

RESEARCH PAPER

Actin-dependent fluid-phase endocytosis in inner cortex cells of maize root apices

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Abstract

The fluorescent dye Lucifer Yellow (LY) is a well-known and widely-used marker for fluid-phase endocytosis. In this paper, both light and electron microscopy revealed that LY was internalized into transition zone cells of the inner cortex of intact maize root apices. The internalized LY was localized within tubulo-vesicular compartments invaginating from the plasma membrane at actomyosin-enriched pit-fields and individual plasmodesmata, as well as within adjacent small peripheral vacuoles. The internalization of LY was blocked by pretreating the roots with the F-actin depolymerizing drug latrunculin B, but not with the F-actin stabilizer jasplakinolide. F-actin enriched plasmodesmata and pit-fields of the inner cortex also contain abundant plant-specific unconventional class VIII myosin(s). In addition, 2,3 butanedione monoxime, a general inhibitor of myosin ATPases, partially inhibited the uptake of LY into cells of the inner cortex. Conversely, loss of microtubules did not inhibit fluid-phase endocytosis of LY into these cells. In conclusion, specialized actin- and myosin VIII-enriched membrane domains perform a tissue-specific form of fluid-phase endocytosis in maize root apices. The possible physiological relevance of this process is discussed.

Key words: Actin, endocytosis, myosin, pit-fields, plasmodesmata, root cortex.

Introduction

Although endocytosis in animal and yeast cells has been extensively studied and is well understood, this important topic lies dormant in the plant field, despite several papers published in the 1980s and early 1990s. Even the existence of endocytosis in plants has often been questioned due to the high turgor pressure and robust cell walls in plants (Cram, 1980; Oparka *et al.*, 1993; for a review see Hawes *et al.*, 1995). Only recently, are receptor-mediated endocytosis and recycling of plasma membrane proteins emerging as processes inherent to higher plant cells (Bahaji *et al.*, 2001; Geldner *et al.*, 2003; for reviews see Low and Chandra, 1994; Robinson *et al.*, 1998; Holstein, 2002; Jürgens and Geldner, 2002). However, fluid-phase endocytosis still awaits characterization and acceptance in plant cells. The most relevant hurdle is that classical fluid-phase markers such as colloidal gold-labelled proteins or horseradish peroxidase penetrate extremely slowly through plant cell walls, imposing serious difficulties for accomplishing critical physiological experiments with these cells (Low and Chandra, 1994). Lucifer Yellow (LY) is a membrane-impermeable and aldehyde-fixable fluorescent dye (Stewart, 1981) which is generally accepted as a reliable marker for fluid-phase endocytosis in a wide range of eukaryotic cells (Swanson *et al.*, 1985; Riezman, 1985; Basrai *et al.*, 1990; Wiederkehr *et al.*, 2001). Sixteen years ago, LY also emerged as a promising marker for fluid-phase endocytosis in plant cells (Oparka *et al.*, 1988; Wright and Oparka, 1989; Hillmer *et al.*, 1989, 1990). However, subsequent studies using cell suspensions and

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Abbreviations: LB, latrunculin B; LY, Lucifer Yellow; BDM, 2,3 butanedione monoxime.

epidermal peels treated with probenecid, an inhibitor of organic anion transporters, indicated that internalization of LY might also occur via non-vesicular pathways (Oparka *et al.*, 1991). Although these latter data were obtained with rather stressed cells isolated from intact tissues, it was suggested that this was a general pathway that operated in all plant cells (Oparka *et al.*, 1993). Later studies performed on intact root apices showed that fluid-phase endocytosis is an inherent feature of root cells (Roszak and Rambour, 1997; Kazmierczak and Maszewski, 1997; Cholewa and Peterson, 2001). Roots are suitable for these studies because they lack impermeable cuticula, present in other plant organs. An important result of these studies is that, in accordance with fluid-phase endocytosis of non-plant cells, F-actin was found to be essential for fluid-phase endocytosis in root cells of different plant species (Roszak and Rambour, 1997; Cholewa and Peterson, 2001). Besides intact actin filaments, the unconventional myosins of class VIII were predicted, and later shown, to reside at, or close to, the plasma membrane (Knight and Kendrick-Jones, 1993; Reichelt *et al.*, 1999; Reichelt and Kendrick-Jones, 2000; Baluška *et al.*, 2000, 2001b). It is reported here that actomyosin-enriched plasmodesmata/pit-fields act as specialized domains of the inner cortex cells accomplishing fluid-phase endocytosis.

Materials and methods

Plant material, drug treatments, and light microscopy of Lucifer Yellow

Maize grains (*Zea mays* L.) of cv. Alarik were soaked for 6 h and germinated in moistened rolls of filter paper for 4 d in the dark at 20 °C. Young seedlings with straight primary roots, 50–70 mm long, were selected. At room temperature, growing roots were placed in a container with aerated nutrient solution composed of the following salts: 1.0 mM NaCl, 0.1 mM KCl, 0.1 mM CaCl₂, and 1.0 mM 2-(*N*-morpholino)ethanesulphonic acid, adjusted to pH 6.5 with TRIS. Lucifer Yellow CH (LY), obtained from Sigma Chemicals (St Louis, MO, USA), was added to this nutrient solution to obtain a final concentration of 1% and root apices were exposed for 2 h. LY has a free hydrazido group which reacts with aliphatic aldehydes at room temperature, binding it firmly to cellular structures (Stewart, 1981). To induce plasmolysis, maize root apices were submerged either into 700 mM mannitol or into 1000 mM of NaCl (mixed with CaCl₂ at a ratio of 9:1) for 2 h.

In order to probe the importance of the cytoskeleton for internalization of LY, roots were pretreated with specific cytoskeletal inhibitors before their exposure to this endocytic tracer. Latrunculin B (Calbiochem, Bad Soden, Germany) was used at a concentration of 10 µM which disintegrates visible F-actin in maize root cells within 30 min (Baluška *et al.*, 2001a). Jasplakinolide, a stimulator of actin polymerization (Spector *et al.*, 1999), was used at 1 µM for 2 h. The inhibitor of myosin ATPase activity, 2,3-butanedione monoxime (for plant cells see Šamaj *et al.*, 2000; Tominaga *et al.*, 2000) was applied for 6 h at 10 mM. Oryzalin (1 µM, 2 h) depolymerizes all microtubules in the cells of maize roots (Baluška *et al.*, 1995). The inhibitor of organic anion transporters probenecid was applied for 2 h at 1 mM concentration (Oparka *et al.*, 1991).

Fixation consisted of the excision of apical root segments (7 mm), encompassing the major growth zones, into 3.7% formaldehyde made up in buffer (50 mM PIPES, 5 mM MgSO₄ and 5 mM EGTA, pH 6.9) for 1 h at room temperature. Following a rinse in SB, the root apices were dehydrated in a graded ethanol series diluted with phosphate buffered saline (PBS). They were embedded in the low melting-point Steedman's wax (Baluška *et al.*, 1992, 1997).

Longitudinal sections (10 µm) were allowed to expand on slides, dewaxed in absolute ethanol, passed through a graded ethanol series diluted with PBS, and then kept in SB for 30 min. The root sections were mounted under coverslips using an anti-fade mountant (Baluška *et al.*, 1992). LY fluorescence was examined with an Axiovert 405M inverted microscope (Zeiss, Oberkochen, Germany) equipped with epifluorescence and standard FITC exciter and barrier filters (BP 450-490, LP 520).

