INTRODUCTION

The plant actin cytoskeleton is known to be required for proper cell growth and division as well as for defining cell shape (Cleary, 1995; Fowler and Quatrano, 1997; Martin et al., 2001; Smith, 2003). Although the plant cytoskeleton has been well studied, little is known regarding what has emerged in recent years as an essential regulator of actin dynamics in several eukaryotic systems: the actin-related protein2/3 (Arp2/3) complex. This seven-subunit complex contains the actin-related proteins Arp2 and Arp3 as well as five novel proteins designated ARPC1-5. The complex was first discovered in Acanthamoeba (Machesky et al., 1994) and has subsequently been found in many diverse eukaryotic organisms. The Arp2/3 complex nucleates the formation of actin filaments (Mullins et al., 1998; Pollard and Borisy, 2003) and has been shown to localize to the branch points in dense actin filaments (Mullins et al., 1998; Pollard and Borisy, 2003) and has been shown to inhibit the elongation of maize (Zea mays) pollen tubes in a concentration-dependent manner (Gibbon et al., 1999). Additionally, studies have shown that inhibition of actin polymerization under conditions that still allow cytoplasmic streaming and vesicle transport stops tip growth in pollen tubes (Vidali et al., 2001; Vidali and Hepler, 2001). Although it has been long established that actin filaments are critical for the myosin-driven delivery of exocytotic vesicles to the growing tip (Pope et al., 1979; Tominaga et al., 2003), the idea that actin dynamics may be critical for tip growth in plants is relatively recent.

Based upon the strong actin filament nucleation activity of the Arp2/3 complex observed in many of the aforementioned model systems, tip-growing cells provide attractive sites for potential Arp2/3 complex activity in plants. Tip growth is known to be dependent upon dynamic actin as established by studies using inhibitors of actin polymerization. Latrunculin B (Lat B), for example, binds actin monomers and has been shown to inhibit the elongation of maize (Zea mays) pollen tubes in a concentration-dependent manner (Gibbon et al., 1999). Additionally, studies have shown that inhibition of actin polymerization under conditions that still allow cytoplasmic streaming and vesicle transport stops tip growth in pollen tubes (Vidali et al., 2001; Vidali and Hepler, 2001). Although it has been long established that actin filaments are critical for the myosin-driven delivery of exocytotic vesicles to the growing tip (Pope et al., 1979; Tominaga et al., 2003), the idea that actin dynamics may be critical for tip growth in plants is relatively recent.

Despite the fact that no functional Arp2/3 complex has yet been purified from plants, homologs of all complex members have been identified in Arabidopsis thaliana. Additionally, functional Arabidopsis homologs of Scar/WAVE family Arp2/3 complex activators have been identified that are capable of activating bovine Arp2/3 complex in vitro (Frank et al., 2004; Basu et al., 2005). Families of Arabidopsis mutants defective in trichome and epidermal cell morphogenesis have proven to be altered in Arp2/3 complex subunits known to exist in a 1:1 stoichiometry with each other (Robinson et al., 2001), knockouts of different subunits in S. cerevisiae, for example, show some variation in their terminal phenotypes (Winter et al., 1999). Such phenotypic differences between subunit knockouts may be attributed to specialized roles of individual subunits within the complex.
Figure 1. *P. patens* Contains ARPC1, a Member of the Arp2/3 Complex.
3 complex subunits or putative complex regulators (Le et al., 2003; Mathur et al., 2003a, 2003b; Basu et al., 2004; El-Assal et al., 2004). In addition, Arabidopsis lines containing T-DNA insertions in Arp2/3 complex members show similar cell morphogenesis phenotypes (Li et al., 2003). In each Arabidopsis Arp2/3 subunit mutant (Arp2, Arp3, ARPC2, and ARPC5), the aberrant cell shapes have been tied to actin defects. Surprisingly, lack of Arp2/3 complex in Arabidopsis does not cause strong phenotypes in tip-growing cells and does not affect the overall growth or developmental pattern of the plant. Because different Arp2/3 complex subunit mutants have given varied phenotypes in other organisms, it is possible that knockouts of other subunits may have stronger tip growth phenotypes in plants. It is also possible that other actin filament nucleators such as formins may make Arp2/3 complex activity redundant in Arabidopsis tip-growing cells.

The moss *Physcomitrella patens* is a multicellular plant with a significantly simpler developmental pattern than that of most higher plants. It consists of two main tissues: leafy gametophores and filamentous protonema, each a single cell layer thick (Cove and Knight, 1993). Tip-growing protonemal filaments are composed of two distinct cell types: chloronema and caulonema. Filaments arise from germinating spores or regenerating protoplasts and initially consist entirely of relatively slow growing chloronemal cells. In response to specific developmental signals, chlornematous cells transition to the more rapidly growing and morphologically distinct caulonemal cell type (Schumaker and Dietrich, 1998). Caulonemal cells can produce side branch initials that are capable of forming either a new filament or a meristematic bud that will grow into a leafy gametophore. Although *P. patens* is an attractive model system because of its predominantly haploidy life cycle and its ability to conduct homologous recombination at a high frequency (Schaefer and Zypri, 1997; Schaefer, 2001, 2002), it is also an excellent system in which to study tip growth because of its abundant supply of tip-growing protonemal filaments.

