Spatial organisation of cell expansion by the cytoskeleton

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Cover: The diameter of the growing tip of *Arabidopsis* root hairs increases after application of low concentrations of the actin depolymerising drug cytochalasin D. The time interval between sequential images is 30 seconds.

Contents

Background

Why study plant cells?

Cells are the building stones of flowering plants, and the shape of a plant is determined by the number of cells, their size, shape and organisation. Plant cells do not move within the cell body because in contrast to animal cells, virtually all plant cells possess cell walls. These mainly polysaccharide structures are made by the plant cell and surround it completely. Since plant cells do not move within the plant tissue, plant development is the accumulation of site, amount and direction of division and growth of single cells. Therefore, the morphogenic plan of all single cells together and in co-operation determines the morphogenesis of a tissue or a complete plant.

The final cell size and shape is determined by cell growth, but the number of cells, required to form an organ is determined at the location where cell division takes place, which are the meristems. Division takes place in a highly organised way so that the total amount of cells that is going to form an organ is being made and the right number of cells will be present at the right locations. After the production of cells, cell expansion takes place. The amount and direction of cell expansion determines the shape and size of organs and eventually the shape of the plant.

The aim of this thesis is to gain insight in how individual cells control the direction of expansion and more specifically, how the cytoskeleton is involved in the control of directionality of cell expansion. The research, presented in this thesis, contributes to a better understanding of the morphogenesis of flowering plants. Once one understands morphogenesis of flowering plants better, one will better be able to optimise it, so that for example in agricultural crops a better yield or a more desirable crop shape can be obtained.

Why study root hairs and pollen tubes?

Plant cell expansion can take place in all cell facets, but is mostly restricted to the walls longitudinal to the plant organ. This type of polar growth is referred to as intercalary or diffuse growth. Cell extension can be localised also to one side of the cell only, referred to as tip growth. Most plant cells expand intercalary. In intercalary

growing cell types, some facets can expand more than others, giving raise to rectangular shaped cells. In that case intercalary growth is polar growth. Tip growth is always polar growth. Examples of tip growing plant cells are root hairs and pollen tubes.

Root hairs are elongate, tubular structures, emerging from certain epidermal cells, the trichoblasts. In *Arabidopsis*, they can reach lengths of over 1mm. In some plants, like *Vicia sativa* and *Medicago truncatula*, all epidermal cells form a root hair and therefore are trichoblasts. In *Arabidopsis*, however, every one in two or tree epidermal cell files normally forms a root hair.

For several reasons, tip-growing cells are an ideal system to study cell expansion. Firstly, the localised growth occurs robustly at one site. Therefore the whole growth machinery is abundantly present. This allows us to compare characteristics of areas where growth takes place and fully-grown areas within one cell. Localised growth implies that all material required for growth has to be present at or delivered to the surface area where cell expansion takes place. This allows us to study the transport towards the area where growth takes place. Furthermore, the growth speed of the expanding cell surface area in a tip growing cell is much higher than the growth speed of a cell that distributes the growth machinery more or less equally over its surface. This allows one to observe changes in growth speed and direction more easily.

How does a plant cell grow?

Plant cell growth is an irreversible increase in cell volume, mainly by water uptake in the central vacuole. Enlargement of cell volume requires increase of plasma membrane and cell wall, and is a finely tuned process. For plant cell growth, cell wall matrix material containing exocytotic vesicles fuse with the plasma membrane and deposit their contents into the existing cell wall. The result is an increase in cell wall volume and an increase in plasma membrane area. This thesis gives insight in how the cell determines where this fusion takes place and thus controls the area where cell growth takes place. Whether by this cell wall deposition process the cell thickens or expands depends on the expandibility of the existing cell wall and the presence of a force that stretches it. Tip growing root hairs of *Arabidopsis* were used as a model

system, and in some occasions, the findings were tested in *Arabidopsis* pollen tubes, the intercalary growing cells of *Tradescantia virginia* stamen hairs and fission yeast.

The cytoskeleton

The cytoskeleton is a complex network of protein filaments, extending through the cytoplasm of cells. These protein filaments can be highly dynamic and reorganise continuously when the cell shape changes or in response to environmental cues. The cytoskeleton in plant cells can be divided in 2 major components: microtubules and actin filaments. The organisation and the dynamics of both components are dependent on proteins that bind or interact with them, the microtubule associated proteins and the actin binding proteins.

Microtubules are hollow tubes with a diameter of 25 nm, consisting of 13 protofilaments, build by polymerisation of α- and β-tubulin dimers, the α-tubulin of one dimer binding to the β-tubulin of the other dimer. This property makes microtubules polar structures, with a plus and a minus end, both ends growing at different speeds. The β-tubulin side of the dimers are localised in the direction of the plus end. Microtubules grow by a process called dynamic instability. When GTP (guanosine 5'-triphospate) is bound to tubulin, the critical concentration of tubulin required for polymerisation is much lower then when a GDP (guanosine 5'-diphospate) group is bound. After incorporation in a microtubule of GTP-tubulin, the GTP is hydrolysed within seconds into GDP. As long as the polymerisation speed exceeds the GTP hydrolysis speed and thus the microtubule has a cap of GTP-tubulin, the microtubule grows. This GTP side is the plus side. GDP-tubulin easily dissociates from microtubule free ends. Thus at the minus side, the microtubule constantly disintegrates. When the polymerisation speed is lower than the speed of GTP-hydrolysis in the microtubule, the cap of GTP-tubulin disappears, causing the GDP—tubulin dimers at the top of the microtubule to dissociate and the microtubule to rapidly depolymerise, called a catastrophy.

In plant cells, microtubules are organised at specific locations and involved in specific processes. In interphase cells, microtubules localise to the cell cortex and in some cell types in the perinuclear region. Just before mitosis, microtubules reorganise into the preprophase band, which is localised at the location where the cell plate, which is the new cell wall between daughter cells, will be formed after mitosis. During

karyokinesis, the mirotubules localise in the spindle, which divides the duplicated chromatin into 2 identical sets of chromosomes. After nuclear division, cell division is completed by phragmoplast formation. Microtubules localise to and are involved in phragmoplast formation. The phragmoplast microtubules are involved in the building of the cell plate.
Different microtubule associated proteins are involved in organising the microtubules during the different phases of the cell cycle. Besides, they are involved upstream and downstream in performing microtubule-regulated processes.

Actin filaments (also called F-actin) are a 6 nm wide, tight helix of uniformly oriented actin molecules, formed by polymerisation of monomeric actin (G-actin). Actin filaments are, like microtubules, dynamic and they are polar structures with a relatively slow growing pointed end and a more rapidly growing barbed end. Actin filaments are thinner and more flexible than microtubules. Actin polymerisation is an ATP (adenosine 5'-triphospate)-dependent process. ATP is bound to actin monomers and hydrolysed when the actin monomer is incorporated into a filament.
Actin filaments localise throughout the cytoplasm of plant cells. They are essential for the cytoplasmic organisation. Furthermore they are present in all cytoskeleton configurations described above for microtubules, the cortical array, the preprophase band, the spindle and the phragmoplast. Different actin binding proteins can change the dynamics of actin, are involved in organising the actin cytoskeleton or use the actin cytoskeleton as scaffold. For example, cytoplasmic streaming is the effect of ATP-dependent movement of the actin binding motor protein myosin, linked to organelles, over actin filaments.
In the different chapters of the thesis, specific functions of the cytoskeleton will be discussed in dept.

Outline of this thesis

In this thesis, I present data that give insight in the role of the cytoskeleton in regulating and facilitating plant cell growth.

In chapter 1, we demonstrate that the actin cytoskeleton plays an important role in specifying the cell surface area where Golgi-derived vesicle fusion can take place

with the plasma membrane, leading to cell expansion. Until now, in plant cell growth, the actin cytoskeleton has only been hypothesized to deliver and retain the Golgi-derived vesicles at the site where exocytosis takes place. The novel finding in this thesis is that very low concentrations of actin depolymerising drugs lead to an increase of the area, free of detectable filamentous actin in the apical zone. This increase correlates with the area where cell expansion takes place in root hairs. Local application of actin depolymerizing drugs onto intercalary growing cells also leads to a local decrease of stability of filamentous actin, correlating with an increase in cell expansion rate in this area, demonstrating that a dynamic (a high amount of (de-)polymerisation) and unstable actin cytoskeleton is required for cell growth more generally in plant cells. Also in tip growing fission yeast, application of low concentrations of actin depolymerising drugs leads to induction of exocytosis.

In chapter 2, we use transmission electron microscope images from median sections through domes of root hairs and pollen tubes, combined with video analysis of the growth speed, to make estimations about exo- and endocytosis rates. By estimating the size and number of exocytotic vesicles and using the obtained values, we theoretically model the exocytosis and endocytosis processes, leading to estimations of the amount of exocytotic vesicles that is being consumed per time-interval. We compared root hairs and pollen tubes of *Arabidopsis* and observed striking differences in the amount of exocytotic vesicles present in the dome, the size of exocytotic vesicles and the amount of vesicle insertion into the plasma membrane per time interval. In growing pollen tubes, we confirmed the estimated number of exocytotic vesicles present in the dome, waiting for consumption by exocytosis, experimentally by inhibiting the delivery of newly produced vesicles to the dome and measuring the time that growth continues. We demonstrate that the estimated value, being 33 seconds and the experimentally obtained value, being 30-40 seconds are comparable.

In tip growing plant cells, the nucleus is always at a distinct distance from the growing tip. This observation has been made for the first time by Haberlandt in 1887. In chapter 3 we present data obtained by using specific cytoskeleton drugs and demonstrate that the positioning of the nucleus in *Arabidopsis* root hairs is an actin regulated process. Besides that, we show by using an optical trap, that proper

positioning is essential for cell growth. Injection of a antibody against plant villin, an actin bundling protein, causes movement of the nucleus towards the tip, demonstrating that a gradient in actin bundling is responsible for maintaining a minimal distance from the growing tip.

Chapters 4 and 5 are reviews. In chapter 4, the organisation and possible functions of microtubules in root hairs are discussed. In Chapter 5, firstly, we discuss different fixation techniques and their consequences for maintenance of correct protein localisation. Secondly, we extrapolate data about the actin and microtubule cytoskeleton and the cytosolic calcium concentration, obtained from root hairs (partially the research presented in this thesis) to intercalary expanding cells as a general discussion.

Chapter 1

Unstable F-actin specifies area and microtubules direction of cell expansion in tip- and intercalary growing cells

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Abstract

Plant cells expand by exocytosis of wall material contained in Golgi-derived vesicles. We examined the role of local instability of the actin cytoskeleton in specifying the exocytosis site. During root hair growth, a specific actin cytoskeleton configuration is present in the cell's sub-apex consisting of fine bundles of actin filaments that become more and more fine towards the apex, where they may be absent (Miller et al. 1999, de Ruijter et al. 1999). Pulse application of low concentrations of the actin depolymerizing drugs cytochalasin D (CD) and latrunculin A (LA) broadened growing root hair tips; i.e. they increased the area of cell expansion, comparable to what has been observed in neuronal cells (Bradke and Dotti (1999). In line with these results, a local application of CD on one side of the dome of a growing hair led to a local swelling at that location. Moreover, CD applied locally onto intercalary growing *Tradescantia* stamen hair cells induced a local outgrowth at the site of application. Addition of CD or LA to a tip-growing fission yeast culture led to an increase in cell width, again implying an unstable actin cytoskeleton to determine the location of exocytosis. Interestingly, recovery from CD led to new growth in the original growth direction, whereas in the presence of oryzalin, a microtubule depolymerizing drug, this direction was altered. Oryzalin alone, in the same concentration, had no influence on root hair elongation. The results are an important step towards understanding the spatial and directional regulation of plant cell growth.

Introduction

Plant cells grow when Golgi-derived vesicles fuse with the plasma membrane, while inserting cellulose synthases (Kimura et al., 1999) into it (Emons and Mulder, 2000) and at the same time deliver cell wall matrix into a flexible cell wall under turgor pressure (Cosgrove, 1997). Root hairs grow at one site, the tip, only. What are the roles of actin filaments and microtubules in specifying growth? Still, the answer remains elusive. In root hairs, a cytoskeleton configuration of subapical, net-axial fine bundles of actin filaments together with a tip area free of detectable actin filaments correlates with root hair tip growth (*Vicia sativa* Miller et al., 1999, *Arabidopsis* Ketelaar et al., 2002). In fully-grown hairs, this typical actin configuration is not present, but in hairs that are terminating growth, it can be re-induced within 3 minutes by a signal molecule (bacterial Nod factor) (De Ruijter et al., 1999). This type of actin configuration is hypothesized to deliver Golgi-derived vesicles to the apical area (Miller et al., 1999; De Ruijter et al., 1999). Recently, cortical fine F-actin has been described to occur also in intercalary growing *Arabidopsis* cells at the site of and correlating with early stages of cell expansion (Fu et al., 2002).

In the *V. sativa* root hairs, the subapical fine bundles of actin filaments, further called fine F-actin is more sensitive to the actin depolymerizing drug cytochalasin D (CD) than the thicker bundles of actin filaments further down in the root hair tube (Miller et al., 1999).

Repeated CD application every 10 minutes led to complete disappearance of the fine F-actin and growth stopped, whereas bundles of actin filaments remained present in the hair tube where cytoplasmic streaming remained normal (Miller et al., 1999). And, indeed, cultured in the actin depolymerizing drug Latrunculin A, plant cell growth is inhibited (Baluška et al., 2001). In the setting up of tip-elongation in axons, low concentrations of actin destabilizing drugs stimulated tip growth (Bradke and Dotti, 1999), and in pancreatic acer cells depolymerization of cortical actin triggered exocytosis (Muallem et al., 1995; Valentijn et al., 1999). In the present study, we investigate whether a local increase in instability (i.e. turnover) of cortical actin can trigger the exocytosis process by which plant cells grow. Therefore, we studied the reaction of *Arabidopsis* root hairs to the actin depolymerizing drugs CD and LA. To investigate our hypothesis for intercalary growing cells, we studied the reaction of growing *Tradescantia* stamen hair cells to local application of CD. The results show

that local increase in instability of the actin cytoskeleton can induce local cell expansion. To test whether actin instability plays a role in determining the location of exocytosis in other cells than plant (this paper) and animal (Bradke and Dotti, 1999, Muallem et al., 1995; Valentijn et al., 1999), we cultured the tip growing yeast *Schizosaccharomyces pombe* in low concentrations of CD and LA. Also in these cells cell expansion took place over a larger area, leading to cells with a wider diameter indicating that actin instability is a conserved mechanism for specifying the area of cell expansion. We further show that the orientation of polarized growth depends on an intact microtubule cytoskeleton, as is known for intercalary growing cells. Altogether, the results re-estimate the widely held view that tip growth is actin based and intercalary growth depends on microtubules (Kropf et al. 1998)

Results

The actin filament configuration in root hairs was studied by 2 different methods: rapid freeze fixation, followed by immunodetection of actin, and phallotoxin staining after an optimized fixation procedure (EAC: ester-aldehyde-choline, Emons and de Ruijter 2000, first used by Miller et al. 1999). Rapid freeze fixation and freeze substitution was followed by treatment with cell wall degrading enzymes to facilitate antibody penetration for immuno-localisation. We used antibodies, because after rapid freeze fixation and freeze substitution, the reactivity of filamentous actin to fluorescently labeled phalloidins was lost for unknown reasons. Both methods gave comparable results, although the immuno-cytochemistry technique after freeze-fixation suffered from the fact that details of the fine F-actin in the sub-apex were obscured, probably by antibody attached to actin monomers (fig 1A), also reported by Miller et al. (1999). The EAC staining method gave a better resolving power, especially in the subapical area of growing root hairs (partial projections: fig 1B-D, refection image: fig 1E, complete projection: fig 1F, deconvoluted complete projection of confocal images fig 1G). In both growing and fully-grown *Arabidopsis* root hairs (Ketelaar et al., 2002) the general configuration of the actin cytoskeleton is the same as described for *V. sativa* (Miller et al., 1999). Growing hairs have a tip area free of detectable bundles of actin filaments, subapical net-axially aligned fine F-

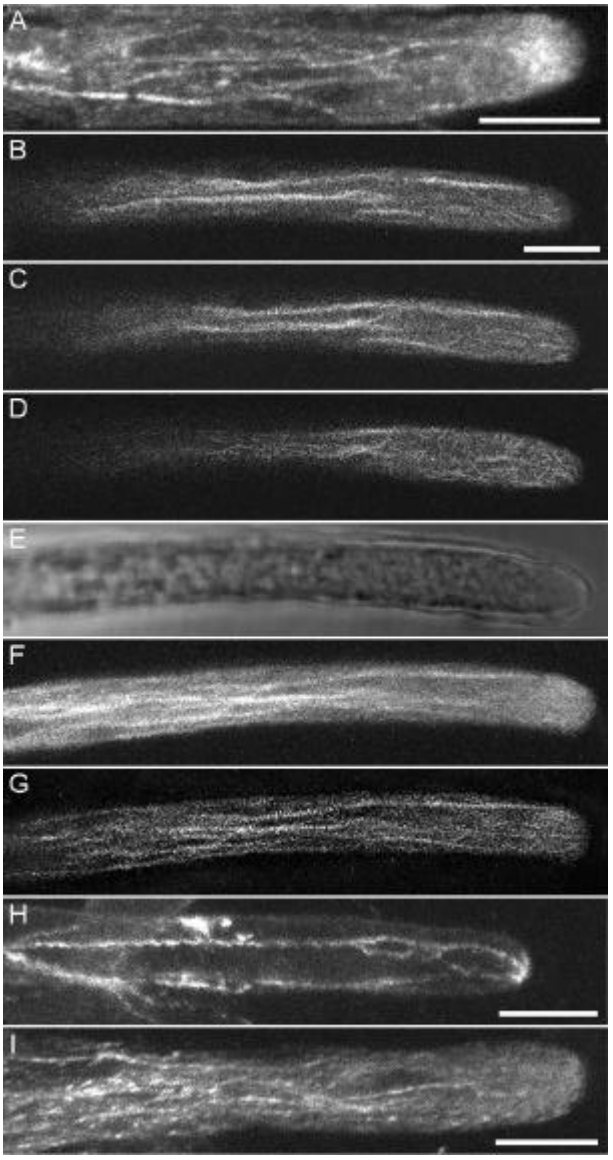

Figure 1. Actin visualisation in *Arabidopsis* root hairs.
1A is an immunofluorescence image of a whole mount, freeze fixed growing *Arabidopsis* root hair, labeled with anti actin antibodies. 1B-G are root hairs, wherein the actin cytoskeleton is visualised by the improved EAC method. B-D are projections of several images from a confocal Z-stack through a whole growing root hair (B and C median and D cortical), E is a reflection image of the root hair and F and G are projections of the whole confocal Z-stack; F without deconvolution and G after deconvolution. A and G show typical images of the actin cytoskeleton in a growing root hair: subapical fine F-actin and thicker bundles of actin filaments in the basal part of the cell. In fully-grown root hairs, actin filaments loop through the tip (H). 5 minutes treatment with 0.1μM CD gives a decrease in fluorescence in the (sub-)apical area whereas the thicker bundles of F-actin towards the root hair base remain intact (I). Bar = 10μm.

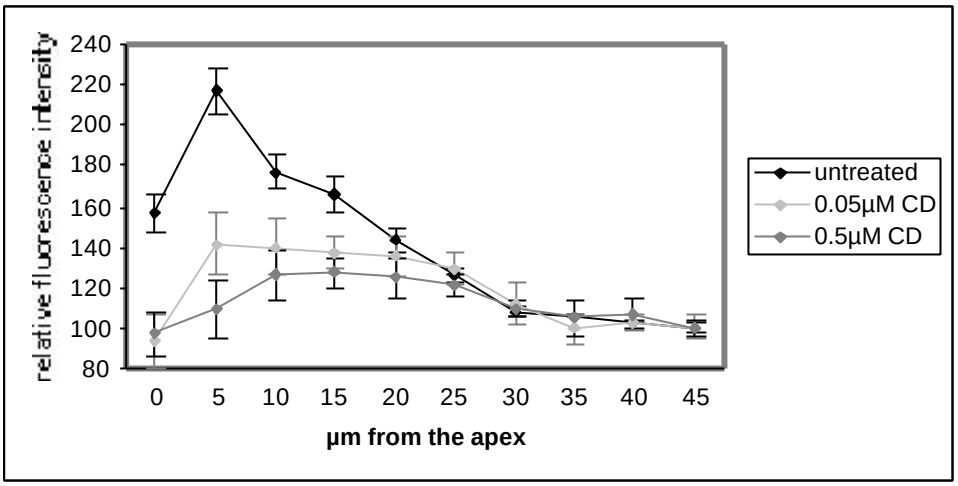

Figure 2. Relative fluorescence intensity plot of an actin immunolabeling, 5 minutes after CD treatment.
The mean intensity of 5µm thick transverse blocks, outlining the root hair shape was measured. The intensity of the block 45-50µm from the tip was set to 100 and the error bars display standard deviations (n=5 per treatment). Application of CD leads to significant decrease in the intensity of actin labeling in the apical area in comparison to the untreated control. The cytoarchitecture of the root hair does not change significantly at the concentrations of CD that were used, therefor the decrease in fluorescence in the apical area cannot be contributed to increased vacuolization.

actin, and thicker bundles of actin filaments basal to the subapical area (fig 1). In fully-grown hairs only the latter are present and loop through the tip (fig 1H). There is debate about the presence of filamentous actin in the apical area of growing root hairs. The expression of GFP-fused to the actin binding site of animal talin (Kost et al., 1998), which binds to actin filaments was studied in *Arabidopsis* root hairs (Baluška et al., 2000). These authors observed fluorescence in the apex of growing root hairs. However, this fluorescence was not localized in filamentous structures, which could be due to insufficient time resolution of our present microscopes for the highly dynamic actin cytoskeleton. Whether there are very dynamic actin filaments with a high turnover rate or no actin filaments at all in the extreme apex of growing root hairs remains to be proven.
In all tested growing root hairs (n=5 per treatment)), a 5 minutes treatment with 0.05 to 0.5 µM CD or 0.01 µM LA caused a decrease in actin fluorescence (fig 1I and 2). This figure shows that the fluorescence intensity of the fine F-actin after CD or LA

treatment is lower than in control cells. CD treatment had no visible effect on cytoplasmic streaming in the basal part of root hairs of these species, as was shown for *V. sativa,* and at this low concentration did not stop cell expansion.
The higher sensitivity of the fine F-actin to a brief treatment with CD or LA, shows that it is less stable than the thicker bundles of actin filaments in the basal part of a growing hair or the actin filament bundles in a fully-grown hair. Thus, the actin cytoskeleton is more unstable at the location where growth takes place.

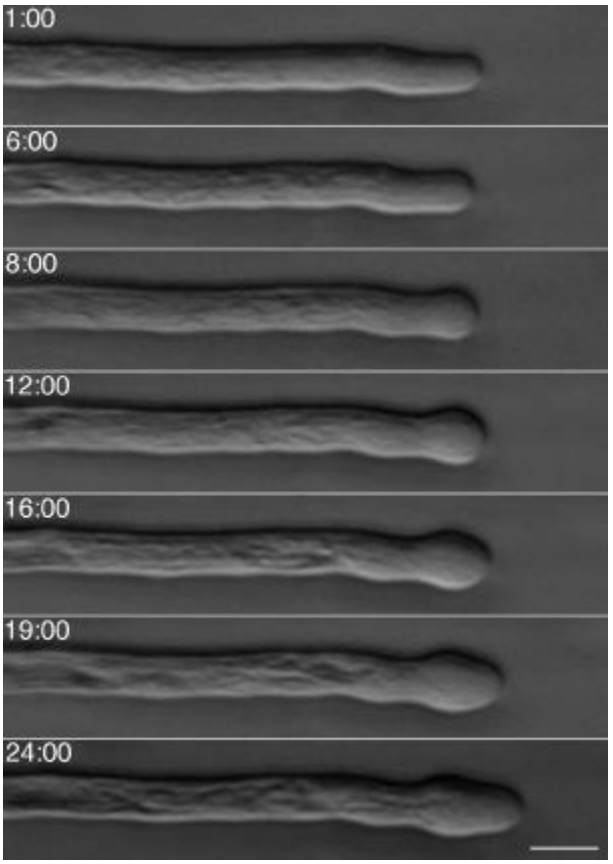

Figure 3. The response of a growing *Arabidpsis* root hair to application of 0.5µM CD.
6 minutes after application, tips of growing root hairs appear more blunt, whereas after 8 minutes, an increase in root hair width can be observed at the growing tip. The diameter of cells continues to increase until around 20 minutes after application, whereafter cells continued to grow with this wider diameter. Bar = 10µm.

To examine changes in the area of cell expansion, we added 0.01-1µM CD or 1nM-10nM LA in medium to roots with living hairs and followed hair growth in time. At 0.5µM CD, we measured the surface expansion rate of growing root hairs in time-lapse recordings from 10 minutes before to 10 minutes after application of CD to the medium. During this time interval, the growth speed after CD application was 1.03 ±

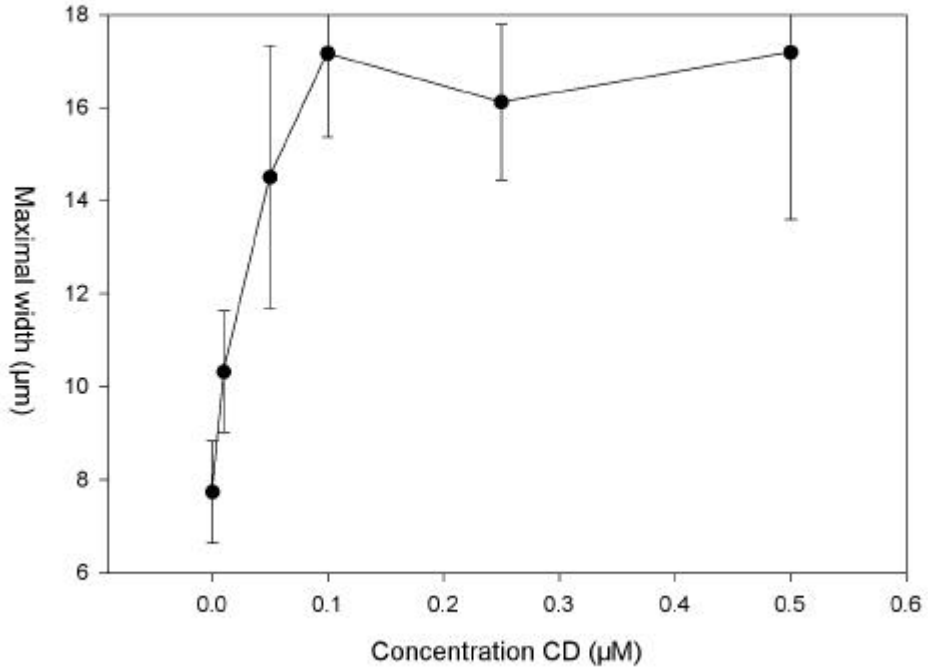

Figure 4. Maximal *Arabidopsis* root hair diameter after application of cytochalasin D (n>20 per concentration, error-bars are standard deviations).

0.10 times the growth speed before application, indicating that the insertion of Golgi-derived vesicles into the plasma membrane of the apical area was not disturbed by the drug treatment. Around 6 minutes after application, tips of growing root hairs became blunt and after 8 minutes, an increase in root hair width was observed at the tips where growth took place (fig 3). At 0.5µM CD, the diameter of cells continued to increase until around 20 minutes after application, thereafter cells continued to grow with this wider diameter for about 45 minutes. At low concentrations (=0.1µM CD or =10nM LA), the diameter of the swelling was concentration-dependent; cell tips reached their maximum width at 0.1µM CD (fig 4). After removal of the CD, root hairs continued to grow and the diameter of the growing tips decreased to normal values (fig 5A). LA treatment (0.1µM) produced a similar increase in tip width in growing root hairs, but root hairs did not recover to normal growth after removal of LA and continued to grow

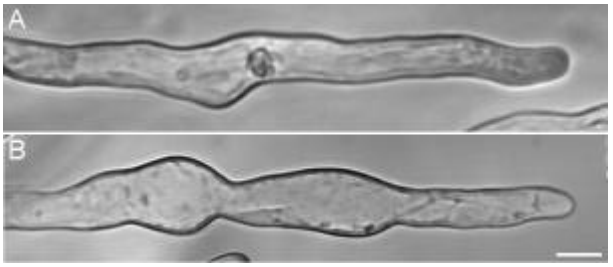

Figure 5. Recovery of root hairs after CD and LA washout.
After washing out CD, root hair growth continues, but the diameter decreases to values as observed in controls (5A). After LA washout, the diameter of growing root hairs remains variable during at least 1 hour after washout (5B). Bar = 5µm.

with irregular diameters (fig 5B). When CD or LA were given repeatedly at 10 minutes intervals, as in *V. sativa* (Miller et al., 1999), the area with fine F-actin shrunk in length and disappeared and tip growth stopped, whereas cytoplasmic streaming continued. Together with the new results, these data show that fine F-actin is needed for vesicle delivery, but not for their insertion into the plasma membrane. At still higher concentrations of CD (>50µM) or LA (>0.5µM) cytoplasmic streaming was arrested in all root hairs and growth was inhibited in growing hairs, caused by complete depolymerization of all filamentous actin as previously reported in root hairs of maize (Baluška et al., 2000) and other cells (Baluška et al., 2001).

