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Journal research area most appropriate:

Signal Transduction and Hormone Action – Associate Editor Bonnie Bartel
Actin-binding proteins implicated in formation of the punctate actin foci stimulated by the self-incompatibility response in *Papaver*

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Revised manuscript for: Plant Physiology: submitted Tuesday, 12 January 2010
Footnotes:

Financial sources:
Work in the lab of V.E.F-T. is funded by the Biotechnology and Biological Sciences Research Council (B.B.S.R.C.). N.S.P. was funded by a B.B.S.R.C. studentship. Preliminary work was carried out by Galina Shevchenko, who was in receipt of a Royal Society NATO Fellowship. Work in the lab of C.J.S. was funded by grants from the US Department of Agriculture-National Research Initiative (2002-35304-12412) and the National Science Foundation (MCB-0130576). Work in the lab of JZR is funded by BBSRC and The Royal Society.

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Abstract

The actin cytoskeleton is a key target for signaling networks and plays a central role in translating signals into cellular responses in eukaryotic cells. Self-incompatibility (SI) is an important mechanism responsible for preventing self-fertilization. The SI system of *Papaver rhoeas* pollen involves a Ca\(^{2+}\)-dependent signaling network, including massive actin depolymerization as one of the earliest cellular responses, followed by the formation of large actin foci. However, no analysis of these structures, which appear to be aggregates of filamentous (F-)actin based on phalloidin staining, has been carried out to date. Here, we characterize and quantify the formation of F-actin foci in incompatible *Papaver* pollen tubes over time. The F-actin foci increase in size over time and we provide evidence that their formation requires actin polymerization. Once formed, these SI-induced structures are unusually stable, being resistant to treatments with latrunculin B (LatB). Further, their formation is associated with changes in the intracellular localization of two actin-binding proteins, cyclase-associated protein (CAP) and actin-depolymerizing factor (ADF). Two other regulators of actin dynamics, profilin and fimbrin, do not associate with the F-actin foci. This study provides 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.
Introduction

The ability to perceive and integrate signals into networks is essential for all eukaryotic cells. The actin cytoskeleton is a major target and integrator of signaling networks in eukaryotic cells. In plants, many extracellular stimuli lead to rapid structural changes in the actin cytoskeleton (Staiger, 2000; Hussey et al., 2006). Although many of the signaling intermediates that regulate actin dynamics are well-defined in animal cells and yeast; see for example, (Iden and Collard, 2008; Thomas et al., 2009), considerably less is known for plants. However, it is generally accepted that actin-binding proteins (ABPs) function as transducers of cellular stimuli into changes in cellular architecture (Hussey et al., 2006; Staiger and Blanchoin, 2006; Thomas et al., 2009). This includes three abundant monomer-binding proteins, profilin, actin-depolymerizing factor (ADF) and cyclase-associated protein (CAP) which function synergistically to stimulate actin turnover in vitro (Chaudhry et al., 2007). Bundling and cross-linking proteins, such as fimbrin, function to stabilize actin filaments into higher-order structures (Kovar et al., 2000; Thomas et al., 2009). These and other regulators of actin turnover are likely targets for signal-mediated changes in actin architecture in response to biotic and abiotic stresses.

Self-incompatibility (SI) is a genetically controlled system to prevent self-fertilization in flowering plants. SI is controlled by a multi-allelic S-locus; S-specific pollen rejection results from the interaction of pollen S- and pistil S- determinants that have matching alleles (Franklin-Tong, 2008). In Papaver rhoeas the pistil S-determinants (previously called S proteins, recently renamed PrsS (Foote et al., 1994; Wheeler et al., 2009), act as ligands, interacting with the pollen S-determinant PrpS (Wheeler et al., 2009), triggering increases in calcium influx and increases in cytosolic-free calcium ([Ca^{2+}]) in incompatible pollen (Franklin-Tong et al., 1993; Franklin-Tong et al., 1997; Franklin-Tong et al., 2002). The Ca^{2+}-mediated signaling network results in rapid inhibition of incompatible pollen tube growth and triggers programmed cell death (PCD) involving several caspase-like activities (Thomas and Franklin-Tong, 2004; Bosch and Franklin-Tong, 2007).

The SI response and the Ca^{2+}-signaling pathway in Papaver stimulates rapid reorganization and massive depolymerization of actin filaments in incompatible pollen tubes (Geitmann et al., 2000; Snowman et al., 2002). Moreover, it has been demonstrated that changes in actin
dynamics are necessary and sufficient for PCD initiation (Thomas et al., 2006). Intriguingly, there seems to be crosstalk between actin and microtubule cytoskeletons in mediating PCD in pollen (Poulter et al., 2008). Thus, there is compelling evidence for signaling to the actin cytoskeleton in mediating PCD during SI; see (Bosch et al., 2008) for a recent review. SI also triggers further changes to the actin cytoskeleton. Small F-actin foci are formed and these increase in size within the first hour after SI-stimulus and remain observable for at least 3 h (Geitmann et al., 2000; Snowman et al., 2002). These aggregates contain F-actin, as they stain with rhodamine phalloidin. The formation of the small actin foci and the larger F-actin structures occurs after cessation of pollen tube growth, so they are unlikely to play a role in pollen inhibition.