We present here the identification of *P. patens* Arp2/3 complex subunit ARPC1 and demonstrate its critical role in tip growth. We used a recently developed system for RNA interference (RNAi) in *P. patens* (Bezanilla et al., 2003, 2005) to generate loss-of-function mutants in ARPC1. Silencing of the ARPC1 transcript results in short aberrantly shaped protonemal cells with abnormal cell division patterns. In addition, *arpc1* RNAi lines fail to differentiate caulonemal cells as evidenced by lack of filament growth in the dark as well as by the absence of bud formation. This deficiency in bud formation results in moss without leafy gametophores. *arpc1* protoplasts are defective in their ability to form a polar extension via tip growth and show an increased sensitivity to osmotic shock during regeneration. The tip-growth phenotype of *arpc1* protoplasts is phenocopied in wild-type protoplasts treated with Lat B, an inhibitor of actin polymerization, and is complemented by expression of the full-length ARPC1 cDNA.

**RESULTS**

**Molecular Characterization of ARPC1**

The full-length ARPC1 cDNA clone was identified from a *P. patens* EST library. The ARPC1 cDNA contains a single open reading frame that encodes a predicted 40-kD polypeptide of 380 amino acids. The ARPC1 homologs from a wide array of organisms (Figure 2A) share between 37 and 60% amino acid identity with ARPC1 from *P. patens*. Solid lines beneath sequences in Figure 2A specify regions of highest similarity among the five plant sequences in the alignment. Dashed lines above sequences specify the regions of greatest similarity across all sequences. The lack of overlap between the solid and dashed lines in Figure 2A indicates that plant ARPC1s have many unique conserved sequences. A comparison of the intron/exon patterning between Arabidopsis and *P. patens* ARPC1 reveals a conservation of the intron/exon junctions as well as the number and size of exons between the two species (Figure 2B). Interestingly, although the size of corresponding exons (boxes in Figure 2B) between Arabidopsis and *P. patens* are almost identical, the sizes of introns in *P. patens* ARPC1 are generally larger than those in Arabidopsis. Eleven of the 14 introns were on average 111 bp larger in *P. patens* than in Arabidopsis.

**Expression Analysis of ARPC1**

To determine the expression pattern of ARPC1 in *P. patens*, we performed quantitative RT-PCR analysis on RNA from spore capsules, protoplasts, protonema, and leafy gametophores (Figure 2A). Approximately equal amounts of transcript were detected in protoplasts, protonema, and gametophores with respect to an internal control. Noticeably less transcript was detected in RNA isolated from capsules. We have currently identified a single full-length *P. patens* ARPC1 sequence, although DNA gel blot analysis (see Supplemental Figure 1 online) indicates the presence of a second copy in the genome. This is supported by initial sequence data from the *P. patens* genomic sequencing project (Joint Genome Institute, U.S. Department of Energy), which has revealed partial sequence of a second highly identical ARPC1 gene. It seems pertinent to note that we have been unable to detect ARPC1 by RNA gel

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**Figure 1.** (continued).

(A) Comparison of an ARPC1 amino acid sequence from *P. patens* with those from other organisms. Amino acid residues in black indicate identity, and those in gray indicate conserved substitutions. Solid lines beneath sequences specify a high degree of similarity among plant sequences. Dashed lines across the tops of sequences denote regions of highest similarity across all organisms. The percentage of amino acid identity of ARPC1 from *P. patens* with ARPC1 from other organisms is as follows: 56% (Arabidopsis-A and Oryza sativa), 60% (Arabidopsis-B and Populus trichocarpa), 45% (Homo sapiens), 44% (Fugu rubripes), 40% (C. elegans), 39% (Schizosaccharomyces pombe and Drosophila melanogaster), 37% (S. cerevisiae).

(B) Comparison of the intron/exon patterning of ARPC1 from *P. patens* and Arabidopsis. Boxes represent exons, and lines represent introns. Exons are numbered with roman numerals.
levels in the apical fraction (Figure 2B). Presenilin (PS) from
and the ARP2, as well as arabinogalactan protein-1 (AGP-1),
apical protoplast fraction and a tip-depleted nonapical pro-
pared the expression of several genes between the tip-enriched
fractions of protoplasts enriched for growing tips. We com-
from protonemal filaments gave us the ability to generate
in 
P. patens
bleach analysis, suggesting that it is a low accumulation transcript
in P. patens.

