Protoplasma (2005) 226: 39–54
DOI 10.1007/s00709-005-0103-9

PROTOPLASMA
Printed in Austria

Endocytosis and vesicle trafficking during tip growth of root hairs

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Received February 7, 2005; accepted March 30, 2005; published online October 20, 2005

Summary. The directional elongation of root hairs, "tip growth", depends on the coordinated and highly regulated trafficking of vesicles which fill the tip cytoplasm and are active in secretion of cell wall material. So far, little is known about the dynamics of endocytosis in living root hairs. We analyzed the motile behaviour of vesicles in the apical region of living root hairs of *Arabidopsis thaliana* and of *Triticum aestivum* by live cell microscopy. For direct observation of endocytosis and of the fate of endocytic vesicles, we used the fluorescent endocytosis marker dyes FM 1-43 and FM 4-64. Rapid endocytosis was detected mainly in the tip, where it caused a bright fluorescence of the apical cytoplasm. The internalized membranes proceeded through highly dynamic putative early endosomes in the clear zone to larger endosomal compartments in the subapical region that are excluded from the clear zone. The internalized cargo ended up in the dynamic vacuole by fusion of large endosomal compartments with the tonoplast. Before export to these lytic compartments, putative early endosomes remained in the apical zone, where they most probably recycled to the plasma membrane and back into the cytoplasm for more than 30 min. Endoplasmic reticulum was not involved in trafficking pathways of endosomes. Actin cytoskeleton was needed for the endocytosis itself, as well as for further membrane trafficking. The actin-depolymerizing drug latrunculin B modified the dynamic properties of vesicles and endosomes; they became immobilized and aggregated in the tip. Treatment with brefeldin A inhibited membrane trafficking and caused the disappearance of FM-containing vesicles and putative early endosomes from the clear zone; labelled structures accumulated in motile brefeldin A-induced compartments. These large endocytic compartments redispersed upon removal of the drug. Our results hence prove that endocytosis occurs in growing root hairs. We show the localization of endocytosis in the tip and indicate specific endomembrane compartments and their recycling.

Keywords: Actin cytoskeleton; *Arabidopsis thaliana*; Brefeldin A; Endocytic pathway; Endosome; *Triticum aestivum*.

Abbreviations: BFA brefeldin A; ER endoplasmic reticulum; F-actin filamentous actin; MVBs multivesicular bodies.

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Introduction

Root hairs are highly polarized tubular extensions from the root epidermis which elongate continuously by cell wall formation occurring mainly at their tips. This requires a highly polarized organization of the cytoplasm and its organelles, similar to pollen tubes expanding from germinating pollen grains (for a recent review, see Hepler et al. 2001 and references therein). The dome-shaped apical region is the most active in secretion of new cell wall; polysaccharides like pectins, hemicellulose, polygalacturonic acid, and proteins are delivered primarily from the Golgi apparatus (Mc Neil et al. 1984, Sherrier and Van den Bosch 1994, Varner and Lin 1989). Hence, the cytoplasm is filled with large numbers of vesicles which are visible by electron microscopy (Bonnet and Newcomb 1966; Emons 1987; Galway et al. 1997, 1999; Ridge 1988, 1995; for a recent review about the ultrastructure of root hairs, see Galway 2000) but are too small to be seen by conventional light microscopy. The tip is therefore also described as a "clear zone" similar to pollen tubes (Lancelle and Hepler 1992).

Exocytosis of Golgi-derived vesicles at the tip brings their content to the extracellular matrix. In addition, it incorporates new membrane into the plasma membrane in order to increase its surface area and to add new proteins. Secretion by exocytosis may occur either by complete fusion of secretory vesicles with the plasma membrane, thereby adding a new patch of membrane, or alternatively secretory vesicles detach from the plasma membrane after partial or full release of their contents (Thiel and Battey 1998). In rapidly growing polarized cells such as root hairs, full fusion of secretory vesicles is expected to be the

dominant form of exocytosis. Substances destined for the cell surface are delivered directly to the plasma membrane in Golgi-derived secretory vesicles and their content is released into the extracellular space by fusion with the plasma membrane. This constitutive secretion, however, so far could not be observed in root hairs and only rarely was reported for pollen tubes (Derksen et al. 1995).

The delivery of new building material to the cell wall and the supply of new membranes to the plasma membrane by secretion is believed to maintain the growth at the tip (Thiel and Battey 1998). However, modulation of the tip-localized calcium gradient with caffeine (Lancelle et al. 1997) and precipitation of arabinogalactan proteins with Yariv reagent (Roy et al. 1998, 1999) in growing pollen tubes showed new important aspects of tip growth. Both treatments inhibit elongation of pollen tubes, although secretion remains unaffected. This latter finding indicates that secretion and growth may be uncoupled from each other and that secretion itself is not sufficient for tip growth. Calculation of the production rates of secretory vesicles in tip-growing pollen tubes indicates that more membrane is added to the plasma membrane than is required for growth (Derksen et al. 1995, Picton and Steer 1983, Steer 1988). Thus, excess membrane is supposed to be retrieved by endocytosis (for recent reviews, see Geldner 2004, Šamaj et al. 2004a, Murphy et al. 2005). Whereas exocytosis is believed to occur in the tip of the apical dome, clathrin-coated vesicles form along the flanks just below the tip of the rapidly growing pollen tube of tobacco (Derksen et al. 1995) or throughout the whole apex of slower pollen tubes of *Arabidopsis thaliana* (Blackbourn and Jackson 1996).

In growing tips of root hairs, coated pits and coated vesicles were detected in varying distributions and concentrations depending on the age and growth rate of the cells (Emons and Traas 1986; Galway et al. 1997, 1999; Ridge 1988; Wymer et al. 1997). In addition, endosomal compartments of the further membrane-trafficking pathway like multivesicular bodies (MVBs) (Tse et al. 2004) and trans-Golgi networks (Uemura et al. 2004) were observed (Galway 2000, Low and Chandra 1994), thus indicating that endocytosis occurs also in growing root hairs. Recent data show that highly motile early endosomes are inherently linked to the tip growth of root hairs (Voigt et al. 2005a). They are abundant in the clear zone only during tip growth and their movement is driven by actin polymerization (Voigt et al. 2005a). So far, however, little is known about the dynamics of endocytosis in living root hairs and about the motility and destination of endosomal compartments.

The study of the dynamic behaviour of vesicles in living cells is hindered by the size of the vesicles due to the resolution limit of conventional light microscopes. Especially in the clear zone, however, observation of their motility is a prerequisite for the understanding of growth regulation processes. Live cell imaging by ultraviolet and video-enhanced contrast microscopy allows the monitoring of exo- and endocytic vesicles in the tip that so far were known mainly from images of fixed cells by electron microscopy (Lichtscheidl and Foissner 1996). We can visualize small vesicles accumulated in the apical region, their dynamic behaviour, and their contacts with the apical plasma membrane with the help of endocytosis markers like the broadly used styryl dyes FM 1-46 and FM 4-64 (Betz et al. 1996, Parton et al. 2001, Camacho and Malhó 2003).

Motility of the organelles including early endosomes in the apex of root hairs is actin-dependent (Voigt et al. 2005a). In addition, a fine net of dynamic F-actin close to the plasma membrane might contribute to endocytosis by drawing coated vesicles away from the plasma membrane (Šamaj et al. 2004a). In pollen tubes, F-actin depolymerization was shown to stop growth (Vidali et al. 2001, Parton et al. 2001, Gibbon et al. 1999). Also in root cells, actin is involved in trafficking of vesicles; it mediates endocytosis, directs the vesicles into endosomal compartments, and brings secretory vesicles back to the plasma membrane (Geldner et al. 2001, Baluška et al. 2002). In this study, we therefore inhibited the actin cytoskeleton by latrunculin B, a substance well established to depolymerize actin filaments and inhibit tip growth in both pollen tubes (Gibbon et al. 1999) and root hairs (Baluška et al. 2000, 2001).

In order to examine a possible involvement of the endoplasmic reticulum (ER) in the fate of endocytosed membranes in tip-growing root hairs, we analyzed the distribution of ER by labelling with the membrane potential dye 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (Quader and Schnepf 1986, Lichtscheidl and Url 1990).

