A comparison of F-actin labeling methods for light microscopy in different plant specimens: multiple techniques supplement each other

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Abstract

Different detection methods for F-actin labeling were compared on a range of plant specimens: cultured cells, whole organ mounts and sectioned material. For cultured cells, microinjection of labeled phalloidin yielded the most detailed picture but careful permeation methods come close, while immunocytochemical methods always gave relatively poor detail, especially on the level of the fine filaments. For whole organ mounts and sectioned material, permeation methods and immunolocalization are the methods of choice, however never reaching the level of resolution of permeation methods in single cells. It is clear that there is no general and universal good method and multiple techniques are needed, especially when working with different specimens and with different aims. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Plant actin; Phalloidin; Glycerol; Microinjection; Confocal laser scanning microscopy

1. Introduction

The fact that actin is ubiquitous in plants as part of the cytoskeleton was accepted while ago and was elegantly demonstrated using labeled phalloidin on formaldehyde fixed plant cells (Parthasarathy et al., 1985). Non-fixation permeation methods, described by Traas et al. (1987) revealed additional details of the actin network in plant cells. During the past decade it became clear that actin might be more important in plant cell physiology than ever expected. The *Arabidopsis* genome contains 10 actin genes, of which at least eight are functional in vegetative and reproductive growth (Meagher et al., 1999). The role of actin in the movement and localization of organelles, such as chloroplasts (Kandasamy and Meagher, 1999), Golgi stacks (Boevink et al., 1998; Nebenführ et al., 1999) and mitochondria (Olyslaegers and Verbelen, 1998) has been underlined. The actin skeleton is also implicated in the succesful establishment of symbiotic plant–bacteria interactions (Miller et al., 1999).

Numerous fluorescence micrographs are published showing actin filaments in plant cells as apparently independent fibers of various sizes and arrangements (e.g. Traas et al., 1987; Schmit and Lambert, 1990; Cleary et al., 1992). On the other hand, Reuzeau et al. (1997a) recently found

evidence for the existence of a very fine fibrillar mesh of actin coating the endomembranes in onion epidermal cells. Being a major component of a putative "endomembrane sheath" supports its overall function in movement and positioning of the endoplasmic reticulum and organelles (Reuzeau et al., 1997a,b). Using wide-field computational optical sectioning fluorescence microscopy, these authors claim that the resolving power of a fluorescence microscope forms a problem when trying to visualize the putative very fine actin meshes. However, the main problem for the demonstration of F-actin is entering a detection probe into the cell while keeping the actin in a state as native as possible. As the possible artifacts of chemical fixation are well known (Mersey and McCully, 1978; Verbelen and Tao, 1998) rapid permeation of the cell membrane or microinjection (Schmit and Lambert, 1990) seemed theoretically the best approaches, at least for single cell use. Elegant freeze fixation methods coupled to different ways of further treatment were also developed for specific purposes (Lancelle and Hepler, 1989; Baskin et al., 1996; Miller et al., 1999). In vivo F-actin labeling in *Arabidopsis* and tobacco cell cultures has been published recently, making use of the expression of a green fluorescent protein (GFP)-mouse talin fusion protein (Kost et al., 1998).

During our ongoing research in plant cell biology, we developed new methods but also used many of the published protocols to visualize F-actin in cultured cells and isolated

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tissues as well as in whole organs. Here we compare and evaluate these different technical approaches for their use in plant cell biology.

2. Materials and methods

2.1. Plant material

Elongated tobacco cells were regenerated from protoplasts which were isolated from leaves of sterile grown *Nicotiana tabacum* L. cv. Petite Havana SR1, as described in Verbelen et al. (1992).

Arabidopsis seedlings (*Arabidopsis thaliana* L., ecotype Columbia) were grown in Petri dishes on a half strength Murashige and Skoog medium including vitamins (4.4 g l^{-1}, Duchefa), supplemented with 10 g l^{-1} sucrose and solidified with 0.4% Gelrite (Duchefa), pH 5.7. After overnight incubation at 4°C, the dishes were placed vertically in the growth chamber at 25°C in a 16 h photoperiod under 40 μmol m^{-2} s^{-1} illumination.

