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Actin depolymerizing factor is essential for viability in plants, and its phosphoregulation is important for tip growth

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Summary

Actin depolymerizing factor (ADF)/cofilin is important for regulating actin dynamics, and in plants is thought to be required for tip growth. However, the degree to which ADF is necessary has been elusive because of the presence of multiple ADF isoforms in many plant species. In the moss Physcomitrella patens, ADF is encoded by a single, intronless gene. We used RNA interference to demonstrate that ADF is essential for plant viability. Loss of ADF dramatically alters the organization of the F-actin cytoskeleton, and leads to an inhibition of tip growth. We show that ADF is subject to phosphorylation in vivo, and using complementation studies we show that mutations of the predicted phosphorylation site partially rescue plant viability, but with differential affects on tip growth. Specifically, the unphosphorylatable ADF S6A mutant generates small polarized plants with normal F-actin organization, whereas the phosphomimetic S6D mutant generates small, unpolarized plants with a disorganized F-actin cytoskeleton. These data indicate that phosphorylation at serine 6 is required for full ADF function in vivo, and, in particular, that the interaction between ADF and actin is important for tip growth.

Keywords: ADF, cofilin, tip growth, phosphorylation, Physcomitrella patens.

Introduction

Tip growth is a form of polarized cell expansion in which growth is confined to the apical portion of the cell. A variety of cells including root hairs, pollen tubes, algal rhizoids and protonemata expand by tip growth. Underlying tip growth are coordinated cellular activities including the spatiotemporal control of ion gradients, distribution of cell-wall material via exocytosis, retrieval of excess materials by endocytosis and maintenance of cytoskeletal dynamics (Hepler et al., 2001).

The actin cytoskeleton has become the focus for understanding tip growth because pharmacological studies using drugs that affect actin dynamics result in the inhibition of tip growth and cytoplasmic streaming (Gibbon et al., 1999; Miller et al., 1999; Vidali et al., 2001). In particular, at low concentrations of actin depolymerizing drugs, where active cytoplasmic streaming still occurs, tip growth is inhibited and a subapical actin network is eradicated (Vidali et al., 2001), suggesting that a dynamic network of actin filaments mediates tip growth. Additional evidence supporting the role of actin dynamics in tip growth comes from studies that alter the cellular concentrations of actin-associated proteins. Aberrant expression of profilin (Vidali et al., 2001), actin-interacting protein (Ketelaar et al., 2004), cyclase-associated protein (Deeks et al., 2007), ROP GTPases (Chen et al., 2003; Molendijk et al., 2001), formins (Cheung and Wu, 2004; Deeks et al., 2005) or Arp2/3 complex proteins (Harries et al., 2005; Mathur et al., 2003a,b; Perroud and Quatrano, 2006) lead to irregular or completely arrested tip growth.

Among actin-binding proteins, the actin depolymerizing factor (ADF)/cofilin family has emerged as a central regulator of actin turnover. ADF/cofilin function is essential in diverse organisms, including Dictyostelium discoideum (Aizawa et al., 1995), budding yeast (Iida et al., 1993; Moon et al., 1993), Drosophila (Gunsalus et al., 1995), Caenorhabditis elegans (McKim et al., 1994) and mouse (Gurniak et al., 2005). Biochemical studies show that ADF/cofilin is capable...
of severing (Andrianantoandro and Pollard, 2006; Maciver et al., 1991; Pavlov et al., 2007) and depolymerizing actin filaments from their pointed ends (Carlier et al., 1997), by binding preferentially to ADP-bound actin subunits (Blanchoin and Pollard, 1999) and inducing a helical twist in the actin filament (McGough et al., 1997). Additional evidence supports a role for ADF/cofilin as a nucleator of actin polymerization when present at high concentrations (Andrianantoandro and Pollard, 2006). These activities are consistent with a role for maintaining a dynamic actin cytoskeleton, by creating new barbed ends from pre-existing filaments, recycling the monomeric actin pool or inducing de novo polymerization of actin filaments.

In plants, various studies have suggested that ADF plays an important role in tip growth. However, because of the presence of multiple isoforms in many plants, it has been difficult to clearly define the role of ADF in tip-growing cells. In Arabidopsis thaliana, overexpression of AtADF1 results in the reduction of root hair length, whereas knock-down of AtADF1 levels using RNA interference (RNAi) increases root hair length (Dong et al., 2001b). In another study, expression of GFP-NtADF in tobacco pollen tubes inhibited tip growth in a dose-dependent manner (Chen et al., 2002). At levels of expression that did not affect growth, GFP-NtADF co-localized with actin filaments in a subapical actin mesh and throughout the pollen tube (Chen et al., 2002). In root hairs, ADF has also been localized to the apical portion of the growing hair (Jiang et al., 1997). Similarly in lily pollen tubes, ADF has been localized by immunofluorescence to a subapical actin fringe, a site believed to consist of dynamic actin filaments (Lovy-Wheeler et al., 2006), suggesting a critical role for ADF in tip growth.