Immunogold electron microscopy of Lucifer Yellow

Sample preparation: Maize roots were treated with 2% LY (for details see above) for 10, 30, or 120 min and washed with distilled water. Subsequently, root tissue pieces were cut and fixed with 5% glutaraldehyde in PBS (100 mM, pH 7.2) for 1.5 h, washed with PBS, and post-fixed with 0.5% OsO₄ for 30 min. Thereafter, samples were dehydrated in a graded ethanol series, embedded in LR White resin (Hard Grade, BioCell, Cardiff, UK), and polymerized for 2 d at 50 °C. Ultrathin sections were made on a Reichert ultramicrotome (Reichert, Vienna, Austria) and collected on formvar-coated Ni grids.

Indirect immunolabelling: To block residual aldehydes and proteins, the sections were treated with 0.05 M glycine in PBS and 5% BSA+5% normal goat serum (NGS), both diluted in PBS for 30 min. Subsequently, sections were washed with 1% BSA+0.1% fish gelatine in PBS for 5 min (WM), incubated for 1.5 h with anti-Lucifer Yellow IgGs raised in rabbit (Molecular Probes, Leiden, The Netherlands) diluted 1:50 in WM, washed 5 times with WM, and incubated with goat anti-rabbit IgGs conjugated to 10 nm gold particles (BioCell, Cardiff, UK) diluted 1:50 for 1.5 h. Thereafter, sections were washed with WM, PBS, and deionized distilled water, and stained with 2% uranyl acetate. As a negative control, sections were treated exactly as described above except that LY antibody was replaced by WM. Sections were observed in a Zeiss EM 10 electron microscope (Zeiss, Oberkochen, Germany) at 60 kV.

Indirect immunolocalization of actin, myosin VIII, and PM H⁺-ATPase

Fixation and handling of root samples was essentially the same as that described above for the localization of the LY. After fixation, they were embedded into Steedman's wax and sectioned (10 µm) longitudinally. To enable efficient penetration of antibodies into root tissues, sections were dewaxed in absolute ethanol, passed through a graded ethanol series diluted with PBS, and kept in SB for 30 min. Then, the sections were transferred to SB for 30 min at room temperature. Dewaxed sections were incubated for 1 h at the room temperature with the following antibodies: monoclonal plasma membrane anti-H⁺-ATPase (Jahn *et al.*, 1998), monoclonal anti-actin (C₄ clone from ICN), polyclonal maize anti-actin obtained from Chris J Staiger (Purdue University, IN, USA), and with the polyclonal anti-myosin VIII (Reichelt *et al.*, 1999), all diluted 1:200 in PBS. After rinsing in SB, the sections were stained with FITC-conjugated anti-mouse (C₄ actin and H⁺-ATPase) and anti-rabbit (maize pollen actin, ATM1 myosin VIII) IgGs raised in goat (Sigma Chemical Co, St Louis, MO, USA) diluted 1:100 in PBS for 1 h at room temperature. A further rinse in PBS (10 min) preceded 10 min in 0.01% Toluidine Blue O. The sections were mounted using an anti-fade mountant (Baluška *et al.*, 1992).

For immunoelectron microscopy, maize root apices were fixed in 4% paraformaldehyde in SB buffer for 90 min at room temperature. After washing in SB and PBS, and dehydration in a graded ethanol series, the tissue was embedded in LR White Resin (Hard grade, Biocell, Cardiff, UK). Ultrathin sections were cut on an ultramicrotome and transferred onto formvar-coated nickel grids. The sections were blocked with 50 mM glycine, 5% BSA, and 5% normal goat serum in PBS for 30 min and washed with wash buffer (WB) made of PBS containing 1% BSA and 0.1% gelatin. They were incubated first with polyclonal myosin VIII antibody ATM1 (diluted 1:50 with WB) alone or with a mixture of ATM1 and monoclonal actin antibody (C₄ clone, ICN), diluted 1:100 in WB, at room temperature for 90 min, extensively washed with WB, and incubated with the second antibody, goat anti-rabbit IgGs–10 nm gold conjugate in the case of myosin and goat anti-mouse IgGs conjugated to 20 nm gold in the case of actin, both diluted 1:100 in WB for 90 min. The sections were washed with WB and PBS, post-fixed with 3% glutaraldehyde for 15 min, and contrasted with ice-cold 2% aqueous uranyl acetate and 1% osmium tetroxide to enhance plasmodesmata visualization. The sections were examined in a Zeiss EM 10 electron microscope at 60 kV.

As a negative control, sections were treated as described above except that the first antibodies were omitted or immunodepleted by the corresponding antigen (in the case of myosin VIII antibody). As a positive control, a range of other proteins (including calreticulin, profilin, ADF, and tubulin) were immunolocalized in the same tissue using the adjacent sections and the same procedure.

Results

Myosin VIII and F-actin accumulate at plasmodesmata/pit-fields in the inner cortex cells

Using a polyclonal antibody raised against maize pollen actin, the association of F-actin with pit-fields of the inner cortex, but not of the middle cortex and endodermis, in cells of the transition zone (Baluška *et al.*, 2001c) was documented (Fig. 1A, B). Polyclonal antibody raised against the myosin VIII tail domain (Reichelt *et al.*, 1999), specific for all myosins of the class VIII (Reichelt and Kendrick-Jones, 2000; Baluška *et al.*, 2001b), localized myosin VIII to the same plasmodesmata and pit-fields of the inner cortex cells (Fig. 1C, D). A closer view of the inner cortex cells reveals that the outer portions of the pit-fields are enriched with myosin VIII while the central parts are relatively depleted (Fig. 1C). In paradermal sections, the characteristic oval shapes of myosin VIII-enriched pit-fields are observed (Fig. 1D). Besides myosin VIII, these pit-fields in the inner cortex cells, but not in other root cells, are also enriched with F-actin which, when viewed in paradermal sections, arrange themselves in the form of stellate structures interconnected with axially organized bundles of actin filaments (Fig. 1E). In cross-sections, pit-field-associated F-actin assemblies resemble pit-fields as recognized with the myosin VIII antibody (Fig. 1F, G).

Differential effect of jasplakinolide on the actin cytoskeleton in cells of the epidermis and cortex

Stabilization of F-actin with the drug jasplakinolide (Spector *et al.*, 1999) makes the association of F-actin

Table 1. Effects of cytoskeletal drugs on LY internalization

Mean number of LY-positive compartments calculated for one inner cortex cell from 10 µm thick sections of root apices treated with LY alone (control) or pretreated with different drugs before LY exposure. Values (±SE) represent the means of 50 inner cortex cells evaluated from 10 adjacent sections from each of three evaluated roots in three independent experiments.

Control +LY	5±2
Probenecid (1 mM, 2 h) +LY	4±2
Latrunculin B (10 µM, 2 h) +LY	0
BDM (10 mM, 6 h) +LY	2±1
Jasplakinolide (1 µM, 2 h) +LY	4±1
Oryzalin (1 µM, 2 h) +LY	7±2

with pit-fields of the inner cortex cells even more evident (Figs 1H, 2B, C). Generally, cells of the stele and inner cortex react differently to jasplakinolide and they maintain intact F-actin arrays (Fig. 2B). On the other hand, the actin cytoskeleton dramatically disintegrates and rearranges into perinuclear short bundles and aggregates in jasplakinolide-treated epidermal cells (Fig. 2A). Intriguingly, cell-to-cell interactions became extremely tight in the inner cortex after jasplakinolide treatment when F-actin was closely associated with the plasma membrane and enriched within pit-fields (Fig. 2C), suggesting that drug-induced stabilization of F-actin at plasmodesmata strengthens cell-to-cell contacts.

