Charles University in Prague
Faculty of Sciences
Department of Plant Physiology

Academy of Science of the Czech Republic
Institute of Experimental Botany
Laboratory of Cell Biology

The secretory vesicles tethering complex exocyst and the auxin transport polarization

Ph.D. Thesis

Mgr. Edita Janková Drdová

Prague 2011

Supervisor:

RNDr. Viktor Žárský, CSc.

Consultant:

doc. RNDr. Fatima Cvrčková, Dr. rer. nat

Acknowledgements

At this place I would like to thank all members of our laboratory, especially to my supervisor Viktor Žárský, for valuable advices and instructions that he gave me during my postgradual study. Many thanks also belong to Hana Soukupová and Tamara Pečenková for big support during writing manuscript and this PhD Thesis.

Furthermore I would like to thank for nice collaboration to colleagues from Oregon State University, John Fowler and Rex Cole and also to Angus Murphy from Purdue University.

At this place I also would like to express my gratitude to my parents and my beloved spouse for their support and encourage that they gave me.

This work was supported by MSMT LC06034 REMOROST, MSM0021620858, MSMT - KONTAKT ME10033. Funds from the Grant Agency of the Czech Republic (P501/11/P853) were used for the final phase of this work

Abbreviations

ARF - ADP-ribosilation factors
BFA – Brefeldin A
BLC – border-like cell
CLMS – confocal laser-scanning microscopy
COG complex - conserved oligomeric Golgi complex
EE – early endosom
FRAP – fluorescent recovery after photobleaching
GAP – GTPase activating protein
GARP complex - Golgi- associated retrograde protein complex
GDI – GDP dissociation inhibitor
GDF – GDI displacement factor
GEF – Guanine exchange factor
GFP – green fluorescent protein
GSV – GLUT4-Specialized Compartment
LE – late endosom
LOF – loss of function
MDCK - Madin-Darby canine kidney cells
PAT – polar auxin transport
PC12 cells - rat pheochromocytoma cells
PGP – P-glycoprotein
PH domain – pleckstrin homology domain
PID – PINOID
PIN1 – PIN FORMED1
PM – plasma membrane
PX domain – Phox homology domain
RE - recycling endosomes
RIC Rop-interactive CRIB domain-containning protein
ROP – Rho of plants
ROS – Reactive Oxygen Species
SNARE - soluble NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein receptor
T-DNA – Transferred DNA
TGN – trans-Golgi network

Declaration

Analyses or experiments that were performed by my collaborators are labeled accordingly in particular paragraphs or chapters. I declare that all collaborators were informed about the presentation of their results and our collective results in my PhD thesis and agreed with this substantiality.

I also declare that this thesis has not been used to graduation at any other university.

Content

1 **INTRODUCTIONS**……..…5

1.1 Exocyst functions in eukaryotic cell polarization……5
1.1.1 Localization and function of exocyst…….....…...6
1.1.2 Molecular structure of exocyst subunits……...…8
1.1.3 Exocyst and the interactors……...…….9
1.1.3.1 Exocyst and interaction with RabGTPase…….....9
1.1.3.2 Exocyst and interaction with Rho GTPase……..10
1.1.3.3 Exocyst and interaction with ArfGTPase……12
1.1.3.4 Exocyst and interaction with SNARE complex……..…12
1.1.4 Exocyst membrane localization……...…13
1.1.5 Plant exocyst……...….14
1.2 Polar auxin transport……..…..16
1.2.1 Regulators changing phosphorylation status of PINs…….….17
1.2.2 Arf-GEFs in PINs trafficking……..…18
1.2.3 Rab5 GTPase pathway in PINs polarization…….....18
1.2.4 Retromer complex sorts PINs from PVC to the TGN…….19
1.2.4 ICR1 and Rop GTPase in PIN polarization…….19
1.3 Defining the hypothesis……...…..20

2 AIMS OF THE THESIS……23
3 **SUMMARY**……..……..…..24
4 **MANUSCRIPT AND APPENDED PUBLICATIONS**……...…26
5 MATERIALS AND METHODS……...…27
6 **RESULTS**……..…..29
6.1 Supporting information to the manuscript…….....….29
6.2 Localization of GFP:EXO70G1…….……30
7 DISCUSSION……...…….34
8 CONCLUSION / SOUHRN…….…….……47
9 LIST OF PUBLICATIONS…….…….…..…49

