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Actin depolymerization is sufficient to induce programmed cell death in self-incompatible pollen

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Self-incompatibility (SI) prevents inbreeding through specific recognition and rejection of incompatible pollen. In incompatible *Papaver rhoeas* pollen, SI triggers a Ca^{2+} signaling cascade, resulting in the inhibition of tip growth, actin depolymerization, and programmed cell death (PCD). We investigated whether actin dynamics were implicated in regulating PCD. Using the actin-stabilizing and depolymerizing drugs jasplakinolide (Jasp) and latrunculin B, we demonstrate that changes in actin filament levels or dynamics play a functional role in initiating PCD in *P. rhoeas* pollen, triggering a caspase-3–like activity. Significantly, SI-induced PCD in incompatible pollen was alleviated by pretreatment with Jasp. This represents the first account of a specific causal link between actin polymerization status and initiation of PCD in a plant cell and significantly advances our understanding of the mechanisms involved in SI.

Introduction

Self-incompatibility (SI) is one of the systems that 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 and Franklin, 2003). In *Papaver rhoeas*, the pistil S proteins (Foote et al., 1994) act as ligands, triggering increases in the cytosolic-free calcium concentration ($[Ca^{2+}]_i$) in incompatible pollen (Franklin-Tong et al., 1993, 1997). The Ca^{2+}-mediated signaling network results in the rapid inhibition of incompatible pollen tube growth. Within a few minutes of SI signals, reorganization and massive, sustained depolymerization of the pollen filamentous actin (F-actin) is induced (Geitmann et al., 2000; Snowman et al., 2002). Although the extent of F-actin depolymerization during SI is clearly sufficient to inhibit pollen tube growth, it appears to be a gross excess over the amount required to achieve this. This suggested possible additional functions for the alteration to actin dynamics.

Unwanted cells are usually removed by programmed cell death (PCD). Many examples of PCD in plant development (Kuriyama and Fukuda, 2002) and in responses to external stimuli (Swidzinski et al., 2002; Danon et al., 2004; Greenberg and Yao, 2004; van Doorn and Woltering, 2005) have been documented. Features of PCD in animal cells include cytochrome *c* leakage from the mitochondria, DNA fragmentation, and caspase activation. In animal cells, apoptosis is mediated by a caspase cascade. Activated caspases cleave numerous substrates, including endogenous nuclease inhibitors, resulting in the fragmentation of nuclear DNA. Although PCD should theoretically involve a caspase-3–like activity, no caspase homologues have been found in plants (Woltering et al., 2002). Despite this, there is good evidence for caspase-like activities in plant cells (Lam et al., 2001; Schaller, 2004). We recently reported that SI triggers a PCD cascade in incompatible *P. rhoeas* pollen, which involves a caspase-3–like activity (Thomas and Franklin-Tong, 2004). This provides a precise mechanism for the specific destruction of incompatible pollen.

As the actin cytoskeleton is a major target and effector of signaling cascades in both animal and plant cells (Schmidt and Hall, 1998; Staiger, 2000), we explored a possible role for actin depolymerization in signaling to PCD. Recent evidence suggests that either stabilization or depolymerization of the actin cytoskeleton is adequate to induce PCD in yeast and some animal cells, depending on the cell type (Levee et al., 1996; Janmey, 1998; Korichneva and Hammerling, 1999; Rao et al., 1999; Morley et al., 2003; Gourlay et al., 2004; for review see Gourlay and Ayscough, 2005). It is postulated that the alteration of actin filament dynamics initiates or modulates the apoptotic signaling cascade (Janmey, 1998; Korichneva and Hammerling,

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Abbreviations used in this paper: CD, cytochalasin D; F-actin, filamentous actin; G-actin, globular actin; GM, germination medium; Jasp, jasplakinolide; LatB, latrunculin B; PCD, programmed cell death; ROS, reactive oxygen species; SI, self-incompatibility.

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Published July 10, 2006

1999; Rao et al., 1999; Morley et al., 2003; Gourlay et al., 2004), thereby committing cells to die (for review see Gourlay and Ayscough, 2005). Because PCD is triggered by SI in incompatible *P. rhoeas* pollen (Thomas and Franklin-Tong, 2004), we hypothesized that early SI-induced actin depolymerization might play a role in acting as an upstream component in PCD activation. We have investigated this possibility using specific drugs to alter actin polymerization status in pollen tubes.

Results

Jasplakinolide stabilizes actin filaments and alleviates SI-induced PCD

Pollen tubes have a characteristic F-actin organization (Fig. 1 A), and SI induces the rapid depolymerization of actin filaments and bundles (Fig. 1 B). To test the hypothesis that SI-stimulated PCD is triggered by actin depolymerization, we used jasplakinolide (Jasp), which stabilizes actin filaments and stimulates polymerization (Bubb et al., 1994; Bubb et al., 2000) and has been shown to inhibit pollen tube growth (Cardenas et al., 2005). We found that 0.5 μM Jasp disrupted *P. rhoeas* pollen tube actin organization, which induced actin filament bundling and aggregation (Fig. 1 C), and inhibited tip growth (unpublished data). The effect of Jasp on actin organization was essentially the opposite of that induced by SI.