Seeds of *Vicia faba* L. and *Zea mays* L. were germinated in moisturized vermiculite at 25°C in a 16 h photoperiod.

Viability of the plant material during and after actin labeling was evaluated after a 5 min incubation in 24 μM of the viability stain fluorescein diacetate (FDA, Serva).

2.2. Methods for actin labeling

2.2.1. Detergent permeation method

Plant tissues were incubated with 0.18 μM Rhodamine-phalloidin (Rh-phalloidin, Molecular Probes, stock dissolved in methanol) in actin stabilizing buffer (ASB; 100 mM PIPES, 10 mM EGTA, 5 mM MgSO$_4$, pH 6.8) to which 5% (v/v) dimethyl sulfoxide and 0.05% (w/v) Nonidet P-40 were added to permeate the cells (Traas et al., 1987). For tobacco cultured cells, 0.3 M mannitol was always included in the ASB. The actin labeling was observed after an incubation period of 15–25 min and 3 h, for tobacco cells and *Arabidopsis* roots, respectively.

2.2.2. Glycerol permeation method

Following the procedure of Olyslaegers and Verbelen (1998), tobacco cells and *Arabidopsis* roots were incubated with 0.18 μM Rh-phalloidin in ASB containing 1% glycerol. Actin started being labeled after 1 h in tobacco cells and after 3 h in *Arabidopsis* roots.

2.2.3. Cytofectene permeation method

Tobacco cells were incubated with 0.18 μM Rh-phalloidin in ASB containing 0.8% (v/v) cytofectene (Quantum Prep® Cytofectene™ Transfection Reagent Kit, Bio-Rad Laboratories). Actin labeling was observed after 30 min and was further evaluated with time.

2.2.4. Microinjection method

Elongated tobacco cells of 5–7 days old were embedded in a very thin film of agarose (1.2% of Seaplaque™ Agarose, Duchefa) in Petri dishes and covered with K$_3$A culture medium (Potrykus and Shillito, 1986). A 15 μl aliquot stock (approximately 6.6 μM) of Alexa 488-phalloidin (Molecular Probes, stock dissolved in methanol) was dried down and re-suspended in 7 μl of filter sterilized injection buffer (5 mM Tris-acetate, 0.2 mM 1,4-dithiothreitol (DTT), pH 7.5, Valster et al., 1997). Injection needles were pulled from 1.0 mm borosilicate glass capillaries with filament (Clark Electromedical Instruments) by using a horizontal pipette puller (P-2000, Sutter Instrument Company). The needles were backfilled with the staining solution and mounted on a pressure injector (CellTram Oil 5176, Eppendorf) filled with water. The microneedle holder was positioned by a joystick-controlled motorized micromanipulator (5171, Eppendorf).

2.2.5. Immunolocalization on cultured cells

Tobacco cells, attached to coverslips by a thin film of agarose, were fixed for 1 h with 4% paraformaldehyde in actin stabilizing buffer (ASB50; 50 mM PIPES, 5 mM EGTA, 2.5 mM MgSO$_4$, pH 6.8) rinsed in ASB50 and treated with 1 mM DTT and with 0.1% NaBH$_4$ in ASB50, both for 15 min. After 5 min in cold methanol (−20°C), cells were re-hydrated in ASB50 and permeated with 1% Triton-X-100 for 10 min, rinsed briefly in ASB50 and treated for 30 min with 1% bovine serum albumin (BSA). Cells were incubated for 2 h in mouse anti-actin Mab (clone C4, ICN), diluted 1:400 in incubation buffer (ASB50 plus 0.1% BSA), followed by several rinses in 0.1% BSA. Then they were incubated for 1 h in FITC conjugated goat anti-mouse IgG (Sigma), diluted 1:100 in incubation buffer, and washed in ASB50.

