Regulation of the Pollen-Specific Actin-Detoplymerizing Factor LlADF1


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Pollen tube growth is dependent on a dynamic actin cytoskeleton, suggesting that actin-regulating proteins are involved. We have examined the regulation of the lily pollen-specific actin-depolymerizing factor (ADF) LlADF1. Its actin binding and depolymerizing activity is pH sensitive, inhibited by certain phosphoinositides, but not controlled by phosphorylation. Compared with its F-actin binding properties, its low activity in depolymerization assays has been used to explain why pollen ADF decorates F-actin in pollen grains. This low activity is incompatible with a role in increasing actin dynamics necessary to promote pollen tube growth. We have identified a plant homolog of actin-interacting protein, AIP1, which enhances the depolymerization of F-actin in the presence of LlADF1 by ~60%. Both pollen ADF and pollen AIP1 bind F-actin in pollen grains but are mainly cytoplasmic in pollen tubes. Our results suggest that together these proteins remodel actin filaments as pollen grains enter and exit dormancy.

INTRODUCTION

The development of pollen grains and their subsequent germination involves some of the most dramatic reorganizations of actin in higher plants. In Narcissus, for example, pollen grains developing in the anthers have a cytoplasmic actin network that breaks down into aggregates and rodlets of actin as the pollen grain dehydrates and enters dormancy. Provided that a suitable environment is encountered, as when the pollen grains alight onto the stigma, for example, the pollen exits dormancy and germinates, and a pollen tube is initiated. Pollen germination is accompanied by the replacement of the aggregates of actin by a filamentous network that converges on the emerging pollen tube and eventually ramifies the pollen tube (Heslop-Harrison and Heslop-Harrison, 1992). The pollen tubes extend by tip growth, which involves the actin network directing vesicle traffic to the tip, where new membrane components are added (Heslop-Harrison and Heslop-Harrison, 1989). These changes in the organization of the cytoskeleton must involve actin-remodeling proteins, and these proteins must be able to respond to environmental stimuli.

The actin-remodeling protein actin-depolymerizing factor (ADF), or cofilin, is involved in these actin reorganizations (Smertenko et al., 2001). In general, the ADF/cofilin group of proteins bind G- and F-actin and increase actin dynamics. They do this by severing actin filaments, thereby providing more ends for polymerization, and increasing the rate of dissociation of actin monomer from the pointed ends (Carlier, 1998; Maciver, 1998; Bamburg, 1999; Chen et al., 2000; Yeoh et al., 2002). There are a variety of mechanisms by which ADF/cofilin activity is regulated. (1) Phosphorylation of a Ser near the N terminus considerably reduces the ability of ADF/cofilin to depolymerize actin filaments. In humans, the kinases responsible are LIM kinases 1 and 2 (Arber et al., 1998; Yang et al., 1998; Bamburg, 1999) and TESK 1 and 2 (Toshima et al., 2001a, 2001b); in maize, a calmodulin-like domain protein kinase (CDPK) is responsible, indicating that maize ADF is controlled indirectly by Ca^{2+} (Smertenko et al., 1998; Allwood et al., 2001). The only ADF/cofilins identified to date that are not controlled by phosphorylation are those from Saccharomyces cerevisiae and Dictyostelium discoideum (Bamburg, 1999). (2) All ADF/cofilins, with the exception of Acanthamoeba actophorin (Maciver et al., 1998) and starfish depactin (Mabuchi, 1983), display pH-sensitive
activity, with ADF/cofilin preferentially binding F-actin at pH 6.0 and G-actin above pH 7.4 (Yonezawa et al., 1985; Hawkins et al., 1993; Hayden et al., 1993; Carlier et al., 1997; Gungabissoon et al., 1998; Yeoh et al., 2002). (3) Many ADF/cofilins are inhibited by the specific phosphoinositides phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and to a lesser extent phosphatidylinositol 4-phosphate (PIP) (Yonezawa et al., 1980; Quirk et al., 1993; Gungabissoon et al., 1998). In addition, ADF/cofilins reciprocally inhibit the breakdown of PIP$_2$ by phospholipase C (Yonezawa et al., 1991; Gungabissoon et al., 1998). PIP$_2$ has long been known as a precursor to the second messengers inositol 1,4,5-triphosphate and diacylglycerol. However, it is now also widely recognized as an important regulator of several signal transduction processes in addition to second messenger production (Takenawa and Itoh, 2001). So although PIP$_2$ may help localize ADF/cofilins to membranes where they affect actin filament dynamics, the ADF/cofilins also may work to modulate PIP$_2$ metabolism.

