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The interface between secretory pathway and cytoskeleton – the exocyst tethering complex and cortical cytoskeleton in plant cell morphogenesis.

Ph.D. Thesis

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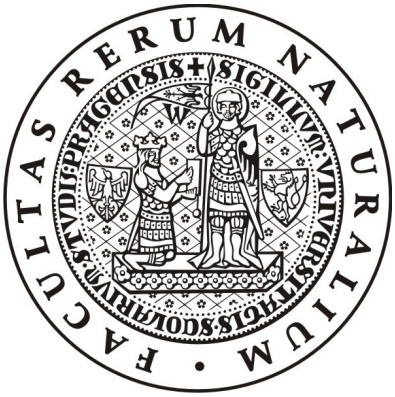

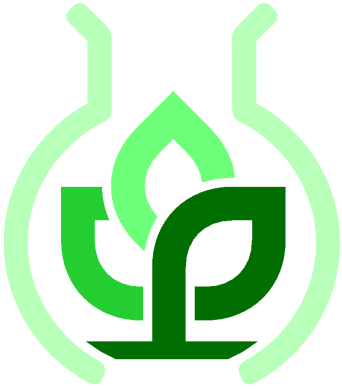

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Declaration

I hereby declare that I worked on this thesis independently while citing all resources used. The people who contributed substantially to this work are noted as coauthors of the respective publications.
This thesis has not been submitted for another qualification to this or any other university.

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Abbreviations:
GOE – specific domain of the group Ie formins
MT – microtubule
PIP2 – phosphatidylinositol 4,5-bisphosphate
PM – plasma membrane
VAEM – variable-angle epifluorescence microscopy
TIRFM – total internal reflection fluorescence microscopy

1. Introduction

All cells in the plant body are polarized, usually along more than one axis. As an example we can consider leaf epidermal cells: pavement cells face at least three different environments – ambient atmosphere, intercellular spaces, and neighbouring cells (moreover, of various kinds – pavement, guard or mesophyll). The cell has to distinguish among these interfaces and differentially communicate with all of them. In the majority of situations, plant cell live interconnected by their cell walls. These cells usually grow by a so called diffuse growth. Diffuse growth is characterized by distribution of the growth along the growing region, whereas tip growth is a spatially focused cell expansion.
Asymmetric distribution of cellular components and structures constitutes cell polarity. Many processes, including signal cascades, cytoskeletal dynamics and intracellular trafficking, are engaged in cell polarity establishing and maintenance. Cell polarity includes differential composition of different membrane domains. Examples of such polarity in plant cells are ubiquitous – auxin transporters PIN, ion transporters (Langowski *et al.*, 2010). Cell polarity is controlled and maintained by protein and other cargo sorting in the endomembrane system, by transport of proteins and cargo to target plasma membrane (PM) domains, and by fusion of the vesicles containing the cargo with the selected PM domain (Mellman and Nelson, 2008). In this thesis, I will focus on the role of formin proteins as potential integrators of (plasma) membrane and both actin and microtubule cytoskeleton, and the exocyst tethering complex that controls the last step of the secretory pathway.

1.1. Agents of cellular polarity – exocytosis and the cytoskeleton

Polarized exocytosis is vital for cell morphogenesis; especially for cells with rigid cell walls. Secretory vesicles are transported and targeted by motor proteins and cytoskeletons, and fuse with the membrane domains defined by tethering proteins. In yeast, a small GTPase from the Rho family, Cdc42, is central to cell polarity; cells lacking this protein are unable to form a bud and create rounded cells (Adams *et al.*, 1990). Cdc42 recruits the formin Bni1p (Evangelista *et al.*, 1997) that nucleates actin filaments and thus attracts the vesicle delivery to the site marked by Cdc42. A component of the exocyst complex Sec3 interacts with Cdc42, and an abolishment of this interaction blocks polarized secretion (Zhang *et al.*, 2001). Sec3 further directly binds to the PM by interaction with the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) (Zhang *et al.*, 2008). The cascade downstream of Cdc42 is thus well described. How is Cdc42 itself targeted to the incipient bud site? Crucial to its polar localization is its guanine nucleotide exchange factor Cdc24 that activates Cdc42 locally (Park and Bi, 2007), Bem1 adaptor protein (Butty *et al.*, 2002) that acts in Cdc42 localization positive feedback loop. Cdc42 is not statically bound to the PM, instead it is very dynamic. Despite this, its polar localization is maintained. This is probably achieved also by localized endocytosis of Cdc42 (Marco *et al.*, 2007) and the action of guanine nucleotide dissociation inhibitor Rdi1 (Slaughter *et al.*, 2009).
In plants, the importance of vesicle trafficking and secretion for cell elongation and polar growth is manifested in many mutants in these processes. For example, mutants in *Arabidopsis* ARF-GEF GNOM, which plays a role in vesicle coat recruitment and

localizes to endosomes are not able to establish polarity of the apical-basal axis (Geldner *et al.*, 2003; Mayer *et al.*, 1993). Mutants in the ECHIDNA gene, coding for a protein necessary for the trans-Golgi network function, fail to elongate their cells (Gendre *et al.*, 2011); mutants in the Rab geranylation machinery RGTB1 (Hála *et al.*, 2010) and the exocyst mutants (Hála *et al.*, 2008) fail to elongate their hypocotyls during etiolation. The exocyst is a heterooligomeric protein complex that acts prior the SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) assembly (see below) and together with small G-proteins of the Rab and Rho families underlies spatial specificity of vesicle-to-membrane fusion (Grote *et al.*, 2000; Guo *et al.*, 1999b; Guo *et al.*, 2001; Zhang *et al.*, 2001). The exocyst localizes to sites of active secretion (Hazuka *et al.*, 1999; TerBush and Novick, 1995). In yeast and mammals, the exocyst consists of eight subunits, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (Guo *et al.*, 1999a; Hsu *et al.*, 1998; TerBush *et al.*, 1996). Plant genomes encode homologs of all exocyst subunits (Eliáš *et al.*, 2003; Koumandou *et al.*, 2007). Most of them are encoded by two or more paralogous genes, the extreme being EXO70, encoded by a family of 23 genes in *Arabidopsis* thaliana (Synek *et al.*, 2006). This situation is dramatically different from Opisthokonts, where the exocyst subunits are encoded usually by single genes. Our group demonstrated (Hála *et al.*, 2008) that the exocyst exists as a complex in plant cells and that is is important for plant development and cell morphogenesis. *Arabidopsis exo70A1* mutants lacking the most abundantly expressed EXO70 isoform display multiple developmental defects (Synek *et al.*, 2006). Mutants in other exocyst subunits are defective in germination and tip growth of pollen tubes (Cole *et al.*, 2005; Hála *et al.*, 2008). Similarly, the maize SEC3 homolog RTH1 mutant produces short root hairs and is of small stature (Wen *et al.*, 2005). The exocyst was also revealed to be crucial for seed coat development (Kulich *et al.*, 2010) and pollen–pistil interaction (Samuel *et al.*, 2009). The study of plant exocyst regulators is only beginning; however, Lavy et al. (Lavy *et al.*, 2007) demonstrated that the SEC3 subunit interacts with plant-specific Rho GTPases via an adaptor protein ICR1 in *Arabidopsis* (see also introductions in Fendrych et al., 2010 and Fendrych et al., 2011).
The actual fusion of the vesicle with the target membrane is executed by the action of SNARE proteins (Söllner *et al.*, 1993) that represent the minimal fusion machinery. These proteins contain a transmembrane region and a coiled coil motif – SNARE motif. Their classification is based on amino acid residue in the centre of the SNARE motif – which is either Q (glutamine) or R (arginine). Q-SNARE are further divided into Qa, Qb, Qc and Qbc families. Q- and R-SNARE differ by their localizations – the Q-SNARE being present at the target membrane, and therefore referred to as t-SNARE also, whereas the R-SNARE is localized in the membrane of the arriving vesicle, and is designated v-SNARE or VAMP (Kato *et al.*, 2010; Lipka *et al.*, 2007). At least two Q- and one R- SNARE molecules form a metastable trans complex, bringing the opposing membranes in close proximity, this further changes into a cis complex, and the resulting energy is used for membrane fusion (Sørensen, 2009).

In plant cells, actin cytoskeleton drives motility and long distance transport of mitochondria, endoplasmic reticulum, Golgi stacks, peroxisomes, chloroplasts (Boevink *et al.*, 1998; Collings *et al.*, 2002; Sparkes *et al.*, 2008), and is also responsible for vesicle trafficking between endomembrane comparments (Grebe *et al.*, 2003) and to and from the PM (Dhonukshe *et al.*, 2008). In fact, in plant cells except for tip growing cells actin serves as the major intracellular cytoskeleton, as the majority of microtubules is located

within the cell cortex (Szymanski and Cosgrove, 2009), where they may act as stabilization anchors for organelles (Crowell *et al.*, 2009; Gutierrez *et al.*, 2009; Romagnoli *et al.*, 2007). According to the literature, the cortical cytoskeleton should be responsible for vesicle delivery to the exocytotic sites during diffuse growth, however, the actual description of these processes is rather scarce (Staiger *et al.*, 2009). What is the actual role of cytoskeleton in growth? Is it general cytoplasm organisation, propelling the Golgi motility, movement of secretory vesicles?

Actin cytoskeleton is vital for cell elongation, as was shown by František Baluška (Baluška *et al.*, 2001) on seedlings grown continuously with latrunculinB. Similarly, phenotype of the ACT2 x ACT7 actin isoforms double mutants ranged from seedling lethality to severe dwarfism (Gilliland *et al.*, 2002). By modulating the expression of an actin depolymerizing factor, another group (Dong *et al.*, 2001) confirmed the importance of filamentous actin for cell expansion. When the ADF was overexpressed, actin cables were not present and the cell size decreased. However, when the ADF was silenced using an antisense transcript, the cell size was greater than in wild-type controls. On the other hand, disruption of microtubule cytoskeleton leads generally to cell swelling (Baskin *et al.*, 1994; Sugimoto *et al.*, 2003).

An example of cytoskeleton involvement in morphogenesis and secretion is the system of interdigitated epidermal lobes of leaf cells. Here, the cells have to coordinate their growth in order to create a jigsaw-puzzle-like cell interlocking. It was hypothesised that microtubules are needed for restricting the growth in indetations, and, on the contrary, actin promotes the lobe growth. The lobe outgrowth is marked by PM localized ROP2 GTPase which is responsible for the F-actin localization into this site. ROP2 activates RIC4 to promote the F-actin assembly. Simultaneously, active ROP2 inactivates RIC1, whose activity promotes ordering of cortical microtubules (Fu *et al.*, 2005). Constitutively active ROP2 expression caused equal distribution of F-actin also outside the lobes. The microtubules restricting growth and actin promoting growth hypothesis seems to be oversimplified, not only because microtubule bundles are found also within the tips of the lobes (Zhang *et al.*, 2011). Recently, plant hormone auxin was implied in regulation of epidermal cell lobes (Xu *et al.*, 2010).

The cytoskeleton might also regulate and modify growth at selected sites within the cell cortex by targeting the Golgi apparatuses to selected locations. The mutant of kinesin 13A produces trichomes with additional branches. This protein localizes to the Golgi apparatus, and probably attaches it to cortical microtubules. The authors suggested a model, where the Golgi is transported to the cell periphery by the actomyosin system, and in the cell periphery, the kinesin13A distributes the Golgi to trichome branches (Lu *et al.*, 2005; Smith and Oppenheimer, 2005). Here, the ANGUSTIFOLIA protein might promote Golgi secretory activity after its interaction with a kinesin KCBP at the desired site (Smith and Oppenheimer, 2005). The trichome branch elongation is dependent on the actin-nucleating Arp2/3 complex; mutations in the latter lead to the distorted phenotypes (Li *et al.*, 2003). Actin is still present in these cells, randomly localized peripheral actin patches are present also, but cytoplasmic actin bundles seem to be affected by the mutation (Saedler *et al.*, 2004). Surprisingly, the Arp2/3 disruption affects mostly trichomes and pavement cells, other cells remain unaffected, including polarly growing root hairs and pollen tubes. The Arp2/3 thus seems to be vital for the trichome diffuse growth. However, disorganised MT in the Arp2/3 mutants also document the interplay between MT and actin networks (Schwab *et al.*, 2003).