In *Saccharomyces cerevisiae* and mammalian cells, changes in endocytosis have been observed after actin depolymerization (Engqvist-Goldstein et al., 1999). In growing root hairs, endocytosis takes place abundantly in the subapical area (Emons and Traas, 1986). Changes in the amount of endocytosis would either lead to disturbance of the equilibrium between exo- and endocytosis or to a change in growth speed. If the equilibrium between exo- and endocytosis would be disturbed, one should be able to observe excess or shortage of inserted plasma membrane, which we do not see. If the amounts of both endocytosis and exocytosis would be similarly affected, one would observe changes in growth speed. We have shown that this is not the case at the concentrations of drugs we used by measuring the growth speed (see above).

Although we have shown that the growth speed during treatment with this low concentration of CD or LA does not change, there may be changes in the area where

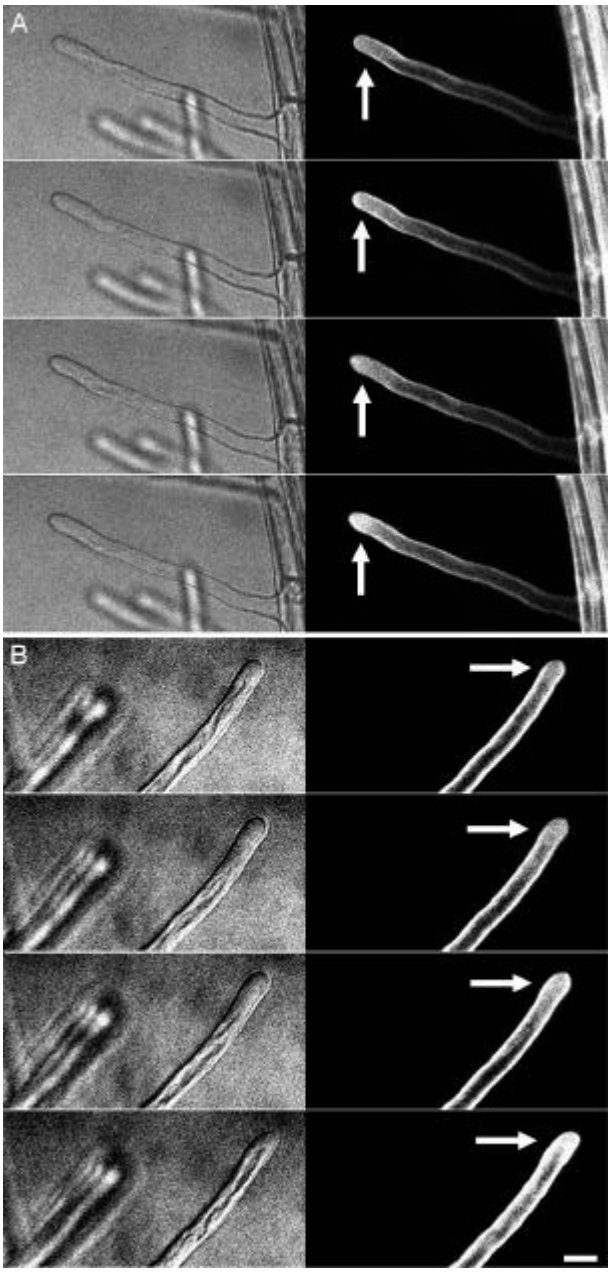

Figure 6. Loading of *Arabidopsis* root hairs with the amphiphilic dye FM4-64. Images were collected on a confocal microscope with completely opened pinhole at 0, 2.5, 5 and 7.5 minutes after application. The right side of separate images shows the fluorescent FM4-64 loading and the left side the reflection image. Image sequence A displays the loading of a control root hair with 2 μM FM4-64. Image sequence B shows a root hair pretreated 1 minute with 0.5μM CD and loaded with 2 μM FM4-64 in the presence of CD. In both sequences, dye loading and imaging did not inhibit root hair growth. Comparison of A and B demonstrates that endocytosis is not inhibited by 0.5μM CD treatment. Bar = 10μm.

endocytosis takes place, leading to changes in the area where exocytosis takes place, and thus a change in cell shape (i.e. an increase in diameter). To test this hypothesis, we loaded Arabidopsis root hairs with 2µM of the amphiphilic styryl dye FM4-64, which is internalized by endocytosis (Fisher-Parton et al., 2000; similar amphiphilic styryl dye FM 1-43: Emans et al., 2002). The loading of root hairs with this dye, pretreated and grown in 0.5µM CD, was not inhibited as expected. In addition, FM4-64 loading did not reveal significant changes in the cellular distribution of the dye (fig 6). We conclude that endocytosis is not inhibited and takes place over a similar area of plasma membrane during drug treatments as in control cells.

Summarizing these results, we observe that a mild treatment with actin filament depolymerizing drugs induces a wider root hair tip. We conclude that a local increase of instability of filamentous actin promotes exocytosis over an expanded cell area.

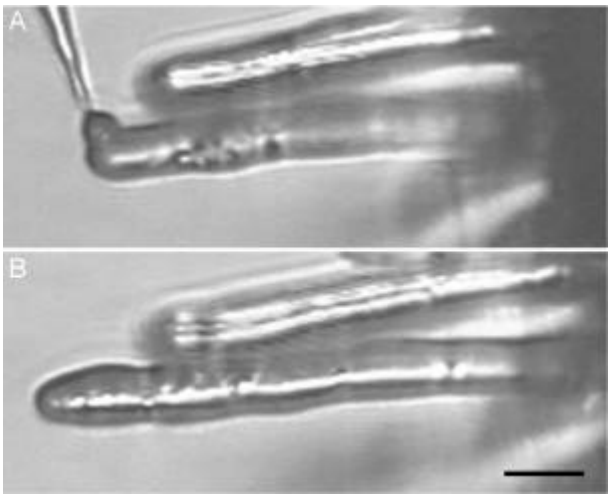

Figure 7. Local application of CD at one side of the dome of growing *Medicago truncatula* root hairs.
A small drop of 0.1 µM CD in water was applied with a microinjection needle on an air-grown root hair (A, n=5). After one hour, a local widening at the site of application had formed (B). Controls, treated with water and 0.1% DMSO did not form a local outgrowth. Bar = 20µm.

To prove that a cell area with unstable actin filaments determines the area of exocytosis, we locally applied 0.1µM CD on the dome of air grown *Medicago truncatula* root hairs. Minutes after application, a local swelling appeared at the site of CD application, showing that only at the location where CD is applied the root hair

width increases (fig 7 and 8, n=5). Controls (local application of water + 0.1% DMSO, the CD solvent; n=4) did not lead to local swelling.

Application of actin depolymerizing drugs did lead to an increase in root hair width, but did not affect root hair growth direction during recovery (fig 4 and 5). If, as suggested by the work of Bibikova et al. (1999), microtubules determine the orientation of root hair growth, the growth direction should change during and after CD treatment in the absence of microtubules. Destruction of the fine F-actin by a CD pulse in the absence of microtubules should lead to root hair tip growth recovery to the original root hair diameter in a random direction. To test this hypothesis, we first tested the effect of oryzalin on root hairs. Oryzalin is a dinitroaniline herbicide that specifically depolymerizes the microtubule cytoskeleton of plant cells (Anthony et al., 1998). 1µM oryzalin had a profound effect on root growth, namely a complete loss of anisotropic growth, as has already been shown by Baskin et al. (1994), but it had hardly any effect on root hair tip growth. We observed a slight waviness of the root hairs (fig 9A) as has also been reported by Bibikova et al. (1999). We then applied 1µM CD to roots in the absence and in the presence of oryzalin for 30 minutes. In the absence of oryzalin, the root hairs continued to grow in the same direction (|7.2 ± 6.4|° deviation ± SD) (fig 9A). However, in the presence of oryzalin, in which no microtubules are present anymore (fig 9B-C), recovery to the original root hair width after 30 minutes CD application took place in a random orientation from the hemisphere (|90.0 ± 24.5|° deviation ± SD) (fig 9D-E). We conclude that although microtubules do not play a role in tip growth itself, they act as a scaffold that imposes the direction of growth during recovery of disturbance of the growth machinery; see cartoon figure 10. Possibly a similar mechanism is involved as has been suggested for neural growth cones, where polymerizing microtubules activate site directed actin filament assembly (Rochlin et al., 1999).

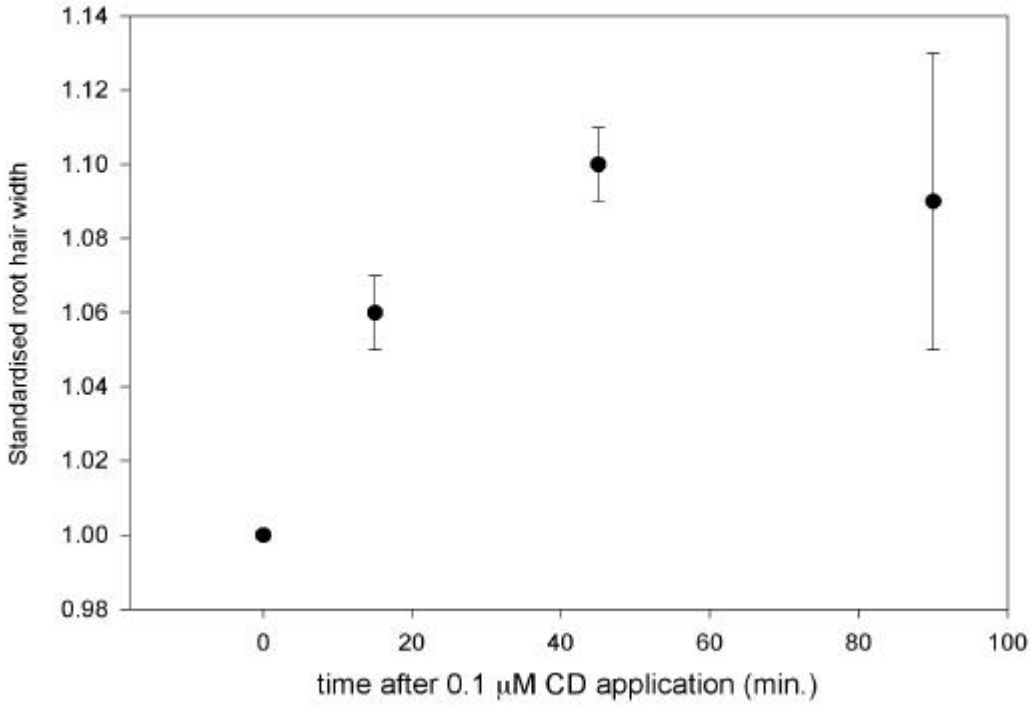

Figure 8. Increase in root hair width at the site of local CD application.
Root hair width was standardized by measuring the width of untreated hairs 10 to 60 µm from the tip in 10 µm intervals and using the mean value as reference for the same measurements 15, 45 and 90 minutes after local application of 0.1µM CD in water at one side of the dome. Since swelling only takes place at the side of application, it is responsible for the complete increase in root hair width. The error-bars are standard deviations (n=5).

To test whether increasing the local instability of actin is sufficient to induce local growth also in an intercalary growing cell type, we applied CD locally on growing *Tradescantia* stamen hair cells. We choose *Tradescantia* stamen hair cells because they are relatively large, growing cells that can be used easily for single cell manipulation. 10 mM CD in DMSO was diluted in 50% w/v Arabic gum to a final concentration of 100 µM. This concentration is high in comparison to the concentrations, as applied to root hairs. However, one should realize that the cell wall properties of the *Tradescantia* stamen hair cells may be different (e.g. walls of these

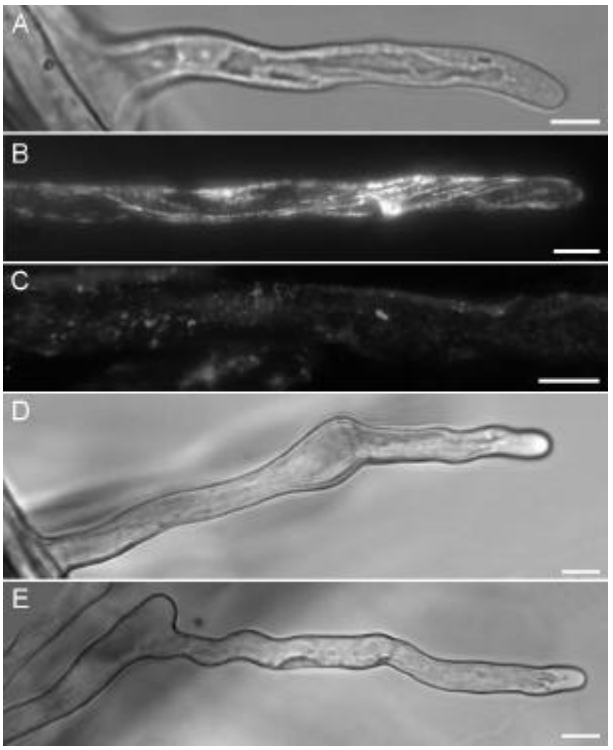

Figure 9. Combined microtubule and actin drug treatment in *Arabidopsis* root hairs. Permanent oryzalin treatment does not change root hair orientation and causes a slight waviness (A). Freeze fixation and immunolocalisation of the microtubules in Arabidopsis root hairs shows that the orientation of the microtubules is net-axial (B) and that the microtubules are depolymerized after 5 minutes 1µM oryzalin treatment (C). When CD is applied in the presence of oryzalin, a 30 minutes 1µM CD pulse leads to an increase in diameter of growing root hairs, as expected, but to recovery of growing tips with normal diameter, now in a random orientation (D-E). Bars = 10µm.

cells contain cutin) and that the time-scale of the experiments is different. It takes hours for a *Tradescantia* stamen hair cell to grow a detectable amount; a time wherein CD breakdown or metabolisation can take place. With a microinjection needle the droplets were placed on cells that were in the developmental stage of elongation growth. Images were taken directly after application (fig 11A) and after 8 hours incubation at room temperature. After 8 hours, phase-dense material had collected at the site of CD application and a local outgrowth could be observed at that site (fig 11B-C). Control cells, only treated locally with Arabic gum and 1% DMSO did not form such outgrowths, showing that the induction of a local outgrowth is a specific

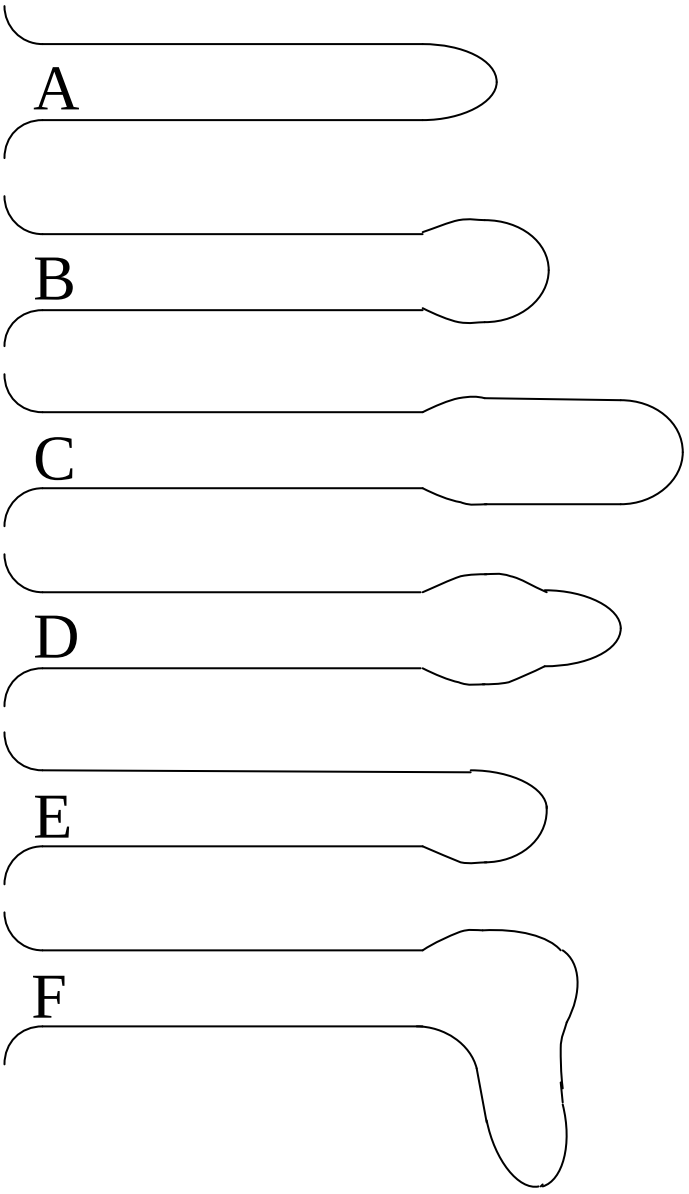

Figure 10. Cartoon showing the effects of CD and oryzalin treatments on growing root hairs. A normal growing root hair (A) responds to treatment with CD by an increase in exocytosis area at the tip (B). After prolonged treatment, the root hair will continue to grow in this wider diameter (C). After removal of the CD, root hairs respond by recovering to their normal width in the original growth orientation (D). Local application of CD leads to a local swelling (E). A CD pulse, in the absence of microtubules, leads to recovery in a random orientation (F).

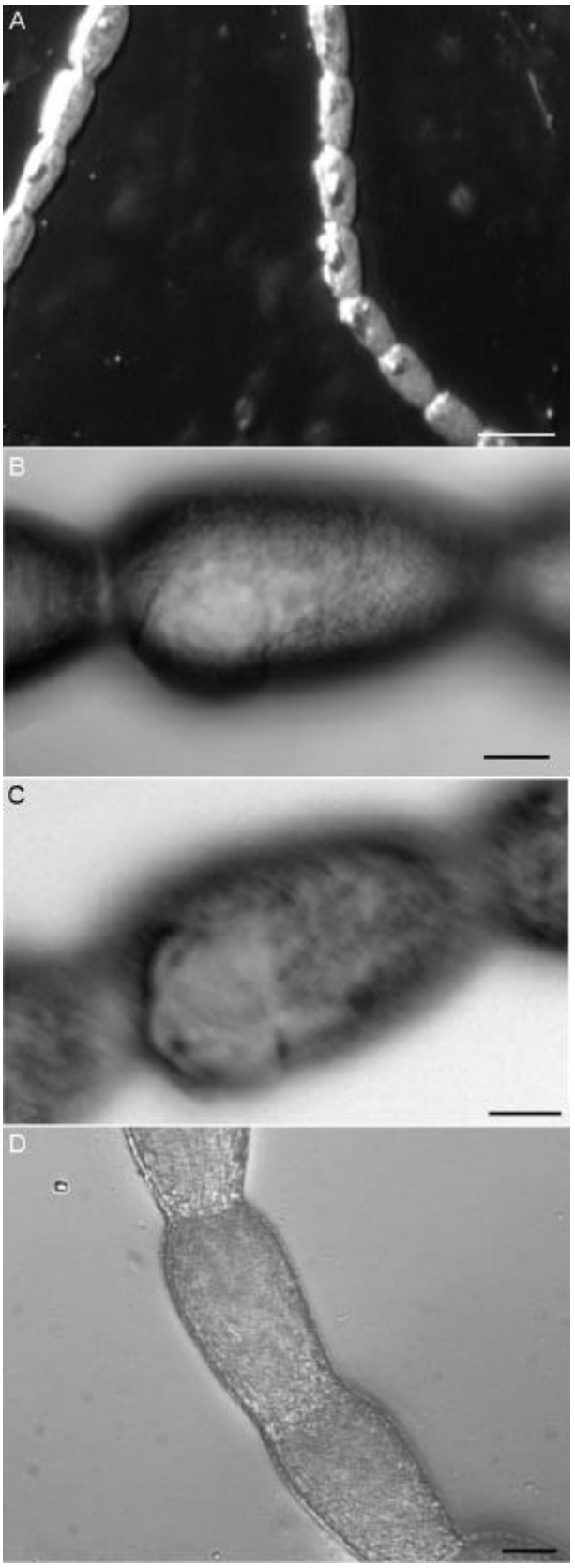

Figure 11. Local CD application on *Tradescantia* stamen hair cells.

Tradescantia stamen hair cells, just after application of drops of 50% (w/v) Arabic gum in water with or without 100µM CD (A). After 8 hours, in 70% of the cells local outgrowths could be observed at the site where the CD containing Arabic gum was applied (n=23, B and C). Control cells, which received the same treatment without the CD, did not show local outgrowths (n=9, D). A: bar = 50µm, others: bar = 10µm.

CD effect (fig 11D). Thus, increasing the local actin instability can trigger local growth in a plant cell.

To demonstrate that increase in actin instability is a general mechanism for increasing the size of the area where exocytosis can take place, we used the fission yeast *Saccharomyces pombe*. Culture of the *S. pombe* strain FYC15 in liquid YEA medium (Hochstenbach et al., 1998) with low concentrations of CD or LA did not inhibit cell division and led to formation of cells with an increased cell width (fig 12 and 13). Thus, an increase in actin instability also leads to an increased area where exocytosis takes place in *S. pombe*, as in animal (Bradke and Dotti, 1999) and plant cells, indicating that the mechanism is common to different organisms.

Discussion

Our results are an important step towards understanding the role of the actin cytoskeleton in the spatial regulation of growth in plant cells. They imply that actin instability specifies the size and location of area of exocytosis. It does that by regulating the area where F-actin is more dynamic. We also show that, in cells that still have flexible cell walls, i.e. growing cells, an artificial local increase of actin instability, which gives rise to a local decrease in the abundance of detectable F-actin by fluorescence microscopy, is sufficient to induce a local increase in exocytosis.

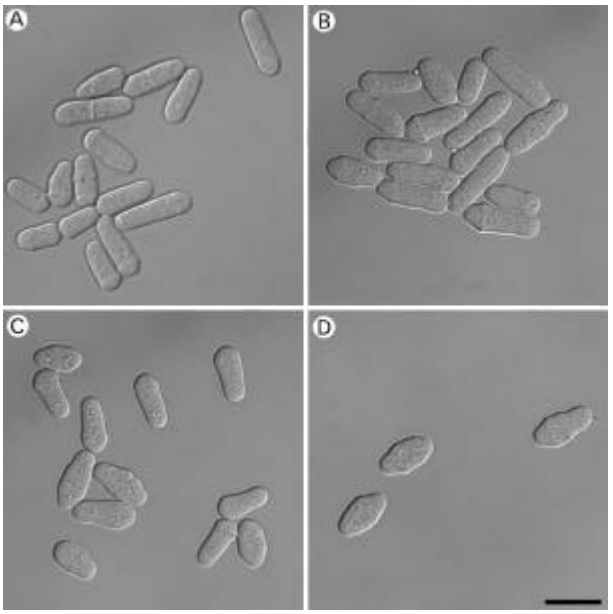

Figure 12. *S. pombe*, cultured overnight in liquid medium with CD.
The medium contained no (A), 0.01µM (B), 0.1µM (C) or 1µM (D) CD. Bar = 10µm.

Miller et al. (1999) have shown that complete depolymerization of the subapical fine F-actin, inhibits root hair growth. This demonstrates that the presence of fine F-actin in the zone adjacent to the growth zone is essential for cell growth. They hypothesize that fine F-actin is involved in delivery of Golgi-derived vesicles to the location where exocytosis takes place. Similar observations have been made by Vidali et al. (2001) in *Lilium longiflorum* pollen tubes. The concentrations of actin depolymerizing drugs we use are lower then the concentration used by Miller et al. (1999) and Vidali et al. (2001). We apparently do not inhibit Golgi-derived vesicle delivery, since expansion proceeds, but increase the cell surface area where expansion takes place. This indicates that the actin cytoskeleton has two distinct functions in cell expansion.

1. It delivers Golgi-derived vesicles to the area where exocytosis takes place and retains them in that area (Miller et al., 1999).
2. It has to be unstable at the plasma membrane surface area where exocytosis takes place (our results).

When CD is locally applied at the surface of a *Tradescantia* stamen hair cell, one creates a surface area with unstable, dynamic actin filaments, fulfilling requirement 2. However, delivery of Golgi-derived vesicles takes place to this area with highly dynamic actin filaments. The bundles of F-actin, that before drug application are

present in the application area, are now replaced by more dynamic fine F-actin that does not form bundles.

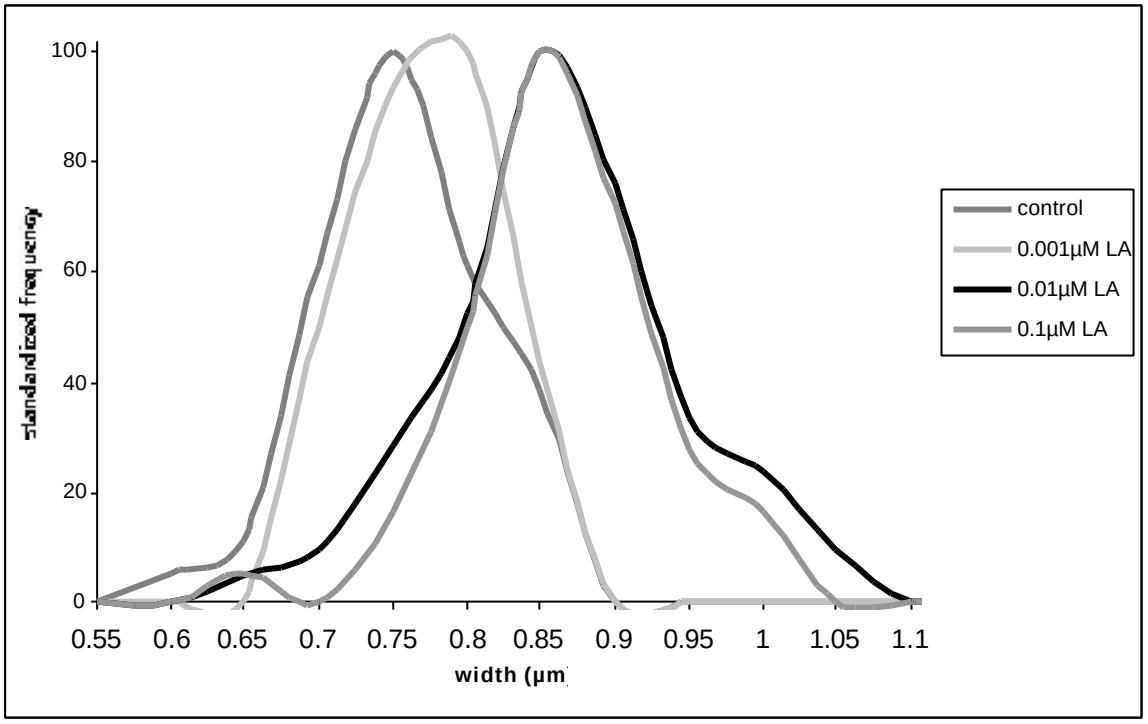

Figure 13. The maximal widths of *S. pombe* after incubation overnight in concentrations of LA.
The measured widths per concentration were categorized in 0.05 µm intervals, the highest frequency set to 100 and the curves were fit to the histograms (n>45 per concentration).

Staiger et al. (1994) and Valster et al. (1997) double-injected *Tradescantia* stamen hair cells. After a profilin injection, fluorescent phalloidin was injected to image the actin cytoskeleton. Both groups reported a dense meshwork of actin filaments surrounding the location where the profilin injection took place. This dense meshwork of actin filaments encloses an area devoid of detectable filamentous actin (figure 14) and is likely to be involved in the delivery of vesicles for localized exocytosis during the wound recovery. It looks similar to the fine F-actin in root hairs and likely fulfills a similar function.
Foissner et al. (1996, 1997) followed the actin cytoskeletal dynamics during the recovery process after wounding of characean internodal cells. After wounding, they observed a rapid local inhibition of cytoplasmic streaming, followed by accumulation of Golgi-derived vesicles at the wounded region. This process correlates with

depletion of detectable filamentous actin at the plasma membrane. After minutes, a dense meshwork of actin is formed around the wounded area. This actin configuration is more sensitive to CD than the thick bundles of actin filaments (Foissner et al., 1997).

Probably, also this actin configuration is involved in delivering the Golgi-derived vesicles, required for wound recovery at the wounding area and maintaining them in this area. The Golgi-derived vesicles perform saltatory and oscillating movements, interspersed by periods of complete immobility when in contact with the plasma membrane. Therefore, the actin configuration in wounded *Chara* cells has similar properties and is likely to perform a similar function as fine F-actin in the subapical

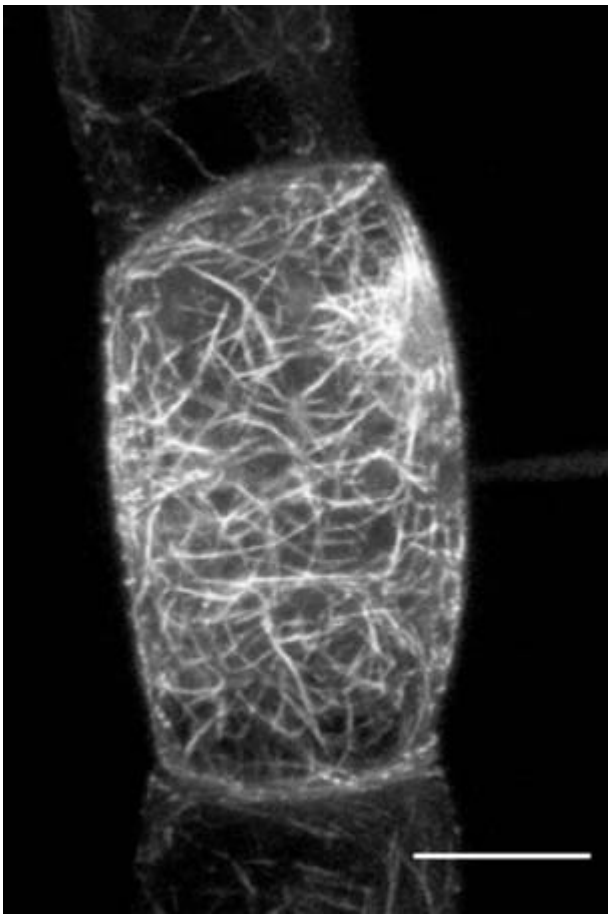

Figure 14. The actin cytoskeleton, visualized with fluorescent phalloidin in double injected *Tradescantia* stamen hair cells.