2.2.6. Immunolocalization on Arabidopsis roots

Roots from 3-day-old seedlings were fixed for 3 h with 3.7% paraformaldehyde and 0.5% glutaraldehyde in ASB50 buffer plus 300 μM phenylmethane sulfonyl fluoride (PMSF), 0.1% Triton X-100 and 100 μM *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). After 15 min in 0.1% NaBH$_4$, the roots were treated with wall degrading enzymes (0.1% pectinase (Serva) and 1% driselase (Fluka) in PBS). Following two rinses with TPPBS (PBS plus 0.02% Tween 20 and 300 μM PMSF), roots were treated with 1% BSA for 10 min. After overnight incubation at 37°C in mouse anti-actin Mab (clone C4, diluted 1:100), roots were rinsed four times and then incubated for 4 h in FITC conjugated goat anti-mouse IgG. After rinsing for 15 min in TPPBS, the roots were mounted on a microscope slide.

2.2.7. Immunolocalization on sectioned material

Three-day-old maize and bean root tips were used according to the procedure of Vitha et al. (1997). Briefly, root tip segments were excised in ASB50, vacuum infiltrated with 100 μM MBS in ASB50 and fixed in 1.5% paraformaldehyde in ASB50 for 1 h. After several rinses in ASB50, they
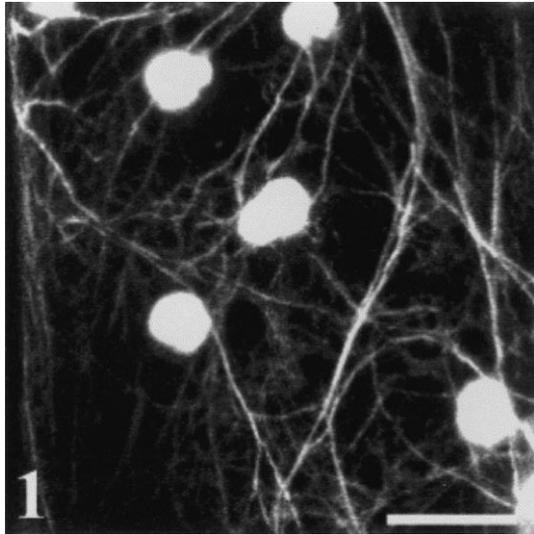
Fig. 1. Confocal laser scanning microscopy (CLSM) extended focus images of actin labeling in cultured tobacco cells obtained by different methods. Note the bright autofluorescence of the chloroplasts in the non-fixed cells. Scale bar indicates 20 μm. Rh-phalloidin labeling by use of the detergent permeation method.

were dehydrated in a graded PBS–ethanol series followed by a step-wise infiltration with Steedman's wax (PEG 400 distearate (Aldrich) and 1-hexadecanol (ICN) in 9:1 (w/w) proportion). After polymerization of the wax, 8 μm thick sections were made from the embedded root segments and stretched on a drop of deionized water on slides coated with glycerol–albumin. The sections were dried overnight, dewaxed in ethanol, re-hydrated in an ethanol–PBS series and washed in ASB50. After 10 min in methanol at −20°C, they were washed in ASB50, then incubated with mouse anti-actin Mab (clone C4, diluted 1:100) for 90 min, washed
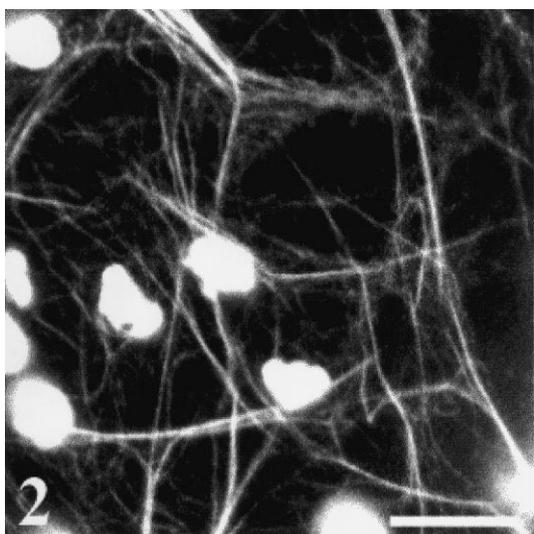
Fig. 2. CLSM extended focus images of actin labeling in cultured tobacco cells obtained by different methods. Note the bright autofluorescence of the chloroplasts in the non-fixed cells. Scale bar indicates 20 μm. Rh-phalloidin labeling by use of the glycerol permeation method.

in ASB50 and incubated for 1 h in FITC-conjugated goat anti-mouse IgG. Slides were washed in PBS and treated with 0.01% Toluidine blue to diminish the autofluorescence.