We examined whether transient treatments with Jasp might "rescue" SI-induced pollen from PCD by counteracting the actin depolymerization. One of the hallmark features of PCD, DNA fragmentation, is triggered by SI and involves a caspase-3–like activity (Thomas and Franklin-Tong, 2004). This was used as a marker for PCD and was assessed using TUNEL (Fig. 1, D and E). The induction of SI, which is caused by exposing incompatible pollen to S proteins for 30 min, produced high levels of DNA fragmentation of 55.2%, which was only slightly lower than levels of 65.4% induced by SI for 8 h and significantly different from control levels of 16.8% ($P < 0.001$; Fig. 1 F). These data indicated that the interaction of incompatible pollen with S proteins for 30 min was sufficient to trigger PCD. As we had previously demonstrated that 10 min of SI stimulated a 65% reduction in F-actin levels (Snowman et al., 2002), we allowed SI to progress for 10 min to ensure that a significant level of actin depolymerization had occurred and then added 0.5 μM Jasp for 20 min (see Pollen treatments). This consecutive treatment significantly reduced DNA fragmentation (Fig. 1 F) by 32% ($P = 0.04$). Simultaneous treatment with SI and Jasp for 30 min also resulted in significantly reduced levels of DNA fragmentation (33.8%; $P < 0.001$; Fig. 1 F). This demonstrates that Jasp can alleviate SI-induced PCD. These data indicate that the actin depolymerization triggered by SI plays a functional role in the initiation phase of PCD in pollen tubes.

Latrunculin B reduces the level of F-actin in *P. rhoeas* pollen

To further investigate whether actin polymerization status is involved in the initiation of PCD in pollen, we attempted to mimic the actin depolymerization induced by SI without the involvement of other SI initiation signals, such as changes to $[Ca^{2+}]_i$.

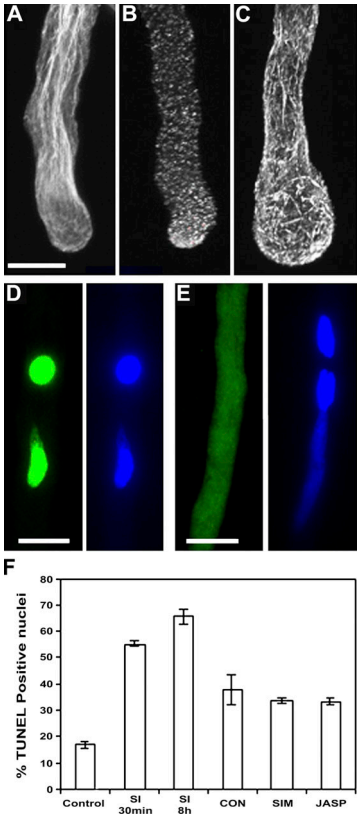

Figure 1. **Jasp alters actin organization and alleviates PCD induced by SI in *P. rhoeas* pollen tubes.** (A) Rhodamine-phalloidin staining shows normal organization of F-actin in an untreated *P. rhoeas* pollen tube. (B) Rhodamine-phalloidin staining shows major disruption of F-actin after 10 min of SI induction. (C) Immunolocalization of actin alterations induced by 0.5 μM Jasp for 10 min shows dramatic rearrangement and stabilization of actin filaments. (D) DNA fragmentation, detected by TUNEL labeling, in a pollen tube treated with 1 μM LatB for 10 min. DAPI staining (right) shows that the TUNEL-positive signal (left) corresponds to nuclear DNA. (E) A typical control pollen tube shows no TUNEL-positive signal (left) corresponding to nuclear DNA (right) despite overexposure revealing the background signal. Bars, 10 μm. (F) Induction of SI for 30 min produced high levels of DNA fragmentation, which was only slightly lower than that achieved by an 8-h treatment (SI, 8 h). Consecutive (CON) treatment, in which 0.5 μM Jasp was added for 20 min to pollen tubes that had previously been treated for 10 min with S proteins, significantly reduced the level of DNA fragmentation. Simultaneous (SIM) treatments of SI and Jasp for 30 min also significantly reduced levels of DNA fragmentation. Untreated pollen tubes (control) produced low levels of DNA fragmentation, whereas 0.5 μM Jasp treatment for 30 min produced higher levels than controls but lower than those induced by 30 min of SI. Values are mean percentages ± SEM (error bars; $n = 3$).

To clarify whether actin depolymerization itself could induce PCD, latrunculin B (LatB; Spector et al., 1983; Coue et al., 1987) was used to depolymerize the actin cytoskeleton in growing *P. rhoeas* pollen tubes. LatB caused the rapid disruption of F-actin organization (Fig. 2, A and B) and the inhibition of pollen tube growth (unpublished data). To confirm actin depolymerization, we measured F-actin levels (Gibbon et al., 1999; Snowman et al., 2002) after 10 min of LatB treatments. LatB caused the depolymerization of pollen tube actin filaments in a concentration-dependent manner (Fig. 2 C). Treatment with 0.1, 1, and 10 μM LatB caused significant reductions ($P < 0.001$) in F-actin levels when compared with controls. Treatment with

Published July 10, 2006

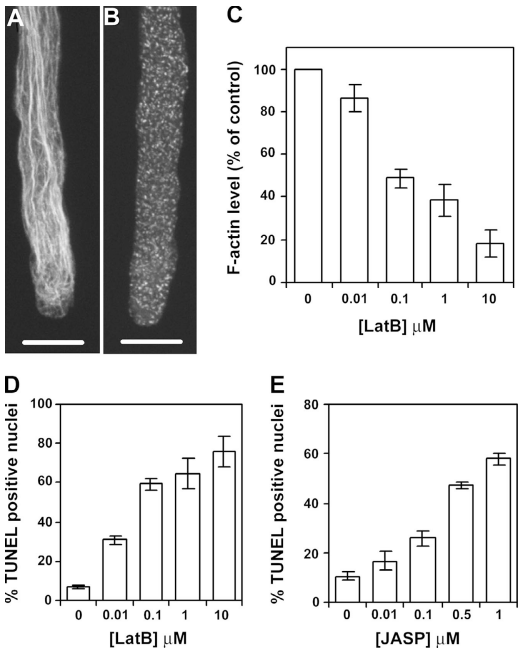

Figure 2. **LatB and Jasp induce DNA fragmentation.** (A) Rhodamine-phalloidin staining of F-actin organization in a normally growing pollen tube. (B) Rhodamine-phalloidin labeling of a pollen tube treated with 1 μM LatB for 10 min showing the reorganization and depolymerization of F-actin. Bars, 10 μm. (C) Treatment of growing pollen tubes with LatB for 10 min caused the concentration-dependent depolymerization of F-actin. Data are mean F-actin levels ± SEM (error bars) as a percentage of the DMSO controls (n = 3). (D) Treatment with LatB for 8 h triggered DNA fragmentation. DMSO controls showed only low levels of DNA fragmentation. (E) Treatment with Jasp for 8 h triggered DNA fragmentation. (D and E) Data are mean percentages of TUNEL-positive nuclei ± SEM from four (D) or three (E) independent experiments.