Punctate F-actin foci are unusual structures and there appears to be a paucity of examples of their formation in any eukaryotic cell type. Actin patches are associated with endocytosis in normally growing yeast (Pelham and Chang, 2001; Kaksonen et al., 2003; Ayscough, 2004; Young et al., 2004) and “actin nodules” are formed during filipodia formation in platelets (Calaminus et al., 2008). Actin bodies are formed when yeast cells enter the quiescent cycle (Sagot et al., 2006) and Hirano bodies are observed in animal cells and Dictyostelium undergoing stress or in the disease state (Hirano, 1994; Maselli et al., 2002). Large star-shaped actin arrays have been observed in pollen tubes growing in vivo (Lord, 1992), but their nature and function is unknown. When we first described the SI-induced structures, we avoided the terminology of patches or bodies, as it was not known whether they were either structurally or functionally comparable to any of these previously characterized actin-based structures.

Studies on the SI-mediated actin responses have, to date, focused on the initial phase of depolymerization, and no analysis of what is involved in the formation of the large punctate actin foci has been made. Here we show that the formation of punctate actin foci requires actin polymerization, but once formed they are unusually stable. Moreover, we find that their formation correlates with changes in the intracellular localization of two ABPs, CAP and ADF, but not with two other key regulators of actin dynamics, profilin and fimbrin.
Results

SI results in a dramatic alteration of actin cytoskeleton organization

Dramatic alterations to the actin cytoskeleton triggered by SI in incompatible pollen tubes have been described previously (Geitmann et al., 2000; Snowman et al., 2002) and are shown in Figure 1 to serve as a baseline for subsequent analyses and interpretation. The filamentous (F-)actin arrays in a normally growing pollen tube comprise longitudinal actin filament bundles and a sub-apical actin collar region (Fig. 1a). This configuration rapidly reorganizes upon SI stimulation. Initially, F-actin is detected in the tip region and there is prominent F-actin labeling adjacent to the plasma membrane (Fig. 1b, c). Later, small F-actin foci are distributed throughout the pollen tube (Fig. 1d & e). The size of these foci increases (Fig. 1e, f, g). In the later stages of SI, many of the large punctate F-actin foci are localized in the pollen tube cortex (Fig. 1f, g). They must contain F-actin as they stain with fluorescent-phalloidin, which is specific for polymeric actin. Moreover, these SI-induced alterations appear to be an active, organized process.

Temporal formation of the SI-induced F-actin foci

We analyzed the appearance and aggregation of F-actin foci triggered by SI quantitatively. The diameter of twenty F-actin foci per pollen tube (n = 10) was measured at various time points after SI-induction (see M&M and Supplemental Information for full details). Prior to SI induction, no F-actin foci were detected. After SI, the diameter of F-actin foci increased over time (Figure 2a). By 30 min post-SI, the average diameter (± s.e.m.) was 0.69 ± 0.01 µm and this increased significantly to 0.82 ± 0.02 µm by 60 min post SI-induction (p<0.001, ***), and to 0.96 ± 0.02 µm by 3 h post SI-induction (p<0.001, ***).

We also determined the average density of these actin foci within pollen tubes (see M&M and Supplemental Information for details). The increased size of the punctate F-actin foci was accompanied by a significant decrease in the abundance of these structures (Fig. 2b). Their mean number more than halved, decreasing from 35 ± 2.6 foci per 100 µm² at 30 min SI to 16 ± 1.2 foci at 3 h (p<0.001, ***; Fig. 2b).
The SI-induced punctate F-actin foci are resistant to depolymerization

‘F-actin bodies’ in quiescent yeast cells and F-actin aggregates in Vero cells, which appear somewhat similar to the SI-induced F-actin foci, are highly resistant to treatments with latrunculin (Sagot et al., 2006; Lazaro-Dieguez et al., 2008). Latrunculins bind to monomeric actin and inhibit polymerization, so they lead to the preferential disassembly of actin filaments and arrays that are undergoing rapid turnover. We therefore used LatB to test the turnover of the F-actin in the foci that had formed in pollen tubes 3 h after SI-induction.

In control pollen tubes, the actin cytoskeleton organization (Fig. 3a) was dramatically affected by treatment with 1 µM LatB for 10 min; most of the F-actin bundles disappeared, (Fig. 3b), consistent with extensive disassembly. This agrees with previous studies where treatment with 1 µM LatB for 10 min resulted in a 47% decrease in actin polymer levels (Thomas et al., 2006). In contrast, large F-actin aggregates in pollen tubes that had undergone SI induction for 3 h (Fig. 3c) were resistant to both 10 min (Fig. 3d) and 30 min (Fig. 3e) treatments with 1 µM LatB. These data demonstrate that the SI-induced F-actin foci are not undergoing rapid turnover and are resistant to disassembly in the presence of LatB. Thus, SI-induced F-actin foci have very different characteristics from the F-actin bundles comprising the major cytoskeletal elements in normally growing pollen tubes.

