ARP2 and ARP3 are localized to sites of actin filament nucleation in tobacco BY-2 cells

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Summary. Complete depolymerization of actin filaments (AFs) at low temperature (0 °C) is followed by the formation of transient actin structures at 25 °C in tobacco BY-2 cells (Nicotiana tabacum L.). Using antibodies against fission yeast actin-related proteins (ARP2 and ARP3), we show here that transient actin structures (dots, dotted filaments, rods) colocalize with epitopes stained by these antibodies and thus are likely to represent sites of actin filament nucleation (SANs). In contrast to the cold-induced disassembly of AFs, no transient actin structures were detectable during recovery of AFs from latrunculin B-induced depolymerization. However, the staining pattern obtained with ARP antibodies in latrunculin B-treated cells was similar to that in controls and cold-treated cells. This suggests that, in addition to the complete depolymerization of AFs, disruption of other cellular structures is needed for the formation of transient actin structures during the early phase of recovery from cold treatment.

Keywords: Nicotiana tabacum; BY-2 cells; Actin filament; Actin polymerization; Cold; Latrunculin B; Immunodetection.

Abbreviations: AFs actin filaments; ARP actin-related protein; SANs sites of actin filament nucleation.

Introduction

Actin filaments (AFs) are essential for plant cells: they participate in processes of cell division, growth and differentiation, determination of cell polarity, and signal transduction. In order to function properly, their distribution and dynamics must be strictly controlled, among others by the spatial regulation of AF nucleation and polymerization. In animal cells, the above-mentioned processes are known to be assisted by the Arp2/3 complex, which nucleates AFs on existing templates at an angle of 70° relative to the parental filament (Mullins et al. 1998, Svitkina and Borisy 1999, Blanchin et al. 2000). ARP2 and ARP3 are the largest of seven subunits composing the multiprotein Arp2/3 complex and share significant structural and sequence similarity with actin (Robinson et al. 2001). The Arp2/3 complex is mostly located in the subcortical region of the cytoplasm in the cells of many species. It colocalizes with highly dynamic AFs in actin patches in yeasts (McCollum et al. 1996, Moreau et al. 1996) or in protruding lamellipodia and filopodia in vertebrate cells (Welch et al. 1997).

All seven proteins of the Arp2/3 complex are encoded in the Arabidopsis thaliana genome (Vantard and Blanchin 2002). In the last few years, genes have been identified and characterized from distorted mutants of A. thaliana that code for proteins of the Arp2/3 complex – ARP2, ARP3, ARPC2, ARPC5 (Mathur et al. 1999, 2003; Le et al. 2003; Li et al. 2003; Saedler et al. 2004) – and for proteins of a potential upstream regulatory pathway, such as NAP125, PIR121, and PIROGI (Basu et al. 2004, Brembu et al. 2004, Deeks et al. 2004). The mutant plants show altered shape in many cell types, including leaf trichomes, pavement cells, and hypocotyl cells. It seems that the reason for the abnormal phenotype is the absence of subcellular cortical domains of fine filamentous actin (F-actin) and the accumulation of denser actin bundles (for a review, see Deeks and Hussey 2003). However, the localization of proteins of the Arp2/3 complex has not been studied in plants, with the exception of a study by Van Gestel et al. (2003), where various heterologous antibodies against animal and fungal ARPs were used and a potential colocal-
ization with AFs was suggested. The mechanism of Arp2/3-based nucleation and branching of AFs appears to be conserved among eukaryotes, but detailed information about its molecular background or subcellular localization in plants remains unclear. Moreover, no direct links between ARPs and the mechanism of AF nucleation have been reported in plants.