Membrane trafficking by exo- and endocytosis in plant cells is also highly sensitive to any conditions interfering with the functional assembly of vesicles and vesicular delivery within the cell. It has been shown that brefeldin A (BFA) inhibits vesicle coat formation and thus prevents vesicle trafficking (Robineau et al. 2000). In root cells, BFA inhibits recycling of a putative auxin efflux carrier PIN1 and affects its polar localization in the plasma membrane (Geldner et al. 2001, Baluška et al. 2002). In growing root hairs, we used BFA in order to get a clearer image of the fate of endocytic vesicles. Our results show active endocytosis at the tip of growing root hairs and further trafficking of the endocytosed membranes through different putative endosomal compartments either back to the plasma membrane or towards the tonoplast. Trafficking of endocytic markers from the tip to

the tonoplast is actin-dependent and BFA-sensitive and does not interact with ER.

Material and methods

Plant material and cultivation

We used growing roots of *Triticum aestivum* and of *Arabidopsis thaliana* for the investigation of endocytosis. Seeds of *Arabidopsis thaliana* were surface sterilized and placed on MS culture medium (Murashige and Skoog 1962) containing vitamins and 1% sucrose that was solidified by 0.8% agar. The pH was adjusted to 5.8. Seeds of *Triticum aestivum* were soaked in water at 4 °C for 24 h on filter paper and then germinated in petri dishes at 22–24 °C under continuous light. 3- or 4-day-old seedlings were transferred to microscopic slides that were modified into thin chambers by coverslips. Chambers were filled with the same liquid medium but without agar (*A. thaliana*) or with 10 mM phosphate buffer, pH 6.5 (*T. aestivum*) and placed in sterile glass cuvettes containing the medium at a level that reached the open lower edge of the chambers. This allows free exchange of medium between chambers and the cuvette. Seedlings were grown in vertical position under continuous light for 12–24 h. During this period, the seedlings stabilized root growth and proceeded in the formation of new root hairs.

Method of treatment and data acquisition

For analysis under the microscope, only seedlings with young root hairs growing at an average growth rate were selected. The chamber was gently and slowly perfused with culture medium during 10 min under microscopic control to make sure that mechanical alteration of the roots was avoided. After perfusion, uptake experiments were performed in the microchambers directly on the microscope stage. The growth medium was removed with a small strip of filter paper that was introduced between slide and coverslip, and slowly the new medium, i.e., the culture medium containing the indicated concentrations of drugs, was applied to the chamber with a micropipette at a loading speed of 10 μl/min.

Tracer dyes for endocytosis

The styryl dyes FM 1-43 (N-(3-triethylammoniumpropyl)-4-(4-[dibutylamino]styryl)pyridinium dibromide) and FM 4-64 (N-(3-triethylammoniumpropyl)-4-(8-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide) at the final concentration of 2 or 4 μM in culture medium were brought into the chambers by perfusion loading for 5 min. Cells were either observed immediately without washing of the dye, or, for pulse-labelling, the dyes were removed after 5 min by plain culture medium. Cells were observed after 10 min washing. $DiOC_6$ was used for labelling of ER at a concentration of 5 μg/ml in culture medium made from stock solution (100 mg/ml prepared in dimethylsulfoxide). Root hairs were pulse treated with 4 μM FM 4-64 for 5 min, then the dye was washed out with culture medium. After 10–30 min cultivation, the root hairs were labelled with $DiOC_6$ for 5 min, washed with culture medium, and observed. Alternatively, both dyes were applied simultaneously for 10 min and washed out before observation.

Inhibitors of tip growth

Latrunculin B, an inhibitor of actin microfilament polymerization, was applied before, during, or after labelling with FM dyes at concentrations of 0.1–1 μM prepared from a stock of 10 μM in water. BFA is supposed to inhibit vesicle recycling. It was applied together with or before FM dyes at a concentration range of 3.5–35 μM.

Microscopy

Seedlings were observed by live cell imaging in order to analyze the dynamic processes during tip growth, as was described in detail by Foissner et al. (1996) and by Lichtscheidl and Foissner (1996). Briefly, we used a Univar microscope (Reichert-Leica, Vienna, Austria) equipped for bright-field and differential interference contrast with a HBO 200 mercury lamp and 40× and 100× plan apo objectives. Additional magnification of ×1.6 or ×2.5 allowed for good visibility of the resolved structures on the monitor screen.

The bright-field pictures were recorded on videotape (mini-DV, Sony and JVC) by a high-resolution video camera (Hamamatsu, Hamamatsu City, Japan), whereas for fluorescence microscopy, we used a low-light-sensitive video camera (Hamamatsu and Photonic Science, Robertsbridge, UK). Enhancement of contrast and gain was possible by the video cameras. For additional information from the object, like the movement of organelles, we used a digital image processor with functions of image addition and trace following (DVS-3000, Hamamatsu). Alternatively to conventional fluorescence microscopy, we used a confocal laser scanning microscope (ICS Leica Microsystems Heidelberg, Mannheim, Germany) equipped with 40× and 63× oil immersion plan apo objectives.

Analysis of organelle motility

Trajectories of organelles were analyzed from videotapes (25 frames per second) by the track following function of the video computer. Every second, third, or fourth frame was displayed on the monitor screen thus forming an integrated picture. Short displacements of single organelles in various directions were levelled out, straight or curvilinear tracks resulted in rows of organelle locations, and static organelles were displayed by strong signals in their positions.

Results

We studied growing root hairs of *A. thaliana* and of *T. aestivum* by the noninvasive methods of video microscopy and fluorescence microscopy (live cell imaging). Video microscopy utilizes the maximum resolution of the light microscope or even improves it by enhancing contrast electronically by video camera and image processor. This approach allows for the visualization and analysis of subresolution details in living cells. Fluorescence microscopy, on the other hand, permits the study of endocytosis and of membrane trafficking after specific labelling of the plasma membrane.

Growing root hairs are tubular cells with a diameter of 9–11 μm in *A. thaliana* and 11–14 μm in *T. aestivum*. Their cytoplasm displays the typical polarized organization that was described for tip-growing cells in general (Schnepf 1986, Sievers and Schnepf 1981), with organelles needed for the formation of new cell wall accumulated in the tip. We therefore concentrated in this study on the analysis of dynamic processes occurring in the tip. It is differentiated into an apical zone proper, i.e., the clear zone, and a subapical region. The apical zone is filled with structures of sizes at the limit of the resolution of the conventional light microscope (0.3 μm or smaller). From electron micrographs, they are supposed to be secretory vesicles destined to export cell wall constituents by exocytosis, and it is still a matter of debate if during the process of cell wall formation, endocytic

vesicles are also formed in order to internalize excess membrane and cell wall material (Galway 2000, Baluška et al. 2002). From their form, we cannot infer whether these structures are endosomes or secretory vesicles derived either from the Golgi apparatus or from other endosomal compartments, and as such we describe them with the general term "vesicles". The subapical region contains all other motile organelles like Golgi stacks, mitochondria, and ER in addition to the different populations of vesicles. We visualized these vesicles in living cells and analyzed their dynamics in the apical dome and in the flanks, as well as their behaviour at the plasma membrane.

Live cell imaging of growing root hairs

The tip of growing root hairs was filled with dynamic vesicles that became visible as dark bodies of spherical, oval, or elongated form. They appeared in different sizes; small vesicles (up to 200 μm) essentially occupied the tip proper, whereas few larger structures (up to 300 μm) occurred mainly at the base of the tip and along the flanks (Fig. 1a). This structural organization was found not only in *A. thaliana* but also in *T. aestivum* root hairs. However, the length of the vesicle-filled clear zone was larger in *T. aestivum* root hairs.

In the tip, vesicles were in constant motion which appeared in many cases chaotic and Brownian-like. In addition, however, we also observed vesicles that displayed a pattern of directed trajectories of the type of saltatory movement; jumps of individual vesicles were interrupted by changes of direction and by short pauses. Vesicles also made close but short contacts with the plasma membrane after which they moved back into the clear zone. This was the most typical mode of contact between the plasma membrane and the vesicles, whereas fusion and subsequent disappearance of vesicles at the plasma membrane occurred only rarely and therefore was rather difficult to document (Fig. 1a).

In the subapical region, all other organelles occurred. This part of the root hairs was regularly invaded by fingerlike projections of the vacuole which were in constant motion and continuously changed their form (Fig. 1a). The large and less dynamic vacuole filled the base of the root hair and restricted the cytoplasm to a thin layer between tonoplast and cell wall. Organelles moved here in straight lines parallel to the axis of the tube. Their direction was towards the tip; large organelles reversed in the subapex and returned towards the base in several strands in the fashion of reverse fountain streaming. Small vesicles, however, did not turn in the subapex but constantly moved from the base of the root hair into the apex and also back (data not shown).