10 REFERENCES…….....……..51

1 INTRODUCTION

1.1 Exocyst functions in eukaryotic cell polarization

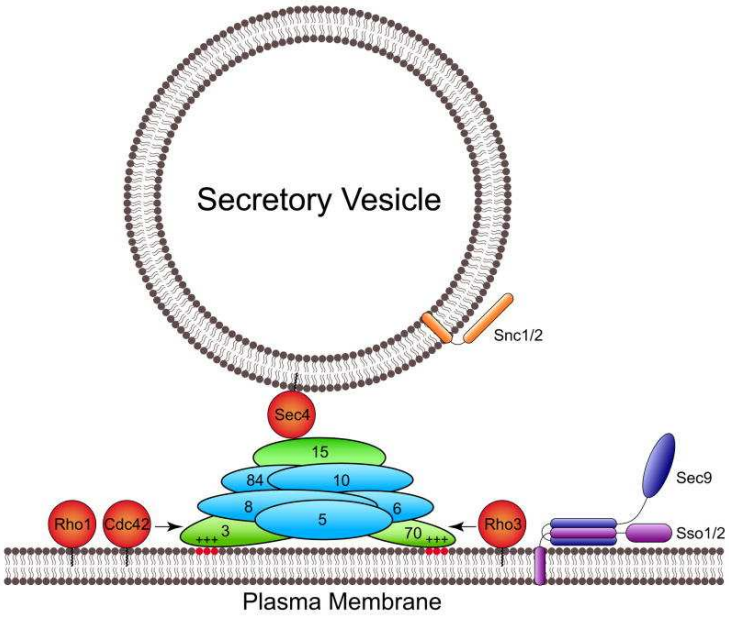
Cell surface is a dynamic system continuously undergoing endocytic and exocytic events, tightly regulated on many different levels. Docking and tethering of secretory vesicles to the plasma membrane employ big machinery of regulatory proteins. First models emphasized the importance of SNARE proteins for specification of vesicles and targeted membrane prior to membrane fusion. Further investigation revealed the promiscuity in SNARE pairing and step by step prove the importance of additional factors, such as small GTPases of the Rab and Rho protein subfamily, regulatory syntaxin-binding protein (Sec1p/Munc18-like, SM) and last but not least specific plasma membrane (PM) tethering complex named exocyst (reviewed in Koumandou et al., 2007; Bassham and Blatt, 2008; Žárský et al., 2010)

The exocyst is an evolutionary conserved octameric complex, consisting of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 subunits, which coordinates tethering of incoming secretory vesicles to the PM. Exocyst subunits Sec3, Sec5, Sec6, Sec8, Sec10, Sec15 were originally discovered in yeast genetic screen focused to secretory defects. This screen was based on measuring the cell density and accumulation of secreted enzymes (invertase) in temperature sensitive mutants of *Saccharomyces cerevisiae* (Novick et al., 1980). Last two subunits Exo70 and Exo84 were discovered later by co-immunoprecipitation with already known exocyst subunits (TerBush et al., 1996; Guo et al.,1999a). Whereas knockout mutant in any of the exocyst subunits is lethal, temperature sensitive mutants blocks exocytosis and accumulate secretory vesicles (Novick et al., 1980; Fringer et al., 1998).

In Opisthokonts, exocyst is localized at specific PM domains, characterized by extensive fusion of post-Golgi derived exocytic vesicles. Depending on the external and internal cues, exocyst localizes also to the TGN and/or different population of endosomes (Vega and Hsu, 2001; Yeaman et al., 2001; Prigent et al., 2003; Folsch et al., 2003; Oztan et al., 2007).

According to the “landmark model”, EXO70 and SEC3 act as a spatial PM landmark for the rest of the exocyst subunits (Boyd et al., 2004; Roumanie et al.,

2005). The polar PM localizalion of EXO70 and SEC3 is independent on actin cytoskeleton and Rho GTPases interaction is proposed to regulate localization of these subunits to the site of exocytosis. The remaining exocyst subunits form subcomplex on the secretory vesicle and via subunit SEC15 interact with RabGTPase. Assembling of the whole exocyst complex enables tethering of exocytotic vesicles to the PM prior the SNARE complex pairing (Boyed et al., 2004). Roumanie et al. (2005) assumed alternative model, where RHO GTPases play the key role in activation of the exocyst complex rather than in localization. The authors proposed that polar distribution of the exocyst in the PM may be a consequence rather than a cause of polarized exocytosis (Roumanie et al., 2005; Wu et al., 2008).



(He and Guo, 2009)

Figure 1 Yeast model showing the role of exocyst complex in the vesicle tethering
SEC3 and EXO70 are regulated by RhoGTPases and function as the landmarks for the remaining exocyst subunits. SEC15 interacts directly with RabGTPases presented on the vesicle. Assembling of the whole complex enables tethering of the vesicles to the targeted membrane prior to SNARE complex formation.

1.1.1 Localization and function of exocyst

In budding yeast exocyst participates in bud-side establishment and later during cytokinesis in separation of doughter and mother cell (TerBush et al., 1996; Finger

and Novick, 1997; Finger et al., 1998). Temperature sensitive mutants result in secretory defects and accumulation of post-Golgi derived vesicles (Novick et al., 1980). Exocyst subunits Sec5p, Sec6p, Sec8p, Sec10p, Sec15p and Exo84p are bound to the secretory vesicle and guided to the place of secretion by actin cytoskeleton. Sec3 is delivered to the place of secretion by vesicle-independent pathway whereas Exo70 can use both pathways to reach the PM (Boyd et al., 2004; Finger et al., 1998).

In fission yeast exocyst localizes and tethers the secretory vesicles to the cell tip and to the division site in dividing cells (Wang et al., 2002; Wang et al., 2003). Interestingly, destabilizing of actin and microtubules in fission yeast do not abolish polar localization of PM proteins, including exocyst (Bendezu and Martin, 2011) Also, disrupting of the exocyst complex is not sufficient to impair polarity of the cell, only simultaneous destabilization of exocyst and cytoskeleton perturbed polarity of the fission yeast and evoke isotropic growth (Bendezú and Martin, 2011). Since exocyst does not affect cytoskeleton organization and vice versa, authors suggested that exocyst and actin cytoskeleton form two independent morphogenetic pathways in fission yeast. In contrast to fission yeast, in HeLa cells it was shown that EXO70 coordinates actin cytoskeleton through interaction with Arp2/3 complex and significantly affects actin-based membrane protrusions and cell migration (Zuo et al., 2006).