ADF/cofilin activity in most organisms is regulated by N-terminal phosphorylation at a highly conserved serine residue. In the unphosphorylated state, ADF/cofilin binds ADP-bound actin with high affinity, and is therefore able to sever and depolymerize actin filaments; conversely, when phosphorylated, ADF/cofilin binding to actin is attenuated and, consequently, severing and depolymerization activities are abolished (Blanchoin et al., 2000; Moriyama et al., 1996; Ressad et al., 1998). In animal cells, phosphoregulation of ADF/cofilin is important for processes such as neurite extension (Endo et al., 2007), maintenance of the lamellipodium (Kučičak et al., 2007; Zebda et al., 2000) and cleavage furrow abscission during cytokinesis (Kaji et al., 2003). In animals, the regulation of ADF/cofilins is coordinated by the activity of slingshot phosphatase (Niwata et al., 2002), and LIM (Arber et al., 1998; Yang et al., 1998) and TES kinases (Toshima et al., 2001). Although a specific plant ADF kinase or phosphatase has not been identified, evidence suggests that ADF activity in plants is also controlled by phosphorylation. A protein extract from French bean containing a calmodulin-like domain protein kinase (CDPK) specifically phosphorylates the conserved N-terminal serine 6 of maize ADF 3 (Allwood et al., 2001). Two-dimensional electrophoresis revealed the presence of both phosphorylated and unphosphorylated forms of ADF in plant extracts from Arabidopsis (Dong et al., 2001b), maize (Jiang et al., 1997) and tobacco pollen (Chen et al., 2003). Additionally, GFP-labelled unphosphorylatable ADF S6A co-localizes with actin filaments, unlike the phosphomimetic GFP-ADF S6D (Chen et al., 2002). A study in tobacco pollen tubes shows that overexpression of ADF S6A, but not of S6D, was able to suppress a tip growth defect caused by ROP GTPase overexpression (Chen et al., 2003). Despite this evidence, without a loss-of-function phenotype, it is unclear whether ADF is required for tip growth, and to what extent phosphoregulation of ADF is physiologically relevant.

Moss protonemata, which expand by tip growth (Menand et al., 2007), are an excellent model for studying this process. Protonemal cells are abundant, easily propagated and can be readily imaged by microscopy. Additionally, the Physcomitrella patens genome has recently been sequenced, making molecular genetic approaches straightforward (Rensing et al., 2008). Here, we use RNAi in moss to demonstrate that ADF is essential for plant viability. Loss of ADF dramatically alters F-actin organization, resulting in an inhibition of tip growth. We performed complementation studies of the ADF RNAi phenotype using unphosphorylatable and phosphomimetic ADF mutants. Our results show that phosphorylation of the N-terminally conserved serine is required for efficient growth and, importantly, for polarization of growth.

Results

Moss has a single ADF gene

We searched the P. patens genome for ADF, and found a single locus containing a predicted gene product highly similar to other ADF proteins (Table S1). Additionally, a single ADF expressed sequence tag (EST) was identified from available databases (Rensing et al., 2008; Nishiyama et al., 2003). The presence of a single ADF gene in moss is strikingly different from other plants, which generally have multiple ADF isoforms (Maciver and Hussey, 2002; Ruzicka et al., 2007). The moss ADF gene structure is also distinct because it lacks introns (Figure 1a). In contrast, Arabidopsis and Oryza sativa ADFs have a conserved gene structure containing two introns (Dong et al., 2001a; Maciver and Hussey, 2002). Despite these differences, moss ADF shares ~50–70% identity and ~70–80% similarity with other plant ADFs at the amino acid level (Table S1), and a sequence alignment with other ADF/cofilins of known structure suggests that it has a conserved tertiary structure (Figure 1b). Moss ADF comprises 142 amino acids with a predicted molecular weight of 16.2 kDa, typical for ADF/cofilin
proteins, which are generally between 113 and 168 amino acids long (Maciver and Hussey, 2002).

ADF is essential for plant viability

We used an established transient RNAi assay (Bezanilla et al., 2005; Vidali et al., 2007) to silence ADF expression in moss protonemal cells. In this assay we use a stable line (NLS-4) that constitutively expresses a nuclear-localized GFP-GUS fusion protein. Transformation of NLS-4 protoplasts with GUS-RNAi, an RNAi construct that targets the GUS sequence in the GFP-GUS fusion transcript, results in silencing of the nuclear GFP-GUS reporter (Bezanilla et al., 2005). The loss of nuclear GFP-GUS expression, and thus GFP fluorescence, offers a quick and reliable method for identifying plants that are actively silencing (Bezanilla et al., 2005).

