Self-Incompatibility-Induced Programmed Cell Death in Field Poppy Pollen Involves Dramatic Acidification of the Incompatible Pollen Tube Cytosol

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Self-incompatibility (SI) is an important genetically controlled mechanism to prevent inbreeding in higher plants. SI involves highly specific interactions during pollination, resulting in the rejection of incompatible (self) pollen. Programmed cell death (PCD) is an important mechanism for destroying cells in a precisely regulated manner. SI in field poppy (Papaver rhoeas) triggers PCD in incompatible pollen. During SI-induced PCD, we previously observed a major acidification of the pollen cytosol. Here, we present measurements of temporal alterations in cytosolic pH ([pH]cyt); they were surprisingly rapid, reaching pH 6.4 within 10 min of SI induction and stabilizing by 60 min at pH 5.5. By manipulating the [pH]cyt of the pollen tubes in vivo, we show that [pH]cyt acidification is an integral and essential event for SI-induced PCD. Here, we provide evidence showing the physiological relevance of the cytosolic acidification and identify key targets of this major physiological alteration. A small drop in [pH]cyt inhibits the activity of a soluble inorganic pyrophosphatase required for pollen tube growth. We also show that [pH]cyt acidification is necessary and sufficient for triggering several key hallmark features of the SI PCD signaling pathway, notably activation of a DEVDase/caspase-3-like activity and formation of SI-induced punctate actin foci. Importantly, the actin binding proteins Cyclase-Associated Protein and Actin-Denpolymerizing Factor are identified as key downstream targets. Thus, we have shown the biological relevance of an extreme but physiologically relevant alteration in [pH]cyt and its effect on several components in the context of SI-induced events and PCD.
increases in cytosolic free calcium ([Ca^{2+}]_{cyt}) are triggered in incompatible pollen tubes (Franklin-Tong et al., 1993), resulting in phosphorylation of soluble inorganic pyrophosphatases (sPPases; Rudd et al., 1996; de Graaf et al., 2006), activation of a Mitogen-Activated Protein Kinase (MAPK; Rudd et al., 2003), and increases in reactive oxygen species (ROS) and nitric oxide (Wilkins et al., 2011, 2014). Most of these components are integrated into a signaling network leading to PCD (Bosch et al., 2008; Wilkins et al., 2014). The actin cytoskeleton is a key target in the field poppy SI response, undergoing depolymerization (Snowman et al., 2002) followed by polymerization into highly stable F-actin foci decorated with the actin binding proteins (ABPs) Actin-Depolymerizing Factor (ADF) and Cyclase-Associated Protein (CAP; Poulter et al., 2010, 2011), with both processes being involved in mediating PCD (Thomas et al., 2006). A major player in SI-mediated PCD is a caspase-3-like/DEVDase-like activity (Thomas and Franklin-Tong, 2004; Bosch and Franklin-Tong, 2007). The SI-induced caspase-3-like/DEVDase exhibits maximum substrate cleavage in vitro at pH 5, with peak activity 5 h after SI induction in vivo (Bosch and Franklin-Tong, 2007). The low pH optimum for this caspase-3-like/DEVDase activity is unusual, because most of the cytosolic plant caspase-like activities identified to date have optimal activity close to normal physiological pH (approximate pH, 6.5–7.0; Korthout et al., 2000; Bozhkov et al., 2004; Coffeen and Wolpert, 2004). Because the SI-induced cytosolic-located DEVDase requires a low pH for activity, this suggested that, during SI, the pollen tube cytosol undergoes dramatic acidification. In vivo pH measurements of the cytosol at 1 to 4 h after SI induction confirmed this, when cytosolic pH ([pH]_{cyt}) had dropped from pH 6.9 to pH 5.5 (Bosch and Franklin-Tong, 2007). This fits the in vitro pH optimum of the caspase-3-like/DEVDase almost exactly, implicating pollen cytosolic acidification as playing a vital role in creating optimal conditions for the activation of the caspase-3-like/DEVDase-like activity and progression of PCD.

Under normal cellular conditions, [pH]_{cyt} is between approximately 6.9 and 7.5 (Kurkdjian and Guern, 1989; Felle, 2001). Pollen tubes, like other tip-growing cells, have [pH]_{cyt} gradients (Gibbon and Kropf, 1994; Feijó et al., 1999). The [pH]_{cyt} of the pollen tube shank is an approximate pH of 6.9 to 7.11 (Fricker et al., 1997; Messerli and Robinson, 1998). There has been much debate about the [pH]_{cyt} gradient, comprising an apical domain with an approximate pH of 6.8 and a subapical alkaline band with an approximate pH of 7.2 to 7.8 in Lilium longiflorum and Lilium formosanum pollen tubes (Fricker et al., 1997; Messerli and Robinson, 1998; Feijó et al., 2001; Lovy-Wheeler et al., 2006). Oscillations of [pH]_{cyt} between approximate pH values of 6.9 and 7.3 have been linked to tip growth in L. formosanum pollen tubes (Lovy-Wheeler et al., 2006). The vacuole and the apoplast have a highly acidic pH between pH 5 and pH 6 (Katsuhara et al., 1989; Feijó et al., 1999). The majority of studies of pH changes in plant cells reports modest, transient changes in [pH]_{cyt} of approximately 0.4 and 0.7 pH units during development, gravitropic responses, decreases in light intensity, and addition of elicitors, hormones, and other treatments. For example, during root hair development in Arabidopsis (Arabidopsis thaliana), root [pH]_{cyt} was elevated from an approximate pH of 7.3 to 7.7 (Bibikova et al., 1998). Root gravitropic responses stimulate small transient [pH]_{cyt} alterations (Scott and Allen, 1999; Fasano et al., 2001; Johannes et al., 2001). More recently, it has been shown that the [pH]_{cyt} drops during PCD controlling root cap development; however, exactly how many units the [pH]_{cyt} decreased was not measured (Fendrch et al., 2014). Other studies investigating [pH]_{cyt} in response to physiologically relevant signals also report small transient alterations. Light-adapted cells respond to a decrease in light intensity with a rapid transient cytosolic acidification by approximately 0.3 pH units (Felle et al., 1986). Addition of nodulation factors resulted in an increase of 0.2 pH units in root hairs (Felle et al., 1998), and abscisic acid increased the [pH]_{cyt} of guard cells by 0.3 pH units (Blatt and Armstrong, 1993). Changes in [pH]_{cyt} are thought to activate stress responses (Felle, 2001). Elicitor treatments resulted in a [pH]_{cyt} drop of between 0.4 and 0.7 pH units in suspension cells (Mathieu et al., 1996; Kuchitsu et al., 1997), a drop of 0.2 pH units in Nitellopsis obtusia cells treated with salt (Katsuhara et al., 1989), and a drop of 0.3 to 0.7 pH units in Eschscholzia californica (Roos et al., 1998).

