Microtubules Are a Target for Self-Incompatibility Signaling in Papaver Pollen

Poulter, Natalie; Vatovec, S; Franklin-Tong, Vernonica

DOI:
10.1104/pp.107.107052

Microtubules Are a Target for Self-Incompatibility Signaling in *Papaver* Pollen[1]

Natalie S. Poulter, Sabina Vatovec, and Vernonica E. Franklin-Tong*

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom

Perception and integration of signals into responses is of crucial importance to cells. Both the actin and microtubule cytoskeleton are known to play a role in mediating diverse stimulus responses. Self-incompatibility (SI) is an important mechanism to prevent self-fertilization. SI in *Papaver rhoeas* triggers a Ca^{2+}-dependent signaling network to trigger programmed cell death (PCD), providing a neat way to inhibit and destroy incompatible pollen. We previously established that SI stimulates F-actin depolymerization and that altering actin dynamics can push pollen tubes into PCD. Very little is known about the role of microtubules in pollen tubes. Here, we investigated whether the pollen tube microtubule cytoskeleton is a target for the SI signals. We show that SI triggers very rapid apparent depolymerization of cortical microtubules, which, unlike actin, does not reorganize later. Actin depolymerization can trigger microtubule depolymerization but not vice versa. Moreover, although disruption of microtubule dynamics alone does not trigger PCD, alleviation of SI-induced PCD by taxol implicates a role for microtubule depolymerization in mediating PCD. Together, our data provide good evidence that SI signals target the microtubule cytoskeleton and suggest that signal integration between microfilaments and microtubules is required for triggering of PCD.

The plant cytoskeleton comprises actin microfilaments and tubulin microtubules that are highly dynamic through their interaction with various actin-binding proteins and microtubule-associated proteins (Erhardt and Shaw, 2006; Hussey et al., 2006). Both actin microfilaments and cortical microtubules play a key role in determining cell shape and growth, and recent work has provided valuable insights (Smith and Oppenheimer, 2005). There is now considerable evidence that the plant actin cytoskeleton plays a key role in modulating signal-response coupling, with many examples of actin mediating various biotic and abiotic responses (Staiger, 2000). Cortical microtubules are also involved in signal-response coupling. It has been shown that abiotic stimuli, such as gravity (Himmelspach et al., 1999), hormones (Shibaoka, 1994), freezing (Bartolo and Carter, 1991), and salt stress (Shoji et al., 2006), result in the reorientation or depolymerization of microtubules. Biotic interactions resulting in microtubule alterations also exist. Plant interactions with pathogenic fungi and symbiotic interactions with mycorrhizal fungi and rhizobia are known to stimulate microtubule reorganization (for review, see Wasteneys and Galway, 2003; Takemoto and Hardham, 2004).

Self-incompatibility (SI) is a genetically controlled system to prevent self-fertilization in flowering plants. A multi-allelic *S*-locus is responsible for specifying *S*-specific pollen rejection to allow discrimination between incompatible and compatible pollen. Interaction of pollen *S*- and pistil *S*-determinants that have matching alleles allows "self" (incompatible) pollen to be recognized and rejected, while compatible pollen is allowed to grow and set seed. In this way, SI provides an important mechanism to prevent inbreeding through specific recognition and rejection of incompatible pollen. Several different SI systems exist; they have quite distinct molecular and genetic control; thus, different mechanisms are involved in SI in different species (for review, see Takayama and Isogai, 2005; McClure and Franklin-Tong, 2006).

In *Papaver rhoeas*, the pistil part of the *S*-locus encodes small, approximately 15-kD proteins that act as signaling ligands named S proteins (Foote et al., 1994). Their interaction with incompatible pollen triggers *S*-specific increases of cytosolic-free calcium concentration ($[Ca^{2+}]_i$; Franklin-Tong et al., 1993). The SI-induced Ca^{2+}-dependent signaling network comprises several intracellular events in incompatible pollen, indicating quite complex networks of interconnected events involved in the SI response. Ca^{2+}-dependent phosphorylation of a cytosolic pollen soluble inorganic pyrophosphatase (sPPase), Pr-p26.1 (de Graaf et al., 2006), inhibits its sPPase activity. As sPPases are important enzymes for driving biosynthesis, they are crucial for cell growth, so SI, by targeting this enzyme, results in pollen tube inhibition. SI also triggers reorganization and depolymerization of the F-actin cytoskeleton (Geitmann et al., 2000; Snowman et al., 2002). As the actin cytoskeleton is required for pollen tube growth (Gibbon et al., 1999), this represents another

[1] This work was supported by the Biotechnology and Biological Sciences Research Council (to V.E.F.-T. and a studentship to N.S.P.). S.V. worked as an undergraduate project student from the University of Ljubljana, Slovenia.

* Corresponding author; e-mail v.e.franklin-tong@bham.ac.uk.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Vernonica E. Franklin-Tong (v.e.franklin-tong@bham.ac.uk).

www.plantphysiol.org/cgi/doi/10.1104/pp.107.107052

mechanism to inhibit incompatible pollen tube growth. SI also triggers programmed cell death (PCD), involving several caspase-like activities (Thomas and Franklin-Tong, 2004; Bosch and Franklin-Tong, 2007). PCD is a conserved mechanism to get rid of unwanted cells and is used to sculpt tissues during development as well as in response to abiotic stress and pathogens (van Doorn and Woltering, 2005). SI activates PCD specifically in incompatible pollen, thereby preventing self-fertilization. Recent investigations revealed that alterations in actin dynamics can push pollen tubes into PCD (Thomas et al., 2006), and an SI-activated mitogen-activated protein kinase (Rudd et al., 1996) is implicated in signaling to PCD (Li et al., 2007). These data suggest that in *Papaver*, these components contribute to an integrated SI signaling network to achieve inhibition and death of incompatible pollen.

While the actin cytoskeleton is well established as being essential for tip growth in plant cells (Gibbon et al., 1999; Staiger, 2000), the role of the microtubule cytoskeleton is more variable, depending to some extent on the cell type. In some tip-growing plant cells, microtubule-disrupting drugs have no effect on tip growth; in others, they result in inhibition of growth, multiple growth initiation sites, or loss of directionality in root hairs (Bibikova et al., 1999) and pollen tubes (Anderhag et al., 2000; Gossot and Geitmann, 2007). However, it is well established that microtubules do not play an obvious role in regulating angiosperm pollen tube growth rate (Heslop-Harrison et al., 1988; Åström et al., 1995; Raudaskoski et al., 2001). Apart from data showing that they help organize the generative cell (GC) and vegetative nucleus (Raudaskoski et al., 2001; Laitiainen et al., 2002), relatively little is known about their function (Cai and Cresti, 2006). As the actin cytoskeleton is known to play a role in SI, we speculatively explored a possible role for the microtubule cytoskeleton in SI-induced signaling. Here, we report that SI stimulates rapid and massive apparent microtubule depolymerization, demonstrating that the pollen microtubule cytoskeleton is an early target for SI signals. Our data implicate signal integration between the microfilament and microtubule cytoskeleton and suggest a role for microtubules in SI-induced PCD.

