Charles University in Prague

Faculty of science

Department of cell and developmental biology

Cell and developmental biology:

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Bc. Juraj Sekereš:

Localization and dynamics of Sec5 subunit of the exocyst complex in plant cells

(Lokalizace a dynamika Sec5 podjednotky komplexu exocyst v rostlinných buňkách)

Diploma thesis

Supervised by RNDr. Viktor Žárský, CSc.

Prague, 2011

Declaration:

I declare, that experimental work this thesis was done by myself. The work was consulted with my supervisor and with my consultant. I wrote this thesis by myself and it includes appropriate references to all scientific literature I adopted information from. The text was revied by and discussed with my supervisor and consultant in the final stage of its creation.

Prague, 29.9.2011

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 29.9.2011

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ABSTRACT

Exocyst is a protein complex involved in tethering of secretory vesicles to cytoplasmic membrane before SNARE-mediated fusion event. Its presence and function in secretory pathway has been confirmed in yeasts, animals and plants. This thesis describes some properties of Sec5, one of the exocyst subunits, in plant model *Arabidopsis thaliana*. Microscopic methods, including VAEM/TIRF microscopy, were used to study subcellular localization and dynamics of Sec5-GFP fusion protein. Sec5 is cytoplasmic protein that also localizes to cytoplasmic membrane, particulary in cells with high secretory activity. It strongly localizes to maturating cell plates during late cytokinesis and its localization to cytoplasmic membrane partially depends on actin cytoskeleton. Generally, obtained results are in agreement with corresponding observations of behavior of other exocyst subunits in plant cells, suggesting, that Sec5 executes its function as part of the exocyst complex.

ABSTRAKT

Exocyst je proteinový complex hrající roli v sekretorické dráze eukaryotických buněk. K plasmatické membráně připoutává váčky, které následně s membránou splynou díky aktivitě SNARE proteinů. V současnosti je jeho funkce studována u kvasinek, živočichů, ale i u rostlin. Tato práce se zabývá studiem podjednotky komplexu Sec5 u modelového rostlinného organismu *Arabidopsis thaliana*. Lokalizace a dynamika tohoto proteinu značeného fluorescenčním proteinem GFP byla studováne na vnitrobuněčné úrovni pomocí mikroskopie, včetně metody VAEM/TIRF. Sec5 je protein lokalizován v cytoplasmě buněk kořene. Protein se také vyskytuje na cytoplasmatické membráně buněk s vysokou sekreční aktivitou a ve zrajících přepážkách v pozdějších fázích buněčného dělení. Doplňování proteinu na membránu částečně závisí na aktinovém cytoskeletu. Závěry práce se shodují s poznatky o dalších podjednotkách komplexu exocyst organismu Arabidopsis thaliana, které byly získány podobnými metodami. Práce přináší výsledky, které spolu s předchozími poukazují na funkci proteinu Sec5 v rámci komplexu exocyst.

LIST OF ABBREVIATIONS

APM	- amiphropos-methyl
BOR4	- high boron requiring 4
CDC42	- Cell division control protein 42 homolog
DRP1C	- dynamin-related protein 1C
FM	- Fei Mao
For3	- formin3
FRAP	- fluorescence recovery after photobleaching
GEF	- guanine nucleotide exchange factor
GFP	- green fluorescent protein
GTP	- guanosin triphosphate
ICR1	- Interactor of Constitutive active ROP1
LB	- Luria Bertani
MAP	- microtubule – associated protein
MS	- Murashige Skoog
Myo52	- myosin 52
PEN3	- PENETRATION3
PI4P	- Phosphatidylinositol 4-phosphate
PIP_2	- Phosphatidylinositol 4,5-bisphosphate
Pob1	- *Schizosaccharomyces pombe boi*-like 1
PRC1	- Interactor of Constitutive active ROP 1
RFP	- red fluorescence protein
RNAi	- RNA interference

Rpm	- rotation per minute
PM	- plasma membrane
SM	- Sec1/Munc18-like
SNARE	- soluble NSF attachment protein receptor
t-SNARE	- target SNARE
TBK1	- TANK-binding kinase 1
TIRF	- total internal reflection microscopy
VAEM	- variable angle epifluorescent microscopy

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1. THEORETICAL INTRODUCTION

1.1 Exocyst composition and assistance in SNARE-mediated fusion

Exocyst (also known as sec6/8 complex) is one of several multisubunit tethering complexes present in eukaryotic cells. These complexes regulate membrane fusion events of various compartments of secretory and endocytic/vacuolar pathway by various means, including bringing target membranes into proximity, catalysis od SNARE complex assembly and binding of vesicel coat proteins (Cai et al., 2007). Most of the exocyst-mediated Eukaryotic endomembrane system, secretory pathway and tethering complexes.The exocyst complex was described in budding yeast for the first time. Most of current understanding of its assembly and interactions comes from studies of this model. It consists of 8 subunits, named Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. Later investigations confirmed presence of these proteins in animals (see for example Kee et al., 1997) and uncovered specialized functions within maintenance of cell and tissue polarity (see further in the text) in multicellular bodies of animals in animals. This nomenclature was initially established for yeast genes but is now used for corresponding animal and plants orthologs as well. Presence of exocyst as a complex in plants was first predicted by recovery of homologous genes in bioinformatic screening (Elias et al., 2002) and later confirmed by genetic, localization and biochemical methods (Hála et al., 2008; Fendrych et al., 2010). Genome investigations confirmed presence of exocyst subunits to be eukaryote-wide. On the other hand, some subunits failed to be recovered from some genomes and reductive evolution of exocyst was suggested for some lineages. It is possible that some of these absences are false-negative results of the screen. On the other hand, loss of subunits cannot be excluded. Exocyst complex has clearly its own evolutionary novelties in well known model species and taxons. Loss of the Sec3 subunit was suggested in lineage leading to fission yeast (*Schizosaccharomyces pombe*), as no homolog of this gene was found in the species, although it is a favorite model in molecular biology with developed exocyst research.

Structure of several exocyst components was determined. They are elongated alfa-helical proteins, presumably associated side-by-side with each other (Munson and Novick, 2006). Purified mammalian exocyst visualized by electron microscopy. Structures of exocyst seen in unfixed and fixed preparations were compared. Unfixed preparations show branched, star-like complex, whereas fixed samles contained more tight particles. Authors propose that exocyst in vivo might be similar to the loose form with accessible binding sites for different interactors and might achieve the closed conformation after activation to bring tether vesicle closer to the target membrane (Munson and Novick, 2006). Strucutures similar to purified

exocyst complexes were detected in dividing plant cells by electron microscopy (Seguí-Simarro et al., 2004; Otegui and Staehelin 2003 and Otegui et al., 2001 see also discussion)

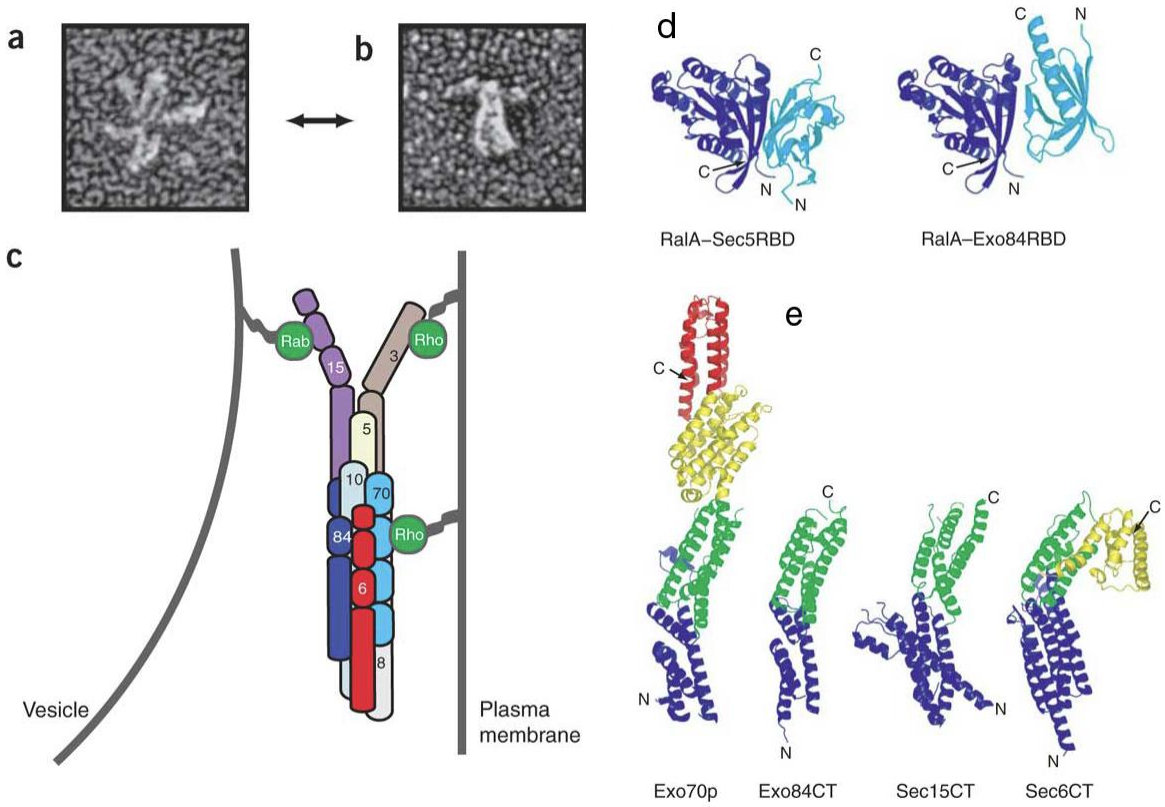


Figure 1.1 Exocyst structure *(derived from Munson adn Novick, 2006). A and B – images of the exocyst complex purified from mammalian brain complex and visualized by quick-freeze/deep-etch electron microscopy. A and B show presumed relieved and „activated" state of the complex, respectively (based on imaging of unfixed and fixed materia). C: schematic depiction of exocyst complex bridging vesicle and PM respecting known structure and interaction of subunits with each other and with small GTPases. D: representations of Ral-binding domains of Exo84 and Sec5 with bound Ral. These domains are specific for metazoan subunits and Ral is a metazoan – specific GTPase. E: representations of solved structures of exocyst subunits – structure of full Exo70 and C-terminal domains of Exo84, Sec15 and Sec6.*

From the above mentioned functions of tethering complexes in general, several clearly apply for the exocyst. For example exocyst and its interactors, not the SNARE proteins, are responsible for spatial specifity of vesicle-fusion events. Membrane t-SNARE proteins turned out to be homogenously distributed throughout PM (Brocker et al., 2010). Exocyst localization pattern, on the other hand, is everything but homogenous in most growing cells. In budding yeast, it localizes to the tip of growing daughter cell during budding process and

localizes to neck of the bud to separate it from mother cell. Its localization is dispersed over PM only during phase of diffuse growth (Boyd et al, 2004). Participation of the exocyst in morphogenesis of animal and plant cells is discussed in corresponding chapters. As mentioned above, one of general functions postulated for tethering complexes is catalysis on SNARE-mediated fusion. Because some subunits are believed to be associated with incoming vesicles and other present at PM, reconstitution of exocyst itself might be an important step for bringing the membranes in proximity and facilitating following SNARE-mediated fusion (Wu et al., 2008). FRAP experiments performed on budding yeast cells suggest, that Sec3 and part of Exo70 subunits locallize to PM independently on intact actin cytoskeleton, whereas rest of subunits localize in actin-dependent manner, most probably coming with secretory vesicles (Boyd et al, 2004).

Furthermore, direct t-SNARE (Sec9p) interaction with Sec6 subunit has been demonstrated in yeast. (Sivaram et al., 2005). Sec1p, which belongs to group of SM proteins, was found to bind exocyst and its overproduction leads to increase in formation of SNARE complexes. Exocyst might thus facilitate SNARE complex assembly via this interaction partner. (Wiederkehr et al., 2004). This was further supported by discovery of interactions between Mso1 protein and several components of tethering and fusion machinery, including Sec1, Sec9p SNARE protein and exocyst component Sec15. Mutations in critical region of the protein, which mediate the interactions, abolish vesicle fusion with membrane (Knop et al., 2005). Weak interaction of Exo70B2 with SNARE SNAP33 has been recently demonstrated in *Arabidopsis* (Pecenkova et al., 2010). Exo70B2 is one of 23 Exo70 paralogs found in *Arabidopsis* genome. Mutation in this subunit does not result in observable cell/organism growth phenotype but it was shown to be involve in responses to pathogen attack (Pecenkova et al., 2010). SNAP33 also seems to have such a specialized function – it localizes to site of pathogen attack together with other SNARE proteins (Yun et al., 2008). This interaction is thus involved in a specific branch of plant secretory pathway (also see further in part 1.4) but similar interactions between other Exo70s and SNARE proteins might exist.

1.2 Exocyst assembly and targeting-insights from yeast model systems

As mentioned above, exocyst plays crucial role in delivery of Golgi-derived vesicle to target site of the PM. Its assembly is thus connected with maturation of secretory vesicles and their fusion site choice. Exocytic vesicles derived from Golgi complex are initially marked with Rab Ypt31/32 GTPase in yeast. This protein recruits Sec2, which is a GEF for Sec4 GTPase (Ortiz et al., 2002). Sec4, in turn, helps recruit its effector Sec15, an exocyst subunit. Sec15 binds both Sec4 and Sec2 in yeast and competes with Ypt31/32 for Sec2 binding. Exchange

of Sec2 binding partners thus occurs during vesicle maturation and association of specific binding partners seems to be facilitated by lipid composition of the vesicle. PI4P formed within Golgi apparatus is bound by Sec2 and inhibits Sec2 interaction with Sec15, thus favoring Sec2-Ypt32 interaction. Loss of membrane PI4P is followed by exchange of binding partners, including Sec15 and other subunits of the exocyst. (Mizuno-Yamasaki et al., 2010) Exocyst assembly is thus regulated by Rab cascade connected with vesicle maturation. Interaction of vesicle-associated Rab with Sec15 has been demonstrated in yeasts, animals and plants (Guo et al., 1999; Langevin et al., 2005 and Toupalová, 2011 – PhD thesis, respectively).