Here, we investigate SI-induced acidification of the cytosol, providing measurements of physiologically relevant temporal alterations in [pH]_{cyt} and identify key targets of this, providing mechanistic insights into these events. The SI-induced acidification plays a pivotal role in the activation of a caspase-3-like/DEVDase activity, the formation of punctate F-actin foci, and ABP localization during SI PCD. We investigate the vacuole as a potential contributor to SI-induced [pH]_{cyt} acidification.

RESULTS

Dramatic and Rapid Acidification of Field Poppy Pollen Tube Cytosol Is Stimulated during SI

We previously reported acidification of field poppy pollen tube cytosol after SI induction (Bosch and Franklin-Tong, 2007), but a temporal characterization of this was not carried out. We, therefore, investigated SI-induced [pH]_{cyt} changes at various time points up to 3 h after SI induction using the ratiometric pH indicator 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) acetoxyethyl ester (AM) and calibrated the [pH]_{cyt} (Supplemental Fig. S1). The time points chosen were 10, 30, 60, and 180 min after SI, because these correspond to when key features of the SI response were observed, particularly the changes in F-actin foci. Typically, small speckles of F-actin are seen at 30 min, and larger F-actin foci are formed between 30 and 60 min post-SI and continue to grow in size (Poulter et al., 2010). Increases in [Ca^{2+}]_{cyt} were virtually instantaneous, and therefore, this is assumed to be the initiating signal; ROS peaks at approximately 5 min and is upstream of actin.
alterations. Three hours was chosen as the end point, because this seemed to be the time point when actin foci had fully formed. DEVDase activity was observed much later; a small (nonsignificant) increase was observed at 90 min, and significant increases in activity were observed later at 3 to 5 h (Bosch and Franklin-Tong, 2007; see Wilkins et al., 2014 and Supplemental Fig. S2 for a recent summary of SI-induced events). The [pH]cyt of untreated, growing pollen tubes at time 0 for all treatments was relatively constant at pH 6.8 ± 0.004 (n = 100; Fig. 1A). There was no significant difference between the [pH]cyt of untreated pollen tubes and that of compatible pollen tubes at t = 0 (P = 0.871, n = 16). The [pH]cyt of untreated pollen tubes did not change significantly over a period of 3 h (P = 0.069, n = 69; Fig. 1A); representative images are shown in Supplemental Figure S3, A and B. SI induction resulted in a rapid drop in [pH]cyt (Fig. 1A). Within 10 min, the mean [pH]cyt was 6.4 ± 0.14, which is significantly different from untreated pollen tube [pH]cyt at the same time point (P = 0.0001, n = 11); 30 min after SI induction, the [pH]cyt of incompatible pollen tubes had decreased to pH 6.0 ± 0.10. Acidification continued for up to 60 min, when it reached [pH]cyt of 5.5 ± 0.12, which was significantly different than that of untreated pollen tubes at 60 min (P = 8.32 × 10−6, n = 16; Supplemental Fig. S3, B and E). The [pH]cyt of incompatible pollen tubes at 60 min post-SI induction was similar to that at 180 min (P = 0.105, n = 26; Fig. 1A), which suggested that the cytosol had reached a [pH]cyt equilibrium. The acidification was SI specific, because the [pH]cyt of compatible pollen tubes treated with PrsS remained at an approximate pH of 7.0 ± 0.04 throughout and did not significantly differ from the [pH]cyt of untreated samples (P = 0.168, n = 8; Fig. 1). These data show that SI induction triggers dramatic and rapid acidification of the pollen tube cytosol in an S-specific manner.

Manipulation of Pollen Tube [pH]cyt Using Propionic Acid Can Mimic or Prevent SI-Induced Acidification

To investigate the role of SI-induced cytolic acidification, 50 mM propionic acid was used to manipulate the pollen tube [pH]cyt. We did not measure the growth rates, because after propionic acid treatment, growth was stopped immediately, although cytoplasmic streaming continued, indicating that pollen tubes were still alive and viable. This has been documented in other publications (Parton et al., 1997), where acidification or alkalinization of the cytoplasmic pH of *Agapanthus* spp. pollen tubes and *Dryopteris* spp. rhizoids using propionic acid completely inhibited growth.

Addition of propionic acid (pH 5.5) resulted in rapid [pH]cyt acidification and mimicked SI-induced acidification (Fig. 1B); within 10 min, the [pH]cyt was 5.3 ± 0.1 (n = 17), significantly different from untreated samples (P = 1.49 × 10−7, n = 22). After 120 min, the [pH]cyt was not significantly different to that of SI-induced pollen tubes (P = 0.691, n = 8). The [pH]cyt of pollen tubes incubated with propionic acid (pH 7) did not significantly change over 120 min compared with that of untreated pollen (P = 0.875, n = 5).

To examine if the SI-induced acidification could be prevented, pollen tubes were treated with 50 mM propionic acid (pH 7) for 10 min before SI induction, and [pH]cyt was monitored. Ten minutes after SI induction, pretreated samples had a [pH]cyt 6.9 ± 0.0 (n = 4); after 120 min, the [pH]cyt was similar to the initial [pH]cyt (P = 1.00, n = 9) and untreated samples (P = 0.705, n = 8; Fig. 1B). The [pH]cyt of SI-induced pollen was significantly different from that of pH 7-pretreated pollen tubes at 120 min post-SI (P = 5.32 × 10−4, n = 8). This shows that propionic acid (pH 7) can prevent SI-induced acidification.

Manipulation of Pollen Tube [pH]cyt Can Trigger or Prevent DEVDase Activity

Caspase-3-like/DEVDase activity is a key feature of SI-induced PCD. Previous characterization revealed that its peak activity, which is observed to increase between 3 and 5 h after SI induction, has a very narrow pH range (approximate pH, 4.5–5.5; Bosch and Franklin-Tong, 2007). Having established that SI-induced [pH]cyt dropped very rapidly, we wished to explore whether this affected pollen tube cytotic DEVDase activity. We used propionic acid to alter [pH]cyt and measured caspase-3-like/DEVDase activity at 5 h post-SI induction using the live-cell caspase-3 probe carboxyfluorescein-DEVD-fluoromethylketone (FAM-DEVD-FMK FLICA). SI-induced pollen tubes at 5 h post-SI induction displayed fluorescence, indicating caspase-3/DEVDase activity (Fig. 2, A and B), unlike untreated pollen tubes (Fig. 2, C and D). We investigated whether the addition of propionic acid (pH 5.5; mimicking SI-induced acidification) could stimulate caspase-3/DEVDase activity and if pretreatment with propionic acid (pH 7) before SI induction prevented it.
Acidi
cification of the pollen tube cytosol can activate or prevent DEVDase/caspase-3-like activity. A. Representative pollen tube after SI induction labeled with FAM-DEVD-FMK FLICA exhibits fluorescence, indicating DEVDase/caspase-3-like activity. B. Bright-field image of A. C. Representative untreated pollen tube labeled with FAM-DEVD-FMK FLICA exhibits no fluorescence. D. Bright-field image of C. All images are 5 h after treatment. Bar = 10 μm. E. Quantitation of 150 pollen tubes scored positive (black bars) or negative (white bars) for fluorescence, indicating DEVDase/caspase-3-like activity. A. Representative pollen tube after SI induction labeled with FAM-DEVD-FMK FLICA exhibits no fluorescence. B. Bright-field image of A. C. Representative untreated pollen tube labeled with FAM-DEVD-FMK FLICA exhibits fluorescence, indicating DEVDase/caspase-3-like activity. A. Representative pollen tube after SI induction labeled with FAM-DEVD-FMK FLICA exhibits no fluorescence. B. Bright-field image of A. C. Representative untreated pollen tube labeled with FAM-DEVD-FMK FLICA exhibits fluorescence, indicating DEVDase/caspase-3-like activity. A. Representative pollen tube after SI induction labeled with FAM-DEVD-FMK FLICA.

