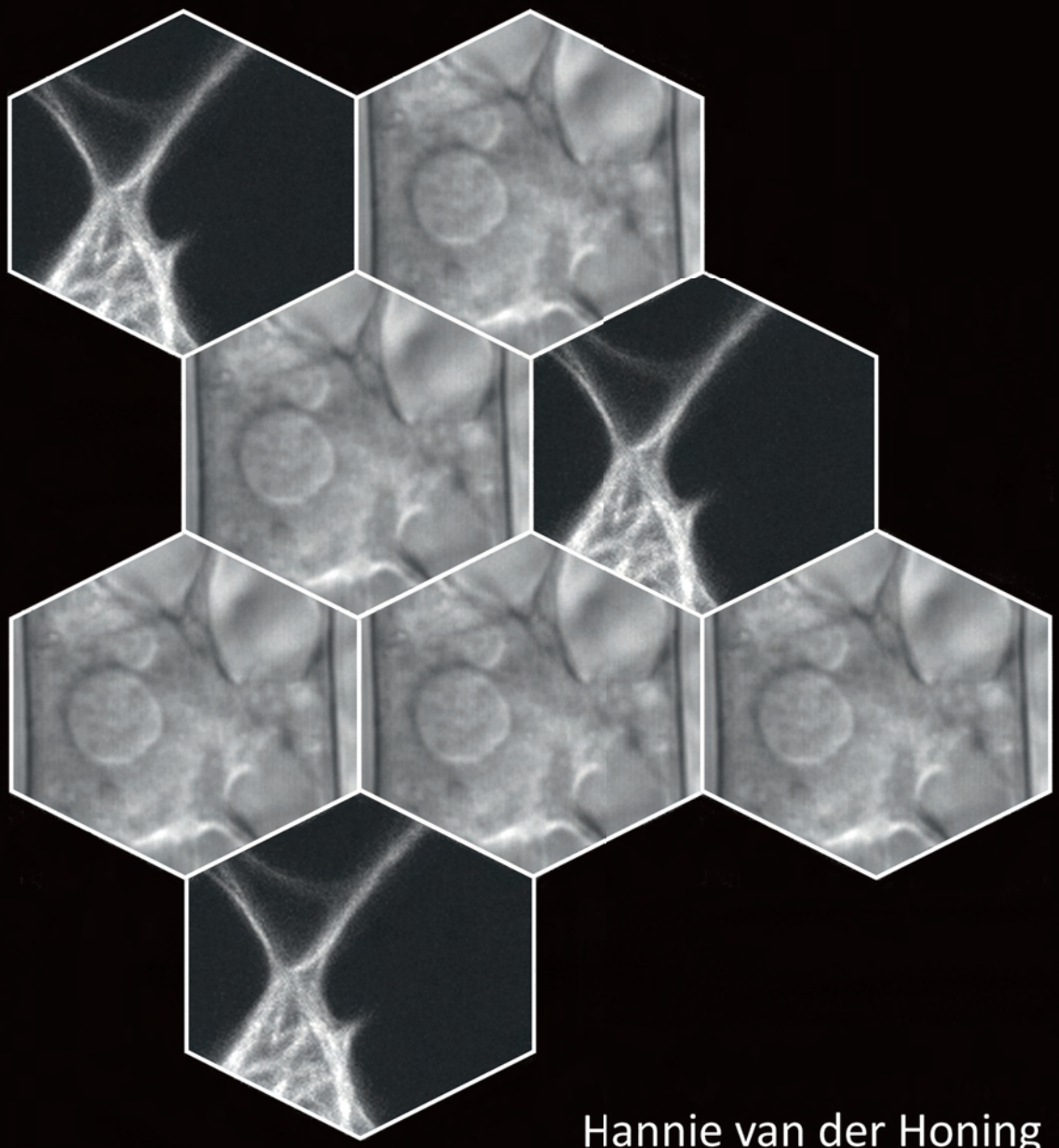
Actin-mediated cytoplasmic organization of plant cells



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Actin-mediated cytoplasmic organization of plant cells

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Thesis

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Outline of the thesis

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In this thesis, I present results that give insight in the role of the actin cytoskeleton in the production of an organized cytoplasm in plant cells, which is, for instance, required for proper cell morphogenesis.

Chapter 1 is a review in which we discuss the possible role of actin-based force generation in the production of an organized cytoplasm in plant cells. We compare the functions of actin binding proteins of three well-studied mammalian model systems that depend on actin-based force generation with the possible functions of their homologues in plants, and predict how these proteins might determine the cytoplasmic architecture of plant cells.

In chapter 2, we describe the use a combined setup of optical tweezers with a confocal laser scanning microscope to study whether stiffness is an actin-related property of plant cytoplasm, and to study parameters involved in the reorganization of the actin cytoskeleton during physical manipulation of the cytoplasm. We used optical tweezers to produce cytoplasmic protrusions that resemble cytoplasmic strands, while imaging the behaviour of the actin cytoskeleton. We determined the trapping force needed to produce cytoplasmic protrusions, and show that the presence of actin filaments stiffens the cytoplasm. The deactivation of a 2,3-butanedione monoxime (BDM)-sensitive factor, probably the molecular motor myosin, stiffens the cytoplasm even more. The observation that actin filaments do not enter the tweezer-formed protrusions during this BDM treatment, suggests that the actin cytoskeleton can reorganize by a myosin-based relocation of actin filaments. Such a myosin-based reorganization of the actin cytoskeleton might be involved in the production of an organized cytoarchitecture in plant cells.

Lifeact:Venus, which consists of the first 17 amino acids from the yeast protein Abp140 fused to a yellow fluorescent protein, is a novel probe for actin filament visualization. In chapter 3, we compare the (re)organization of the actin cytoskeleton visualized with Lifeact:Venus with that of the actin cytoskeleton visualized with GFP:FABD2, a commonly used marker for filamentous actin in plants that consists of GFP fused to the second actin binding domain of *Arabidopsis* FIMBRIN1. We show that Lifeact:Venus reduces remodeling of the actin cytoskeleton in *Arabidopsis* root epidermal cells, as well as concomitant reorganization of the cytoplasm. Nonetheless, expression of Lifact:Venus neither affects cytoplasmic organization, nor plant growth and development. The data imply that the organization of the actin cytoskeleton, but not its dynamic relocation over time, is a determining factor in plant cell growth, and show that Lifeact should be used with caution when studying reorganization of actin filaments.

In cytoplasmic strands, actin filaments are organized in thick bundles. The actin bundling protein villin is involved in maintaining these bundles. In chapter 4, we analyze the role of VLN2 and VLN3, two members of the villin protein family in *Arabidopsis*, and show that mutations in the genes encoding these villins result in a decrease in the number of thick actin filament bundles. Double mutant plants have abnormal leaves, stems, siliques and roots. The wavy, twisted appearance of these organs in the double mutant shows that the coordination of cell expansion is affected. Furthermore, the rotational movements

(circumnutation) of *vln2 vln3* inflorescences have larger amplitudes than those of wild type Col-0 inflorescences and are less regular. The data show that VLN2 and VLN3 are involved in the generation of thick actin filament bundles, and suggest that these bundles are important for coordinated cell expansion.

Chapter 5 is the general discussion of the thesis. We discuss research in which actin binding proteins that could play a role in cytoplasmic organization have been described. In this chapter, we have included our initial data about the role of the actin bundling protein fimbrin on actin organization. We further discuss how manipulation of cytoplasmic organization by optical tweezers can give insight into physical properties of actin filaments in the plant cytoplasm.

Chapter 1

Actin based processes that could determine the cytoplasmic architecture of plant cells

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Abstract

Actin polymerisation can generate forces that are necessary for cell movement, such as the propulsion of a class of bacteria, including *Listeria*, and the protrusion of migrating animal cells. Force generation by the actin cytoskeleton in plant cells has not been studied. One process in plant cells that is likely to depend on actin-based force generation is the organisation of the cytoplasm. We compare the function of actin binding proteins of three well-studied mammalian models that depend on actin-based force generation with the function of their homologues in plants. We predict the possible role of these proteins, and thus the role of actin-based force generation, in the production of cytoplasmic organisation in plant cells.

Introduction

The actin cytoskeleton is present in all eukaryotic cells and is essential in many cellular processes. The actin cytoskeleton is highly dynamic. A pool of monomeric actin (G-actin) and filamentous actin (F-actin) are simultaneously present in the cytoplasm. G-actin can polymerise into F-actin, which in turn can depolymerise into G-actin. Actin filaments are polarised structures, with one end referred to as the barbed (plus) end, and the other end referred to as the pointed (minus) end. Polymerisation preferably takes place on the barbed ends of F-actin, whereas depolymerisation takes place preferably on the pointed end. The formation and dynamics of F-actin depend on the interactions of the filaments with actin-binding proteins (Hussey et al., 2006). One important function of the actin cytoskeleton in animal cells is the localised exertion of a force on the plasma membrane by coordinated actin nucleation and polymerisation. In this way, extensions of the plasma membrane are locally formed, which enables animal cells to alter their shape and to move. The actin-binding proteins that are involved in this system are known, and homologues of most of these proteins are present in plant cells. Since plant cells contain a cell wall, their shape depends on the local deposition of cell wall material. Actin filaments are important in this process, because they deliver the exocytic vesicles that contain cell wall material itself, or enzymes for its production, to the location of cell elongation (Emons and Mulder, 2000; Hussey et al., 2006). However, it is unlikely that actin-based force generation is involved in determining the shape of these cells, since the force that is generated is likely to be insufficient to stretch the cell wall. Summarising: all the classes of proteins that are needed for force generation by actin nucleation and polymerisation are present in plant cells, but actin-based force generation is not likely to be involved in determining the shape of these cells. Could actin-based force generation play another role in plant cells?

The tonoplast is the vacuolar membrane. Mature plant cells possess one or several large vacuoles, which can occupy over 90% of the total cell volume (Kutsuna and Hasezawa, 2002; Ruthardt et al., 2005). The cytoplasm fills up the rest of the cell interior, and surrounds the vacuole(s). The cytoplasmic organisation of plant cells varies with its developmental stage. Usually, a layer of cytoplasm is located in the cortical and perinuclear areas of the cell. These two areas of cytoplasm are interconnected by strands of cytoplasm that traverse the vacuole: the transvacuolar or cytoplasmic strands, bounded by the tonoplast (Fig. 1). Cytoplasmic strands are thought to function as transport routes for transcripts, proteins and organelles. The majority of all intracellular transport in plant cells occurs over actin filaments, and this active movement of organelles is called cytoplasmic streaming (reviewed in Shimmen and Yokota, 2004). This streaming is likely to be facilitated by myosin XI coated organelles that move over bundles of F-actin throughout the cytoplasm (Holweg and Nick, 2004). Cytoplasmic strands are constantly changing in shape and location (Ruthardt et al., 2005). It is not clear what causes this constant reorganisation, but since actin filaments are the backbone of cytoplasmic strands, rearrangements of the actin cytoskeleton are thought to be responsible for this dynamic behaviour (Hoffmann and Nebenfuhr, 2004). In addition, there are indications that myosins play a role in the reorganisation of existing cytoplasmic strands (Hoffmann and Nebenfuhr, 2004).

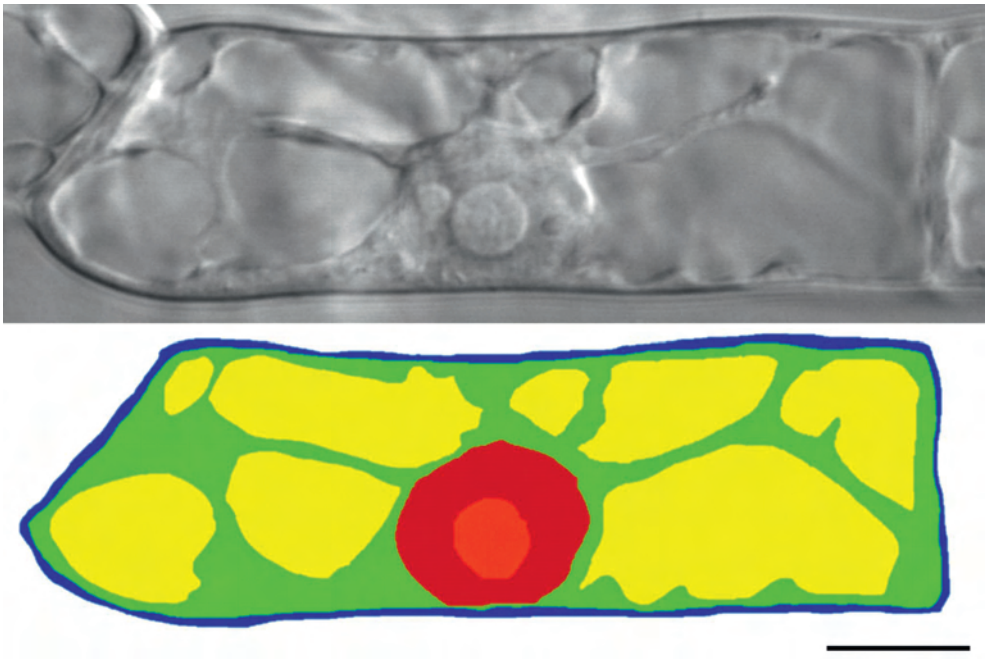

Figure 1. Differential interference contrast image of a tobacco bright yellow 2 suspension cell. Cytoplasmic strands (green) traverse the vacuole (yellow), and connect the cortical and perinuclear cytoplasm (cytoplasm is green; nucleus is dark red; nucleolus is light red). The cell wall (blue) encases the cell. Scale bar, 10 µm.

As stated above, cytoplasmic strands are bounded by the tonoplast. The shape of the tonoplast is determined by the actin cytoskeleton: when F-actin is depolymerised, cytoplasmic strands disappear (Staiger et al., 1994; Shimmen et al., 1995; Valster et al., 1997; Hussey et al., 1998; Van Gestel et al., 2002). Cytoplasmic dense areas, such as those typical for the apical and subapical area of growing root hairs, also dissipate when F-actin is depolymerised (Miller et al., 1999; Ketelaar et al., 2002; Ketelaar et al., 2003). Thus, F-actin not only serves as a transport route, but is also the backbone of cytoplasmic strands and cytoplasmic dense areas. The tonoplast is not fortified by a cell wall and its shape is determined by the actin cytoskeleton. Could the formation of cytoplasmic strands and cytoplasmic dense areas in plant cells depend on a process similar to the coordinated nucleation and polymerisation of actin filaments in animal cells?

In this review, we will look at actin based force generation in animal cells, and review the results on actin-binding proteins that are involved in this process. We will relate the findings in animal cells to the properties of the actin cytoskeleton in plant cells, and speculate about the function of plant homologous proteins in the formation of cytoplasmic organisation.

Model systems of force generation by actin nucleation and polymerisation

There are three model systems in which actin nucleation and polymerisation generate forces: the propulsion of unicellular bacteria through the cytoplasm of their host cells, first described for *Listeria monocytogenes* (Tilney et al., 1992), protrusion of lamellipodia, important for the crawling motion of animal cells over a substrate (Abercrombie, 1980), and the formation of filopodia, thin cylindrical extensions that are present between lamellipodia (reviewed in Wood and Martin, 2002). These three systems have been extensively investigated, and it was shown that polymerisation of a dense network of actin filaments that branch from each other at the surface of bacteria and at the plasma membrane of lamellipodia results in a force generation that is sufficient for motility/protrusion (reviewed in Goldberg, 2001; Small et al., 2002; Carlier et al., 2003; Plastino and Sykes, 2005). A prerequisite for force generation by unbundled actin filaments is that the filaments are relatively short. Short filaments are less flexible, and because of their stiffness, force is exerted more effectively (Upadhyaya and Van Oudenaarden, 2003). Indeed, the spacing between branches is in the order of tens to a few hundred nanometers (Mullins et al., 1998; Upadhyaya and Van Oudenaarden, 2003) and it was theoretically shown that the length of a pushing filament must be 30–150 nm (Mogilner and Oster, 1996; Upadhyaya and Van Oudenaarden, 2003), which is far shorter than the persistence length of an actin filament—the length at which an actin filament starts to bend spontaneously due to thermal fluctuations (i.e. about 15 μm (Ott et al., 1993)). The force that is generated by protruding bacteria has been estimated between 0.01 to up to 10 nN by different groups that used different techniques (Giardini et al., 2003; McGrath et al., 2003; Upadhyaya et al., 2003; Wiesner et al., 2003; Boukellal et al., 2004; Marcy et al., 2004; Parekh et al., 2005). The force production by lamellipodium protrusion has been estimated to lie in the nN range - a value that is comparable to the force production during *Listeria* propulsion (Abraham et al., 1999). The molecular components of actin-based force generation have been identified, and it was proven that homologous proteins play important roles in both systems, supporting the idea that a similar molecular mechanism is responsible for the propulsion of pathogens and lamellipodium formation at the leading edge of crawling cells (Beckerle, 1998; Cameron, 2000; Carlier et al., 2003).

Filopodium protrusion exceeds the maximal length of a pushing filament before it starts buckling. By forming a bundle of 10–30 actin filaments (Svitkina et al., 2003; Mogilner and Rubinstein, 2005; Atilgan et al., 2006), a stiffer structure is formed that does not buckle until it reaches a far larger length than a single actin filament could achieve before buckling. It was theoretically shown that although a filopodium contains a bundle of actin filaments, the maximal length that it can reach is still limited by buckling of the bundle, showing that the length of actin filaments is limited in order to allow force production (Mogilner and Rubinstein, 2005). Since filopodia formation depends on the presence of a bundle of actin filaments, the molecular mechanism underlying this process is different from the molecular mechanisms responsible for bacterial propulsion and lamellipodia formation. Next, we will discuss the different molecular mechanisms of coordinated actin polymerisation that are responsible for force generation in these three model systems.

Actin-based motility of Listeria bacteria

A breakthrough in the attempts to understand the mechanism of actin-based force production was the finding that polystyrene beads coated with ActA, which is present at the surface of *Listeria*, formed comet tails when placed in actin-rich cell-extracts, resulting in a directional movement of the beads, comparable to the motion of *Listeria* through its host cells (Cameron et al., 1999). By mimicking the intracellular environment in these extracts, but only adding a limited number of proteins, the essential components required for actin-based motility could be identified (Loisel et al., 1999). Only a limited number of actin binding proteins is required to be present for the formation of a comet tail: the Arp2/3 complex, ADF/cofilin, capping protein (such as gelsolin) and ActA (Fig. 2A). ActA, which is asymmetrically distributed on the surface of *Listeria* (Kocks et al., 1993), is the only bacterial protein that is required for propulsion. Below, we discuss how the different actin binding proteins contribute to actin-based force generation.

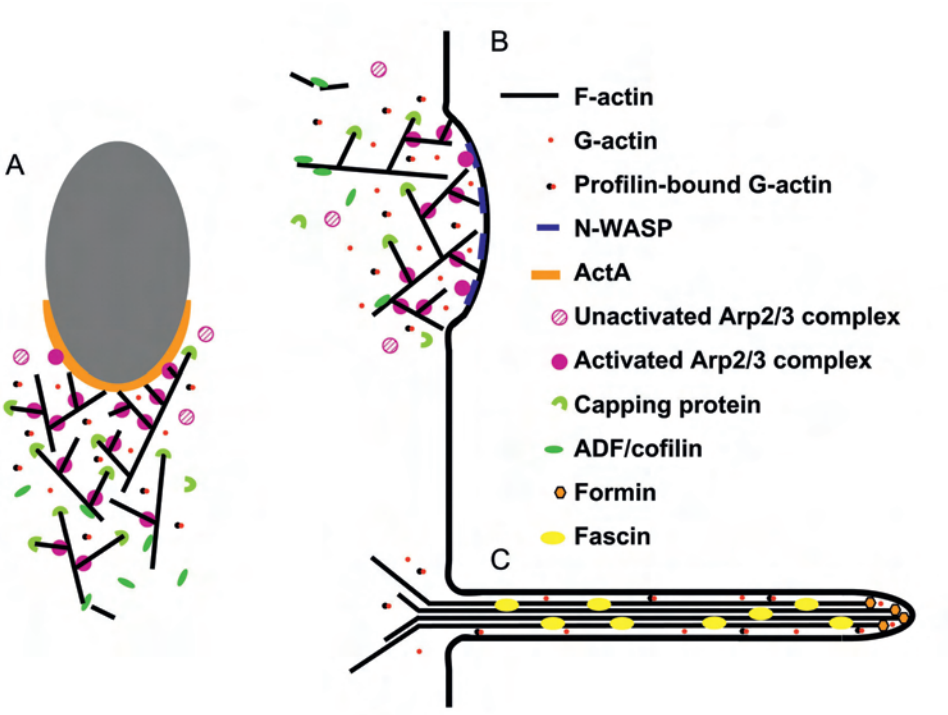

Figure 2. Schematic diagram representing the three model systems for actin-based force generation. (A) *Listeria* propulsion. ActA, which is distributed asymmetrically on the bacterial surface, activates the Arp2/3 complex, which nucleates actin-filaments at the side of existing filaments. Polymerisation of the barbed ends at the bacterium surface produces the force needed for propulsion. Capping protein and ADF are needed for the regulation of actin dynamics. (B) Lamellipodium extension. Activated N-WASP at the membrane surface activates the Arp2/3 complex, which results in the formation of a similar, branched actin network as in (A). (C) Filopodium extension. Polymerisation of actin filaments in association with formins produces the force needed for filopodium extension. Fascin is required to bundle the long, linear filaments, to prevent them from buckling. The image is not to scale and not all proteins that are known to affect these systems are included.

The ARP2/3 complex

The Arp2/3 complex is a highly conserved protein complex that consists of seven subunits, two of which are the actin binding proteins Arp2 and Arp3. The complex is concentrated in actin comet tails (Welch et al., 1997). Activated Arp2/3 complex nucleates actin filaments by promoting barbed-end assembly while capping the pointed end (Machesky and Way, 1998; Mullins et al., 1998; Welch et al., 1998; Machesky and Gould, 1999; Pollard and Beltzner, 2002). This nucleation occurs only when Arp2/3 is bound to the flank of an existing filament, so that the newly formed actin filament grows at a fixed angle of 70 ° from the mother filament (Mullins et al., 1998). The Arp2/3 complex can be activated by different classes of activators, which all transmit signals from different pathways to the actin cytoskeleton. *Listeria* bacteria have their own Arp2/3 activator, ActA. Actin nucleation occurs so that the barbed ends of the nucleated actin filaments are pointing in the direction of the surface of propelling bacteria. By creating a dense cloud of branched actin filaments, which is subsequently becoming polarised into a comet tail, a sufficient force is generated for propulsion of bacteria. *Shigella* and the vaccinia virus, other pathogens that undergo actin-based propulsion, activate the N-WASP protein (one of the members of the Wiscott–Aldrich Syndrome Protein family of proteins) of their hosts by cell surface proteins. In *Shigella*, N-WASP is activated by IcsA (Bernardini et al., 1989), and in the vaccinia virus, a pathway that involves the integral membrane protein A36R (Frischknecht et al., 1999) activates N-WASP. N-WASP in turn induces Arp2/3 complex activation (reviewed in Frischknecht and Way, 2001; Gouin et al., 2005).

Capping protein

In addition to the Arp2/3 complex and an Arp2/3 activator, capping proteins are required for actin-based propulsion of *Listeria* (Loisel et al., 1999). This class of proteins is represented by the gelsolin family of proteins, which perform numerous additional functions outside the scope of this review (reviewed in Silacci et al., 2004). Capping proteins tightly bind to the barbed end of actin filaments, thus preventing both polymerisation and depolymerisation at this end. By decreasing the number of free barbed ends, capping protein increases the polymerisation rate of the few remaining uncapped filaments (Carlier and Pantaloni, 1997). Due to the combination of nucleation of free barbed ends by the Arp2/3 complex, and rapid capping of these free barbed ends by capping proteins, a comet tail of interconnected, short actin filaments will form. Since Arp2/3 mediated nucleation continues when actin filaments are capped, the density of branches increases with the concentration of capping protein (Wiesner et al., 2003). The presence of capping proteins only, however, is not sufficient to reach the high propulsion rates of bacteria, as continued actin polymerisation exhausts the source of G-actin (Carlier and Pantaloni, 1997).

Actin depolymerising factor

Actin depolymerising factor (ADF/cofilin) is the component that increases the amount of G-actin that is needed for fast barbed end growth (Carlier et al., 1999; Loisel et al., 1999). ADF binds to both G-actin and F-actin, enhances the turnover of actin filaments by increasing depolymerisation at the pointed end and severs existing filaments (Carlier et al., 1997; Bamburg, 1999). Since polymerisation at the barbed end produces the force that is needed for propulsion, sufficient available actin monomers are required

for this polymerisation. An enhanced turnover of actin filaments increases the number of available actin monomers for polymerisation and thus the rate of movement.

Proteins that enhance the efficiency of motility

Although the presence of the Arp2/3 complex, ADF/cofilin, capping protein, and ActA are sufficient for *Listeria* propulsion, several other actin binding proteins enhance the effectiveness of motility: profilin, α-actinin and VASP. Profilin specifically binds G-actin. When bound to profilin, spontaneous nucleation and incorporation at the pointed end are inhibited, whereas growth at the barbed end occurs at normal rates. Profilin by itself does not increase actin filament turnover rates. However, profilin synergises with ADF, increasing the rate of treadmilling from 25 fold for ADF alone to 125-fold when both ADF and profilin are present (Didry et al., 1998). This increases the rate of movement. Alpha-actinin cross-links actin filaments, thereby affecting the tail morphology, which becomes more rigid, leading to a more persistent movement (Dold et al., 1994; Loisel et al., 1999). VASP, a member of the Ena/VASP family, greatly enhances the rate of propulsion in *Listeria*. Ena/VASP proteins are known to enhance ActA-induced Arp2/3 nucleation in *Listeria* and to decrease the number of branches in the F-actin array in actin tails (Skoble et al., 2001), possibly by facilitating the dissociation of the Arp2/3 induced branch junction from the ActA that coats the bacteria surface, which is a rate limiting step (Samarin et al., 2003). The exact working mechanism of the protein is not known (Krause et al., 2003; see below) and more research is needed to elucidate the exact mechanism by which VASP increases propulsion rates.

Lamellipodium protrusion

The molecular mechanism involved in the formation of lamellipodia is very similar to that of *Listeria* propulsion (Fig. 2B). In contrast to the *Listeria* system, in which the Arp2/3 complex is activated by ActA, N-WASP activates the Arp2/3 complex during lamellipod formation. N-WASP itself is activated by Rho family GTPases (reviewed in Vartiainen and Machesky, 2004). Activation of the Arp2/3 complex generates a densely branched array of actin filaments with their barbed ends directed to the leading edge that pushes the membrane forward. Also capping protein is required (Mejillano et al., 2004). ADF promotes filament disassembly, predominantly at the rear of the lamellipodium, since actin filaments within a narrow zone at the leading edge are protected from depolymerisation (Svitkina and Borisy, 1999). In contrast to *Listeria* propulsion, which can occur in the absence of ADF, the protein is required for lamellipod extension: inhibition of ADF is sufficient to inhibit lamellipod extension, even when high concentrations of G-actin are present (Zebda et al., 2000). To explain this, Zebda et al. (Zebda et al., 2000) hypothesized that the function of ADF in lamellipodial protrusion not only concerns an enhanced turnover of actin filaments, but also the production of free barbed ends by actin severing (Zebda et al., 2000). Indeed, ADF was proven to generate free barbed ends in vivo (Ghosh et al., 2004). As in the *Listeria* propulsion system, Ena/VASP family proteins have a function in membrane protrusion: lamellipodia lacking Ena/VASP protrude slower, but the protrusion persists longer. Lamellipodia with excess Ena/VASP contain an F-actin array with a decreased density

of branches (Bear et al., 2002). In vitro, Ena/VASP antagonises the effect of capping protein, by protecting barbed ends from capping protein (Barzik et al., 2005). In this way, elongation of actin filaments increases, thus leading to a decreased number of branches in the F-actin array (Bear et al., 2002). However, the role of Ena/VASP proteins is not fully understood (review Ena/VASP proteins: Krause et al., 2003).

Formation and extension of filopodia

A third system that depends on actin-based force generation is the formation of filopodia (Fig. 2C). Filopodia are fingerlike extensions, expanding between the lamellipodia at the leading edge of motile cells. They function to explore the local environment (reviewed in Wood and Martin, 2002). As in lamellipodia, the membrane of filopodia is pushed forward by polymerisation of actin filaments that are oriented with their barbed ends towards the leading edge (Mallavarapu and Mitchison, 1999; Wood and Martin, 2002; Faix and Rottner, 2006). Extension of filopodia happens by polymerisation of a bundle of 10–30 tightly bundled linear, parallel running actin filaments (Svitkina et al., 2003; Mogilner and Rubinstein, 2005; Atilgan et al., 2006), suggesting that the Arp2/3 complex, which nucleates branched actin filaments, does not play a role in filopodium growth. Indeed, although Arp2/3-mediated actin nucleation has been proposed (Svitkina et al., 2003; Vignjevic et al., 2003) and shown (Biyasheva et al., 2004) to be required for filopodium initiation, the Arp2/3 complex is absent from most filopodia once they are established (Svitkina and Borisy, 1999). Furthermore, in a recent study, filopodia formation was unaffected by the absence of Arp2/3 mediated actin nucleation (Steffen et al., 2006). Although these studies are contradictive, it is sure that filopodium growth depends on a different molecular mechanism of force generation by the actin cytoskeleton than the Arp2/3 complex dependent mechanism responsible for *Listeria* propulsion and lamellipodium protrusion. Several actin-associated proteins are known to be enriched in filopodia, but the function of some of these proteins in the formation and extension of filopodia is still unknown (Schirenbeck et al., 2005a). We will discuss the actin-associated proteins that have a known function in filopodium growth.

Formin

Besides the Arp2/3 complex, formins are a second major group of proteins that stimulate the nucleation of actin filaments. Formins are, like the Arp2/3 complex, conserved among eukaryotic organisms, and are known to be involved in a wide range of actin-based processes, including cell polarisation and cytokinesis of fungi, invertebrates and vertebrates (Evangelista et al., 2003). Formins bind at or very near to the barbed end of actin filaments (Pruyne et al., 2002; Evangelista et al., 2003; Kovar et al., 2003; Zigmond et al., 2003), in this way preventing complete blocking of the barbed end by capping proteins. Furthermore, addition of profilin-sequestered actin monomers to the barbed end is accelerated (Romero et al., 2004), and de novo nucleation of actin filaments is promoted (reviewed in Zigmond, 2004a). In vivo, formins might produce linear actin filaments that can continue elongation, as formins remain bound to the barbed end during elongation (Zigmond, 2004b; Fig. 2C). Indeed, formins have recently been proven to play a role in filopodia formation: in a null mutant of a formin that is enriched in filopodial tips, fewer filopodia were formed, that were

shorter than wild type filopodia (Schirenbeck et al., 2005a; Schirenbeck et al., 2005b). In addition, overexpression of these formins caused an increase in the frequency of filopodium formation and filopodium length (Schirenbeck et al., 2005a; Schirenbeck et al., 2005b). Polymerisation of actin filaments in association with formins has been shown to produce a force of at least 1.3 pN per filament (Kovar and Pollard, 2004). This supports the idea that formins could also mediate some protrusive forces in cells (Wallar and Alberts, 2003; Higashida et al., 2004; Watanabe and Higashida, 2004).

Capping proteins

In the absence of capping protein, the formation of filopodia is highly increased (Mejillano et al., 2004), since actin filaments are allowed to continue elongating, leading to the bundle of parallel aligned linear actin filaments that is needed for filopodium formation.

Ena/VASP

Ena/VASP not only plays a role in the protrusion of lamellipodia; it is also targeted to filopodial tips (Rottner et al., 1999). In the absence of Ena/VASP, filopodium formation and elongation is inhibited in neurons (Lebrand et al., 2004) and in *Dictyostelium* (Han et al., 2002). Ena/VASP has been proposed to antagonise the effect of capping protein in filopodia (Bear et al., 2002), by inhibiting barbed end capping (Barzik et al., 2005), which would promote barbed end filament elongation and thus filopodium formation in vivo. Also in vitro, the antagonising effect of Ena/VASP on capping protein has been found (Barzik et al., 2005). Profilin enhances the ability of Ena/VASP to protect the barbed ends from capping protein (Barzik et al., 2005). However, when both capping protein and Ena/VASP are absent, filopodia formation is rare, and instead, cells switch to ruffling (Mejillano et al., 2004), indicating that in actin based ruffling, Ena/VASP and capping protein are not involved. When Ena/VASP is re-expressed, filopodia are formed again, proving that in addition to antagonising the effect of capping protein (Bear et al., 2002), Ena/VASP has a function in filopodium formation downstream of actin elongation. This function could be the bundling of actin filaments, as a recent study (Schirenbeck et al., 2006) shows that the actin filament bundling activity of VASP is crucial for formin-mediated filament elongation. In contrast with the hypothesis of Bear et al. (Bear et al., 2002), this recent study (Schirenbeck et al., 2006) shows that VASP does not compete with capping proteins or block depolymerisation from the barbed ends. The exact function of Ena/VASP thus remains to be elucidated.

Bundling proteins

In addition to the nucleation and elongation of the actin filaments, bundling of the linear, parallel running filaments is required for filopodium extension, in order to prevent buckling of the long filaments. There are several proteins with actin-bundling activity, but fascin is thought to be the most likely protein that bundles actin filaments in filopodia (Svitkina et al., 2003; Vignjevic et al., 2003).

Plant homologues of proteins involved in actin-based force production

The Arp2/3 complex

Although homologues of all components of the Arp2/3 complex (reviewed in (reviewed in Deeks and Hussey, 2003; Deeks and Hussey, 2005; Mathur, 2005), and a potential Arp2/3 activator, the SCAR complex (Egile et al., 1999; Deeks and Hussey, 2003; Deeks and Hussey, 2005; Mathur, 2005), are present in plant cells, there are no known homologues of the WASP and ActA family proteins in plants (Deeks and Hussey, 2003; Deeks and Hussey, 2005), and the presence of the Arp2/3 complex is not required for plants to survive (reviewed in Deeks and Hussey, 2005).

To analyse the function of the Arp2/3 complex in plants, *Arabidopsis* lines with null-mutations in Arp2/3 complex subunits have been used. These mutants are characterised by a surprisingly mild phenotype: trichomes are disturbed in their development, resulting in the development of twisted and/or short branches (Le et al., 2003; Li et al., 2003; Mathur et al., 2003a; Mathur et al., 2003b; El-Din et al., 2004) with an altered cytoplasmic organisation (Le et al., 2003; Mathur et al., 2003a; Mathur et al., 2003b). In addition, a decrease in actin-dependent cytoplasmic streaming was observed in these cells (Mathur et al., 2003b). Other cell types that are affected are epidermal cells of leaves, in which lobe extension is inhibited, and epidermal cells of dark-grown hypocotyls, which lose contact with their neighbours and curl out of the epidermal plane (Li et al., 2003; Mathur et al., 2003a; Mathur et al., 2003b; El-Din et al., 2004). Root hair growth in Arp2/3 mutants is disturbed; root hairs of these mutants are somewhat wavy and have a variable diameter. All these effects point to actin cytoskeleton defects, such as less or mislocalised fine F-actin (Deeks and Hussey, 2003; Le et al., 2003; Li et al., 2003; Mathur et al., 2003a; Mathur et al., 2003b; El-Din et al., 2004; Mathur, 2005). Summarising, the Arp2/3 complex seems to be involved in the organisation of the subapical fine F-actin array in rapidly growing cells (dark-grown hypocotyl epidermal cells) or cells with cell expansions that take place over a limited surface area (trichomes, root hairs and leaf epidermal cells), but its role does not seem to be of major importance in other cell types. The Arp2/3 complex is therefore hypothesized to only contribute to the nucleation of a small fraction of the total F-actin within higher-plant cells (Deeks and Hussey, 2005).