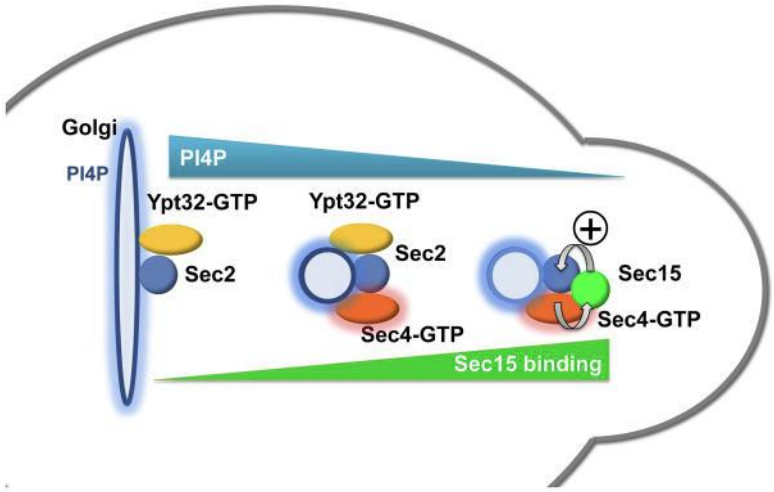


Figure 1.2 Exocyst maturation *– Golgi – associated GTPase Ypt-32 recruits Sec2 to membrane. Sec2 itself is a positive regulator (GEF) of another GTPase – Sec4. Sec4 recruits exocyst subunit Sec15 in its active state. Sec2 is able to bind both Ypt-32 and Sec15 with the interactions being competitive. Sec2-Ypt-32 interaction is favored by PI4P – rich environment, characteristic for Golgi apparatus and early Golgi – derived vesicles. PI4P concentration gradually drops during vesicle maturation and in mature vesicles, Sec2-Sec15 interaction is favored. Cascade of small GTPases is thus involved in vesicle maturation and exocyst recruitment.*

Sec3 and Exo70 are able to localize to membrane independently on actin cytoskeleton and vesicle delivery in yeast (Boyd et al., 2004). Both subunits bind PIP_2 – Sec3 via its polybasic region in N-terminal domain (Zhang et al., 2008) and Exo70 with basic residues at C-terminus (He et al., 2009). Exo70-mediated PIP_2 interaction is essential for the protein membrane localizations and can properly polarize localization of other subunits even in absence of Sec3p. The phospholipid interaction is specific for PIP_2 (it is much lower for other phosphoinositides) (He et al, 2007). After reduction of PIP_2 content in PM, exocyst fails to

properly localize (He et al., 2009). Furthermore, both subunits interact with various small GTPases in different systems, including budding yeast. Here, they are bound and regulated by members of Rho family, namely Rho1, Rho3 and Cdc42. These proteins are known regulators of actin cytoskeleton and cell polarity (Wu et al., 2008). Two models supposing different role of GTPases and exocyst subunits Sec3 and Exo70 have been formulated. (reviewed by Wu et al., 2008). Older landmark model postulates that exocyst localization is regulated by specific binding of its „landmark" subunits to target sites by interactions with specific GTPases and PM phospholipids. Role of the GTPase is recruitment of the exocyst in such a case. Sec3 recruitment by its interactor Rho1 is an example of regulation via this mechanism. Sec3 fails to localize properly in rho1 mutant (this mutant is specific for exocyst localization and its other properties, like actin cytoskeleton regulation are not affected) (Guo et al., 2001). Complementary model postulates local allosteric activation of the exocyst by GTPases. There is no „landmark" in this scenario and increased localization of exocyst subunit is perceived as a result of enhanced secretion, not as its cause. Cdc42 acts as an exocyst activator in phase of the cell cycle corresponding to early stages of bud formation (Adamo et al., 2001). Defect in Cdc42 impairs exocyst function but not its localization. cdc42 mutant shows strong genetic interaction with rho3 mutation. Overexpression of one gene suppresses mutation in the other one. Similar observations as in case of Cdc42 mutants have actually been done in rho3 mutant yeasts. Namely, exocyst localization does not change with respect to wildtype but it there is defect in secretion (Roumanie et al., 2005). Rho3, an Exo70 interactor, thus seems to act differently than Rho1 in exocyst regulation. In agreement with this, there is no redundancy between Rho3-Exo70 and Rho1-Sec3 pathway (Roumanie et al., 2005). Rho3-Exo70 interaction, contrary to Exo70- PIP_2 interaction, is in deed not essential for Exo70 membrane association (He et al, 2007). Both Cdc42 and Rho3 were shown to activate exocyst in GTP-hydrolysis independent manner (Roumanie et al., 2005). Thus, allosteric activation is the most probable scenario in this case (Wu et al., 2008;Roumanie et al., 2005). It is evident that some GTPases activate exocyst but do not have role in its localization, whereas other GTPases and specific phospholipids mediate its interaction with membrane.

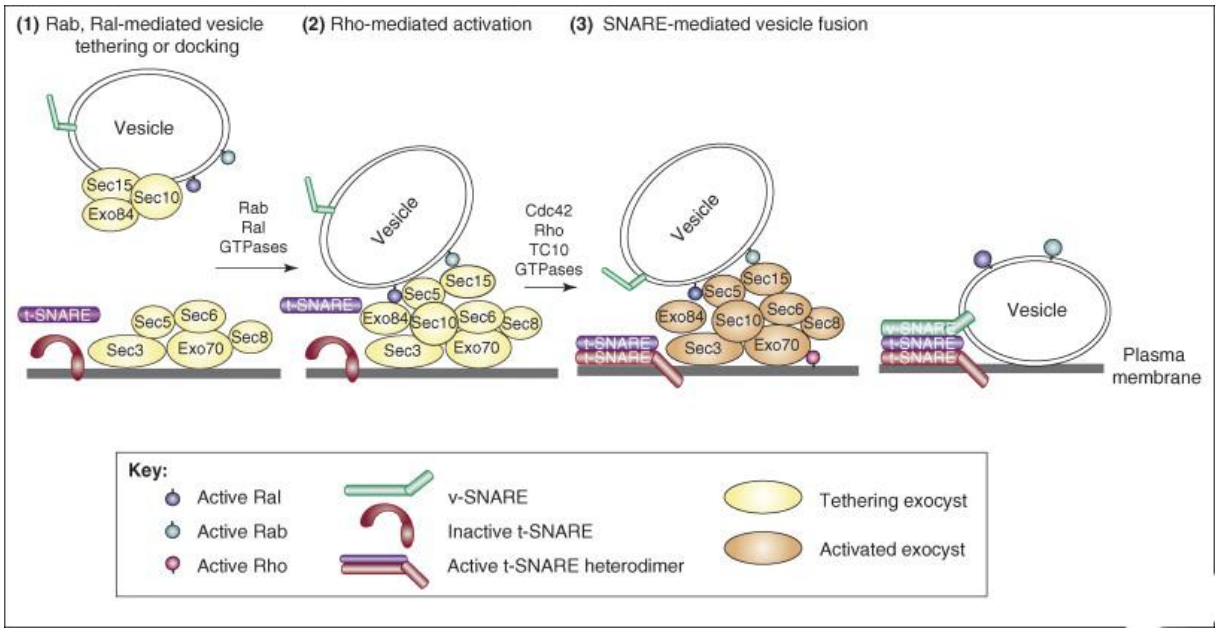


Figure 1.3 Exocyst – mediated vesicle tethering *(adopted from Wu et al., 2008). Some subunits of exocyst associate with target site at PM while other bind arriving vesicle. Note, that for other subunit than Sec3, Sec15 and Exo70, the particular distribution between PM and vesicle depicted is highly speculated. Vesicle tethering assisted by exocyst assembly brings the two membrane together. Small GTPases help localize exocyst, as well as mediate steps of its assembly (see also text of the publication and of this thesis for landmark and activation concept of small GTPase action)*

Evidences that the exocyst-localization models accepted for budding yeast might not be appliable on all model organisms are accumulating. Several recent studies show, that in another widely used fungal model, *Schizosaccharomyces pombe*, actin cytoskeleton and exocyst act in parallel and their presence is functionally redundant for polar growth. Disrupting of either pathway itself does not lead to a major defect in cell polarity (Bendezú et al., 2011; Nakano et al., 2010). On the other hand polar growth is disrupted in double mutants with defect in both pathways: mutation in either of myosin myo52, a molecular motor transporting vesicles along actin cables, and formin for3, an actin polarizing factor, results in loss of polarity when combined with loss of Exo70 or Sec8 (Bendezú et al., 2011). Latrunculin treatment, while disrupting actin cytoskeleton, does not result in loss of exocyst localization polarity. This result is in contrast with FRAP experiments performed on *Saccharomyces cerevisiae* showing dependence of exocyst-subunits membrane localization on intact actin cytoskeleton (probably via vesicle delivery) (Boyd et al., 2004). Simultaneous drug-disruption of both actin cytoskeleton and microtubules does not cause loss of polarized growth in *S.pombe* (Bendezú et al., 2011). Both pathways, while acting in parallel, act

downstream of common regulator – the small GTPase Cdc42. Its location remains intact in cells with simultaneous actin and exocyst impairment, while localization of exocyst components and activity and localization of formin For3 both depend on Cdc42 (Bendezú et al., 2011). Another factor, Pob1, was also shown to be required for both For3-mediated actin formation and Sec8 localization (Nakano et al., 2010). Simulataneous mutation in For3 and Sec8 phenocopies Pob1 mutation *pob1-664* with severly altered cell shape. Mechanism of exocyst regulation by Pob1 is not yet knowm, but its binding via a common interactor is suggested. Pob1 homologs from *S.cerevisiae* (Boi1p and Boi2p) both bind cell polarity protein Bem1p, which in turn binds Sec15. Scd2, a protein structuraly related to Bem1p, was suggested as a possible Pob1-exocyst link. Cells mutant in this gene are round, supporting the above mentioned hypothesis. (Nakano et al., 2010). Interestingly, Pob1 interacts with Cdc42 and its overexpression suppresses cdc42 temperature-sensitive mutant (Nakano et al., 2010). Pob1 thus acts together with Cdc42 as an upstream polarity regulator of pathways involved in polar growth: localized vesicle tethering and actin cable polarization. The vesicle tethering branch also contains Mug33. This protein assists exocyst function in yet unknown way and shows similar genetic interactions with members of the actin pathway (For3, Myo52), as mutant genes for exocyst subunits do. (Snaith et al., 2011)

1.3 Exocyst in animal cells – secretory functions and beyond

As in yeasts, exocyst specifically binds to PIP_2 via the Exo70 subunit and prevention of this association abolishes membrane binding of Exo70 (Liu et al., 2007). Also, GTPases are important regulators of exocyst action in animal cells. Different interactions between exocyst subunits and GTPases than those in yeasts exist in mammals, though. For example, mammalian Sec3 and Exo70 do not bind Rho and Cdc42 GTPases but mammalian Exo70 does bind TC10 – a Cdc42 family protein (Wu et al., 2008). This interaction is involved in insulin-activated trafficking of glucose transporters (see also below).

In metazoan systems, interaction with small GTPase Ral is extremely important for exocyst assembly and localization regulation (reviewed by van Dam and Robinson, 2006, see also below). It directly interacts with both Sec5 and Exo84. Several events involving regulated exocytosis have been proven to be exocyst dependent. Trombin-stimulated platelet dense granule secretion triggers exocyst association with Ral. Inhibition of Sec5-Ral association prevents dense granule secretion, demonstrating importance of this interaction. (Kawato et al., 2008). Similarly, insulin-stimulated GLUT4 transport to membrane in adipocyte is dependent on Ral-mediated exocyst recruitment (Chen et al., 2007). Similar process has been suggested to be involved in release of insulin itself. Insulin release is a regulated, Ral-

mediated event occurring in pancreatic beta-cells (Jamie et al, 2008). Above mentioned events together comprise for regulated exocytotic events involving exocyst in animal cells.

Exocyst is also involved in spatial regulation of trafficking in at least some animal cell types. In epithelia, the complex is localized at cell-cell contacts (apical-junctional complex) and mediates transport of cargo to basolateral domain of epithelial cells. Intracellular application of antibodies binding Sec8 prevents further addition of proteins to basolateral membrane but not apical membrane (Grindstaff et al., 1998). Exocyst-mediated trafficking to basolateral domain is maintained also during epithelial remodelling events. Sec10 overexpression increases delivery to basolateral (but not to the apical) membrane. Moreover, it promotes tubulogenesis, an event in which epithelial sheets form tubes with apical membrane facing lumen and basal membrane placed outward, having increased surface when compared to the original state (Lipschutz at al, 2000).

Several exocyst properties act synergistically in formation of membrane processes during cell migration. Besides contractions driven by actin-myosin interactions, continuous membrane trafficking from rear to front plays is important in ameboid movement of animal cells (Hertzog and Chavrier, 2011). Ral-exocyst interactions are necessary for induced filopodia formation and for exocyst recruitment to leading edge of migrating cells. Sec5 or Sec6 knockdown interferes with adhesion-mediating integrin delivery (Hertzog and Chavrier, 2011). Exocyst also interacts with atypical protein kinase C, an important component of animal cell polariation machinery, via scaffold protein kibra. Interestingly, Exo70 directly interacts wit Arp2/3 complex, an actin-nucleating protein. It might thus facilitate actin polymerization at the leading edge of migrating cells (Hertzog and Chavrier, 2011). This interaction is also important for formation of invadopodia, structures formed by migrating tumor cells with ability to degrade surrounding extracellular matrix. Interestingly, animal exocyst is able to bind microtubules and both exocyst as complex and Exo70 subunit alone destabilize microtubules in vitro and in NRK cells (Wang at al., 2004). In the cells Exo70 overexpression also promotes formation of filopodia-like protrusions. Addition of taxol, a microtubule-stabilizing agent, cancels this effect. Thus, microtubule have inhibitory effect on formation of actin-containing protrusions, with exocyst being a mediator of this regulation (Wang at al., 2004). Developing neuron processes have similar structure as migrating cells and it is thus not unexpected, that exocyst is required for dendrite/axon growth and synaptic maturation (Murthy et al., 2003). Observations of *Drosophila sec5* mutants uncovered, however, exocytosis independent on functional exocyst in synaptic transmission (Murthy et al., 2003). Although exocyst is necessary for growth of neuron processes and trafficking of specialized proteins to their ends in order to form functional synapses, release of

neurotransmitters at once formed synapses is exocyst independent. Neurotransmitters are released from special type of vesicles that arise through recycling of membrane material and filling with new content directly in axon terminals (Murthy et al., 2003). One might wonder, whether such results indeed do uncover exocyst functions or apply specifically to the Sec5 subunit.

Big surprise in the field of endomembrane system and trafficking biology was brought by a publication demonstrating exocyst function in autophagosome assembly (Bodemann et al., 2011). RalB activation was shown to be involved in autophagosome assembly and its activation sufficient for autophagosome induction. Binding of RalB to Exo84 occurs during initial steps of autophagosome formation, and key molecular components bind directly to Exo84. Interestimgly, these components are bound to Sec5 in inactive state and their interaction with Sec5 inhibits autophagy. Thus, presence of different subcomplexes with opposite role in regulation of formation of autophagosome was supposed.Both subcomplexes interact would interact with RalB – one via Sec5 and other via Exo84. Specific model for exocyst role in tethering or other memrane regulating event during autophagosome formation was not introduced.

1.4 Exocyst and secretory pathway in plant cells

Many vital functions of plant cells involve coordinated vesicle-fusion events. Plant cell are enclosed inside firm cell walls, so changes of their size and shape either occur while the cell wall is still young and less rigid or involve weakening of cell wall by localized secretion of lytic enzymes. Some plant cells exhibit type of growth referred to as tip growth. It differs from common, so called diffuse growth (Martin et al., 2001) which involves addition of new material to all parts of the growing cell. Typical examples of tip-growing structures in angiosperms are root hairs and pollen tubes. Mechanisms underlying their growth share many similarities and they both involve highly-localized extensive secretion (Martin et al., 2001). Several membrane domains with different protein composition coexist in a plant cell (Grebe M., 2010). Plant cell plate can also be viewed as a specialized secretory compartment. It is formed by a coordinated fusion of Golgi-derived (and probably also endocytic – see) vesicles, followed by reorganization involving endocytosis and additional fusion events. Capability of polarized targeting of proteins is important for both specific interaction with external environment and signalling between cells in plant tissues. Immune response of plant to a pathogen attack also involves localized and polarized secretory response (Yun et al., 2008). One of the most striking differences between opisthokont and plant exocyst in presence of multiple paralogs coding exocyst subunits in plant genomes. There are at least two paralogs for most subunits and striking 23 Exo70 paralogs in

Arabidopsis thaliana genomes (Elias et al., 2003). Exact number of copies differs among plant species but presence of multiple copies of genes for subunits with highest number of copies coding Exo70s is a general trend (Chong et al., 2009). Exo70A1, the dominant Exo70 in most tissues, is clearly involved in both tip and diffuse growth of plant cells, including root hairs, stigmatic papillae and elongating hypocotyl (Synek et al., 2008). Regulated degradation of Exo70A1 resulting into impaired growth of involved stigmatic papillae was suggested to be an important factor in self-sterility response (Samuel et al., 2009). Exocyst is indispensable for pollen tube growth, as has been demonstrated by genetic studies (Cole et al. 2005, Hála et al., 2008). Polarized secretion of pectin in seed coat is also mediated by exocyst, since sec8 and Exo70a1 mutants exhibit defect in pectin deposition (Kulich et al., 2010). Genetic and localization studies have demonstrated role of exocyst in cytokinesis as well (Fendrych et al, 2010). Based on fact that exocyst is involved in regulated secretion in nonplant species (see previous chapters), plant cells perform many different events involving exocytosis and requiring regulation in time and space and that plant genomes contain many alternative Exo70 subunits, concept of specialized recycling membrane domains present in parallel in a plant cell has been proposed (Žárský et al., 2009). Different Exo70 variants might interact with membrane and protein interactors, including GTPases. C-terminal region responsible for PIP_2 binding in yeast is present in plant Exo70s but slightly differs between particular paralogs. It is one of regions potentially involved in location to target membrane domain. Specialization of other components of secretory pathway is expected as well. In fact, examples of such proteins are already known - like cell-type specific Rab proteins involved in root hair or pollen tip growth (Preuss et al., 2004 and Szumlanski et al. 2009, respectively). As mentioned above, specialized variants of membrane SNARE proteins accumulate around site of pathogen attack **(**Yun et al., 2008) and recruit secretory machinery. Exo70B2 interacts with one of these SNARE proteins and was shown by mutant studies, together with Exo70H1, to participate response to a pathogen attack (Pecenkova et al., 2010). Immune response is thus first example of regulated exocyst-involving secretory event with specialized Exo70 variant participating. Current knowledge of Sec5 protein in plants is scarce. Two paralogs of Sec5 are present in *Arabidopsis* genome with one of them preferentially transcribed in most plant tissues (Chong et al., 2009). Genetic experiments imply interaction of Sec5a with Exo70A1 subunit and necessity of at least one functional Sec5 allele (at least one copy of either Sec5a or Sec5b) for pollen tube growth (Hála et al. 2009). Nuclear-enriched phosphoprotein extract sequenced by contained putative fragment of Sec5. Authors admit that the peptide might be rather contamination than nucleus-derived. Their discovery, however, implies regulation of plant Sec5 protein by phospohorylation. Further investigation of Sec5, mainly of its localization and dynamics, is the main topic of this thesis.