Dolja and colleagues (Prokhnevsky *et al.*, 2008) studied mutants in *Arabidopsis* myosins

class XI, and discovered that these have problems with cell elongation and root hair growth. Authors observed impeded motility of organelles, including the Golgi apparatuses. The authors suggest to replace the concept of cytoplasmic streaming which distributes organelles within the cells, by cytoplasmic stirring, where the organelles move independently using associated motors (Avisar *et al.*, 2008) and their saltatory movements propel movement of the surrounding cytoplasm (Ueda *et al.*, 2010).
The "Golgi delivery model" resembles the "recycling domains" model (Žárský *et al.*, 2009). In this model, a delimited domain on the PM is connected to a set of endosomes, trans-Golgi networks and Golgi apparatuses by the actin cytoskeleton. Sub-lethal doses of actin disrupting drugs would thus lead to disruption of such recycling domains and to defects in cell polarity.
The most prominent example of the interplay between cytoskeleton and shape creation, morphogenesis, is represented by microtubules and cellulose-synthase complexes. The cell wall is anisotropic – possesses different mechanical properties in different directions – mainly due to anisotropic alignment of cellulose microfibrils. The alignment is directed by the cytoskeleton, especially by microtubules (Chan *et al.*, 2007; Paredez *et al.*, 2006). The relationship between microtubules and cellulose synthase complexes has been a subject of a debate, because in some cases, ordered microfibrile arrays are formed also in the absence of microtubule cytoskeleton (Pickard, 2008; Sugimoto *et al.*, 2003). The cellulose-synthases are transmembrane enzymes that catalyse synthesis of cellulose, which is further embedded in a polysaccharide matrix secreted into the wall by Golgi derived vesicles. The cellulose-synthases travel along microtubules and are usually deposited by the Golgi/trans-Golgi network directly onto a microtubule in the cell cortex (Crowell *et al.*, 2009; Gutierrez *et al.*, 2009; Paredez *et al.*, 2006; Wightman and Turner, 2008), and thus microtubules are important not only for the trafficking of the complexes, but also for recruitment of cellulose-synthase complexes to the PM. Microtubules also underline sites of massive secretion of pectic mucilage in *Arabidopsis* seed coat cells (McFarlane *et al.*, 2008), and accompany secretion sites in spiral thickening in protoxylem development (Fukuda, 1997; Roberts *et al.*, 2004).
Secretory system and cytoskleletons closely interact also in another type of plant cell growth – the tip growth. A pollen tube provides a nice example of an actin function diversification within the same cell: longitudinal actin cables serve as tracks and scaffold for cytoplasmic streaming. The apical actin fringe is crucial for tip growth but its precise function has not been elucidated (Staiger *et al.*, 2010). The apical fringe of actin might coordinates the vesicle delivery and fusion to the pollen apex (Lee and Yang, 2008). This happens in a ROP1 dependent manner, where ROP1 downstream target RIC4 promotes actin accumulation that in turn results in vesicle accumulation in the tip. On the other hand, the ROP1 target RIC3 increases cytosolic Ca++ , leading to actin depolymerization and to exocytosis of the accumulated secretory vesicles (Lee and Yang, 2008). The very tip of the pollen tube, so called clear zone, is believed to be devoid of filamentous actin, or, more precisely, probably some actin filaments are presents, but definitively less than in the actin fringe (Staiger *et al.*, 2010). Therefore, the pollen tube tip is a nice example of a domain that might be free of cytoskeleton. Actin cytoskeleton might deliver secretory vesicles and organelles producing secretory vesicles to the clear zone (Cheung *et al.*, 2010). In this zone, secretory vesicles might be recognized by tethering complexes and fuse with the PM.

So far, I have presented examples of situations where cytoskeleton is necessary for

organelle and vesicle trafficking and delivery to or delimitation of zones of active growth. Does the cytoskeleton play any role in the process of exocytosis itself? It is known that actin is crucial for endocytosis, it localizes to endocytotic sites and probably plays a role in endocytic vesicle separation from the donor membrane (Taylor *et al.*, 2011). In the case of exocytosis, the situation is more obscure, and I am aware of only a few reports where actin plays a direct role in exocytosis. In many cases, actin is necessary for the secretory vesicle delivery (see above) or organization of secretory machinery, as in the case of chromaffin cells (Torregrosa-Hetland *et al.*, 2011). It is believed that actin acts as a barrier against docking of secretory insulin granules (Wang and Thurmond, 2009). Another work reports actin interaction with Syntaxin 4 that should negatively regulate exocytosis (Jewell *et al.*, 2008). On the other hand, actin may push out the content of lamellar bodies in lung cells during exocytosis (Miklavc *et al.*, 2010). Also actin based motor myosins were reported to participate in exocytosis. Myosin 5a was implicated in docking of secretory granules, this protein also interacts with the t-SNARE syntaxin-1i (Loubéry and Coudrier, 2008). Myosin VI has a role in vesicle fusion, colocalizes with secretory vesicles at PM, and its depletion increases the number of unsuccessful vesicle fusions in animal cells (Bond *et al.*, 2011). The role of myosin could inhere in the fusion pore expansion, similarly to dynamins (Anantharam *et al.*, 2011).

1.2. Interconnection between actin and microtubule cytoskeletons

Cortical cytoskeleton is of a special importance in plant cells as the centre of cells is often occupied by a large vacuole. Actin and microtubule cytoskeletons are coordinated, which is prominent during intracellular transport (Romagnoli *et al.*, 2007), plant cell division (Li *et al.*, 2010b), diffuse growth (Gutierrez *et al.*, 2009), pathogen defense (Hardham *et al.*, 2008). One could expect that interference with one type of cytoskeleton would affect also the other cytoskeletal system. Examples of this phenomenon are present in the literature (Saedler *et al.*, 2004; Schwab *et al.*, 2003). A few proteins connecting actin and microtubule cytoskeletons were described. These include motor proteins with the affinity for the other type of cytoskeleton – kinesins with calponin homology domain from cotton and rice (Frey *et al.*, 2009; Preuss *et al.*, 2004; Xu *et al.*, 2009).
Arp2/3 and formins are known to nucleate actin (Michelot *et al.*, 2005; Welch *et al.*, 1997). Formins can generate unbranched actin filaments by nucleation achieved by their FH2 domains, and act as processive, leaky caps (Kovar and Pollard, 2004). In plants, it was suggested that, after the nucleation event, AtFH1 moves to the side of the actin filament and remain attached here and can form actin bundles (Michelot *et al.*, 2006). In addition to their actin nucleation/binding activity, animal formins mDia1, mDia2, FMN1 and INF1, CAPU associate with microtubules (Bartolini *et al.*, 2008; Palazzo *et al.*, 2001; Rosales-Nieves *et al.*, 2006; Zhou *et al.*, 2006) and regulate their dynamics. Overexpression of mDia1 and mDia2 stabilized microtubules at their plus ends, and also these proteins colocalized with a subset of microtubules (Bartolini and Gundersen, 2010; Bartolini *et al.*, 2008; Palazzo *et al.*, 2001). Another link of mDia to MT is represented by its interaction with plus end binding protein CLIP170 (Lewkowicz *et al.*, 2008).
Further level of coordination in the cortical area would be represented by proteins that associate with the PM and interact with cytoskeletons. Already in the first electron micrographs of plant cortical cytoplasm, it was obvious that microtubules are closely associated with the PM (Ledbetter and Porter, 1963), and later, electron dense junctions, most likely proteins, crosslinking microtubules and PM were observed (Barton *et al.*,

2008; Hardham and Gunning, 1978). Plant PIP2 dependent Phospholipase D beta is associated with PM and is able to interact with microtubules (Gardiner *et al.*, 2001). This protein also harbours an actin interacting site and therefore it might serve as an "intelligent anchor" whose activity is stimulated by F-actin and inhibited by G-actin (Pleskot *et al.*, 2010). Some plant formins, unlike their Opisthokont counterparts (with exceptions in Nematoda and Annelida), posses an N-terminal secretory signal sequence followed by a transmembrane domain. Angiosperm formins belong to two distinct subfamilies (Cvrčková, 2000; Grunt *et al.*, 2008). Class I formins have a putative signal peptide and predicted N-terminal transmembrane domain (the only exception is the formin AtFH7 that lacks the transmembrane domain), whereas class II formins have a PTEN-like domain that also associates these protein with the PM (Vidali *et al.*, 2009). If the microtubule binding activity were conserved between Opisthokonts and plants, formins could link PM and microtubule cytoskeleton. Analogously, formins are able to integrate actin nucleating/binding activity with membrane localization. AtFH6 localized in the PM and involved in giant cell formation during nematode infection (Favery *et al.*, 2004), AtFH5 is localized to the cell plate and participates in endosperm cellularization (Ingouff *et al.*, 2005). In the male gametophyte – the pollen tube – the role of membrane localized formin AtFH5 in subapical actin assembly was demonstrated. AtFH5-GFP localized to the tip PM of pollen tubes, and from here it nucleated the subapical actin structure – the actin fringe (Cheung *et al.*, 2010), and AtFH1 overexpression caused excess actin filament formation at the pollen tube membrane, though the expression level of this protein in wild type pollen is negligible (Cheung and Wu, 2004). These findings implicate formins as likely candidates for the integration of PM, microtubule and actin cytoskeletons.

1.3. Exocyst and cytoskeleton

In *S. cerevisieae*, the actin cytoskeleton and associated motor proteins are vital for polarization of the cell and for the delivery of secretory vesicles to the growing bud (Pruyne *et al.*, 1998), and also the recruitment of 6 out of 8 exocyst subunits to the bud is dependent on the actin cytoskeleton (Boyd *et al.*, 2004). The situation is obscured by the fact that in yeast, mRNAs for polarity factors such as exocyst subunits are often transported to the bud by the acto-myosin system (Yakir-Tamang and Gerst, 2009), which further tangles interpretation of the results. The situation might differ dramatically in other model organisms. In another yeast, *S. pombe*, neither inactivation of actin cytoskeleton by generation of a triple formin mutant, nor further elimination of microtubules by drug treatment abolished the ability of the cells to elongate. The *S. pombe* cells are able to perform polar growth in the absence of long distance vesicle transport. When the exocyst was disrupted by mutation, the situation was similar – cytoskeletons were able to sustain polar growth, albeit for a limited time. This means that the either of the pathways – cytoskeletons and the exocyst – is able to support the vesicle delivery and fusion with the cell poles. Only the simultaneous destruction of actin and exocyst lead to isotropic swelling of the *S. pombe* cells, i.e. to failure of the polarization of vesicle cargo delivery. Even in this situation, Cdc42 remained polarized to cell poles. The ability of the exocyst to localize to the correct site was independent of actin and MT cytoskeleton, but depended on PIP2 and Cdc42 (Bendezú and Martin, 2011). PIP2 probably provides means to anchor at the PM whereas Cdc42 constitutes the localization clue (See also the introduction section in Fendrych et al., 2011). Conversely, the actin

module members (For3, Myo52 and actin cables) were present and correctly localized in sec8 mutant cells. In conclusion, the "mantra" that the exocyst rides the vesicles on actin cables does not seem to be valid in *S. pombe*.
Apart from indirect interactions, subunits of the exocyst complex were shown to interact with cytoskeletons directly. In mammalian cells, Wei Guo and colleagues (Zuo *et al.*, 2006) discovered that the EXO70 exocyst subunit interacts with the Arp2/3 complex. Downregulation of EXO70 led to defects in cell motility. It is however difficult to separate the effects of impaired secretory pathway and modified actin polymerization. On the other hand, overexpression of the EXO70, and not other exocyst subunits, stimulated formation of membrane protrusions, suggesting the role of this protein in actin nucleation. The exocyst was also observed to coimmunoprecipitate with microtubules in rat brain cells (Vega and Hsu, 2001) and to colocalize with microtubule cytoskeleton and mitotic spindles in normal rat kidney cells (Wang *et al.*, 2004). The authors tested the influence of an *in vitro* reconstituted exocyst complex on tubulin dynamics, and showed that it inhibits tubulin polymerization. The inhibition activity was dependent on the presence of Sec5, Sec6, Sec15 and Exo70 subunits, the latter being able to inhibit polymerization on its own. Overexpression of Exo70 disrupted the microtubule network in the cells, and, as was mentioned above, led to filopodia-like protrusion formation. These results suggest that the exocyst subunits are often bi- or multi-functional and further underline the intimate relationship between exocytosis and cytoskeletons that might be mediated by the exocyst complex.

1.4. Plant cell division as a polarity case study

Plant cells often fulfil their roles via their cell walls, the extreme examples being leaf trichomes or xylem vasculature. The plant cell wall can be perceived as a vital part of a plant cell – it participates in perceiving pathogen attacks (Galletti *et al.*, 2009), its properties including porosity can be regulated (Willats *et al.*, 2001), its components are recycled in various instances, including cell division (Baluška *et al.*, 2005; Dhonukshe *et al.*, 2006) and stomatal closure (Apostolakos *et al.*, 2010). The cell wall should be thus understood as another cell compartment (Szymanski and Cosgrove, 2009), however, its presence impedes mobility of plant cells. Therefore the orientation of cell division is of great importance. Cell division is a fascinating process during which the cell duplicates or multiplicates itself. During cell separation, the distribution of structures and organelles must be regulated so the daughter cells remain viable. In plant cells, cytoskeletons are crucial for determining the plane of cell division, for the action of the mitotic apparatus, and for the execution of cytokinesis. The division plane is determined by the preprophase band, consisting mainly of microtubules and also actin (Fowler and Quatrano, 1997; Panteris *et al.*, 2009). This structure arises at the end of the G2 phase of the cell cycle. It predicts the plane of cell division, the nucleus migrates into the middle of the preprophase band. This plant specific structure disappears in prophase/metaphase. Mutants FASS/ TONNEAU2, in which the regulatory B subunits of protein phosphatase PP2A complex is impaired, lack the preprophase band, and consequently, cell division planes are chaotic (Camilleri *et al.*, 2002; Traas *et al.*, 1995). This likely demonstrates the importance of phosphorylation for the microtubular dynamics regulation during preprophase band formation. In a way, the preprophase band can be perceived as an extreme of cortical microtubule array, which gives rise to the band by altered microtubule dynamics. It was speculated that the preprophase band leaves a trace, or locally modifies the membrane/cell

wall so it is later capable of fusion with the growing cell plate (Mineyuki and Gunning, 1990). There are a few markers that characterize the preprophase band site after its disassembly. It is the so called actin-depleted zone, or more precisely, zone with less abundant actin (Panteris, 2008). This zone is also depleted in kinesin KCA1 (Vanstraelen *et al.*, 2006). After the band disassembly, the site is continuously marked by TANGLED protein (Walker *et al.*, 2007), whose mutations lead to disorganized cell divisions. In onion epidermal cells, the proprophase band is a localized centre of active endocytosis, on the other hand, no increase in exocytosis was observed (Karahara *et al.*, 2009). The function of the preprohase band seems to be linked with division orientation in multicellular tissues, as it is absent in algae, in gametophytic and syncytial cytokinesis and also in some cell cultures (in Van Damme and Geelen, 2008). Also the fact that the TON2/FASS mutants still divide, albeit chaotically, stresses the importance of the preprophase band for cell division in the tissue context.