Capping protein

A gelsolin-like protein has been isolated from *Papaver* pollen (Huang et al., 2004). This protein tightly binds to the barbed ends of actin filaments in vitro, in this way preventing polymerisation and depolymerisation at the barbed ends in a calcium dependent way. The gelsolin-like protein also has actin filament nucleation and severing properties. The *Arabidopsis* genome, however, does not contain sequences for gelsolin-like proteins (Huang et al., 2004). The closest sequence homologues in *Arabidopsis* to gelsolin are villin-like proteins. Plant villins have been shown to bundle actin filaments (Tominaga et al., 2000; Ketelaar et al., 2002; Huang et al., 2005; Yokota et al., 2005). The actin bundling activity of some villins is calcium-dependent (Tominaga et al., 2000) and of others not (Huang et al., 2005). Villin-like proteins from lily can inhibit growth of barbed ends at high calcium concentrations, which could be caused by actin capping activity (Yokota et al., 2005). Huang et al. (Huang et al., 2003)

demonstrated that in plants, a capping protein is present that binds to the barbed ends of actin filaments, in this way preventing polymerisation and depolymerisation. The capping protein forms heterodimers and binding of this protein to the barbed end is regulated by phosphatidic acid (PA): in the presence of PA, the actin-binding activity of the capping protein is inhibited, which leads to extensive actin filament growth (Huang et al., 2006). It is not known whether the presence of capping proteins is required for Arp2/3 complex-dependent growth processes in plants (Hussey et al., 2006).

ADF

Although plant ADF, and proteins that control the activity of ADF, differ somewhat from animal ADF (Hussey et al., 2004), plant ADF has been shown to increase the turnover of actin filaments, as animal ADF does. Indeed, microinjection of pollen-specific ADF in *Tradescantia* stamen hair cells led to the depolymerisation of F-actin in cytoplasmic strands, which caused cytoplasmic strands to disappear (Hussey et al., 1998). When ADF is overexpressed in *Arabidopsis*, thick actin bundles disappear in different cell types, and cell expansion and organ growth are reduced. In contrast, inhibition of AtADF expression led to an increased number of actin cables, a stimulation of cell expansion and organ growth, and a delay in flowering (Dong et al., 2001). Furthermore, ADF has been shown to localise primarily at the tip of emerging and elongating maize root hairs (Jiang et al., 1997), and to play a critical role in pollen tube growth by regulating actin dynamics (Chen et al., 2002). Similarly to the animal systems that we discussed above, the role of ADF in plants likely constitutes of an enhanced turnover of actin filaments. This turnover generates monomeric actin that is required for continued actin polymerisation and thus continued reorganisation of the cytoplasm. Thus, the phenotypes that are caused by changes in the levels of ADF expression are likely to be caused by changes in the amount of available monomeric actin.

Profilin

Profilin is a protein with a conserved function throughout eukaryotes (Valenta et al., 1991). Profilin specifically binds to monomeric actin. When bound to profilin, G-actin cannot incorporate at the pointed end of actin filaments, but incorporation at the barbed end happens at normal rates (Pollard et al., 2000; Hussey et al., 2006). Animal and fungal profilin can accelerate the exchange of ADP for ATP on G-actin, thus accelerating F-actin polymerisation at the barbed end (Lu and Pollard, 2001). Plant profilins do not have this activity (Perelroizen et al., 1996; Kovar et al., 2000). The lack of the nucleotide exchange ability of plant profilins may be substituted by an increase of the intrinsic nucleotide exchange activity of plant actin, which is 10 to 20 fold higher than animal actin (Kovar et al., 2001). *Arabidopsis* plants have been generated that over-and under-express profilin (Ramachandran et al., 2000). Underexpressing plants were smaller and flowered earlier, whereas overexpressing plants had longer roots and root hairs. Immunolabeling of profilin shows an enrichment in the tips of growing root hairs (Braun et al., 1999; Baluska et al., 2000); however, this might just reflect the available cell volume. It is likely that profilin is involved indirectly in the generation of actin based forces in the cytoplasm, as a decrease in available monomeric actin for polymerisation would lead to a decrease in actin polymerisation.

Formins

In *Arabidopsis*, formin homologues have been identified (Deeks et al., 2002). The actin nucleating function of plant formins is conserved, including the capacity to associate with the growing barbed end of actin filaments while allowing profilin-bound actin monomers to incorporate at this end (Cheung and Wu, 2004; Ingouff et al., 2005; Michelot et al., 2005; Yi et al., 2005). The formin family in *Arabidopsis* is represented by two subclasses, the group I formins, which contains 11 members in *Arabidopsis*, and the group II formins, which contains 10 members in *Arabidopsis* (Deeks et al., 2002). Most of the group I formins possess an N-terminal trans-membrane domain. The large family of formins in *Arabidopsis* makes it difficult to identify cellular and/or developmental defects in knockout lines, as there is likely to be a high degree of redundancy between the different proteins. Nonetheless, several research groups have studied the function of formins in plant development. Ingouff et al. (Ingouff et al., 2005) have shown that the group I formin AtFH5 localises to newly formed cell plates and that an fh5 knockout line is disturbed in cell plate formation in the seed endosperm. Deeks et al. (Deeks et al., 2005) show that the group I formins AtFH4 and AtFH8, which together represent a distinct clade, localise to distinct patches of the plasma membrane where cotyledon cells are in direct contact with their neighbouring cells. When an fh8 construct, without an FH2 domain that is responsible for actin nucleation, was expressed under its endogenous promoter, root hair development was inhibited. Another study shows that overexpression of AtFH8 dramatically changes root hair development (Yi et al., 2005). These changes, ranging from short and wavy root hairs to tip-swollen and branched root hairs, correlate with an altered distribution of the actin cytoskeleton (Yi et al., 2005). Cheung and Wu (Cheung and Wu, 2004) over-expressed both the intact group I formin AtFH1 and an FH1 + FH2 fragment (which does not contain the regulatory domain of the formin and is constitutively active) of this protein in pollen tubes. They show that at low levels of over-expression, growth is stimulated. At higher levels of over-expression, pollen tube tips broaden and growth arrests. GFP-fusions to AtFH1 localised to the apical plasma membrane of pollen tubes. Finally, Favery et al. (Favery et al., 2004) show that the group I formin AtFH6 associates to the plasma membrane of giant cells that are induced by parasitic nematodes. These authors suggest that this formin might be involved in the growth of these cells. Altogether, these observations suggest that group I formins play a role in the generation and/or the maintenance of cell polarity, for which specific cytoplasmic organisation is required. The function of group II formins has not yet been identified.

Actin bundling proteins

In plants, two families of actin bundling proteins have been identified: the villins and the fimbrins. In addition, it has been shown that the formin AtFH1 is able to bind the side of existing actin filaments in vitro and bundle actin filaments (Michelot et al., 2005). The first plant homologue of villin that was described, was isolated from lily pollen (Vidali et al., 1998). This plant villin bundles F-actin in vitro (Yokota et al., 1998) in a calcium dependent fashion (Yokota et al., 2005), although not all plant villins are calcium dependent (Huang et al., 2005). The *Arabidopsis* genome contains 5 copies of villin. Each of these genes is expressed in a wide range of tissues (Klahre et al., 2000; Staiger and Hussey, 2004). This is in contrast with the expression pattern of mammalian villin, which is restricted to the microvilli of brush border cells (Staiger and Hussey, 2004).

Plant villin is involved in organising the cytoplasm in root hairs. Injection of antibodies against villin resulted in disintegration of the actin filament bundles (Tominaga et al., 2000; Ketelaar et al., 2002), followed by disappearance of transvacuolar strands (Tominaga et al., 2000). This indicates that bundles of actin filaments are essential for continued existence of transvacuolar strands. Fimbrins, the other family of actin bundling proteins in plants, are ubiquitously expressed in *Arabidopsis* (McCurdy and Kim, 1998). The actin binding of fimbrins is calcium dependent, whereas the actin bundling activity of fimbrin is not calcium dependent (Kovar et al., 2000). Cellular or developmental defects in fimbrin knockout lines have not been reported. This could be caused by redundancy of fimbrins in *Arabidopsis*.

Comparing the role of actin in the generation of cytoplasmic organisation of plant cells with its role in the model systems for actin based force generation in animal cells

It seems likely that at least some of the plant homologues of the mammalian proteins that we discussed are involved in actin-based force generation in plant cells. We will discuss the possible role of formin and Arp2/3 complex mediated actin polymerisation mechanisms in determining the generation of two types of plant cytoarchitecture: the cytoplasmic dense area in the apex and subapex of tip growing cells, and cytoplasmic strands in all plant cells.

Cytoplasmic dense areas

In pollen tubes (Geitmann et al., 2000) and root hairs (Miller et al., 1999; Ketelaar et al., 2002; Ketelaar et al., 2003), there is a network of fine F-actin that supports the subapical cytoplasm. A logical candidate for generating a network of fine F-actin would be the Arp2/3 complex, since it has been shown to generate branched arrays of actin filaments that can push a membrane forward during lamellipodium protrusion (Svitkina and Borisy, 1999). The Arp2/3 complex may be involved in the organisation of cytoplasm in growing cells, where cell expansion takes place locally, since the development of those cell types is disturbed in plants in which the Arp2/3 complex is non-functional. In favour of this, the Arp3 subunit was immunolocalised to the tip of growing root hairs (Van Gestel et al., 2003). The network of fine F-actin is, however, present near the tip of growing root hairs, and further away from the root hair tip, the actin filaments become increasingly bundled (Miller et al., 1999; Tominaga et al., 2000; Ketelaar et al., 2002; Ketelaar et al., 2003; Mathur, 2004; Mathur, 2005). It therefore seems unlikely that Arp2/3 mediated actin filament nucleation is directed towards the tonoplast to keep the vacuole away from the root hair tip and to maintain the cytoplasmic dense area in the apex. Furthermore, pollen tube development, which also depends on cell expansion over a small surface area, is unaffected by the absence of a functional Arp2/3 complex (Le et al., 2003; Li et al., 2003; Mathur et al., 2003a). Therefore, the Arp2/3 complex is unlikely to be the (only) key player in generating an actin network that builds and maintains the cytoplasmic dense area in the apex/subapex of tip-growing cells.

Cytoplasmic strands

Although the Arp2/3 complex is not able to nucleate the long, bundled actin filaments that are required for the existence of cytoplasmic strands (Tominaga et al., 2000), Arp2/3 complex activity directed towards the tonoplast *could* be involved in the initial formation of cytoplasmic strands, in the event of new strand formation. The Arp2/3 complex could nucleate a branched actin filament network, which elongates at the front, but is bundled continuously at its base (Fig. 3A). A similar process has been proposed for filopodium formation (Vignjevic et al., 2003) [although a recent study (Steffen et al., 2006) contradicts this hypothesis] and actin tail formation behind the intracellular pathogen *Rickettsia* (Jeng et al., 2004) [especially during the initial stages of movement (Gouin et al., 2005)]. This process differs from the mechanism employed by the intracellular pathogens that we have discussed above (Gouin et al., 2004; Gouin et al., 2005). Capping proteins, ADF and profilin could be involved in the generation of such a branched array (Fig. 3A), as they are in lamellipodia formation and bacteria protrusion.

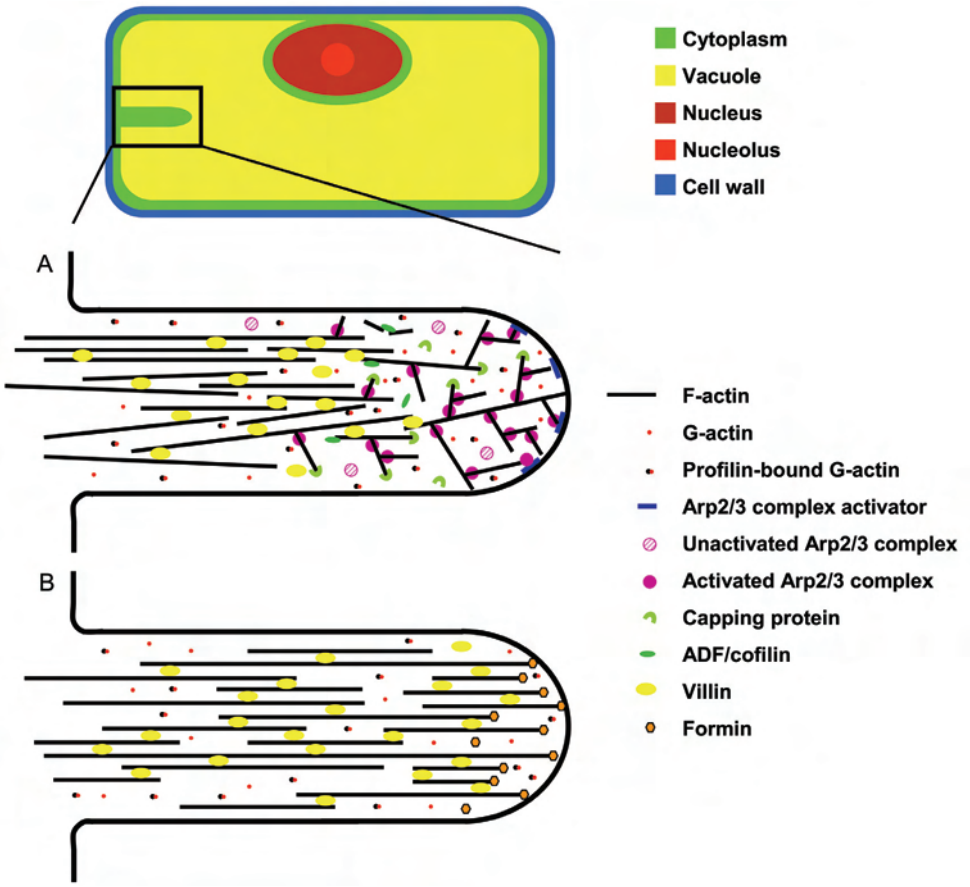

Figure 3. Hypothetical mechanisms of force generation by the actin cytoskeleton, resulting in the formation of cytoplasmic strands. The elongation of cytoplasmic strands may be achieved by Arp2/3 complex (A) or by formin mediated nucleation of actin filaments (B). In (A), capping proteins, ADF and profilin could be involved in the generation of the branched array. Elongation of cytoplasmic strands requires bundling of actin filaments by bundling proteins (A, B), which are represented by villin. The inset shows a typical location from which a cytoplasmic strand would appear. The image is not to scale.

Even if the Arp2/3 complex is needed for the initial formation and the elongation of cytoplasmic strands, the maintenance of cytoplasmic strands is unlikely to be a process that is mediated by Arp2/3 based actin nucleation, because stable, thick bundles of F-actin are required. A careful analysis of the cytoarchitecture in Arp2/3 knockouts would be required to show its role in cytoplasmic organisation.

Another possibility would be that formins nucleate the actin filaments that are needed for the initial formation of cytoplasmic strands (Fig. 3B). In contrast to the Arp2/3 complex, formins would be able to nucleate actin filaments and continue actin polymerisation over long distances, immediately resulting in the long, linear filaments that are known to be present in cytoplasmic strands. This situation resembles the filopodium protrusion system, in which nucleation of long, linear actin filaments results in the formation of thin cylindrical extensions of a plasma membrane.

As discussed above, the Arp2/3 complex, formins, or another, yet unknown, class of actin nucleating proteins could well be responsible for the initial formation of a cytoplasmic strand. However, in existing strands, long bundles of linear actin filaments continuously support the cytoplasmic strand (Tominaga et al., 2000; Ketelaar et al., 2002). Thus, apart from actin filament polymerisation at one end, filament bundling is required during cytoplasmic strand elongation. Since group 1 formins from *Arabidopsis* have been shown to bundle actin filaments in vitro (Michelot et al., 2005) and perhaps in vivo (Cheung and Wu, 2004), these proteins would be good candidates to facilitate the formation of a cytoplasmic strand. Though plant formin can bundle actin filaments in vitro (Michelot et al., 2005), from injections of antibodies against villin in root hairs (Tominaga et al., 2000; Ketelaar et al., 2002), we know that formin is, if involved at all, not the only actin bundling protein that is involved in elongation and continued existence of cytoplasmic strands.

In summary, establishment of the cytoplasmic organisation in vacuolated plant cells is likely to depend on actin nucleating proteins, actin polymerisation and actin bundling.[1]

Prospects

Force generation by actin filaments in mammalian cells is the subject of intense study, since it is clearly of importance for motility of cells. In plant cells the force that is generated by the actin cytoskeleton is likely to be insufficient to protrude the plasma membrane, because the cell is surrounded by the cell wall. The cytoplasmic organisation of cells, which is of importance for cell structure and required for cell growth and

1. Note added after publication: recent data have shown that existing actin filaments can be reorganized in a myosin-dependent way (Staiger et al., 2009; Van der Honing et al., 2010). Myosin motor activity could therefore play a role in actin-based force generation in plant cells, which, in turn, may contribute to cytoplasmic organization.

development, is at least partially organised by the actin cytoskeleton. It will be just as interesting and important to analyse the molecular basis of cytoplasmic organisation in plant cells with, as a basis, the available working hypotheses that we present here. In the years to come, cytoplasmic organisation in plant cells, a determining factor in cell growth, is likely to become a better understood system with similarities and differences when compared to membrane protrusion and bacterial propulsion in mammalian cells.

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Chapter 2

Actin and myosin regulate cytoplasm stiffness in plant cells: a study using optical tweezers

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Summary

- Here, we produced cytoplasmic protrusions with optical tweezers in mature BY-2 suspension cultured cells to study the parameters involved in the movement of actin filaments during changes in cytoplasmic organization and to determine whether stiffness is an actin-related property of plant cytoplasm.
- Optical tweezers were used to create cytoplasmic protrusions resembling cytoplasmic strands. Simultaneously, the behavior of the actin cytoskeleton was imaged.
- After actin filament depolymerization, less force was needed to create cytoplasmic protrusions. During treatment with the myosin ATPase inhibitor 2,3-butanedione monoxime, more trapping force was needed to create and maintain cytoplasmic protrusions. Thus, the presence of actin filaments and, even more so, the deactivation of a 2,3-butanedione monoxime-sensitive factor, probably myosin, stiffens the cytoplasm. During 2,3-butanedione monoxime treatment, none of the tweezer-formed protrusions contained filamentous actin, showing that a 2,3-butanedione monoxime-sensitive factor, probably myosin, is responsible for the movement of actin filaments, and implying that myosin serves as a static cross-linker of actin filaments when its motor function is inhibited. The presence of actin filaments does not delay the collapse of cytoplasmic protrusions after tweezer release.
- Myosin-based reorganization of the existing actin cytoskeleton could be the basis for new cytoplasmic strand formation, and thus the production of an organized cytoarchitecture.

Introduction

The organization of the cytoplasm is crucial for correct plant cell functioning. For instance, in plant cell growth, it plays a fundamental role, as the position of cytoplasmic components is a determining factor in the delivery of cargo to the surface area at which growth occurs (Miller et al., 1997). In plant cells that elongate by intercalary growth (i.e. expansion that takes place over the whole longitudinal cell axis), cytoplasm is present around the nucleus and in the cell's periphery. These two regions are connected by strands of cytoplasm, called cytoplasmic or transvacuolar strands, that cross the vacuole. New cytoplasmic strands are formed after cytokinesis (Kutsuna & Hasezawa, 2002), during and after cell elongation (Sheahan et al., 2007), and after recovery from treatment with the actin cytoskeleton depolymerizing drug latrunculin (Fiserova et al., 2006). The presence of interphase cytoplasmic strands depends on an intact actin cytoskeleton: cytoplasmic strands of interphase cells contain actin filaments, and disappear on actin filament depolymerization (Staiger et al., 1994; Shimmen et al., 1995; Valster et al., 1997; Hussey et al., 1998; Van Gestel et al., 2002; Higaki et al., 2006; Sheahan et al., 2007). The mechanisms by which actin filaments regulate the maintenance, localization and reorganization of cytoplasmic strands have not yet been elucidated, although it has been shown that the actin bundling protein villin is essential to maintain cytoplasmic strand size and number (Tominaga et al., 2000b; Ketelaar et al., 2002), and there are indications that myosins are responsible for the relocation of existing cytoplasmic strands (Hoffmann &Nebenfuhr, 2004; Higaki et al., 2006; Sheahan et al., 2007). Little is known about the structural properties of the acto-myosin system of cytoplasmic strands. Does the actin cytoskeleton provide strength to the strands and prevent strand collapse? Does myosin have the capacity to play a role in the sliding of actin filaments (bundles) relative to each other?

Optical tweezers (Ashkin, 1970) are useful for the study of cytoplasmic organization. Using the radiation pressure of a focused laser beam, one can manipulate small particles (Ashkin & Dziedzic, 1987; Ashkin et al., 1987; Block, 1992; Grier, 2003). A high numerical aperture lens is used to create a diffraction-limited spot in which particles in the (sub-)micrometer range, with a higher refractive index than the surrounding medium, are trapped. With infrared light, controlled forces can be applied inside living cells (Ashkin et al., 1987; Ashkin & Dziedzic, 1989), which can be employed to manipulate intracellular organization (Ashkin & Dziedzic, 1989; Grabski et al., 1994). We produced cytoplasmic protrusions into the vacuolar space using optical tweezers and showed that actin filaments entered these strands, probably by myosin motor activity. Making cytoplasmic protrusions is easier when actin filaments are depolymerized, and harder when myosin motor activity is inhibited. Although actin filaments support naturally occurring cytoplasmic strands, the presence of actin filaments does not delay the collapse of cytoplasmic protrusions after tweezer release. These data provide insight into the mechanisms that underlie cytoplasmic organization.

Materials and methods

Plant material and green fluorescent protein (GFP) marker transformations

Tobacco BY-2 suspension cultured cells were cultured in standard BY-2 medium (Nagata et al., 1992; Nagata & Kumagai, 1999) on a rotary shaker (120 rpm) at 25°C in darkness. The cultures were subcultured weekly (1–2 ml of the old culture in 38–39 ml of fresh medium) in 250 ml Erlenmeyer flasks. *Agrobacterium tumefaciens* strain LBA4404 was used for transformation of the 35S::GFP:FABD2 (Ketelaar et al., 2004) and 35S::GFP:TuA6 (Ueda et al., 2003) constructs into BY-2 cells; 100 µl of an overnight culture of LBA4404 carrying a binary plasmid containing one of the above constructs (grown on a rotary shaker at 180 rpm, 28°C) was co-cultured with 1 ml of 3–4-d-old tobacco BY-2 suspension cultured cells on a flat surface in darkness at 25°C. After 3 d, the cultures were washed three times with BY-2 medium and plated onto solid BY-2 medium containing 1% agarose supplemented with cefotaxime (240 mg l^{-1}) and selective antibiotic (GFP:FABD2, glufosinate ammonium, 25 g l^{-1}; GFP:TuA6, kanamycin, 50 mg l^{-1}). Calli were subcultured every 2–4 wk. Lines with correct localization of the fluorescent fusion proteins were selected, and used to start liquid suspension cultures.

Experiments were performed with cells 8–13 d after subculturing, which were diluted 1 : 10 in BY-2 medium before observation. Mitochondria were visualized by complementing the medium with 10 nM Mitotracker Red CMX-Ros (Invitrogen, Breda, The Netherlands).

Drug treatments

Stocks of latrunculin B (LatB; 10 mM; Sigma-Aldrich, Zwijndrecht, The Netherlands) and oryzalin (20 mM; Merck, Amsterdam, The Netherlands) were prepared in dimethylsulfoxide (DMSO), and diluted to the desired concentration in the suspension culture. The final DMSO volume did not exceed 0.1% (v / v) of the total volume. For recovery experiments, LatB was removed from the suspension culture by washing the cells three times (at least 1 min per wash) with fresh BY-2 medium using a 50 µm mesh filter. 2,3-Butanedione monoxime (BDM) (Sigma-Aldrich, Steinheim, Germany) was freshly dissolved in BY-2 medium before each experiment, yielding a final concentration of 25 mM. Staining for esterase activity with 0.005% fluorescein- diacetate (FDA; Sigma, St Louis, MO, USA) was used to confirm cell viability during the 30 min BDM treatment. Tweezer-formed cytoplasmic protrusion formation Molecular Machines and Instruments (MMI, Glattbrugg, Switzerland) optical tweezers, consisting of an NdYAG laser (1064 nm, 3000 mW CW, Newport Spectra-Physics Ltd, Didcot, Oxfordshire, UK) and x–y galvo scanner, were connected to an Axiovert 200 M microscope (Zeiss, Jena, Germany) with a Zeiss LSM510 META confocal scanning system. The focal point of the YAG laser was adjusted to the visual focus of the microscope with the CellManipulator software (MMI). Laser intensity measurements indicated that the YAG laser intensity was between 15 and 150 mW through the objective lens. Cytoplasmic protrusions were formed by keeping the laser stationary and moving the XY SCAN IM 120–100 stage (Märzhauzer, Wetzlar, Germany) at a constant velocity of 600 nm s^{-1}, through a defined displacement (160–200 steps; 75 nm step size). Confocal imaging was performed during the formation and maintenance of cytoplasmic protrusions.

To determine the force required to create cytoplasmic protrusions, trapping force ranges of $0.3F_{max}$, $0.5F_{max}$, $0.7F_{max}$, $0.9F_{max}$ and F_{max} were used. When a protrusion could be formed at 600 nm s^{-1}, and maintained for 20 s successfully within three attempts at a certain laser power, we scored this as a successful trapping event. When protrusion formation failed three times at the same laser power, we scored this as an unsuccessful trapping event. When protrusion formation failed, we either increased the laser power by $0.2F_{max}$, or chose another cell, and repeated the protrusion formation procedure until maximal laser power was reached, or until a laser power was reached at which 100% of the trapping attempts were successful.

To determine the trap force required to maintain protrusions, we formed cytoplasmic protrusions and reduced the laser power by $0.01F_{max}$ per second until the protrusion collapsed. The moment of force reduction depended on the initial trapping force, and was chosen such that the laser power was always $0.5F_{max}$, 1 min after protrusion formation. During these experiments and the experiments performed to determine the collapse velocity of protrusions, sometimes, instead of a rapid retraction from the tweezers, the protrusions sagged away from the tweezers and shrunk slowly, similar to the observations of Ashkin & Dziedzic (1989). As it was difficult to determine the moment and velocity of collapse in these cases, these data are not included.

Microscope imaging

Tobacco BY-2 suspension cultured cells were imaged in gas-permeable microchambers, as described by Vos et al. (2004), at room temperature. For confocal imaging, we used a 30 mW Ar laser (488 nm) and a 1 mW HeNe laser (543 nm). The laser intensities and mirror settings were as follows: green, 488 nm 3–5%, DM488/ 543, EM BP505- 530; red, 543 nm 35–80%, DM488/ 543, EM LP560. The pinhole size was 2–2.5 Airy Units (200-250 μm). Transmission images were collected simultaneously. For simultaneous imaging and optical trapping, light from the tweezers was removed by an 850 nm beamsplitter before detection. A Zeiss x 63 α-Plan Fluar oil immersion objective (NA 1.4) was used for all experiments.

Image analysis and statistics

Zeiss LSM image examiner (version 3.5.0.223) was used to determine the width of the cytoplasmic protrusions (determined 2–3 min after protrusion formation, in the middle of the protrusion), the velocity of organelles inside the protrusions and the collapse velocity after trap release. Student's *t*-tests ($\alpha = 0.05$) were performed to determine the significance of differences in these parameters for the different treatments.

Results

Interphase cytoplasmic strands contain actin filaments, and disappear on actin filament depolymerization

For all experiments, we used tobacco BY-2 suspension cultured cells from an 8–13-d-old culture in which the nucleus was positioned at the cell's periphery (Fig. 1). In these mature cells, the vacuole occupies more than 90% of the cell volume. Cytoplasmic strands in cells of this developmental stage still relocated and, in these strands, the cytoplasmic streaming velocity was 0.30 ± 0.03 μm s^{-1} (average and SE of 36 organelles from 22 cells). We studied the organization of the cytoskeleton in cytoplasmic strands of mature tobacco BY-2 suspension cultured cells stably expressing GFP:FABD2 (Ketelaar et al., 2004) or the microtubule marker GFP:TuA6 (Ueda et al., 2003)].

Cortical microtubules were abundantly present (Fig. 1a, inset) but, in cytoplasmic strands, microtubules were not visible (Fig. 1a). Treatment with the microtubule depolymerizing drug oryzalin (20 μM) led to the disappearance of all detectable microtubules within 30 min, but cytoplasmic strands were still present (Fig. 1b) and strand relocation, as well as cytoplasmic streaming, continued normally.

All cytoplasmic strands contained actin filaments (Fig. 1c). When actin filaments were fully depolymerized by a 12–16 h treatment with 500 nM LatB (Spector et al., 1983; Coue et al., 1987), cytoplasmic strands disappeared (Fig. 1d), showing that actin filaments are necessary for the maintenance of these strands. After LatB treatment, the cytoplasm was present only in the cell's periphery and around the nucleus, and cytoplasmic streaming had stopped. Within 30 min after washing away LatB, actin filaments, cytoplasmic strands and cytoplasmic streaming were observed again, showing that the cells recovered rapidly from the treatment. This is in agreement with previous studies in other cell types, showing that actin filaments (Staiger et al., 1994; Shimmen et al., 1995; Valster et al., 1997; Hussey et al., 1998; Van Gestel et al., 2002; Higaki et al., 2006; Sheahan et al., 2007), and not microtubules (Van Gestel et al., 2002; Sheahan et al., 2007), are the structural basis of cytoplasmic strands in interphase plant cells.

Creation of cytoplasmic protrusions

When the infrared laser beam of the optical tweezers is focused on the cytoplasm, refractive organelles, such as mitochondria, are easily trapped. We trapped an organelle (often a mitochondrion) in the perinuclear cytoplasm and, whilst keeping the organelle trapped, moved the microscope stage at a constant velocity, so that the trapped organelle was pulled into the volume occupied by the vacuole. As a result, the vacuolar membrane curved inwards, creating a cytoplasmic protrusion impinging the vacuole. Although the appearance of these protrusions resembled that of cytoplasmic strands, tweezer-formed protrusions are attached to the rest of the cytoplasm by one side, whereas the other side is supported by the optical tweezers. Protrusions instantly contained cytoplasm and remained connected to the region from which the organelle was trapped originally (Fig. 2). After trap release, the protrusions collapsed back into the perinuclear region.

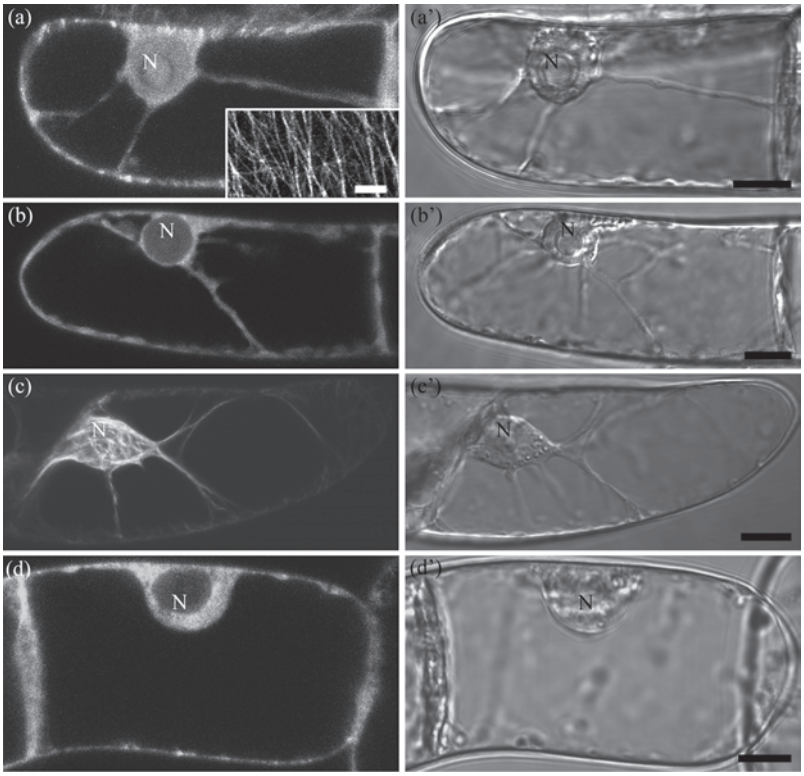

Figure 1. Actin filaments, and not microtubules, are the structural basis of cytoplasmic strands in mature tobacco BY-2 suspension cultured cells. (a,a',b,b') GFP:TuA6 expressing tobacco BY-2 suspension cultured cell from a 12-day-old culture (a, maximal projection of three images taken at 1.4 µm z-steps; a', transmission image). Note the cross-sectioned (bundles of) microtubules in the cortical region of the cell. Inset: cortical plane showing the presence of cortical microtubules. (b,b') When microtubules are depolymerized (20 µm oryzalin, 30-120min), cytoplasmic strands remain present, and lateral movements of these strands continue (b, maximal projection of three images taken at 1.4 µm z-steps; b', transmission image). (c,c',d,d') GFP:FABD2 expressing tobacco BY-2 suspension cultured cell from an 11-day-old culture (c, maximal projection of eight images taken at 1.2 µm z-steps; c', transmission image). Cytoplasmic strands of interphase cells contain actin filaments (c). (d,d') When actin filaments are depolymerized [500 nM latrunculin B (LatB), 12-16 h], cytoplasmic strands in interphase cells disappear and filamentous actin is not detectable (d, confocal image of median plane; d', transmission image). N, nucleus. Bars, 10 µm, except for inset (5µm).

Protrusion formation with optical tweezers and collapse after tweezer release are reproducible in cells of different species: spring onion epidermal cells (Ashkin & Dziedzic, 1989) and Tradescantia stamen hair cells (N. C. A. de Ruijter, A. M. C. Emons and T. Ketelaar, unpublished). When we traversed the whole vacuole with the trapped organelle and reached the opposite side of the vacuole in BY-2 cells, the strands always collapsed. In Tradescantia stamen hair cells, the protrusion occasionally connects to the distal tonoplast membrane, so that both ends of the protrusion are connected to the pool of cytoplasm. These strands remained intact after release of the tweezers (N. C. A. de Ruijter, A. M. C. Emons and T. Ketelaar, unpublished).

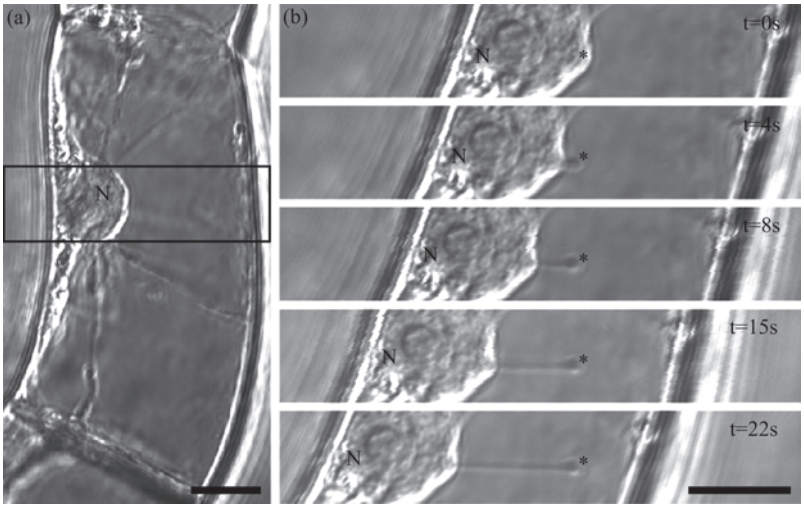

Figure 2. Formation of a tweezer-formed cytoplasmic protrusion. (a) Tobacco BY-2 suspension cultured cell from an 11-day-old culture. The area indicated by the rectangle is enlarged in (b). (b) Optical tweezers-mediated displacement of a trapped organelle from the perinuclear cytoplasm through the vacuole results in the formation of a cytoplasmic protrusion. *Tweezers position; N, nucleus. Bars, 10 μm.

For our experiments, we arrested the movement of the stage once a trapped organelle was dislocated 10–19 μm into the vacuolar space, resulting in cytoplasmic protrusions of that same length. Protrusions could be maintained by the optical tweezers for at least 10 min. During the formation and maintenance of protrusions, the velocity of organelles inside the cytoplasmic strands was not affected, and we did not observe any changes in the organization of the cytoplasm, nor did exposure to the optical tweezer laser beam visibly affect the cell. Thus, it is likely that optical trapping, including the formation and maintenance of cytoplasmic protrusions, is not harmful to the cells.