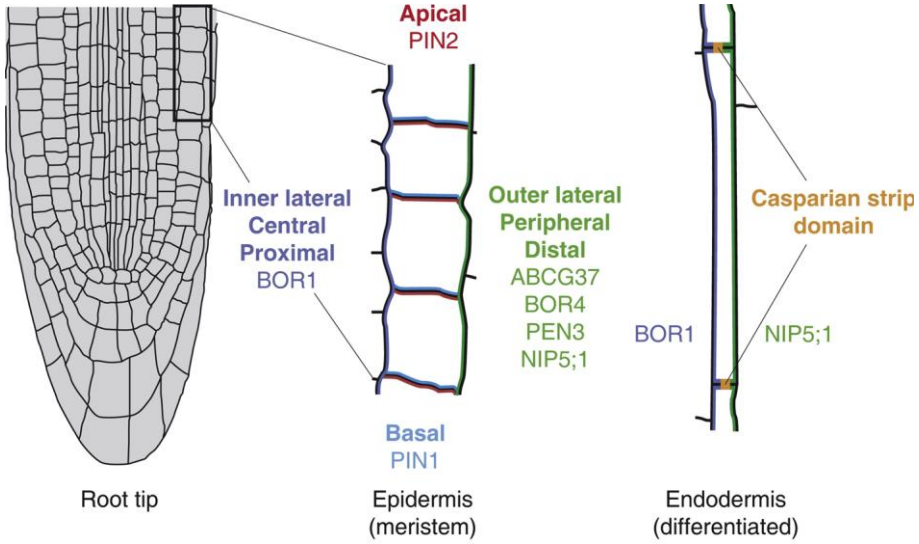


Figure 1.4 Membrane domains of a plant cell *schematic depiction of plat tissue with proteins experimentally confirmed to be specific for particular membrane domains, derived from Grebe M.,*

2. AIMS OF THIS THESIS

1. To prepare transgenic *Arabidopsis* plants expressing Sec5a-GFP exocyst subunit under the control of the constitutive 35S promoter

2. To study subcellular localization and dynamics of Sec5a in various *Arabidopsis* cells and during cytokinesis by confocal microscopy and to analyze Sec5a-GFP localization and dynamics by TIRF/VAEM microscopy

3. To compare results with previous studies of other subunits of the exocyst complex in *Arabidopsis*

3. MATERIALS AND METHODS

3.1 Transient transformation of tobacco leaves by infiltration

Agrobacterium tumefaciens (strain GV3110) cells were transformed with gateway binary vector pGWB6 vector carrying Sec5a-GFP under 35S constitutive viral promoter (Nakagawa et al., 2007; the construct had been previously prepared by Mgr.Matyáš Fendrych) by Electroporation at 1700V using Eppendorf Electroporator 2510. Transformed bacteria were grown on plates with solid LB medium containing kanamycin and hygromycin as selective antibiotics. After 3 days of incubation at 28°C, single colony grown on selection medium was transferred into 5mL of liquid LB medium, containing hygromycin and kanamycin, both at concentration of 50μg/ml. *Agrobacterium* cells were further cultured in a shaking incubator until the suspension reached OD_{600} 0.3-0.5 at 1:5 dilution. 1mL of culture was than transferred into Eppendorf tube and pelleted at 4000rpm for 5 minutes. Bacterial pellet was washed twice with 1mL of infiltration medium with acetosyringone added. Then, cells were centrifuged at 4000 rpm for 5 minutes. Pellet was resuspended in infiltration medium to OD_{600} 0.01-0.05. Bacterial suspension was injected into the abaxial epidermis (it contains stomata, allowing entering of the solution) of *Nicotiana tabacum* plants with 1mL plastic syringe by holding the leaf against the syringe and gradually applying pressure. Spread of entering liquid resulted into darkening of corresponding leaf area. Infiltrated area was marked with a permanent pen for facilitating further orientation. Infiltrated plants were incubated for 2 days at 28°C. For microscopic observation, squares of transformed leaf area were cut and mounted in distilled water.

3.2 Preparation of stable Sec5a-GFP expressing *Arabidopsis* line

Arabidopsis thaliana plants (ecotype Columbia) were grown at 22°C under long-day conditions (16h light, 8h dark) in pots with diameter of approximately 10 cm. For soil growth of *Arabidopsis* plants, hydrated peat pellets produced by Jiffy group were used. Primary inflorescences were then cut, in order to induce growth of bushier secondary inflorescences with more flowers. Such cut plants were left in cultivation room for one week to produce the secondary inflorescences. *Agrobacterium tumefaciens* cells were transformed and grown on plates with the binary vector as described in part M.1 about one week prior transformation of plants. 3-days old colony grown on antibiotics was transfered to 2ml of liquid LB medium and grown overnight. On the following day, this 2mL of medium with grown bacteria were trensfered to 20mL of the same medium with antibiotics and left to grow overnigth again. The procedure was then repeated one more time, to prepare 200mL of overnight-grown suspension for the transformation procedure. Cells were centrifuged at 3700g and 4°C for 15 minutes. Pellet was resuspended in small amount of liquid that remained in falcon tubes after

removal of supernatant and in 5% sucrose. Silwett L-77 (Ambersil Ltd. UK) was then added up to a final concentration of 0,05%. The exact volume of sucrose solution was adjusted, so that there would be enough liquid in pot used for floral dip. Prior to transformation, all opened and immature siliques are removed from plants, so that only closed would remain at the tips of stalks. Whole shoots were dipped into solution for about one minute and left overnight in a dark place covered with plastic sac. They were uncovered and put back into the cultivation room in the following morning. After ripening and drying of siliques, seeds from the transformed plants were sterilized and vernalized (see below). Vernalized seeds were plated on solid ½MS medium containing kanamycin as a selection agent and claforan to kill remaining *Agrobacterium* cells on horizontal plates. One week old seedlings were carefully observed and those resistant to kanamycin (resistant plants, contrary to nonresistant, have long roots growing deep into medium and are larger) further examined for GFP fluorescence. About twenty plants exhibiting various level of fluorescence were planted to soil and grown until production of seeds under the same conditions as described above for *Arabidopsis* growth. Thus, about 20 different lines were prepared. One of them was further used for microscopic observations and inhibitor treatments described in the thesis.

3.3 Plant material used for microscopic observations.

Besides Sec5a-GFP line prepared as a part of work on this thesis two other *Arabidopsis thaliana* lines were used, namely plants expressing Sec6-GFP under control of native promoter and plants expressing Exo70A1 under control of constitutive 35S promoter. (Fendrych et al., 2010)

For observations and experiments, plants were grown accordingly: after seed sterilization and vernalizaiton (see below), they were planted on vertical plates with solid MS medium. Individual seeds in 3-4 rows were separated by approximately 1 cm spaces to ensure enough space for growth of seedlings. Manipulation with seeds was done in flowbox to prevent contamination of MS medium. Vertical plots were sealed with parafilm (Pechiney plastic packaging) and kept in cultivation rooms with light-cycling conditions described above. 4-5 days old seedlings were used for microscopic observations and drug treatments.

For observation of root hairs, plants were moved, together with underlying piece of solid medium, to chambers used for confocal microscopic observations approximately 12 hours prior to examination. These chambers were than positioned vertically and left in cultivation room. Prior to microscopy, mechanical manipulation with plants was reduced to careful transfer of the whole chamber with plant under the confocal microscope.

For FM4-64 staining, plants were carefully moved to liquid MS medium with 1:500 dissolved FM4-64 stock solution (500mM FM4-64 in DMSO, Invitrogen, stored at -4°C). After 10 minutes of staining, plants were observed. For observaiton and signal recovery measurement of plants with disrupted cytoskeleton, 1:1000 dilutions of 100mM APM (Sigma) in ethanol and 10mM latB (Molecular probes) in DMSO (stocks were stored at -20°C) were used for microtubule and actin disruption, respectively. Stocks were dissloved in liquid 1/2MS medium for treatment. Seedlings were treated with cytoskeleton-disruption agents for 1 hour. Control plants were treated with corresponding concentrations of ethanol (as a control for APM treatment) and DMSO (as a control for latB treatment) dissolved in liquid MS medium, because these substances are used as solvents for stocks of the drugs.

3.4 Seed sterilization and vernalization

Prior planting in sterile conditions (for selection of transformants and microscopic observations), seeds were treated followingly: They were incubated in 70% ethanol for 4 minutes and than twice in 10% bleach for 3 minutes. Seeds were washed with sterile water 6 times afterwards. Vernalization was achieved by keeping sterilized seeds in water at 4°C for 3-4 days.

3.5 Preparation of media used for bacteria and plant growth:

½MS medium (1L): following amounts of contents are dissolved in distilled water: 2,2g of Murashige and Skoog medium (stored at 4°C), 10g of sucrose, 100mg of myo-inositol, 500mg of MES, medium is supplemented with 1ml of vitamin stock solution (stored at -20°C) containing thiamin (1mg/ml), nicotinacid (0,5mg/ml) and pyridoxin (0,5mg/ml). After preparation of solution, its pH is adjusted to 5,7 by KOH addition. For preparation of solid ½MS medium, agar is added to the solution after pH adjustment. 16g of plant agar is added per 1L of medium for vertical plates and 8g of plant agar for the same amount of medium for horizontal plates.

Medium is autoclaved and let to cool to room temperature prior to use. It is stored at room temperature. If solid medium with antibiotics is needed, they are added after autoclaved medium reaches room temperature but before it solidifies. Medium is poured to plates and let to solidify inside a running flowbox. Following final concentration of antibiotics are used: 100µg/ml claforan, 25µg/ml kanamycin.

Infiltration medium (100mL) MES 10mM, $MgCl_2$ 10mM, 200µM acetosyringone (added from a 200mM stock stored in alliqots at -20°C).

LB medium (1L): Luria broth base 15,5g, NaCl 4,75g, 7.5g of micro agar (for solid LB medium).

Medium is autoclaved and let to cool to room temperature prior to use. It is stred at room temperature. If solid medium with antibiotics is needed, they are addad after autoclaved medium reaches room temperature but before it solidifies. Medium is poured to plates and let to solidify inside a running flowbox. Following final concentration of antibiotics are used: 50µg/ml hygromycin, 50µg/ml kanamycin.

3.6 Microscopic observations

Olympus BX51 epifluorescence microscope was used for preliminary observations, for selection of seedlings with GFP signal during Sec5a-GFP line preparation and for selection of plants with reasonable level of fluorescence for confocal microscopy and TIRF/VAEM observations.

For confocal microscopy (which involved all observations published in results besides TIRF/VAEM microscopy), Zeiss LSM5 DUO confocal laser scanning microscope with Zeiss C-Apochromat x 40/1.2 water corrected objective was used. For GFP excitation, lasers with wavelength 488 and 489nm were used. For FM4-64 excitation laser with wavelength of 561nm was used.

For TIRF/VAEM microscopy, we used the Leica DMI6000 microscope with TIRF illumination and a HCX PL APO x100.0/1.46 OIL objective. We used laser with wave length of 488nm for GFP excitation.

3.7 FRAP experimental setup

FRAP experiments were performed with the same instrumental setup as other confocal microscopy observations. All images analyzed in FRAP experiment were acquired with identical settings. The image size was 500x200 pixels and the region of interest was large 70x20 pixels. It was bleached with 10 iterated excitations by 489 nm laser at 100% power. 5 and 35 frames were acquired prior and post bleaching, respectively

3.8 Image analysis and software

images were analyzed using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011). The root growth occurring during FRAP experiment was compensated using the CorrectStackDrift.txt macro (http://rsbweb.nih.gov/ij/macros/examples/).

After correction of FRAP images for root tip growth,resultus were normalized according to following formula:

$$I_t = \left|\left(A_t / C_t\right) - \left(A_b / C_b\right)\right| / \left|av - \left(A_b / C_b\right)\right|$$

where A_t represents the intensity in the bleached area in the time *t*, *Ct* intensity in the control non-bleached area, A_b and C_b intensity in the bleached area immediately following the bleaching, *av* represents average A_t/C_t intensity in 5 frames prior to bleaching.

For quantification of punctae density, Cell Counter ImageJ plugin was used. Punctae in 5O μm^2 rectangles from 10 cells were used.

For lifetime distribution analysis of Sec5 punctae, 5 pixel-wide regions of images of TIRF/VAEM time-series were selected and trimmed time-lapse images (with images captured in 500ms intervals) were assembled into kymographs with single images following from left to right. Lifetime of approximately 500 punctae was than manually counted.

Z-stacks for long-term distribution of exocyst punctae were constructed from images captured every 500ms for approximately 5 minutes. Z-stacks of whole observation, first, second and last third of each event were reconstructed (see results and discussion).

Figures in this thesis were prepared with ImageJ and Adobe Photoshop.