In neurons exocyst resides in the tip of neurite and facilitates its outgrowth, branching and synaptogenesis (Vega and Hsu, 2001; Hazuka et al., 1999). In non polarized MDCK epithelial cells exocyst is localized on trans-Golgi at the perinuclear region (Yeaman et al., 2001; 2004). In polarized MDCK cells, Sec8 and Sec6 reside on the TGN and the PM of tight junction (Grindstaff et al., 1998; Yeaman et al., 2001; 2004). More recent work done by Oztan and coworkers revealed the localization of exocyst subunits Sec6, Sec8 and Exo70 in the multiple populations of endosomes in polarized MDCK cells. They proposed exocyst requirement in several endocytic pathways including basolateral recycling, apical recycling and basolateral-to-apical transcytosis (Oztan et al., 2007).

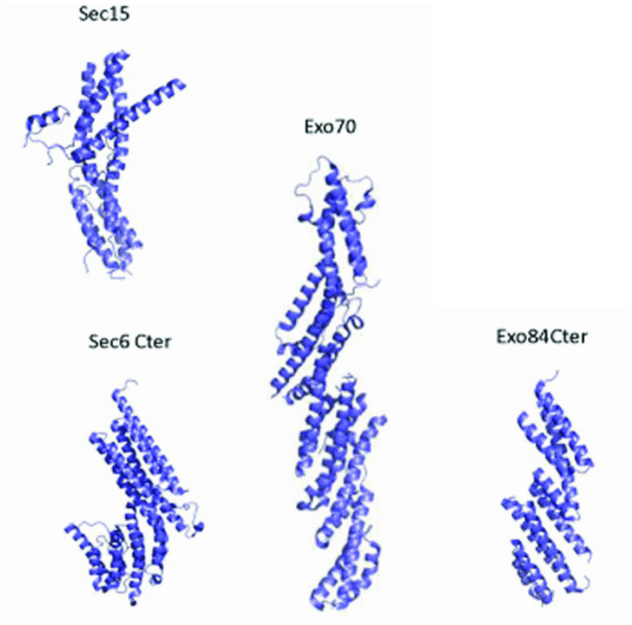
In addition to the role of exocyst complex in delivering material to the site of the polar growth exocyst also participate in targeting various transporters and receptors to the PM. Studies of neuronal cells showed that exocyst subunits facilitate transport of vesicles carrying several synaptic membrane proteins such as neurotransmitter transporter GAT1 (Farhan et al., 2004), glycine transporter GLYT1 (Cubelos et al.,

2005), NMDA (N-methyl -D-aspartate) receptor (Sans et al., 2003) and AMPA-type glutamate receptor (Gerges et al., 2006). In adipocytes well described is tethering of vesicles carrying glucose transporter GLUT4 (Inoue et al., 2003).

1.1.2 Molecular structure of exocyst subunits

The size of exocyst subunits ranges from 70 - 144 kDa (TerBush et al., 1996; Guo et al., 1999). Despite the fact that sequence identity between exocyst genes is only about 10%, their secondary and tertiary structures are remarkably similar (Dong et al., 2005; Sivaram et al., 2006; Wu et al., 2005; Moore et al., 2007).

It was predicted that exocyst subunits contain repeats of helical bundle that are folded in rod-like structure. Almost complete crystal structure of EXO70 and partial structure of EXO84, SEC3, SEC5, SEC6 and SEC15 confirmed previous prediction (Dong et al., 2005; Jin et al., 2005; Baek et al., 2010; Yamashita et al., 2010; Fukai et al., 2003; Hamburger et al., 2006). This structure is quite unique and was proposed and further described in evolutionary exocyst related tethering complexes COG, GARP and DSL1 (Bonifacio and Hierro, 2011). The whole exocyst complex is probably assembled by parallel alignment of individual exocyst subunits together.



(Hertzog and Chavrier, 2011)

Figure 3 Structure of exocyst subunits

The model showing EXO70, SEC15, SEC6 C-terminus and SEC84 C-terminus folded into the rod-like structure.

1.1.3 Exocyst and the interactors

1.1.3.1 Exocyst and interaction with RabGTPase

RabGTPases represents one of the major factors ensuring organelle identity. Rab binding to the membrane is enabled by prenylation on the C terminus or plant-specific myristoylation on the N terminus of RabGTPase (reviewed in Woollard and Moore, 2008). Rabs are able to bind the membrane only in active GTP form. RabGTPase in the inactive GDP bound state is caged in cytoplasma by RabGDI (GDP dissociation inhibitor), which masks the prenyl or myristoyl group. At the target membrane, RabGDF (GDI displacement factor) catalyses the dissociation of RabGTPase from GDI and enables anchoring of prenyl group to the membrane. Rab is activated by RabGEF (Guanine exchange factor) catalyzing replacement of bound GDP for GTP. Activated RabGTPase recruits to the membrane a large number of effector proteins (Bassham and Blatt, 2008) including tethering complexes (factors) CORVET, HOPS,

TRAPPI, II, III, COG, GARP, exocyst (see review Brocker et al., 2010), enzymes of phosphatidyl-inositol metabolism (phosphatidylinositol 4 kinase, phosphatidylinositol 3 kinase, phosphatidylinositol 5- and phosphatidylinositol 4-phosphatases) (Shin et al., 2005), molecular motor proteins (myosines, kinesins) and regulators of SNARE complex (Grosshans et al., 2006). The final regulation step represents hydrolysis of GTP bound to the activated Rab. This hydrolytic reaction is accelerated by RabGAP (GTPase activating protein) and leads to inactivation of RabGTPase. Inactivated Rab is localized to the cytosol and prepared for new round of the RabGTPase cycle. Above described RabGTPase activation, inactivation and cycling between membrane and cytosol via GEF, GDF, GAP, GDI is analogous to all small GTPase (Vernoud et al., 2003).

