Actin-Binding Proteins Implicated in the Formation of the Punctate Actin Foci Stimulated by the Self-Incompatibility Response in *Papaver*

Natalie S. Poulter, Christopher J. Staiger, Joshua Z. Rappoport, and Vernonica E. Franklin-Tong*

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom (N.S.P., J.Z.R., V.E.F.-T.); and Department of Biological Sciences and the Bindley Bioscience Center, Purdue University, West Lafayette, Indiana 47907–1392 (C.J.S.)

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. Furthermore, their formation is associated with changes in the intracellular localization of two actin-binding proteins, cyclase-associated protein and actin-depolymerizing factor. Two other regulators of actin dynamics, profilin and fimbrin, do not associate with the F-actin foci. This study provides, to our knowledge, 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.

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 (Ieden 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., 2000b; 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 multiallelic 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; Wheel et al., 2009) act as ligands, interacting with the pollen S-determinant PrpS (Wheel et al., 2009), triggering increases in calcium influx and increases in cytosol-free calcium in incompatible pollen (Franklin-Tong et al., 1993, 1997, 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* stimulate 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 cross talk 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 (for a recent review, see Bosch et al., 2008). 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 are 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 to date have 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 F-actin arrays in a normally growing pollen tube comprise longitudinal actin filament bundles and a subapical 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. 1, B and C). Later, small F-actin foci are distributed throughout the pollen tube (Fig. 1, D and E). The size of these foci increases (Fig. 1, E–G). In the later stages of SI, many of the large punctate F-actin foci are localized in the pollen tube cortex (Fig. 1, F and 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 20 F-actin foci per pollen tube (n = 10) was measured at various time points after SI induction (for

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, At 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 the 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 and 2 h after SI induction. F and G, At 3 h 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. Bar = 10 μm.
full details, see “Materials and Methods”; Supplemental Information S1). Prior to SI induction, no F-actin foci were detected. After SI, the diameter of F-actin foci increased over time (Fig. 2A). By 30 min post SI, the average diameter (±SE) 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 (for details, see “Materials and Methods”; Supplemental Information S1). 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 was more than halved, decreasing from 35 ± 2.6 foci per 100 μm² at 30 min post 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. Therefore, we used latrunculin B (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 preexisting 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 was 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 the formation of F-actin foci. Thus, when added during the first 10 min of SI (prior to the formation of the foci) and SI was allowed to proceed for 3 h, 10 μM LatB resulted in a reduction in the number of F-actin foci formed (Fig. 3G), 100 μM LatB treatments completely prevented the formation of F-actin foci, and no other F-actin structures were
A Subset of ABPs Colocalize with F-Actin Foci

Because the formation and maintenance of these F-actin aggregates most likely involve 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 inferred by intracellular localization, to further understand the formation and stability of these SI-induced F-actin foci, we examined the presence and colocalization of four major regulators of actin dynamics with the SI-induced F-actin foci. These regulators were CAP, 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 approximately 55 kD (Fig. 4A). The fimbrin antibody, anti-AtFIM1, recognized an approximately 75-kD protein (Fig. 4B); rabbit anti-LiADF cross-reacted with poppy ADF at approximately 18 kD (Fig. 4C), as did mouse anti-LiADF (Allwood et al., 2002; Fig. 4D). The anti-ZmPRO5 (Kovar et al., 2000a) recognized poppy profilin (approximately 14 kD; 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 colocalization 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 at 3 h post SI (i.e. with large actin foci). After SI induction, very distinctive alterations in

**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 of 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 (3 h SI + 10' 1 μM LatB). E, Treatment of a 3-h SI-treated pollen tube with 1 μM LatB for 30 min also resulted in no detectable effect on formation of F-actin foci (3 h SI + 30' 1 μM LatB). F, Treatment with 1 μM LatB at 10 min post SI induction (prior to the formation of foci) and incubation for 3 h had no detectable effect on F-actin foci formation (10' SI + 170' 1 μM LatB). G, LatB (10 μM) added 10 min post SI induction had a detectable effect on the formation of F-actin foci at 3 h of SI (10' SI + 170' 10 μM LatB). H, LatB (100 μM) added 10 min post SI induction completely prevented the formation of F-actin foci at 3 h of SI (10' SI + 170' 100 μM LatB). All images are full projections of confocal sections of actin visualized with rhodamine phalloidin. Bar = 10 μm.