The phragmoplast is another plant specific structure, that distantly resembles the midbody of animal cells. It is a cytoskeletal – membrane structure. Both microtubules and actin participate in phragmoplast functioning (Higaki *et al.*, 2008). The role of phragmoplast is to create a new cell wall between daughter cells. Phragmoplast grows centrifugally towards the sites marked previously by the preprohase band, lying foundations of a new cell wall, termed the cell plate, during its growth, in its centre. After chromosome separation, membrane vesicles arrive at the division plane into a so called cell plate assembly matrix to form a late anaphase vesicle cloud. Vesicles are transported into the middle of the phragmolast by motor proteins including kinesins (Vanstraelen *et al.*, 2006). Fused vesicles are stretched into dumbbells, fuse with other vesicles, and further join together giving rise to a tubulo-vesicular network. The network ultimately forms a fenestrated sheet that is consolidated with callose. Involvement of several SNARE-type proteins in cell plate vesicle fusion was documented (Heese *et al.*, 2001; Lauber *et al.*, 1997; Seguí-Simarro *et al.*, 2004). There has been a long debate about the source of the vesicles for the cell plate. These include vesicles derived from the Golgi apparatus, but also vesicles containing recycled material from the PM, including lipids and polysaccharides (Baluška *et al.*, 2005; Dhonukshe *et al.*, 2006; Reichardt *et al.*, 2007; Samuels *et al.*, 1995; Seguí-Simarro *et al.*, 2004). This is exemplified by the fact that the endocytotic tracer FM4-64 stains growing cell plates rapidly, although this stain does not label Golgi apparatus (Bolte *et al.*, 2004*; Chow *et al.*, 2008). It is believed that the cell plate has a trans-Golgi-like identity, more specifically a Rab-A2/A3 compartment identity (Chow *et al.*, 2008).

The final step of the cell plate growth is its insertion into the parent cell wall. A few factors have been identified in this process, including the T-PLATE protein, involved in endocytosis (Van Damme *et al.*, 2006; Van Damme *et al.*, 2011), microtubule associated protein AIR9 (Buschmann *et al.*, 2006), and an extensin RSH (Hall and Cannon, 2002).

Successful cytokinesis requires coordination of cytoskeletons with membrane trafficking. The timing of cell plate growth must be precise, as well as its targeting to the site previously marked by the preprophase band (Dhonukshe *et al.*, 2005), and at this site, the cell plate must fuse with the parent membrane and physically separate the daughter cells.

* In this work, authors report Golgi labelling by FM4-64, but the Golgi markers they used actually stain the trans-Golgi network.

1.5. High resolution imaging of secretory vesicles at the plasma membrane

Fluorescence microscopy and tagging proteins of interest with green fluorescent protein (and other fluorescent proteins) utterly changed cell biology. Visualization of proteins within their native environment in real time in vivo is invaluable for elucidating their roles. Microtubule cytoskeleton is readily observable using confocal microscopy, whereas actin is more dynamic and its visualization by this technique is slower than necessary (Staiger *et al.*, 2009). Secretory and endocytic vesicles are practically invisible in confocal microscopy, due to their small size and ubiquitous background fluorescence. For this reason, widefield total internal reflection microscopy (TIRFM) is used (Axelrod, 2003), that illuminates object that are in close proximity (approximately 100 nm) to the coverslip. This method has been used to visualize single vesicles and secretion of their cargo in mammalian cells (Burchfield *et al.*, 2010; Verhage and Sørensen, 2008). In a breathtaking study, Christien Merrifield with colleagues (Taylor *et al.*, 2011) decoded the recruitment dynamics of 34 proteins at the endocytic site during clathrin mediated endocytosis. They took advantage of the sensitivity of fluorophores to changes in pH, and by changing the pH of the medium by perfusion every 2 seconds, they were able to distinguish the precise moment when the vesicle pinch of the PM and use this time point as a reference point. However, plant cells are embedded in their cell walls, which presents a substantial disadvantage models for similar experiments. Cell walls not only prevent similar manipulation of extracellular medium but also thwart the usage of TIRF microscopy, as their thickness is larger than the reach of the exciting light in this method. Therefore a modification of the TIRFM is used for plant studies – variable-angle epifluorescence microscopy (VAEM; (Konopka and Bednarek, 2008b). Clathrin endocytosis has been studied in plants with the help of this technique, and the assembly of clathrin and dynamin-related proteins at single endocytic sites in *Arabidopsis* was visualized (Fujimoto *et al.*, 2010; Konopka and Bednarek, 2008a; Konopka *et al.*, 2008). However, the secretory pathway and visualization of secretory vesicles remains obscure. The function of the exocyst tethering complex inheres in its precisely defined localization. The available data present localizations of the bulk of exocyst proteins to membrane domains associated with high secretion activity both in animal and plant cells (Fendrych *et al.*, 2010; Finger *et al.*, 1998; Gromley *et al.*, 2005; Lipschutz *et al.*, 2000) but description of the exocyst localization at high resolution is scarce in animal cells (Tsuboi *et al.*, 2005) and absent in plant cells. In the third paper included in this thesis, we used the available constructs of the exocyst subunits and attempted to elucidate their behaviour during exocytosis.

2. Questions, aims of the thesis

What is the role of formins in the molecular machinery interconnecting the two types of cortical cytoskeletons and plasma membrane in plant cells?

Plant cytokinesis depends on coordinated action of cytoskeleton and focused secretion into the developing cell plate. What is the role of the exocyst in this process?

The exocyst localizes to highly secretory active plasma membrane domains. Is it possible to visualize subunits of the exocyst complex at exocytic sites using a high resolution imaging technique?

3. Results

3.1. Title: The plant formin AtFH4 interacts with both actin and microtubules, and contains a newly identified microtubule-binding domain

Authors:
Michael J. Deeks*, Matyáš Fendrych*, Andrei Smertenko, Kenneth S. Bell, Karl Oparka, Fatima Cvrčková, Viktor Žárský, and Patrick J. Hussey

* equal contribution

Summary:
The dynamic behaviour of the actin cytoskeleton in plants relies on the coordinated action of several classes of actin-binding proteins (ABPs). These ABPs include the plant-specific subfamilies of actin-nucleating formin proteins. The model plant species *Arabidopsis* thaliana has over 20 formin proteins, all of which contain plant-specific regions in place of the GTPase-binding domain, formin homology (FH)3 domain, and DAD and DID motifs found in many fungal and animal formins. We have identified for the first time a plant- specific region of the membrane-integrated formin AtFH4 that mediates an association with the microtubule cytoskeleton. In vitro analysis shows that this region (named the GOE domain) binds directly to microtubules. Overexpressed AtFH4 accumulates at the endoplasmic reticulum membrane and co-aligns the endoplasmic reticulum with microtubules. The FH1 and FH2 domains of formins are conserved in plants, and we show that these domains of AtFH4 nucleate F-actin. Together, these data suggest that the combination of plant-specific and conserved domains enables AtFH4 to function as an interface between membranes and both major cytoskeletal networks.

My contribution:
I performed part of the experiments together with Mike Deeks (subcloning of some of the AtFH4 fragments, confocal imaging of transiently transformed *N. benthamiana* and *A. thaliana*, protein expression and purification, cosedimentation assays, “bead assay”).
I participated in writing of the text by editing the manuscript prepared by Mike Deeks.

Supplemental material:
Fig. S1 – S8 and Movie 1 – 3 are are part of Deeks et al., 2010

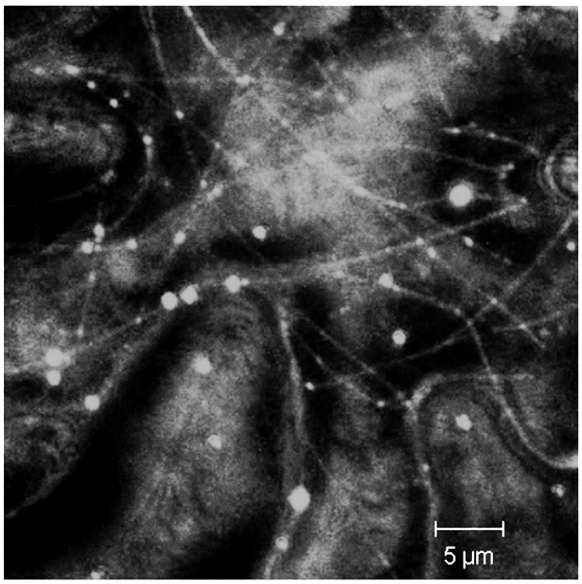

Fig. S1. GFP-AtFH4Δ1 binds to *A. thaliana* microtubules. Construct GFP-AtFH4Δ1 was transformed into *A. thaliana* leaf epidermis using particle bombardment.

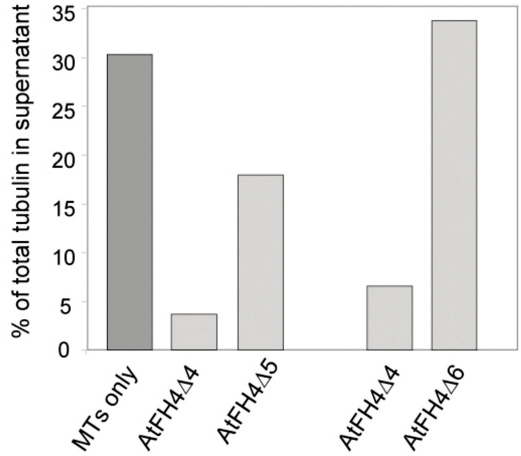

Fig. S2. Fragments of AtFH4 that bind microtubules (MT) promote MT precipitation in co-sedimentation assays. The histogram shows the proportion of tubulin present in the supernatant fraction after co-sedimentation in the presence of AtFH4 recombinant protein fragments. The first three bars represent measurements taken from the gel in the upper panel of Fig. 4C, whereas the last two bars show measurements taken from the gel in the lower panel of Fig. 2C. AtFH4Δ4 (containing the GOE and FH1 domains) reduces tubulin in the supernatant over fivefold when compared with MTs alone or the non-MT-binding fragment AtFH4Δ6.

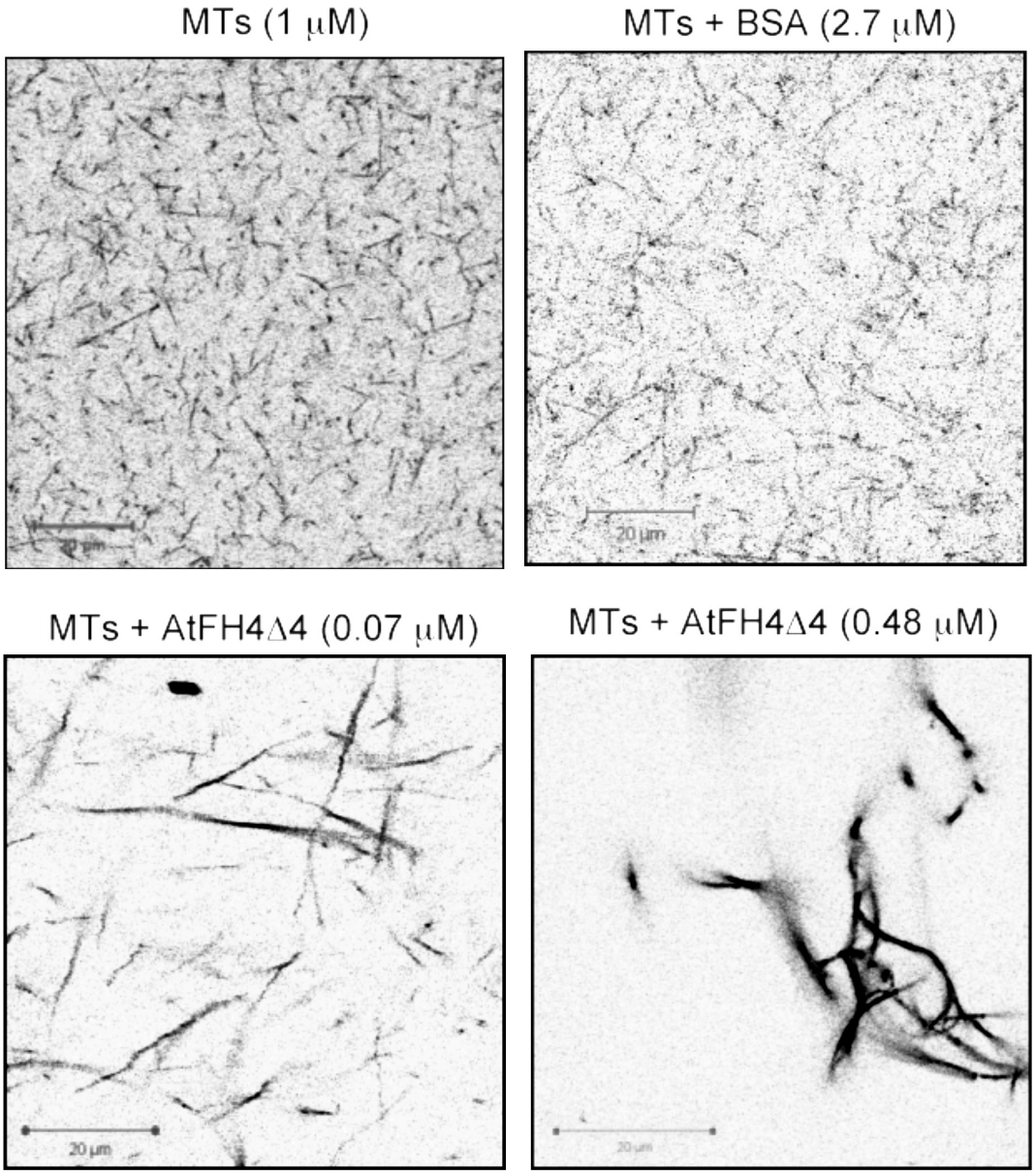

Fig. S3. AtFH4 can stimulate the bundling of taxol-stabilised microtubules. Taxol-stabilised microtubules labelled with Oregon Green were incubated with AtFH4Δ4. Increasing concentrations of AtFH4Δ4 are associated with increased aggregation of microtubules. Similar results were also obtained using AtFH4Δ1 (not shown).