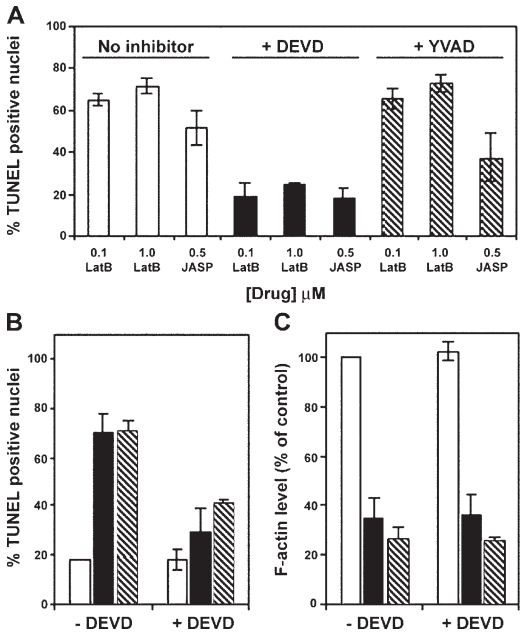

Figure 3. **Alterations in the actin polymerization status trigger a caspase-like/DEVDase activity upstream of DNA fragmentation.** (A) LatB- and Jasp-induced DNA fragmentation is dependent on a caspase-like/DEVDase activity. LatB and Jasp (white bars) induced high levels of DNA fragmentation. Pretreatment with DEVD significantly reduced the levels of DNA fragmentation (black bars), whereas pretreatment with YVAD (crosshatched bars) had no significant effect on DNA fragmentation induced by LatB and Jasp. (B) DEVD significantly reduced levels of DNA fragmentation in pollen tubes treated with LatB. (A and B) Data are mean percentages of TUNEL-positive nuclei ± SEM (error bars) from three independent experiments. (B and C) Untreated pollen, white bars; + 1 μM LatB for 10 min, black bars; + 1 μM LatB for 60 min, crosshatched bars. (C) DEVD had no effect on LatB-induced actin depolymerization induced in growing pollen tubes; similar levels of F-actin were measured in the presence and absence of DEVD. Data are means ± SEM from four independent experiments.

1 μM LatB, which induced 61.5% F-actin depolymerization, gave a similar reduction to that stimulated by SI (69%; Snowman et al., 2002).

Actin depolymerization is sufficient to induce PCD

To test whether LatB-induced actin depolymerization could trigger PCD, pollen tubes were treated with LatB, and levels of DNA fragmentation were assessed after 8 h. LatB stimulated DNA fragmentation in a concentration-dependent manner (Fig. 2 D). The incidence of DNA fragmentation at all concentrations of LatB was significantly different from control pollen tubes ($P < 0.001$) and increased from 6.9% in controls to 30.7% in the presence of 0.01 μM LatB and to 75.8% with 10 μM LatB (Fig. 2 D). Assuming that LatB does not have side effects, this indicated that actin depolymerization triggered DNA fragmentation independently of other SI signals. We also examined whether actin filament stabilization or net assembly of filaments from the profilin–actin pool might also induce PCD in pollen. Jasp stimulated DNA fragmentation in a concentration-dependent manner (Fig. 2 E). The incidence of DNA fragmentation in Jasp-treated pollen tubes increased from 10.4% in control samples to 58% in the presence of 1 μM Jasp (Fig. 2 E). As Jasp binds the phalloidin-binding site of actin, we could not quantify actin polymer levels for these treatments. Given the assumptions regarding the lack of Jasp and LatB side effects, the results indicate that changes in actin filament dynamics can induce DNA fragmentation.

A hallmark feature of apoptosis and PCD is the involvement of caspases, which are responsible for initiating and executing cell death. We showed previously that SI-induced PCD is mediated by a caspase-3–like/DEVDase activity (Thomas and Franklin-Tong, 2004). Therefore, we tested whether the DNA fragmentation stimulated by LatB and Jasp involved such an activity. We used the tetrapeptide Ac-DEVD-CHO (DEVD), which is a caspase-3 inhibitor (Garcia-Calvo et al., 1998; Richael et al., 2001; Danon et al., 2004; Thomas and Franklin-Tong, 2004). Pollen was pretreated with either DEVD or the caspase 1 inhibitor Ac-YVAD-CHO (YVAD), which acts as a negative control, for 1 h before the addition of LatB or Jasp for 8 h. Pollen tubes pretreated with DEVD had significant (65.1–71.6%) reductions in the levels of DNA fragmentation compared with controls without pretreatment ($P < 0.001$; Fig. 3 A). Pretreatment with YVAD had no significant effect on the levels of DNA fragmentation induced by LatB or Jasp ($P = 0.2$; Fig. 3 A). Because DEVD can prevent LatB- or Jasp-induced DNA fragmentation, this implies that both actin depolymerization and stabilization or polymerization can stimulate the activation