**Figure 4.** The ABP antibodies cross-react with poppy pollen proteins. A, The anti-AtCAP1 antiserum (1:2,000) recognized a prominent band of approximately 55 kD, representing poppy CAP (arrowhead). B, The anti-AtFIM1 antiserum (1:2,000) recognized a prominent band of approximately 75 kD, representing poppy fimbrin (arrowhead). C, The anti-LiADF rabbit antiserum (1:2,000) identified a prominent band of approximately 18 kD, representing poppy ADF (arrowhead). D, The anti-LiADF mouse antiserum (1:1,000) identified the same approximately 18-kD band, representing poppy ADF (arrowhead). E, The anti-ZmPRO5 antiserum (1:2,000) identified a band of approximately 14 kD, representing poppy profilin (arrowhead).
ABP localization were observed. In untreated pollen tubes, CAP appeared as fine speckles scattered throughout the pollen tube (Fig. 5A), with no detectable colocalization 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 colocalization with F-actin cables (Fig. 5C). At 3 h post SI, a significant proportion of the ADF population had reorganized into large foci that colocalized with F-actin, but there also remained a strong cytosolic signal (Fig. 5D). The actin filament-stabilizing and -bundling protein, fimbrin (Kovar et al., 2000b), had two major distributions in untreated, growing poppy pollen tubes: fine speckles distributed throughout, and a colocalization along the major actin filament cables (Fig. 5E; Supplemental Fig. S1). After 3 h post SI, fimbrin still appeared as fine speckles and its distribution was altered, but there was no colocalization with F-actin foci (Fig. 5F). In untreated pollen tubes, profilin appeared largely cytosolic, with some possible colocalization with F-actin bundles (Fig. 5G). At 3 h post SI induction, the localization of profilin was distinctly altered, with profilin forming small foci-like aggregates; however, these did not colocalize with the F-actin foci (Fig. 5H).

Thus, although all four ABPs showed alterations in localization after SI induction, only CAP and ADF colocalized 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. S2). Together, these data suggest that both CAP and ADF play roles in the formation/maintenance of these punctate actin foci, at least during the later stages of SI induction.

**CAP and ADF Colocalize with F-Actin in a Similar Temporal Manner**

As both CAP and ADF appeared to be virtually completely colocalized with the F-actin foci at 3 h post SI, we wished to ascertain the spatiotemporal dynamics of their distribution. Therefore, we performed double labeling for actin and CAP (Supplemental Fig. S3) or actin and ADF (Supplemental Fig. S4) at various time points after SI induction and used confocal imaging with single optical sections to assess the temporal progression of colocalization.

In untreated pollen tubes, CAP was distributed as scattered small speckles that did not colocalize with F-actin (Supplemental Fig. S3, A–C). By 10 min post SI, when most F-actin was found adjacent to the plasma membrane (Supplemental Fig. S3E), CAP localization was not detectably altered (Supplemental Fig. S3D) and only modest colocalization with actin was detected (Supplemental Fig. S3F). By 30 min post SI, CAP had started to form small foci (Supplemental Fig. S3G), as had F-actin (Supplemental Fig. S3H), and some CAP colocalized with the small actin foci (Supplemental Fig. S3I). By 3 h post SI, CAP had formed large foci throughout the pollen tube (Supplemental Fig. S3, J and M) that colocalized with the large actin foci (Supplemental Fig. S3, K and N). Although there was considerable association of CAP with the large F-actin foci, some diffuse labeling remained (Supplemental Fig. S3, L and 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. S4).

**Quantitative Analysis of ABP and F-Actin Colocalization Alterations after SI Induction**

Although imaging indicated that ADF and CAP colocalized 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 five pollen tubes. The ROIs were transferred, and the fluorescence signal of the ABP for the corresponding region was measured to estimate colocalization of the ABP signal within the ROIs (for full details, see “Materials and Methods”; Supplemental Information S1; Supplemental Fig. S5).