Interaction between exocyst and RabGTPase was first reported in yeast. It was shown that exocyst subunit Sec15p is a downstream effector of Sec4p- RabGTPase presented on the secretory vesicles derived from the Golgi apparatus (TerBush et al., 1996; Guo et al., 1999b; Fielding et al., 1998). In addition, Sec15p interacts also with Sec2p, protein with dual role, functioning as an effector for Rab Ypt32p and also as a GEF for above mentioned Rab Sec4p (Medkova et al., 2006). Later the interplay between SEC15 and RabGTPase was confirmed in several mammalian cell types (Zhang et al., 2004; Oztan et al., 2008; Bryant et al., 2010). For example in epithelial MDCK cells Sec15A interacts with Rab11 and regulate basolateral-to-apical transcytosis (Oztan et al., 2007).Wu group found that Sec15-C terminus can interact *in vitro* with several Rabs (Rab3, Rab8, Rab11, Rab27) and emphasized the strongest interaction with Rab11 (Wu et al., 2005). Rab11 is the major Rab involved in TGN to PM trafficking. In *Drosophila* they revealed colocalization between Sec15 and Rab11 in photoreceptor cell and described their common action in trafficking of vesicles containing rhodopsin (Wu et al., 2005).

1.1.3.2 Exocyst and interaction with Rho GTPase

The family of RhoGTPase represents a key regulator in cell polarization. This is achieved mainly by organization of action cytoskeleton and vesicle trafficking. In animals, family of RhoGTPase can by subdivided into three major subfamilies - Rac, Cdc42 and Rho. Interestingly no clear homologues of these subgroups were found in plants, however plants possess specific subfamily of RhoGTPase called ROP (Rho Of

Plants) with the highest similarity to the Rac subfamily (reviewed in Mucha et al., 2011).

One of the identified downstream effectors of activated RhoGTP was the exocyst complex. In yeast, three out of six Rho family members (RHO1-5, and CDC42) associate and regulate the exocyst complex. Rho3 and Cdc42 are interactors of exocyst subunit Exo70 (Robinson et al., 1999; Hamburger et al., 2006; Wu et al., 2010). Rho1 and Cdc42 associate with the subunit Sec3 (Boyd et al., 2004; Roumanie et al., 2005; Zhang et al., 2008). Whereas *rho1* or *cdc42* mutant displays mislocalization of exocyst subunits including Sec3p and Exo70p, *rho3* mutant do not affect exocyst subunits localization (Guo et al., 2001; Zhang et al., 2001).

In mammalian cells, interaction between Exo70 and RhoGTPase is well described on trafficking of glucose transporter GLUT4 to the PM of adipocytes (Inoue et al., 2003). After insulin stimulation, active GTP-bound form of Rho GTPase TC10 (homologue of yeast Cdc42), associates with Exo70 and leads to relocalization of Exo70 to the PM. Consequently, Exo70 accelerates tethering of GLUT4 vesicles and increase glucose uptake into the cell (Inoue et al., 2003; 2006).

Plant specific RopGTPases (Rho Of Plants) are crucial players in plant signaling, polar growth, cell division and plant-microbe interactions. The exact mechanism of Rop action in those processes is not completely uncovered, but there is for example strong evidence for Rop mediated cytoskeleton dynamics, intracellular Ca^{2+} flux and ROS production (reviewed in Brembu et al., 2006; Yang and Fu, 2007). RopGTPases regulate cellular processes through interaction with plant unique Rop-interactive CRIB domain-containing proteins (RIC). These proteins function probably as adaptor proteins enabling the interaction with downstream effectors (Vernoud et al., 2003). However despite the intensive study no downstream effectors of RIC have been identified till now, except for several other novel scaffolding proteins uncovered in Y2H screen (reviewed in Mucha et al., 2011). It seems that in contrast to the direct interaction between exocyst subunit and RhoGTPase in yeast and animals, the interaction between exocyst subunit Sec3 and Rop in plants is mediated through additional adaptor protein ICR1 (Interactor of Constitutive active ROPs 1) (Lavy et al., 2007). ICR1can interact with constitutively active Rop6, Rop10 and Sec3A exocyst subunit in Y2H. Additionally interaction between ICR1 and Sec3A was further confirmed by pulldown assay. Transient expression in *Nicotiana benthamiana* revealed that ICR1 make a complex with AtSEC3A in cytoplasm and after

coexpression with AtRop9, the preformed complex is recruited to the PM (Lavy et al., 2007). Since ICR1 contains two coiled coil domains and no other catalytic or structural domains were identified, authors predicted that ICR1 functions as the adaptor protein scaffolding the interaction between exocyst subunit Sec3 and Rops (Lavy et al., 2007).

1.1.3.3 Exocyst and interaction with ArfGTPase

ADP-ribosilation factors (ARFs) represent a key player in vesicle trafficking. Its main role is recruiting coat proteins to the membrane of forming vesicle (reviewed in Vernoud et al., 2003). There are several lines of evidence showing interaction between exocyst and ArfGTPase in animals. In epithelial MDCK cells Prigent and coworkers (2003) described relocalization of Sec10 from the TGN and recycling endosomes (RE) to the PM via interaction with activated ARF6 (Prigent et al., 2003). Furthermore Fielding et al. (2005) performed detailed study focused on regulation of mammalian cytokinesis using human cancer cell lines. Based on acquired results they proposed that family of Rab 11 interacting protein-FIP3 and FIP4 may serve to tether RE to the PM of the cleavage furrow and midbody of dividing cells by forming ternary complex with endosomal localized Rab11 and PM localized exocyst subunit EXO70 and ARF6 (Fielding et al., 2005).