In our transient RNAi assay, we simultaneously silence the nuclear GFP-GUS reporter and ADF. To do this, we fused a region of GUS to the moss ADF coding sequence to generate an RNAi construct that targets GUS and the coding sequence of ADF (CDS-RNAi; Figure 1a). When this construct is transformed into NLS-4, silenced plants, as marked by the loss of nuclear GFP fluorescence, are severely stunted and often have diminished chlorophyll fluorescence, indicative of cell senescence (Figure 2a). As tip growth is the only form of growth for protonemal moss tissue (Menand et al., 2007), the stunted phenotype resulting from the CDS-RNAi construct suggests that ADF is essential for tip growth. The extent of growth inhibition was determined by quantifying...
the area of GFP-deficient plants using intrinsic chlorophyll fluorescence seven days after transformation. CDS-RNAi plants are smaller in area compared with GUS-RNAi controls (Figure 2b), and this difference is highly significant ($P < 0.0001$).

A nearly identical phenotype was observed when transforming NLS-4 with an RNAi construct that targets both GUS and a region of the non-coding, 3′-untranslated region (UTR) sequence of ADF (UTR-RNAi; Figures 1a and 2a). The area of UTR-RNAi plants is significantly different from that of GUS-RNAi plants ($P < 0.0001$), but not from that of CDS-RNAi plants (Figure 2b, $P = 0.8738$). Scanning electron micrographs show that GUS-RNAi plants maintain polarized, filamentous structures with numerous branches, whereas UTR-RNAi plants are small and lack developed branches (Figure 2c). Interestingly, CDS-RNAi plants were more likely to show weak chlorophyll fluorescence and death, compared with UTR-RNAi plants, especially 8–10 days after transformation with the RNAi construct. This suggests that RNAi targeting the coding sequence of ADF is more toxic than RNAi targeting the 3′ UTR. Control GUS-RNAi plants were never observed to undergo a loss of chlorophyll fluorescence.

Moss ADF is subject to phosphorylation in vivo

To determine whether ADF is phosphorylated in moss, we raised a polyclonal antibody against moss ADF, and used it for 2D western blot analysis. The affinity purified polyclonal antibody binds exclusively to an ~18-kDa protein in wild-type protein extracts (Figure 3a, lane 1), and to a purified recombinant His$_6$PpADF protein (Figure 3a, lane 2). Over-expression of ADF in moss extracts leads to a specific increase in the ADF signal at the same molecular weight (data not shown), indicating that the antibody is specific for ADF.

We used the affinity purified ADF antibody to probe blots containing total protein extracts separated by 2D electrophoresis. Extracts from wild-type protonemal tissue isolated in the presence of phosphatase inhibitors exhibited two spots, with the more acidic spot present at considerably lower levels.
Moss actin depolymerizing factor (ADF) is phosphorylated in vivo. (a) The affinity purified anti-ADF antibody binds exclusively to ADF in moss. Lane 1 contains 6.5 μg of wild-type total protein extract, and lane 2 contains 40 ng of purified His-PpADF. Protein samples were blotted onto nitrocellulose and were probed with affinity purified anti-ADF polyclonal antibodies. Both lanes are from the same blot. (b) ADF and phospho-ADF are detectable in moss. 2D western blots probed with affinity purified anti-ADF antibody. A 100-μg sample of total protein from wild-type protonemal tissue was treated with or without phosphatase inhibitors (−Pi). For each panel the acidic pI is to the left, and the basic pI is to the right.

Phosphoregulation of ADF is required for efficient tip growth

We performed a complementation analysis to ensure that the specific knock-down of ADF is responsible for the loss of plant viability and tip growth. We co-transformed NLS-4 with UTR-RNAi and the ADF coding sequence under the control of the strong constitutive maize ubiquitin promoter. The ADF expression construct has a nopaline synthase (NOS) terminator in place of the ADF 3′-UTR sequence, and is therefore not targeted by UTR-RNAi. Rescued plants phenocopied GUS-RNAi plants with very similar average areas (Figure 4; Table S2). Like control plants, complemented plants were never observed to undergo a loss of chlorophyll fluorescence. This indicates that the senescence phenotype is specific to the loss of ADF function.

We used this complementation assay to determine the physiological significance of ADF phosphorylation. Phosphorylation of ADF/cofilin was shown to significantly reduce the interaction between ADF and actin (Blanchoin et al., 2000; Chen et al., 2002). Although plant area of UTR-RNAi plants could be fully rescued by expression of wild-type ADF, expression of unphosphorylatable ADF S6A or phosphomimetic ADF S6D only partially rescued plant area (Figure 4a,b; Table S2). These data strongly indicate that ADF phosphorylation is important for efficient tip growth.