When tip growth terminated, the polarization of the cytoplasm gradually disappeared. Large organelles invaded the tip, and finally the tip was entered also by a large vacuole. It became surrounded by only a thin cytoplasmic layer that contained the small and large organelles in even distribution. They moved in a rotation-like streaming and through cytoplasmic transvacuolar strands (Fig. 1b).

Rapid endocytosis visualized with FM dyes

Growing root hairs were labelled with FM 1-43 and FM 4-64, today's most widely used dyes for the plasma membrane recycling. Both dyes gave similar results. However, FM 1-43 yielded sufficient fluorescence intensity at 4 μM, whereas FM 4-64 needed longer time or higher concentrations for obtaining the same results. For proper observation of the fate of endocytosed plasma membrane, roots were pulse labelled, i.e., root hairs were incubated for 5 min and the dye was

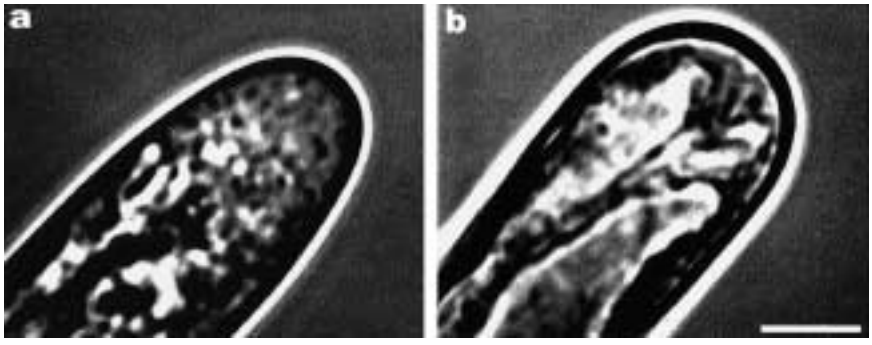


Fig. 1 a, b. Live cell microscopy of growing (**a**) and growth-terminating (**b**) root hairs of *A. thaliana* with electronic enhancement of the contrast. **a** The apex of a growing root hair is differentiated into the clear zone filled with vesicles and the subapical region containing also other larger organelles and thin protrusions of the vacuole. **b** The apex of a root hair during termination of tip growth. The vesicle-rich zone disappeared and the vacuole invaded into the tip. Bar: 5 μm

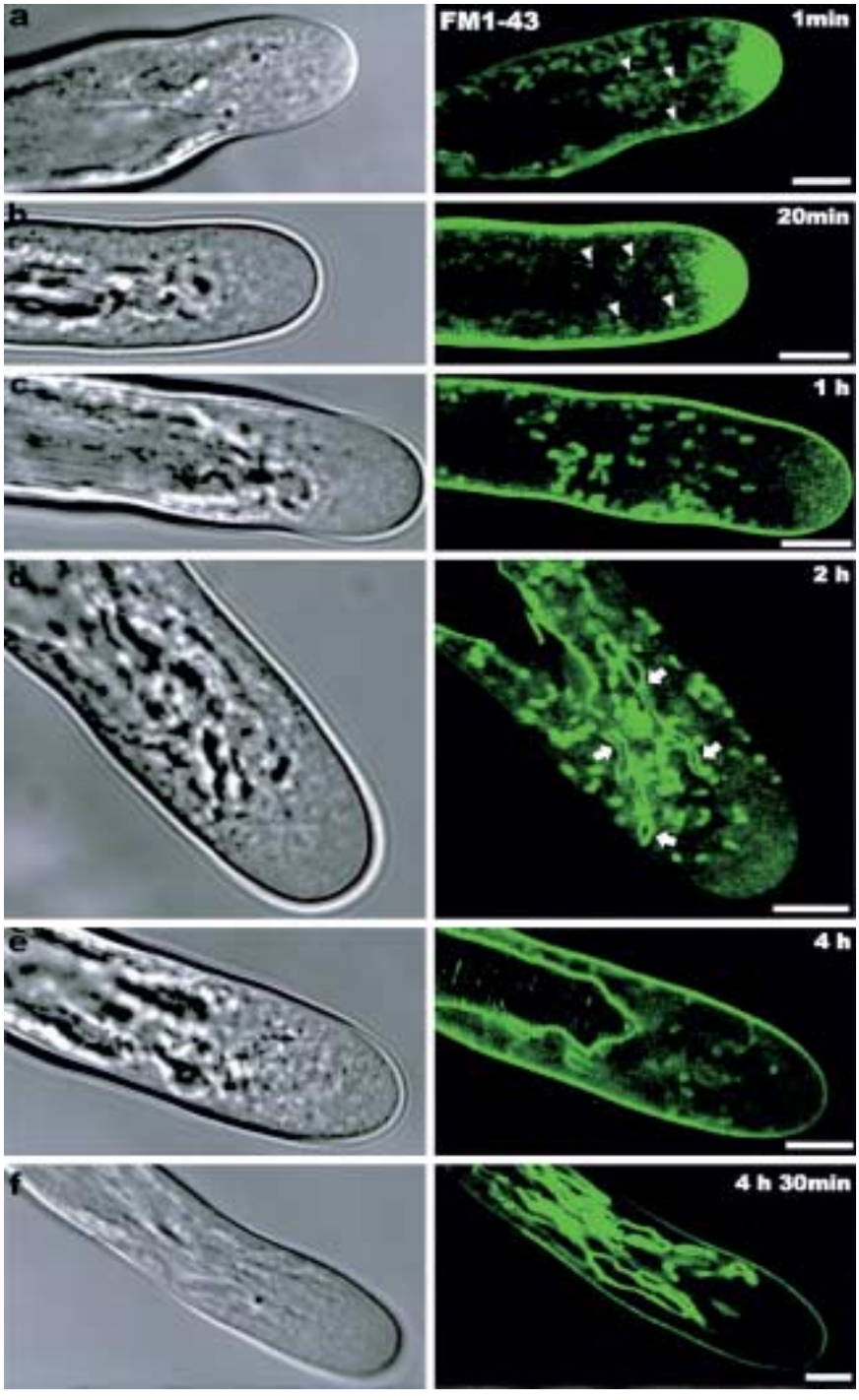


Fig. 2 a–f. Pulse treatment of root hairs of *A. thaliana* with 4 μM FM 1-43 for 5 min followed by washing. **a** 1 min after washing. **b** 20 min after washing. The plasma membrane is prominently labelled, as well as the clear zone and punctuate structures (the putative early endosomes) which move in the apical and subapical region (arrowheads). **c** 1 h after washing. Plasma membrane in the apex was fainter and the marker dye was concentrated in larger, slowly moving, endosome-like compartments (the putative late endosomes). Note that the putative late endosomes were excluded from the clear zone. **d** 2 h after washing. Putative late endosomes were still labelled but the dye was further transferred to the tonoplast. The central vacuole protruded into the subapical zone by dynamic fingerlike projections (arrows). **e** 4 h after washing. The labelling intensity of the tonoplast increased and the number of FM-positive putative late endosomes declined. **f** 4 h and 30 min after washing. Staining of the putative late endosomes disappeared, only the tonoplast had bright fluorescence. Note the dynamic and polymorphic nature of the central vacuole. Bars: 5 μm

washed out and substituted with the normal culture medium for 10 min before first observations. The time course of membrane trafficking is shown in Fig. 2.

Incubation resulted in strong staining of the plasma membrane followed by a gradual increase of fluorescence in the cytoplasm which derived from tiny fluorescent vesicles that apparently pinched off from the labelled plasma membrane. It culminated in strong labelling of the clear zone in a lens-shaped form of 8–12 μm length already within 5 min. Staining of the clear zone persisted after washout of the dye for more than 40 min. Labelled vesicles populated the tip cytoplasm so densely that the whole apex

appeared diffusely stained. Due to their constant motion, a clear image was difficult to obtain (Fig. 2a, b). Already during the washing step, the first fluorescence of putative early endosomes appeared. They had a larger size (ca. 500 nm in the fluorescence image) and accumulated mainly below the tip in the subapical region but occasionally were traced also within the tip. From here, they moved into the tube, where they travelled together with the other organelles and also came back to the tip (Fig. 2a, b).

1 h after washing, all labelled compartments had fused into large endosome-like compartments that were rod-shaped or oval and had a length of approximately 2 μm. They were distributed evenly throughout the cytoplasm with the exception of the apical region, which was still intact and growing. They moved together with the other organelles. At the same time, the bright staining of the tip decreased, only few vesicles at the limit of the resolution of the confocal microscope remained visible (Fig. 2c).