RESULTS

Microtubule Cytoskeleton Organization in Growing *Papaver* Pollen Tubes

The microtubule cytoskeleton organization in normally growing *P. rhoeas* pollen tubes, using immunolocalization and probing with α-tubulin (Fig. 1), has previously been described (Gossot and Geitmann, 2007). The microtubule arrangement is very similar to that described previously (Åström et al., 1995; Gossot and Geitmann, 2007). The tip region is relatively microtubule-free; behind this region are arrays of short, longitudinally organized microtubule bundles (Fig. 1A). Further back, in the shank region, there are longer, more regularly organized longitudinal microtubule bundles (Fig. 1, A and B), which are mainly cortical (Fig. 1, B and C). Pollen tubes have a vegetative nucleus and a GC, which has a distinctive population of spindle-shaped GC microtubules (Fig. 1B).

SI Triggers Microtubule Depolymerization

To establish whether microtubules are a target for SI signaling, we examined the microtubule cytoskeleton using immunolocalization at various time points after incompatible SI induction (Fig. 2). Typical microtubule and microfilament organization was seen in control pollen tubes (Fig. 2, A and B). The microtubule cytoskeleton was rapidly altered after SI induction. As early as 1 min after SI, cortical microtubule bundles were virtually undetectable in incompatible pollen tubes; much weaker staining suggested that they had depolymerized (Fig. 2C). The GC spindle-shaped microtubules remained relatively intact at this time point (Fig. 2D). F-actin also dramatically reorganized by 1 min and accumulated in the tip, where it is not normally detected; many of the filament bundles had disappeared (Fig. 2E). At 3 min, the cortical microtubule bundles were virtually undetectable (Fig. 2F), and F-actin appeared disintegrated (Fig. 2G). At 30 min, cortical microtubules remained depolymerized (Fig. 2H), the GC spindle-shaped microtubules were still evident but disintegrating (Fig. 2I), and F-actin was aggregating (Fig. 2J). These data demonstrate that SI induces very rapid alterations to the cortical microtu-

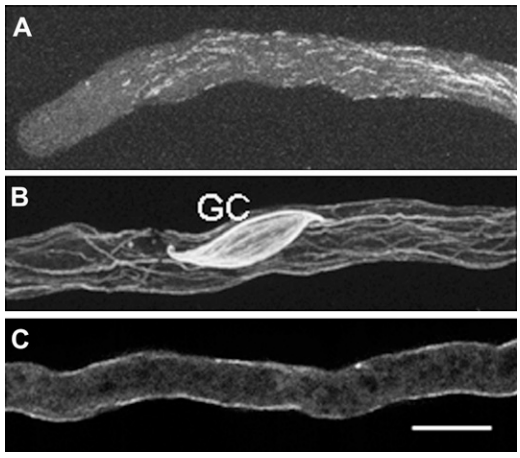

Figure 1. Microtubule organization in untreated *Papaver* pollen tubes. A, The apical region is relatively microtubule free; behind this is a region comprising shorter microtubule bundles, and behind this are longer arrays of cortical microtubule bundles. B, In the shank region, cortical microtubules are longitudinally arranged; the GC has a distinctive population of spindle-shaped microtubules. C, A single confocal optical section (0.5 μm) shows that microtubules are primarily cortical. A and B, Full projections of confocal optical sections. Microtubules were detected using immunolocalization with anti-α-tubulin antibody clone B-5-1-2. Scale bar = 10 μm.

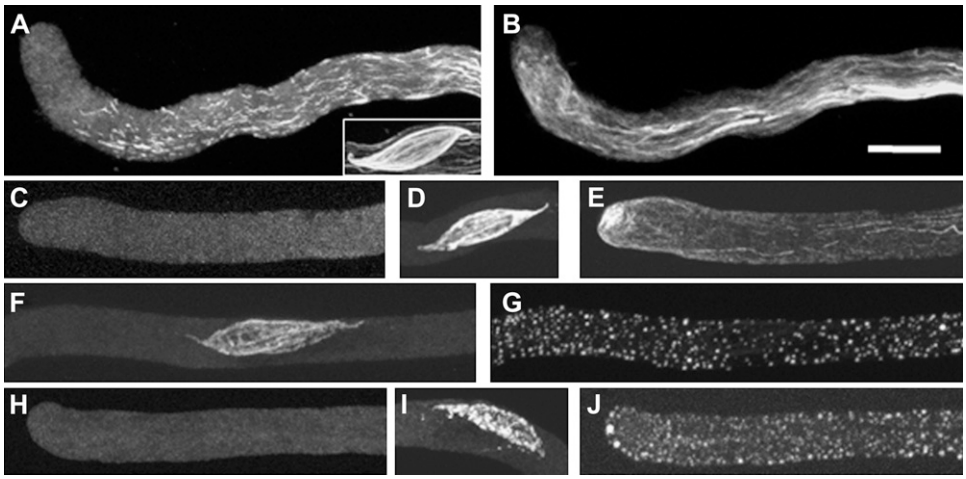

Figure 2. SI stimulates rapid apparent depolymerization of cortical microtubules coinciding with actin depolymerization. A, Cortical microtubules in an untreated pollen tube. Inset, GC microtubules. B, F-actin in an untreated pollen tube. C, At 1 min after SI induction, cortical microtubules are apparently virtually completely depolymerized. D, At 1 min after SI, GC microtubules are more or less intact. E, At 1 min after SI, F-actin is in the apical region; many F-actin bundles have disappeared. F, At 3 min after SI, cortical microtubules are undetectable; spindle-shaped microtubules show signs of disintegration. G, At 3 min after SI, F-actin has formed small punctate foci. H, At 30 min after SI induction, cortical microtubules are undetectable. I, At 30 min after SI induction, GC microtubules are further degraded. J, At 30 min after SI induction, F-actin comprises larger punctate foci. Microtubules were detected using immunolocalization with anti-α-tubulin; F-actin was colocalized using rhodamine-phalloidin. Images are full projections of confocal sections. Scale bar = 10 μm.

bule cytoskeleton of incompatible pollen tubes, which appeared to be depolymerized. The spindle-shaped microtubules were much more stable and were still apparent at 60 min post-SI but were disintegrating. These comparisons between SI-induced microtubule and microfilament responses show that although both respond very rapidly, they are quite distinct responses.

Although the rapidity of the alterations to the microtubules argued against degradation of total tubulin and suggested tubulin depolymerization, we wished to establish whether this was the case. To address this question, we examined the overall levels of α-tubulin in SI-induced pollen tubes at various time points, using western blotting. The overall amount of α-tubulin in the pollen tubes remained virtually constant for at least 60 min after SI induction (Fig. 3), although cortical microtubules detected using immunolocalization disappeared within 1 min of SI induction. This strongly suggests that the SI-induced cortical microtubule disappearance is due to tubulin depolymerization rather than degradation.