In addition to the controls of activity described above, it has been found that a WD-repeat protein also can modulate ADF. This protein is actin-interacting protein1 (AIP1), which was first identified in yeast two-hybrid screens using actin as a bait (Amberg et al., 1995). This WD-repeat protein has been identified by genetic screens for extragenic suppressors of yeast cofilin mutants, by two-hybrid screens using ADF/cofilin as a bait, and by affinity purification of ADF-interactors of yeast cofilin mutants, by two-hybrid screens using ADF as a bait (Iida and Yahara, 1999; Rodal et al., 1999). AIP1 has been shown to bind to and depolymerize actin filaments weakly in vitro, but in the presence of ADF, it greatly increases depolymerization. Similarly, AIP1 is unable to sever actin filaments itself, but in the presence of ADF, actin severing is increased markedly (Aizawa et al., 1994). AIP1 has been shown to bind to and depolymerize ADF in vivo. For example, AIP1 and ADF colocalize in the cleavage furrow of the first division cycle of *X. laevis* (Okada et al., 1999). Moreover, AIP1 is an important regulator of actin filament organization in *Caenorhabditis elegans* (Ono, 2001), and yeast mutants in which AIP1 is not present show colocalization of ADF to actin cables rather than just to cortical actin patches (Rodal et al., 1999).

The potential role of a pollen-specific ADF/cofilin in the actin reorganization that occurs as pollen grains enter and exit dormancy has been described (Smertenko et al., 2001). Pollen ADF binds the actin array in maturing pollen grains and is proposed to break down this array into actin/ADF rosettes, which may be the storage form of actin in dehydrated pollen in readiness for pollen germination. As the pollen grains germinate, the ADF apparently is released from the actin and the new actin array is formed (Smertenko et al., 2001). A pollen-specific ADF has been shown to bind G- and F-actin and to increase actin dynamics, but at considerably lower rates than an ADF expressed solely in vegetative tissues (Gungabissoon et al., 1998; Smertenko et al., 2001), which was more similar to human ADF in this respect (Smertenko et al., 1998). These data suggest that pollen ADF decorates actin arrays because it has a high affinity for filaments but inefficient depolymerizing activity. However, the release of pollen ADF from F-actin aggregates on pollen germination implies that its depolymerizing activity is increased (Smertenko et al., 2001). Because the extension of the pollen tube is dependent on a dynamic actin cytoskeleton, we wondered how an otherwise weak pollen ADF in vitro could be activated in vivo. Here, we examine the regulation of LIADF1 by pH, phosphoinositides, phosphorylation, and another protein, AIP1.

## RESULTS

### Pollen ADFs Have Conserved Sequences

Previously, four pollen-specific ADFs were identified, two from maize (ZmADF1 and ZmADF2), one from lily (LIADF1), and one from *Brassica napus* (BnADF) (Kim et al., 1993; Chung et al., 1995; Rozycka et al., 1995; Lopez et al., 1996; Smertenko et al., 2001). In previous articles, we have shown that these four pollen ADFs show a greater degree of identity with each other than with the maize vegetative tissue ADF, ZmADF3, suggesting a common role for an evolutionarily conserved ADF in pollen development and function (Maciver and Hussey, 2002). Here, we show that polyclonal antiserum raised against maize or lily pollen ADFs identifies only the pollen ADFs on protein gel blots (Figures 1A and 1B, respectively), whereas antiserum raised against ZmADF3 identifies ADF in all maize tissues examined with the exception of pollen. These results demonstrate structural distinctions between the pollen ADFs and the maize and lily vegetative ADFs as well as differential tissue localization. Moreover, in a phylogenetic analysis of all known plant ADFs (Maciver and Hussey, 2002), the four pollen ADFs fall in one clade, and no ADFs that are known to be expressed in vegetative tissues (i.e., ZmADF3, AtADF5, AtADF6, and PeADF) fall within this group (Mun et al., 2000; Dong et al., 2001a; Maciver and Hussey, 2002).

### LIADF1 Is Not Regulated by Phosphorylation

To determine the biochemical characteristics of a pollen ADF, we used the lily pollen ADF, LIADF1, because it produces stable, soluble recombinant protein when expressed in, and purified from, bacteria (Smertenko et al., 2001). We first assessed whether LIADF1 could be phosphorylated by plant kinases and therefore whether LIADF1 was likely to be controlled by this mechanism. LIADF1 has the conserved
Ser at position 6, which has been shown to be phosphorylated in ZmADF3. The CDPK known to phosphorylate ZmADF3 (Allwood et al., 2001) does not phosphorylate LlADF1 in in vitro phosphorylation assays, as shown in Figure 2A. To determine whether another kinase could phosphorylate this pollen ADF, we performed in-gel phosphorylation assays. In these assays, maize pollen, maize leaf, lily pollen, and lily leaf extracts all were shown to contain at least one protein kinase that was capable of phosphorylating ZmADF3, but neither homologous nor heterologous extracts contained a kinase that was capable of phosphorylating LlADF1 (Figure 2B). We conclude that lily pollen ADF is not controlled by reversible phosphorylation.

