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Demonstration of prominent actin filaments in the root columella

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Abstract. The distribution of actin filaments within the gravity-sensing columella cells of plant roots remains poorly understood, with studies over numerous years providing inconsistent descriptions of actin organization in these cells. This uncertainty in actin organization, and thus in actin's role in graviperception and gravisignaling, has led us to investigate actin arrangements in the columella cells of *Zea mays* L., *Medicago truncatula* Gaertn., *Linum usitatissimum* L. and *Nicotiana benthamiana* Domin. Actin organization was examined using a combination of optimized immunofluorescence techniques, and an improved fluorochrome-conjugated phalloidin labeling method reliant on 3-maleimidobenzoyl-*N*-hydroxy-succinimide ester (MBS) cross-linking combined with glycerol permeabilization. Confocal microscopy of root sections labeled with anti-actin antibodies revealed patterns suggestive of actin throughout the columella region. These patterns included short and fragmented actin bundles, fluorescent rings around amyloplasts and intense fluorescence originating from the nucleus. Additionally, confocal microscopy of MBS-stabilized and Alexa Fluor-phalloidin-labeled root sections revealed a previously undetected state of actin organization in the columella. Discrete actin structures surrounded the amyloplasts and prominent actin cables radiated from the nuclear surface toward the cell periphery. Furthermore, the cortex of the columella cells contained fine actin bundles (or single filaments) that had a predominant transverse orientation. We also used confocal microscopy of plant roots expressing endoplasmic reticulum (ER)-targeted green fluorescent protein to demonstrate rapid ER movements within the columella cells, suggesting that the imaged actin network is functional. The successful identification of discrete actin structures in the root columella cells forms the basis for advancing studies on the role of actin in gravity perception and signaling.

Key words: Actin – Columella – Confocal microscopy – Gravitropism (gravity sensing) – Phalloidin – Root

Introduction

The characterization of actin distribution within the gravity perceiving cells of a plant remains a significant goal. In roots, the putative gravity sensing cells are found in the center of the root cap. These cells, referred to as columella cells, show a distinct structural polarity of their organelles. In vertically growing roots, the nucleus is located at the wall closest to the meristem (proximal wall), while endoplasmic reticulum (ER) is peripheral but predominantly associated with the distal cell wall where the amyloplasts (statoliths) settle (Sack 1997). In recent studies of the organization of the actin cytoskeleton in roots, it was shown that the columella cells lack prominent actin bundles in comparison to the extensive actin arrays found in other plant cell types (Baluška et al. 1997a,b). It was proposed that this reduction in actin was functionally significant for gravity sensing, allowing amyloplasts to sediment more freely (Baluška et al. 1997a; Baluška and Hasenstein 1997). In contrast, earlier reports proposed a direct role for actin in gravity sensing. These reports suggested that amyloplasts interact with actin filaments directly, and that amyloplast redistribution following gravistimulation results in tension being imparted on the actin cytoskeleton, with this being part of the mechanism through which the root perceives a reorientation (Sievers et al. 1989, 1991). Thus, a better understanding of the process of root gravitropism should follow from a better knowledge of the distribution of actin in the columella.

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Abbreviations: ER = endoplasmic reticulum; GFP = green fluorescent protein; MBS = 3-maleimidobenzoyl-*N*-hydroxy-succinimide ester; PME = 50 mM Pipes, 4 mM $MgSO_4$, 10 mM EGTA

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Despite several attempts to localize actin in the columella cells using fluorescence microscopy, conclusive demonstration of discernible actin structures in these cells has been difficult. For example, labeling actin with rhodamine-phalloidin in fixed *Zea mays* root caps that have been enzymatically separated, demonstrated only sparse longitudinal bundles (Vaughan and Vaughn 1987; Staves et al. 1997) or finer transverse cortical arrays (White and Sack 1990). This pattern, however, was difficult to interpret because cell separation resulted in the loss of spatial information. Furthermore, only cap cells without amyloplasts showed visible actin while cells containing amyloplasts did not (White and Sack 1990). While rhodamine-phalloidin labeling of permeabilized but unfixed *Lepidium sativum* root caps revealed the presence of actin strands throughout the columella cells, these strands were poorly resolved (Hensel 1989). Similarly, rhodamine-phalloidin labeling of aldehyde-fixed roots of *Z. mays* (Blancaflor and Hasenstein 1997) and *Lens culinaris* (Driss-Ecole et al. 2000) showed diffuse fluorescence in the columella, and an absence of the actin bundles as typically found in other cell types.

Similar to methods employing fluorescently tagged phalloidin, immunolabeling using actin-specific antibodies has not been successful in demonstrating prominent actin bundles in the columella. For example, immunofluorescence labeling of fixed *L. sativum* roots using monoclonal actin antibodies revealed no distinct filaments and only occasionally showed amyloplasts surrounded by a ring of fluorescence (Koropp and Volkmann 1994). In another report, only diffuse labeling localized to the nucleus was observed in the columella of fixed *L. sativum* roots (Hensel 1989). Interestingly, Baluška et al. (1997a) also found diffuse actin labeling in the columella of sectioned *Z. mays* and *L. sativum* root caps although actin was detected elsewhere in the root. Therefore, direct experimental evidence for the presence of prominent actin bundles in columella cells remains equivocal and the continued inability to image distinct actin filaments in these cells has made it difficult to pursue further work on the involvement of actin in gravity sensing.

Observations indicative of actin functionality, however, provide indirect evidence for the presence of discrete actin structures in living columella cells, although contradictory results also exist. For example, cytoplasmic streaming was reported in living columella cells of *Z. mays* and *Hordeum vulgare* (Sack et al. 1986; White and Sack 1990) but was reported to be absent from columella cells of *Z. mays*, *Lycopersicon esculentum*, *L. sativum* and *Phleum pratense* (Hensel 1988; Baluška et al. 1997a). In contrast, the adjacent, mucilage-secreting cells of the peripheral root cap consistently showed cytoplasmic streaming and contained an extensive actin cytoskeleton (White and Sack 1990) that presumably functions in secretory-vesicle delivery to the plasma membrane (Mollenhauer and Morré 1976; Vaughan and Vaughn 1987).

Similar contradictory accounts also exist describing the effects of actin disrupting drugs on gravitropic bending. While cytochalasin had no observable effect on root curvature in *Oryza sativa*, *Z. mays* and *L. sativum* (Staves et al. 1997), it has been reported to marginally increase bending in *Z. mays* roots (Blancaflor and Hasenstein 1997), to reduce or delay curvature in *L. sativum* and *P. pratense* (Wendt et al. 1987; Monshausen et al. 1996), and to induce random bending in *Z. mays* (Guikema and Gallegos 1992). Cytochalasin may also induce other changes in the root cap, with treatments being reported to disrupt the polarized distribution of plastids (Hensel 1985) and nuclei (Lorenzi and Perbal 1990), to increase the rate of plastid sedimentation (Sievers et al. 1989), and to block or delay gravity-induced changes in membrane potential and proton effluxes (Sievers et al. 1995; Monshausen et al. 1996). In summary, the varied effects of cytochalasin generally suggest that actin may function in the perception and transduction of gravity; however, conclusive proof of this has yet to be achieved.

