

The Role of ARPC4 in Tip Growth and Alignment of the Polar Axis in Filaments of *Physcomitrella patens*

Pierre-François Perroud and Ralph S. Quatrano*

Department of Biology, Washington University in St. Louis,
St. Louis, Missouri

When the actin related protein 2/3 (Arp2/3) complex member *arpc4* was deleted in *Physcomitrella patens* (moss), the resulting null mutant ($\Delta arpc4$) was viable and revealed no gross changes during morphogenesis of filaments into gametophores. However, we observed a striking reduction of filamentous tip growth, resulting in smaller, denser colonies. Although polar responses of $\Delta arpc4$ filaments to unilateral white light were unaffected, these mutant filaments were defective in their response to polarized white light. These observations strongly suggest a specific role of the Arp2/3 complex as a downstream target for signals regulating oriented tip growth. Insertion of YFP-ARPC4 into $\Delta arpc4$ rescued the mutant phenotypes and localized ARPC4 exclusively to the tip cell of filaments, the site of actin dynamics and polarized growth. The ability of $\Delta arpc4$ to perform some but not all cellular responses will allow the study of its function in orientation of tip growth in response to directional cues (e.g. light) in a viable but mutated background. Cell Motil. Cytoskeleton 63:162–171, 2006. © 2006 Wiley-Liss, Inc.

Key words: Arp2/3 complex; moss; cytoskeleton; actin; polarity

INTRODUCTION

The actin related protein 2/3 (Arp2/3) complex plays a crucial role in shaping the actin cytoskeleton [Mullins et al., 1998]. Its localization at the leading edge of many extending pseudopods in a variety of animal cell types reflects the importance of actin dynamics for directed cell movement [Higgs and Pollard, 2001]. Although plant cells are clearly different, they undergo a similar form of extension growth, with dynamic actin localized at the leading tip of single-celled filaments found in algae, bryophytes, and certain cells of seed plants, e.g. root hairs, pollen tubes [Fowler and Quatrano, 1997; Mathur, 2005]. This type of tip growth and its orientation to environmental cues require actin dynamics [Quatrano, 1997; Staiger, 2000; Cove and Quatrano, 2004]. For example, actin plays a key role in the establishment of a polar axis as well as the orientation of tip growth in response to unilateral light in zygotes of the brown algae [Quatrano, 1997] and in regenerating protoplasts of the moss *Ceratodon purpureus* [Cove et al., 1996].

However, the possible role and intracellular localization of the Arp2/3 complex, or any of its seven members, in tip growth and its orientation has not been directly investigated. Although the plant Arp2/3 complex is not characterized, all subunits of the complex can be found in the genomes of Arabidopsis (<http://www.arabidopsis.org/>), rice (<http://www.shigen.nig.ac.jp/rice/oryzabase/top/top.jsp>), and the moss *Physcomitrella*

The supplemental materials described in this article can be found at <http://www.interscience.wiley.com/jpages/0886-1544/suppmat>

Contract grant sponsor: The National Science Foundation; Contract grant number: (IBN-0112461).

*Correspondence to: Ralph S. Quatrano, Ph.D., Department of Biology CB#1137, 1 Brookings Drive, Washington University, St. Louis, MO 63130-4899, USA. E-mail: rsq@wustl.edu

Received 19 September 2005; Accepted 14 December 2005

Published online 31 January 2006 in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cm.20114

patens (<http://www.mossgenome.org>). Furthermore, members of the Scar/WAVE family of proteins from maize [Frank et al., 2004] and Arabidopsis [Basu et al., 2005] can activate bovine Arp2/3 complex [Mathur, 2005; Szymanski, 2005].

Extensive analysis has been documented for T-DNA insertional mutants in most members of the Arabidopsis Arp2/3 complex [Li et al., 2003; Mathur, 2005; Szymanski, 2005]. Phenotypes of these lines belong to the DISTORTED class of mutants that are characterized as exhibiting cell shape and cell expansion defects in epidermal cells, as well as in actin filament organization [Mathur, 2005; Szymanski, 2005]. All of these mutants are viable and set seed since their defects are exhibited in intracellular processes only in selected cell types. However, no member of the Arp2/3 complex has been localized in Arabidopsis to a cellular site that would suggest a role of the Arp2/3 complex or its members in the affected processes. However, ARP2 and ARP3 have been immunolocalized to the tips of algal rhizoids [Hable and Kropf, 2005] and root hairs of tobacco and maize [van Gestel et al., 2003], but there was no specific loss of function mutant in these genes to confirm the role of these proteins in tip growth. The *in vivo* localization of any Arp2/3 member has not been reported in any plant system.

Our interest is in the process whereby polar tip growth can be oriented and aligned to directional cues such as light and gravity [Quatrano, 1997; Cove and Quatrano, 2004]. We chose *P. patens* as our experimental system not only because its filaments undergo polarized tip growth and respond to various directional cues [Cove et al., 1997], but also for its ability to be transformed and to undergo efficient homologous recombination [Schaefer, 2002]. Furthermore, we can observe and manipulate tip growth more directly since these individual filaments arise directly from single regenerating protoplasts. Our hypothesis is that since actin is required for tip growth in *P. patens* [Harries et al., 2005], an Arp2/3 complex or any of its putative member(s) are targets for the signaling pathway that would link a signal (e.g. light) to the response (e.g. polar tip growth).

To determine the role of the Arp2/3 member proteins (and indirectly the complex), our approach was to use targeted gene disruption and RNAi [Bezanilla et al., 2005] to delete a gene or reduce the level of a specific mRNA(s), respectively. We have shown that RNAi lines with reduced transcript levels for the multicopy *ARPC1* gene in *P. patens* show severe tip growing phenotypes, supporting in part our hypothesis. However, these RNAi lines were unable to develop into the rapidly elongating caulonemal filaments as well as the three-dimensional bud and leafy gametophores. Because of this severe phenotype, we were unable to adequately assess the role of

the Arp2/3 complex in polar axis orientation through ARPC1 [Harries et al., 2005].