In our previous study, we used tobacco BY-2 cells to investigate cold-induced depolymerization of AFs followed by their subsequent reassembly at normal temperature (Pokorná et al. 2004). We observed that AFs reassembled quickly from distinct sites localized on the nuclear envelope and in the cortical cytoplasm. A complete reconstitution of AFs was preceded by the formation of transient actin structures, such as actin dots and branched filaments with a dotted signal. In this study, we used low temperature (0 °C) and a specific inhibitor of AF polymerization, latrunculin B, to temporarily degrade AFs in BY-2 cells. During the subsequent recovery of AFs, the cells were processed for simultaneous visualization of AFs and the main components of the Arp2/3 complex with heterologous antibodies against ARP2 and ARP3. Differences were found between the modes of AF nucleation occurring after destabilization caused by latrunculin B or cold treatment, suggesting a possible role of other cellular structures, e.g., the endomembrane system or microtubules (MTs), in the processes of nucleation and polymerization of AFs.

Material and methods

Plant material

The tobacco cell line BY-2 (Nicotiana tabacum L. cv. Bright Yellow 2 [Nagata et al. 1992]) was cultured in a liquid medium containing Murashige–Skoog (MS) salts, 1 mg of thiamine, 200 mg of KH₂PO₄, 100 mg of inositol, 30 g of sucrose, and 0.2 mg of 2,4-dichlorophenoxyacetic acid (2,4-D) per liter, pH 5.8 (all chemicals obtained from Sigma-Aldrich, St. Louis, Mo., U.S.A.). Every seven days, 1.5 ml of cells were transferred to 30 ml of fresh medium and cultured in darkness at 25 °C on a horizontal shaker (IKA KS501; IKA Labortechnik, Staufen, Federal Republic of Germany; 120 rpm; orbital diameter, 30 mm).

Cold and latrunculin B treatment and recovery experiments

Tobacco BY-2 cells from cultures in the exponential phase of growth (3-day-old) were used in all experiments. Cultivation conditions and cold treatment were carefully optimized to exclude any additional stresses. Cell suspensions in Erlenmeyer flasks were placed into an ice water bath (0 °C) and shaken on a horizontal shaker at 100 rpm (IKA KS501) in darkness. After 12 h, flasks were taken out from the ice water bath and the cells were immediately collected by filtering on a nylon mesh (pore diameter, 20 µm). They were then resuspended in medium at the control temperature (25 °C) and further cultivated at this temperature.

For treatments with latrunculin B (LatB, Sigma-Aldrich), appropriate volumes of a 2.5 mM stock solution in dimethyl sulfoxide (Sigma-Aldrich) were added to the cell suspension to give final concentrations of 100 or 500 nM LatB (final concentration of dimethyl sulfoxide less than 0.015%). To depolymerize AFs for subsequent recovery experiments, 500 nM LatB was applied for 12 h. Subsequently, the cells were collected by filtering on a nylon mesh (pore diameter, 20 µm), washed at least three times in fresh MS medium to remove LatB and resuspended in fresh medium. They were then further cultivated for the desired period (5–60 min). Samples of cell culture were collected for cytological observations during cold and LatB treatment and subsequent recovery.

Determination of cell viability

Cell viability was assessed by staining with fluorescein diacetate (Sigma-Aldrich), according to the method of Widholm (1972) as described by Pokorná et al. (2004).