Formation of the punctate F-actin foci requires actin polymerization

We investigated whether the formation of these SI-induced F-actin foci requires actin polymerization. If actin foci formation is inhibited in the presence of LatB, we can assume that they result from new filament assembly. If they form in the presence of LatB, we can conclude that they predominantly originate from aggregation of pre-existing filaments and/or by filament stabilization. To test this, we added 1, 10 or 100 µM LatB to SI-induced pollen tubes prior to formation of the F-actin foci (at 10 min after SI) and allowed SI to progress for a total of 3 h (for 170 min in the presence of LatB). We subsequently observed the F-actin cytoskeleton using rhodamine-phalloidin staining. When 1 µM LatB was added to pollen tubes prior to formation of the foci and SI allowed to progress for a total of 3 h, the large F-actin foci were still formed (Fig 3f). However, higher concentrations of LatB had a marked effect on formation of F-actin foci. Thus, when added during the first 10 min of SI (prior to
the formation of the foci) and SI allowed to proceed for 3 h, 10 µM LatB resulted in a reduction in the number of F-actin foci formed (Fig 3g) and 100 µM LatB treatments completely prevented the formation of F-actin foci, and no other F-actin structures were detectable in any of these pollen tubes (Fig 3h). Assuming specificity of action of LatB (see discussion), these data demonstrate that formation of the F-actin foci requires actin polymerization.

A subset of ABPs co-localize with F-actin foci
Because the formation and maintenance of these F-actin aggregates most likely involves the concerted action of several ABPs, we investigated the possible involvement of a number of likely candidates in this process. As protein function can be implicated by intracellular localization, to further understand the formation and stability of these SI-induced F-actin foci, we examined the presence and co-localization of four major regulators of actin dynamics with the SI-induced F-actin foci. These comprised: cyclase-associated protein (CAP), actin-depolymerizing factor/cofilin (ADF), fimbrin and profilin.

Western blot analyses on total protein extracts from pollen established that the antibodies cross-reacted with the corresponding ABPs in Papaver pollen (Fig. 4). The anti-AtCAP1 (Chaudhry et al., 2007) identified poppy CAP at ~55 kDa (Fig. 4a). The fimbrin antibody, anti-AtFIM1, recognized a ~75 kDa protein (Fig. 4b); rabbit anti-LlADF cross-reacted with poppy ADF at ~18 kDa (Fig. 4c), as did mouse-anti-LlADF (Allwood et al., 2002) (Fig. 4d).

The anti-ZmPRO5 (Kovar et al., 2000) recognized poppy profilin (~14 kDa; Fig. 4e). Although there was some modest cross-reactivity with minor proteins or degradation products, this confirms that the antibodies were suitable for use in immunolocalization studies.

In order to perform co-localization of ABPs with F-actin, we used rhodamine phalloidin in conjunction with each antiserum. Initially, actin organization was examined in normally growing, untreated pollen tubes and in pollen tubes after 3 h SI (i.e. with large actin foci). After SI induction, very distinctive alterations in ABP localization were observed. In untreated pollen tubes, CAP appeared as fine speckles scattered throughout the pollen tube (Fig. 5a) with no detectable co-localization along the prominent F-actin bundles. At 3 h post-SI, a substantial amount of CAP colocalized with the large F-actin foci (Fig. 5b), but a
substantial proportion of the CAP signal remained as fine speckles (Fig. 5b). In untreated pollen tubes, ADF gave a diffuse cytosolic signal, with a stronger signal in the cortical regions, and no obvious co-localization with F-actin cables (Fig. 5c). At 3 h post-SI, a significant proportion of the ADF population had reorganized into large foci that co-localized with F-actin, but there also remained a strong cytosolic signal (Fig. 5d). The actin-filament stabilizing and bundling protein, fimbrin (Kovar et al., 2000), had two major distributions in untreated, growing poppy pollen tubes: fine speckles distributed throughout, and a co-localization along the major actin filament cables (Fig. 5e; Supplemental Fig. 1). After 3 h SI, fimbrin still appeared as fine speckles and its distribution was altered, but there was no co-localization with F-actin foci (Fig. 5f). In untreated pollen tubes, profilin appeared largely cytosolic, with some possible co-localization with F-actin bundles (Fig. 5g). After 3 h SI induction, the localization of profilin was distinctly altered, with profilin forming small foci-like aggregates; however, these did not co-localize with the F-actin foci (Fig. 5h). Thus, although all four ABPs showed alterations in localization after SI induction, only CAP and ADF co-localized with the large F-actin foci.

To test whether CAP and ADF were present in the same structures, we performed dual immunolocalization with a mouse anti-LlADF serum and rabbit anti-AtCAP1. Confocal imaging confirmed that these two proteins were present in the same foci after SI induction (Supplemental Fig. 2). Together these data suggest that both CAP and ADF play a role in the formation/maintenance of these punctate actin foci, at least during the later stages of SI induction.

CAP and ADF co-localize with F-actin in a similar temporal manner

As both CAP and ADF appeared to be virtually completely co-localized with the F-actin foci at 3 h post-SI, we wished to ascertain the spatio-temporal dynamics of their distribution. We therefore performed double labeling for actin and CAP (Supplemental Fig. 3) or actin and ADF (Supplemental Fig. 4) at various time points after SI induction and used confocal imaging with single optical sections, to assess the temporal progression of co-localization.