In this article, we targeted the single-copy *arpc4* gene for deletion because both the model of Arp2/3 complex assembly [Zhao et al., 2001] and the *in vivo* reconstitution experiments of this complex [Gournier et al., 2001] place this subunit at the initial step of Arp2/3 complex assembly. Rogers et al. [2003] also targeted ARPC4 in *D. melanogaster* S2 cells by RNAi that resulted in abnormal lamella formation. Hence, its selective and complete removal by targeted gene deletion should prevent the formation of the complex and result in a loss of its function. We demonstrate here that deletion of the *arpc4* dramatically reduces tip growth of the caulonemal filaments but does not disrupt the transition from two-dimensional filamentous growth to three-dimensional growth of buds and leafy shoots. Homologous recombination allowed us to insert an YFP-tagged wild type *arpc4* gene (*yfp-arpc4*) into the deleted line ($\Delta arpc4$), to show its complementation and then to visualize the specific and exclusive localization in living cells of ARPC4 to the tip cell of the elongating caulonemal filament. Furthermore, $\Delta arpc4$ lines were unable to properly orient their polar axis in polarized light. These results with ARPC4 in *P. patens* support our hypothesis that a member(s) of the Arp2/3, or the complex itself, plays a pivotal role in the translation of directional cues into polar growth.

MATERIALS AND METHODS

Plant Material, Culture Conditions, and Treatments

Physcomitrella patens (Gransden wild type) tissue was placed on cellophane disks overlaying 0.7% agar (Plant Cell Culture Agar, Sigma; A-9799) in Petri dishes (9 cm) containing minimal medium and 0.5 g/l (di)ammonium tartrate. These cultures were grown at 25°C under a long day/light cycle [16 h light ($130 \mu\text{mol m}^{-2} \text{s}^{-1}$) and 8 h dark]. Hygromycin (25 mg/l; Sigma, St. Louis, MO) was added to the media to select for antibiotic-resistant cells. Phenotypic analyses were completed on minimal media without addition of (di)ammonium tartrate, while protoplast regeneration assays under polarized white light were completed on minimal medium [Ashton and Cove, 1977] plus 0.5 g/l (di)ammonium tartrate and 8.5% mannitol. Polarized light was generated using a polarizer grey filter (Edmund Scientific, Tonawanda, NY). The fluency under polarization was set at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ for high light condition and $5 \mu\text{mol m}^{-2} \text{s}^{-1}$ for low light condition.

Molecular Procedures

All PCR cloning was performed using the pGEM Teasy kit (Promega, Madison, WI). RNA was routinely

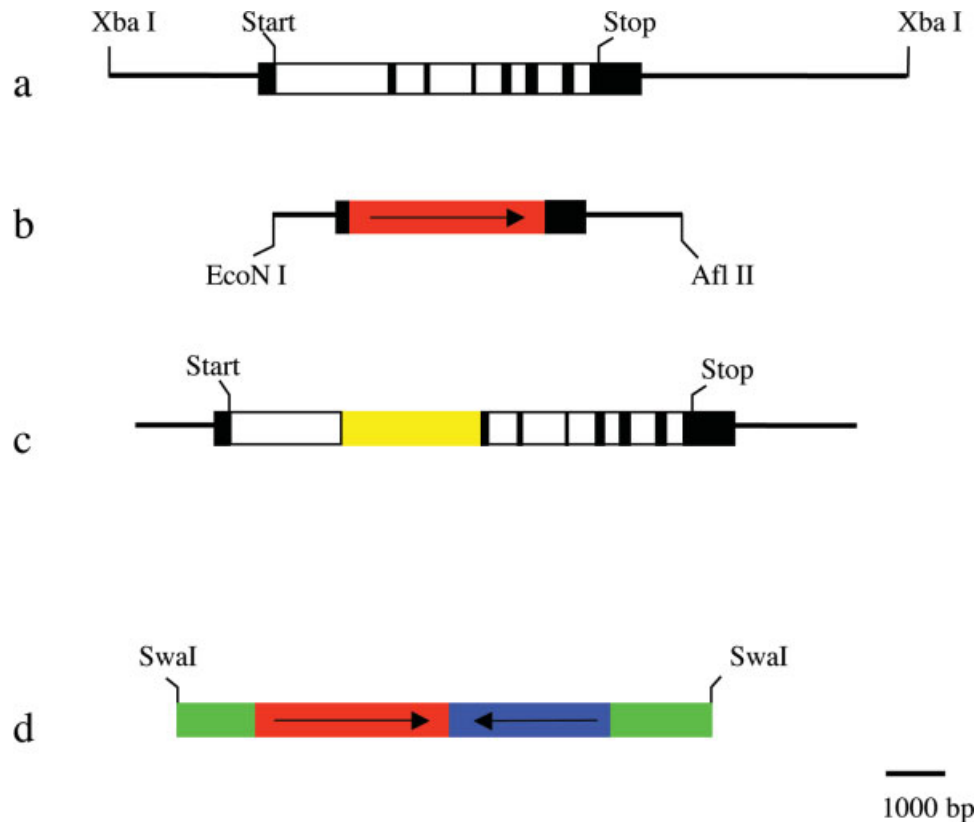


Fig. 1. Schematic representations of the ARPC4 locus (*arpc4*) and the transformation vectors used in this study. (a) Wild type genomic locus of ARPC4. Black boxes represent exons, white boxes introns, while the line corresponds to the 5' and 3' flanking regions of the gene. (b) Full deletion vector. The solid black boxes correspond to the 5' and 3' transcribed regions of the ARPC4 gene (a), with the open reading frame completely removed and substituted by a resistance cassette

(red) consisting of *Lox* sites flanking a 35S hygromycin gene and the Cauliflower Mosaic Virus terminator (see Materials and Methods). (c) Gene tagging vector. Two copies of YFP gene (yellow box) have been inserted at this 5' site of the ARPC4 genomic locus (a). (d) Over-expressing complementation vector. Genomic sequences of the 108 targeting locus (green) are flanking the resistance cassette (red) and the cDNA expression cassette *OsACT1::PpARPC4* (blue).