During the next 2 h, these compartments gradually disappeared and at the same time the tonoplast became stained so that the large vacuole and the fingerlike projections in the subapical region became obvious (Fig. 2d). Dynamic fingerlike projections reached the subapical region but not the clear zone, indicating an exclusion limit not only for large organelles but also for the vacuole. However, the dynamics and the penetration ability of the apical part of the tonoplast clearly demonstrate the highly motile and dynamic nature of the central vacuole in growing root hairs (Figs. 1a and 2d, e, f).

Larger endosome-like compartments were often distributed in close proximity with the plasma membrane in the cortical cytoplasm, but despite their closeness, fusion never was observed (Fig. 2c, d). Furthermore, the labelling intensity of the plasma membrane never increased during this time, indicating no fusion of large endosome-like compartments with the plasma membrane. The dynamic tonoplast, on the other hand, gradually increased in fluorescence and the number of large endosome-like compartments decreased at the same time (Fig. 2d). Finally, the large endosome-like compartments disappeared, whereas the tonoplast became brightly stained (Fig. 2e, f). Fluorescence derived most probably from the consumption of endosome-like compartments into the tonoplast, because we regularly observed docking of large endosome-like compartments with the dynamic tonoplast and subsequent fusion events. Immediately after fusion, this part of the tonoplast glowed brightly, but the fluorescence spread out quickly (Fig. 3). In addition to the tonoplast, many membranes inside the vacuole were also brightly labelled (not shown). This indicates that membrane trafficking ends in the vacuole by fusion of the large endosome-like compartments with the tonoplast (Fig. 2e, f). Recycling of the labelled membranes from late endosome-like compartments or from the tonoplast into secretory vesicles within the clear zone or to the plasma membrane could not be observed within observation times of up to 5 h (Fig. 2e, f).

Inhibition of rapid endocytosis and vesicular trafficking by latrunculin B

In yeast and animal cells, actin filaments, myosins, and other actin-binding proteins were shown to be essential for multiple stages of clathrin-mediated endocytosis (Osterweil et al. 2005, Toshima et al. 2005, Yarar et al. 2005). Similarly, plant endocytosis requires intact F-actin (Baluška et al. 2002, Geldner et al. 2003, Grebe et al. 2003) and myosin VIII activities (Baluška et al. 2004). We therefore inhibited actin filaments by latrunculin B at relatively high concentrations ranging between 100 nM and 100 μM, which have immediate and clear effects on the movement of organelles. Here we present results obtained with the concentration of 100 nM latrunculin B.

Organelles in the subapical zone were the first to slow down movements; within 2 min after application, they only slightly vacillated in their positions. At the same time, dynamic vacuolar elements penetrated into the tip, became inflated, and stopped their movements. It was remarkable that the tonoplast of these round apical vacuoles became decorated by small stationary vesicles so that the membranes looked like chains of pearls (Fig. 4).

Whereas the directional movement of the large organelles in the subapical zone and the tube shank was affected almost immediately after latrunculin B treatment, the small vesicles in the apical zone continued temporally to move and to touch the plasma membrane, although they gradually lost their directionality. Where they were in close contact with the plasma membrane, they were static (Fig. 5). The movement finally ceased (Fig. 5c–h), but as long as it occurred, the apical zone still elongated slightly.

Uptake of FM 4-64 as well as distribution and motility of endocytic compartments (Fig. 6a) were changed by treatment with latrunculin B. Vesicles accumulating FM 4-64 were still present in the clear zone, but with prolonged action of latrunculin B they gradually stopped moving. Finally, they were immobilized and aggregated at the plasma membrane, so that the apical plasma membrane became lined with stationary vesicles (Fig. 6b). Putative endosomes were rather enlarged and aggregated, and their movement was also reduced. Labelling of the plasma membrane was

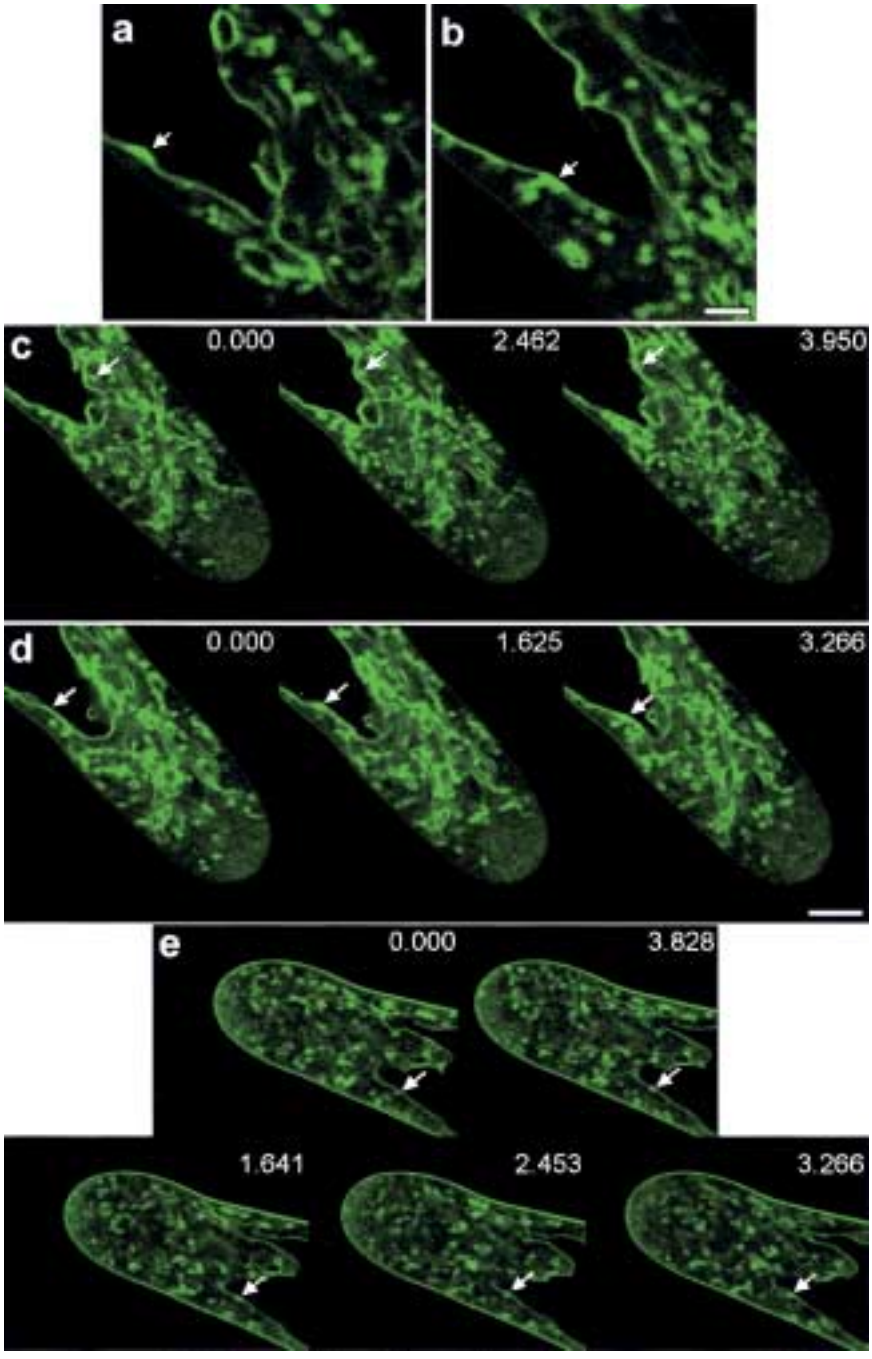


Fig. 3 a–e. Fusion of putative late endosomes with the tonoplast. **a** and **b** Fusion events in root hairs of *A. thaliana*. Fluorescence of the putative late endosomes after docking was incorporated in localized portions of the tonoplast (arrows). **c–e** Serial pictures from real-time video microscopy show approaching, docking (**c**), and fusion (**d** and **e**) of putative late endosomes with the tonoplast. Putative late endosomes are pointed out by arrows. Timing of the serial pictures is indicated in seconds. Bar: a and b, 2 μm; c–e, 5 μm

considerably reduced after perfusion with latrunculin B, but recovered after some time (Fig. 6c).

ER not involved in rapid endocytosis

ER was visualized by fluorescence labelling of the cells with $DiOC_6$. It consisted of thin tubules and lamellae that were interlaced and formed a relatively stable net in the cortical cytoplasm of the root hair flanks between cell wall and vacuole (data not shown). In the apical and subapical regions, the ER net was very dense but motile. Dynamic ER from the subapex invaded the clear zone temporarily in the cortex, whereas the center was free of ER (Fig. 7). In addition, several roundish or elongated structures were stained.