The uncertainty about the function of actin in gravitropism coupled with the inability to localize distinct actin bundles within the columella cells warrants a more careful characterization of actin structure and distribution within the root columella. Key to this characterization is a consideration of alternative actin labeling methods. In the current study, we tried several approaches to demonstrate the presence of discrete actin structures in the columella cells of several plant species. This included immunofluorescence methods that successfully labeled actin bundles in *Z. mays* pulvinal cells (Collings et al. 1998a) and modified permeabilization/cross-linking techniques that avoid aldehyde fixation (Olyslaegers and Verbelen 1998; Sonobe and Shibaoka 1989). Our study shows that columella cells do contain prominent actin strands, and, with the use of confocal microscopy, we clearly resolve actin bundles in the columella, apparently for the first time. Furthermore, because the distribution and movement of subcortical ER in plant cells is an actin-dependent process (Quader et al. 1987; Boevink et al. 1998), we suggest that the imaged actin bundles are functional by observing bulk ER movement in the columella cells through the use of an ER-targeted green fluorescent protein (GFP).

Materials and methods

Plant materials

Seeds of *Zea mays* L. cv. Merit (maize), *Medicago truncatula* Gaertn. cv. Jemalong, line A17 (barrel medic), *Linum usitatissimum* L. (flax) and *Nicotiana benthamiana* Domin. (muntju) were germinated and grown vertically at 22 °C as previously described (Blancaflor and Hasenstein 1997). Similarly, seed of a stably transformed line of *N. benthamiana* expressing the ER-targeted construct mGFP5 (Haseloff et al. 1997) was obtained from Dr. David Baulcombe (The Sainsbury Laboratory, John Innes Centre, Norwich, UK), and grown for 5–7 d on Phytagel plates containing MS salts as previously described for *Arabidopsis thaliana* (Scott and Allen 1999). Roots that had reached a length of 15–25 mm were used for all experiments (2–4 d after germination depending on the plant species).

3-Maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) cross-linking and Alexa Fluor-phalloidin labeling of actin

Labeling of actin filaments using fluorescently conjugated phalloidin was modified from Olyslaegers and Verbelen (1998) and Sonobe and Shibaoka (1989). Briefly, the terminal 2 mm of vertically growing roots was cut and immediately attached to a Vibratome-1000 (Technical Products International, St. Louis, Mo., USA) using a thin film of cyanoacrylate (superglue). Median longitudinal sections (60 μm thick) were collected and immediately immersed into PME (50 mM Pipes, 4 mM $MgSO_4$, 10 mM EGTA) buffer (pH 6.9) containing 300 μM MBS for 30 min. The sections were then incubated in PME buffer (without MBS) containing 0.1 μM Alexa Fluor-phalloidin (Molecular Probes, Eugene, Ore., USA), 0.3 M mannitol and 2% glycerol (v/v). After 5–10 min, root sections were washed once with PME buffer, mounted in this buffer and immediately observed under a confocal microscope. Some sections were counterstained using either 10 μM propidium iodide (Sigma) or 4′, 6-diamidino-2-phenylindole (DAPI, Molecular Probes) for 10 min to stain DNA.

Variations in the above method included fixation in 2–3% (v/v) formaldehyde in PME buffer for 30 min to 1 h after MBS cross-linking. Control experiments described by Traas et al. (1987) were conducted to test whether the applied Alexa Fluor-phalloidin induces artificial actin polymerization. This included incubating sections in a low concentration of Alexa Fluor-phalloidin (0.01 μM). To determine the specificity of phalloidin binding, sections were incubated in unlabeled phalloidin (1 μM) prior to labeling with fluorochrome-conjugated phalloidin.

Indirect immunofluorescent labeling of actin

Vertically grown roots were fixed for 2 h to overnight in PME buffer containing 0.1% (v/v) Triton X-100, 200 μM phenylmethylsulfonylfluoride, 400 μM MBS (giving final dimethylsulfoxide and ethanol concentrations of 1.0%), 3–8% (v/v) formaldehyde and 0.5–1% (v/v) glutaraldehyde. Roots were then washed extensively in phosphate-buffered saline (PBS; 2.68 mM KCl, 1.47 mM KH_2PO_4, 13.69 mM NaCl, 0.81 mM Na_2HPO_4, pH 7.4), and sectioned in a vibratome at a thickness of 50–100 μm. Median longitudinal sections were selected under a dissecting microscope, and were treated sequentially with 1.0% (v/v) Triton X-100 in PBS (20 min), methanol at –20 °C (10 min) and 1 mg ml^{-1} $NaBH_4$ (10 min) with PBS washes after each treatment, and then incubated in blocking buffer (5% w/v BSA, 0.05% v/v Tween-20 in PBS) for 30 min. Sections were labeled overnight with either polyclonal anti-maize actin (gift of Dr. Chris Staiger, Purdue University; Gibbon et al. 1999) diluted 1/200 in incubation buffer (1% BSA, 0.05% Tween-20 in PBS) or monoclonal anti-pea actin antibody (gift of Dr. Richard Cyr, Pennsylvania State University) used as described previously (Andersland et al.1994). The sections were then washed with PBS for several hours and incubated in secondary antibodies (either Cy-3-labeled goat anti-rabbit IgG diluted 1/3000 in incubation buffer or Oregon green-labeled goat anti-mouse IgG diluted 1/2000 in incubation buffer; Jackson, West Grove, Pa., USA and Molecular Probes respectively). After extensive washing in PBS, sections were mounted between two coverslips (allowing imaging from both sides) in PBS (pH 8.5) containing 0.1% (w/v) *p*-phenylenediamine as an antifade agent.

Confocal microscopy

Fluorescence in the root sections was imaged with a confocal microscope (1024ES: Bio-Rad, Hercules, Calif., USA; or TCS NT or TCS SP: Leica, Wetzlar, Germany) with 20 × N.A. 0.7 dry, 40 × N.A. 1.3 oil- and 63 × N.A. 1.2 water-immersion objectives. For actin labeling, optical stacks were recorded using sections of approximately 0.3–1 μm thickness, with each plane being averaged two to four times. For time-lapse imaging of GFP, images were taken every 10 s with each plane being averaged two times. 4′, 6-Diamidino-2-phenylindole was excited at 351 and 361 nm with emission recorded from 380 to 450 nm, fluorescein isothiocyanate (FITC), Alexa Fluor, Oregon green and GFP at 488 nm with emission from 500 to 550 nm, and rhodamine, propidium iodide and Cy-3 at 568 nm with emission from 580 to 620 nm. Concurrent transmitted light images were recorded with differential interference contrast (DIC) optics and a helium/neon laser (633 nm) by placing a red transmission filter in front of the transmitted light detector. All images were processed using Adobe Photoshop 5.0 by applying standard methods of image optimization such as contrast and brightness adjustments. All images were enhanced equally and printed on a Kodak 8670 PS thermal printer (Eastman Kodak, New York, USA).

Results

Due to the inability of previous studies to demonstrate prominent actin bundles in the columella region using conventional aldehyde fixation techniques (Baluška and Hasenstein 1997 and references therein), we devised a cell permeabilization protocol that combined the glycerol permeabilization methods used by Olyslaegers and Verbelen (1998) for onion epidermal and cultured tobacco cells, and the MBS cross-linking technique of Sonobe and Shibaoka (1989) used for tobacco suspension cells. These methods gave labeling of actin arrays in cultured tobacco cells similar to that seen with antibodies in aldehyde-fixed cells (Collings et al. 1998b) or to living tobacco cells transiently expressing a GFP-talin fusion protein (Kost et al. 1998).