Two additional mutants were analyzed to further address the site of ADF phosphorylation. We were concerned about the presence of two adjacent N-terminal serine residues in the moss ADF sequence (Figure 1b), and the possibility that one serine might be regulated in the absence of a phosphorylation site at the other serine. To address this, we generated a serine 5 to alanine and serine 6 to alanine (S5AS6A) double mutant to prevent phosphorylation at both sites. Complementation of UTR-RNAi with S5AS6A was indistinguishable from S6A, indicating that the residual complementing activity of S6A is not caused by a second phosphate acceptor site at serine 5 (Figure 4a,b). We also generated a serine 6 to threonine (S6T) mutation to maintain an amino acid with similar biochemical properties to serine, including the potential capability of being phosphorylated. Although complementation with S6T did not fully rescue plant area, it exhibited a greater degree of rescue compared with S6A, S5AS6A and S6D (Figure 4; Table S2). This evidence suggests that a threonine at the sixth position of moss ADF can also be phosphorylated, enabling more efficient tip growth.

We noticed differences in plant morphology resulting from complementation with the different ADF constructs. The ADF- and S6T-rescued plants resembled control plants with many filamentous outgrowths, whereas the S6A and S5AS6A plants had fewer and shorter filamentous extensions (Figure 4a). In contrast, the S6D plants resembled the UTR-RNAi plants with an overall rounded plant morphology, lacking polarized extensions and suggestive of an inhibition of tip growth. Notably, S6D plants never exhibited chlorophyll senescence. The fact that S6D plants were viable, but unable to perform tip growth, strongly suggests that ADF plays an essential role in tip growth.

To quantify these morphological differences, we measured two morphometric parameters: circularity and...
Solidity. Briefly, circularity is a measure of the degree of polarization, where a value of 1 represents a perfect circle whereas values approaching 0 have a more linear structure. Solidity is used to estimate the degree of outward cell branching, where a plant having no branches is solid and has a value of 1, whereas a plant with many branches has empty space between the branches lowering the solidity value. Using these parameters, we found that the unphosphorylatable ADFs, S6A and S5AS6A, rescued plant morphology to a greater degree when compared with the S6D mutant (Figure 4c,d; Table S2). Based on work from other systems, the alanine mutants should be capable of binding actin, whereas the phosphomimetic mutant should have a decreased affinity for actin (Blanchin et al., 2000; Chen et al., 2002; Ressad et al., 1998). Taken together, this implies that ADF actin binding activity is required for tip growth, and that the regulation of actin binding is necessary for efficient growth. Furthermore, morphometric analysis provides additional evidence that serine 6 is the site of phosphorylation. Complemented ADF and S6T plants are statistically indistinguishable; moreover, S6A and S5AS6A plants were rescued to a similar extent (Figure 4c,d; Table S2).

Expression constructs restore ADF protein levels to control levels

The inability of unphosphorylatable and phosphomimetic ADF mutants to fully rescue plant size or morphology
may be a consequence of reduced ADF expression levels. We tested this by performing immunofluorescence with the affinity purified anti-ADF antibody on RNAi plants with and without ADF expression constructs (Figure 5a,b). Only a fraction of transformed plants are actively silencing, marked by the loss of the nuclear GFP-GUS reporter. Thus, it is very difficult to obtain enough material to analyze protein levels using western blots. Instead, we used the immunofluorescence of individual plants to estimate the protein levels present in actively silenced plants. All images were acquired using identical camera settings to enable quantification of fluorescence intensity as a measure of protein expression level. As a background control, GUS-RNAi plants were probed with IgG. We determined that ADF protein levels in GUS-RNAi plants are indistinguishable from levels in ADF, S6A and S6D plants (Figure 5; Table S3), indicating that under these conditions, expression from the maize ubiquitin promoter restores wild-type ADF levels. In contrast, UTR-RNAi plants have a significantly reduced ADF signal (Figure 5; Table S3), indicating that the RNAi construct effectively reduces ADF protein levels. Furthermore, these data show that the phenotypes observed with S6A and S6D are specific to the introduced mutation, and are not caused by a lack of expression from the constructs.

Figure 5. Actin depolymerizing factor (ADF) complementation restores protein to control levels.
(a) Determination of relative ADF expression levels. GFP-deficient plants were immunostained with affinity purified anti-ADF antibodies. Panels with a plus sign represent plants transformed with UTR-RNAi plus the indicated expression construct. The left panel shows chlorophyll fluorescence, the middle panel is the immunofluorescence signal from either rabbit IgG, as a control for background fluorescence (top row), or affinity purified anti-ADF rabbit polyclonal antibody (all other rows). Right panels are the merged images. Scale bar: 100 μm, and applies to all panels.
(b) Mean fluorescence was determined as a fraction of GUS-RNAi plants immunostained with affinety purified ADF antibody. Background values obtained from GUS-RNAi IgG immunostained plants were subtracted from all anti-ADF immunostained plants. Error bars represent standard error of the mean from four experiments (GUS-RNAi, n = 25; UTR-RNAi, n = 30; +ADF, n = 26; +S6A, n = 30; +S6D, n = 44; n = number of plants). P-values are shown in Table S3.