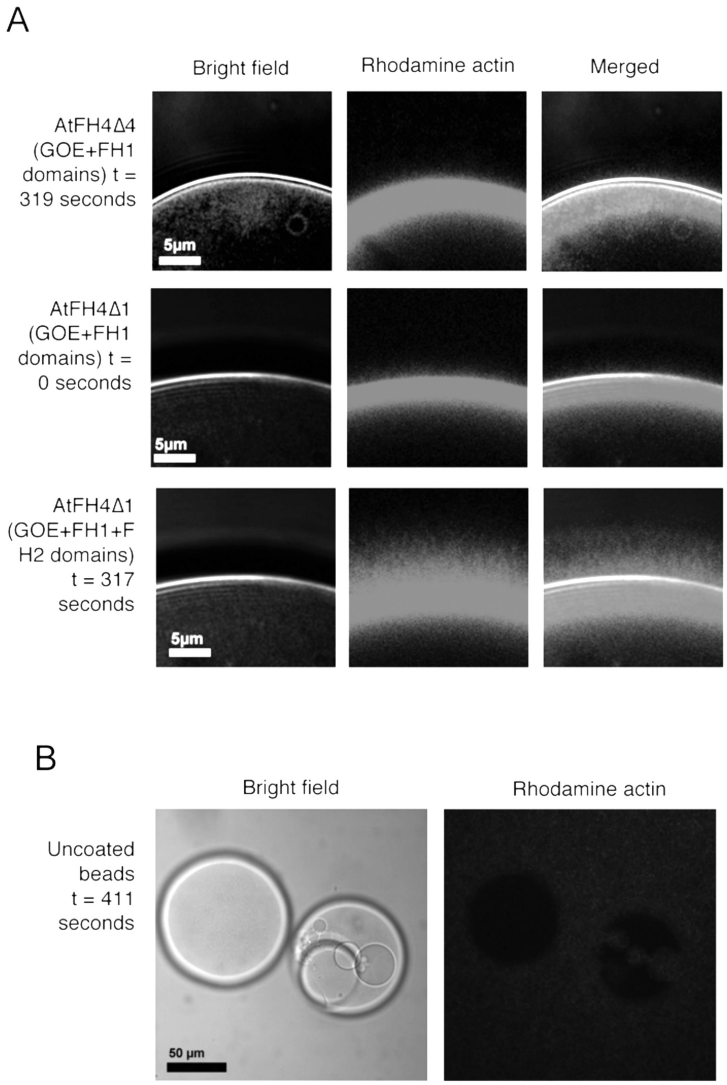

Fig. S4. Efficient seeding of actin filaments on Ni-NTA beads requires the complete cytosolic domain of AtFH4. (A) The left column shows contrast-enhanced bright-field images of Ni-NTA beads. The central column shows rhodamine-actin fluorescence at the time points indicated. Merged images are presented in the right column. An actin filament corona does not develop on beads coated with AtFH4Δ4. This fragment contains the GOE domain and FH1 domain, but lacks the FH2 domain. Images of beads coated with AtFH4Δ1 (the complete cytosolic fragment, including the GOE, FH1 and FH2 domains) taken at an equivalent time point show the association of an F-actin corona that was not present at time 0 (also see supplementary material Movie 1). (B) Uncoated Ni-NTA beads do not seed filaments or associate with rhodamine-actin monomers. Rhodamine fluorescence is excluded from the beads.

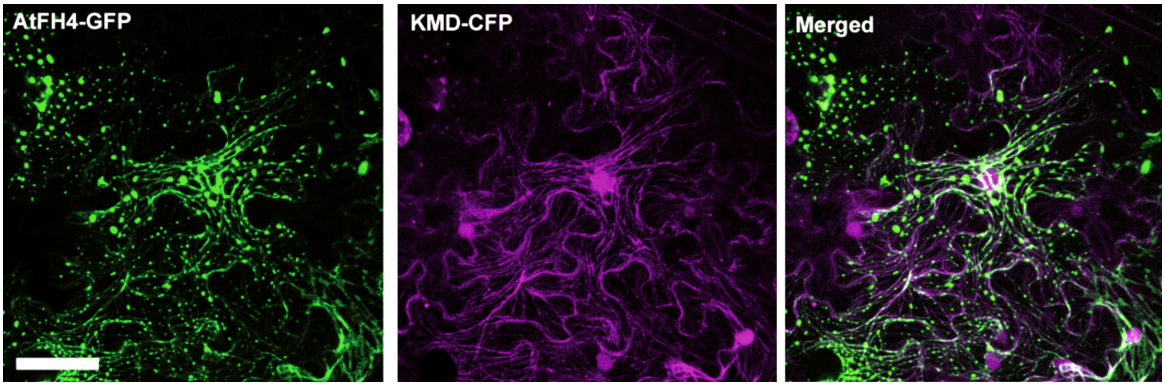

Fig. S5. AtFH4-GFP can have a globular appearance, but consistently co-aligns with the microtubule cytoskeleton. Epidermal cells are co-transformed with AtFH4-GFP and KMD-CFP. Scale bar: 50 µm.

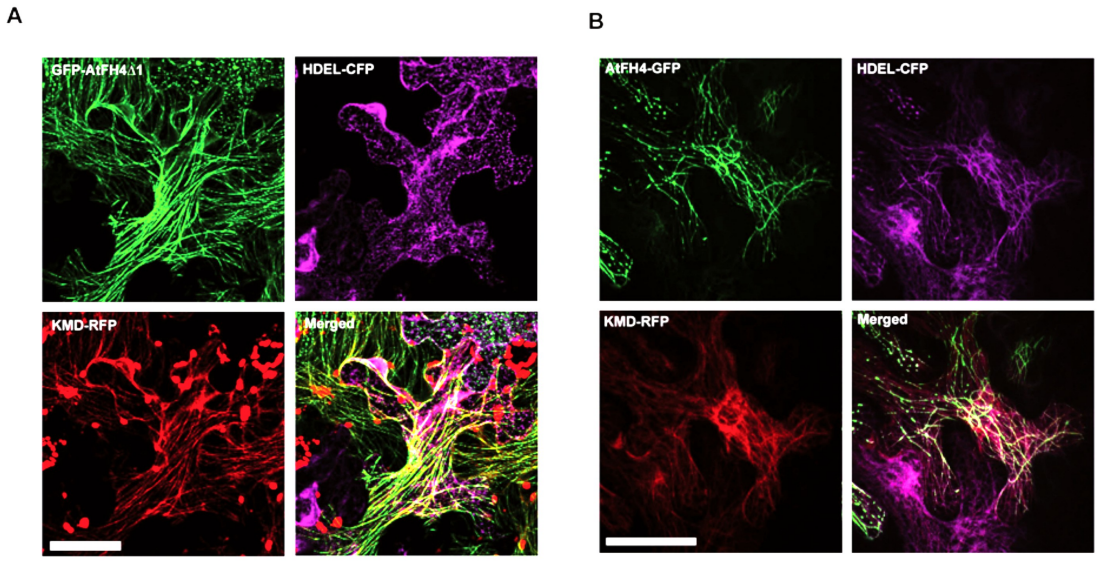

Fig. S6. Low-magnification images of AtFH4-GFP, KMD-RFP and HDEL-CFP coexpression within epidermal cells. (A) Cells expressing the control construct GFP-AtFH4Δ1, containing the cytosolic domains of AtFH4. (B) Cells expressing transmembrane protein AtFH4-GFP. The ER network does not align with the microtubule cytoskeleton in the presence of GFP-AtFH4Δ1, but cotransformation with AtFH4-GFP results in ER-microtubule co-alignment. Scale bar: 50 µm.

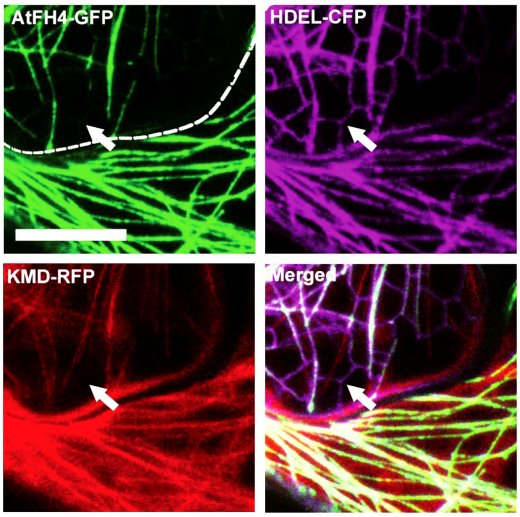

Fig. S7. Zones of the ER co-aligned with microtubules in the presence of AtFH4-GFP can be connected together by ER tubules that are not associated with microtubules. The two epidermal cells in this image are transformed with AtFH4-GFP, HDEL-CFP and KMD-RFP. The boundary between the cells is shown as a white dashed line within the AtFH4-GFP panel. ER tubules connecting two sections of MTs co-aligned with ER are indicated by a white arrow. AtFH4-GFP is enriched in sections of the ER that are co-aligned with MTs. Scale bar: 10 µm.

Fig. S8. Nucleotide sequence of KMD probe:
ATGGCGACAAGACAAGCGAAATCCCAAAAATTGAGCTCAAGAGTAGCAAATT
CACCTGCGTCATCAACCACATCATCATCTAAGCAGTTTCCAGAAAATTCCATAG
ACGGCGTGAGCTCGCCGGCGTCCTCATCAGCGAGAAGTAAGCCACAGTACTAT
TACTCGGAGAGTGCGTCCGCAGACACAACGGAGAGATCCAAAGAGAATGTTA
CAGTGACAGTGCGGTTCCGACCTCTAAGTCCGAGGGAGATACGACAAGGGGA
GGAAATCTCATGGTATGCAGATGGAGAAACTATTGTTAGAAATGAGCATAATCC
TACGCTAGCGTATGCATATGATAAGGTTTTTGGTCCTACCACCACAACACGTCAT
GTCTATGATATTGCTGCTCAGCATGTTGTTGGTGGTGCCATGGAAGGCATCAAC
GGCACGATCTTCGCGTATGGGGTTACAAGCAGTGGGAAAACCCATaCCATGCAT
GGTGATCAAAGATCTCCTGGTATTATACCATTGGCTGTGAAGGATGCCTTCAGC
ATTATTCAAGAGACTCCGGACCGAGAATTTCTGCTTCGCCTCTCATACCTGGAG
ATCTACAATGAGGTTGTTAATGACTTGCTAAATCCAGCTGGGCAGAATTTAAGA
ATCCGAGAGGACACTCAGGGTACCTTTGTGGAAGGAATTAAGGAGGAGGTCG
TTTTATCCCCTGCTCATGCTCTATCCCTTATTGCTGCAGGGGAAGAGCATAGGCA
TGTTGGTTCCACAAACTTCAATCTACTCAGTAGCAGAAGTCATACAATATTTAC
GCTGACAATAGAAAGTAGCCCCTGTGGTGAATATAGTGAAGGAGGGGCAGTTA
CCTTGTCACAGCTGCATCTTATTGATTTGGCAGGTTCAGAGAGCTCAAAGGCTG
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ATATAGAGATTCAAAATTGACCAGGCTTCTTCAATCCTCATTAAGTGGTCAAGG
ACGTGTATCTTTAATCTGCACTGTGACTCCTTCGTCAAGCAATTCTGAAGAGAC
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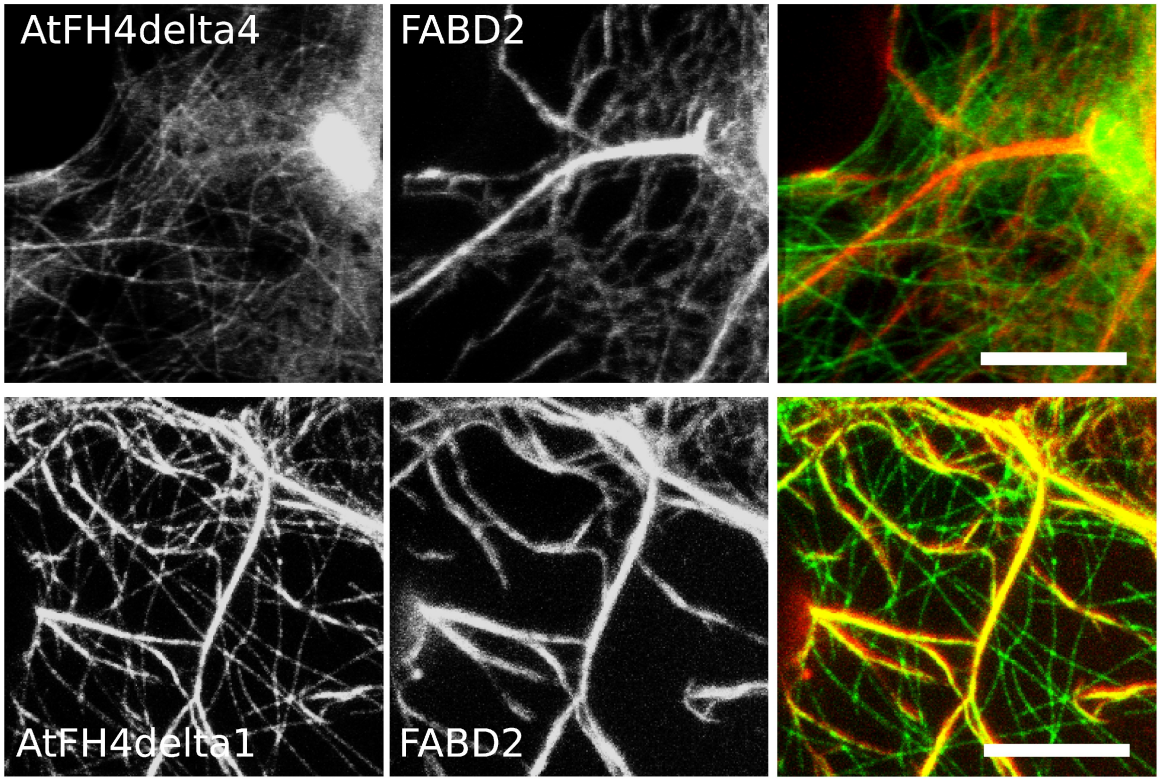

Fig. S9. AtFHΔ1 occasionally induced coalignment of MT and actin labelled by dsRed-FABD2; this was not observed with AtFH4Δ4; scale bars: 10 µm.