2.3. Microscopy

An inverted microscope (Diaphot TMD, Nikon) equipped with brightfield and fluorescence illumination was used for microinjection. All the imaging was performed with a Bio-Rad MRC-600 confocal system mounted on a Zeiss Axioskop microscope, equipped with a 40× (NA 0.9) water-immersion and a 63× (NA 1.2) oil-immersion objective, using the 488 and 514 nm laser lines to visualize the Alexa 488-phalloidin and Rh-phalloidin labeled actin, respectively.

3. Results

3.1. Cultured cells

In single cells, the actin filaments can be stained very well using the detergent permeation method. The resulting picture (Fig. 1) shows thick bundles of actin filaments and a dense mat of fine filaments. Pictures have however to be registered exactly at the moment of optimal staining, which is generally after 15–25 min, dependent from cell to cell. Afterwards, the structure of the actin filament network deteriorates very fast. As a consequence, very often the stained actin filaments are more wavy or tortuous than in Fig. 1.

The use of glycerol as a membrane permeant, instead of detergents, improves significantly the quality and the shelf-life of the preparations. Especially the very fine actin filaments are more abundant in glycerol treated cells, but they remain however difficult to capture in an image (Fig. 2). In general the actin filaments are less wavy and tortuous than after detergent permeation and the deterioration of the cell structure is minimal. Probably therefore, permeation with glycerol — in contrast to detergents — of cells having GFP-labeled mitochondria does not result in the release of GFP out of the mitochondria, allowing co-localization of actin filaments and mitochondria in these cells (results not shown). The two permeation methods have one strong limitation: in older cells that have a thicker cell wall, the success rate of the staining drastically decreases. Cells of 20 days in age will very seldom exhibit a positive stain.

Alternatively, cells were chemically fixed and assayed using immunocytochemistry. This approach resulted in a poor picture of the actin skeleton (Fig. 3). Some thick bundles could be recognized while there remained only small stretches of putative thin filaments; sometimes these remnants had a net-like shape. Labeling was also found at the periphery of the chloroplasts, however amorphous in appearance. The autofluorescence of the chloroplasts in chemically fixed cells is almost negligible (Fig. 3), in contrast to the non-fixed cells where they are very conspicuous as big fluorescent bodies (Figs. 1, 2 and 4–6). In the
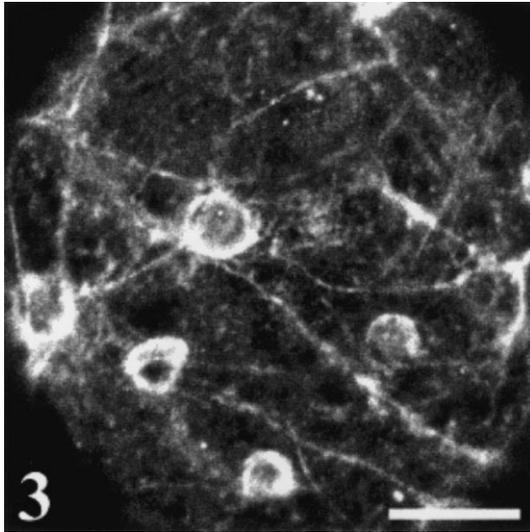
Fig. 3. CLSM extended focus images of actin labeling in cultured tobacco cells obtained by different methods. Note the bright autofluorescence of the chloroplasts in the non-fixed cells. Scale bar indicates 20 μm. Immunolabeling of actin.

Fig. 5. CLSM extended focus images of actin labeling in cultured tobacco cells obtained by different methods. Note the bright autofluorescence of the chloroplasts in the non-fixed cells. Scale bar indicates 20 μm. Microinjected cultured tobacco cell at low magnification; only a part of the actin network, close to the injection point (arrow) is stained by Alexa-phalloidin.

immunolocalization method, the cell wall forms even more a barrier than in the permeation method. For cells older than 7 days, the use of wall degrading enzymes, which affect the final result negatively, is necessary. In the three approaches discussed above, the loss of cell integrity and thus the loss of viability is a prerequisite for actin labeling.