In untreated pollen tubes CAP was distributed as scattered small speckles that did not co-localize with F-actin (Supplemental Fig. 3a-c). By 10 min post-SI, when most F-actin was
found adjacent to the plasma membrane (Supplemental Fig. 3e), CAP localization was not detectably altered (Supplemental Fig. 3d) and only modest co-localization with actin was detected (Supplemental Fig. 3f). By 30 min post-SI, CAP had started to form small foci (Supplemental Fig. 3g), as had F-actin (Supplemental Fig. 3h), and some CAP co-localized with the small actin foci (Supplemental Fig. 3i). By 3 h SI, CAP had formed large foci throughout the pollen tube (Supplemental Fig. 3j & m) that co-localized with the large actin foci (Supplemental Fig. 3k & n). Although there was considerable association of CAP with the large F-actin foci, some diffuse labeling remained (Supplemental Fig. 3l & o), indicating that a proportion of the CAP remained cytosolic. ADF and F-actin displayed a similar pattern of alterations after SI induction (Supplemental Fig. 4a-o).

Quantitative analysis of ABP and F-actin co-localization alterations after SI induction

Although imaging indicated that ADF and CAP co-localized to the F-actin foci, quantification of these changes was considered important, as this would provide a measure of the alterations and would also allow the temporal dynamics to be analyzed in more detail. Briefly, fifty regions of interest (ROIs) where F-actin was brightly decorated with rhodamine phalloidin were selected from a single optical section of each of 5 pollen tubes. The ROIs were transferred, and the fluorescence signal of the ABP for the corresponding region measured to estimate co-localization of the ABP signal within the ROIs (see Materials and Methods and Supplemental Information 1 and Suppl. Fig 5 for full details).

Quantitation of the co-localization of CAP and ADF with F-actin showed that the association of these ABPs with F-actin was relatively low in untreated pollen tubes (mean ± s.e.m.; 16.8 ± 4.0%, 20 ± 3.3% respectively; n = 250), whereas profilin and fimbrin showed higher levels of co-localization (31.2 ± 3.0% and 46.4 ± 8.1% respectively; Fig. 6a). After SI induction, large alterations in the amount of CAP and ADF associated with F-actin were observed. Overall, the percentage co-localization of CAP and ADF with F-actin at 3 h post-SI rose by 77.9% and 74.7%, respectively (Fig. 6a), which was significantly different from controls (p<0.001). In contrast, by 3 h post-SI the co-localization of fimbrin and profilin with F-actin decreased significantly (p<0.05; Fig. 6a).
Quantitative analysis of CAP and ADF association with F-actin over the time-course of SI-induction examined, revealed that the trend for co-localization was very similar. However, ADF showed a slightly faster association with F-actin compared to CAP, although this was just below a statistically significant level (p=0.055; Fig. 6b). There were significant increases in the level of co-localization of ADF with F-actin at 10 min and 30 min post-SI induction (p<0.001 and p<0.01, respectively). CAP and F-actin co-localization also increased significantly at each time interval examined, until 1 h after SI-induction (p<0.05 for each time point). After 1 h SI-induction, the extent of CAP and ADF co-localization with F-actin was virtually identical, with nearly all of the F-actin foci co-localizing with both ABPs. No further significant changes in alteration were observed after 1 h SI, suggesting that most alterations were complete by 60 min post-SI. This co-localization was maintained for at least 3 h post SI-induction (Fig. 6b). Together, these data confirm the imaging data, and provide a good description of the timing and extent of the alterations in ABP localization induced by the SI response. This firmly establishes that CAP and ADF co-localize with F-actin punctate foci.

**Discussion**

Here we have investigated the composition, formation and dynamics of the punctate actin foci formed during the later stages of SI in incompatible *Papaver* pollen. The early phase of SI-induced actin reorganization comprises massive actin depolymerization in incompatible pollen, which will result in inhibition of pollen tube growth (Snowman et al., 2002) and has been shown to be necessary and sufficient for triggering PCD in poppy pollen (Thomas et al., 2006). In this study, we have examined subsequent SI-induced actin alterations, comprising the formation of large punctate actin foci, and show that a subset of known regulators of actin dynamics, CAP and ADF, associate with these structures. These studies provide the first insights into the mechanisms involved in the formation of these intriguing structures, which appear to be actively formed during the SI response in both pollen grains and pollen tubes (Geitmann et al., 2000; Snowman et al., 2002).

**The nature, formation and stability of the SI-induced punctate F-actin foci**

The current study establishes that SI triggers F-actin polymerization to form highly stable actin foci. These actin structures, once formed, are highly resistant to disassembly by LatB
under conditions that remove almost all detectable F-actin in normally growing pollen tubes; see (Snowman et al., 2002; Thomas et al., 2006). This sets them apart from many other actin-based structures, such as actin patches in yeast (Pelham and Chang, 2001; Kaksonen et al., 2003; Ayscough, 2004; Young et al., 2004) and “actin nodules” in platelets (Calaminus et al., 2008), which are very dynamic. However, it is clear that some cells under certain circumstances form very stable actin aggregates. Examples of structures with little or no actin- and ABP-turnover include actin bodies in quiescent yeast cells (Sagot et al., 2006), large F-actin aggregates in Vero cells (Lazaro-Dieguez et al., 2008), and now, SI-induced actin foci in pollen tubes. Currently there are no explanations for the formation or unusual dynamic properties of these structures. One possibility is that binding of specific ABPs stabilizes these structures against disassembly; for example, many side-binding proteins stabilize actin filaments from depolymerization (Thomas et al., 2009). Moreover, when actin filaments are highly decorated or saturated with ADF, severing of the filaments is not observed (Andrianantoandro and Pollard, 2006). We discuss the possible implications of CAP and ADF localization below.