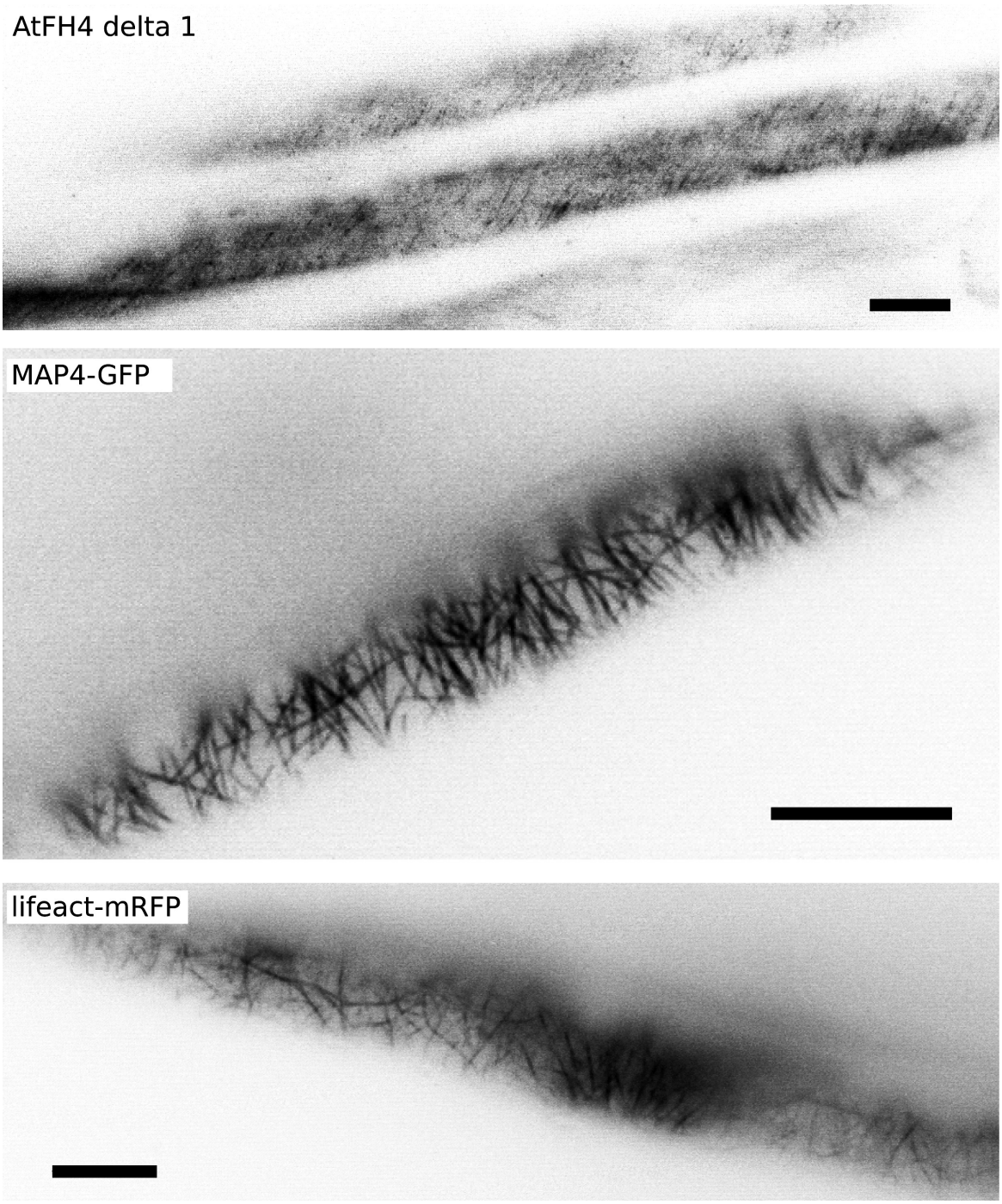

Fig. S10. AtFH4Δ1 decorated a MT like network in root epidermal cells of stably transformed *A. thaliana* (upper image). Localization of MT binding MAP4-GFP (Marc *et al.*, 1998) and lifeact-mRFP (see Fendrych et al., 2011) are shown. All images were obtained using the VAEM technique (see Fendrych et al., 2011), scale bars: 10 µm.

Movie 1. AtFH4Δ1-decorated microtubules are dynamic. A time series of microtubules labelled with CFP-AtFH4Δ1 was taken at intervals of 6 seconds. Elongating microtubules and microtubules undergoing catastrophe are both visible.

Movie 2. Dynamic F-actin associates with AtFH4-coated sepharose beads. Images were taken every 100 seconds and are replayed at 12 frames per second. Beads coated with shorter subfragments containing the FH2 domain (AtFH4Δ2 and AtFH4Δ3) do not associate with F-actin.

Movie 3. Latrunculin B inhibits F-actin polymerisation associated with formin-coated beads. The specific action of 10 μM latrunculin B demonstrates that the increased rhodamine fluorescence presented in Movie 1 is derived from F-actin formation associated with beads coated with AtFH4.

Movie 4. AtFH4Δ1 decorates MT like structures in root epidermal cells of *A. thaliana*. Imaged by VAEM, scale bar: 10 μm.

Movie 5. MAP4-GFP (Marc *et al.*, 1998) localization to MT in root epidermal cells of *A. thaliana*. Imaged by VAEM, scale bar: 10 μm.

Movie 6. lifeact-mRFP (see Fendrych et al., 2011) decorates actin cytoskeleton in root epidermal cells of *A. thaliana*. Imaged by VAEM, scale bar: 10 μm.

3.2. Title: The Arabidopsis Exocyst Complex Is Involved in Cytokinesis and Cell Plate Maturation

Authors:
Matyáš Fendrych, Lukáš Synek, Tamara Pečenková, Hana Toupalová, Rex Cole, Edita Drdová, Jana Nebesářová, Miroslava Šedinová, Michal Hála, John E. Fowler, and Viktor Žárský

Summary:
Cell reproduction is a complex process involving whole cell structures and machineries in space and time, resulting in regulated distribution of endomembranes, organelles, and genomes between daughter cells. Secretory pathways supported by the activity of the Golgi apparatus play a crucial role in cytokinesis in plants. From the onset of phragmoplast initiation to the maturation of the cell plate, delivery of secretory vesicles is necessary to sustain successful daughter cell separation. Tethering of secretory vesicles at the plasma membrane is mediated by the evolutionarily conserved octameric exocyst complex. Using proteomic and cytologic approaches, we show that EXO84b is a subunit of the plant exocyst. *Arabidopsis thaliana* mutants for EXO84b are severely dwarfed and have compromised leaf epidermal cell and guard cell division. During cytokinesis, green fluorescent protein–tagged exocyst subunits SEC6, SEC8, SEC15b, EXO70A1, and EXO84b exhibit distinctive localization maxima at cell plate initiation and cell plate maturation, stages with a high demand for vesicle fusion. Finally, we present data indicating a defect in cell plate assembly in the *exo70A1* mutant. We conclude that the exocyst complex is involved in secretory processes during cytokinesis in *Arabidopsis* cells, notably in cell plate initiation, cell plate maturation, and formation of new primary cell wall.

My contribution:
I designed and performed most of the experiments (characterisation of *exo84b* mutants, EXO84b cloning, coimmunoprecipitations, electron microscopy observations, confocal imaging of EXO84b-GFP), and I was the principal author of the text.

3.3. Title: Visualization of the Exocyst Complex Dynamics at the Plasma Membrane of Arabidopsis thaliana

Authors:
Matyáš Fendrych, Tamara Pečenková, Juraj Sekereš, Lukáš Synek, and Viktor Žárský

Summary:
Polarized exocytosis is crucial for plant cell morphogenesis. Prior to their fusion with the plasma membrane, secretory vesicles are tethered at exocytic sites by the exocyst, an octameric protein complex. Accordingly, exocyst subunits localize to secretory active regions of the plasma membrane of yeast, animal, and plant cells. Such a region is exemplified by the outer epidermal domain of *Arabidopsis* root cells that delimits the root-soil interface. We employed the high resolution VAEM microscopy to visualize exocyst subunits SEC6, SEC8, EXO70A1, and EXO84b. These colocalized in distinct foci at the plasma membrane, likely corresponding to single exocytic sites, which were distinct from sites of endocytosis marked by dynamin-related protein 1C. We observed considerable decrease of SEC6-GFP foci density at the plasma membrane in *Arabidopsis* exocyst subunit mutant *exo70A1*. Exocyst foci partially overlapped with bona fide vesicles visualized by membrane fluorescent dye FM4-64 and by v-SNARE VAMP721. The recorded dynamics of exocyst foci plausibly represents dynamics of secretory vesicle tethering at the plasma membrane in *Arabidopsis* cells. Finally, we show that the exocyst foci localization was independent on microtubule and actin cytoskeleton, although prolonged actin disruption led to changes in exocyst polarization within the cells. This work provides a background for high resolution studies of *Arabidopsis* exocytosis.

My contribution:
I designed and performed the majority of experiments (VAEM imaging, cloning of VAMP721, most of the FRAP experiments), and I was the main author of the text.

Supplemental material:

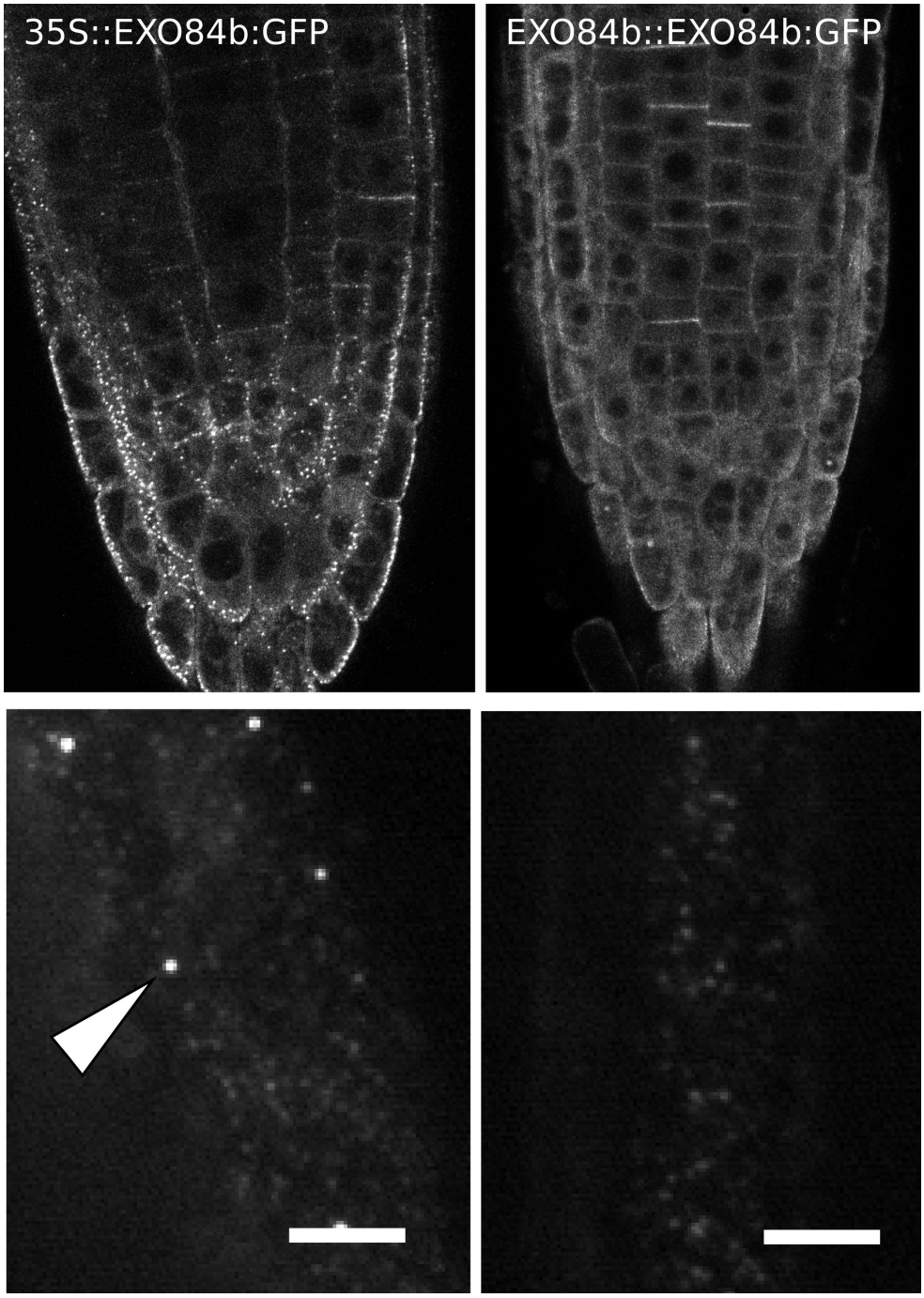

Fig. S11.
Comparison of EXO84B-GFP localization when expressed under the control of the 35S and genomic promoter. Upper row: *A. thaliana* root tip imaged using confocal microscopy. Lower row: VAEM images of root epidermal cells. In the case of the constitutive 35S promoter driven expression, membrane localized spots are visible both in confocal and VAEM images (arrowhead). Scale bars: 3 μm.

Supplementary Movie 1:
EXO84b-GFP foci in elongating epidermal cells of *Arabidopsis* root. Time is indicated in seconds, scale bar represents 10 μm.