4. RESULTS

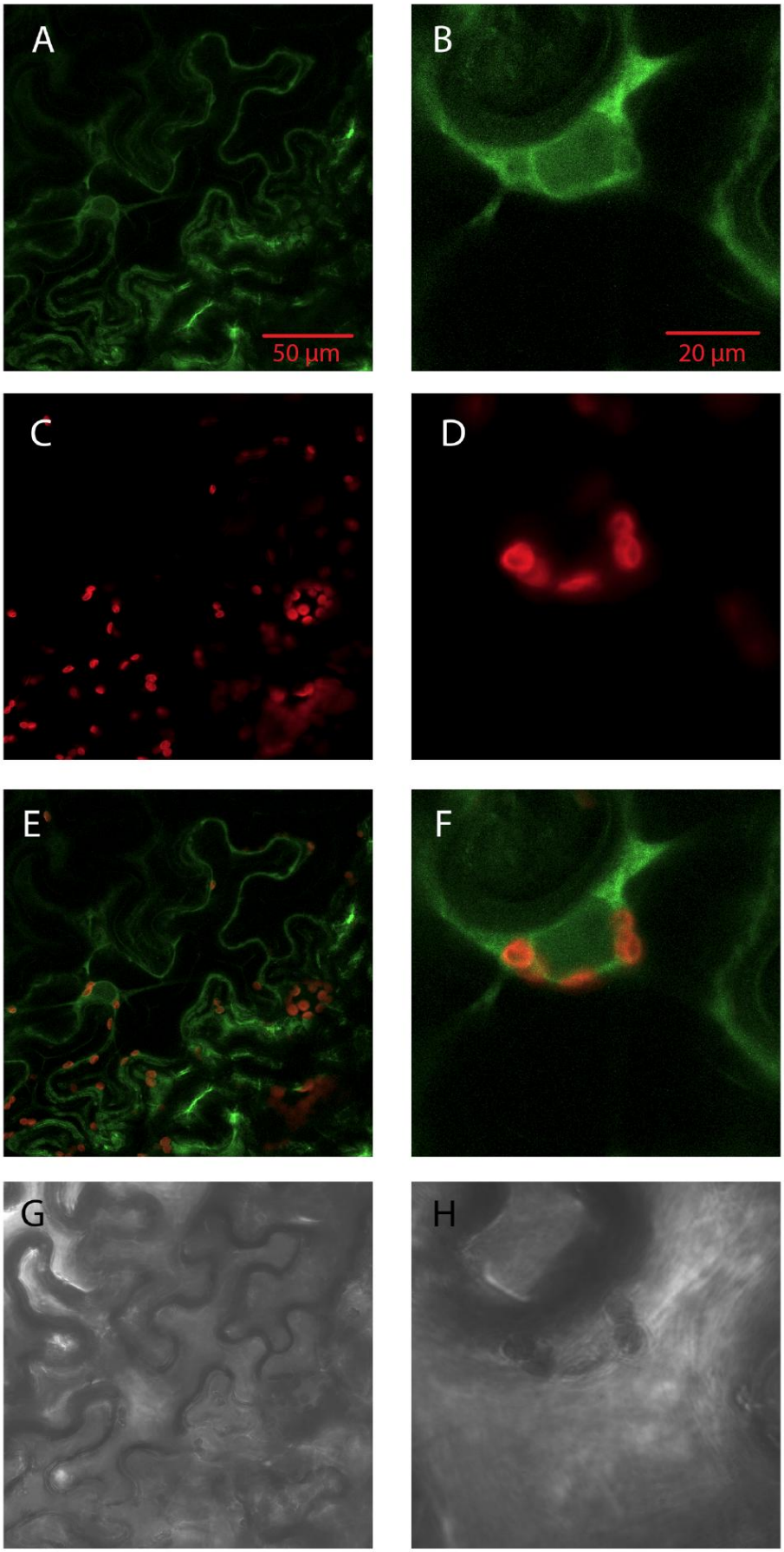
4.1 Transient transformation of tobacco leaves with Sec5a-GFP under the control of the 35S promoter

To investigate subcellular localization of Sec5a protein, we first performed transient transformation of tobacco (*Nicotiana benthamiana* leaves with Sec5a-GFP under 35 promoter. The protein localized to cytoplasm, including cell cortex, transvacuolar strands and area around nucleus and chloroplasts. As the exocyst is involved in vesicle tethering at PM, I expected PM localization. PM localization is possible but could not be demonstrated due to relatively strong signal of cortical cytoplasm. Fluorescent protein was not detected in nucleus. Figure 1. shows transformed tobacco (*Nicotiana benthamiana)* epidermal cells observed with confocal scanning laser microscope.

4.2 reparation of stably-transformed *Arabidopsis* plants expressing Sec5a-GFP under the constitutive viral 35S promoter

To get a deeper insight into Sec5a protein localization and dynamics, I transformed *Arabidopsis thaliana* plants, to prepare stable lines. Based on resistance to kanamycin, 20 offspring plants/transformants were selected for further propagation. Plants of several lines exhibited GFP fluorescence in the second generation. One of these lines was used for further observations and measurements described in this thesis. Interestingly, sibling seedlings exhibited variable levels of fluorescence, although being derived from the same parental plant. It is possible that Sec5a-GFP gene under 35S promoter was inserted into an epigenetically unstable part of the genome and/or was differentially silenced due to presence of the viral promoter (see discussion). Plants with reasonable level of fluorescence were selected for observations under the confocal scanning laser microscope and for TIRF/VAEM microscopy. None of the observed plants exhibited extremely high level of fluorescence (as for example plants expressing free GFP do).

Figure 1. Transient expression of Sec5-GFP in tobacco leaf epidermal cells (next page). *A:whole cell signal in GFP channel showing cytoplasmic localization, including transvacuolar strands and perinuclear area. It is excluded from nucleus and chloroplasts. C:chloroplast autofluorescence. E:merged image formed from A and C, G:the same cell in brightfield. Detail of perinuclear area and chloroplasts is shown in right column (B:GFP, D:chloroplasts, F:merged, H:brightfield.*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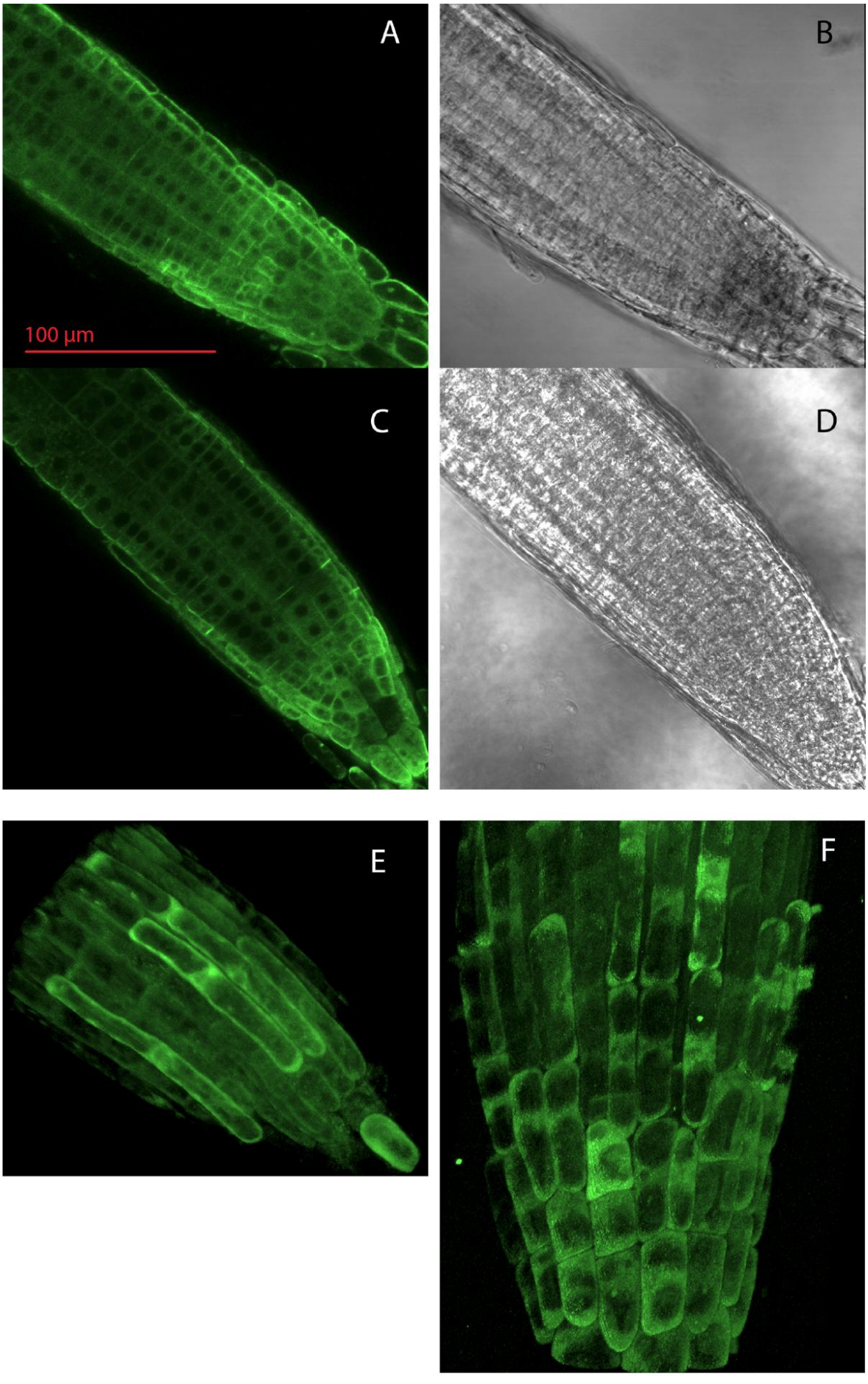
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Figure 2. Sec5-GFP in root of stable Arabidopsis transformants (previous page) *A and C: optical section of two different roots showing signal in various cell types, including columella cells, lateral root cap cells, root apical meristem, root epidermal cells and ground tissue. Strong signal at cell plates of dividing cells is also visible. B and D: brightfield images of the same roots. E:3D reconstruction of root tip, reconstructed from optical sections obtained by confocal microscopy. Most of the cells visible belong to root cap, which covers surface of the root tip. F:3D image of root tip cells in layers under the lateral root cap, obtained by reconstruction of optical sections from deeper layers.*

4.3 Sec5a-GFP is localized in cytoplasm and decorates the cytoplasmic membrane-cell wall interface in *Arabidopsis* root cells, with enrichment at outer lateral domain of epidermal cells and lateral root cap cells

Sec5a-GFP shows rich cytoplasmic localization in cells of the root tip. An overall picture of Sec5a-GFP localization in the root tip is shown in Figure 2. Most prominent cytoplasmic signal was observed in columella cells at the very tip of the root. Most cells showed stronger signal in basal and apical membrane area when compared with the cytoplasmic signal. Lateral root cap cells and root epidermal cells showed strong signal enrichment at the outer lateral membrane domain – place of rich pectin secretion. The protein was excluded from nuclei. Occasionally, cells detached from root cap can be observed near a root tip. These border-like cells (see Discussion) showed strong expression of Sec5a-GFP. Figure 3. shows details of the localization of Sec5a-GFP in *Arabidopsis* root cells.

4.4 Sec5a-GFP collocalizes with membrane marker FM4-64 dye

In order to prove the PM localization of Sec5a-GFP, we stained Sec5a-GFP expressing plants with the fluorescent endocytotic dye FM4-64, which exhibits fluorescence only in hydrophobic environment and is used to visualize membranes, including endosomes and other internal membrane compartments (Bolte et al, 2004). Figure 4. shows colocalization of FM4-64 signal and Sec5a-GFP signal in epidermal cells. Sec5a-GFP signal in the PM area will be further referred to as membrane signal, although FM4-64 staining and collocalization evaluation was not done during all observations. Endosomal compartments visualized by internalized dye have almost no fluorescence in GFP channel. Thus, Sec5a protein localized at PM does not enter endocytic compartments/endosomes and probably cycles between membrane and cytoplasm (see results of FRAP experiment and discussion).

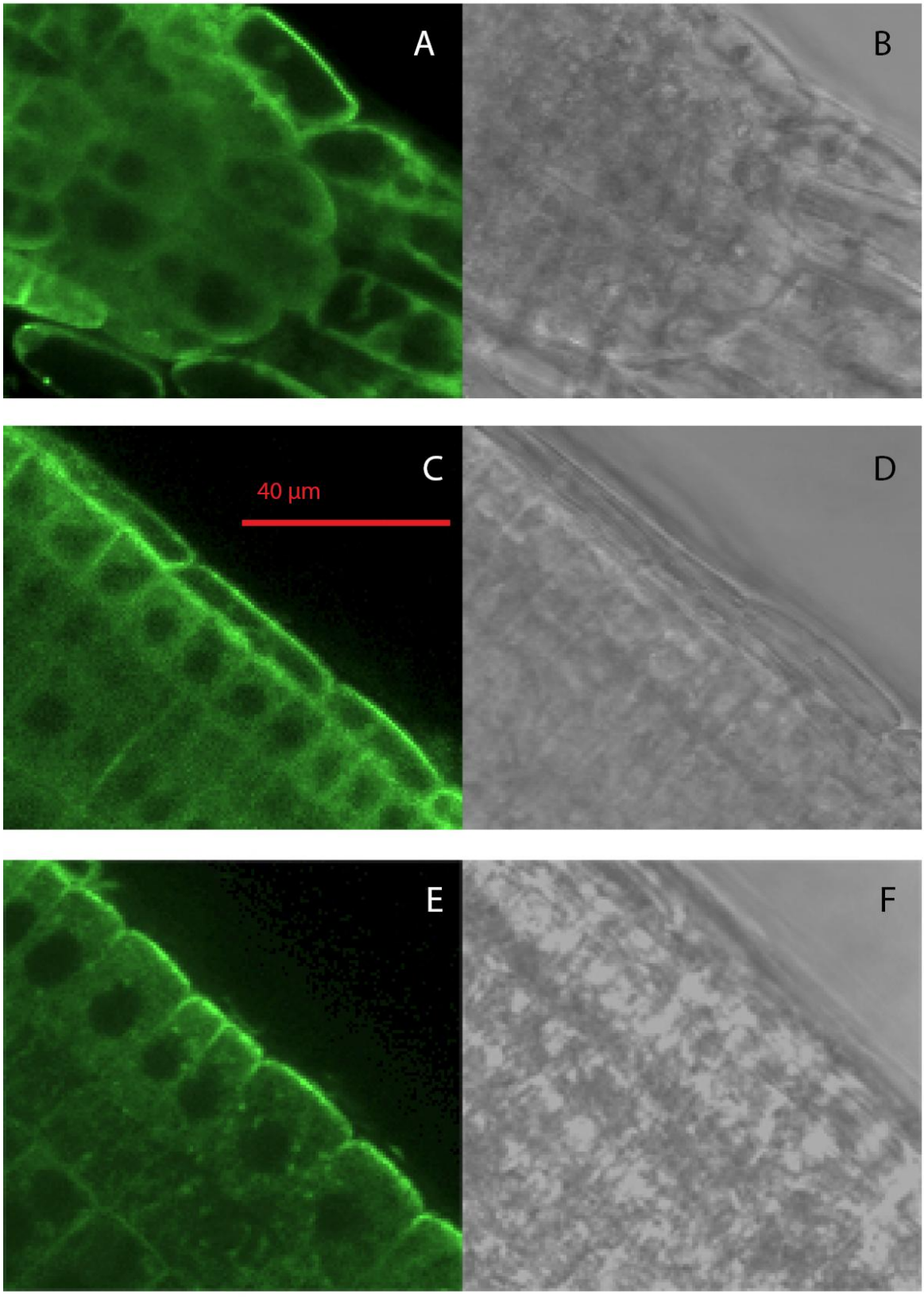
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Figure 3. Detail images of Sec5 localization in various root cells. (previous page) *A: the very tip of root with columella cells. C: root epidermal cells with outer polar domain showing enhanced signal. These cells are covered with lateral root cap cells. E: root epidermal cells from region farther from root tip, not covered with lateral root cap. B,D and F: corresponding brightfield images.*