Colabelling with FM 4-64 detected endocytic compartments. Most of the putative endosomes occupied the

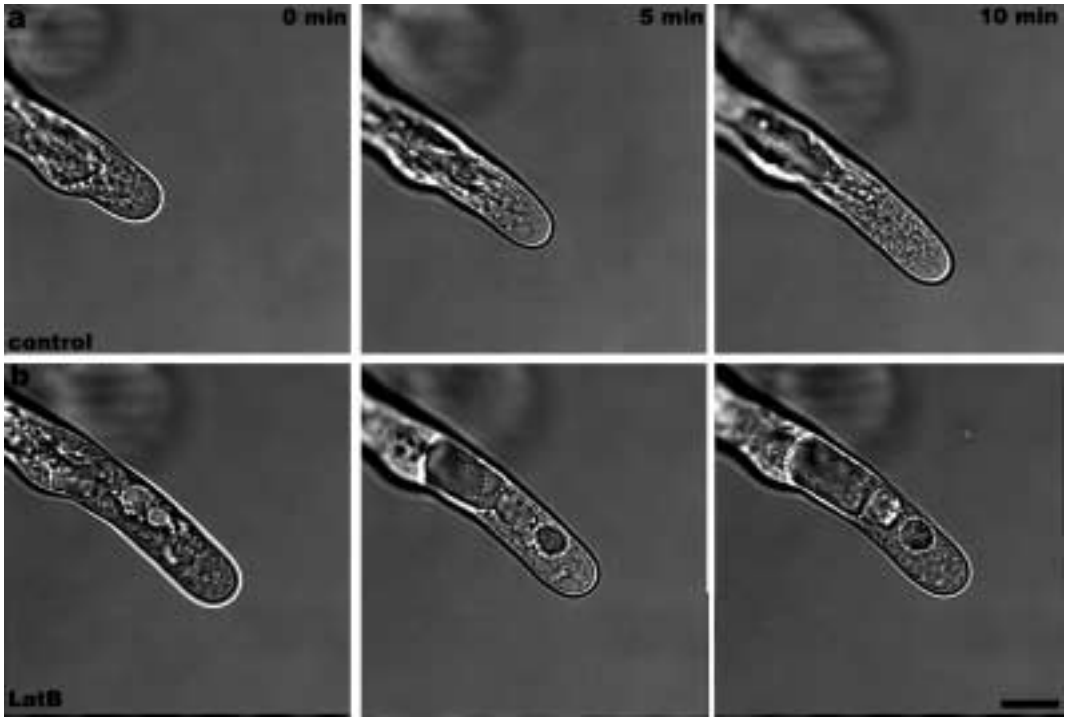


Fig. 4 a, b. Cytoarchitecture of the root hair of *A. thaliana* treated by 100 nM latrunculin B. **a** Control growing root hair. Nonvacuolated apex was filled with dense cytoplasm. **b** Latrunculin B-induced gradual cessation of tip growth within 5 min and protrusion of the vacuole into the subapical zone. Note the radical change of the vacuole form from dynamic and polymorphic (**a**) to round and static (**b**) with tonoplast decorated by small stationary vesicles (arrows). Bar: 10 μm

subapical, ER-rich zone (Fig. 7), and although ER profiles were abundant in this part of the root hairs, they never colocalized. ER and putative endosomes form two separate compartments (Fig. 7).

Inhibition of vesicle trafficking but not rapid endocytosis by brefeldin A

BFA is an effective inhibitor for vesicular trafficking. However, BFA does not interfere with endocytosis at the plasma membrane level in plant cells; uptake of endocytic markers continues unaffectedly or it can be even stimulated by BFA, but further delivery to the vacuole is inhibited (Emans et al. 2002). In order to characterize the effect of BFA on the distribution of early endocytic compartments in root hairs, we applied this drug before or together with FM dye. Data presented here are representative for the treatment with 35 μM BFA. BFA alone or in a mixture with FM dyes inhibited growth of root hairs in a concentration-dependent manner within minutes (data not shown). At the same time, BFA treatment stimulated the formation of so-called BFA compartments (Šamaj et al. 2004a) in the main tubular part of the root hair; these are accumulations of FM-positive compartments that form dense clusters of 6–9 μm in diameter (Fig. 8a).

While growth of root hairs was arrested and BFA compartments appeared, at the same time the bright staining of the apical zone decreased. Typical FM-containing vesicles in the clear zone as well as putative early endosomes disappeared until only the plasma membrane and those large BFA compartments remained labelled (Fig. 9a).

Typically, 2 or 3 BFA compartments were present in most growing root hairs. Although they were large, they were compact enough to form distinct motile structures. In the subapex their movement was less pronounced and they adopted more or less constant positions, but in the vacuolated part of the root hairs, BFA compartments travelled for long distances via circulation streaming (Fig. 8a). Velocity of the moving BFA compartments varied but was generally comparable with the motility of larger organelles (e.g., plastids) present in the cortical sheet of the cytoplasm of control root hairs. Morphological characterization of BFA compartments from optical sections revealed their nonuniform arrangement; aggregated membranous organelles formed a compact core that fanned out and became fenestrated towards the periphery (Fig. 8b).

Effects of BFA were fully reversible upon removal of the drug. This was observed partly also in root hairs treated with a mixture of BFA and FM 1-43 for 30 min (Fig. 9a).