Supplementary Movie 2:
GFP-SEC8 foci in epidermal cells in the root hair zone of *Arabidopsis* root. Time is indicated in seconds, scale bar represents 10 μm.

4. Discussion

Arabidopsis thaliana genome encodes genes for more than 20 formin isoforms, whose domain organisation differs dramatically between opisthokonts and plants. These can be divided according to sequence similarity and domain structure into two distinct groups (groups I and II) (Grunt *et al.*, 2008). Study of plant formins has been complicated by the fact that mutations in formin genes often lead to very subtle phenotypic defects. This is caused probably by a high redundancy among formin proteins. There are, however, a few publications reporting phenotypic demonstrations of a formin mutation. The mutant in AtFH5 has a delayed endosperm cellularization, possibly due to cytokinetic defects. Consistently, GFP tagged AtFH5 localized into developing cell plates, and also to intracellular compartments (Ingouff *et al.*, 2005). Given the protein´s behaviour during cytokinesis (compare with Fendrych et al., 2010, and the associated discussion), these compartments might represent the trans-Golgi network. As a typical Class I formin, and similarly to AtFH4, AtFH5 has an N-terminal signal peptide and therefore it should be inserted into the ER membrane and possibly also to the PM. Its localization in the endomembrane compartments could mean it was not properly trafficked to the PM. Hai-Yun Ren and her collaborators recently demonstrated the importance of AtFH8, a close paralogue of AtFH4, for the root development (Xue *et al.*, 2011). Mutants in AtFH8 showed hypersensitivity to treatment with latrunculinB; the drug reduced cell numbers in the root meristem. By a forward genetic screen, another group identified a rice mutant FH5/BENT UPPERMOST INTERNODE that exhibits a cell expansion defect (Yang *et al.*, 2011b). Authors of both of these studies show that the morphology of filamentous actin is altered in the mutants. These results must be interpreted with an extreme caution, as the plants differ morphologically from wild type, and therefore it is difficult to discover the proximal cause of changes in the actin cytoskeleton appearance. Mutants in the classII formin AtFH14 have defects in the male gametophyte meiosis (Li *et al.*, 2010b). All these phenotypes are most probably caused by an altered cytoskeletal dynamics.

4.1. AtFH4 associates with actin and microtubule cytoskeleton

We have deliberately eliminated the transmembrane domain of AtFH4 to observe its behaviour within the cell and discover potential cytoplasmic localizations. Considering the canonical role of formins as actin nucleators, which act as processive caps (Kovar and Pollard, 2004), localization *along* actin filaments should not be expected. Instead, formins should associate with the actin barbed end, and such a situation is likely to be invisible by the classical confocal microscopy. *In vivo*, the association of AtFH1 with actin filaments was not observed (Cheung and Wu, 2004), although this formin binds along actin filaments following nucleation *in vitro* (Michelot *et al.*, 2006). Localization of AtFH4 to actin network MT was observed only occasionally as an actin-MT coalignment. This was dependent on the marker used to visualize the actin cytoskeleton. As mentioned in the publication (Deeks et al., 2010), the lifeact-GFP marker never showed coalignment with AtFH4 labelled MT. The situation differed in the case of the fimbrin actin binding domain 2 (FABD2, Voigt *et al.*, 2005) fused to dsRED. When AtFH4Δ1 was coexpressed with FABD2, partial coalignment of actin bundles with AtFH4 labelled MT was observed, and this was never observed in the case of AtFH4Δ4, a construct lacking the FH2 domain

(Fig. S9). Because, in our hands, the FABD2 labelled actin bundles were not sensitive to actin disrupting drugs, and because the coalignment was not observed always (data not shown), we decided not to include the coalignment data into the publication. However, these results confirm the ability of AtFH4 to simultaneously associate with MT and actin *in vivo*.

We developed a simple coated sepharose bead assay to assess the ability of recombinant AtFH4 to influence actin dynamics *in vitro*. A corona of actin developed around beads coated with AtFH4Δ1 but not around beads coated with AtFH4Δ4 – a construct, which associated with MT but lacks the FH2 domain. The corona growth was sensitive to latrunculinB. These results demonstrated the MT and actin binding activities are located within separate domains of the protein. We were not able to determine whether we observed actin filament binding or actin nucleation. To distinguish between these we would have to employ more elaborated biochemical methods, for example observation of actin behaviour *in vitro* by the TIRF microscopy. We were, however, not able to use this technique in the time of the article preparation. AtFH4 was previously shown to reduce the lag phase of actin polymerization, indicating that it nucleates actin filaments (Deeks *et al.*, 2005).

To visualize the behaviour of AtFH4 *in vivo*, we used the transient expression system employing *Agrobacterium* mediated transformation of *Nicotiana benthamiana* leaves. Constructs containing the GOE domain decorated MT. To confirm this result in *A. thaliana*, we transiently transformed *A. thaliana* leaves by particle bombardment and observed MT like network. We further prepared stable *A. thaliana* transformants expressing AtFH4Δ1. Using confocal microscopy, we observed cytoplasmic localization (data not shown). Only with the usage of the variable-angle epifluorescence microscopy (VAEM, Konopka and Bednarek, 2008b, see also below), we were able to distinguish the localization of AtFH4Δ1 to a MT-like cortical network (Fig. S10 and Movie 4). This result is of a great significance because it demonstrates the ability to bind MT in a homologous system.

We mapped the MT binding domain into the region between the transmembrane domain and the FH1 domain. This domain on its own did not exhibit the strongest association with MT, therefore we assume that the neighbouring FH1 domain also helps the MT association. The GOE domain has no homologues outside the group Ie formins. Another member of this group, AtFH8, localized to the nuclear envelope when expressed in *A. thaliana* root cells, and the authors did not observe MT localization for constructs that contained the GOE domain (Xue *et al.*, 2011). On the first sight, this might contradict our results. However, protein localizations presented in the study of Xue et al. are of a low quality, and so the potential MT localization might not be discernible. Also in the case of AtFH4 stably transformed into *A. thaliana*, we were able to observe the MT localization only with the help of the VAEM technique. The localization reported by Xue et al. should be revised using the same technique. A ClassII formin, AtFH14, was shown recently to localize to the preprophase band, spindle, and phragmoplast, structures composed of actin and MT cytoskeletons (Li *et al.*, 2010b). Similarly to AtFH4, AtFH14 interacted with MT and actin *in vitro*. The authors provide a functional evidence for an *in vivo* role of AtFH14 in coordinating MT and actin during cell division. Unlike in the case of AtFH4, the MT binding activity of AtFH14 lies somewhere within the FH2 domain, and MT compete with actin for binding this domain.

What is the advantage of formin being able to communicate with both actin and MT systems? Theoretically, this connection might integrate trafficking along the two

networks, or the two network might be co-aligned by the action of formins. It is still not evident though, whether formins can bind to actin and MT simultaneously (Chesarone *et al.*, 2010). Alternatively, actin and MT binding could be mutually exclusive. MT and actin binding sites overlap in the case of AtFH14, metazoan mDia1, mDia2 and *Drosophila* CAPU (Bartolini *et al.*, 2008; Li *et al.*, 2010b; Rosales-Nieves *et al.*, 2006); mDia1 also contains a N-terminal region in an analogous position to the AtFH4 GOE domain that is essential for mitotic spindle association (Kato *et al.*, 2001). MT binding domain is located outside the FH2 domain in the case of AtFH4, and metazoan FMN1, and INF1 (Young *et al.*, 2008; Zhou *et al.*, 2006); FMN1 binds to MT via its 2nd exon, creating a splice variant FMN1-Ib.

The fact that plant and opisthokont FH2 domains are capable of MT binding might suggest that the association of this domain with MT was present already in their common ancestor. The convergent tendency to acquire further MT binding domains is evident in both opisthokont and plant formins.

4.2. AtFH4 is able to simultaneously bind membranes and microtubules

The overexpression of full-length AtFH1 within pollen tubes caused excessive bundling and membrane-associated accumulation of filamentous actin in tip-growing cells (Cheung and Wu, 2004). When we expressed the full length AtFH4 in *N. benthamiana* epidermal cells, the signal decorated MT network, but also a membrane compartment – endoplasmic reticulum (ER). Apparently, the formin was not able to be inserted to the PM. There are a few possible explanation of this phenomenon. The transmembrane domain of the protein should be inserted into the ER membrane, but the GOE, FH1, and FH2 domains should protrude into cytoplasm, and in this configuration, the protein should be trafficked through the secretory pathway (Martinière *et al.*, 2011). It is therefore possible that the protein attaches to cortical MT and thereby inhibits its own trafficking through the secretory pathway. Overexpression of the protein caused MT-ER coalignment, and this must be reflected in the ER functioning as this organelle is normally moved by the actomyosin system (Boevink *et al.*, 1998; Ueda *et al.*, 2010). The other possibility is that AtFH4 indeed indigenously localizes to the ER. Similarly, mammalian formin INF2 was found to associate with the ER periphery of Swiss 3T3 cells (Chhabra *et al.*, 2009). Speaking against this hypothesis is the presence of a signal peptide in the AtFH4 N-teminus (Cvrčková, 2000). AtFH4 localized to cell boundaries in shoot cells when visualized by immunolocalization (Deeks *et al.*, 2005), but the pictures do not actually demonstrate a membrane localization; in these cells ER and PM localization could result in a very similar staining pattern. To discover the native localization of AtFH4, a fluorescently tagged protein should be expressed under the control of its genomic promoter and observed using high resolution fluorescent microscopy (see Fig.S9 and Movies 4-6). Nevertheless, the MT-ER coalignment caused by AtFH4 demonstrates that this protein is capable of a strong attachment to MT. This attachment, mediated by the GOE domain, was robust enough to override the force generated by the actomyosin system. We therefore suggest that AtFH4 serves as a molecular bridge between PM, MT and actin cytoskeleton.

In an elegant publication, John Runions and colleagues (Martinière *et al.*, 2011) demonstrated that AtFH1 inserts into the cell wall, spans the PM and associates with actin in the cytoplasm. A conserved extracellular region immobilizes this protein within the cell wall. In the cytoplasm, AtFH1 bundles actin filaments. AtFH1 labels PM and shows an

inverse pattern to MT network, thus it obviously does not interact with MT. The negative staining of MT is surprising, as MT thickness is approx. 30 nm (Ledbetter and Porter, 1963), a distance that should not be resolvable by the confocal microscope (the XY resolution of a confocal microscope is approximately 200 nm – see e.g. Schermelleh *et al.*, 2010). Therefore, the negative staining should not result merely from the physical presence of the MT.
Together with Martinière et al, (2011) our results imply plant formins as possible candidates for communicators between apoplastic space/PM and cytoskeletal systems. According to publicly available expression data (www.genevestigator.com), AtFH4 is strongly pathogen responsive, and its expression is predicted (www.atted.jp) to be correlated with components of the secretory pathway connected with pathogen defense – exocyst subunit EXO70B2 (Pečenková *et al.*, 2011), SNAP33 (Kwon *et al.*, 2008), SYP121 and SYP122 (Assaad *et al.*, 2004). AtFH4 could thus participate in actin and MT cytoskeleton reorganisation during pathogen attack (Hardham *et al.*, 2008), and this way attract vesicle trafficking and secretion to the pathogen attack site (Assaad *et al.*, 2004; Pečenková *et al.*, 2011).

4.3. Future AtFH4 experiments

In future experiments, GFP-tagged AtFH4 should be expressed under the control of its genomic promoter in *A. thaliana*. Localization of the protein should be examined in detail, special attention should be devoted to observation of AtFH4, AtFH4Δ1 and AtFH4Δ4 localization during cell division. Also, the localization to cortical microtubules and preprophase band should be examined using the VAEM technique. AtFH4 should be colocalized with a marker for actin cytoskeleton (lifeact-mRFP) using VAEM to achieve high spatio-temporal resolution. Future work should also address the regulation of AtFH4 actin and MT nucleation/binding activity.

4.4. Exo84b mutant is a dwarfed plant with cytokinetic defects

Mutations in subunits of the exocyst complex interfere with normal plant development. The *roothairless1* maize mutant (a SEC3 exocyst subunit homologue) and *Arabidopsis exo70A1* mutant have problems with the root hair elongation (Synek *et al.*, 2006; Wen *et al.*, 2005; Wen and Schnable, 1994). *Arabidopsis* sec6*, sec8*, *sec15a, exo70C1* mutants and *sec5a/sec5b* double mutants cause a male specific transmission defect. In these mutants, pollen germination and pollen tube growth are impaired (Cole *et al.*, 2005; Hála *et al.*, 2008; Li *et al.*, 2010a). These effects are caused by the inability of exocyst mutants to support the rapid tip growth of pollen tubes and root hairs. Diffuse growth is affected as well, demonstrating the importance of exocytosis for this process. Dark grown exocyst mutants have substantially shorter hypocotyls, and exhibit pleiotropic phenotypic defects including dwarf stature (Hála *et al.*, 2008; Synek *et al.*, 2006; Wen *et al.*, 2005; Wen and Schnable, 1994).
In the sporophyte, the phenotypic defect of *exo84b* mutants was more severe than that of other viable plant exocyst mutants. One possible explanation is that EXO84b is particularly important for the exocyst complex functioning. Alternatively, other known mutations might only incompletely eliminate the function of the affected exocyst subunit due to a genetic and functional redundancy among paralogous genes (EXO70s, SEC5, and RTH1) or due to partial elimination of their function (weak *sec8* alleles; Cole *et al.*,

2005). The C-terminally located insertion in the *EXO84b* gene exerted more severe phenotypic deviation than the N-terminally located one, which is the opposite situation to the *sec8* alleles (Cole *et al.*, 2005). It is possible that in the N-terminal insertion, *EXO84b* transcripts were spliced correctly at a very low frequency, or that the more severe allele produced a partial protein that interfered with the exocyst function. Both lines had similar phenotypic defects and could be complemented by expression of a GFP-tagged EXO84b. The e*xo84b* mutants were dwarfed and sterile; their epidermal pavement cells lobing was decreased, although some cells still exhibited the typical jigsaw puzzle interlocking. Mutation in the **i**nteractor of **c**onstitutive active **R**OPs 1 (ICR1) that interacts also with the SEC3 also lead to a decreased epidermal cell expansion (Lavy *et al.*, 2007). It is, however, difficult to separate a pleiotropic growth defect from a defect in pavement cell expansion in the case of *exo84b.*

Obviously, both gametophyte and sporophyte generations are affected by exocyst mutations. Somewhat surprisingly, female gametophyte seems to be unaffected. Male gametophytic phenotypic deviation is limited to the pollen tube growth, a secretory demanding process. Microgametogenesis including the post-meiotic cytokinesis are also unaffected by exocyst mutations (Cole *et al.*, 2005). This could imply that (especially) female gametophyte secretion needs are limited, or that tethering of secretory vesicles is achieved by other tethering factors in the gametophyte (Okamoto *et al.*, 2004); or that the gametophyte uses proteins originating from the large somatic megaspore mother cell. I am not aware of a study of exocytic activity of the female gametophyte, this issue could be tested by secretory markers including exocyst subunits and v-SNARE GFP fusions (see Fendrych et al., 2011). Further research is necessary to distinguish among these hypotheses.

