalloidin staining. Prior to SI induction, no F-actin foci were detected. Quantification of imaging data showed that the F-actin foci significantly increased in size over time and their apparent size is surprisingly large – up to approx. 1 μm in diameter. Data suggested that their formation requires actin polymerization (Poulter et al., 2010). Strikingly, analysis demonstrated that, once formed, these SI-induced structures are unusually stable. Treatments with extremely high levels of LatB (1 μM for 10 min), which is more than sufficient to inhibit pollen tube growth and results in an approx. 50% decrease in actin polymer levels in normally growing pollen tubes.
(Thomas et al., 2006), had no effect on SI-induced actin punctate foci. Longer treatments (1 μM for 30 min) also had no effect (Poulter et al., 2010). This provided clear evidence that these structures have very different properties to those exhibited by normal actin arrays in unchallenged pollen tubes. This suggests that they are likely to be associated with different ABPs.

To investigate this possibility, immunolocalization was used in conjunction with rhodamine phalloidin to establish whether these unusual structures were associated with the intracellular localization of several ABPs known to be major regulators of actin dynamics. These comprised CAP, ADF/cofilin, fimbrin and profilin. The localization of these proteins was examined in untreated pollen tubes (Fig. 2A, C, E, G) as well as after 3-h SI, when the foci had formed to their maximum size (Fig. 2B, D, F, H). In normally growing pollen tubes, CAP showed no detectable co-localization with the prominent F-actin bundles, and was distributed as fine speckles (Fig. 2B). After SI, a large proportion of CAP co-localized with the large F-actin foci, but there was still a large proportion of CAP remaining as fine speckles (Fig. 2B). In untreated pollen tubes, ADF also had no co-localization with F-actin bundles, but there was a strong signal in the cortical regions, together with a diffuse cytosolic signal (Fig. 2C). At 3-h post-SI, a significant proportion of the ADF population was co-localized with large F-actin foci, but there was also a strong background cytosolic signal (Fig. 2D). In untreated pollen tubes, fimbrin visualized as fine speckles, together with some co-localization along the major actin filament cables (Fig. 2E). After SI, fimbrin had altered distribution, but there was no co-localization with...
F-actin foci (Fig. 2F). In untreated pollen tubes, profilin was largely cytosolic (Fig. 2G). Although the localization of profilin was distinctively altered after SI, forming small foci-like aggregates, these did not co-localize with the F-actin foci (Fig. 2H). Thus, two of these ABPs, CAP and ADF, displayed altered localization and associated with these distinct F-actin structures. Two other key regulators of actin dynamics, profilin and fimbrin, did not associate with the F-actin foci (Poulter et al., 2010).

These data provide the first insights into the actin-binding proteins and mechanisms involved in the formation of these intriguing structures, which appear to be actively formed during the SI response. Double labelling for actin and CAP or ADF combined with confocal imaging, using single optical sections, was used to confirm the co-localization and its progression (see Poulter et al., 2010). Quantitative analysis of images documenting these events over a time series between 0 and 180 min provided a good description of the temporal–spatial alterations of actin and these two ABPs that was induced by the SI response. This showed that the association of both CAP and ADF with F-actin was relatively low in untreated pollen tubes, and after SI there was very rapid and significant co-localization of both these ABPs with the forming punctate actin foci (Fig. 2I). It was calculated that the percentage co-localization of CAP and ADF with F-actin at 3-h post-SI increased by 77.9% and 74.7%, respectively, compared with their localization in untreated control pollen tubes (P < 0.001). Although we had hoped to identify which ABP associated with the foci first, our analysis showed that the co-localization patterns for these two ABPs were very similar, with significant increases in the level of co-localization of both CAP and ADF with F-actin at 10-min and 30-min post-SI induction. After 1-h SI-induction, nearly all of the F-actin foci co-localized with CAP and ADF and no further significant changes in alteration were observed, suggesting that the major alterations relating to cytoskeletal reorganization took place within the first hour after SI induction. This organization was maintained for at least 3 h (Fig. 2I).

**MASS SPECTROMETRY OF PROTEINS ISOLATED WITH THE SI-INDUCED F-ACTIN FOCI**

Although our studies using immunolocalization to characterize ABPs that co-localized with the large SI-induced punctate F-actin foci provided valuable and interesting data, these studies were limited by the choice and availability of antisera that cross-reacted with poppy pollen ABPs, as well as by the time-consuming nature of such a study. Moreover, other proteins (such as signalling proteins) are likely to bind actin and modulate its properties during the SI response. To identify novel proteins, or proteins not previously known to interact with F-actin, a two-step purification procedure to enrich for the SI-induced F-actin was used and then mass spectrometry was performed on the proteins in this complex. Figure 3 outlines what is currently known about the SI-induced F-actin foci and their associated proteins, and questions relating to this and how this approach using mass spectrometry may help answer some of these unknowns. CAP and ADF have been shown to co-localize with the F-actin foci and the approach described in this section aimed to identify other potential F-actin binding partners and possible signalling proteins. The proposed functions of the proteins identified may reveal currently unknown processes that relate to the SI response that could be the basis of future studies.

The first step involved an enrichment of F-actin using ultracentrifugation. Pollen tube proteins from both untreated and 3-h SI treatments were extracted in the presence of phalloidin to stabilize the F-actin during the extraction procedure. After the removal of pollen grains and cell wall material, the extracts were centrifuged at 435 000 g for 15 min to pellet the majority of the F-actin present in the extract. The total protein in the crude extract, the supernatant and pellet fractions were separated by SDS–PAGE and subjected to western blot analysis to confirm enrichment for F-actin and the ABPs previously analysed (Fig. 4). The amount of actin in the pellet fraction was considerably greater than in the total protein extract in both the untreated and SI samples (Fig. 4A); this provides evidence that this ultracentrifugation step enriched for F-actin. We therefore examined which ABPs previously studied using immunolocalization co-sedimented with the F-actin in the pellet, as co-sedimentation suggests interaction. Immunolocalization had indicated CAP, ADF and profilin mainly localized to the cytoplasm in untreated pollen tubes and fimbrin showed some co-localization with the F-actin. During SI, CAP and ADF both co-localized with the F-actin foci, whereas profilin and fimbrin did not. Western blot analysis (Fig. 4A) revealed the presence of CAP and ADF in both the supernatant and pellet fractions, indicating they were present in the cytoplasm as well as being associated with the F-actin in untreated and SI-induced samples. ADF has been localized to the F-actin in lily and tobacco pollen tubes (Chen et al., 2002). Thus, ADF and, probably, CAP are likely to interact with F-actin to some extent in poppy under normal growing conditions which accounts for their presence...
Profelin was only found in the supernatant, as expected, as it is an actin monomer-binding protein. Fimbrin was found predominantly in the supernatant, with higher levels detected in the pellet fraction of the untreated compared with the SI-induced sample, which confirmed the immunolocalization data (Poulter et al., 2010).