Figure 6. Actin depolymerizing factor (ADF) localizes to the cytoplasm in moss cells. Localization of ADF in moss tip cells. A transgenic line expressing GFP was immunostained with the affinity purified anti-ADF rabbit polyclonal antibody. Two representative cells are shown. Scale bar: 10 μm, and applies to all panels.

ADF localizes to the cytoplasm in moss protonemal cells
We used the affinity purified anti-ADF antibody to determine the subcellular localization of ADF in moss protonemal cells.
Under conditions that preserve the actin cytoskeleton (see below), we observed that ADF is diffusely localized to the cytoplasm (Figure 6). We performed immunofluorescence on wild-type plants (data not shown) and on a transgenic line expressing GFP (Figure 6). The GFP line allowed us to analyze the localization of ADF with respect to GFP, another cytosolic protein. As is the case for GFP, which easily diffuses in and out of the nucleus in moss cells, ADF is also found in the nucleus, but perhaps to a lesser extent (compare ADF and GFP panels in Figure 6). ADF also appears as punctae on the chloroplasts (Figure 6).

ADF is required for proper organization of the actin cytoskeleton

To determine how a loss of ADF affects the actin cytoskeleton, we labelled the actin cytoskeleton with Alexa-488-phalloidin in cells actively undergoing RNAi (Figure 7). In GUS-RNAi control cells, we observed a subapical cortical ‘fringe’ structure (Vidali et al., 2007). This structure is composed of parallel actin bundles closely associated with the cell cortex (Figure 7, square brackets). Behind the fringe, actin is generally longitudinally oriented in control cells, and is also found tightly surrounding chloroplasts (Figure 7, arrows). Strikingly in UTR-RNAi cells, the subapical cortical fringe structure is absent and instead prominent actin structures, which are composed of actin bundles appearing to emanate from a point on the cell cortex, are visible (Figure 7, arrow heads). These structures resemble ‘stars’, and many UTR-RNAi cells contained more than one actin ‘star’. In addition to these prominent cortical actin structures, UTR-RNAi cells had actin filaments throughout the cell. In contrast to controls cells, actin filaments in UTR-RNAi cells did not appear to have a uniform orientation. The actin filament organization was rescued in UTR-RNAi plants complemented with wild-type ADF (Figure 7).

We observed a variety of actin filament organizations in plants rescued with S6A and S6D. In general, actin filaments in S6A plants more closely resembled those of GUS-RNAi or ADF-rescued plants. The subapical cortical fringe was detected in most cells, even in cells that were smaller than controls (Figure 7, +S6A middle panel). In contrast, the S6D rescued plants had a wider variety of actin filament organization. In some cases, we observed cortically associated actin filament ‘bars’ or patches (Figure 7, +S6D middle and right panels, respectively). Surprisingly S6D plants did not

Figure 7. Actin depolymerizing factor (ADF) is required for proper F-actin organization. F-actin organization in moss tip cells. Alexa-488 phalloidin was used to label F-actin in actively growing moss cells. Three representative cells are shown for each treatment. Panels with a plus sign represent plants transformed with UTR-RNAi plus the indicated expression construct. Arrows indicate actin filaments associated with chloroplasts. Square brackets denote the cortical fringe. Arrowheads denote the actin ‘stars’ present in UTR-RNAi. Scale bar: 10 μm, and applies to all panels.
have the actin ‘stars’ observed in UTR-RNAi plants, suggesting that the presence of S6D appears to partially rescue the actin cytoskeletal organization. In some S6D plants that had cells with normal morphology, we observed wild-type actin filament organization (Figure 7, +S6D left panel). Taken together, these data suggest that ADF is essential for the proper organization of actin filaments in moss tip growing cells, and that phosphorylated ADF is unable to properly organize the actin cytoskeleton.

Discussion

ADF is essential for plant viability and is required for tip growth

Using the moss *P. patens* as a model system, we have shown that ADF is essential for viability, and have provided evidence that ADF is important for tip growth. Until this study, only ADFs from seed plants had been characterized, and these plants typically contain multiple ADF isoforms. For example, Arabidopsis has twelve isoforms, many of which are expressed and some of which show tissue-specific expression patterns (Dong et al., 2001a; Maciver and Husey, 2002; Ruzicka et al., 2007). In contrast, we identified a single ADF gene in moss. It appears that the number of ADF genes has increased from non-vascular to vascular plants over the ~400 million years of evolution separating mosses from seed plants. To investigate this, we searched for ADF genes in the lycophyte *Selaginella moellendorfii*, a member of the oldest living vascular plant division. We discovered that the genome of *S. moellendorfii* (http://moss.nibb.ac.jp) has two loci highly similar to ADF, suggesting that as plants evolved a vasculature, more ADF genes became necessary. The presence of multiple ADF isoforms in vascular plants, with differential expression patterns in seed plants, suggests tissue-specific roles for each ADF that were not necessary in early plant evolution.