The fact that apical tip protoplasts are the first to be released
from protonemal filaments gave us the ability to generate
fractions of protoplasts enriched for growing tips. We com-
pared the expression of several genes between the tip-enriched
apical protoplast fraction and a tip-depleted nonapical pro-
oplast fraction by RT-PCR. Arp2/3 complex members ARPC1
and ARP2, as well as arabinogalactan protein-1 (AGP-1),
which localizes to apical tips (R.S. Quatrano, personal commu-
nication), all showed at least a threefold increase in transcript
which localizes to apical tips (R.S. Quatrano, personal commu-
nication), all showed at least a threefold increase in transcript

Figure 2. P. patens ARPC1 Expression Analysis.
(A) RT-PCR expression analysis of the ARPC1 transcripts in various
tissues of P. patens. A ubiquitin internal control is shown for each
sample.
(B) RT-PCR analysis of ARPC1 transcript levels in apical versus non-
apical protoplast fractions from P. patens protonemal tissue. A positive
fold change indicates increased expression in the apical fraction, and
a negative fold change indicates an increase in transcript in nonapical
cells. Controls are P. patens genes AGP-1 and PS. Fold change was
calculated as the fold difference in mean band intensity between apical
and nonapical fractions. Each band intensity was adjusted for differ-
ences in a ubiquitin internal control. Bars and error bars indicate means
of 10 experiments ± SE.

Reduction of ARPC1 Transcripts by RNAi
To look at loss of ARPC1 function in P. patens, we used a recently
developed method for RNAi in moss (Bezanilla et al., 2005). This
RNAi technique takes advantage of loss of nuclear green
fluorescent protein (GFP) as an internal control for silencing.

We transformed NLS4, a kanamycin-resistant line that uses the
3SS promoter to uniformly express a β-glucuronidase (GUS)-
GFP fusion in the nucleus, with either an ARPC1 RNAi construct
(containing both ARPC1 and GFP inverted repeats) or a GFP
RNAi control plasmid. Because arpc1 RNAi lines generate
double-stranded RNA that is a fusion of ARPC1 and GFP
sequences, both transcripts should be targeted for degradation.
All RNAi constructs were driven by the ubiquitin promoter and
contained a hygromycin resistance cassette. Silenced trans-
formants were identified by the absence of GFP in their nuclei
and by their hygromycin resistance. Two arpc1 RNAi lines were
isolated from independent transformations that gave stable
phenotypes. However, both lines were subsequently found to
lack kanamycin resistance, the marker for nuclear localization
signal (NLS)-GFP-GUS. This suggested that the ARPC1 RNAi
construct may have integrated into the genome at the NLS-GFP-
GUS locus by homologous recombination. A recombination
event at this locus would not be surprising given that the NLS-
GFP-GUS and RNAi constructs share identical plasmid back-
bones. We verified the elimination of the NLS-GFP-GUS insert
using DNA gel blot analysis (see Supplemental Figure 2 online),
and we confirmed the presence of the RNAi construct by PCR
and by testing the arpc1 RNAi lines for hygromycin resistance
(data not shown). Additionally, we tested the ability of the arpc1
lines to silence a transformed GFP construct driven by the 3SS
promoter using particle bombardment. We found both arpc1 and
gfp RNAi lines silenced a transformed GFP (transformation
confirmed by dsRed cobombardment), while wild-type controls
did not (Figure 3A). This result demonstrates the presence of
a functional and actively silencing RNAi construct in these lines.

We used RT-PCR to show that although the gfp RNAi control
line showed no significant decrease in ARPC1 transcript com-
pared with NLS4, both arpc1 lines were reduced in their ARPC1
transcript levels by >79% compared with the controls (Figure
3B). The low level of ARPC1 transcript shows that the arpc1 lines
represent a knockdown rather than a knockout of ARPC1.
Complete loss of ARPC1 function may be lethal because we
were unable to isolate any lines with greater reduction of ARPC1
transcript.

Phenotypic Analysis of arpc1 Lines
The colony morphology of arpc1 lines is altered due to the
absence of extension growth of protonema as well as the lack of
leafy gametophores (Figures 4A to 4D). arpc1 lines also show
a dramatic reduction in cell length as well as abnormal cell shapes
and division patterns in protonemal filaments. Figures 4A to 4H
illustrate the dramatic difference in arpc1 colony and filament
morphology in comparison with NLS4 or gfp RNAi controls. This
abnormal appearance is due to filaments composed of short
irregularly shaped cells (Figure 4H, arrow). In addition, cell
branching patterns are altered in these lines. We regularly see
two side branches initiated on opposite sides of a single cell to form a t-junction (Figure 4G, arrows). This unusual branching pattern is not present in wild-type filaments (Figures 4E and 4F).

We used Calcofluor staining to better elucidate the individual cell shapes as well as the patterning of cell division. Figures 4M to 4P show fluorescence images of Calcofluor-stained filaments, and Figures 4I to 4L show the corresponding bright-field images. The \textit{arpc1} lines (Figures 4O and 4P) show atypical division patterns with cell walls laid down at irregular intervals as well as in abnormal locations (Figure 4O, arrows) in comparison with controls (Figures 4M and 4N). The arrows in Figure 4P show two side branches initiated from a single initial cell.