Internalization of the fluid phase endocytosis marker LY into cells of the inner cortex

When growing maize root apices were submerged into the LY solution for 2 h, they readily took up this low-molecular weight fluorescent probe into their apoplast. Although the largest amount of LY was found in the surface slime layer covering the outer tangential cell walls of the epidermis (Fig. 3A), LY was also found within all cell walls, with side-walls being more intensely labelled than cross-walls (Fig. 3A–C). This pattern of LY fluorescence reveals that the cell walls of maize roots are freely accessible to molecules within the size range of LY.

Cells of the two innermost layers of the inner cortex internalized apoplastic LY into distinct cytoplasmic compartments which were distributed in the form of fluorescent spots throughout the cytoplasm (Fig. 3D, E). Importantly, probenecid pretreatment did not prevent LY internalization (Fig. 3D). Such LY internalization was especially conspicuous in cells of the transition zone, although slightly less prominent LY-positive compartments also occurred in the inner cortex cells of the basal part of the meristem (data not shown). The central vacuole did not accumulate LY even after prolonged incubation of roots with the dye solution. Quantification of LY-positive compartments within 10 µm thick sections revealed that, within the transition zone cells of the inner cortex, there are

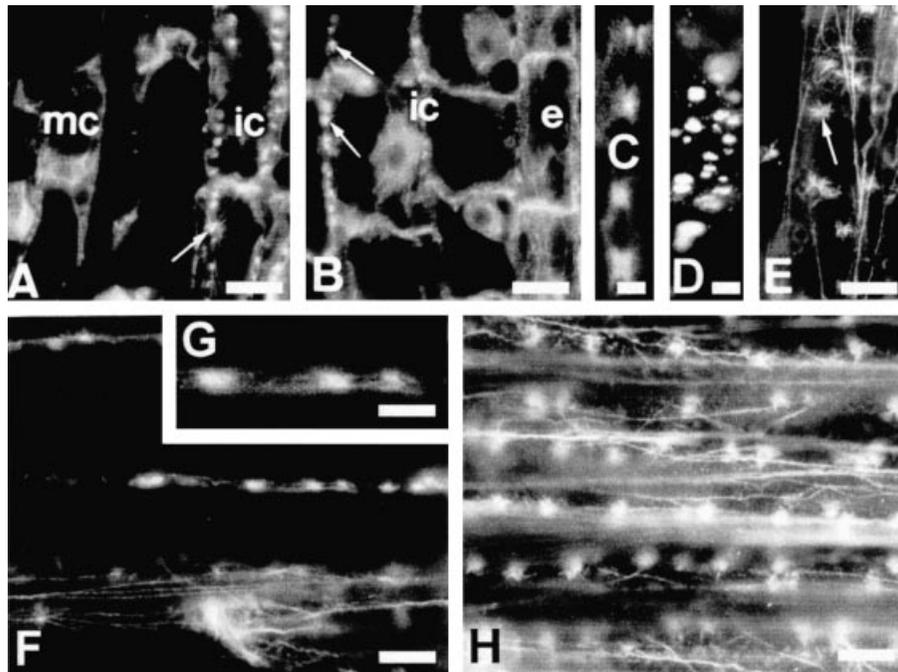


Fig. 1. F-actin and myosin VIII localization at pit-fields in the inner cortex cells of maize root apices. (A, B) Significant amounts of actin are localized in plasmodesmata grouped into prominent pit-fields at the side-walls (parallel with the apical-basal root axis) of the inner cortex (ic), but not middle cortex (mc) and endodermis (e). (C) A closer view of pit-fields reveals that myosin VIII is particularly localized at the outer portions of pit-fields. (D, E) Paradermal view of myosin VIII (D) and actin (E) enriched pit-fields shows their lens-shaped form. (F, G) In cross-sections, F-actin-rich pit-fields resemble those enriched with myosin VIII rich pit-fields shown in (C). (H) Jasplakinolide treatment does not change the association of F-actin with pit-fields although F-actin cables are more prominent. Bars=10 μm (A, B), 15 μm (D, E, F, G), 20 μm (C).

about five compartments per section through one cell (Table 1).

Depolymerization of F-actin, but not of microtubules, inhibits internalization of LY

Tissue- and development-specific internalization of LY was also found, with a slight increase in the abundance of internalized LY-positive compartments, in roots pretreated with the anti-MT drug oryzalin (Fig. 3F; Table 1) or with the F-actin stabilizer jasplakinolide (Fig. 4G; Table 1). Similar data were also obtained with cytochalasin D (data not shown) which does not depolymerize F-actin as effectively as latrunculin B (Baluška *et al.*, 1997, 2001a). By contrast, roots pretreated with the F-actin-depolymerization drug latrunculin B (LB) did not internalize LY into these LY-positive cytoplasmic compartments (Fig. 3H; Table 1). The general myosin inhibitor 2,3-butanedione monoxime (BDM) partially inhibited internalization of LY (Fig. 3I; Table 1). These results suggest that LY was internalized via acto-myosin dependent, but MT-independent, fluid-phase endocytosis.

Immunogold EM analysis reveals fluid-phase endocytosis of LY at plasmodesmata/pit-fields

Using a polyclonal antibody raised against LY to immunolocalize the dye at the ultrastructural level, the

highest accumulation of gold particles was found within plasmodesmata and peripheral vacuoles (Fig. 4A). Typically, clusters of LY gold particles abundantly decorated plasmodesmata (Fig. 4B, C) of the two innermost cell files of the inner cortex. As a negative control, the LY antibody was omitted and sections were treated only with the secondary antibody conjugated to colloidal gold (Fig. 4D).

Unique osmotic properties of the inner cortex cells

Plasma membrane H^+ -ATPase is highly abundant in epidermal cells, moderately abundant in cells of the outer cortex, but depleted within cells of the inner cortex (Fig. 5A–B). This finding highlights the uniqueness of cells of the inner cortex with respect of energization of their plasma membranes. The plasma membrane H^+ -ATPase is highly abundant in cells of the endodermis and pericycle (Fig. 5C). Accordingly, the characteristic feature of the inner cortex cells is that only these cells are prone to plasmolyse if apical root segments are dehydrated more harshly due to fewer and shorter steps of graded ethanol series during sample dehydration (Fig. 5G). This indicates that the root inner cortex cells are naturally coping with a mild osmotic stress.