Published July 10, 2006

Table I. **Quantification of actin polymer level and DNA fragmentation**

Treatment	Actin polymer level at end of treatment	Actin polymer level after washing	Percent TUNEL-positive nuclei
DMSO	100	100	16.4 ± 1.4
0.1 μM LatB for 5 min	ND	ND	19.5 ± 2.2
0.1 μM LatB for 10 min	77.0 ± 8.9	125.9 ± 7.3	24.3 ± 0.6
0.1 μM LatB for 30 min	ND	ND	57.1 ± 8.2
0.1 μM LatB for 60 min	49.8 ± 5.5	107.3 ± 9.0	64.0 ± 4.0
1 μM LatB for 5 min	ND	ND	58.5 ± 9.9
1 μM LatB for 10 min	46.9 ± 3.8	93.5 ± 10.1	56.4 ± 11.8
1 μM LatB for 30 min	ND	ND	68.2 ± 6.2
1 μM LatB for 60 min	30.8 ± 4.2	70.1 ± 5.2	70.5 ± 6.3

Results are means ± SEM ($n = 3$). The actin polymer level is expressed as a percentage of the amount measured in the control (i.e., control is 100%). TUNEL data are expressed as the percentage of nuclei showing TUNEL labeling.

of a caspase-like enzyme upstream of DNA fragmentation. These data indicate that changes in actin filament dynamics are sufficient to induce a caspase-like (DEVDase) activity that results in PCD in pollen.

Transient F-actin depolymerization is sufficient to induce PCD

To examine whether transient changes in actin filament levels or dynamics could serve as a signal involved in the initiation of PCD or whether sustained alterations are required, "washout" experiments were conducted using LatB treatments, as this most closely mimicked the effect of SI. Pollen tubes were treated with 0.1 and 1 μM LatB for 10 or 60 min, after which the drug was washed out and the incidence of DNA fragmentation was assessed. Actin filament levels were determined at the end of the LatB incubation period to demonstrate that depolymerization had taken place and also after washouts to establish whether repolymerization had occurred. As expected, F-actin levels were reduced in a concentration- and time-dependent manner (Table I). The level of actin polymer after treatment with 0.1 μM LatB was reduced by 23.0% ($P = 0.03$) at 10 min and was further reduced by 50.2% ($P < 0.001$) by 60 min. The higher concentration of 1 μM LatB reduced actin levels by 53.1% ($P < 0.001$) at 10 min and by 69.2% ($P < 0.001$) at 60 min. After washouts, with the exception of the 1-μM 60-min treatment, F-actin levels returned to similar levels as found in untreated pollen, demonstrating that F-actin was only depolymerized transiently (Table I). Pollen tubes treated with 0.1 μM LatB also resumed normal growth (unpublished data).

DNA fragmentation correlated with the duration and extent of actin depolymerization. After 5- and 10-min treatments with 0.1 μM LatB, the effects were similar, and only small increases in DNA fragmentation were detected ($P = 0.3$; NS; Table I). With longer treatments of 0.1 μM LatB, DNA fragmentation increased to 57.1% at 30 min ($P = 0.02$) and to 64.0% at 60 min ($P = 0.001$). The 1-μM LatB treatments induced higher levels of DNA fragmentation than those found in the controls for all four time points ($P < 0.001$). However, there was no significant difference between the four treatments ($P = 0.6$; Table I), indicating that a threshold level of DNA fragmentation had been triggered.

Taking these F-actin quantification and DNA fragmentation data into account, reducing F-actin levels to ∼50% for either 10 or 60 min gave a high incidence of DNA fragmentation even though F-actin levels returned to normal after washing. This indicates that during a brief 10-min period of actin depolymerization to this level, an irreversible "decision-making" step is made, pushing pollen into PCD. Because a DEVDase/caspase-like activity is involved, this implicates actin depolymerization in PCD initiation. Treatments that did not reduce F-actin to this level (e.g., 0.1 μM LatB for 10 min) had a low incidence of DNA fragmentation (24.3%), which was only slightly higher than the control level ($P = 0.02$). Therefore, we can say with some confidence that here, the reduced incidence of DNA fragmentation was the result of a transient actin depolymerization that was insufficient to cause PCD. Thus, the threshold for PCD induction is somewhere between a 23 and 46.9% reduction in F-actin levels for as little as 10 min. Our data indicate that <50% actin depolymerization for 10 min is sufficient to induce PCD, achieving levels of DNA fragmentation very similar to that induced by 8 h of LatB ($P = 0.4$; NS). Notably, this threshold level of actin depolymerization required to initiate PCD using LatB is somewhat lower than the 58 and 69% reduction in F-actin levels resulting from 5- and 10-min SI inductions, respectively (Snowman et al., 2002).

LatB-induced PCD is mediated by a caspase-3–like activity

As LatB treatments appeared to mimic the SI–PCD response, we examined whether LatB-induced DNA fragmentation relied on a caspase-like/DEVDase activity similar to that induced by SI (Thomas and Franklin-Tong, 2004). DNA fragmentation was normally induced by 1-μM LatB treatments, whereas DEVD pretreatment prevented this (Fig. 3 B), and levels of DNA fragmentation were not significantly different from untreated samples ($P = 0.13$). These data provide strong evidence that even quite transient LatB-induced actin depolymerization is sufficient to induce a caspase-3–like/DEVDase activity upstream of DNA fragmentation. Because DEVD had no significant effect on the amount of actin depolymerization caused by LatB (Fig. 3 C), this established that DEVD does not interfere with the action of LatB.