Quantitation revealed that almost all (97%) SI-induced pollen tubes exhibited caspase-3-like/DEVDase activity, unlike untreated pollen tubes (3%; Fig. 2E). Addition of propionic acid (pH 5.5) resulted in many pollen tubes with caspase-3-like/DEVDase activity (not significantly different from SI-induced samples; P = 0.331). Treatment with propionic acid (pH 7) alone was not significantly different from untreated samples (P = 0.26). Importantly, pretreatment with propionic acid (pH 7) before SI induction gave lower numbers (5%) of pollen tubes with caspase-3-like/DEVDase activity, significantly different from those treated with propionic acid (pH 5.5; P = 1.6 × 10⁻⁵; Fig. 2E). These data show that acidification of the pollen tube cytosol is required for the activation of caspase-3-like/DEVDase activities and identifies a strong link between pollen tube [pH]cyt and initiation of PCD.

Acidi
cification of Field Poppy Pollen Tube Cytosol Triggers F-Actin Foci

We previously showed that the actin cytoskeleton is a key target in the field poppy SI response, undergoing depolymerization (Snowman et al., 2002) followed by polymerization into highly stable F-actin foci (Poulter et al., 2010, 2011), with both processes being involved in mediating PCD (Thomas et al., 2006). We wondered if the SI-induced acidification might play a role in mediating some of these changes. To investigate this, we used propionic acid to manipulate the [pH]cyt of pollen tubes. Normally growing pollen tubes had typical longitudinal bundled actin filament organization (Fig. 3A). Incompatible pollen tubes after 3 h of SI induction had many F-actin foci (Fig. 3B). Pollen tubes treated with propionic acid (pH 5.5) for 3 h also had numerous F-actin foci (Fig. 3C). Pollen tubes treated with propionic acid (pH 7) had an actin configuration similar to untreated pollen tubes (Fig. 3D) as did pollen tubes pretreated with propionic acid (pH 7) before SI induction. Quantification revealed that propionic acid (pH 5.5) resulted in significantly more pollen tubes with F-actin foci than untreated samples (P = 1.08 × 10⁻¹⁵) or those treated with propionic acid (pH 7; P = 5.98 × 10⁻⁴; Fig. 3E). Preventing SI-induced acidification with propionic acid (pH 7) resulted in significantly fewer pollen tubes with F-actin foci than observed in SI-induced samples (P = 8.30 × 10⁻⁸; Fig. 3E), but it was not significantly different from those treated with propionic acid (pH 7) alone (P = 0.114). Thus, lowering the pollen tube [pH]cyt to pH 5.5 can trigger the formation of F-actin foci. Moreover, preventing acidification in SI-induced pollen tubes prevented their formation. This shows the functional importance of acidification in the SI response in field poppy pollen tubes and gives us an insight into mechanisms involved in their formation.

Acidi
cification of the Cytosol Triggers Colocalization of CAP and ADF with F-Actin Foci

To understand mechanisms involved in F-actin foci formation, we used propionic acid to investigate the effect of pH on the localization of two ABPs: ADF/cofilin and CAP (Poulter et al., 2010). Pollen was treated with propionic acid, and F-actin was visualized using rhodamine-phalloidin together with immunolocalization with antibodies directed against either CAP or ADF. Normally growing pollen tubes had typical longitudinal bundled actin filament organization and cytosolic-localized CAP and ADF (Supplemental Fig. S4, A–F). Incompatible pollen tubes after 3 h of SI induction had large F-actin foci that colocalized with CAP and ADF (Supplemental Fig. S4, G–L). Pollen tubes treated with propionic acid (pH 7) had an actin configuration similar to untreated pollen tubes (Supplemental
Fig. S4, M–R) as did pollen tubes pretreated with propionic acid (pH 7) before SI induction. Pollen tubes treated with propionic acid (pH 5.5) for 3 h also had large F-actin foci, typical of SI induction that colocalized with CAP and ADF (Supplemental Fig. S4, S–X).

Quantification of F-actin and CAP colocalization (Fig. 4A) showed that, in untreated pollen tubes, colocalization of F-actin and CAP was minimal (3.7% of untreated pollen tubes showed some colocalization) and significantly different from SI-induced pollen tubes (80%; $P = 9.88 \times 10^{-2}$). Pollen treated with propionic acid (pH 5.5) resulted in high levels of F-actin foci and CAP colocalization, which was significantly different from untreated pollen ($P = 1.23 \times 10^{-5}$). Pretreatment of pollen tubes with propionic acid (pH 7) before SI induction resulted in significantly lower occurrence of F-actin foci and CAP colocalization compared with SI alone (45% of SI-induced pollen tubes; $P = 8.25 \times 10^{-4}$) and compared with pollen treated with propionic acid (pH 5.5; $P = 0.004$). Propionic acid (pH 7) gave low levels of F-actin foci, similar to untreated pollen tubes. Similar results were obtained for colocalization of F-actin and ADF for these treatments (Fig. 4B). In untreated pollen tubes, colocalization of F-actin foci and ADF was minimal (4%) and significantly different from that in SI-induced pollen tubes (80%; $P = 6.44 \times 10^{-6}$). Addition of propionic acid (pH 5.5) gave high levels of F-actin foci, which was significantly different to untreated pollen ($P = 4.06 \times 10^{-4}$). Pretreatment with propionic acid (pH 7) before SI induction resulted in a lower occurrence of F-actin with colocalized ADF foci compared with SI-induced pollen tubes (48% of SI samples; $P = 1.86 \times 10^{-2}$) and compared with pH 5.5-treated pollen tubes ($P = 0.002$). Pollen treated with propionic acid (pH 7) alone had similar levels of actin foci with colocalized ADF to untreated samples ($P = 0.100$). This shows that lowering the [pH]cyt to 5.5 stimulates both the formation of F-actin foci and the colocalization of both CAP and ADF to these structures, providing in vivo evidence that the cellular localization of both CAP and ADF is affected by [pH]cyt acidification.