isolated from a 1-week-old culture of *P. patens* using RNeasy Plant Mini Kit (Quiagen, Valencia, CA). *P. patens* genomic DNA was isolated from tissue using the Nucleon Phytopure Plant DNA Extraction Kit (Amersham Biosciences, UK). *P. patens* ARPC4 cDNA (*PpARPC4*) was isolated using the 3'RACE system for rapid amplification of cDNA ends (Invitrogen). Two nested degenerated primers dP1 (5' CAGAAATTYCCYTGYCAAGARGT-3') and dP2 (5'-AAGGIAAGCARGCIGAYGARYTDGA-3'), designed from a eukaryotic ARPC4 cDNA nucleotide sequence alignment, resulted in the isolation of a partial *PpARPC4*. A subsequent round of 5'RACE, using specific nested primers P3 (5'-CCGTAAGTACAATTAGCGGGA-AATTGACC-3') and P4 (5'-TGACCTACAAATCGGGGAGGGTGAC-3'), resulted in the full-length cDNA sequence (*PpARPC4*). Partial genomic sequence of *arpc4* was determined by PCR on *P. patens* genomic DNA using specific primer gP5 (5'-TGACCTACAAATCGGGGAGGGTGAC-3') and gP6 (5'-ATGGTTGGAGAACATTCTAGC-3') designed on *PpARPC4*. This subsequent genomic fragment was used to screen a BAC library. A resulting 8.41 kb *Xba*I

restriction fragment from a BAC clone contained the *arpc4* which included a 1.5 kb of 5' sequence, the full open reading frame (ORF) of *PpARPC4*, and 3.5 kb of 3' sequence. This *Xba*I genomic fragment (*arpc4*) is used as the reference for different constructs and probes used in this study (Fig. 1a). Southern analysis was routinely performed with 1.0 μ g of genomic DNA per digestion. After digestion with specific restriction enzymes, the reactions were resolved on a 0.6% agarose gel and transferred to N-Hybond (Amersham Biosciences, UK). Hybridization and detection were performed with the nonradioactive method DIG (Roche Diagnostic, Mannheim, Germany). The PCR DIG synthesis kit (Roche Diagnostic) was used to make probes. Following the gene specific hybridization, the membrane was always stripped and tested for the presence of nonspecific plasmid insertion with a probe designed for the resistance gene.

Transformation Vector Constructs

The gene deletion vector (Fig. 1b) was designed to remove the full ORF of *arpc4*. The resistance cassette,

Lox-355::hphCaMV-Lox, is flanked by regions 683–1732 and 5970–6894 of the *XbaI* fragment. Double crossing-over with this construct would result in the total deletion of the *arpc4* ORF. Overexpressing complementation vector is constructed to overexpress *PpARPC4* (Fig. 1d). *PpARPC4* cDNA is subcloned behind the Act1-F fragment of the rice actin 1 promoter [McElroy et al., 1990]. This overexpressing cassette is incorporated in a vector containing the hygromycin resistance cassette and the 108 genomic locus used as targeting sequence [Schaefer and Zrýd, 1997]. The tagging vector (Fig. 1c) was constructed to tag and complement the mutant obtained with the full deletion vector. It contained the region 683–6894 of the *XbaI* genomic *arpc4* sequence into which had been inserted the 2XeYFP coding sequence (position 2937). This vector did not contain any resistance cassette and therefore a visual screen was required to determine whether complementation occurred.

Transformation of *P. patens*

PEG-mediated protoplast transformation was carried out as described by Schaefer and Zrýd [1997]. To increase the efficiency of transformation, each construct was linearized with appropriate restriction enzymes; *AflIII* and *EcoNI* for the full deletion vector (Fig. 1b) and *SwaI* for an overexpressing vector (Fig. 1d). Alternatively, the tagging construct has been generated by a PCR (Fig. 1c). To eliminate any episomal resistant colony [Ashton et al., 2000], two rounds of selection were undertaken using the appropriate antibiotic. Resistant colonies were then homogenized and plated in duplicate. After 1 week of growth, one of the two cellophane pads was transferred to a medium containing the antibiotic while the other pad remained on antibiotic-free medium. After an additional week of growth, any difference in growth of a specific clone of tissue on these different media resulted in that specific clone being discarded (i.e. a nonintegrated clone). Removal of the selection cassette was achieved by the transient expression of the Cre recombinase [Chakhparonian, 2001].

Microscopic Observations

All photographs were taken with a Spot RT Slider camera (Diagnostic Instruments, Sterling Heights, MI) mounted on either an inverted Zeiss microscope or an Olympus dissecting microscope. Images were then processed using Image J and Adobe Photoshop software. Confocal microscopy was carried out on a Zeiss LSM 510 META NLO microscope using a laser excitation line at 514 nm and a detection band between 520 and 530 nm. Images were then processed using either Bitplane AG, Imaris software (Switzerland) or ImageJ software.

RESULTS

The full-length ARPC4 cDNA from *P. patens* (*PpARPC4*) is 1103 base pairs long, coding for a 169-amino acid protein, while the corresponding ARPC4 genomic locus contained eight exons and seven introns (Fig. 1a). *PpARPC4* transcripts were detected in all cell types investigated (gametophore protonema and isolated protoplasts) at similar level (Fig. S1a). An amino acid sequence alignment of the deduced protein from *PpARPC4* showed high similarity to other known plant and animal ARPC4 proteins (Fig. 2). Amino acid identity along the full length of the protein was very high when compared with that of rice (88%) and Arabidopsis (83%), and remains high (>55%) when compared with that of nonplant eukaryotes. Although the intron/exon pattern and borders were similar to other plant sequences, the overall size of the introns is larger in *P. patens* (data not shown).

The genomic locus of *arpc4* was targeted with a replacement vector aimed to remove the full ORF (Fig. 1b). After regeneration of stable transformants, we confirmed the proper targeting by the absence of a transcript (Fig. S1b) and by Southern analysis (Fig. S2). We applied the transient Cre procedure [Chakhparonian, 2001] to remove the selection cassette so as to rule out any possible interference on the phenotype observed. These true null lines ($\Delta arpc4$) maintained their distinctive phenotype. Regeneration of a 3-week-old colony from a single $\Delta arpc4$ protoplast was characterized by tissue that was much reduced in size when compared with that of the wild type (WT) (Figs. 3a and 3b). We also followed growth from a single protoplast at 4, 7, and 13 days in parallel with a regenerating WT protoplast (Figs. 3d–3l). After 4 days of regeneration, no obvious morphological difference was observed between the WT and $\Delta arpc4$ (Figs. 3d and 3e). Moreover, the percentage and rate of protoplast regeneration rate were not affected by the deletion (data not shown). However, the difference between WT and $\Delta arpc4$ was striking at 7 and 13 days (Figs. 3g and 3h, and 3j and 3k, respectively), i.e. protonemal cells failed to elongate properly, and the distinctive characteristics between caulonema and chloronema filamentous cells [Cove et al., 1997; Schumaker and Dietrich, 1998] were absent in $\Delta arpc4$. Furthermore, the single cell type of $\Delta arpc4$ had an average cell size of only 40 μm , while caulonema and chloronema cells of 14–21-day-old WT tissue were 150 and 75 μm respectively. After 2 weeks of growth, buds normally develop at a frequency of one bud/20 caulonema cells and start the formation of the gametophores, a three-dimensional leafy shoot supported by rhizoids. This developmental process can be specifically stimulated by treatment with cytokinin, with bud formation occurring in every caulonema cell.