LIADF1 Is Regulated by pH

The activity of plant ADFs is affected by pH (Carlier et al., 1997; Gungabissoon et al., 1998). To determine whether this is the case for the lily pollen ADF as well, pure recombinant LIADF1 was incubated for 20 min at room temperature with F-actin over a range of pH values and centrifuged at 365,000g to pellet the actin. The amount of ADF that remained bound to F-actin and therefore was found in the pellet was determined by SDS-PAGE and densitometry and plotted graphically (Figure 3). As with the ZmADF3 control, LIADF1 was more able to depolymerize F-actin at higher pH, releasing monomeric actin and ADF itself into the supernatant. At lower pH, a greater proportion of the LIADF1 remained in the pellet bound to the F-actin, indicating that at these pH values, the actin-depolymerizing activity of LIADF1 was low (Figure 3), although the effect was not as great as with ZmADF3. These data show that LIADF1 has pH-sensitive activity.

LIADF1 Is Inhibited by Phospholipids

Members of the ADF/cofilin group, including ADFs from plants, are known to bind specific phospholipids, and this interaction inhibits F-actin binding. Moreover, the presence of PIP2 in membranes at the tips of pollen tubes (Dong et al., 2001b) is consistent with a regulatory role of the phosphoinositide signal transduction pathway in ADF activity and hence actin dynamics. To test for specific phosphoinositide binding, LIADF1 was preincubated with a range of phospholipids before being run on a nondenaturing gel (Figure 4A). In the presence of phospholipids that are not able to bind, LIADF1 ran as a single distinct band similar to the control, but preincubation with PIP or PIP2 caused it to form a smear on the gel, indicating binding between the phospholipid and ADF. These results are similar to those observed for ZmADF3. In addition, LIADF1 inhibited the activity of phospholipase C (Figure 4B), confirming that LIADF1 is able to sequester PIP2 in vitro. Incubation of LIADF1 with either phosphatidylcholine or varying amounts of PIP2 before performing a cosedimentation assay showed that PIP2 inhibited the binding of LIADF1 to F-actin and that this inhibition increased between a 0- and 50-fold molar excess of PIP2 over LIADF1. By contrast, a 50-fold molar excess of phosphatidylcholine had no effect on the binding of LIADF1 to F-actin (Figure 4C). These data indicate that LIADF1 activity is inhibited by PIP2.
Identification of the Plant Homolog of Yeast AIP1p

So far, we have established that although the lily pollen ADF does not appear to be regulated by phosphorylation, it can be regulated by pH and may be inhibited by phospholipids. However, neither of these methods of regulation can explain how the relatively low levels of pollen ADF activity observed in vitro could account for the high actin turnover that would be required to extend a pollen tube if, as postulated for root hairs, active ADF is essential for tip growth (Jiang et al., 1997; Smertenko et al., 2001). Therefore, it seems likely that another method of regulating the pollen ADF occurs in vivo. One regulatory protein that enhances ADF activity is the WD-repeat protein AIP1. Screening of the Arabidopsis databases revealed a large number of WD-repeat proteins. The sequence that showed the highest identity with *X. laevis* AIP1 was isolated from an Arabidopsis flower cDNA library. This Arabidopsis sequence showed 34 and 31% identity with *X. laevis* and yeast AIP1, respectively. To determine whether this protein was AIP1 like, we performed two-hybrid assays in which the putative AIP1, an actin, and an ADF were inserted into both the activation and the DNA binding domains of yeast two-hybrid vectors, and the various combinations were cotransformed into yeast. The results show that the Arabidopsis AIP1-like protein was able to interact with actin and ADF but not with itself (Figure 5A). Moreover, the WD-repeat structure was essential, because deletion of the seventh of the nine WD repeats to generate the mutant AIPΔ7 abolished any interaction with ADF or actin (Figure 5A). These results confirm that the WD-repeat protein cloned from the Arabidopsis cDNA library has AIP1-like activity.

**Reproductive and Vegetative/Constitutively Expressed AIP1 Genes**

Screening of the completed Arabidopsis genome sequence revealed only one other AIP1-like gene, AtAIP1-2.

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**Figure 2.** LlADF1 Is Not Phosphorylated.

(A) In vitro phosphorylation assays of recombinant ADF by CDPK. Autoradiograph and corresponding colloidal silver-stained gel of assays using ZmADF3 or LlADF1 in the presence of CDPK are shown. (B) In-gel phosphorylation assays using either ZmADF3 or LlADF1 incorporated into the gel. Colloidal silver-stained blots showing equal loading of the various extracts onto the gel and corresponding autoradiographs are shown.