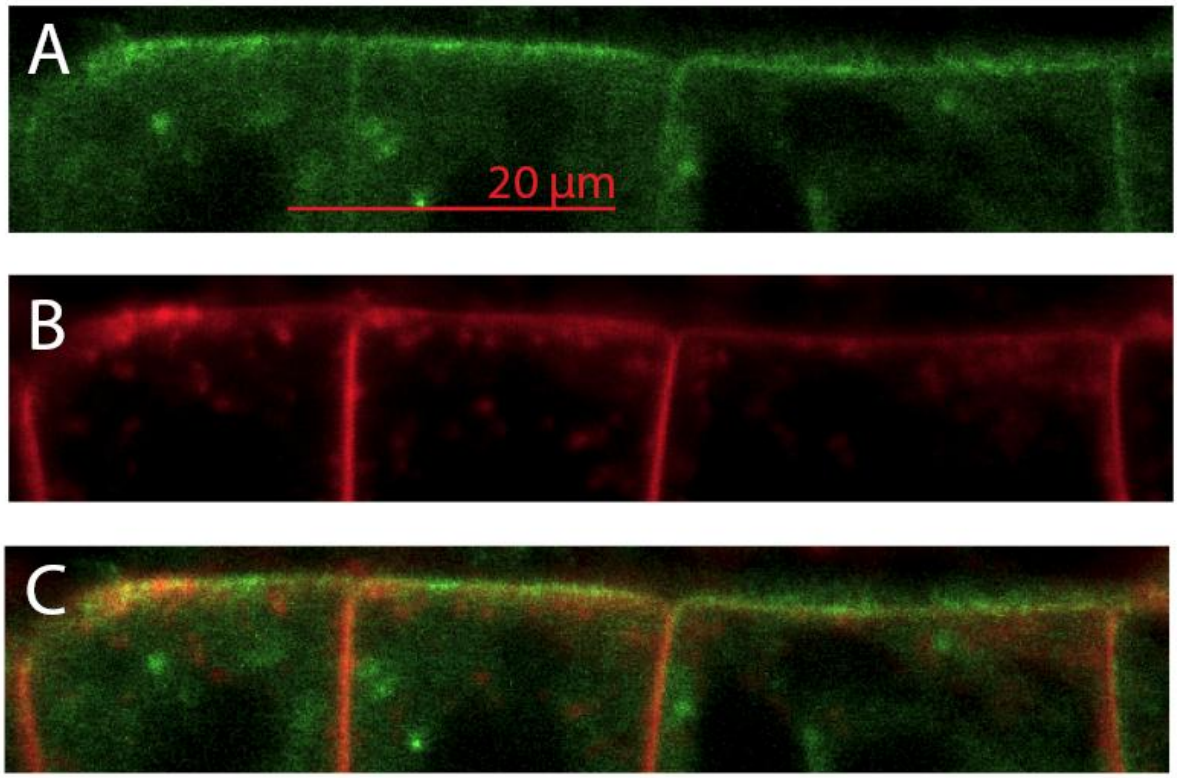


Figure 4. Colocalization of FM4-64 dye and Sec5-GFP signal. *A:GFP signal, B:FM4-64 signal, C:merged. Signals colocalize at membrane. FM4-64 also stains endocytic compartments but these do not colocalize with Sec5 signal. Larger vesicle-like structures visible in Sec5 channel do not colocalize with Sec5.*

4.5 Sec5a-GFP is localized in cytoplasm, PM area and is enriched near tips in growing root hairs

Root hairs are cells exhibiting an extensive tip growth. As such, they continuously transport material by vesicles to the growing tip with extensive fusion events. I was interested, whether Sec5 preferentially localizes to the area, where secretory vesicles meet the extending PM. To investigate Sec5a localization in trichoblasts and root hair cells, seedlings were grown for several hours in chambers used for confocal microscopy observations (see Methods). Possible damage of growing root hairs due to manipulations was thus minimalized. Only young and actively growing root hairs were observed and analyzed. Viability of the root hairs was assessed by time-lapse microscopy showing growth of a hair from the observed area. In

trichoblasts with newly outgrowing root hairs from bulges and in growing root hairs, signal is mostly cytoplasmic with enrichment in PM area. Figure 6 shows Sec5-GFP signal on optical sections and 3D reconstructions of root hairs. Supplementary video 1 shows growth of one root hair from the observed area.). Root hairs in the fig. 6, as well as the hair on the **supplementary video 1** show cytoplasmic localization of the Sec5-GFP signal with enrichment in the PM area. Moving fluorescent structures visible inside the growing hair in GFP channel are most probably starch grains.

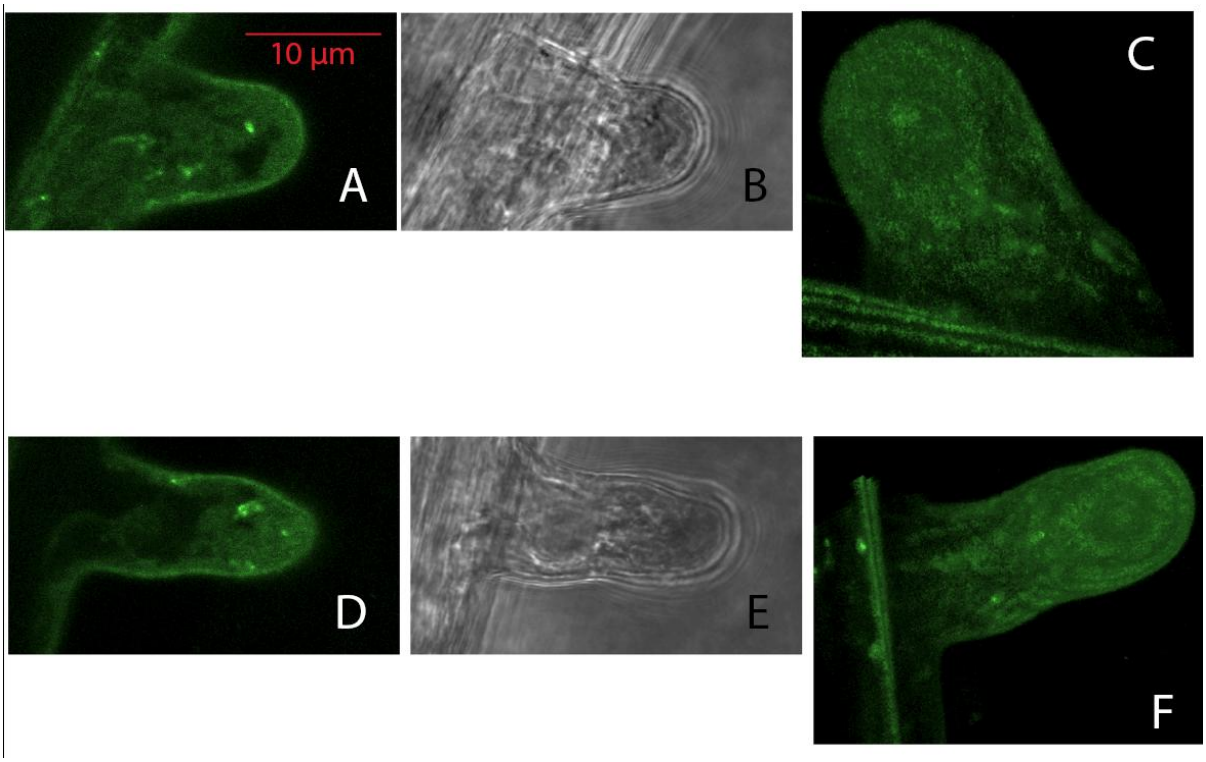


Figure 5. Sec5 localization in young root hairs. *A: Sec5 in root hair around browth initiation stage. Signal is in cytoplasm and at membrane, where it is stronger. B: brightfield image of root hair shown in A, C: 3D reconstruction of the corresponding root hair prepared from optical sections. D: slightly older hair than that shown in A. Cytoplasmic signal is stronger at the tip and less apparent towards the base. Membrane area shows stronger signal than cytoplasm. E: brightfield image of root hair shown in D, F:3D reconstruction of GFP signal of the same hair.*

4.6 Sec5a-GFP is strongly localized to the newly formed and maturing cell plates

Cell plate in cytokinesis and cortical area between some cells in root meristematic zone shows strong signal. Sec5a protein apparently localizes to the maturating post-cytokinetic cell plates, as other components of exocyst in plant cells do (Fendrych et al., 2010). To investigate dynamics of this localization, time lapse imaging of Sec5a-GFP in dividing root

meristematic cells was performed. Figure 6. shows Sec5a-GFP signal at the growing cell plate at various time points. Signal persists at mature cell plates/fresh cell wall domains between newly divided cells for some time. Sec5a-GFP co-localizes with FM4-64 signal at cell plates (Fig.5). Besides expanding and maturing cell plates with clear GFP signal, earlier plates with FM4-64 signal but without GFP signal can be seen. Localized spots of Sec5a-GFP signal can be also seen at the very beginning of cytokinesis. These signals quickly disappear and stage with FM4-64 stained cell plate without Sec5-GFP signal follows. Sec5-GFP signal reappears only in later stages of cell plate growth described above. Thus, it appears that Sec5a takes part in initial fusion event initiating/establishing cell plate and in final stages of cell plate maturation, possibly including fusion and maturation with PM of mother cell.

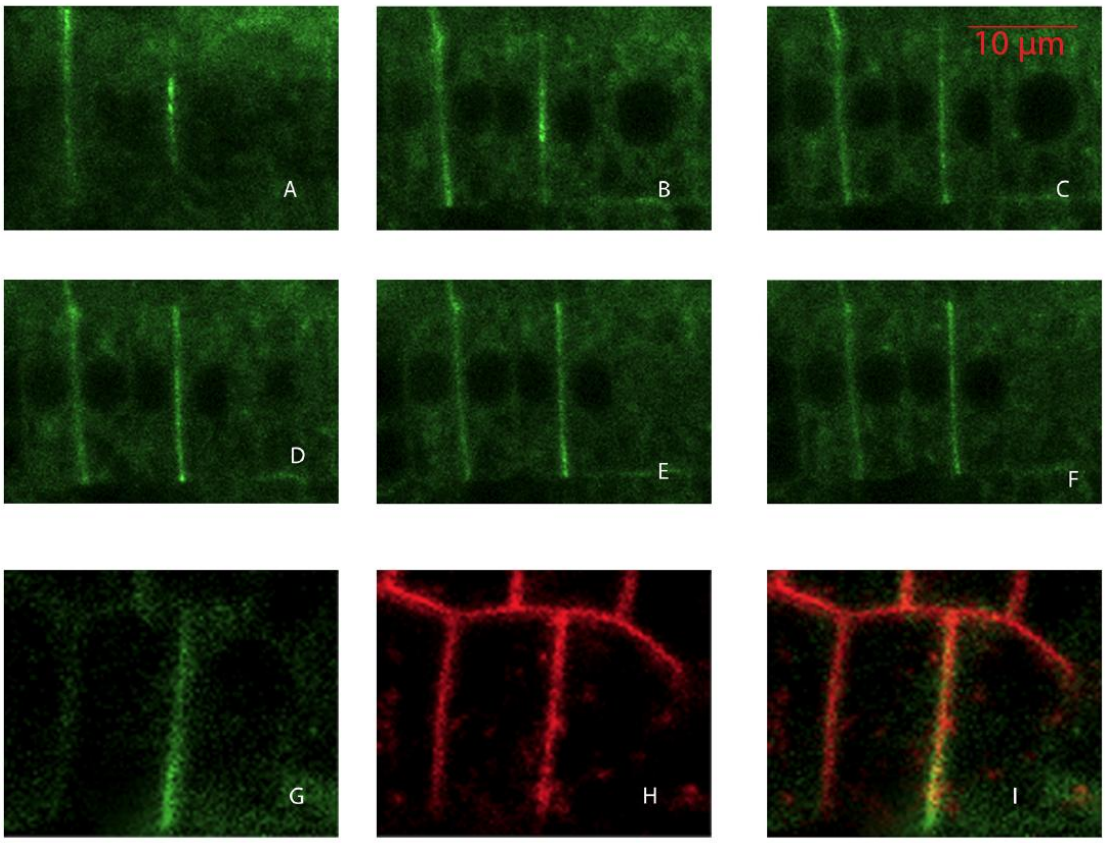
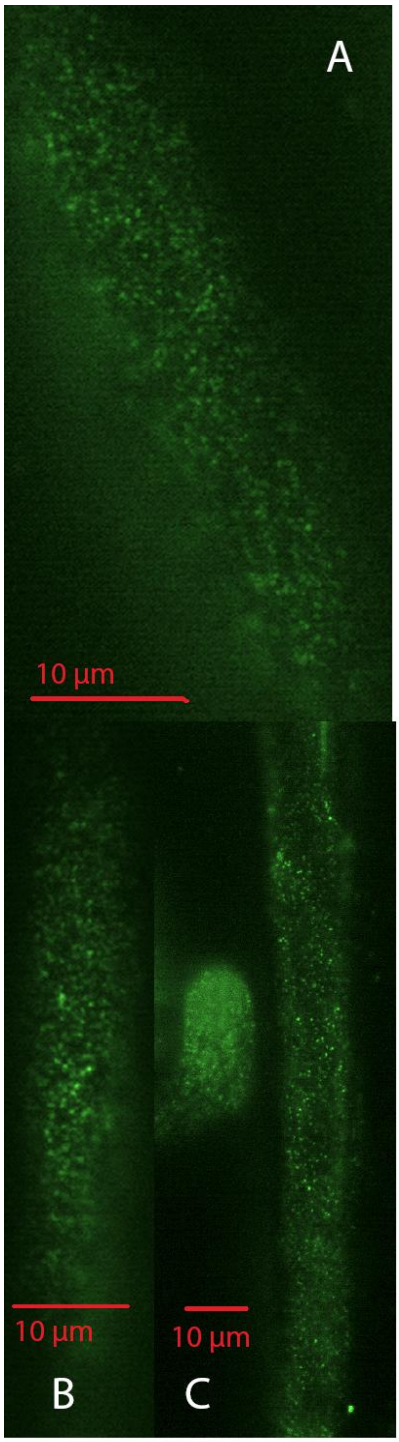


Figure 6. Sec5 in cytokinesis. *A-F: Sec5-GFP localization at growing cell plate during later stages of its maturation and fusion with mother cell's membrane. G-I: colocalization of membrane endocytosis marker FM4-64 with Sec5-GFP signal in maturating cell plate (G:GFP signal, H:FM signal, I:merge).*

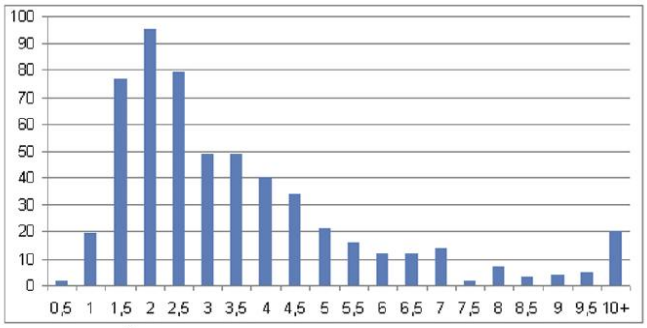
4.7 Sec5-GFP signal observed by TIRF/VAEM microscopy localizes to distinct dynamic spots in PM area of root cells and root hairs

Because recent breakthrough study showed that TIRF/VAEM microscopy can be used for observation of exocytotic machinery (Fendrych – PhD thesis, 2011), including several exocyst subunits, we decided to use the method for more precise Sec5a-GFP behavior determination, because it provides better signal to noise ratio and higher resolution. Observation revealed, that Sec5a-GFP localizes into distinct small punctae in PM. They can be observed in various root epidermal cells , including trichoblasts and root hairs. Density of these punctae, determined by manual counting in images of more than 10 cells (see Methods), was approximately 1,62 per square micrometer. Observed punctae were highly dynamic in respect to appearance and disappearance from particular PM region. They also displayed some, but limited, lateral mobility. I also calculated lifetime of about 500 punctae to get further insight in their dynamics. Figure 7. shows TIRF/VAEM images of different cells and graph with lifetime distribution of the punctae. Most of punctae were rather short-lived, disappearing after 2-4 seconds after arrival to PM area observed. Visualized root hairs might not have been viable due to transport of seedlings from plates to chambers used for microscopic observations. However, punctae in the observed hairs exhibited similar dynamics (when observed and assessed by observation of time-lapse signal from the microscope. No quantitative analysis has been made in this respect) as punctae in root epidermal cells. Movement of Sec5-GFP punctae captured in time by TIRF microscope is shown on **supplementary video 2**.