Actin Depolymerization Results in Alterations to the Microtubule Cytoskeleton

Because SI stimulated rapid actin depolymerization (Snowman et al., 2002), we wondered whether this might be responsible for alterations to the microtubules. We used latrunculin B (LatB) to examine the effect of F-actin depolymerization on the pollen microtubule cytoskeleton. Pollen was treated with 1 μM LatB for various time periods; we then imaged the effect of this treatment on both F-actin and microtubule populations. These relatively high concentrations were employed, as we wished to mimic the SI effect of rapid, complete depolymerization within a couple of minutes as closely as possible (Thomas et al., 2006). Typical untreated control pollen tubes are shown in Figure 4, A to D. After 5 min treatment with 1 μM LatB (Fig. 4, E–H), F-actin appeared fragmented, with a few short actin microfilament bundles remaining (Fig. 4, E and F). Although apical microtubule organization was altered by LatB treatment, the changes were not as observed for actin. Microtubules were present in the apical region and showed a distinctive random organization (Fig. 4G), but cortical microtubules in the shank appeared largely unaffected (Fig. 4H), as were the GC spindle-shaped microtubules (data not shown). Longer treatments with 1 μM LatB (Fig. 4, I–L) resulted in depolymerized F-actin (Fig. 4, I and J) and loss of virtually all cortical microtubules in the apical region (Fig. 4K) and shank (Fig. 4L). These data show that actin depolymerization results in apparent depolymerization of cortical microtubules, confirming data from Gossot and Geitmann (2007). This provides evidence for signaling between these two cytoskeletal

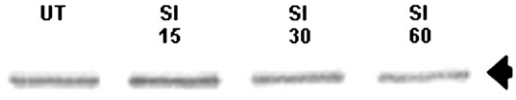

Figure 3. SI does not trigger tubulin degradation. Western blot of extracts from untreated pollen tubes (UT) and extracts from SI-induced pollen tubes at 15, 30, and 60 min after SI, probed with anti-α-tubulin antibody clone B-5-1-2. Overall α-tubulin levels (equal loading of samples; arrowhead) did not significantly change.

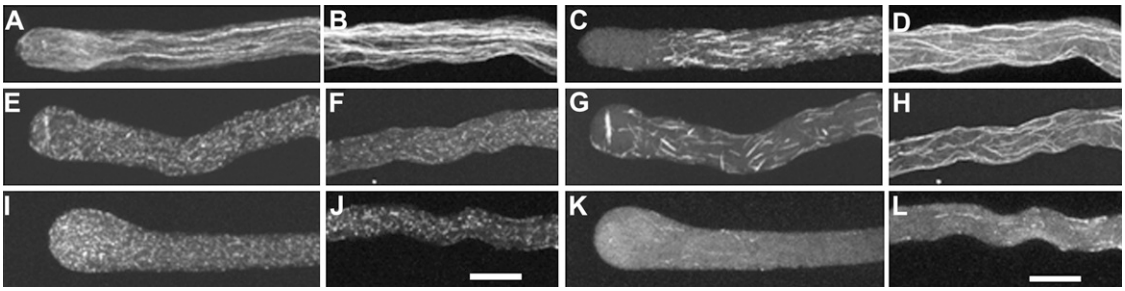

Figure 4. Actin depolymerization triggers changes in microtubule organization and apparent depolymerization. A to D, Typical untreated pollen tube cytoskeleton organization. A and B, F-actin; C and D, cortical microtubules. E to H, Pollen tubes treated with 1 μM LatB for 5 min. F-actin in the apical (E) and shank (F) region is fragmented. G and H, Cortical microtubules in the apical and mid-region are short and disorganized (G), while cortical microtubules in the shank region are relatively undisturbed (H). I to L, Pollen tubes treated with 1 μM LatB for 30 min. F-actin in the apical (I) and shank (J) region is extensively fragmented, indicating depolymerization. K, Microtubules in the apical and mid-region are virtually undetectable. L, Microtubules are virtually undetectable, with a few fragmented bundles remaining. Microtubules were detected using anti-α-tubulin; F-actin was colocalized using rhodamine-phalloidin. Images are full projections. Scale bars = 10 μm.

components, though this does not necessarily involve direct interactions between actin and tubulin.

Actin Stabilization Prevents or Delays SI-Induced Microtubule Depolymerization

To investigate further whether actin depolymerization plays a role in the SI-induced apparent microtubule depolymerization, we stabilized F-actin using jasplakinolide (Jasp) and then induced SI. We reasoned that if actin depolymerization was important for microtubule depolymerization, stabilizing actin should prevent or delay this event. Untreated pollen tubes showed normal microtubule configurations (Fig. 5A); 30 min treatment with 0.5 μM Jasp, which causes bulbous tips due to actin stabilization/reorganization (Thomas et al., 2006), stimulated reorganization, but not depolymerization, of microtubules (Fig. 5B). After SI, microtubules were rapidly depolymerized by 1 to 3 min (see Fig. 2, C and F). Ten minutes after SI, microtubules were completely depolymerized (Fig. 5C), but with a pretreatment of 0.5 μM Jasp 30 min prior to SI induction, at 10 min post-SI significant remnants of microtubules remained (Fig. 5D). Thus, Jasp-mediated stabilization of F-actin alleviated or delayed SI-induced microtubule depolymerization, providing further evidence consistent with the notion that F-actin depolymerization signals to microtubule depolymerization during SI.

Microtubule Depolymerization Is Not Required for Actin Alterations

Because actin depolymerization results in microtubule depolymerization, this suggested cross talk between actin and tubulin. As the response was rapid, we wondered whether microtubules might signal to actin. We therefore examined the effect of microtubule depolymerization on the pollen tube actin cytoskeleton, using oryzalin to artificially depolymerize tubulin. The relatively high concentrations used were to ensure that the SI effect of rapid depolymerization within a couple of minutes was mimicked as closely as possible. After 5 min treatment with 10 μM oryzalin, no cortical microtubules were evident (Fig. 6A); there was no detectable effect on the actin cytoskeleton (Fig. 6B). Even after 30 min treatment with oryzalin, when cortical microtubules were undetectable (Fig. 6C), F-actin organization appeared normal (Fig. 6D). To confirm that oryzalin did not affect actin, we measured pollen tubes, as actin depolymerization inhibits pollen tube growth (Gibbon et al., 1999). For pollen tubes treated with 10 μM oryzalin for 60 min, mean lengths were 293 $\pm$ 10 μm, compared with 314 $\pm$ 11 μm for untreated controls (n = 3 independent experiments). These values were not significantly different from each other (P = 0.156, nonsignificant), establishing that oryzalin had no effect on actin. Our data demonstrate that microtubule depolymerization does not stimulate actin depolymerization in pollen tubes, confirming data from Gossot and Geitmann (2007). Because these high levels of oryzalin do not affect actin or growth, we can be reasonably sure that possible side-effects are not an issue. This suggests there is one-way cross talk from actin to tubulin cytoskeleton, but not vice versa.