**Figure 3.** Effect of pH on LlADF1 Binding to F-Actin.

F-actin and LlADF1 (diamonds) or ZmADF3 (squares) cosedimentation assays were performed in the pH range 6.0 to 9.0. The percentage of ADF bound to F-actin and found in the pellet is plotted.
The two sequences share 67% identity with each other at the amino acid level. A phylogenetic tree was generated of all of the known AIP1 sequences (Figure 5B). Interestingly, the sequences cluster according to their taxonomic groups. Moreover, to date, no more than one AIP1 homolog per organism has been found in the databases, with the exception of the nematode *C. elegans* and the plant *Arabidopsis*, for which two forms are found. Plant actin, profilin, and ADF isoforms can be divided into those expressed in reproductive tissues and those expressed constitutively and/or in vegetative tissues (Christensen et al., 1996; Huang et al., 1996; Lopez et al., 1996; Kandasamy and Meagher, 1999). RNA gel blot analysis showed that the AtAIP1-1 transcript was present in floral tissues but not in vegetative tissues, whereas the AtAIP1-2 transcript was found in all tissues examined (Figure 5C). Thus, there are reproductive and vegetative/constitutively expressed AIP1 genes in plants.

**AtAIP1-1 Enhances the F-Actin Depolymerization Activity of LIADF1**

The AtAIP1-1 cDNA was expressed in *Escherichia coli*, and the recombinant protein was purified. The effect of AtAIP1-1 and LIADF1 on F-actin was investigated using cosedimentation assays (Figure 6A). As expected, the bulk of the F-actin control was found in the pellet, whereas only small amounts of ADF and AIP1-1 were seen in the pellets. When F-actin was mixed with AtAIP1 or LIADF1, there was a small increase in the amount of soluble actin in the supernatant, indicating that both the AIP1 and the ADF can solubilize F-actin. However, incubation of F-actin with both LIADF1 and AtAIP1-1 resulted in a massive 60% increase in the amount of actin found in the supernatant (Figure 6B), indicating that the AIP and the ADF cooperated to depolymerize the F-actin. This finding strongly supports the hypothesis that AIP1 may cooperate with ADF to increase actin dynamics in vivo.

![Figure 4](image-url)  
*Figure 4. Regulation of Pollen ADF by Phospholipids.*  
(A) Native gels of either LIADF1 or ZmADF3 mixed with a 50-fold molar excess of purified phospholipids. (–)PL, control without phospholipid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; IP3, inositol 1,4,5-triphosphate; OAG, 1-oleoyl-2-acetylglycerol.

(B) Effect of LIADF1 on plant phospholipase C activity. The activity of plant phospholipase C was measured in the presence of increasing concentrations (0 to 12.5 μM) of LIADF1 (diamonds) or ZmADF3 (squares).

(C) Inhibition of LIADF1 binding to F-actin by PIP2. F-actin and LIADF1 cosedimentation assays were performed in the presence of a 50-fold molar excess of phosphatidylethanolamine (PC) as a control and a 50- to 0-fold molar excess of PIP2. The percentage of LIADF1 remaining in the supernatant is plotted. The data shown are means of three replicate experiments, and the bars represent standard errors.
AIP1 and ADF Are Located Similarly in Pollen Grains and Pollen Tubes

For AIP1 to cooperate with pollen ADF, it should be expressed in pollen. Polyclonal antiserum raised against the AtAIP1-1 recombinant protein stained a band of ~67 kD on one-dimensional protein gel blots of total protein extracts from Arabidopsis pollen and leaf and lily pollen and leaf (Figure 7A). These data indicate that this antiserum detects both vegetative/constitutively expressed and reproductive AIP1 proteins and cross-reacts with AIPs from other plants.

Figures 7B and 7C show that pollen ADF binds to bundles and aggregates of F-actin in pollen grains, and Figures 7F, 7G, 7J, and 7K show that when pollen grains exit dormancy and germinate, the ADF is located mainly in the cytoplasm. When these pollen grains and pollen tubes are stained with anti-AIP1 antiserum, a similar pattern of staining is observed (Figures 7D, 7E, 7H, 7I, and 7L). We used two fixation protocols (conventional formaldehyde fixation [Figures 7F to 7I] and freeze substitution [Figures 7J to 7L]) for the pollen tubes, and both gave similar results, although the freeze substitution gave a better preservation of actin filaments and pollen tubes (Cresti et al., 1987). This similar localization of pollen AIP1 and ADF, coupled with the observation that AIP1 enhances the activity of ADF, suggests that these proteins may work as a pair and cooperate in vivo.

DISCUSSION

Here, we demonstrate that the binding of actin to the lily pollen ADF, LIADF1, is pH sensitive and is inhibited by the specific phosphoinositides PIP and PIP$_2$. Moreover, LIADF1 cannot be phosphorylated by plant protein kinases, indicating that reversible phosphorylation does not control this pollen ADF. LIADF1 has weak actin-depolymerizing activity compared with human ADF and a plant vegetative ADF, but we show that this is increased massively by the presence of AIP1. Both pollen ADF and pollen AIP1 localize to actin filament bundles in pollen grains but are mainly cytoplasmic in pollen tubes.