The second step in the isolation of F-actin from pollen protein extracts was an F-actin pull-down, using the pellet fraction from the ultracentrifugation step. Phalloidin conjugated to biotin was added to the re-suspended pellet fraction to allow the phalloidin to bind the F-actin. The biotin–phalloidin and any bound proteins were then pulled down using streptavidin MagneSphere paramagnetic particles (SA-PMPs – magnetite beads coated with streptavidin; Promega). Unbound proteins were washed from the SA-PMPs and the resulting SA-PMPs, which were binding F-actin and its associated proteins, were subjected to SDS–PAGE. Although some of the actin in the pellet fraction did not bind to the SA-PMPs, a substantial amount of actin was pulled-down by this procedure (Fig. 4B). Coomassie blue staining of duplicate gels showed equal loading of samples (Fig. 4C) and that the SA-PMP-bound fraction contained many pollen proteins that might be putative F-actin interactors (Fig. 4D). These were analysed using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS).

FT-ICR-MS is an ideal approach for identifying proteins from a complex mixture of peptides as it has a high resolution that is able to distinguish between ions of a very similar mass (Pinto et al., 2002). The phalloidin–biotin-bound samples were digested with trypsin and subjected to FT-ICR-MS. The resulting peptides were searched against an arabidopsis protein database using Mascot software (Matrix Science). Actin was identified in both samples (seven different peptides for untreated and 16 peptides for SI). To get an overview of the types of proteins identified, the peptides identified as ‘hits’ for both the SI and untreated samples were categorized into ten functional groups according to their predicted general functions, as shown in Fig. 5. Major categories in untreated pollen included energy production pathways (18 %), metabolism (31 %) and protein synthesis (18 %). Other groups included proteins involved in signal transduction, cytoskeleton, heat-shock and chaperone proteins, protein degradation, DNA and RNA processes,
membrane-associated proteins and protein transport; others were placed into an ‘uncharacterized’ category. Of the proteins identified in the SI sample, 53% were found in the SI sample and not in the untreated sample. However, 56% of these proteins had proteins of similar function in the untreated sample, such as different sub-units of the same complex or different isoforms. Bearing in mind that *P. rhoeas* genomic data are not currently available and this analysis has been carried out against arabidopsis sequences, our analysis should be considered with caution. Analyses comparing the types of proteins identified in the SI and untreated samples revealed that the proportion of proteins involved in metabolism and energy production were similar in untreated samples and SI samples 3 h after SI induction (Fig. 5). In contrast, there was a huge drop in the number of proteins involved in protein synthesis in the SI sample; the untreated sample had three times as many of its proteins involved in protein synthesis compared with SI samples. This is intuitively what might be expected, as the pollen tubes in this sample would be actively growing, and pollen tube growth is biosynthetically highly active. Several of the protein hits identified by FT-ICR-MS were associated with mitochondria (15 in SI and 10 in untreated). This could implicate interaction of mitochondria with F-actin. This is perhaps significant, and merits further investigation. Other systems have found important evidence for links between actin and mitochondria. In yeast, mitochondria have been shown to physically interact with bundles of F-actin cables; a role for actin in mediating gating of mitochondrial membrane permeability has been demonstrated. Moreover, actin disruption results in aberrant mitochondrial distribution, morphology, and PCD (for a review, see Franklin-Tong, 2008). Compared with the untreated control, the SI sample showed increased levels of proteins compared with controls in three major groupings (see Fig. 5): heat-shock and chaperone proteins (10% in the SI sample versus 5% in untreated pollen), cytoskeleton proteins (13% versus 7%) and signal transduction proteins (12% versus 8%).

As we were interested in potential signalling links to the formation of the SI-stimulated F-actin foci, we analysed more...
thoroughly the proteins identified by FT-ICR that might be implicated in signalling. The SI sample exhibited an increase in proteins involved in signal transduction, heat-shock proteins and chaperonins (Table 1). Notably, 14-3-3 proteins, Ras-like proteins, heat-shock proteins and chaperonins were more abundant in the SI-induced samples. The 14-3-3 family of proteins are highly conserved and are involved in many cellular processes, including regulating cytoskeletal dynamics (Jin et al., 2004). The SI sample contained five different 14-3-3 protein hits, with a total of 16 different peptides. Although the untreated sample also contained some 14-3-3 proteins, there were fewer protein hits and the number of different peptides identified was less (three proteins and nine different peptides, three of which were unique to the untreated sample), suggesting that fewer 14-3-3 proteins interacted with actin in the untreated sample. This is supported by the emPAI (exponentially modified Protein Abundance Index), which gives an estimate of the relative quantitation of the proteins in a mixture (Ishihama et al., 2005). The emPAI scores for the five 14-3-3 proteins identified in the SI-induced sample were higher than for the three 14-3-3s in the untreated sample (SI emPAI scores: 0.91, 0.73, 0.51, 0.58 and 0.24 compared with untreated emPAI scores: 0.24, 0.26 and 0.11), indicating these proteins were more abundant in the SI sample. This might implicate a role for 14-3-3 proteins in SI-induced signalling to actin.