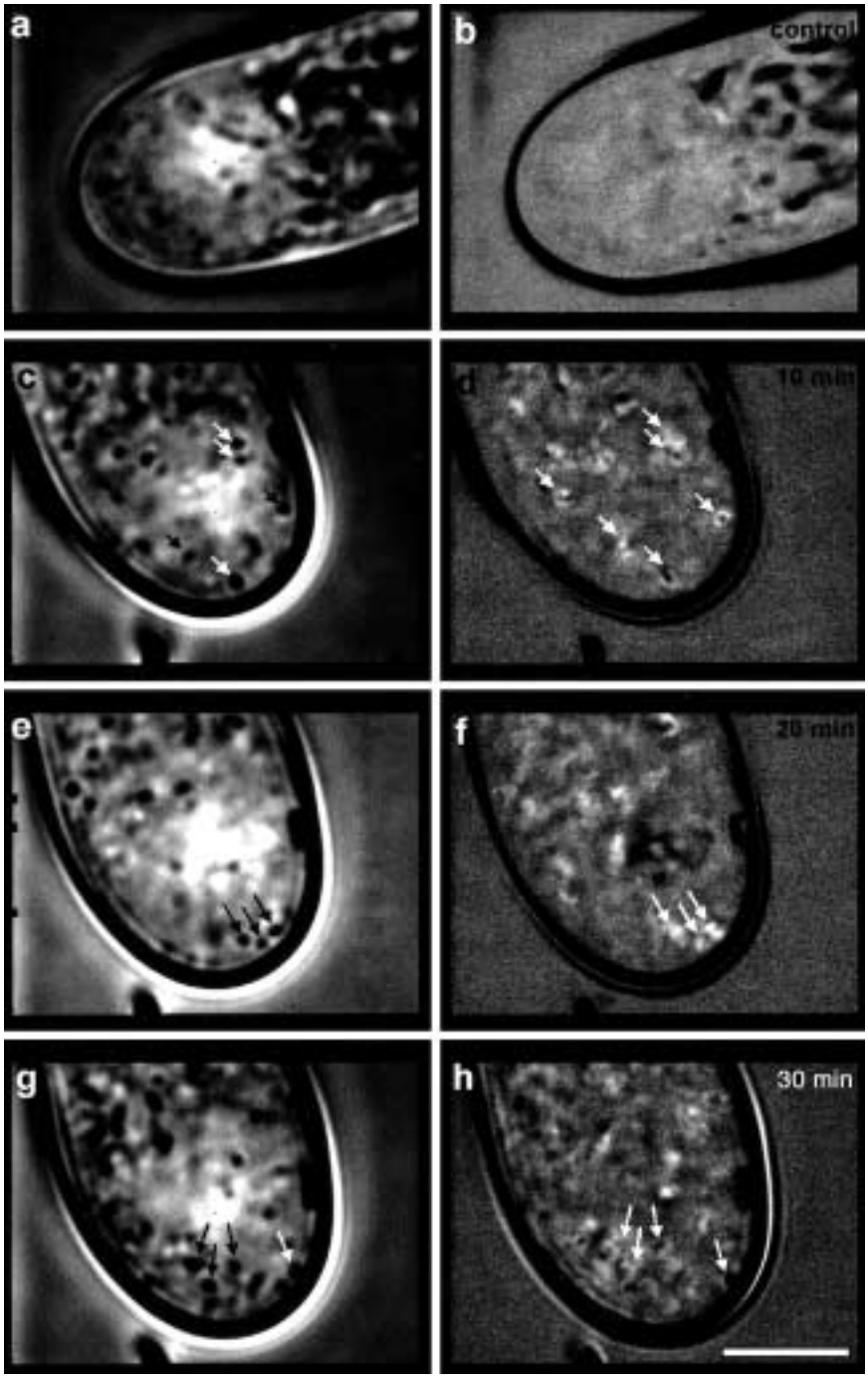


Fig. 5 a–h. Motility of the vesicles in root hairs of *A. thaliana* treated by 100 nM latrunculin B. Tracking of the particle movement by video microscopy. **a** and **b** Highly motile vesicles in the clear zone of control root hair. **c** and **d** Reduced movement of some vesicles and endosome-like compartments (arrows) 10 min after latrunculin B treatment. **e** and **f** Directional movement was partly resumed in the subapical zone 20 min after latrunculin B treatment, but vesicles in the clear zone (arrows) moved still only by Brownian movement. **g** and **h** Oscillating vesicles (arrows) in the clear zone 30 min after latrunculin B treatment. Note the vesicles in contact with the plasma membrane which were completely static. Control (a and b), and latrunculin B treated (c–h) root hairs shown in single frames from the video sequence (a, c, e, and g) or as integrated pictures of the whole video sequence (b, d, f, and h). Bar: 5 μm

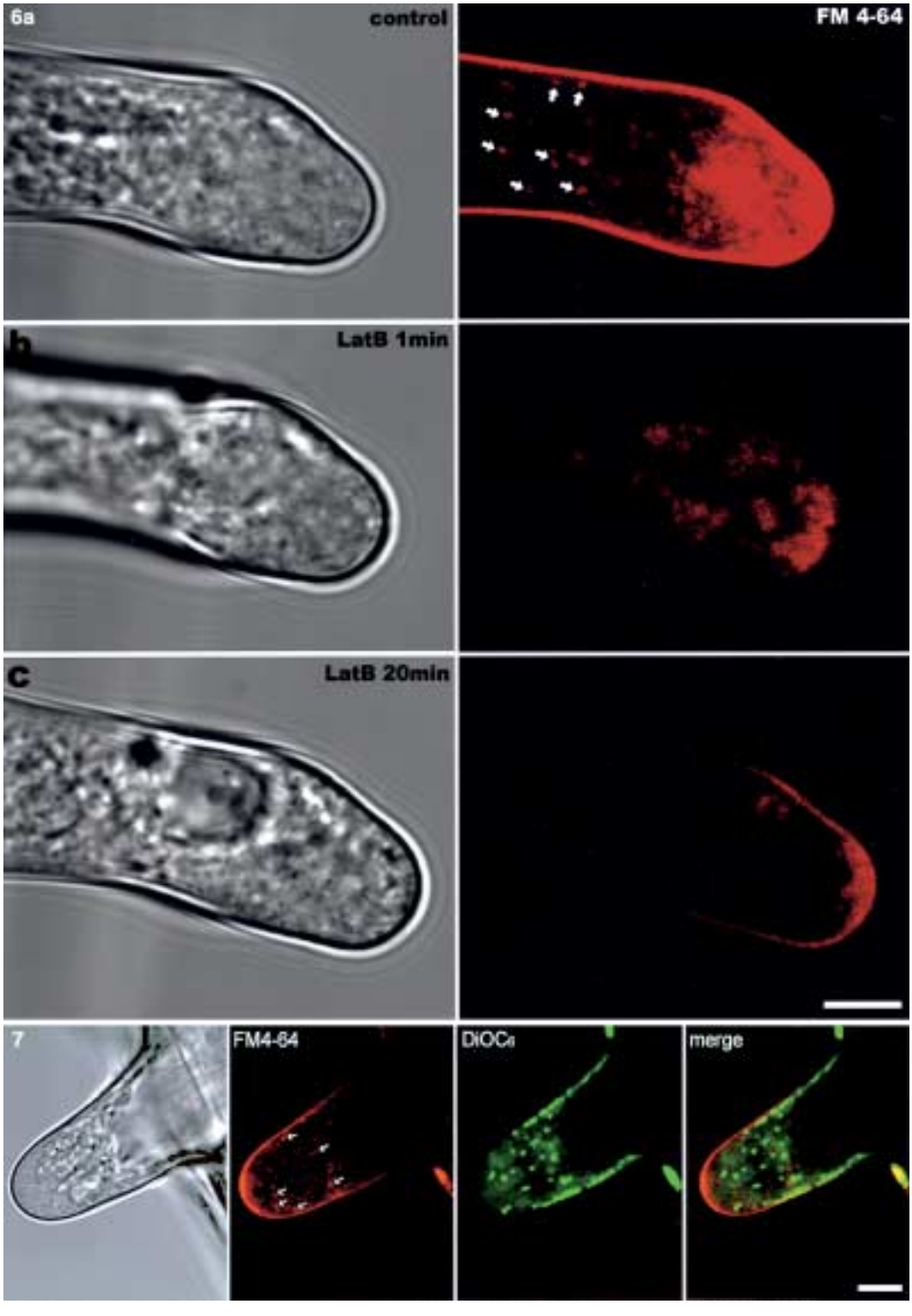
Removal of BFA by washing out with the culture medium caused gradual disintegration of the BFA compartments within 10 min. Small individual spots and endosome-like structures reappeared and they circulated again separately through the whole cytoplasm (Fig. 9 b–d). Growth of the root hairs, however, was not resumed and neither was the vesicle-rich apical clear zone reconstructed.

Discussion

We studied endocytosis in growing root hairs of *A. thaliana* and *T. aestivum* by video and fluorescence microscopy. These noninvasive methods visualized vesicles of subresolution size in living cells and allowed to partly identify them by staining with membrane-specific dyes. The fate of the endocytosed

membrane could thus be explored and the dependence of endocytosis on the actin cytoskeleton was established.

According to the conventional model of plant tip growth (Hepler et al. 2001), the clear zone is populated by secretory vesicles of Golgi origin for cell wall formation and by endocytic vesicles regarded as vehicles for internalization of excess of membranous material. Very recently we showed, however, that membrane-trafficking processes in this clear



zone are more complex; in addition to secretory and endocytic vesicles, numerous putative early endosomes also move into the clear zone (Voigt et al. 2005a and this study).

The study of vesicle movement showed that small vesicles and putative early endosomes can move freely into and out of the clear zone, whereas large endosome-like compartments are excluded and restricted to the shaft. In the tip, vesicles moved either in chaotic Brownian motion or in directed trajectories and often reached out into the very periphery of the cytoplasm towards the cell wall. We expect that the vesicles contacted the plasma membrane, but for practical reasons this cannot be resolved in living cells. Sometimes, the vesicles disappeared but more often they bounced back into the cytoplasm and continued to travel there. Contact of vesicles with the plasma membrane is expected and needed for the secretion of the cell wall by exocytosis. Cell wall constituents are exported, and at the same time new membrane is incorporated into the plasma membrane in order to increase the surface of the growing cell and to supply it with the necessary components. We were astonished to observe, however,