Actin filaments, but not microtubules, enter tweezer-formed protrusions

It is known that microtubules are absent from naturally occurring cytoplasmic strands (Van Gestel et al., 2002; Sheahan et al., 2007). Indeed, in the mature cells used, we did not observe microtubules in cytoplasmic strands (Fig. 1a). We investigated whether microtubules, which are present in the cytoplasm surrounding the nucleus, were also absent from cytoplasmic protrusions. Protrusions were created in tobacco BY-2 cells that stably expressed GFP:TuA6, and were maintained for 5–6 min. During protrusion creation and maintenance, the GFP:TuA6 fluorescence was evenly distributed, and filamentous structures were not observed (Fig. 3a, n = 10). To determine whether the GFP signal in protrusions was free GFP:TuA6, 20 μM oryzalin was applied. After 30 min, all cortical microtubules had depolymerized. The GFP distribution in the cytoplasmic protrusions of these oryzalin-treated cells was similar to that of cytoplasmic protrusions in untreated cells (Fig. 3b). We performed photobleaching experiments to confirm that the fluorescence in tweezer-formed protrusions was produced by freely diffusing GFP:TuA6 (Supporting Information Supplemental figure 1). Thus, microtubules were absent from cytoplasmic protrusions produced from the cytoplasm around the nucleus, as they are absent from cytoplasmic strands in interphase cells.

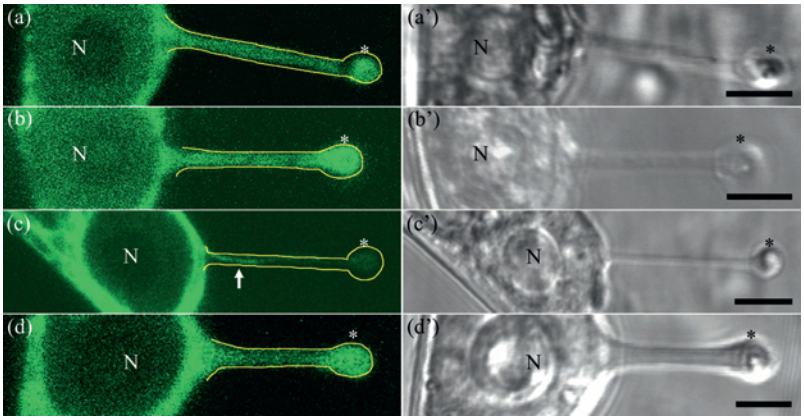

Figure 3. Actin filaments, but not microtubules, enter tweezer-formed cytoplasmic protrusions. Tweezer-formed cytoplasmic protrusions in GFP:TuA6 (a, b) and GFP:FABD2 (c, d) expressing tobacco BY-2 suspension cultured cells (a-d, confocal images of GFP fluorescence; a'-d', transmission images). (a,b) Microtubules are absent from cytoplasmic protrusions. The GFP fluorescence in the protrusions is similar in untreated (a,a') and 20 µM oryzalin-treated (b,b') cells, showing that this fluorescence represents free GFP-tubulin. (c,d) Actin filaments are present in cytoplasmic protrusions. In untreated cells (c,c'), GFP:FABD2 decorates actin filaments in cytoplasmic protrusions (arrow). By contrast, the GFP:FABD2 signal is evenly distributed in protrusions of cells in which the actin cytoskeleton has been fully depolymerized by an overnight treatment with 500 nM LatB (d,d'). *Tweezers position; N, nucleus. Bars, 5 µm.

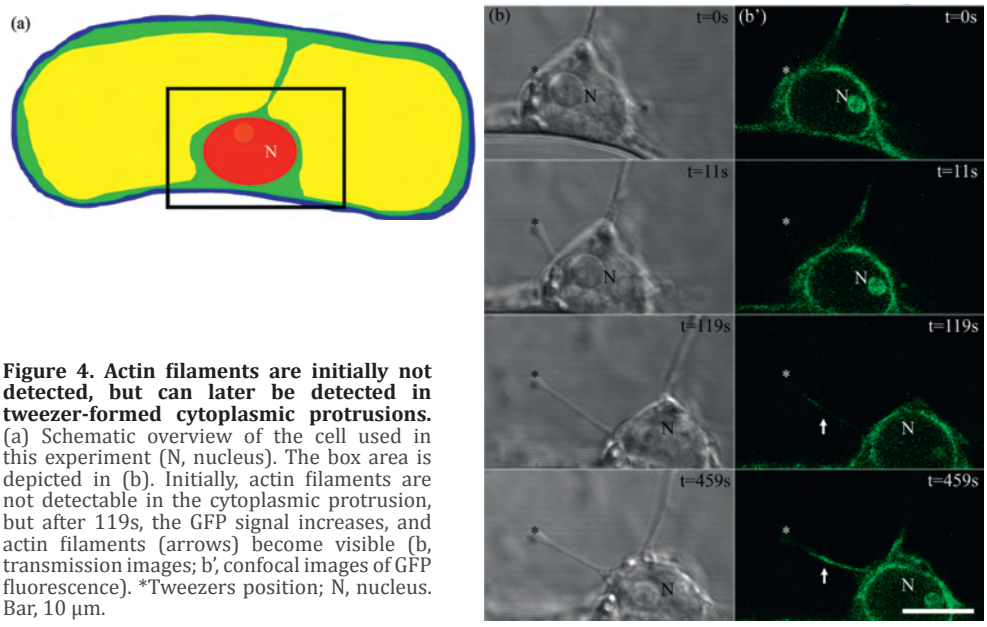

Figure 4. Actin filaments are initially not detected, but can later be detected in tweezer-formed cytoplasmic protrusions. (a) Schematic overview of the cell used in this experiment (N, nucleus). The box area is depicted in (b). Initially, actin filaments are not detectable in the cytoplasmic protrusion, but after 119s, the GFP signal increases, and actin filaments (arrows) become visible (b, transmission images; b', confocal images of GFP fluorescence). *Tweezers position; N, nucleus. Bar, 10 µm.

In interphase cells, cytoplasmic strands contain actin filaments, and these actin filaments are the structural basis for their existence. We investigated whether cytoplasmic protrusions also contained actin filaments. We pulled protrusions in tobacco BY-2 suspension cultured cells that expressed GFP:FABD2, and maintained them for 6 min. In 74% of these protrusions (n = 39), actin filaments (bundles) were visible within 3 min after protrusion formation (Fig. 3c). Actin filaments were detected 71 ± 10 s (average with SE) after protrusion formation (i.e. after stage movement was arrested, 20–25 s after the start of protrusion creation), but were often not visible immediately after protrusion creation (Fig. 4). In order to verify that the observed increase in fluorescence results from the presence of actin filaments, and not from unbound GFP:FABD2, protrusions were created in the absence of actin filaments (12–16 h treatment with 500 nM LatB). In these LatB-treated cells, diffuse fluorescence was evenly distributed throughout the whole volume of the protrusions (Fig. 3d), whereas fluorescently labeled filaments were observed in protrusions of untreated cells (Fig. 3c, 4). We conclude that most cytoplasmic protrusions, like all naturally occurring cytoplasmic strands in interphase cells, hold actin filaments.

Organelle transport over actin filaments occurs in cytoplasmic protrusions

To assess whether active organelle transport occurred over the actin filaments in cytoplasmic protrusions, we investigated organelle movement within them. Mitochondria (stained by the application of 10 nM Mitotracker Red) move into and inside protrusions (Fig. 5), and also other moving organelles were observed in the protrusions with differential interference contrast microscopy. Organelles that can be observed – with this magnification – are, besides the stained mitochondria, probably plastids. The number of visible organelles moving inside the protrusions ranged from 0 to 10 per protrusion during the 6 min experiment. Most organelles moved towards the trap but, in 21% of the protrusions (n = 29), organelles moved in both directions. The net organelle velocity in protrusions was 0.26 ± 0.02 μm s^{-1} (Fig. 5b). This is similar to the organelle velocity in naturally occurring cytoplasmic strands in cells of this developmental stage (0.30 ± 0.03 μm s^{-1}). In cytoplasmic protrusions of cells in which all actin filaments had been depolymerized (500 nM LatB; 12–16 h), organelles jiggled and net organelle displacements over at least 2.5 lm occurred at a velocity of 0.07 ± 0.01 μm s^{-1} (average with SE; Fig. 5b), significantly lower than the net organelle displacement velocity in protrusions pulled in untreated cells (0.26 ± 0.02 μm s^{-1}). A partial depolymerization of the actin cytoskeleton (100 nM LatB; 10–90 min) also resulted in a reduced organelle velocity within the protrusions (Fig. 5b). These data confirm that the actin filaments in tweezer-formed cytoplasmic protrusions support cytoplasmic streaming.

Most protrusions contain fluorescently decorated actin filaments within 3 min after their formation but, immediately after protrusion formation, actin filaments are often not detectable. As GFP:FABD2 does not show fine dynamic (bundles of) actin filaments in the subapex of growing root hairs in a confocal laser scanning microscope (Ketelaar & Emons, 2009), we tested the presence of filamentous actin during protrusion creation by determining the organelle velocity. The velocities of organelles that were immediately present (0.27 ± 0.08 μm s^{-1}, average with SE; Fig. 5c) and of organelles that entered the protrusion when actin filaments were detectable (0.26 ± 0.04 μm s^{-1})

do not differ significantly (t-test; $P < 0.05$) from the normal organelle velocity (0.30 ± 0.03 μm s^{-1}). Thus, actin filaments, although not detectable with GFP:FABD2, are immediately present and functional. The velocity of organelles did not change during protrusion maintenance.

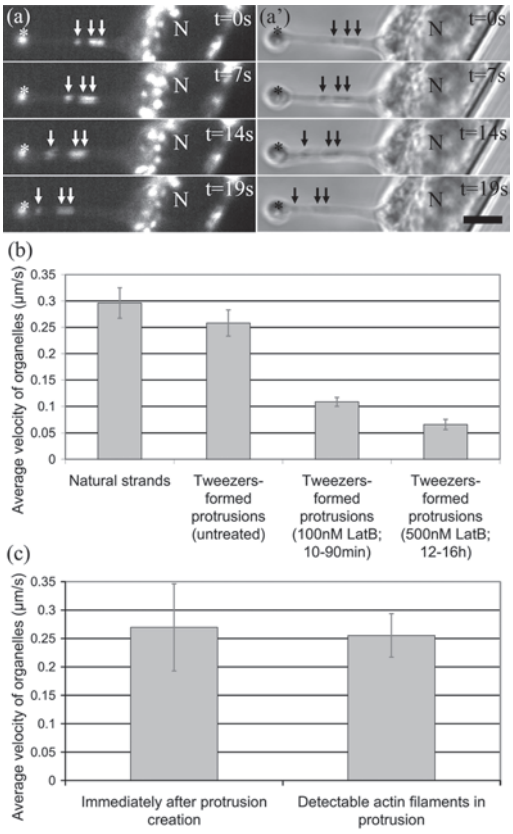

Figure 5. Organelle movement in tweezer-formed cytoplasmic protrusions. (a,a') Movement of mitochondria (arrows) in cytoplasmic protrusions (a, confocal images of Mitotracker Red (10 nM)-stained mitochondria; a', transmission images). *Tweezers position. N, nucleus. Bar, 5 μM. (b) Tweezer-formed cytoplasmic protrusions of both untreated (n = 46) and latrunculin B (LatB)-treated (100 nM, n = 28; 500 nM, n = 12) cells contain moving organelles. However, in untreated cells, the average velocity of these organelles is higher, and similar to the organelle velocity in naturally occuring cytoplasmic protrusions (n = 36). (c) The average velocity of organelles that are immediately present (n = 9) in tweezer-formed protrusions is similar to that of organelles that enter the protrusions at a later stage (n = 18), when actin filaments are detected. Error bars represent SE.

The actin cytoskeleton increases the force required to create cytoplasmic protrusions, but actin filaments in protrusions do not slow down their collapse

Our optical tweezers set-up is able to measure the absolute forces needed to dislocate beads in fluid medium. As the complexity of cytoplasm and its properties, such as viscosity, are unknown, it is at present impossible to measure the absolute forces in living cells. Therefore, we used fractions of the maximal laser power employed ($0.3F_{max}$, etc.) to compare relative forces. The infrared laser output was linear over the power range used in these experiments. For a range of laser power ($0.3F_{max}$, $0.5F_{max}$, $0.7F_{max}$, $0.9F_{max}$ and F_{max}), we scored the number of successful protrusion creations (see Materials and Methods) in experimental conditions to investigate the contribution of the actin cytoskeleton to the force needed for protrusion creation and maintenance (Table 1). In cells with a completely depolymerized actin cytoskeleton (500 nM LatB; 12-16 h

treatment), the force required to pull protrusions was less than in untreated cells (Table 1). The trap power at which all creations succeeded was $0.7F_{max}$ in untreated cells, but $0.5F_{max}$ for LatB-treated (500 nM; 12–16 h) cells. At $0.3F_{max}$, the fraction of successful trapping attempts was also higher in LatB-treated (500 nM; 12–16 h) cells (72%) than in untreated cells (12%). These results show that, in the presence of actin filaments, more force is required to pull cytoplasmic protrusions. To investigate whether, in the presence of actin filaments, more trap power is also needed to maintain the protrusions, we pulled protrusions, and reduced the trap power by $0.01F_{max}$ per second once a protrusion had been successfully formed, until the trapped organelle escaped and the protrusion collapsed (see Materials and Methods). The trap power at which the protrusions collapsed in LatB-treated cells ($0.20 \pm 0.01F_{max}$; average and SE of 12 replicates) was significantly lower than that of untreated cells ($0.26 \pm 0.01F_{max}$; n = 12; t-test, $P < 0.05$; Fig. 6a). Thus, the maintenance of cytoplasmic protrusions requires a higher force when actin filaments are present in the cell. In addition, protrusions in cells with a depolymerized actin cytoskeleton are wider in diameter than those in control cells (Fig. 6b). A partial depolymerization of the actin filament pool (100 nM LatB; 10–90 min) also resulted in wider protrusions (Fig. 6b) and an intermediate force required to create (Table 1) and maintain (Fig. 6a) them. This greater ease of protrusion creation and maintenance when filamentous actin is absent, and its dose dependence, show that, in plant cells, the actin cytoskeleton stiffens the cytoplasm.

	$0.3\ F_{max}$	$0.5\ F_{max}$	$0.7\ F_{max}$	$0.9\ F_{max}$	F_{max}
GFP:FABD untreated	12% (n=17)	89% (n=36)	100% (n=20)		
100 nM LatB (10-90 min)	40% (n=10)	92% (n=12)	100% (n=10)		
500 nM LatB (12-16 h)	72% (n=18)	100% (n=22)			
25 mM BDM (5-30 min)		0% (n=10)	40% (n=10)	48% (n=21)	58% (n=43)

Table 1. The percentage of successful attempts to create tweezer-formed cytoplasmic protrusions is shown for a range of laser power of the optical tweezers. The force required to create cytoplasmic protrusions is lower than in control cells in the absence of actin filaments, and higher when myosin motor activity is inhibited.

Filamentous actin is essential to prevent the disappearance of naturally formed cytoplasmic strands (Staiger et al., 1994; Shimmen et al., 1995; Valster et al., 1997; Hussey et al., 1998; Van Gestel et al., 2002; Higaki et al., 2006; Sheahan et al., 2007). Thus, actin filaments are a stabilizing factor. Tweezer-formed cytoplasmic protrusions, however, can be formed in cells with an intact and depolymerized actin cytoskeleton and, after trap release, all protrusions collapse back into the perinuclear region from which they were pulled (Fig. 7a). Does the presence of actin filaments influence the collapse velocity of protrusions? In other words, do actin filaments serve as a stabilizing factor that delays the collapse of cytoplasmic protrusions? Protrusions were pulled in untreated cells and in cells in which all filamentous actin had been depolymerized (500 nM LatB for 12–16 h). We released the trap 6 min after protrusion formation,

and determined the collapse velocity by measuring the length of protrusions as the percentage of their initial length. This length was plotted over time, with t = 0 being the time point of trap release (Fig. 7b). When the actin cytoskeleton was completely depolymerized (500 nM LatB; 12–16 h), the collapse velocity was reduced compared with untreated cells. Although the difference in collapse velocity is small, it is nevertheless significant for nine of 14 time points after trap release ($P < 0.05$). Partial depolymerization of the actin cytoskeleton (100 nM LatB; 10–90 min) did not cause a significant decrease in collapse velocity. We conclude that actin filaments do not slow down the collapse of protrusions, indicating that actin filaments only support a protrusion when this strand is connected at both ends.

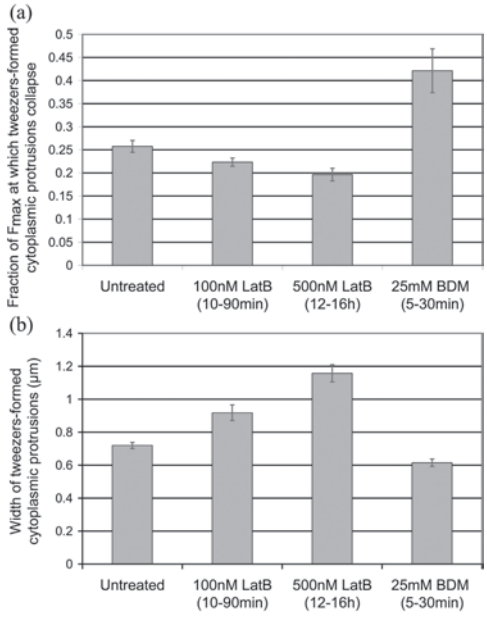

Figure 6. The force required to maintain a cytoplasmic strand with optical tweezers decreases in the absence of actin filaments, and increases when myosin motor activity is inhibited. (a) By gradually reducing the laser power once protrusions were successfully formed, the force required to maintain these protrusions was determined, *i.e.* the force at which they collapsed back into the perinuclear region [n = 8 for 2,3-butanedione monoxime (BDM)-treatment and n = 12 for the other treatments]. Force is expressed as fractions of the maximal laser power (F_{max}). (b) The width of cytoplasmic protrusions differs among the treatments [n = 50 for untreated cells, n = 23 for 100 nM latrunculin B (LatB)-treated cells, n = 12 for 500 nM LatB- and 25 mM BDM-treated cells). Error bars represent SE.

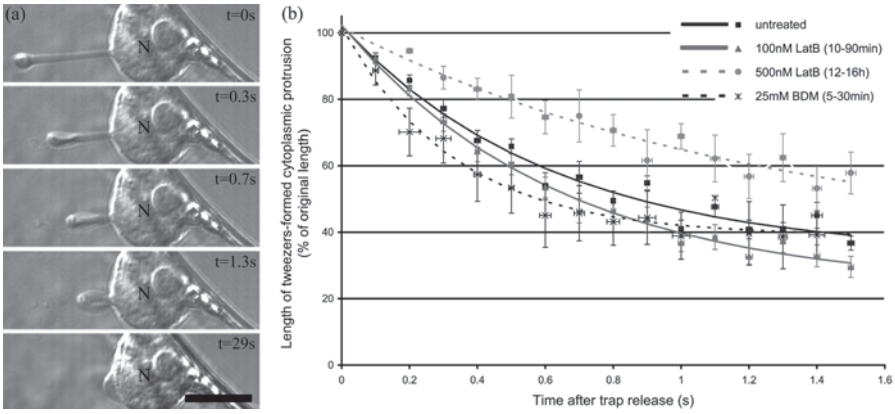

Figure 7. Actin filaments in tweezer-formed cytoplasmic protrusions do not delay the collapse of these protrusions. (a) After trap release, cytoplasmic protrusions collapse back into the perinuclear cytoplasm from which they were drawn. N, nucleus. Bar, 10 µm. (b) Collapse velocity of cytoplasmic protrusions, plotted as the decrease in length after trap release [n = 25 for untreated cells, n = 19 for 100 nM latrunculin B (LatB)-treated cells, n = 9 for 500 nM LatB treated cells, n = 5 for 25 mM 2,3-butanedione monoxime (BDM)-treated cells]. Trap release was performed 6 minutes after protrusion formation. Error bars represent SE.

A BDM-sensitive factor, probably myosin, facilitates the creation of cytoplasmic protrusions, and does not influence their collapse

Class XI myosins are plant-specific, actin-based motor proteins that are responsible for organelle movement (Reddy, 2001; Shimmen & Yokota, 2004; Shimmen, 2007). There are indications that myosins play a role in the reorganization of naturally occurring cytoplasmic strands by reorganizing the actin cytoskeleton (Hoffmann & Nebenfuhr, 2004; Sheahan et al., 2007). This conclusion was derived from experiments with BDM. To investigate whether myosin motor activity is required for the entrance of actin filaments into cytoplasmic protrusions, we treated the cells with BDM. BDM is a myosin ATPase inhibitor (Higuchi & Takemori, 1989; Yagi et al., 1992) that is known to inhibit the activity of at least some myosins in plant cells (Tominaga et al., 2000a). A 15 min treatment of the cells with 25 mM BDM resulted in an inhibition of cytoplasmic streaming and cytoplasmic strand reorganization, as expected. Thus, BDM indeed inhibits the activity of a factor, which is probably a myosin, involved in these processes. We tested whether 25 mM BDM treatment changes the force required to pull protrusions. Only at a laser power of 0.7Fmax or higher were protrusions formed successfully. At 0.7Fmax, only 40% of the trapping attempts in BDM-treated cells were successful, whereas trapping attempts at this laser power never failed in untreated cells. At Fmax, only 58% of the trapping attempts were successful in BDM-treated cells (Table 1). Thus, BDM increases the force required to pull protrusions, suggesting that the action of a BDM sensitive factor, probably myosin, facilitates protrusion formation. To investigate whether it is also harder to maintain protrusions when myosin activity is inhibited, we created protrusions in BDM-treated cells at Fmax and reduced the laser power until the protrusions collapsed (see Materials and Methods). This laser power (0.42 ± 0.05Fmax; average and SE of eight replicates; Fig. 6a) was significantly higher (t-test; $P < 0.05$) than that of untreated cells (0.26 ± 0.01Fmax), showing that the force counteracting the tweezers is higher, and thus the cytoplasmic deformability is lower, in cells with inactivated myosin. The fact that protrusions in BDM-treated cells are thinner than those in untreated cells (t-test; $P < 0.05$; Fig. 6b) confirms this. We conclude that myosin motor activity is a factor facilitating protrusion creation. Although filamentous actin restricts the amount of cytoplasm pulled by the tweezers, myosin motor activity in the presence of filamentous actin aids the displacement of cytoplasm by optical tweezers. We also measured the protrusion collapse velocity in cells with inactivated myosin (Fig. 7b). The collapse velocity of protrusions in cells that had been treated with 25 mM BDM was similar to that of protrusions in untreated cells: only for one of 13 time points after trap release did the length of protrusions differ (t-test; $P < 0.05$). Thus, although inactivation of myosin stiffens the cytoplasm, making it less expandable, this stiffening does not slow down or accelerate protrusion collapse when the tweezers are released.

Entrance of actin filaments into tweezer-formed cytoplasmic protrusions depends on myosin motor activity

The myosin dependence of actin filament translocation in cytoplasmic protrusions was investigated by pulling them in cells 5–30 min after the addition of 25 mM BDM, a concentration that inhibited cytoplasmic streaming and strand reorganization within 15 min. None of the protrusions created during BDM treatment ($n = 12$) contained filamentous actin (Fig. 8). Altogether, we observed only one organelle in such

protrusions, its velocity being similar to that of the passively displacing organelles in protrusions in cells without actin filaments (500 nM LatB; 12–16 h). As the specificity of BDM for plant myosins has not been carefully investigated (McCurdy, 1999; Hoffmann & Nebenfuhr, 2004), these data must be interpreted with caution. Nevertheless, these data strongly suggest that actin filaments enter cytoplasmic strands by a myosin-dependent translocation of existing actin filaments. To prove that actin filaments can enter and move through cytoplasmic protrusions, independent of the force exerted by displacement of the optical tweezers, we studied their displacement before and after protrusion formation. In 28% of the actin-containing protrusions in untreated cells, fluorescent filaments (bundles) with higher fluorescence intensity alternated with lower intensity fragments. In Fig. 9, such a highly fluorescent fragment is present near the tweezer position (Fig. 9a', arrow). When the length of the protrusion was increased (Fig. 9a), the fluorescent fragment remained present at the same position during 22 s, showing that it is not passively pulled into the protrusion by the sucking force of the tweezers. After 22 s, this fragment started to move towards the tweezers (Fig. 9a',b). The kymograph of the fluorescence distribution in the protrusion shows that the movement of the fluorescent fragment (Fig. 9b) is not dependent on displacement of the tweezers.

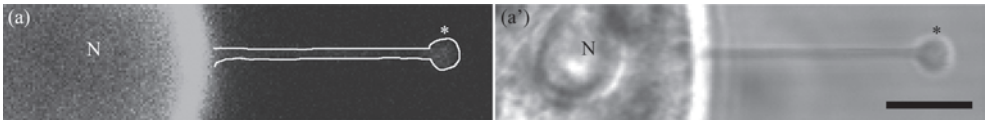

Figure 8. Actin filaments enter tweezers-formed cytoplasmic protrusions in a myosin-dependent manner. (a,a') Actin filaments are absent from cytoplasmic protrusions when myosin motor activity is inhibited by 25 mM 2,3-butanedione monoxime (BDM) for 5-30 minutes (a, confocal image; a', transmission image). *Tweezers position; N, nucleus. Bar, 5 μm.

Discussion

In plant cells, cytoplasmic strands devoid of actin filaments have not been shown to exist. When the actin cytoskeleton is depolymerized, strands disappear (Staiger et al., 1994; Shimmen et al., 1995; Valster et al., 1997; Hussey et al., 1998; Van Gestel et al., 2002; Sheahan et al., 2007). Regarding strand stabilization, the tweezers experiments show two properties of the cytoplasm. First, when dragging an organelle with optical tweezers through the vacuolar space, a cytoplasmic protrusion can be made when actin filaments are absent, and the formation of cytoplasmic protrusions requires more force when actin filaments are present in the cytoplasm, showing that actin filaments stiffen the cytoplasm. Second, tweezer-formed protrusions, which are only connected to the rest of the cytoplasm at their base, collapse back into the perinuclear cytoplasm when the trap force is released, showing that actin filaments do not stabilize them in this situation.

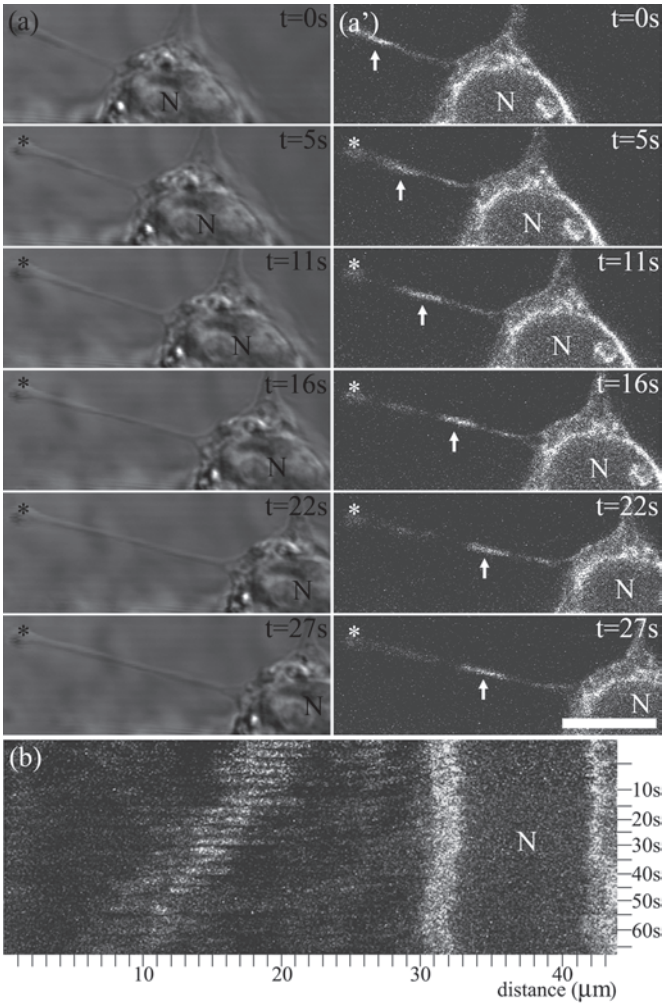

Figure 9. Actin filaments can enter and move through tweezer-formed protrusions independent of the tweezers displacement. (a,a') Tweezer-formed protrusion in an untreated cell. When the length of the protrusion was increased (a), the highly fluorescent fragment shown in a' (arrow) remained present at the same position for 22 s, after which it started to move towards the tweezers (a, transmission images; a', confocal images of GFP fluorescence). *Tweezers position; N, nucleus. Bar 10 µm. (b) A kymograph of the fluorescence distribution in the tweezer-formed protrusion shown in (a) shows the movement of fluorescent fragments. The upper line in the kymograph (b) corresponds to a', 22 s. N, nucleus.

There are three ways in which actin filaments can enter protrusions: through actin nucleation / polymerization; through myosin-based displacement of actin filaments; and in a motor- and polymerization-independent manner, i.e. by diffusion. In yeast, a cross-linked network of actin filaments polymerizes at one side of mitochondria, steering them through the cytoplasm. The Arp2 / 3 complex nucleates new free barbed ends of actin filaments for polymerization in this process (Boldogh et al., 1998). Our observation that short (bundles of) actin filaments move through protrusions even 5 min after protrusion formation demonstrates that actin filaments enter protrusions independent of the tweezer force. Although we cannot exclude the possibility that actin polymerization occurs during the migration of actin filaments into tweezer-formed cytoplasmic protrusions, we have shown that motor protein-based displacement of actin filaments over other actin filaments is likely to be responsible for the appearance of fluorescent actin filaments in the protrusions during and after protrusion formation.

Although the precise mode of action of BDM on plant myosins is not known, it has been shown to inhibit plant organelle movement, a myosin-based process (Tominaga et al., 2000a), and has been used as a myosin inhibitor in plant cells (Radford & White, 1998; McCurdy, 1999; Samaj et al., 2000; Molchan et al., 2002; Oertel et al., 2003; Funaki et al., 2004; Hoffmann & Nebenfuhr, 2004; Frantzios et al., 2005; Higaki et al., 2006; Paves & Truve, 2007; Esseling-Ozdoba et al., 2008). Myosin motor activity is important for cytoplasmic reorganization, such as fusion and lateral displacements of cytoplasmic strands (Hoffmann & Nebenfuhr, 2004; Sheahan et al., 2007). The tweezer-formed protrusions in BDM-treated cells never contained actin filaments, pointing to a role for myosin activity in the entry of actin filaments into protrusions. Myosins could interconnect (bundles of) actin filaments and cause sliding of these (bundles of) actin filaments along each other. As the migration of actin filaments into new protrusions is inhibited by BDM, it is probably myosin dependent. Our results suggest that myosin-dependent movement of actin filaments is not only responsible for the relocation of existing cytoplasmic strands (Hoffmann & Nebenfuhr, 2004), but also for the formation of new strands.

Grabski et al. have studied the tension produced by the actin cytoskeleton in naturally occurring cytoplasmic strands by laterally displacing the strands with optical tweezers. They showed that this tension is reduced in the presence of actin filament depolymerizing drugs (Grabski et al., 1994), but increased in the presence of BDM (Grabski et al., 1998). We visualized the actin organization in tweezer-formed cytoplasmic protrusions and showed that the deformation capability of the cytoplasm depends on the actin cytoskeleton that supports it. This resembles the results from deformation experiments of actin networks in vitro, which show that an actin network functions as an elastic structure (Liu et al., 2006; Tharmann et al., 2007). Our experiments show that a BDM-sensitive factor, probably myosin, serves as a sliding cross-linker that influences the deformation competence of the actin network, and thus the cytoplasm, of plant cells. The BDM treatment resists the deformation of the cytoplasm during protrusion creation, probably by inhibiting the sliding capacity of myosin motor proteins, so that only the actin filament cross-linking activity remains and the cytoplasmic organization is frozen.

Myosin motor activity along actin filaments could be an efficient method to restructure the cytoplasm, as, for instance, occurs during cytokinesis in plant cells. Future work should clarify the mechanisms that underlie natural cytoplasmic strand formation. Manipulation of the cytoplasm with optical tweezers, combined with confocal microscopy, specific drugs, RNA interference and mutants, will be important tools in these studies.

Acknowledgements

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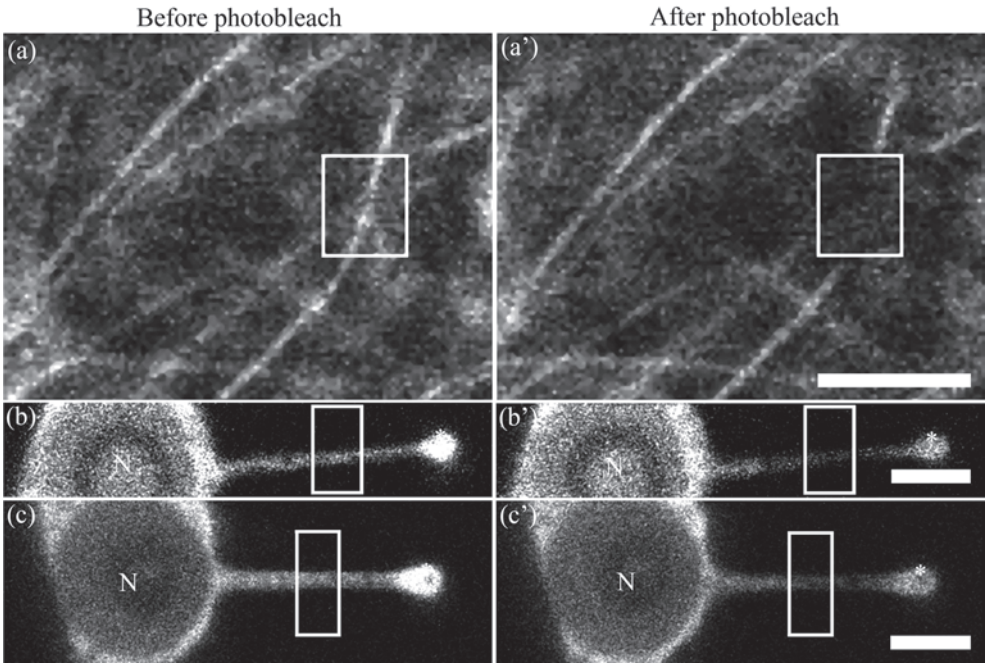
Supporting Information



Supplemental figure 1. Microtubules are absent from tweezer-formed cytoplasmic protrusions. Photobleaching experiments were performed to prove the absence of microtubules in tweezer-formed protrusions. (a) We performed photobleaching in the cortical region of a cell, where microtubules are abundant. Immediately after photobleaching (a'), the fluorescence intensity in the bleached region (depicted by the box) had decreased, but outside the bleached area, the GFP-tubulin-alpha, which is incorporated in microtubules, still showed filamentous structures (arrow). (b,c) Photobleaching of tweezer-formed cytoplasmic protrusions in a control cell (b) and a cell treated with oryzalin (c). After the formation of a tweezer-formed cytoplasmic protrusion, a box in the middle of the strand was photobleached at least 2.5 minutes after protrusion formation. This not only caused a decrease in fluorescence in the photobleached area, but also immediately led to a decrease in fluorescence in the surrounding unbleached areas of the protrusion (b,b'). The same phenomenon was observed in tweezer-formed protrusions that had been formed in the presence of oryzalin (c,c'), and these results were reproducible (n = 8 for untreated cells, and 6 for oryzalin-treated cells). Loss of fluorescence outside of the photobleached area can only occur by diffusion or transport of bleached fluorophores from the bleached area, showing that only GFP-tubulin monomers are present in the protrusion. *Tweezers position; N, nucleus. Bar, 10 μm.