Mass spectrometry analysis also identified Rab-proteins in both the SI and untreated samples. Seven different Rab-proteins were identified in the SI sample, with 19 different peptides, compared with only three proteins (one unique) and six different peptides in the untreated sample. This, together with higher emPAI numbers for the SI sample, could indicate that although the Rab-proteins are associated with F-actin in the untreated sample, they were more abundant in SI. The proteins identified by this analysis all belong to the Rab family of small GTPases found in plants. Rab-GTPases function in endocytosis and intracellular membrane trafficking (for a review, see Stenmark, 2009). Their possible role in SI will be discussed later.

Another interesting class of identified proteins, that exhibited a higher frequency in the SI compared with the untreated sample, was the group of heat-shock proteins and chaperonins. Heat-shock proteins are a family of proteins that are up-regulated in response to stress and act as intra-cellular chaperones for other proteins. In the SI sample five heat-shock/chaperone proteins were identified that were not present in the untreated sample, suggesting these proteins may play a role in the SI response. The heat-shock protein that was unique to the SI sample was heat-shock protein 81-3 (HSP81-3), with six different peptides. This protein is a

Table 1. Identification of proteins in the F-actin enriched samples using FT-ICR

(A) Signalling proteins identified to associate with F-actin in the 3-h SI-induced samples

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein name</th>
<th>Score</th>
<th>No. of peptides</th>
<th>Cellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>14332_ARATH</td>
<td>14-3-3-like protein GF14 epsilon</td>
<td>146</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>14333_ARATH</td>
<td>14-3-3-like protein GF14 protein 1</td>
<td>145</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>14312_ARATH</td>
<td>14-3-3-like protein GF14 protein 2</td>
<td>99</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>14311_ARATH</td>
<td>14-3-3-like protein GF14 protein 3</td>
<td>99</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>14310_ARATH</td>
<td>14-3-3-like protein GF14 protein 4</td>
<td>83</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Bipolarized  | RABA1c Ras-related protein  | 68    | 5               | TGN-PGV               |
| RABA2a_ARATH | Ras-related protein RABA2a   | 68    | 4               | TGN-PGV               |
| ARA3_ARATH   | Ras-related protein ARA-3    | 55    | 3               | Polarized secretion ER−Golgi
| RABD1_ARATH  | Ras-related protein RABD1    | 52    | 4               | ERP−Golgi            |
| RBH1B_ARATH  | Ras-related protein RBH1B    | 52    | 2               | Golgi−ER retrograde transport TGN-PGV |
| ARA4_ARATH   | Ras-related protein ARA-4    | 50    | 2               |                       |
| RAA1A_ARATH  | Ras-related protein RAA1A    | 50    | 3               | TGN-PGV               |


The cellular localization of the Rab proteins is according to Vernoud et al. (2003).

Table 1 Continued

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein name</th>
<th>Score</th>
<th>No. of peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP73_ARATH</td>
<td>Heat-shock cognate 70-kDa protein 3</td>
<td>74</td>
<td>6</td>
</tr>
<tr>
<td>HSP71_ARATH</td>
<td>Heat-shock cognate 70-kDa protein 1</td>
<td>67</td>
<td>4</td>
</tr>
<tr>
<td>HSP72_ARATH</td>
<td>Heat-shock cognate 70-kDa protein 2</td>
<td>212</td>
<td>9</td>
</tr>
<tr>
<td>HSP83_ARATH</td>
<td>Heat-shock protein 81-3</td>
<td>34</td>
<td>9</td>
</tr>
<tr>
<td>CH60B_ARATH</td>
<td>Chaperonin CPN60-like 1</td>
<td>34</td>
<td>9</td>
</tr>
<tr>
<td>CH60A_ARATH</td>
<td>Chaperonin CPN60, mitochondrial</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>BIP1_ARATH</td>
<td>Luminal-binding protein 1</td>
<td>66</td>
<td>4</td>
</tr>
<tr>
<td>BIP2_ARATH</td>
<td>Luminal-binding protein 2</td>
<td>66</td>
<td>4</td>
</tr>
<tr>
<td>ENPL_ARATH</td>
<td>Endoplasmin homologue</td>
<td>52</td>
<td>12</td>
</tr>
<tr>
<td>TCPE_ARATH</td>
<td>T-complex protein 1 subunit epsilon</td>
<td>28</td>
<td>3</td>
</tr>
</tbody>
</table>

Proteins indicated in bold were only found in the SI sample; others were found in both untreated and SI samples.

Protein ID: the protein identification number in the UniProt database.
Score: the score retrieved by the MASCOT programme: the higher the score the more confidence we have in the protein hit. Only significant hits were reported, so all protein hits in this analysis were significant (P < 0.05). No. of peptides: the number of different peptides identified by the MS analysis.
member of the HSP90 family of heat-shock proteins (Milioni and Hatzopoulos, 1997), which in mammalian cells have been implicated in cross-linking branched actin filaments (Koyasu et al., 1986; Park et al., 2007). Another potentially interesting hit was heat-shock protein HSP70, a protein normally up-regulated in response to stress. Although this protein was identified in both untreated and SI samples, the number of peptides identified in the SI sample was far greater than in the untreated (21 compared with 9 peptides) which suggests it is more abundant after SI was induced. In addition, three chaperonins, chaperonin CPN60, CPN60-like 1 and t-complex polypeptide 1 (TCP-1) subunit epsilon, which are involved in polypeptide folding, were found in the SI sample and not in the untreated pollen sample. Together, these data suggest that SI triggers a stress response in incompatible pollen, and this involves chaperonins and heat-shock proteins. Although this finding is intuitively not very surprising, given what we already know generally about this SI response, these data provide the first evidence implicating a possible role for chaperonins and heat-shock proteins in the SI response.

DISCUSSION

We have described how SI stimulates a variety of striking alterations to both the actin and microtubule cytoskeleton. SI not only triggers depolymerization, but also F-actin polymerization to form highly stable actin foci. Here we discuss the candidate proteins that are potentially implicated in mediating SI-induced actin alterations, focusing especially on those newly implicated in interacting with the stable actin foci.