Epidermal cells of the *exo84b* mutant displayed cell wall stubs, indicating a failure in cytokinesis. The guard cell cytokinesis and morphogenesis was also adversely affected.
exo84b is, however, not a canonical cytokinetic mutant (Assaad *et al.*, 1996; Lukowitz *et al.*, 1996; Sollner *et al.*, 2002), instead, the cytokinetic defects is mild. The *exo84b* cytokinetic phenotype is very similar to other stomatal cytokinesis mutants – *scd1* and *cyd1* (Falbel *et al.*, 2003; Yang *et al.*, 1999). SCD1 is also involved in vesicle trafficking, and it is also involved in innate immunity response against bacteria (Korasick *et al.*, 2010). Surprisingly, the *CYD1* gene has not been identified yet to my knowledge, although it was recently used to study spindle morphology (Yang *et al.*, 2011a). It would be extremely interesting to identify this gene, the mutation was mapped to the interval between nga225 and nga249 in chromosome 5 (Yang *et al.*, 1999) (and this region does not encompass the *EXO84b* locus). On the subcellular level, a portion of mutant cells accumulated a high concentration of vesicles, consistent with the idea of exocyst function as a secretory vesicle tethering complex. This is reminiscent of ultrastructural defect of the vesicle accumulation in first yeast exocyst mutants identified in original sec-screen (Novick *et al.*, 1980) and later found also in yeast exo84 mutant (Guo *et al.*, 1999a). Vesicles accumulated in the exo84b mutant are mostly filled with the fibrous content suggesting possible pectin and xyloglucan nature of the cargo. Ivan Kulich with colleagues (Kulich *et al.*, 2010) demonstrated the role of the exocyst complex in cell wall biogenesis, more precisely in the seed coat development. Considering the localization of the exocyst during cytokinesis (see bellow), I conclude that the cytokinetic defect of *exo84b* results from defective maturation of the new cell wall.

4.5. Localization of the exocyst complex during cytokinesis

We observed localizations of GFP-tagged SEC6, SEC8, SEC15b, EXO70A1, and EXO84b exocyst subunits in root cells of stably transformed *A. thaliana*. All subunits observed exhibited very distinct localization during cytokinesis: they localized to the cell plate in the moment of its assembly. During subsequent growth, the intensity of the cell plate labelling by the exocyst subunits decreased. Finally, the exocyst localized into the cell plate insertion site and labelled the new cell wall throughout its maturation. I am not aware of any other protein that shares this dynamic localization with the exocyst. Instead, the expanding cell plate is believed to be a "default" target for a plethora of proteins. Although this issue was not addressed in detail, we did not see significant staining of the preprophase band site. This is consistent with previous results (Karahara *et al.*, 2009).

Although the canonical role of the exocyst inheres in secretory vesicle tethering to the PM (TerBush *et al.*, 1996), the localization to the initial phase of cell plate assembly strongly implicates an additional, plant-specific role for the exocyst complex. Andrew Staehelin and colleagues observed protein linkages between vesicles during the cell plate formation using electron tomography. In early cytokinesis, vesicles arrive at the equatorial plane into the "cell plate assembly matrix" (a cocoon-like, ribosome-excluding domain around the forming cell plate) to form the late anaphase vesicle cloud. Here, the vesicles were connected by Y shaped linkers resembling the exocyst complexes, whereas outside the matrix, the vesicles carried L-shaped protein appendages (Seguí-Simarro *et al.*, 2004). The authors suggested that the Y-shaped linkages represent assembled exocyst complexes, and the L-shaped appendages were partially assembled exocyst complexes. To test the involvement of the exocyst in the cell plate assembly, we analysed morphology of the cell plate in the available exocyst mutants. The *exo84b* mutants grow slowly, and thus divide less frequently, and it was therefore difficult to find enough dividing cells for a thorough analysis of cell plate morphology. However, the few cell plates we saw appeared normal. On the other hand, the nascent cell plates of the *exo70A1* mutant (Synek *et al.*, 2006) had usually a gap in their centres. In the *exo70A1* mutant plants, the exocyst mediated vesicle tethering is decreased (Fendrych et al., 2011). These results support the role of the exocyst in the cell plate assembly. An alternative explanation would be that in the mutant, the centre of the cell plate is composed of vesicles not stained by the dye used for cell plate staining, or that the phragmoplast morphology is altered.

It is widely accepted that the cell plate exhibits a trans-Golgi network-like identity (Chow *et al.*, 2008; Viotti *et al.*, 2010). Recently it was shown that the TRAPPII is involved in the cell plate development (Jaber *et al.*, 2010; Thellmann *et al.*, 2010). TRAPPII is a guanine nucleotide exchange factor complex which tethers vesicles at the trans-Golgi network (Bröcker *et al.*, 2010). Considering the low abundance of the GFP-tagged exocyst subunits in the expanding cell plate, the identity of exocyst-like complexes observed by electron tomography (Seguí-Simarro *et al.*, 2004) should be confirmed using exocyst-specific antibodies. Based on the behaviour of tethering factors during the cell plate assembly and expansion, and on the available data, I deduce that the cell plate assembly is triggered with the help of the exocyst complex. In the subsequent growth phase, the cell plate acquires a trans-Golgi-derived identity and the exocyst is not necessary for this stage of cytokinesis.

In yeast, the exocyst is linked to the PM by direct binding of its Exo70 and Sec3 subunits to PIP2 (He *et al.*, 2007; Zhang *et al.*, 2008). PIP2 localization during cytokinesis was described, although the data should be perceived as preliminary: the cell plate is strongly

labelled by phosphatidylinositol 4-phosphate (Vermeer *et al.*, 2009). Shortly prior to or concurrently with its contact with the mother membrane, the rim of the cell plate is labelled by a PIP2 marker (van Leeuwen *et al.*, 2007), although the figure documenting this is not absolutely convincing. The reported PIP2 localization differs substantially from that of the exocyst subunits, and thus PIP2 should not involved in the exocyst subunit recruitment to the cell plate. However, the localization of PIP2 in plant cells and the exocyst subunits should be compared further and also the VAEM technique should be employed (see below).

Exocyst subunits localized to the cell plate insertion and subsequently labelled the entire surface of the new cell wall. This localization pattern implicates a role in the cell plate attachment to the mother wall and in closing of the remaining gaps in the cell wall by the emerging secondary cell plate assembly matrices (Seguí-Simarro *et al.*, 2004).
The exocyst could play a role in the recognition of markers laid by the preprophase band, such as the TANGLED protein (Walker *et al.*, 2007). Arguing for this hypothesis is the fact that the types of cytokinesis, where the preprophase band is not formed*, namely microsporogenesis and megasporogenesis (Verma, 2001) , are not affected by exocyst mutations (Cole *et al.*, 2005; Hála *et al.*, 2008; Synek *et al.*, 2006). Similarly, in the dividing guard cells of the moss *Funaria,* the preprophase band is missing, the guard mother cell divides imperfectly, just enough to create a stomatal pore, and the guard cell cytoplasms remain connected (Sack and Paolillo Jr, 1985). This strikingly resembles the phenotype of the *exo84b* mutant (and *cyd1* mutant) stomata, where a portion of stomata develops a pore without complete ventral walls. On the other hand, I believe that the principal role of the exocyst in cytokinesis inheres in the tethering of secretory vesicles carrying components of the new cell wall.
During the cell plate maturation, which is triggered by its contact with the mother wall (Mineyuki and Gunning, 1990), the exocyst could act in trafficking of vesicles containing cell wall components and cellulose synthase complexes, which are assembled in the Golgi apparatus and then deposited to the PM (Crowell *et al.*, 2009; Gutierrez *et al.*, 2009). A few proteins had similar localization to that of the exocyst subunits. The RSH extensin accumulated at the cell plate insertion site (Hall and Cannon, 2002). AIR9 was associated with cortical microtubules in interphase cells (Buschmann *et al.*, 2006). During cytokinesis, it was recruited to the phragmoplast microtubules, then it relocated to the insertion site and later appeared more centrally in the cell plate. Another cytoskeleton associated protein – a class VIII myosin – localized to the post-cytokinetic cell wall, and it was implicated in the cell plate maturation process (Reichelt *et al.*, 1999). The formin AtFH8, a close paralogue of AtFH4, localized to postcytokinetic walls on *Arabidopsis* roots (Xue *et al.*, 2011). On the other hand, AtFH5 shows an inverted localization during cytokinesis – this protein labelled the developing cell plate, but the signal weakened after the cell plate insertion (Ingouff *et al.*, 2005). The T-PLATE protein (probably involved in endocytosis, Van Damme *et al.*, 2011) is present in the growing cell plate, and is most strongly localized around the area of cell plate insertion site (Van Damme *et al.*, 2006). Other proteins are recruited to the newly developed PM only after its insertion to the PM, including PM H-ATPase (Lauber *et al.*, 1997).
Cutler and Ehrhardt (2002) suggest that there are two phases of the cell plate development: before and after its contact with the cell cortex. The behaviour of the

* cell divisions that lack the preprophase band include also the first asymmetric division of the embryo and endosperm cellularization (Verma, 2001)

exocyst subunits and the above mentioned proteins demonstrates a clear distinction between the cell plate pre- and post-insertion phases, and these results imply that the membrane of the developing cell plate acquires the PM identity following the cell plate insertion into the mother cell wall.
In *S. pombe*, the exocyst is necessary for hydrolytic enzyme delivery necessary for cell separation (Martín-Cuadrado *et al.*, 2005). The exocyst and associated hydrolytic enzymes localize to a cortical ring that surrounds the septum. Remarkably, the exocyst localizes to a peripheral ring in the midbody in mammalian cells and probably act in the fusion of vesicles with the PM (Gromley *et al.*, 2005). The midbody and phragmoplast share a substantial set of features, and are likely homologous structures (Eme *et al.*, 2009; Otegui *et al.*, 2005). Also the exocyst might play a similar role in both of them – the final separation of the mammalian cells and cell wall maturation between the plant cells.

4.6. TIRFM vs. VAEM

In the third paper of this thesis, we used the Variable-angle epifluorescent microscopy (VAEM) to characterize the behaviour of exocyst subunits at the PM of *Arabidopsis* cells. TIRFM is believed not to be usable in plant cells as the cell wall is believed to be thicker than the reach of the evanescent wave. Therefore the VAEM is employed, where light penetrates the sample in an oblique, subcritical angle (Konopka and Bednarek, 2008b). The penetration depth of the evanescent wave can be estimated (Tsuboi *et al.*, 2005) and depending on the angle and refractive indexes can range up to 400 nm. The cell wall thickness is variable – ranging from 300 nm in elongating dark-grown hypocotyl to 800 nm in elongating light-grown hypocotyl (Derbyshire *et al.*, 2007), and thus in some cases the cortical cytoplasm might theoretically lay in the reach of the evanescent wave. Putative TIRFM was used to visualize vesicles in *Picea* pollen tubes (Wang *et al.*, 2006) and a PM marker in *Arabidopsis* leaf epidermis (Sparkes *et al.*, 2011). The question remains whether and at which interface total internal reflection occurs. To distinguish between TIRFM and VAEM, we should determine the critical angle for total internal reflection for our system. This angle is determined by the equation:

$$\theta_c = \sin^{-1}\left(n_1 / n_3\right)$$

(Axelrod, 2003), where n_1 is the refractive index of the medium and n_3 that of glass. The n of glass is approximately 1.5, the n of water 1.3, and therefore the critical angle equals approximately 61°. However, if the cell wall is in a direct contact with the glass, total internal reflection could occur at the glass/cell wall interface. In the latter case, the critical angle will be dependent on the actual n of the cell wall. The n of the cell wall was measured to vary between 1.4 to 1.5 in various soybean leaf cell walls (Woolley, 1975), approximately 1.4 in *Lemna triscula* cell walls (Gabryś, 1978). For the n = 1.4, the critical angle would be approximately 67°, which is larger than angles we used in this study. If the n of the cell wall equals to 1.5, the light will penetrate the cell wall and the total internal reflection will occur at the cell wall/cytoplasm interface; this was also suggested in a recent methodological paper (Sparkes *et al.*, 2011). As we are not able to determine the cell wall refractive index, it would maybe be more correct to designate the method TIRFM/VAEM, however, I decided to stick to the established term VAEM for the publication.