Close inspection revealed that \textit{arpc1} lines clearly lacked the buds necessary to form gametophores. Bud formation in \textit{P. patens} is dramatically enhanced in both number and speed by the addition of exogenous cytokinin (Brandes and Kende, 1968). To test the ability of the \textit{arpc1} lines to form buds, we treated the tissue with cytokinin, which strongly induced bud formation. A \textit{gfp} RNAi line produced buds identical to the NLS4 control (Figures 5A and 5B, arrows). \textit{arpc1} RNAi lines lacked buds after 3 d on cytokinin (Figures 5C and 5D). To further test the ability of \textit{arpc1} lines to form leafy gametophores, we treated for 4 d with cytokinin and then transferred to normal media lacking hormone. This type of cytokinin pulse is optimal for producing gametophores because continual exposure to the hormone actually inhibits the development of buds into full gametophores. Although control lines produced abundant gametophores 14 d after transfer to normal media, \textit{arpc1} lines produced none (data not shown). Additionally, \textit{arpc1} tissue was grown for up to 5 months in standard culture media without producing gametophores.

Because it is known that only caulonemal filament cells are capable of producing buds (Schumaker and Dietrich, 1998), we wanted to test for the presence of the caulonemal cell type in our \textit{arpc1} lines. We used the ability of caulonemal but not chloronemal cells to grow in the dark (Cove et al., 1978) as an assay for the presence of caulonemal cells in our RNAi lines. Figures 5E to 5H show moss lines grown in the dark for 17 d. Plates were grown upright to aid visualization because it has been shown that in the absence of light signals, \textit{P. patens} filaments will orient their growth with respect to gravity (Jenkins and Cove, 1983). Although both NLS4 and \textit{gfp} RNAi lines showed substantial growth in the dark (Figures 5E and 5F), neither \textit{arpc1} RNAi line demonstrated any detectable growth (Figures 5G and 5H), indicating the absence of the caulonemal cell type.

**Polar Growth in \textit{arpc1} Lines**

By definition, polar tip-growing cells restrict their elongation to the very apex of the cell (Heath, 1990). To determine whether \textit{arpc1} lines were inhibited in their ability to undergo polar tip extension, we used time-lapse video microscopy to monitor the growth of apical protonemal filament cells. Representative time-lapse images (Figure 6A) demonstrate the dramatic reduction in growth observed in \textit{arpc1} tip-growing cells compared with wild-type chloronemal tips. To quantitate the difference in growth observed between wild-type and \textit{arpc1} RNAi tip cells, we calculated the growth rate of apical cells (Figure 6B) in both lines. We found a significant reduction in the growth rate of \textit{arpc1} tip cells (1.9 m m/h) in comparison with wild-type chloronemal tips (9.0 m m/h).

Because \textit{arpc1} lines were found to be defective in tip growth, we wanted to examine the ability of these lines to initiate apical growth. Regenerating protoplasts provide an excellent model to study establishment of a polar axis followed by the initiation of a polar filament outgrowth (Cove et al., 1996). We found that \textit{arpc1} protoplasts failed to establish a proper polar outgrowth after 4 d of regeneration (Figure 7), whereas NLS4 and \textit{gfp} RNAi control lines had already initiated a filament of four cells in...
length. *arpc1* RNAi lines undergo cell divisions (Figure 7, arrows) but are lacking the polar outgrowth seen in the control lines. After transferring the regenerating protoplasts from mannitol to normal growth media at the end of day four, we were surprised to find that by day six, 99% of the mutant protoplasts were dead, whereas the NLS4 control showed no noticeable drop in viability (data not shown). This widespread mortality of the *arpc1* lines indicates an inability of these lines to absorb the osmotic shock associated with the transfer to regular media. Of the surviving *arpc1* regenerants, all continued to divide. However, cell shape and size were drastically altered in comparison with the wild type after 8 d of regeneration (Figure 7).

To determine the role of actin in the observed *arpc1* protoplast regeneration defects, we looked at the effect of Lat B, an inhibitor of actin dynamics, on regenerating protoplasts. Wild-type protoplasts were overlaid with a 100 μm Lat B solution at 1 d (Figure 8A, panel b) or 5 d (Figure 8A, panel f) after protoplasting and photographed 3 d after Lat B treatment. Treatment of wild-type protoplasts with lower concentrations of Lat B did not noticeably affect growth (data not shown). *arpc1* lines (Figure 8A, panels c, d, g, and h) and a wild-type control (Figure 8A, panels a and e) were treated with the same concentration of DMSO. Although DMSO treatments did not seem to have any significant effect on protoplast regeneration, Lat B–treated wild-type protoplasts closely resembled DMSO-treated *arpc1* protoplasts at both 4 and 8 d after the release of protoplasts. Both Lat B–treated wild-type protoplasts and *arpc1* control protoplasts failed to regenerate a proper polar filament outgrowth at 4 d and were characterized at 8 d by chains and clumps of short irregularly shaped cells. We found that transfer of protoplasts to fresh media lacking Lat B reversed the inhibition of tip elongation within 24 h (data not shown).