We have also used the caspase-3 substrate Ac-DEVD-AMC to establish this caspase-like activity more directly. Pollen tubes treated with 1 μM LatB for 6 h exhibited an increase in

Published July 10, 2006

DEVDase activity of 54 ± 5% (n = 4) compared with control samples containing germination medium (GM) only. This level of caspase activity was similar to that induced by either SI or 0.5-μM Jasp treatments (unpublished data). Thus, LatB-stimulated pollen extracts exhibited markedly increased levels of caspase-3–like activity, indicating cleavage of the substrate by a DEVDase activity. The activation of caspases commits the cell to die; therefore, our data implicate alterations in actin depolymerization as playing a functional role in stimulating or regulating the PCD cascade in pollen.

Inhibition of tip growth does not induce PCD in pollen tubes

Because both LatB and Jasp inhibit tip growth (Gibbon et al., 1999; Vidali et al., 2001; Snowman et al., 2002; Cardenas et al., 2005), we wished to establish that PCD induction was not indirectly caused by the cessation of tip growth. We used caffeine, which perturbs tip growth (Pierson et al., 1996) but does not affect the actin cytoskeleton of pollen tubes (Geitmann et al., 2000; Snowman et al., 2002). Pollen tubes treated with 3 mM caffeine for 10 or 60 min did not significantly alter F-actin levels compared with controls (112 ± 12 and 112 ± 2%, respectively; n = 3; P = 0.06). Caffeine-treated pollen tubes had low DNA fragmentation levels that were not significantly different from controls (15 ± 3% at 10 min, P = 0.2; and 23 ± 4% at 60 min, P = 0.06; n = 3). This demonstrates that the LatB/Jasp-induced DNA fragmentation is not merely a consequence of the inhibition of growth but is caused by changes in actin polymer levels or assembly dynamics.

Jasp counteracts LatB-induced PCD

We also investigated whether counteracting the effect of LatB-induced depolymerization with Jasp might prevent progression into PCD. Pollen tubes were treated in a similar manner to the SI experiment. They were first treated with 0.1 μM LatB for 10 min to ensure that actin depolymerization had occurred; 0.5 μM Jasp was then added for 20 min, after which the drugs were washed out. Thus, the total length of time that the pollen was exposed to LatB for this consecutive treatment was 30 min. This treatment resulted in a 45% reduction (P = 0.002) in the level of DNA fragmentation compared with LatB alone for 30 min (Fig. 4). We also performed treatments whereby LatB and Jasp were added simultaneously; this had a similar significant effect with a 51.7% (P = 0.007) reduction in the incidence of PCD to 19.8%, which was not significantly different (P = 0.2) from control levels of 11.3% (Fig. 4). These data show that Jasp can counteract the actin depolymerization induced by LatB and, thereby, rescues pollen from entry into PCD.

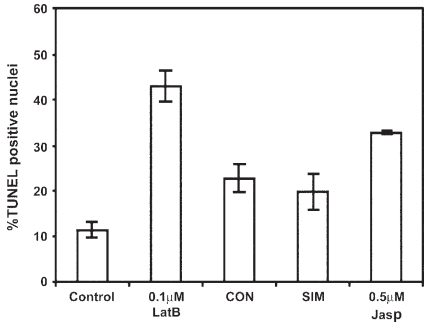

Figure 4. **Actin depolymerization induces PCD, which can be alleviated by altering the actin polymerization status.** The effect of counteracting LatB-induced depolymerization with Jasp was assessed by the extent of DNA fragmentation. Untreated pollen tubes (control) produced low levels of DNA fragmentation; treatment with 0.1 μM LatB for 30 min produced increased levels of DNA fragmentation, and 0.5 μM Jasp for 30 min produced increased but lower levels of DNA fragmentation. With combined LatB-Jasp treatments (consecutive [CON] treatments [10 min with 0.1 μM LatB and then 0.5 μM Jasp for a further 20 min] and simultaneous [SIM] treatments [0.1 μM LatB and 0.5 μM Jasp for 30 min], the level of DNA fragmentation was significantly reduced compared with that observed with LatB or Jasp alone. Data are mean percentages of TUNEL-positive nuclei ± SEM (error bars) from four independent experiments.

Discussion

The actin cytoskeleton has been identified as a major target and effector of signaling cascades in both animal and plant cells (Schmidt and Hall, 1998; Staiger, 2000). SI may be viewed as the triggering of a signaling cascade that takes the cell into PCD. In this study, we provide the first account of a causal link between actin polymer levels or dynamics and the initiation of PCD in a plant cell.

Our study provides a significant advance in our understanding of the mechanisms involved in early SI and of the initiation of PCD in plant cells because very little is known about the early events involved in PCD in plants. Our data provide evidence that relatively transient but substantial F-actin depolymerization can trigger PCD, which is mediated by a caspase-3–like activity. We previously demonstrated that the SI response in *P. rhoeas* pollen results in a 69% reduction in F-actin levels within 10 min (Snowman et al., 2002) and, independently, that SI also induces PCD (Thomas and Franklin-Tong, 2004). In this study, we have demonstrated that a transient reduction in F-actin levels of <50%, which is independent of SI induction, is sufficient to induce PCD. This provides evidence for a link between the signaling cascades involving actin dynamics and PCD, showing that actin depolymerization is sufficient to induce a caspase-like/DEVDase activity resulting in PCD. Furthermore, together with previous data (Snowman et al., 2002), they demonstrate that during an incompatible SI response, the degree of actin depolymerization is more than adequate to act as an intermediary signal to PCD. Because Jasp can alleviate SI-induced DNA fragmentation, presumably by interfering with or counteracting the actin depolymerization induced by SI, this provides further, more direct evidence for the involvement of actin depolymerization in SI-induced PCD. Thus, rapid depolymerization of the actin cytoskeleton induced by SI not only inhibits pollen tip growth but also acts upstream of a signaling cascade that is involved in initiating PCD. Our data support the hypothesis that the F-actin depolymerization and PCD observed during SI function together in a signaling network that prevents incompatible pollen from affecting fertilization.