The wound of the first injection is surrounded by a dense meshwork of actin filaments, enclosing an area where no actin filaments can be observed at this magnification. Bar = 10µm.

area of root hairs, where vesicles perform similar saltatory and oscillatory movements (Sieberer and Emons, 2000). In the wounded area, the vesicles are retained and brought in contact with the plasma membrane. Since there is no cytoplasmic streaming at that site, it is likely that there are no stable, continuous actin filaments at the location where exocytosis takes place.
Baluška et al. (2001) grew plants in latrunculin, which led to complete actin filament depolymerization and inhibition of cell growth. Our hypothesis readily explains their results. Since the fine F-actin, responsible for Golgi-derived vesicle delivery to the location of the cell surface where expansion takes place is depolymerized, cell expansion stops.
In pollen tubes, which are tip-growing cells, a comparable actin configuration has been reported as in root hairs: an apical area without detectable actin filaments, followed sub-apically by an area with a dense actin meshwork (Miller et al., 1996; Doris and Steer, 1996, Geitmann et al., 2000). Miller et al. (1996) confirm by immunogold electron microscopic analysis after freeze substitution that the apical area indeed is essentially free of filamentous actin. However, recently Fu et al. (2001) demonstrated that very dynamic short actin bundles (SAB), correlating with growth, are present in the apex of *Nicotiana tabacum* pollen tubes when expressing LAT52::GFP(S65C)-mTalin. The performed experiments suggest that Rop is involved in regulating polar growth of pollen tubes at least partially through the control of the organization of dynamic F-actin at the apex of pollen tubes. Both our results and the results of Fu et al. (2001) demonstrate the importance of the dynamics of the (sub)apical actin cytoskeleton in determining the area of cell expansion . In root hairs, overexpression of constitutively active Rop2 has been demonstrated to increase the diameter of the tube, while forming a network of fine F-actin in the tip (Jones et al., 2002), indicating that Rop2 is likely involved in the control of the subapical actin cytoskeleton.

Localized actin instability, leading to local decrease of stabile bundles of filamentous actin, combined with an underlying meshwork of fine F-actin, could provide a general mechanism for determination of the area of cell growth, whereas microtubules delimit the growth orientation in plant cells.

Methods

Plant growth, drug treatment and bright field microscopy

Roots of *Arabidosis thaliana* plants (ecotype Columbia) were grown between glass slides in 1/2 strength Murashige and Skoog medium including vitamins (Duchefa, Haarlem, The Netherlands) for 5 days at 25 °C, 16 h light, 8 h darkness. Drugs ware applied by gradually replacing the medium by identical medium with added drugs in the culture room to avoid temperature shocks. CD and LA were dissolved as 10mM stocks in DMSO. The medium of control plants was replaced by medium containing similar percentages DMSO as the experimentally treated plants. Root hairs were analyzed by placing the plant growth chamber on a Nikon Diaphot 200 microscope equipped with Hoffman Modulation Contrast. Time lapsed images were recorded with a Hamamatsu Argus-20 system and processed with Adobe Photoshop 7.0 (Adobe Systems Inc., Mountain View CA, USA).

Calculation of root hair surface increase

High-resolution images were taken with 30 seconds time intervals as described above, 10 minutes before drug application to 10 minutes after drug application. Sequential images of the root hairs were aligned and every µm, starting at 10 µm from the apex of the first image, a transverse line was placed until the apex was reached. The increase in root hair surface was estimated by measuring the maximal diameter of the root hair in every individual slice, thereafter the obtained values were extrapolated to the 3-dimentional circular circumference and the individual slide surfaces were summed. The total increase in cell surface before and after local CD application was calculated. The standard deviation was taken from the differences in cell surface increase between the individual images and the changes in growth speed did not exceed the standard deviation.

Immunocytochemistry, improved EAC fixation followed by fluorescently labeled phallotoxin staining and fluorescence microscopy

3-5’ After drug application, plants were either fixed with the EAC method and stained with Bodipy-FL phallacidin as described before (Ketelaar et al., 2002) or rapidly frozen in liquid propane and freeze substituted in a freeze substitution device, (Freezy SUB, Plant Cell Biology, Wageningen), for at least 24 h in acetone + 0.5% glutaraldehyde at -90°C, after which the temperature was stepwise increased over 6 hour periods (via -60°C and -30°C) to 0°C. After allowing the freeze substituted material to reach room temperature, the freeze substitution medium was replaced gradually by phosphate buffered saline (PBS) with 4% paraformaldehyde and 0.1% glutaraldehyde. After washing in PBS, the material was incubated in a saturated driselase (Fluka, Buchs SG, Switserland) and macerozyme R10 (Serva, Heidelberg, Germany) suspension in MES buffer (30°C, pH 5.5, 30’). The enzymes were washed away, free aldehydes were reduced in 0.1M NH3OHCl in PBS and proteins blocked in 0.1% acetylated BSA (Aurion, Wageningen, The Netherlands). As primary antibody either the monoclonal anti plant actin 3H11 (Andersland et al., 1994) or monoclonal anti α-tubulin (clone DM1a, Sigma, St. Louis MO, USA) was used and as secondary antibody GaM-IgG-Cy3 (Molecular Probes, Leiden, The Netherlands). After labeling, the material was washed in PBS-citifluor and mounted in Citifluor-glycerol (Citifluor UKC, Canterbury, UK). Images were acquired on a BioRad MRC-600 confocal laser scanning microscope, equipped with an 5mW Argon Krypton laser. A neutral density filter was used to obtain 1% transmission intensity. The 568nm line was used to excite the Cy3 and a DM 568 BA 585 EFLP filterblock was used. Z-series were obtained with 1μm intervals. Images were processed with Confocal Assistant (Bio-Rad, Hemel Hempstead, UK) and Adobe Photoshop 7.0 (Adobe Systems Inc., Mountain View CA, USA). Deconvolution was performed by opening the raw CLSM data in OpenLab 3.0 (Improvision, Coventry, UK) and performing 25 iterations of the Volume Deconvolution function.

FM4-64 staining

Arabidopsis roots in a growth chamber were placed on a Bio-Rad MRC-600 confocal laser scanning microscope. The medium was gradually replaced with medium containing 2μM FM4-64, whereafter the root hairs were imaged using the DM 488 BA 522 DF35 filter block. Imaging was done with completely opened pinhole to image the whole root hair.

Local CD application

CD was locally applied on *Medicago truncatula* root hairs, grown in air on the surface of an 0.6% agar plate by placing a small drop of water, containing CD with a water pressure microinjection device (water pressure device: Gilmont, Barrington IL, USA; needle holder: Eppendorf, Merck Eurolab BV, Amsterdam, The Netherlands) at one side of the dome of a growing root hair. Images were acquired in the reflection channel of a Bio-Rad MRC 600 confocal microscope.

S. pombe culture and drug treatments

S. pombe strain FYC15 was obtained from dr. F.M. Klis (University of Amsterdam) and cultured in 70ml liquid YEA medium (Hochstenbach et al., 1998) in 200 ml conical flasks on a shaker (160 rpm at 25°C). CD and LA were applied to the medium before inoculating the flasks with 1 ml from a 3 day old culture. Drug treatments were carried out overnight by culturing at the conditions as described above. Controls contained comparable amounts of DMSO in the medium as the drug treated *S. Pombe*. Microscopical analysis was carried out the next morning with a Nikon Microphot FXA equipped with a 100x NA 1.4 oil immersion objective and a Photometrics Quantix cooled CCD camera, combined with IP-Lab spectrum 3.1 image analysis software (Scanalytics, Fairfax VA, USA).

Statistical background figure 13

In the table below, the averages and standard deviations of the maximal width as measured in μm are given. The average width increases by maximally 13%. However, the standard deviations are not very small. Therefore, the increase in width between the control and 0.001 μM LA and the increase in width between 0.01 and 0.1 μM LA not statistically meaningful. However, the total increase in width is statistically meaningful. The standard deviations are relatively large because there are yeast cells in the medium that were fully-grown when the medium was inoculated and the concentration of the LA presumably goes down during the culture period. The latter will lead to growth of cells with lower diameters over time in the medium

with lower concentrations LA. The lack of difference between 0.01 and 0.1 μM LA may be explained by the observation of a maximal width in *Arabidopsis* root hairs. Because of these reasons, we believe, although not all data are statistically meaningful, that the graph as presented is the clearest way to present the data.

Table. Values as used to create figure 13

[LA]	average maximal width	standard deviation
0 μM	0.7322	0.04478
0.001 μM	0.75326	0.04652
0.01 μM	0.84042	0.07711
0.1 μM	0.83923	0.05776

Acknowledgements

The double injected *Tradescantia* stamen hair cell in figure 14 is courtesy of dr. E.S. Pierson. We are grateful for her permission to use this image. We thank dr A.H.J. Bisseling, dr M. Dogterom and dr A.R. van der Krol for discussion, dr F.M. Klis for providing *S. pombe* and dr R.J. Cyr for providing the plant actin antibody.

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Chapter 2

A study into exocytosis and endocytosis rates in growing *Arabidopsis* pollen tubes and root hairs

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Abstract

We measured the growth speed of *Arabidopsis* pollen tubes and root hairs, using video microscopy and the diameter of vesicles located in the tip of these cell types in longitudinal thin transmission electron microscopic sections. The obtained values were used to estimate the number of exocytotic vesicles required for cell wall expansion, and the excess membrane inserted into the plasma membrane to be internalised. In pollen tubes, we obtained an exocytosis rate of 3238 vesicles per minute and an endocytosis rate of 21265 vesicles per minute. In root hairs the exocytosis rate was 4202 vesicles per minute and the endocytosis rate 6901 vesicles per minute.

From counting of the number of vesicles, present in thin EM-sections through the pollen tube tip, we calculated the number of vesicles, present in the pollen tube dome. This latter number was used to caculate the time a cell would be able to grow without new delivery of vesicles. The obtained value of 33 seconds is comparable to that of the 30-40 seconds found experimentally, by arresting vesicle delivery to the vesicle rich area with cytochalasin D and measuring the subsequent amount of cell elongation, i.e. the consumption of residual exocytotic vesicles. In addition, this result confirms that filamentous actin is not required for the insertion of the Golgi-derived vesicle membrane into the plasma membrane, the exocytosis process proper (Chapter 1 of this thesis).

Introduction

Plant cell growth requires newly delivered cell wall matrix material and plasma membrane at the site of growth (Roberts, 1994). Cell wall matrix material is required for cell wall extension and additional membrane is required for plasma membrane extension in expanding cells. The plasma membrane is not elastic; increase in surface can only take place by insertion of new plasma membrane (Wolfe et al., 1986; plant cells: Homann, 1998; Homann and Tester, 1998). The cell wall matrix and plasma membrane are delivered in the form of Golgi-derived vesicles. When exocytotic Golgi-derived vesicles fuse with the plasma membrane, the membrane of these vesicles supplies new plasma membrane whereas at the same time the interior of the exocytotic vesicles, the cell wall matrix, is deposited into the existing cell wall (review: Miller et al., 1997). In this way, plant cells need exocytosis to expand. However, exocytosis also occurs in plants without cell expansion, e.g. for excretion (review: Battey et al., 1999).

Exocytotis is a complex process. Before the actual exocytosis can occur, exocytotic vesicles have to be produced in the Golgi cisternae, whereafter they have to be targeted and delivered to the growth area to become inserted into the plasma membrane. The actin cytoskeleton is involved in the delivery (Miller et al., 1999, De Ruijter et al. 1999, Chapter 1 of this thesis). Specific proteins, like the v-SNARE and t-SNARE proteins, are involved in docking and fusion of the exocytotic vesicles with the plasma membrane (for review see e.g. Battey et al., 1999). Also a tip focussed gradient of cytosolic Ca^{2+} is involved in exocytosis in pollen tubes and root hairs (pollen tubes: Malhó and Trewavas, 1998; root hairs: Wymer et al., 1997, De Ruijter 1998).
Since three-dimansional membrane structures deliver approximately two-dimensional cell wall plates, the fusion of exocytotic vesicles with the plasma membrane leads to a surplus of inserted plasma membrane in comparison to inserted cell wall matrix. The cell compensates this surplus by recycling plasma membrane by endocytosis. At the same time, delivery of trans-membrane proteins to the plasma membrane and recycling of these proteins takes place by respectively exocytosis and endocytosis (Battey et al., 1996, 1999). There are indications that incomplete fusion with the plasma membrane can take place, wherein the vesicle membrane is recycled

immediately after deposition of its contents into the wall (Staehelin and Chapman, 1987, Emons et al., 1992); reviews: Battey et al., 1996, 1999). In work on mammalian cell types, this incomplete fusion of vesicles with the plasma membrane, which allows them to drop their contents into the extracellular lumen, followed by retrieval of the vesicle membrane is described as ‘kiss-and-run’ exocytosis (reviews: Nichols and Lippincott-Schwartz, 2001; Valtorta et al., 2001). We do not address the type of actual plasma membrane retrieval process here, which is not relevant for the amount of membrane internalized. In this article we call all plasma membrane internalization endocytosis.

Pollen tubes and root hairs are ideal cell types to study exocytosis, because they are tip-growing cells in which all cell growth, and thus all the exocytosis required for cell growth, takes place robustly at one location in the cell, the tip. This implies that one can study the total cell expansion by examining a relatively small area of the cell surface. We used growing pollen tubes and root hairs of the model plant *Arabidopsis*. We measured growth speed and made median Transmission Electron Microscopic (TEM) sections through domes of freeze fixed, growing pollen tubes and root hairs. These sections were used to count vesicles and measure their diameter, the thickness of the cell wall and the width of the cell lumen. The obtained values were used to make estimations about the exo- and endocytosis rates.

The exocytosis rate together with an estimation of the number of exocytotic vesicles, allowed to calculate how long cell growth can be sustained when delivery of exocytotic vesicles to the dome is inhibited. The calculated time of 33 seconds for 1222 vesicles is comparable to the experimentally found value of 30-40 seconds, obtained by measuring the amount of cell elongation after depolymerising the actin cytoskeleton reponsible for vesicle delivery to the vesicle rich area.

Assumptions

For analysis, we assume steady state cell growth and a cylindrical shape of the root hair and pollen tubes. The increase in cell wall volume is assumed to be due exclusively to deposition of the contents of exocytotic vesicles into the cell wall, which implies the assumption that cellulose microfibrils do not contribute significantly to the

cell wall volume in the apical area of pollen tubes and root hairs. Every exocytotic vesicle that fuses with the plasma membrane is assumed to contain cell wall matrix material, which is completely deposited into the cell wall, where it maintains the same volume, but not the same shape. The measured diameter of the vesicles on TEM sections is inaccurate; because a section may not contain the median plane of a vesicle. However, since few vesicles exceeded a diameter value of 150 nm, in the calculation all measured values are assumed to be median diameters in the 100-150nm thick TEM sections. The diameter of endocytotic vesicles is assumed to be 60 nm without coat. This is the only reported value for root hairs, in *Equisetum hyemale,* and is comparable with the diameter of endocytotic vesicles in intercalary growing plant cells (Emons and Traas 1986). The dome of a root hair or pollen tube is defined as the apical area wherein the width of the fully-grown pollen tube/hair has not yet been reached. For estimation of the amount of exocytotic vesicles in the pollen tube and root hair dome, the dome is assumed to be hemispherical, which is a simplification. All vesicles in the dome are assumed to be exocytotic vesicles. This is not necessarily true, because it has been demonstrated that coated pits are present in the dome of growing root hairs (reviewed by Galway, 2000), although the coated pits are present over a much larger area than the exocytosis area (Emons and Traas, 1986). Since we did not observe many vesicles with diameters of approximately 60 nm in the domes of pollen tubes and in root hairs, this simplification is not likely to cause major changes in the obtained values, unless there are non-coated pit mediated forms of endocytosis in plants, in which exocytotic and endocytotic vesicles have similar diameters.

The exocytotic vesicle distribution in the dome is assumed to be random. The area of pollen tube or root hair filled with vesicles is highly variable during cell elongation, but approximately coincides with the dome of the cell. Therefore, we assumed that the whole stock of exocytotic vesicles capable of exocytosis is localised within the dome after blocking delivery of newly synthesised vesicles.

Results and discussion

Arabidopsis pollen tube and root hair growth speed

We measured the growth speed of expanding pollen tubes and root hairs by time-lapsed imaging. The increase in pollen tube length was measured over a period of 5

minutes. In the conditions specified in the methods section, the mean growth speed was 4.07±0.92µm (n=10) per minute. Root hairs were measured over a period of 10 minutes. Their mean growth speed was 0.93±0.17µm (n=10) per minute.

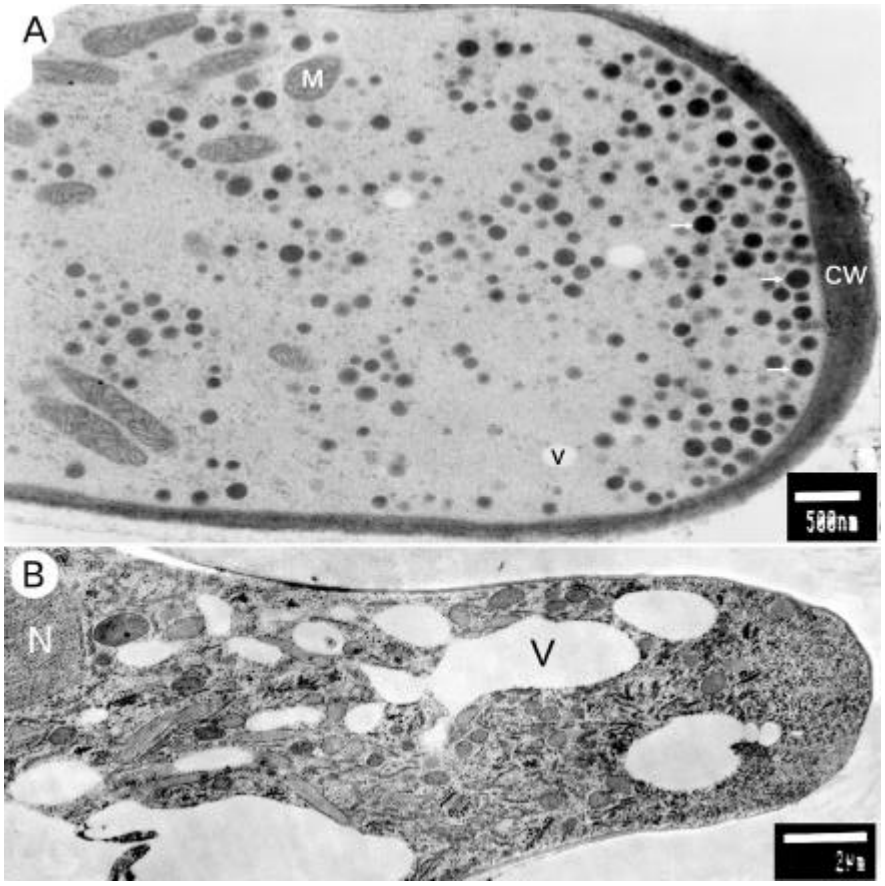

Figure 1. Sections through a dome of a growing *Arabidopsis* pollen tube (a) and a growing root hair (b). The dark, circular spots in the apex are the vesicles (Arrows) and the cell is surrounded by the dark cell wall (CW). At the left side of figure 1a, some mitochondria are visible (M). The white spots are vacuoles (V). In figure 1b, the nucleus is visible (N).

Calculation of volume and surface area of Golgi-derived vesicles in the *Arabidopsis* pollen tube and root hair dome

The volume and surface area of Golgi-derived vesicles in pollen tubes and root hairs were estimated by measuring their diameter in TEM sections (pollen tube: figure 1A; root hair: figure 1B). Measurement of vesicle diameter is only reliable if the fixation/preparation procedure does not influence the size or localisation of the vesicles. Therefore, we used rapid freeze fixation and freeze substitution.

We measured the cell lumen width and the width of the cell wall in median thin TEM sections of pollen tubes or root hairs. In pollen tubes, the mean width of the cell lumen in the subapical area was 4.53±0.36 µm (4 tubes, $n \geq 2$ per pollen tube) and the mean cell wall width in the subapical area was 0.143±0.014 µm (4 tubes, $n \geq 2$ per pollen tube). In root hairs, the mean width of the cell lumen in median sections was 6.29±0.67 µm, the width of the cell wall was 0.07±0.01 µm.
The mean measured diameter (d) of vesicles in thin TEM sections was $0.171 \pm 31\ \mu m$ in pollen tubes (7 pollen tubes, n=97) and $0.085 \pm 16\ \mu m$ in root hairs (4 root hairs, n=86).

Estimation of the exo- and endocytosis rate

Exocytosis rate

In tip growing cells, the increase of cell membrane surface area per unit time ($\delta A_{membrane}$) can be estimated by (1)

$$dA_{membrane} = p\,Dv$$

Wherein D is the diameter of the inner volume (lumen) of the tube and v is the growth speed. Likewise, the increase of cell wall volume per unit time ($dV_{cell\ wall}$) is given by the difference between the increase of the outer (lumen + cell wall) volume and the inner volume of the cell, i.e. (2)

$$dV_{cellwall} = \left\{ \frac{1}{4}p\,(D + 2w)^2 - \frac{1}{4}pD^2 \right\} v = pw(D + w)v$$

Wherein w is the thickness of the cell wall. This rate can also be expressed in the exocytosis rate through (3)

$$dV_{cellwall} = N_{exo} V_{exo}$$

Wherein N_{exo} is the number of exocytosis events per unit time and V_{exo} is the volume of an exocytotic vesicle. The volume of the exocytotic vesicle is given by (4)

$$V_{exo} = \frac{1}{6}pd_{exo}^3$$

Wherein d is the diameter of the exocytotic vesicle. Its surface area is given by (5)

$$A_{exo} = p\ d_{exo}^2$$

This allows the exocytosis rate to be determined as (6)

$$N_{\text{exo}} = \frac{V_{\text{cellwall}}}{V_{\text{exo}}} = \frac{6w(D+w)}{d_{\text{exo}}^3} v$$

Endocytosis rate

The increase in cell membrane surface area arises from a balance between the surface delivered by exocytosis and the surface removed by the endocytosis, or internalised otherwise (7)

$$dA_{\text{membrane}} = N_{\text{exo}} A_{\text{exo}} - N_{\text{endo}} A_{\text{endo}}$$

Wherein N_{endo} is the number of endocytotic events per time unit and A_{endo} is the mean surface of an endocytotic vesicle. Comparing equation 1 and using equation 6, we find (8)

$$N_{\text{endo}} = \frac{N_{\text{exo}}A_{\text{exo}} - dA_{\text{membrane}}}{A_{\text{endo}}} = \frac{v}{d_{\text{endo}}^2}\left\{\frac{6w(D+w)}{d_{\text{exo}}} - D\right\}$$

Note that the endocytosis rate would become negative if the exotytotic vesicle diameter were larger than the critical value (9)

$$d^* = 6\frac{w(D+w)}{D}$$

If the exocytotic vesicle diameter exceeds d^*, the amount of membrane surrounding the contents of the exocytotic vesicle is not sufficient to cover the area of newly deposited cell wall by one exocytotic vesicle with plasma membrane.

Using equation 1, we find a $\delta A_{\text{membrane}}$ of 57.85 µm^2 per minute in pollen tubes and 18.38 µm^2 per minute in root hairs. Equation 2 gives a $dV_{\text{cell wall}}$ of 8.52 µm^3 per minute in pollen tubes and 1.37 µm^3 per minute in root hairs, leading to 3238 exocytotic events per minute in pollen tubes and 4202 exocytotic events in root hairs (equation 3). In pollen tubes, V_{exo} (equation 4), the volume of the vesicle, is 2.63*10^{-3} µm^3 and A_{exo} (equation 5), the vesicle diameter is 0.09 µm^2.

The amount of endocytosis can be estimated by using equation 8, leading to a N_{endo} of 21265 endocytotic vesicles per minute in pollen tubes. In root hairs, V_{exo}, after correction for sectioning is 3.27*10^{-4} µm^3, A_{exo} is 2.23*10^{-2} µm^2 and N_{endo} = 6901 endocytotic vesicles per minute. In both root hairs and pollen tubes, the percentage of the newly inserted plasma membrane that is recycled by endocytosis is 81% ($A_{endo}N_{endo}/A_{exo}N_{exo}$). The critical diameter of exocytotic vesicles, above which the amount of membrane of an exocytotic vesicle is not sufficient to cover the area of newly deposited cell wall by its contents with plasma membrane (d_x) is 0.883 µm in pollen tubes and 0.448 µm in root hairs of *Arabidopsis* (equation 9).

In similar studies to ours, calculations have been made to estimate the number of vesicles required for exocytosis and/or endocytosis. Miller et al. (1997) calculated that in *Equisetum hyemale* root hairs, in which the growth speed is ~40µm per hour (Emons and Wolters-Arts, 1983), the extension of the plasma membrane requires 8900 exocytotic vesicles with a measured diameter of 300 nm (Emons, 1987) per hour. In combination with an estimated membrane turnover time of 20-40 minutes, calculated from the amount of coated pits (Emons and Traas, 1986), this leads to a requirement of 26700 exocytosed vesicles per hour or 445 vesicles per minute to sustain membrane recycling and cell expansion. In this calculation, 67% of the inserted membrane needs to be recycled, lower than the 81% in *Arabidopsis* pollen tubes and root hairs, but in the same magnitude.

In *Lilium* pollen tubes, Morré and van der Woude (1974), estimated the amount of exocytotic vesicles by assuming that the volume of the exocytotic vesicles contributed to the cell wall expansion stoichiometrically, similarly to one of our assumptions. They obtained a value of ~1000 vesicles per minute, a lower value than the values we obtained.

Picton and Steer (1983) used a different approach. Using 0.3µg/ml (~0.6µM) cytochalasin D, they inhibited growth of *Tradescantia* pollen tubes. The inhibition of growth is caused by the inability to deliver newly synthesised Golgi-derived vesicles to the growing tip. However, CD treatment does not arrest the production of Golgi-derived vesicles immediately, leading to accumulation of non-useable Golgi-derived vesicles in the cytoplasm. After 5 minutes, they calculated the increase in the density of Golgi-derived vesicles in the cytoplasm and extrapolated this value to the number

of Golgi-derived vesicles that would be consumed for pollen tube growth during that time. Their estimate of 3000-5000 exocytotic vesicles per minute approaches our value.

Estimation of the amount of exocytotic vesicles in the domes of pollen tubes and root hairs

Knowing the total number of exocytotic vesicles in a dome will enable one to calculate the time required to insert the supply of exocytotic vesicles in growing pollen tubes and root hairs after their delivery to the vesicle area has stopped by using the dimensions of the cell wall and the vesicles as obtained in the previous section.

If the distance from the centre of one vesicle to the centre of the next is considered as a unit, one can calculate the diameter of a dome, expressed in this unit by counting the number of vesicles in a median longitudinal section through a dome. In the following calculations, we will define this unit as ‘distance’.

In pollen tubes, the mean number of counted exocytotic vesicles was 139±41 (n=5 median sections through domes), leading to a diameter of 18.8 distances ($½\pi 1/2d_{(vesicle)}^2$= 139). In root hairs, the mean number of exocytotic vesicles in a median section of a dome was 915±132 (n=4 median sections through domes). From this value, a diameter of 48.2 distances can be calculated. By calculating the volume of a hemisphere, expressed in distances ($¾\pi 1/2d^3*½$), the total number of vesicles in the vesicle rich region estimates 1222 in pollen tubes. Therefore, the time before the exocytotic vesicles in the dome will have been consumed is 1222/2208=33 seconds. In root hairs, an estimated amount of 19328 exocytotic vesicles are present, which is consumed in 19328/9631=120 seconds of normal cell expansion.

Experimental determination of the time required to insert the accumulated supply of exocytotic vesicles present in the vesicle rich region of pollen tubes

To test whether and for how long cell growth is sustained when the delivery of exocytotic vesicles to the vesicle rich region of a pollen tube is inhibited, we blocked

new delivery of exocytotic vesicles with cytochalasin D (CD). CD changes actin dynamics, leading to net-depolymerisation of actin filaments (Cooper, 1987). Also in *Arabidopsis*, at 5µM, this actin drug selectively depolymerises only and completely the subapical fine F-actin (Chapter 3 of this thesis), known to be involved in delivery of exocytotic vesicles to the dome of *Vicia sativa* root hairs (Miller et al., 1999). High magnification time-lapsed microscopy allowed us to follow the increase in length of pollen tubes in detail. Application of CD led to growth arrest, 30-40 seconds after CD application (figure 2).