Interestingly, the non-ionic osmoticum mannitol induced plasmolysis within maize root apices only in cells of

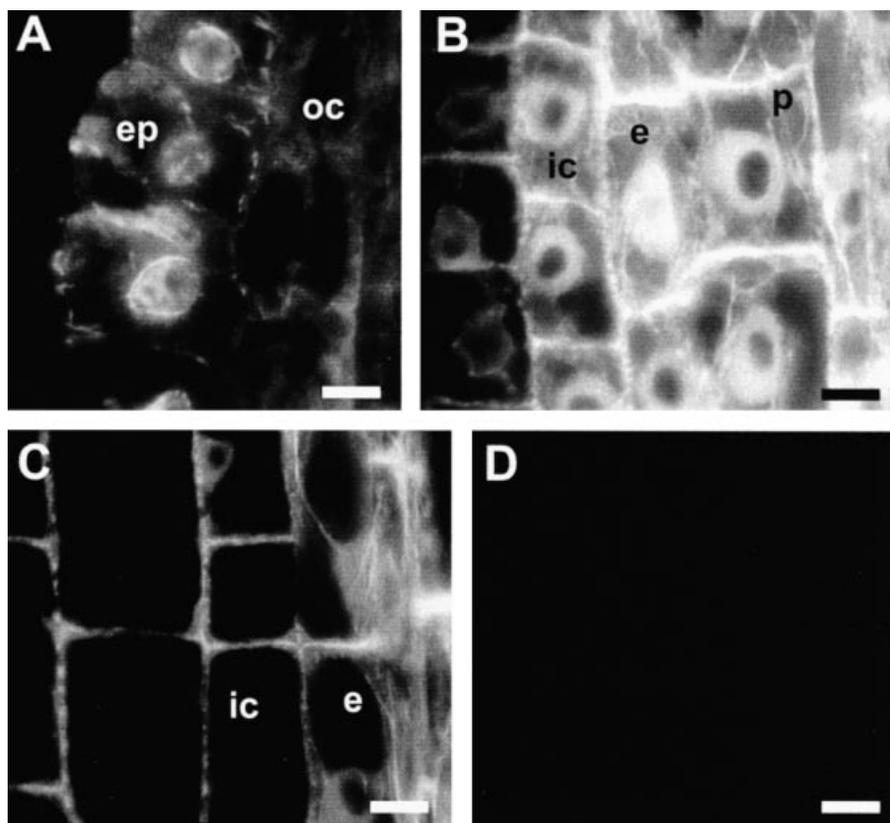


Fig. 2. Immunolocalization of F-actin in cells of roots treated with jasplakinolide for 2 h. (A) F-actin arrays were transformed into short bundles that accumulated around nuclear surfaces and at cellular peripheries in the epidermis (ep). Cells of the outer cortex (oc) lost F-actin completely. (B) The innermost cortical cell file (ic), as well as all cells of the endodermis (e) and pericycle (p), preserved apparently intact arrays of F-actin. (C) After 2 h of jasplakinolide treatment and subsequent 12 h of growth, cells of the inner cortex showed unusually tight cell-to-cell contacts. Omitting (D) the first antibody resulted in no signal at all, confirming the specificity of immunofluorescence labelling. Bars=10 μ m.

the epidermis and cortex (Fig. 5D) whereas cells of the endodermis (Fig. 5E, F) and stele (data not shown) effectively resisted this osmoticum. By contrast with mannitol, ionic osmotica induced plasmolysis of all root cells, irrespective of their position within the maize root apex (data not shown). Those few cells of the roots exposed to mannitol which lost their plasma membrane integrity diffusely accumulated large amounts of LY in the cytoplasm and, typically, their nuclei became the most prominent fluorescent compartments (Fig. 5E; Palevitz and Hepler, 1985; Hillmer *et al.*, 1989, 1990). Importantly, the accumulation of LY within the cytoplasm and nucleus in this case was not affected by the BDM and LB pretreatments (data not shown).

Discussion

In the present study, tissue-specific fluid-phase endocytosis has been documented at distinct subcellular domains of the inner cortex cells of the transition zone of maize root apices. Both epifluorescence and immunogold electron

microscopy revealed the accumulation of the membrane-impermeable and low molecular weight fluorescent dye LY at the plasmodesmata and pit-fields of the transition zone of maize root apices. Pharmacological analysis revealed that this LY internalization was actomyosin-dependent and probenecid-insensitive, confirming the fluid-phase endocytic nature of the LY internalization.

Immunogold EM reveals the accumulation and internalization of LY at plasmodesmata/pit-fields

Using immunogold EM, LY was found within tubulovesicular invaginations of the plasma membrane at F-actin- and myosin VIII-enriched plasmodesmata and pit-fields. LY also accumulated within vesicles, tubules, and small peripheral vacuoles. However, LY never accumulated within the central vacuole. Similar ultrastructural membranous localization of LY was reported for nutrient absorptive trichomes of a carnivorous bromeliad (Owen *et al.*, 1991).

Although the formation of tubulovesicular invaginations of the plasma membrane at the plasmodesmata and