Although LatB or cytochalasin D (CD) has previously been reported to implicate actin depolymerization in PCD during embryogenesis in plants (Smertenko et al., 2003), the drug treatments extended over a long period of time (6 d), and

Published July 10, 2006

disruption of the actin cytoskeleton for this length of time will almost certainly result in defects in embryogenesis; this in itself could result in PCD. Thus, although the data suggested a possible involvement of the actin cytoskeleton in PCD, they provided no clear evidence for a functional role for actin depolymerization in PCD.

Our findings establish a fundamentally important role for the actin cytoskeleton as a sensor of cellular stress that is common to many eukaryotic cells, as various links between changes in actin dynamics and PCD induction have previously been reported for animals and yeast (Levee et al., 1996; Janmey, 1998; Korichneva and Hammerling, 1999; Suria et al., 1999; Morley et al., 2003; Gourlay et al., 2004). Several studies have shown that alterations to the actin cytoskeleton (either stabilization or depolymerization, depending on the cell type involved) can play a functional role as an effector in the initiation of PCD. For example, CD resulted in DNA fragmentation and caspase-3 activity in Jurkat T cells and hippocampal neurons (Suria et al., 1999), and Jasp enhanced and accelerated apoptosis induced by cytokine withdrawal in the IL-2–dependent T cell line CTLL-20 and induced a caspase-3 activity (Posey and Bierer, 1999). Because these data demonstrate that presumed changes to actin polymer levels or dynamics can stimulate caspase-3 activity, which is an effector caspase triggered early in PCD, they provide evidence that actin depolymerization or stabilization is sufficient to act as an intermediary signal early in the PCD signaling cascade. Other studies in yeast and some mammalian cells have also shown that decreasing actin filament turnover (by using Jasp to stabilize actin) induces apoptosis (Posey and Bierer, 1999; Rao et al., 1999; Odaka et al., 2000; Gourlay et al., 2004). Thus, it has been proposed that altering actin dynamics or the rates of assembly and disassembly rather than the level of polymeric actin in cells is sufficient to signal the initiation of apoptosis (Janmey, 1998; Korichneva and Hammerling, 1999; Rao et al., 1999; Suria et al., 1999; Odaka et al., 2000; Cioca and Kitano, 2002; Kim et al., 2003; Morley et al., 2003; Gourlay et al., 2004; for review see Gourlay and Ayscough, 2005).

Interestingly, although actin stabilization stimulates PCD in yeast, actin depolymerization has the reverse effect, leading to increased viability (Gourlay et al., 2004), which is not the case in pollen. This suggests that although there are clear parallels, the specific mechanisms are likely to differ between plants, animals, and yeast. This is borne out by several studies with mammalian cells, in which actin depolymerization can induce or promote apoptosis (Korichneva and Hammerling, 1999; Posey and Bierer, 1999; Suria et al., 1999; Cioca and Kitano, 2002; Morley et al., 2003), whereas actin stabilization can inhibit or delay apoptosis (Korichneva and Hammerling, 1999; Genesca et al., 2006). Indeed, it has been shown in Jurkat T cells that either increased actin depolymerization or polymerization using CD or Jasp can enhance apoptosis (Posey and Bierer, 1999; Morley et al., 2003). This appears to be the situation in pollen, in which either LatB or Jasp can induce PCD. Furthermore, it has recently been shown that Jasp can alleviate apoptosis in ischemic kidney cells; ischemia normally correlates with a substantial depolymerization of the actin cytoskeleton (Genesca et al., 2006). Similarly, phalloidin treatment, which stabilizes F-actin, prevented cisplatin-mediated actin depolymerization and apoptosis in porcine kidney proximal tubule cells (Kruidering et al., 1998). This is exactly what we have demonstrated here; Jasp effectively reverses the effects of S-protein treatment, presumably by stabilizing actin filaments against depolymerization or counteracting the calcium-induced disassembly.

Therefore, it would appear that a precise level of actin polymer or exact flux of subunits through the polymer pool is necessary for normal growth and cell viability of pollen (Gibbon et al., 1999; Vidali et al., 2001); when this is substantially perturbed, PCD is initiated. It has been proposed that it is the alteration of actin dynamics (i.e., the rate of actin polymerization and depolymerization) that modulates the transduction of the apoptotic signal (Morley et al., 2003). The alterations to actin providing the sensory mechanism could involve either changes in polymer levels, changes in the flux of actin through the filament pool, or both. However, although in some mammalian cells, alterations to F-actin status alone (either polymerization or depolymerization, depending on the cell type) are sufficient to induce apoptosis, in other cell types, other apoptotic stimuli are required in addition to drug treatments affecting actin dynamics (Korichneva and Hammerling, 1999; Posey and Bierer, 1999; Morley et al., 2003). Thus, there are likely to be quite important and subtle differences in the mechanisms operating to initiate apoptosis/PCD in different cell types. One notable and important difference between plant pollen and yeast or mammalian cells is the high ratio of globular actin (G-actin) to F-actin in actively growing cells. Measurements from maize and *P. rhoeas* pollen indicate that just 5–10% of the total actin protein is present in the filamentous form (Gibbon et al., 1999; Snowman et al., 2002). In comparison, budding yeast cells are considered to have the majority of the total actin pool in filamentous form (Karpova et al., 1995). These observations further suggest that certain differences in apoptosis initiation and/or responses to cytoskeletal drugs may relate to the endogenous balance between monomer and polymer in different eukaryotic cells.