Figure 7. Sec5 visualized by TIRF microscope. (next page) *A and B: Sec5 punctae in two different root epidermal cells. C: Sec5 punctae in root hairs. Tip of one root hair, with high density of Sec5 punctae, is visible on the left part. Body of another root hair is visible on the right. Left part of figuer shows distribution of different lifetime lengths among 500 individually examined Sec5 punctae in root epidermal cells.*



Lifetime distribution of Sec5 punctae

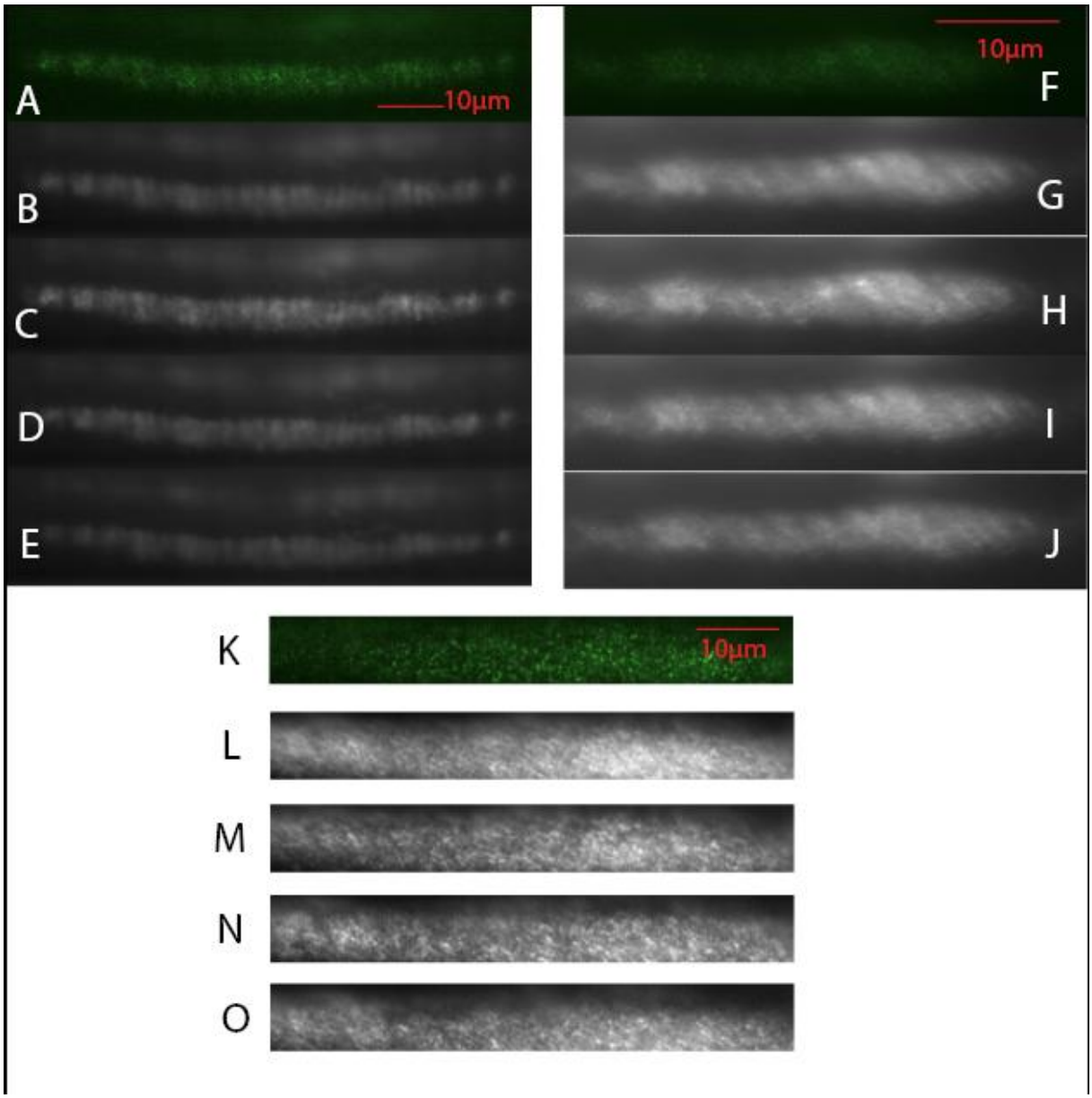


seconds

4.8 Distribution of exocyst punctae visualized by TIRF/VAEM microscopy is not random in time and space.

We were further interested, whether the exocyst subunit punctae visualized by VAEM microscopy and possibly showing tethering events of arriving vesicles arrive to the PM in a homogenous manner to evenly the whole cell surface or whether areas with more frequent arrivals of punctae exist. Therefore, I performed time-lapse imaging of plants stably expressing Exo70A1-GFP, plants expressing Sec6-GFP and plants expressing Sec5-GFP. At least two different plants and at least 4 cells from each plant were observed. Distribution of punctae incidence was than assessed via Z-stacking of images captured in several hundreds of consecutive time moments. Each cell was imaged every 500ms for about 5 minutes. To avoid bias towards events captured earlier (which should provide stronger signal, due to yet unbleached fluorophores), Z-stacks of first, middle and last third of 5-minute long capturing were also assembled into individual Z-stacks. Z-stacks from later phases of capturing events are more fuzzy, due to higher noise-to signal ratio after bleaching during previous phases of time-lapse excitation and image capture. Nevertheless, they approve overall observed pattern. Most of the Z-stacks show non-homogenous distribution of exocyst punctae. This phenomenon is particularly distinctive in the case of Exo70A1. Such „hot-spots“ of exocyst localization are rather stable at the timescale of performed observations. When Z-stacks from three phases of the particular cell observation are compared, sites with higher punctae density change their location only slightly or do not change location at all. Regular patterning of high and low signal stripes along the cell PM in case of Exo701A1-GFP and Sec6-GFP is well observable at Fig. 8 and implies possible involvement of cortical cytoskeleton (possibly especially MTs) in constraining long term exocytosis domains at the PM (see also the Discussion). This phenomenon will be addressed further in the future using anti-cytoskeletal drugs.

Figure 8. Heterogenous distribution of exocyst subunits over time. (next page) *A-E:Exo70: A: distribution of Exo70 punctae at the beginning of time-lapse observation. B: normalized sum of signal of punctae localized at membrane in all time points captured. C, D, E: normalized sum of signal of punctae at membrane during first, second and last third of time-lapse observation, respectively, F-J:Sec6: F: distribution of Exo70 punctae at the beginning of time-lapse observation. G: normalized sum of signal of punctae localized at membrane in all time points captured. H, I, J: normalized sum of signal of punctae at membrane during first, second and last third of time-lapse observation, respectively, K-O: Sec5: K: distribution of Exo70 punctae in the beginning of time-lapse observation. L: normalized sum of signal of punctae localized at membrane in all time points captured. M, N, O: normalized sum of signal of punctae at membrane during first, second and third third of time-lapse observation, respectively*



4.9 Sec5-GFP shows rapid signal recovery to PM after photobleaching and its recovery is independent on microtubule cytoskeleton and only partially dependent on actin cytoskeleton

Exocyst serves supposedly for tethering of freshly delivered exocytotic vesicles prior to their fusion with the PM. As such, it should be highly dynamic and constantly move towards and from the PM. Sec5a-GFP dynamics and rate of localization to PM was measured employing fluorescent recovery after photobleaching (FRAP). After the normalization to non-bleached cell areas (see Methods), fluorescence intensity was ploted against time, as seen in figure 9.. Sec5a-GFP signal indeed quickly returns to the PM after photobleaching, with half-time of return being 16,5 +- 1,65 seconds. Localization of exocyst subunits is known to be dependent on actin cytoskeleton in budding yeasts (Boyd et al., 2004). To test role of

cytoskeleton in Sec5a-GFP localization, we performed a FRAP experiment on seedlings treated with cytoskeletondisrupting drugs and compared results with measurements in control plants. One hour long microtubule disruption does not have any effect on protein localization in observed cells. Neither it influences Sec5a-GFP signal recovery after photobleaching compared with the control. The only difference in respect to control plants is the absence of a visible signal at cell plates (which is apparently caused by requirement of intact microtubules for cell division). On the other hand, after 1 hour long actin disruption, previously evenly distributed protein forms clutches/patches – often but not always in cell corners (fig. 9). Sec5a-GFP recovery after photobleaching is significantly delayed when compared with the control.

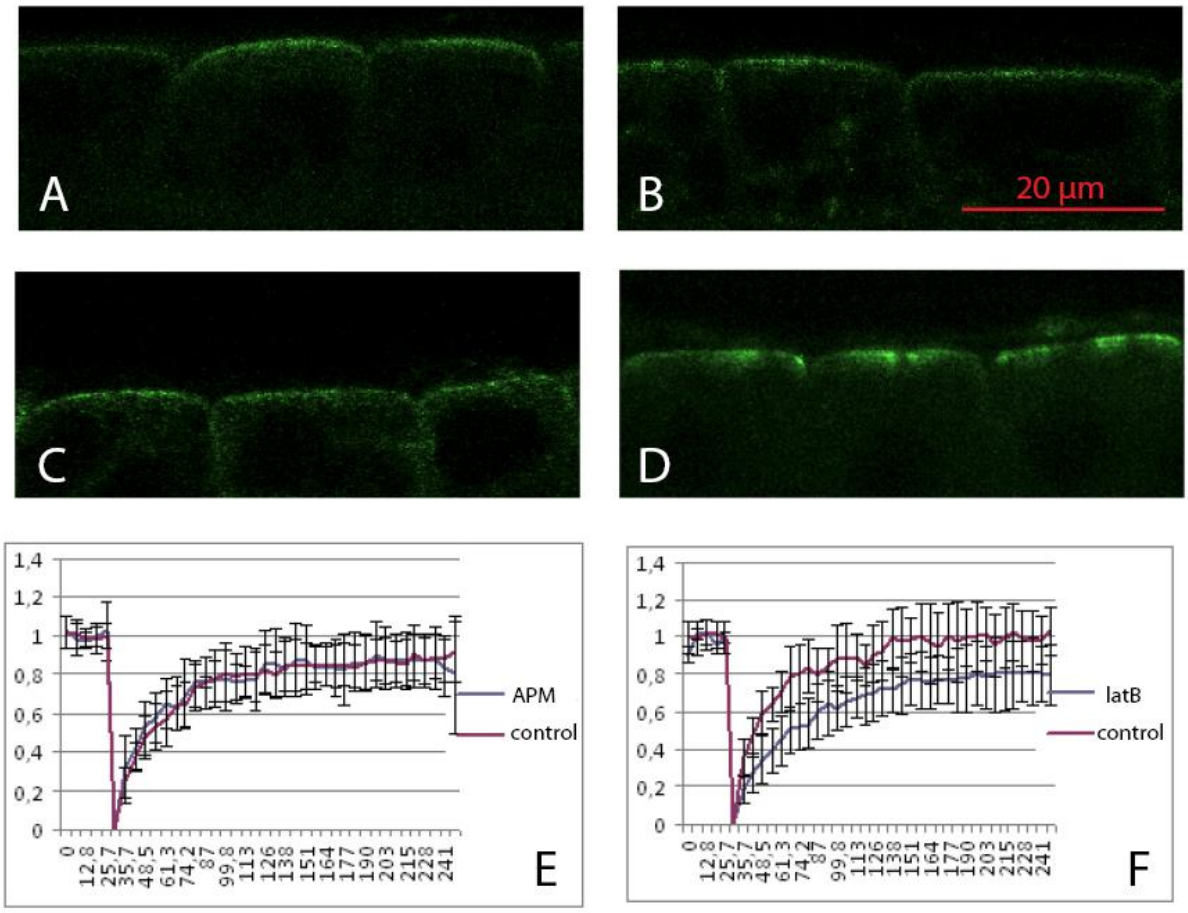


Figure 9. FRAP-measured dynamics of Sec5 relocalization to membrane and effect of cytoskeleton disruption. *A: Sec5-GFP in epidermal cells of control plants for micro-tubule disruption (plants were treated with ethanol, so that final concentration of ethanol would be same as for plants treated with APM, which is stored dissolved in ethanol) B: Sec5-GFP in epidermal cells of control plants for actin disruption (plants were treated with DMSO, so that final concentration of DMSO would be same as for plants treated with APM, which is stored dissolved in DMSO). C: Sec5-GFP signal of cells from APM-treated roots. The signal does*

not differ from that in corresponding control. D: Sec5-GFP signal of cells from latrunculin-treated roots. The Sec5 forms clutches, often localized incell corners, rather than being evenly distributed at membrane like in control. E and F: recovery of Sec5 signal after photobleaching in APM/latrunculin-treated cells versus control, respectively. While APM does not affect recovery of Sec5 to membrane, actin disruption by latrunculin partially inhibits recovery.

Supplementary videos

Supplementary part of this thesis contains video of growing young root hair with Sec5a-GFP signal and time-lapse serie of Sec5a-GFP signal observed with VAEM microscopy.

5. DISCUSSION

5.1 Sec5a-GFP localizes to cytoplasm in transiently expressed tobacco cells.

We observed cytoplasmic localization of Sec5-GFP in transformed tobacco leaves. Cytoplasmic localization is in agreement with data obtained later in stably-expressed *Arabidopsis* lines and in agreement with *Arabidopsis* lines stably expressing other exocyst subunits fused with GFP (Fendrych 2010; Janková – PhD thesis, 2011; Toupalová – PhD thesis, 2011). These data are in contrast with previously described results of tobacco cell culture transient biolistic transformation with Sec5-GFP expressed under 35S promoter (Cheng et al., 2009). Here Sec5-GFP, as well as other exocyst subunits, was described to localize into large globular perinuclear structures. These structures are supposed to be components of endocytic pathway. We have, however, observed such structures neither in transiently transformed tobacco cells, nor in stably transformed *Arabidopsis* cells. Bright field images of transformed tobacco cells suggest that the cells are damaged (see Cheng et al., 2009) and the observed localization could be caused by stress with possible contribution of autofluorescence.

5.2 Sec5a-GFP line under the 35S promoter displays variable level of fluorescence

All observations were performed on material where Sec5-GFP expression was driven by a viral promoter. Sec5a is transcribed in all *Arabidopsis* tissues tested (GENEVESTIGATOR and Chong et al., 2010) and so the SEC5a protein is most probably present in significant amount in most *Arabidopsis* tissues. Most importantly, in plants used for observation, the SEC5a-GFP fluorescence was rather low, thus we assume that localization and dynamics of fluorescent protein corresponds to a large extent to normal Sec5a behavior *in vivo*. The stable transgenic lines exhibited variable fluorescence even in the second generation of transformed plants. Such behavior is not usual (Fendrych, Synek, personal communication). It is possible, that the random insertion of transgene occurred in some epigenetically unstable genomic region. Alternatively, multiple insertions might have occurred, causing stochastic silencing by methylation in the plants. Such changes in gene expression of genes under the 35S promoter were observed in various plant models (Chalfun-Junior A, 2003, Yamasaki S., 2011). Several plants with reasonable fluorescence level were kept for further maintenance of the line and behavior of the third generation of transformants will be observed. A line expressing Sec5a-GFP under control of the native promoter of *Arabidopsis thaliana* Sec5a will be prepared. I have already cloned the putative promoter (sequence long about 800 bases separating the beginning of Sec5a open reading frame and the stop codon

of the preceding gene, according to *Arabidopsis* T-DNA express database (http://signal.salk.edu/cgi-bin/tdnaexpress) and such a line will be an important tool in the future (for example for crossoing Sec5-GFP plants with plants expressing other fluorescent proteins under e.g. 35S or ubiquitin promoters; presence of 35S promoter at both transgenes might lead to silencing).