The actin binding and depolymerizing activity of LIADF1 is pH sensitive and inhibited by specific phosphoinositides.
Fluctuations in pH have been reported in pollen tubes, with a constitutive alkaline band (pH 7.6) in the subapical region and an acidic region (pH 6.0) at the tip (Feijo et al., 1999). The alkalinization in the subapical domain of the pollen tube could present a microenvironment in which this pollen ADF would be most active in depolymerizing and severing actin filaments to provide seeds and monomer for actin polymerization at the tip and the subsequent pollen tube extension. 

Fu et al. (2001) recently showed that F-actin is present at the tips of pollen tubes and that it is highly dynamic and regulated by Rop GTPase. However, with the tip at pH 6.0, ADF itself is unlikely to be active. Moreover, there is evidence for the presence of PIP$_2$ in the membrane at the tip of the pollen tube (Dong et al., 2001b), and coupled with the acidic microenvironment in this region, this may enhance the inhibition of ADF activity. Because external stimuli are known to lead to changes in local concentrations of PIP$_2$ (Smolenskasym and Kacperska, 1994; Gawer et al., 1999), inhibition of pollen ADF by PIP$_2$ may provide a fundamental link between external stimuli and the regulation of the actin cytoskeleton in this region. Artificially increasing PIP$_2$ in other organisms has been shown to affect the actin cytoskeleton in a way that leads to filament bundles. In CV1 cells (an African green monkey kidney cell culture line), an increase in PIP$_2$ inhibits actin-severing proteins such as gelsolin, ADF/cofilin, capping proteins, and profilin (Witke et al., 1995; Azuma et al., 1998), resulting in longer filaments that are more readily cross-linked to form stress fibers. However, similar experiments with the rat embryo fibroblast cell line REF52 and mouse Swiss 3T3 cells expressing PI(4)P 5-kinase (and therefore having higher levels of PIP$_2$) show diminished stress fibers and the formation of actin comets (Rozelle et al., 2000). It has been suggested that the difference between the responses of these cell lines to increased PIP2 levels reflects the activation of another actin binding protein, WASP. WASP is absent from plant genomes, which suggests that an increase in PIP2 in plants would lead to the formation of more stress fibers.

We have shown that lily pollen ADF cannot be controlled by reversible phosphorylation because LIADF1 cannot be phosphorylated by the CDPK that phosphorylates ZmADF3 or by any kinase in pollen and vegetative extracts from lily or maize. These data indicate that the control of LIADF1 is more like that of fungal and slime mold ADFs in that these also are not regulated by phosphorylation, although the sequence of LIADF1 is more similar to those of other plant ADFs than to those of fungal and slime mold ADFs (Maciver and Hussey, 2002).

We have identified AIP1 in Arabidopsis and cloned the cDNAs corresponding to the two genes, AtAIP1-1 and AtAIP1-2. These show 67% identity with each other at the amino acid level; one is expressed in floral organs, and the other is expressed in all organs examined. We have shown that recombinant AtAIP1-1 increases the depolymerizing activity of LIADF1 on F-actin to an extent considerably greater than the effect of either protein on its own. This raises the possibility that AIP1-1 and ADF cooperate in vivo to promote actin dynamics. For this to happen, they must occupy similar locations within the cell. Using panreactive antibodies raised against recombinant AtAIP1-1, we found that pollen AIP1 and pollen ADF were localized with F-actin bundles in pollen grains but were mainly cytosolic in the pollen tube. We propose that as pollen grains enter dormancy, ADF and AIP1 bind actin filaments into bundles, presumably in cooperation with actin-bundling proteins (Maciver et al., 1991; Gungabissoon et al., 2001). In pollen tubes, on the other hand, actin reorganization and polymerization are essential for pollen tube growth. We propose that in the subapical region of the pollen tube, where alkalinization occurs,
Figure 7. Localization of Pollen AIP1.

Protein gel blots of Arabidopsis and lily pollen and leaf total protein extracts probed with the anti-AtAIP1-1 antibody. Immunofluorescence of Narcissus pollen grains (B to E) and pollen tubes (F to L) stained for actin using rhodamine-conjugated phalloidin (B, D, F, and H) or anti-actin (J), for pollen ADF using anti-LlADF1 (C, G, and K), or for AIP1 using anti-AtAIP1-1 (E, I, and L). Pollen grains and pollen tubes shown in (B) to (I) were fixed directly using formaldehyde, and pollen tubes in (J) to (L) were prepared for fixation using the freeze-substitution method.
the depolymerizing activity of LIADF1 is activated and that this activity is enhanced further by interaction with pollen AIP1-1, making actin monomers or short oligomers available for the assembly of the long filament bundles characteristic of the pollen tubes. The pH of the subapical region in lily pollen tubes is 7.6 (Feijo et al., 1999), which corresponds well with increased LIADF1 activity at this pH in vitro.