To test the specificity of the *arpc1* phenotypes, we transformed RNAi protoplasts with an ARPC1 overexpression construct driven by the maize ubiquitin promoter. We found that ARPC1 overexpression strongly complemented the RNAi protoplast regeneration phenotype (Figure 8B, panels c and g) and resulted in elongated, branched filaments lacking any of the morphological defects observed in the *arpc1* lines. Additionally, overexpression of ARPC1 rescued the sensitivity to osmotic

**Figure 4.** Morphological and Cell Division Phenotypes of *P. patens arpc1* Lines.

Images demonstrate the phenotypic differences among an untransformed NLS4 control, a *gfp* RNAi control, and two *arpc1* lines. (A) to (D) Colony morphology. Bars = 5 mm. (E) to (H) Filament morphology. Arrows indicate irregular branching patterns (G) and abnormal cell shapes (H). Bars = 0.2 mm. (I) to (L) Cell morphology. Bars = 50 μm. (M) to (P) Fluorescent images of (I) to (L) stained with Calcofluor. Arrows in (O) and (P) denote abnormal cell divisions. Bars = 50 μm.
shock clearly observed in RNAi protoplasts (data not shown). By contrast, arpc1 lines transformed with a control plasmid retained a clumped morphology (Figure 8B, panels d and h) and failed to undergo the rapid tip extension characteristic of the wild type (Figure 8B, panels a, b, e, and f).

**DISCUSSION**

**Characterization of ARPC1 in P. patens**

We show that P. patens contains the 40-kD Arp2/3 complex subunit ARPC1 and demonstrate that it is has a similar amino acid content to ARPC1 proteins from a set of widely divergent organisms (Figure 1). The identification of seven WD40 repeat domains within the P. patens amino acid sequence using conserved domain BLAST (Marchler-Bauer et al., 2003) provides additional support for the identification of this protein as an ARPC1 (data not shown). All ARPC1 proteins identified to date have been shown to contain seven WD40 repeats that based upon the crystal structure of the mammalian Arp2/3 complex are known to fold into a seven-bladed β-propeller (Robinson et al., 2001). Although WD40 repeat-containing proteins range widely in their functionality, they are known primarily for their involvement in protein–protein interactions (van Nocker and Ludwig, 2003; Madrona and Wilson, 2004). These repeats may therefore be critical for ARPC1’s interactions with other proteins that may bind the Arp2/3 complex. Neer et al. (1994) point out that despite the wide variety of WD40 repeat-containing proteins, the majority may be said to have regulatory functions. In light of recent evidence from the yeast S. cerevisiae demonstrating ARPC1’s strong interaction with the VCA domain of WASP/SCAR-family Arp2/3 complex activators, it is tempting to predict a possible role for ARPC1’s WD40 repeats in this regulatory interaction (Pan et al., 2004). The recent identification of four Arabidopsis SCAR homologs containing VCA domains capable of activating bovine Arp2/3 complex in vitro raises the possibility for an ARPC1–VCA interaction in plants (Frank et al., 2004; Basu et al., 2005). It is interesting to note that the plant proteins identified as containing a VCA domain all have a conserved Trp in the acidic region that was shown to be necessary for efficient binding of ARPC1 to VCA in yeast (Pan et al., 2004). Although the domain(s) of ARPC1 necessary for interaction with VCA-containing proteins are not known, the presence of sequences unique to plant ARPC1 (Figure 1A, solid lines) provide possible candidate domains for plant-specific interactions.

**Role of ARPC1 in Tip Growth**

We used RNAi interference to study loss of ARPC1 function and to examine the role of ARPC1 in several tip growth–related processes in moss filaments that are likely influenced by actin dynamics. These include establishing a polar axis, polar tip (extension) growth, cell division, filament branching, and the switch from two- to three-dimensional growth (i.e., bud formation).

We found arpc1 protoplasts to be defective in their ability to extend a polar outgrowth during protoplast regeneration (Figure 7). To regenerate properly, protoplasts from P. patens must successfully define and fix a polar axis before extending a polar outgrowth via tip growth (Cove et al., 1996). arpc1 lines were clearly defective in this important step, which suggested a specific role for ARPC1, and thus the Arp2/3 complex, in polarized cell extension.
Interestingly, a high percentage (99%) of protoplasts generated from arpc1 lines were unable to withstand the osmotic shock associated with transfer from protoplast regeneration media (containing mannitol) to normal growth media. This high level of sensitivity to osmotic shock suggests defects in cell wall architecture or composition in these lines. An altered cell wall in arpc1 lines would not be surprising given the known role of actin in the exocytotic deposition of cell wall components during tip growth (Wasteneys and Galway, 2003).

The complementation of the arpc1 protoplast regeneration phenotype by ARPC1 overexpression (Figure 8B) demonstrates that the observed phenotype indeed results from loss of ARPC1 function. Additionally, this result reveals that the observed RNAi phenotypes are specific to the ARPC1 gene.

In addition to problems with protoplast regeneration, arpc1 lines exhibit cell growth defects in protonemal filaments. Although chloronemal filaments form, cells within these filaments are abnormally short with aberrant branching and cell division patterns. In addition, tip-growing cells in these lines elongate at a significantly reduced rate compared with the wild type. Most interesting, however, was the observation that arpc1 lines were found to be blocked in their ability to differentiate caulonemal filament cells as evidenced by lack of growth in the dark and absence of bud formation, even in response to exogenous cytokinin treatment. The lack of this critical developmental transition to the rapidly elongating, branching filament stage results in no buds and, hence, no leafy gametophores. This blockage also has the effect of preventing sporophyte formation and, thus, completion of the life cycle (Figure 9).