Therefore, there must have been a store of vesicles enough for 30-40 seconds continuous growth. This experimentally obtained value affirms the estimation of 33 seconds of potential cell elongation from the store of exocytotic vesicles calculated to be present in growing pollen tube tips. The fact that the estimated and the real values are similar in addition proves that filamentous actin is not involved in the exocytosis process per se.

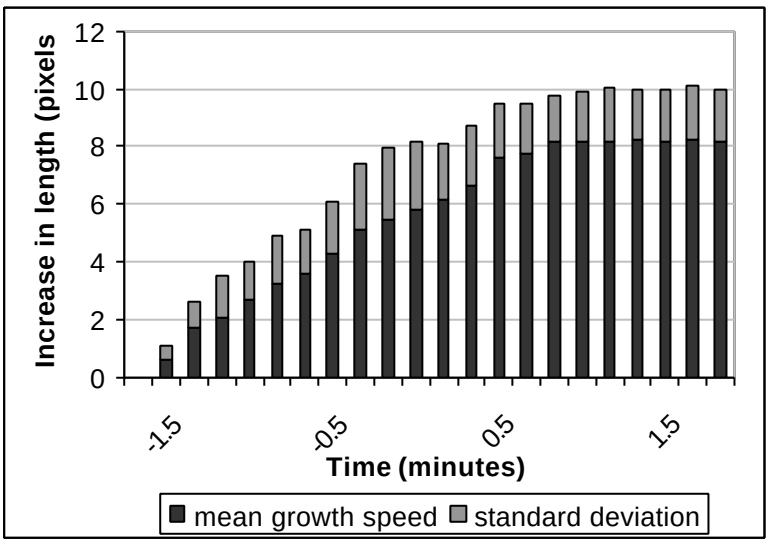

Figure 2.The increase in length of pollen tubes, before and during treatment with 5µM cytochalasin D. For measurements, means of the values obtained for 3 different pollen tubes were used. The standard deviation was calculated from the differences in length increase between the different pollen tubes. At the start of the measurement, the length was set to 0. Pollen tubes were allowed to grow until time-point 0, where cytochalasin D was applied to the medium. The pollen tubes continued to grow for 30-40 seconds after application, whereafter growth is inhibited.

Methods

Pollen tube and root hair growth

Arabidopsis ecotype Columbia pollen was germinated on dialysis-tubing, flattened on top of solid pollen tube growth medium, which contained 1mM $CaCl_2$, 1mM $Ca(NO_3)_2$, 1mM $MgSO_4$, 0.01% boric acid, 18% sucrose and 0.5% agarose, pH 7.0 at 20°C.
To obtain root hairs, *Arabidopsis* ecotype Columbia plants were germinated in liquid medium with their roots between glass slides, as described before (Ketelaar et al., chapter 1).

CD treatment

Pollen was germinated on solid medium as described above with a top layer of identical liquid medium without agarose. CD was offered by sucking away the liquid medium with a piece of filter paper from the side and simultanously providing new medium with CD from the opposite side. We used a 60x LWD objective on an inverted Nikon microscope and imaged the growing pollen tubes with the Hamamatus Argus-20 image processor, linked to a PC.

Freeze fixation and substitution

After 3-6 hours germination, small pieces of dialysis tubing, containing pollen tubes, were grabbed from the medium and immediately plunged into liquid propane. For root hairs, 4-5 day old seedlings were grabbed with tweezers and plunged into liquid propane. Freeze substitution was performed in acetone + 2% OsO_4 at –90°C for 40 hrs, whereafter the samples were slowly warmed up to room temperature over 6 hours. The pieces of dialysis tubing with the attached pollen tubes and the complete roots were embedded in Spurr's resin; Samples with pollen tubes were immediately tranferred from the freeze substitution solution into 100% resin, which was refreshed 3*12 hours. Root samples were taken through a graded series of 25, 50, 75 and 100% v/v acetone/Spurrs resin before washing 3*12 hous in 100% resin. The resin, contianing the samples, was polymerized at 70°C for 24 hours. Sections were made on a Reichert ultramicrotome and poststained with 2% uranil acetate and 3% lead citrate.

Electron Microscopy

The sections were examined in a Jeol TEM and photographed, whereafter the negatives were scanned. Measurements were carried out with the NIH Image software package.

The calculations were carried out with as many decimals as possible. In the results section, for clarity the numbers are rounded off.

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Chapter 3

Positioning of nuclei in *Arabidopsis* root hairs: an actin regulated process of tip growth

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Abstract

In growing *Arabidopsis* root hairs, the nucleus locates at a fixed distance from the apex, migrates to a random position during growth arrest and moves from branch to branch in a mutant with branched hairs. Consistently, an artificial increase of the distance between nucleus and apex by entrapment of the nucleus in a laser beam, stops cell growth. Drug studies show that microtubules are not involved in the positioning of the nucleus but that sub-apical fine F-actin between the nucleus and the hair apex is required for maintaining the nuclear position with respect to the growing apex. Injection of an antibody against plant villin, an actin filament-bundling protein, leads to actin filament unbundling and movement of the nucleus closer to the apex. Thus, the bundled actin at the tip side of the nucleus prevents the nucleus from approaching the apex. In addition we show that the basipetal movement of the nucleus at root hair growth arrest requires protein synthesis and a functional actin cytoskeleton in the root hair tube.

Introduction

Root hairs are tubular structures that emerge from certain root-epidermal cells (Haberlandt, 1883). They expand by localized exocytosis of cell wall matrix contained in Golgi-derived vesicles at the cell apex into a plastic cell wall, a phenomenon referred to as tip growth (review: Derksen and Emons, 1990). During the process of tip growth, a distinct organization of the cell can be observed. The apical area of the root hair is cytoplasm dense with endoplasmic reticulum (ER), Golgi cisternae and mitochondria, whereas in the extreme apex, a high density of vesicles is present (Emons, 1987, Ridge 1988, Galway et al., 1997; Miller et al., 2000). Net-axially aligned fine bundles of actin filaments (fine F-actin), which are involved in the delivery of Golgi-derived vesicles to the apex, are present in the sub-apical area (Miller et al., 1999). Another typical observation in root hairs involves the position of the nucleus. In growing root hairs, the nucleus is located at a distinct distance from the apex (Haberlandt, 1887, reviewed in Miller et al., 1997). This precise positioning of the nucleus during the growth process suggests that the nucleus is part of the growth machinery involved in root hair elongation. How the nucleus maintains a fixed position from the growing tip and how it is involved in the tip growth machinery is not clear.

Mutants can be helpful tools to gain insight into various processes. In this study, we used the *cow1-2* mutant (Grierson et al., 1997), which produces branches in approximately 20% of its root hairs. The length of the branches in a branched *cow1-2* root hair can be greater than the distinct distance between nucleus and tip; therefore, the behavior of the nucleus in a branched root hair could provide valuable insight into the role of the nucleus in tip growth.

Here we demonstrate that the presence of the nucleus at a fixed distance from growing root hair tips is essential for *Arabidopsis* root hair growth and that the positioning is dependent on the actin cytoskeleton, but not the tubulin cytoskeleton, during hair growth and during growth arrest. Furthermore, we show that actin filament bundling is involved in the positioning of the nucleus in root hairs and that the movement of the nucleus away from the hair tip during growth arrest is both actin-based and dependent on new protein synthesis.

Results

Position of the nucleus during root hair growth and growth arrest

To determine the movement of nuclei in root hairs, time-lapse recordings were made of developing hairs (Figure 1). The distance between the nucleus and apex of growing *Arabidopsis thaliana* ecotype Columbia root hairs was (77±15 µm (19%) SD; n=54), whereas in fully-grown root hairs, the nucleus was located at a random position in the hair (247±134 µm (54%) SD; n=57). In Figure 2, the position distribution of nuclei in growing versus fully-grown root hairs is displayed. The nucleus remains at a fixed distance from the apex of growing hairs, and moves back when growth terminates at a speed between 0 and 60 µm per minute (12±23 µm per minute SD; n=25), so that it eventually obtains a random position in the hair. Chytilova et al. (2000) report occasional division of nuclei into subnuclear structures in root hairs of *Arabidopsis*, which remain connected by thread-like structures. We did not observe such divisions using Hoffman Modulation Contrast or differential interference contrast microscopy of living hairs. However, they used a green fluorescent protein (GFP)-β-glucuronidase fusion construct with a nuclear localization sequence that accumulates in the nucleoplasm. The loading of the nucleoplasm with the GFP-β-glucuronidase protein may be the reason for the creation of subnuclear structures. The fixed distance between the nucleus and the apex in a growing root hair can be observed clearly in the supplemental data online of Chytilova et al. (2000).

Behavior of the nucleus in mutant with branching root hairs

The *cow1-2* mutant (Grierson et al., 1997) has shorter and wider root hairs than those of wild-type plants and reduced growth speed. It produces a branch in ~20% of its root hairs. We studied the position of the nucleus during the branch formation by time-lapse video microscopy. In nonbranching root hairs of the *cow1-2* mutant, the

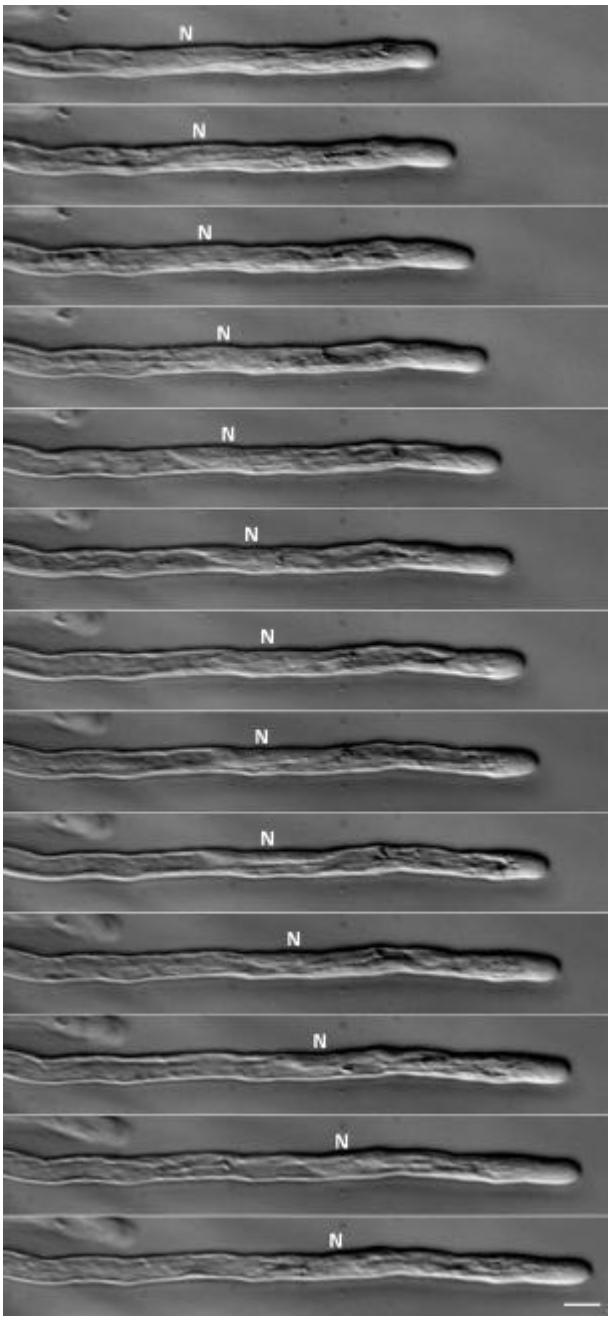

Figure 1. Hoffman Modulation Contrast images from a time-lapse recording of a growing *Arabidopsis* root hair.

Photographs were taken every 4 minutes. The nucleus (N) is indicated. The sequential images demonstrate that the distance between the nucleus and the tip in growing root hairs is more or less fixed. Bar=10µm.

positional behavior of the nucleus was comparable to that of wild-type root hairs in that the distance was smaller and more constant in growing root hairs than in fully-grown root hairs (distance nucleus-apex in growing root hairs: 31±6 µm (19%) SD (n=57), fully-grown root hairs: 106±70 µm (66%) SD (n=61). The shorter distances

between nucleus and apex in the mutant correlates with the reduced length and increased width of *cow1-2* root hairs.

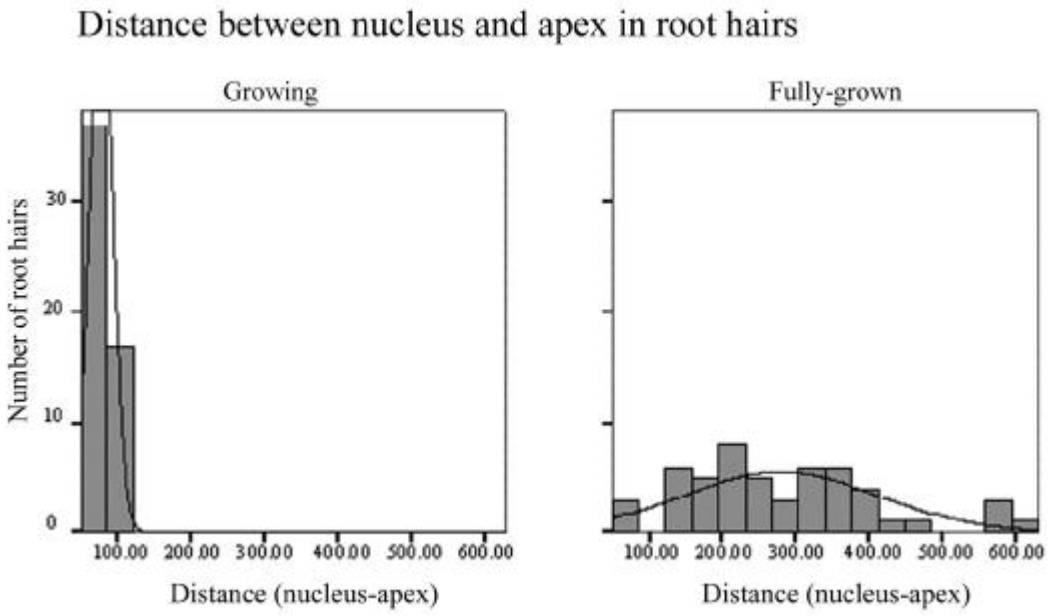

Figure 2. Distribution of the distance between apex and nucleus in growing and fully-grown root hairs of *Arabidopsis*
The bars represent the frequency distribution, and the lines represent a fitted normal distribution curve.

Branches develop from the side of growing root hairs (Figure 3), a process preceded by movement of the nucleus to that site. Subsequently, the nucleus moves from one hair tip to the other and is located within 31 μm from the growing tip between 30-70 % of the time (n=20). When the nucleus moves back from a tip, that tip continues to elongate until the smooth area at the tip, containing the Golgi-derived vesicles, has disappeared. The tip to which the nucleus moves starts to elongate when such a smooth area has been built up. We conclude that in the *cow1-2* mutant, the presence of the nucleus at a fixed distance from a growing root hair tip correlates with sustained growth of that tip.

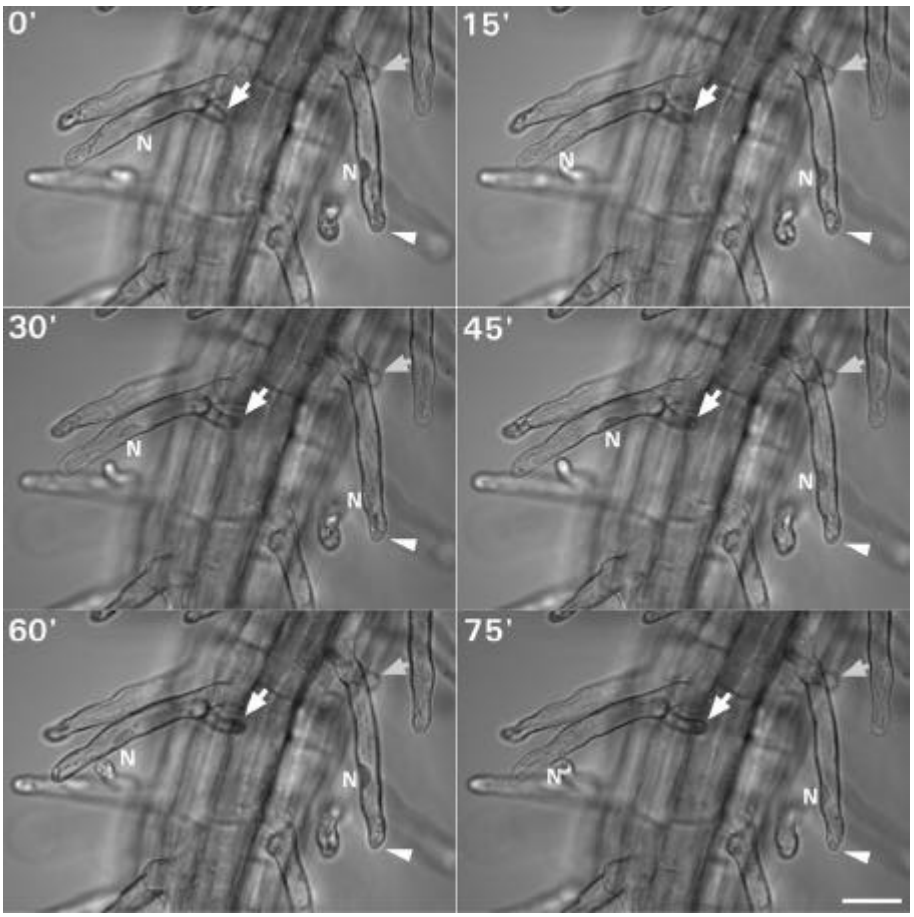

Figure 3. Sequential images from a time-lapse recording of growing *cow1-2* root hairs that develop branches.

One image was taken every 15 minutes. The nuclei are marked with a N. During this time-lapse, both tips of the left root hair are growing, and the nucleus is moving between the two branches. In the right root hair, only one tip is growing. The nucleus is at a fixed distance from this tip. The branches of the root hairs have been marked with arrows and in the root hair where only one tip grows, the growing tip is marked with an arrowhead.

Prolonged mechanical inhibition of the movement of the nucleus by optical trapping leads to growth inhibition

Because the presence of the nucleus at a fixed distance from a growing tip is correlated with growth and the nucleus alternates between the growing tips of the *cow1-2* mutant, we studied the effect of direct manipulation of the position of the nucleus in growing wild-type root hairs. To achieve non-invasive manipulation of the nucleus, an optical trap was used. This technique allows the trapping of a micrometer-sized object with an index of refraction greater than the surrounding

medium in a focused laser beam. In practice, we trapped the nucleolus, which has a higher density and hence a higher index of refraction than the nucleus, and also is smaller (specific conditions of the optical trap are given in methods). During the experiments, we caught the nucleolus in the laser beam and were able to keep it at a fixed position, so that movement of the nucleolus, and thus the nucleus, was inhibited. It is not feasible to trap the nucleus and observe the root hair tip simultaneously because of the large distance between nucleus and hair tip at the high magnification required. Therefore it was not possible to check for root hair growth before the optical trap was released, so root hair growth was checked before and after optical trapping of the nucleus.

When the nucleolus was held in a fixed position, in the first minutes of the experiments, the position of the nucleolus in the nucleus changed, because the nucleus moved forward at the speed of growth, maintaining the fixed distance to the growing tip. After 1-3 minutes, when the nucleolus was located at the rear end of the nucleus, nuclear movement was inhibited and the distance between nucleus and tip increased at the speed of cell growth.

When the trap was released after less than 10 minutes, root hair growth continued. After 10-15 minutes of continued trapping, the nucleus suddenly struggled to move toward the root hair tip for about half a minute. When the trap was released after this time root hair growth was found to have stopped. Since a root hair grows 10-15 μm in 10-15 minutes, the nucleus struggles to move out of the trap when the distance between the nucleus and the tip has increased by 10-15μm. This increased distance correlates very well with a value slightly greater than the largest known distance between the nucleus and the tip in growing root hairs. In addition, inspection of the cyto-architecture of the root hair at this time revealed that the sub-apical cytoplasm dense region found in growing root hairs was disappearing, and that the cyto-architecture of the hair was becoming more similar to that of a fully-grown hair. On a few occasions, the generated force on the nucleus in the basipetal direction was so great that the nucleolus escaped from the trap. However, it never escaped towards the tip, but only backwards After this basipetal movement of the nucleus, its position in the root hair did not alter for at least 30 minutes (n=5).

To determine whether optical damage of the nucleolus, or its movement inside the nucleus, could have caused the root hairs to terminate growth, we trapped the

nucleolus and moved the trap in the direction of growth every 30 seconds. The nucleolus relocated within the nucleus during the trapping time, but recovered its original position once the trap was released. During this experiment, the nucleus followed the growing tip, and this treatment did not lead to inhibition of growth. This experiment demonstrates that there is no relevant damage caused by optical adsorption and relocation of the nucleolus inside the nucleus. In addition, pointing the optical trap to a fixed position in the sub-apical cytoplasm did not lead to changes in growth speed and nuclear positioning, showing that the trapping procedure does not disturb the tip growth cytoarchitecture. In table 1, the results of the different treatments are listed. We conclude that the location of the nucleus plays a role in the growth of root hairs and that the sub-apical cytoplasm dense area plays a role in the localization of the nucleus in wild-type growing root hairs.

Table 1. Mechanical increase of the distance between the nucleus and the tip by trapping the nucleolus in a laser beam in growing root hairs led to inhibition of growth after 10-15 minutes.

Growth before trapping	Trapping time (min)	Treatment	Growth after trapping (μm/minute)
+	5:00	Nucleolus	0.90
+	5:00	Nucleolus	0.86
+	6:00	Nucleolus	0.96
+	10:00	Nucleolus	0.03
+	12:00	Nucleolus	0.05
+	13:00	Nucleolus	-0.01
+	17:00	Nucleolus	0.04
+	26:00	Nucleolus	0.00
+	10:00	Follow	0.87
+	10:00	Follow	0.93
+	15:30	Follow	0.93

The treatments are trapping of the nucleolus at a fixed position (Nucleolus) and the control as described in the text (Follow). Pointing the trap for at least 15 minutes to a location in the cytoplasm did not lead to growth inhibition (n=5 traps of cytoplasm).

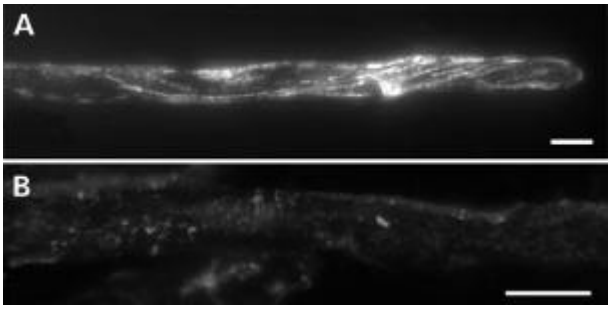

Figure 4. The microtubule cytoskeleton in *Arabidopsis* root hairs

Microtubules form a shallow helix in *Arabidopsis* root hairs and are cortically located (A). Application of 1µM oryzalin led to depolymerization of all microtubules within 5 minutes (B).

Role of the cytoskeleton in maintaining a fixed distance between the nucleus and the hair tip

Microtubules form a shallow helix in the cortical cytoplasm of *Arabidopsis* root hairs (Figure 4A). Addition to the medium of 1µM oryzalin, which specifically binds to tubulin dimers and prevents their incorporation into microtubules (Anthony et al., 1998), led to complete depolymerization of all microtubules in root hairs within 5 minutes (Figure 4B). Time-lapse imaging shows that during and after microtubule depolymerization, root hair growth continued at the same speed of ~1µm (0.96±0.15 µm compared to 0.93±0.17 µm in untreated root hairs) per minute. The cyto-architecture of the cells did not change dramatically, but the root hairs became slightly wavy, as has been reported previously (oryzalin: Bibikova et al., 1999; *mor1* mutant: Whittington et al., 2001). Consistently, the position of the nucleus was not significantly altered by the microtubule-depolymerizing drug (67±14 µm (21%) SD, 10 minutes after oryzalin application, compared to 77±15 µm (19%) SD in untreated growing root hairs; Kolmogorov-Smirnov test). We conclude that microtubules are not essential for nuclear positioning in growing *Arabidopsis* root hairs.

Using GFP fused to the actin-binding domain of mouse talin (Kost et al., 1998), Baluška and co-workers (2000) have published the occurrence of filamentous actin in living root hairs of *Arabidopsis.* In this work the labeling of filamentous actin in the sub-apex was not very distinct, possibly as a result of the dynamics of the actin cytoskeleton or the inability of the GFP-actin binding domain of mouse talin fusion to bind to the sub-apical fine F-actin. We have previously reported that actin filament staining with fluorescent phalloidin of root hairs of *Vicia sativa* after an optimized

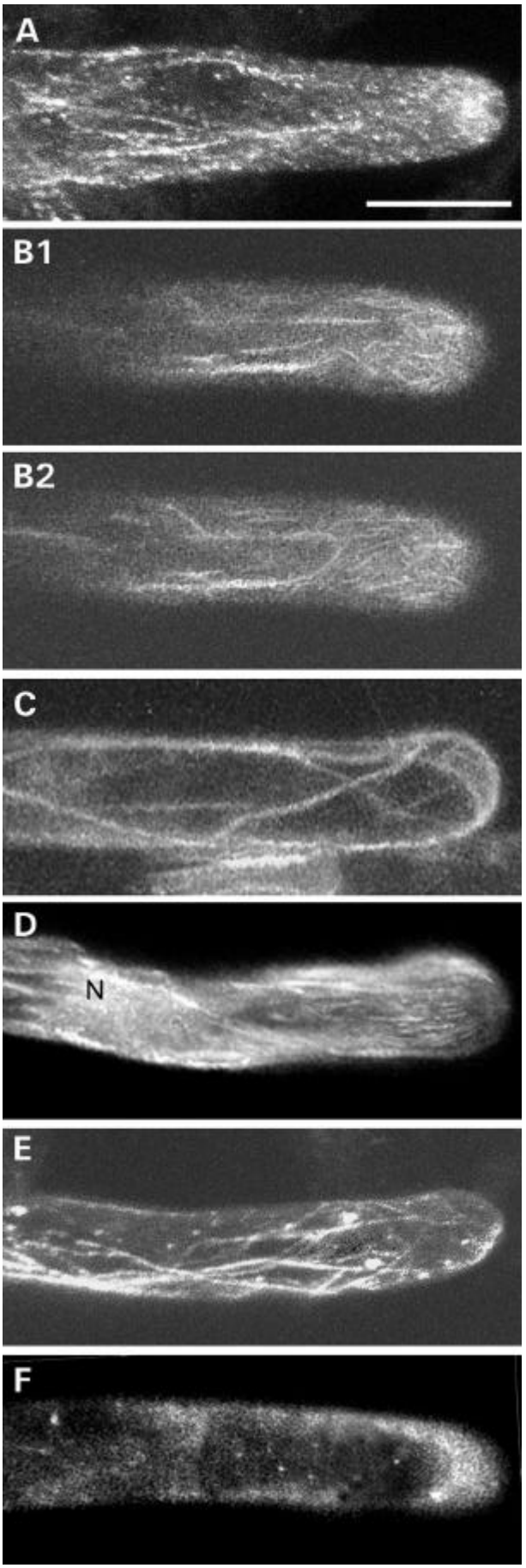
A
B1
B2
C
D
N
E
F

Figure 5. The actin cytoskeleton in *Arabidopsis* root hairs.
(A) Full projection of freeze fixed root hair labeled with anti-actin antibody. Flat-projection of a z-series of confocal images shows net-axially oriented, thick bundles of actin filaments in the root hair base that branch into a sub-apical network of thinner bundles of actin filaments. In the extreme apex of growing root hairs an area was present where actin filament bundles could not be detected. (B1 and B2) Partial projections (depth of 2 μm) of confocal images of EAC fixed and fluorescently labeled phallacidin stained growing root hair show thin bundles of actin filaments in the cortical plane (B1) and the median plane (B2) of the sub-apical cytoplasm. (C) Fully-grown root hair stained with fluorescently labeled phallacidin shows thick bundles that loop through the tip, while fine sub-apical actin filament bundles were absent. (D) A living root hair injected with fluorescently labeled phalloidin at 5 min after injection. The nucleus (N) is indicated. (E) 5 minutes after the application of 5 μM CD, the bundles of actin filaments in the root hair tube are intact, but the sub-apical fine F-actin has disappeared (freeze fixed root hair, labeled with anti-actin antibody). (F) The application of 1 μM LA led to depolymerization of all the filamentous actin within 1 min (freeze fixed root hair, labeled with anti-actin antibody).

fixation procedure (EAC: ester-aldehyde-choline, Emons and De Ruijter, 2000) gives the same results as immuno-cytochemistry after rapid freeze fixation and freeze substitution, although with more detail (Miller et al., 1999). We have now used both techniques with *Arabidopsis* root hairs. The immuno-cytochemistry technique after freeze-fixation suffered from the fact that the fine bundles of actin filaments in the sub-apex were obscured (Figure 5A), probably by antibody attached to actin monomers, as reported by Miller et al., (1999). The EAC method gave the most crisp images of the actin cytoskeleton. In growing hairs an area at the extreme tip was free of bundles of actin filaments, followed by a sub-apical area with fine actin filaments (fine F-actin) (Figure 5B1 cortex, B2 median plane of the same cell), typical of growing root hairs (Miller et al., 1999). Fine F-actin was not present in fully-grown root hairs (Figure 5C). In addition, we injected fluorescently labeled phalloidin into *Arabidopsis* root hairs, with similar results (Figure 5D). The organization of the actin cytoskeleton in the injected living hairs was similar to those fixed with the EAC procedure. Although hairs stayed alive, they mostly stopped growing, making microinjection of labeled phalloidin an unsuitable laboratory procedure for determination of the actin cytoskeleton configuration in growing root hairs.