Altered pH Dramatically Affects sPPase Activity

We previously identified two pollen-expressed sPPases, Prp26.1a and Prp26.1b, that are modified by phosphorylation early in the SI response (Rudd et al., 1996; de Graaf et al., 2006). We wished to establish the effect of the SI-induced pH alterations on these important cytosolic enzymes by testing their activities within the range of SI-induced pH alterations in incompatible pollen
of inorganic phosphorous (Pi) released was measured (5–7.5) in the presence of 2 mM Na₄P₂O₇ as a substrate, and the amount of inorganic phosphorous (Pi) released was measured (n = 4).

Figure 5. pH dramatically affects sPPase activity. Recombinant p26.1a and p26.1b sPPase samples were assayed in 50 mM propionic acid (pH 5–7.5) in the presence of 2 mM Na₄P₂O₇ as a substrate, and the amount of inorganic phosphorous (Pi) released was measured (n = 4).

At the normal physiological pH of pollen tubes, there was high sPPase activity (77.82 ± 2.8 and 90.4 ± 1.6 for Prp26.1a and Prp26.1b, respectively; Fig. 5). At an approximate pH of 6.5, which corresponds to the [pH]cyt of incompatible pollen tubes approximately 10 min post-SI, the sPPase activities of Prp26.1a and Prp26.1b were reduced to 40.4% and 58.3%, respectively, of their original activities (P = 0.029 and P ≤ 0.001, respectively; Fig. 5). At pH 6.0, the [pH]cyt at 30 min post-SI, the sPPase activities were reduced further to 36.0 ± 2.7 and 36.0 ± 3.8, respectively (P ≤ 0.001 and P < 0.001, respectively). At pH 5.5 (the [pH]cyt at 60 min post-SI), sPPase activities were negligible at only 11.4% and 13.2%, respectively, of their original activity at pH 7 (both P ≤ 0.001; Fig. 5). This shows that the SI-induced acidification has a dramatic effect on sPPase activities and most likely, many other important cytosolic enzymes. This suggests that, within a few minutes of the SI response, many key enzymes required for pollen tube function are likely to be inactivated.

SI Triggers Vacuolar Reorganization and Disintegration

Vacuolar rupture is implicated as a key, irreversible step in several plant PCD systems (van Doorn et al., 2011). Because the vacuole is a major acidic organelle in plant cells, we wished to investigate whether it might be involved in the SI PCD response. We used the vacuolar marker carboxy-5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA) to label the pollen tube vacuole. The vacuole of untreated growing pollen tubes has a reticulate structure throughout the pollen tube shank, absent in the apical region (Fig. 6A; Supplemental Movie S1). SI-induced pollen tubes displayed vacuolar reorganization within 15 min with small aggregations (Fig. 6A). As SI progressed, the typical reticulate structure was lost (Fig. 6A); later, there was additional aggregation and a decrease in vacuolar labeling, suggesting breakdown. After 118 min, very little intact structure remained (Fig. 6A). Scoring vacuolar morphology revealed that 80% of pollen tubes had undergone some form of reorganization within 15 min (n = 63), and within 30 to 50 min, 77% (n = 35) had undergone extensive breakdown (Supplemental Fig. S5). To be confident that the carboxy-DCFDA probe was reporting the vacuole, δ-Tonoplast Intrinsic Protein (TIP)-GFP, which labels the vacuolar membrane (Hicks et al., 2004), was used. The δ-TIP-GFP reported a similar reticulate pattern to that observed with carboxy-DCFDA, and by 89 min after SI induction, there was very little intact vacuolar signal remaining (Fig. 6B; Supplemental Movie S2). This provides confidence that carboxy-DCFDA reports the vacuole and shows that SI triggers vacuolar reorganization/breakdown.

Artificial Manipulation of [pH]cyt of Pollen Tube Triggers Vacuolar Alterations

We wished to examine the relationship between the vacuole and cytosolic acidification further. We used propionic acid to alter [pH]cyt and monitored vacuolar organization using carboxy-DCFDA. No reorganization was observed in untreated pollen tubes within 80 min (Fig. 6C; Supplemental Fig. S6A). SI induction resulted in reorganization and apparent breakdown of the vacuole after 85 min (Fig. 6C). Pollen tubes treated with propionic acid (pH 5.5) showed dramatic disintegration of the vacuole (n = 6; Fig. 6C; Supplemental Fig. S6B). This shows that cytosolic acidification has a major effect on the pollen tube vacuole. Contrary to our expectation that vacuolar breakdown would result in acidification of the [pH]cyt, acidification of the cytosol actually triggered vacuolar breakdown. This suggests that there may be an earlier acidification event before vacuolar breakdown. We also investigated whether maintaining the [pH]cyt might prevent vacuolar breakdown. Pollen tubes treated with propionic acid (pH 7) for 10 min before SI induction exhibited only slight reorganization of the carboxy-DCFDA vacuolar signal (Fig. 6C; Supplemental Fig. S6C). This provides evidence that maintaining the pollen tube [pH]cyt can prevent vacuolar breakdown but not reorganization and suggests that there is a signal upstream of vacuolar alterations, which triggers breakdown. Together, these data suggest that the initial SI-induced acidification of the cytosol is upstream of SI-induced vacuolar breakdown.

The Ca²⁺ Ionophore A23187 Triggers Cytosolic Acidification and Vacuolar Reorganization

Because the [pH]cyt alterations were unexpectedly rapid, we wondered whether increases in [Ca²⁺]cyt might trigger acidification, because SI triggers almost instantaneous, transient increases in pollen tube [Ca²⁺]cyt and Ca²⁺ influx (Franklin-Tong et al., 1997; Wu et al., 2011). Pollen
tubes were treated with the Ca\(^{2+}\) ionophore A23187, and the [pH\text{cyt}] was monitored. Addition of A23187 resulted in rapid acidification of the [pH\text{cyt}] (Fig. 7A). Within 30 min, there was a significant difference in [pH\text{cyt}] of A23187-treated pollen tubes compared with untreated pollen tubes (\(P = 0.003, n = 12\)). After 60 min, [pH\text{cyt}] had dropped to pH 5.69 ± 0.17 (\(n = 8\)), and after 120 min, the [pH\text{cyt}] had dropped further and was significantly different to that of untreated pollen tubes at the same time point (\(P = 6.33 \times 10^{-9}\); Fig. 7A). This shows that the Ca\(^{2+}\) ionophore A23187 can trigger rapid major acidification, suggesting that the influx of Ca\(^{2+}\) (and possibly other ions) is upstream of acidification and may be implicated in triggering SI-induced acidification of the pollen tube cytosol.