We have proposed that the activation of pollen ADF in pollen tubes is by pH and by AIP1-1, but there also may be control of the ADF:AIP1-1 pair through AIP1-1 itself. For example, it is possible that AIP1-1 may be controlled by other factors, such as pH. Interestingly, AIP1 has been shown to interact with cyclase-associated protein in a yeast two-hybrid system (Drees et al., 2001), suggesting that there may be a link between AIP1 and cAMP-activated signaling pathways. Moreover, a pollen-specific protein with adenylate-cyclase activity has been identified, and fluctuations in the levels of cAMP in pollen tubes can change growth and reorientation (Moutinho et al., 2001). The further study of pollen AIP1 may provide exciting insights into the regulation of the actin cytoskeleton in pollen activation, germination, and tube growth.

METHODS

Protein Extraction, Electrophoresis, and Protein Gel Blot Analysis

Protein extracts were prepared, fractionated on one-dimensional gels, and blotted onto nitrocellulose membranes as described by Hussey et al. (1988). The membrane was probed with mouse anti-pollen actin-depolymerizing factor (ADF) antiserum (ZmADF1 or LIADF1), rabbit anti-ZmADF3 diluted 1:1000, or mouse anti-AtAIP1-1 diluted 1:200 in TBST (10 mM Tris, pH 7.4, 140 mM NaCl, and 0.05% Tween 20) buffer including 1% (w/v) fat-free dried milk. Anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Amersham Pharmacia) were used at a dilution of 1:2000 in TBST-milk buffer. Protein bands cross-reacting with anti-ADF or anti-AIP1 were visualized using the enhanced chemiluminescence detection kit (Amersham Pharmacia). After enhanced chemiluminescence detection, nitrocellulose membranes were washed in water and then stained with colloidal silver.

Phylogenetic Tree

The phylogenetic tree was prepared using ClustalW to align sequences (http://www.ebi.ac.uk/clustalw/), followed by TreeView PPC version 1.5.3. (created by Roderic D.M. Page, http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

Protein Phosphorylation

In vitro phosphorylation was performed using partially purified calmodulin-like domain protein kinase, as described by Allwood et al. (2001). In-gel phosphorylation assays were performed as follows. To prepare extracts containing kinases, cells were harvested and homogenized in 50 mM Hapes-KOH, pH 7.6, 2 mM DTT, 2 mM EDTA, 20 mM β-glycerophosphate, 20% (v/v) glycerol, 1 mM Na3VO4, 1 mM NaF, and 50 μg/μL protease inhibitors (complete; Boehringer Mannheim). The extracts were centrifuged at 14,000 g for 20 min, and a 40 to 70% ammonium sulfate fraction was prepared and then desalted using 2-μL Sephadex G25 coarse-spin columns. A 10% SDS-PAGE gel to which 7 μM ADF was added immediately before polymerization was prepared, and the extracts were run through the gel. After electrophoresis, the gel was washed in 25 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 1 mM Na3VO4, 5 mM NaF, 0.5 mg/mL BSA, and 0.1% Triton X-100 with gentle shaking at room temperature for three separate 30 min washes. The gel then was equilibrated with 25 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 1 mM Na3VO4, and 5 mM NaF at 4°C overnight to renature the proteins. After renaturing, the gel was incubated for 60 min at room temperature in reaction buffer but without ATP (25 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 1 mM Na3VO4, 2 mM EDTA, 12 mM MgCl2, and 1 mM CaCl2). The gel was incubated in 15 mL of fresh reaction buffer, this time containing 50 μCi of γ32P-ATP, for 60 min with gentle shaking. The majority of the unincorporated γ32P was removed by rinsing in electrotransfer buffer several times for a few seconds each, and the gels were subjected to electroblotting for 3 h at 400 mA. Proteins from the extracts that were run through the gel were transferred onto nitrocellulose membranes in this way, whereas ADF that was incorporated into the gel before polymerization remained fixed on the gel. Gels were stained with Coomassie Brilliant Blue R250, destained, and after electrophorization in 5% methanol and 20% glycerol, dried onto paper. Gels and blots were exposed to x-ray film overnight. After exposure, blots were stained with colloidal silver.

Actin Binding and Enzyme Assays

Cosedimentation assays, phospholipid binding assays, and assay of phospholipase C activity were performed as described by Gungabissoon et al. (1998). To measure the extent of ADF binding to F-actin over a range of pH values, F-actin and ADF, each at 10 μM, were mixed in buffer F (12 mM Bis-Tris-Propane, 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM CaCl2, 1 mM NaN3) in the pH range 6.0 to 9.0 and centrifuged at 386,000g. The proteins in the pellets and supernatants were separated by SDS-PAGE. The amounts of protein in the supernatants and pellets were quantified by densitometric scanning of the SDS-PAGE gels. In buffer F, Bis-Tris-Propane (Sigma) was used to buffer pH values at 6.0 to 9.0, making use of the fact that this buffer has two pKv values, 6.8 and 9.0.