Immunostaining of AFs and actin-related proteins

AFs and ARPs were visualized by the modified method of Collins et al. (1998). Briefly, 3-day-old cell culture was washed in actin-stabilizing buffer (ASB) (100 mM piperazine-N,N'-bis-2-ethanesulfonic acid) [Sigma-Aldrich], 5 mM EGTA [Sigma-Aldrich], 10 mM MgSO₄ [P-Lab, Prague, Czech Republic], pH 6.4, 25 °C) supplemented with 400 µM N-succinimidyl 3-maleimidobenzoate (Fluka, Buchs, Switzerland) for 20 min, subsequently fixed in 2% (w/v) paraformaldehyde (Serva Electrophoresis GmbH, Heidelberg, Federal Republic of Germany) and 1% (w/v) glutaraldehyde (Fluka) in ASB supplemented with 0.05% Triton X-100 (Sigma-Aldrich) and 200 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich) for 20 min. The cells were then washed in ASB twice for 10 min, digested with 0.05% (w/v) pectolyase Y-23 (Kyowa Chemical Products Co. Ltd, Tokyo, Japan) in ASB containing 0.4 M mannitol (Sigma-Aldrich) for 20 min and rinsed again with ASB (twice for 10 min). After dehydration in cold methanol (−20 °C, 10 min), the cells were washed in phosphate-buffered saline (PBS) (0.15 M NaCl, 2.7 mM KCl, 1.2 mM KH₂PO₄, 6.5 mM Na₂HPO₄, all components obtained from Sigma-Aldrich) and attached to poly-L-lysine-coated coverslips (poly-L-lysine; P-Lab) and incubated with mouse monoclonal anti-actin 69100 clone C4 antibody (ICN Biomedicals Inc., Irvine, Calif., U.S.A.) (1 mg/ml, 20 min), followed by a wash in 1% (w/v) bovine serum albumin (Fluka) in PBS for 30 min and incubated with mouse monoclonal anti-actin 69100 clone C4 antibody (ICN Biomedicals Inc.) and rabbit polyclonal sera against Schizosaccharomyces pombe Arp2 or Arp3 (McCollum et al. 1996; kind gift of Dr. Kathleen L. Gould, Vanderbilt University, Nashville, Tenn., U.S.A.) for 2 h at 25 °C (both diluted 1 : 400 in PBS). After washing with PBS (30 min), secondary fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (Sigma-Aldrich) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit antibody (Sigma-Aldrich) (diluted in PBS 1 : 30 and 1 : 100, respectively) were applied for 2 h at 25 °C. Specimens were washed in PBS, embedded in 50% glycerol supplemented with Hoechst 33258 (bisbenzimide, 2-(4-ethylhydranyloyl)-5-(4-methyl-1-piperazinyl)-2′,3′-bi-1H-benimidazole, 0.1 µg/ml; Sigma-Aldrich) to stain nuclei and observed immediately.

Rhodamine-phalloidin staining of AFs

AFs were visualized by the method of Kakimoto and Shibaoa (1987) modified according to Olyslaegers and Verbelen (1998), as described previously (Pokorná et al. 2004).

Microscopy and image analysis

Preparations were observed with an epifluorescence microscope (Olympus Provis AX 70; Olympus Optical Co., Ltd., Tokyo, Japan) equipped with standard filter sets for FITC, TRITC, and Hoechst 33258 fluorescence detection, and Nomarski differential interference contrast (DIC).
Fluorescence images were grabbed with a monochrome integrating charge-coupled-device camera (Cohu 4910; Cohu, Inc., San Diego, Calif., U.S.A.) and digitally stored with Lucia image analysis software (Laboratory Imaging, Prague, Czech Republic). Confocal microscopy was performed using a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica TCS NT; Leica, Heidelberg, Federal Republic of Germany) equipped with an ArKr laser using filter sets for FITC (excitation at 488 nm, emission at 500–530 nm) and TRITC (excitation at 543 nm, emission at 545–590 nm). An objective lens Plan Apo (magnification, ×63; numerical aperture, 1.2) was used for all observations.

**Determination of the angles of branched AFs**

In two independent cold-recovery experiments, 50 optical fields containing 55 cells with branched AFs were scanned with an epifluorescence microscope (Olympus Provis AX 70) and stored and analyzed by Lucia image analysis software. A total of 156 angles were analyzed. The measured values were sorted into nine categories at 10° increments, and the frequencies in each category were plotted.