Next, Alexa-labeled phalloidin was microinjected into the same type of cultured cells. Actin staining was visible some minutes after the injection and it had the best resolution we ever obtained (Fig. 4). During the first 15 min after injection of the probe, the cell recovers by forming a wound plug around the needle tip. In this way, the cell keeps its

integrity and natural appearance, this is in contrast with the collapsed look of the permeated cells. Probably as a consequence of this integrity, the actin filaments appear as straight flowing lines, they are never tortuous or wavy as in the permeated cells. The staining depends however on the amount of labeled phalloidin injected into the cell, and the concentration of the probe inside the cell. Very often, only a part of the cytoskeleton, close to the point of injection, was stained (Fig. 5). Although there were no visible signs of cell impairment due to microinjection, we found that successfully stained cells soon lost their viability.

Fig. 4. CLSM extended focus images of actin labeling in cultured tobacco cells obtained by different methods. Note the bright autofluorescence of the chloroplasts in the non-fixed cells. Scale bar indicates 20 μm. Actin revealed by microinjection of Alexa-phalloidin.
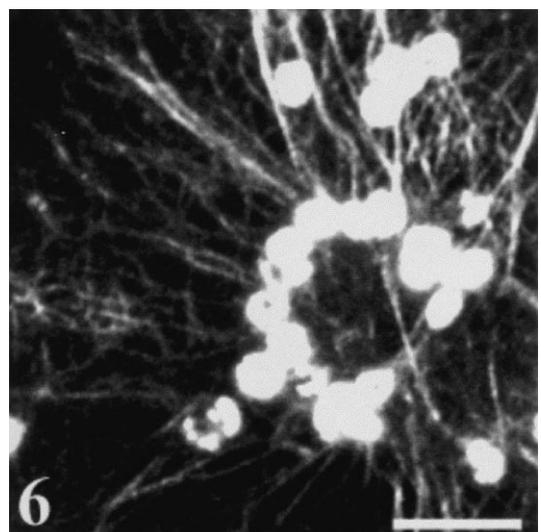
Fig. 6. CLSM extended focus images of actin labeling in cultured tobacco cells obtained by different methods. Note the bright autofluorescence of the chloroplasts in the non-fixed cells. Scale bar indicates 20 μm. Rh-phalloidin labeling by use of the cytofectene permeation method.
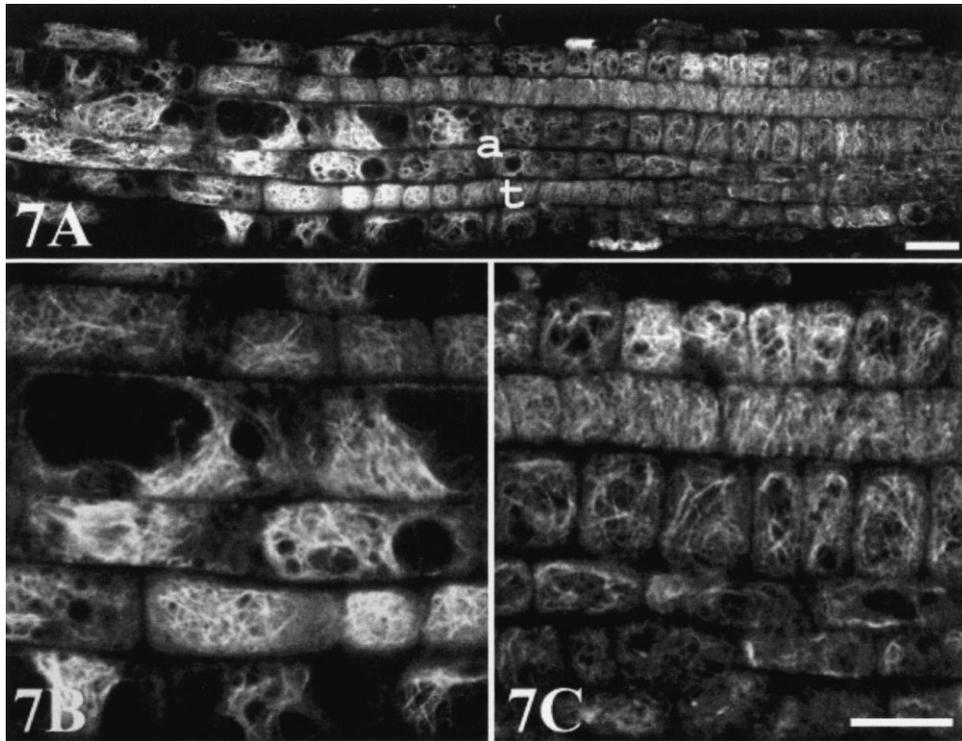
Fig. 7. CLSM images of Rh-phalloidin labeled actin in detergent permeated whole mount *Arabidopsis* roots. (A) Actin staining in epidermal cells in the division and the elongation zones. The difference in actin network organization between the trichoblast cell files (t) and the atrichoblast cell files (a) is obvious; scale bar indicates 10 μm. (B) Detail of (A), showing actin labeling in epidermal cells of the elongation zone. (C) Detail of (A), showing actin labeling in epidermal cells of the division zone. Scale bar indicates 10 μm.