Chapter 3

Lifeact reduces dynamic reorganization of actin filaments in *Arabidopsis thaliana*

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Submitted

Abstract

Lifeact is a novel probe for life cell actin filament visualization. Fused to a fluorescent protein, this probe labels actin filaments in a wide range of cell types and organisms. In this study, we compared the localization and reorganization of Lifeact:Venus labeled actin filaments in *Arabidopsis* root hairs and root epidermal cells with that of actin filaments labeled with GFP:FABD2, a commonly used probe in plants. Unlike GFP:FABD2, Lifeact:Venus labeled the highly dynamic fine F-actin in the subapical region of tip-growing root hairs. Lifeact:Venus, however, reduced reorganization rates of (bundles of) actin filaments in root epidermal cells. Reorganization rates of cytoplasmic strands, which reflect reorganization of the actin cytoskeleton, were reduced in Lifeact:Venus expressing cells compared to both wild type and GFP:FABD2 expressing cells. Furthermore, Lifeact:Venus decorated actin filaments were more resistant to depolymerization by latrunculin B than GFP:FABD2 decorated actin filaments. We conclude that Lifeact:Venus reduces remodeling of the actin cytoskeleton in *Arabidopsis*, and that Lifeact should be used with care when studying actin reorganization.

Introduction

The actin cytoskeleton in eukaryotic cells is essential in many processes. It is capable to rapidly form a plethora of structures, such as cross-linked networks and bundles of various thicknesses. The organization of the actin cytoskeleton is regulated by the intrinsic properties of actin and by actin binding proteins. To study the dynamic reorganization of the actin cytoskeleton, live cell imaging is an important experimental procedure. Adding a fluorescent protein directly to actin reduces retrograde flow in lamellipodia of fibroblast cells and slows down chemotactic dendritic cell migration (Riedl et al. 2008). In plant cells, fluorescently labeled actin does incorporate into actin filaments, but those do not form the plant specific actin cytoskeleton configurations (Van der Krogt et al. 2006). To circumvent this problem, several genetic fusions of fluorescent probes to actin binding domains of actin binding proteins are available for live cell actin cytoskeleton visualization. For live cell actin visualization in plants, GFP:FABD2 (Ketelaar et al. 2004a; Sheahan et al. 2004), consisting of GFP fused to the second actin binding domain of *Arabidopsis* fimbrin1, and GFP:mTalin (Kost et al. 1998), consisting of GFP fused to the actin binding domain of mouse talin, have been frequently used. Expression of any fluorescently tagged actin binding domain is likely to cause competition with native actin binding proteins for actin binding. This clearly is the case in GFP-mTalin expressing plants. In *Arabidopsis* expressing GFP-mTalin, growth is reduced and morphology is altered (Sheahan et al. 2004). This is thought to be caused by a reduced cytoplasmic streaming velocity, over-stabilization of the actin cytoskeleton (Holweg 2007) and a reduction of ADF/cofilin mediated actin depolymerization in the presence of GFP-mTalin (Ketelaar et al. 2004b). GFP:FABD2 expression only causes a slight reduction in organelle velocity in *Arabidopsis* (Holweg 2007) and plant growth is not inhibited (Voigt et al. 2005). GFP:FABD2 is therefore considered to be the best choice for visualization of actin filament organization and dynamics in plant cells. GFP:FABD2 does, however, not appear to decorate the actin fringe in the apical region of pollen tubes (Wilsen et al. 2006), and the dynamic (bundles of) actin filaments in the subapex of growing root hairs (Ketelaar and Emons 2009). Furthermore, in moss cells, GFP:FABD2 expression results in growth abnormalities and cytoskeletal anomalies (Vidali et al. 2009). Thus, even though GFP:FABD2 is considered as the best available probe for actin visualization in plant cells, it has drawbacks.

Lifeact is a novel probe for live cell actin visualization. Fused to a fluorescent probe, this short peptide, consisting of the first 17 amino acids from the yeast protein Abp140, clearly visualizes filamentous actin in eukaryotic cells and tissues (Riedl et al. 2008). Lifeact affects neither actin nucleation, elongation and depolymerization rates *in vitro*, nor cytoskeletal functions in living mammalian cells (Riedl et al. 2008). Since there are no sequences homologous to Abp140 in plants and vertebrates, it is thought that the peptide is likely to have no or little side effects on actin organization and dynamics in vertebrate cells (Riedl et al. 2008). Besides labeling actin filaments in vertebrate cells (Estecha et al. 2009; Riedl et al. 2008), Lifeact labels actin filaments in filamentous growing fungi (Berepiki et al.) and in different plant species ranging from the liverworth *Marchantia* and the moss *Physcomitrella* to the seed plants *Arabidopsis*, lily and tobacco (Era et al. 2009; Vidali et al. 2009). In contrast to GFP:FABD2, Lifeact does decorate fine F-actin in the subapical area of tip-growing pollen tubes (Vidali

et al. 2009). Based on the above data, Lifeact appears a very good choice for live cell visualization of actin filaments in a wide range of cell types and organisms.

In this study, we compared the organization and dynamics of the actin cytoskeleton in GFP:FABD2 and Lifeact:Venus expressing *Arabidopsis* plants. Unlike GFP:FABD2, Lifeact:Venus labeled the highly dynamic fine F-actin configuration in the subapical region of tip growing root hairs. Expression of Lifeact:Venus, however, reduced the reorganization rate of actin filaments in root epidermal cells when compared to GFP:FABD2. Cytoplasmic strand reorganization rate, which reflects remodeling of the actin cytoskeleton, was similar in wild type and GFP:FABD2 expressing cells, but reduced in Lifeact:Venus expressing cells, indicating that GFP:FABD2 does not enhance, but that Lifect:Venus reduces actin reorganization. Moreover, in Lifeact:Venus expressing cells, the actin cytoskeleton was more resistant to actin depolymerization with latrunculin B than in GFP:FABD2 expressing cells. Together, these results demonstrate that Lifeact reduces remodeling of the actin cytoskeleton in *Arabidopsis*.

Materials and methods

Plant material and growth conditions

Seeds were sterilized for 1 minute with 70%-ethanol, followed by a 3-5-minute treatment with 15-20% household bleach (4% hypochlorite) and 0.05% triton X-100. After sterilization, the seeds were washed 4 times with ddH_2O, and stratified at 4°C for at least 2 days. For root hair imaging, seeds were sown on tilted coverslips containing a thin 0.7% agarose layer of Hoaglands' medium, covered with biofoil (Vivascience, Göttingen, Germany). Root hairs grew along the coverslip, and were imaged with a spinning disk confocal microscope 3-4 days after planting. For root epidermal cell visualization experiments, seeds were germinated on 0.5 MS plates containing 1.5% agarose. The plates were placed at an oblique angle (approximately 15-30° off vertical). All plants were grown at 25°C (16 h light, 8 h darkness).

Life cell imaging and drug treatments

For all experiments, 3-5 day old plants were used. Root hairs were imaged with a I-LAS Spinning Disk Confocal System (Roper Scientific SAS, France) on a Nikon Eclipse Ti microscope using a 100x (N.A. 1.49) oil immersion objective (Gutierrez et al. 2009). Root epidermal cells were imaged and FRAPped with an Axiovert 200M microscope (Zeiss, Jena, Germany) connected to a Zeiss LSM510 META confocal scanning system equipped with a 63x N.A. 1.4 oil immersion objective (Van der Honing et al. 2010). The pinhole size was 1 Airy Unit (98 (GFPFABD2) / 102 (Lifeact:Venus) µm). Transmission images were collected simultaneously.

Fluoresceindiacetate (FDA; Sigma, St Louis, MO, USA; 0.5% stock in acetone) was diluted to a final concentration of 0.01% in ddH_20. A 10 mM-stock (in DMSO) of latrunculin B (Sigma-Aldrich, Zwijndrecht, The Netherlands) was diluted to a final concentration of 1 µM in ddH_20.

Fluorescence Recovery After Photobleaching (FRAP)
Pre-bleach (always 5 images) and post-bleach imaging occurred at 5% excitation at 488 nm (GFP:FABD2) or 514 nm (Lifeact:Venus). Photobleaching was performed using both the 488 nm and the 514 nm laser lines at 100% laser power (10 iterations) using a box size of 50 - 190 μm^2. Fluorescence intensity values of the bleached region were normalized, and the average time for 50% recovery ($t_{1/2}$) was determined from fitting recovery curves.

Analysis of actin reorganization
Images of the cortical region of root epidermal cells were collected at 5 s intervals. After running an Image J macro (Van Bruaene et al. 2004) (see results), we averaged the number of red, green, yellow and black pixels for the first 3 images (*i.e.* the first 10 s) of each image sequence. Actin filament reorganization was quantified by determining the percentage of fluorescent pixels that newly appeared at a location that was not fluorescent in the previous frame, *i.e.* (number of green pixels) / (number of green pixels + number of yellow pixels) * 100%.

Protein Gel Blot Analysis
Arabidopsis seedlings were grown on plates with Hoaglands' medium and 1% agarose. Seedlings of similar age as the ones used for imaging were harvested and processed as described by Ketelaar et al. (2002). SDS-PAGE was performed on a 10% acrylamide gel. The blot was probed with antiserum against GFP (Abcam ab290) diluted 1:1000, followed by goat anti-rabbit IgG alkaline phosphatase diluted 1:3000 (Abcam ab6722).

Results

Expression of GFP:FABD2 or Lifeact:Venus does not dramatically affect plant growth
To determine the consequences on plant growth of GFP:FABD (Ketelaar et al. 2004a) or Lifeact:Venus (Era et al. 2009) expression, *Arabidopsis* Col-0 plants expressing GFP:FABD2 or Lifeact:Venus, both driven by the CaMV 35S promoter, were grown on potting compost together with *Arabidopsis* Col-0 plants without actin marker. Plant growth and organ development of GFP:FABD2 and Lifeact:Venus expressing plants occurred at similar rates as those of wild type plants, and the plants of all lines are similar in size and stature (Fig. 1 A, B). Root growth rates of Lifeact:Venus expressing plants were similar to those of wild type plants (Fig. 1 C), but those of GFP:FABD2 expressing plants grew slightly, but significantly, faster. To investigate the consequences of GFP:FABD2 and Lifeact:Venus expression at cell level, we determined root hair growth rates of both lines. Root hair tip growth is sensitive to changes in actin organization and dynamics (Ketelaar et al. 2003; Ketelaar and Emons 2009; Miller et al. 1999). Altered root hair growth velocities can be indicative for defects in the organization or dynamics of actin filaments. Thus, we measured root hair growth velocities in GFP:FABD2 and Lifeact:Venus expressing plants. Root hair growth rates of both GFP:FABD2 and Lifeact:Venus expressing plants were, however, similar to those of wild type plants (Fig. 1 D). Thus, except for the slightly higher root growth rates of GFP:FABD2 expressing plants, both fusion proteins express at levels that do not affect plant development and cell growth.

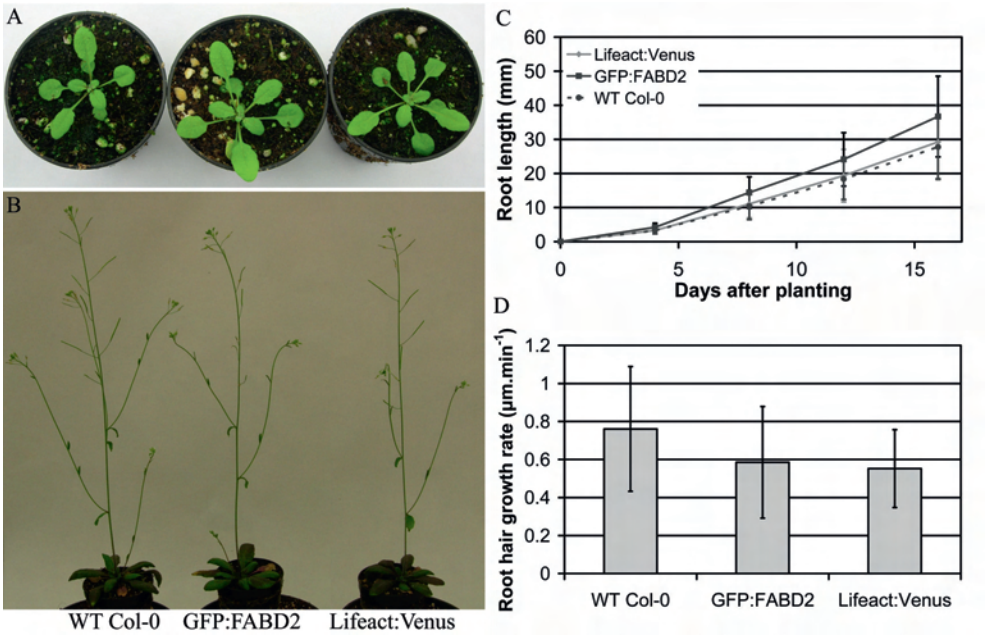

Figure 1. Expression of Lifeact:Venus or GFP:FABD2 does not dramatically affect plant growth and morphology. 25 day old (A) and 35 day old (B) wild type (left), GFP:FABD2 expressing (middle) and Lifeact:Venus expressing (right) plants are similar in size and stature. (C) Root growth rates of Lifeact:Venus expressing plants are similar to those of wild type plants (t-test, p = 0.56 at day 16), but those of GFP:FABD2 expressing plants are slightly higher (t-test, p = 0.002 at day 16). (D) Root hair growth rates of Lifeact:Venus expressing (n=12) and GFP:FABD2 expressing (n=10) plants do not differ significantly from those of wild type plants (n=15) at α = 0.05 (p = 0.07 for Lifeact:Venus, and 0.19 for GFP:FABD2). Error bars represent standard deviations.

Expression levels of GFP:FABD2 and Lifeact:Venus are similar

Although plant development and cell growth were not affected by the expression levels of GFP:FABD2 or Lifeact:Venus that we used, we further tested the expression levels of both probes by Western blotting. The expression level of GFP:FABD2 is slightly higher than that of Lifeact:Venus, but the amounts of protein are similar in both lines (Fig. 2).

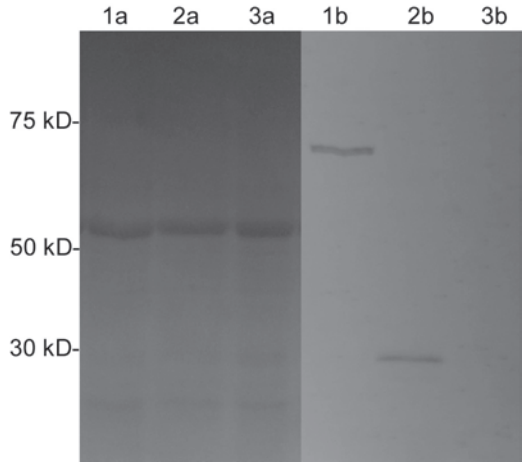

Figure 2. Western blotting with a GFP antibody shows that expression levels of GFP:FABD and Lifeact:Venus are similar. Equal amounts of protein from seedlings expressing GFP:FABD2 (1), Lifeact:Venus (2) and wild type Col-0 plants (3) were separated by SDS-PAGE and the blot was probed with an antibody against GFP which is equally sensitive to all GFP variants. The lanes labeled with (a) show a total protein stain that confirms equal loading and the lanes labeled with (b) show the same lanes probed with the GFP antibody. The sizes of the bands are similar to the predicted molecular mass of GFP:FABD2 (68 kD) and Lifeact:Venus (29 kD).

Lifeact:Venus labels fine F-actin in the apical region of growing root hairs of *Arabidopsis*

To test the quality of Lifeact:Venus in labeling the subapical, dynamic fine F-actin in growing root hairs, we compared the actin organization in *Arabidopsis* root hairs expressing Lifeact:Venus with that in root hairs expressing GFP:FABD2. From phalloidin stained and actin immunolabeled fixed root hairs, the actin organization in growing root hairs is known. In the non-expanding root hair tube, thick, more or less longitudinally oriented bundles of actin filaments are present. In the (sub)apical area, these flare out in thinner bundles of actin filaments, which configuration we have named fine F-actin (Ketelaar et al. 2003). We compared the above actin organization with that in GFP:FABD2 and Lifeact:Venus expressing root hairs. In the root hair tube, the actin organization in root hairs expressing GFP:FABD2 or Lifeact:Venus was similar to that in fixed cells (Fig. 3). However, in the (sub)apical area of GFP:FABD2 expressing root hairs, only few actin filaments were detectable (Fig. 3 A, B). This does not resemble the fine F-actin in fixed root hairs. In Lifeact:Venus expressing root hairs, highly dynamic fine F-actin was visible in the (sub)apex (Fig. 3 D, E). The fine F-actin resembles the fine F-actin in fixed root hairs. Growth, morphology and cytoarchitecture of root hairs expressing either GFP:FABD2 or Lifeact:Venus were normal. Summarizing, Lifeact:Venus, unlike GFP:FABD2, labels the highly dynamic fine F-actin in the apical region of root hairs.

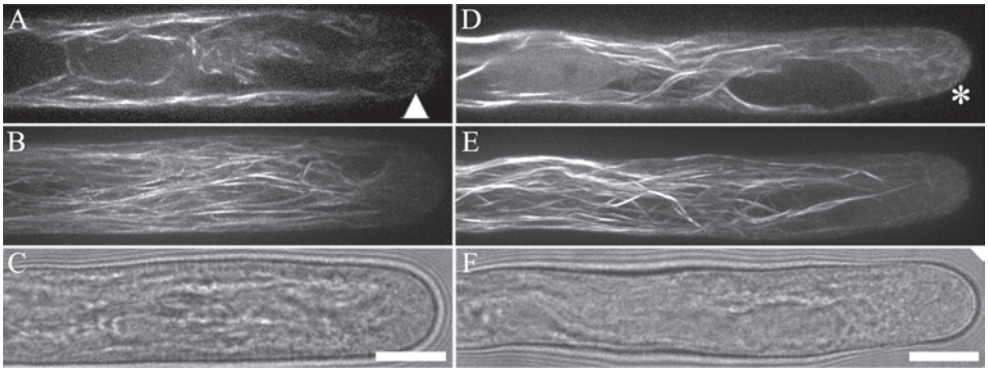

Figure 3. Unlike in GFP:FABD2 expressing root hairs, fine F-actin is detectable in the subapical region of root hairs expressing Lifeact:Venus. In the root hair tube, thick, more or less longitudinally oriented bundles of actin filaments are similarly present in GFP:FABD2 (A, B) and Lifeact:Venus (D, E) expressing root hairs. Unlike in GFP:FABD2 expressing root hairs (A, B; arrowhead), actin filaments are detectable in the subapical region of Lifeact:Venus expressing root hairs (D, E; asterisk). A, D: confocal images of median plane; B, E: maximal projections of z-stacks collected using a 0.5 μm z-step size; C, F: transmission images (taken afterwards). Scale bars are 10 μm.

Actin filament (bundle) relocation over time in Lifeact:Venus expressing cells is slower than that in GFP:FABD2 expressing cells

To study the dynamic reorganization of the actin cytoskeleton over time in GFP:FABD2 and Lifeact:Venus expressing cells, image sequences of the cortical actin cytoskeleton of growing root epidermal cells (situated between the root apical meristem and the root hair zone, with a length of 30 - 85 μm) were collected at 5 s time intervals. In both GFP:FABD2 and Lifeact:Venus expressing cells, the cortical actin cytoskeleton was clearly labeled, and these actin filament (bundle)s relocated over time (Fig. 4 A-H).

To quantify the relocation over time of actin filament (bundle)s of both lines, we first applied a threshold in order to obtain a binary image, in which fluorescent regions, representing actin filaments, appeared white, and in which non-fluorescent regions appeared black (Fig. 4 I, J). An Image J macro (Van Bruaene et al. 2004) was used to compare each image frame of this binary image sequence to the subsequent image frame in the time series. The output of this macro resulted in an image sequence in which fluorescence at the same location in two subsequent images appeared yellow, whereas fluorescence present in only one of the two subsequent images appeared either red (when fluorescence was present only in the first frame) or green (when fluorescence was present only in the second frame). Regions with no fluorescence in either of the frames appeared black (Fig. 4 K, L).

We determined the percentage of actin filament (bundles) that relocated within 5 s (Fig. 4 M). On average, 58 ± 5 % of actin filament (bundle) fluorescence in GFP:FABD2 expressing cells were located at a different position after a 5 s interval, whereas only 17 ± 5 % of Lifeact:Venus labeled actin filament (bundle) fluorescence had relocated within 5 s. The relocation velocity of the two lines differed significantly (t-test, $p = 0.00$). Thus, actin filament (bundle) relocation in Lifeact:Venus expressing cells occurs at lower rates than that in GFP:FABD2 expressing cells.

Analysis of cytoplasmic strand reorganization shows that actin filament reorganization in Lifeact:Venus expressing plants is reduced in rate when compared to wild type and GFP:FABD2 expressing plants

The different rate of actin filament (bundle) relocation in GFP:FABD2 and Lifeact:Venus expressing cells could result from a reduction in actin filament (bundle) relocation due to Lifeact:Venus expression, or from an increase in actin filament (bundle) relocation due to GFP:FABD2 expression. To discriminate between these possibilities, we followed cytoplasmic strand relocation of both lines, and compared this with cytoplasmic strand relocation in wild type plants. Actin filaments are the backbone of cytoplasmic strands (Higaki et al. 2006; Hussey et al. 1998; Sheahan et al. 2007; Shimmen et al. 1995; Staiger et al. 1994; Valster et al. 1997; Van der Honing et al. 2010; Van Gestel et al. 2002), and consequently cytoplasmic strand dynamics result from a reorganization of the actin cytoskeleton. The relocation rate of cytoplasmic strands therefore reflects actin filament remodeling.

FDA (0.01%) was used to fluorescently stain the cytoplasm of root epidermal cells, and image sequences were collected at 5 s intervals. In root epidermal cells, cytoplasmic strands constantly changed in location (Fig. 5), reflecting a continuous remodeling of the actin cytoskeleton. Cytoplasmic strands in wild type and GFP:FABD2 expressing cells relocated at similar frequencies (Fig. 5 A-F), implying a similar rate of actin filament relocation. In Lifeact:Venus expressing cells, the location of cytoplasmic strands hardly changed over time (Fig. 5 G-I), implying a reduced rate of actin filament relocation when compared to wild type plants. These data show that Lifeact:Venus reduces actin filament reorganization over time.

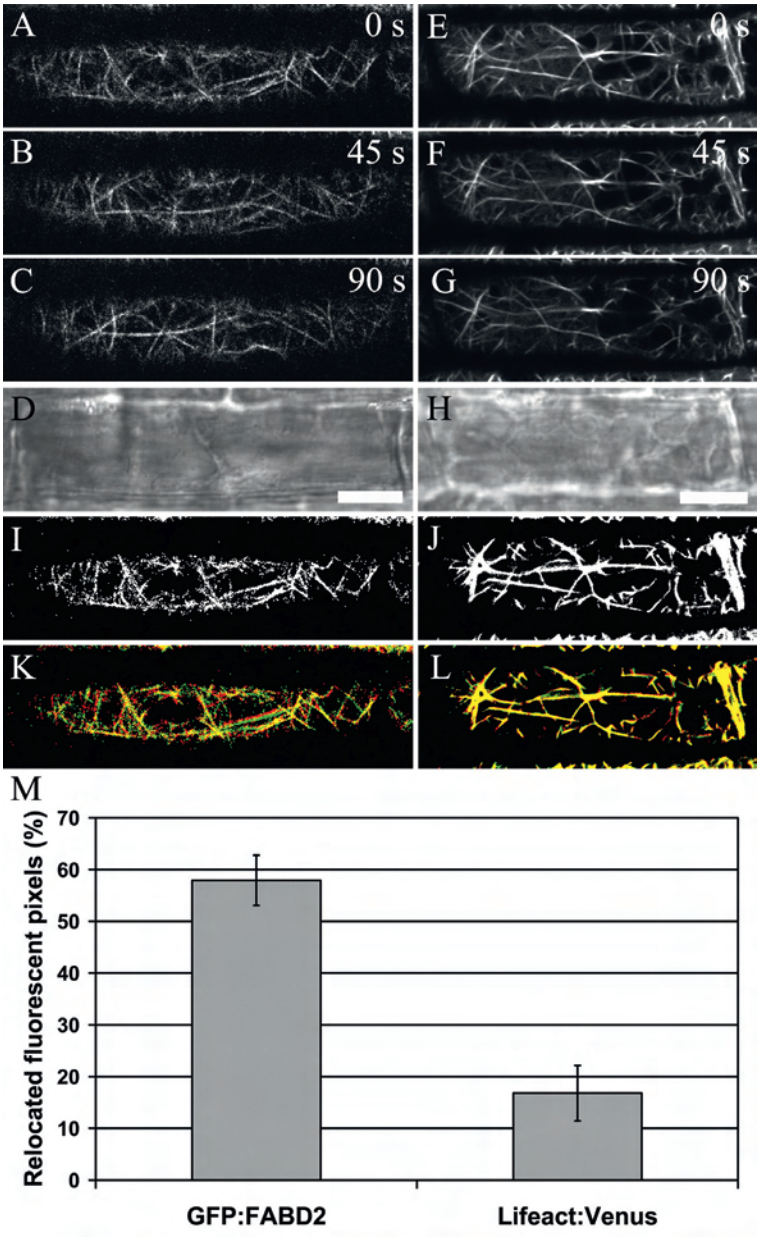

Figure 4. Reorganization of (bundles of) actin filaments in Lifeact:Venus expressing root epidermal cells occurs more slowly than in GFP:FABD2 expressing cells. Both GFP:FABD2 (A-C) and Lifeact:Venus (E-G) labeled actin filaments reorganize over time. (I, J) After thresholding, a binary image was obtained, in which white pixels represent filamentous actin. (K, L) Running an Image J macro (Van Bruaene et al. 2004) resulted in an image sequence in which yellow regions represent actin filaments that have not reorganized within a 5 s time interval, and in which red and green regions represent actin filaments that have reorganized within a 5 s time interval (green: pixels in which fluorescence newly appeared at a location where fluorescence was absent in the previous frame; red: pixels in which fluorescence disappeared from a location where fluorescence was present in the previous frame). M: In GFP:FABD2 expressing cells, the percentage of fluorescently labeled filamentous actin that reorganizes within a 5 s interval is significantly higher than in Lifeact:Venus expressing cells (n = 9 for GFP:FABD2 expressing cells, and n = 10 for Lifeact:Venus expressing cells). A-C and E-G: confocal images of cortical plane; D, H: transmission images. Error bars represent standard deviation. Scale bars are 10 μm.

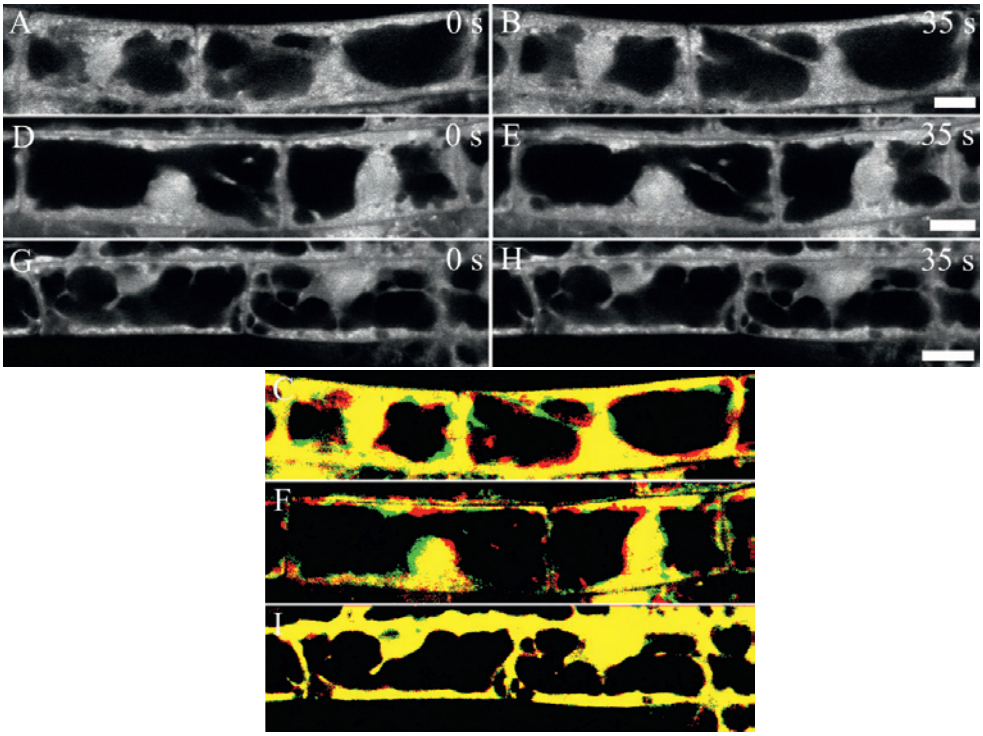

Figure 5. Cytoplasmic strand reorganization rates are reduced in Lifeact:Venus expressing root epidermal cells, but not in GFP:FABD2 expressing cells when compared to wild type cells. In wild type (A, B) and GFP:FABD2 expressing (D, E) root epidermal cells, cytoplasmic strands continuously change in location, whereas in Lifeact:Venus expressing cells (G, H), the localization of the cytoplasm is much more static. C, F, I: Running the same Image J macro (Van Bruaene et al. 2004) as used in Fig. 4 shows that in Lifeact:Venus expressing root epidermal cells, the amount of cytoplasm that reorganizes within a 35 s time interval (represented by green or red pixels) is lower than in wild type and GFP:FABD2 expressing root epidermal cells. Cytoplasm was visualized by fluoresceindiacetate staining (0.01%). Scale bars are 10 μm.

Latrunculin B induced depolymerization of actin filaments is reduced by Lifeact:Venus

To test the sensitivity to actin filament depolymerization of both lines, we added 1 μm latrunculin B to roots of GFP:FABD2 and Lifeact:Venus expressing plants, and collected confocal z-stacks of root epidermal cells. After a 5-minute latrunculin B treatment, most (bundles of) actin filaments had depolymerized in GFP:FABD2 expressing cells (Fig. 6 A). The few remaining (bundles of) actin filaments continued to depolymerize until, after a 30-minute treatment with latrunculin B, almost all filamentous actin had disappeared (Fig. 6 C). In contrast, Lifeact:Venus labeled actin filament depolymerization occurred more slowly over time (Fig. 6 E-G), and 30 minutes after latrunculin B addition, many actin filament (bundle)s were still present. Thus, actin filament depolymerization in Lifeact:Venus expressing plants is slower than in GFP:FABD2 expressing plants.

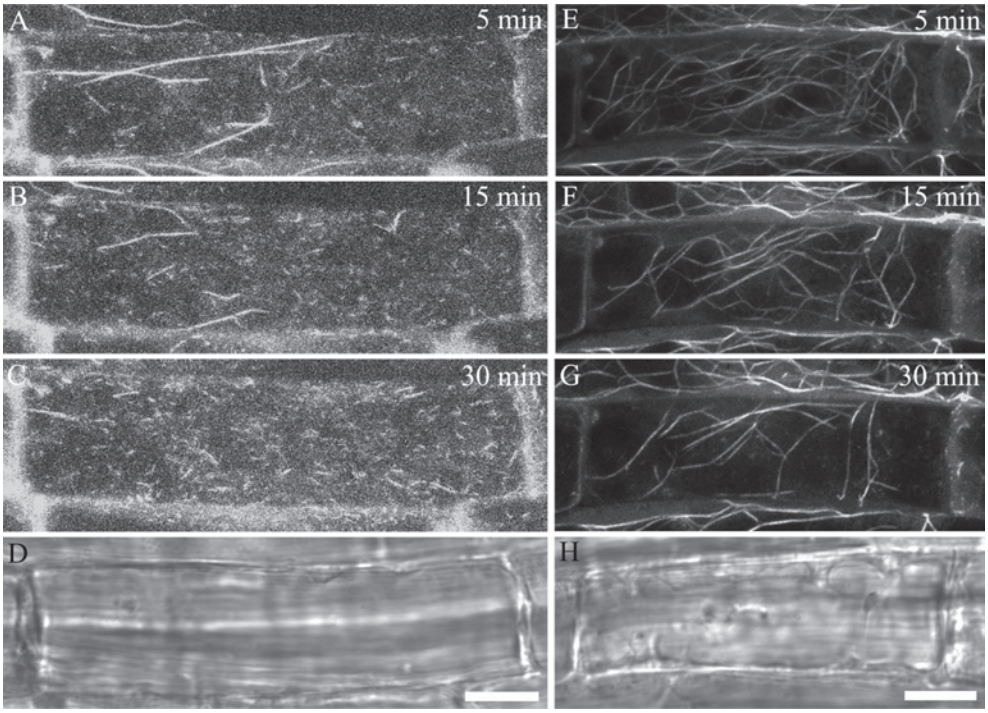

Figure 6. Latrunculin B mediated actin filament depolymerization in Lifeact:Venus expressing cells is slower than in GFP:FABD2 expressing cells. Latrunculin B (1 µm) induces depolymerization of actin filaments in both GFP:FABD2 (A-C) and Lifeact:Venus (E-G) expressing root epidermal cells. After a 30 minute treatment with latrunculin B, almost all GFP:FABD2 labeled filamentous actin has disappeared (C), whereas many (bundles of) actin filaments are still present in Lifeact:Venus expressing cells (G). A-C and E-G: maximal z-projections of 19 frames taken at 0.6 µm z-steps. D and H: transmission images. Scale bars are 10 µm.

Lifeact dissociates more rapidly from actin filaments than FABD2

FRAP was used to analyze dissociation rates of Lifeact:Venus and GFP:FABD2 from actin filaments in root epidermal cells (Fig. 7). We bleached a box in the cortical region (Fig. 7 B, G) and determined the average time for 50% fluorescence recovery ($t_{1/2}$) (Fig. 7 K, L). For GFP:FABD2, we found a $t_{1/2}$ of 6.9 ± 2.7 s. Fluorescence recovery of Lifeact:Venus occurred more rapidly, with a $t_{1/2}$ of 2.7 ± 1.4 s. A t-test showed that the difference in fluorescence recovery time between GFP:FABD2 and Lifeact:Venus is significant ($p = 0.00$). This shows that Lifeact:Venus has a faster exchange rate from actin filaments than GFP:FABD2.

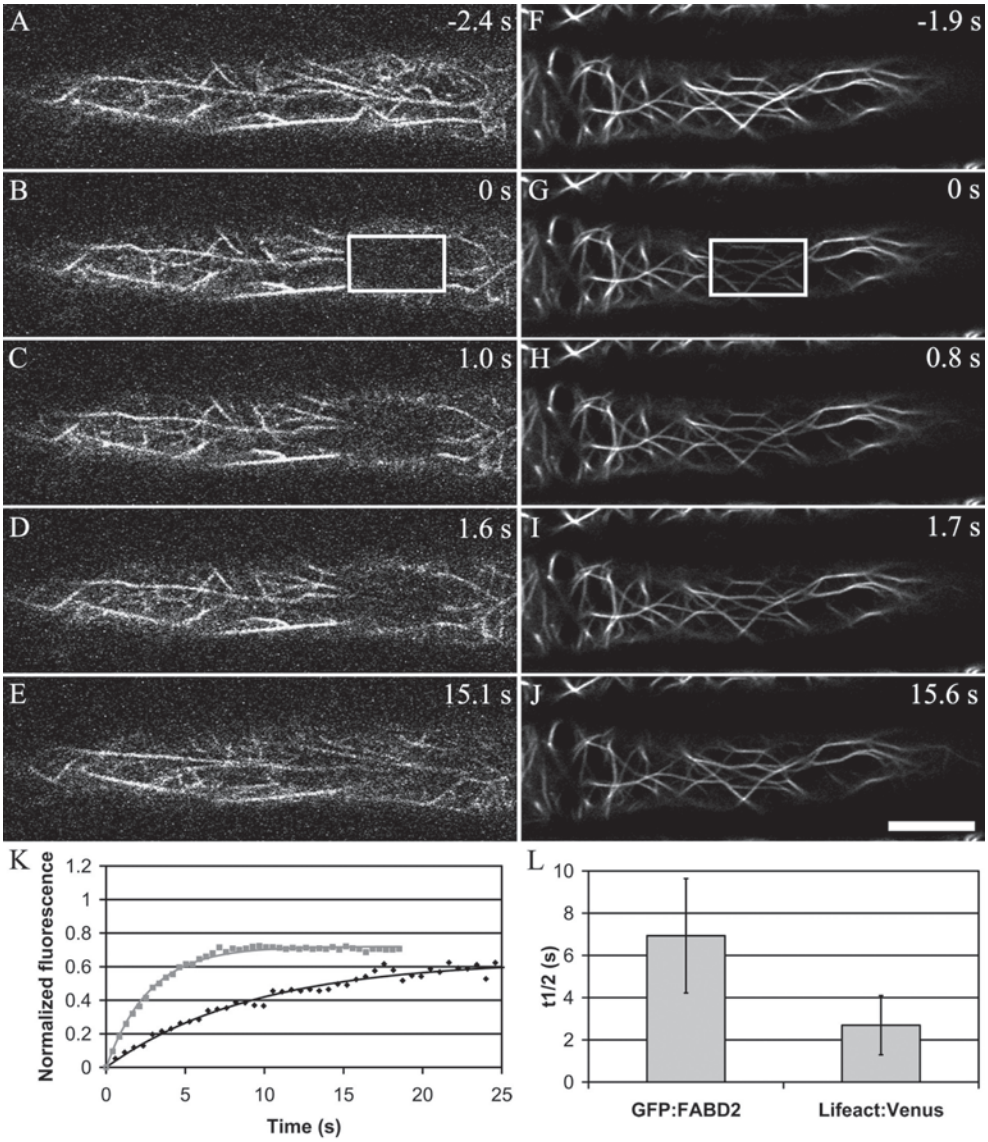

Figure 7. Lifeact exchanges faster than actin filaments than FABD2. A-J: Fluorescence recovery after photobleaching in GFP:FABD2 (A-E) and Lifeact:Venus (F-J) expressing root epidermal cells. The box indicates the area that has been FRAPped. K: Typical fluorescence recovery curves of a bleached region in a Lifeact:Venus (grey squares) and a GFP:FABD2 (black diamonds) expressing root epidermal cell. The pre-bleach fluorescence intensity has been set to 1, and the fluorescence intensity directly after bleaching (t = 0 s) has been set to 0. Lines represent the fitted fluorescence recovery curves. L: The average time for 50 % fluorescence recovery ($t_{1/2}$) of Lifeact:Venus is significantly lower than that of GFP:FABD2 (n = 11 for both lines). Error bars represent standard deviation. Scale bar is 10 µm. Box size in B and G is 11.3 x 6.7 µm.