To establish whether increases in [Ca\(^{2+}\)]\text{cyt} might trigger vacuolar changes, we investigated the effect of A23187 on the pollen tube vacuole using carboxy-DCFDA (Fig. 7B). Seven minutes after addition of A23187, the reticulate structure observed in untreated pollen tubes had undergone some reorganization. Within 25 min, the vacuolar signal had formed large aggregates, and an increased cytosolic-located signal suggested leakage of the dye and vacuolar rupture. By 46 and 77 min after the addition of A23187, there was very little vacuolar signal remaining (Fig. 7B). A23187-stimulated breakdown of the vacuole appeared similar to the SI-induced alterations that were observed. This places Ca\(^{2+}\) influx and increases in [Ca\(^{2+}\)]\text{cyt} (as well as other ions; possibly H\(^{+}\)) as very early signals that occur upstream of vacuolar breakdown.

**DISCUSSION**

**SI Triggers a Dramatic Drop in [pH\text{cyt}]**

We previously reported dramatic cytosolic acidification triggered by SI (Bosch and Franklin-Tong, 2007). This was the first report, to our knowledge, of PCD-associated cytosolic acidification in plants. Here, we have investigated SI-induced acidification of the cytosol in detail. We measured the temporal alterations in [pH\text{cyt}] and provide evidence showing its biological relevance and identifying targets and intracellular consequences of the SI-induced acidification. We show that acidification of the [pH\text{cyt}] plays a pivotal role in SI-induced PCD by creating optimal conditions for activation of the DEVDase/caspase-3-like activity, which has optimal activity between pH 4.5 and pH 5.5 and negligible activity above pH 6.0 (Bosch and Franklin-Tong, 2007). Acidification also plays a crucial role in the formation of punctate F-actin foci, most likely by altering the localization and activity of at least two ABPs, ADF and CAP. The scale of the drop in [pH\text{cyt}] triggered by SI is notable. Although many measurements of alterations in [pH\text{cyt}] exist in various plant cell systems, most of the alterations are rather small and on the scale of <1.0 pH unit. Below, we consider some of the cellular processes that are affected by this major change in cellular homeostasis.
Pollen tubes were treated with 10 μM A23187, and calibrated with the ratiometric pH indicator BCECF AM. B, was added to field poppy pollen tubes growing in vitro, and pH was measured and calibrated with the ratiometric pH indicator BCECF AM. A similar methodology was used to show that the pH dropped during root cap developmental PCD (Fendrych et al., 2014). However, neither study was calibrated to give an indication of the actual [pH]cyt range. Our study confirms that acidification can trigger PCD in another plant system, suggesting that it may be a general phenomenon worth investigating in other PCD systems to determine how widespread this control might be.

![Figure 7](image_url)

**Figure 7.** Ca²⁺ ionophore A23187 triggers cytosolic acidification and vacuolar reorganization of field poppy pollen tubes. A, Fifty micromolar A23187 (black symbols; n = 27) or growth medium (white symbols; n = 25) was added to field poppy pollen tubes growing in vitro, and pH was measured and calibrated with the ratiometric pH indicator BCECF AM. B, Pollen tubes were treated with 10 μM A23187 and labeled with carboxy-DCFDA to visualize the vacuole. Numbers indicate the length of treatment time in minutes: 7 min after the addition of A23187, the vacuole still shows some reticulate appearance; 25 min after the addition of A23187, there is major reorganization with appearance of collapse/aggregation; 46 min after the addition of A23187, little structure is apparent; and 77 min after the addition of A23187, reduced signal suggests extensive breakdown of the vacuole. UT, Typical untreated pollen tube exhibits a reticulate structure. Bar = 10 μM.

### A Drop in [pH]cyt Is Necessary and Sufficient for PCD

We have shown using propionic acid that a drop in [pH]cyt to pH 5.5 is sufficient to trigger a caspase-3-like/DEVDDase activity in field poppy pollen tubes and that maintaining [pH]cyt at pH 7 is sufficient to prevent entry into PCD. This shows that the drop in [pH]cyt is a pivotal event in the SI-induced PCD. Contrary to expectation, there are surprisingly few measurements of [pH]cyt during PCD in any plant system. To our knowledge, with the exception of our previous study (Bosch and Franklin-Tong, 2007) and the results described here, no measurements of [pH]cyt exist to show the extent of the acidification of [pH]cyt during PCD in plants. One of the few studies to monitor pH alterations during PCD in plants is a recent study by Young et al. (2010), which measured loss of fluorescence of the pH-sensitive Yellow Fluorescent Protein probe as an indication of change in [pH]cyt. A similar methodology was used to show that the [pH]cyt dropped during root cap developmental PCD (Fendrych et al., 2014). However, neither study was calibrated to give an indication of the actual [pH]cyt range. Our study confirms that acidification can trigger PCD in another plant system, suggesting that it may be a general phenomenon worth investigating in other PCD systems to determine how widespread this control might be.

### The Involvement of the Vacuole in Cytosolic Acidification and SI-Mediated PCD

The vacuole is a very acidic organelle, with a pH of approximately 5 (Shen et al., 2013). Vacuolar breakdown is a common feature of PCD in plants (Fukuda, 2000; Jones, 2001; Hara-Nishimura and Hatsugai, 2011) and one of two major classes of plant PCD (van Doorn et al., 2011). It is thought that collapse of the vacuole is a key, irreversible step in several plant PCD systems, and vacuolar rupture is often used to mark the death of a cell. However, how this is achieved and what processes are involved remain unknown. Vacuoles accumulate hydrolases, which are released into the cytoplasm upon vacuolar rupture. This is thought to be important for eliminating the cell corpse and recycling cellular material (Jones, 2001). During tracheary element differentiation, the vacuole ruptures virtually instantaneously and marks a point of no return, releasing enzymes into the cytosol that contribute to the degradation of the cell (Obara et al., 2001; Rotari et al., 2005). During the hypersensitive response in cryptogein-induced cell death in tobacco (Nicotiana tabacum) Bright Yellow-2 cells, disruption of vacuolar organization with formation of bulb-like structures and alterations in F-actin organization were observed (Higaki et al., 2007). This latter report seems similar to the SI-induced events, which involve the actin cytoskeleton (Snowman et al., 2002; Wilkins et al., 2014).

We, therefore, expected to observe alterations in the vacuole during the SI response and initially thought that vacuolar breakdown might be responsible for the dramatic acidification of the cytosol. Surprisingly, before the study by Fendrych et al. (2014) and this study, this does not seem to have been investigated. Although vacuolar breakdown occurs during SI, to our surprise, significant cytosolic acidification occurred very early and before observable vacuolar breakdown. Moreover, alterations in [pH]cyt can mediate vacuolar breakdown. This suggests that the initial acidification of the cytosol may not be caused by loss of vacuolar integrity. Thus, although the vacuole is expected to contribute to SI-induced acidification of the cytosol, a drop in [pH]cyt initiates events. Our findings confirm those in Fendrych et al., 2014, which recently showed that the [pH]cyt of the root cap...
cells dropped before vacuolar collapse, suggesting that PCD in these two systems is pH activated. This merits examination in additional PCD systems to establish if acidification is a general phenomenon and how it is achieved.