As a last alternative, the cationic lipid formulation cyto-fectene was used to bring the labeled phalloidin inside the cell. Cells older than 7 days were difficult to stain, again as a consequence of the thickness of the cell wall. The results are comparable to the glycerol approach: first, the actin skeleton and especially the fine filaments are well preserved and stained (Fig. 6) and second, the use of cytofectene also allows co-localization of actin and mitochondria in cells having GFP-labeled mitochondria (results not shown). It turns out however that the effect of cytofectene is a locally limited entrance of phalloidin, often resulting in cells having only parts of their cytoskeleton stained. Also the cells stained by use of cytofectene lost their viability during staining.

3.2. Whole mounts and sectioned roots

The detergent permeation method was applied to excised living roots of *Arabidopsis* seedlings. In contrast with cultured cells, a quite long incubation time in the staining solution was necessary. After 1.5 h, several epidermal cells were already stained, but a complete epidermal staining was only obtained after 3 h (Fig. 7A). This method results in a very nice and clear actin staining in the whole epidermal layer almost without background (Fig. 7B and C). In Fig. 7A, the difference in actin network organization between the trichoblast cell files (shorter cell length and low rate of vacuolation; indicated by 't') and the atrichoblast cell files (indicated with a) is obvious. The protocol worked for roots of different ages but the exact labeling time varies among the specimens.

The use of glycerol instead of detergents on *Arabidopsis* roots gave similar results (data not shown), the labeling time is more or less the same as for the detergent method.

In order to label actin in root cells underneath the
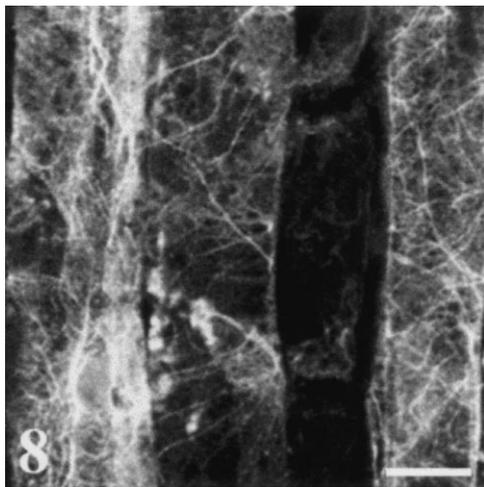
Fig. 8. CLSM extended focus image of immunolabeled actin in a part of a whole *Arabidopsis* root. Scale bar indicates 10 μm.
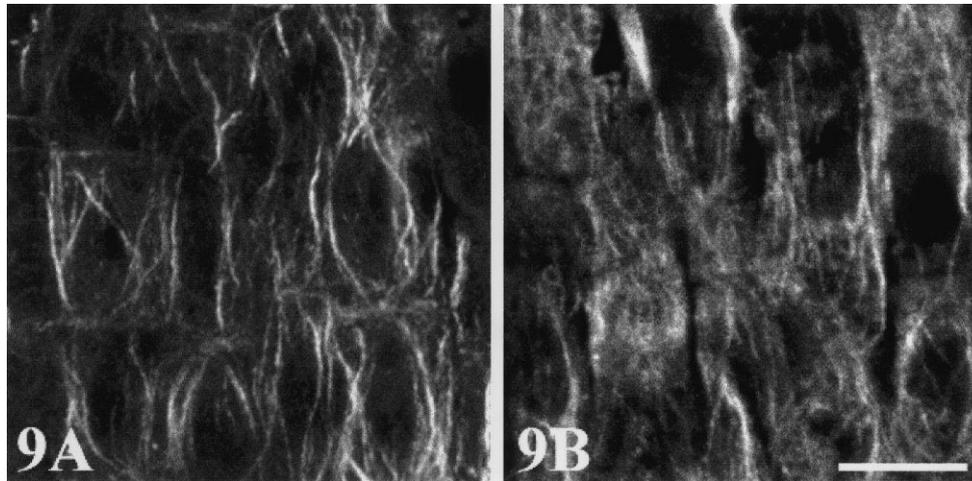
Fig. 9. CLSM extended focus images of immunolabeled actin in sectioned Steedman's wax embedded roots. (A) Actin labeling in a longitudinal section of a maize root. (B) Actin labeling in a longitudinal section of a bean root. Scale bar indicates 10 μm.