Discussion

Here we show that in Lifeact:Venus expressing plants, at expression levels that do not affect plant development, the actin organization looks similar to the actin organization in cells expressing a similar amount of GFP:FABD2. In root hairs, the organization of Lifact:Venus labeled actin filaments is similar to that described for fixed root hairs, and, unlike GFP:FABD2, Lifeact:Venus labels fine F-actin in the subapical region. Lifeact:Venus, however, reduces reorganization rates of actin filaments in *Arabidopsis* root epidermal cells. Further, Lifeact:Venus labeled actin filaments are more resistant to depolymerization by latrunculin B than actin filaments labeled with GFP:FABD2.

Rapid dissociation of Lifeact:Venus does not prevent interference with actin filament reorganization rate

The rapid dissociation of Lifeact from actin filaments is consistent with its low affinity for actin filaments *in vitro* (Riedl et al. 2008). This low affinity of Lifeact for actin filaments, however, does not prevent it from altering actin reorganization. The rate of actin filament reorganization is reduced quite dramatically by Lifeact, and this reduction in actin filament (bundle) reorganization is also reflected in a reduced reorganization rate of cytoplasmic strands. GFP:FABD2 dissociates slower from actin filaments than Lifeact:Venus, consistent with previous results (Riedl et al. 2008; Sheahan et al. 2004). Nevertheless, cytoplasmic strand reorganization rate of GFP:FABD2 expressing cells is similar to that of wild type cells. Our results show that low affinities of fusion proteins for actin filaments do not necessarily relate to reduced effects on remodeling of the actin cytoskeleton.

A reduction in reorganization rate of actin filament (bundle)s and cytoplasmic strands does not affect plant cell growth and plant morphology

The reorganization rate of actin filament (bundle)s and, consequently, cytoplasmic strands, is reduced in Lifeact:Venus expressing cells. Surprisingly, this reduction in cytoplasmic strand reorganization rate does not affect plant cell growth and plant morphology. Lifeact:Venus expressing plants grow at rates comparable to those of wild type plants, and plant growth and organ development are similar. Moreover, plant size and stature are not affected by Lifeact:Venus expression. Even root hair growth, which is very sensitive to changes in actin organization and dynamics (Ketelaar et al. 2003; Ketelaar and Emons 2009; Miller et al. 1999), is unaffected by the reduction in actin filament (bundle) reorganization. Despite the effect of Lifeact:Venus on cytoplasmic strand reorganization rate, the cytoplasmic organization is not visibly affected in root hairs and root epidermal cells. Since plant cell elongation depends on an organized actin cytoskeleton (Collings et al. 2006) our finding implicates that the organization of the actin cytoskeleton, but not its dynamic relocation over time, is a determining factor in plant cell growth.

Labeling of fine F-actin in the subapex of root hairs with Lifeact:Venus may result from the reduced actin filament (bundle) reorganization rate

We show that Lifeact:Venus expression reduces actin filament reorganization rate, and that Lifeact:Venus, unlike GFP:FABD2, reveals fine F-actin in the subapical region of root hairs. Fine F-actin is highly dynamic (Ketelaar and Emons 2009). Either fine

F-actin may be too dynamic for GFP:FABD2 binding, or the rapid reorganization of GFP:FABD2 decorated fine F-actin in root hairs may be too fast for detection with current microscopes. In the latter case, the reduced actin filament reorganization rate in Lifeact:Venus expressing plants might enable microscopic detection.

Lifeact is used as a marker for actin filament imaging in different types of eukaryotic cells. In animal cells, Lifeact has been shown not to label some forms of stress-induced twisted F-actin (Munsie et al. 2009), and these authors state that Lifeact may not be a universal marker to study actin filament dynamics. In the moss *Physcomitrella*, it has been shown that although high expression levels of Lifeact:Venus result in reduced growth rates, the actin labeling looks similar to lines with lower expression levels, which are not affected in their growth rate (Vidali et al. 2009). We show that despite the high exchange rate of Lifeact from actin filaments, it dramatically reduces reorganization rates of actin filaments and cytoplasmic strands in *Arabidopsis*, even though plant growth and morphology are not affected. The reduction in actin reorganization may be a universal property of Lifeact, independent of the organism in which it is used. Lifeact should therefore be used with care when studying actin reorganization.

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Chapter 4

Arabidopsis VILLIN2 and VILLIN3 are required for the generation of thick actin filament bundles and for directional organ growth

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Submitted

Abstract

In plant cells, actin filament bundles serve as tracks for myosin-dependent organelle movement and play a role in the organization of the cytoplasm. Although virtually all plant cells contain actin filament bundles, the role of the different actin bundling proteins remains largely unknown. In this study, we investigated the role of the actin bundling protein villin in *Arabidopsis thaliana*. We used *Arabidopsis* T-DNA insertion lines to generate a double mutant in which *VLN2* and *VLN3* transcripts are truncated. Leaves, stems, siliques and roots of *vln2 vln3* double mutant plants are twisted. Microscopy analysis of the actin cytoskeleton showed that in these double mutant plants, thin actin filament bundles are more abundant, while thick actin filament bundles are virtually absent. *In vitro* experiments show that villin's headpiece region is essential for villins bundling capacity. Indeed, in contrast to full-length VLN3, truncated VLN3 lacking the headpiece region does not rescue the phenotype of the *vln2 vln3* double mutant. Our results show that villin is involved in the generation of thick actin filament bundles in several cell types, and suggest that these bundles are involved in the regulation of coordinated cell expansion.

Introduction

The plant actin cytoskeleton plays an essential role in cell division, cytoplasmic organization, cytoplasmic streaming, cell growth, and consequently plant morphogenesis. Actin binding proteins modulate the formation and dynamics of filamentous actin, and its configuration. Among these proteins are the actin bundling proteins, which are able to cross-link adjacent actin filaments, resulting in bundles consisting of several parallel actin filaments (Thomas et al., 2009). In plant cells, bundling of actin filaments occurs (Thomas et al., 2009), which is likely mediated by actin bundling proteins. There are four known families of actin bundling proteins in plants: villins (Vidali et al., 1998; Klahre et al., 2000; Tominaga et al., 2000; Yokota et al., 2003; Huang et al., 2005; Yokota et al., 2005; Khurana et al., 2010; Zhang et al., 2010; Zhang et al., 2011), fimbrins (Kovar et al., 2000; Kovar et al., 2001), formins (Cheung and Wu, 2004; Favery et al., 2004; Michelot et al., 2005; Ye et al., 2009), and LIM proteins (Thomas et al., 2006; Thomas et al., 2008; Wang et al., 2008; Papuga et al., 2010). In addition, elongation factor 1 alpha (Collings et al., 1994; Gungabissoon et al., 2001) has been shown to have actin filament bundling properties as well. The presence of these different actin bundling proteins suggests that their combined actions can result in several types of actin filament bundles, which differ in form and function (Thomas et al., 2009).

Although the role of the different actin bundling proteins in the generation of actin filament bundles is not yet known, it is clear that actin filament bundles fulfill several functions in plant cells. Actin filament bundles serve as the preferred tracks for myosin-dependent movement of organelles (Miller et al., 1999; Ketelaar et al., 2003; Holweg, 2007; Ye et al., 2009). Next to their function in cytoplasmic streaming, actin filament bundles have been shown to play a role in keeping the nucleus at a fixed position from the root hair tip (Ketelaar et al., 2002). Furthermore, actin filament bundles structure the cytoplasm. Their depolymerization causes collapse of cytoplasmic strands (Staiger et al., 1994; Shimmen et al., 1995; Valster et al., 1997; Hussey et al., 1998; Van Gestel et al., 2002; Higaki et al., 2006; Sheahan et al., 2007; van der Honing et al., 2010), and unbundling results in more, but thinner cytoplasmic strands (Tominaga et al., 2000; Ketelaar et al., 2002). Thus, actin filament bundles are required to maintain cytoplasmic strand size and number, i.e. the overall organization of the cytoplasm of plant cells.

The genome of *Arabidopisis thaliana* contains five villin genes, and the villins encoded by these genes are highly expressed in several tissues of *Arabidopsis* (Klahre et al., 2000). Plant villins are similar to vertebrate villins (Staiger and Hussey, 2004). Villins consist of a core (made up of six tandem subdomains) and a distinct C-terminal domain, which is referred to as the headpiece. Villin's core shares structural homology to the actin binding protein gelsolin, which has Ca^{2+}-regulated actin filament nucleation, severing, and barbed end capping activity (Bryan and Kurth, 1984; Way et al., 1989). Both the core and the headpiece contain an actin filament binding domain, and the headpiece region of vertebrate villins has been shown to be essential for actin filament bundling (Glenney and Weber, 1981). This led to the hypothesis that villin bundles actin filaments by acting as a monomer, with the two actin filament binding domains – one present in the core, the other one in the headpiece – cross-linking two adjacent

actin filaments (Glenney et al., 1981b). However, one study suggests the presence of a third actin binding domain, which is present in the core (Hampton et al., 2008), while another study suggests that villin acts as a dimer (George et al., 2007). In addition to their ability to bundle actin filaments, vertebrate villins also show Ca^{2+}-dependent actin filament severing, nucleating and capping activity (Bretscher and Weber, 1980; Glenney et al., 1981a; Glenney and Weber, 1981). Plant villins also possess actin filament barbed-end capping (Yokota et al., 2005; Zhang et al., 2010), nucleating (Yokota et al., 2005) and severing (Khurana et al., 2010; Zhang et al., 2010) activities *in vitro*. In plants, villin has been shown to play a role in organizing the cytoplasm (Tominaga et al., 2000; Ketelaar et al., 2002), as well as in nuclear positioning in root hairs (Ketelaar et al., 2002).

In this study, we analyzed the role of two *Arabidopsis* villins using lines with a T-DNA insertion in *VLN2*, *VLN3*, or both *VLN2* and *VLN3*. The *vln2 vln3* double mutants show a clear anomaly in the growth direction of organs, suggesting problems with coordinated cell elongation. The actin cytoskeleton in the double mutants has a finer appearance, and thick bundles of actin filaments are virtually absent. GFP:VLN3 rescued the morphological phenotype and localizes to actin filament bundles in all cell types studied. We further show that the headpiece region is important for the bundling activity of VLN3, and for the regulation of directional organ growth. The data show that villin is involved in the generation of thick actin filament bundles and suggest that these bundles are important for coordinated cell expansion.

Results

T-DNA insertions in VLN2 and VLN3 result in a truncated transcript for both genes

A cladogram based on cDNA sequences shows that *VLN2* (At2g41740) and *VLN3* (At3g57410) belong to the same clade (Figure 1A), suggesting that they have arisen from a relatively recent genome duplication. They share 84 % similarity in their cDNA sequences, and 80 % similarity in their amino acid sequences. Both *VLN2* and *VLN3* are expressed in all organs (www.bar.utoronto.ca), with similar expression levels for both villins in most organs. VLN2 has a slightly higher expression level in mature pollen grains.

To test the biological role of VLN2 and VLN3, lines homozygous for T-DNA insertions in *VLN2* and *VLN3* were identified. The T-DNA insertions for both *vln2* and *vln3* (*i.e.* SAIL_813_H02 and SALK_117097, respectively) are located in exons (Figure 1B), at locations corresponding to the G6 domain in *vln2*, and to the linker domain between the G6 and headpiece domain in *vln3* (Figure 1C) according to Klahre et al. (Klahre et al., 2000). The presence of *VLN2* and *VLN3* transcripts was tested using RT-PCR in both azygous and mutant plants from the same population. Primer combinations before and after the inserts were used to test if transcripts were present, truncated, or absent. For both *vln2* and *vln3*, transcripts corresponding to coding regions before the insert were present, but the region after the insert was not transcribed (Figure 1D). Thus, the presence of the inserts results in truncated transcripts for both *VLN2* and *VLN3*. We generated a double mutant of these lines with truncated mRNA for both genes (Figure 1D).

Although the presence of the T-DNA inserts results in truncated transcripts for both *VLN2* and *VLN3*, the corresponding proteins could be absent. We tested this by probing a protein gel blot of wild type Columbia-0 (Col-0), *vln2*, *vln3*, and *vln2 vln3* root extracts with a polyclonal anti-lily villin antibody (Tominaga et al., 2000; Ketelaar et al., 2002; Khurana et al., 2010). This resulted in a band at a height corresponding to the predicted mass of VLN3 (107 kD) in Col-0 and *vln2* extracts, but in *vln3* and *vln2 vln3* extracts, no band was visible (Figure 1E). The absence of a band at a height corresponding to a smaller protein shows that *vln3* and *vln2 vln3* do not contain a truncated version of VLN3. Thus, although these lines contain a truncated *VLN3* transcript (Figure 1D), the VLN3 protein is absent. The absence of a villin band in the root extract of the *vln3* plant shows that the antibody does not recognize the VLN2 protein. We conclude that while *vln2* and *vln2 vln3* might contain a truncated version of VLN2, *vln3* and *vln2 vln3* do not contain the VLN3 protein.

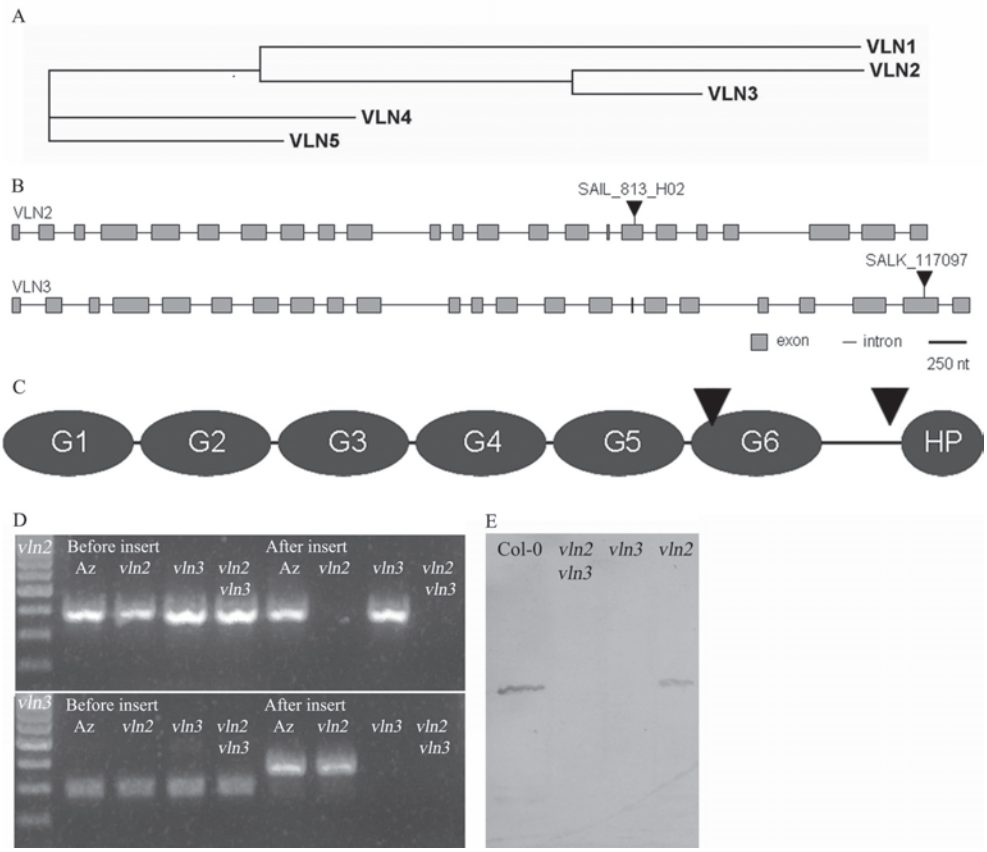

Figure 1. Characterization of the *Arabidopsis* villin gene family and T-DNA insertions in *vln2* and *vln3*. (A) Cladogram of the Arabidopsis villins, based on cDNA sequences. (B) Locations of T-DNA inserts in *vln2* and *vln3*. Grey boxes represent exons, and horizontal lines represent introns. T-DNA inserts (arrowheads) are not drawn to scale. (C) Domain structure of villin. Arrowheads show locations corresponding to the locations of T-DNA inserts of *vln2* and *vln3*. (D) T-DNA insertions result in truncated transcripts in *vln2*, *vln3*, and *vln2 vln3*. Products could be amplified using a cDNA template using *VLN* specific primers before the inserts, but when both primers (*vln2*) or the reverse primer (*vln3*) were designated for coding regions after the insert (see Supplemental Figure 2 and Table 1), products could not be amplified. (E) A protein gel blot of Col-0, *vln2*, *vln3*, and *vln2 vln3* root extracts probed with lily anti-villin antibody (Tominaga et al., 2000) shows that *vln3* and *vln2 vln3* do not contain (a truncated version of) the VLN3 protein.

Arabidopsis plants homozygous for a T-DNA insertion in two villin genes display defects in directional organ growth

Seedlings of single mutants of *vln2* and *vln3* do not show any developmental defects or delays, but those of *vln2 vln3* double mutants have curly roots (Figure 2A). Plant growth and organ development in the double mutant occur at similar rates as those of azygous plants and single mutants, and production of viable seeds is unaffected by the presence of T-DNA insertions in both genes. However, rosette leaves of the double mutant are twisted (Figure 2B). In addition, stems of the *vln2 vln3* double mutant are curly, and inflorescences show complete turns (Figure 2C, D). Both single mutants do not show this phenotype. The tops of the growing inflorescences of the double mutant are often (41 %; n = 17) oriented downward, while this never occurs in Col-0 plants (n = 22) and single mutants (n = 19 for *vln2*, and 17 for *vln3*; Figure 3A). Time lapse recording of Col-0 and *vln2 vln3* plants shows that the rotational movements (circumnutation) of *vln2 vln3* inflorescences differ from those of Col-0 inflorescences: in the double mutant, periods of normal circumnutation alternate with periods in which the circumnutation movements show larger amplitudes than those of Col-0 (Figure 2E). These data suggest that the coordination of cell expansion in the organs is affected in *vln2 vln3*, resulting in the curly phenotype of roots, leaves, and inflorescences. Siliques and fruit stalks of *vln2 vln3* are also curly (Figure 2C, D), and 59 % of the siliques were oriented at angles smaller than 90° with respect to the plant axis (n = 80), while this rarely occurred (with a maximum of 6 %) in Col-0 plants (n = 49) or single mutants (n = 50; Figure 3B). Despite the curly phenotype of leaves and fruit stalks, only the width of fruit stalk epidermal cells is slightly, but significantly, higher in the double mutant (0.99 ± 0.25 µm in *vln2 vln3* compared to 0.88 ± 0.23 µm in Col-0; Student's t test, P = 0.01). The surface area, perimeter and circularity of leaf pavement cells (n = 26 for Col-0 plants, and 61 for *vln2 vln3*; Figure 3C) and the surface area, perimeter, length and circularity of fruit stalk epidermal cells (n = 68 for Col-0 plants, and 67 for *vln2 vln3*; Figure 3D) are not affected by the mutations in *VLN2* and *VLN3*.

Although *VLN2* and *VLN3* are both expressed in root hairs (www.bar.utoronto.ca), we did not observe differences from Col-0 plants in root hair morphology (Figure 4A), nucleus to tip distance (Figure 4B), and growth rate (Figure 4C) of elongating root hairs of the double mutant. Thus, the mutations in *VLN2* and *VLN3* do not affect the growth and morphology of individual cells, but do result in defects in directional growth of roots, shoots, leaves and siliques. This suggests that the mutations affect coordinated cell elongation.

To confirm that the observed morphological phenotype is caused by the presence of the T-DNA inserts in *VLN2* and *VLN3*, we complemented the mutant phenotype with genomic *VLN2* and *VLN3*, under their endogenous promoters, and with *PVLN3:VLN3* cDNA. All these constructs rescued the mutant phenotype. These results confirm that the defects in directional organ growth are caused by the combined mutations in *VLN2* and *VLN3*. Thus, VLN2 and VLN3 play a redundant role in the regulation of directional organ growth.

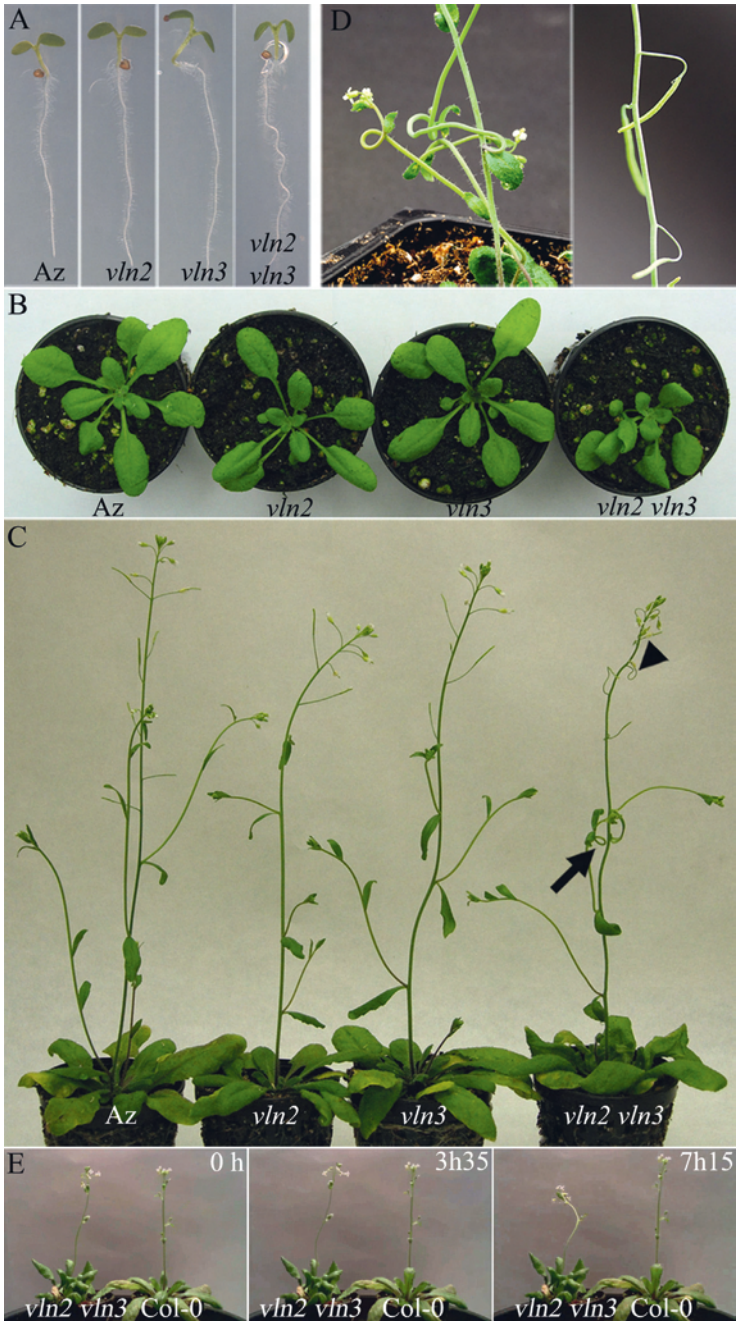

Figure 2: Phenotype of *vln2 vln3*. (A) Root morphology of azygous, *vln2*, *vln3* and *vln2 vln3* plants. Roots of both single mutants have the same appearance as azygous roots, but roots of double mutants grow in a curly, wavy manner. (B) Phenotype of 2-week-old plants. Leaves of the *vln2 vln3* double mutant are twisted, but in single mutants this twisting is absent. (C) Phenotype of 5-week-old plants. Branches of single mutants grow straight, similar to those of azygous plants, but in the double mutants, branches are curly, and even show complete twists (e.g. arrow). This twisting also occurs in the fruit stalks (e.g. arrowhead). (D) Twisting of double mutant branches and fruit stalks shown at a higher magnification. (E) The rotational movements (circumnutation) of *vln2 vln3* inflorescences have larger amplitudes than those of Col-0 inflorescences and are less regular.

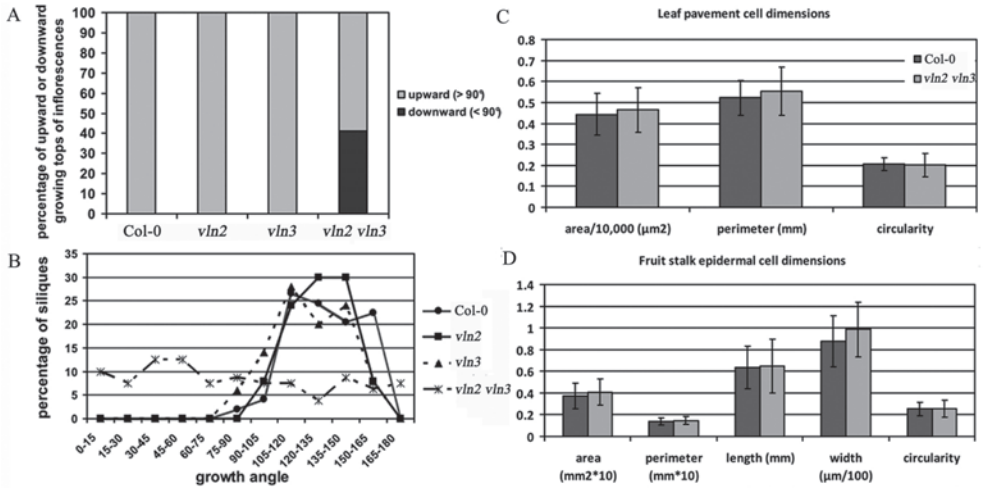

Figure 3: Quantification of *vln2 vln3* phenotype. (A) 41 % (n = 17) of the tops of inflorescence meristems of *vln2 vln3* grow downward, while this never occurs in Col-0 (n = 22) and single mutant (n = 19 for *vln2*, and 17 for *vln3*) plants. (B) The angle of siliques with respect to the plant axis of the *vln2 vln3* double mutant is less regular than that of Col-0 and single mutant plants: siliques of *vln2 vln3* grow in all directions at similar frequencies, while those of Col-0 and single mutant plants preferentially grow upward at an oblique angle. (C, D) Leaf pavement (C; n = 26 for Col-0 plants, and 61 for *vln2 vln3*) and fruit stalk epidermal (D; n = 68 for Col-0, and 67 for *vln2 vln3*) cell dimensions of *vln2 vln3* are similar (Student's t tests, P > 0.05) to those of Col-0 plants, except for fruit stalk epidermal cell width, which is significantly higher (Student's t test, P = 0.01) in *vln2 vln3*. Circularity reflects the ratio of cell area to cell perimeter, and is defined as $4\pi Area/Perimeter^2$ (Vidali et al., 2007). Error bars in C and D represent standard deviations.

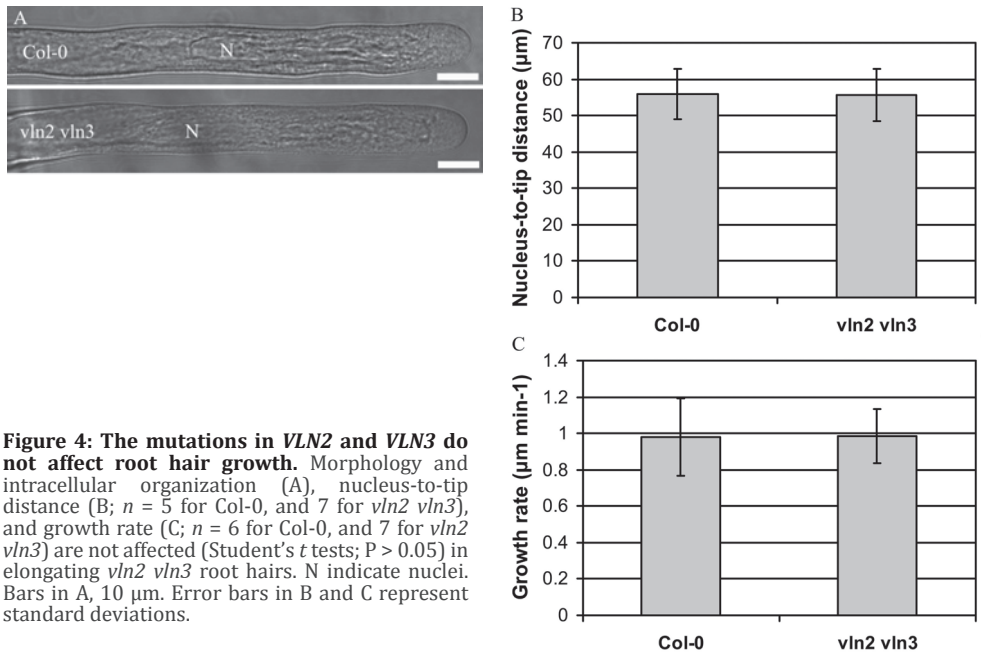

Figure 4: The mutations in *VLN2* and *VLN3* do not affect root hair growth. Morphology and intracellular organization (A), nucleus-to-tip distance (B; n = 5 for Col-0, and 7 for *vln2 vln3*), and growth rate (C; n = 6 for Col-0, and 7 for *vln2 vln3*) are not affected (Student's t tests; P > 0.05) in elongating *vln2 vln3* root hairs. N indicate nuclei. Bars in A, 10 µm. Error bars in B and C represent standard deviations.

Thick actin filament bundles are virtually absent in *vln2 vln3*, while thin bundles are more abundant

To investigate if the actin organization is affected by the presence of the T-DNA insertions in *VLN2* and *VLN3*, we used *GFP:FABD2* (Ketelaar et al., 2004) expression to visualize actin filaments in cells of the single mutants and the double mutant, and compared the actin organization with that of Col-0 cells. The actin organization in hypocotyl epidermal cells of the single mutants is similar to that of wild type cells: thick, predominantly longitudinal actin filament bundles are interspersed with a more complex network of thinner bundles (Figure 5A-I). In the double mutant, however, the thick, longitudinal actin filament bundles are absent, and thinner bundles are more abundant. Root and leaf epidermal cells of the double mutant also appear to contain more thin bundles of actin filaments, while thick actin filament bundles are absent (Figure 5J-L).

To quantify the observed differences in actin organization, we created intensity profiles of GFP fluorescence intensities of *P35S:GFP:FABD2* expressing hypocotyl cells (n = 8 for Col-0 and *vln2 vln3*), in the middle of Z-projections of the cortical cytoplasm, perpendicular to the longitudinal cell axis (excluding the bright cell edges; Figure 5M-O). In these intensity profiles, high peaks represent brightly labeled actin filament bundles, while low peaks represent weakly labeled actin filament bundles (or perhaps single actin filaments, although it is unlikely that single actin filaments are detectable with the used set-up). We counted the number of peaks per µm, and distributed these peaks in three classes: high, medium and low grey levels (Figure 5P, Q).

The frequency distribution of the number of peaks across the three classes was clearly different between Col-0 and *vln2 vln3* hypocotyl cells. 17 % of the peaks in Col-0 cells belonged to the class with the highest intensity levels, representing thick actin filament bundles, while in *vln2 vln3*, only 2 % of the peaks represented this class (Figure 5P). Peaks with a low fluorescence intensity were more abundant in *vln2 vln3* (70 %) then in Col-0 (47 %) cells. A Pearson's chi-square test showed that the frequency distribution across the three classes was significantly different between Col-0 and *vln2 vln3* cells ($p < 0.001$). The average number of peaks per µm was higher (t-test; $p = 0.04$) in the double mutant (0.82 ± 0.29) then in Col-0 cells (0.51 ± 0.26; Figure 5Q). We conclude that cells of the double mutant contain more thin bundles of actin filaments than those of Col-0 cells, while thick actin filament bundles are virtually absent.

GFP:VLN3 labels some (bundles of) actin filaments

To determine the subcellular localization of villin, we complemented the double mutant with *GFP:VLN3*, expressed under control of the endogenous promoter (*PVLN3:GFP:VLN3* genomic). Expression of this construct in the mutant rescued the phenotype, showing that the fusion protein is functional. GFP:VLN3 is present in all investigated cells: leaf, hypocotyl, and root epidermal cells, including root hairs (Figure 6). In all these cell types, GFP:VLN3 partly shows a cytoplasmic localization. Besides this cytoplasmic localization, GFP:VLN3 localizes to filamentous structures resembling (bundles of) actin filaments both in the cortical cytoplasm and in cytoplasmic strands of the cells studied (Figure 6A-D). Coexpression of *PVLN3:GFP:VLN3* and *P35S:mCherry:FABD2* in tobacco (*Nicotiana benthamiana*) leaves demonstrates that GFP:VLN3 and mCherry:FABD2 colocalize (Figure 6E-G), confirming that GFP:VLN3 localizes to actin filaments. Villin appears not to label all actin filament bundles equally strong (Figure

6E-G). In growing root hairs, GFP:VLN3 localizes to the long actin filament bundles oriented longitudinally to the cell's long axis in the root hair tube (Figure 6D). These actin filament bundles do not penetrate the (sub)apical region. Image sequences of root epidermal cells collected at 5 s intervals (Figure 6H-J), or of root hairs at 30 s intervals (Figure 6K-M) show that the actin filament bundles to which VLN3 localizes, relocate over time.