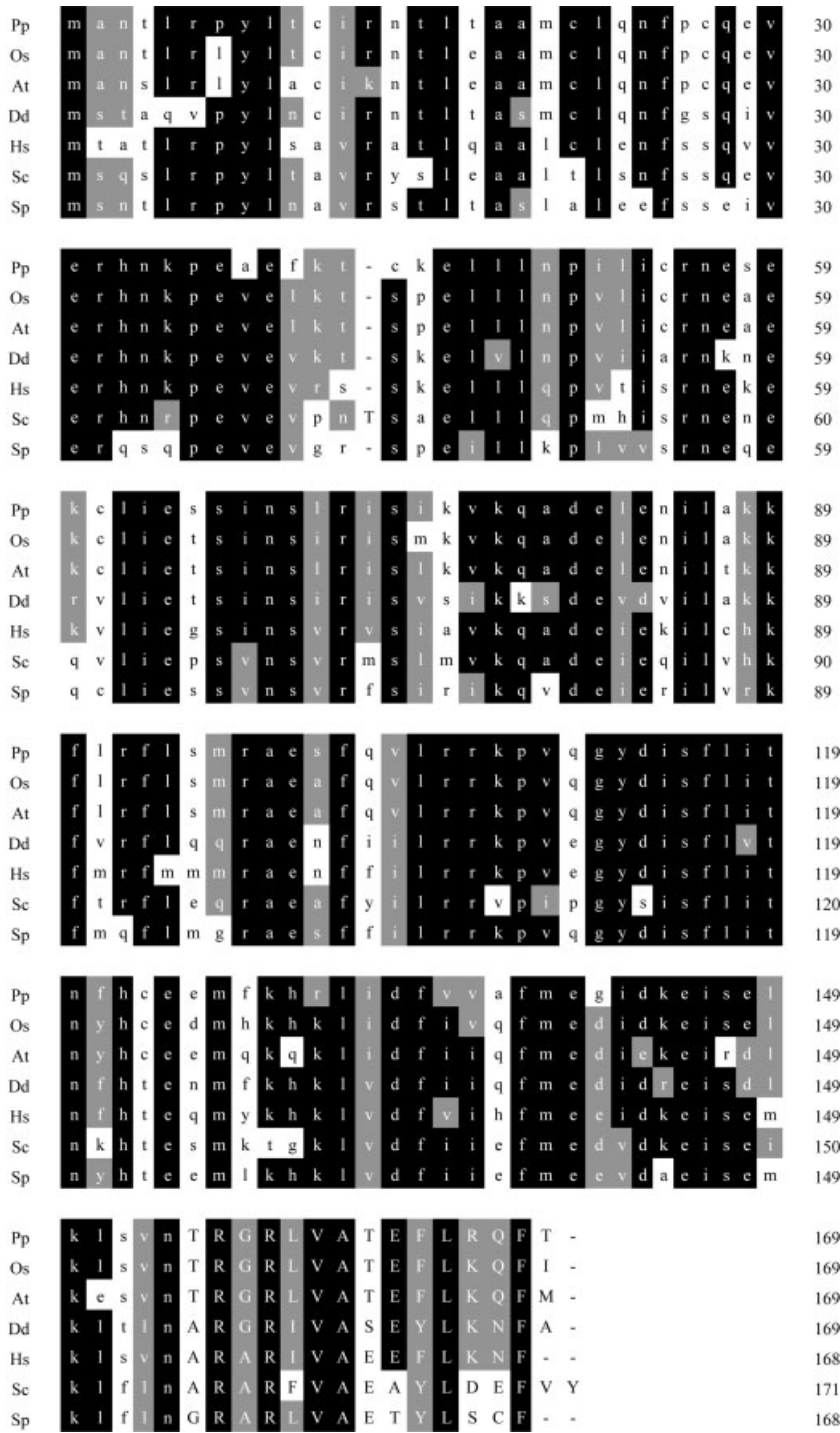


Fig. 2. Comparison of *PpARPC4* amino acid sequence in selected eukaryotes. The amino acid residues in black indicate identity while the gray areas represent similarity compared to *Physcomitrella patens* (Pp). Percentages indicate identity to Pp. Os, *Oryza sativa* (88%); At, *Arabidopsis thaliana* (83%); Dm, *Drosophila melanogaster* (67%); Dd, *Dictyostelium discoideum* (66%); Hs, *Homo sapiens* (66%); Sp, *Schizosaccharomyces pombe* (59%); Sc, *Saccharomyces cerevisiae* (57%).

nema cell [Cove et al., 1997]. The number of cells, the cytokinin-induced pattern of budding, and the formation of the gametophore are unchanged in $\Delta arpc4$ (data not shown). However, the overall structure of the gametophore was stunted in $\Delta arpc4$, as evidenced by the number of leaf-like structures per millimeters of gametophore (Fig. 4 right and center). Rhizoids that develop

from the $\Delta arpc4$ gametophores showed even stronger defects; cells fail to elongate and development is aborted after four to six cell divisions (data not shown). However, we were unable to detect differences in actin network when viewed with GFP-talin labeling [Kost et al., 1998] in intercalary cells. Hence, $\Delta arpc4$ was not lethal; normal morphogenesis of buds and gametophores