**Actin and the arpc1 Phenotype**

We found that treatment of wild-type protoplasts with Lat B closely phenocopied the protoplast regeneration phenotype of arpc1 lines (Figure 7). The ability of an inhibitor of actin polymerization to copy the arpc1 phenotype provides strong evidence for the fact that the observed RNAi phenotypes do in fact result from some disruption of the ability of the Arp2/3 complex to promote actin polymerization in these lines. Additionally, the

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**Figure 6.** Growth Rate Is Reduced in arpc1 Apical Cells.

(A) Time-lapse images illustrating the reduction in growth in arpc1 apical cells compared with wild-type chloronemal apical cells over a 4-h period. Blue arrows indicate the original position of the tip.

(B) Growth rate of wild-type chloronemal tip cells and arpc1 tip cells. Error bars indicate SE. The difference in growth rate between the two samples is statistically significant as determined by t test analysis (P < 0.0001) (n = 10).

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**Figure 7.** arpc1 Lines Are Defective in Proper Protoplast Regeneration.

All images represent structures regenerated from a single-celled protoplast. NLS4 and gfp RNAi controls as well as arpc1 lines are shown at 4 and 8 d of protoplast regeneration. NLS4 and gfp RNAi controls show polar outgrowths, and arpc1 RNAi lines divide (arrows) but without polar extension. Bars for 4 d = 50 μm; bars for 8 d = 100 μm.
inhibition of tip growth in \textit{arpc1} lines implicates the important role that dynamic actin likely plays at the cell apex. Interestingly, treatment of \textit{arpc1} protoplasts with Lat B did not alter the existing RNAi phenotypes, thus suggesting that actin polymerization is the main target of ARPC1 function.

It is important to note that disruption of Arp2/3 complex–mediated polymerization does not necessarily correspond to gross defects in the organization of the actin cytoskeleton. We observed no difference between the patterning of actin microfilaments in \textit{arpc1} and wild-type protonema (data not shown). Although this may be surprising given the large body of work correlating Arp2/3 complex subunit mutants with defects in the actin cytoskeleton, this result likely reflects the action of the Arp2/3 complex on a very specific subset of dynamic actin filaments in \textit{P. patens}. The recent localization of a fluorescently tagged version of the endogenous ARPC4 in \textit{P. patens} supports this notion. In protonemal filaments, ARPC4 was weakly expressed and was found to be restricted to the extreme apex of growing tips (P.F. Perroud, personal communication). This is supported by our finding that ARPC1 transcript is enriched in apical cells. Given the localization of ARPC4, it seems very plausible to suggest that in \textit{P. patens} protonemal filaments and possibly in all plant tip-growing cells, the Arp2/3 complex may function to regulate the actin dynamics of a small subset of filaments adjacent to the tip, leaving the general structure of the actin cytoskeleton unperturbed. Although the localization of actin filaments to the apex of tip-growing cells has been a matter of debate (Perdue and Parthasarathy, 1985; Miller et al., 1996; Kost et al., 1998), the presence of the Arp2/3 complex in this region certainly seems to confirm the existence of dynamic actin at the tip. Additionally, our finding that tip elongation is significantly reduced in \textit{arpc1} lines reiterates the apparent importance of dynamic actin at the cell apex. Although the precise role that actin dynamics may play at the tip remains unknown, it is tempting to speculate that the polymerization of actin at the apex may act to drive membrane extension as is known to be the case in motile animal cells (Pollard et al., 2001; Mathur, 2005). However, the presence of the cell wall in plants may preclude this possibility. Alternatively, actin polymerization at the tip may be in some way required for efficient positioning or activity of the cell’s exocytic machinery. Whatever the role of dynamic actin in tip growth, the strong \textit{arpc1} morphological defects emphasize its importance.

Differential Subunit Function

It will be interesting to determine whether reduction or loss of other Arp2/3 complex subunits in \textit{P. patens} give similar or
related phenotypes to those observed in *arpc1* lines. It is possible that loss of different subunits may give differing severities of phenotypes. This may not be surprising given the fact that individual subunits likely have specialized roles within the complex. It is known in budding yeast, for example, that ARPC1 may be critical for strong interactions with Arp2/3 complex activating proteins (Pan et al., 2004). Additionally, it was recently observed in Drosophila bristles that a mutation in ARPC1 actually acts to enhance the phenotypes associated with a capping protein mutant. This is in contrast with loss of other Arp2/3 complex subunits that act to suppress the capping protein phenotypes (D. Frank, R. Hopmann, and K. Miller, personal communication).