Alexa Fluor-phalloidin labeling of actin in the meristem of permeabilized roots

When freshly cut root vibratome sections were incubated with MBS and permeabilized with glycerol in PME buffer containing Alexa Fluor-phalloidin, actin was extensively labeled throughout the root (Figs. 1–3). This method successfully labeled actin in the quiescent center and root meristem of the four species studied (Fig. 1). Randomly oriented cytoplasmic (Fig. 1A) and cortical (Fig. 1B) actin filaments were observed in cells of the quiescent center. Double labeling of root sections with propidium iodide and Alexa Fluor-phalloidin showed that *Z. mays* (Fig. 1C) and *L. usitatissimum* (Fig. 1D) cells at metaphase contained actin in the spindle. Furthermore, actin labeling was seen during the early stages of phragmoplast formation in *Z. mays* cells at late anaphase while dense cytoplasmic strands surrounded the nucleus (Fig. 1E). In addition to the actin arrays in dividing cells, transverse arrays of cortical actin filaments (Fig. 1F), and a more random array of cytoplasmic actin bundles (Fig. 1G) were observed in epidermal cells located in the meristematic region of *Z. mays* roots. Transverse patterns of cortical actin were also well preserved in a majority of non-dividing cells in the meristem of *Z. mays* (Fig. 1H), *M. truncatula* (Fig. 1I), *L. usitatissimum* and *N. benthamiana* (data not shown).

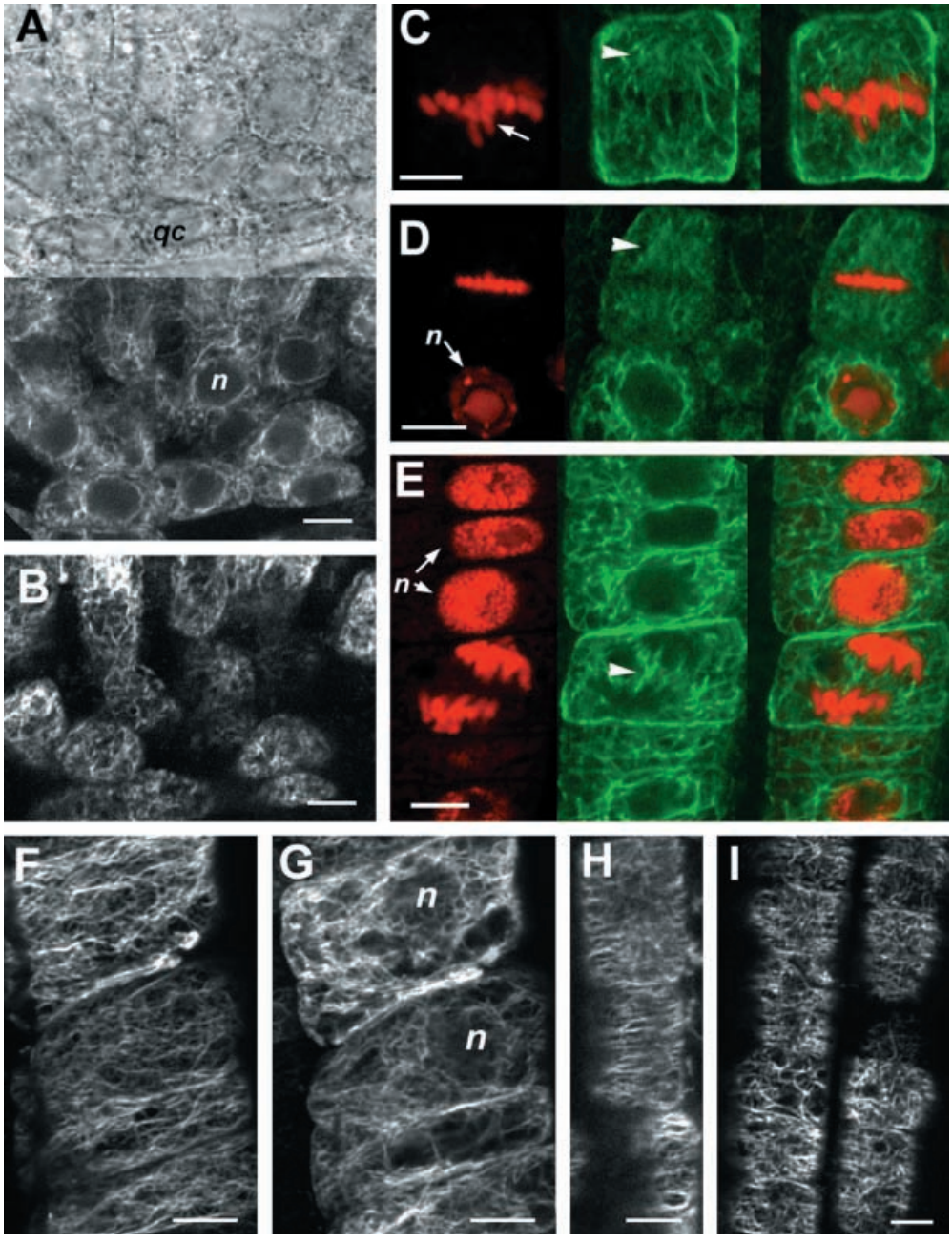


Fig. 1. Organization of actin filaments in the meristem region of *L. usitatissimum* (**A, B, D**), *Z. mays* (**C, E-H**) and *M. truncatula* (**I**). Root sections were processed using the MBS cross-linking/glycerol permeabilization method and imaged with a confocal microscope. All images are single optical sections and representative of 5–10 different roots. **A, B** Brightfield image and corresponding fluorescence image of the quiescent center (*qc*) of *L. usitatissimum*. Randomly oriented cytoplasmic actin bundles (**A**) and cortical actin filaments (**B**) were detected in these cells. **C–E** Mitotic cells double-labeled for DNA with propidium iodide (*red*, at left) and for actin with Alexa Fluor-phalloidin (*green*, center) (*false-color overlay*, right). Optical sections reveal the nuclei (*n*), condensed chromosomes (*arrow*), and prominent actin bundles. Metaphase cells of *Z. mays* (**C**) and *L. usitatissimum* (**D**) show actin in the spindle (*arrowheads*). **E** A *Z. mays* cell at late anaphase showing actin filaments in the early stages of phragmoplast formation (*arrowhead*). Extensive actin arrays also surround the phragmoplast. **F, G** Epidermal cells in the meristematic region of *Z. mays* roots. An optical section at the surface of the cells reveals transverse actin filaments (**F**) while a more random actin pattern occurs at the plane of the nuclei (*n*) in the same cells (**G**). **H, I** Transverse actin filaments, close to the cell surface, were also observed in non-dividing cortical cells of *Z. mays* (**H**) and *M. truncatula* (**I**). Bars = 10 μm