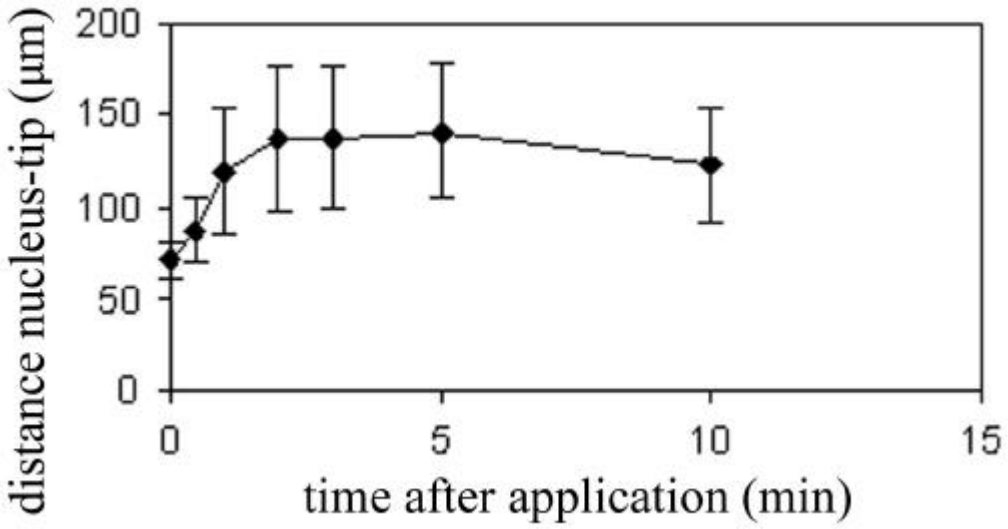

Figure 6. Positioning of the nucleus in *Arabidopsis* root hairs after the application of 5µM CD to the medium.
After CD application, the distance between the nucleus and the apex increases, whereas root hairs terminated growth. The bars represent standard deviations. n = 15 per time point.

To answer the question of whether the sub-apical fine F-actin is involved specifically in keeping the nucleus following the growing hair tip, we used cytochalasin D (CD). At a concentration of 1-5 µM, CD depolymerized fine F-actin in growing *Arabidopsis* root hairs, and kept the thicker bundles in the root hair tube (Figure 5E), and thus cytoplasmic streaming, intact as demonstrated by Miller et al. (1999) for *Vicia sativa* root hairs. The CD-induced fine F-actin depolymerization gave immature root hairs a cytoarchitecture similar to that of fully-grown root hairs (Figure 5C) and arrested their growth. In this experiment, the nucleus behaved similar to normal growth terminating root hairs and migrated away from its fixed distance from the apex back toward the root hair base (Figure 6). We conclude that in the absence of the sub-apical fine F-actin, the nucleus does not maintain its position in relation to the growing root hair tip.

Chytilova et al. (2000) demonstrated that CD-induced actin depolymerization inhibited random movement of GFP-tagged nuclei in *Arabidopsis* intercalary growing root cells. However, these experiments were carried out with high concentrations of this actin drug (50 and 100 µM cytochalasin B and D), which in our hands, leads to a complete arrest of cytoplasmic streaming. Results obtained with such high

concentrations of cytochalasin should be compared to results obtained with 1µM LA, in which case all actin filaments are depolymerized (Gibbon et al., 1999).

Role of the cytoskeleton in the basipetal movement of the nucleus when growth terminates

In growth terminating root hairs, the basipetal movement of the nucleus occurred normally in the absence of microtubules (oryzalin treatment). In the absence of the sub-apical fine F-actin (CD treatment), the nucleus behaved as in normal growth terminating root hairs and migrated from its fixed position near the hair tip backward towards the hair base. To determine whether filamentous actin is required for backward movement of the nucleus during growth arrest, we completely depolymerized the actin cytoskeleton by application of latrunculin A (LA) (Figure 5F). LA binds 1:1 to G-actin and prevents incorporation of the protein into filaments, leading to a change in the equilibrium between G- and F-actin and thus inducing actin depolymerization (Gibbon et al., 1998). The application of 1µM LA to *Arabidopsis* root hairs led to the arrest of cell growth and of cytoplasmic streaming within 1 minute. After complete actin depolymerization, the position of the nucleus did not change (mean change in position in µm from the tip at the moment of and 10 minutes after LA application was 0.23±0.16 µm (n=15)). From these data, it can be concluded that the movement of the nucleus in root hairs is based on filamentous actin. More importantly, at growth arrest, the backward movement of the nucleus from its position in the subapex of the hair did not occur in the absence of bundles of actin filaments caused by LA treatment. Together, the data obtained with actin drugs together show that during root hair growth arrest, the movement of the nucleus away from its position in the sub-apex is related to the disappearance of the sub-apical fine F-actin and that the bundles of actin filaments in the root hair tube are required for this process.

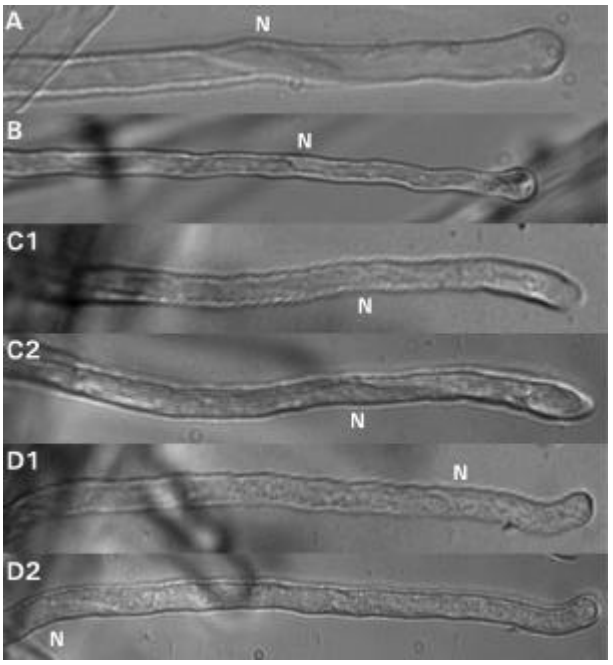

Figure 7. The nucleus does not migrate basipetally during or after growth termination if inhibition of gene transcription or protein synthesis starts during cell growth, but it migrates if inhibition starts after growth has stopped.

In root hairs that had terminated growth before the inhibition of gene transcription or translation, the basipetal movement of nuclei was not inhibited. A is a micrograph of a root hair treated for 4 hrs with 10µg/ml of the translation blocker CHI. The nucleus (N) was still located at the fixed distance from the tip. Root hair B has been treated for 4 hrs with 20µM ActD, an inhibitor of gene transcription. C1 and C2 are micrographs of a growing root hair, immediately after (C1) and 2 hrs (C2) after application of 20µM ActD. The nucleus was still located at the same distance from the tip. D1 (immediately after application) and D2 (after 2 hrs) are images of a fully-grown root hair, undergoing the same treatment. In fully-grown root hairs, the nucleus continued to migrate when formation of new proteins was blocked. During protein synthesis inhibition in growing root hairs, we observed a slight flattening of the root hair tip, which we cannot explain.

Protein synthesis is required to initiate basipetal movement of the nucleus when growth terminates

The speed of the movement of the nucleus towards the root hair base when growth terminates in naturally growth terminating root hairs varies between 0 and 1 µm per second. The initial release takes place when the root hair is still growing, but the length of the cytoplasm dense area is decreasing. Once the basipetal movement is

initiated, the nucleus moves randomly throughout the root hair, alternating with intervals wherein it does not move, as has been demonstrated by Chytilova et al. (2000).

To determine whether the release of the nucleus from its fixed position from the apex at growth arrest is an active process, we added to the medium either 10 μg/ml cycloheximide to block mRNA translation or 20 μM actinomycin D to block gene transcription. Two minutes after application of cycloheximide and 20 minutes after application of actinomycin D, root hair growth had terminated (Figure 7), but cells remained alive for the 5 hours during which they were observed and cytoplasmic streaming continued. During protein synthesis inhibition in growing root hairs, we observed a slight flattening of the root hair tip, which we cannot explain. Within the first hour, the sub-apical cytoplasmic dense area disappeared. The nucleus did not move in the basipetal direction but maintained its fixed distance from the root hair tip (62 +/- 10μm (17%) SD; n=20), (Figure 7). In root hairs that had terminated growth before application of the drugs, normal basipetal movement of the nucleus was not inhibited (Figure 7). Figure 7A shows a root hair that had been treated for 4 hrs with 10 μg/ml of the translation blocker CHI. The nucleus is still located at the fixed distance from the tip, which is normal for a growing cell. The root hair shown in Figure 7B had been treated for 4 hrs with 20 μM ActD, an inhibitor of gene transcription. The nucleus is in its place, which is normal for a growing cell. The photograph of the growing root hair shown in Figure 7C1 was taken immediately after application of 20 μM ActD and the one in Figure 7C2 was taken 2 h later. The nucleus is still located at the same distance from the tip. Figures 7D1 (photograph taken immediately after application) and 7D2 (photograph taken after 2 h) are images of a fully-grown root hair undergoing the same treatment. In fully-grown root hairs, the nucleus continues to migrate when the formation of new proteins is blocked. These results show two interesting phenomena. First, when growth is inhibited, the accumulation of sub-apical fine F-actin stops, but its bundling continues without new protein synthesis. Secondly, protein synthesis seems to be required for the release of the nucleus from its fixed position when growth terminates.

Actin filament bundling is involved in maintaining the distance between the nucleus and the tip

Because sub-apical fine F-actin is required for keeping the nucleus at a fixed distance from the growing hair tip, because for its movement away from the tip at growth arrest the production of new protein is needed, and because for the actual backward movement bundles of actin filaments are required, we wondered whether actin bundling is involved in keeping the nucleus in place in growing hairs and/or its movement away from the tip during growth arrest.

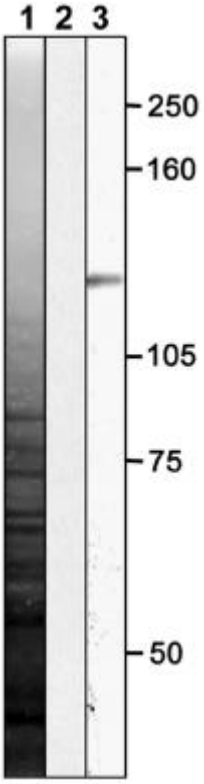

Figure 8. Protein gel blot of *Arabidopsis* root extract, labeled with lily anti-villin antibody.
On Arabidopsis root extract, the antibody gave a band of about 135kD (lane 3), comparable to the mass of lily villin. Lane 1 is the silver stained nitrocellulose membrane and lane 2 has been labeled with rabbit pre-immune serum.

In common with *Vicia sativa* root hairs (De Ruijter et al., 1999), progressive bundling of actin filaments, from the apex to the nucleus, occurred in the sub-apical area of growing *Arabidopsis* root hairs (Figure 5A, B, C, D). The elongated nucleus is surrounded by actin filaments and located in the basal part of this area. To determine whether bundling of actin filaments is involved in maintaining the fixed distance

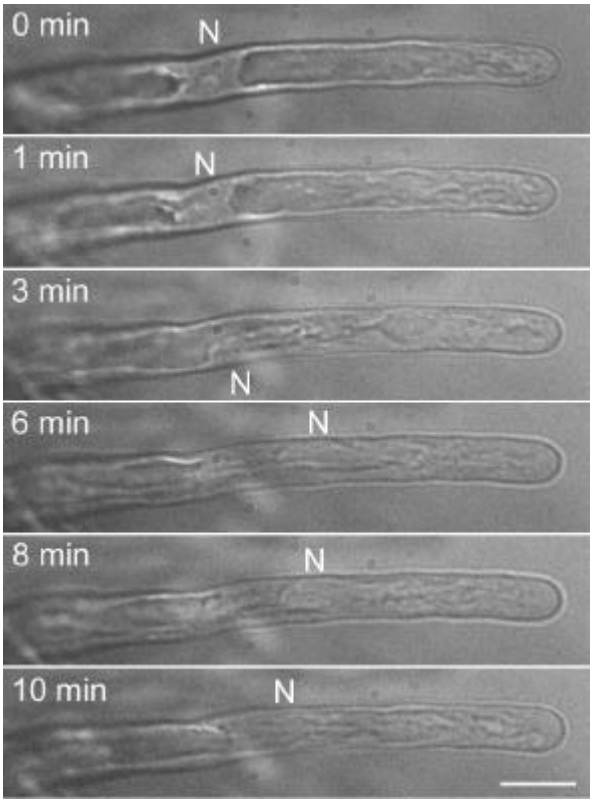

Figure 9. Injection of lily anti-villin antibody causes an increase in the number of cytoplasmic strands.

During the first minutes after injection, newly formed cytoplasmic strands emerged through the vacuolated area of the root hair as a result of the unbundling of the bundles of actin filaments. 3 minutes after injection, the nucleus moves towards the apex, until it stopped approaching the apex after 6-8 minutes (see figure 10). Nuclei (N) are indicated. Bar=10μm.

between the nucleus and the growing tip in root hairs, we injected an antibody against a villin-like actin binding protein derived from plants.

Villin-like actin binding proteins are ubiquitously expressed in *Arabidopsis* (Klahre et al., 2000). Yokota and Shimmen (1999) raised an antibody against one such protein of 135kD from lily. Injection of this antibody in *Hydrocharis* root hairs demonstrated that the protein has actin bundling activity. After injection of the antibody against lily villin-like protein, fusion of transvacuolar cytoplasmic strands into one transvacuolar strand was inhibited, leading to numerous transvacuolar cytoplasmic strands (Tominaga et al., 2000). Because actin filaments are the backbones of cytoplasmic strands (Valster et al., 1997), one can infer from this result that lily villin-like protein bundles actin filaments. The lily anti-villin antibody recognises specifically a band of approximately 135kD in *Arabidopsis* root protein extracts (Figure 8). We tested

whether actin bundling is involved in determining the position of the nucleus in growing root hairs by injecting lily anti-villin antibody into *Arabidopsis* root hairs and studying the positioning of the nucleus. Injection of anti-villin into *Arabidopsis* root hairs increased the number of cytoplasmic strands, as did injection of this antibody into *Hydrocharis* root hairs (Figure 9). Interestingly, during the unbundling process after anti-villin injection, the nucleus moved forward 10-20 μm, 3 to 5 minutes after injection, after which it oscillated around that position and moved slightly backwards but did not reach its original position (Figure 10). Injection of pre-immune rabbit serum did not lead to significant relocation of the nucleus or other

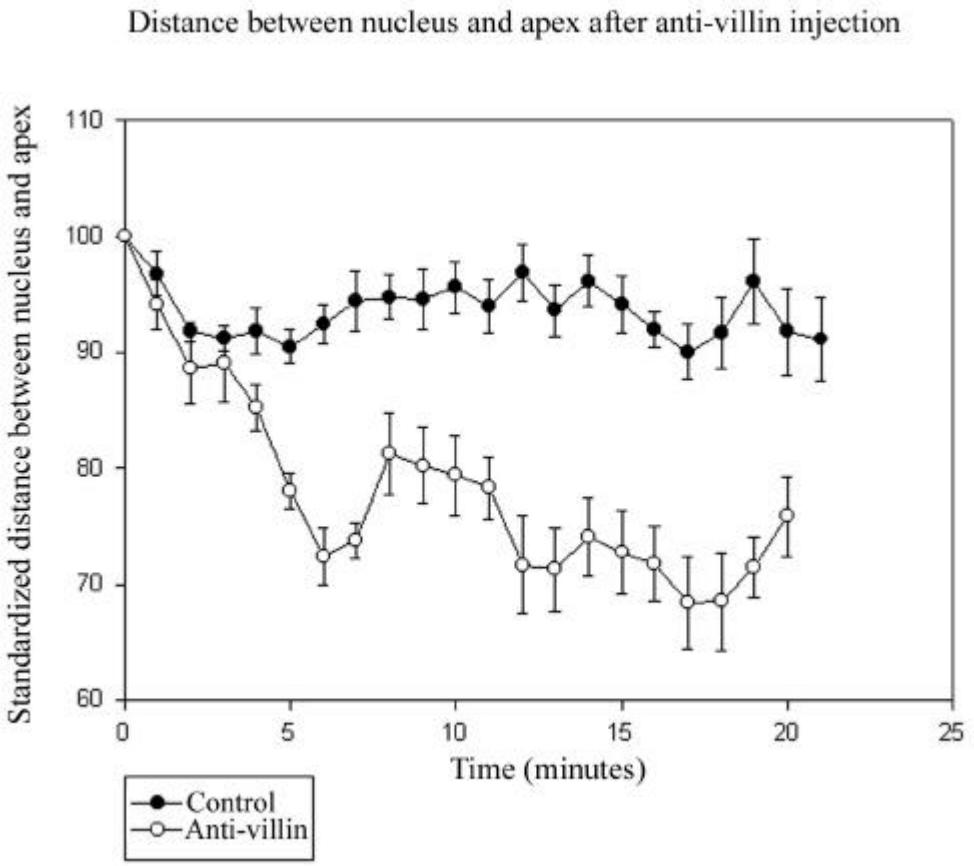

Figure 10. Injection of lily anti-villin leads to a decrease in the distance between the nucleus and the tip.

The distance between the nucleus and apex after injection of a 1:10 dilution of anti-villin antibody into growing *Arabidopsis* root hairs (n=6). As control 1:10 diluted rabbit pre-immune serum was used (n=4). The distance between the nucleus and the tip was standardized by setting the distance before injection to 100 in all observed root hairs. Error-bars indicate standard errors.

cellular changes. During injections of either anti-villin or pre-immune rabbit serum, growth continued in most root hairs (80%; N=10) during and after injection. The root hairs that terminated growth after injection were evenly distributed among anti-lily villin and control injections (1 in 6 for the experiment, 1 in 4 for the pre-immune serum control), indicating that the anti-villin antibody does not inhibit growth. Figure 11 shows the positions of nuclei in relation to the actin cytoskeleton in growing root hairs and after anti-villin microinjection.

The results lead us to conclude that the actin binding villin-like protein bundles the sub-apical fine F-actin into thicker bundles of actin filaments in the root hair tube in a gradient away from the hair tip. The forward movement of the nucleus during the inhibition of actin bundling shows that actin bundling is involved in maintaining a fixed distance between the nucleus and the growing apex. Actin filament bundling appears to prevent the nucleus from approaching the growing root hair tip.

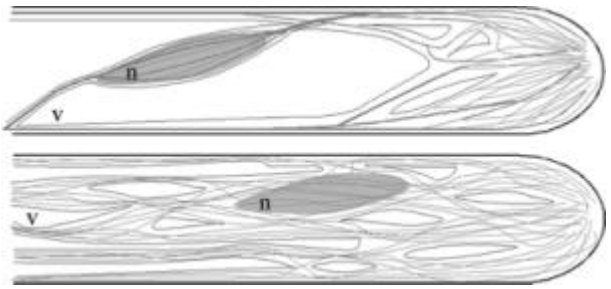

Figure 11. Effects of unbundling of the actin filaments.
Actin cytoskeleton configuration and the location of the nucleus (N) in control root hairs (top) and after injection of anti-villin antibody (bottom). Vacuoles (V) are indicated.

Discussion

In tip growing root hairs, growth occurs abundantly at one side of the cell and the nucleus follows the growing cell tip at a certain distance. This fact has been observed many times and the advantage for a cell to keep the nucleus in the vicinity of the site of abundant protein production for plasma membrane growth and new wall deposition is obvious. As in other species studied, in growing *Arabidopsis* wild type root hairs, the nucleus moves relative to the root hair base, but does not move relative to the

root hair tip; it keeps pace with the advancing tip. How is this distance maintained? An obvious possibility could involve the microtubule cytoskeleton.

It has been shown that without microtubules (oryzalin treatment: Bibikova et al., 1999; *mor1* mutant: Whittington et al., 2001) *Arabidopsis* root hairs grow normally, though with a wavy morphology. Here we show in addition that the nucleus persists in following the growing tip. The physical link that keeps the nucleus tethered in the sub-apex does not seem to be microtubule cytoskeleton based in *Arabidopsis*. However, data obtained with legume root hairs indicate that microtubules do play a role in nuclear positioning. Lloyd and co-workers (1987) reported that microtubules are responsible for the connection of the nucleus with the tip in growing root hairs of *Vicia hirsuta*. More recently, Sieberer et al. (2002) reported endoplasmic microtubules in the sub-apex of *Medicago truncatula* root hairs. After depolymerization of these microtubules, they observed decreased growth speeds and backward movement of the nucleus. These observations indicate that differences between species may occur.

Sub-apical fine F-actin as the agent that keeps the nucleus in the vicinity of the growing hair tip

One specific type of actin configuration that could be a good candidate for maintaining the nuclear position is sub-apical fine F-actin. Sub-apical fine F-actin has been previously described as a configuration of fine bundles of actin filaments (FB-actin), which are progressively more and more bundled away from the hair tip (*Vicia sativa*: Miller et al., 1999; *Arabidopsis*: Figure 5A, B, C, D). Sub-apical fine F-actin is enhanced by lipochito-oligosaccharides from *Rhizobium* bacteria (De Ruijter et al., 1999), molecules that reinitiate growth in growth terminating legume root hairs (De Ruijter et al., 1998). During root hair growth the sub-apical area with fine F-actin maintains a more or less constant length of 63±17 μm in *Arabidopsis*. This is achieved by constant bundling between the actual sub-apical fine F-actin and the nucleus and constant elongation of the sub-apical fine F-actin at the hair tip where the Golgi-derived vesicles accumulate. From this we deduce that in the sub-apical fine F-actin the filaments point with their plus sides toward the hair tip, however this idea remains has to be proven.

Pharmacological experiments provide good evidence that the maintenance of the nucleus with the growing root hair tip is an process, involving the sub-apical fine F-actin cytoskeleton. CD, which caps growing actin filaments (Cooper, 1987), at low (1-5 μM) concentration depolymerizes the sub-apical fine F-actin only, as has been shown for vetch root hairs (Miller et al., 1999). This results initially in cessation of the delivery of Golgi-derived vesicles and consequently an arrest of cell elongation, although cytoplasmic streaming continues. Similarly, LA, a G-actin binding molecule, at low concentration initially depolymerizes sub-apical fine F-actin and arrests root hair growth (data not shown), but this drug also depolymerizes the thicker bundles of actin filaments in the remainder of the hair and consequently stops cytoplasmic streaming. These results indicate that sub-apical F-actin and its continuation of actin filaments around the nucleus is (part of) the physical link between nucleus and hair tip.

The link between nucleus and tip is severed, or "snaps" when the distance between nucleus and tip is experimentally increased by trapping of the nucleus. However, this direct physical link may include the sub-apical longitudinal endoplasmic reticulum that also encircle the nucleus (Miller et al., 2000). This type of ER configuration is supported and probably produced and configured by the sub-apical fine F-actin. The "snapping" occurs after the nucleus is trapped for 10-15 minutes, which is exactly the time in which a root hair grows 10-15 μm. When a root hair elongates 10-15 μm, the distance between the hair tip and the nucleus just exceeds the maximal distance known from the growing control hairs. This suggests that the trapping of the nucleus causes the disappearance of the sub-apical area containing fine F-actin in a way that is comparable to physiological growth arrest and is not an unphysiological rupture of the actin cytoskeleton. Detailed analysis of the actin cytoskeleton during trapping would be advantageous but is not yet possible because of the limited ability to visualize the sub-apical fine F-actin in living cells (GFP-talin: Baluška et al., 2000).

We do not know if and where fine F-actin attaches to the plasma membrane of the hair tip. To our knowledge no work has reported the presence of filamentous actin in the extreme tip of growing root hairs, but with the procedures used here, the thinnest bundles or single filaments may be invisible in the confocal laser scanning microscope. Therefore, we do not know whether actin polymerization takes place at

the plasma membrane, as has been suggested for animal cells (recent review: Sechi and Wehland, 2000). Interestingly, using the new technique of single molecule speckle analysis of actin filament turnover in lamellipodia, Watanabe and Mitchison (2002) have shown that also in these cells, contrary to previous expectations, the majority of actin filaments are generated away from the very tip. Another possibility would be that the sub-apical fine F-actin attaches to and/or polymerizes from the Golgi-derived vesicles in the cell's apex, or that the sub-apical longitudinal ER is the site of actin filament nucleation. These are important questions to answer in future work.

Actin bundling as the agent that inhibits the nucleus from reaching the hair tip

As discussed above, at the location of the hair tip the sub-apical fine F-actin elongates constantly in growing root hairs, and on the other side actin filaments are bundled constantly. Therefore, we asked whether actin bundling also is involved in keeping the nucleus in place. Villin is an actin bundling protein. Villin-like proteins are known to be present in *Arabidopsis* (Klahre et al., 2000). Therefore we attempted to increase actin bundling in our system by injecting an antibody against a plant villin-like protein into the *Arabidopsis* root hair. The injection of lily anti-villin antibody has previously been shown to unbundle bundles of actin filaments in *Hydrocharis* root hairs (Tominaga et al., 2000), and lily anti-villin antibody cross-reacted with a polypeptide of similar molecular mass in an *Arabidopsis* root extract (Figure 8). Indeed, injection of this antibody unbundled the sub-apical cytoplasmic strands of growing *Arabidopsis* root hairs further and increased the length of this area such that the sub-apical fine F-actin containing cytoplasmic strands between original sub-apical fine F-actin and nucleus also became unbundled. Consequently, the nucleus moved forward toward the hair tip. This result is direct proof that bundling of the actin cytoskeleton plays a role in nuclear positioning, keeping the nucleus at a fixed distance from the growing root hair tip.

In vivo, the actin cytoskeleton is under control of many actin interacting proteins, some of which are sensitive to the concentration of specific ions, fore example calcium. Therefore, as has been shown in pollen tubes, ion gradients may play an

indirect or direct role in the organization of the actin cytoskeleton (review: Hepler et al., 2001) and thus the positioning of the nucleus.

Requirement of bundles of actin filaments and new protein synthesis for nuclear movement during growth arrest

What could happen during physiological growth arrest? During growth arrest, the sub-apical fine F-actin area becomes progressively shorter because fine F-actin no longer elongates on the tip side, whereas bundling of actin filaments and, consequently, cytoplasmic strands, continues on the other side. After the sub-apical fine F-actin has disappeared, no new vesicles are delivered and the residual Golgi-derived vesicles are consumed at the plasma membrane of the root hair tip after which cell growth stops. Thus, actin filament polymerization stops, although actin filament bundling continues. The study of the *cow1-2* mutant supports this hypothesis. In this mutant with branched root hairs, the nucleus oscillates between the two tips. The tip it is closest to is the one that elongates, although with a certain lag time. The tip from which the nucleus moves still elongates slightly, possibly because the Golgi-derived vesicles already present in the vesicle rich area are still being used. Similarly, it could take time before a newly approached tip elongates because first the Golgi-derived vesicles needed for cell elongation may have to be delivered. If this hypothesis is true, the results from the *cow1-2* mutant would demonstrate that the nucleus approaches the tip for vesicle delivery, but not for vesicle insertion, the growth process per se. This phenomenon also is seen when cell growth is terminated by sub-apical fine F-actin breakdown by CD or when the nucleus is artificially kept at an greater distance from the tip by trapping it in a laser beam.

The experiments in which LA was used show that a functional actin cytoskeleton in the root hair tube, consisting of the thicker bundles of actin filaments, is required for the movement of the nucleus at growth arrest. The experiments with transcription/translation inhibitors provide an interesting clue about the mechanism of growth arrest. They show that for the hair base-directed movement of the nucleus during growth arrest, gene transcription and translation is required. Because we

found that the bundling of actin filaments and the fusion of Golgi-derived vesicles goes on during growth arrest, but the sub-apical fine F-actin area becomes shorter and vanishes, the newly formed protein should stop, directly or indirectly, the polymerization of fine F-actin at the plasma membrane/ER/Golgi-derived vesicle membrane.

A tip growth scenario

The purpose of our study was to investigate how the cell accomplishes its persistent localization of the nucleus at a set distance from the advancing cell tip and, more specifically, the role of the cytoskeleton in this process. Our results indicate the following: (I) fine F-actin, a subset of actin filaments in the cell's sub-apex between nucleus and hair tip (i.e., distal from the nucleus) functions in keeping the nucleus following the growing hair tip. (II) actin bundling between sub-apical fine F-actin and nucleus inhibits the nucleus from reaching the hair tip. (III) New protein synthesis is required to move the nucleus back from the tip at root hair growth arrest. (IV) Bundles of actin filaments in the hair's tube are needed for this movement of the nucleus away from the hair tip at growth arrest.