SI STIMULATES F-ACTIN AND MICROTUBULE DEPOLYMERIZATION

Although it has been shown that the actin cytoskeleton in plant cells can undergo significant reorganizations, for instance at the penetration sites following a pathogen infection (Takemoto et al., 2003; Almeida Engler et al., 2004) or mechanical stimulation (Hardham et al., 2008), examples of physiological stimuli triggering actin depolymerization in plants are scarce. It has been shown that fusccocin-induced cell death in sycamore (Acer pseudoplatanus) cultured cells induces a massive depolymerization of actin filaments that is prevented by NO scavengers (Malerba et al., 2008). Fusccocin-induced stomatal opening is also accompanied by depolymerization of actin filaments in guard cells of Commelina communis (Eun and Lee, 2000). To date, the SI-induced actin depolymerization response observed in Papaver represents a unique example for a physiological stimulus triggering such an extremely rapid and dramatic actin depolymerization in plants.

The SI response triggers a Ca\(^{2+}\)-dependent signalling network that stimulates the rapid reorganization and massive depolymerization of actin filaments (Geitmann et al., 2000; Snowman et al., 2002). This strongly suggests the involvement of Ca\(^{2+}\)-dependent ABPs in the depolymerization of F-actin. To date, two Ca\(^{2+}\)-dependent candidate ABPs have been identified that may be involved in the SI-mediated actin depolymerization – profilin and gelsolin. Previously, a possible role for profilin in the early SI-induced depolymerization events had been examined (Snowman et al., 2002) as it was a good candidate protein due to its calcium-regulated actin-sequestering activity (Kovar et al., 2000a). Profilin is a small cytosolic actin monomer-binding protein, which exhibits increased actin-sequestering activity in the presence of calcium (Kovar et al., 2000a) and is a major regulator of actin dynamics (Kovar et al., 2006; Staiger and Blanchoin, 2006) However, the increased sequestering activity of profilin, triggered by the SI-induced increases in cytosolic calcium, is insufficient to account for the high level of F-actin depolymerization observed during SI (Snowman et al., 2002). Thus a second ABP, a gelsolin-like protein, PrABP80, is also thought to contribute to the F-actin depolymerization in incompatible pollen tubes (Huang et al., 2004; Snowman et al., 2002). Reassuringly, our recent results show no association of profilin with the SI-induced F-actin foci, which supports the idea that, once formed, these structures predominantly constitute F-actin, and there is likely to be little actin turnover occurring within them.

The other ABP implicated in the rapid SI-stimulated F-actin depolymerization is PrABP80 (Papaver rhoeas ABP80), an 80-kDa, Ca\(^{2+}\)-dependent ABP from poppy pollen, with properties consistent with it being a gelsolin (Huang et al., 2004). It was co-purified with poppy actin, and mass spectrometry revealed peptide sequences that identified it as a member of the villin family. There are several plant villin family members, and many are expressed in pollen. In recent years our knowledge about their properties has greatly improved (for a recent review, see Staiger et al., 2010). Villins from Lilium longiflorum, 135-ABP and 115-ABP, bundle F-actin in a Ca\(^{2+}\)-dependent manner (Yokota et al., 1998). Arabidopsis VILLIN1 (AtVLN1) only bundles actin filaments and has been shown to protect filaments against depolymerization (Huang et al., 2005), but most villins, like the pollen expressed arabidopsis VILLIN5 (VLN5), typically display barbed-end capping, filament bundling and calcium-dependent severing (Zhang et al., 2010). Recent studies have shown that arabidopsis villin isoforms have overlapping but distinct activities that contribute to the turnover of actin filament bundles (Khurana et al., 2010). Characterization of the biochemical properties of PrABP80 in vitro revealed that it is likely to be gelsolin, a member of the villin family that lacks the villin headpiece and therefore the properties of villin. It exhibits potent actin filament severing activity in the presence of Ca\(^{2+}\) but it cannot cross-link actin filaments (Huang et al., 2004). Although no gelsolin sequence has been identified in plants, PrABP80 exhibits all the biochemical classic hallmark features for it being a gelsolin. Its properties fit a model that suggests it may play a role in the very rapid SI-mediated actin depolymerization, perhaps in partnership with profilin, and together these two proteins could account for up to 50 % F-actin depolymerization, which is in line with the levels seen during SI (Snowman et al., 2002; Huang et al., 2004). However, as PrABP80 has not been cloned, it is not yet possible to test its role in the SI response.

The SI-induced apparent microtubule depolymerization response is also extremely rapid and dramatic. Other incidences of relatively rapid apparent depolymerization of microtubules in plant cells have been reported during plant-
pathogen and plant root–Nod factor interactions (Gross et al., 1993; Timmers et al., 1999; Binet et al., 2001; Cahill et al., 2002; Weerasinghe et al., 2003). It is likely that MAPs play a role in these depolymerisation events. Several putative MAPs (e.g. AtEB1, katanin, AtMAP-65, MOR1 and others) have been identified in the arabidopsis genome, and some are beginning to be characterized (for recent reviews, see Gardiner and Marc, 2003; Sedbrook, 2004; Petrásek and Schwarzerová, 2009). Regulation of microtubule dynamic instability is thought to play a major role in the reorganization of plant microtubule arrays, and arabidopsis MOR1 is a good candidate for playing a role in this. Another recently identified MAP, SB401, from Solanum, is pollen-specific; it binds and bundles microtubules in vitro and co-localizes with microtubules in pollen tubes (Huang et al., 2007). Many of the MAPs identified to date act to bundle microtubules (e.g. MAP-65, MOR1, WVD2), and here we are interested in candidates that act to depolymerize microtubules. To date, only two MAPs from plants, the p60 and p80 katanin subunits and MAP18, have been shown to sever and depolymerize microtubules (Burk et al., 2001; Burk and Ye, 2002; Stoppin-Mellet et al., 2002; Bouquin et al., 2003; Wang et al., 2007). These are candidates that may potentially play a role in the SI-mediated microtubule depolymerization. Other cellular components also interact with microtubules. Phospholipase D (PLD) in tobacco BY-2 cells is involved in their association with the plasma membrane and has been shown to bind to cortical microtubules (Gardiner et al., 2001). Activation of PLD with n-butanol led to detachment of the microtubules from the membrane and partial depolymerization (Dhomoukshe et al., 2003). As calcium can also activate PLD, it is tempting to speculate that the SI-induced calcium influx could stimulate PLD-mediated release of microtubules from the membrane where they could be depolymerized by other MAPs.