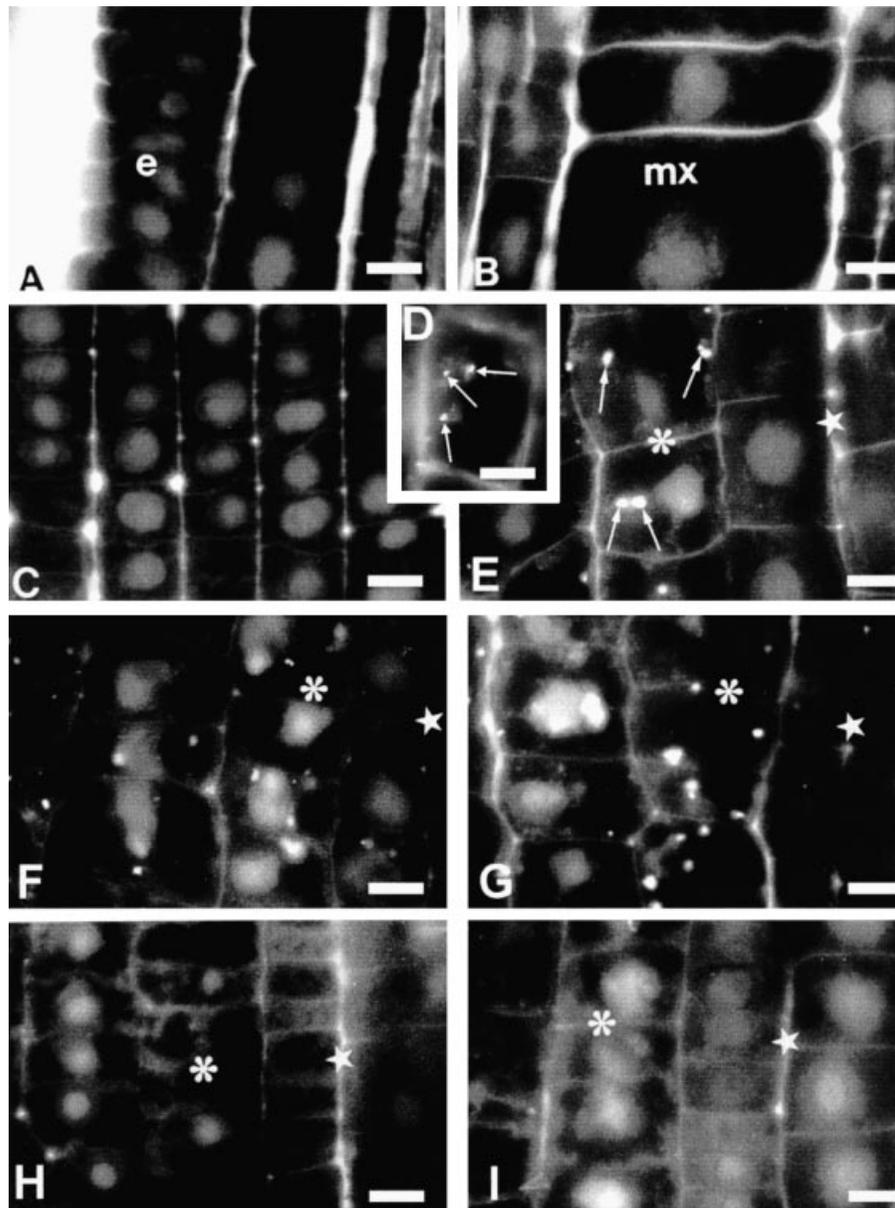


Fig. 3. Fluorescence light microscopy analysis of the LY distribution throughout root apices. (A, C) Apoplastic fluorescence was clearly visible throughout the root apices, as shown in the epidermis (A, e), in the metaxylem (B, mx), and in the outer-middle cortex (C). (D, E) The cells of the two innermost cortex files (asterisk) are unique as they internalized LY into small compartments (arrows) scattered throughout their cytoplasm. Probenecid pretreatment did not prevent internalization of LY (D). (F, G) The anti-MT drug oryzalin (F) and actin filament stabilizer jasplakinolide (G) induced slight increases in the number of LY-positive compartments in cells of the two–three innermost cortical cell files. By contrast, pretreatments with the F-actin drug latrunculin B (H) and the general myosin inhibitor BDM inhibited internalization of LY (I). Stars (E, F, H, I) indicate the position of longitudinal walls between the pericycle and endodermis. Asterisks (E, F, G, H, I) indicate the position of the innermost cell file of the cortex. Bars=10 μ m.

pit-fields observed here might appear unusual, similar invaginations of the plasma membrane at plasmodesmata and pit-fields have been reported by other authors for both root and leaf cells (Warmbrodt, 1985; Evert *et al.*, 1977a, b). Besides plasmodesmata and pit-fields, prominent plasmatabules were found in pollen tubes growing through pistil tissues, but not in pollen tubes grown *in vitro* (Kandasamy *et al.*, 1988), as well as in host–parasite

interactions between *Melicope–Korthalsella* (Coetzee and Fineran, 1989) and *Uromyces–Vigna* (Stark-Urnau and Mendgen, 1995). These invaginations of the plasma membrane at plasmodesmata/pit-fields remotely resemble the plasmatabules that were found particularly at sites where high solute flux from the apoplast into symplast is known to occur (Chaffey and Harris, 1985; Harris and Chaffey, 1986; Kandasamy *et al.*, 1988; Derksen *et al.*,

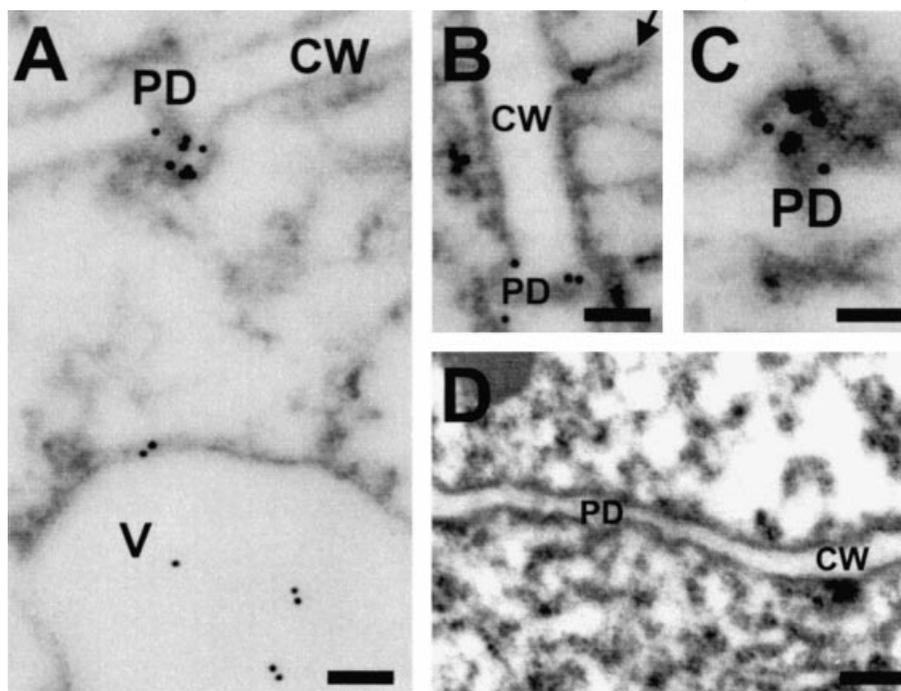


Fig. 4. Immunogold electron microscopy visualization of LY using polyclonal antibody raised against LY. (A–C) In cell walls (CW) of the innermost cortex cells, LY gold particles localized preferentially to plasmodesmata (PD). Besides these tubular invaginations near plasmodesmata and pit-fields (B, arrow), small vacuoles were also labelled (A, v). (D) No labelling was found in sections were treated only with the secondary antibody conjugated to colloidal gold. (A–C, I) Bar=150 nm; (D, F–H) bar=100 nm; (E) bar= 50 nm.

2002). This concept is highly attractive for large apices of maize roots for which the available symplastic routes cannot satisfy their large demand for a continuous nutrient supply (Bret-Harte and Silk, 1994). Plasma membrane-associated tubules implicated in endocytosis were also reported for cells of rice root apices (Nishizawa and Mori, 1977). Even larger pleiomorphic invaginations of the plasma membrane are typical for bulging domains during root hair initiation (Čiamporová *et al.*, 2003) and for the tips of emerging root hairs (Robertson and Lyttleton, 1982).

Actin and myosin VIII enriched at plasmodesmata/pit-fields are involved in LY internalization

Actomyosin-based forces are essential for endocytosis in non-plant cells (Munn, 2001; Morris *et al.*, 2002). In plants, these forces can be viewed as an ideal tool for invagination of the plasma membrane, especially in the case of walled cells which have a high internal turgor pressure (for a review on turgid yeast cells see Munn, 2001). Clearly, such a cytoskeleton-driven internalization mechanism would oppose the long-standing notion that plant cells are unable to accomplish endocytosis (Cram, 1980; Oparka *et al.*, 1993). Myosin-based forces, working with the plasma membrane-associated actin filaments, could be involved in the critical internalization step accomplished against the high turgor pressure of plant cells. These results support such a model. They show that

plasmodesmata and pit-fields in the inner cortex of maize root apices contain significant amounts of F-actin filaments and myosin VIII which obviously internalize LY by actomyosin-dependent fluid-phase endocytosis. The connection between myosin VIII and fluid-phase endocytosis, as described here for the inner cortex cells of maize root apices, might prove to be of general validity as myosin VIII accumulates at pit-fields, simple pits, and bordered pits, all of which are also enriched with F-actin, in diverse cell types of secondary vascular tissues of angiosperm and gymnosperm trees (Chaffey and Barlow, 2001, 2002).