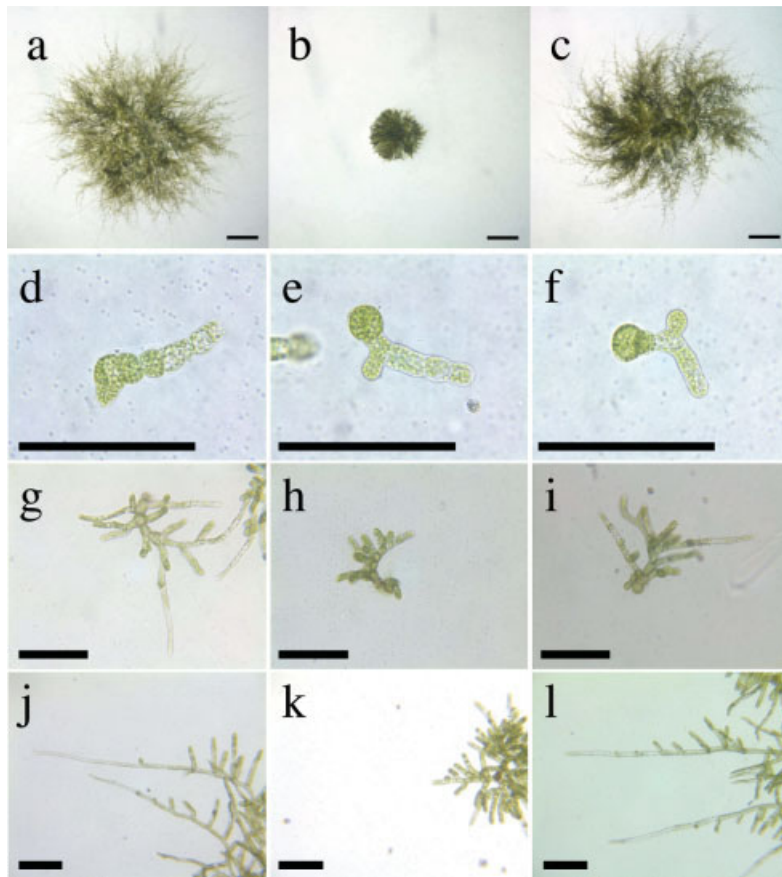


Fig. 3. Protonemal growth of different lines initiated from a single protoplast. (a), (d), (g), and (j) Wild type; (b), (e), (h), and (k) Null line, $\Delta arpc4$; (c), (f), (i), and (l) Complemented line $arpc4-OsACT1::P-pARPC4$. (a–c) 18 days (Scale bar = 500 μm); (d–f) 4 days (Scale bar = 200 μm); (g–i) 7 days (Scale bar = 200 μm); (j–l) 13 days (Scale bar = 200 μm).

occurred, but tip extension of the rhizoid and caulonemal filaments was specifically affected without any major change in the distribution of actin filaments.

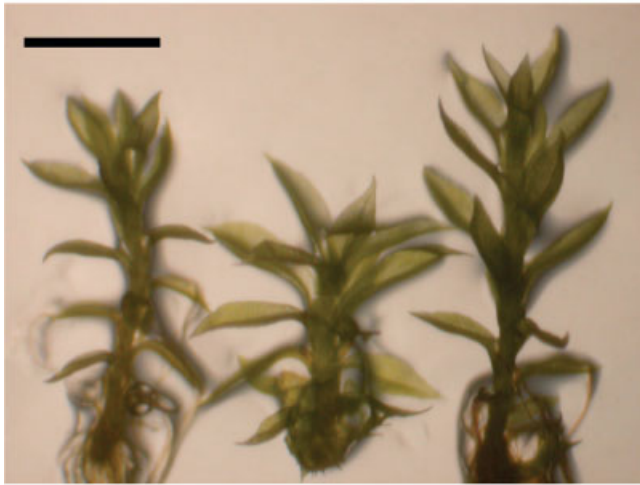
Complementation of the phenotype was observed by overexpressing the *arpc4* full-length cDNA in $\Delta arpc4$ ($\Delta arpc4 -ACT1::PpARPC4$) (Fig. 1d). To avoid any interference with the endogenous ARPC4 locus and to increase the frequency of stable transformants, we targeted this overexpressing cassette to a known genomic locus [Schaefer and Zrýd, 1997]. After transformation and regeneration of stable colonies, all the resistant clones showed a WT phenotype. A regenerated colony from a single protoplast in the complemented line grew to its normal size (Fig. 3c), was composed of well-defined caulonema and chloronema cells, and all cells elongated to their proper size (Figs. 3f, 3i, and 3l). The gametophore was restored to the WT appearance (Figure 4 right), as were the rhizoids (data not shown).

To determine whether the localization of ARPC4 was related to the phenotype and to the postulated site of

dynamic actin, we reintroduced the full ORF of the genomic sequence coupled to a double YFP, to the deleted site of *arpc4* (Fig. 1c). We screened stable transformants by Southern analysis for a double crossing over event, as well as a corresponding single copy insertion (Fig. S2). We isolated several lines that were morphologically indistinguishable from the WT (data not shown) and chose one line, *yfp-arpc4*, for further analysis. When observed under the confocal microscope, the YFP signal was clearly present at the elongating end of the caulonema cell, forming a cap around the tip of the cell (Fig. 5). The same accumulation was observed at the tip of the rhizoids (data not shown). In contrast, we did not detect the YFP signal in any cells of the gametophore where this specialized form of tip growth is absent.

Given the role of ARPC4 in rapid extension growth and its localization, we asked whether polar growth of filaments from regenerating protoplasts to light vectors was affected [Jenkins and Cove, 1983]. $\Delta arpc4$ showed a normal polar growth response toward unilateral white

a



b

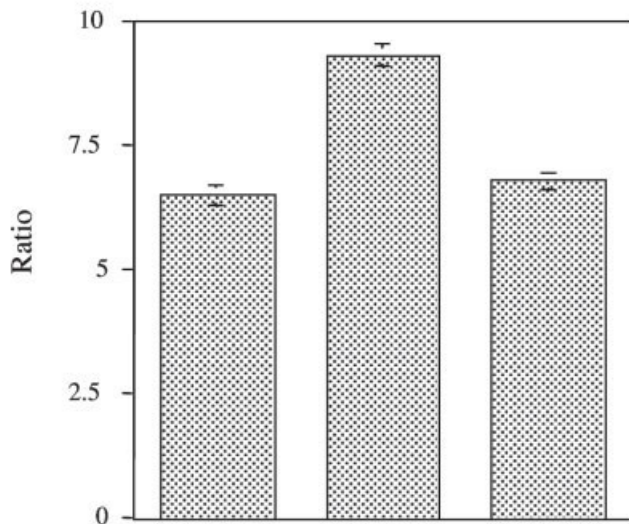


Fig. 4. Six-week-old gametophores. (a) From left to right: wild type, the null line ($\Delta arpc4$), and the complemented line ($\Delta arpc4 -OsACT1::PpARPC4$). Scale bar = 500 μ m. (b) Measurements (+/- SE) of the ratio of the number of leaves per millimeter of gametophore. From left to right: wild type, the null line ($\Delta arpc4$), and the complemented line ($\Delta arpc4 -OsACT1::PpARPC4$). Scale bar = 500 μ m.

light (data not shown). However, when we exposed both WT and $\Delta arpc4$ to two different intensities of polarized white light, striking differences were apparent. At low light (5 μ mol $m^{-2} s^{-1}$), there was no difference between the two lines, i.e. both grow perpendicular to the plane of polarized light (Figs. 6a and 6b). At high light (50 μ mol $m^{-2} s^{-1}$), the WT line changes the orientation of filaments from perpendicular to parallel (Fig. 6d), similar to previous results [Jenkins and Cove, 1983].