Signaling to SI-Induced PCD

Ca\(^{2+}\) operates as a second messenger in many signaling cascades in both plant and animal systems, including PCD (Rudd and Franklin-Tong, 1999). [Ca\(^{2+}\)]\(_{\text{cyt}}\) is known to play a pivotal signaling role in incompatible field poppy pollen; increased [Ca\(^{2+}\)]\(_{\text{cyt}}\) is the earliest event triggered by SI (Franklin-Tong et al., 1993; Wu et al., 2011) that involves Ca\(^{2+}\) influx (Wu et al., 2011). Vacular collapse in several PCD systems requires increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\), artificially inducing Ca\(^{2+}\) influx can trigger the collapse of the vacuole in cells competent to undergo PCD (Jones, 2001). Here, we have shown that A23187 can trigger both cytosolic acidification and vacuolar breakdown. [Ca\(^{2+}\)]\(_{\text{cyt}}\) is known to play a pivotal signaling role in incompatible field poppy pollen. The SI signaling events can be classified into two categories. Signal initiation events include increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Franklin-Tong and Franklin, 1993; Franklin-Tong et al., 1997), Ca\(^{2+}\) and K\(^+\) influx (Wu et al., 2011), inhibition of sPPase activity (Rudd et al., 1996; de Graaf et al., 2006), depolymerization and stabilization of F-actin (Snowman et al., 2002; Thomas et al., 2006; Poulter et al., 2008, 2011), and rapid inhibition of incompatible pollen tube growth. These feed into later suicide signaling events involved in commitment to PCD through the activation of caspase-3-like/DEVDase activities. Events include activation of the p56 MAPK (Rudd et al., 2003; Li et al., 2007), increases in ROS (Wilkins et al., 2011), and formation of F-actin foci (Poulter et al., 2010, 2011; Wilkins et al., 2011; for review, see Wilkins et al., 2014). Collectively, these signals trigger suicide signaling events leading to a gateway, around 10 min, that incompatible pollen must pass to become irreversibly inhibited and killed. We have previously shown that PrsS can be applied and washed out and that the response of the initial Ca\(^{2+}\) influx can inhibit or activate enzyme activity and that the configuration of the F-actin cytoskeleton can be altered by a drop in pH. Our data provide important information about some of the early events that are affected and instrumental in pushing a cell into a PCD pathway and irreversible death. Below, we discuss some of the targets for the dramatic SI-induced reduction in [pH]\(_{\text{cyt}}\).

We found that the activity of a key enzyme required for cellular biosynthesis and growth, an sPPase p26.1a/p26.1b, was drastically inhibited at pH 6.0 (achieved within 30 min of SI induction) and virtually completely inhibited at pH 5.5. We previously identified this protein as a target for SI signals, being rapidly modified by phosphorylation and inhibited by increases in Ca\(^{2+}\). Here, we have identified a third SI mechanism whereby this sPPase can be inhibited, namely pH. Although we have only measured the effect of a drop in [pH]\(_{\text{cyt}}\) on the activity of just one enzyme, it is likely that not just sPPase activity is affected by the SI-induced acidification. Many enzymes operate within a narrow pH range, close to the physiological norm of an approximate pH of 7; not surprisingly, they will be inhibited if the [pH]\(_{\text{cyt}}\) is shifted so dramatically. This very obvious event has not previously been widely examined or discussed in the context of PCD.

Another target of SI-mediated PCD is the actin cytoskeleton, which undergoes rapid depolymerization (Snowman et al., 2002) and then, accumulation and stabilization into punctate actin foci (Poulter et al., 2010); both of these processes seem to be intimately involved in PCD (Thomas et al., 2006). The SI-induced F-actin foci are unusually stable and a hallmark feature of SI PCD (Poulter et al., 2010). Reconfiguration of the actin
cytoskeleton is modulated by ABPs, and we previously showed that, within 10 min of SI induction, ABPs ADF/ coflin and CAP colocalized with these highly stable F-actin foci (Poulter et al., 2010). It is rather surprising to observe ADF and CAP associated with the stable foci, because they are well-characterized key mediators of actin filament depolymerization (Staiger et al., 2010). However, numerous in vitro studies have shown that ABPs exhibit pH-sensitive activity. In general, ADF binding alters actin dynamics by severing actin filaments, providing more ends for polymerization, and increasing the rate of dissociation of actin monomer from the pointed ends (Carlier et al., 1997; Bamberg et al., 1999; Maciver and Hussey, 2002). The activity of most ADFs, including those in plants, is pH sensitive, with preferential binding of F-actin at pH 6.0 to pH 6.5 and G-actin above pH 7.4 (Carlier et al., 1997; Gungabissoon et al., 1998; Allwood et al., 2002). In vitro studies showed that maize (Zea mays) ZmADF3 is pH sensitive, cosedimenting with F-actin at pH 6 (Gungabissoon et al., 1998). The activity of LIADF1 from L. longiflorum pollen is pH sensitive; at pH 6, LIADF1 was predominantly in the pellet bound to F-actin, indicating low actin-depolymerizing activity, and tobacco NtADF1 depolymerized F-actin in vitro more efficiently at pH 8 than at pH 6 (Chen et al., 2002). ADF-depolymerizing activity increased at more alkaline pHs (Allwood et al., 2002; Chen et al., 2002). A hypothesis proposed is that, in a physiologically relevant, cellular context, local spatial and temporal alkalinization in subapical regions might render ADF more active, resulting in increased actin polymerization and growth (Gungabissoon et al., 1998). However, unexpectedly, GFP-NtADF1 has been observed to colocalize with actin filament bundles in pollen tubes; overexpression of GFP-NtADF1 caused bundles or patches of F-actin and resulted in inhibited growth (Chen et al., 2002). It was proposed that local H+ gradients in the pollen tube apex were likely to affect its actin-depolymerizing activity, contributing to actin remodeling, and that this regulation of the stability and organization of this ADF-rich actin mesh in elongating pollen tubes might play a role in regulating both growth rate and growth orientation. This was experimentally examined by altering the [pH]cyt of L. formosanum pollen tubes (Lovly-Wheeler et al., 2006); data suggested that intracellular pH might act as a regulator of oscillatory pollen tube growth by acting on ADF activity.

Here, we show large acidification of [pH]cyt in pollen tubes undergoing SI. Our observation that ADF decorates F-actin foci in SI-induced and artificially acidified pollen provides in vivo evidence that the actin-depolymerizing activity of ADF is altered by SI-induced [pH]cyt alterations. Our data are consistent with the idea that SI-induced acidification alters ADF activity, so that it has considerably reduced actin-depolymerizing activity and instead, decorates F-actin. This provides a good explanation for the extraordinary stability of these F-actin structures, which are resistant to 1 μM latrunculin B, whereas F-actin in growing pollen tubes is completely destroyed by this treatment (Poulter et al., 2010). Thus, in the context of SI, our data are consistent with the idea that ADF plays a pivotal role in the formation and stabilization of the SI-induced actin foci caused by acidified [pH]cyt. The timing of this fit very nicely with the start of the major shift in pH measured at 30 and 60 min. We propose that this may play an important role in making SI irreversible. Exactly how F-actin aggregation is mediated to allow the formation of the distinctive SI-induced F-actin foci is currently not known. However, it is clear that ADF (perhaps in association with other ABPs) plays an important role in modulating this event through a pH-regulated alteration to its usual activity in growing pollen tubes.