We have evidence that there is cross-talk from the actin to the microtubule cytoskeleton in poppy pollen tubes (Poulter et al., 2008), suggesting that there are proteins that link the two cytoskeletons. Microtubules and F-actin are often closely associated and there is good evidence for their interaction in plant cells. For example, transverse cortical microtubules and microfilaments in diffusely elongating cells can influence each other’s organization (Collings and Allen, 2000), and fern cell microtubules were affected by the actin-depolymerizing drug, cytochalasin B (Kadota and Wada, 1992), suggesting that actin dynamics can influence microtubule dynamics. How these interactions between actin and tubulin may be achieved is not clear, but identification of microtubule-associated proteins (MAPs) that can also interact with actin provide a starting point to suggest functional interactions between microtubules and F-actin in plants, which are well established in animal cells and yeast (see Goode et al., 2000). It is currently thought that interactions between actin filaments and microtubules may be mediated by their respective motor proteins. In plants, the calponin homology domain, known to be important for binding actin filaments, has been identified in class-14 kinesins in several plant species. For example, a 190-kDa polypeptide from tobacco BY-2 cells, two actin-binding kinesins, GhKCH1 and GhKCH2, from cotton fibres and a pollen-specific protein SB401 have been shown to interact with both microtubules and F-actin (Igarashi et al., 2000; Preuss et al., 2004; Huang et al., 2007; Liu et al., 2009; Xu et al., 2009). Moreover, in yeast there is evidence that the MAP EB1 interacts through other microtubule plus-end-binding proteins with the ABP formin (Martin et al., 2005). In plants, the pollen-specific MAP, SB401, has been shown to bind and bundle both actin filaments and microtubules in vitro and to co-localize with microtubules in pollen tubes (Huang et al., 2007). More detail can be found in a recent review of microtubule and actin interactions by Petrásek and Schwarzerová (2009). Thus, there are several proteins that may be candidates for actin–microtubule interactions to be explored in future studies.

These proteins represent the first firm evidence for links between these two dynamic cytoskeletal components in plant cells. Therefore, during SI, the microtubule cytoskeleton could potentially detect the SI-induced F-actin depolymerization through similar protein interactions. There are also candidates signalling components that link actin to microtubules to mediate alterations in cytoskeletal organization. PLD6 may interact with both actin filaments and microtubules or with their monomers, perhaps acting to integrate them into the downstream signalling pathways (Gardiner et al., 2001; Ho et al., 2009). Moreover, pull-down assays, using GFP-PLD6 as a bait, identified actin 7 and β-tubulin as interacting partners, as well as other components of intracellular trafficking machinery (Ho et al., 2009). This suggests that PLD6 may be involved in signalling to cytoskeletal remodelling. ROP GTases are also candidate signalling components that may be potentially involved in microtubule depolymerization. ROP2 has been shown to regulate both the F-actin and cortical microtubule cytoskeletons in arabidopsis pavement cells through interaction with the Rop-interactors RIC1 and RIC4 (Fu et al., 2005). RIC1 bundles microtubules and is inactivated via ROP2 (Fu et al., 2005), which could act to destabilize the microtubule cytoskeleton, making it more susceptible to depolymerization. It has also been suggested that phosphoinositide signalling may play a role in mediating F-actin and microtubule remodelling (Wasteneys, 2003; Wasteneys and Galway, 2003). Further identification of pollen MAPs and signalling components that interact with microtubules and actin filaments may, in the future, provide answers to how SI achieves rapid cytoskeletal depolymerization.

THE NATURE OF THE PUNCTATE ACTIN FOCI AND ABPs ASSOCIATED WITH THEM

Not only depolymerization is triggered by SI. Strikingly, after the rapid actin depolymerization is achieved, further actin reorganization, involving the formation of punctate actin foci, is a hallmark feature of SI. Microfilaments are normally quite dynamic, but once formed, the SI-induced punctate actin foci are very stable and are not disassembled by LatB treatments that remove virtually all F-actin in normally functioning cells (Poulter et al., 2010). This unusual characteristic is shared with actin bodies formed in quiescent yeast cells (Sagot et al., 2006) and large F-actin aggregates in Vero cells (Lazarro-Diequez et al., 2008), which form stable actin aggregates which have little actin turnover.

Until recently we had no idea which ABPs may be involved in mediating the unusual dynamic properties of the SI-induced
actin foci. We recently examined whether certain ABPs may bind or are associated with F-actin in SI-stimulated pollen tubes, as it seemed likely that binding of specific ABPs might stabilize them against disassembly; e.g. many side-binding proteins stabilize actin filaments from depolymerization (Thomas et al., 2009). The use of immunolocalization to characterize ABPs that co-localized with the large punctate F-actin foci has provided valuable and interesting data, identifying two important ABPs – ADF and CAP (Poulter et al., 2010). ADF usually mediates actin depolymerization by severing filaments and facilitating subunit loss from minus ends (Bamburg and Bernstein, 2008). However, ADF can bind to both G- and F-actin, and there are instances where ADF has been seen to associate with F-actin in vivo, e.g. in Vero cells (Lazaro-Dieuguez et al., 2008), in tobacco pollen tubes expressing GFP–NtADF1 (Chen et al., 2002) and in fixed lily pollen tubes where it is associated with the actin collar (Lový-Wheeler et al., 2006). So the SI-induced colocalization between ADF and F-actin structures, while unusual, is not unique. ADF activity can be regulated by phosphorylation, polyphosphoinositides and pH (Bamburg, 1999). Changes in the intracellular pH may be the most likely mechanism for regulating the actin-binding activity of ADF during SI. Most ADFs, including those from plants, preferentially bind to F-actin at a pH of approx. 6-0 and to G-actin at a pH of >7.4. High F-actin depolymerizing activity is observed at an alkaline pH in vitro (see Allwood et al., 2002, Carlier et al., 1997; Gungabissoon et al., 1998). It has been hypothesized that these properties may be linked to physiological changes in intracellular pH. For example, in pollen tubes, where it has been shown that the apical region is highly acidic, with the pH of the tip being at approx. 6-0, it has been suggested that ADF is unlikely to be active, and that ADF in the ‘alkaline band’ subapical region is likely to be highly active in depolymerizing and severing actin filaments (Allwood et al., 2002). However, this has not been directly explored in any plant system to date, to our knowledge. Thus, it appears that when the pH is acidic, ADF binds to, and stabilizes, F-actin. As dramatic SI-induced acidification of the pollen tube cytosol from pH 6-8 to approx. 5-5 was recently demonstrated (Bosch and Franklin-Tong, 2007), it seems likely that acidification may play an important role in enhancing the association of ADF with the F-actin foci. Moreover, when F-actin is highly decorated or saturated with ADF, filament severing is no longer observed (Andrianantoandro and Pollard, 2006). Thus SI-induced acidification may alter ADF’s activity and this is likely to ensure the stability of the foci. This hypothesis will have to be tested in future studies.