4.7. The genuine exocyst localization?

The exocyst localization has been described in various model organisms as intensive signal in the yeast bud tip and bud neck (Finger *et al.*, 1998), as distinct domains in membrane of neurons (Hazuka *et al.*, 1999), localization to primary cilium and tight junctions in Madin-Darby canine kidney cells (Lipschutz *et al.*, 2000; Rogers *et al.*, 2004), localization to microtubules and the leading edge in normal rat kidney cells (Wang *et al.*, 2004; Zuo *et al.*, 2006), localization to midbody in human cells (Gromley *et al.*, 2005). Further, the exocyst was localized in the endomembrane system (Prigent *et al.*, 2003), in the tip vesicles/cytoplasm in tobacco pollen tubes (Hála *et al.*, 2008), in the Spitzenkörper in growing hypha of Aspergillus (Taheri-Talesh *et al.*, 2008), colocalized with insulin vesicles in pancreatic cells (Tsuboi *et al.*, 2005), localized to various intracellular and endomembrane spots in tobacco BY2 cells (Chong *et al.*, 2010) and to PM and postcytokinetic membranes in *Arabidopsis* (Fendrych *et al.*, 2010). Most of these localizations represent membrane domains that are secretory active, significance of others is not as straightforward, for example the microtubule localization or intracellular localization in the case of BY2 cells. Our study (Fendrych et al., 2011) and the work of Tsuboi et al. present data showing the exocyst associated with individual vesicles; an expected feature of the exocyst. *Arabidopsis* exocyst subunits were also shown to localize into double membrane compartments, distinct from multivesicular endosomes and phagosomes (Wang *et al.*, 2010). The authors claim that these compartments should fuse with the PM and thus release a single membrane-bound vesicle into the apoplast. Considering a lack of electron microscopic data documenting such compartments in the literature, and our results (Fendrych et al., 2011), I suggest that these compartments might be induced by the overexpression of the EXO70E2 protein. Similarly, I observed localization of the EXO84b-GFP protein into unidentified PM-associated spots, larger than and distinct from the exocyst foci, when the expression was driven by the constitutive 35S promoter (see Fig. S11); these spots were not present when the expression was driven by the EXO84b genomic promoter. A subset of exocyst subunits was recently shown to participate in the autophagosome assembly (Bodemann *et al.*, 2011), and so these compartments could be part of the autophagic machinery, although the authors refuse this hypothesis.

We identified the exocyst in PM localized foci, representing probably the exocytic sites. We showed that the SEC6, SEC8 and EXO84b positive foci colocalize. We were not able to colocalize EXO70A1 with the other subunits due to silencing of the EXO70A1 transgene, but based on similar behaviour and dynamics, and on the fact that the SEC6 foci density was dramatically decreased in the *exo70A1* mutant, I assume that EXO70A1 foci are identical to other exocyst foci. Although the Sec5 exocyst subunit localized to clathrin coated vesicles in the oocytes of *Drosophila melanogaster* (Sommer *et al.*, 2005), we did not observe substantial colocalization of the exocyst foci (represented by SEC8) with endocytic sites marked by Dynamin related protein 1C (Konopka and Bednarek, 2008a) at the time resolution achieved.
To test whether exocyst foci colocalize with membrane-bound vesicles when dwelling on the PM, we labelled the seedlings with the membrane fluorescent dye FM4-64. This dye is quickly endocytosed and labels recycling endosomes and trans-Golgi networks (Dettmer *et al.*, 2006; Dhonukshe *et al.*, 2006). Subsequently, vesicles derived from these compartments are also labelled by the dye and can be visualized (Calì *et al.*, 2008). The

second attitude inhered in visualization of the vesicle-localized R-SNARE protein (Allersma *et al.*, 2004), specifically VAMP721 (Uemura *et al.*, 2004). The exocyst foci colocalized with both of these markers, confirming that it indeed is vesicle tethering that we observe. We also attempted to visualize secretory vesicles using the secreted GFP as a *bona fide* vesicle marker (Batoko *et al.*, 2000), but we were unable to visualize secretory vesicles.

In the *Arabidopsis* root, the exocyst strongly decorated the outer PM of elongating epidermal cells, the most easily accessible domain for the VAEM imaging. It is not possible to observe deeper cell layers with this technique. The density of the foci decreased with the distance from the meristem, likely reflecting a decreasing secretory activity. In postmitotic cells, the exocyst re-focused from the lateral domain onto the maturing cell wall (Fendrych *et al.*, 2010). Considering the fact that the compositions of the post-cytokinetic and lateral cell wall must differ, a question is coming to one's mind: is the refocusing of the exocyst reflecting a temporarily separated secretion of vesicles with distinct content? It would be interesting to observe the exocyst localization during other type of a sudden change of secretion, for example a pathogen attack or a symbiotic fungus penetration (Genre *et al.*, 2008). We can also ask a complementary question: is a plant cell able to *simultaneously* create and secrete vesicles with different content?

The answer is probably *yes* in the case of proteins; a plethora of proteins localize to distinct PM domains (Alassimone *et al.*, 2010; Langowski *et al.*, 2010), the mechanism of their localization includes also selective endocytosis (Boutté *et al.*, 2010; Geldner *et al.*, 2003; Men *et al.*, 2008; Paciorek *et al.*, 2005) and differential phosphorylation (Friml *et al.*, 2004; Michniewicz *et al.*, 2007). By the electron tomography approach, two types of vesicles were identified within the cell plate assembly matrix based on their size and electron density (Seguí-Simarro *et al.*, 2004) and two types of secretory (light and dark) vesicles in the rhizoids of *Chara* (Limbach *et al.*, 2008). It was shown that secretory marker proteins are trafficked to the PM by a pathway distinct from that of polysaccharides targeted to the cell wall (Leucci *et al.*, 2007). To my knowledge, very little is known about vesicles carrying different soluble cargoes to distinct PM domains. The PM domain recognition machinery could involve EXO70 subunits of the exocyst complex. *Arabidopsis* encodes a family of 23 paralogues EXO70 genes (Eliáš *et al.*, 2003; Synek *et al.*, 2006), and the various EXO70 subunits could function in targeting the exocyst complexes to various PM domains (Žárský *et al.*, 2009). The mechanism recognizing the vesicle cargo by EXO70 has not been postulated, however. An indirect recognition could be mediated by the palette of Rab GTPases (Rutherford and Moore, 2002), which could be loaded onto specific vesicles concurrently with the vesicle creation at the source compartment. The relocalization of exocyst subunits in *Arabidopsis* root epidermal cells represents another possible way how to establish distinct secretion domains within a single cell – by a temporal separation of exocytosis of the distinct cargoes.

We further tested exocyst foci colocalization with cytoskeletons and their dependence on actin and MT. We did not observe a significant colocalization between exocyst foci and the cytoskeletons, and MT disruption had no obvious effect on the foci appearance. Also a short term actin disruption did not prevent the exocyst to form the exocytic foci at the PM, which suggests that filamentous actin is not required for the mechanism of exocytosis *per se*. This is in agreement with the work of František Baluška (Baluška *et al.*, 2001) who showed that plant cells are able to grow and divide even in the absence of detectable filamentous actin, albeit in distorted orientations. Also in fission yeast, polarity

and morphogenesis are cooperatively, yet in a redundant fashion, guided by the actin and the exocyst (Bendezú and Martin, 2011). A prolonged actin disruption led to exocyst foci clustering and resulted probably in relocalization of the upstream exocyst effectors and changes in cell polarity. Actin disruption led to Golgi bodies and VHA1-positive endosomes aggregation and to subsequent uneven distribution of cellulose synthase complexes at the PM (Crowell *et al.*, 2009). These results support the hypothesis that positioning and regular distribution of the donor compartments within the cell cortex is crucial both for focused secretion and diffuse growth. Therefore, the seemingly chaotic actin network (see Movie 6) should be perceived in longer time scales as a means of distributing the secretory vesicle donor compartments within the cell cortex.

5. Conclusions

Plant cell morphogenesis is largely dependent on the coordination of cytoskeletal elements, plasma membrane, and vesicle trafficking. Formin proteins are nucleators of the actin cytoskeleton. Plant Class I family formins are integral membrane proteins and thus have the ability to coordinate cytoskeletal dynamics with the plasma membrane localization. We identified *Arabidopsis thaliana* formin AtFH4 as a microtubule associated protein. The binding is conferred by a novel domain located between the transmembrane domain and the formin homology 1 domain. The protein associated with actin in *in vitro* conditions. Overexpressed AtFH4 accumulated in the endoplasmic reticulum, and induced coalignment of endoplasmic reticulum membranes with microtubules. Together, these data suggest that the combination of plant-specific and conserved domains enables AtFH4 to function as an interface between membranes and both major cytoskeletal networks .

Secretory pathways supported by the activity of the Golgi apparatus play a crucial role in cytokinesis in plant cells. Prior to their fusion with the plasma membrane, secretory vesicles are tethered at exocytic sites by the exocyst, an octameric protein complex. We analysed the mutant in the EXO84b exocyst subunit, and discovered that the mutant plants were dwarfed and exhibited cytokinetic defects. The dwarf stature of the mutants is probably caused by a general secretion defect. The cytokinetic deviations resulted from a post-cytokinetic cell wall maturation defect; this we inferred from the behaviour of GFP-tagged SEC6, SEC8, SEC15b, EXO70A1, and EXO84b exocyst subunits during cytokinesis. These exhibited distinctive localization maxima at the cell plate initiation and cell plate maturation stages, suggesting that the exocyst is also involved in the assembly of the cell plate. Accordingly, the cell plate assembly of *exo70A1* mutants was defective. We conclude that the exocyst complex is involved in secretory processes during cytokinesis in *Arabidopsis* cells; in cell plate initiation, cell plate maturation, and formation of new primary cell wall.

The available data present localizations of the bulk of exocyst proteins to plasma membrane domains associated with high secretion activity. We used variable-angle epifluorescent microscopy that provides higher signal-to-background ratio than confocal microscopy to visualize exocyst subunits at the plasma membrane. Exocyst subunits localized into distinct foci at the plasma membrane, likely corresponding to the individual exocytic sites, which were distinct from sites of endocytosis marked by the dynamin-related protein 1C. Exocyst foci partially overlapped with *bona fide* vesicles markers – the membrane fluorescent dye FM4-64 and v-SNARE VAMP721. We further analysed

colocalization of the exocyst foci with actin and microtubule cytoskeletons. These results provide first insights into the dynamics of single exocytic events at the plant plasma membrane in plant cells, and at the same time provide further experimental evidence for the exocyst function in the vesicle tethering process in plants.

6. Souhrn

Morfogeneze rostlinných buněk úzce souvisí s koordinací plasmatické membrány, cytoskeletu a exocytózy. Forminy jsou proteiny regulující tvorbu vláken aktinového cytoskeletu. Rostlinné forminy náležející do třídy I jsou integrální membránové proteiny, mohou tedy regulovat dynamiku cytoskeletu z pozice membránového proteinu. Zjistiti jsme, že formin AtFH4 z *Arabidopsis thaliana* se váže na mikrotubulární cytoskelet. Doména zodpovědná za tuto vazbu se nachází mezi transmembránovou a „formin homology 1“ doménou. V *in vitro* podmínkách vázal AtFH4 protein aktinová vlákna. AtFH4 se lokalizoval do membrán endoplasmatického retikula, když byl overexprimován v buňkách tabáku. Zároveň tato lokalizace způsobila rozmístění membrán endoplasmatického retikula podél mikrotubulárního cytoskeletu. Z těchto výsledků vyvozujeme, že AtFH4 je schopný být současně umístěn v membráně a svoji vazbou na aktin a mikrotubuly propojovat tyto dva typy cytoskeletu.
Rostlinná cytokineze závisí na sekretorické dráze; na exocytóze i endocytóze. Před tím, než sekretorické váčky splynou s plasmatickou membránou, jsou k ní poutány „poutacím komplexem“ zvaným exocyst, složeným z osmi podjednotek. Analyzovali jsme mutanty v jedné z podjednotek exocystu *A. thaliana* – EXO84b. Mutantní rostliny byly zakrslé, nejspíše kvůli porušené sekretorické dráze, a část jejich buněk vykazovala cytokinetické defekty – nedokončená buněčná dělení. Soudě podle lokalizace podjednotek exocystu během buněčného dělení bylo příčinou těchto defektů špatné dozrávání buněčné destičky. Podjednotky SEC6, SEC8, SEC15b, EXO70A1 a EXO84b značené zeleným fluorescenčním proteinem se masivně lokalizovaly do buněčné destičky v okamžiku jejího vzniku a poté do zrající buněčné destičky. Úlohu exocystu ve vzniku tohoto útvaru navíc podporuje poškozené skládání buněčné destičky u rostlin mutantních v podjednotce EXO70A1. Tyto výsledky napovídají, že exocyst je klíčkový pro procesy buněčného dělení – skládání buněčné destičky a dozrávání nové buněčné stěny mezi dceřinými buňkami.
Lokalizace exocystu je většinou popisována jako intenzivní signál v oblastech plasmatické membrány spojených s aktivní exocytózou. V této práci jsme použili tzv. „variable-angle epifluorescent microscopy“, která vyniká vysokým podílem signálu ku pozadí, čehož je dosaženo částečným osvětlením vzorku. Pomocí této metody jsme pozorovali umístění exocystu do teček na plasmatické membráně buněk *A. thaliana*. Abychom potvrdili, že tyto tečky představují jednotlivé exocytické váčky navázané na plasmatickou membránu, označili jsme je pomocí membránové barvy FM4-64 a proteinu VAMP721 a zjistili jsme částečnou kolokalizaci mezi exocystem a takto značenými váčky. Exocyst nekolokalizoval s endocytickými váčky označenými pomocí dynamin-related proteinu 1C. Dále jsme zjistili, že tečky značené exocystem přímo nekolokalizují s aktinovým ani mikrotubulárním cytoskeletem. Tyto výsledky představují jedny z prvních pokusů popsat ve vysokém rozlišení lokalizaci a dynamiku vázání a exocytózy váčků u rostlinných buněk a zároveň podporují představu exocystu jako poutače váčků na plasmatické membráně rostlinných buněk.

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