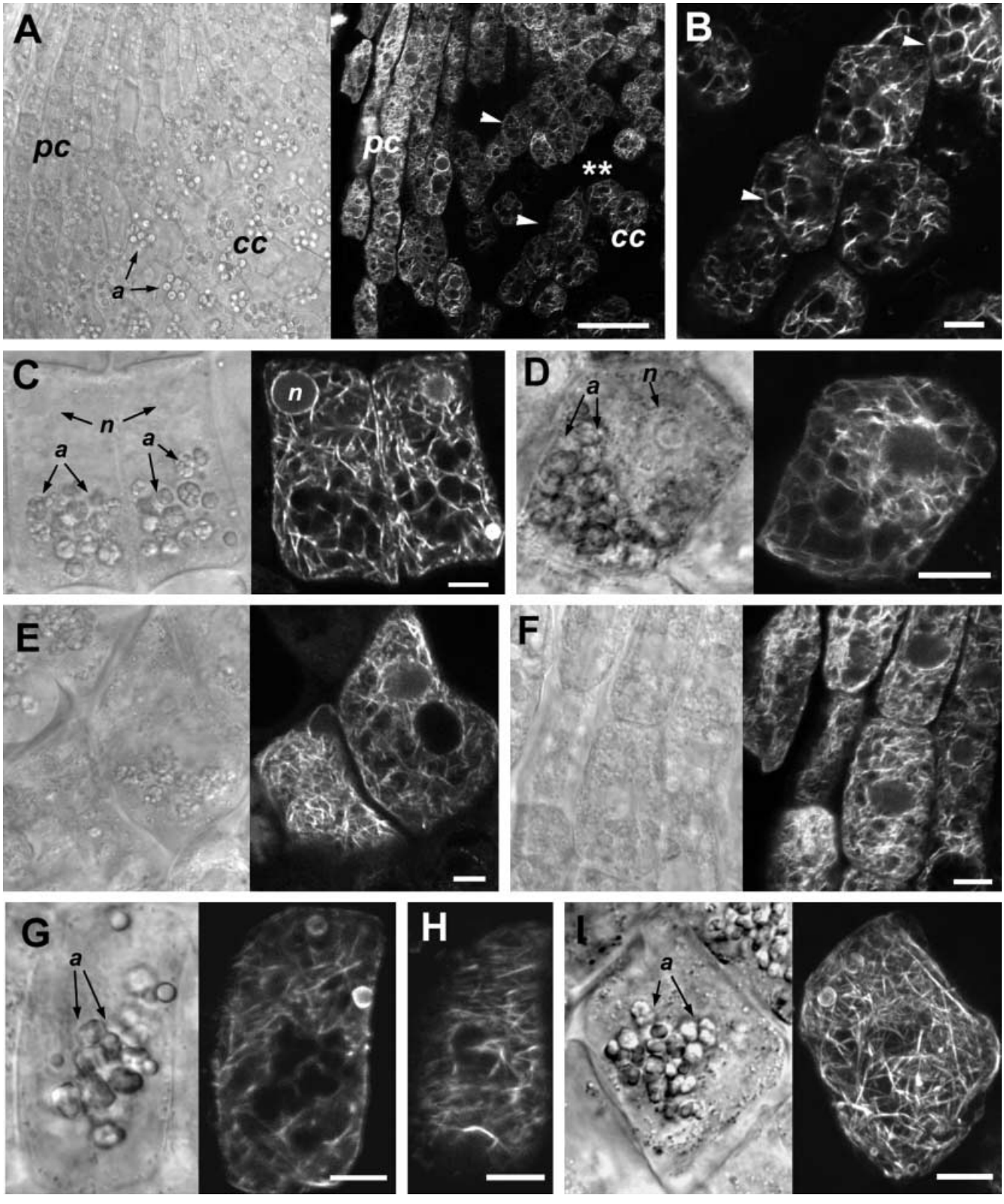
Alexa Fluor-phalloidin labeling of actin in permeabilized root cap cells

A more noteworthy result using our method was the dramatic improvement in the quality of actin labeling in the columella cells of all the plant species that we examined (Fig. 2). At lower magnification, the fluorescence signal emanating from the columella region was less intense than that in the adjacent peripheral cap cells (Fig. 2A), but despite this weaker fluorescence signal, distinct cytoplasmic actin bundles were clearly observed in individual columella cells when viewed at higher magnification (Fig. 2B). In columella cells of *M. truncatula*, the cytoplasmic actin bundles appeared to surround the amyloplasts (Fig. 2B), with this pattern of actin arrangement also evident in the columella cells of *L. usitatissimum* (Fig. 2C), and *Z. mays* (Fig. 2D). Distinct actin strands were also seen to radiate from the proximally positioned nucleus and fan out toward the cell periphery in all plant species examined (e.g. Fig. 2C,D). Single optical sections and higher-magnification images of the peripheral cap cells of *L. usitatissimum* (Fig. 2E) and *M. truncatula* (Fig. 2F) showed a more extensive network of randomly oriented actin arrays compared to the amyloplast-containing columella cells. This observation is consistent with the usually brighter fluorescence signal that we observed in the peripheral cap region when viewed at lower magnification (see Fig. 2A).

The method of labeling we employed was not limited to localizing actin in the subcortical cytoplasm as it also yielded images of fine transversely oriented actin in the

cortex. A single optical section from a columella cell of *L. usitatissimum* revealed the randomly oriented network of cytoplasmic actin bundles surrounding the amyloplasts (Fig. 2G) while a single optical section at the surface of the same cell shows finer transverse actin arrays (Fig. 2H).

The transversely oriented actin present at the columella cell surface was more difficult to image than the thicker cytoplasmic actin bundles. Often, the fluorescence from these arrays would bleach rapidly making image capture problematic. To obtain images of this very delicate actin arrays, the laser power and aperture



settings of the confocal microscope had to be higher than the settings used to capture images of the cytoplasmic actin bundles. Since the transverse cortical actin arrays in the columella were very faintly labeled, the extensive network of randomly oriented cytoplasmic actin bundles often obscured them when several optical sections from a single columella cell were projected (Fig. 2I). Nevertheless, the projected image demonstrates the extent of prominent actin bundles present in the central columella cells of *L. usitatissimum* (Fig. 2I), with similar observations possible in *Z. mays*, *N. benthamiana* and *M. truncatula* (data not shown).

Control experiments

Phalloidin stabilizes actin filaments. Therefore, the introduction of labeled phalloidin into permeabilized cells might artificially polymerize actin, and account for observations of actin bundles in the columella (Baluška and Hasenstein 1997). Thus, we conducted several control experiments, as initially proposed by Traas et al (1987), to confirm that our modified permeabilization technique was not inducing actin polymerization (Fig. 3). First, pretreatment with MBS prior to Alexa Fluor-phalloidin labeling was omitted. This substantially decreased the amounts and preservation quality of actin in columella cells (Fig. 3A) compared to cross-linking with MBS (Fig. 3B; Fig. 1). Second, lowering the Alexa Fluor-phalloidin concentration to 0.01 μM reduced the signal, but did not change the pattern of filamentous staining (Fig. 3C,D). As an additional control for the specificity of the actin probe, root sections were incubated in unconjugated phalloidin prior to Alexa Fluor-phalloidin application. This resulted in very weak or no labeling in roots of all species examined (e.g. Fig. 3E).

Actin in the cap and meristem of aldehyde-fixed roots

To confirm the actin localization patterns obtained in permeabilized root cells, and to determine whether we could improve on labeling actin in the columella using standard aldehyde fixation techniques, we used an immunolabeling protocol that was successfully used to label actin in the *Z. mays* pulvinus (Collings et al. 1998a). These methods relied on polyclonal or monoclonal antibodies raised against plant actin, and involved aldehyde fixation coupled with MBS stabilization. When entire root tips, labeled with either polyclonal anti-maize actin or monoclonal anti-pea actin, were imaged with a confocal microscope, fluorescence was observed from all areas of the root tip (Fig. 4A). In the meristem, developing vascular tissue showed random to longitudinal actin bundles (data not shown) whereas epidermal cells retained transverse arrays of finer actin bundles or single filaments (Fig. 4B). Actin was also observed in division-specific arrays such as the phragmoplast (Fig. 4C). These structures were essentially identical to those seen with the permeabilization method (see Fig. 1), although actin was not observed in the mitotic spindle in antibody-labeled preparations (data not shown).

However, as with the observations made with phalloidin labeling, there was a substantially lower fluorescent signal from cells of the central columella in comparison to other cells in the meristem and root tip (Fig. 4A). Labeling in the columella was usually in the form of diffuse fluorescence throughout the cytoplasm and a few fragmented actin bundles, so that although columella initials and cells in the quiescent center were extensively labeled (Fig. 4A,D), distinct actin strands were observed less frequently than in MBS-stabilized and permeabilized root sections (Fig. 4D). In some cases, columella cells displayed fluorescent rings around the amyloplasts and an intense diffuse fluorescence originating from the nucleus (Fig. 4E). The reduced fluorescence and scarce actin in the central columella cells was also observed in *Z. mays* and *L. usitatissimum* using both the monoclonal and polyclonal antibodies (data not shown). The polyclonal anti-maize actin antibody was slightly more successful than the monoclonal antibody in revealing actin bundles in the columella cells of *L. usitatissimum* (Fig. 4F) although the labeling was not as extensive as was the labeling obtained in MBS-stabilized roots (see Fig. 1). Interestingly, the actin bundles that were successfully preserved also appeared to surround the amyloplasts (Fig. 4F). Like the labeling pattern obtained with the MBS cross-linking technique, actin in the peripheral cap cells was well stained and displayed prominent actin bundles (data not shown).