Although no ARPC1 mutants have been previously studied in seed plants, several Arp2/3 complex subunit mutants have been characterized in Arabidopsis (Le et al., 2003; Li et al., 2003; Mathur et al., 2003a; El-Assal et al., 2004; Saedler et al., 2004). These mutants show changes in fine F-actin structures resulting in dramatic cell shape defects in trichome and pavement cells as well as problems with cell adhesion in elongating hypocotyl epidermal cells. The fact that the Arp2/3 complex is required for proper growth in these cell types has revealed an unexpected role for the complex in cells where expansion is known to be driven by turgor pressure. In these cells, shape is determined by the composition of the cell wall and its ability to contain the cell’s internal pressure. This correlates well with our results showing increased sensitivity of *arpc1* protoplasts to osmotic shock because both turgor-driven expansion and osmotic sensitivity are dependent upon the competing forces of internal cellular pressures and cell wall strength and rigidity.

Surprisingly, Arabidopsis tip-growing cells appear relatively insensitive to loss of Arp2/3 complex subunits. In Arp2, Arp3, and ARPC5 mutants, root hairs show relatively minor growth defects when challenged to rapidly elongate (Mathur et al., 2003a, 2003b). Pollen tube defects have not been noted in any of the Arabidopsis Arp2/3 mutants to date. In *P. patens*, where the major growth is filamentous via tip growth, the strong phenotypes exhibited in the *arpc1* lines visibly underscore the importance of Arp2/3-dependent actin polymerization in the process of tip growth. It is possible that the milder tip-growth phenotypes in Arabidopsis cells are due to some functional redundancy of Arp2/3 complex members or other actin nucleators such as formins. Arabidopsis formin, for example, was recently shown to be a regulator of actin-dependent growth in pollen tubes (Cheung and Wu, 2004). It will be interesting to see whether ARPC1 mutants will show a stronger phenotype in tip-growing Arabidopsis cells than the other Arp2/3 subunit mutants identified to date. If Arabidopsis ARPC1 proves to be more critical for key cell elongation steps in pollen tubes and root hairs than other Arp2/3 subunits, this might denote some specialized function for this subunit either within or apart from the Arp2/3 complex.

**Table 1. Primer Sequences Used in This Study**

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<thead>
<tr>
<th>Sequence Amplified</th>
<th>Primer 1</th>
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<tbody>
<tr>
<td>ARPC1 genomic probe</td>
<td>5’-AGCGCTGTGCAACATCT-3’</td>
<td>5’-CGTTGCTCATACTTTGCTAC-3’</td>
</tr>
<tr>
<td>NPTII PCR probe</td>
<td>5’-ACAGTCGATGAATCCAGAA-3’</td>
<td>5’-TTAATTGAATGAAATCGCGTCGTCGATG-3’</td>
</tr>
<tr>
<td>ARPC1 RT-PCR fragment</td>
<td>5’-GCTAGAACAATTGGTTTCCG-3’</td>
<td>5’-GAAACGACATACGCGAGGG-3’</td>
</tr>
<tr>
<td>Ubiquitin RT-PCR fragment</td>
<td>5’-AGTTCTTGGCAAGTCGCGAGG-3’</td>
<td>5’-CTGGTGACATACACGACGAG-3’</td>
</tr>
<tr>
<td>Arp2 RT-PCR fragment</td>
<td>5’-GAAACGCTCATTGTTTGCGA-3’</td>
<td>5’-AGTCAGGAGGATACCGTTCAG-3’</td>
</tr>
<tr>
<td>AGP-1 RT-PCR fragment</td>
<td>5’-ACAGAGCAGTAGATTGTTTCA-3’</td>
<td>5’-TCAGAAAAACAGATAGTGGACAT-3’</td>
</tr>
<tr>
<td>PS RT-PCR fragment</td>
<td>5’-ACGGTACGTTAAGCCC-3’</td>
<td>5’-AGTTAAATATGACGTAGACGCAC-3’</td>
</tr>
<tr>
<td>ARPC1 RNAi fragment</td>
<td>5’-AGCGCTGTGCAACATCT-3’</td>
<td>5’-CGTTGCTCATACTTTGCTAC-3’</td>
</tr>
</tbody>
</table>