In addition to fewer ADF isoforms, the moss ADF gene structure is divergent from those found in seed plants because it lacks introns. ADFs in seed plants typically possess two introns at conserved positions, where the first intron has been shown to be critical for proper expression (Jeong et al., 2007; Mun et al., 2002). Interestingly, the two ADF genes in *S. moellendorfii* have a single intron at the second conserved position. The presence of introns in vascular plant ADFs suggests that the more complex tissue and developmental patterns of these plants may require regulation of ADF gene expression. This may be accomplished by the presence of introns and/or multiple isoforms of ADF. In contrast, the lack of introns in the single ADF gene in moss may enable higher levels of expression, as has been found in one study comparing genes in *P. patens*, where shorter gene structures equated to higher expression levels (Stenoien, 2007).

We used RNAi to investigate the consequences of reducing ADF levels in moss tip-growing protonemal cells. We observed inhibition of tip growth when employing either the CDS-RNAi or UTR-RNAi constructs to target either the coding sequence or the 3’-UTR of the ADF transcript, respectively. This result differs from previous studies of ADF in Arabidopsis, where reduction of ADF1 resulted in enhanced tip growth in root hairs (Dong et al., 2001b). A possible explanation for this is that not all ADF function was reduced in the root hairs because of the presence of other ADF isoforms. In moss, we also observed cell death as a result of loss of ADF function, demonstrated by a reduction or complete loss of chlorophyll fluorescence, indicating that ADF is essential for plant viability.

Additionally, we observed that plants lacking ADF function were unable to properly organize their F-actin cytoskeleton. Instead of longitudinally oriented actin filaments and a prominent subapical actin fringe, UTR-RNAi cells exhibited striking cortical actin structures resembling ‘stars’. Interestingly, these structures were typically observed on the cortex of the cell most distal to the neighbouring cell. This suggests that polarizing machinery may still be in place in these cells, but in the absence of ADF activity, the cortical fringe is unable to organize itself. Perhaps the cortical ‘stars’ are precursors to the fringe structure observed in control cells.

The loss of the cortical actin fringe in UTR-RNAi cells suggests that ADF may have a direct role in organizing this cortical structure. Indeed in pollen tubes, ADF appears to localize specifically to the cortical fringe or collar (Chen et al., 2002; Lovy-Wheeler et al., 2006). However, we did not observe any specific localization of ADF to this region of the cortex. Instead, ADF appears to be localized diffusely to the cytoplasm. One region where ADF may co-localize with actin is on the chloroplasts, as we observe that ADF and actin both associate with chloroplasts.

The use of the UTR-RNAi construct allowed us to perform complementation studies by co-expressing the coding sequence of ADF under the control of a strong constitutive promoter. Co-expression of ADF fully rescued plant viability and actin cytoskeleton organization, demonstrating that the UTR-RNAi phenotype is not a consequence of silencing another essential gene.

ADF phosphoregulation is physiologically relevant for tip growth

Analysis of protein extracts suggests that moss uses phosphorylation to control ADF activity. Wild-type protein extracts isolated in the presence of phosphatase inhibitors exhibited both ADF and phospho-ADF spots. The phospho-ADF spot is present at significantly lower levels compared with the non-phosphorylated ADF isoform. This implies that a balance between active and inactive ADF isoforms is maintained in vivo, and suggests that kinases and
phosphatases regulating ADF are present in moss. The kinases and phosphatases involved in regulating ADF/cofilin in animals do not appear to be present in plants (Allwood et al., 2001; Bamburg, 1999); instead, in vitro evidence supports a role for a calcium-dependent CDPK in regulating plant ADF (Allwood et al., 2001; Smertenko et al., 1998). Calcium gradients are regulated spatially and temporally in tip-growing cells (Hepler et al., 2001), and CDPKs have been shown to be important for tip growth (Yoon et al., 2006); thus, CDPKs appear to be likely candidates for regulating the activity of ADFs during tip growth.

The ability to complement the UTR-RNAi phenotype with the coding sequence of ADF allowed us to investigate the relevance of ADF phosphoregulation in planta. The unphosphorylatable (S6A) and phosphomimetic (S6D) mutants rescued plant area to a similar extent, but have significant differences in their rescue of plant morphology and actin cytoskeletal organization. Interestingly, we did not observe the prominent F-actin ‘stars’ that were present in UTR-RNAi cells, but we did observe cortical actin patches and ‘bars’ in the S6D rescued plants. This suggests that S6D may have some residual function in helping to organize the subapical cortical fringe. Importantly, the observed rescue of S6D and S6A is not a result of aberrant expression of the mutants, because protein levels are indistinguishable from control plants. These data demonstrate that both phosphorylation and dephosphorylation of ADF are important for tip growth.