Appropriate controls for the different antibodies were negative. Under identical imaging and printing condi-

Fig. 2. Organization of actin in cap cells of *M. truncatula* (**A, B, F**), *Z. mays* (**D**) and *L. usitatissimum* (**C, E, G–I**). Actin filaments were labeled with Alexa Fluor-phalloidin after glycerol permeabilization of MBS-fixed vibratome sections and viewed with a confocal microscope. Each panel, except **B**, shows a brightfield image and corresponding fluorescence image. All images are single optical sections, except **I**, and representative of 5–10 different roots. **A** Brightfield image and corresponding fluorescence image of peripheral cap cells (*pc*) and amyloplast-containing central columella (*cc*) cells of *M. truncatula*. Although the columella region showed unstained areas (**) and lower fluorescence intensity compared to the peripheral cap region (*pc*), the columella cells (*arrowheads*) were clearly labeled. **B** A higher-magnification image showing several columella cells in the root cap of *M. truncatula*. Prominent actin cables appear to enclose the amyloplasts (*arrowheads*). **C, D** The central columella cells of *L. usitatissimum* (**C**) and *Z. mays* (**D**), also show distinct cytoplasmic actin cables forming cages around the amyloplasts and connect the proximally located nucleus (*n*) to the cell periphery. **E, F** In *L. usitatissimum* (**E**) and *M. truncatula* (**F**), a denser actin network can be observed in peripheral cap cells, which consisted of both thick and thin bundles. **G, H** Single optical sections show that in addition to the actin bundles within the columella cell cytoplasm (**G**), fine transverse arrays of actin occur at the cell cortex (**H**). **I** Projection of 20 optical sections taken at 0.3-μm intervals reveals the extensive network of actin filament bundles in a central columella cell of *L. usitatissimum*. *a*, amyloplasts. Bars = 50 μm (**A**), 10 μm (**B–I**)

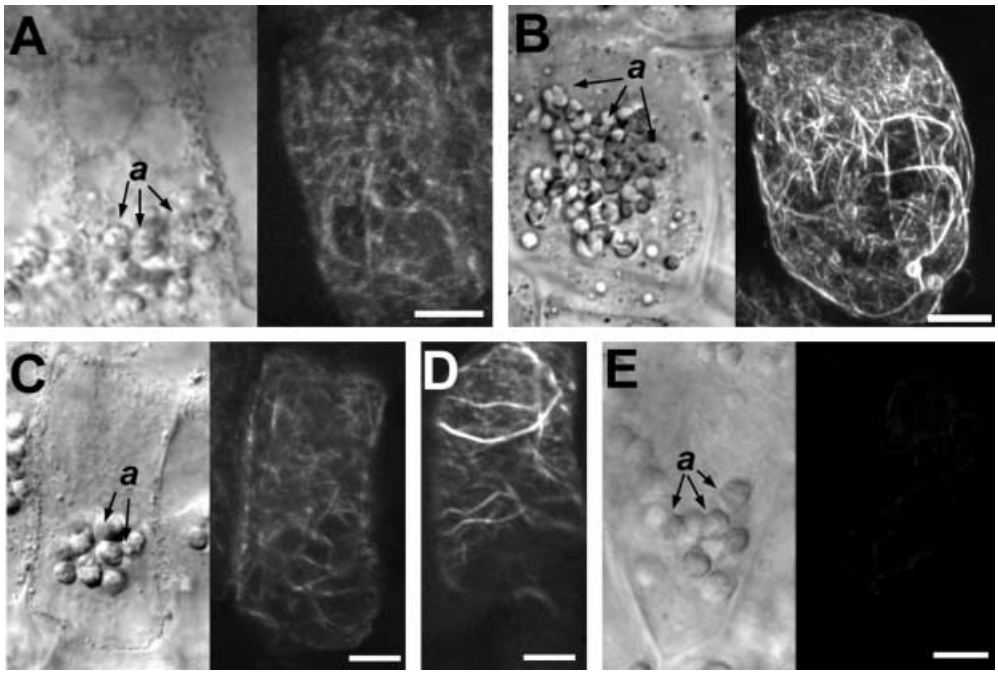


Fig. 3A–E. Control experiments. **A, B** Columella cells from *L. usitatissimum* root sections, labeled with 0.10 μM Alexa Fluor-phalloidin, that were either directly labeled without MBS pretreatment (**A**) or pretreated with MBS prior to labeling (**B**). Columella cells from root sections pretreated with MBS displayed better resolution and more abundant and brighter actin strands than columella cells from root sections not treated with MBS. **C, D** Columella cells from *L. usitatissimum* root sections labeled with 0.01 μM Alexa Fluor-phalloidin. Although labeling was generally weak, actin organization was essentially similar to the pattern obtained when higher probe concentrations were used. Randomly oriented cytoplasmic actin (**C**) and transverse cortical actin (**D**) were detected. **E** A columella cell from a *L. usitatissimum* root section treated with 1-μM unconjugated phalloidin prior to Alexa Fluor-phalloidin staining. Only a few, if any actin bundles were observed. Panels **A** and **B** are projections of 25 optical sections taken at 0.3-μm intervals while panels **C**–**E** are single optical sections. Images are representative of at least three different roots. *a*, amyloplasts. Bars = 10 μm

tions, controls using the pre-immune serum or controls that omitted the primary antibody showed little fluorescence throughout the root (data not shown).

Aldehyde fixation degrades actin in the columella

In aldehyde-fixed roots, the lower quality of actin labeling in the columella cells compared to other cells could indicate that the actin arrays in the columella were not fully preserved, as has been reported for certain other cell types (Traas et al. 1987; Blancaflor and Hasenstein 2000). Therefore, the full extent of the actin cytoskeleton is not revealed in the columella by aldehyde fixation (Traas et al. 1987), given the fact that we observed an extensive actin network in the columella cells of MBS-stabilized root sections after glycerol permeabilization.

To improve actin filament labeling in the columella of aldehyde-fixed root tissue and further confirm that our labeling with phalloidin was not artificially forming actin bundles, we pretreated freshly cut root vibratome sections in MBS for 30 min followed by fixation in 3% formaldehyde for another 30 min. 3-Maleimidobenzoyl-*N*-hydroxy-succinimide ester has been shown to protect the delicate actin filaments from the adverse effects of aldehydes (Sonobe and Shibaoka 1989) and could therefore confer the same advantages for the actin in the columella. Aldehyde-fixed roots stained with Alexa Fluor-phalloidin without MBS pretreatment showed only weak and diffuse labeling throughout the columella region (Fig. 5A). On the other hand, root sections pretreated with MBS prior to aldehyde fixation revealed a few columella cells with well-preserved actin (Fig. 5B–D). Although the quality of labeling was not as good as labeling seen from roots that were not aldehyde-fixed, the staining was markedly improved over non MBS-pretreated roots. Despite the differences in the quality of actin preservation in the columella between MBS cross-linked/non-aldehyde treated and MBS cross-linked and aldehyde-fixed sections, some general patterns of actin organization were consistent. For example, the actin bundles surrounding the amyloplasts were also observed in columella cells of *L. usitatissimum* (Fig. 5B,C) and *M. truncatula* (Fig. 5D), while nuclear-associated actin bundles were occasionally preserved (Fig. 5C). However aldehyde fixation of MBS cross-linked sections did not preserve transversely oriented actin in the cortex.