epidermal cell layer, we cracked the *Arabidopsis* roots by freeze-shattering before incubation in the staining solution, as described by Wasteneys et al. (1997). Only some cells were stained, giving a very poor image of the general actin organization in roots (data not shown).

Obtaining an overview of actin organization in the whole epidermis as in Fig. 7 was not possible with immunofluorescence on whole mount *Arabidopsis* roots because only part of the epidermis of the elongation zone could be stained (Fig. 8). Cells in other zones did not show significant staining at all. In the stained cells, the technique revealed some fine actin filaments but most of them were overshadowed by a strong background fluorescence.

To obtain actin immunolabeling in subsequent layers of root cells, we applied the "Steedman's wax" sectioning technique, described by Vitha et al. (1997), on maize and bean root segments. With this method large arrays of cells are stained in maize root sections (Fig. 9A), however only thick bundles of F-actin are clearly visible while the network of fine filaments is not revealed; in bean roots the resolution of the staining is even worse than in sections from maize (Fig. 9B).

4. Discussion

We used different approaches to stain actin filaments in cultured cells, roots and sectioned roots. The quality of the staining was evaluated by the abundance of the fine filaments and the straightness of the thick bundles. These features are characteristic for the in vivo labeling reported by Kost et al. (1998) as represented on a cover photograph of the journal issue.

For both cultured cells and roots, the most rapid and complete actin staining is obtained by the permeation methods, where the use of glycerol in cultured cells instead of detergents gives a more detailed and long lasting actin stain-

ing. Both thick bundles and thin actin filaments are well preserved during the labeling procedure, but especially in cultured cells their pattern of organization can be affected by the loss of cell integrity. Phalloidin is an actin stabilizing protein which can artificially enhance actin polymerization, but when using a Rh-phalloidin concentration of approximately 10^{-7} M, this problem can be ruled out (Traas et al., 1987). In whole roots, the actin of epidermal cells can be stained easily by just incubating long enough (3 h). The difference in stability of the phalloidin-labeled actin filaments between single cells and whole organs is striking. Using detergents the staining pattern deteriorates already strongly after 30 min in single cells, while in whole roots it is optimal only after 3 h and it keeps the optimal appearance for hours. As far as we know, we are the first to demonstrate the usefulness of the simple permeation method for labeling actin in root epidermis in situ. With this method, subepidermal cell layers are however not accessible for the staining solution. To overcome this problem, *Arabidopsis* roots were freeze-shattered, but the quality of the resulting actin staining was poor. Freeze-shattering can be an effective method to make the cells of a multicellular specimen accessible for staining (Wasteneys et al., 1997), but in a complex organ consisting of different types of very small cells, like the *Arabidopsis* roots, one cannot control the site of shattering. This technique was not suitable for our investigation.