In mammalian kidney cells (COS7 cell lines) EXO70 controls recycling of transferrin receptor (TfnR) through interaction with endosomal localized ARF GEF BIG2 (Shen et al., 2006). Involvement of EXO70 along with other exocyst subunits in TfnR recycling was confirmed in several different cell types (Folsch et al., 2003; Oztan et al., 2007).

1.1.3.4 Exocyst and interaction with SNARE complex

The last regulatory step during vesicle fusion with target membrane is provided by SNARE complex, which specifically tightens two adjacent membranes downstream of exocyst complex. SNARE complex is tetramer composed of Qa-, Qb-, Qc-SNARE and R-SNARE. Alternatively Q-SNAREs are frequently called t-SNAREs due to the association with target membrane and R-SNAREs are called v-SNARE due to the association with vesicles. Assembling of the SNARE complex enables the fusion of the vesicle membrane with the targeted membrane. Several regulatory proteins were shown to control this process. SM family protein

(Sec1p/Munc18-like) is required for activation of Qa-SNARE (Sudhof and Rothman, 2009). The link between SNAREs and RabGTPases was studied by Grosshans and coworkers (2006). They established that vesicle associated RabGTPase SEC4 interacts indirectly with t-SNARE SEC9 via SRO7 protein (Grosshans et al., 2006).

The interplay between exocyst and SNARE proteins was first reported in yeast. Overexpression of either Sec4p, Sec1p or Sro7p bypass the inviability of *sec3, sec5* or *exo70* strains (Wiederkehr et al., 2004; Zhang et al., 2005; Grosshans et al., 2006) as well overexpression of t-SNAREs Sso2p and Sec9p can partially suppress secretion defect of *sec3* mutant (Grosshans et al., 2006). Moreover Sro7p was shown to interact with Exo84p (Zhang et al., 2005). In mammalian cell N-terminal domain of Exo70 competes with t-SNAREs SNAP23 for Snapin binding. Snapin interacts directly with t-SNARE SNAP23 and SNAP25 and its depletion decreased insulin stimulated glucose uptake (Bao et al., 2008). Those data support the hypothesis, that exocyst probably act prior to SNARE assembly and might be involved in SNARE complex regulation.

1.1.4 Exocyst membrane localization

Membrane phospholipids composition significantly influences membrane trafficking. Membrane composition adjust binding of regulatory proteins to the membrane and vice versa same regulatory proteins from small GTPase family have a potential to regulate phospholipid composition of the membrane by recruiting enzymes of phosphatidyl-inositol metabolism to the membrane (Shin et al., 2005). Each compartment maintains original membrane composition, which is dynamically changing but possesses certain characteristic to ensure organelle identity. Phosphatidylinositol-3-phosphate (PI3P) are localized mainly in the early endosomal membrane, phosphatidylinositol-3,5-bisphosphate ($PI(3,5)P_2$) in the late endosomal membrane and phosphatidylinositol-3-phosphate (PI4P) is in the membrane of GA (reviewed in Behnia and Munro, 2005). PI4P is also present on the PM and serves as a substrate for synthesis of phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$)- the main PI in the PM (McLaughlin et al., 2002). Rapid turnover of phospholipids is managed by specific PI phosphatases, kinases and phospholipases (reviewed in Behnia and Munro, 2005). Interestingly, the ratio PI4P/$PI(4,5)P_2$ in the PM is much lower in plants (10:1) than in animals and yeast (1:1) (Thole and Nielsen, 2008; Perera et al.,

2005). In *Arabidopsis* genome 70 genes contain predicted phospholipid binding pleckstrin homology (PH) domain, Phox homology (PX) domain or FYVE domain (Leeuwen at al., 2004).

Direct interaction between exocyst and PM was described for exocyst subunits SEC3 and EXO70 in yeast and mammals. N-terminus of SEC3 contains a new subclass of pleckstrin homology domain, responsible for binding membrane lipids and small GTPase Cdc42 (Baek et al., 2010). This domain carries cluster of positive residues, that are responsible for phospholipid binding, predominantly phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) (Baek et al., 2010).

Positively charged patch is formed also on the well conserved C-terminus of EXO70 in yeast and mammals and is directly liable for interaction with negatively charged phospholipides (Liu et al., 2007; He et al., 2007). Some of the plant EXO70s have well conserved this motif, suggesting that at least some plant EXO70 isoforms could bind directly the PM (Žárský et al., 2009). N-terminal region of mammalian EXO84 also carries putative PH domain. However the interaction of EXO84 with the PM was not experimentally proved (reviewed in Hertzog and Chavrier, 2011).

Alternative to the "spatial landmark" model is "exocytic signal" model. The authors of this model assume that local activation of RHO GTPase via the PI(4,5)P2 dependent exocytic signal, organize actin cytoskeleton and coordinate exocyst function (Yakir-Tamang and Gerst, 2009).