We also established that CAP associates with the SI-induced F-actin foci. Although this may seem surprising, as plant CAP is primarily an actin monomer sequestering protein (Barrero et al., 2002; Chaudhry et al., 2007; Deeks et al., 2007), preliminary data suggest that AtCAP1 can also bind to F-actin (Deeks et al., 2007). In mammalian cells, CAP can associate with F-actin stress fibres, where it plays a positive role in F-actin assembly (Freeman and Field, 2000). In yeast cells, Srv2p/CAP has been shown to localize to actin patches via an interaction with the F-actin-binding protein Abp1 (Lila and Drubin, 1997; Balcer et al., 2003) and is required for the formation of F-actin aggregates (Gourlay et al., 2004). Furthermore, Srv2p/CAP is also present in the actin ‘bodies’ in quiescent yeast (Sagot et al., 2006). The SI-stimulated association of CAP with F-actin represents a further example, and may provide clues about actin turnover in these structures. In arabidopsis, CAP catalyses the exchange of ADP for ATP on monomeric actin (Chaudhry et al., 2007). This generates a pool of assembly-competent monomers that are available to profilin, which in turn, is able to incorporate the monomer onto the end of a growing filament, allowing actin polymerization to take place. In this way, CAP could play a role in the formation of the SI actin foci. CAP is also an important component of several signalling pathways so could provide clues about potential signalling networks in operation during SI. In the yeast S. cerevisiae, Srv2p/CAP binds to adenylyl cyclase and facilitates the initiation of the cAMP/PKA signalling cascade (Franklin-Tong and Gourlay, 2008). Furthermore, studies of a CAP T-DNA mutant in arabidopsis suggested that plant CAP has evolved to attain plant-specific signalling functions (Deeks et al., 2007). Therefore, CAP represents an appealing candidate for linking signalling networks to actin reorganization. It is tempting to speculate that the formation of the large F-actin foci and their association with CAP is an active process that is involved in downstream signalling events during SI.

Fimbrins might be expected to colocalize with the SI-induced actin foci, as they cross-link and stabilize F-actin in plant cells. The yeast fimbrin/Sac6p has been found to associate with the stable actin bodies in quiescent cells and is necessary for their formation or maintenance (Sagot et al., 2006). Moreover, there are several pollen-expressed fimbrins. A pollen-expressed protein, AtFim1, has been shown to act as a major cross-linker that can stabilize actin against depolymerization (Kovar et al., 2000b). However, fimbrin did not co-localize with the SI-induced F-actin foci, so is not implicated in their formation. A possible explanation for this irregularity is the pH sensitivity of fimbrin. Mammalian fimbrin has reduced F-actin-binding capabilities at pH 6-5 (Glenney et al., 1981) and, therefore, the SI-induced cytosolic acidification (Bosch and Franklin-Tong, 2007) may inhibit the F-actin binding ability of fimbrin, which may explain why we did not detect fimbrin binding to the F-actin foci. It is highly likely that other additional ABPs are involved in the formation of the SI-induced actin foci. The punctate actin foci are very large structures (up to approx. 1 μm in diameter), so actin cross-linking or bundling proteins are likely to be involved in their formation. Several ABPs in plants can bundle or cross-link existing filaments. These include villins, LIM domain-containing proteins and fimbrins (for a recent review, see Staiger et al., 2010). Several ABPs merit investigation in this context. In particular, formins, which can stimulate nucleation of new actin filaments and can bundle existing actin filaments (Michelot et al., 2006; Ye et al., 2009). Another candidate for bundling the F-actin filaments is villin, which, as mentioned earlier, is one of the major bundling factors identified in eukaryotic cells. It bundles actin filaments in a Ca$^{2+}$-dependent manner and has been shown to protect filaments against ADF-mediated depolymerization (Yokota et al., 1998; Huang et al., 2005). As mentioned earlier, several plant villin family members have been...
characterized (see Khurana et al., 2010; Staiger et al., 2010; Zhang et al., 2010). Most villins have been shown to sever, cap and bundle actin filaments in vitro. VLN5 is proposed to be a major actin filament stabilizing factor in pollen tubes (Zhang et al., 2010). Future studies should examine the localization of members of the villin family to see if they play a role in the formation of the SI-induced F-actin foci. Another candidate ABP that could be involved in the initiation of foci formation is the actin-nucleating protein complex, Arp2/3. Although homologues of all of the Arp2/3 complex components have been identified in the arabidopsis genome, as yet, no information on the localization of Arp2/3 is available in plants, due to problems with generating antisera. Biochemical evidence suggests that it is localized at the plasma membrane (Kotchoni et al., 2009). F-actin-capping proteins, which bind in a Ca$^{2+}$-independent manner to the fast growing ends of actin filaments, thereby blocking the exchange of subunits at these ends (Lee and Dominguez, 2010), are also likely to be involved as the dynamics of the actin foci during SI appear to be much reduced compared with other F-actin structures. Therefore, further analysis of the ABPs potentially involved in interacting with the SI-induced actin foci is required in the future.