By contrast with latrunculin B-mediated depolymerization of F-actin, the stabilization of F-actin with jasplakinolide (Spector *et al.*, 1999) did not prevent the internalization of LY. This finding might seem to be surprising as jasplakinolide was reported to disrupt the organization of the actin cytoskeleton in dividing plant suspension cells (Ou *et al.*, 2002). However, jasplakinolide has been reported to reorganize actin arrays in other plant cells (Sawitzky *et al.*, 1999). Our previous study reported that jasplakinolide can rearrange thin F-actin arrays into thick F-actin bundles in root hairs (Šamaj *et al.*, 2002). Moreover, the present study reveals that root tissues differ in their response to jasplakinolide. Cells of the inner cortex, which accomplish the fluid-phase endocytosis of LY, do not disintegrate the actin cytoskeleton. This finding is in agreement with data obtained using Madin–Darby canine kidney cells in which jasplakinolide allowed, even

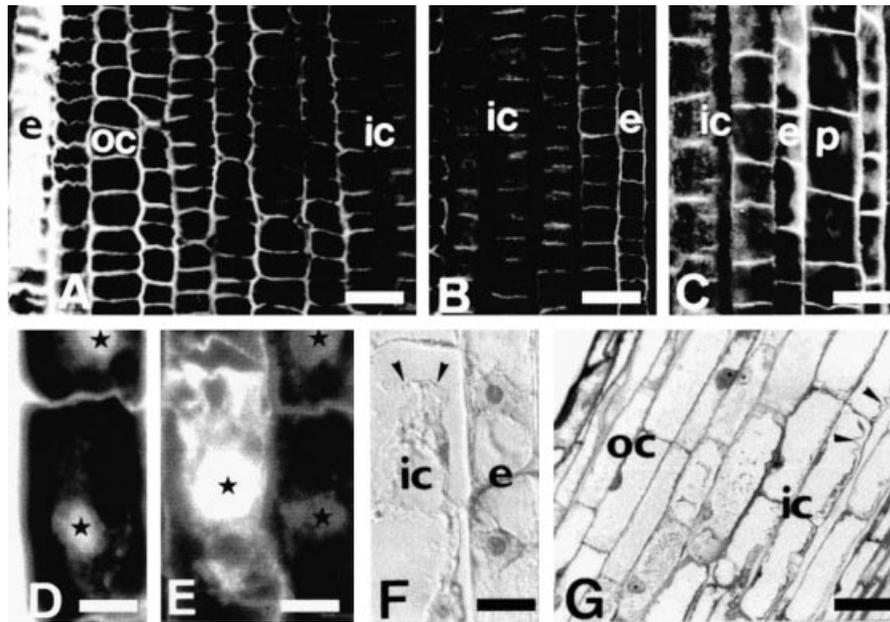


Fig. 5. Depletion of PM- H^+ -ATPase and unique osmotic properties of the inner cortex cells. (A–C) Cells of the inner cortex (ic) are depleted of the plasma membrane H^+ -ATPases. Strong labelling was found especially at the epidermis (A, e) which gradually decreased towards the inner cortex–endodermis interface. Stronger labelling was recorded in cells in the endodermis (B, C, e), pericycle (C, p), and in the adjacent stele parenchyma. (D–F) If mannitol was used as osmoticum (700 mM, 2 h), plasmolysis of cells occurred only in cells of the epidermis (D), outer cortex (E), and inner cortex (F). On the other hand, cells of endodermis (F, e), pericycle and stele (not shown) resisted this treatment and did not show plasmolysis. Stars in (D) and (E) indicate the position of nuclei. A few cells in the mannitol-treated root apices lost their viability and accumulated LY diffusely in their cytoplasm and nuclei (star in the left part of E), the latter becoming the most fluorescent compartment. (G) In harshly dehydrated samples, only cells of the innermost cortex cell files (G, ic) suffered from plasmolysis (arrowheads in G) while cells of other tissues (oc is for the outer cortex) did not undergo plasmolysis. (A, B, G) Bar=30 μ m, (C) bar=35 μ m, (D, E) bar=15 μ m, (F) bar=20 μ m.

promoted in a polarized manner, fluid-phase endocytosis (Shurety *et al.*, 1998). Cell-to-cell contacts are tighter in jasplakinolide-treated root apices. This finding suggests that jasplakinolide-induced actin polymerization at plasmodesmata and pit-fields has an impact on cell-to-cell contacts in plant tissues. In accordance with this notion, it is well-known that cells of the endodermis, pericycle, and stele periphery not only have very tight cell-to-cell contacts but also show the highest amount of F-actin (Baluška *et al.*, 1997, 2000).

BDM (2,3-butanedione monoxime) is emerging as an effective inhibitor of myosin ATPase activity (Šamaj *et al.*, 2000; Tominaga *et al.*, 2000) although its effectiveness in plants is still an open issue and needs to be tested further (McCurdy, 1999). It is reported here that BDM only partially inhibited the uptake of LY into inner cortex cells. The absence of more specific myosin drugs prevents this question being addressed in the current study. So far, only one of the class VIII plant myosins has been shown to be localized at the plasma membrane (Reichelt *et al.*, 1999; Reichelt and Kendrick-Jones, 2000). Therefore, if myosins are involved in plant endocytosis then myosins of class VIII represent the best candidates for this role. Myosin VIII is localized to the plasma membrane domains of plasmodesmata and pit-fields in transition zone cells of the inner cortex which accomplish fluid-phase endocytosis.

Thus, plant-specific myosins of class VIII are strong candidates for endocytic motors of plant cells.

Internalization of LY was not inhibited with a drug that depolymerizes microtubules (MTs) which is in accordance with previous studies showing that endocytosis is MT-independent in plants (Geldner *et al.*, 2001; Baluška *et al.*, 2002). Similarly, the MT-independent nature of fluid-phase endocytosis of LY shown here for maize roots corresponds well with data obtained from animal cells where fluid-phase endocytosis was reported to be unaffected by MT disruption whereas clathrin-based endocytosis was found to be dependent on intact MTs (Jin and Snider, 1993; Subtil and Dautry-Varsat, 1997).

Physiological implications of fluid-phase endocytosis in the inner cortex

Why should fluid-phase endocytosis be restricted to the two-to-three innermost cell files of the cortex in maize root apices? Apart from the fact that myosin VIII and F-actin are enriched at the pit-fields of the inner cortex cells of the root apex transition zone, **these cells contain additional specific features**. They show rather sparse networks of cortical MTs (Baluška *et al.*, 1992, 1993b) and a **surprisingly low abundance of plasma membrane-associated H^+ -ATPases** (this study; but see also Jahn *et al.*, 1998). The location of these cells near unloading phloem elements,

which in root apices exploit apoplastic pathways (Schulz, 1994), together with inefficient energization of their plasma membranes, are likely to contribute to the lowering of turgor pressure in these cells. This local lowering of turgor pressure might allow fluid-phase endocytosis to take place (Gradmann and Robinson, 1989), especially when combined with actomyosin forces. In fact, if plant cells are exposed to a high concentration of extracellular sugars these are then, as predicted by Gradmann and Robinson (1989), readily taken up into plant cells via an LY-accessible fluid-phase endocytic pathway (Jitsuyama *et al.*, 2001) to lower their osmotic stress. High amounts of extracellular and intracellular sugars in the cells of the transition zone might also contribute to cold acclimation and protection against chilling injury (Baluška *et al.*, 1993a; Uemura and Steponkus, 2003).

It can be expected that endocytic uptake of phloem-unloaded assimilates not only relieves the mild osmotic stress imposed on these specialized inner cortex cells near the phloem unloading sites, but this process apparently also provides an additional route for the transport of assimilates into the apical meristem. This suggestion would agree with the earlier data of Bret-Harte and Silk (1994) who calculated that the available symplastic routes are insufficient to provide the high carbon demands requested by robust maize root apices. Not surprisingly, maize is one of the largest annual plants and fluid-phase endocytosis emerges as an important nutrition pathway for nourishing their very large apical root meristems.