Endoplasmic reticulum dynamics in the columella of Nicotiana benthamiana demonstrate a functional actin cytoskeleton

Cytoplasmic streaming, and the bulk movement of subcortical ER, are actin-dependent processes and, as such, are inhibited with compounds that disrupt actin

filaments (Quader et al. 1987; Boevink et al. 1998). To confirm the presence and functionality of actin filaments in the columella, we used confocal microscopy to image roots of *N. benthamiana* lines stably expressing ER-targeted GFP. The small size of *N. benthamiana* primary roots allowed us to obtain a single optical section at the plane of the columella revealing the large and dense amyloplasts characteristic of these cells (Fig. 6A). The pattern of ER fluorescence obtained from the columella was different from the pattern in the peripheral cap cells. While peripheral cap cells contained the ER arrays typically seen in other plant cell types, including subcortical strands throughout the center of the cell and reticulate cortical arrays (Boevink et al. 1998), ER in columella cells was primarily located in the cell periphery and was more abundant along the distal wall where it was positioned below the sedimented amyloplasts (Fig. 6A,B). This pattern of ER in the columella revealed by GFP is consistent with published reports using transmission electron microscopy (Sack 1997). Time-lapse imaging of the columella cells over several minutes demonstrated vigorously undulating ER tubules and rapidly changing fluorescence patterns, including the distal ER adjacent to the amyloplasts. These pattern changes were evident even over periods as short as 10 s (Fig. 6B). Similar observations were made in *Arabidopsis thaliana* roots expressing a similar ER-targeted GFP construct (data not shown). Labeling of actin using the glycerol permeabilization technique described above confirmed that prominent actin bundles occur in the columella cells of *N. benthamiana* (Fig. 6C). Because of the small size of *N. benthamiana* roots, sectioning with the Vibratome was more difficult. Thus, actin labeling was not as good as the labeling obtained in the other plant species, and the application of this technique to smaller roots such as *Arabidopsis* would be even more technically demanding.

Discussion

Despite the proposal that the actin cytoskeleton is involved in gravity sensing (Sievers et al. 1989, 1991), a reliable way to image actin filaments in the columella cells of plant roots has yet to be developed. Previous attempts at localizing actin filaments in the columella cells have been fraught with difficulties, as illustrated by the number of studies showing poorly resolved actin strands or diffuse actin labeling in the columella cell cytoplasm (Hensel 1986, 1989; Korropp and Volkmann 1994; Baluška et al. 1997a,b; Baluška and Hasenstein

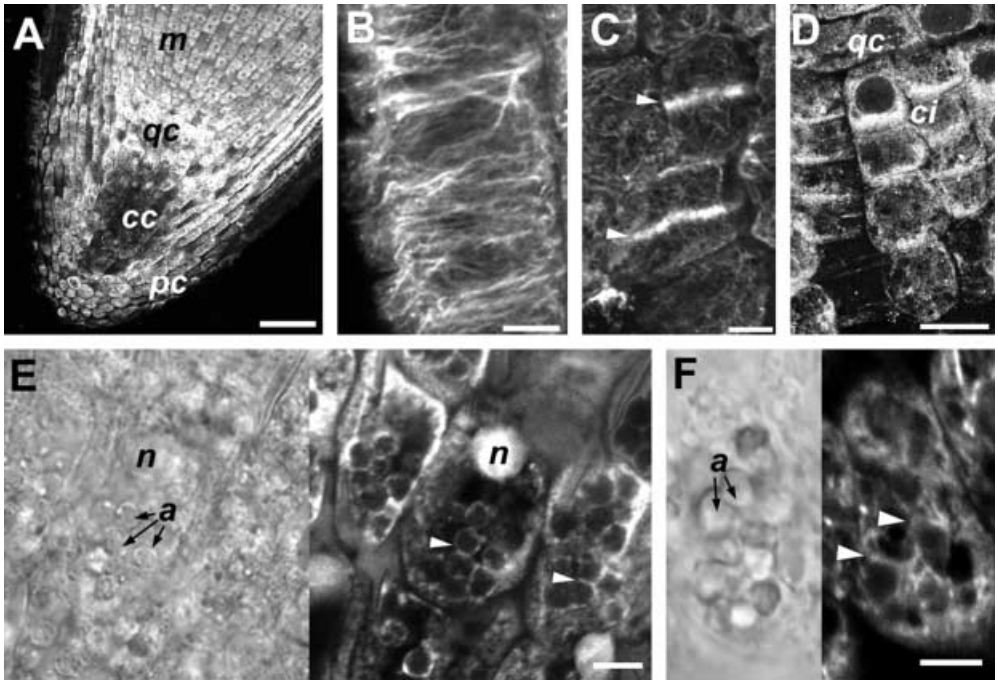


Fig. 4. Immunofluorescence labeling of actin filaments in roots of *M. truncatula* (**A, E**), *Z. mays* (**B–D**) and *L. usitatissimum* (**F**) using polyclonal anti-maize actin (**A, C–F**) and monoclonal anti-pea actin (**B**) antibodies. Panel **A** is a projection of 20 optical sections taken at 1-μm intervals; panel **D** a projection of 15 optical sections at 0.8-μm intervals, while **B, C, E** and **F** are single optical sections. **A** Although the peripheral cap (*pc*), quiescent center (*qc*) and meristem (*m*) are strongly labeled, the central columella (*cc*) region shows a less intense fluorescent signal. **B, C** Different cell types in the meristematic region of *Z. mays* show different arrays. Transverse actin was observed in the epidermal cells (**B**) while cells undergoing division displayed randomly oriented cytoplasmic actin and division-specific arrays such as the phragmoplast (*arrowheads* **C**). **D** In maize roots, an intense fluorescent signal is observed in all cells of the quiescent center (*qc*), columella initials (*ci*) and columella cells at an early stage of development. **E** Higher-magnification image of the central columella cells depicted in **A**. Fluorescent rings surround the amyloplasts (*a*, *arrowheads*) and an intense diffuse fluorescence is associated with the nucleus (*n*). **F** A columella cell of *L. usitatissimum* labeled with anti-maize actin polyclonal antibody reveals only a few distinct actin bundles surrounding the amyloplasts (*arrowheads*). Bars = 100 μm (**A**), 20 μm (**D**), 10 μm (**B, C, E, F**)

1997; Staves et al. 1997; Driss-Ecole et al. 2000). This inability to image actin filaments in the columella cells led to the proposal that these cells indeed lack prominent actin bundles which was then interpreted to be important for gravity sensing because it allowed amyloplasts to sediment more readily (Baluška and Hasenstein 1997; Baluška et al. 1997a).

However, we argue that the inability to label distinct actin filaments in the columella results from poor preservation and inadequate preparative techniques, and that the studies reporting diffuse actin labeling in the columella did not thoroughly explore this possibility. The need to test a variety of tissue processing methods for labeling actin in the columella becomes even more important in view of reports indicating that the actin cytoskeleton in some plant cell types is sensitive to aldehyde fixation (Traas et al. 1987; Blancaflor and Hasenstein 2000). We therefore conducted this study in order to optimize actin labeling in the columella cells of a few selected plant species.