As they require actin polymerization for assembly and label with fluorescent phalloidin, the SI-induced actin foci must contain F-actin. However, we cannot discount the possibility that they are composed of a mixture of G- and F-actin. As LatB prevents their formation, this suggests that they are formed through polymerization. Although we cannot discount that this may be due to a non-specific effect of the large amount of LatB required for this effect, these concentrations are likely to be what is required to completely block polymerization. This is because the G-actin pool in pollen is extremely large, perhaps as great as 100–200 µM (Gibbon et al., 1999; Snowman et al., 2002), providing a huge reservoir of actin subunits for polymerization. Although much lower concentrations of LatB inhibit pollen tube growth, this is not fully understood, and could be due to a variety of mechanisms. Our finding that actin polymerization is triggered, appears slightly at odds with our earlier observation that actin filament levels fell dramatically by 1 h post-SI (Snowman et al., 2002). However, it is possible that increases in polymer levels were not detected in the earlier quantitative assays (Snowman et al., 2002) because ADF co-localizes with these structures. ADF bound to F-actin is likely to interfere with the phalloidin-binding, and ADF and phalloidin binding on actin filaments is mutually exclusive (McGough et al., 1997).
ADF associates with the SI-induced F-actin foci

Here we have shown that SI induces ADF co-localization with the F-actin foci with increasing percentage of co-localization as aggregation progressed. ADF can bind to both G- and F-actin, mediating actin depolymerization by severing filaments and facilitating subunit loss from minus ends (Bamburg and Bernstein, 2008). ADF is cytosolic in Narcissus pollen tubes (Smertenko et al., 2001; Allwood et al., 2002); our data confirm this in normally growing poppy pollen tubes. However, reports that ADF colocalizes with F-actin structures exist. For example, in Vero cells, F-actin aggregates contain ADF (Lazaro-Dieguez et al., 2008). Tobacco pollen tubes expressing GFP-NtADF1 show ADF association with filamentous actin (Chen et al., 2002). Although it has been suggested that this might be a consequence of overexpression of the fusion protein (Wilsen et al., 2006), regardless of why the association was observed, this clearly demonstrates that under certain conditions, ADF can associate with F-actin in vivo.

ADF’s ability to regulate actin dynamics is altered by phosphorylation, polyphosphoinositides and pH; see (Bamburg, 1999). In plants, phosphorylation of ADF is Ca\(^{2+}\)-activated (Smertenko et al., 1998; Allwood et al., 2002); this decreases its actin-binding activity (Smertenko et al., 1998; Chen et al., 2002). As SI triggers large increases in [Ca\(^{2+}\)]\(_{cyt}\), ADF may be phosphorylated. However, as SI stimulates ADF association with F-actin, it suggests that ADF’s actin-binding activity is not inactivated. Thus, it appears unlikely that phosphorylation of ADF is responsible for regulating these alterations. Most ADFs, including those from plants, show a greater ability to depolymerize F-actin at alkaline pH in vitro (Carlier et al., 1997; Gungabissoon et al., 1998; Ressad et al., 1998; Allwood et al., 2002; Hayden et al., 2002) and at acidic pHs, ADF binds to and stabilizes F-actin. This is highly relevant to the SI-induced events, as we recently demonstrated dramatic SI-induced acidification of the pollen tube cytosol to ~pH 5.5 (Bosch and Franklin-Tong, 2007). Thus, acidification is a possible mechanism that may play a role in triggering the SI-induced alteration in localization of ADF from cytosol to F-actin and the association might alter the properties of the F-actin foci (e.g., enhance their stability). This will need to be explored in future studies.
CAP co-localizes with the SI-induced F-actin foci

The finding that CAP associates with the SI-induced F-actin foci initially appears somewhat surprising, as plant CAP binds with moderate affinity to G-actin (Barrero et al., 2002; Chaudhry et al., 2007; Deeks et al., 2007), though preliminary data suggest that AtCAP1 can also bind to F-actin (Deeks et al., 2007). However, (Deeks et al., 2007) there are several examples of CAP binding to F-actin structures. For example, in budding yeast (S. cerevisiae), Srv2p/CAP localizes to actin patches via interactions with the F-actin binding protein Abp1 (Lila and Drubin, 1997; Balcer et al., 2003); the formation of F-actin “bodies” in yeast is dependent on Srv2p/CAP activity (Gourlay et al., 2004), and actin bodies in quiescent yeast cells also contain Srv2p/CAP (Sagot et al., 2006). Here we describe another example of CAP associated with F-actin structures, though we cannot discount the possibility that these structures may also contain G-actin.