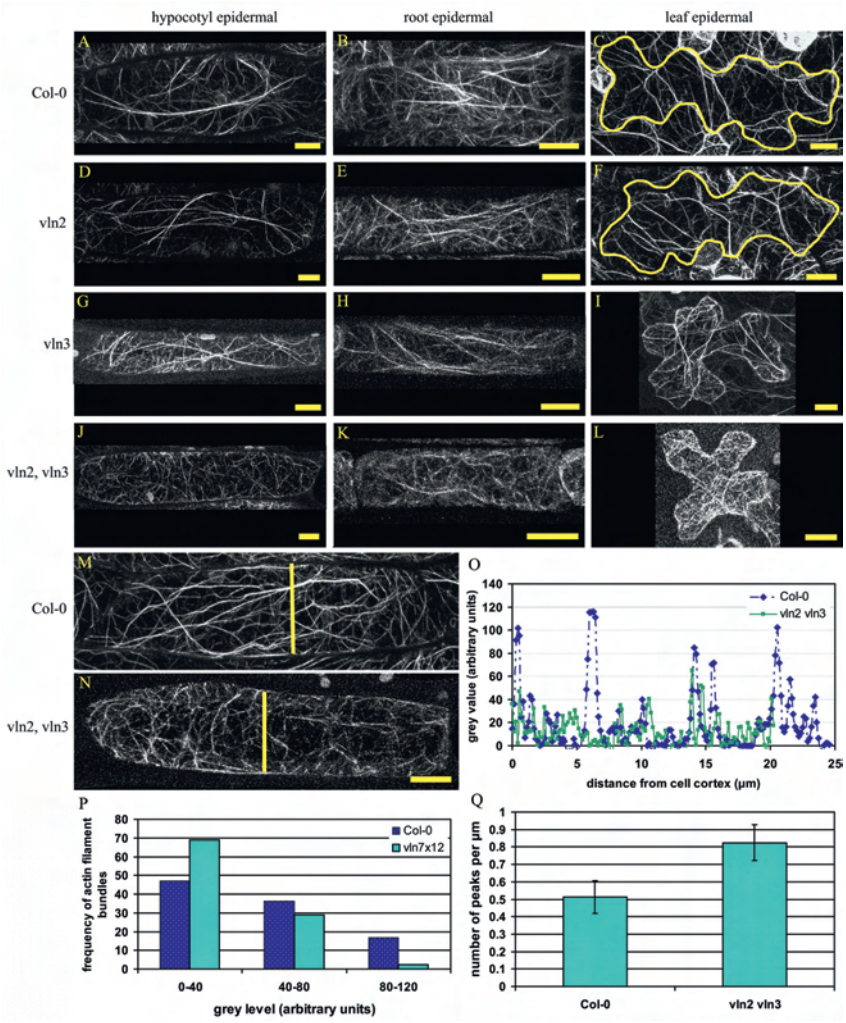

Figure 5: Thick actin bundles are absent in *vln2 vln3*, but thin bundles of actin filaments are more prominent. (A-L) The actin organization (visualized with GFP:FABD2) in cells of both single mutants (D-I) is similar to that in Col-0 cells (A-C): thick bundles of actin filaments are alternated by a more complex network of thin (bundles of) actin filaments. In the double mutant (J-L), thick actin filament bundles appear to be absent, while thin actin filament bundles seem more prominent. (M-O) Representative intensity profiles of fluorescence intensity in a Col-0 (M) and a *vln2 vln3* (N) hypocotyl cell. High peaks represent thick actin filament bundles, while lower peaks represent thinner bundles. The yellow lines in M and N show the location of the intensity profile in figure O. (P) Frequency distribution of peaks belonging to 3 fluorescence intensity classes (determined for 6 cells for each genotype) in Col-0 and *vln2 vln3*. In Col-0 cells, peaks with a fluorescence intensity of 80-120 (representing thick actin filament bundles) are more abundant than in *vln2 vln3*, while peaks with a fluorescence intensity of 0-40 (representing thin(ner) actin filament bundles) are more abundant in *vln2 vln3*. (Q) The number of peaks per micrometer shown for Col-0 and *vln2 vln3*. *vln2 vln3* cells contain significantly more (Student's *t* test; p = 0.04) actin filament bundles than Col-0 cells. Bars in A-N, 10 µm. Error bars in Q represent standard errors.

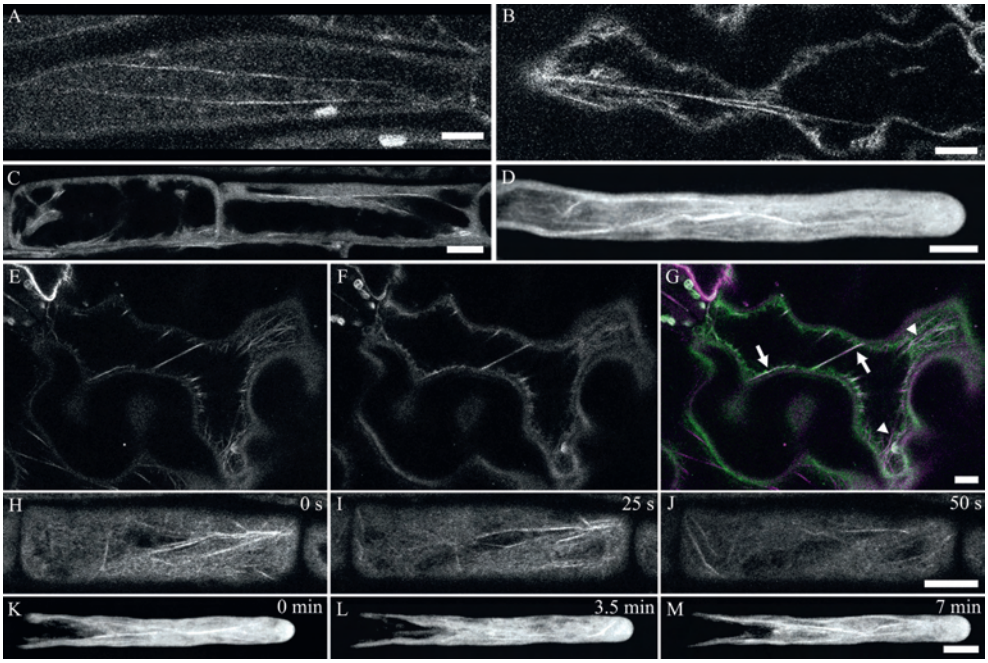

Figure 6: GFP:VLN3, expressed under control of the *VLN3* promoter, localizes to (bundles of) actin filaments. Representative images of complemented *vln2 vln3* plants show that besides a cytoplasmic localization, GFP:VLN3 decorates (bundles of) actin filaments in hypocotyl epidermal (A), leaf epidermal (B) and root epidermal (C, D) cells. In root hairs (D), GFP:VLN3 localizes to the long actin filament bundles oriented longitudinally to the cell's long axis in the root hair tube. (E-G) Coexpression of *PVLN3:GFP:VLN3* and *P35S:mCherry:FABD2* in tobacco (*Nicotiana benthamiana*) demonstrates that GFP:VLN3 (F) and mCherry:FABD2 (E) colocalize (e.g., arrows) in leaf epidermal cells, although GFP:VLN3 does not localize to all actin filaments (e.g., arrowheads). (G) Overlay of E and F (GFP:VLN3: green; mCerry:FABD2: magenta). Image sequences of root epidermal cells (H-J; K-M: root hairs) of complemented *vln2 vln3* plants show that GFP:VLN3 localizes to (bundles of) actin filaments that reorganize over time. Bars, 10 µm.

The headpiece region of VLN3 is required for bundling of actin filaments

Next to its actin filament bundling capacity, which is independent of Ca^{2+} levels (Khurana et al., 2010), VLN3 has actin filament severing properties, and this activity is Ca^{2+}-dependent (Khurana et al., 2010). Since the mutations in *VLN2* and *VLN3* result in an actin cytoskeleton organization that is virtually devoid of thick actin filament bundles, we propose that the absence of villin's bundling activity plays a major role in causing the morphological phenotype. The fact that the mutations affect the actin cytoskeleton organization also at locations where Ca^{2+} is at the basal level, while VLN3 shows only severing activity at high Ca^{2+} concentrations (Khurana et al., 2010), is in agreement with the hypothesis that villin's bundling rather than its severing activity causes the developmental problem in the double mutant. It is likely that plant villins require the headpiece region for actin filament bundling. Both the core and the headpiece region of vertebrate villins can bind to filamentous actin, and the headpiece region of vertebrate villin is crucial for its bundling capacity (Glenney and Weber, 1981). We therefore hypothesized that in plant cells, villin's headpiece region plays an important role in the generation of actin filament bundles. To obtain more insight into the function of the headpiece region of *Arabidopsis* VLN3, we bacterially expressed and purified both full-length VLN3, as well as a truncated version lacking the headpiece region (VLN3-HP) to perform biochemical experiments.

A high-speed cosedimentation assay was performed to determine whether VLN3 and VLN3-HP can bind to actin filaments. Filamentous actin was combined with either VLN3 or VLN3-HP, and centrifuged at 135.000 g. At this speed, 91 ± 8 % (n = 3) of the actin was pelleted (Figure 7), showing that most actin had polymerized. In the absence of filamentous actin, both VLN3 (93 ± 6 %; n = 3) and VLN3-HP (100 ± 0 %; n = 2) largely remained in the supernatant fraction (Figure 7). Although a limited amount (7 ± 10 % (n = 2)) of VLN3-HP was present in the pellet when cosedimented with filamentous actin, this amount was not significantly different (Student's t test; P = 0.42) from the amount of VLN3-HP that was pelleted in the absence of filamentous actin. On the other hand, 76 ± 14 % (n = 3) of VLN3 cosedimented with filamentous actin, a significantly higher amount (Student's t test; P = 0.002) then the amount of VLN3 that was pelleted in the absence of filamentous actin. These data show that VLN3 can bind to filamentous actin.

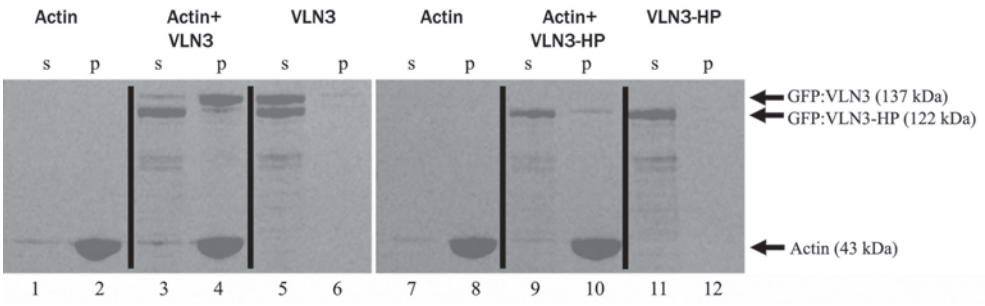

Figure 7: VLN3 binds to filamentous actin. Full-length VLN3 (lanes 3 and 4) and VLN3-HP (lanes 9 and 10) were combined with filamentous actin, and, after a 20 min incubation, centrifuged at 135.000 g. Actin alone (lanes 1, 2, 7 and 8), VLN3 alone (lanes 5 and 6), and VLN3-HP alone (lanes 11 and 12) were used as controls. The proteins present in the resulting supernatants and pellets were separated by SDS-PAGE. In the absence of actin, both VLN3 (n = 3) and VLN3-HP (n = 2) largely remained in the supernatant fraction. In the presence of polymerized actin, a limited amount of VLN3-HP was present in the pellet, but this amount was not significantly different (Student's t test; P = 0.42) from the amount of VLN3-HP that was pelleted in the absence of filamentous actin. A significantly higher (Student's t test; P = 0.001) amount of VLN3 cosedimented with filamentous actin then the amount of VLN3 that was pelleted in the absence of actin filaments, showing that VLN3 can bind to actin filaments.

To investigate the ability of full-length VLN3 and VLN3-HP to bundle actin filaments, we performed a low-speed co-sedimentation assay. Polymerized actin was combined with VLN3 or VLN3-HP, and centrifuged at 13.500 g. At this speed, the presence of an actin binding protein that is capable of bundling or cross-linking filaments into networks will result in a higher amount of filamentous actin that is present in the pellet. When incubated without VLN3 or VLN3-HP, 61 ± 5 % (n = 2) of the polymerized actin was present in the pellet (Figure 8A). When combined with VLN3-HP, the amount of polymerized actin that was present in the pellet (50 ± 3 %; n = 2) was similar (Student's t test; P = 0.12) to that of the control (Figure 8A). On the other hand, when polymerized actin was combined with VLN3, a significantly higher amount (93 ± 5 %; n = 2; Student's t test; P = 0.02) of the actin was present in the pellet (Figure 8A), indicating that VLN3 bundles actin filaments.

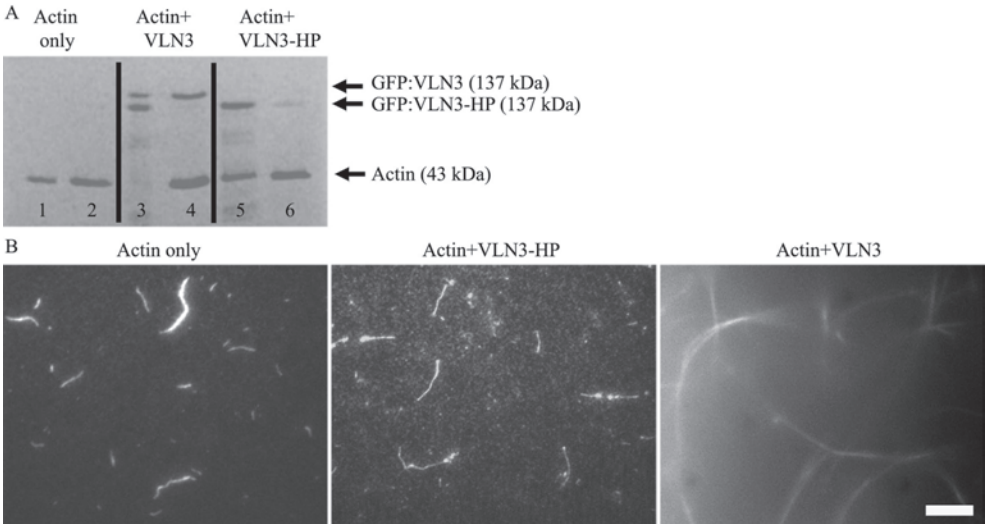

Figure 8: VLN3, but not VLN3-HP, is able to cross-link actin filaments. (A) Low-speed co-sedimentation assay. Actin was allowed to polymerize for 1 h, and then combined with either VLN3 or VLN3-HP. After a 20-minute incubation, the samples were centrifuged at 13.500 g. The proteins present in the resulting supernatants and pellets were separated by SDS-PAGE. In the presence of VLN3, significantly more actin (*n* = 2; Student's *t* test; P = 0.02) was present in the pellet (lane 4) then the amount that pelleted in the absence of VLN3 (*n* = 2; lane 2), while in the presence of VLN3-HP (lane 6), the concentration of actin in the pellet (*n* = 2) was similar (Student's *t* test; P = 0.12) to that of actin alone. (B) *In vitro*, rhodamine-actin or rhodamine-actin combined with VLN3-HP resulted in single actin filaments, while in the presence of VLN3, rhodamine-actin organized into a cross-linked network. Bar, 10 µm.

We used TIRF and wide-field microscopy to visualize the actin filament bundling capacity of VLN3. Rhodamine-labeled actin was allowed to polymerize, after which we added VLN3 or VLN3-HP. When combined with VLN3-HP, the length and number of actin filaments were similar to those in the control situation (Figure 8B). In the presence of full-length VLN3, however, cross-linked networks of actin filaments were observed (Figure 8B). The combined results from the cosedimentation assays and microscopy analysis show that VLN3 is able to cross-link actin filaments, and that the headpiece region of VLN3 is required for this cross-linking capacity.

To prove the importance of villin's bundling capacity, which depends on the presence of the headpiece domain, for directional organ growth, we performed a complementation analysis with 3 different constructs lacking the DNA that encodes the villin headpiece region, all driven by the endogenous *VLN3* promoter: *PVLN3:VLN3-HP* genomic, *PVLN3:GFP:VLN3-HP* genomic, and *PVLN3:GFP:VLN3-HP* cDNA. All these constructs were unable to rescue the phenotype. In addition, in contrast to GFP:VLN3, which localizes to (bundles of) actin filaments, GFP:VLN3-HP fluorescence is equally distributed throughout the cytoplasm (Figure 9). We conclude that VLN3 requires the headpiece region for a correct localization to actin filament bundles, for actin filament bundling, and for its function in directional organ growth.

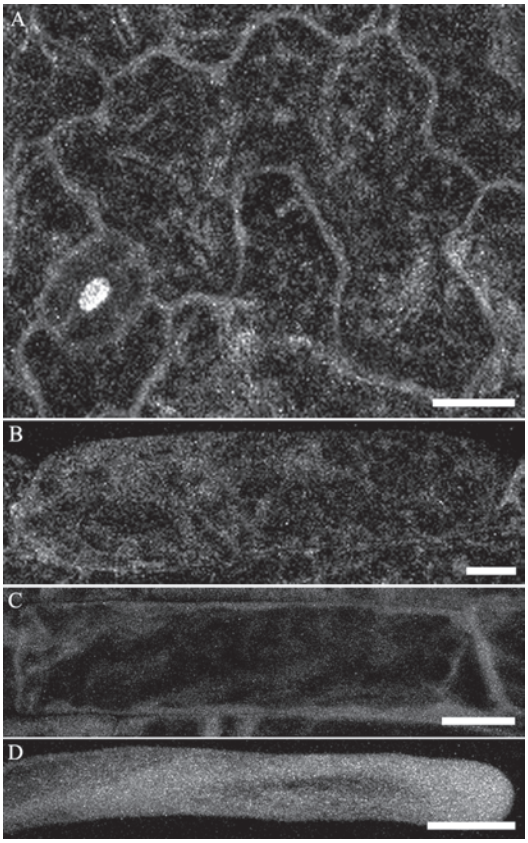

Figure 9: GFP:VLN3-HP, expressed under control of the *VLN3* promoter, shows a cytoplasmic localization. Representative images of leaf epidermal (A), hypocotyl epidermal (B), and root epidermal (C, D) cells (D: root hair) of *vln2 vln3* plants in which *GFP:VLN3-HP* is expressed. In these plants, which are not rescued, GFP:VLN3-HP fluorescence is equally distributed throughout the cytoplasm. Bars, 10 µm.

Discussion

The actin cytoskeleton plays a key role in plant cell growth and morphogenesis. Although in virtually all plant cells actin filament bundling occurs (Thomas et al., 2009), it is unknown how actin filament bundles are generated by actin bundling proteins. In this study, we investigated the role of two villins in *Arabidopsis*, and show that the absence of these villins results in a low abundance of thick actin filament bundles. *Vln2 vln3* plants have twisted leaves, stems, siliques and roots, implying an important role for villin in the regulation of directional organ growth. Truncated VLN3 lacking the headpiece region is, in contrast to full-length VLN3, not able to rescue the phenotype, and *in vitro* experiments show that the headpiece region is essential for actin filament bundling. These data show that villin is involved in the generation of thick actin filament bundles, and suggest that villin-mediated actin filament bundling is required for the regulation of coordinated cell expansion.

VLN2 and VLN3 play a role in actin filament organization in *Arabidopsis*

The presence of the T-DNA insertions in *VLN2* and *VLN3* affects the actin organization in several cell types. In cells of the double mutant, thick actin filament bundles are virtually absent, whereas thin bundles are more abundant. The fact that the double mutant still contains thin actin filament bundles points to the combined action of VLN2 and VLN3 with that of another actin bundling protein in the generation of actin filament bundles in plant cells. Since *VLN5* is preferentially expressed in pollen (Zhang et al., 2010), VLN1 and VLN4 are good candidates to work cooperatively with VLN2 and VLN3. Alternatively, another class of actin bundling proteins could be involved in the generation of actin filament bundles in plant cells. In vertebrate cells, also different actin bundling proteins are generally present in the same actin filament bundles (Tilney et al., 1998; Bartles, 2000) and these proteins do not act redundantly. *In vitro* experiments showed that small rigid actin-bundling proteins can generate small bundles with a finite thickness of approximately 20 filaments (Claessens et al., 2008). Other actin bundling proteins were shown to be able to link these small bundles into larger bundles of several hundreds of actin filaments (Claessens et al., 2008). In plants, actin filament bundles could be generated in a comparable way. Villins might work coordinately with fimbrins (Kovar et al., 2000), formins (Cheung and Wu, 2004; Favery et al., 2004; Michelot et al., 2005; Ye et al., 2009), LIM proteins (Thomas et al., 2006; Thomas et al., 2007), and/or elongation factor 1 alpha (Collings et al., 1994) in the formation of thick actin filament bundles. Consistent with this idea, fimbrin has been proposed to cross-link actin filament bundles generated by other actin bundling proteins, such as villin (Matova et al., 1999; Wu et al., 2010).

Besides villin's role in actin filament bundling, it is likely to play additional roles in actin organization. In addition to its bundling capacity, which is independent on Ca^{2+} levels, VLN3 has been shown to have actin filament severing properties, and this activity is Ca^{2+}-dependent (Khurana et al., 2010). VLN4, which is expressed in root hairs (Zhang et al., 2011), and VLN5, which is highly expressed in pollen tubes (Zhang et al., 2010), have similar properties: they both bundle actin filaments in a Ca^{2+}-independent manner, but have actin filament severing capacity only at high (micromolar and millimolar) Ca^{2+} concentrations (Zhang et al., 2010). In addition, these villins have actin filament capping activity. The lily villin P-135-ABP has been shown to have actin filament nucleating, depolymerizing, and capping activity, and these activities were Ca^{2+}/calmodulin-dependent. Although the authors state that the nucleating capacity is probably not relevant *in vivo* (since the nucleation was not accelerated when G-actin was saturated with profilin, which is the case in plant cells), the depolymerizing and capping activity might enhance actin dynamics in the apical region of tip-growing cells, where Ca^{2+} is abundant (Yokota et al., 2005). Zhang et al. (2011) predicted that VLN4, which is involved in the generation and/or maintenance of actin filament bundles in the shank of root hairs (Zhang et al., 2011), participates in the regulation of actin cytoskeleton organization in the subapical and apical region of root hairs by its bundling, capping and/or severing activity. Likewise, VLN5 has been proposed to bundle actin filaments in the shank of pollen tubes, while enhancing actin dynamics in the apical region, by severing and capping of actin filaments (Zhang et al., 2010). VLN3 (and perhaps also VLN2) could, besides being involved in the generation of actin filament bundles, locally also play a role in enhancing actin dynamics.

Although the localization of GFP:VLN3 to (bundles of) actin filaments in the shank of root hairs shows that *VLN3* is expressed in root hairs, root hair growth and morphology, which are very sensitive to changes in actin filament organization, are not affected by the mutations in *VLN2* and *VLN3*. This might mean that the proteins act redundantly with another villin in root hairs. VLN5 is preferentially expressed in pollen and pollen tubes (Zhang et al., 2010), and therefore not likely to act redundantly with VLN2 and VLN3 in root hairs. VLN1, which is Ca^{2+}- independent, has only actin filament bundling capacity (Huang et al., 2005), and VLN3 can sever actin filament bundles in the presence of VLN1 (Khurana et al., 2010), showing that the activities of VLN1 and VLN3 are not completely redundant. If VLN2 and VLN3 act redundantly with another villin in root hairs, VLN4, which is involved in actin filament bundling in root hairs (Zhang et al., 2011), would therefore be the best candidate. Alternatively, the fact that root hair growth and morphology are not affected by the mutations in *VLN2* and *VLN3* could mean that these villins are not essential for root hair growth and morphology. In intercalary growing cells, VLN2 and VLN3 are essential for the organization of actin filaments. Thick actin filament bundles are virtually absent in cells of the *vln2 vln3* double mutant, and our data show that villin requires the headpiece region for its bundling capacity *in vitro*, and for localizing to (bundles of) actin filaments *in vivo*. This implies that although villin may play additional roles in actin organization, for instance by actin filament severing, villin's bundling capacity plays a major role in its function in actin filament organization.

Actin filament organization is required for plant growth polarity

The actin cytoskeleton plays a key role in plant cell growth. It plays a fundamental role in the delivery of growth materials to exocytosis sites (Miller et al., 1997; Geitmann and Emons, 2000; Vidali and Hepler, 2001), not only because (bundles of) actin filaments serve as tracks for cytoplasmic streaming, but also because they optimize the cytoplasmic organization for cell growth. In addition, fine F-actin is thought to be important for the filtering and delivery of Golgi-derived vesicles (Miller et al., 1999) that contain cell wall matrix materials in their lumen and the enzymes for callose and cellulose production in their membrane. Our data show that the activities of VLN2 and VLN3 are required for the organization of the actin cytoskeleton. In the absence of VLN2 and VLN3 proteins, thick actin filament bundles are virtually absent, while fine bundles are more abundant. Cell shapes and sizes, and plant growth rates are similar in Col-0 and double mutant plants. This shows that the thick actin filament bundles that are absent in the double mutant are not essential for cell and plant growth. However, the wavy, twisted, appearance of several organs in the double mutant, and the larger amplitudes of the rotational movements (circumnutation) of double mutant inflorescences, point to a role for VLN2 and VLN3 in coordinated cell elongation. Although we were, due to the large variation in cell sizes, not able to determine differences in cell sizes between Col-0 and double mutant plants (except for a small difference in fruit stalk epidermal cell width), it is likely that the organ twisting in *vln2 vln3* results from subtle changes in cell sizes in opposite locations of the organs. It is not clear how villin-mediated actin filament bundling regulates coordinated cell expansion. We show that it does so by altering the organization of the actin cytoskeleton. The altered actin cytoskeleton organization in the double mutant might have effects on the direction of transport routes, and/or the proper allocation of Golgi vesicles in the vicinity of the plasma

membrane. In conclusion, our results show that villin is involved in the generation of thick actin filament bundles and suggest that these bundles are, directly or indirectly, important for coordinated cell expansion.

Material and methods

Growth conditions, plant strains, allele characterization and creation of double mutants

Seeds were sterilized for 1 minute with 70%-ethanol, followed by a 3-5-minute treatment with 15-20% household bleach (4% hypochlorite) and 0.05% triton X-100. After sterilization, the seeds were stratified at 4°C for 2-4 days, and germinated on 0.5 MS plates containing 0.7% agarose. After 1 week, seedlings were transplanted to potting compost. For live cell visualization of root epidermal cells, hypocotyl epidermal cells and leaf pavement cells, seeds were germinated on 0.5 MS plates containing 1.5% agarose, which were placed at an oblique angle (approximately 15-30° off vertical). For root hair imaging, seeds were sown on tilted coverslips containing a thin 0.7% agarose layer of Hoaglands' medium, covered with biofoil (Vivascience, Göttingen, Germany). Root hairs grew along the coverslip, and were imaged 3-4 days after planting. Colocalization of GFP:VLN3 and mCherry:FABD2 was performed by *Agrobacterium tumefaciens*-injection in tobacco (*Nicotiana benthamiana*) leaves as described by Bouwmeester et al. (Bouwmeester et al., 2011). All plants were grown at 25°C (16 h light, 8 h darkness).

The T-DNA insertion lines (both in a Col-0 background) for VLN2 (SAIL_813_H02) and VLN3 (SALK_117097) were obtained from the Nottingham Arabidopsis Stock Centre (NASC; (Scholl et al., 2000)). 4-6-week old leaves were used to isolate genomic DNA, which was used to confirm the T-DNA insertions (Supplemental Figure 1) by PCR using T-DNA left-border-specific primer LB3 (for SAIL_813_H02) or LBA1 (for SALK_117097) and *VLN* specific primers (Supplemental Figure 2 and Table 1) flanking the insertions. Homozygous mutants were identified in F3 progeny.

To analyze the expression of VLN2 and VLN3, RNA was extracted from leaves of the homozygous T-DNA insertion mutants using a QUIAGEN RNeasy Mini Kit. Total RNA was reverse transcribed into cDNA with Superscript II Reverse Transcriptase (Invitrogen), and eluted in 20 µL DEPC-treated H_2O. A volume of 1 µL of the total cDNA was used in RT-PCR reactions using primer combinations designed for coding regions before and after the T-DNA inserts (Supplemental Figure 2 and Table 1).

Complementation analyses

Primers that included GATEWAY sequences (Invitrogen) were used to amplify genomic VLN3 including the promoter region (2299 bp upstream of the ATG) and terminator region (1228 bp including the stop codon) as annotated by The Arabidopsis Information Resource (TAIR; www.arabidopsis.org), which was recombined into pDONR207 (Invitrogen), followed by recombination into pMDC99 (Curtis and Grossniklaus, 2003). Genomic VLN3 including the promoter but lacking the headpiece encoding region and terminator region (which lacks the last 606 bp of coding region of VLN3 including

introns) was recombined into pDONR207, followed by a recombination into pMDC32 (Curtis and Grossniklaus, 2003), from which we deleted the 2 x 35S promoter. The same adapted version of pMDC32 was used for recombination of VLN2 including the promoter (3902 bp upstream of the ATG) but lacking the stop codon and terminator region.

To express GFP:VLN3 and GFP:VLN3-HP in the *vln2 vln3* double mutant, genomic VLN3 lacking the promoter and terminator region, as well as genomic VLN3 lacking the promoter, headpiece encoding region, and terminator region were amplified by PCR and recombined into pDONR207, followed by a recombination into pMDC43 (Curtis and Grossniklaus, 2003) of which the 2 x 35S was replaced by the endogenous VLN3 promoter. The same adapted version of pMDC43 was used for recombination (using pDONR221 (Invitrogen) as entry clone) of coding sequences of VLN3 and VLN3-HP, which were amplified from cDNA. All constructs were transformed into the *vln2 vln3* double mutant by *Agrobacterium tumefaciens* mediated transformation using the floral dip method (Clough and Bent, 1998). Primer sequences are shown in Supplemental Table 2.

Phenotype analysis and confocal microscopy

To visualize the actin cytoskeleton in the single mutants, we crossed the *vln2* and *vln3* single mutants with wild-type Col-0 plants expressing *P35S:GFP:FABD2* (Ketelaar et al., 2004). In the F2-generation, homozygous lines were identified by genotyping and selected for *GFP:FABD2* expression. A double mutant line with *GFP:FABD2* expression was obtained by *Agrobacterium tumefaciens* mediated transformation of *P35S:GFP:FABD2* into the *vln2 vln3* double mutant using the floral dip method (Clough and Bent, 1998).

For live cell imaging of GFP:FABD2 and GFP:VLN3 localization, 3-5 day old plants were used. Root hairs were imaged with a I-LAS Spinning Disk Confocal System (Roper Scientific SAS, France) on a Nikon Eclipse Ti microscope using a 60x (N.A. 1.4) oil immersion objective. Root epidermal cells, hypocotyl epidermal cells, leaf pavement cells, and GFP:VLN3 and mCherry:FABD2 colocalization were imaged with an Axiovert 200M microscope (Zeiss, Jena, Germany) connected to a Zeiss LSM510 META confocal scanning system equipped with a 63x (N.A. 1.4) oil immersion objective. Cell dimensions of leaf and fruit stalk epidermal cells were imaged with a Nikon Eclipse 80i microscope, using a 10x (N.A. 0.3) objective, and traced in image J. Circularity reflects the ratio of cell area to cell perimeter, and is defined as $4\pi Area/Perimeter^2$ (Vidali et al., 2007).

Thickness of actin filament bundles was quantified by creating intensity profiles of GFP fluorescence intensities of *P35S:GFP:FABD* expressing hypocotyl cells, in the middle of Z-projections of the cortical cytoplasm, perpendicular to the longitudinal cell axis (excluding the bright cell edges). To correct for differences in GFP:FABD2 intensity, we selected an area in which no actin filaments were visible, and subtracted the mean fluorescence intensity of this region from the fluorescence intensities of the intensity profile. This resulted in a new plot profile, which was used to distribute the peaks in three classes: low (0-40 arbitrary units), medium (40-80 arbitrary units) and high (8-120 arbitrary units) grey levels (8-bit files were used). Only peaks that were at least 10 units higher in fluorescence intensity then the intensities of the left and right basis of the peaks were included.

Protein expression and purification

We adapted the bacterial expression vector pET28a (Novagen) by ligation of GATEWAY recombination sites (amplified from pMDC43) using the NdeI and Xho1 restriction sites. This resulted in a kanamycin resistant vector which adds an N-terminal 6xHIS:GFP to the recombinant protein. Coding sequences of VLN3 and VLN3-HP were amplified from cDNA using primers that also included GATEWAY sequences and then recombined into pDONR221, followed by recombination into the adapted version of pET28. For expression in *Escherichia coli*, the strain BL21-DE3 (Promega) was used. 6 mL of an overnight culture of bacteria were poured into 200 mL lysogeny broth (LB) media and cultured at 37°C for 4 h, after which they were transferred to 15 °C and induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight. HIS-tagged proteins were immobilized on Ni-NTA agarose (Qiagen) and eluted with 20 mM NaH_2PO_4, 250 mM NaCl, and 500 mM imidazole.

Cosedimentation assays and Total Internal Reflection Fluorescence (TIRF) microscopy

0.1 volume of 10x KME buffer (500 mM KCl, 10 mM $MgSO_4$, 10mM EGTA, 100 mM imidazole pH 6.5) was added to skeletal muscle actin or rhodamine muscle actin (derived from rabbit, Cytoskeleton; resuspended to 0.5 mg ml^{-1} in buffer G (2mM TRIS pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.02% NaN_3, 0.5 mM DTT)) to induce polymerization of actin filaments at room temperature for 1 h. Purified proteins (pre-clarified at 135.000 g for 30 min) were combined with filamentous actin (actin:protein ratio was approximately 10:1 for binding assays and *in vitro* visualization, and 5:1 for bundling assays) and incubated for 20 min at room temperature, after which the samples were centrifuged at room temperature for 1 h at 135.000 g (Beckman Airfuge) for actin filament binding assays, and at 13.500 g (Eppendorf 5415C Centrifuge) for actin filament bundling assays. For *in vitro* visualization, samples were diluted to a final actin concentration of 0.5 μg ml^{-1} before observation with a TIRF setup based on a Nikon Eclipse Ti inverted microscope. Both TIRF and epi illumination were provided by a 561nm laser (Cobolt AB). Samples were excited through a Nikon 100x TIRF objective (NA=1.49). Emission light was collected with the same objective, filtered using a custom made dichroic mirror and emission filter (transmission bands: 480nm - 550nm, 570nm - 625nm and 650nm - 800nm, Chroma) and imaged on a QuantEM EMCCD camera (Photometrics).

Accession numbers

Sequence data from this article can be found in GenBank/EMBL data libraries or The Arabidopsis Information Resource (TAIR) under accession numbers At2g41740 or NP_565958.1 (*Arabidopsis VLN2*) and At3g57410 or NP_567048.1 (*Arabidopsis VLN3*).

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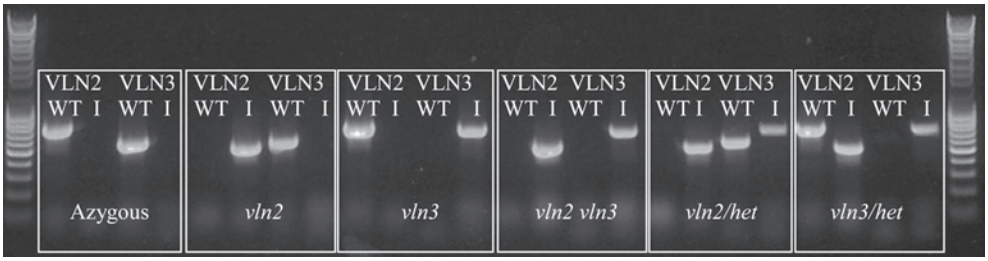
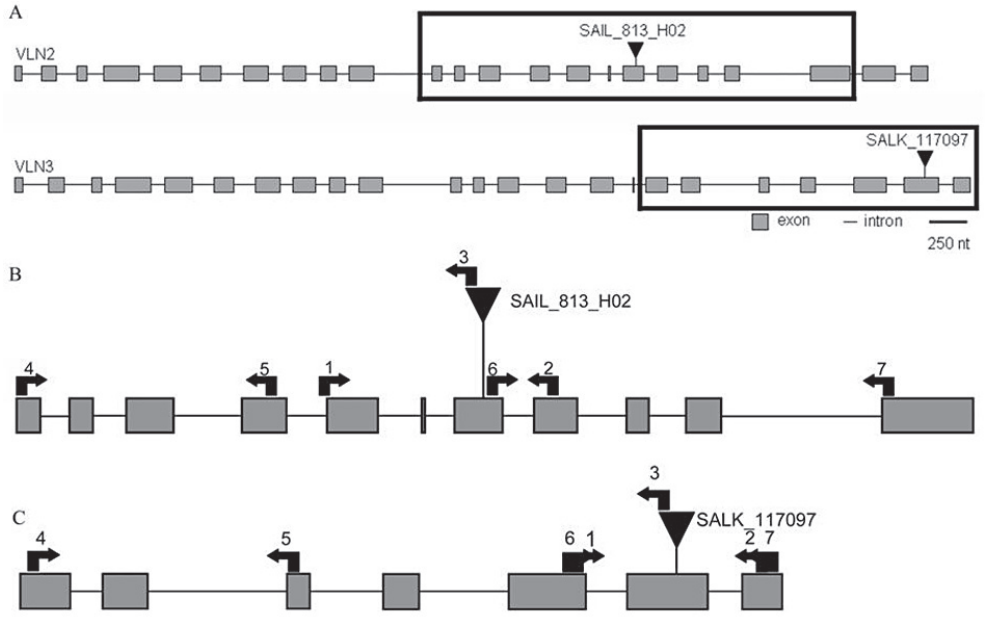
Supplemental data



Supplemental Figure 1: Molecular characterization of villin T-DNA insertion alleles. For all lines, a combination of wild type primers was used for both VLN2 and VLN3, resulting in a band only for plants carrying the wild type (WT) allele, and a combination of insert primers was used for both VLN2 and VLN3, resulting in a band only for plants carrying the insert (I) allele. Azygous plants show wild-type alleles for both VLN genes, the single mutants show one wild-type allele and one insert allele, and the double mutants show insert alleles for both VLN genes.