**Protein electrophoresis and immunoblotting**

Cells were harvested by filtration on nylon mesh as described above, and 1 g of biomass was homogenized immediately in liquid nitrogen with a pestle and mortar. The frozen powder was mixed 1:1 (w/v) with twice-concentrated extraction buffer (25 mM 2-morpholinoethanesulfonic acid, 5 mM EGTA, 5 mM MgCl₂, 1 M glycerol, 1 mM GTP, 1 mM dithiothreitol, 1 mM PMSF, 1 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin, pH 6.9). The mixture was allowed to thaw on ice and was then centrifuged at 3000 g for 15 min at 4 °C. The supernatant was centrifuged again at 100,000 g for 1 h at 4 °C. The resulting supernatant represented the soluble fraction and the pellet the membrane fraction. All fractions were precipitated with 10% trichloracetic acid and diluted in denaturing buffer (50 mM Tris-HCl, pH 6.9, 2% [w/v] sodium dodecyl sulfate [SDS], 36% [w/v] urea, 30% [w/v] glycerol, 5% [w/v] β-mercaptoethanol, 0.5% [w/v] bromophenol blue). Protein concentration was determined after staining with amido black. Protein samples were vortexed, boiled for 5 minutes and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% (w/v) acrylamide gels. Separated proteins were either visualized by staining with Coomassie Brilliant Blue (Sigma-Aldrich) or transferred onto polyvinylidene difluoride membranes (ICN Biomedicals Inc.) by semidyry electroblotting for probing with antibodies. Mouse monoclonal anti-actin 69100 clone C4 was used at a dilution of 1:4000, and rabbit polyclonal anti-ARP2 or anti-ARP3 antibodies were used at a dilution of 1:1000. After incubation with horseradish peroxidase-conjugated secondary antibody (ICN Biomedicals Inc.), the reactions were visualized with a chemiluminescence ECL detection kit (Amersham Biosciences, Uppsala, Sweden) on X-ray films (Foma, Hradec Králové, Czech Republic).

Fig. 1 A–J. Localization of ARP2 and ARP3 in BY-2 cells from 3-day-old cultures. Immunofluorescence staining with anti-actin (green channel) and anti-ARP2 (red channel) antibodies. Fluorescence microscopy (A–H) and confocal microscopy (I–J). Details boxed in panels A, E, and I are shown in panels B–D, F–H, and J, respectively. A–D Colocalization of ARP2 and AFs at the sites of AF branching (arrows) and along AFs (arrowhead) in the cortical cytoplasm. E–H Colocalization of ARP2 and AFs at the sites of AF branching (arrows) and along AFs (arrowhead) in the cortical cytoplasm. E–H Colocalization of ARP2 and AFs in cytoplasmic strands and in the perinuclear region. I and J Confocal section showing colocalization of ARP3 and AFs at the site of AF branching. Bars: 10 μm.
To test for cross-reactivity between the anti-actin and anti-ARP2 antibodies, proteins from the soluble fraction were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. One line of separated proteins was cut longitudinally into 3 strips and each strip was incubated separately with anti-actin, anti-ARP2, and anti-ARP3 antibodies. Strips were then placed back together before visualization with the ECL detection kit.

Results

ARP2 and ARP3 localize as dots along AFs and at filament branching points in control BY-2 cells

To visualize the distribution of ARPs in control BY-2 cells, heterologous antibodies against ARP2 and ARP3 from fission yeast were used. Both antibodies produced a similar staining pattern within the cells, appearing as dots in all regions of AF occurrence, namely, in the cortical layer of the cytoplasm (Fig. 1A–D), around the nucleus and in cytoplasmic strands (Fig. 1E–J). The ARP dots in the cortical layer of the cytoplasm often colocalized with AFs (Fig. 1B–D) and were frequently found at points of AF branching (approximately 40–60% of all dots; Fig. 1B–D). Isolated ARP dots without any contact with AFs were observed very rarely, approximately for 5% of all dots. The colocalization of ARP dots and AFs was further confirmed on confocal optical sections (Fig. 1I, J).

Fig. 2 A–J. ARP2 colocalization with transient actin structures formed after 12 h at 0 °C and 5 min at 25 °C. Immunofluorescence staining with anti-actin (green channel) and anti-ARP2 (red channel) antibodies. Details boxed in panels A and E are shown in panels B–D and F–H, respectively. A–D ARP2 and actin colocalization in dots. E–H ARP2 and actin colocalization in branchings (F–H, arrows) and along AFs (E, white arrowheads). E Black arrowheads indicate ARP2 dots that do not colocalize with actin. I and J Angles formed by two branches of newly formed AFs. I Branching of thin AFs with a dotted signal formed in the cortical cytoplasm. Rhodamine-phalloidin staining. J Frequency of branching angles of defined size categories. The angle of branching was measured for 156 cases in a total of 50 optical fields. Data are from one representative experiment. Bar: 10 μm
ARP2 and ARP3 colocalize with sites of AF nucleation in cells recovering from cold-induced depolymerization of AFs