We also investigated whether stabilizing microtubules with taxol might affect actin reorganization. Taxol inhibits microtubule dynamics, causing stabilization of microtubules (Blagosklonny and Fojo, 1999), and is effective in plant cells (Baskin et al., 1994; Collings et al., 1998). Taxol does not dramatically affect microtubule organization, but some bundling is generally observed (Collings et al., 1998). Taxol, as expected, did not stimulate any major alterations to the organization of either the microtubule (Fig. 7, A and B) or actin microfilament (Fig. 7, C and D) cytoskeleton of pollen tubes, but the microtubule bundles were slightly larger and brighter, suggesting stabilization. The concentrations of taxol used are in line with other studies (see, e.g. Collings et al., 1998). To confirm that

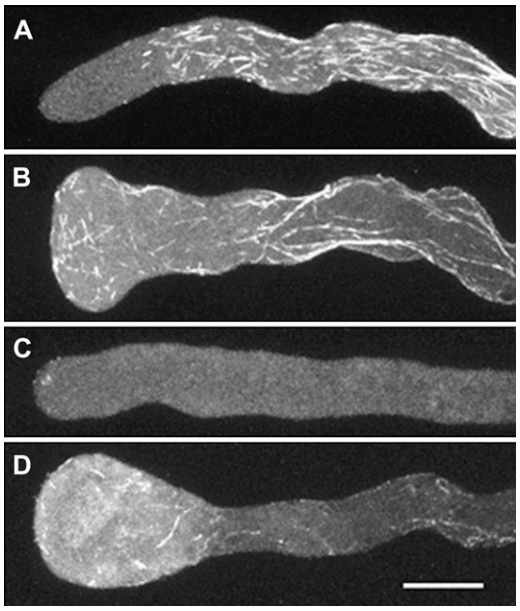

Figure 5. Actin stabilization by Jasp alleviates or delays SI-induced apparent microtubule depolymerization (A). Typical untreated pollen tube microtubule organization. B, Microtubule organization 30 min after Jasp treatment. C, Microtubules were completely depolymerized 10 min after SI. D, Microtubules were detectable after 30 min Jasp pretreatment followed by 10 min SI induction. Microtubules were detected with anti-α-tubulin. Images are full projections. Scale bar = 10 μm.

taxol had no effect on the actin cytoskeleton, we measured pollen tubes after treatment with 5 and 10 μM taxol for 1 h. The mean pollen tube lengths were 194.6 $\pm$ 9.8 μm and 194.0 $\pm$ 9.1 μm, respectively, compared to 190.0 $\pm$ 8.1 μm for untreated controls (n = 3). Thus, taxol had no significant effect on pollen tube growth (P = 0.717, 0.744, respectively, nonsignificant), consistent with taxol not having an effect on the actin cytoskeleton. To investigate whether stabilizing microtubules affected the ability of actin to depolymerize, we pretreated pollen tubes with 5 μM taxol for 30 min and then added 1 μM LatB for 30 min (Fig. 7, E–H). Although the organization of the microtubules in the apical region was disturbed (which is expected, as LatB inhibits pollen tube growth), the shank microtubules appeared relatively normal (Fig. 7, E and F), but F-actin depolymerized as normal (Fig. 7, G and H). Thus, microtubule depolymerization, although it accompanies actin depolymerization, is not required for actin depolymerization in pollen tubes. This confirms our data suggesting one-way signaling from actin to tubulin cytoskeleton.

Disruption of Microtubule Dynamics Does Not Trigger PCD

We previously demonstrated that actin depolymerization or stabilization can trigger PCD in pollen tubes (Thomas et al., 2006). Because SI also stimulated apparent microtubule depolymerization, we wondered whether microtubule depolymerization might also signal to PCD. We investigated this using oryzalin to depolymerize, or taxol to stabilize, pollen tube microtubules. Pollen tubes were treated with 10 μM oryzalin or 5 μM taxol and extracts tested for caspase-3-like activity using Ac-DEVD-AMC, a caspase-3 substrate, which we have used previously (Bosch and Franklin-Tong, 2007; Li et al., 2007). Untreated pollen tube extracts exhibited low DEVDase activity. The DEVDase activities in oryzalin- and taxol-treated pollen tube extracts were not significantly different from the untreated controls (P = 0.9581 and 0.6286, respectively; n = 4). Thus, microtubule depolymerization or stabilization alone clearly does not trigger PCD in *Papaver* pollen.

SI-Induced PCD Requires Depolymerization of Microtubules to Progress

Although changes in microtubule dynamics alone are not sufficient to signal to PCD, we wondered whether tubulin depolymerization might be required in conjunction with actin depolymerization to allow progression into SI-induced PCD. As microtubule depolymerization accompanies actin depolymerization, this was an important point to establish. We investigated whether pollen tubes with stabilized microtubules prior to SI-induced actin depolymerization affected entry into PCD. Pollen tubes were pretreated with 5 μM taxol, SI was induced, and extracts were

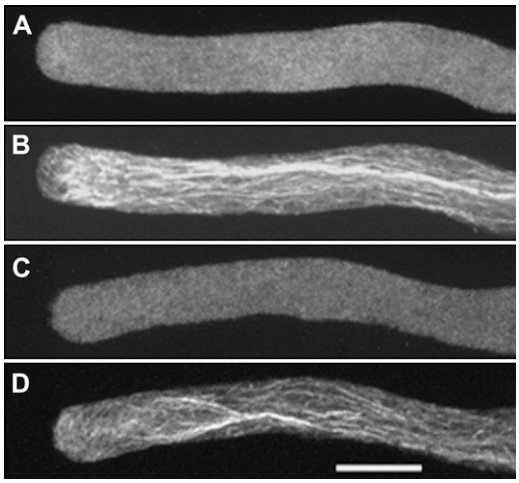

Figure 6. Microtubule depolymerization does not trigger alterations to the actin cytoskeleton. A and B, A 5-min treatment with 10 μM oryzalin. A, Apparent complete depolymerization of cortical microtubules. B, No apparent effect on F-actin organization. C and D, A 30-min treatment with 10 μM oryzalin. C, Cortical microtubules are apparently completely depolymerized. D, No apparent effect on F-actin organization. Microtubules were detected with anti-α-tubulin; F-actin was colocalized using rhodamine-phalloidin. Images are full projections. Scale bar = 10 μm.

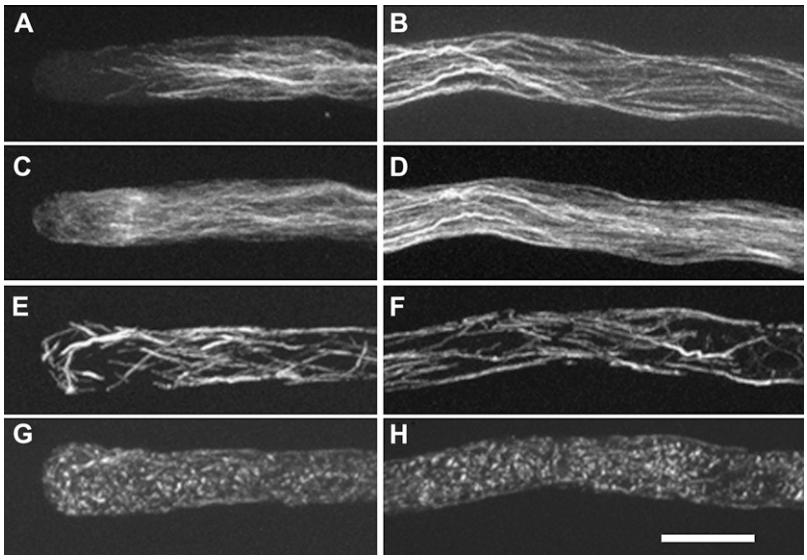

Figure 7. Microtubule depolymerization is not required for actin depolymerization. A to D, A 5-μM taxol treatment for 60 min. A and B, Cortical microtubule arrangement appears stabilized. C and D, F-actin organization was not significantly affected. E to H, Thirty-minute consecutive treatments of 5 μM taxol and 1 μM LatB. Microtubule organization in the tip was altered (E), but microtubules remained stabilized and intact (E and F). Actin microfilaments in the tip (G) and shank (H) were depolymerized. Microtubules were detected with anti-α-tubulin, F-actin with rhodamine-phalloidin. Images are confocal full optical projections. Scale bar = 10 μm.