Supplemental Figure 2: Locations of primers used for molecular characterization of villin T-DNA insertion alleles. (A) Overview of the *VLN2* and *VLN3* gene structure. The boxed areas in A are shown in detail in (B) and (C). Grey boxes represent exons, horizontal lines represent introns. Primers (arrows) and T-DNA inserts (arrowheads) are not drawn to scale. Primer numbers correspond to the primer sequences in Supplemental Table 1.

Primer number	VLN2: SAIL_813_H02	VLN3: SALK_117097
1	CAGCCCGGGATTACTCTCAAGCATG	GAGAAGAAGAAATCACCTGATACCAGCC
2	GGGAACGAACCATGCTTCTTCACC	GAATCATTCTACAAATTGCCTGGATGG
3	TAGCATCTGAATTTCATAACCAATCTCGATACAC (=LB3)	TGGTTCACGTAGTGGGCCATCG (=LBA1)
4	GGACCAAGATACAGCAATTCGACTGGC	GAGATTCACAACTTTGATCAAGATGACC
5	CGCATGAACAGCTAGAACTGGCCAC	GCAAGGGAATTCCTACCAGAAGAAGGC
6	GTGTGGAGCCTAAGGAAAAGCAAACTG	CTGCTGAGAAGAAGAAATCACCTGATACC
7	GATTAAACGAAAGTCACGACGGCCC	GCCTGGATGGAAACAAGACTTACTG

Supplemental Table 1: Sequences of primers used for molecular characterization of villin T-DNA insertion alleles. For genotyping, presence of wild type alleles was tested using primer 1 and 2, and presence of insert alleles was tested using primers 1 and 3. Primer combinations 4 and 5, and 6 and 7 were used to test whether transcripts are present, truncated, or absent in mutant plants. Primer numbers correspond to the numbers in Supplemental Figure 2.

PCR product	Fw primer (5'- 3')	Rv primer (5'- 3')
VLN3 genomic including promoter and terminator region	GGGG**ACAAGTTTG-TACAAAAAAGCAGGCT**AAACC-*CGAACCGGCAACATATATTCAAAG-TATATGG*	GGGG**ACCACTTTGTACAAG-AAAGCTGGGT***AGACCTGTCTCGCT-CAAAGCAACGTC*
VLN2 genomic including promoter but lacking the stop codon and terminator region	GGGG**ACAAGTTTG-TACAAAAAAGCAGGCT**AAACC-*CGGTGTGGACATTGCACTCTCTT-TATTC*	GGGG**ACCACTTTGTACAAG-AAAGCTGGGT***AGAACAAGTC-GAACTTCTTCTTAAGCAGATC*
VLN3 genomic (without promoter and terminator region) and VLN3 coding region (for bacterial expression)	GGGG**ACAAGTTTG-TACAAAAAAGCAGGCT**CCAC-*CATGTCTGGGTCAACAAAAGTAT-TGGATCC*	GGGG**ACCACTTTGTACAAG-AAAGCTGGGT**A*GAATAAGTT-GAATTTCTTCTTCAGTAAGTCTTGTT-TCC*
VLN3 genomic lacking the promoter, headpiece encoding and terminator region, and VLN3 coding region lacking the promoter, headpiece encoding and terminator region (for bacterial expression)	GGGG**ACAAGTTTG-TACAAAAAAGCAGGCT**CCAC-*CATGTCTGGGTCAACAAAAGTAT-TGGATCC*	**GGGGACCACTTTGTACAA-GAAAGCTGGGT**A*TGCTGCT-GCTCTTTGTGACGCCTGGC*
VLN3 promoter	CCCCCTGCAGG*CGAACCGGCAA-CATATATTCAAAGTATATGG*	CCCGGTACC*TTTGTATTAGTGGC-TAATCTCTTCCTTCAAGAG*

Supplemental Table 2: Sequences of primers used for complementation experiments and bacterial protein expression. Gateway sequences are shown in bold, restriction sites are underlined, and villin DNA is shown in italic. For bacterial expression of VLN3 and VLN3-HP coding regions, a stop codon (TAA) was added in the reverse primer.

Chapter 5

General discussion

Molecular and physical aspects of the actin cytoskeleton that are involved in the generation of an organized cytoplasm

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Summary

In interphase plant cells, the actin cytoskeleton is essential for intracellular transport and cytoplasmic organization. To fully understand how the actin cytoskeleton functions as the structural basis for cytoplasmic organization, both molecular and physical aspects of the actin cytoskeleton have to be considered. First, we discuss literature that gives insight in how cytoplasmic organization is achieved and literature in which actin binding proteins have been identified that play a role in this process. In this part, the roles of the actin bundling proteins villin (chapter 4) and fimbrin (new results) receive special attention. Next, we discuss how physical properties of the actin cytoskeleton in the cytoplasm of live plant cells, such as deformability and elasticity, can be probed by using optical tweezers. This technique allows non-invasive manipulation of cytoplasmic organization. Optical tweezers, integrated in a confocal microscope, can be used to manipulate cytoplasmic organization while studying actin dynamics (chapter 2). By combining this with mutant studies and drug applications, insight can be obtained about how the physical properties of the actin cytoskeleton, and consequently the cytoplasmic organization, are influenced by different cellular processes.

Introduction

The actin cytoskeleton is of pivotal importance for many cellular processes, including, amongst others, cell shape formation, intracellular transport, cytoplasmic organization and signalling. It is as such a key coordinator of development of eukaryotes. Actin filaments are capable to rapidly form a plethora of structures, such as cross-linked gels and linear bundles. The organization of actin networks is dependent, besides the intrinsic properties of actin, on a large number of actin binding proteins (ABPs; (Hussey et al., 2006)). The combined actions of these proteins result in adaptation of the actin organization in response to intra- and extra-cellular cues. In this review we will focus on an actin configuration that is prominently present in plant cells: the thick actin filament bundles that play a role in the formation and maintenance of cytoplasmic strands in plant cells (Staiger et al., 1994; Valster et al., 1997; Tominaga et al., 2000). We will discuss how the formation and organization of these actin filament bundles is regulated (work reported in chapter 4), and show our initial data about the role of the actin bundling protein fimbrin on actin organization. Next, we discuss how physical manipulation of cytoplasmic organization with optical tweezers (work reported in chapter 2) can help to gain insight in cytoplasmic organization.

1. Cytoplasmic organization in highly vacuolated plant cells

During cell growth, plant cells become highly vacuolated. Mature plant cells contain one or several large vacuoles that can occupy over 90% of the cell volume (Kutsuna and Hasezawa, 2002; Ruthardt et al., 2005). In intercalary growing plant cells (i.e. cells in which expansion takes place over the whole longitudinal cell axis, resulting in cell elongation), cytoplasm is located around the nucleus (perinuclear cytoplasm) and in the periphery of the cell (cortical cytoplasm). These cytoplasmic areas are interspaced by the vacuole. Strands of cytoplasm bounded by the tonoplast (vacuolar membrane), called cytoplasmic or transvacuolar strands, cross the vacuole to connect the perinuclear and cortical cytoplasm (figure 1). They are formed after cytokinesis (Kutsuna and Hasezawa, 2005), during and after cell elongation (Sheahan et al., 2007), and during recovery from actin depolymerization (Fiserova et al., 2006). Cytoplasmic strands are highly dynamic: they constantly change in shape and location (Hoffmann and Nebenfuhr, 2004; Ruthardt et al., 2005).

Cytoplasmic strands are thought to function as transport routes for transcripts, proteins and organelles. This transport is visible as cytoplasmic streaming, the myosin-mediated movement of organelles over actin filaments and the hydrodynamic flow induced by this movement (Houtman et al., 2007; Esseling-Ozdoba et al., 2008). All interphase cytoplasmic strands contain actin filaments, and upon actin depolymerization, not only cytoplasmic streaming is inhibited, but also cytoplasmic strands disappear (Staiger et al., 1994; Shimmen et al., 1995; Valster et al., 1997; Hussey et al., 1998; Van Gestel et al., 2002; Higaki et al., 2006; Sheahan et al., 2007; Van der Honing et al., 2010 [chapter 2]). Thus, actin filaments not only serve as transport routes, but are also the backbone of cytoplasmic strands. In this chapter, we will focus on the structural function of actin filaments in forming, maintaining and reorganizing cytoplasmic strands.

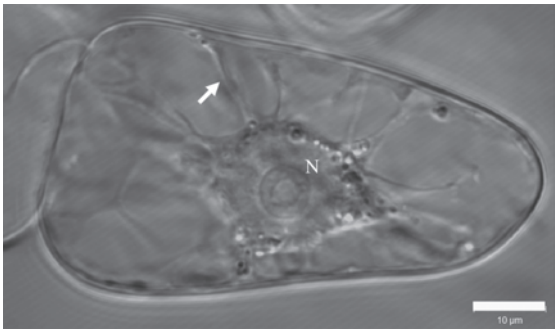

Figure 1. The cytoplasmic organization in a tobacco BY-2 suspension cultured cell. Around the nucleus, the perinuclear cytoplasm is present and in the periphery of the cell, the cortical cytoplasm is present. These two pools of cytoplasm are interconnected by cytoplasmic strands (e.g. arrow) that impinge the central vacuole. The organization of cytoplasmic strands is highly dynamic; the localisation of strands constantly changes and they fuse and branch. N indicates nucleus. Bar: 10 µm.

2. Biogenesis and maintenance of cytoplasmic strands

Two alternative hypotheses have been proposed for the de novo formation of cytoplasmic strands, which are outlined below.

I. Protrusions of cytoplasm extend into the vacuolar space and may eventually connect to the peripheral cytoplasm at the other side of the vacuole (Higaki et al., 2006; Van der Honing et al., 2007 [chapter 1]). In tobacco BY-2 suspension cultured cells, these protrusions originate from the perinuclear cytoplasm during and just after cytokinesis (figure 2). We propose that these protrusions could be formed by two alternative mechanisms:

- **a.** existing bundles of actin filaments that are positioned against the tonoplast are displaced towards it by myosin motor activity, indenting the tonoplast
- **b.** coordinated polymerization of actin filaments with their barbed ends towards the tonoplast pushes the tonoplast forward, similar to the protrusion of the plasma membrane of mammalian cells during formation of lamellipodia or filopodia (Van der Honing et al., 2007 [chapter 1])

II. A sheet of the cortical cytoplasm forms an invagination into the vacuolar space, where after the lateral connection between the sheet and the cortical cytoplasm disappears and the sheet becomes a strand, which is connected to cortical or perinuclear cytoplasm at two sides (Ruthardt et al., 2005; Szymanski and Cosgrove, 2009). Szymanski and Cosgrove (Szymanski and Cosgrove, 2009) present data that show that this type of strand formation indeed occurs in plant cells and propose that the underlying molecular mechanism is myosin activity in the periphery of the cell which pulls a peripheral bundle of actin filaments into the vacuolar space.

To decipher how new cytoplasmic strands are formed, the three dimensional organisation of the cytoplasm would need to be studied over time. Due to the continuous, rapid reorganization of the cytoplasmic organization that occurs within seconds (Ruthardt et al., 2005), high-speed time series, combined with quantitative data analysis would be required for conclusive answers.

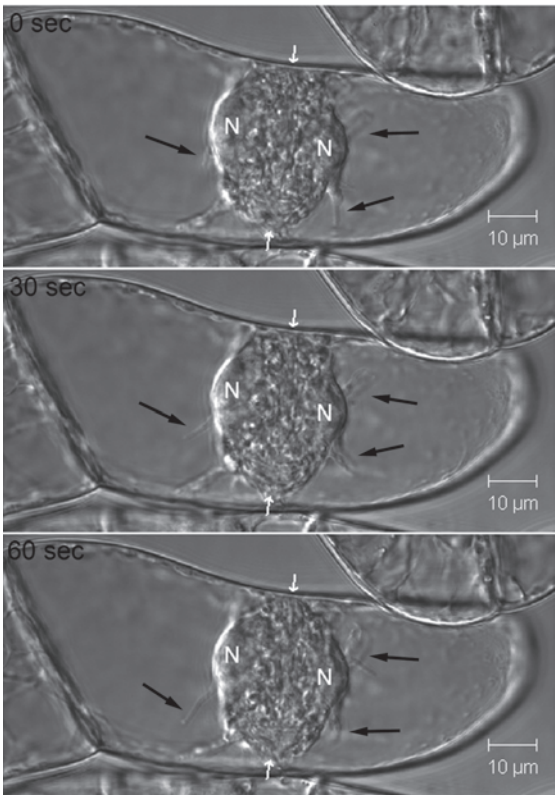

Figure 2. Highly dynamic cytoplasmic protrusions into the vacuolar space are formed during and just after cytokinesis. Three images with 30 seconds intervals showing the protrusions (black arrows). White arrows indicate the direction of the forming cell plate. N indicate nuclei. Bar: 10 µm.

Once cytoplasmic strands have been formed, they constantly reposition, split and fuse (Hoffmann and Nebenfuhr, 2004). When myosin motor activity is inhibited, the cytoplasmic organization is frozen, which indicates that myosin based displacement of the actin filament bundles that support the cytoplasmic strands is responsible for the reorganization of cytoplasmic strands (Hoffmann and Nebenfuhr, 2004; Van der Honing et al., 2010 [chapter 2]). The reaction of cells on the application of low concentrations of actin depolymerizing drugs suggests that actin polymerization does not play a role in the reorganization of existing cytoplasmic strands, other than providing the actin filament bundles that can be displaced by myosin activity (Hoffmann and Nebenfuhr, 2004; Van der Honing et al., 2010 [chapter 2]). Although the number and dynamicity of cytoplasmic strands is known to decrease when cells mature, it is not known how this happens.

3. Molecular players in strand formation, maintenance and reorganization

a. Actin bundling proteins

Actin filaments in cytoplasmic strands are organized as thick bundles. The actin bundling protein villin is involved in maintaining these bundles. Injection of an anti villin antibody in root hairs of *Hydrocharis* (Tominaga et al., 2000) and *Arabidopsis* (Ketelaar et al., 2002) causes cytoplasmic strands to fall apart into many thinner cytoplasmic strands, which eventually disappear. This indicates that villin-mediated actin filament bundling at least partially determines the number and the size of

cytoplasmic strands. We found that *Arabidopsis* VLN3 localizes to actin filaments in cytoplasmic strands and in the cortical cytoplasm of root, hypocotyl, and leaf epidermal cells (chapter 4), and in root hairs (figure 3; chapter 4). Phenotypic analysis of a *vln2 vln3* double mutant (resulting in a truncated transcript for both genes) showed that villin is involved in the regulation of directional organ growth, and that thick bundles of actin filaments are less abundant in the cells of the double mutant. Furthermore, the headpiece domain of VLN3 is required for its actin filament bundling capacity, as well as for its functionality in organ growth polarity (chapter 4). Thus, VLN2 and VLN3 are involved in the generation of actin filament bundles that are likely to be important for coordinated cell expansion. Loss of function of VLN4 resulted in an alteration of cytoplasmic streaming routes and velocities in root hairs (Zhang et al., 2011), implying an altered organization of the cytoplasm. Although the cytoplasmic organization in the *vln2 vln3* double mutant is not visibly affected at the light microscopy level, subtle changes in cytoplasmic organization and/or cytoplasmic streaming could be responsible for the defects in directional organ growth.

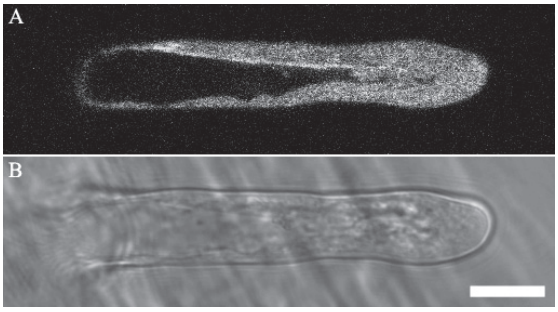

Figure 3. (A) GFP:VLN3 localizes to actin filaments in cytoplasmic strands of root hairs. (B) transmission image. Bar: 10 µm.

Next to its actin filament bundling capacity, which is independent of Ca^{2+} levels (Khurana et al., 2010), VLN3 has actin filament severing properties, and this activity is Ca^{2+}-dependent (Khurana et al., 2010). Although not all plant villins are Ca^{2+}-dependent (Huang et al., 2005), the Ca^{2+}-dependency of some plant villins (Yokota et al., 2005; Khurana et al., 2010; Zhang et al., 2010) indicates that the free cytoplasmic concentration of Ca^{2+} could be involved in controlling the locations and amount of actin filament bundling in plant cells. For example, in the cytoplasm of the apical region of tip-growing cells, which contains a high concentration of free cytoplasmic Ca^{2+}, actin filament bundles have not been detected (Kost et al., 1998; Miller et al., 1999), while in the shank of pollen tubes and root hairs, at lower Ca^{2+} concentrations, thick, more or less longitudinally oriented bundles of actin filaments are present (Kost et al., 1998; Miller et al., 1999). VLN4, which is expressed in root hairs (Zhang et al., 2011) and VLN5, which is highly expressed in pollen and pollen tubes (Khurana et al., 2010), both bundle actin filaments in a Ca^{2+}-independent manner, just as VLN3 does, but have actin filament severing activity only at high (micromolar and millimolar) Ca^{2+}-concentrations (Zhang et al., 2010), a condition that is present in the apical zone of tip-growing cells (Pierson et al., 1996; Wymer et al., 1997; De Ruijter et al., 1998). Besides these activities, VLN4 and VLN5 have actin filament capping activity (Zhang et al., 2010; Zhang et al., 2011). VLN4, which is involved in the generation and/or maintenance of

actin filament bundles in the shank of root hairs (Zhang et al., 2011), was predicted to participate in the regulation of actin cytoskeleton organization in the subapical and apical region of root hairs by its bundling, capping and/or severing activity (Zhang et al., 2011). Zhang et al. (2010) proposed that VLN5 binds to and stabilizes (bundles of) actin filaments in the shank of pollen tubes by bundling and capping actin filaments, while regulating actin dynamics in the apical region of the pollen tube, where Ca^{2+} is abundant, by capping and severing actin filaments. VLN2 and VLN3 might perform similar functions in tip-growing cells, although their function may be redundant with that of VLN5 in pollen tubes, and VLN4 in root hairs. In conclusion, villins are, besides regulating the amount of actin filament bundling, and in this way one of the actors determining the number and width of cytoplasmic strands, likely to play additional roles in actin organization and dynamics, by their actin filament nucleating, severing, and barbed end capping activities.

Besides villins, several other types of proteins with actin filament bundling capacity have been identified in plant cells: fimbrins (Kovar et al., 2000; Kovar et al., 2001), formins (Cheung and Wu, 2004; Favery et al., 2004; Michelot et al., 2005; Ye et al., 2009), LIM domain proteins (Kovar et al., 2001; Thomas et al., 2006; Thomas et al., 2008; Wang et al., 2008; Papuga et al., 2010), and elongation factor 1α (Collings et al., 1994; Gungabissoon et al., 2001). The actin nucleating formin AtFH1 is able to bind the side of existing actin filaments *in vitro* so that the newly formed actin filaments form an actin filament bundle together with the existing filament (Michelot et al., 2005). Michelot et al. (Michelot et al., 2005) suggest that this activity may be the basis of actin filament bundle formation in plant cells. Indeed, overexpression of AtFH1 increases the number of actin filament bundles in pollen tubes, especially in the apical and subapical region (Cheung and Wu, 2004). On the other hand, down-regulation of AtFH3 resulted in the disappearance of actin filament bundles in pollen tubes, which led to an altered direction and velocity of cytoplasmic streaming in these cells (Ye et al., 2009). This implies that formin-mediated actin filament bundling is involved in cytoplasmic organization.

LIM domain proteins are small (~200 amino acids) actin bundling proteins that bind to, stabilize and bundle actin filaments *in vitro* (Thomas et al., 2006; Wang et al., 2008). The genome of plants only contains a limited number of LIM domain proteins, in contrast to animal genomes, which contain many (Arnaud et al., 2007; Thomas et al., 2009). The six *Arabidopsis* LIM domain proteins, which were shown to have actin filament bundling activity *in vitro*, all localize to actin filaments in cytoplasmic strands and the cortical cytoplasm of different cell types (Papuga et al., 2010). PLIM2C, the only *Arabidopsis* LIM domain protein that bundles actin filaments in a Ca^{2+}-dependent way (its bundling activity is inhibited at a high Ca^{2+} concentration of 5 μM), localizes to long actin filament bundles in the pollen tube shank that do not penetrate the subapical region, and occasionally localizes to the subapical actin fringe (Papuga et al., 2010). The Ca^{2+} concentration is, however, not the only factor regulating the amount of actin filament cross-linking by LIM domain proteins: the actin filament bundling activity of all three AtPLIMs (which are exclusively or preferentially expressed in pollen and pollen tubes) are inhibited at pH values above 6.8 (values that are present in the subapical region of pollen tubes (Feijo et al., 1999)). The lily pollen LiLIM1 was

also shown to be pH-dependent: this protein preferentially binds to actin filaments under pH values below 6.8, that are present in the shank of pollen tubes (Wang et al., 2008). Over-expression of the tobacco and lily LIM domain proteins NtWLIM1 and LiLIM1 cause hyper-bundling of actin filaments and a decrease in the number of actin filament bundles (Thomas et al., 2006; Thomas et al., 2008; Wang et al., 2008; Papuga et al., 2010). These results indicate that LIM domain proteins, in addition to villins, are involved in forming and/or maintaining actin filament bundles and thus could be involved in the regulation of the number and size of cytoplasmic strands. LIM domain protein knockouts so far have failed to produce lines that exhibit developmental defects (Thomas et al., 2009).

Elongation factor 1α (EF-1α) is a protein that functions in binding aminoacyl-tRNA to ribosomes in eukaryotes. In different species, EF-1α binds to microtubules or actin filaments. Gungabissoon et al. (Gungabissoon et al., 2001) show that Maize EF-1α is capable of bundling actin filaments *in vitro* at low pH; this activity is enhanced by the presence of maize actin depolymerizing factor, ADF3.

The actin cross-linking protein fimbrin is ubiquitously expressed in *Arabidopsis* (McCurdy and Kim, 1998). The actin binding activity of FIM1 is Ca^{2+}-independent (Kovar et al., 2000). Kovar et al. (Kovar et al., 2001) showed that microinjection of Oregon green labeled FIM1 in *Tradescantia* stamen hair cells causes the formation of a finer and denser actin filament network in these cells. Microinjection of native FIM1 caused inhibition of cytoplasmic streaming and an increased resistance against profilin induced actin depolymerization (Kovar et al., 2000). However, when FIM1, fused to GFP, was over-expressed in *Arabidopsis*, it appeared to decorate the actin cytoskeleton in different cell types, but did not modify the actin organization (Wang et al., 2004).

We started experiments to investigate the role of *Arabidopsis* FIM5 on actin filament organization, which we present here. FIM5 is preferentially expressed in pollen, and FIM5 loss of function results in a delay in pollen germination and inhibition of pollen tube growth, while no other phenotypic malformations were described (Wu et al., 2010). Our data show, however, that the gene is expressed throughout the plant. GFP:FIM5 (expressed under its endogenous promoter) has a predominant cytoplasmic localization in leaf, hypocotyl, and root epidermal cells, but also localizes to filamentous structures resembling (bundles of) actin filaments in the cortical cytoplasm and in cytoplasmic strands of these cell types (figure 4). Rhodamine-Phalloidin-staining of fixed cells expressing pFIM5::GFP::FIM5 shows that fimbrin colocalizes with actin filaments (figure 5). A comparison of the localization of GFP:FIM5 with that of GFP:FABD2 (figure 4) shows that this fimbrin does not localize to all actin filaments in the cell. Time lapse series show that the (bundles of) actin filaments to which FIM5 localizes, reorganize over time (figure 6). High- and low-speed co-sedimentation assays showed that this fimbrin has actin filament binding and bundling capacity (figure 7). The data suggest that fimbrins could play a role in determining the amount of cross-linking between different (bundles of) actin filaments. Consistent with this idea, Wu et al. showed that in pollen grains of a *fim5* mutant, actin filament bundles are thicker, and distributed more randomly and irregularly than in wild type pollen grains (Wu et al., 2010). They further showed that FIM5 stabilizes actin filaments *in vitro*, and

their results suggest that the protein also stabilizes actin filaments *in vivo*. Wu et al. (2010) explain the increase in actin filament bundle thickness in the *fim5* mutant as follows: fimbrin, which may be involved in the cross-linking of actin filament bundles generated by other actin bundling proteins, could decrease the distance between adjacent actin filaments in these bundles, in this way generating stabilized, thinner and denser actin filament bundles (Wu et al., 2010). Likewise, assembly of actin filament bundles in intestinal microvilli has been proposed to be a stepwise process: according to this hypothesis, villin acts first to bring actin filaments together, and fimbrin acts as a second bundling protein to pack them more tightly (Matova et al., 1999). Consistent with this hypothesis, we show that VLN3 and FIM5 colocalize in leaf epidermal cells (figure 8), showing that villin and fimbrin are present in the same actin filament bundles. It is conceivable that actin filament bundling by fimbrin could have an effect on the number and/or width of cytoplasmic strands by regulating the amount and/or thickness of actin filament bundles. Indeed, *fim5* pollen tubes show an altered pattern of cytoplasmic streaming (Wu et al., 2010), implying an altered organization of the cytoplasm. More work is needed to determine if and how fimbrins contribute to the actin filament bundles involved in formation and maintenance of cytoplasmic strands.

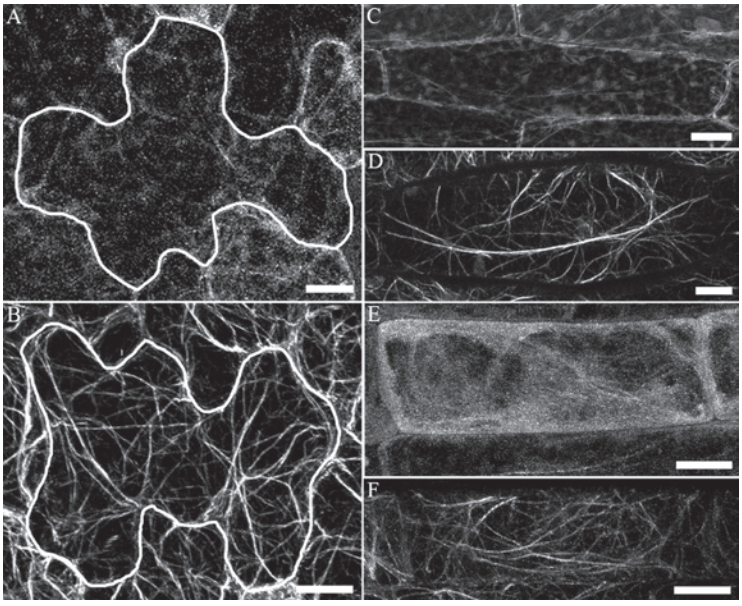

Figure 4. GFP:FIM5 localizes to filamentous structures resembling actin filaments in leaf (A), hypocotyl (C) and root (E) epidermal cells. A comparison of the localization of FIM5 with that of GFP:FABD2 (B, D, F) shows that FIM5 does not localize to all actin filaments in the cell. Bars: 10 µm.

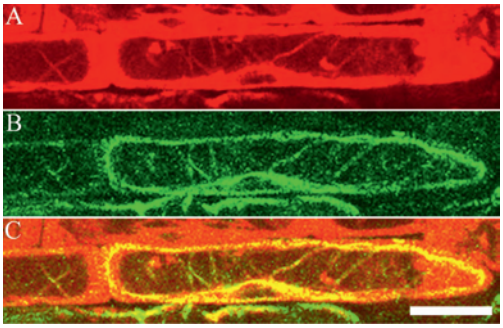

Figure 5. Rhodamine-phalloidin staining of fixed root epidermal cells of pFIM5::GFP:FIM5 expressing plants shows that GFP:FIM5 colocalizes with actin filaments. A: GFP:FIM5; B: Rhodamine-phalloidin stained actin filaments; C: overlay (red: actin filaments; green: GFP:FIM5).
Five day old seedlings were fixed for 2 min with 100 µM MBS-ester in 1 % para-formaldehyde and 0.025 % glutaraldehyde, followed by 200 µM MBS-ester in 2 % para-formaldehyde and 0.025 % glutaraldehyde for 10 min. The plants were post fixed for 20 min in a final concentration of 3 % paraformaldehyde and 0.075 % glutaraldehyde in 0.5 x Actin Stablizing buffer.

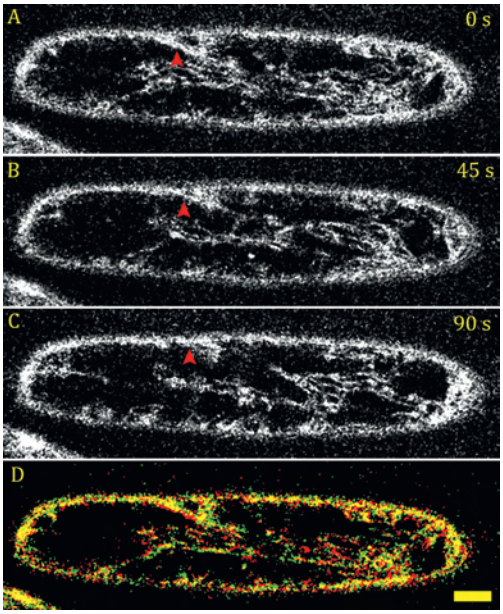

Figure 6. FIM5 localizes to (bundles of) actin filaments that reorganize over time. (A-C) Time lapse series of GFP:FIM5 in an elongating hypocotyl epidermal cell. FIM5 localizes to (bundles of) actin filaments that reorganize over time (e.g. arrowhead). (D) An Image J macro (Van Bruaene et al., 2004) was used to compare (A) and (B), resulting in an image in which fluorescence at the same location in the two images appears yellow, whereas fluorescence present in only one of the two images appears either red (when fluorescence is present only in the first frame) or green (when fluorescence is present only in the second frame). Regions with no fluorescence in either of the frames appear black. Bar, 10 µm.

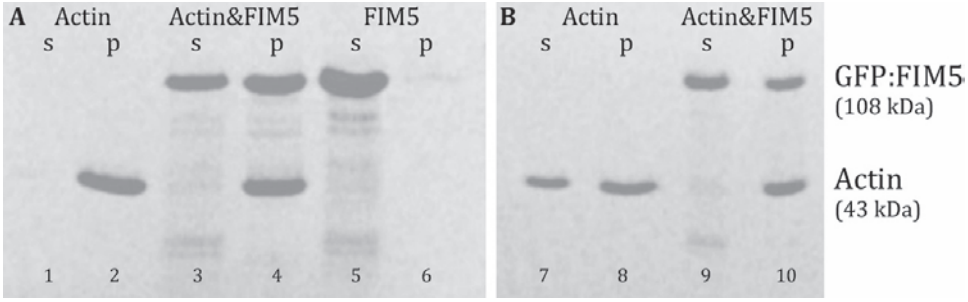

Figure 7. FIM5 has actin filament binding and bundling capacity. (A) High-speed co-sedimentation assay. GFP:FIM5 was combined with filamentous actin (actin:FIM5 was approximately 2:1), and, after a 20 min incubation, centrifuged at 135.000 g. The proteins present in the resulting supernatants and pellets were separated by SDS-PAGE. In the absence of actin, 97 % of FIM5 remained in the supernatant fraction, but in the presence of polymerized actin, 54 % of the protein was present in the pellet, showing that FIM5 is able to bind to actin filaments. (B) Low-speed co-sedimentation assay. Actin was allowed to polymerize for 1 h, and then combined with FIM5 (actin:FIM5 was approximately 2:1). After a 20-minute incubation, the samples were centrifuged at 13.500 g. The proteins present in the resulting supernatants and pellets were separated by SDS-PAGE. In the presence of FIM5, more actin (83 %, versus 59 % in the absence of FIM5) was present in the pellet, showing that FIM5 is able to bundle actin filaments.

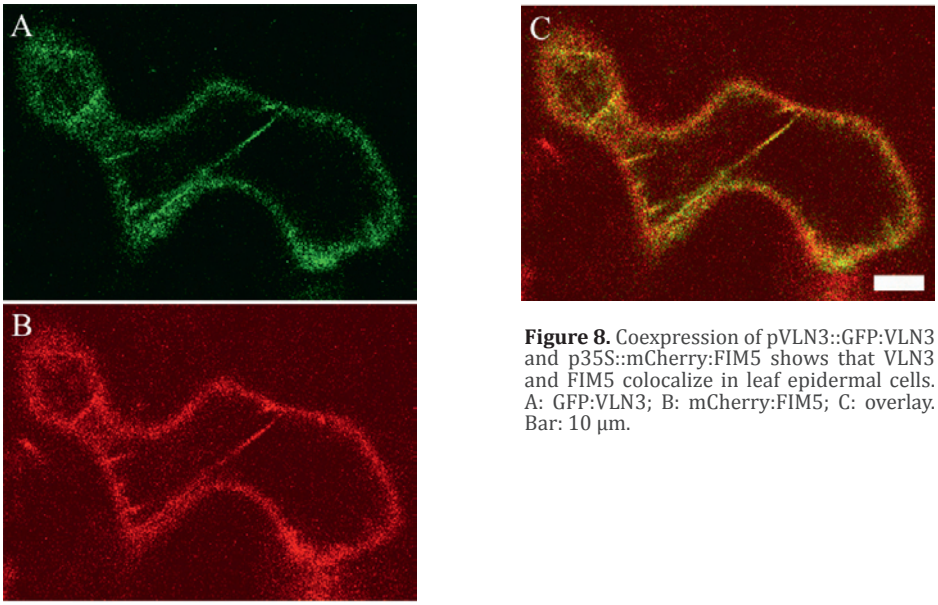

Figure 8. Coexpression of pVLN3::GFP:VLN3 and p35S::mCherry:FIM5 shows that VLN3 and FIM5 colocalize in leaf epidermal cells. A: GFP:VLN3; B: mCherry:FIM5; C: overlay. Bar: 10 µm.

Although it is likely that besides villins, (some of) these other classes of actin bundling proteins play a role in the formation and maintenance of actin filament bundles and thus cytoplasmic strands, disruption of villin activity appears to be sufficient to induce unbundling of actin filament bundles and disintegration of cytoplasmic strands in root hairs (Tominaga et al., 2000; Ketelaar et al., 2002). This suggests that either villins are essential for the maintenance of actin filament bundles that are also supported by other actin bundling proteins, or that other actin bundling proteins have functions in processes other than the maintenance of actin filament bundles in cytoplasmic strands. For example, formins could play a role only during the formation of actin filament bundles, and not during their maintenance (Michelot et al., 2005).

b. Myosins

The genomes of seed plants contain two distinct groups of myosins, myosin VIII and myosin XI. Myosin VIII localises to plasmodesmata and the newly formed cell wall after cell division, endosomes and the endoplasmic reticulum (ER; (Reichelt et al., 1999; Avisar et al., 2008a; Golomb et al., 2008; Sattarzadeh et al., 2008) and could thus be involved in different steps of endocytosis, ER tethering and plasmodesmatal activity (Golomb et al., 2008). It is unlikely that myosin VIII plays a role in forming or maintaining cytoplasmic strands.