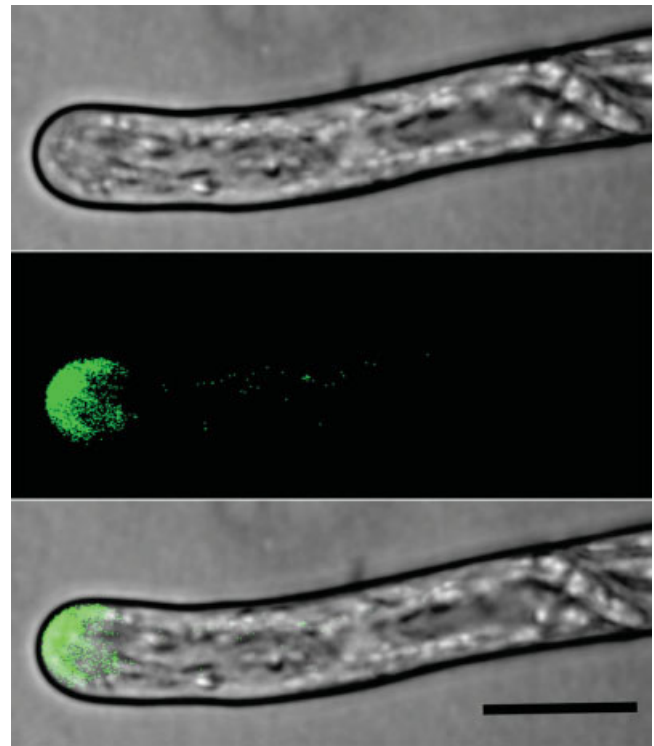


Fig. 5. Localization of ARPC4 with YFP (*yfp-arpc4*) (see Fig. 1c). Tip of a caulonema apical cell in white light (top), the same cell viewed for the YFP signal (middle), and the merged images (bottom). Scale bar = 40 μ m.

However, $\Delta arpc4$ lacked the ability to alter its plane of polar growth from perpendicular to parallel, continuing to grow perpendicular to the polarized light (Fig. 6e). There was no difference in the amount or speed of growth between the two lines under the two different light regimes, i.e. only the orientation of polar growth was affected. Complementation with *yfp-arpc4* (Fig. 1c) showed the normal polar response to high light (Fig. 6f). Hence, deletion of *arpc4* showed a specific effect on the ability of the filaments from regenerating protoplasts to orient parallel to high-intensity polarized light as did WT filaments.

DISCUSSION

Several results from our study point to a specific and specialized role of ARPC4 in tip growth and its orientation to polarized light. First, the transition from two-dimensional to three-dimensional growth (i.e. bud formation and development into gametophores), as well as growth responses to cytokinin and auxin (data not shown) were not affected by the absence of ARPC4. However, ARPC4 appeared to be essential for the characteristic rapid extension of caulonemal and rhizoid filaments. The lack of these elongated filaments in proto-

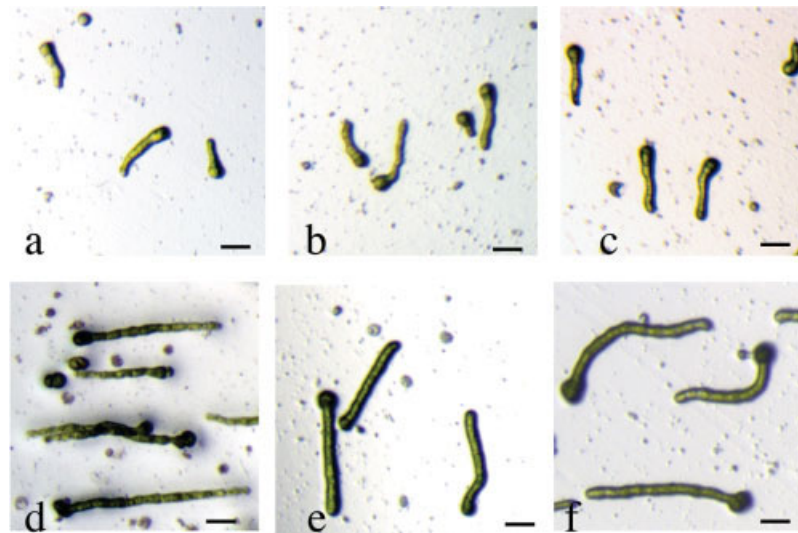


Fig. 6. Protoplast regeneration in different intensities of polarized white light with the electric vector parallel to the scale bar (60 μm). (a–c) Low light, 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$; (d–f) High light, 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$. (a and d) Wild type; (b and e) Null line $\Delta arpc4$; (c and f) Complemented line *yfp-arpc4*.

nema resulted in a very characteristic phenotype at the colony and cell level, i.e. a dense, compact colony with smaller cells of only one cell type. These results are similar to the T-DNA insertional mutants in Arabidopsis; that is, plants are morphologically normal but show defects in specific cell types (e.g. trichomes) [Mathur, 2005; Szymanski, 2005].

The results showing ARPC4 specifically localized at the site of tip growth and required for proper responses to polarizing light are consistent with its role in tip growth. There have been other reports of Arp2/3 members localized at the site of tip growth of algal rhizoids [Hable and Kropf, 2005] and root hair cells [van Gestel et al., 2003]. Colocalization of ARP2 and actin at the rhizoid tip also tracked to the site on the surface of the algal zygote where polarized growth was going to occur [Hable and Kropf, 2005]. However, our article reports the first in vivo localization of an Arp2/3 member in plants using gene replacement methods. The *yfp-arpc4* was able to fully complement the $\Delta arpc4$ line, highlighting the usefulness and importance of gene replacement techniques to identify conclusively the site of ARPC4 localization.

Harries et al. [2005] showed that in *P. patens* a reduction in transcripts of *arpc1*, another member of the Arp2/3 complex, results in a phenotype similar but more severe than that with the selective removal of ARPC4. Using RNAi [Bezannilla et al., 2005], they observed that tip cells of chloronemal filaments had a reduced growth rate by over 4-fold and caulonemal cells were not formed. But, unlike the deletion of $\Delta arpc4$, this reduction of *arpc1* transcripts resulted in a more severe phenotype, i.e. the complete absence of buds, gameto-

phores and rhizoids. Also, only a few percent of protoplasts were able to regenerate in the ARPC1-deficient lines because of an apparent sensitivity to the osmoticum in the regeneration medium. Those protoplasts that regenerated did not exhibit normal polar outgrowths and displayed abnormal cell shapes and patterns of branching and cell division [Harries et al., 2005]. Unlike the null mutant of *arpc4* from this study, the lack of development beyond the chloronemal stage for the ARPC1 RNAi lines does not allow one to study further the response network involved with orienting polar growth. Although similar phenotypes (i.e. abnormal extension growth) were observed with the reduction or elimination of transcripts for ARPC1 and ARPC4 respectively, it is clear each member appears to have more specific and/or severe effects on tip growth from regenerating protoplasts and on orientation to directional cues. This is similar to the results from budding yeast where deletions of different subunits 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 putative complex, or possibly their role in tip growth independent of their presence in a complex.