In order to improve the immunodetection of plant actin, antibodies were raised against plant actin. Andersland et al. (1994) prepared a monoclonal antibody against pea actin, which could label extensively actin filaments in pollen tubes, stamen hairs and *Arabidopsis* roots (Baskin et al., 1996). Kandasamy et al. (1999) characterized the different actin isovariants in *Arabidopsis* and produced specific antibodies having a strong reactivity with the respective actin subclasses. As these antibodies were not available, we used the monoclonal actin antibody clone C4 (raised again

chicken gizzard actin), known for its capacity to recognize plant actins (Lessard, 1988). This monoclonal actin antibody was used before to visualize actin filaments perfectly in freeze-shattered trichomes (Szymanski et al., 1999) and root hairs (Braun et al., 1999). However, in maize roots rather poor images were obtained after immunolabeling with the same monoclonal antibody (Blancaflor and Hasenstein, 1997). Baskin et al. (1996) showed that cryofixation combined with freeze-substitution and resin embedding, instead of chemical fixation, could enhance the antigenicity in multicellular specimens. This nice method requires the appropriate equipment and is time consuming. For single cells, immunolabeling of the actin skeleton gave really poor results. Some filaments were preserved, but the majority of the fine actin fibers was lost. The amorphous fluorescence seen around the chloroplasts can be interpreted as an actin cage, which is protected against breakdown by interaction with actin binding proteins. Immunolabeling on whole *Arabidopsis* roots always provides less detailed information on actin organization than the permeation method. Actin immunolabeling on sections of maize and bean gave data comparable with those obtained by Vitha et al. (1997). However, at high magnification only thick bundles are resolved. A first explanation for the general poor quality of the actin detected with immunofluorescence in both single cells and roots, can be that some of the delicate actin filaments did not survive the sequence of chemical treatments typical for the immunolabeling procedure. Even MBS, known for its actin preserving effect (Sonobe and Shibaoka, 1989), was of limited use. For single cells, the pre-treatment with MBS was omitted because it did not improve actin preservation at all. Detergents, necessary for penetration of the MBS into the single cells, are able to damage the huge vacuoles resulting in a release of proteolytic enzymes, which can affect the stability of the actin network (Staiger and Schliwa, 1987). A second possible reason is that the monoclonal antibody used (clone C4) is not perfectly binding on the whole array of plant actin filaments. The use of plant specific antibodies, as described above, could potentially eliminate this problem.

In huge and highly vacuolated plant cells, like the cultured elongated cells, microinjection is not an evident technique, but the results are satisfactory giving the best visualization we ever obtained. Until now, most of the microinjection work on plant cells has been done on easily injectable cell types, like wall-less *Haemanthus* endosperm cells (Schmit and Lambert, 1990) and protoplasts (Kost et al., 1995) or small *Tradescantia* stamen hair cells (Cleary et al., 1992; Hussey et al., 1998). In these specimens, it is easier to control the injected volume and/or there is a faster spreading of the probe throughout the whole cell. Using microinjection with labeled phalloidin, cells can keep a dynamic actin network and thus survive, only if the right cellular concentration of the probe is reached. When this condition is not fulfilled, microinjection still is a very good — if not the best — in vitro technique to label the actin network. For real in vivo actin labeling, the use of GFP-constructs is potentially the best solution. As plant material expressing actin-labeling GFP-constructs was not accessible, we could not evaluate this approach.

The choice of cytofectene as a vector to bring the labeled phalloidin inside the cell, is based on the fact that it can accomplish the transfection of animal cell lines with plasmids without endangering the cell viability. In contact with labeled phalloidin, cytofectene encapsulates this probe resulting in a kind of liposomes that are able to migrate through the plasma membrane of the cultured plant cells, only if the cell wall is permeable. Unfortunately, also with this method the stained cells lose their viability but the deterioration of the cell structure is minimal. In young cells, the cytofectene method gives results with a staining quality comparable to the glycerol permeation method.

To date there is no general and universal good method for actin labeling in plant cells. The choice of a method will depend on the plant species, the cell or tissue type, the required resolution, the available equipment and the time one can spend. We can recommend the detergent and glycerol permeation methods for single cells and roots because of their low input/output ratio, where glycerol is preferable to detergents when working with plant material containing GFP-labeled organelles, and we suggest microinjection for the best quality obtainable.

Acknowledgements

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