OTHER PROTEINS THAT MAY INTERACT WITH SI-INDUCED F-ACTIN PUNCTATE FOCI

We took the approach of analysing an enriched F-actin sample from pollen, using mass spectrometry to identify further proteins associated with these unusual SI-stimulated F-actin structures. These proteins are of interest as they may represent previously unknown signalling components or other proteins involved in mediating the formation of the SI-induced foci.

14-3-3 proteins may interact with F-actin during SI

One group of candidate proteins identified as potential F-actin interactors in poppy pollen during the SI response were the 14-3-3 family. These proteins mediate the cellular effects of protein kinases by binding specific phosphorylated peptide motifs. They have been shown to play a role in various cellular processes such as cytoskeleton reorganization, stress response and apoptosis. A large group of 14-3-3 binding partners have been shown to regulate cytoskeletal architecture and cellular morphology in animal cells (Jin et al., 2004). As SI in poppy is a stress response that involves cytoskeletal reorganization, 14-3-3 proteins may be candidates, previously unidentified, that could be involved in SI signalling to cytoskeletal reorganization. Indeed, a phosphorylation-dependent interaction between ADF/cofilin and 14-3-3ζ in animal cells has been demonstrated. Data suggest that 14-3-3ζ proteins may play a dynamic role in the regulation of cellular actin structures by modulating ADF’s actin depolymerizing and/or severing functions (Gohla and Bokoch, 2002). Moreover, studies in animal cells suggest 14-3-3 proteins are a target of caspase-3/DEVDase activity during apoptosis, and that the cleavage of 14-3-3 proteins promotes cell death (Won et al., 2003). Thus, investigating a role for 14-3-3 proteins in SI may be a fruitful avenue to explore in the future.

Potential involvement of Rab-GTPases

A significant increase in the number of Rab-proteins was identified as associated with F-actin in the SI-induced sample. Rab-GTPases are a large family of the GTP-binding proteins, involved in membrane trafficking between organelles. Rab GTPases ensure that cargoes are delivered to their correct destinations and they regulate vesicle budding, uncoating, motility and fusion through the recruitment of effector proteins (for a recent review, see Stemmark, 2009). Moreover, Rab-GTPases have been demonstrated to be important for pollen tube growth, as overexpression in tobacco pollen tubes resulted in inhibition of pollen tube growth and loss of directionality (Cheung et al., 2003; de Graaf et al., 2005) and loss of pollen-expressed RABA4D resulted in a disruption of polar growth and altered cell wall patterning (Szumalanski and Nielsen, 2009). A high proportion of the large SI-stimulated punctate F-actin foci are associated with the cortical region of the pollen grain and tube, although many are also present in the cytosol (Geitmann et al., 2000). As activated Rabs are associated with membranes, the increase in number of Rabs associated with F-actin in the SI-induced sample suggests that they may be playing a role in SI and also potentially implicates changes in membrane dynamics during the SI response. This may be due to the binding of PrsS ligand to the membrane-localized pollen S-determinant, PrpS. As most of the intracellular transport in pollen tubes occurs along the F-actin filament bundles (Cai and Cresti, 2009), this may be an explanation why some Rabs were identified as potential F-actin interactors in untreated pollen tubes. Further work is needed to clarify the association of Rab-proteins with the F-actin in SI-induced pollen tubes and the potential functional implications.

Chaperonins are implicated as F-actin interactors during SI

An unexpected finding was that chaperonins were associated with SI-stimulated F-actin. Their possible involvement in the SI response has not previously been implicated or studied, and this finding opens up potential new avenues to explore in the future. Chaperonins (also known as Hsp60) are a class of molecular chaperones found in prokaryotes and eukaryotes and have an important function in facilitating ATP-dependent protein folding of non-native polypeptides (Frydman, 2001). Chaperonins are large multi-subunit toroidal complexes formed by the oligomerization of 60-kDa proteins. The toroid is made of two rings placed back-to-back with each ring enclosing a cavity where folding occurs (Ellis, 1996). These molecular chaperones act on a large variety of proteins using a mechanism that involves the recognition of the unfolded polypeptide by hydrophobic residues at the entrance of the chaperonin’s central cavity (Fenton et al., 1994).

It is well established that chaperonins, including CPN-60, a mitochondrial chaperonin, and the cytoplasmic chaperonin CCT (chaperonin containing t-complex polypeptide 1, TCP-1), display high affinity for denatured, unfolded actin and tubulin intermediates. They play a key role in catalysing the folding of denatured actin and tubulin intermediates in eukaryotic cells (Gao et al., 1992; Melki and Cowan, 1994). Both in vitro and in vivo studies have shown that the TCP-1 complex, an oligomeric 900-kDa cytosolic particle consisting of TCP-1 and four or five
related polypeptides of similar size (55–60 kDa), plays an essential role in the folding of both cytoskeletal proteins, actins and tubulins (Gao et al., 1992; Sternlicht et al., 1993). Thus, the TCP-1 complex is implicated in mediating a cytosolic pathway for folding tubulin and actin in vivo. The Arabidopsis thaliana genome sequence contains 29 genes predicted to encode members of the chaperonin family of chaperones (CPN60 and CCT), their associated cochaperonins and the cytoplasmic chaperonin cofactor prefoldin (Hill and Hemmingsen, 2001). Thus, the machinery for the pathway of chaperonin-assisted folding is present in plants. More recently it has been shown that, in maize, myosin XI colocalized with TCP-1 (Wang and Pesacreta, 2004), implicating colocalization with actin. However, to date there is relatively little information indicating a link between actin and chaperonins in plant cells.

Although chaperonin-assisted folding is implicated in the biogenesis of actin and tubulin from de-novo synthesized monomeric subunits, it is well known that chaperonins are important in stress responses by re-establishing normal protein conformation and thus cellular homeostasis. Many molecular chaperonines are stress-inducible and assist in refolding of proteins denatured by heat and related stress (reviewed in Ellis and Vandervies, 1991). In animal cells, it has been found that levels of CCT subunit proteins were up-regulated under chemical stress, though total levels of actin and tubulin remained constant. Moreover, both actin and tubulin co-immunoprecipitated with CCT (Yokota et al., 2000). Thus, it has been proposed that CCT plays an important role in the recovery of cells from protein damage by assisting in the folding of proteins that are actively synthesized and/or renatured during the stress (Yokota et al., 2000). As there is good evidence that TCP-1 interacts with denatured actin and tubulin, our finding that it was present in our pull-downs of SI-induced F-actin suggests that the highly stable F-actin in the punctate foci are likely to contain denatured actin. This suggests that there are cellular mechanisms triggered by SI that are attempting to undo the assumed damage that SI has inflicted upon cellular actin. Future studies will be needed to explore the involvement of chaperonins and their role in the SI response to gain a fuller picture of events triggered.