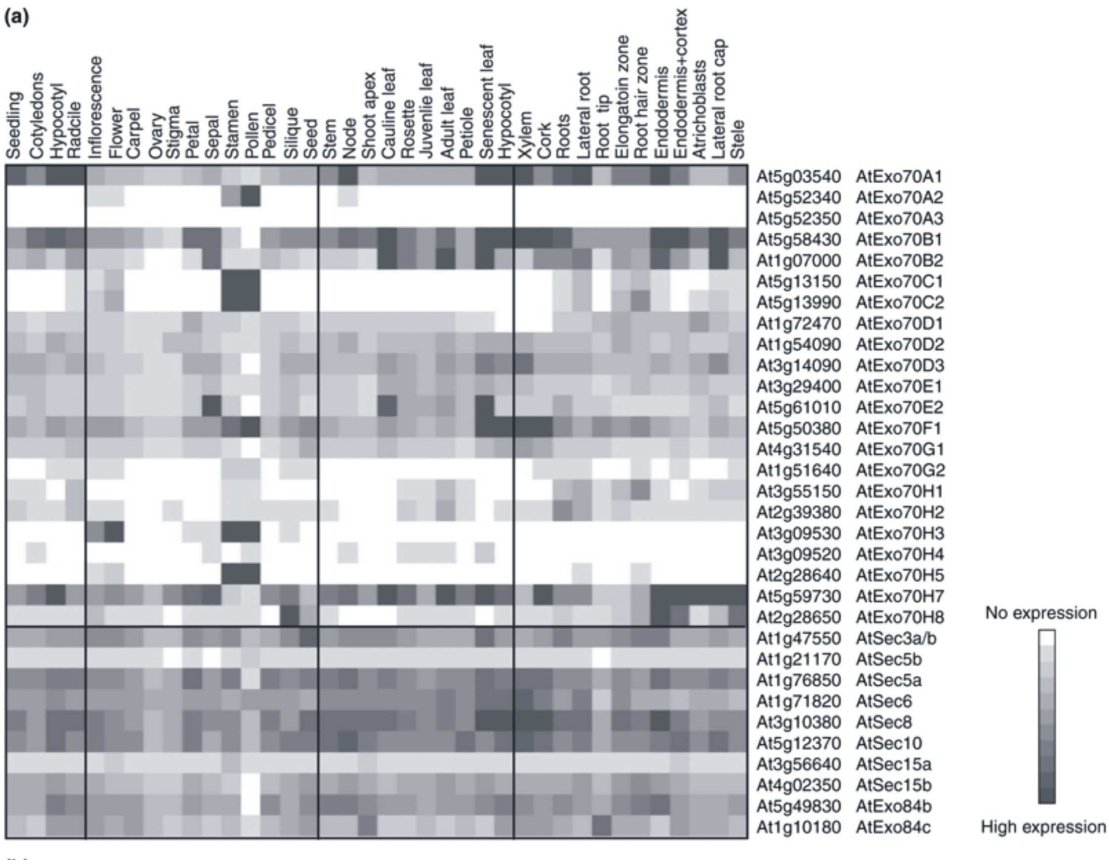
1.1.5 Plant exocyst

Homologues to all eight exocyst subunits were found in plant genomes (Cvrčková et al., 2001; Eliáš et al., 2003). Recently, it was proved that exocyst functions as a complex in plants and facilitates polarized secretion in growing pollen tubes and in hypocotyl elongation of etiolated seedlings in *Arabidopsis* (Hála et al., 2008). Interestingly, plant genome contains a big family of Exo70 genes- e.g. 23 members in *Arabidopsis thaliana* (Eliáš et al., 2003), while yeast and animals have a single copy of Exo70. This fact points to the possibility of plant-specific functions or tissue specialization of individual EXO70 isoforms. 23 isoforms of *Arabidopsis* Exo70s can be divided into the 3 classes: EXO70.1, EXO70.2 and EXO70.3. These classes possibly evolved from three Exo70s that might have been present in common ancestor of mosses and vascular plants. In *Arabidopsis*, EXO70.1 class possesses the highest homology to the Exo70 in yeast and mammals and contains two members of

the cluster EXO70A. One of the genes, Exo70A1 is highly expressed in sporophyte, whereas the second, Exo70A2 is expressed specifically in the pollen (Chong et al., 2009). Class EXO70.2 is subdivided to the clusters B, C, D, E, F, H and contains 19 *Arabidopsis* Exo70s. (Eliáš et al., 2003). *Arabidopsis* EXO70.3 class compasses cluster EXO70G containing 2 members - Exo70G1 expressed in all sporophytic tissues and Exo70G2 specifically expressed in the pollen and root tissue (Chong et al., 2009). *Arabidopsis* T-DNA insertional mutant in Exo70G1gene did not show any discernible phenotype (Synek L., Ph.D. Thesis).

Based on expression patterns of Exo70s in *Arabidopsis* and phenotypic analysis of several *exo70* mutants we have indicated that the EXO70A1 isoform plays a major role in the sporophyte (Synek et al., 2006). T-DNA insertional mutants in Exo70A1 exhibit short stature, loss of apical dominance, delayed senescence, delayed lateral root initiation, reduced number of cells, defects in elongation of root hairs and stigmatic papillae as well as in etiolated hypocotyls, and aberrant pollen development, leading to nearly complete sterility (Synek et al., 2006). Cole at al. (2005) demonstrated that several T-DNA insertions in Sec8 gene cause partial or complete pollen transmission defect. Mutant pollen grains complete microgametogenesis but were unable to germinate. This defect can by complemented by the expression of Sec8 gene under the pollen specific promoter LAT52 (Cole et al., 2005). Furthermore mutants in other exocyst subunits (*sec3*, *sec5*, *sec6*, *sec15*) exhibit similar defects in germination and polar growth of pollen tubes (Cole et al., 2005; Hála et al., 2008).