Root columella cells contain extensive and functional actin arrays

The data presented in this paper clearly show that the columella cells of *Z. mays*, *L. usitatissimum*, *M. truncatula* and *N. benthamiana* contain prominent actin filament arrays. The method that gave the greatest success for labeling distinct actin arrays in the columella involved treating freshly cut root vibratome sections in MBS, a protein cross-linker that has been shown to improve actin labeling in various plant cell types (Sonobe and Shibaoka 1989; Blancaflor and Hasenstein 2000), followed by glycerol permeabilization of Alexa Fluor-phalloidin (Olyslaegers and Verbelen 1998). With this technique, predominantly random arrays of actin filaments were detected in the columella cell cytoplasm. Some of the cytoplasmic actin bundles were observed to be in association with both the nucleus and amyloplasts, while fine transverse actin was imaged at the cell surface.

The existence of organelle-associated actin arrays in the columella has been alluded to before in proposed models on gravity sensing (Sievers et al. 1991). More recently, immunoelectron microscopy using anti-actin antibodies showed that immunogold particles were localized very close to the membranes of the nucleus and amyloplasts, indicating that these organelles were connected to actin filaments (Driss-Ecole et al. 2000). While the association of the nucleus to the actin network could aid in maintaining its proximal position in the cell (Lorenzi and Perbal 1990), the linkage between amyloplasts and the actin cytoskeleton could have important implications in gravity sensing and transduction. Rearrangement of the actin network upon gravistimulation due to amyloplast displacement could constitute one of the initial acts of gravity sensing or trigger the primary signal transduction events leading to root curvature (Sievers et al. 1989, 1991). The ability to image distinct actin filament bundles, as shown in this study, will allow this hypothesis to be tested.

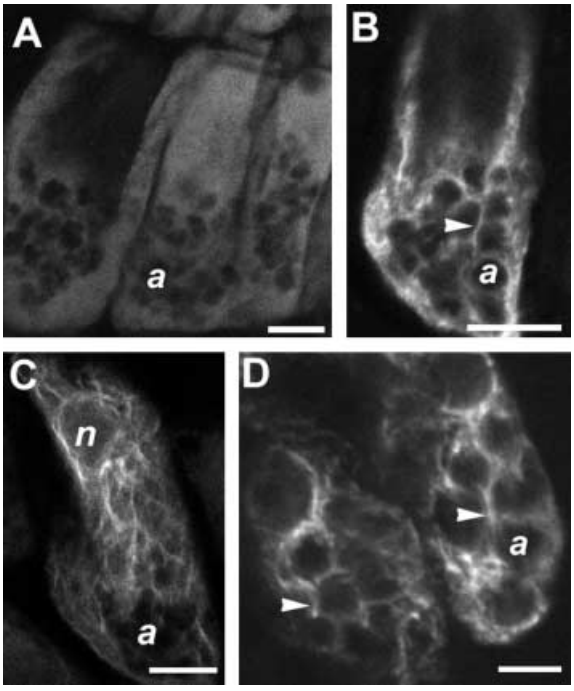


Fig. 5A–D. Actin labeling with Alexa Fluor-phalloidin in the columella of aldehyde-fixed root sections after MBS cross-linking. All images are single optical sections and representative of three different roots. **A** An *L. usitatissimum* columella cell from a root fixed in 3% formaldehyde without MBS pretreatment and labeled with Alexa Fluor-phalloidin shows very weak diffuse fluorescence and the absence of distinct actin structures. **B, C** Columella cells from an *L. usitatissimum* root section pretreated with MBS prior to aldehyde fixation. Actin bundles can be seen around the amyloplasts (*arrowhead*, **B**) and radiating from the nuclear surface (*n*, **C**). **D** Several columella cells from *M. truncatula* also display actin bundles forming rings around the amyloplasts (*a*, *arrowheads*). Bars = 10 μm

This study shows that the actin cytoskeleton within the columella is functional. In verifying electron microscopy studies that showed ER distributed to the cell periphery and adjacent to sedimented amyloplasts (Sack 1997), our observations of highly dynamic ER in living *N. benthamiana* and *A. thaliana* columella cells confirm that these cells contain an extensive actin cytoskeleton, and more importantly, demonstrate that this actin is functional. Furthermore, an assay for actin functionality within the columella allows actin's involvement in gravitropism to be tested in a more rigorous manner. Observations of ER dynamics in the columella will provide a critical measure for the uptake and washout of actin-disrupting agents such as cytochalasin or latrunculin that has not previously been possible.

Our study also shows that columella cells contain fine transversely oriented actin filaments adjacent to the plasma membrane. Similar transverse actin arrays also occurred in the peripheral root cap, and in non-dividing epidermal and cortical cells of the root proper (see Figs. 1F–I and 4B), and have been demonstrated in a variety of cell types (Traas et al. 1987; Blancaflor and Hasenstein 2000; Collings and Allen 2000). While White and Sack (1990) observed similar transverse arrays in cells of the *Z. mays* root cap, the absence of amyloplasts

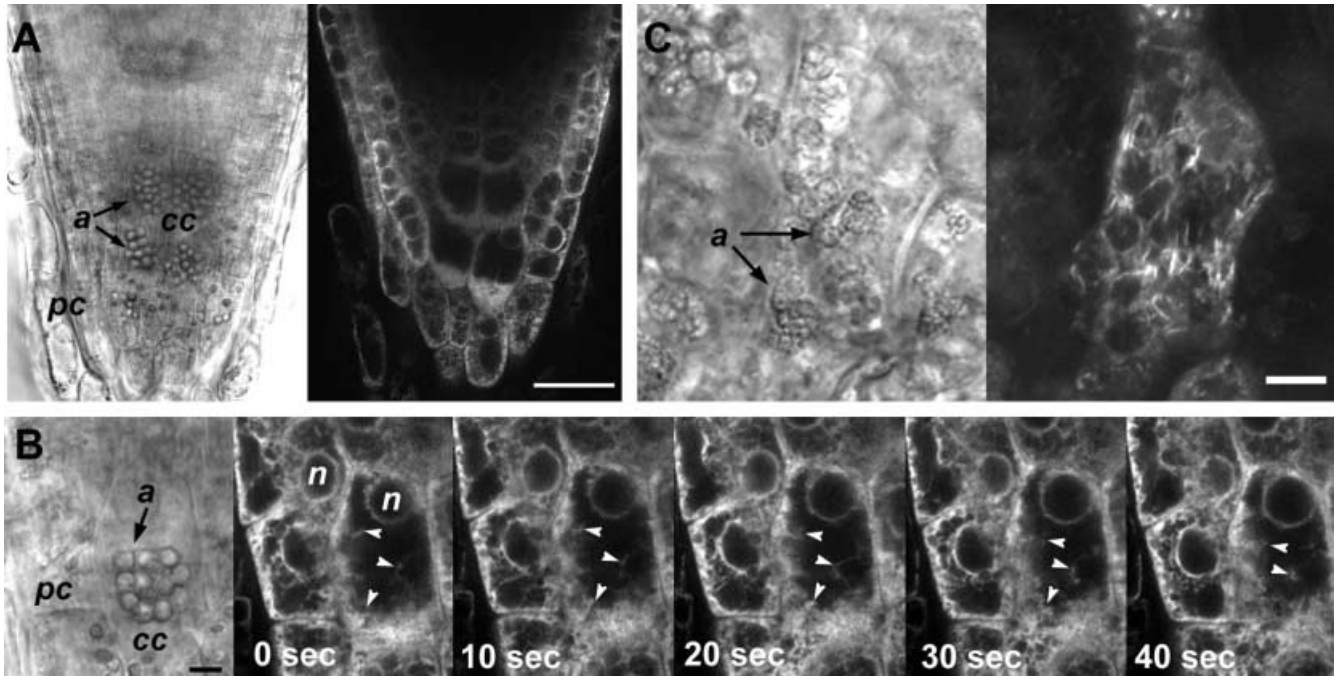


Fig. 6A–C. Confocal fluorescence microscopy of GFP targeted to the ER in *N. benthamiana* root caps. The presence of vigorous movement of ER is indicative of an extensive and functional actin cytoskeleton, in both the peripheral root cap (*pc*) and columella cells (*cc*). **A** Low-magnification brightfield image and corresponding fluorescence image at a single focal plane showing the general pattern of ER in a whole cap of *N. benthamiana*. **B** Brightfield image and corresponding sequential fluorescence images demonstrate that substantial changes in ER patterning occur in the columella cells over time (*arrowheads*). Animations of the sequential fluorescence images also demonstrate this motion (data not shown). This image also shows that the nucleus (*n*) in an *N. benthamiana* columella cell remains in its typical, proximal position while nuclei in the peripheral root cap (*pc*) show variability in nuclear location. Columella cells also show reduced levels of ER compared to adjacent peripheral root cap cells, and have ER concentrated along the cell periphery, being particularly enriched at the distal wall. **C** Actin filaments in a columella cell of *N. benthamiana* labeled using MBS cross-linking and glycerol permeabilization. *a*, amyloplasts. Bars = 50 μm (**A**), 10 μm (**B–C**)