A possible scenario to be studied further is the following. Actin filament polymerization is nucleated at the plasma membrane/ER/Golgi-derived vesicle membranes in the vesicle-rich area of the root hair tip. An actin bundling protein produces a gradient of more and more bundled actin filaments with in between ER cisternae away from the growing tip. In these fine F-actin supported ER cisternae the nucleus is caught. The nucleus follows the tip because it is connected to the elongating fine F-actin at the hair tip, but bundling of actin filaments at the base of the sub-apical fine F-actin keep the nucleus from approaching the very tip. Our study has clarified some details about mechanisms of nuclear positioning during tip growth, and has laid the basis for further studies leading to discovery of proteins involved in plant cell (tip) growth.

Methods

Plant growth and drug treatment

Seeds of *Arabidopsis thaliana* plants (ecotype Columbia) were germinated on top of a chamber of a glass slide and a coverslip so that the roots grew into the chamber. Germination was carried out in 1/2 strength Murashige and Skoog medium (Duchefa, Haarlem, The Netherlands) for 5 days at 25 °C, 16 hrs light, 8 hrs darkness. Drugs were applied by gradually replacing the medium by medium with added drugs in the growth room. Oryzalin (Greyhound, Birkenhead, UK), cytochalasin D (Sigma, St. Louis MO, USA) and latrunculin A (Molecular Probes, Leiden, The Netherlands) stocks were made in DMSO and at least 1000 fold diluted in the medium. Cycloheximide and actinomycin D stocks were made in methanol.

Immuno-cytochemistry

For immuno-fluorescence, plants were fixed by rapid freezing in liquid propane. Freeze substitution was carried out in methanol + 0.5% glutaraldehyde and for actin labeling in 0.4 mM MBS-ester (Sigma) at -90°C. After fixation, the temperature was stepwise increased (via -60°C and -30°C) over 10 hrs to 0°C. The freeze substitution medium was replaced stepwise (10%, 30%, 50%, 70%, 90%, 100%v/v) by buffer (for actin labeling actin stabilizing buffer (ASB, Miller et al., 1999) and for microtubules phosphate buffered saline (PBS)) with 2% paraformaldehyde and 0.05% glutaraldehyde. After washing in buffer, the material was incubated in a saturated suspension of driselase (Kyowa Hakko Kogyo, Tokyo, Japan) and macerozyme R10 (Serva, Heidelberg, Germany) in MES (30°C, pH 5.8, 40'). The enzymes were washed away in buffer and the samples were blocked in 0.1% acetylated BSA (Aurion, Wageningen, The Netherlands) for at least 3 min. As primary antibody either the monoclonal anti plant actin clone 3H11 (Andersland et al., 1994) or DM1a, a monoclonal anti α-tubulin (1:300 diluted, Sigma) was used and as secondary antibody GaM-IgG-Cy3 (Molecular Probes). Before microscopic analysis, the material was washed in PBS-citifluor (Citifluor, Canterbury, UK) and mounted in Prolong antifade (Molecular Probes).

Staining of actin filaments after improved EAC fixation

Five days old roots of *Arabidopsis thaliana* were fixed for 2 min with 100 µM m-maleimido benzoyl N-hydroxysuccinimide (MBS) ester in 1% freshly prepared paraformaldehyde and 0.025% glutaraldehyde in demineralized water, followed by 200 µM ester in 2% paraformaldehyde and 0.025% glutaraldehyde in demineralized water for 10 minutes. Roots were post-fixed for 20 min in a final concentration of 3% paraformaldehyde and 0.075% glutaraldehyde in Actin Stabilizing Buffer (ASB: 50 mM Pipes pH 6.8, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 37 mM KCl). Subsequently, root hairs were permeabilized for 2-3 min with 100 µg/ml L-α-lyso-phosphatidylcholine in ASB. Actin filaments were stained with 0.33 µM Bodipy-FL (505/512)-phallacidin (Molecular Probes B607) in ASB supplemented with 0.05% acetylated bovine serum albumin (BSA_{ac}) (Aurion, Wageningen, NL) to lower aspecific binding. Roots were mounted in glycerol.

Microinjection

For antibody injection, growing root hairs were pressure-microinjected with a 1:10 dilution of lily anti-villin antibody or pre-immune serum in PBS, and for actin visualisation, root hairs were injected with 0.66 µM Alexa-488-phalloidin (Molecular Probes) in 1/2 strength PBS. Root hairs were grown in a thin layer of 0.1% Phytagel (Sigma) in 1/2 strength Murashige and Skoog medium and injected with micropipettes, pulled with a vertical pipette puller (David Kopf Instruments, model 700C) from borosilicate glass capillaries containing an internal filament (World Precision Instruments). Root hairs were injected at the base with a water pressure microinjection device (water pressure device: Gilmont, Barrington, IL, USA; needle holder: Eppendorf, Merck Eurolab BV, Amsterdam, The Netherlands).

Western blotting

Arabidopsis plants were grown on 1/2 strength Murashige and Skoog medium (Duchefa, Haarlem, The Netherlands) containing 0.6% agar for 3 weeks at 20 °C, 16 hrs light, 8 hrs darkness. Immediately after they had been cut from the plants, roots were frozen in liquid nitrogen, ground with pestle and mortar and transferred to boiling SDS sample buffer (0,0625M Tris-HCl, (pH 6.8), 2.3% w/v SDS, 5% 3-

mercaptoethanol, 10% glycerol, 0.00125% bromophenolblue) for 3 minutes. SDS-PAGE was performed on a 6% acrylamide gel (Laemmli, 1970), whereafter the proteins were electrophoretically transferred to nitrocellulose blotting membrane (BDH).
The blot was probed with either 1:1000 diluted antiserum against ABP 135 (the lily villin) or 1:100 diluted rabbit pre-immune serum, followed by 1:5000 anti-rabbit IgG horseradish peroxidase (Amersham). The signal was detected with ECL (Amersham).

Microscopy and image processing

For measurement of the distance between apex and nucleus, plants were prefixed with 1 μM m-maleimido benzoyl N-hydroxysuccinimide ester (MBS-ester) to arrest cytoplasmic streaming and fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in medium. Nuclei did not displace during this procedure. After fixation, nuclei were stained with 1 μg/ml DAPI and the distance from the middle of the nucleus to the apex was measured. Images were acquired either on an Nikon Microphot FXA equiped with a Photometrics Quantix cooled CCD camera, combined with IP-Lab spectrum 3.1 image analysis software (Scanalytics, Fairfax VA, USA), or on a BioRad MRC-600 confocal laser scanning microscope (CLSM) equipped with an Argon Krypton laser with a 60x 1.4 NA oil objective. A neutral density filter was set to obtain 10% transmission intensity of a 5 mW 488 nm laserbeam to excite the Bodipy-FL or a 5mW 568 nm laserbeam to exite the Cy3. For imaging a DM 488 BA 522 DF 35 filter was used for Bodipy-FL and a DM 568 BA 585 EFLP filter for Cy3. Images were obtained as z-series with 0.5-1 μm intervals with a moderately closed pinhole (setting 3 or 4), with high gain settings (80-100) and an average of 2-3 scans (Kalman filter). Images were processed with Confocal Assistant (Bio-Rad Software, Hertfortshire, UK) and Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA, USA).

Time-lapse sequences were acquired by placing the plant growth chamber in a inverted Nikon Diaphot 200 microscope, equipped with Hoffman Modulation Contrast.

Trapping of the nucleolus with optical tweezers

A Nd:YVO4 laser beam (1064 nm CW, Spectra Physics) was introduced into a 100x/1.3 N.A. oil immersion objective (DMIRB inverted microscope, Leica), for wich a dichroic mirror was used, positioned below the objective. The trap position within the specimen plane was controlled using a lens telescope mounted on a XYZ translation stage in a conjugate plane with the back focal plane of the objective. The multiple laser spots were created by time-sharing the laser beam with two acousto-optical deflectors (IntraAction) digitally controlled by computer (Visscher et al., 1996). The standard laser power used to hold the nucleolus was about 300-400 mW in the sample.

Upon request, all novel material described in this publication will be made available in a timely manner for non-commercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use on non-commercial research purposes.

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Chapter 4

The role of microtubules in root hair growth and cellulose microfibril deposition

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1 Introduction, cortical microtubules in plant cells

Cortical microtubules (CMT) were first observed in plant cells (Ledbetter and Porter 1963) by transmission electron microscopy. In intercalary growing cells, CMT have always been found oriented with a right angle to the elongation axis of the cell (reviewed in Cyr 1994). Moreover, if CMT are depolymerized by addition of pharmalogical agents, cells expand isotropically (colchicine: Green 1962; dinitroaniline herbicides: Upadhyaya and Noodén 1977; Baskin et al 1994). Stabilization of CMT by taxol also leads to more isotropic growth (Baskin et al 1994). Furthermore, growth regulators affect the orientation of the CMT (Van Spronsen et al 1995). Most researchers agree that the orientation of the CMT determines the direction of cell expansion.

One hypothesis as to how the CMT could determine the orientation of cell expansion, namely by orienting the deposition orientation of the cellulose microfibrils (CMF) of the cell wall (Heath 1974). The CMT could perform this process by channeling the cellulose synthases through the plasma membrane (Giddings and Staehelin 1991). This is a plausible hypothesis, since in all growing cells the CMT and the nacsent CMF are found in parallel to each other, both directed tranverse to the orientation of cell elongation (reviewed in Cyr 1994). One can imagine that abundant transverse hoops of stiff cellulose microfibrils would limit cell elongation to the direction transverse to the hoops (Gertel and Green 1977). However, there is no agreement among researchers that CMT determine the orientation of the CMF by default (Emons et al 1992).

Some of the reasons for the lack of agreement are that the pharmacological agents that depolymerize microtubules have many different effects on the orientation of the CMF (review: Robinson and Quader 1982; Emons et al 1992), such as random deposition (Schnepf et al 1975), deposition is swirls (Hepler and Fosket 1971), ordered deposition without regular changes in the orientation that occurs without treatment (Green at al 1970) or no change in deposition (Emons et al1990; Schmid and Meindl 1992). Furthermore, a co-alignment of CMT and CMF is often not found in secondary walls which have an even deposition along the plasma membrane (review: Emons et al 1992). Since in the absence of CMT ordered deposition occurs, one expects an ordering mechanism independent of CMT. A drawback in many

studies is that researchers did not check whether the microtubules were really depolymerized, or whether growth really continued; and in many studies appropriate methods for the visualization of CMF were not used. Uranyl acetate and lead citrate post staining after glutaraldehyde osmium tetroxide fixation does not reliably stain the CMF. Stripes may be seen on the images, which have no relationship whatsoever with the CMF (Emons 1988). Appropriate visualization methods for CMF are (Emons 1988): freeze fracturing, cleaving and shadowing with or without wall matrix extraction (Chapter xx), staining of sections of matrix-extracted material, and specific polysaccharide staining of fixed cells.

Experiments with drugs that inhibit CMF formation indicate that vice versa the CMF provide spatial cues for CMT organization (Fisher and Cyr 1998). Thus, CMT determine the orientation of cell elongation, and this process may be mediated by CMF. However, such correlations do not prove that CMT regulate the orientation of CMF deposition by default. As mentioned above, in growing cells the orientation of the CMF and the orientation of the CMT both correlate with the direction of growth. Therfore, these cells are not suited to discriminate between the function of the CMT in cell growth, whether or not mediated by CMF, and CMF deposition orientation itself.

Because root hairs are tip-growing cells, the wall deposited at the tip is a primary cell wall. A secondary cell wall is deposited in the tube of the hair, against this primary cell wall. The new wall deposited in the hair tube is a secondary wall by definition, since in the tube cell expansion does not take place. Furthermore, since this secondary wall is deposited sequentially from root hair base to root hair tip, all stages of wall development are represented in a single root hair. Since the deposited CMF in this non-growing tube do not change position, they form a record of the deposition history. This makes root hairs, especially those with non-uniform CMF deposition angle, ideal cells to study the function of the CMT in CMF deposition orientation, independently from their function in cell growth.

In this chapter, we will review our knowledge of interphase microtubules in root hairs. We will discuss the role of the CMT in tip growth and show that there is no role for CMT in CMF orientation during deposition. Therefore, other possible functions will be discussed as well as the role of the occurrence and possible role of endoplasmic CMT.

2 Cortical microtubules in root hairs

2.1 Growing root hair tip

Newcomb and Bonnett (1965) and Seagull and Heath (1980) reported an absence of CMT in the tip of radish root hairs. However, they used chemical fixation, which is now known to cause fixation artifacts. Lloyd and Wells (1985) used various chemical fixation protocols and showed that the CMT in tips of radish root hairs are sensitive to fixation conditions. With improved chemical fixation they could detect CMT at the tip of growing root hairs (Lloyd and Wells 1985). Currently, the most reliable fixation method is freeze fixation, where the first fixation step is physical. In freeze substitution studies of Equisetum hyemale root hairs (Emons and Derksen 1986; Emons 1987), Emons (1989) has shown that CMT are present at the plasma membrane of the tips of growing hairs. CMT have a random orientation in the hemisphere. The microfibrils in the hemisphere are also randomly oriented (Fig. 1) (Emons 1989). Thus, in the root hair tip, like in other expanding cells, the nascent CMF lie parallel to the CMT, perpendicular to the direction of expansion. Expansion in a semi-hemisphere is in all orientations. This is the only study so far in which the microtubule cytoskeleton was investigated in depth in tips of growing root hairs with freeze substitution electron microscopy. In published micrographs of another study using freeze substitution electron microscopy, but not primarily directed to the investigation of CMT, CMT can be observed in areas where tip growth takes place (Galway et al 1997). We conclude that in the growing area of root hairs, just like in other growing cells, the orientation of both CMT and CMF is perpendicular to the direction of growth, and may determine the orientation of expansion.

2.2 Root hair tube

2.2.1 Techniques for visualisation of CMT, orientation of CMT

The CMT in the root hair tube of a variety of species have been analyzed with many different techniques. Radish (*Raphanus sativus*) root hairs were one of the first cell types in which the CMT were studied (Newcomb and Bonnett 1965). Like in all early ultrastructural studies, the cells were chemically fixed, followed by resin embedding

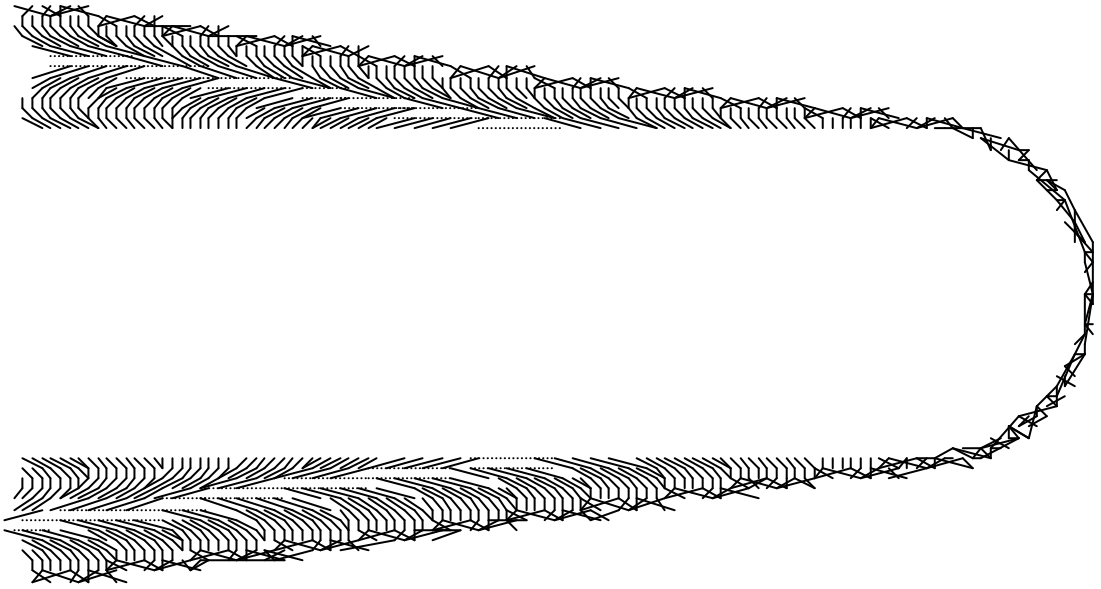

Figure 1. Cartoon, displaying the different stages of helicoidal cell wall development which are present in the tube of a growing *Equisetum hyemale* root hair.

and post staining. High concentrations of glutaraldehyde (up to 6%) were used, in combination with osmium tetraoxide. In grazing sections of the cortical cytoplasm of the root hair tube, microtubules were observed in the electron microscope in axial orientations. These observations were confirmed by Seagull and Heath (1980). In *Raphanus sativus* (Newcomb and Bonnett 1965; Seagull and Heath 1980) and *Equisetum hyemale* (Emons 1982; Emons and Wolters-Arts 1983) net-axially oriented CMT were observed in chemically fixed electron microscope sections. Two different orientations of CMT in root hairs have been reported with immunofluorescence after chemical fixation. Net-axial CMT were found in *Equisetum hyemale*, *Limnobium stoloniferum, Lepidium sativum*, *Ceratopteris thalictroides* (Traas et al 1985), *Raphanus sativus* (Lloyd and Wells 1985; Traas et al 1985) and helical CMT were found in *Allium cepa* (Lloyd 1983; Lloyd and Wells 1985; Traas et al 1985), and *Urtica dioica* (Traas et al 1985; Van Amstel and Derksen 1993). *Equisetum hyemale* and *Raphanus sativus* root hairs were studied with dry cleaving, which confirmed their net-axial orientation (Traas et al 1985).

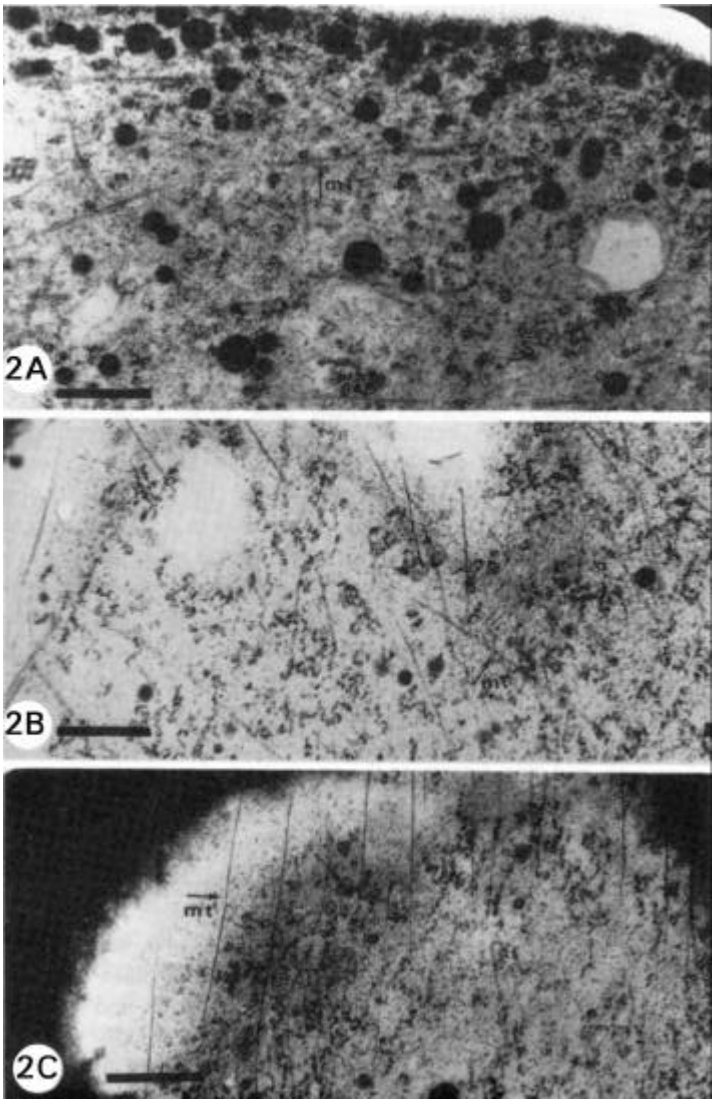

Figure 2. The orientation of cortical microtubules (CMT) at different distances from the growing tip of a *Equisetum hyemale* root hair. A. Random orientation in the dome; B. Subapically a change in orientation to net-axial; C. Net-axially oriented CMT in the tube

The only freeze substitution study on the CMT cytoskeleton carried out previously has been done on *Equisetum hyemale* (Emons 1989). This paper confirmed the net-axial orientation seen before with chemical fixation. However, CMT were more straight and diverted less from the axial orientation in comparison to the orientations found in chemically fixed cells. Light microscopic freeze fixation studies, which have given good results for the actin cytoskeleton (Miller et al 1999), are promising for CMT, but remain to be done.

2.2.2 Relationship of CMT and CMF in root hair tubes?

As mentioned above, secondary cell wall formation takes place in the non-expanding tube of the root hair. Therefore, the role of the CMT in CMF deposition orientation can be studied in this part of the cell independently of the role of the CMT in cell expansion. In most terrestrial plant species studied, the CMF (Emons and Van Maaren 1987) in the root hair tube run net-axially, as do the CMT (Traas et al 1985) (Table 1). This observed correlation, however, does not mean that the one orients the other. Root hairs with helicoidal walls (Pluymaekers 1982) would be the perfect cells to study the relation between CMT and nascent CMF. Helicoidal cell walls consist of one microfibril thick lamellae. The microfibrils in every subsequent lamella make a constant angle with the CMF in the previous lamella. During growth at the tip, all stages of secondary wall development are present in the hair tube (Emons and Wolters-Arts 1983). Therefore, lamellae having microfibrils in different orientations are present along the plasma membrane (Emons 1989). If microtubules would orient the microfibrils during deposition, the microtubules should lie in those orientations as well. However, the microtubules are net-axially oriented in the whole hair tube (Fig 2.) (electron microscopy: Emons 1982; Emons and Wolters-Arts 1983; immunofluorescence: Traas et al 1985; freeze substitution electron microscopy Emons 1989). Figure 2a is an immunofluorescence micrograph of a growing root hair of *Equisetum hyemale* with net-axial microtubules. CMT in the tube of other root hairs with helicoidal wall texture (*Hydrocharis morsus ranae*: Emons and van Maaren 1987; *Ceratopteris thalictroides*: Meekes 1985; *Limnobium stoloniferum*: Traas et al 1985) are net-axial as well. Although in growing cells or cells parts like root hair tips, CMT may have a role in orienting the CMF, we conclude that CMT do not function in determining the orientation of depostition of cellulose microfibrils by default.

2.2.3 Function of the cortical microtubules in the root hair tube

In expanding cells or cell parts such as the tips of growing root hairs, the CMT may be linked to the plasma membrane by Microtubule Associated Proteins (MAP). MAP have been found in plant cells (Chang Jie and Sonobe 1993; Bokros et al 1995; Durso et al 1996; Rutten et al 1997). CMT have a very specific halo around them,

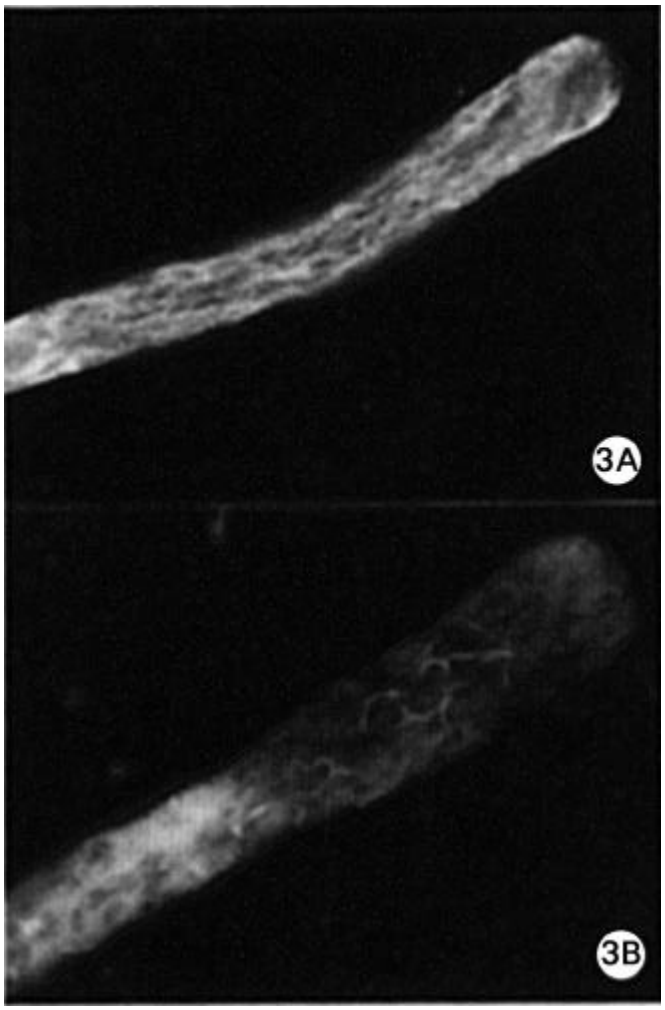

Figure 3a. Immunofluorescence micrograph of a growing root hair of *Equisetum hyemale* with net-axial microtubules.

Figure 3b. Immunofluorescence micrograph of root hair of *Equisetum hyemale* treated with 10 mM colchicine. The part of the hair that had been growing in the colchicine solution is wider and has no or abnormal microtubules.

seen well in electron micrographs. This halo and the distance of the core of the microtubule to the plasma membrane is not different in transverse sections of the tube of root hairs of *Equisetum hyemale* (Emons unpublished) and other plant cells. Therefore, the electron microscope studies do not provide evidence for a difference in function between the CMT in growing cells and in full-grown cells.

The function of the CMT in the tube of root hairs remains unclear. A few observations point to a possible role. Emons et al. (1990) have shown that when the CMT are depolymerised by colchicine at such a concentration that root hair growth proceeds

Table 1. Orientations of cortical microtubules (CMT) and texture of the recently deposited cellulose microfibrils (CMF) in the cell wall.

Species	Microfibril deposition (references)	Microtubule orientation (references)
Equisetum hyemale		
Growing tip	Random (1,2, 11)	Random (1,2, 11)
Tip-300μm	Random (1,2, 11)	Axial (1,2, 11)
300-3000μm	Helicoidal (1,2, 11)	Axial (1,2, 11)
Full-grown	Helical (1,2, 11)	Axial (1,2, 11)
Old	Axial (13)	Axial (11)
Ceratopteris thalictroides		
Growing tip	Random (3)	?
Tip-base	Helicoidal (3,12)	Axial (3,12)
Limnobium stoloniferum		
Growing tip	Random (3,4)	Random (3,4)
Tip-base	Helicoidal (3,4)	Axial (3,4)
Raphanus sativus, Lepidium sativum		
Growing tip-25μm	Random (3,5)	?
25μm-base	Axial (3,5)	Axial (3,5)
Urtica dioica		
Growing tip	Random (3)	
Tip-base	Helical (3,6)	Net-axial (3,6)
Allium cepa		
Growing tip	Random (7,9)	Helical or absent (9)
Tip-base	Helical (7) or Helicoidal (9)	Helical (7,10)
Hydrocharis morsus ranae		
Tip-base	Helicoidal (8)	Axial (8)

References: (1) Emons (1982); (2) Emons and Wolters-Arts (1983); (3) Sassen et al (1981); (4) Pluymaekers (1982); (5) Newcomb and Bonnett (1965); (6) Van Amstel and Derksen (1993); (7) Lloyd and Wells (1985); (8) Emons and Van Maaren (1987); (9) Traas et al (1985); (10) Lloyd (1983); (11) Emons (1989); (12) Meekes (1985); (13) Emons, unpublished

(10 mM), the new tube has a larger diameter than the exsisting tube. Figure 2b shows a root hair of *Equisetum hyemale* treated with colchicine.
The part of the hair that had been growing in the colchicine solution has no CMT and is wider. Bibikova et al. (1999) conducted a pharmacological study to decipher the role CMT in root hairs of *Arabidopsis thaliana*. Both CMT depolymerization by the drug oryzalin and CMT stabilization by the drug taxol cause a wavy root hair phenotype, and taxol is able to induce branching, indicating that CMT are involved in the determination of growth direction of root hairs. Tominaga et al. (1997) show that cytoplasmic streaming does not recover in the proper orientation after actin filament depolymerization by cytochalasin B, when microtubules are not present. They conclude that functioning microtubules are required for recovery of cytoplasmic streaming after actin depolymerization. This indicates that the CMT may be involved in directing newly formed actin filaments. Obviously more work is needed in this field.