Myosin XI isoforms localize to different organelles such as the ER (Samaj et al., 2000; Yokota et al., 2009), mitochondria (Van Gestel et al., 2002; Romagnoli et al., 2007), plastids (Wang and Pesacreta, 2004) and peroxisomes (Hashimoto et al., 2005; Reisen and Hanson, 2007). The movement of these myosin XI decorated organelles over actin filaments is responsible for cytoplasmic streaming (Avisar et al., 2008b; Peremyslov et al., 2008; Prokhnevsky et al., 2008; Sparkes et al., 2008; Avisar et al., 2009). Myosin XI isoforms appear not to be specific for single organelles and analysis of T-DNA

insertion lines shows that the functions of different myosin XI isoforms are greatly redundant. Peremyslov et al. generated triple and quadruple knockout mutants. In these mutants, processive movement of Golgi stacks and peroxisomes was almost completely eliminated (Peremyslov et al., 2010). By application of the myosin ATPase inhibitor 2,3-Butanedione monoxime (BDM), which freezes cytoplasmic organization, it has been shown that myosins may play a role in the relocation of cytoplasmic strands (Hoffmann and Nebenfuhr, 2004; Higaki et al., 2006; Sheahan et al., 2007; Van der Honing et al., 2010 [chapter 2]). This suggests that the restructuring of the actin filament bundles that are the backbone of cytoplasmic strands is mainly performed by myosin-based sliding of existing actin filaments, rather than by actin (de-)polymerization. Myosin XI has indeed been shown to be involved in organizing the actin cytoskeleton (Peremyslov et al., 2010; Ueda et al., 2010). In myosin XI knockout mutants, thick, longitudinal actin filament bundles are absent in midvein epidermal cells (Peremyslov et al., 2010). In root hairs, the thick actin filament bundles that are normally excluded from the apical region (Miller et al., 1999) projected into the cell apices. In another study, actin filament bundles were randomly oriented in epidermal cells of the petioles of cotyledons and of etiolated hypocotyls of myosin XI knockout mutants, while thick, longitudinally oriented actin filament bundles are normally present in these cells (Ueda et al., 2010). Furthermore, these mutants exhibited defects in the development of cytoplasmic strands. These data show that myosin XI is involved in the organization of actin filament bundles and cytoplasmic strands. To understand how myosins exactly contribute to the (re)structuring of the actin filament bundles in cytoplasmic strands, more information about the identity, localization and the activity of these myosins is important.

4. Manipulation of cytoplasmic organization

To fully understand cytoplasmic organization, knowing its molecular aspects is not sufficient. Besides molecular characteristics of the molecules that are involved in the organization of cytoplasm, also physical aspects have to be taken into account. Processes that contribute to cytoplasmic organization not only depend on the molecules involved; they also depend on the physical properties of these molecules, and their assemblages. For example, a single actin filament (>30 – 150 nm long) will buckle when pushed against a membrane, whereas a bundle of actin filaments is able to push a membrane forward (Svitkina et al., 2003; Mogilner and Rubinstein, 2005; Atilgan et al., 2006). Also the degree of cross-linking and bundling dramatically changes the physical properties of an actin filament network (Liu et al., 2006; Tharmann et al., 2007). Physical aspects of actin filament networks can be studied *in vitro* or within living cells. So far, physical properties of the plant actin cytoskeleton only have been probed within living cells.

Using optical tweezers (Ashkin, 1970), physical properties of cytoplasmic organization can be probed in a non-invasive manner (Grabski et al., 1994; Grabski et al., 1998; Van der Honing et al., 2010 [chapter 2]). Optical tweezers function by focussing a high intensity laser beam on a small particle, during which the laser's radiation pressure constrains the particle to the centre of the laser. Lateral displacement of the focussed laser beam allows non-invasive dislocation of these particles (Ashkin and Dziedzic, 1987; Ashkin et al., 1987; Block, 1992; Grier, 2003). Requirements for successful optical trapping are a high numerical aperture lens and the presence of structures in the

(sub-)micrometer range, with a higher refractive index than the surrounding medium close to the coverslip. Generally, lasers that emit infrared light are used, since these wavelengths do not interfere with imaging and are not perceived by cells. Thus, optical tweezers can be employed to produce controlled forces inside living cells (Ashkin et al., 1987; Ashkin and Dziedzic, 1989) to manipulate intracellular organization (Ashkin and Dziedzic, 1989; Grabski et al., 1994; Sparkes et al., 2009; Van der Honing et al., 2010 [chapter 2]).

Optical tweezers have been used to measure the tension in naturally occurring cytoplasmic strands (Grabski et al., 1994; Grabski et al., 1998) and have been employed to alter cytoplasmic organization by the production of new cytoplasmic strands or cytoplasmic protrusions (Van der Honing et al., 2010 [chapter 2]). The tension in naturally occurring cytoplasmic strands was studied by trapping an organelle in one of these strands with the optical tweezers and performing a series of rapid lateral displacements at different laser powers (Grabski et al., 1994). When performing these experiments in the presence of the actin filament depolymerizing drug cytochalasin D (20 μM), tension in cytoplasmic strands was reduced (Grabski et al., 1994), whereas in the presence of the myosin inhibitor BDM (10 mM) the tension in cytoplasmic strands was increased (Grabski et al., 1998). These experiments show that tension in cytoplasmic strands is produced by actin filaments and suggest that myosin-based sliding of actin filaments is responsible for the deformation competence of cytoplasmic strands, which fits with the idea that myosin-based sliding of actin filaments is responsible for cytoplasmic restructuring (Hoffmann and Nebenfuhr, 2004; Szymanski and Cosgrove, 2009; Van der Honing et al., 2010 [chapter 2]).

Besides measuring tension in existing cytoplasmic strands, optical tweezers can also be employed to modify cytoplasmic organization by trapping an organelle and displacing the trapped organelle into the space occupied by the vacuole. This results in the formation of a cytoplasmic protrusion into the vacuole, bounded by tonoplast membrane (Van der Honing et al., 2010 [chapter 2]). When the tweezers with a trapped organelle at the top of such a cytoplasmic protrusion were moved to the tonoplast at another side of the vacuole, we have made two different observations: (1) when the organelle is released, for example by switching off the trap, the cytoplasmic protrusion shoots back to its origin and disappears, and (2) the tonoplast membrane of the protrusion fuses with the tonoplast membrane at the other side of the vacuole, resulting in a cytoplasmic strand that remains intact when the tweezers are switched off (Norbert de Ruijter, Anne Mie C. Emons and Tijs Ketelaar, unpublished results). The first observation has been seen in tobacco Bright Yellow-2 suspension cultured cells; in *Tradescantia* stamen hair cells, both reactions were observed, and in epidermal peals of onion skin, the second reaction predominantly occurs. Thus, the possibility of tweezer-formed strands to fuse with the tonoplast is cell type and/or species dependent.

We have produced tweezer-formed cytoplasmic protrusions in cells in which the actin cytoskeleton had completely been depolymerized (treatment with 500 nM latrunculin B for 12-16 hours), or in which the myosin activity had been inhibited (25 mM BDM for 30 minutes; (Van der Honing et al., 2010 [chapter 2])). It requires more force to produce cytoplasmic protrusions when myosin motor activity is inhibited and less force when

the actin cytoskeleton is depolymerized. Thus, the presence of actin filaments decreases the deformation capacity of the cytoplasm and myosin motor inhibition makes the cytoplasm even harder to deform. We studied the actin filament localization during and after physical manipulation of cytoplasmic organization. In control experiments, visible actin filaments had entered most tweezer-formed strands after several minutes. This suggests that actin filaments occupy free cytoplasmic space, either by active targeting or by random movement. The entry of actin filaments was inhibited by application of BDM (Van der Honing et al., 2010 [chapter 2]), but not by partial depolymerization of actin filaments with 100 nM latrunculin B or 100 μM cytochalasin D (Hannie S. van der Honing, Anne Mie C. Emons and Tijs Ketelaar, unpublished results). This suggests that myosin-mediated displacement of existing actin filaments, and not actin polymerization, is responsible for the entry of actin filaments in these strands. Sliding of actin filaments over other actin filaments by myosin activity could be, besides being responsible for reorganization of existing cytoplasmic strands, a mechanism by which actin filaments are pushed against the tonoplast to generate the force to deform the vacuolar membrane during the formation of new cytoplasmic strands.

Although actin filaments support naturally occurring cytoplasmic strands and appear in tweezer-formed protrusions, the presence of actin filaments does not delay the collapse of cytoplasmic protrusions after release of the tweezers, indicating that actin filaments do not support tweezer-formed cytoplasmic protrusions, whereas they do support naturally occurring cytoplasmic strands. Apparently, actin filaments can only support a cytoplasmic strand when they are held in place by connections to other actin filaments at both sides of the strand, or else by membrane continuity.

5. Cytoplasmic organization: prospects

An integrated optical trapping and confocal microscopy system is a powerful tool for the investigation of cytoplasmic organization and the underlying behaviour of the actin cytoskeleton. In combination with the use of mutants and drugs to manipulate actin organization it is possible to directly link changes in physical aspects of intracellular organization to the action of specific proteins. Ideally, these experiments should be complemented with experiments using the same techniques in systems with reduced complexity, such as *in vitro* experiments with purified proteins. Besides these 'wet' experiments, the understanding of a complex process such as intracellular organization would likely benefit from an approach in which known aspects of the system are incorporated in models that simplify the complexity, explain the observations and make predictions about unknown aspects of cytoplasmic organization.

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Summaries and Acknowledgements

Summary, Samenvatting, Acknowledgements

Summary

The organization of the cytoplasm, the cytoarchitecture, is crucial for plant development, since for instance plant cell growth depends on the proper allocation of growth substances to cell elongation sites. The production and maintenance of an organized cytoplasm is regulated by the actin cytoskeleton and the activities of several actin binding proteins that modulate the dynamics and organization of actin filaments. In this thesis, results are presented that give insight into the regulation of the production of an organized cytoplasm in plant cells by the actin cytoskeleton. We examine physical properties of the actin cytoskeleton by deformation of the cytoplasm with optical tweezers. Further, we examine the role of the actin bundling protein villin in the organization of the actin cytoskeleton and discuss the possible role of several actin binding proteins in the production of an organized cytoplasm.

In **chapter 1**, we review the role of actin binding proteins in actin-based force generation in three well-studied mammalian model systems: the propulsion of *Listeria* through animal cells, and the formation of filopodia and lamellipodia during the migration of animal cells. We compare the functions of these actin-binding proteins with those of their homologues in plants. Force generation by polymerization of actin filaments has not been studied in plant cells, but may play a role in the production of an organized cytoplasm. We predict that the Arp2/3 complex and/or formins are responsible for the nucleation of actin filaments that are required for the initial formation of cytoplasmic strands. Organizing these newly formed actin filaments into the long actin filament bundles that are present in cytoplasmic strands depends on the activity of actin bundling proteins, such as formins or villins. It seems likely that these proteins, in cooperation with other actin binding proteins, play a role in actin-based force generation in plant cells, which, in turn, is likely to be involved in the production of an organized cytoplasm.

To understand cytoplasmic organization, physical properties of the underlying actin network have to be investigated. In **chapter 2**, we describe a study in which optical tweezers were used to study physical properties of the cytoplasm in a non-invasive manner. We used optical tweezers to deform the cytoplasm of Tobacco BY-2 suspension cultured cells, while studying the behaviour of the actin cytoskeleton by confocal laser scanning microscopy. An organelle in the perinuclear area was trapped and displaced into the vacuolar space. Since the vacuolar membrane remained intact, this resulted in the formation of a cytoplasmic protrusion that resembles a cytoplasmic strand. When actin filaments were depolymerized by Latrunctulin B, it was easier to deform the cytoplasm, while inhibition of myosin motor activity by BDM increased the force required to form cytoplasmic protrusions. In control experiments, actin filaments entered the tweezer-formed protrusions within several minutes after their formation. In BDM-treated cells, however, actin filaments were never observed in the protrusions. These data suggest that existing actin filaments can be reorganized in a myosin-dependent way. Myosin-based relocation of actin filaments therefore is expected to play a role in the (re)organization of the cytoplasm of plant cells.

Lifeact is a novel probe for live cell actin filament visualization. Fused to a fluorescent probe, this short peptide, consisting of the first 17 amino acids from the yeast protein Abp140 (Actin binding protein 140), clearly visualizes filamentous actin in eukaryotic cells. In **chapter 3**, results are presented that describe the (re)organization of the actin cytoskeleton in root epidermal cells of *Arabidopsis thaliana* visualized with Lifeact:Venus. We compared the (re)organization of the actin cytoskeleton visualized with Lifeact:Venus with that of the (re)organization of the actin cytoskeleton visualized with GFP:FABD2, a commonly used marker for filamentous actin (F-actin) in plant cells that consists of GFP (green fluorescent protein) fused to the second actin binding domain of *Arabidopsis* FIMBRIN1 (fimbrin actin-binding domain 2). Unlike GFP:FABD2, Lifeact:Venus labeled highly dynamic fine F-actin in the subapical region of tip-growing root hairs. The reorganization rate of (bundles of) actin filaments in root epidermal cells was, however, significantly reduced in Lifeact:Venus expressing cells. By comparing cytoplasmic strand reorganization of both lines with that of wild type Col-0 cells, we show that this difference is caused by a decrease in reorganization rate of the actin cytoskeleton in cells expressing Lifeact:Venus, rather than by an increase in reorganization rate in cells expressing GFP:FABD2. Despite the effect of Lifeact:Venus on cytoplasmic strand reorganization rate, the cytoplasmic organization was not visibly affected in these cells. Furthermore, expression of Lifeact:Venus did not affect plant growth and development. This implicates that the organization of the actin cytoskeleton, but not its dynamic relocation over time, is a determining factor in plant cell growth. We conclude that Lifeact:Venus reduces remodeling of the actin cytoskeleton in *Arabidopsis*, and that this probe should be used with caution when studying this aspect of cells.

Actin filaments in cytoplasmic strands are organized in thick bundles. The actin bundling protein villin is involved in maintaining these bundles. The role of villin in the generation of actin filament bundles and in plant development was explored in **chapter 4**. We used *Arabidopsis* T-DNA insertion lines to generate a double mutant in which *VLN2* and *VLN3* transcripts are truncated. These *vln2 vln3* double mutants showed a clear anomaly in directional organ growth, suggesting problems with coordinated cell elongation. The rotational movements (circumnutation) of *vln2 vln3* inflorescences appeared more random than those of wild type Col-0 inflorescences: periods of normal circumnutation were alternated with periods in which the circumnutation showed larger amplitudes than those of wild type Col-0, confirming problems with coordinated cell elongation in the double mutants. Microscopy analysis showed a higher abundance of thin actin filament bundles in several cell types of the double mutants, while thick bundles were virtually absent. The data show that villin is involved in the generation of thick bundles of actin filaments and suggest that such bundles are – directly or indirectly – important for coordinated cell expansion.

Chapter 5 is a general discussion. We review research in which actin binding proteins that could be involved in cytoplasmic organization are described, and discuss how physical properties of actin filaments in the plant cytoplasm can be studied by manipulation of cytoplasmic organization by optical tweezers. The chapter focuses on the structural function of actin filaments (influenced by actin binding proteins) in the formation, maintenance and reorganization of cytoplasmic strands. In this chapter,

we have included our initial data about the role of the actin bundling protein fimbrin on actin filament organization. We show that this protein has actin filament bundling capacity *in vitro*, and that it localizes to (bundles of) actin filaments *in vivo*. The data suggest that fimbrins could contribute to the formation of actin filament bundles that are involved in cytoplasmic organization. In the second part of this chapter, we discuss how an integrated optical trapping and confocal microscopy system can be used to investigate cytoplasmic organization and the underlying behaviour of the actin cytoskeleton. In the future, this setup will, in combination with the use of mutants and drugs to manipulate the actin cytoskeleton, likely result in an increased insight into the physical aspects of intracellular organization, which depends on the action of specific (actin binding) proteins.

Samenvatting

De organisatie van het cytoplasma, ook wel cytoarchitectuur genoemd, is cruciaal voor de ontwikkeling van planten. Zo is plantencelgroei, bijvoorbeeld, afhankelijk van de juiste localisatie van groeisubstanties op plaatsen waar de cel groeit. De productie en handhaving van een georganiseerd cytoplasma wordt gereguleerd door het actine-cytoskelet en de activiteiten van verschillende actine-bindende eiwitten die de dynamiek en organisatie van actinefilamenten beïnvloeden. In dit proefschrift worden resultaten gepresenteerd die inzicht geven in de regulatie van de productie van een georganiseerd cytoplasma in plantencellen door het actine-cytoskelet. We onderzoeken fysische eigenschappen van het actine-cytoskelet door middel van het vervormen van het cytoplasma met een optisch pincet. Verder onderzoeken we de rol van het actine-bundelende eiwit villine in de organisatie van het actine-cytoskelet, en bediscussiëren de mogelijke rol van verschillende actine-bindende eiwitten in de productie van een georganiseerd cytoplasma.

In **hoofdstuk 1** bespreken we de rol van actine-bindende eiwitten in actine-gebaseerde krachtgeneratie in drie goed bestudeerde dierlijke modelsystemen: de voortstuwing van de *Listeria* bacterie in dierlijke cellen, en de vorming van filopodia en lamellipodia tijdens de voortbeweging van dierlijke cellen. We vergelijken de functies van deze actine-bindende eiwitten met die van hun homologe eiwitten in planten. De krachtgeneratie door polymerisatie van actinefilamenten is niet bestudeerd in plantencellen, maar zou een rol kunnen spelen in de productie van een georganiseerd cytoplasma. We voorspellen dat het Arp2/3 complex en/of formines verantwoordelijk zijn voor de nucleatie van actinefilamenten die nodig zijn voor de initiële vorming van cytoplasmadraden. Het organiseren van deze nieuwgevormde actinefilamenten in de lange bundels van actinefilamenten die aanwezig zijn in cytoplasmadraden is afhankelijk van de activiteit van actine-bundelende eiwitten, zoals formines of villines. Het lijkt aannemelijk dat deze eiwitten, in samenwerking met andere actine-bindende eiwitten, een rol spelen in actine-gebaseerde krachtgeneratie in plantencellen, en dat deze krachtgeneratie op haar beurt een rol speelt in de productie van een georganiseerd cytoplasma.

Om de organisatie van het cytoplasma te begrijpen, moeten fysische eigenschappen van het onderliggende actine-netwerk onderzocht worden. In **hoofdstuk 2** beschrijven we een studie waarin een optisch pincet gebruikt is om fysische eigenschappen van het cytoplasma te bestuderen op een zodanige manier dat celprocessen intact blijven. We gebruikten een optisch pincet om het cytoplasma van suspensiecellen van de BY-2 cellijn van tabak te vervormen, terwijl het actine-cytoskelet tegelijkertijd bestudeerd werd door middel van confocale laser scanning microscopie. Een organel in de perinucleaire ruimte werd gevangen, en verplaatst naar de vacuolaire ruimte. Doordat het vacuolemembraan intact bleef, resulteerde dit in de vorming van een cytoplasmatische tubulaire extensie die lijkt op een cytoplasmadraad. Het was gemakkelijker om het cytoplasma te vervormen wanneer actinefilamenten gedepolymeriseerd (afgebroken) waren door Latrunculine B, terwijl remming van myosine motor-activiteit door BDM de kracht die nodig is om de cytoplasmatische extensies te vormen, verhoogde. In controle-experimenten kwamen actinefilamenten de cytoplasmatische extensies binnen enkele minuten na hun vorming binnen. In BDM-behandelde cellen werden

echter nooit actinefilamenten geobserveerd in de extensies. Deze resultaten wekken de suggestie dat bestaande actinefilamenten kunnen worden gereorganiseerd op een myosine-afhankelijke wijze. Wij verwachten daarom dat myosine-gebaseerde relocatie van actinefilamenten een rol speelt in de (re)organisatie van het cytoplasma van plantencellen.

Lifeact is een nieuwe merker voor het visualiseren van actinefilamenten in levende cellen. Dit korte peptide, bestaand uit de eerste 17 aminozuren van het gisteiwit Abp140 (Actin binding protein 140), visualiseert, wanneer het gekoppeld is aan een fluorescente probe, duidelijk filamenteus actine in eukaryotische cellen. In **hoofdstuk 3** worden resultaten gepresenteerd die de (re)organisatie van het actine-cytoskelet beschrijven in wortelepidermiscellen van *Arabidopsis thaliana*, gevisualiseerd met Lifeact:Venus. We vergeleken de (re)organisatie van het actinecytoskelet wanneer dit gevisualiseerd werd met Lifeact:Venus met dat van de (re)organisatie van het actinecytoskelet gevisualiseerd met GFP:FABD2, een vaak gebruikte merker voor filamenteus actine (F-actine) in plantencellen, bestaand uit GFP (green fluorescent protein) gekoppeld aan het tweede actine-bindende domein van *Arabidopsis* FIMBRIN1 (fimbrin actin-binding domain 2). In tegenstelling tot GFP:FABD2, labelde Lifeact:Venus het zeer dynamische fijne F-actine in de subapicale regio van topgroeiende wortelharen. De reorganisatiesnelheid van (bundels van) actinefilamenten in wortelepidermiscellen was echter significant lager in cellen waarin Lifeact:Venus tot expressie kwam. Door de reorganisatie van cytoplasmadraden van beide lijnen te vergelijken met die van wildtype Col-0 cellen, laten we zien dat dit verschil veroorzaakt wordt door een verlaagde reorganisatiesnelheid van het actine-cytoskelet in cellen waarin Lifeact:Venus tot expressie komt, en niet door een verhoogde reorganisatiesnelheid in cellen waarin GFP:FABD2 tot expressie komt. Ondanks het effect van Lifeact:Venus op de reorganisatiesnelheid van cytoplasmadraden, was de organisatie van het cytoplasma niet zichtbaar beïnvloed in deze cellen. Bovendien beïnvloedde expressie van Lifeact:Venus niet de groei en ontwikkeling van de planten. Dit impliceert dat de organisatie van het actine-cytoskelet, maar niet haar dynamische relocatie in de tijd, een bepalende factor is in plantencelgroei. We concluderen dat Lifeact:Venus de remodellering van het actine-cytoskelet in *Arabidopsis* vermindert, en dat voorzichtigheid geboden is bij het gebruik van deze probe als dit aspect van cellen bestudeerd wordt.

Actinefilamenten in cytoplasmadraden zijn georganiseerd in dikke bundels. Het actine-bundelende eiwit villine is betrokken bij het onderhouden van deze bundels. De rol van villine bij de vorming van actinefilamentbundels en bij plantenontwikkeling werd verkend in **hoofstuk 4**. We gebruikten *Arabidopsis* T-DNA insertielijnen om een dubbelmutant de genereren waarin *VLN2* en *VLN3* transcripten verkort zijn. Deze *vln2 vln3* dubbelmutanten vertoonden een duidelijke onregelmatigheid in gerichte orgaangroei, wat problemen met gecoördineerde celelongatie suggereert. De roterende bewegingen (circumnutatie) van *vln2 vln3* bloeiwijzen bleken meer willekeurig dan die van wildtype Col-0 bloeiwijzen: periodes van normale circumnutatie werden afgewisseld met periodes waarin de circumnutatie grotere amplitudes liet zien dan die van wildtype Col-0, wat de problemen met gecoördineerde celelongatie in de dubbelmutanten bevestigt. Een microscopische analyse liet zien dat er meer

dunne actinefilamentbundels aanwezig waren in verschillende celtypes van de dubbelmutanten, terwijl dikke bundels vrijwel afwezig waren. De data laten zien dat villine betrokken is bij de vorming van dikke bundels van actinefilamenten en suggereren dat deze bundels - direct of indirect - belangrijk zijn voor gecoördineerde celexpansie.

Hoofdstuk 5 is een algemene discussie. We bespreken onderzoeken waarin actine-bindende eiwitten die een rol zouden kunnen spelen in de organisatie van het cytoplasma worden beschreven, en bediscussiëren hoe fysische eigenschappen van actinefilamenten in plantencytoplasma kunnen worden bestudeerd door manipulatie van de cytoplasmatische organisatie met behulp van een optisch pincet. Het hoofdstuk concentreert zich op de structurele functie van actinefilamenten (beïnvloed door actine-bindende eiwitten) in de vorming, onderhouding, en reorganisatie van cytoplasmadraden. In dit hoofdstuk hebben we onze eerste data over de rol van het actine-bundelende eiwit fimbrine bij actinefilament organisatie opgenomen. We laten zien dat dit eiwit *in vitro* actine-bundelende aciviteit heeft, en dat het *in vivo* gelocaliseerd is in/op (bundels van) actinefilamenten. De data suggereren dat fimbrines een bijdrage kunnen leveren aan de vorming van actinefilamentbundels die betrokken zijn bij cytoplasma-organisatie. In het tweede deel van dit hoofdstuk bediscussiëren we hoe een optisch pincet geïntegreerd met een confocale microscoop gebruikt kan worden bij het onderzoeken van cytoplasma-organisatie en het onderliggende gedrag van het actine-cytoskelet. In de toekomst zal deze techniek, in combinatie met het gebruik van mutanten en drugs om het actine-cytoskelet te manipuleren, waarschijnlijk resulteren in een verhoogd inzicht in de fysische aspecten van intracellulaire organisatie, welke afhankelijk is van de werking van specifieke (actine-bindende) eiwitten.

Legenda

Celwand
Vacuole
Cytoplasma
Celkern
Kernlichaampje

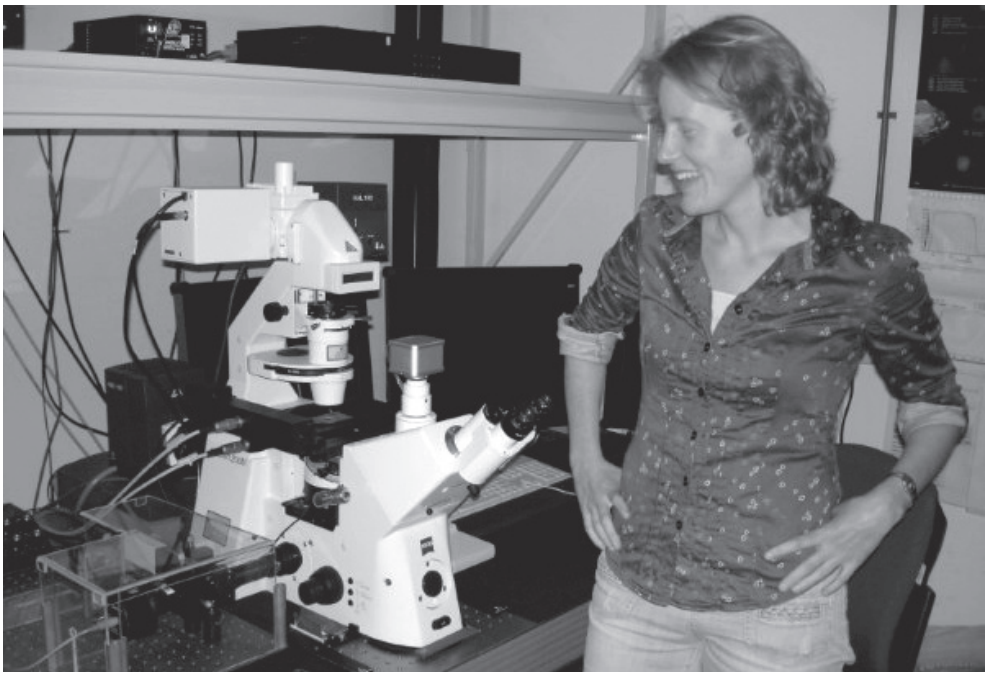
Schematisch overzicht van een plantencel

Cytoplasmadraden (rood) lopen door de vacuole (paars) en verbinden het cytoplasma wat om de celkern (groen) ligt met het wandstandige cytoplasma. De celwand omgeeft de cel.

About the author

***Curriculum Vitae*, List of publications, Education statement**

Curriculum Vitae



Hannie van der Honing werd op 22 april 1981 geboren in het Friese plaatsje Winsum. Na het doorlopen van het gymnasium op het Christelijk Gymnasium Beyers Naudé te Leeuwarden begon zij in 1999 met de studie bos- en natuurbeheer aan Wageningen Universiteit. In 2000 stapte ze over op de studie biologie. Na het afronden van haar eerste afstudeervak bij de leerstoelgroepen plantenfysiologie en erfelijkheidsleer (Wageningen Universiteit) ontdekte Hannie haar enthousiasme voor de plantencelbiologie tijdens haar tweede afstudeervak, bij de leerstoelgroep plantencelbiologie (Wageningen Universiteit). Haar stage deed zij in het laboratorium van plantencelbioloog Geoffrey Wasteneys, in Vancouver, Canada. Tijdens haar studie was zij actief als bestuurs- en commissielid binnen de studentenatletiekvereniging Tartlétos en de Nederlandse Studenten Atletiek Federatie Zeus. In 2005 studeerde zij *cum laude* af, en begon ze bij de leerstoelgroep plantencelbiologie (Wageningen Universiteit) aan het promotieonderzoek beschreven in dit proefschrift. Naast haar onderzoekswerkzaamheden was Hannie als docent betrokken bij de uitvoering van verschillende BSc vakken. In het schooljaar 2008-2009 was ze tevens werkzaam als biologiedocent aan het Rembrandt College te Veenendaal. In 2010 kreeg zij een tijdelijke aanstelling als docent aan Wageningen Universiteit in het kader van onderwijsvernieuwing voor het vak Celbiologie I. Met ingang van augustus 2011 is Hannie werkzaam als docent biologie en ANW op het Beekdal Lyceum in Arnhem.

List of publications

Publications in international journals

Van der Honing HS, de Ruijter NC, Emons AMC, Ketelaar T, 2010. Actin and myosin regulate cytoplasm stiffness in plant cells: a study using optical tweezers. New Phytologist 185: 90-102.

Ketelaar T, **Van der Honing HS,** Emons AMC, 2010. Probing cytoplasmic organization and the actin cytoskeleton of plant cells with optical tweezers. Biochemical Society Transactions 38: 823-828.

Sakai T*, **Van der Honing HS*,** Nishioka N, Uehara Y, Takahashi M, Fujisawa N, Saji K, Seki M, Kazuo S, Jones MA, Smirnoff N, Okada K, Wasteneys GO, 2008. Armadillo repeat-containing kinesins and a NIMA-related kinase are required for epidermal cell morphogenesis in *Arabidopsis*. Plant Journal 53, 157-171.

Van der Honing HS, Emons AMC, Ketelaar T, 2007. Actin based processes that could determine the cytoplasmic architecture of plant cells. Biochimica et Biophysica Acta 1773, 604-614.

Submitted manuscripts

Van der Honing HS, Van Bezouwen LS, Emons AMC, Ketelaar T. Expression of Lifeact at levels that do not affect plant development can reduce dynamic reorganization of actin filaments in *Arabidopsis thaliana* (*submitted*).

Van der Honing HS, Lansky Z, Kieft H, Anne Mie C. Emons AMC, Ketelaar T. *Arabidopsis* VILLIN2 and VILLIN3 are required for the generation of thick actin filament bundles and for directional organ growth (*submitted*).

* Equal contribution

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Hannie van der Honing
Date: 20 October 2011
Group: Laboratory of Plant Cell Biology, Wageningen University & Research Centre

1) Start-up phase	*date*
• **First presentation of your project**	
Towards understanding cytoplasmic organisation, a prerequisite for organised cell expansion	Oct 07, 2005
• **Writing or rewriting a project proposal**	
• **Writing a review or book chapter**	
Van der Honing et al., 2007: Actin based processes that could determine the cytoplasmic architecture of plant cells (Biochimica et Biophysica Acta, 1773: 604-614)	Aug 01, 2006
• **MSc courses**	
• **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7,5 credits**

2) Scientific Exposure	*date*
• **EPS PhD student days**	
EPS PhD student day 2006	Sep 19, 2006
EPS PhD student day 2007	Sep 13, 2007
EPS PhD retreat 2008, Wageningen	Oct 03, 2008
• **EPS theme symposia**	
EPS theme 1 symposium 2006, Leiden	May 12, 2006
EPS theme 1 symposium 2007, Wageningen	Oct 11, 2007
• **NWO Lunteren days and other National Platforms**	
Annual Dutch Meeting on Cellular and Molecular Biophysics	Oct 10-11, 2005
ALW-meeting, Lunteren	Apr 03-04, 2006
Annual Dutch Meeting on Cellular and Molecular Biophysics	Oct 09-10, 2006
ALW-meeting, Lunteren	Apr 02-03, 2007
Annual Dutch Meeting on Cellular and Molecular Biophysics	Oct 01-02, 2007
ALW-meeting, Lunteren	Apr 07-08, 2008
Annual Dutch Meeting on Cellular and Molecular Biophysics	Sep 28-29, 2009
ALW-meeting, Lunteren	Apr 19-20, 2010
• **Seminars (series), workshops and symposia**	
Flying Seminars	
Joseph Ecker	Oct 02, 2005
Phil Benfey	Oct 24, 2005
Simon Gilroy	May 19, 2008
Seminar series Physics and physical processes, Seminar series Mathematics and/or Physics in (Plant) Cell Biology, Seminar series Mathematics in Biology, and other seminars	
Seminar Bela Mulder	Oct 14, 2005
Seminar Helmut Schiessel	Nov 25, 2005
Seminar Marileen Dogterom	Nov 25, 2005
Seminar Erika Eiser	Feb 17, 2006
Seminar Veronica Albers Grieneisen	Jun 16, 2006

	Seminar Chris Hawes	Apr 27, 2006
	Seminar Jiri Friml	Jun 14, 2006
	Seminar David Ehrhardt	Oct 11, 2006
	Seminar Christian Tischer	Oct 13, 2006
	Seminar Alison Roberts	Oct 17, 2006
	Seminar Viola Willemsen	Nov 24, 2006
	Seminar Anne-Catherine Schmit	Dec 12, 2006
	Seminar Farhah Assaad	Feb 16, 2007
	Seminar Anja Geitmann	Oct 21, 2007
	Seminar Gijsje Koenderink	Feb 09, 2007
	Seminar Lacey Samuels	Oct 05, 2007
	Seminar Lacey Samuels	Oct 09, 2007
	Seminar Staffan Persson	Nov 23, 2007
	Seminar Michael Lampson	Feb 01, 2008
	Seminar Marcel Janson	Feb 23, 2008
	Seminar Simon Tindemans	Apr 11, 2008
	Seminar Remco Offringa	Sep 12, 2008
	Seminar Astrid van der Horst	Sep 19, 2008
	Seminar Anna Akhmanova	Oct 24, 2008
	Seminar Minako Kaneda	Sep 18, 2009
	Seminar Mireille Claessens	Nov 27, 2009
	Seminar Chris Hawes	Aug 20, 2010
•	**Seminar plus**	
•	**International symposia and congresses**	
	Gordon Research Conference on Plant and Fungal Cytoskeleton, Andover, NH, USA	Aug 20-25, 2006
•	**Presentations**	
	Poster presentation Annual Dutch Meeting on Cellular and Molecular Biophysics	Oct 01, 2005
	Poster presentation GRC on Plant and Fungal Cytoskeleton, Andover, NH, USA	Aug 20-25, 2006
	Poster presentation Annual Dutch Meeting on Cellular and Molecular Biophysics	Oct 1-2, 2007
	Oral presentation EPS theme 1 symposium 2007, Wageningen	Oct 11, 2007
	Oral presentation EPS PhD retreat 2008, Wageningen	Oct 03, 2008
	Poster presentation Annual Dutch Meeting on Molecular and Cellular Biophysics	Sep 28, 2009
	Oral presentation ALW-meeting, Lunteren	Apr 20, 2010
	Oral presentation University of British Columbia, Vancouver, Canada	Jul 12, 2010
•	**IAB interview**	Dec 12, 2008
•	**Excursions**	
	Subtotal Scientific Exposure	*19,5 credits**

3) In-Depth Studies		*date*
•	**EPS courses or other PhD courses**	
	Systems biology course: Principles of ~omics data analysis	Nov 07-10, 2005
	Models take root: the role of mathematics in plant biology	Feb 19-24, 2006
•	**Journal club**	
	Literature discussion group PCB (monthly)	2005-2010
•	**Individual research training**	
	Subtotal In-Depth Studies	*4,7 credits**

4) Personal development		*date*
•	**Skill training courses**	
	Scientific writing	2006
	Career orientation	2008
•	**Organisation of PhD students day, course or conference**	
	Organisation of EPS PhD students day 2006	2006
•	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*4,8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**36,5**

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* A credit represents a normative study load of 28 hours of study.

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