assayed for DEVDase/caspase-3-like activity. Untreated pollen tube extracts exhibited low DEVDase activity, while SI induced high DEVDase activity (72.5% higher than untreated samples), which was significantly different from the controls ($P < 0.001$, ***; $n = 10$). In pollen tubes pretreated with taxol prior to SI induction, the level of DEVDase activity was significantly reduced; 41% lower compared to SI alone ($P = 0.0256$, *; $n = 10$). The reduction in DEVDase activity by taxol firmly implicates that microtubule depolymerization plays a role in mediating SI-induced PCD in addition to actin depolymerization. Moreover, when pollen tubes were pretreated with oryzalin for 30 min prior to SI induction, there was no significant difference in the DEVDase activity compared with SI-induced samples ($P = 0.7079$; $n = 5$). Together with the results from the taxol treatment, this is consistent with the idea that microtubule depolymerization is involved in SI-induced PCD, but suggests that an optimal threshold level of caspase activation is already achieved by SI-induced actin depolymerization.

In summary, our data provide good evidence that SI targets the microtubule cytoskeleton and implicate signal integration between microfilament and microtubule cytoskeleton. They reveal that SI-induced microtubule disruption is very different from that of actin. Altering microtubule dynamics did not stimulate F-actin depolymerization, suggesting one-way signaling from actin to microtubules. While actin microfilament depolymerization is sufficient to trigger PCD in pollen tubes via activation of a caspase-3-like/DEVDase activity, microtubule depolymerization alone is not. However, stabilization of microtubules reduced SI-induced caspase-like activity, suggesting that microtubule depolymerization, although on its own is insufficient to trigger PCD, is not just a consequence of SI signaling but is required for SI-induced PCD to progress.

DISCUSSION

Temporal Dynamics of the SI-Mediated Microtubule Alterations

Here, we show that in *Papaver*, although like other angiosperm pollen tubes, microtubules do not play an obvious role in regulating pollen tube growth rate (Heslop-Harrison et al., 1988; Raudaskoski et al., 2001), they are clearly responding to SI signals. Moreover, as our data demonstrate that the cortical microtubule cytoskeleton is a very early target for SI signals, it suggests that these alterations are not just a consequence of events but are likely to play a role in mediating SI. SI induces very rapid alterations to the cortical microtubule cytoskeleton, which are apparently depolymerized within approximately 1 min. Although both microtubule and microfilament SI-induced responses are very rapid, they are quite distinct responses. In contrast to F-actin, which also depolymerizes very rapidly, the microtubules remain depolymerized, while F-actin reorganizes and aggregates into punctate foci later.

One problem with fixation and such rapid responses is that it is difficult to establish exactly how rapid these changes to the cytoskeleton are and how they interrelate. Our data, and those of Gossot and Geitmann (2007) using LatB to artificially trigger actin depolymerization, show consequent apparent microtubule depolymerization, suggesting that SI-induced actin depolymerization triggers microtubule depolymerization. As stabilizing actin using Jasp prevents complete microtubule depolymerization, this further suggests a causal link. However, because of their rapidity, it is difficult to ascertain the order of these events definitively using fixation and immunolocalization. Live-cell imaging of microtubule- and microfilament-localized GFP fusion proteins would help establish the timing and nature of cytoskeletal organization and dynamics.

This would aid elucidation of the relationship between the actin and microtubule networks, especially during these early, rapid responses; we will address this in future studies.

Signal-Mediated Cortical Microtubule Reorganization/Depolymerization

Because cortical microtubules are intimately associated with the plasma membrane, where numerous receptors reside, they are implicated as targets of signaling networks (Gilroy and Trewavas, 2001; Wasteneys and Galway, 2003). Our data contribute to the evidence for this, demonstrating that the *Papaver* pollen tube microtubules are an early target of the SI-signaling network. Here, we have shown that a specific recombinant protein stimulus, involved in a biologically relevant phenomenon, has a very distinctive effect on pollen tube microtubules. The SI-induced apparent microtubule depolymerization response is extremely rapid and dramatic, far more so than any physiological response previously reported in a plant cell, to our knowledge.

Microtubule reorganization and/or apparent depolymerization occurs in response to specific abiotic stimuli (Bartolo and Carter, 1991; Himmelspach et al., 1999; Shoji et al., 2006). Several examples of the microtubule cytoskeleton alterations in response to biotic stimuli, such as infection by pathogenic fungi or symbiotic interactions with mycorrhiza or rhizobia, exist (for review, see Takemoto and Hardham, 2004). These interactions generally involve reorganization and/or focusing of the microtubule cytoskeleton around the infecting organism. However, rapid apparent depolymerization of microtubules has also been reported, for example, in parsley (*Petroselinum crispum*)- and soybean (*Glycine max*)-*Phytophthora* interactions and in elicitor-treated tobacco (*Nicotiana tabacum*) cells (Gross et al., 1993; Binet et al., 2001; Cahill et al., 2002). Nod factor signaling also stimulates rapid localized apparent depolymerization of microtubules in root hairs and later increases in microtubule arrays (Timmers et al., 1999; Weerasinghe et al., 2003). Thus, biotic interactions involve specific alterations to the microtubule cytoskeleton (for review, see Takemoto and Hardham, 2004). Our data provide evidence for signaling to the microtubule cytoskeleton from another physiologically relevant system.

Microtubule Depolymerization Plays a Functional Role in SI-Mediated PCD

We previously showed that stabilizing F-actin using Jasp partially alleviates SI-induced PCD (Thomas et al., 2006) to about the same extent as taxol in this study. Although we did not know it at the time, actin depolymerization also stimulates microtubule depolymerization. Thus, our finding that stabilization of actin by Jasp also partially stabilizes microtubules implicates a role for microtubule depolymerization in mediating PCD. We provide a simple model outlining our understanding of the cytoskeletal events triggered by SI in order to clarify the relationship between microfilaments and microtubules (Fig. 8). Although microtubules are rapidly depolymerized by SI induction, microtubule depolymerization alone does not trigger PCD in pollen tubes. This is in contrast to actin depolymerization, which plays a key role in initiating PCD in pollen (Thomas et al., 2006). Despite this, stabilization of microtubules using taxol alleviates SI-induced PCD, suggesting that microtubules play a role in mediating PCD. Microtubule depolymerization, which we and others (Gossot and Geitmann, 2007) have shown occurs as a consequence of actin depolymerization, is effectively reduced by taxol. As we show here that taxol does not inhibit pollen tube growth, SI-induced actin depolymerization should progress normally in the presence of taxol. Thus, normal levels of SI-induced caspase induction should be triggered in the presence of taxol if microtubules play no role and are depolymerized merely as a consequence of SI-induced actin depolymerization. However, as taxol alleviates PCD, this clearly demonstrates that preventing microtubule depolymerization is important for progression of PCD (Fig. 8). This strongly suggests that the microtubules are not just onlookers, but that they play a role in mediating caspase activation.