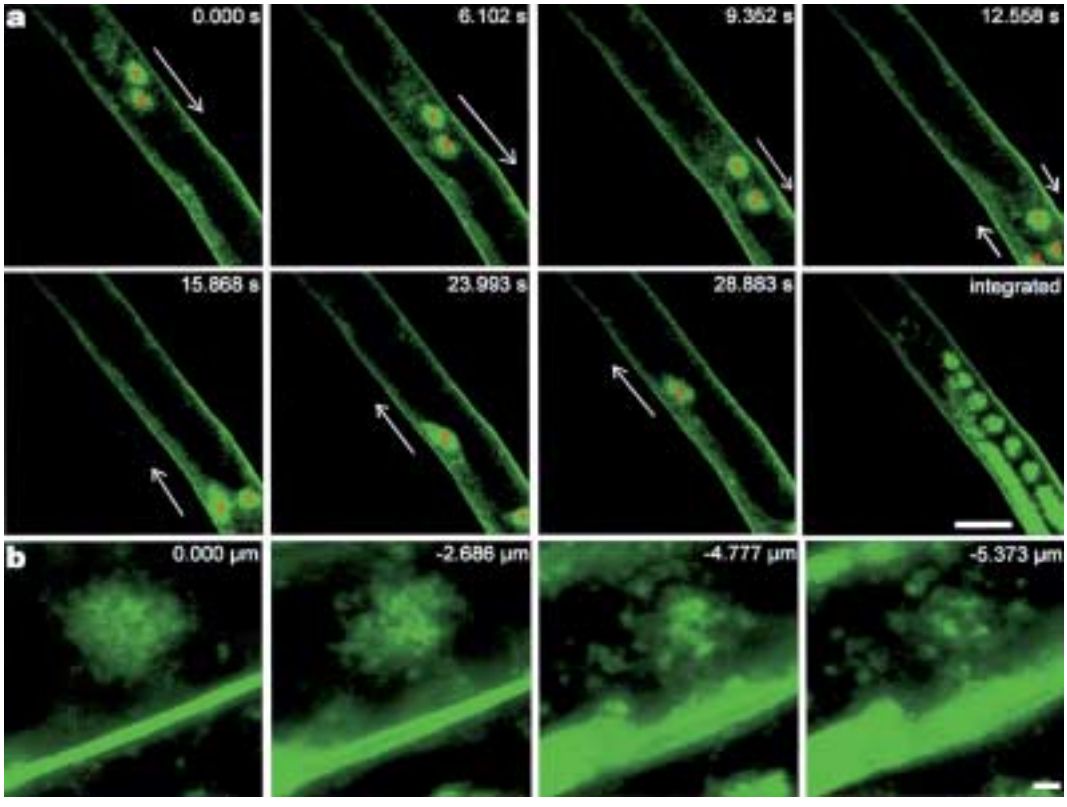


Fig. 8. Motility and structure of BFA-induced compartments in root hairs of *T. aestivum* (**a**) and *A. thaliana* (**b**). **a** Real-time video sequences of one individual root hair treated with a mixture of 35 µM BFA and 4 µM FM 1-43 for 30 min showing motility of three BFA compartments. Directionality and extent of the movement are shown by arrows. Timing of the serial pictures is indicated in seconds. **b** Spatial view on the structure of the BFA compartment in a root hair treated by 35 µM BFA for 10 min followed by 4 µM FM 1-43. Positions of optical sections within the Z-stack series are indicated in micrometers. Bar: a, 10 µm; b, 1 µm

Fig. 6 a–c. Endocytosis in root hairs of *A. thaliana* treated by 100 nM latrunculin B. **a** Uptake of FM 4-64 in a control root hair. Plasma membrane, vesicles in the clear zone, and putative early endosomes (arrows) in the apex and subapical region were brightly stained. **b** Latrunculin B reduced the labelling of the plasma membrane and induced an aggregation of FM-positive compartments. **c** Aggregated vesicles and putative early endosomes were immobilized at the plasma membrane. Control root hair loaded with 4 µM FM 4-64 for 15 min (**a**) was perfused with 100 nM latrunculin B for 10 min. Pictures were taken 1 min (**b**) and 20 min (**c**) after latrunculin B perfusion. Bar: 5 µm

Fig. 7. Labelling of ER in root hairs of *A. thaliana* by $DiOC_6$. FM 4-64 accumulated in the clear zone and in putative early endosomes. They were distributed within the apical and subapical region of the root hair (arrows). ER membranes arranged in dense and dynamic meshwork and roundish structures were abundant in the basal portion of the clear zone and in subapex of the root hair. There was no colocalization of FM-positive putative endosomes with the network of dense profiles of ER (merge). Bar: 5 µm

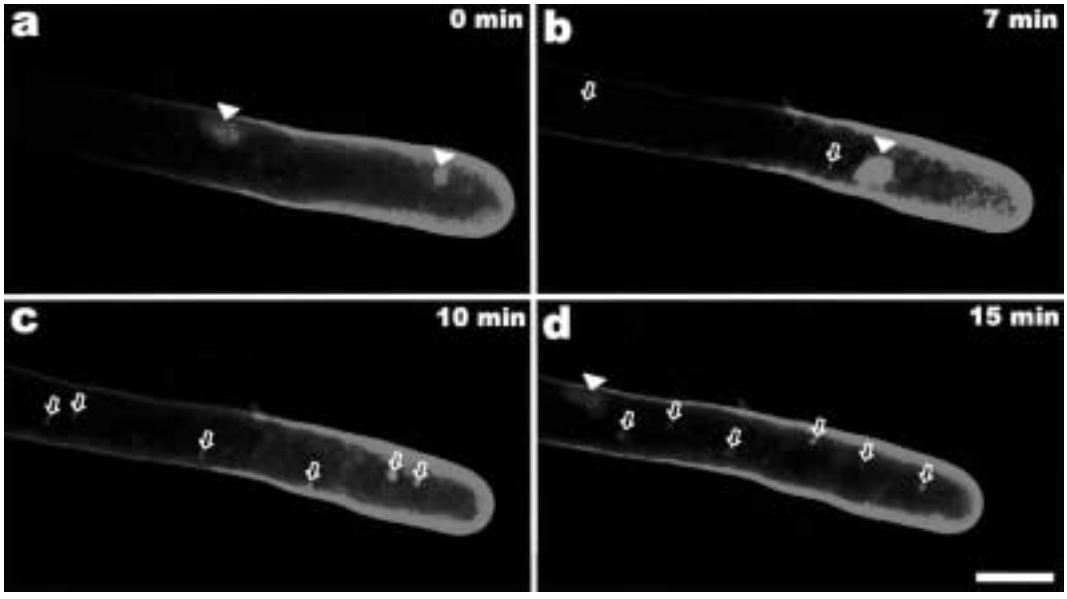


Fig. 9 a–d. BFA-induced agglomeration of vesicles and putative endosomes into large compartments was reversible upon removal of BFA. **a** Root hair of *T. aestivum* treated with a mixture of 35 μM BFA plus 4 μM FM 1-43 for 30 min. Two BFA-induced compartments were present (arrowheads). **b–d** After washing, big BFA compartments (arrowheads) dissociated and small putative endosomes (arrows) were gradually recovered within 7 (**b**), 10 (**c**), and 15 min (**d**) after washing. Bar: 10 μm

that vesicles so frequently jumped back into the cytoplasm after contact with the plasma membrane, thus suggesting a "kiss-and-run" mode of exocytosis.

Rapid uptake of FM-labelled plasma membrane at growing root hairs

For the investigation of endocytosis, we used the well-known endocytic tracer dyes FM 1-43 and FM 4-64 (Bolte et al. 2004), which proved to be useful for visualization of plant endosomes (Ueda et al. 2001, Geldner et al. 2003, Grebe et al. 2003, Voigt et al. 2005a). These dyes have weak fluorescence in aqueous medium, but they shine brightly when inserted into membranes (Betz et al. 1996). So far, they were shown to be membrane impermeative with no other uptake mechanism than endocytosis of membrane vesicles throughout the eukaryotic superkingdom. In tip-growing pollen tubes, they were reported to rapidly label most vesicles of the clear zone (Parton et al. 2001, 2003; Camacho and Malhó 2003). This phenomenon was confirmed also for root hairs (Voigt et al. 2005a, this study).

Labelling of the root hair plasma membrane with FM dyes gave clear evidence of internalization of the plasma membrane and further trafficking to specific endomembrane compartments. First staining appeared very rapidly in the clear zone by endocytic vesicles. Together with this strong signal that appeared rather diffuse, due to the size of the tiny vesicles, brightly stained dotlike structures appeared. These particles moved rapidly within the clear zone and also in the subapical cytoplasmic regions. From their pattern of motility, they closely resembled early endosomes visualized with the GFP-FYVE construct (Voigt et al. 2005a). Within 1 h, the endocytic pathway proceeded further; larger endosome-like compartments of uniform size were marked by both FM dyes. They were excluded from the clear zone, indicating that they no longer participated in membrane recycling to the tip. It seems that these latter structures belong to the late endocytic compartments, possibly MVBs (Tse et al. 2004). Further progress of the endocytic pathway resulted in staining of the largest endocytic compartment, the tonoplast of the central vacuole, and finally ended in bright condensed clumps.