The partial rescue of plant area with S6D is surprising, as we expected that this mutant would not interact with actin (Moriyama et al., 1996). This observation suggests that S6D may still have some actin-binding activity, which is supported by biochemical studies of actophorin, the Acanthamoeba castellanii ADF/cofilin protein (Blanchin et al., 2000). Although we were unable to find distinct differences between S6A- and S6D-expressing plants regarding total plant area, we did notice obvious morphological differences. This indicates that ADF’s actin binding activity is critical for determining polarization.

An alternative explanation for the partial rescue by S6D is that ADF may bind to another protein independently of its N-terminus, enabling partial function and a limited level of growth, but with a distinct loss of polarization. This interaction would be absent upon ADF depletion, as occurs for UTR-RNAi plants. A possible candidate is actin interacting protein (AIP) – a known binding partner to both ADF and actin (Okada et al., 1999; Rodal et al., 1999). In combination, ADF and AIP were shown to synergistically increase actin disassembly to far higher levels than either protein could achieve on its own (Allwood et al., 2002). Thus, interaction with AIP may still be possible with the S6D mutant.

As threonine can often be recognized and phosphorylated by serine/threonine kinases, we generated the S6T mutant to provide further evidence that regulation of the phospho-state of ADF is necessary for tip growth. LIMK, the kinase responsible for phosphorylation of ADF/cofilin proteins in animal cells, was shown to phosphorylate both serine and threonine, but not tyrosine, residues using an in vitro phosphorylation assay (Okano et al., 1995). Notably, Arabidopsis ADF5 has a threonine in place of the conserved N-terminal serine, further suggesting that an N-terminal threonine can be phosphorylated (Figure 1a; Dong et al., 2001a). Although the S6T ADF mutant did not fully rescue plant area, it showed much greater partial rescue compared with the S6A and S6D plants, suggesting that phosphorylation is necessary for efficient tip growth, and that serine 6 is the site of phosphorylation.

As ADF is essential, we have been able to address the physiological significance of ADF phosphorylation in plants. By performing complementation of the loss-of-function phenotype, we show that phosphorylation of ADF is required for efficient tip growth. This suggests that optimal rates of growth require the presence of a kinase that negatively regulates ADF function. In addition, the phosphomimetic mutant inhibits tip growth in the absence of endogenous ADF, and this inhibition is not a result of death. Thus, we provide strong evidence that ADF is essential for tip growth via the ability to interact with actin and organize the F-actin cytoskeleton. Now we are poised to study the molecular basis of ADF function and regulation in plant cell tip growth.

Experimental procedures

Protein sequence alignment

Actin depolymerizing factor/cofilin family proteins with available crystal or NMR structures were downloaded from the Protein Data Bank (http://www.pdb.org). Swiss-ProtView (Guex and Peitsch, 1997) was used to generate an alignment based on tertiary structures. All sequences were fitted to the Arabidopsis ADF 1 crystal structure. ADF protein sequences from various species without available structures were obtained from the Swiss-Prot database (http://www.expasy.org/sprot), whereas the P. patens ADF protein sequence was determined based on the cDNA sequence. These ADF sequences were manually fitted to the structural alignment.

Generation of constructs

The ADF RNAi constructs were created by PCR amplification of either the coding sequence or the 3′-UTR of ADF from P. patens cDNA using the primers indicated in Table S4. PCR fragments were cloned into pENT-TOPO (Invitrogen, http://www.invitrogen.com) following the manufacturer’s recommendations, and the resulting constructs were sequenced. LR clonase (Invitrogen) reactions were used to transfer either the coding sequence or 3′-UTR sequence into inverted-repeat GUS-Gateway cassette fusions of the destination vector pUGGi (Bezanilla et al., 2005) to generate the CDS-RNAi or UTR-RNAi constructs, respectively. Restriction enzyme digestion was used to verify these constructs. The vector pUGi (Bezanilla et al., 2005) was the GUS-RNAi control.
Expression constructs were generated by PCR amplifying the ADF coding sequence from *P. patens* cDNA using a 5′-CACC sequence on the specific primers for cloning into the pENT-TOPO vector (Invitrogen). The S6A, SAAS6A, S6D and S6T mutations were introduced by site-directed mutagenesis (Weiner et al., 1994) using the primers listed in Table S4. All mutant and wild-type ADF constructs were sequenced and transferred via LR clonase to a pTHUBI-Gate destination vector (kindly provided by P.-F. Perroud and R.S. Quatrano, Washington University in St Louis, http://www.wustl.edu), which drives expression using the constitutive maize ubiquitin promoter (Bezanilla et al., 2005).

For moss transformations, plasmids were purified using the GenElute HP Plasmid MaxiPrep kit (Sigma-Aldrich, http://www.sigmaaldrich.com) following the manufacturer’s recommendations, or with a conventional maxiprep protocol (Sambrook et al., 1989) without the chloroform extraction.