Quantitation of the colocalization 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 ± se: 16.8% ± 4.0% and 20% ± 3.3%, respectively; n = 250), whereas profilin and fimbrin showed higher levels of colocalization (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 colocalization 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 colocalization 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 colocalization was very similar. However, ADF showed a slightly faster association with F-actin compared with CAP, although this was just below a statistically significant level (P = 0.055; Fig. 6B). There were significant increases in the level of colocalization of ADF with F-actin at 10 and 30 min post SI induction (P < 0.001 and P < 0.01, respectively). CAP and F-actin colocalization also increased significantly at each time interval examined, until 1 h after SI induction (P < 0.05 for each time point). After 1 h post SI induction, the extent of CAP and ADF colocalization with F-actin was virtually identical, with nearly all of the F-actin foci colocalizing with both ABPs. No further significant changes in alteration were observed at 1 h post SI, suggesting that most alterations were complete by 60 min post SI. This colocalization 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 colocalize 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 the 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, to our knowledge, 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

Figure 6. Quantitation of colocalization of ABPs with F-actin. A, Colocalization of CAP, ADF, fimbrin, and profilin with F-actin in untreated pollen tubes (white bars) compared with their localization in pollen tubes that had been SI induced for 3 h (black bars). Values are means ± se. CAP and ADF showed significant increases in colocalization with F-actin in the 3-h SI treatment compared with the untreated tubes (*** P < 0.001). Fimbrin and profilin both showed a significant reduction in colocalization with F-actin in the 3-h SI tubes compared with the untreated pollen tubes (* P < 0.05). B, CAP or ADF colocalization with F-actin at the given time points after SI induction. Values are means ± se. In each case, 50 F-actin ROIs for each of five pollen tubes (a total of 250 spots) per treatment were analyzed for each of the ABPs.
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 (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; Kakhosan 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 (Lazar-Dieguex 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 nonspecific effect of the large amounts 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 to completely block polymerization. This is because these concentrations are likely to be what is required for the formation of 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 nonspecific 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 to 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 colocalizes with these structures. ADF bound to F-actin is likely to interfere with the phalloidin binding as ADF and phalloidin binding on actin filaments are mutually exclusive (McGough et al., 1997).

**ADF Associates with the SI-Induced F-Actin Foci**

Here, we have shown that SI induces ADF colocalization with the F-actin foci, with increasing percentage of colocalization 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 (Lazar-Dieguex et al., 2008). Tobacco (Nicotiana tabacum) 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 (Wilson 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 (Bamburg, 1999). In plants, phosphorylation of ADF is Ca2+ activated (Smertenko et al., 1998; Allwood et al., 2002); this decreases its actin-binding activity (Smertenko et al., 1998; Chen et al., 2002). ASI triggers large increases in cytosolic Ca2+ concentration, 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 pH, 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 approximately 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 Colocalizes 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), although preliminary data suggest that AtCAP1 can also bind to F-actin (Deeks et al., 2007). However, there are several examples of CAP binding to F-actin structures. For example, in budding yeast (Saccharomyces 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 forma-
F-Actin Foci Stimulated by Self-Incompatibility

The finding that CAP colocalizes with actin foci may provide clues about actin turnover in these structures. In Arabidopsis (Arabidopsis thaliana), 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 colocalization also provides clues about potential signaling networks. In budding yeast, Srv2p/CAP binds to adenylate cyclase and facilitates cAMP/PKA activation (for a recent review, see Franklin-Tong and Gourlay, 2008). 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 the association of CAP to these structures is an active process that may signal to downstream SI events. Interestingly, jasplakinolide stabilization of F-actin did not stimulate the reorganization of CAP in poppy pollen tubes (Natalie S. Poulter, Christopher J. Staiger, and Veronica E. Franklin-Tong, unpublished data), which indicates that it is perhaps not F-actin stabilization per se that is responsible for the CAP association.

Are the Punctate Actin Foci Involved in Stress or PCD?

Actin and ABPs are convincingly placed within signaling networks regulating commitment to apoptosis (for a recent review, see Franklin-Tong and Gourlay, 2008). 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 the 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). This 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 roles in regulating SI-induced 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.