While Exo70A1 is expressed homogenously in all sporophytic tissue some other Exo70 genes display tissue specific expression. For example 14 out of the 23 *Arabidopsis* Exo70s are expressed in roots (Geneinvestigator; Li et al., 2010). For details see figure 2. Expression profile further revealed that some Exo70s are significantly up regulated during different stress condition such as pathogen infection (B2, H1, H4), wounding (B1, E1), cold (H4,H1,E2), salt (H2, B2, D3, B1, E1) osmotic (G2, F1, H8), drought (H8) or heat stress (H3, H2, H5) (*Arabidopsis* eFP Browser).



(Chong et al., 2010)

Figure 2 Expression analysis of exocyst subunits

1.2 Polar auxin transport

Plant hormones (phytohormones) are low molecular weight organic compounds that regulate plant growth and development. Distribution and concentration of phytohormones reflect environmental as well as internal signals. Phytohormones modulate signalling cascades leading to changes in gene expression, and consequently to physiological responses. Auxin has a prominent position among plant hormones, as it is the only one known to be transported in a polar manner along the shoot-root axis, creating an important component of positional information within the plant body. On the organ level, auxin promotes adventive and lateral root formation, vascular development, apical dominance establishment and maintenance, gravitropic and phototropic responses (reviewed in Benjamins and Scheres, 2008; Vanneste and Friml, 2009). On the cellular level, auxin stimulates cell division, elongation and differentiation.

Auxin distribution is controlled by coordination of its synthesis, conjugation, degradation and transport. Auxin is produced especially by the shoot apical region and move toward the root base by active polar pathway through parenchymal cells and by a passive non-polar pathway through the phloem. In the root tip, the auxin flow changes direction and passes actively through epidermal cells back to the elongation zone (reviewed in Benjamins and Scheres, 2008; Vanneste and Friml, 2009). The direction of auxin flow is determined by polarly localized auxin efflux carriers PINs. Expression pattern and polar localization of PINs are dependent on developmental stage of the plant and change in response to external and internal cues. For example PIN1 is localized mainly on the basal PM in the root protophloem cells (Galweiler et al., 1998) whereas PIN2 resides on the apical PM in epidermal root cells and basal PM in the root cortex cells (Muller et al, 1998). The polar localization of PIN proteins is connected to their constitutive cycling between the plasma membrane and endosomal compartments (Geldner et al., 2001; Dhonukshe et al., 2007; 2008). PIN proteins are initially delivered to the PM in a non-polar manner and their polarity is established by subsequent endocytic recycling (Dhonukshe et al., 2008), which depends on F-actin, clathrin-mediated endocytosis and exocytosis (Geldner et al., 2003; Dhonukshe et al., 2008).

Polar PIN localization is known to determine the direction of intercellular auxin flow however the precise mechanisms generating PIN polarity remain unclear. Only few regulators of PIN polar localization have been described so far:

1.2.1 Regulators changing phosphorylation status of PINs

Phosphorylation status of protein often represents an important aspect in their activation and function, the same is true for PIN proteins. There is strong evidence showing that AGC3 serine/threonin kinase PINOID (PID) can phosphorylate central hydrophilic loop of PIN proteins and therefore promote apical PM localization of the PINs (PIN1, PIN2, PIN4) (Kleine-Vehn et al., 2009; Dhonukshe et al., 2010; Zhang et al., 2010). Additionally Dhonukshe and coworkers uncovered that also other two AGC3 kinases WAG1 and WAG2 undertake the similar function as PID (Dhonukshe et al., 2010). On the other hand protein phosphatase 2A (PP2A) acts antagonistically to the mentioned serine/threonin kinases and instruct trafficking of PINs to the basal PM (Friml et al., 2004; 2007; Michniewicz et al., 2007) (Figure 4).

1.2.2 Arf-GEFs in PINs trafficking

In general ADP-ribosilation factor (ARF) is coordinated by ARF-GEF and ARF-GAP and all together regulate recruitment of protein coat on the secretory and endocytic vesicles. In plants ARF-GEF GNOM was the first discovered ARF-GEF involved in PIN recycling (Steinmann et al., 1999). It was shown that GNOM is localized to the endosomes and instruct trafficking of vesicles carrying PINs to the PM (Geldner et al., 2003). Since that time several other ARF-GEFs involved in PIN recycling were identified- the closest GNOM homologue GNL1 (GNOM LIKE1) placed on the PM and VAN3 (VASCULAR NETVORK DEFECTIV 3) localized to the TGN (Robinson et al., 2008). Using variable angle epifluorescence microscopy (VAEM) Naramoto and coworkers (2010) were able to localize GNOM and VAN3 to partially overlapping discrete foci at the plasma membranes that are regularly associated with the endocytic vesicle coat clathrin. Weak mutant alleles for GNOM and null alleles for GNL1 and VAN3 confirmed impaired endocytosis of PM proteins, including PINs (Naramoto et al., 2010).

To study endomembrane system ARF GEF specific inhibitor brefeldin A (BFA) is widely used. In *Arabidopsis* root tip cells, BFA inhibits several ARF GEFs including GNOM and induces aggregation of endosomes and TGN in so called BFA bodies. The core of BFA body is composed of Rab-A2/A3 membranes, surrounded by VHAa1 containing membrane (TGN), GNOM endosomes and the most of outer membrane is composed from Golgi stacks and PVC (SNX1 endosomes) (Chow et al., 2008). Whereas GNOM is BFA-sensitive and represents the major ARF GEF regulating PINs recycling, GNL1 is BFA-resistant and in case of BFA treatment, GNL1 partly compensates GNOM function in PIN recycling (Naramoto et al., 2010; Teh and Moor 2007) (Figure 4).