The other ABP associated with the SI-induced punctate foci is CAP. CAP sequesters actin monomers, prevents them from polymerization in vitro, and also, promotes the severing of actin filaments in association with ADF (Barrero et al., 2002; Chaudhry et al., 2007; Deeks et al., 2007; Ono, 2013). Plant CAP is likely to be a key regulator of actin dynamics using a mechanism unique to plants (Staiger et al., 2010; Ono, 2013). The loss-of-function cap1 mutant in Arabidopsis has major defects in pollen germination and tube growth, with altered actin configuration consistent with a major role for CAP in regulating actin dynamics (Deeks et al., 2007). Regarding a role for pH in regulating plant CAP activity, to our knowledge, there are no data. Animal ADF/cofilins efficiently sever actin filaments at basic pH but not neutral or acidic pHs (Yonezawa et al., 1985; Hawkins et al., 1993; Hayden et al., 1993). This might hint that, in SI-induced pollen, acidification might prevent CAP’s actin severing activity. Confusingly, a recent study has shown that, in Listeria monocytogenes, CAP alone can sever actin filaments at an acidic pH but not a neutral pH, but together, CAP1 and ADF/cofilin promote severing of actin filaments within a wide pH range (Normoyle and Brieher, 2012). This seems to be an unlikely scenario in the pollen SI system, where we see both CAP and ADF associated with stable F-actin structures under conditions of low [pH]cyt. This study suggests that CAP monomer binding and actin severing activity are reduced under acidic conditions. However, interestingly, in L. monocytogenes, it has recently been suggested that, at acidic pHs, CAP’s actin severing factor may serve to produce filament ends that could seed actin assembly reactions (Normoyle and Brieher, 2012). This observation suggests a potential role for CAP under SI conditions: as the cytosol acidifies, CAP might be important in producing filament ends to seed actin assembly by ADF under acidic conditions. This will be something to test in the future.

We have shown here that shifting the [pH]cyt is both necessary and sufficient for triggering PCD. This has also been recently reported by Fendrych et al. (2014). Our finding that alterations in [pH]cyt also trigger alterations in actin, ADF, and CAP localization is unique and places these in a unique, biologically relevant scenario involving PCD in pollen. It is of interest that overexpression of wild-type CAP1 in several animal cells stimulated cofilin/ADF-induced apoptosis (Wang et al., 2008). Although it is relatively well established...
that actin can act as both a sensor and a mediator of cell death, translating stress signals into alterations in actin polymerization status (Franklin-Tong and Gourlay, 2008; Desouza et al., 2012), relatively few studies have examined this in plant cells with respect to a mechanistic understanding. Moreover, the occurrence of F-actin foci has not been reported in many systems. They seem to be similar to Hirano bodies found in animal cells undergoing neurodegenerative diseases or cellular stresses, which are associated with ADF/cofilin (Bamburg and Wiggan, 2002). In Brewer’s yeast (Saccharomyces cerevisiae), CAP (Srv2p in yeast) localizes to actin patches (Lila and Drubin, 1997) and is required for the formation of F-actin bodies (Gourlay et al., 2004) in stressed, quiescent Brewer’s yeast (Sagot et al., 2006). Several plant systems have been observed to exhibit F-actin reorganization during PCD, and it is thought that the polymeric status of actin acts as a dynamic, integrated feedback mechanism for the health status of a cell, with PCD signaling major alterations (Smertenko and Franklin-Tong, 2011). Drawing comparisons with a study providing a link between actin alterations and signaling to apoptosis in lymphocytes (Hao and August, 2005), it has been suggested that a similar scenario may operate in plant cells and that alterations to the plant actin cytoskeleton could potentially signal directly to PCD (Tian et al., 2009).

However, mechanistically, we have no idea of what might be involved. It would be of considerable interest to examine other plant systems undergoing PCD for alterations in [pH]cyt, actin, ADF, and CAP organization to see if this is a more general phenomenon.

**MATERIALS AND METHODS**

**Growth of Pollen Tubes and Treatments**

Field poppy (Papaver rhoas) pollen was hydrated and grown at 10 mg mL$^{-1}$ in liquid germination medium [GM; 13.5% (w/v) Suc, 0.01% (w/v) H$_3$BO$_3$, 0.01% (w/v) KNO$_3$, and 0.01% (w/v) Mg(NO$_3$)$_2\cdot$6H$_2$O] for at least 60 min before the addition of any treatments, which has been previously described in Snowman et al. (2002). For SI treatments, recombinant PrsS proteins were produced by cloning the nucleotide sequences specifying the mature peptide (Franklin-Tong and Gourlay, 2007). Pollen tubes were loaded with 1 μM BCECF-free acid (Invitrogen) in a 1:1 mixture of GM and 0.01% (w/v) KNO$_3$, and 0.01% (w/v) Mg(NO$_3$)$_2\cdot$6H$_2$O for at least 60 min.

**Measurement of [pH]$_{cyt}$ of Pollen Tubes**

Intracellular [pH]$_{cyt}$ was monitored in living pollen tubes with AM of the pH-sensitive fluorophore BCECF (Invitrogen) as described by Bosch and Franklin-Tong (2007). Pollen tubes were loaded with 1 μM BCECF for 3.5 min followed by a wash with GM. Pollen tubes were only imaged within 5 to 10 min after the addition of BCECF because this time frame allowed accurate reporting of [pH]$_{cyt}$. Samples could not be used after this 10-min period because of dye sequestration by organelle compartments. Images were taken sequentially using a Leica DMIRE2 Confocal Microscope under the following microscope settings: pH-dependent wavelength, excitation at 488 nm and 7% power; and pH-independent wavelength, excitation at 458 nm and 12% power. Emission was collected at 510 to 550 nm for both images. Using ImageJ software, a 50 × 50-pixel box was used to measure the mean intensity of the area of each pair of images (488 and 458 nm). A measurement was taken from each pollen tube image (488- and 458-nm images) at the same position on each image. Four pairs of measurements were collected for each tube. Each pair of measurements was used to create a ratio (pH-dependent/pH-independent ratio of 488:458 nm). The mean ratio values of each pollen tube were then used to determine the [pH]$_{cyt}$ of the pollen tube using a reference calibration curve with a pseudocyttoplasm calibration set.