The T-DNA insertion lines for Arabidopsis ARP2, ARP3, ARPC2, and ARPC5 [Le et al., 2003; Mathur et al., 2003a,b; El-Assal et al., 2004; Basu et al., 2005] each have certain unique phenotypes exhibited primarily in epidermal hairs (trichomes) and pavement cells as well as hypocotyl cells and root hairs. However, *P. patens* has no equivalent organs that form trichomes, pavement or hypocotyls cells, and so direct comparisons to these phenotypes are difficult. However, ARPC4 mutants of the

CROOKED class from *Arabidopsis* showed root hair cells that are significantly shorter, thicker, and more sinuous when compared with those of WT [Le et al., 2003; Mathur et al., 2003b; El-Assal et al., 2004; Basu et al., 2005], a phenotype in a cell type more analogous to filaments of *P. patens*. These defects in epidermal cells and trichomes in *Arabidopsis* were accompanied by alterations in actin filaments and their localization. However, we observed no such alterations in actin filaments or cables, either in distribution or in structure (data not shown). Several possibilities might account for this difference, given the fact that in both systems the mutant phenotypes are viable. In plants, other actin filament nucleators (e.g. formins) may substitute for and/or be at equilibrium with Arp2/3 for many actin structure/functions [Brembu et al., 2004]. Also, our results, including *in vivo* localization, might also suggest that the effect of our deletion of *arpc4* may specifically affect actin dynamics, i.e. processes involved with actin filament formation rather than disruption of filaments/cables already formed. ARPC4 may be required for rapid extension growth, as characterized by caulonemal cells of *P. patens*, and less involved with other expansion functions of chloronemal cells and cells in gametophores. It would be interesting to compare the localization of ARPC4 in root hairs and pollen tubes with our results (obtained using tip growing filaments from *P. patens*), and also to know whether the structure/subcellular localization of actin in root hairs and pollen tubes are affected in the T-DNA insertional mutant of ARPC4.

Our results with polarizing light have identified ARPC4, and possibly the Arp2/3 complex, as being associated with the perception of specific light signals, and/or the subsequent signaling cascades that lead to directed polar cell development. When filaments from *Δarpc4* protoplasts were grown in high-intensity polarizing light, they were not able to link this light signal to the direction of growth in the same manner as did WT, i.e. parallel to the electric vector. Therefore, ARPC4 either alone or as a member of the Arp2/3 complex could be directly linked to the reception of the polarizing light signal and/or its transduction to polarized growth. We are investigating further the interaction between ARPC4 and this response to polarizing light.

Finally, we must remember that an Arp2/3 complex that functions to nucleate actin and to initiate filament formation and branching of filaments in many other systems [Harries et al., 2005] has not been biochemically purified from any plant and therefore not assayed for actin function. Hence, we cannot directly determine whether deletion of one of its likely members is able to render the complex nonfunctional, whether the complex exists, or its contribution to actin structure or function. Furthermore, the situation in *P. patens* with respect to this question might be quite different than in seed plants;

that is, the complex may be a more advanced adaptation and present in seed plants but not in bryophytes. Also, it is not clear whether the Scar/WAVE family of proteins identified in *Arabidopsis* and maize are true activators of a plant complex or essential for plant Arp2/3 activity, since it was recently shown that yeast Arp2/3 did not require activating proteins for yeast actin polymerization [Wen and Rubinstein, 2005].

In summary, our results point to a role of the ARPC4 specifically in tip growth and its orientation in response to polarizing signals. The ability of *Δarpc4* to perform many cellular responses and developmental events is unlike the results of similar deletions of Arp2/3 members in animals and yeast [Winter et al., 1999] but similar to those in *Arabidopsis* [Mathur, 2005]. Since we can observe and manipulate tip growth directly in individual filaments without surrounding cells/tissue, the *P. patens* system will uniquely allow us to use allele replacement for the rescue of true nulls to determine their *in vivo* localization as we did with ARPC4. This will help to dissect the specific role(s) of Arp2/3 members in tip growth of plant cells. The completely sequenced genome of *P. patens* will aid in all structure/function comparisons (<http://www.mossgenome.org>) between Arp2/3 members in *P. patens* and *Arabidopsis* and other seed plants.

ACKNOWLEDGMENTS

We are grateful to H. Berg (Donald Danforth Plant Science Center) for his help with the confocal microscopy and D.S. Schaefer (Université de Lausanne, Switzerland) for the generous gift of the plasmid containing the hygromycin resistance cassette flanked by Lox sites, as well as the recombinase overexpressing plasmid *OsACT1::CRE*. We thank Magdalena Bezanilla, David Cove (Leeds, UK), and Aihong Pan for their helpful discussions and suggestions, as well as John Cooper and Kathryn Miller for their constructive comments on this manuscript. P.-F.P. was a recipient of a Young Scientist Fellowship from the Swiss National Science Foundation.

REFERENCES

- Ashton NW, Cove DJ. 1977. The isolation and preliminary characterisation of auxotrophic and analogue resistant mutant of the moss *Physcomitrella patens*. *Mol Gen Genet* 154:87–95.
- Ashton NW, Champagne CEM, Weiler T, Verkoczy LK. 2000. The bryophyte *Physcomitrella patens* replicates extrachromosomal transgenic elements. *New Phytol* 146:391–402.
- Basu D, Le J, El-Essal SE-D, Huang S, Zhang C, Mallery EL, Koliantz G, Staiger CJ, Szymanski DB. 2005. DISTORTED3/SCAR2 is a putative *Arabidopsis* WAVE complex subunit that activates the Arp2/3 complex and is required for epidermal morphogenesis. *Plant Cell* 17:502–524.