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References

- Bahaji A, Cornejo MJ, Ortiz-Zapater E, Contreras I, Aniento F. 2001. Uptake of endocytic markers by rice cells: variations related to the growth phase. *European Journal of Cell Biology* **80**, 178–186.
- Baluška F, Barlow PW, Hauskrecht M, Kubica Š, Parker JS, Volkmann D. 1995. Microtubule arrays in maize root cells. Interplay between the cytoskeleton, nuclear organization, and post-mitotic cellular growth patterns. *New Phytologist* **130**, 177–192.
- Baluška F, Barlow PW, Volkmann D. 2000. Actin and myosin VIII in developing root cells. In: Staiger CJ, Baluška F, Volkmann D, Barlow PW, eds. *Actin: a dynamic framework for multiple plant cell functions*. Dordrecht, The Netherlands: Kluwer Academic Publishers, 457–476.
- Baluška F, Brailsford RW, Hauskrecht M, Jackson MB, Barlow PW. 1993b. Cellular dimorphism in the maize root cortex: involvement of microtubules, ethylene and gibberellin in the behavior in post-mitotic growth zones. *Botanica Acta* **106**, 394–403.
- Baluška F, Cvrcková F, Kendrick-Jones J, Volkmann D. 2001b. Sink plasmodesmata as gateways for phloem unloading. Myosin VIII and calreticulin as molecular determinants of sink strength? *Plant Physiology* **126**, 39–46.
- Baluška F, Hlavacka A, Šamaj J, Palme K, Robinson DG, Matoh T, McCurdy DW, Menzel D, Volkmann D. 2002. F-actin-dependent endocytosis of cell wall pectins in meristematic root cells: insights from brefeldin A-induced compartments. *Plant Physiology* **130**, 422–431.
- Baluška F, Jásik J, Edelmann HG, Salajová T, Volkmann D. 2001a. Latrunculin B induced plant dwarfism: plant cell elongation is F-actin dependent. *Developmental Biology* **231**, 113–124.
- Baluška F, Parker JS, Barlow PW. 1992. Specific patterns of cortical and endoplasmic microtubules associated with cell growth and tissue differentiation in roots of maize (*Zea mays* L.). *Journal of Cell Science* **103**, 91–200.
- Baluška F, Parker JS, Barlow PW. 1993a. The microtubular cytoskeleton in cells of cold-treated roots of maize (*Zea mays* L.) shows tissue-specific responses. *Protoplasma* **172**, 84–96.
- Baluška F, Vitha S, Barlow PW, Volkmann D. 1997. Rearrangements of F-actin arrays in growing cells of intact maize root apex tissue: a major developmental switch occurs in the postmitotic transition region. *European Journal of Cell Biology* **72**, 113–121.
- Baluška F, Volkmann D, Barlow PW. 2001c. A polarity crossroad in the transition growth zone of maize root apices: cytoskeletal and developmental implications. *Journal of Plant Growth Regulation* **20**, 170–181.
- Basrai MA, Naider F, Becker JM. 1990. Internalization of Lucifer Yellow in *Candida albicans* by fluid phase endocytosis. *Journal of General Microbiology* **136**, 1059–1065.
- Bret-Harte MS, Silk WK. 1994. Non-vascular, symplasmic diffusion of sucrose cannot satisfy the carbon demands of growth in the primary root tip of *Zea mays* L. *Plant Physiology* **105**, 19–33.
- Chaffey NJ, Barlow PW. 2001. The cytoskeleton facilitates a three-dimensional symplasmic continuum in the long-lived ray and axial parenchyma cells of angiosperm trees. *Planta* **213**, 811–823.
- Chaffey NJ, Barlow PW. 2002. Myosin, microtubules, and microfilaments: co-operation between cytoskeletal components during cambial cell division and secondary vascular differentiation in trees. *Planta* **214**, 526–536.
- Chaffey NJ, Harris N. 1985. Plasmotubules: fact or artefact? *Planta* **165**, 185–190.
- Cholewa E, Peterson CA. 2001. Detecting exodermal Casparian bands *in vivo* and fluid-phase endocytosis in onion (*Allium cepa* L.) roots. *Canadian Journal of Botany* **79**, 30–37.
- Čiamporová M, Dekánková K, Hanáčková Z, Ovečka M, Baluška F. 2003. Structural aspects of root hair initiation in *Vicia sativa* roots treated with F-actin polymerization inhibitor latrunculin B. *Plant and Soil* **255**, 1–7.
- Coetzee J, Fineran BA. 1989. Translocation of lysine from the host *Melicope simplex* to the parasitic dwarf mistletoe *Korthalsella lindsayi* (Viscaceae). *New Phytologist* **112**, 377–381.
- Cram WJ. 1980. Pinocytosis in plants. *New Phytologist* **84**, 1–17.
- Derksen J, Knuiman B, Hoedemaekers K, Guyon A, Bonhomme