**Figure 9. Developmental Differences in arpc1 Lines.**

Illustration of typical *P. patens* developmental progression from a single-celled spore or protoplast to a mature colony with leafy gametophores. Center and left columns represent the wild-type life cycle. The right column shows *arpc1* RNAi phenotypes at various developmental stages. Red Xs indicate blockages, and the red dash denotes inhibition of particular developmental processes.
RT-PCR Analysis
RNA from various tissues was isolated using the RNeasy plant mini kit (Qiagen, Valencia, CA). Spore capsules were mature and contained spores. Protoplasts were generated from 1-week-old protonemal filamentary tissue by standard methods (Schaefer et al., 1991). Five micrograms of total RNA was treated with five units of amplification grade DNase I (Invitrogen, Carlsbad, CA). One microgram of DNase-treated RNA was then used to generate cDNA with Invitrogen’s Thermoscript RT-PCR system. Oligo(dT) nucleotides were used for the first-strand cDNA synthesis. A 1/50 volume of the cDNA was subsequently used as template for PCR. The primers used for PCR are listed for each gene in Table 1. To optimize each primer set to be in the linear range for amplification, the following standard PCR program was modified for annealing temperature (X), extension time (Y), and cycle number (Z) depending on primer set used: (A) 94°C 30 s initial denaturation, (B) 94°C 30 s denaturation, X°C 30 s annealing, 68°C Y s extension. Part (B) was repeated for Z number of cycles. For ARPC1, X = 54, Y = 60, Z = 32; for Arp2, X = 55, Y = 30, Z = 31; for Arp4, X = 52, Y = 60, Z = 32; for ATP8, X = 55, Y = 60, Z = 31; for ubiquitin, X = 57, Y = 30, Z = 30. All PCR was performed with Elongase Enzyme Mix (Invitrogen). TIF images of DNA gels were taken with a Gel-Doc imager (Bio-Rad, Hercules, CA). ImageJ (http://rsb.info.nih.gov/ij/) software was used to determine band intensities for comparison across all samples. All intensities were adjusted for differences in the ubiquitin internal control between apical and nonapical samples. Percentage of expression was determined by calculating each line’s mean RT-PCR band intensity as a percentage of the highest ARPC1 expressing line (GFP-RNAi) (Figure 5).

Moss Transformation and Protoplasting
Moss protoplast transformation, growth, and isolation of transgenic moss was performed as described previously (Schaefer et al., 1991). Transformed lines were assayed for stability by blending tissue and plating onto two plates containing cellophane and media without selection. After the second wash, protoplast pellets were flash frozen in liquid nitrogen and stored at −80°C. Nonapical protoplasts were generated by transferring the tissue retained on the filter used for the apical protoplast isolation into fresh Driselase. The digestion was then continued for 45 min at room temperature. Nonapical protoplasts were filtered, washed, and stored identically to the apical fraction. Transient transformation by particle bombardment was performed as previously described (Bezanilla et al., 2003).

DNA Constructs
We amplified a 500-bp fragment of ARPC1 cDNA with primers that added an AvrII site to the 5′ end and a PacI site to the 3′ end of the amplified fragment (Table 1). A second identical 500-bp fragment was amplified with SnaBI restriction sites on both ends. These two fragments were cloned into pUFI RNAi vector (Bezanilla et al., 2005) on either side of the GUS loop to form inverted repeats. The orientation of the fragments in pUFI was confirmed by sequencing. For overexpression, the full ARPC1 cDNA was amplified with primers that added a Nol site on the 5′ end and a HindIII site on the 3′ end. The resulting fragment was cloned in pMKubi vector containing the maize (Zea mays) ubiquitin promoter.

DNA Gel Blot Analysis
Total genomic DNA was isolated using the Nucleon Phytopure DNA extraction kit (Amersham Biosciences, Piscataway, NJ). One microgram of DNA was digested and run on a 0.8% agarose gel (Seakem LE agarose; Cambrex Bio Science, East Rutherford, NJ) and transferred to a nitrocellulose membrane by standard methods (Sambrook and Russel, 2001). ARPC1 genomic probe was prepared using the DIG probe synthesis kit with Expand Long Template Enzyme Mix (Roche, Indianapolis, IN). The NPTII probe was similarly prepared but did not require the use of Long Template Enzyme Mix. Primers used to amplify a 3-kb internal portion of the ARPC1 genomic sequence and a 0.6-kb portion of the NPTII gene are listed in Table 1. Hybridization washes and detection were performed following the manufacturer’s recommendations.

Cytokinin and Lat B Treatment
Cytokinin treatment was conducted by transferring cellophane containing 6-d-old homogenized tissue to standard media supplemented with 3 μM benzylaminopurine (Sigma-Aldrich). Protoplasts were prepared and embedded in top-agar on cellophane disks placed on the appropriate growth medium as described previously (Schaefer et al., 1991). Lat B was prepared by diluting a 1 mM Lat B stock (dissolved in 100% DMSO) to 100 μM with water. Five hundred microliters of 100 μM solution was added to the top of each plate and dispersed evenly.

Microscopy
All images were acquired on either a Zeiss compound microscope (Jena, Germany) or an Olympus dissecting microscope (Tokyo, Japan), each capable of fluorescence imaging. Filters suitable for viewing calcofluor fluorescence or GFP were used to image cell walls and bombarded GFP, respectively. For time-lapse microscopy, protonemal tissue was grown overnight under continuous light in a modified tissue cell containing liquid medium (Ashton and Cove, 1977). Images were collected every 20 min. All images were captured using a SPOT RT Slider camera (Diagnostic Instruments, Sterling Heights, MI) and were processed using ImageJ and Adobe Photoshop software (Mountain View, CA).

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers AAN18136 (Arabidopsis-A), AAC26262 (Arabidopsis-B), XM_468407 (O. sativa), AY156053 (P. patens), Q29747 (H. sapiens), CA730207.1 (S. pombe), NP_000793.1 (S. cerevisiae), CAB54510 (C. elegans), C08578-PA (D. melanogaster), gracilis.0012007101 (P. trichocarpa, Joint Genome Institute),
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