To study the mechanism of actin nucleation, we used low temperature (0 °C) to depolymerize AFs in exponentially growing BY-2 cells. Changes in the inner architecture of the cell and in AFs caused by the cold treatment and during subsequent recovery were similar to those described previously (Pokorná et al. 2004). Briefly, AFs in the cortical cytoplasm depolymerized gradually during the cold treatment and were undetectable after 12 h of treatment. During subsequent recovery at 25 °C, reassembly of AFs started quickly, and in the early stages of this process, various forms of polymerized actin were transiently detected (dots, dotted filaments, rods). These actin dots may represent potential sites of AF nucleation (SANs), while branched filaments with dotted signal may represent a subsequent phase of actin reassembly.

To test whether transient actin structures represent early phases of AF nucleation and correspond to SANs, we performed double immunostaining of ARPs and actin during the early phase of recovery after cold-induced depolymerization. As in the control cells, both ARP2 and ARP3 antibodies stained dots in the cortical layer of cytoplasm (Fig. 2 for anti-ARP2 antibody). These ARP dots colocalized with transient actin dots (Fig. 2A–D) and junctions of Y-shaped actin structures (Fig. 2F–H), and were localized along newly formed cortical AFs (Fig. 2E). Isolated ARP dots without any colocalization with AFs were rarely observed (Fig. 2E).

Fig. 3 A–L. Localization of AFs and ARP3 after LatB treatment and subsequent recovery of AFs in BY-2 cells from 3-day-old cultures. A and B Spatial organization of cytoplasm in control cells that formed many cytoplasmic strands (A) and after 12 h treatment with 500 nM LatB, when degradation of cytoplasmic strands was visible (B), as observed by Nomarski DIC. C–H Rhodamine-phalloidin staining. C AFs in cells treated with 100 nM LatB for 12 h. D AFs in cells treated with 500 nM LatB for 12 h. E–G Progressive reassembly of AFs during recovery from 500 nM LatB treatment. Note the absence of obvious SANs. H Normal arrangement of AFs recovered after 60 min in most cells. I–L Immunofluorescence staining with anti-actin (green channel) and anti-ARP3 (red channel) antibodies. ARP3 detected along AFs is indicated by arrowheads and ARP3 colocalizing with AFs branching is indicated by arrows. Details boxed in panel I are shown in panels J–L. Bars: 10 μm
Angle between newly formed AFs

The Arp2/3 complex is known to assist in the nucleation and branching of AFs at an angle of 70° in animals (Mullins et al. 1998, Svitkina and Borisy 1999). To test the hypothesis that the branched AFs observed during recovery from their cold-induced degradation (Fig. 2I) (previously described in Pokorná et al. [2004]) have resulted from the activity of the Arp2/3 complex, we measured the angle between two branches of newly formed AFs. All values (0–90°) were categorized into nine groups at 10° increments. In most cases, the angle between branches was in the range between 50° and 80° (Fig. 2J).

No transient actin structures in cells recovering from LatB-induced depolymerization of AFs

LatB was used as a tool to induce specific depolymerization of AFs (Fig. 3). First, we investigated the effect of LatB on cell architecture and viability of BY-2 cells from 3-day-old cultures. Neither of the LatB concentrations tested (100 and 500 nM for 12 h) affected the viability of cells significantly, which ranged from 90 to 95% in all experiments (details not shown). Control cells formed many radially oriented cytoplasmic strands that connected the cortical and perinuclear regions (Fig. 3A). When treated with 500 nM LatB, gradual disruption of the cytoplasmic strands occurred, the vacuolar complex fused into one big central vacuole, and the nucleus shifted into the layer of the cortical cytoplasm. After 12 h, no or only one thick cytoplasmic strand connected the nuclear region with the cortical cytoplasm (Fig. 3B).