Importantly, our study, which used washouts to achieve short-term actin depolymerization or stabilization, is, to our knowledge, the first to demonstrate that transient (10–60 min) alterations to actin dynamics can trigger PCD. Most of the aforementioned studies have used rather long-term treatments with Jasp or LatB for between 8 and 48 h to induce apoptosis (Posey and Bierer, 1999; Suria et al., 1999; Gourlay et al., 2004). To our knowledge, this is the only study to quantify the changes in F-actin levels required to trigger PCD. Only two other studies attempted to quantify the actin polymerization status (Rao et al., 1999; Morley et al., 2003). One of these studies reports that measurements after CD treatment of CTLL-20 and Jurkat T cells indicated an increase in the amount of monomeric or G-actin and a decrease in the amount of F-actin, suggesting the promotion of actin filament disruption and depolymerization (Morley et al., 2003). However, no data are shown. The other study (Rao et al., 1999) measured G- and F-actin levels after camptothecin-induced apoptosis in HL-60 cells and found that G-actin was decreased in TUNEL-negative cells at 2 h, indicating that polymerization was increased early in apoptosis.

Published July 10, 2006

In TUNEL-positive cells (in which PCD had already occurred), G-actin levels were increased, indicating depolymerization. These data are consistent with camptothecin- and Jasp-induced actin polymerization occurring before PCD. Our data go further than this because we have estimated the levels of actin depolymerization required to induce PCD in pollen. In this study, we show that 50% depolymerization of the actin cytoskeleton for just 10 min is sufficient to trigger substantial numbers of cells to undergo PCD.

The alterations to actin could involve either changes in polymer levels, changes in the flux of actin through the filament pool, or both. Because virtually no biochemical analysis of actin dynamics leading to PCD has been performed, it would be of considerable interest to establish which mechanisms are involved and whether they vary depending on cell type. Similarly, several different actin-binding proteins have been suggested to play a role during the initiation or execution phases of PCD, but cause and effect relationships are difficult to decipher. We recently identified pollen gelsolin, PrABP80, which is a calcium-stimulated actin filament severing and depolymerizing protein that could potentially be involved in SI-mediated actin depolymerization (Huang et al., 2004). This is of interest, as gelsolin is implicated in modulating apoptosis in animal cells (for review see Kwiatkowski, 1999).

How exactly actin polymerization status can affect apoptosis induction is not yet established in any organism. However, there is evidence that changes to actin dynamics interact with apoptotic signaling cascades. It has been suggested that disruption of the cytoskeleton might promote the release of caspases, enabling their activation or, alternatively, disrupting mitochondria and causing the release of cytochrome *c*, caspases, or caspase activators (Suria et al., 1999). In mammalian cells, there is evidence that either actin depolymerization (Suria et al., 1999) or stabilization (Posey and Bierer, 1999; Cioca and Kitano, 2002; Genesca et al., 2006) can induce caspase-3 activation; actin depolymerization using CD has been shown to regulate nitric oxide–stimulated apoptosis by modulating PI3-kinase, PKC, and MAPK signaling (Kim et al., 2003). In yeast, it has been proposed that the actin cytoskeleton could act as a regulator for reactive oxygen species (ROS) release from mitochondria, as Jasp can stimulate changes in the levels of ROS (Gourlay et al., 2004). We have shown here that actin depolymerization or polymerization is sufficient to induce a caspase-3–like activity in pollen. Whether ROS is involved in SI is not yet known, but it is known that the rapid disruption of mitochondria is triggered by SI (Thomas and Franklin-Tong, 2004). Furthermore, we have previously shown that a MAPK, p56, is activated by SI (Rudd et al., 2003). These provide possible targets for signaling links between the actin cytoskeleton and PCD in pollen. Thus, our data clearly demonstrate that actin acts as a sensor for signals and that its dynamics play a key role in signaling to initiate PCD in plant cells.

Materials and methods

Pollen treatments

Pollen of *P. rhoeas*, the field poppy, was germinated and grown in vitro in liquid GM (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% sucrose) as described previously (Snowman et al., 2002) at 25°C. Pollen was grown for 1 h before any treatments were applied.

For SI treatments, recombinant proteins were produced by cloning the nucleotide sequences specifying the mature peptide of the S_1, 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 were performed as described previously (Kakeda et al., 1998). SI was induced by adding recombinant S proteins (final concentration of 5 μg/ml) to pollen growing in vitro as described previously (Snowman et al., 2002).

For DNA fragmentation experiments with actin inhibitors, LatB or Jasp (both from Calbiochem) were added to growing pollen tubes at various concentrations. Pollen was grown for a total of 8 h, after which it was fixed in 2% PFA and assayed for DNA fragmentation (see next section). For washout experiments, after short treatments with LatB or Jasp, pollen tubes were washed extensively in GM (six washes of 5 ml for 5 min each). Pollen tubes were resuspended in GM and incubated for the remainder of the 8-h period. Controls were comprised of the addition of DMSO at a final concentration of 0.1% (vol/vol).

Caffeine (Sigma-Aldrich) treatments consisted of the addition of the drug to pollen tubes at a final concentration of 3 mM; pollen was incubated for 10 or 60 min before washouts as described above. Controls were comprised of the addition of GM alone.

For the SI-Jasp experiments, pollen tubes were subjected to two antagonistic treatments with incompatible S proteins and Jasp. To ascertain a suitable Jasp treatment, washout experiments were conducted. Treatments of 0.5 μM Jasp for 10 and 30 min followed by washouts only stimulated levels of DNA fragmentation between 31.2 ± 2.5 and 33.0 ± 0.4%, respectively. This provided a suitable concentration of Jasp for attempts to counteract the PCD induced by LatB. Consecutive SI-Jasp treatments consisted of the addition of S proteins for 10 min followed by the addition of 0.5 μM Jasp for 20 min. Simultaneous treatments involved the addition of S proteins and Jasp at the same time and incubation for 30 min. Immediately after each treatment, the pollen tubes were washed extensively with GM as described above and were incubated for the remainder of the 8-h period before assaying for DNA fragmentation. Control treatments were comprised of 30 min in 0.1% DMSO or 0.5 μM Jasp. The LatB-Jasp experiments were performed in a similar manner.