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., 2000b, 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\(^{2+}\) independent, so it is unlikely to be affected by SI. Fimbrin did not colocalize 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 approximately 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 Blanchin, 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). This 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 constitute F-actin, and there is little actin turnover occurring in these foci.
MATERIALS AND METHODS

Pollen Treatments

Pollen of the field poppy *Papaver rhoas* was germinated and grown in vitro in liquid germination medium [GM; 0.01% H2BO3, 0.01% KNO3, 0.01% Mg(NO3)2-6H2O, 0.036% CaCl2-2H2O, and 13.5% Suc] 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 S3 and S4 alleles of the S gene (pPRBS100 and pPR300) into the expression vector pMS19 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 of 10 μg·mL−1) to pollen growing in vitro (Snowman et al., 2002).

For the cytoskeleton drug treatments, LatB (Calbiochem), at the concentration detailed in the text, or 0.5 μM jaspалaminolide (Calbiоchem) was added to growing pollen tubes.

Fixation and Immunolocalization

Pollen tubes were fixed in 400 μL 3-maleimidoobenzoic acid N-hydroxy-succinimide ester (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 artifact, 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 (100 μM Pipes, pH 6.8, 1 mM MgCl2, 1 mM CaCl2, and 75 mM KC1) and 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 methylmethanesulfonyl fluoride and 1% bovine serum albumin for 15 min. Cells were washed once in MES and then twice in Tris-buffered saline (TBS) and then incubated in blocking solution (1% bovine serum albumin in TBS) for 30 min at room temperature. Pollen was incubated with primary antibody (for details, see Supplemental Table S2) 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 + 4,6-diamidino-2-phenylindole (Vector Laboratories) and coverslips sealed with nail varnish. For colocalization studies, F-actin was stained using 66 μM rhodamine phalloidin (Invitrogen).

Imaging

Images were collected using a Bio-Rad Radiance 2000 laser-scanning system (50-μW argon laser, 488-nm line, and 1.5 μW helium-neon laser, 543 nm) with a 60× Plan-Apo 1.4 numerical aperture oil objective (Nikon). Full z-series stacks or single section scans of the pollen tubes were taken using the following microscope settings: 512 × 512 pixel box, a 166 line speed scan, and a 3.9-μm pixel size.

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-× 30-pixel boxes, each comprising a 3.9-× 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 colocalization are provided in Supplemental Information S1 and Supplemental Figure S5. Briefly, for the colocalization analysis, 50 F-actin ROIs for each of five pollen tubes were taken using the following microscope settings: 512 × 512 pixel box, a 166 line speed scan, and three Kalman scans. Images were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/) and archived as TIF files.

Protein Extraction and Western Blotting

Pollen was hydrated and grown as described. Pollen tubes were collected by centrifugation, and some of the liquid GM was removed. Pollen proteins were extracted by homogenization in 2× Tris buffer (100 mM Tris-HCl, pH 8, 200 mM NaCl, 2 mM EDTA, 1 mM Suc, and 2× protease inhibitor cocktail [Roche; Complete mini, EDTA free]) and analyzed using SDS-PAGE and western blot. Blots were probed with the dilutions of the primary antibodies detailed in Supplemental Table S2 and detected using alkaline phosphatase.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Fimbrin colocalizes with some of the large F-actin bundles.

Supplemental Figure S2. CAP and ADF colocalize at 3-h SI.

Supplemental Figure S3. CAP localization is altered during the SI response.

Supplemental Figure S4. ADF localization is altered during the SI response.

Supplemental Figure S5. Quantification of colocalization using MetaMorph.

Supplemental Figure S6. Bleed through did not give false positives for colocalization quantitative data.

Supplemental Table S1. Equations used to calculate colocalization of actin foci and ABPs.

Supplemental Table S2. Details of the antibodies used.

Supplemental Information S1. Methodology for image analysis using MetaMorph.

ACKNOWLEDGMENTS

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.

Received December 8, 2009; accepted January 13, 2010; published January 15, 2010.

LITERATURE CITED


Kovar DR, Staiger CJ, Weaver EA, McCurdy DW (2000b) AffiGel is an actin filament crosslinking protein from Arabidopsis thaliana. Plant J 24: 625–636