Microtubule reorganization triggered by pathogen infection hints at a possible microtubule involvement in PCD in plant cells. Our data are consistent with a model whereby microtubules, in concert with actin, somehow play a functional role in integrating signals involved in regulating PCD. However, a direct connection between microtubule reorganization and triggering of PCD remains to be elucidated.

Notably, the GC spindle-shaped microtubules were not dramatically affected by SI and remained relatively intact for a considerable time; these microtubules showed signs of disintegration but were still apparent at 60 min post-SI. This suggests that either the SI signals are specifically targeted to the cortical microtubules and/or that the GC-associated microtubule population is protected. Thus, it is the cortical microtubule population that is primarily affected and participates in this response. Interestingly, the GC appears to be a target for caspase-3-like/DEVDase activity 2 to 3 h after SI induction (Bosch and Franklin-Tong, 2007).

Evidence for Cross Talk between Actin and Tubulin

It is evident from our data that there is cross talk between microfilaments and microtubules in pollen tubes during SI. We have shown that SI triggers both actin depolymerization (Snowman et al., 2002) and apparent microtubule depolymerization. Moreover, depolymerizing actin with LatB triggers microtubule depolymerization, while depolymerizing microtubules with oryzalin has no effect on actin organization, as also previously shown by Gossot and Geitmann (2007). This suggests the actin depolymerization triggers microtu-

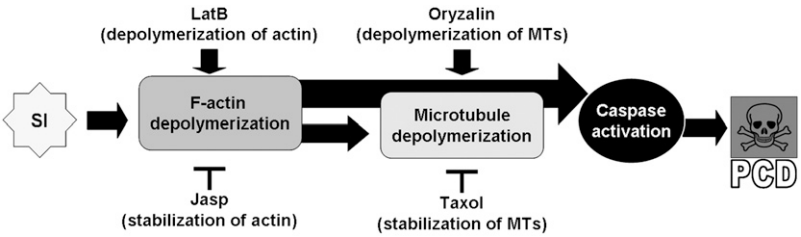

Figure 8. Model for integration of cytoskeletal events triggered by SI. SI triggers actin depolymerization, which is sufficient to trigger caspase activation and PCD (Thomas et al., 2006). LatB causes actin depolymerization, caspase activation, and PCD (Thomas et al., 2006). Treatment with Jasp after SI induction alleviated the extent of PCD (Thomas et al., 2006). Thus, partial prevention of actin depolymerization gives some protection from PCD. SI also triggers microtubule depolymerization (this study). Use of LatB showed that actin depolymerization also triggers microtubule depolymerization (Gossot and Geitmann, 2007; this study). This suggests that during SI, microtubule depolymerization is a consequence of actin depolymerization. Use of oryzalin showed that microtubule depolymerization on its own is not sufficient to trigger caspase activation and PCD (this study). This raises the question of whether microtubule depolymerization is actually required for PCD, or whether it is just a consequence of actin depolymerization. Use of taxol, which alleviated the extent of PCD, showed that preventing microtubule depolymerization is somehow involved in regulating PCD (this study). This implicates a functional role for both actin and tubulin in signaling to PCD.

bule depolymerization, but not vice versa, providing evidence for one-way signaling between these two cytoskeletal components in pollen tubes. As actin stabilization by Jasp delays or prevents microtubule depolymerization, this further suggests that actin influences microtubule polymerization status (Fig. 8).

Microtubules and actin microfilaments are often closely associated; in animal and yeast cells, there is no question that actin microfilament and microtubule cytoskeletons interact, and there is substantial evidence that this is also the case in plant cells. For example, transverse cortical microtubules and microfilaments in diffusely elongating cells can influence each other's organization (Collings and Allen, 2000). Drug-induced microtubule disassembly in Characean internodal cells (Foissner and Wasteneys, 2000) and root hairs (Tominaga et al., 1997) exacerbate the effects of actin-targeted drugs, suggesting that microtubule dynamics can influence actin dynamics. In fern cells (Kadota and Wada, 1992; Collings et al., 2006) and pollen tubes (Gossot and Geitmann, 2007), actin-depolymerizing drugs affect cortical microtubules. Thus, there is good evidence for signaling and interplay between microtubules and microfilaments, but the direction of the signaling varies. In SI, both actin depolymerization (Thomas et al., 2006) and microtubule depolymerization play a role in PCD, providing evidence for an integrated signaling network between these components.

Emerging data are beginning to provide some clues about how interactions between actin and tubulin are achieved. Identification of proteins bridging these interactions has confirmed functional interactions between microtubules and microfilaments in animals and fungi (for review, see Goode et al., 2000). In plants, proteins that interact with both microtubules and actin microfilaments are beginning to be identified (Igarashi et al., 2000; Preuss et al., 2004; Huang et al., 2007), providing the first firm evidence for how these two dynamic cytoskeletal components are linked in plant cells. There is clearly much remaining to be explored in the future, and the SI-induced responses reported here appear to represent an excellent model system in which to examine interactions between microtubules and microfilaments.

MATERIALS AND METHODS

Pollen Treatments

Pollen of *Papaver rhoeas* was germinated and grown in vitro in liquid germination medium [0.01% H_3BO_3, 0.01% KNO_3, 0.01% $Mg(NO_3)_2.6H_2O$, 0.036% $CaCl_2$-$2H_2O$, 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 S_1, S_3, and S_8 alleles of the *S* gene (pPRS100, pPRS300, and pPRS800) into the expression vector pMS119 as described previously (Foote et al., 1994). Expression and purification of the proteins was performed as described by Kakeda et al. (1998). SI was induced by adding recombinant S proteins (final concentration 10 $\mu g\ mL^{-1}$) to pollen that had been grown for 1 h in vitro (Snowman et al., 2002).

For the cytoskeleton drug treatments, 1 μM LatB, 0.5 μM Jasp (Calbiochem), 5 or 10 μM taxol, or 10 μM oryzalin (Sigma-Aldrich) was added to pollen tubes grown for 1 h. Controls comprised addition of dimethyl sulfoxide at a final concentration of 0.1% (v/v). For the drug-SI experiments, pollen tubes were subjected to a consecutive treatment of the relevant drug for 30 min, followed by the addition of incompatible S proteins for 5 h.

Immunolocalization

Pollen tubes were prefixed using the cross-linker 3-maleimodobenzoic acid *N*-hydroxysuccinimide ester (MBS; 400 μM; Pierce) for 6 min at 20°C, followed by 2% formaldehyde (1 h, 4°C), as described by Thomas et al. (2006); we used 2% formaldehyde as a compromise. Actin preservation was indistinguishable from what we previously obtained using 4% formaldehyde following MBS (Geitmann et al., 2000). MBS has been reported to stop cytoplasmic streaming within seconds (Ketelaar and Emons, 2001). The treatment times indicated in the text are the time point after treatment that MBS was added. Cells were washed in actin-stabilizing buffer (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 in MES buffer containing 0.1 mM phenylmethylsulfonyl fluoride and 1% bovine serum albumin for 10 min. Washes in MES, then Tris-buffered saline (TBS), were followed by permeabilization in

0.1% Triton X-100/TBS for 10 min, and blocking in TBS/1% bovine serum albumin for 30 min.