3 Endoplasmic microtubules in root hairs

The observation of endoplasmic microtubules in tips of root hairs of *Vicia sativa* by Bakhuizen (1988), together with the absence of CMT is an indication that this observation could be an artifact of the chemical fixation of the root hairs. Bakhuizen suggests that the microtubules radiating from the nuclear envelope could be the result of activation of microtubule organizing centers at or near the nuclear envelope. In the legume *Vicia hirsuta*, Lloyd et al. (1987) found bundles of endoplasmic microtubules which link the nuclear region to the apical dome, where they fountain out upon the cortex. The microtubules were visualized by chemical fixation and immunolocalization. Such a localization has never been reported for other species. Nuclei seem to follow the advancing tip of root growing root hairs (Ridge 1992). Endoplasmic microtubules around the nucleus may function in nuclear movement. In a number of studies in different organisms, varying from yeast to higher plant cells, a relationship has been found between nuclear movement and the microtubule cytoskeleton. In budding yeast, interphase nuclear movement can be inhibited by nocodazole treatment (Shaw et al 1997; review: Reinsch and Gönczy 1998). In sea urchin and *Xenopus* egg extracts, nuclei have been found to move over microtubules by dynein motor activity (reviewed in: Reinsch and Gönczy 1998). During branching of tip growing moss protonemata, endoplasmic microtubules are found to connect the

nucleus to the prospective division site (Doonan et al. (1986), whereas in filamentous fungi cytoplasmic dynein has been found to be involved in nuclear migration **(**Xiang et al 1994, 1995; Plamann et al 1994; Bruno et al 1996; Inoue et al 1998). Additionally, microtubule nucleation sites have been found on nuclei of higher plants (maize cultured cells and tobacco BY-2 cells: Stoppin et al. (1994, 1996); tobacco cultured cells: Mizuno (1993); maize root in vivo: Baluška et al. (1992)). Lastly, in tip growing pollen tubes of *Nicotiana sylvestris*, the vegetative nucleus and generative cell dislocate when microtubules are depolymerized by colchicine treatment, indicating that microtubules are involved in their positioning (Joos et al 1994). These observations suggest that endoplasmic microtubules may play a role in nuclear positioning in root hairs, but additional studies need to be done to obtain more clarity.

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Note added in thesis

Recently, Sieberer et al. (2002) have reported the presence of endoplasmic microtubules (EMTs) besides cortical microtubules (CMTs) in growing root hairs of *Medicago truncatula*. The EMTs are localised in the subapical cytoplasmic dense region between the basal part of the nucleus and the tip of the hair. They form a densely structured 3-dimensional array. In growth arresting root hairs, these endoplasmic microtubules disappeared.

Application of the microtubule depolymerising drug oryzalin at concentrations up to 1μM led to a disappearance of specifically the EMTs, while the CMTs remained present. In treated root hairs, the positioning of nuclei was affected as nuclei followed the apex at a distance of 120-160μm in comparison to 30-40μm in control root hairs. Also the growth speed of oryzalin treated root hairs decreased over the first hour to approximately 40% of the initial growth rate and the cytoplasmic dense area became shorter. Interestingly 1μM taxol did not alter the cyto-architecture, including the position of the nucleus, but lowered the growth speed dropped to the value observed in oryzalin-treated hairs. These observations indicate that EMTs function in keeping cyto-architecture and consequently in cell polarity establishment.

So far, EMTs have only be reported in legumes (Lloyd et al., 1987; Sieberer et al., 2002). EMTs may have been missed in other species, but there is also a possibility that the EMTs are specific for legumes, possibly to control the root hair curling and infection threat formation during interaction with rhizobium bacteria.

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Chapter 5

The cytoskeleton in plant cell growth: lessons from root hairs

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Summary

In this review, we compare expansion of intercalary growing cells, in which growth takes place over a large surface, and root hairs, where expansion occurs at the tip only. Research that pinpoints the role of the cytoskeleton and the cytoplasmic free calcium in both root hairs and intercalary growing cells is reviewed. From the results of that research, we suggest experiments to be carried out on intercalary growing cells to test our hypotheses on plant cell expansion. Our main hypothesis is that instability of the cortical actin cytoskeleton determines the location where expansion takes place and the amount of expansion.

I. How do plant cells expand their surface?

Cell growth is an irreversible increase in cell surface. Plant cells can only increase their surface by increasing the total surface area of their plasma membrane and cell wall. Since the plasma membrane can only elastically stretch to additional 2% of its total surface (Wolfe & Steponkus, 1981, 1983), continuous delivery of plasma membrane has to take place to allow for increase in cell surface (Miller et al., 1997). Though the cell wall in growing plant cells does increase in surface, it does not become thinner during the growth process (Roberts, 1994; root hairs: Miller et al., 2000). This implies that plant cell growth and wall deposition are finely-tuned combined processes.

Addition of new cell wall and new plasma membrane takes place by means of exocytosis of vesicles, derived from Golgi bodies. These Golgi vesicles supply the essentials for cell wall synthesis; they contain cell wall matrix and cell wall synthesizing enzymes, while the vesicle membrane delivers cellulose-synthesizing complexes into the plasma membrane.

Though the plasma membrane is a semifluid continuum, the cell wall is not. Cell wall material does not shift horizontally from one part of the cell to another. Therefore exocytosis has to occur at the area of the cell where growth takes place.

In plant cells, expansion can take place: over the whole cell surface, referred to as isodiametrical growth; primarily over longitudinal wall facets thus causing the cell to expand anisotropically, more in one direction than in the other, referred to as diffuse growth or intercalary growth; or local, for example tip growth (as occurs in root hairs). Cell expansion can only take place if the cell wall is flexible.

Root hairs are cylindrical, uniaxial structures, emerging from special root epidermal cells that only expand at their apical dome (Derksen & Emons, 1990; Shaw et al., 2000). The extensive exocytosis occurring in this dome, implies that Golgi vesicles have to be delivered to and inserted into the plasma membrane of the apex. This localized abundant exocytosis makes root hairs excellent cells to study the process of cell expansion in plant cells.
In this review we discuss the role of the cytoskeleton and calcium in polarized growth of root hairs: how are Golgi vesicles directed to the area where growth takes place, how is this area specified and thus: what determines the cell shape? We extrapolate the findings from root hairs to intercalary and isodiametrically growing plant cell types, to hypothesize about mechanisms that are involved in controlling cell expansion in these cell types.

II. Immunolocalization of epitopes in fixed root hairs for light-microscopy

To study the role of the cytoskeleton in cell expansion, visualization of its exact localization and configuration is essential. If changes occur during the fixation/labelling/visualization procedure, incorrect conclusions can be drawn.
As discussed extensively in a recent review from our laboratory (Esseling et al., 2000), in recent years rapid freeze fixation and optimized chemical fixation have been developed and shown to result in the optimal visualization of actin in root hairs. However, in other publications (Vitha et al., 2000; Braun et al., 1999), fixation techniques, that in our hands do not preserve the localization of dynamic structural proteins properly, and thus may lead to fixation artifacts, are still being used.
We will discuss the critical points in the fixation procedure and advances in fixation techniques that have been achieved over recent years and supplement this discussion with light-microscopic images of the cytoarchitecture after different fixation methods.

Aldehyde fixation is an easy, fast and cheap way to fix tissues. However, it is a relatively slow fixation method, not suitable for highly polarized and dynamic parts of cells, because during the slow fixation process changes in the cytoarchitecture and

thus in protein localization can take place. Several techniques have been developed to circumvent this problem and improve the preservation of the cytoarchitecture during fixation. The fixation speed is increased by cryofixing the tissue. One method is plunging the material in liquid propane and thus rapid freezing of the tissue (Baskin et al., 1995). This method only works for cell layers in direct contact with the liquid propane. Root hairs are ideal cells for this method since they form the outermost cell layer of the root. Deeper cell layers are slower to freeze due to the insulating properties of peripheral cell layers. The resulting slower decrease in temperature will cause freeze damage, that is it will allow the formation of ice crystals that will destroy the cellular structure. For cryofixation of these deeper cell layers, good results can however, be achieved with high pressure freezing methods (Müller & Moor, 1984; Kiss et al., 1990, Thijssen et al., 1998). Normally after freeze fixation, the water in the cytoplasm is substituted by acetone or methanol. Sometimes, a trace of water-free glutaraldehyde or 2,2,-dimethoxypropane (DMP; chemically reacts with water molecules producing acetone and methanol) (Thorpe & Harvey, 1979; Dierichs & Dosche, 1982) is added.

After freeze substitution, the material can either be rehydrated and processed as whole mount, or embedded in a resin for sectioning. For whole mount processing, we rehydrate in a graded series of stabilizing buffer plus aldehyde fixative, whereafter we use enzymatic digestion of the cell wall to facilitate antibody penetration, followed by immunolabeling. During the procedure, the tissue is handled with care, and only straight, intact root hairs with a well-preserved cytoarchitecture, checked under the microscope, are analyzed.

Resin embedding of freeze substituted tissue in our laboratory is carried out using the method described by Baskin et al. (1992). Unfortunately, reactivity of fluorescently labeled phalloidins to actin is lost during the freeze substitution (Tang et al., 1989), but antigenicity is maintained.

A way to circumvent fixation artifacts caused by chemical fixation is fixation of the actin cytoskeleton by a brief prefixation with the cross-linker m-maleimidobenzoyl N-hydroxylsuccinimide ester (MBS-ester; Sonobe & Shibaoka, 1989) before aldehyde fixation. MBS-ester effectively crosslinks actin subunits in actin filaments, arrests the cytoplasmic streaming within seconds and preserves the typical cytoarchitecture in growing root hairs; therefore no changes in cytoarchitecture take place during a subsequent aldehyde fixation (Miller et al., 1999; reviewed in Esseling et al., 2000). In

Fig. 1, a comparison is made of aldehyde fixation, with and without MBS-ester pretreatment. After fixation, the tissue can either be permeabilized with a mild membrane-permeabilizing agent, L-α-lysophosphatidylcholine (LP-choline), which facilitates entry of fluorescently labeled phalloidins into the cell (Miller et al., 1999) or with cell wall degrading enzymes, followed by antibody labeling. The effects of LP-choline treatment are time- and tissue-dependent. Short treatment will not allow phallotoxin penetration and prolonged treatment will destroy the cell. Therefore, we monitor the cytoarchitecture microscopically during cell fixation steps. After subsequent labeling with fluorescent phalloidin, the cells can be studied for several hours before the fluorescent signal dissociates from the actin filaments.
Immunolabeling with antibodies can be carried out in a similar way to after freeze substitution. We carefully compared immunolabeling on sections of freeze fixed material and whole mounts, fixed by the optimized chemical fixation method. Both methods give similar actin localization at the light microscopic level (Miller et al., 1999). However, in freeze fixed and substituted root hairs that are immunolabeled whole mount, a fluorescent signal can be observed in the apical cytoplasm. This fluorescence is not organized in filamentous structures and disappears after washing. It is likely caused by the presence of the antibody, bound to actin monomers. Similar observations have been made by Baluška et al. (2000) when studying root hairs of *Arabidopsis* plants, transformed with a construct of Green Fluorescent Protein (GFP), fused to the actin binding sequence of mouse talin (Kost et al., 1998). The authors likely misinterpret the apical fluorescence caused by free cytoplasmic fusion-protein as GFP-talin, bound to filamentous actin.
Freeze shattering (Wasteneys et al., 1997) is a crude fixation method, wherein the cells are aldehyde fixed, followed by 'cracking' of the cell walls by freezing the tissue in liquid nitrogen and using glass slides as a 'nut cracker'. This is a very efficient method for quick screening of stable cytoskeletal elements, like cortical microtubules, but not suitable for the highly dynamic and extractable actin at tips of root hairs, since the first step is a slow aldehyde fixation.

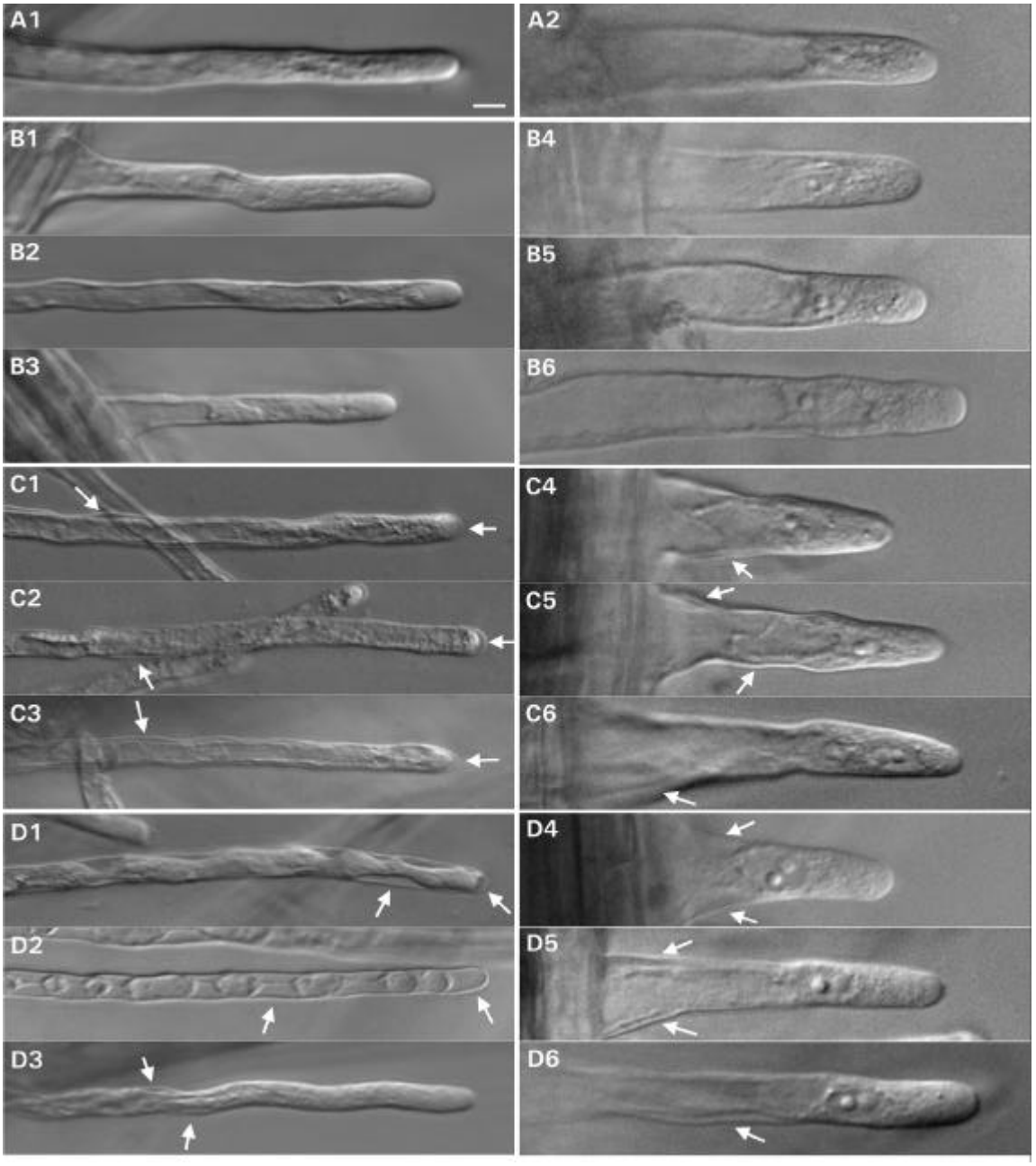

Figure 1. Comparison between different fixation methods applied to *Arabidopsis thaliana* and *Medicago truncatula* root hairs grown in liquid medium between glass slides. (a1) An unfixed *A. thaliana* root hair; (a2) an unfixed *M. truncatula* root hair; (b1-b3) *A. thaliana* root hairs, treated with 1mg ml^{-1} m-maleimidobenzoyl N-hydroxylsuccinimide ester (MBS-ester) in growth medium for 1 min, followed by fixation with 4% paraformaldehyde (PFA) and 0.05% GA in phospate buffered saline (PBS); (b4-b6) *M. truncatula* root hairs after the same treatment; (c1-c3) *A. thaliana* root hairs, immediately fixed with 4%PFA and 0.05% GA for 15 min in PBS; (c4-c6) *M. truncatula* root hairs after the same treatment. The *A. thaliana* root hairs in d1-d3 are fixed in 1.5% PFA in actin stabilizing buffer. The *M. truncatula* root hairs in d4-d6 underwent the same treatment. In both treatments c and d the cellular organization changed during the fixation procedure and plasmolysis occurred, although the amount of

plasmolysis could be decreased by gradual application of the fixative to the medium. Slower application of the fixative increases the time wherein cytoplasmic rearrangements can occur during the fixation process. Arrows indicate some of the locations where plasmolysis did take place during the fixation procedure. Bar, 10μm.

Generally, care should be taken to prevent cytoplasmic rearrangements during the fixation procedure, leading to fixation artifacts. Fixation should be rapid and during the whole procedure, the cytoarchitecture should be monitored.

III. The cytoskeleton in growing root hairs

1. Microtubules

In contrast to animal cells, plant cells posses cortical microtubules (CMTs) (Green, 1962; Ledbetter & Porter, 1963). Heath (1974) hypothesized that CMT determine the orientation of the cellulose microfibrils (CMFs) in the cell wall and thus the direction of cell expansion. The CMT could perform this process by channeling the cellulose synthase complexes through the plasma membrane (Giddings and Staehelin 1991). For a long time, this seemed a plausible hypothesis, since the CMTs and CMFs are both mostly transversely oriented to the growth axis and thus parallel to each other. However, recently, detailed observations of CMTs and CMFs in expanding cells show that the spatial relationship is not always present (reviewed in Wasteneys, 2000). Only in *Equisetum hyemale* have both CMTs and CMFs of growing root hairs been reliably studied by means of freeze fixation and substitution (Emons, 1989). In the hemispherically shaped tip of these hairs, the orientation of both CMTs and CMFs (Emons, 1989; Ketelaar & Emons, 2000) is random, thus also in this expanding part of the cell, CMTs may be in the same orientation as the CMFs (reviewed in: Ketelaar & Emons, 2000). It has been hypothesized that transversely oriented CMF could limit cell expansion to the direction transverse to the hoops (Gertel & Green, 1977). Since expansion of the root hair tip occurs in all directions, the random orientation of the CMFs may also determine the direction of root hair tip expansion (Ketelaar & Emons, 2000). In root hair tubes, which do not expand, CMTs are oriented net-axially or helically and CMFs are deposited in an axial, helical or helicoidal organization (review: Ketelaar & Emons, 2000).

Therefore in root hair tubes the CMTs do not always coalign with the orientation of the CMFs, for example during helicoidal cell wall deposition. Absence of coalignment of CMTs and CMFs has been found in more nongrowing cells (Emons et al., 1992). Therefore, in nonexpanding cells CMTs do not determine the orientation of deposition of CMFs by default (Ketelaar & Emons, 2000).

In the absence of microtubules, plant cells expand isodiametrically (Green, 1962). Since the hemispherical tips of root hairs expand in all directions, one would expect no changes in cell tip morphology during microtubule depolymerization. Bibikova et al. (1999) applied the microtuble depolymerizing drug oryzalin to *Arabidopsis* root hairs. Indeed, treatment with this drug did not inhibit cell growth and did not even change the diameter of the cell. However, cells did grow in a more wavy fashion. The mor1 mutant, disturbed in microtubule organization, also normally initiates root hairs at restrictive temperature, they are slightly wavy, similar to root hairs grown in the presence of oryzalin (Whittington et al., 2001). Since the growth speed of root hairs in the absence of microtubules remains the same, we conclude from these data that microtubules do not contribute to or limit Golgi vesicle delivery or insertion, but have a role in determining the growth direction of root hairs.

The drug paclitaxel (taxol) stimulates polymerization of microtubules (Morejohn, 1991) by decreasing the critical concentration of tubulin necessary for polymerization, also leading to increased stability (Bokros et al., 1993). Application of low concentrations of paclitaxel leads to a more severe wavy phenotype of *Arabidopsis* root hairs (Bibikova et al., 1999) than described above for oryzalin. Moreover, local UV activation of injected ionophore and touch stimulus, were sufficient to form an artificial calcium gradient and a new growth point at the site of and in the direction of the new gradient in paclitaxel treated cells. In untreated cells only a transient reorientation of growth could be observed after UV activation of injected ionophore and touch stimulus (Bibikova et al., 1999). Unfortunately, the mode of action of paclitaxel is not well defined. From immunocytochemical (Baskin et al., 1994) and biochemical (Bokros et al., 1993) data, it is known to stabilize microtubules, but to what extend the stabilized microtubules are functional is not clear. Disturbance of the calcium gradient or touch stimulus (Bibikova et al., 1999) may cause a disturbance or reorientation of the actin cytoskeleton, responsible for Golgi vesicle delivery. The presence of functional microtubules is required for recovering the Golgi vesicle delivery system in the proper direction. The results of Tominaga et al. (1997) point to

the same direction. The authors state that microtubules may be involved in determining the direction of newly formed actin filaments after actin depolymerization in the subapical area of *Hydrocharis* root hairs.

2. Actin filaments

In plant cells, actin filaments appear to be the backbone of cytoplasmic strands and the basis of cytoplasmic streaming (Valster et al., 1997; reviewed in: Esseling et al., 2000). In growing root hairs, the cytoplasmic streaming pattern is reversed fountain-like (Sieberer & Emons, 2000). Thick bundles of longitudinally oriented actin filaments run through the cortical cytoplasm of the vacuole filled tube and branch into thinner and thinner bundles in the more cytoplasmic dense subapical area, where a high density of fine bundles of net-axially oriented actin filaments (FB-actin) is present (Miller et al., 1999). Other bundles of actin filaments loop back through a cytoplasmic strand running through the vacuole, where bundling takes place (Tominaga et al., 2000). Most FB-actin points to the apical area, devoid of detectable actin filaments (Miller et al., 1999). In this area, Emons (1987) found a cytoplasmic streaming speed of 0μm s^{-1}, suggesting that indeed no actin filaments were present. The subapical FB-actin has been hypothesized to not only deliver vesicles to the vesicle rich area, but also to serve as a buffer to retain the vesicles in the apical area and as a sieve to inhibit penetration of other organelles into the apical area (De Ruijter & Emons, 1999). Miller et al. (1999) demonstrated that the subapical FB-actin is more sensitive to the actin filament capping drug cytochalasin D than more basal actin filament bundles. Continued application of cytochalasin D leads to disappearence of the FB-actin, the area free of detectable actin filaments and growth, while cytoplasmic streaming is normal. This proves that the typical actin configuration is required for root hair growth, that is for correct vesicle delivery. Fig. 2 shows a cartoon of the actin organization in a typical root hair.

Cárdenas et al. (1998) demonstrated that changes in the actin cytoskeleton in the subapical area of root hairs of *Phaseolus vulgaris* take place within minutes after application of nodulation factors, signalling molecules of *Rhizobium* bacteria. De Ruijter et al. (1999) showed that these changes in *Vicia sativa* root hairs are due to an increase in the density of subapical FB-actin. These authors hypothesize that this

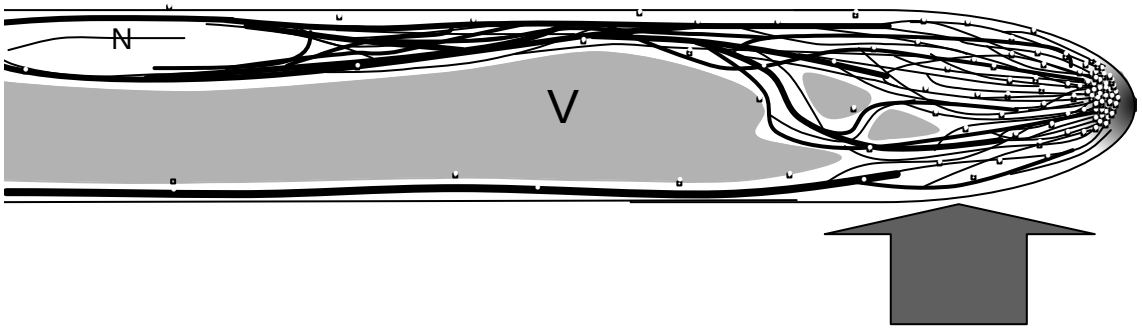

Figure 2. A cartoon, displaying the organization of the actin cytoskeleton in a growing root hair. Towards the apex, the actin branches into thinner bundles of actin filaments, the FB-actin, indicated with an arrow. In the apical area, which is essentially free of detectable actin filaments, the darkening displays the gradient of increased cytosolic calcium. Golgi-vesicles, which are produced throughout the cytoplasm and travel over the actin filaments towards the apex, accumulate in this area, where at the membrane exocytosis takes place. The nucleus is marked with a N and the vacuoles with a V.

increase in the density of FB-actin is caused by debundling of thicker bundles of actin filaments, increased polymerization in different directions or new nucleation and polymerization of fine bundles of actin filaments. Since these signal molecules, together with actin unbundling, induce a reinitiation of tip growth in growth terminating root hairs that have a lower amount of FB-actin (De Ruijter et al., 1998), this work shows that a minimal number of fine bundles of actin filaments is required for root hair tip growth.

In full grown root hairs the cytoplasmic polarity of the cell is lost and thick bundles of actin filaments loop through the tip (Miller et al., 1999), reflected in the circulation type of cytoplasmic streaming (Sieberer & Emons, 2000). The absence of the FB-actin and the minimum amount required for cell growth strongly suggests that it plays a role in cell expansion.

3. Free cytoplasmic calcium concentration

The apical area of growing root hairs contains a gradient of high concentration of free cytoplasmic calcium (Wymer et al., 1997; Felle & Hepler, 1997; Bibikova et al., 1997; De Ruiter et al., 1998; Cárdenas et al., 1999), which more or less colocalizes with the area without detectable actin filaments. This locally increased calcium concentration disappears during termination of growth (Wymer et al., 1997, De Ruijter et al., 1998).

Schiefelbein et al. (1992) demonstrated that calcium influx from the extracellular environment is a prerequisite for growth of root hairs. The calcium influx takes only place at the tips of growing root hairs (Herrman & Felle, 1995; Felle & Hepler, 1997). The high $[Ca^{2+}]_c$ is probably required for exocytosis (Carroll et al., 1998). The role of the $[Ca^{2+}]_c$ gradient in root hair growth is demonstrated by experiments, carried out by Bibikova et al. (1999). They demonstrated that local UV activation of injected ionophore and touch stimulus, were sufficient to form a new calcium gradient, resulting in a transient reorientation of the growth point at the site of and in the direction of the new gradient.

The calcium influx at the apex of growing root hairs has been shown to be inhibited by several calcium channel blockers (nifedipine (Schiefelbein et al., 1992; Herrmann & Felle, 1995), verapamil (Wymer et al., 1997), La^{3+} (Herrmann & Felle, 1995; Felle & Hepler, 1997)) leading to an inhibition of growth.

Until recently, depolarization-activated calcium channels were thought to be a good candidate for the calcium influx (Thion et al., 1998). However, by patch-clamping experiments, Véry & Davies (2000) showed that hyperpolarization-activated calcium channels are present in cytoplasts derived from tips of growing root hairs, whereas such channels are down regulated in cytoplasts, derived from full grown root hairs, as well as from the subapical area of growing root hairs. These results offer the possibility to enable a new hypothesis about calcium influx, with stronger evidence than previous hypotheses, wherein channel activity is dynamically regulated by voltage and internal calcium (Véry & Davies, 2000). Depolarization-activated calcium channels were not found, but even if they were present, they generally are not active at potentials more negative than -140mV, which suggests that their involvement in continuous uptake of extracellular calcium at the root hair apex is not likely (Véry & Davies, 2000). In pollen tubes, it has been suggested that stretch-activated calcium channels are logical candidates for the apical calcium influx. Since pollen tubes, similarly to root hairs, only expand at the apex, the probability of activation is highest for the channels localized at the apex (Feijó et al., 1995; Pierson et al., 1996. Reviewed in Feijó et al., 2001). Obviously, if stretch activated calcium channels are present in root hairs, they may function in a way similar as has been suggested in pollen tubes.

IV. The role of cytoskeletal elements and cytoplasmic free calcium in intercalary expanding root cells

1. Microtubules

In young elongating *Arabidopsis* root cells, the CMTs are oriented, more or less, perpendicular to the primary growth direction (Sugimoto et al., 2000). The direction of cell expansion is thought to be regulated by the orientation of the cortical microtubules. Indeed, in several mutants with disturbed CMTs cells expand less anisotropicly. The fass mutant does have CMTs, but they do not organize in cortical arrays. This leads to isodiametrically expanding cells (McClinton & Sung, 1997). Also the temperature sensitive mor1 mutant and the ton, botero1 and fat root mutants have disrupted CMTs and their cells expand isodiametrically (ton: Traas et al., 1995; mor1, botero1 and fat root: Wasteneys, 2000). CMT organization is at least partially under the control of microtubule associated protein (MAP) regulation (Sawano et al, 2000), as reported for mammalian cells (Maccioni & Cambiazo, 1995; Mandelkow & Mandelkow, 1995). The observation that application of inhibitors of specific protein phosphatases, leading to inhibition of protein phosphorylation, results in isodiametrical cell expansion in *Arabidopsis* roots supports these findings (Smith et al., 1994).

Pharmacological data confirm the importance of microtubules in anisotropic intercalary cell expansion. Depolymerization of microtubules leads to development of isotropically swollen cells (Green, 1962; Baskin et al., 1994), and application of paclitaxel also leads to isodiametrical expansion, similar to oryzalin treatment (Baskin et al., 1994). This suggests that microtubule function is disturbed during paclitaxel treatment. However, transient stabilization of microtubules in developing trichomes of the *Arabidopsis* stichel and zwichel mutants leads to recovery of the branching phenotype of trichomes (Mathur & Chua, 2000), whereas microtubule depolymerization does not, indicating that transient microtubule stabilization by short treatments with paclitaxel could be mimicking a physiological event. From all these data, it is clear that functional CMTs are essential in anisotropic cell expansion, though it is not clear how they perform the role they play in this process. Some reports demonstrate that the relationship between CMT orientation and expansion

speed and direction is not always present; therefore it should be remarked that functional CMT are essential, but not sufficient for controlling growth anisotropy (Wasteneys, 2000).