5.3 Sec5a-GFP localization in root epidermal cells mirrors their secretory activity - it localizes to the outer lateral domain of cells facing external environment

Sec5a-GFP is expressed in all cells of the root tip. Similarly to transiently transformed tobacco cells, *Arabidopsis* cells have a high amount of the protein in cytoplasm. The localization is most prominent in young cells in which the large vacuole has not formed yet. Cells in the meristematic and elongation zone need a prominent secretory pathway, in order to supply material for PM surface increase and intercalary (diffuse) cell wall growth (Dolan and Davies, 2004; Martin et al., 2001). Prominent cytoplasmic signal is also visible in columella cells. The cells of the root cap constitute a tissue with high secretory activity, producing mucilage that reduces friction during root growth, as well as compounds used for interactions with soil microbiota – namely suppression of pathogens and interaction with rhizobacteria (Arnaud et al., 2010). Cells of columella are gradually being displaced and detached from root apex, as new cells are formed (Driouich et al., 2006). In *Arabidopsis*, these cells remain connected in a file of so-called border-like cells („true“ border cells of other plant species detach from root cap as single cells). I was occasionally able to observe such detached cells adjacent to the investigated root using the confocal microscope. They exhibited strong fluorescence, comparable with that of root cap cells. Ultrastructural studies of border-like cells have shown that they contain many Golgi stacks and Golgi-derived vesicles, supporting idea that these cells are highly secretory active. (Vicré et al., 2005). Border cells from different plant species have been shown to further influence soil environment in favour of the growing root and continue with secretion of compounds, that influence local microbiota. They can divide and form callus in vitro under appropriate conditions (Vicré et al., 2005).

Fluorescent signal of SEC5a-GFP was stronger near and at the PM than in the cytoplasm. It is further enriched at the apical and basal PM in cells of root ground tissue. Strongest signal, however, can be consistently observed at the outer lateral PM domains of root epidermal cells and cells of the lateral root cap. Epidermal cells facing outer space, as well as those covered with lateral root cap, have strong Sec5-localization at the outer lateral domain. It is however difficult, in case of the covered epidermal cells, to strictly say that the membrane – associated signal belongs to these cells and not to the neighbouring lateral root cap cell

membranes. Lateral membrane is a distinct plant cell domain and some proteins are localized exclusively to this domain and not to other PM domains (Grebe, 2010). Lateral domain serves as a barrier contacting outer environment, absorbing water and nutrients as well as extruding toxic material. It is also in contact with various biotic stressors. Indeed, two of the proteins showed to specifically localize to this domain are boron uptake transporter BOR4 and PEN3 protein involved in pathogen response (Langowski et al., 2010). Proteins specific for outer lateral membrane are transported to their destination by a distinct and largely unknown pathway. The proteins specific for the lateral outer domain are always localized towards root-soil interface (not neccesarily at lateral sides of cells. It can be „basal" in those cells, where the closest root-soil interface is near the „basal" PM) Thus, this specific membrane domain always faces outer environment, so that they would be always in the membrane part facing the outer environment (Langowski et al., 2010). Interestingly, Sec5-GFP signal enrichment at the outer lateral PM is comparable to that of some of these lateral-membrane specific proteins. It is unclear, whether this is just consequence of higher bulk-flow of secretory material to outer domain compared with the other domains of corresponding cells or whether Sec5 (and possibly other exocyst subunits) themselves play some regulatory role in recognition of outer domain and discrimination between this domain and other membrane areas. The former scenario is more probable, because exocyst is believed to be involved as a complex in most exocytotic events of a plant cell as is the case in fungi and animals (Murthy et al., 2005; TerBush et al., 1996). Still, it is possible, that special type of exocyst involving some of yet uncharacterized Exo70 paralogues specifies vesicle trafficking to the outer lateral domain, consistent with hypotheses of subfunctionalization of different Exo70s (Synek et al., 2006) and presence of multiple recycling domains in a single plant cell (Žárský et al., 2009). Root epidermal cells also secrete substantial amount of pectin into the outer space (Willats et al., 2001). Exocyst, with its components strongly localized at lateral membrane, is probably involved in this process. Exocyst subunits Sec8 and Exo7OA1 have been already shown to play a role in polarized pectin secretion in seed coat volcano cells (Kulich et al., 2010). Epidermal cells covered with lateral root cap and inner neighbouring cells of columella probably target proteins involved in border-like cell detachment to the outer domain. Detachment of border-like cells is an example of dehiscence, a process when part of plant tissue falls apart from the rest of plant. These events require partial degradation of the middle lamella connecting tissues and thus involve polarized secretion of hydrolytic enzymes (Lewis et al., 2006). During separation of border-like cells, homogalacturonan, that plays part in adhesion of neighbouring cells, is specifically degraded between separating cell file and the rest of the root but not at cell wall sides connecting border-like cells together (Durand et al., 2009). Thus, this process involves polarized secretion of hydrolytic enzymes to specific PM and therefore also cell wall domains without affecting other sides of the cell. It is

probable, that exocyst-driven secretion plays a significant role in the process. Besides the strong localization of Sec5-GFP and other subunits to area connecting vertical cell files in root tissues, Exo70A1 mutant plants seem to have strongly altered root cap structure, possibly because of the impaired cell separation (Janková – PhD thesis, 2011).

5.4 Sec5a-GFP localizes to the plasma membrane

The colocalization of Sec5a-GFP and FM4-64 signal at the PM was apparent - on the other hand, there was no GFP signal on the endocytic compartments stained with FM4-64. Thus, association of Sec5 (and probably other subunits) do not follow the PM into endosomes after the endocytosis. This observation is in contrast with a study showing Sec5 endocytosis in *Drosophila* oocytes, with possible role in endocytic trafficking (Sommer et al., 2005). As the Sec5 signal rapidly recovers after photobleaching and also shows transient localization under membrane when visualized by VAEM/TIRF, the protein most probably cycles between membrane and cytoplasm.

5.5 Sec5a is localized in root hairs with enrichment at the membrane

It was shown that the exocyst is involved in root hair and pollen tube tip growth. *Arabidopsis* plants with defective Exo70A1 subunit and maize plants with defective Sec3 subunit have defective root hair growth (Synek et al., 2006; Wen et al., 2005, respectively). *Arabidopsis* mutants in several exocyst subunits exhibit defective pollen-tube growth and male transmission defect. (Cole et al, 2005; Hála et al., 2008; Toupalová 2011). This also applies to pollen with both Sec5 paralogues defective (pollen from single mutant homozygots is viable and without any phenotypic difference). Exocyst components were also shown to strongly localize to the tip of pollen tubes by immunocytochemistry (Hála et al., 2008). Exocyst is also involved in tip growth of fungal cells, which is most probably an evolutionary convergent process to plant tip growth, but shares with it many molecular components. SECC, a Sec3 homologue, was shown to localize precisely at the growing tips of *Aspergillus nigrans* hyphae (Jones and Sudbery, 2010) and exocyst role in tip growth was also demonstrated in other fungi (2010 Köhli et al., 2008; Taheri-Talesh et al, 2008).

Thus, the exocyst seems to be involved in tip growth in various eukaryotic organisms and it is not surprising, that we could observe Sec5-GFP localization in growing root hairs. Growth of a young root hair from the observed zone was documented, as a proof of young root hair viability under the particular treatment. The Sec5-GFP localized to the cytoplasm of young root hairs, with a stronger signal at PM. Sec5-GFP was also observed in root hairs using TIRF/VAEM microscopy. Similarly to epidermal cells, Sec5-GFP localized to distinct dynamic punctae in root hair cells (see fig. 7). The root hairs observed under TIRF microscope might

have been damaged during plant treatment, so the obtained data should be taken with caution. However, dynamics of foci in root hairs resembled that in epidermal cells, so the hairs might be viable even after mechanical treatment during transfer from plate, where seedlings had been grown.

5.6 Sec5a-GFP localizes distinctly to forming and maturing cell plates in a distinct steps

During cell division, every eukaryotic cell faces a problem, how to separate new daughter cells. This process involves not only increase of membrane surface area, but also addition of new cell wall material, at least in cells that are surrounded by a rigid cell wall. Such events involve special case of polarized material delivery by secretory machinery. It was shown long time ago in a canonical eukaryotic cell model, budding yeast, that exocyst is involved in daughter cell separation.

Budding yeast exocyst is localized to specific parts of the cell, mirroring actual need for expansion and cell wall addition. As such, it is first localized into the tip of an expanding bud, but later relocalized to mother-bud neck, in order to facilitate separation of mother and daughter cell. (Boyd et al., 2004). A dominant negative Sec10 line was prepared, that is viable and grows well, but forms elongated cells instead of budding. This Sec10 carboxy-terminal domain does not bind other exocyst subunits and does not disrupt exocyst but prevents exocyst relocalization to the bud neck during cytokinesis (Roth et al., 1998). Fission yeast cells forms three-layered septae during cytokinesis. The middle layer is subsequently degraded by locally-targeted enzymes. Exocyst is involved in this targeting and mild sec8 mutant (which has only the cell-separation phenotype) accumulates secretory vesicles around septum in the restrictive temperature (Spitzki, 2007).

The midbody is a structure localized between separating animal cells. It is localized at spindle microtubules between two nuclei of daughter cells. Microtubule-binding proteins of MAP65/PRC1 family are involved in plant cell plate formation as well as animal cytokinesis. The proteins are dispensable for contraction phase but essentail for the abscission during animal cytokinesis. Midbody and vesicle-fusion stage of animal cytokinesis discovery has led to an overall understanding of underlying similarities between animal and plant cytokinesis (Otegui et al., 2005, Lloyd and Chan, 2006). Exocyst components and SNARE proteins were shown to localize to the midbody and exocyst disruption prevents the abscission (Gromley et al., 2006).

Cellularization of *Drosophila* embryo comprises special type of animal cytokinesis. The embryo develops up to the 13th cell cycle as a syncytium, thus undergoing repetitive rounds

of mitosis without cytokinesis. All nuclei are afterwards separated during one massive event. Mutant embryos bearing a temperature-sensitive Sec5 allele develop normally until cellularization above restrictive temperature but fail to proceed in development further. Sec5 localizes to edges of growing membranes during cellularization. Thus, Sec5 and exocyst are indispensable for proper cellularization (Murthy et al., 2010).

In plant cytokinesis, there are at least two mayor stages involving massive vesicle fusion events. In early cytokinesis, vesicles arrive at the equatorial cell plane. After a massive fusion event, tubulo-vesicular network of interconnected membrane compartments forms (Van Damme et al., 2008; Jurgens G., 2005). This further undergoes intensive rearrangement into planar sheet stage, which is a fenestrated cell plate. Dynamin–like proteins are involved in both tube and sheet rearrangement and endocytosis connected with plate maturation. Fenestrae are closed during further maturation. In the second massive fusion event, projections from growing plate connect with membrane of the mother cell accomplishing cell division (Jurgens G., 2005; Staehelin and Helper, 1996). Electron tomographic reconstruction of cell plate formation in meristematic cells has reveled presence of „exocyst-like“ tethers (they resemble previously electron-microscopy visualized purified yeast exocyst complexes) connecting vesicles prior to fusion event (Seguí-Simarro et al., 2004).

Recent study of exocyst functioning during plant cytokinesis (Fendrych et al, 2010) describes a cytokinetic defect in Exo84 mutant plants and shows localization of multiple exocyst subunits at maturating cell plates. Strong exocyst signal can be observed during two particular stages of cytokinesis that most probably correspond to the initial fusion event of vesicles and the cell plate maturation. Strong localization of exocyst subunits persists at cell plate after completion of cytokinesis. Exocyst might contribute to closure of perforations in new cell plate and to cell wall maturation. The study also shows altered, somehow defective, cytokinetic plate assembly in Exo70A1 mutant background. Although alterations are observed, affected cells eventually accomplish cytokinesis and Exo70A1 mutant does not show such deleterious defects as those caused by mutation in Exo84. However clear defect in the initiation of cell plate was described in Arabidopsis *exo70A1* mutant plants (Fendrych et al, 2010). Studies of phenotype in plants bearing a non-lethal mutation of other exocyst subunit (for example weak *sec8* mutations, see Cole et al., 2005) could be helpful for distinguishing between specific functions of particular exocyst subunits in cytokinesis and general effect of nonlethal exocyst disruptions of various severity.

Sec5 localization described in this thesis is in full agreement with previously published observations of other subunits. Strong and rather shortlived Sec5a-GFP signal can be observed in cells entering cytokinesis. The signal than disappears and growing cell plate can

be observed by FM-64 dye staining but not via Sec5a-GFP fluorescence. In later stages, signal reappears and colocalizes with FM-64 along the whole plate. It is very strong (compared with interphasic signal at corresponding apical and basal membranes in root ground tissue) and persists during cell plate fusion with the parental membrane and cell wall, as well as after this event. It is not absolutely clear what happens in the phase without or with a weak exocyst signal at cell plate, bud it is possible, that remodelling of new compartment and its extension into a plate is more prominent than addition of new membrane material in that phase. As referred above, a lot of endocytosis occurs during this stage of cell plate maturation, possibly even decreasing absolute mass of membrane material. Further addition of vesicles to growing plate probably corresponds to reappearance of the exocyst subunits at cell plate, which continues into fusion with mother cells membrane. Sec5 and other exocyst subunits localize to cell plate in various cells, including leaf epidermis and most probably play role in all plant cytokinetic events. Even special cases of cytokinesis like endosperm cellularization and male meiotic cytokinesis are variations of somatic cytokinesis. Although they might superficially resemble *Drosophila* cellularization, new membrane is not added centripetally but multiple „mini-phragmoplasts“ and „mini-cell plates“ form to start cytokinesis (Jurgens G., 2005). Therefore, exocyst participation in these events can be anticipated. Consistently with such idea, the above mentioned „exocyst-like“ tethers were also observed to participate endosperm cellularization and male meiotic cytokinesis in ultrastructural studies. (Otegui et al., 2001 and Otegui and Staehelin, 2003, respectively).

Recently, the paradigm implying exlusively Golgi-derived vesicles in cell plate formation has been challenged (Dhonukshe P. et al., 2006). Several experiments (including careful time-lapse FM4-64 staining observation and detection of material of extracellular origin in vesicles) show that material derived by endocytosis from mother cells wall and membrane substantially contributes to the growing cell plate. Observations and experiments underlying importance of endocytosis during cytokinesis were also made in animal cells (Schweitzer et al., 2005). It is, however, unclear, whether requirement for endocytosis in dividing animal and plant cells shows common underlying principle or whether it is only a superficial similarity. Endocytosis in animal cells occurs during late cytokinesis and may well remove some regulatory molecules from cleavage furrow area or physically assist ring contraction and abscission (Schweitzer et al., 2005). I did not observe FM4-64 Sec5-GFP co-localization of endocytic vesicles containing Sec5 during cytokinesis. If the exocyst complexes (with Sec5) participate in addition of any material of endocytic origin to cell plates, Sec5 proteins involved are most probably derived from the cytoplasmic pool.

5.7 TIRF/VAEM microscopy of plant cells and exocyst

It has been argued, that TIRF microscopy cannot be used for visualization of plant cell structures, because cell walls are so thick, that the evanescent wave cannot reach fluorophores under membrane (Konopka and Bednarek, 2008). Several recent studies, however, involved life-cell imaging of plant cells with TIRF microscope. True total internal reflection probably might not occur in such cases. Instead, fluorophores might be excited as in VAEM (variable angle epifluorescence) microscopy (Konopka and Bednarek, 2008). The method is refered to as TIRF/VAEM microscopy in this thesis, due to partial unambiguity about the mechanism of GFP excitation during observations under TIRF microscope.