The finding that CAP co-localizes with actin foci may provide clues about actin turnover in these structures. In Arabidopsis, CAP acts as a nucleotide exchange factor for plant actin, catalyzing exchange of ADP for ATP on monomeric actin, potentially stimulating actin polymerization (Chaudhry et al., 2007). Thus, CAP may provide a pool of assembly-competent monomers, thereby playing a role in the formation of actin foci through this mechanism. CAP co-localization also provides clues about potential signaling networks. In budding yeast Srv2p/CAP binds to adenylate cyclase and facilitates cAMP/PKA activation; see (Franklin-Tong and Gourlay, 2008) for a recent review. Moreover, it has been proposed that AtCAP1 is part of a plant-specific signaling pathway in Arabidopsis (Deeks et al., 2007). Thus, CAP is an attractive candidate that could link signaling networks to actin reorganization. If this is the case, it suggests that the formation of the punctate actin foci and association of CAP to these structures is an active process that may signal to downstream SI events. Interestingly, Jasp-stabilization of F-actin did not stimulate reorganization of CAP in poppy pollen tubes (NSP, CJS & VEF-T, unpublished data), which indicates that it is perhaps not F-actin stabilization per se that is responsible for the CAP association.
**Fimbrin and profilin do not associate with the punctate F-actin foci**

Fimbrin cross-links and stabilizes actin filaments in plant cells (Kovar et al., 2000; Kovar et al., 2001) and might be expected to colocalize with the SI-induced actin foci. In quiescent yeast cells, fimbrin/Sac6p associates with stable actin bodies and is necessary for their formation or maintenance (Sagot et al., 2006). However, Arabidopsis fimbrin bundling activity is Ca\textsuperscript{2+}-independent so is unlikely to be affected by SI. Fimbrin did not co-localize with the SI-induced F-actin foci and does not appear to be involved in the formation of the SI-induced F-actin foci. As F-actin binding by mammalian fimbrin is reduced at pH 6.5 (Glenney et al., 1981), the SI-induced cytosolic acidification to ~pH 5.5 (Bosch and Franklin-Tong, 2007) could explain why fimbrin did not bind F-actin during the SI response.

Profilin, a cytosolic actin-monomer binding protein, is another major regulator of actin dynamics (Staiger and Blanchoin, 2006) and has previously been implicated in the early SI-induced depolymerization events (Snowman et al., 2002). Profilin together with a gelsolin-like protein, PrABP80, is thought to contribute to the rapid depolymerization of F-actin stimulated by SI in incompatible pollen tubes (Snowman et al., 2002; Huang et al., 2004). The current study shows that profilin and F-actin do not exhibit any significant overlap in normal or SI-treated pollen tubes. This supports the idea that these foci, once formed, predominantly comprise F-actin and there is little actin turnover occurring in these foci.

**Are the punctate actin foci involved in stress or PCD?**

Actin and ABPs are convincingly placed within signaling networks regulating commitment to apoptosis; see (Franklin-Tong and Gourlay, 2008) for a recent review. The formation of highly stable F-actin structures is associated with cells undergoing apoptosis (Song et al., 1997; Sagot et al., 2006), but this unusual stability is not always associated with apoptosis (Maselli et al., 2002; Sagot et al., 2006), but is thought to be a stress response. We do not know if the SI-induced aggregates are a plant equivalent of these structures, but SI could represent a stress response, and it would be interesting to explore this in future studies. ADF and CAP are implicated in mediating PCD (Chua et al., 2003; Wang et al., 2008). CAP plays a functional role in progression of apoptosis (Wang et al., 2008), and accumulation of F-actin aggregates triggers actin-mediated apoptosis in yeast, which is dependent on CAP/Srv2p’s actin-binding activity (Gourlay and Ayscough, 2006; Franklin-Tong and Gourlay, 2008). It is
known that SI triggers PCD and that the SI-mediated depolymerization plays a role in PCD (Thomas and Franklin-Tong, 2004; Bosch and Franklin-Tong, 2007). Moreover, both F-actin depolymerization and stabilization using LatB and jasplakinolide could trigger PCD in pollen tubes (Thomas et al., 2006). The current study, showing that SI also has an actin polymerization/stabilization phase, comprising the formation of large, punctate actin foci that are associated with CAP, raises the possibility that both phases of actin reorganization may play a role in regulating PCD. The possible role of CAP signaling to PCD when stable actin aggregates are formed will be the focus of future studies.

In summary, we have shown that the punctate actin foci formed during SI have both ADF and CAP, but not profilin or fimbrin, prominently associated with them. This association of actin with a subset of ABPs probably contributes to their increased stability. Our data suggest that SI upsets the normal cellular actin dynamics, disturbing the delicate balance between the actin cytoskeleton and the ABPs that modulate responses to less extreme stimuli. We propose that the extreme SI-triggered actin depolymerization followed by stabilization is a stress response, modulated by these ABPs. Future studies will attempt to elucidate the function of the punctate actin foci and also examine if these structures are formed during SI in vivo.
MATERIALS AND METHODS

Pollen treatments
Pollen of *P. rhoeas*, the field poppy, was germinated and grown *in vitro* in liquid GM (0.01% H$_3$BO$_3$, 0.01% KNO$_3$, 0.01% Mg(NO$_3$)$_2$-6H$_2$O, 0.036% CaCl$_2$-2H$_2$O, and 13.5% sucrose) as described previously (Snowman et al., 2002) at 25°C. Pollen was grown for 1 h before any treatments were applied.

For SI treatments, recombinant proteins were produced by cloning the nucleotide sequences specifying the mature peptide of the $S_1$ and $S_3$, alleles of the $S$ gene (pPRS100, pPRS300) into the expression vector pMS119 as described previously (Foote et al., 1994). Expression and purification of the proteins was performed as described previously (Kakeda et al., 1998). SI was induced by adding recombinant $S$ proteins (final concentration 10 µg.ml$^{-1}$) to pollen growing *in vitro*; see (Snowman et al., 2002).