To assess the effect of phospholipids on ADF activity, similar cosedimentation experiments were performed in buffer F at pH 8.0 with the following modifications. ADF (2 μM) was incubated with 100 μM phosphatidylinositol, phosphatidylinositol 4-monophosphate, phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylethanolamine, phosphatidylincholine, phosphatidylserine, inositol 1,4,5-triphosphate, or 1-oleoyl-2-acetylgllycerol for 20 min at room temperature. F-actin then was added at a final concentration of 5 μM and incubated for 40 min at room temperature. The amount of ADF in the supernatants after sedimentation was determined by densitometric scanning, as described above.

To assess the effect of actin-interacting protein (AIP) on the depolymerization of F-actin, cosedimentation assays were performed at pH 8.0 using 10 μM actin and 5 μM AIP and ADF. This experiment
was repeated three times, and the results shown in Figure 6 are typical of the data obtained in each experiment.

To assess the binding of phospholipids to ADF, native gels were used as described by Gungabissoon et al. (1998), using a modified method based on that described by Maciver and Weeds (1994). A total of 2 μg of ADF was loaded with phosphatidylinositol, phosphatidylinositol 4-monophosphate, PIP$_2$, phosphatidylethanolamine, phosphatidylcholine, phosphatidyserine, inositol 1,4,5-trisphosphate, or 1-oleoyl-2-acetylglucero (Sigma) at a molar ratio of 1:5 in 5% Suc. Six percent gels were prepared in half-concentrations of gel buffer containing 0.2 mM ATP, 0.2 mM EGTA, and 0.5 mM DTT; the tank buffer also was at half concentration. These gels were run at 10 mA for 40 min.

ADF inhibition of phospholipase C activity was assayed as described by Gungabissoon et al. (1998). After temperature equilibrium, phospholipase C activity of plasma membranes (5 μg of protein) was assayed at 25°C in 50 μL of phospholipase C buffer (50 mM Tris/malate, pH 6.0, and 10 μM CaCl$_2$) containing a sonicated micellar suspension of PIP$_2$ (50 μM) spiked with 0.86 kBq 3H-PIP$_2$ (specific activity of 325.6 GBq/mmol). Various concentrations of ADF were preincubated with the PIP$_2$ micelles in phospholipase C buffer (10 min at 25°C), and the assays were started by the addition of plasma membranes. After 6 min of incubation, the reaction was stopped by the addition of 1 mL of chloroform:methanol (2:1, v/v), and tubes were placed on ice for 5 min. HCl (0.25 mL of 0.6 N) was added to facilitate phase separation, and the tubes were vortexed vigorously and centrifuged at 14,000 g for 2 min. Aliquots of the aqueous top phase were removed from each tube, and radioactivity was determined by liquid scintillation spectrometry after the addition of the scintillation fluid (Hionic-Fluor; Hewlett-Packard, Bracknell, UK).

Cloning of AtAIP1-1

AtAIP1-1 was identified in the Arabidopsis thaliana genome database in a BLAST search using the Xenopus laevis AIP1 sequence. The predicted protein showed 34% identity at the amino acid level. An Arabidopsis cdNA library (CD4-6 flow cdNA library from the ABRC [Columbus, OH]) (Weigel et al., 1992) was used as a template for PCR to amplify the 5’ region of the coding sequence (245 bp). This PCR product was used to screen the same library, and a full-length clone was isolated. Standard molecular biological techniques were used for PCR and library screens (Ausubel et al., 1992).

Two-Hybrid Assays

PCR was used to generate the coding sequences of AtAIP1-1 and AtADF2 with Ncol-BamHI ends incorporated into the 5’ and 3’ primers, respectively. Products were subcloned initially into pCR2.1 (Invitrogen, Carlsbad, CA) and then transferred to the Ncol-BamHI sites of either the GAL4 activation domain vector pACT2 or the GAL4 DNA binding domain vector pAS2-1 (Clontech, Palo Alto, CA). The actin cdNA (mouse) in pACT2 and pAS2-1 was kindly provided by I. Hauser-Hahn (Bayer, Leverkusen, Germany). All vector constructions were checked by restriction analysis and DNA sequencing.

All assays were performed using the GAL4-based two-hybrid system (Clontech). For qualitative assays, pairwise combinations of vectors were cotransformed into yeast colonies (HF7c strain) and were plated on the same selective medium to test for protein–protein interaction. The test used a β-galactosidase activity assay on a filter with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside or growth in the absence of His in medium containing 5 to 10 mM 3-aminotriazole.

RNA Gel Blot Analysis

Arabidopsis total RNA was prepared as described by Jordan et al. (1994), fractionated on a 1% formaldehyde-agarose gel, and transferred to a nylon membrane (Hybond-N; Amersham). Each lane on the gel contained 30 μg of RNA, as quantified by $A_{260}$ spectrophotometric assays supplemented by comparison of rRNAs on ethidium bromide–stained agarose gels. Genes–specific 3’ untranslalted regions for both AIP1-1 and AIP1-2 were used to generate riboprobes with high specific activity (using the Promega Riboprobe In Vitro Transcription System).