in the labeled cells raises the question as to whether these cells were indeed columella cells. Our results convincingly show that amyloplast-containing columella cells do contain fine transverse actin filaments. Transverse cortical actin filaments mirror the alignment of cortical microtubules (Collings and Allen 2000), and an interaction between actin filaments and microtubules at the plasma membrane has been demonstrated recently (Collings et al. 1998b). Interestingly, transverse arrays of cortical microtubules were also imaged in columella cells of *Z. mays* (Baluška et al. 1997a). Although it is unclear what the functions of the transverse actin arrays in the columella cell are, their co-alignment with cortical microtubules could indicate a role in columella cell development. As cortical microtubules play a role in cellulose deposition, which in turn leads to the directional expansion of the cell, it is possible that the actin filaments at the plasma membrane could cooperate with microtubules to direct cellulose deposition (Collings and Allen 2000).

Alexa Fluor-phalloidin permeabilization accurately reports actin organization of columella cells

One argument raised against using fluorescently labeled phalloidin to label actin in cells that are not aldehyde-fixed is the possibility that the applied phalloidin could lead to artificial actin polymerization (Baluška et al. 1997a,b; Baluška and Hasenstein 1997). However, numerous observations in our experiments concerning the actin cytoskeleton in both the columella and root proper argue against this possibility, instead demonstrating that the observed patterns reflect the real distribution of actin in these cells. First, Alexa Fluor-phalloidin at either 0.01 or 0.1 μM gave similar labeling patterns with these concentrations being 10- to 100-fold lower than the known concentrations that could induce artificial actin polymerization (Traas et al. 1987). Second, MBS is a protein cross-linker that is thought to stabilize actin by inducing monomer cross-linking within filaments (Sonobe and Shibaoka 1989). However, coupling of actin monomers to other proteins will also occur, thereby reducing or eliminating the pool of polymerizable G-actin available for polymerization induced by phalloidin (Sonobe and Shibaoka 1989; Driss-Ecole et al. 2000). As we observed an increase in the amount of actin present after MBS stabilization, whether by Alexa Fluor-phalloidin in permeabilized cells (Figs. 1–3), or by actin antibodies (Fig. 4), it seems highly unlikely that polymerization artifacts resulting from phalloidin could explain our observations. Third, root sections cross-linked with MBS followed by formaldehyde fixation also showed an extensive network

of actin filaments in the columella (Fig. 5). As these roots had been fixed in formaldehyde it is very unlikely that addition of Alexa Fluor-phalloidin induced artificial actin polymerization. Fourth, indirect immunofluorescence experiments revealed actin-labeling patterns in the meristem and peripheral cap cells that were also observed in MBS cross-linked and glycerol-permeabilized root cells. These included actin arrays in the phragmoplasts and fine transversely oriented actin at the surface of epidermal and cortical cells (see Figs. 1 and 4; see also Blancaflor and Hasenstein 1997). The detection of these fine actin arrays using both preparative methods makes it unlikely that actin polymerization was artificially induced in these cell types. Immunofluorescence experiments are also indicative of extensive actin within the columella, although as discussed below, preservation was not as good in these cells. And fifth, the presence of active ER movement in the columella is indicative of an extensive and functional actin cytoskeleton (Quader et al. 1987; Hensel 1989).

In addition to the columella, Baluška et al. (1997a,b) reported that cells of the quiescent center and columella initials were depleted of actin filament bundles. This is in contrast to our results showing distinct actin arrays in the quiescent center cells and columella initials using both immunofluorescence and phalloidin staining (see Figs. 1A,B and 4A). Again, the discrepancy in these results may be explained by the differences in the procedures used for tissue preparation. The chances that the actin bundles we observed in the columella initials and quiescent center resulted from artificial actin polymerization are even more unlikely since both aldehyde-fixed and MBS-cross-linked roots showed similar labeling patterns.

The differences between our results and those of previous investigators may be due to poor preservation, possibly resulting from slow penetration of fixatives into the columella, and because of the inherent instability of actin with these aldehyde-based fixatives (Blancaflor and Hasenstein 2000). Slow fixative penetration, possibly due to differences in the extracellular matrix of the cap, may also explain why we also consistently observed a reduction in fluorescence intensity from the columella, compared to cells in the adjacent peripheral cap, quiescent center and meristematic regions. However, it is also possible that while these cells contain an actin cytoskeleton similar to other plant cell types, differences in the dynamic nature and intrinsic stability of actin filaments within these densely cytoplasmic cells make fixation more difficult (Baluška and Hasenstein 1997). Such a dynamic actin cytoskeleton within the columella would provide a suitable intracellular environment for amyloplasts sedimentation, which appears to play a key role in gravity perception (Sack 1997). Additional work will be needed to test these possibilities. The application of actin-binding GFP fusion proteins (e.g. Kost et al. 1998) to this question will likely be beneficial. However, in such experiments, care that the fusion protein does not modify actin dynamics, and controls for the expression of the protein on gravitropic bending, would be needed.

Implications for models of the gravity response pathway

Our results depicting distinct actin structures in the columella are significant in view of recent molecular genetic studies identifying genes that play important roles in gravitropism (Chen et al. 1999). For example, the *ARG1* gene which when mutated altered graviresponsiveness, encodes a DnaJ-like protein whose C-terminal domain contains coiled-coil motifs with sequence similarity to cytoskeletal-binding proteins, including tropomyosin, myosin and kinesin. Thus, it was proposed that sedimenting amyloplasts, enmeshed in a network of actin filaments, could transmit a signal to plasma-membrane receptors facilitated by the *ARG1* gene product (Sedbrook et al. 1999). Recent molecular genetic evidence has also renewed interest in auxin as a signaling molecule in gravitropism (Chen et al. 1998), with actin potentially involved in the regulation of auxin transport. Actin co-purifies with an unknown protein that modulates the activity of the auxin efflux carrier, with this interaction being inhibited following the disruption of actin with cytochalasin (Butler et al. 1998). Based on the model proposed by Sievers et al. (1989, 1991), amyloplast displacement in the columella might induce tensional changes within an interlinked actin network. These tensional changes could lead to modulation of auxin transport activity within the root cap by cortical actin, thus beginning the transmission of the gravity signal to the elongation zone. Alternatively, reorganization of the actin cytoskeleton in the columella upon gravistimulation could modulate ion channel (Hwang et al. 1997) or pump activity resulting in the influx (or efflux) of ions, which then generates an early gravitropic signal (Monshausen et al. 1996; Scott and Allen 1999). A combination of cell biological and molecular genetic approaches should help resolve these issues.

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