**Tissue culture and protoplast transformation**

All moss tissue culture was performed as previously described (Bezanilla et al., 2003). Protoplast transformation procedures are described in Appendix S1.

**Scanning electron microscopy**

One-week-old plants were analyzed by scanning electron microscopy as described previously (Vidali et al., 2007).

**Recombinant protein purification**

Glutathione-S-Transferase (GST)-ADF and His<sub>6</sub>-ADF were constructed by transferring the coding sequence of ADF from the pENT-ADF vector into the pDEST15 and pDEST17 vectors, respectively (Invitrogen) with an LR clonase reaction. A detailed description of protein purification is given in Appendix S1.

**Antibody production and purification**

Polyclonal antibodies were generated in a rabbit injected with GST-ADF isolated from a polyacrylamide gel and boosted with GST-ADF in solution, following standard antibody production methods (Harlow, 1988). Affinity purification of the ADF polyclonal antibody is described in Appendix S1.

**Western blotting**

A detailed description of protein extraction and 2D electrophoresis is presented in Appendix S1. Western blotting procedures were performed as described previously (Vidali and Hepler, 1997). Protein isoelectric points were predicted using the pl/MW program (Bjellqvist et al., 1993) from the Swiss Institute of Bioinformatics (SIB).

**Morphometric analysis**

Four days after transformation, plants were transferred to plates containing hygromycin (15 μg ml<sup>−1</sup>). On day 7, images of GFP-deficient plants were captured and analyzed as previously described (Vidali et al., 2007). Plants covering ≥500 μm<sup>2</sup> were counted in the analysis. The plant area is determined from the total number of adjacent pixels corresponding to a GFP-deficient plant, whereas circularity is defined as 4π(area perimeter<sup>2</sup>)/area, and solidity is defined as the area/convex hull area of those same plants.

**Immunofluorescence**

For quantification of protein levels in 1-week-old plants, we followed the procedure described in Vidali et al., (2007), with the following modifications. All plants were incubated overnight with 1 μg ml<sup>−1</sup> affinity purified ADF antibody. For quantification of nonspecific binding, GUS-RNAI plants were separately incubated with 1 μg ml<sup>−1</sup> rabbit IgG (Jackson ImmunoResearch, http://www.jacksonimmuno.com). For immunolocalization of ADF, either wild type or a moss line stably expressing GFP was cross-linked directly on the agar growth medium in 0.3 mM m-maleimobenzoyl-N-hydroxysuccinimide ester (MBS; Pierce, http://www.piercenet.com) and 1 mM ethylene glycol bis(succinimidyldimethanolate; EGS; Pierce) in 5 ml of PME buffer (100 mM PIPES, pH 6.8, 5 mM MgSO<sub>4</sub>, 10 mM EGTA). After 15 min, gluteraldehyde (Electron Microscopy Sciences, http://www.emsdiasm.com) was added to a final concentration of 0.5% and incubated for 25 min. Plants were recovered into 15-mL conical tubes and 10 ml of PME was added. After centrifugation at 300 g for 10 min, 12.5 ml of supernatant was removed and reconstituted with 12.5 ml of PME. Plants were immobilized in 0.7% low-melting-point agarose, type VII (Sigma-Aldrich) in PME. All subsequent solutions were added to the plants attached to the coverslip. After two 5-min washes in PME, plants were incubated in 1% saponin in PME for 30 min. All subsequent steps were identical to the immunofluorescence treatment described above, except that TBSS (125 mM NaCl, 25 mM Tris–HCl, pH 8, 0.1% saponin) was used in place of TBST. Plants were imaged with a Nikon confocal microscope (Nikon D-Eclipse-C1; Nikon, http://www.nikon.com) on an inverted stand (Nikon Eclipse-TE2000-S) fitted with a 60× water immersion 1.2 numerical aperture objective. A 488 argon laser was used to excite GFP, whereas a 543 helium-neon laser excited CY3. Confocal sections were taken at 0.5-μm intervals, typically through to at least the bottom half of the cell.

**F-actin labelling**

One-week-old plants were fixed and stained for actin using the same procedure as described previously (Vidali et al., 2007).

**Statistical analysis**

We performed statistical analysis as described previously (Vidali et al., 2007).

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**Supplementary Material**

The following supplementary material is available for this article online:
Table S1. Actin depolymerizing factor (ADF)/cofilin family protein sequence comparison.
Table S2. Statistical analyses of area and morphology from complementation experiments.
Table S3. Statistical analysis comparison of actin depolymerizing factor (ADF) immunofluorescence levels.
Table S4. Primers used in this study.

Appendix S1. Tissue culture and protoplast transformation.

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References


Accession numbers: A list of accession numbers are provided in Appendix S1.