Samples were incubated with anti-α-tubulin antibody (clone B-5-1-2; Sigma-Aldrich; 1:1,000 dilution) overnight at 4°C. They were washed in TBS, then incubated for 1.5 h at room temperature in anti-mouse fluorescein isothiocyanate antibody (1:300 dilution). Following TBS washes, rhodamine-phalloidin (66 nM) was added. Pollen tubes were mounted with 5 μL of Vectashield (Vector Laboratories). Images were collected using a Bio-Rad Radiance 2000 laser-scanning system (50-mW argon laser, 488-nm line, and 1.5-mW HeNe laser, 543 nm) with a 60× plan-Apo 1.4 NA oil objective (Nikon). z-Series of 0.5-μm optical slices were captured. Images were analyzed using ImageJ and archived as TIF files.

Protein Extraction and Western Blotting

SI was induced and pollen tubes collected by centrifugation in HEPES buffer (50 mM HEPES, pH 7.4, 10 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol) and samples snap-frozen in liquid N_2. Proteins were extracted by sonication (2 × 10 s, 10 amps) and analyzed using SDS-PAGE and western blotting. Samples were measured using the Bio-Rad protein assay; equal amounts were loaded and checked by Ponceau staining of blots. Blots were probed with a 1:4,000 dilution of the monoclonal anti-α-tubulin antibody clone B-5-1-2 (Sigma-Aldrich), then probed with an anti-mouse alkaline phosphatase secondary antibody and detected using alkaline phosphatase.

Pollen Tube Length Measurements

Pollen tubes were grown for 1 h, then samples were treated as specified in the text, and pollen tubes fixed in 2% formaldehyde for 1 h, washed in TBS, and mounted on glass slides. Thus, before treatment, all mean pollen tube lengths were similar. Fixed pollen tubes were imaged using a Nikon Eclipse TE-300 microscope attached to a SenSys camera, using a Quips PathVysion image analysis system (Applied Imaging International). Final pollen tube lengths were measured (40 tubes for each of three independent treatments) using IPlab software. Lengths indicated are total lengths of the pollen tubes (i.e. 1 h pretreatment time plus treatment time with the relevant drug). Statistical analysis comprised a *t* test analysis.

Caspase Assays

PCD was assessed using a fluorogenic caspase-3/7-amino-4-trifluoromethyl coumarin substrate, Ac-DEVD-AMC, to measure caspase-like activity. Pollen tubes were subjected to treatments for 5 h and protein extracts made by grinding and sonicating pollen tubes in caspase extraction buffer (50 mM sodium acetate, 10 mM L-Cys, 10% [v/v] glycerol, and 0.1% [w/v] CHAPS, pH 6.0). Assays containing 10 μg of protein extract at 1 μg μL^{-1} and 50 μM substrate were performed in caspase extraction buffer, pH 5.0. Release of fluorophore by cleavage was measured (excitation 380 nm, emission 460 nm) using a FLUOstar OPTIMA reader (BMG Labtechnologies) at 27°C for 5 h. Background relative fluorescent unit readings for control samples were subtracted from test samples. All assays were performed on at least four independent samples, each measured in duplicate. *P* values were calculated using a two-way ANOVA.

ACKNOWLEDGMENTS

We thank the horticultural staff for growing the plants and helping harvest material. Thanks to Tobias Baskin for helpful advice on tubulin antibodies, and to Harpal Pooni and Mike Kearsey for statistical advice.

Received August 8, 2007; accepted December 15, 2007; published January 11, 2008.

LITERATURE CITED

Anderhag P, Hepler PK, Lazzaro MD (2000) Microtubules and microfilaments are both responsible for pollen tube elongation in the conifer *Picea abies*. Protoplasma **214:** 141–157

Åström H, Sorri O, Raudaskoski M (1995) Role of microtubules in the movement of the vegetative nucleus and generative cell in tobacco pollen tubes. Sex Plant Reprod **8:** 61–69

Bartolo ME, Carter JV (1991) Microtubules in mesophyll cells of non-acclimated and cold-acclimated spinach: visualization and responses to freezing, low temperature, and dehydration. Plant Physiol **97:** 175–181

Baskin TI, Wilson JE, Cork A, Williamson RE (1994) Morphology and microtubule organization in Arabidopsis roots exposed to oryzalin or taxol. Plant Cell Physiol **35:** 935–942

Bibikova TN, Blancaflor EB, Gilroy S (1999) Microtubules regulate tip growth and orientation in root hairs of *Arabidopsis thaliana*. Plant J **17:** 657–665

Binet MN, Humbert C, Lecourieux D, Vantard M, Pugin A (2001) Disruption of microtubular cytoskeleton induced by cryptogein, an elicitor of hypersensitive response in tobacco cells. Plant Physiol **125:** 564–572

Blagosklonny MV, Fojo T (1999) Molecular effects of paclitaxel: myths and reality (a critical review). Int J Cancer **83:** 151–156

Bosch M, Franklin-Tong VE (2007) Temporal and spatial activation of caspase-like enzymes induced by self-incompatibility in *Papaver* pollen. Proc Natl Acad Sci USA **104:** 18327–18332

Cahill D, Rookes J, Michalczyk A, McDonald K, Drake A (2002) Microtubule dynamics in compatible and incompatible interactions of soybean hypocotyl cells with Phytophthora sojae. Plant Pathol **51:** 629–640

Cai G, Cresti M (2006) The microtubule cytoskeleton in pollen tubes: structure and role in organelle trafficking. *In* R Malho, ed, The Pollen Tube: A Cellular and Molecular Perspective. Plant Cell Monographs. Springer, Berlin/Heidelberg, pp 157–175

Collings D, Allen NS (2000) Cortical actin interacts with the plasma membrane and microtubules. *In* FB CJ Staiger, D Volkmann, PW Barlow, eds, Actin: A Dynamic Framework for Multiple Plant Cell Functions. Kluwer Academic, Dordrecht, The Netherlands, pp 145–164

Collings DA, Asada T, Allen NS, Shibaoka H (1998) Plasma membrane-associated actin in bright yellow 2 tobacco cells. Evidence for interaction with microtubules. Plant Physiol **118:** 917–928

Collings DA, Lill AW, Himmelspach R, Wasteneys GO (2006) Hypersensitivity to cytoskeletal antagonists demonstrates microtubule-microfilament cross-talk in the control of root elongation in *Arabidopsis thaliana*. New Phytol **170:** 275–290

de Graaf BHJ, Rudd JJ, Wheeler MJ, Perry RM, Bell EM, Osman K, Franklin FCH, Franklin-Tong VE (2006) Self-incompatibility in Papaver targets soluble inorganic pyrophosphatases in pollen. Nature **444:** 490–493

Erhardt DW, Shaw SL (2006) Microtubule dynamics and organization in plant cortical array. Annu Rev Plant Biol **57:** 859–875

Foissner I, Wasteneys GO (2000) Microtubule disassembly enhances reversible cytochalasin-dependent disruption of actin bundles in characean internodes. Protoplasma **214:** 33–44