For the cytoskeleton drug treatments Latrunculin B (LatB; Calbiochem, UK), at the concentration detailed in the text, or 0.5 µM Jasplakinolide (Jasp; Calbiochem, UK) was added to growing pollen tubes.

Fixation and Immunolocalization
Pollen tubes were fixed in 400 µM 3-maleimodobenzoic acid N-hydroxysuccinimide ester (MBS, Pierce) for 6 min at 20°C, followed by 2% formaldehyde in GM (1 h, 4°C). Although these cells are fixed, we can be sure that the F-actin foci are not an artefact, as they are not found in untreated samples or at earlier time points after SI induction, and all samples were treated in the same way. Cells were washed in actin-stabilizing buffer (ASB: 100 mM Pipes, pH 6.8, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 75 mM KCl) then in MES buffer (15 mM MES, pH 5.0), then incubated in 0.05% cellulose/0.05% macerozyme with 0.1% Triton X-100 in MES buffer containing 0.1 mM PMSF and 1% BSA for 15 min. Cells were washed once in MES, then twice in TBS and then incubated in blocking solution (1% BSA in TBS) for 30 min at room temperature. Pollen was incubated with primary antibody diluted in blocking solution overnight at 4°C. Following TBS washes pollen was then incubated with the secondary antibody for 1.5 h at room temperature followed by further TBS washes. Pollen tubes were
mounted on slides with 5 μL of Vectashield + DAPI (Vector Laboratories, USA) and coverslips sealed with nail varnish. For co-localization studies, F-actin was stained using 66 nM rhodamine-phalloidin (Invitrogen).

Sources/citations and dilutions of antibodies

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Titre for western blot</th>
<th>Titre for immuno-localization</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-AtCAP1</td>
<td>1:2000</td>
<td>1:500</td>
<td>(Chaudhry et al., 2007)</td>
</tr>
<tr>
<td>Rabbit anti-LlADF</td>
<td>1:2000</td>
<td>1:500</td>
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<tr>
<td>Rabbit anti-AtFIM1</td>
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<tr>
<td>Rabbit anti-ZmPRO5</td>
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<td>(Kovar et al., 2000)</td>
</tr>
<tr>
<td>Mouse anti-LlADF1</td>
<td>1:1000</td>
<td>1:300</td>
<td>(Allwood et al., 2002)</td>
</tr>
</tbody>
</table>

Secondary antibodies were as follows: Goat anti-mouse IgG (Fₐb-specific) -FITC (Sigma-Aldrich, UK). Titre: 1:300; Goat anti-rabbit IgG-FITC (Sigma-Aldrich, UK). Titre: 1:200; Sheep anti-rabbit IgG-Cy3 (Sigma-Aldrich, UK). Titre: 1:300.

Imaging

Images were collected using a BioRad Radiance 2000 laser-scanning system (50-mW Ar laser, 488-nm line and 1.5 mW HeNe laser, 543 nm) with a 60x plan-Apo 1.4 NA oil objective (Nikon). Full z-series stacks or single section scans of the pollen tubes were taken using the following microscope settings: 512 x 512 pixel box, a 166 line speed scan and 3 Kalman scans. Images were analyzed using ImageJ software (Rasband, W.S., ImageJ, NIH, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2008) and archived as TIF files.

Quantification of imaging data

To make comparisons between the average density of F-actin foci at different time-points after SI-induction, equivalent regions of pollen tubes (four 30 x 30 pixel boxes, each comprising a 3.9 x 3.9 μm area, placed within the first 60 μm of the pollen tube to ensure comparison of equivalent regions in all cases) were assessed. Full methodological details for quantification of co-localization are provided in Supplemental Information 1 and Suppl. Fig 5). Briefly, for the co-localization analysis, 50 F-actin ROIs for each of 5 pollen tubes
were assessed for co-localization with each of the four ABPs for each treatment. Single optical sections were used for analysis. No significant bleedthrough was detected (see Supplemental Information 1, Fig 6), giving confidence that co-localization was real. We used MetaMorph software (Molecular Devices) to perform these analyses, and developed a new method to allow quantification of the co-localization, as standard co-localization algorithms examine the level of co-localization across a whole image, whereas we were interested in analyzing co-localization specifically with the F-actin structures. Moreover, the structures we wished to analyze changed from long filamentous structures to rounded foci. See Supplemental Information 1 for full details.

Protein extraction and Western blotting
Pollen was hydrated and grown as described. Pollen tubes were collected by centrifugation and some of the liquid GM removed. Pollen proteins were extracted by homogenization in 2xTris buffer (100 mM Tris-HCl (pH 8), 200 mM NaCl, 2 mM EDTA, 1 M sucrose, 2x protease inhibitor cocktail (Roche, Complete mini, EDTA-free)) and analyzed using SDS–PAGE and western blot. Blots were probed with the dilutions of primary antibodies detailed above and detected using alkaline phosphatase.

Acknowledgements
We thank the horticultural staff for growing the plants and helping harvest material. We are grateful to Patrick Hussey for kindly providing the LIADF antibody, and for being involved in preliminary studies related to this topic. Preliminary work was carried out by Galina Shevchenko, who was in receipt of a Royal Society NATO Fellowship. The studies described were carried out by N.S.P. as part of her PhD studies, supported by a B.B.S.R.C. studentship.