The endocytic compartments and their distribution closely resemble the pictures shown in yeast and animal cells (Vida and Emr 1995, Murkherjee et al. 1999), as well as in plant suspension cells (Emans et al. 2002, Kutsuna and Hasezawa 2002, Kutsuna et al. 2003). In tip-growing systems, staining with FM 4-64 labelled the apical Spitzenkörper in filamentous hyphae (Fischer-Parton et al. 2000, Hoffmann and Mendgen 1998), which is a vesicle-rich structure comparable to the clear zone (Šamaj et al. 2004a). The clear zone of both pollen tubes (Parton et al. 2001, 2003) and root hairs (Voigt et al. 2005a, this study) accumulates these endocytic tracers within the first minutes of incubation. After prolonged expo-

sures of plant cells, FM dyes accumulate at the tonoplast and within the vacuolar lumen.

Ultrastructural analysis of the endocytic pathway with electron-dense markers in plant cells, mainly in isolated protoplasts, has suggested a primary role of clathrin-coated vesicles (Joachim and Robinson 1984, Tanchak et al. 1984) that are further processed by a partially coated reticulum (Pesacreta and Lucas 1985) and through MVBs (Tanchak and Fowke 1987). In rapidly growing tobacco and lily pollen tubes, clathrin-coated endocytic vesicles were observed in the electron microscope mainly at the base of the tip (Derksen et al. 1995, Lancelle and Hepler 1992), and clathrin was distributed over the whole apex (Blackbourn and Jackson 1996). In tip-growing root hairs, clathrin-coated pits and vesicles were distributed evenly over the whole surface of the apical dome (Galway et al. 1997), indicating that endocytosis may occur along the whole tip. This mode of internalization can be directly labelled and monitored by FM dyes.

Recycling vesicles abundant at the tip

Pulse labelling of the plasma membrane with FM dyes gives evidence for recycling of endosomal structures before their final degradation. The growing tip is not just populated by secretory and by endocytic vesicles, as is generally accepted, but in addition endocytic vesicles fuse to form putative early endosomes which are inherently linked with the clear zone of growing root hairs (Voigt et al. 2005a). Together, they accumulate in the tip for more than 20 min after formation. Obviously, they shuttle back to the plasma membrane several times, because fluorescence is transferred to the newly formed plasma membrane even when no dye is in the medium.

The secretory role of such "recycling" vesicles could be performed by a "kiss-and-run" mode of secretion at the plasma membrane (Thiel and Battey 1998); although it is difficult to understand that the short contacts with the membrane could be sufficient for the exchange of membrane and for the transfer of the dye (Parton et al. 2001).

Nature of vesicles filling the clear zone: brefeldin A provides the clue

The most dramatic response of the root hairs to BFA was the formation of BFA-induced compartments (Satiat-Jeunemaitre and Hawes 1993, Baluška et al. 2002, Geldner et al. 2003), similar to growing pollen tubes (Parton et al. 2001, 2003). It is accompanied by a stop of tip growth and by a reduction of the clear zone and a disappearance of the vesicle pool from the tip. These BFA-induced compartments consist of agglomerates of vesicular structures of endosomal origin (Baluška et al. 2002, Geldner et al. 2003, Grebe et al. 2003, Šamaj et al. 2004a, Voigt et al. 2005a). The formation of BFA compartments is usually interpreted as a result of the inhibition of secretion. However, recent studies revealed that BFA affects also the actin cytoskeleton at tips of growing root hairs (Šamaj et al. 2002) and blocks secretion based on putative recycling endosomes (Geldner et al. 2003).

BFA not only inhibited root hair tip growth (Šamaj et al. 2002) but also affected the processes of site selection and root hair initiation (Grebe et al. 2002). Thus, inhibition of root hair initiation and tip growth might be mediated by BFA inhibition of vesicle recycling from putative endosomes. One could, therefore, propose that the aggregation of putative endosomes and endocytic vesicles into the newly formed compartments would be the most apparent reason for the depletion of vesicles from the tip. In the presence of BFA, endocytic vesicles receive the FM marker from the plasma membrane and then transfer the dye into putative endosomes; however, both are effectively trapped within the BFA-induced compartments. New FM-positive endocytic vesicles are still generated and directed to the BFA compartment because plant endocytosis is not inhibited by BFA (Emans et al. 2002).

Endocytic pathway in root hairs does not cross ER

ER is abundant in the apical portion of emerging root hairs (Baluška et al. 2000), and short ER profiles accumulate in the apex and subapical zone of the root hairs as was detected by electron microscopy (Galway et al. 2000, Čiamporová et al. 2003). However, studies of endocytosis in plant cells with FM dyes did not show an involvement of the ER in the endocytic pathway (Robinson et al. 1998, Geldner et al. 2003). In root hairs of *A. thaliana* and of *T. aestivum*, the subapical zone was very rich in ER; it protruded into the basal part of the clear zone, where it was highly dynamic. Formation of endocytic vesicles in the clear zone was independent of ER. Vesicles and the fluorescent putative early endosomes occupying the clear zone were clearly separated from ER, and transfer of membrane-resident dye was never observed between them. This indicates the structural and functional independence of the two important organelles of the endomembrane system.

Rapid endocytosis of plasma membrane needs actin cytoskeleton

Motility of organelles and vesicles, as well as the stability of the apical cytoplasm, are the most important functions of the

cytoskeletal elements actin and myosin in tip-growing systems (for recent reviews, see Hepler et al. 2001; Vidali and Hepler 2001; Šamaj et al. 2004a–c). Growing root hairs possess a highly dynamic, unstable fine actin meshwork and fine bundles of actin filaments in the apical and subapical region (Voigt et al. 2005b). Basal parts of growing root hairs with active cytoplasmic streaming contain thicker actin filaments (Baluška et al. 2000, De Ruiter et al. 2001, Esseling et al. 2000, Ketelaar et al. 2003, Šamaj et al. 2004a).

We probed the function of the actin cytoskeleton in tip-growing root hairs through experiments interfering with the kinetics of F-actin polymerization. Latrunculin B at the rather low concentration of 100 nM interrupted the endocytic pathway. It induced a gradual stop of tip growth and elicited morphological changes as well as modifications of the dynamic properties in the tip. Motility of organelles was reduced and the complex pattern of the movement of vesicles and small endosomes in the clear zone was lost. Slowing down of the vesicles which were in contact with the plasma membrane and tonoplast and long-time decoration of these membranes by small vesicles indicate in addition the involvement of dynamic and highly latrunculin-sensitive F-actin in vesicle docking, vesicle fusion, and endocytic vesicle formation. Moreover, transformation of dynamic tubular vacuoles into a static round vacuole in the subapical region indicates that the motility of the vacuole also depends on F-actin. This corresponds well with observations from root cells, where actin is involved in both the directing of endocytic vesicles into endosomal compartments and the recycling of vesicles back to the plasma membrane (Geldner et al. 2001, Baluška et al. 2002). In tip-growing root hairs, our recent data revealed an essential role of F-actin in driving the motility of endosomes in the clear zone (Voigt et al. 2005a). Depolymerization with cytochalasins or latrunculins inhibits rapidly the hair tip growth (Baluška et al. 2000, 2001), and this effect is concentration dependent. Low concentrations of latrunculins target specifically the fine and dynamic F-actin meshworks in the apical and subapical region; tip growth is arrested, but cytoplasmic streaming is unaffected (Gibbon et al. 1999, Ketelaar et al. 2003).

All these observations indicate that the actin cytoskeleton is indispensable for vesicular trafficking needed for tip growth, i.e., for targeted delivery and localized recycling of vesicles. However, the mechanistic understanding of the functions of the actin cytoskeleton in the organization of the clear zone (Šamaj et al. 2004a, b) and in driving tip growth (Voigt et al. 2005a, b) requires further experimental studies. A promising approach for attaining new information is live cell imaging of the actin cytoskeleton in growing root hairs of transgenic *A. thaliana* lines expressing GFP-tagged actin binding proteins (Sheahan et al. 2004, Wang et al. 2004, Voigt et al. 2005b).

Conclusions

Endocytosis of FM-labelled plasma membrane occurs in root hairs during tip growth. Endocytic vesicles cohabit the clear zone of the tip together with other kinds of vesicles and endosome-like structures. Our results support the existence of vesicles in the tip of growing root hairs, which are probably responsible not only for uptake of the FM dyes from the plasma membrane and direct delivery of the dye to putative early endosomes but also for recycling of the dye back to the plasma membrane. This mode of action could indicate a "recycling" nature of vesicles in the tip. Subsequent degradation via MVBs ends in the vacuole but does not involve the ER.

Acknowledgments

We thank Gregor Eder for technical assistance. This work was supported by the European Commission, Research Project of Human Potential Programme TIPNET (HPRN-CT-2002-00265), and by grant nr. 2/5085/25 from the Grant Agency VEGA.

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