Expression and Purification of AIP1-1

AtAIP1-1 was amplified by PCR using primers that incorporated 5’ Ndel and 3’ HindIII restriction sites and cloned into pET28a. The vector construct was checked by restriction digests and sequencing. The protein was expressed with a His tag in Escherichia coli strain BL21 (DE3). Luria-Bertani medium (1 L) was inoculated with 5 mL from an overnight culture of transformed cells, and the culture was grown to an $OD_{600}$ of 0.6 at 37°C. Then, the flasks were transferred to 10°C, and the temperature was allowed to equilibrate for 1 h. The cells were induced with 1 mM isopropylthio-β-D-galactoside for 24 h before being harvested. Cell extract was prepared, and recombinant epitope-tagged protein was purified using a His-Trap column (Amersham Pharmacia) according to the manufacturer’s instructions. Briefly, the cells were pelleted after induction by centrifugation at 6000g for 5 min, and the pellet was resuspended in START buffer (10 mM imidazole, pH 7.4, 10 mM Na$_2$HPO$_4$, 10 mM NaH$_2$PO$_4$, and 400 mM NaCl). The cells were disrupted by sonication (20 × 2 s over 1 min), and the extract was centrifuged at 13.5g for 45 min to pellet the cell debris. Extract was filtered through a 0.2-μm filter, loaded onto a Pharmacia His-Trap column previously loaded with nickel, and then equilibrated with START buffer. The column was washed with START buffer and then with 5 mL each of a series of wash buffers containing START buffer and imidazole concentrations of 40, 60, and 100 mM. Recombinant protein was eluted in elution buffer (START buffer containing 500 mM imidazole). Soluble recombinant protein was eluted in the first 2 mL of elution buffer. For biochemical assays, this protein was used within 60 min.

Immunofluorescence Microscopy

Narcissus pollen grains were germinated in vitro as described by Heslop-Harrison and Heslop-Harrison (1992). The pollen grains and pollen tubes were prepared for indirect immunofluorescence microscopy as described by Smertenko et al. (2001). The anti-LiADF1 and anti-AtAIP1-1 antisera were diluted 1:500 in PBS containing 0.5% BSA. The anti-mouse IgG fluorescent isothiocyanate conjugate (Sigma) was used as a secondary antibody in a dilution of 1:100. Actin filaments were stained with 0.66 μM rhodamine-phalloidin in PBS (Molecular Probes, Leiden, The Netherlands) for 20 min immediately before microscopy, as described by Smertenko et al. (2001) (for Fig-
ures 7B and 7D to 7F). Alternatively, anti-actin antibodies (C4 mouse monoclonal antibody) were used as described by Jiang et al. (1997) (for Figure 7J). It should be noted that no staining was observed with either the preimmune mouse serum or the secondary antibody alone. Also, the single staining of actin on the red channel generated no signal on the green channel and vice versa.

For freeze-substitution fixation, daffodil pollen grains were attached to 3-mm² pieces of dialysis tubing placed on the surface of solidified germination medium. After 3 h of germination, the material was immersed rapidly in liquid propane and prepared for immunofluorescence microscopy as described by Cresti et al. (1987). Rhodamine-phalloidin did not decorate actin filaments after freeze-substitution fixation, so mouse monoclonal anti-actin IgG clone C4 (ICN, Basingstoke, UK) antibody was used (at a dilution of 1:200). The secondary antibody was anti-mouse IgG fluorescein isothiocyanate conjugate.

All fluorescently stained samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined with a Bio-Rad Radiance 2000 laser confocal microscope. The images digitally captured were processed with Adobe Photoshop 3 software (Mountain View, CA).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for non-commercial research purposes.

Accession Numbers

The accession numbers for AtADF2 are AL162459 (EMBL) and At3g46000 (TAIR). Other accession numbers (NCBI unless indicated otherwise) are as follows: C. elegans AIP1-2, AAC11613.1; C. elegans AIP1-1, CAB03187.1; Drosophila melanogaster AIP1, AAF49822.1; X. laevis AIP1, AAD22662; Gallus gallus AIP1, AAD05042.1; Homo sapiens AIP1, AAI00201.1; Mus musculus AIP1, AAD05043.1; Schizosaccharomyces cerevisiae AIP1, P46680; Schizosaccharomyces pombe AIP1, CAB11489.1; Neurospora crassa AIP1, T49741; Dictyostelium discoideum AIP1, AAB05881.1; Physarum polycephalum AIP1, AAC26321.1; Oryza sativa AIP1, BAB21186; Arabidopsis AIP1-1, AC006200 (GB) and At2g01330 (TAIR); and Arabidopsis AIP1-2, AB020749 (EMBL) and At3g18060 (TAIR).

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