Foote HCC, Ride JP, Franklin-Tong VE, Walker EA, Lawrence MJ, Franklin FCH (1994) Cloning and expression of a distinctive class of self-incompatibility (S) gene from *Papaver rhoeas* L. Proc Natl Acad Sci USA **91:** 2265–2269

Franklin-Tong VE, Ride JP, Read ND, Trewavas AJ, Franklin FCH (1993) The self-incompatibility response in *Papaver rhoeas* is mediated by cytosolic-free calcium. Plant J **4:** 163–177

Geitmann A, Snowman BN, Emons AMC, Franklin-Tong VE (2000) Alterations in the actin cytoskeleton of pollen tubes are induced by the self-incompatibility reaction in *Papaver rhoeas*. Plant Cell **12:** 1239–1251

Gibbon BC, Kovar DR, Staiger CJ (1999) Latrunculin B has different effects on pollen germination and tube growth. Plant Cell **11:** 2349–2363

Gilroy S, Trewavas A (2001) Signal processing and transduction in plant cells: the end of the beginning? Nat Rev Mol Cell Biol **2:** 307–314

Goode BL, Drubin DG, Barnes G (2000) Functional cooperation between the microtubule and actin cytoskeletons. Curr Opin Cell Biol **12:** 63–71

Gossot O, Geitmann A (2007) Pollen tube growth: coping with mechanical obstacles involves the cytoskeleton. Planta **226:** 405–416

Gross P, Julius C, Schmeltzer E, Hahlbrock K (1993) Translocation of cytoplasm and nucleus to fungal penetration sites is associated with depolymerisation of microtubules and defense gene activation in infected, cultured parsley cells. EMBO J **12:** 1735–1744

Heslop-Harrison J, Heslop-Harrison Y, Cresti M, Tiezzi A, Moscatelli A (1988) Cytoskeletal elements, cell shaping and movement in the angiosperm pollen tube. J Cell Sci **91:** 49–60

Himmelspach R, Wymer CL, Lloyd CW, Nick P (1999) Gravity-induced reorientation of cortical microtubules observed in vivo. Plant J **18:** 449–453

Huang S, Jin L, Du J, Li H, Zhao Q, Ou G, Ao G, Yuan M (2007) SB401, a pollen-specific protein from Solanum berthaultii, binds to and bundles microtubules and F-actin. Plant J **51:** 406–418

Hussey PJ, Ketelaar T, Deeks MJ (2006) Control of the actin cytoskeleton in plant cell growth. Annu Rev Plant Biol **57:** 109–125

Igarashi H, Orii H, Mori H, Shimmen T, Sonobe S (2000) Isolation of a Novel 190 kDa Protein from tobacco BY-2 cells: possible involvement in the interaction between actin filaments and microtubules. Plant Cell Physiol **41:** 920–931

Kadota A, Wada M (1992) The circular arrangement of cortical microtubule around the subapex of tip growing fern protonemata is sensitive to cytochalasin B. Plant Cell Physiol **33:** 99–102

Kakeda K, Jordan ND, Conner A, Ride JP, Franklin-Tong VE, Franklin FCH (1998) Identification of residues in a hydrophilic loop of the *Papaver rhoeas* S protein that play a crucial role in recognition of incompatible pollen. Plant Cell **10:** 1723–1731

Ketelaar T, Emons AMC (2001) The cytoskeleton in plant cell growth: lessons from root hairs. New Phytol **152:** 409–418

Laitiainen E, Nieminen KM, Vihinen H, Raudaskoski M (2002) Movement of generative cell and vegetative nucleus in tobacco pollen tubes is dependent on microtubule cytoskeleton but independent of the synthesis of callose plugs. Sex Plant Reprod **15:** 195–204

Li S, Samaj J, Franklin-Tong VE (2007) A mitogen-activated protein kinase signals to programmed cell death induced by self-incompatibility in *Papaver* pollen. Plant Physiol **145:** 236–245

McClure B, Franklin-Tong V (2006) Gametophytic self-incompatibility: understanding the cellular mechanisms involved in ''self'' pollen tube inhibition. Planta **224:** 233–245

Preuss ML, Kovar DR, Lee YRJ, Staiger CJ, Delmer DP, Liu B (2004) A plant-specific kinesin binds to actin microfilaments and interacts with cortical microtubules in cotton fibers. Plant Physiol **136:** 3945–3955

Raudaskoski M, Astrom H, Laitiainen E (2001) Pollen tube cytoskeleton: structure and function. J Plant Growth Regul **20:** 113–130

Rudd JJ, Franklin FCH, Lord JM, Franklin-Tong VE (1996) Increased phosphorylation of a 26-kD pollen protein is induced by the self-incompatibility response in *Papaver rhoeas*. Plant Cell **8:** 713–724

Shibaoka H (1994) Plant hormone-induced changes in the orientation of cortical microtubules: alterations in the cross-linking between microtubules and the plasma membrane. Annu Rev Plant Physiol Plant Mol Biol **45:** 527–544

Shoji T, Suzuki K, Abe T, Kaneko Y, Shi H, Zhu JK, Rus A, Hasegawa PM, Hashimoto T (2006) Salt stress affects cortical microtubule organization and helical growth in *Arabidopsis*. Plant Cell Physiol **47:** 1158–1168

Smith LG, Oppenheimer DG (2005) Spatial control of cell expansion by the plant cytoskeleton. Annu Rev Cell Dev Biol **21:** 271–295

Snowman BN, Kovar DR, Shevchenko G, Franklin-Tong VE, Staiger CJ (2002) Signal-mediated depolymerization of actin in pollen during the self-incompatibility response. Plant Cell **14:** 2613–2626

Staiger CJ (2000) Signalling to the actin cytoskeleton in plants. Annu Rev Plant Physiol Plant Mol Biol **51:** 257–288

Takayama S, Isogai A (2005) Self-incompatibility in plants. Annu Rev Plant Biol **56:** 467–489

Takemoto D, Hardham AR (2004) The cytoskeleton as a regulator and target of biotic interactions in plants. Plant Physiol **136:** 3864–3876

Thomas SG, Franklin-Tong VE (2004) Self-incompatibility triggers programmed cell death in *Papaver* pollen. Nature **429:** 305–309

Thomas SG, Huang S, Li S, Staiger CJ, Franklin-Tong VE (2006) Actin depolymerization is sufficient to induce programmed cell death in self-incompatible pollen. J Cell Biol **174:** 221–229

Timmers ACJ, Auriac MC, Truchet G (1999) Redefined analysis of early symbiotic steps of the *Rhizobium-Medicago* interaction in relationship with microtubule cytoskeleton rearrangements. Development **126:** 3617–3628

Tominaga M, Morita K, Sonobe S, Yokota E, Shimmen T (1997) Microtubules regulate the organization of actin filaments at the cortical region in root hair cells of Hydrocharis. Protoplasma **199:** 83–92

van Doorn WG, Woltering EJ (2005) Many ways to exit? Cell death categories in plants. Trends Plant Sci **10:** 117–122

Wasteneys GO, Galway ME (2003) Remodelling the cytoskeleton for growth and form: an overview with some new views. Annu Rev Plant Biol **54:** 691–722

Weerasinghe RR, Collings DA, Johannes E, Allen NS (2003) The distributional changes and role of microtubules in Nod factor challenged *Medicago sativa* root hairs. Planta **218:** 276–287