- Bezanilla M, Perroud P-F, Pan A, Klueh P, Quatrano RS. 2005. An RNAi system in *Physcomitrella patens* with an internal marker for silencing allows for rapid identification of loss of function phenotypes. *Plant Biol* 7:251–257.
- Brembu T, Winge P, Seem M, Bones AM. 2004. NAPP and PIRP encode subunit of a putative wave regulatory protein complex involved in plant cell morphogenesis. *Plant Cell* 16:2335–2349.
- Chakhparonian M. 2001. Développements d'outils de la mutagenèse ciblée par recombinaison homologue chez *Physcomitrella patens*. PhD Thesis, Université de Lausanne, Lausanne. <http://www2.unil.ch/lpc/docs/publications.htm>
- Cove DJ, Quatrano RS. 2004. The use of mosses for the study of cell polarity. In: Cove DJ, editor. *New Frontiers of Biology*. Netherlands: Kluwer, p 183–203.
- Cove DJ, Quatrano R, Hartmann E. 1996. The alignment of the axis of asymmetry in regenerating protoplasts of the moss, *Ceratodon purpureus*, is determined independently of axis polarity. *Development* 122:371–379.
- Cove DJ, Knight CD, Lamparter T. 1997. Mosses as model systems. *Trends Plant Sci* 2:99–105.
- El-Assal SE-D, Le J, Basu D, Mallery EL, Szymanski DB. 2004. Arabidopsis GNARLED encodes a NAP125 homolog that positively regulates ARP2/3. *Curr Biol* 14:1405–1409.
- Fowler JE, Quatrano RS. 1997. Plant cell morphogenesis: plasma membrane interactions with the cytoskeleton and cell wall. *Annu Rev Cell Develop Biol* 13:697–743.
- Frank M, Coumaran E, Dyachok J, Djakovics S, Nolasco M, Li R, Smith LG. 2004. Activation of Arp2/3 complex-dependant actin polymerisation by plant proteins distantly related to Scar/WAVE. *Proc Natl Acad Sci USA* 101:16379–16384.
- Gournier H, Goley ED, Niederstrasser H, Trinh T, Welch MD. 2001. Reconstitution of human Arp2/3 complex reveals critical roles of individual subunits in complex structure and activity. *Mol Cell* 8:1041–1052.
- Hable WE, Kropf DL. 2005. The Arp2/3 complex nucleates actin arrays during zygote polarity establishment and growth. *Cell Motil Cytoskeleton* 61:9–20.
- Harries PA, Pan A, Quatrano RS. 2005. Arp2/3 complex component ARPC1 is required for proper cell morphogenesis and polarized cell growth in *Physcomitrella patens*. *Plant Cell* 17:2327–2339.
- Higgs HN, Pollard TD. 2001. Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu Rev Biochem* 70:649–676.
- Jenkins GI, Cove DJ. 1983. Phototropism and polarotropism of primary chloronemata of the moss *Physcomitrella patens*: response of the wild type. *Planta* 158:357–364.
- Kost B, Spielhofer P, Chua N-H. 1998. A GFP-mouse talin fusion protein labels plant actin filaments *in vivo* and visualizes the actin cytoskeleton in growing pollen tubes. *Plant J* 16:393–401.
- Le J, El-Assal SE-D, Basu D, Szymanski DB. 2003. Requirements for *Arabidopsis* ATARP2 and ATARP3 during epidermal development. *Curr Biol* 13:1341–1347.
- Li S, Blanchoin L, Yang Z, Lord EM. 2003. The putative *Arabidopsis* Arp2/3 complex controls leaf cell morphogenesis. *Plant Physiol* 132:2034–2044.
- Mathur J. 2005. The ARP2/3 complex: giving plant cells a leading edge. *Bioessays* 27:377–387.
- Mathur J, Mathur N, Kernebeck B, Hulskamp M. 2003a. Mutations in actin-related proteins 2 and 3 affect cell shape development in *Arabidopsis*. *Plant Cell* 15:1632–1645.
- Mathur J, Mathur N, Kirik V, Kernebeck B, Srinivas BP, Hulskamp M. 2003b. *Arabidopsis* CROOKED encodes for the smallest subunit of the ARP2/3 complex and controls cell shape by region specific fine F-actin formation. *Development* 130:3137–3146.
- McElroy D, Zhang W, Cao J, Wu R. 1990. Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* 2:163–171.
- Mullins RD, Heuser JA, Pollard TD. 1998. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc Natl Acad Sci USA* 95:6181–6186.
- Quatrano RS. 1997. Cortical asymmetries direct the establishment of cell polarity and the plane of cell division in *Fucus* embryos. In: *Cold Spring Harbor Symposium on Quantitative Biology*, Cold Spring Harbor, NY. Vol. LXII, p 65–70.
- Rogers SL, Weidemann U, Stuurman N, Vale RD. 2003. Molecular requirements for actin-based lamella formation in *Drosophila* S2 cells. *J Cell Biol* 162:1077–1088.
- Schaefer DG. 2002. A new moss genetics: targeted mutagenesis in *Physcomitrella patens*. *Annu Rev Plant Biol* 53:477–501.
- Schaefer DG, Zryđ JP. 1997. Efficient gene targeting in the moss *Physcomitrella patens*. *Plant J* 11:1195–1206.
- Schumaker KS, Dietrich MA. 1998. Hormone-induced signaling during moss development. *Annu Rev Plant Physiol Plant Mol Biol* 49:501–523.
- Staiger CJ. 2000. Signaling to the actin cytoskeleton in plants. *Annu Rev Plant Physiol Plant Mol Biol* 51:257–288.
- Szymanski DB. 2005. Breaking the WAVE complex: the point of *Arabidopsis trichomes*. *Curr Opin Plant Biol* 8:103–112.
- van Gestel K, Slegers H, von Witsch M, Samaj J, Baluska F, Verbelen J-P. 2003. Immunological evidence for the presence of plant homologues of the actin-related protein Arp3 in tobacco and maize: subcellular localization to actin-enriched pit fields and emerging root hairs. *Protoplasma* 222:45–52.
- Wen K-K, Rubinstein PA. 2005. Acceleration of yeast actin polymerization by yeast Arp2/3 complex does not require an Arp2/3-activating protein. *J Biol Chem* 280:24168.
- Winter DC, Choe EY, Li R. 1999. Genetic dissection of the budding yeast Arp2/3 complex: a comparison of the *in vivo* and structural roles of individual subunits. *Proc Natl Acad Sci USA* 96:7288–7293.
- Zhao X, Yang Z, Qian M, Zhu X. 2001. Interactions among subunits of human Arp2/3 complex: p20-Arc as the hub. *Biochem Biophys Res Commun* 280:513–517.