The concentration of LatB required for complete depolymerization of AFs was tested, and AFs and ARPs were visualized in the subsequent recovery process. Treatment with 100 nM for 12 h did not result in complete depolymerization of AFs (Fig. 3C), and only treatment with 500 nM for 12 h was sufficient (Fig. 3D).

After 12 h of treatment with 500 nM LatB, the cells were returned to normal media to allow reassembly of AFs. During the initial 5–15 min of recovery, no structures resembling the transient actin structures seen during the cold recovery experiments described above, such as actin dots or filaments with a dotted signal, were observed. Instead, reassembly of AFs in the cortical layer commenced with the formation of short AFs that quickly elongated and sometimes branched (Fig. 3E–G). The reassembly process continued gradually and, as in the cold experiments, normal arrangement of AFs was reestablished within 1 h in most cells (Fig. 3H). In cytoplasmic strands, AFs began to recover within 5–10 min after restoration of normal conditions and were fully developed within 30–60 min in most cells.

In some experiments with cells recovering from the LatB treatment, the AFs and ARPs were double-stained (Fig. 3I–L). In the cortical cytoplasm, ARP2 and ARP3 formed dots along cortical AFs (Fig. 3L) and in the junctions of Y-shaped AFs (Fig. 3L). This distribution is similar to that in the control cells as well as in the cells recovering from cold treatment (see Figs. 1B–D and 2F–H).

Immunoblotting of ARPs

To test for potential cross-reactivity of antibodies against actin and the ARPs, we performed immunoblot analysis with soluble and membrane protein fractions from BY-2 cells in the exponential phase of growth (Fig. 4A, B). The antibody against ARP2 recognized a protein with an apparent molecular mass of about 44 kDa in the soluble fraction but not in the membrane fraction (Fig. 4A). The antibody against ARP3 recognized a protein with an apparent molecular mass slightly larger than 45 kDa in the membrane fraction but not in the soluble fraction (Fig. 4A). The anti-actin antibody detected a band with an apparent molecular mass that was distinct from those of ARP2 and ARP3 (approximately 45 kDa; Fig. 4B).

Discussion

ARP2 and ARP3 colocalize with AFs in control BY-2 cells

The staining patterns of anti-ARP2 and anti-ARP3 antibodies were identical. In control cells, the antibodies
recognized protein epitopes in the form of dots closely associated with AFs in the cortical cytoplasm, cytoplasmic strands, and around the nucleus. ARP dots were often localized at junctions of branched AFs, particularly in the cortical cytoplasm where branching of filaments was seen more clearly than in other regions of the cell. Similar distribution of ARPs was also observed in cells reassembling their actin network during recovery after cold and LatB treatments. These results are consistent with the hypothetical branching function of ARPs described by Mullins et al. (1998) and Svitkina et al. (2003).

Colocalization of ARP3 and AFs was shown earlier by Van Gestel et al. (2003) for isolated membranes derived from tobacco leaf protoplasts. Using anti-ARP3 antibody, the authors observed punctate and filamentous signals colocalizing with actin structures. In contrast, we never observed a filamentous pattern. This slight difference in staining pattern could be explained by the protoplasting procedure used in the experiments of Van Gestel et al. (2003). It is likely that a substantial reorganization in the cortical cytoplasm occurs during protoplast preparation, resulting in an altered association of actin and associated proteins.

ARPs have been shown to localize to sites with high actin dynamics (Moreau et al. 1996, Welch et al. 1997, Bretschneider et al. 2004). Thus, the occurrence of ARPs described in our experiments may reflect high AF nucleation activity in the metabolically active, exponentially growing cells. The ARP dots localized along AFs may represent Arp2/3 complexes with potential branching activity, the rarely observed single ARP dots that did not colocalize with actin possibly represent inactive Arp2/3 complexes. Alternatively, ARPs composing the isolated dots might participate in other, yet unknown, processes.