Part of this study involved observation of Sec5a, another exocyst subunit by the TIRF microscope. Overall appearance of observed signal was similar to that of other exocyst subunits (Exo70A1, Sec6, Sec8 and Exo84b). Density of the Sec5-GFP spots, being approximately 1,62 spots per square micrometer, also agrees with density of the other four subunits (Fendrych - PhD thesis, 2011). Another feature shared by Sec5a and previously described exocyst subunits is limited motility within the plane of the PM. In contrast, life-time distribution of observed Sec5 punctae (see Fig. 7 in results) is shifted towards shorter times with most punctae residing at membrane for about two seconds. This difference is quite unexpected and its significance is unclear. It might be an artifact caused be weaker fluorescence of the plants observed, that would not allow detection of initial phase of spot lifetime at the PM, due to its weaker signal than during later stages. It is also possible, that Sec5 is added to other subunits later during some assembly event or dissociates earlier after successful tethering of a vesicle. Biochemical properties of exocyst complex suggest, that Sec5 is associated with the complex more loosely that other subunits (Hála et al., 2008). This hypothesis could be supported or falsified by analysis of Sec5-GFP colocalization with RFP-fused exocyst subunits in plants expressing both proteins, as has been done for other exocyst subunit pairs (Fendrych - PhD thesis, 2011).

5.8 Time-lapse observations uncover more frequent arrival of exocyst subunits, particularly Exo70A1, to specific areas of membrane

Time-lapse TIRF/VAEM observations of three different exocyst subunits showed nonhomogenous localization of exocyst punctae at the PM, most notably in case of Exo70A1-GFP subunit, but clearly also in the case of Sec6-GFP. Potential „hotspots“ of exocyst activity showed only slow movement/displacement during 5 minutes of observation. Longer observation with the same frequency of image capture (every 500ms) were not possible, due to gradual bleaching of the GFP signal. Observation with less frequent capture

could, however, uncover long-term behavior of the „hotspots“. It is currently unclear, why Exo70A1-GFP shows more distinct heterogeneity of localization. It is possible, that „hotspots“ correspond to microdomains of different membrane composition, preferentially bound by the Exo70A1 subunit. The domains at the PM are clearly organized into the relatively regular stripes in case of Sec6-GFP and Exo70A1-GFP. This opens an extremely interesting question if the cortical cytoskeleton (and esp. microtubules) does – in contrast to short single spot dynamics – influence long term exocytotic maxima and minima. Experiments addressing this question using phramacological disruption of cytoskeleton (including stabilization by taxol and jasplakinolide) are in progress. Difference in distribution of remaining subunits would, however, imply that not all observed punctae correspond to protein in a complex. Previously examined colocalization of individual subunits showed to be stable during whole lifetimes of punctae (Fendrych - PhD 2011). However, colocalization of Exo70A1 with other subunits was not assessed in the study. Exo70A1-GFP plants express the transgene under the 35S viral promoter, as well as all used exocyst-RFP-expressing lines do. It was not possible to prepare *Arabidopsis* lines expressing both transgenes, probably due to silencing caused by presence of more 35S promoters in one genome (Fendrych - PhD 2011). Besides simultaneous time-lapse observations of two exocyst subunits, it would be interesting to compare the long-term localization of exocyst punctae and endocytotic sites, marked by DRP1C, a dynamin-related protein marking sites of endocytosis (Konopka et al., 2008). Plants coexpressing DRP1C with Sec8-GFP have already been prepared (Fendrych - PhD thesis 2011). The „hotspots“ where exocyst punctae accumulate vary in size, bud theoretically, some of them are large enough to be seen by conventional confocal microscopy. This would require careful observation of PM in root epidermal cells. However, it is possible that exocyst subunits do associate with different membrane parts in a selective manner but are distributed more homogenously in underlying cortical cytoplasm. Such homogenous cortical distribution would produce noise and „hotspots“ would not be visible by confocal microscopy.

5.9 There is a rapid flow of Sec5 from cytoplasm to the PM and it is partially dependent on actin cytoskeleton

To assess dynamics of the Sec5a-GFP protein at the PM and its dependence on cytoskeletal machinery, FRAP experiments were performed. Results show, that Sec5a-GFP is highly dynamic, with half-time of recovery being approximately 16,5 +- 1,65 seconds. Because Sec5a-GFP spots observed by TIRF/VAEM microscopy display only very limited lateral mobility, most of the Sec5-GFP protein returning to the bleached area does not diffuse there from non-bleached parts of the PM but arrives with secretory vesicles and/or from cytoplasm (see below). Dynamics of Sec5 behavior is similar to that of other exocyst subunits examined before by the same method (Fendrych - PhD thesis 2011). Dependence of Sec5a-GFP

recovery on cytoskeleton was investigated by FRAP measurement after actin and microtubule disruption. Whereas microtubule disruption has no effect on Sec5a-GFP recovery even after 1 hour long treatment with APM, actin disruption affects Sec5a-GFP recovery after 1 hour. Effect of shorter treatment was not evaluated but it does not influence Exo84b-GFP behavior under same experimental setup (Fendrych - PhD thesis, 2011).

Canonical model of exocyst assembly in budding yeast implies transfer of all exocyst subunits except Sec3 and Exo70 towards cytoplasmic membrane together with secretory vesicles (Boyd et al., 2004). Actin disruption prevents delivery of remaining subunits to PM and partially affects recovery of Exo70. It is possible, that there are two populations of Exo70 – one of them being associated with the rest of the complex at vesicle, since Exo70 was also detected, together with rest of the complex, at vesicles by electron microscopy (Boyd et al., 2004). Budding yeast Sec3 and Exo70 subunits serve as a landmarks for exocyst arrival and both interact with small GTPases and PIP_2 and their localization to the PM is supposed to be largely F-actin independent (He et al., 2007; Wu et al, 2010; Zhang et al., 2008). Components of exocyst localization machinery are somehow redundant – mutations in the targeting machinery often have to be combined to achieve severe phenotypes (Zhang et al., 2008). Nevertheless, actin disruption alone is sufficient to abolish both vesicle delivery and arrival of most exocyst subunits to the membrane (Boyd et al, 2004).

Recent studies of polarity pathways in *Schizosaccharomyces pombe* point to differences among biological systems in regard to exocyst and actin cytoskeleton interplay (Bendezú and Martin, 2011, Nakano et al., 2010, Snaith et al., 2011). Results of these studies favor model postulating that neither actin disruption nor mutations in exocyst subunits alone prevent polarized vesicle transport and morphogenesis in *Schizosaccharomyces pombe*. Both pathways seem to act in a redundant manner but use common upstream polarization clues, namely Cdc42 GTPase (Bendezú and Martin, 2011) and Pob1 (Nakano et al., 2011). In these upstream cues are defective, growth still occurs, nut cells are spherical. Cdc42 and Pob1 act in localization of the secretory pathway rather than its activation, as *pob1* mutant cells do expand but lose polarity and Cdc42 seems to be an important landmark of cell growth, being itself regulated by other proteins (Kelly D. and Paul Nurse, 2011). Only disruption of both pathways results in full loss of polarity. Mutation in major phosphatidylinositol 4-phosphate 5-kinase, which causes reduced levels of PIP_2, results in loss of polarity in formin For3 mutant background, indicating role for PIP_2 in exocyst-driven pathway.

Interestingly, actin disruption by prolonged latrunculin B treatment only partially interferes with the delivery of both Exo84b-GFP (Fendrych - PhD thesis 2011) and Sec5a-GFP (this

thesis) to the PM in Arabidopsis cells. Actin disruption causes the otherwise evenly distributed signal to form clutches at the PM. This is reminiscent of Exo70 behavior in budding yeast cells upon latrunculin treatment (Boyd, 2004). In fission yeast, Sec6 still localizes to cell cortex, although in a less polar manner, upon latrunculin-imposed actin disruption. Part of Sec6-GFP relocalizes to the PM after the bleaching (Bendezú and Martin, 2011). It thus seems, that actin cytoskeleton is not always necessary for membrane localization of exocyst subunits in some biological systems, including plants. There is, however, a pool of subunits that do not localize to PM and could be bound to secretory vesicles, as in budding yeast (Boyd et al., 2004). Reduced and altered - but still present - distribution of Exo84b-GFP at the PM after the latrunculin treatment can be also observed by TIRF/VAEM microscopy (Fendrych - PhD thesis 2011). One possible explanation of localization of actin-independent pool to PM is diffusion-mediated transport to target sites stabilized by interaction with polarity clues, particularly with Exo70 and/or Sec3 subunits, known as landmarks for exocyst in non-plant systems. In plants, Sec3, was shown to interact with small GTPases via interaction scaffold protein ICR1. (Lavy et al., 2007). It would be interesting to study FRAP of fluorescent exocyst subunits in *exo70A1* mutant backgroud (Sec6-GFP in this background is already available, see Fendrych - PhD thesis 2011) after latrunculin tB treatment. Larger pool of non-recovering Sec6-GFP could be expected, if Exo70 helps to localize actin-independent pool of subunits to the PM. Observation of the same subunits in Sec8 mutant background (mild mutant alleles of SEC8 exist, see Cole et al., 2005), on the other hand, should not show larger relative decrease between latrunculin untreated and treated plants than does occur in wild-type background. Alternatively, it is possible that part of secretory vesicles themselves reaches membrane independet of functional actin cytoskeleton and that „actin-independent pool„ of Sec5 is bound to these vesicles. Potential secretory events have been documented by study of co-localization dynamics of exocyst subunits and vesicle-associated SNARE protein. (Fendrych - PhD thesis 2011). Similar observations could be made after latrunculin treatment, to asses, whether spots of exocyst signals will still co-localize with SNARE proteins and be present during the entire supposed fusion event. This would favor model, in which exocyst spots visible under TIRF/VAEM microscopy after latrunculin treatment (Fendrych - PhD thesis 2011) correspond to secretory vesicles, which must have reached the PM without presence of intact actin cytoskeleton.

5.10 Sec5 as a part of exocyst complex in plants

From the very beginning of research involving yeasts, Sec5 is known to be subunit of the complex (TerBush et al., 1996). In plants, several approaches were utilized to proof Sec5 association with other exocyst subunits. Pollen grains with mutant alleles of both Sec5

paralogues have defective growth and fail to fertilize eggs (Hála et al, 2008). Male transmission defect and impaired pollen tube growth are phenotypes shared by plants with mutations in different exocyst subunits, suggesting their role in common processes. Although having no specific phenotype in single mutants, Sec5a mutant allele has a synthetic effect resulting in strongly impaired hypocotyl elongation of double-mutant plants (Hála et al, 2008). No phenotypic deviation has been found in homozygous Sec5a or Sec5b mutant plants so far. Although Sec5b shows much lower level of expression on a transcriptional level in most tissues (Chong et al., 2009), it is possibly sufficient to supply Sec5 function in *sec5a* mutant plants and *vice versa*. It might be interesting to compare Sec5b transcript levels in wild type and *sec5a* mutant plants to show, whether such low Sec5b expression (as wild type plant microarray data show) is sufficient for proper exocyst function. On the other hand, it is not possible to prepare *sec5a/sec5b* double-mutant plants due to the male transmission defect. Study of phenotype deviations caused by Sec5 disruption is thus difficult. In the future, RNAi approach could help solve the problem. Small silencing RNA expressed under an inducible promoter (the method is already being widely used in plant molecular biology, as reviewed by Wielopolska et al., 2005) could be used for disruption of Sec5 function in desired phases of ontogenesis without impairing Sec5 function in pollen, where it is crucial. Moreover, short complementary RNAs can be designed to bind and downregulate expression of multiple paralogues of the same gene. Sec5 was also found to co-fractionate in the same complex with other exocyst subunits on protein blot after size exclusion chromatographic separation of protein lysate from suspension-cultured *Arabidopsis* cells (Hála et al. 2008). Yeast two-hybrid screen has uncovered interaction of Sec5a with both Exo70H1 and Exo7OB2 (Pečenková et al., 2010). These subunits perform a specialized function during response of a plant to a pathogen attack. Sec5 thus seems to be part of the exocyst complex with Exo70A1 subunit, responsible for many processes connected with the general growth and secretion like hypocotyl elongation, as well as part of complexes containing other Exo70 paralogues reserved for more specific functions. It is possible that double mutants bearing the defective Sec5a allele and Exo70H1 or Exo70B2 will show stronger defect than Exo70H1/B2 single mutants. Arabidopsis *sec5a* mutant plants were crossed with *exo70h1* mutant plants and will be analyzed in the future. This thesis shows localization data of Sec5a protein, mostly similar to those of other exocyst subunits, including intracellular localization in various root cells, dynamics during cytokinesis, kinetics of transport to membrane in intact plants and plants with disrupted cytoskeleton and behavior at the PM visualized by TIRF/VAEM microscopy. The data gathered here thus further imply common function with other exocyst subunits in secretion and cytokinesis.

5.11 Sec5 as a part of exocyst complex in animal, yeasts and plants – a comparison

Knowledge about the exact function of Sec5 inside exocyst in general is still scarce. Most data come from animal models, where it clearly mediates interaction of the exocyst with its refulator Ral during various events (van Dam et al., 2006). Sec5-Ral interaction has been well characterized at molecular and structural level (Fukai et al., 2003). Most animal exocyst studies actually involve experimental work on Sec5. The results are usually interpreted in a way suggesting that exocyst, as a whole complex, is responsible for the particular function. Some studies also involve performing of complementary experiments with other exocyst subunits to prove this assumption (see for example Murthy et al., 2006). To my knowlegde, no study describing a Sec5 function proved that Sec5 does not perform the particular function as a part of the exocyst. Sec5 function outside exocyst and vesicle tethering was suggested in a signaling pathway mediating innate immune response and cell survival (Mantovani and Balkwill, 2006). This pathway involves the well known Sec5 interactor RalB and kinase TBK1, which directly associates with Sec5. Clearly, Sec5 particitates many cellular events as an intagral part of exocyst and many of them are regulated by Ral GTPases. These GTPases are, however, specific for metazoans (van Dam et al., 2006). Little is known about specific Sec5 functions in yeasts. Sec5 was suggested to participate in mediating interaction of SNARE proteins, because *sec5* mutation (and also exo70 and sec3 mutations but not mutations in other subunits) can be rescued by overexpression of Sec1p, a protein known to mediate SNARE complex assembly (Wiederkehr et al., 2004). Sec5 mutant can be rescued by Sec4 (GTPase involved in exocyst assembly) overexpression as well. Thus, Sec5 may contribute to exocyst stabilization and assembly. Plant Sec5, as it is known, collocalizes with other subunits and mutation in the subunit have similar, sometimes synergic, defects to defects in other subunits (this thesis and Hála et al., 2008). Dynamics and interactions mediating plant exocyst assembly are not well known (but differ between animals, plants and yeasts – see Novick and Munson 2006 and Hála et al., 2008) and do not imply specific function for Sec5. It is possible that besides stabilization of exocyst, Sec5 could have specific interactors mediating exocyst functions in plants, comparable to function of Ral in animals. Unfortinately, plant Sec5 interactors outside the exocyst remain undescribed.

6. CONCLUSIONS

1. Sec5 localizes to cytoplasm and PM in plant cells. Its incidence monitored by translational GFP-fusion is most prominent in cells with high secretory activity and it is highly localized to outer lateral domain of root cell. Sec5 is also enriched at the tip of growing root hairs, implying its function in the tip growth.

2. Sec5 localizes to growing cell plate during cytokinesis - esp. at the initial phase and during the cell plate fusion with the parental PM and keeps the localization at the junction for some time after the cytokinesis was completed. Sec5 localizes to cell plate in different cell types, including developing epidermal leaf cells.

3. Sec5 forms distinct dynamic foci at the PM visualized with TIRF/VAEM microscopy. Their presence is short lived, as they rapidly appear and disappear in the cytoplasmic membrane area but their lateral mobility is limited. Recovery of Sec5 to PM after the photobleaching is microtubule-independent and only partially actin cytoskeleton dependent.

4. TIRF/VAEM-visualized exocyst subunits (including Sec5), particularly Exo70A1 and Sec6, occupy preferentially some areas of the membrane on several-minutes long time scale. Banded pattern of the exocyst signal maxima allows speculation of cortical cytoskeleton involvement in long term exocytosis localization in plant cells.

5. Most of Sec5 localization and dynamics observed in this thesis matches previously described properties of other exocyst subunits in Arabidopsis and confirm common function with other subunits as a part of the complex.

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