To complicate the situation, there are indications for a feedback mechanism from CMFs to the CMTs. Inhibition of CMF synthesis leads to disturbance of the normal CMT alignment in tobacco suspension cells (Fisher & Cyr, 1998) and when *Arabidopsis* root cells start to elongate CMFs obtain a transverse orientation before the CMTs (Sugimoto et al., 2000). On the other hand, *Arabidopsis* mutants disturbed in cellulose synthesis, have expanding cells with a radially swollen phenotype, a heavily disturbed CMF patterning and normal transversely oriented CMTs (Hauser et al., 1995, Sugimoto et al., 2000). Wasteneys (2000) proposes that maximal coalignment between CMTs and CMFs could optimize anisotropy during periods of rapid expansion.

Experimental data are available suggesting that, in some cases, an intact actin cytoskeleton is essential for normal orientation of the CMTs. During recovery of cytoplasmic streaming in wounded, full-grown *Nitella* internodal cells, CMTs demonstrate a passive alignment into the direction of the cytoplasmic streaming in areas where the cytoplasmic streaming creates sufficient hydrodynamic forces on the CMTs (Foissner & Wasteneys, 1999). But the authors also demonstrate that the distance between actin filaments and CMTs in these cells is too big for cross-linking. Therefore, a direct interaction is not likely. Wang & Nick (1998) found that the coleoptyles of the Yin-Yang rice mutant show a combined increased sensitivity to cytochalasin D and a resistance to microtubule depolymerizing drugs during cell elongation. Furthermore, cytochalasin D treatment in Azuki bean epicotyls led to an accelerated reorientation of CMTs into the longitudinal direction and slowed down the reorientation into the transverse direction (Takesue & Shibaoka, 1998). However, the CMTs reorientation in the research of Wang & Nick (1998) and Takesue & Shibaoka (1998) is auxin dependent, and auxin transport has been found to decrease by actin depolymerization (Butler et al., 1998). Therefore, the observed effects during actin depolymerization may be caused by changes in auxin concentration.

During root hair initiation and in growing root hairs, CMTs do not determine whether or not root hairs are initiated or growth takes place, but do codetermine the growth direction of the cell, and therefore the exact site where exocytosis takes place.

Deduction to intercalary growing cell types would indicate that CMTs do not determine whether or not growth takes place, but do determine the cell site where exocytosis takes place, that is which wall facets expand, thereby imposing polarity of growth direction. It suggests that in intercalary growing cells treated with oryzalin, or in mutants with disturbed CMTs, expansion should be in all directions, which is indeed the case (mutants: McClinton & Sung, 1997; Traas et al., 1995; Wasteneys, 2000, oryzalin: Baskin et al., 1994).

2. Actin filaments

As discussed above, in elongating root cells, similar to root hairs, CMTs are involved in determining the direction of cell expansion. Cell expansion takes place by exocytosis of cell wall matrix containing Golgi vesicles, which are not necessarily produced in the vicinity of the membrane and have to be kept at the membrane until inserted, and not swept away. This implies that they have to be targeted and delivered to the area of the plasma membrane where exocytosis occurs. Mathur et al. (1999) and Szymanski et al. (1999) demonstrated that a functional actin cytoskeleton is essential for proper morphogenesis in intercalary growing cells. Both groups show that disturbance of the actin cytoskeleton in *Arabidopsis* trichomes leads to disturbed spatial patterning of cell expansion and altered cell morphogenesis, possibly because the delivery of Golgi vesicles to the location where normally exocytosis takes place is disturbed.

Staiger et al. (1994) and Valster et al. (1997) demonstrate that injection of the G-actin binding protein profilin in *Tradescantia* stamen hair cells depolymerizes actin filaments, breaks down cytoplasmic strands, and stops cytoplasmic streaming. Valster et al. (1997) found the cortical actin filaments to be more resistant to depolymerization, and even when cytoplasmic streaming was inhibited completely, still cortical filamentous actin remained present. From this observation, we conclude that the remaining cortical actin network is not involved in cytoplasmic streaming. Delivery of Golgi vesicles to the plasma membrane will not take place if the actin cytoskeleton is depolymerized so that the cytoplasmic streaming is inhibited. Therefore, except for exocytosis of residual Golgi vesicles, which have already been delivered to the plasma membrane, growth should be inhibited upon depolymerization of the actin cytoskeleton.

Are vesicles actively targeted to the plasma membrane at the site where the cell expands, or are they moving over the actin highways throughout the cell, until they arrive in an area of the cell where exocytosis can take place? In the latter case, the actin highways could be actively directed to this area. In root hairs, the actin highways lead to the subapical area of FB-actin into an area without detectable actin filaments, no cytoplasmic streaming and high $[Ca^{2+}]_c$. The dense area of FB-actin is hypothesized to prevent the Golgi vesicles from running back and the high $[Ca^{2+}]_c$ enables calcium dependent exocytosis. In root cells, a similar mechanism could be present: actin highways that transport the vesicles to an area with more diffuse actin filaments, bordering the site of exocytosis. This site is physiologically different so that the actin filaments either end there so that the vesicles run off or continue but allow the vesicles to fall off. This process could be mediated by numerous cellular compounds, for example the local $[Ca^{2+}]_c$ or the activation of proteins. Indeed, recently Fu et al. (2001) demonstrated that a Rho of plants (Rop) GTPase is involved in controlling polar growth by regulating the dynamics of the actin cytoskeleton in the apical area of tobacco pollen tubes.

In mammalian cells, the role of the actin cytoskeleton in exocytosis has been better established. In nerve cells, Bradke & Dotti (1999) have demonstrated that treatment with low concentrations of actin inhibitors leads to formation of an increased amount of axons. Local application of low concentrations of actin inhibitors on one growth cone lead to formation of an axon at that point. They hypothesize that the actin inhibitors increase the instability of the actin cytoskeleton, leading to an increase in exocytosis. However, absense of the actin cytoskeleton for a longer period leads to inhibition of exocytosis. Pendleton & Koffer (2001) demonstrated that 1 h exposure to 40 $\mu g\ ml^{-1}$ latrunculin B led to 85% inhibition of agonist induced secretion in rat peritoneal mast cells. Short term depolymerization of the actin cytoskeleton leads to an increase of exocytosis in mammalian cell types. Depolymerization of the actin cytoskeleton leads to increase in exocytotic activity in pancreatic acer cells (Muallem et al., 1995). Stabilization of the actin cytoskeleton by jasplakinolide, on the other hand, inhibits granule exocytosis in murine oocytes (Terada et al., 2000). The changes in the cortical actin cytoskeleton are at least partially caused by the calcium activated actin filament severing protein scinderin in mouse pancreatic beta-cells (Bruun et al., 2000). Scinderin has calcium independent actin nucleating activity and

it severs actin filaments after calcium dependent activation (Trifaro et al., 2000; Trifaro, 1999). In digitonin-permeabilized GH3 cells, calcium has also been found to be responsible for actin disassembly (Yoneda et al., 2000). To summarize: in animal cells, actin filaments deliver secretory vesicles to the plasma membrane, and a high local $[Ca^{2+}]_c$ at the plasma membrane activates proteins like scinderin, leading to an increase in actin instability, so that exocytosis at that location can take place.
We propose that in plant cells, a similar mechanism could be regulating cell expansion. In root hairs, the growing tip has a high $[Ca^{2+}]_c$ and no detectable filamentous actin. If one could increase the instability of the actin cytoskeleton in an artificial way, as Bradke & Dotti (1999) did by application of low concentrations of actin filament disrupting drugs, changes in root hair expansion rates or the area where exocytosis can take place are to be expected. In root hairs, application of actin inhibitors, in concentrations lower than the concentrations De Ruijter et al. (1999) used, so that growth is not inhibited, has never been published. We hypothesize that an increase of the instability of the dynamic subapical FB-actin or cortical actin in root hairs will lead to an increased apical area without filamentous actin, and therefore changes in the root hair shape. If this hypothesis is also valid for intercalary expanding cell types, depolymerization of cortical actin in intercalary growing cells such that delivery of vesicles to the plasma membrane is not inhibited but the cortical actin is disturbed, would lead to an increase in the amount of exocytosis.
In root hairs, growth is confined to one point and the growth speed is very high. These properties make root hairs perfect cells to study short-term changes in growth speed and cytoarchitecture. In intercalary expanding cells, a similar amount of growth as in root hairs takes place over the whole cell surface and thus the growth speed over the surface area where growth takes place is much slower, making it harder to observe changes in growth speed and cytoarchitecture. Therefore, it would be helpful to study changes in growth speed within different areas in one intercalary growing cell, because detection of local changes in expansion rate within a cell is easier than to compare expansion rates of different cells. We are currently developing techniques that allow us to locally apply drugs to the cell surface of individual cells. If our hypothesis is true, local application of low concentrations of actin perturbing drugs at the cell surface should lead to a local increase in instability of the cortical actin and thus a local increase in the amount of exocytosis. This should result in a local cell expansion.

3. Free cytoplastic calcium concentration

In elongating root cells, as in root hairs, hyperpolarization-activated calcium currents have been found (Kiegle et al., 2000). Also, depolarization activated calcium channels have been reported, which are stimulated by microtubule-depolymerizing drugs and more active in the *Arabidopsis* ton mutant (Thion et al., 1998). Although it has been shown that sulfate efflux (Frachisse et al., 1999) may be responsible for the depolymerization activated current, and not calcium (Kiegle et al., 2000), there are changes in the current after depolymerizing the cortical microtubules (Thion et al., 1998).

Since, as discussed above, in root hairs a high $[Ca^{2+}]_c$ is required for cell growth, and similar calcium channels have been found in intercalary expanding root cells, a similar increased $[Ca^{2+}]_c$ at the plasma membrane where growth takes place could be present in intercalary growing cells. No such a gradient has been reported yet in intercalary growing cells, possibly because obtaining experimental evidence is difficult. To gain insight in the role of calcium channels in cell expansion, local application of specific calcium channel activators or blockers on the expanding area of a cell and subsequent observation of the cell morphology would be a useful experiment. Blocking the calcium influx could lead to polymerization of the cortical actin pool, so that no exocytosis at that location can take place, and therefore a local inhibition of growth should occur.

Obviously, we are far from understanding the process of plant cell expansion, although over recent years, the involvement of the cytoskeleton has become clearer. At this moment, with the availability of genomic databases, unraveling of the molecular control of the cytoskeleton in cell expansion and its upstream control is within our reach.

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Samenvatting

In dit proefschrift wordt onderzoek gepresenteerd dat bijdraagt aan een beter begrip van de rol van het cytoskelet in het reguleren van expansie van plantencellen. Wortelharen, die uitsluitend groeien aan de top, werden gebruikt als modelsysteem. Voor het onderzoek zijn hoofdzakelijk wortelharen van de plant *Arabidopsis thaliana* gebruikt. In verschillende hoofdstukken komen verschillende functies van het cytoskelet in wortelhaar groei aan de orde.

Hoofdstuk 1 behandelt de rol van het actine cytoskelet in het bepalen van de locatie waar celexpansie, de fusie van Golgi vesikels met de plasma membraan ofwel exocytose, plaatsvindt. In groeiende *Arabidopsis* wortelharen is het actine cytoskelet georganiseerd zoals is beschreven voor wortelharen van andere plantensoorten: longitudinale bundels ten opzichte van de lengte-as in de wortelhaarbuis, in de (sub-)apex vertakkend in een dicht netwerk van over het algemeen longitudinaal georienteerde dunne bundels. In de uiterste top kon met licht-microscopische technieken geen filamenteus actine worden aangetoond.

Wanneer actine depolymeriserende chemikalien (cytochalasine D (CD) of latrunculine A (LA)) worden toegevoegd aan het medium waar de wortels van de planten in groeien, destabiliseren de actine filamenten vanuit de richting van de haartop en tegelijkertijd neemt de diameter van de toppen van groeiende wortelharen toe (het oppervlakte waar celgroei plaats vindt neemt toe). Dit suggereert dat tijdens wortelhaargroei een subapicale, lokale toename in instabiliteit van het actinecytoskelet de grootte van het gebied reguleert waar celexpansie plaatsvindt. Lokale applicatie van CD aan een zijde van de top van groeiende wortelharen leidt tot een lokale toename in de hoeveelheid celgroei op die plek. Om de hypothese te testen dat de hoeveelheid instabiliteit van het (sub-)corticale actine cytoskelet ook in intercalair groeiende cellen de hoeveelheid groei bepaalt op een bepaalde plek, hebben we lokaal CD aangebracht op intercalair groeiende *Tradescantia* meeldraadhaarcellen. Dit leidde tot een lokale uitgroei op de plek van aanbrengen, hetgeen onze hypothese bevestigde. Ook in een polair-groeiende gist (*Schizosaccharomyces pombe*) leidt het aanbieden van CD of LA tot een toename in diameter. Dit geeft aan dat het actine cytoskelet ook in deze gist een vergelijkbare rol speelt.

In hoofdstuk 2 combineren we metingen, aan structuren in transmissie electronen microscopische coupes en groeisnelheid metingen van *Arabidopsis* wortelharen en pollenbuizen om een schatting te maken van het aantal exocytose- en endocytose-gebeurtenissen dat plaatsvindt in beide cel-typen. In wortelharen vinden we 4202 exocytose gebeurtenissen en 6901 endocytose gebeurtenissen per minuut. In pollenbuizen zijn de gevonden waarden 3238 exocytose gebeurtenissen en 21265 endocytose gebeurtenissen per minuut.
Actine depolymerisatie, zodat de aanlevering van nieuwe exocytose vesicles in de pollenbuis top verhinderd wordt, leidt tot het stoppen van groei in 30-40 seconden. In deze tijd worden geen nieuwe vesikels aangevoerd en alle aanwezige vesikels in de plasmamembraan gebracht. Deze waarde komt overeen met de theoretisch geschatte waarde van 33 seconden tot alle exocytose vesicels zijn geconsumeerd die aanwezig zijn in de ronding van de celtop.

Sinds lange tijd is bekend dat de kern altijd aanwezig is op een vaste afstand van de top in groeiende wortelharen. In hoofdstuk 3 laten we zien dat een intact actine cytoskelet noodzakelijk is voor correcte positionering van de kern door middel van gebruik van CD en LA.
Injectie van een antilichaam tegen een villine-achtig eiwit leidt tot ontbundeling van gebundelde actine filamenten en beweging van de kern in de richting van de apex. Aangezien bekend is dat dit eiwit actinefilamenten bundelt, blijkt hieruit dat bundeling van actine filamenten de kern op een minimale afstand van de top houdt. Er zou naast actinebundeling een andere kracht kunnen zijn die de kern in de richting van de groeiende top trekt. Welke kracht dit is, is niet bekend. Optical trapping van de kern laat zien dat correcte positionering van de kern essentieel is voor topgroei van *Arabidopsis* wortelharen.

In *Arabidopsis* wortelharen zijn microtubuli vooral gelocaliseerd in de cel-cortex in een net-axiale orientatie. Depolymerisatie van microtubuli leidt niet tot veranderingen in groeisnelheid en niet tot veranderingen in top diameter. Hieruit blijkt dat microtubuli, net als in intercalair groeiende cellen, geen belangrijke rol spelen bij het bepalen van de hoeveelheid exocytose die plaatsvindt, en evenmin een belangrijke

rol spelen bij het bepalen van de grootte van de plaats waar celgroei plaatsvindt in wortelharen.
Wortelharen die groeien in de afwezigheid van microtubuli zijn licht golvend in plaats van recht ten opzichte van de wortelas. Dit suggereert dat microtubuli wel een rol spelen in het bepalen van de locatie van het gebied waar exocytose plaatsvindt.

In hoofdstuk 4 geven we een overzicht van mogelijke functies die microtubuli in wortelharen zouden kunnen spelen. Uit gepubliceerde literatuur blijkt dat in veel weefsels de corticale microtubuli in de cellen dezelfde orientatie hebben als de laatst afgezette cellulose microfibrillen in de celwand. In wortelhaarbuizen is dit niet altijd het geval. De hypothese dat corticale microtubuli de cellulose microfibrillen richten doordat ze in de zelfde richting georienteerd zijn als cellulose microfibrillen is niet correct. Omdat in de top van groeiende wortelharen zowel de corticale microtubuli als de cellulose microfibrillen een random orientatie hebben, zijn de gegevens over wortelharen voor groeiende cellen niet in tegenspraak met de bovenstaande hypothese.

Hoofdstuk 5 is een overzichtsartikel waarin de rol van het actine cytoskelet, microtubuli en de concentratie van vrije calcium ionen in het cytoplasma tijdens wortelhaargroei worden besproken. De data, verkregen uit publicaties over wortelharen worden geextrapoleerd naar intercalair groeiende plantencellen. Door data, verkregen uit gepubliceerd onderzoek aan dierlijke cellen te gebruiken maken we een hypothese die de rol van het actine cytoskelet in het bepalen van de plaats waar celgroei plaatsvindt in plantencellen beschrijft. Tevens wordt het belang van een goede fixatie besproken wanneer men het actine cytoskelet wil visualiseren in wortelharen.

Summary

In this thesis, research is presented that contributes to a better understanding of the function of the cytoskeleton in cell expansion. We use tip growing root hairs, mostly from *Arabidopsis thaliana*, as a model system. In the different chapters, different functions of the cytoskeleton in root hair growth are being covered.
Chapter 1 covers the role of the actin cytoskeleton in determining the location where cell expansion, i.e. the fusion of Golgi-derived vesicles with the plasma membrane, exocytosis, takes place. In growing *Arabidopsis* root hairs, the actin cytoskeleton is organised similarly as in root hairs of *Vicia sativa*: from base to tip thick bundles, running mostly longitudinally to the root hair axis, gradually branching in a meshwork of thinner bundles, also mostly longitudinally oriented. In the extreme apex, we did not detect filamentous actin when using immuno-fluorescence methods.
The actin depolymerizing drugs cytochalasin D (CD) and latrunculin A (LA) induce depolymerization of the tip-directed ends of actin filaments. Pulse application to the roots of low concentrations of these drugs broadened growing root hair tips (i.e. the area where cell expansion takes place increases). This demonstrates that during root hair growth, a local increased instability of the subapical actin cytoskeleton specifies the size of the area in the apex where growth takes place.
Accordingly, local application of CD to one side of the dome of a growing hair leads to a local increase in the amount of cell expansion at that location.
To test the hypothesis that the degree of instability of the (sub-)cortical actin cytoskeleton also in intercalary growing cells leads to a local increase of cell expansion at that location, we locally applied CD onto intercalary growing *Tradescantia* stamen hair cells. At the side of application, a local outgrowth formed, confirming our hypothesis. Also in a tip-growing yeast (*Schizosaccharomyces pombe*) application of CD or LA led to an increase in cell width, indicating that the actin cytoskeleton plays a similar function in the regulation of exocytosis in this yeast.

In chapter 2, we collect data from transmission electron microscopic sections and growth speed measurements of *Arabidopsis* root hairs and pollen tubes for measurements of the dimensions of exocytotic vesicles, cell wall width and growth speed. The obtained data are being used to calculate the number of exocytotic and endocytotic events, taking place in both tip-growing cell types. In root hairs there

were 4202 exocytotic events and 6901 calculated endocytotic events per minute. In pollen tubes, calculated values were 3238 exocytotic events and 21265 endocytotic events per minute.
Actin depolymerisation, so that delivery of new exocytotic vesicles to the pollen tube tip is inhibited, leads to growth inhibition after 30-40 seconds. This value coincides with the theoretically calculated value of 33 seconds until all vesicles present in the dome of a pollen tube, growing under the microscope, at room temperature, are consumed.

It has been known for a long time now that the nucleus is always present at a fixed position from the tip of growing root hairs. In chapter 3, we demonstrate that an intact actin, but not microtubule, cytoskeleton is essential for proper positioning of the nucleus by using CD, LA and oryzalin in concentrations that specifically depolymerize particular parts of the cytoskeleton of actin and microtubules.
Injection of an antibody against a villin-like protein leads to debundling of bundled actin filaments and simultanous apex directed movement of the nucleus. This demonstrates that the nucleus is being kept at a minimal distance from the growing tip by actin filament bundling. Besides bundling, another force has to be present, drawing the nucleus in the direction of the root hair tip. What causes this force is not well understood. Optical trapping of the nucleus indicates that proper positioning of the nucleus is essential for tip growth.

In *Arabidopsis* root hairs, microtubules localise net-axally in the cell cortex. Depolymerisation of microtubules does not lead to changes in growth speed and tip diameter, showing that microtubules do not play an important role in determining the amount of exocytosis that takes place and neither play an important role in determining the size of the area where exocytosis takes place. Root hairs, growing in the absence of microtubules are slightly wavy. This suggests that microtubules do play a role in determining the location where exocytosis takes place.

In chapter 4, we review possible functions of microtubules in root hairs. Published research reveals that in many tissue types the cortical microtubules are in the same orientation as the latest deposited cellulose microfibrils in the cell wall. In root hair tubes, this is not always true. Therefore, the hypothesis that cortical microtubules are

by default oriented in the same direction as the latest deposited cellulose microfibrils is not valid. Since in tips of growing root hairs, both cortical microtubules and cellulose microfibrils are randomly oriented. Data on root hairs do not contradict this paradigm for elongating cells or cell parts.

Chapter 5 is a review wherein the roles of the actin cytoskeleton, microtubules and the concentration of free calcium ions in the cytoplasm in root hair growth are discussed. The data obtained from publications about root hairs are extrapolated to intercalary growing plant cells. By using data, obtained from published research on mammalian cells, we build a hypothesis about the role of the actin cytoskeleton in determining the location where cell expansion takes place in plant cells. Besides, the importance of a proper fixation for actin visualisation in root hairs is discussed.

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Tijs, 14-10-2002

Curriculum Vitae

Tijs Ketelaar werd op 16 mei 1973 geboren te Doetinchem. In 1991 behaalde hij het diploma VWO aan het Isala College te Silvolde. In het najaar van dat jaar startte hij de opleiding Plantenveredeling (T13) aan de toenmalige Landbouwuniversiteit te Wageningen. Tijdens zijn studie in Wageningen ontdekte Tijs al vrij snel zijn liefde voor de plantencelbiologie. Hij deed dan ook zijn eerste afstudeervak binnen dit vakgebied op de vakgroep Planten Cytologie en -Morphologie: 'Localization of spectrin like epitopes in nuclei of *Pisum sativum*' onder begeleiding van Dr. Jan Schel en ing. Norbert de Ruijter, hetgeen leidde tot een publicatie.

Na een tweede afstudeervak voltooid te hebben aan de vakgroep Plantenveredeling, 'Introduction of sense and antisense fragments of the cassava (*Manihot escuenta* Crantz) AGPase B gene in potato', begeleid door Dr. Richard Visser en MSc. Tichafa Munyikwa, besloot hij, mede door het enhousiasme van Dr. Anne Mie Emons ook een stage te volgen binnen de plantencelbiologie. Deze stage werd uitgevoerd op het Department of Plant Cell Biology aan de University of Minnesota in de Verenigde Staten. Tijs werkte aan 'Comparison of alterations in tubulin isoform composition after chilling of a cold tolerant and a cold sensitive sweetcorn (*Zea mays*) variety' onder begeleiding van Dr. Sue Wick. Tijdens deze stage solliciteerde hij naar een AIO positie bij Dr. Anne Mie Emons waarvoor hij werd aangenomen. Het promotieonderzoek werd uitgevoerd in de periode 1996 tot 2001. Tijdens zijn promotie volgde Tijs de prestigieuze cursus Physiology aan het Marine Biological Laboratory te Woods Hole MA in de Verenigde Staten en won daar financiering voor een onderzoeksproject na afronding van de cursus. Ook sprak hij op verschillende nationale en internationale congressen over zijn werk, zoals de NATO Advanced Research Workshop ‘Cell biology of Plant and Fungal Tip Growth’ en op de 16de Meeting of the European Cytoskeleton Forum. Het promotieonderzoek resulteerde in de totstandkoming van dit proefschrift.

Vanaf juni 2001 is Tijs werkzaam als postdoctoraal onderzoeker aan de University van Durham in het Verenigd Koninkrijk, in het laboratorium van Dr. Patrick Hussey. Dit laboratorium is gespecialiseerd in het cytoskelet van plantencellen en eiwitten die het cytoskelet beinvloeden of gebruiken om signalen door te geven. Hij werkt er onder andere aan een familie eiwitten die bindt aan microtubuli.

List of publications

De Ruijter, NCA, Ketelaar, T, Blumenthal, SS, Emons, AMC, Schel, JH. (2000)
Spectrin-like proteins in plant nuclei
Cell Biol Int. 24, 427-438

Ketelaar, T, Emons, AMC (2000)
The role of microtubules in root hair growth and cellulose microfibril deposition
In: Ridge RW and Emons AMC, eds. *Root hairs. Cell and molecular biology.* Tokyo Berlin Heidelberg New York: Springer-Verlag, 17-28

Ketelaar, T, Emons, AMC (2001)
The cytoskeleton in plant cell growth: lessons from root hairs
New Phytologist 152, 409-418

Ketelaar, T, Faivre-Moskalenko, C, Esseling, JJ, De Ruijter, NCA, Grierson, CS, Dogterom, M, Emons, AMC (2002)
Positioning of nuclei in Arabidopsis root hairs: an actin regulated process of tip growth
Plant Cell 14, in press

Ketelaar, T, De Ruijter, NCA, Emons, AMC. (2003)
Unstable F-actin specifies area and microtubules direction of cell expansion in Arabidopsis root hairs
Plant Cell 15, accepted

Stellingen

1. Omdat exocytose een proces is dat in cellen van alle eukaryotische organismen voorkomt, is het voor de hand liggend dat het op een geconserveerd mechanisme berust.
(Dit proefschrift, hoofdstuk 1; Ketelaar et al., Plant Cell, accepted)

2. Men kan complexe processen, zoals de positionering van kernen in wortelharen, alleen doorgronden als men ook alle krachten die erbij betrokken zijn en hun waarden kent.
(Dit proefschrift, hoofdstuk 3; Ketelaar et al., (2002) Plant Cell 14: 2941-2955)

3. In een groeiende plantencel is lokale toename van actine-instabiliteit voldoende om de hoeveelheid celgroei op die locatie te vergroten.
(Dit proefschrift, hoofdstuk 1; Ketelaar et al., Plant Cell, accepted)

4. In een polair groeiende cel, zoals een wortelhaar, zijn fixatie-artefacten van het actinecytoskelet duidelijker zichtbaar dan in intercalair groeiende cellen, maar een snelle fixatie is even essentieel voor beide typen cellen.
(Dit proefschrift, hoofdstuk 5; Ketelaar and Emons (2001), New Phytologist 152: 409-418)

5. Het bestuderen van het actinecytoskelet met green fluorescent protein (GFP) gekoppeld aan het actinebindend domein van taline uit muizen (Kost et al, 1998) is voor veel plantencellen de enige manier om het actine cytoskelet in levende cellen te bestuderen. Echter de binding van een eiwit zo groot als een actinemonomeer aan filamenteus actine kan tot veranderingen leiden in de dynamische eigenschappen van het actinecytoskelet, waardoor de observaties met dit construct waarschijnlijk niet volledig overeenkomen met de werkelijke situatie.
(Kost et al. (1998), Plant J. 16: 393-401)

6. De vondst van een actine-achtig eiwit in bacteriën dat betrokken is bij bepaling van de celvorm (Van den Ent et al., 2001) geeft aan dat ook in prokaryoten een actine-achtig eiwit een rol zou kunnen spelen in het exocytose proces.
(Van den Ent et al. (2001), Nature 413: 39-44)

7. De relatief kleine hoeveelheid geld die jaarlijks door de overheid wordt uitgetrokken voor fundamenteel wetenschappelijk onderzoek getuigt van gebrek aan langetermijnvisie.

8. De kwaliteit van een proefschrift is in belangrijke mate afhankelijk van de wetenschappelijke kwaliteit van de onderzoeksgroep waarin de promovendus werkzaam is.

9. Als grote lengte de enige parameter zou zijn die de kwaliteit van een basketbalspeler bepaalt, dan zou Nederland - gezien de gemiddelde lengte van de inwoners - één van de beste basketbalteams van de wereld moeten hebben.

10. Een werkdiscussie onder het genot van een stevige borrel leidt tot een stroom aan originele ideeën, waarvan tenminste een gedeelte bruikbaar is in het onderzoek van de aanwezige wetenschappers.

11. De neiging van veel Nederlanders om de kroket als culinair hoogtepunt van de Hollandse keuken op te voeren leidt tot onderwaardering van de Nederlandse keuken door buitenlanders.

12. Indien bij het berekenen van de prestatie van openbaar vervoer de vertraging per afgelegde kilometer als norm zou worden genomen, zou het Nederlandse openbaar vervoer internationaal gezien beduidend slechter voor de dag komen dan nu het geval is.

13. Het veroorzaken van vervuiling door de mensheid maakt niet het milieu maar de mensheid de grote verliezer.

Stellingen behorende bij het proefschrift
'Spatial organisation of cell expansion by the cytoskeleton'

Tijs Ketelaar, Wageningen, 9 december 2002.