**ARP3s colocalize with hypothetical SANs in cells recovering from cold treatment**

In our previous study, we showed that the system of cold-induced, reversible degradation of AFs in tobacco BY-2 cells represents a unique tool for investigating the reassembly processes (Pokorná et al. 2004). The present study confirms that proteins of the Arp2/3 complex are indeed components of the transient actin structures. Hence, we hypothesize that the sites of transient actin structure formation represent SANs.

Both ARP2 and ARP3 colocalized with transient actin dots, as well as with branches of newly formed AFs that were observed in cells recovering from cold-induced AF depolymerization. Further, ARP dots also appeared along AFs and only rarely as single, isolated dots. The occurrence of components of the Arp2/3 complex in potential SANs is of particular interest and, to our knowledge, has not been described before. The colocalization of ARPs with transiently formed actin structures may reflect their function in de novo AF nucleation (previously reported in Ma et al. [1998], Mullins and Pollard [1999], Rohatgi et al. [1999]) and AF nucleation on a filament template, resulting in a network of branched AFs (for a review, see Welch and Mullins 2002). We suggest that, as in control cells, ARPs localized along reassembling AFs or as isolated dots during recovery after depolymerization represent the initiation of branching or nucleation points.

**Angle of AF branching**

The Arp2/3 complex is believed to initiate polymerization of a new actin filament from the side of a mother filament at the typical angle of 70° (Blanchoin et al. 2000, Svitkina et al. 2003). The AFs in the cortical cytoplasm of tobacco BY-2 cells form a complex branched network. The branching of AFs and formation of Y-shaped structures was seen most clearly in the cortical cytoplasm of cells recovering from cold-induced depolymerization of AFs (Pokorná et al. 2004). Most AFs in recovering cells branched at an angle of 50–80°. These results, however, should be interpreted with consideration of the methodological approach. If the plane of analyzed branched structures is not collinear with the plane of observation, the measurement of the angle is not precise. Although epifluorescence microscopy did not allow us to examine the precise spatial arrangement of AFs, our analysis showed that most of the measured branches formed an angle close to 70°, suggesting that an Arp2/3-based mechanism was involved in the branching of these AFs.

**Depolymerization of AFs by itself not necessarily sufficient for detection of SANs**

Treatment with 500 nM LatB for 12 h induced complete depolymerization of AFs, as far as could be detected by optical microscopy. After the drug had been washed out, AFs started to reassemble quickly, and within 60 min of recovery, a control-like actin network was re-formed. However, transient actin structures – potential SANs (actin dots or actin dots localized along AFs) – were never observed during any stage of LatB recovery. This indicates that simple actin depolymerization by itself is not sufficient for the formation of the transient structures during actin recovery.
Low temperature has a great impact on subcellular structures. It mainly affects membranes (Murata and Los 1997) and the architecture of the vacuome (Pokorná et al. 2004), and causes microtubules to depolymerize (Pokorná et al. 2004). It has been shown that the Arp2/3 complex plays an important role in vacuolar organization in Arabidopsis trichomes (Mathur et al. 2003) and localizes to the vacuolar membrane and regulates vacuolar fusion in Saccharomyces cerevisiae (Eitzen et al. 2002). Thus, actin nucleation and polymerization might occur in close cooperation and interaction with cellular membranes such as the tonoplast, plasma membrane, or endoplasmic reticulum. Moreover, it is known that AFs physically interact with microtubules and that both cytoskeletons affect each other (Collings et al. 1998). Further, Abdrakhamanova and co-workers (2003) have suggested that microtubules have a role in the cold acclimation of winter wheat, leading to freezing tolerance. Thus, cold-induced degradation of microtubules may represent another factor affecting the dynamics of the actin cytoskeleton.