Heat-shock proteins are implicated in interactions with F-actin during SI

The other notable class of proteins more highly represented in SI-induced pollen was that of the heat-shock proteins (HSPs). HSPs represent an important cellular protective device. They act as molecular chaperonines by assisting in the refolding of misfolded proteins or in getting rid of irreversibly damaged proteins. Although separate from the chaperonins, they fall into a similar functional grouping as they also act in a similar cytoprotective role. Some HSPs act as intracellular chaperonines to prevent denaturation of proteins; others target protein aggregates for proteolytic degradation or dissociate them (Vierling, 1991; Liang and MacRae, 1997). The expression level of HSPs is generally up-regulated in response to stress. Some of the heat-shock proteins identified in our F-actin pull-down experiment have previously been shown to interact with actin in other cell types. HSP83-1, the heat-shock protein that was unique to the SI sample, is a member of the HSP90 group of heat-shock proteins. In mammalian cells, HSP90 and HSP100 have been shown to co-precipitate with actin under actin-polymerizing conditions (Koyasu et al., 1986). The reported F-actin cross-linking ability of mammalian HSP90 and HSP100 (Koyasu et al., 1986; Park et al., 2007) is interesting in the context of SI. The F-actin foci formed during SI appear to be high-order structures that would require proteins to cross-link the filaments to maintain their structure. Thus, HSP90 (HSP83-1) could potentially be involved in the formation of the foci by cross-linking the filaments. HSP70, more abundantly pulled down in the SI sample, is also upregulated in response to stress. A study by Macejak and Luftig (1991) on human cell lines (HEp-2) demonstrated that infection of these cells with adenovirus resulted in stabilization of the actin filaments, making them resistant to depolymerization by latrunculin. The authors proposed that HSP70 was responsible for this F-actin stabilization. Their study also showed that heat-shock of HEp-2 and HeLa cells resulted in rearrangement of F-actin around the nucleus, which was also resistant to latrunculin-induced depolymerization, and that HSP70 could stimulate actin polymerization from monomers in vitro. Our studies have shown that the SI-induced F-actin foci are resistant to concentrations of latrunculin that would normally depolymerize most of the F-actin in the cell. HSP70 could therefore be implicated in playing a role in the formation of the F-actin foci and could also be responsible for their increased stability. Moreover, HSP70 has a potential link to PCD, as it has been shown that Hsp70 can inhibit apoptosis by interfering with target proteins involved in animal cell apoptosis (Ravagnan et al., 2001).

Our data, suggesting preferential binding of HSPs to actin in SI-induced samples, provide the first evidence for a possible role of heat-shock proteins in the SI response. As SI is a form of stress, it is perhaps not surprising that heat-shock proteins were detected in the SI sample. Although considerably more work needs to be carried out into the potential involvement of the HSPs in the SI response, these studies point to a possible role that should be investigated further.

SI, ABPs AND A POSSIBLE ROLE IN PCD?

It is well established that normally functioning cells maintain a delicate balance of actin dynamics. Tight control of the dynamic equilibrium between monomeric G-actin and F-actin is important for maintenance of cellular function, and recent studies have placed actin and ABPs within signaling cascades that regulate apoptosis (for a recent review, see Franklin-Tong and Gourlay, 2008). It had previously been shown that the SI-mediated F-actin depolymerization plays a key role in SI-induced PCD (Thomas and Franklin-Tong, 2004). Artificially inducing F-actin depolymerization but also artificially stabilizing F-actin could trigger PCD in Papaver pollen tubes (Thomas and Franklin-Tong, 2004). Our recent data, showing that initial actin depolymerization is followed by an actin polymerization/stabilization phase, leading to the formation of large punctate actin foci to which CAP and ADF are associated, raises the possibility that both phases of actin reorganization are involved in regulating SI-induced PCD. Although not common, there are a few examples of the formation of very stable F-actin structures associated with stressed cells and cells undergoing apoptosis (Song et al., 1997; Sagot et al., 2006). Of considerable interest
is the finding that both ADF/cofilin and CAP, which were found to be associated with the SI-induced punctate actin foci, have been implicated in mediating PCD in mammalian cells where they require a functional actin-binding domain to promote apoptosis (Chua et al., 2003; Wang et al., 2008). Also, in yeast cells, the accumulation of F-actin aggregates triggers actin-mediated apoptosis, which is dependent on CAP/Srv2p’s actin-binding activity (Franklin-Tong and Gourlay, 2008). As SI could be considered as a stress response, it would be of considerable interest to explore the potential role of the formation of these F-actin aggregates in future studies to see if they play a role in the SI-induced PCD.

In summary, our studies reveal that Papaver SI uses a complex signalling network involving both the actin and microtubule cytoskeletons. Both are rapidly targeted and the organization of both is altered within minutes of SI induction. There is evidence for interplay between the actin and microtubule cytoskeletons that appears to play a functional role in mediating the outcome of PCD signals. We speculate that this may integrate signals that contribute to SI-mediated inhibition and death of incompatible pollen tubes through activation of several caspase-like activities, although further research is required to identify the exact mechanisms involved. Moreover, our recent studies provide some of the first insights into the actin-binding proteins and other proteins that may bind the previously largely uncharacterized SI-induced F-actin structures, including the possible involvement of the 14-3-3 proteins in the SI signalling network. The increase in numbers of HSPs and chaperonin associated with the F-actin foci suggest that these structures are composed of denatured and misfolded actin. Together our results provide a starting point for future studies that aim to explore possible mechanisms involved in the formation of these intriguing structures, which appear to be actively formed during the SI response.

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LITERATURE CITED