A calibration curve was carried out for each individual imaging session. In vitro calibration was performed using 40 μM BCECF-free acid (Invitrogen) in a pseudocytosol (100 mM KCl, 10 mM NaCl, 1 mM MgSO$_4$, 10 mM MES, and 10 mM HEPES adjusted to desired pH). Images were taken sequentially using the same microscope settings as those used for in vitro pollen tube measurements (488 and 458 nm). A pair of images was taken at each of the following pH values to create a calibration curve: pH 5.5, pH 6, pH 6.5, pH 7, pH 7.5, and pH 8. Using ImageJ software, intensities were measured for each pair of images, and a ratio value was calculated. These ratios were plotted to give a calibration curve to pH ratio, which was used to calculate the pH of individual pollen tubes after various treatments. Statistical analysis (Wilcoxon rank sum test) was carried out; where statistical comparisons are made, they are between samples at the same time point.

**Visualisation of F-Actin and ABPs in Field Poppy Pollen Tubes**

Field poppy pollen tubes were fixed with 400 μM 3,3-diaminobenzocic acid N-hydroxysuccinimide ester (Pierce; 10 μM stock in dimethyl sulfoxide) for 6 min at room temperature followed by 2% (w/v) formaldehyde freshly prepared from paraformaldehyde for 1 h at 4°C. Pollen was collected, and supernatant was removed. The pollen pellet was washed three times in 1× Tris-buffered saline (TBS; pH 7.6) and resuspended in 100 μL of TBS. Actin was stained using 66 nm rhodamine phalloidin, which only binds F-actin and not G-actin. The fixed pollen samples were incubated with rhodamine phalloidin overnight at 4°C. For quantification, pollen F-actin was assessed as described by Pouiller et al. (2010). For each of seven independent experiments, 50 pollen tubes were scored for actin configuration for each treatment; 100 pollen tubes were scored for each treatment in another three experiments, and in total, 3,800 pollen tubes were scored per treatment. Statistical analysis was carried out using ANOVA. For ABP colocalization experiments, pollen tubes were stabilized, fixed, and subsequently, incubated in 0.05% (w/v) celllose/0.05% (w/v) macrozyme with 0.1% (w/v) Triton X-100 in MES buffer containing 0.1 mM phenylmethylsulfonyl fluoride and 1% (w/v) bovine serum albumin for 15 min. Cells were washed in MES and then TBS, and they were incubated in 1% (w/v) bovine serum albumin in TBS for 30 min. Samples were incubated with either rabbit anti-AICAPI or rabbit anti-LADP primary antibody at 1:500 at 4°C. After TBS washes, pollen was then incubated with the secondary antibody goat anti-rabbit IgG fluoro-rescein isothiocyanate (1:300; Sigma-Aldrich) and 66 nm rhodamine phalloidin for 1.5 h. In total, 700 pollen tubes were scored for actin foci and ABP colocalization over three independent experiments. Colocalization of actin foci with the ABPs was assessed in overlaid confocal images by manually identifying the actin foci in a region of interest in the red channel and then, scoring for the presence or absence of the ABP in the region of interest in the green channel. Results are reported as percentages of pollen tubes with foci that colocalized with the ABP.

**Caspase Activity Assays**

Caspase-3-like/DEVDase activity in living, growing pollen tubes was visualized with the cell permeant ImageIT Live Caspase 3 & 7 Detection Kit (Invitrogen). Pregrown pollen was incubated with the 0.1X solution of FAM-DEVD-FMK FLICA regent in GM for 60 min. Pollen was subsequently washed in GM and imaged using an epifluorescence microscope using fluorescein isothiocyanate filters. The level of fluorescence in pollen tubes indicated the presence and level of caspase-3 and caspase-7 activity in the cell. Only pollen tubes scored as pollen grains display strong autofluorescence; 50 pollen tubes were scored for each treatment, and three independent replicates were performed. Student’s t test was used for statistical analysis.
Pyrophosphatase Assays on Recombinant p26.1a and p26.1b at Different pHs

Activity assays for sPPase activity at different pH were performed using recombinant Pr-p26.1a and Pr-p26.1b proteins. The p26.1a and p26.1b constructs with an N-terminal His-Tag were transfected into Escherichia coli (BL21 strain), and expression was induced with 1 mM isopropyl-β-D-thiogalactoside and purified using Ni-agarose following the manufacturer’s protocol (QIAGEN). To determine pyrophosphatase (sPPase) activity, a standard curve was constructed using different concentrations of NaH2PO4. For the assays, recombinant p26.1a and p26.1b were diluted to 10 μM into basic medium (50 mM HEPES KOH, pH 8.0, 50 μM EGTA, 2 mM MgCl2 supplemented with 2 mM Mg++, and 50 μM EGTA). To determine activity at different pHs, the buffer pH was adjusted before assaying. Fiske-Subbarow reagent was added to both standard curve solution and enzyme assay solution and kept at room temperature. The absorbance of each sample was read at optical density at 691 nm, and activities were calculated from the standard curve using known concentrations of NaH2PO4.

Vacular Labeling

Carboxy-DCFDA (Invitrogen) was used for vacular visualization. Pollen tubes were labeled with 1 μM carboxy-DCFDA (Invitrogen) for between 15 and 30 min and protected from light. Samples were washed in GM and imaged using a Leica DM IRE2 Confocal Microscope exciting with 488-nm lasers, and 30 min and protected from light. Samples were washed in GM and imaged using confocal microscopy as previously described.

ACS kinase assayed using tungsten particles (1.3 μm) performed using the helium-driven PDS-1000/He Biolistic System (Bio-Rad) aged using a Leica DM IRE2 Confocal Microscope exciting with 488-nm lasers, and 30 min and protected from light. Samples were washed in GM and imaged using confocal microscopy as previously described.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Calibration of the [pH]cyt of field poppy pollen tube with the pH indicator BCECF AM.

Supplemental Figure S2. Cartoon showing a model of the integrated SI PCD signaling network in field poppy pollen.

Supplemental Figure S3. Ratiometric imaging of BCECF-labeled, SI-induced pollen tubes reveals cytosolic acidification.

Supplemental Figure S4. Acidification of the pollen tube cytosol triggers the formation of punctate F-actin foci and colocalization of CAP and ADF.

Supplemental Figure S5. Quantification of SI-induced alterations in vacuolar morphology.

Supplemental Figure S6. Artificial manipulation of [pH]cyt of pollen tube triggers alterations in vacuolar organization.

Supplemental Movie S1. Visualization of typical vacuolar movement in a field poppy pollen tube vacuole labeled with carboxy-DCFDA.

Supplemental Movie S2. Visualization of vacuolar alterations after SI in a field poppy pollen tube vacuole using 6-TIP-GFP.

ACKNOWLEDGMENTS

We thank Natasha Raikhel for providing the 6-TIP construct, Patrick Hussey for the LlADF antibody, and Chris Staiger for the CAP antibody. We also thank Natasha Raikhel for providing the LAT52–TIP construct (a gift from Natasha Raikhel) and cloned before assaying. Fiske-Subbarow reagent was added to both standard curve solution and enzyme assay solution and kept at room temperature. The absorbance of each sample was read at optical density at 691 nm, and activities were calculated from the standard curve using known concentrations of NaH2PO4.

SI triggers Cytosolic Acidification