Interestingly, the subcellular distribution of ARPs, which we show to be localized to potential SANs, was not affected by either cold or LatB treatment. We suggest that the nucleation of AFs observed in recovering BY-2 cells affected by low temperature is a two-phase process. During the first phase, actin accumulates around SANs. The nature of SANs has to be further investigated, but here we show that ARPs are components of these structures and that their distribution in a cell is not affected by cold or LatB treatment. During the second phase, polymerization of AF occurs. We propose that the polymerization process requires an interaction between AFs and other cellular structures, as mentioned above. During early phases of recovery from cold treatment, these structures (probably membranes or microtubules) would be unable to serve as the support or “cellular memory” for actin polymerization and reorganization. A massive accumulation of actin around SANs would then reflect the lag phase between actin nucleation and polymerization, before the support structures recover from cold treatment.

During the recovery from LatB treatment, however, no obvious actin accumulation around SANs was detected. In these cells, the effect of LatB was restricted to AFs and the inner architecture of the cell (apart from changes directly linked to disrupted actin cytoskeleton such as collapsed cytoplasmic strands), the system of cellular membranes and particularly the microtubular cytoskeleton remained unaffected. Thus, reassembly of AFs could occur immediately with the support of other cellular structures. Accumulation of nonpolymerized actin around SANs was either too quick to be seen or did not occur at all.

An alternative explanation is that a population of very short filaments whose character or amount was below the detection limit of the staining method may have remained in the cells even after the exposure to 500 nM LatB for 12 h and acted as nucleation “seeds”. LatB alters the globular actin interface by preventing nucleotide exchange and polymerization to filaments (Morton et al. 2000). This means that AF depolymerization occurs as a consequence of inefficient binding of actin monomers to AFs. Under these conditions, constant turnover of AFs finally leads to their depolymerization. However, stable filaments with very low turnover should be more resistant to LatB, and these filaments may create new barbed ends for fast reassembly after the drug has been washed out. Under these circumstances, actin would not accumulate around SANs and hence would not appear as actin dots under the microscope.

**Anti-ARP antibodies do not cross-react with actin**

The cross-reactivity test on immunoblots showed that the two anti-ARP antibodies raised against fission yeast ARP2 and ARP3 recognized proteins with molecular masses different from that of actin. Therefore, the antibodies did not cross-react with actin, indicating that both antibodies also recognized epitopes other than actin in immunofluorescence staining. The molecular mass of the protein band recognized by anti-ARP2 in the soluble protein fraction, but not in the membrane fraction, corresponds to the putative molecular masses of ARP2 from Arabidopsis thaliana (44 kDa, 389 amino acids, GenBank accession nr. AF507910) and Lycopersicon aesculentum (44 kDa, 391 amino acids). In the membrane protein fraction, but not in the soluble protein fraction, anti-ARP3 recognized a protein slightly larger than 45 kDa, which corresponds to the predicted molecular masses of Arabidopsis thaliana (47.6 kDa, 427 amino acids, GenBank accession nr. BT006584) and Lycopersicon aesculentum (47.8 kDa, 429 amino acids) ARP3 proteins. Since both proteins are known to exist in the same protein complex but cosediment with different protein fractions, they may have different roles within this complex. The ARP3 protein, cosedimenting only with the membrane fraction, could mediate the interactions of the Arp2/3 complex with membranes. This hypothesis is consistent with studies of mammalian cells. ARP3 has been shown to bind to Golgi membranes in an ADP-ribosylation factor 1-dependent manner (Chen et al. 2004, Matas et al. 2004), mediating regulation of retrograde (Golgi-to-endoplasmic reticulum) (Luna et al. 2002) and anterograde (endoplasmic reticulum-to-Golgi) membrane trafficking at the endoplasmic reticulum–Golgi interface (Fucini et al. 2002). A primarily membrane
function of ARP3 in tobacco cells was also suggested by Van Gestel et al. (2003).

Conclusion

Considering the evolutionary conservation of the structure and function of the Arp2/3 protein complex in animals and fungi, it is highly probable that a similar convergence exists in plants. In our previous study, we described the incidence of transient actin structures during early phases of AF reassembly after their cold-induced depolymerization (Pokorná et al. 2004). In this study, we propose that these structures represent SANs around which actin accumulates during the early phases of AF recovery. We show that ARP2 and ARP3 colocalize with these hypothetical SANs, suggesting that they indeed represent sites where actin polymerization is initiated.

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