- S, Pierson ES. 2002. Growth and cellular organization of *Arabidopsis* pollen tubes *in vitro*. *Sexual Plant Reproduction* **15**, 133–139.
- Evert RF, Eschrich W, Neuberger DS, Eichhorn SE. 1977a. Tubular extension of the plasmalemma in leaf cells of *Zea mays* L. *Planta* **135**, 203–205.
- Evert RF, Eschrich W, Heyser W. 1977b. Distribution and structure of the plasmodesmata in mesophyll and bundle-sheath cells of *Zea mays* L. *Planta* **136**, 77–89.
- Geldner N, Friml J, Stierhof Y-D, Jürgens G, Palme K. 2001. Auxin-transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**, 425–428.
- Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Müller P, Delbarre A, Ueda T, Nakano A, Jürgens G. 2003. The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* **112**, 219–230.
- Gradmann D, Robinson DG. 1989. Does turgor prevent endocytosis in plants? *Plant, Cell and Environment* **12**, 151–154.
- Harris N, Chaffey NJ. 1986. Plasmaturbules—real modifications of the plasmalemma. *Nordisk Journal of Botany* **6**, 599–607.
- Hawes K, Crooks K, Coleman J, Satiat-Jeuematrie B. 1995. Endocytosis in plants: fact or artefact? *Plant, Cell and Environment* **18**, 1245–1252.
- Hillmer S, Hedrich R, Robert-Nicoud M, Robinson DG. 1990. Uptake of Lucifer Yellow CH in leaves of *Commelina communis* is mediated by endocytosis. *Protoplasma* **158**, 142–148.
- Hillmer S, Quader H, Robert-Nicoud M, Robinson DG. 1989. Lucifer Yellow uptake in cells and protoplasts of *Daucus carota* visualized by laser scanning microscopy. *Journal of Experimental Botany* **40**, 417–423.
- Holstein SEH. 2002. Clathrin and plant endocytosis. *Traffic* **3**, 614–620.
- Jahn T, Baluška F, Michalke W, Harper J, Volkmann D. 1998. Plasma membrane H⁺-ATPase in the root apex: evidence for strong expression in xylem parenchyma and asymmetric localization within cortical and epidermal cells. *Physiologia Plantarum* **104**, 311–316.
- Jin M, Snider MD. 1993. Role of microtubules in transferrin receptor transport from the cell surface to endosomes of the Golgi complex. *Journal of Biological Chemistry* **268**, 18390–18397.
- Jitsuyama Y, Suzuki T, Harada T, Fujikawa S. 2001. Loading process of sugars into cabbage petiole and asparagus shoot apex cells by incubation with hypertonic sugar solutions. *Protoplasma* **217**, 205–216.
- Jürgens G, Geldner N. 2002. Protein secretion in plants: from the trans-Golgi network to the outer space. *Traffic* **3**, 605–613.
- Kandasamy MK, Kappler R, Kristen U. 1988. Plasmaturbules in the pollen tubes of *Nicotiana glauca*. *Planta* **173**, 35–41.
- Kazmierczak A, Maszewski J. 1997. Uptake of fluorescent-labelled BSA into root cells: Endocytosis? *Acta Societatis botanicorum Poloniae* **66**, 319–324.
- Knight A, Kendrick-Jones J. 1993. A myosin-like protein from a higher plant. *Journal of Molecular Biology* **231**, 148–154.
- Low PS, Chandra S. 1994. Endocytosis in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **45**, 609–631.
- McCurdy DW. 1999. Is 2,3 butanedione monoxime an effective inhibitor of myosin-based activities in plant cells? *Protoplasma* **209**, 120–125.
- Morris SM, Arden SD, Roberts RC, Kendrick-Jones J, Cooper JA, Luzzio JP, Buss F. 2002. Myosin VI binds to and localizes with Dab2, potentially linking receptor-mediated endocytosis and the actin cytoskeleton. *Traffic* **3**, 331–341.
- Munn AL. 2001. Molecular requirements for the internalization step of endocytosis: insights from yeast. *Biochimica et Biophysica Acta* **1535**, 236–257.
- Nishizawa N, Mori S. 1977. Invagination of plasmalemma: its role in the absorption of macromolecules in rice roots. *Plant and Cell Physiology* **18**, 767–782.
- Oparka KJ, Murant EA, Wright KM, Prior DAM, Harris N. 1991. The drug probenecid inhibits the vacuolar accumulation of fluorescent anions in onion epidermal cells. *Journal of Cell Science* **99**, 557–563.
- Oparka KJ, Robinson D, Prior DAM, Derrick P, Wright KM. 1988. Uptake of Lucifer Yellow CH into intact barley roots: evidence for fluid-phase endocytosis. *Planta* **176**, 541–547.
- Oparka KJ, Wright KM, Murant EA, Allan EJ. 1993. Fluid-phase endocytosis: do plants need it? *Journal of Experimental Botany* **44**, 247–255.
- Ou GS, Chen ZL, Yuan M. 2002. Jasplakinolide reversibly disrupts actin filaments in suspension-cultured tobacco BY-2 cells. *Protoplasma* **219**, 168–175.
- Owen TP, Platt-Aloia KA, Thomson WW. 1991. Ultrastructural localization of Lucifer Yellow and endocytosis in plant cells. *Protoplasma* **160**, 115–120.
- Palevitz BA, Hepler PK. 1985. Changes in dye coupling of stomatal cells of *Allium* and *Commelina* demonstrated by microinjection of Lucifer Yellow. *Planta* **164**, 473–479.
- Reichert S, Kendrick-Jones J. 2000. Myosins. In: Staiger CJ, Baluška F, Volkmann D, Barlow PW, eds. *Actin: a dynamic framework for multiple plant cell functions*. Dordrecht, Netherlands: Kluwer Academic Publishers, 29–44.
- Reichert S, Knight AE, Hodge TP, Baluška F, Šamaj J, Volkmann D, Kendrick-Jones J. 1999. Characterization of the unconventional myosin VIII in plant cells and its localization at the post-cytokinetic cell wall. *The Plant Journal* **19**, 555–569.
- Riezman H. 1985. Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis. *Cell* **40**, 1001–1009.
- Robertson JG, Lyttleton P. 1982. Coated and smooth vesicles in the biogenesis of cell walls, plasma membranes, infection threads and peribacteroid membranes in root hairs and nodules of white clover. *Journal of Cell Science* **58**, 63–78.
- Robinson DG, Hinz G, Holstein SEH. 1998. The molecular characterization of transport vesicles. *Plant Molecular Biology* **38**, 49–76.
- Rozak R, Rambour S. 1997. Uptake of Lucifer Yellow by plant cells in the presence of endocytotic inhibitors. *Protoplasma* **199**, 198–207.
- Šamaj J, Peters M, Volkmann D, Baluška F. 2000. Effects of myosin ATPase inhibitor 2,3-butanedione 2-monoxime on distributions of myosins, F-actin, microtubules, and cortical endoplasmic reticulum in maize root apices. *Plant Cell Physiology* **41**, 571–582.
- Šamaj J, Ovecka M, Hlavacka A, et al. 2002. Involvement of the mitogen-activated protein kinase SIMK in regulation of root hair tip-growth. *EMBO Journal* **21**, 3296–3306.
- Sawitzky H, Liebe S, Willingale-Theune J, Menzel D. 1999. The anti-proliferative agent jasplakinolide rearranges the actin cytoskeleton of plant cells. *European Journal of Cell Biology* **78**, 424–433.
- Schulz A. 1994. Phloem transport and differential unloading in pea seedlings after source and sink manipulations. *Planta* **192**, 239–248.
- Shurety W, Stewart NL, Stow JL. 1998. Fluid-phase markers in the basolateral endocytic pathway accumulate in response to the actin assembly-promoting drug jasplakinolide. *Molecular Biology of the Cell* **9**, 957–975.
- Spector I, Braet F, Shochet NR, Bubb MR. 1999. New anti-actin drugs in the study of the organization and function of the actin cytoskeleton. *Microscope Research Techniques* **47**, 18–37.
- Stark-Urnau M, Mendgen K. 1995. Sequential deposition of plant

- glycoproteins and polysaccharides at the host–parasite interface of *Uromyces vignae* and *Vigna sinensis*. Evidence for endocytosis and secretion. *Protoplasma* **186**, 1–11.
- Stewart WW.** 1981. Lucifer dyes—highly fluorescent dyes for biological tracing. *Nature* **292**, 17–21.
- Subtil A, Dautry-Varsat A.** 1997. Microtubule depolymerization inhibits clathrin coated-pit internalization in non-adherent cell lines while interleukin 2 endocytosis is not affected. *Journal of Cell Science* **110**, 2441–2447.
- Swanson JA, Yirinec BD, Silverstein SC.** 1985. Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. *Journal of Cell Science* **100**, 851–859.
- Tominaga M, Yokota E, Sonobe S, Shimmen T.** 2000. Mechanism of inhibition of cytoplasmic streaming by a myosin inhibitor, 2,3-butanedione monoxime. *Protoplasma* **213**, 46–54.
- Uemura M, Steponkus PL.** 2003. Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from *Arabidopsis thaliana* leaves. *Plant, Cell and Environment* **26**, 1083–1096.
- Warmbrodt RD.** 1985. Studies on the root of *Hordeum vulgare* L.—ultrastructure of the seminal root with special reference to the phloem. *American Journal of Botany* **72**, 414–432.
- Wiederkehr A, Meier KD, Riezman H.** 2001. Identification and characterization of *Saccharomyces cerevisiae* mutants defective in fluid-phase endocytosis. *Yeast* **18**, 759–773.
- Wright KM, Oparka KJ.** 1989. Uptake of Lucifer Yellow CH into plant cell protoplasts: a quantitative assessment of fluid-phase endocytosis. *Planta* **179**, 257–264.