DNA fragmentation assay

Fixed pollen tubes were labeled with a Deadend Fluorometric TUNEL kit (Promega) according to the manufacturer's instructions. Pollen tubes were scored for DNA fragmentation (50 tubes per treatment; $n = 3$) using a fluorescence microscope (T300; Nikon) and a 60× plan-Apo 1.4 NA oil objective (Nikon). Capture and analysis of images was performed at 20°C using a camera (SenSys KAF1400-G2; Photometrics) and an image analysis system (Quips PathVysion; Applied Imaging). Composite images were prepared using Adobe Photoshop 8.0.

Caspase assays

To test whether DNA fragmentation was dependent on a caspase-like activity, pollen was grown in the presence of 100 μM DEVD or YVAD (Calbiochem) for 1 h before the addition of the cytoskeletal drugs for 8 h. Samples were then tested for DNA fragmentation using TUNEL. Inhibition of DNA fragmentation by DEVD was taken as evidence for caspase-3–like activity.

We also used the fluorogenic caspase-3 substrate Ac-DEVD-AMC (Calbiochem) to establish this caspase-like activity more directly. Pollen tubes were treated with 1 μM LatB and 0.5 μM Jasp or GM for 6 h, and protein extracts were made from them by grinding in extraction buffer (50 mM sodium acetate and 10 mM L-cysteine, pH 6.0). 10 μg of total protein was incubated with 50 μM Ac-DEVD-AMC at 27°C, and fluorescence was monitored at 460 nm using a time-resolved fluorescence plate reader (FLUOstar OPTIMA; BMG LABTECH). Fluorescence at 480 nm indicated cleavage of the substrate by a DEVDase activity. Relative fluorescence units were expressed as the percent increase relative to the control after 4 h.

F-actin imaging

Pollen was grown on GM medium solidified with 1.2% wt/vol agarose, treated with actin inhibitors at various concentrations and different times as described in the figure legends and text, and fixed with 400 μM 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS; Pierce Chemical Co.) for 6 min followed by 2% PFA for 40 min. Pollen tubes were washed three times in actin-stabilizing buffer (100 mM Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 75 mM KCl) and permeabilized with TBS-Tween-DTT (50 mM Tris, pH 7.4, 200 mM NaCl, 0.05% Tween 20, and 5 mM DTT) for 15 min.

Published July 10, 2006

Except for Jasp-treated pollen tubes, in which the phalloidin-binding site was masked, F-actin was labeled by treatment with 500 nM rhodamine-phalloidin (Invitrogen) for 20 min at RT. All images were collected using a 60× plan-Apo 1.4 NA oil immersion objective (Nikon). Capture and analysis of images was performed at 20°C using a laser-scanning system (Radiance 2000; Bio-Rad Laboratories) and the 543-nm excitation line of a 1.5-mW HeNe laser. Images are optical projections of z series taken at 0.5-μm sections, and each section was the average of three Kalman scans. Images were exported, and composite images were prepared using Adobe Photoshop 8.0.

For Jasp-treated pollen, pollen tubes were grown, treated with 0.5 μm Jasp for 10 min, fixed with PFA and MBS, and prepared for immunolocalization. Samples were washed three times in actin-stabilizing buffer and once in MES buffer (5 mM MES, pH 5.0, 5 mM EGTA, and 0.4 M mannitol). Cell walls were digested with 0.1% cellulase and 0.1% macerozyme in MES buffer containing 0.1 mM PMSF for 10 min. Pollen tubes were washed once in MES, washed twice in TBS, permeabilized in 0.1% Triton X-100 in TBS for 10 min, and washed in TBS + 1% BSA. Samples were then incubated with a 1:250 dilution of anti-actin antibody (Gibbon et al., 1999) overnight at 4°C. Unbound primary antibody was washed out, and the pollen tubes were incubated for 2 h at RT in anti–rabbit FITC antibody (1:200 dilution). All images were collected at 20°C with a 60× plan-Apo 1.4 NA oil objective (Nikon) using a Radiance 2000 laser-scanning system with the 488-nm excitation line of a 50-mW Ar laser. Images are optical projections of z series (0.5-μm sections). Images were exported, and composite images were prepared using Adobe Photoshop 8.0.

F-actin quantification

Pollen F-actin levels were determined using the modified phalloidin-binding assay described previously (Gibbon et al., 1999). In brief, LatB-treated pollen tubes were fixed (1.55 M sucrose, 0.1% NP-40, and 600 μM MBS) for 1 h and washed in GM + 0.05% NP-40. Fixed pollen tubes were gradually exchanged into TBS + sucrose (TBSS; 50 mM Tris, pH 7.4, 200 mM NaCl, and 400 mM sucrose) plus 0.05% NP-40 and 2 mM DTT. The TBSS was aspirated from pollen samples, and 50 μl of 2 μM AlexaFluor 488 phalloidin (Invitrogen) in TBSS + 0.05% NP-40 was added to label F-actin at 4°C overnight. After washing, bound phalloidin was extracted in 1 ml methanol overnight at 4°C. The amount of F-actin in the samples was determined by fluorimetry with excitation at 492 nm and emission at 514 nm using a spectrofluorometer (QM-2000-SE; Photon Technology International). Fluorescence values were calculated per 100,000 pollen grains/tube and expressed as the percentage of the relevant control ± SEM. F-actin levels from control samples without LatB treatment were normalized to 100%.

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