Literature Cited


Young ME, Cooper JA, Bridgman PC (2004) Yeast actin patches are networks of branched actin filaments. J. Cell Biol. **166**: 629-635
**Figure legends**

**Figure 1. F-actin organization is altered during the SI response**

(a) F-actin organization in an untreated pollen tube showing prominent actin filament cables in the shank of the tube and a subapical actin filament collar.
(b) 10 min after SI induction, much of the F-actin was depolymerized, but a population of F-actin adjacent to the plasma membrane remained.
(c) Small actin fragments were detected at 30 min.
(d) Coincident with formation of small F-actin foci, the distinctive continuous cortical F-actin was lost around 30 min post-SI.
(e) The cortical F-actin foci increased in size, to form large punctate foci between 1-2 h after SI induction.
(f) and (g) At 3h post-SI, large F-actin “foci” had formed.

All images (except (g), which is a full projection) are single optical sections using confocal microscopy. F-actin was detected with rhodamine-phalloidin. Scale bar, 10 µm.

**Figure 2. SI stimulates increases in size and decreases in the number of F-actin foci in incompatible pollen tubes**

(a) The diameter of the SI-induced actin foci increased over time. Values are the mean diameter (µm ± s.e.m) of 20 F-actin foci per pollen tube for 10 individual pollen tubes.

(b) The mean number of actin foci in a 100 mm² area of the pollen tube decreased over time. Quantification was carried out on single optical sections and values are the mean (± s.e.m) of 4 boxes per tube for 10 individual pollen tubes, as described in Supplemental Methods.

The significance values (*** = p<0.001) relate to t-tests carried out between the time point indicated and the previous time point.

**Figure 3. The SI-induced F-actin foci are stable structures and do not require actin polymerization for formation**

(a) F-actin organization in an untreated (UT) pollen tube.
(b) After treatment of a normal pollen tube with 1 µM LatB for 10 min, the majority of the F-actin bundles had disappeared, indicating depolymerization.
(c) After 3 h SI induction in the absence of LatB, numerous large F-actin foci were observed.
(d) No apparent alteration was observed when 3 h SI-treated pollen tubes were subsequently treated with 1 µM LatB for 10 min (3h SI + 10’ LatB).
(e) Treatment of a 3 h SI-treated pollen tube with 1 µM LatB for 30 min (3h SI + 30’ 1 µM LatB) also resulted in no detectable effect on formation of F-actin foci.
Treatment with 1 μM LatB at 10 min post SI-induction (prior to formation of actin foci) and incubation for 3 h had no detectable effect on F-actin foci formation (10’ SI + 170’ 1 μM LatB).

10 μM LatB added 10 min post-SI induction had a detectable effect on the formation of F-actin foci at 3h SI (10’ SI + 170’ 10 μM LatB), and

100 μM LatB added 10 min post-SI induction completely prevented the formation of F-actin foci at 3h SI (10’ SI + 170’ 100 μM LatB).

All images are full projections of confocal sections of actin visualized with rhodamine-phalloidin. Scale bar, 10 μm

**Figure 4. The ABP antibodies cross-react with poppy pollen proteins**

a) The anti-AtCAP1 (1:2000) recognized a prominent band ~55 kDa, representing poppy CAP (arrow).
b) The anti-AtFim1 (1:2000) recognized a prominent band ~75 kDa, representing poppy fimbrin (arrow).
c) The anti-LlADF rabbit antiserum (1:2000) identified a prominent band ~18 kDa, representing poppy ADF (arrow).
d) The anti-LlADF mouse antiserum (1:1000) identified the same ~18 kDa band, representing poppy ADF (arrow).
e) The anti-ZmPRO5 (1:2000) identified a band ~14 kDa, representing poppy profilin (arrow).

**Figure 5. A subset of ABPs co-localizes with the F-actin foci.**

CAP (a, b), ADF (c, d), fimbrin (e, f) and profilin (g, h) localization (green), and F-actin localization (red) in pollen tubes that were either growing normally (untreated; a, c, e, g) or after 3h SI induction (SI; b, d, f, h).

In untreated (UT) pollen tubes CAP and ADF (green) were cytosolic, whereas profilin and fimbrin (green) co-localized to some extent with F-actin (red). After 3 h SI induction, CAP & ADF co-localized to the punctate F-actin foci (b & d; yellow signal); profilin & fimbrin did not (f & h).

(a-h) are confocal sections showing F-actin stained with rhodamine phalloidin (red) with the corresponding ABP immunolocalization (green; see M&M for details of antisera used); co-localization is shown as yellow. Scale bar, 10 μm.
Figure 6. Quantitation of co-localization of ABPs with F-actin

(a) Co-localization of CAP, ADF, fimbrin and profilin with F-actin in untreated pollen tubes (white bars) was compared with their localization in pollen tubes that had been SI induced for 3 h (black bars). Values are mean ± s.e.m.. CAP and ADF showed significant increases in co-localization with F-actin in the 3 h SI treatment compared to the untreated tubes (*** = p<0.001). Fimbrin and profilin both showed a significant reduction in co-localization with F-actin in the 3 h SI compared to untreated pollen tubes (* = p<0.05).

(b) CAP or ADF co-localization with F-actin at the given time points after SI induction. Values are mean ± s.e.m..

In each case, fifty F-actin ROIs for each of 5 pollen tubes (a total of 250 spots) per treatment were analyzed for each of the ABPs.
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