1.2.3 Rab5 GTPase pathway in PINs polarization

The importance of endocytosis in PINs polarization was emphasized by Dhonukshe et al., (2009). They demonstrated that disturbing Rab5 pathway blocks endocytosis of PIN proteins, which consequently leads to the unpolarized localization of PINs in the PM. Interestingly, in embryogenesis, this defect provokes homeotic transformation of cotyledons to roots (Dhonukshe et al., 2009). Later in the plant development defect in Rab5 pathway perturbed gravitropic reaction and impaired

lateral root initiation. Rab5 pathway was disturbed by induction of dominant negative version (DN-Ara7) lacking Rab5 in the active state or by using mutated weaker allele of Rab5 GEF AtVps9a-2. Since all three *Arabidopsis* RAB5 homologues reside on the endosomes, the authors suggested that impaired RAB5 pathway provoke accumulation of internalized vesicles, which in turns block endocytosis through regulation feedback (Dhonukshe et al., 2009).

1.2.4 Retromer complex sorts PINs from PVC to the TGN

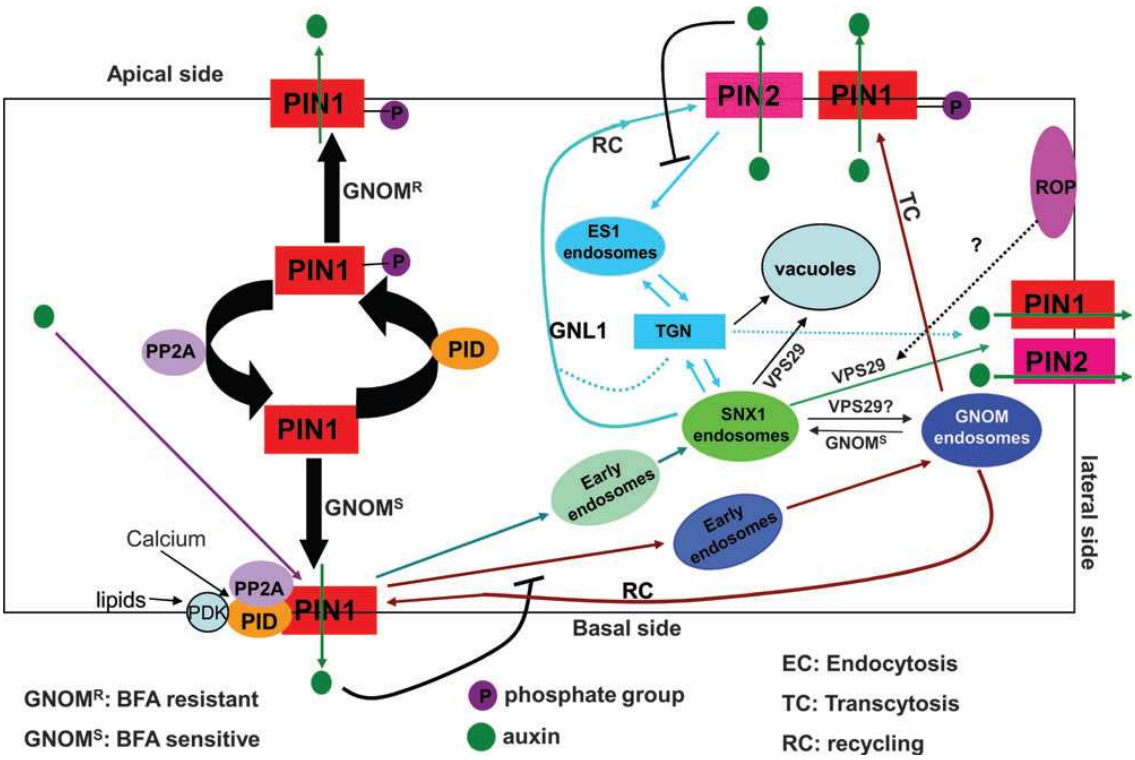
Important part in controlling auxin flow represents regulated accumulation of PINs in lytic vacuoles and their further degradation. Certain conditions such as gravitropic stimulation and/or dark treatment provoke increased degradation of PINs (Laxmi et al., 2008; Kleine-Vehn et al., 2008). Jillais and coworkers described involvement of putative retromer subunits SNX1 and VPS29 in retrieval of PINs from PVC to TGN (Figure 4). Furthermore they demonstrated colocalization of SNX1 and VPS29 at the PVC (pre-vacuolar compartment) downstream of GNOM endosomes (Jaillais et al., 2007; 2008). In *vps29* mutant PIN1 and PIN2 recycling was inhibited and PINs were accumulated in aberrant SNX1 endosomes (Jaillais et al., 2007; Jallais and Gaude, 2007). Kleine-Vehn et al., (2008) confirmed these data and further demonstrated, that *snx1* and *vps29* mutants carry less PIN1 and PIN2 on the PM and accumulate PIN2 in the vacuole-like structure (Kleine-Vehn et al., 2008). It is worth mentioning that in contradiction to the previous research Nimes detected retromer component SNX1 at the TGN/EE in BY2 cells as well as in *Arabidopsis* root cells (Nimes et al., 2010).

The final sorting of PINs into the internal vesicles of the MVB is managed by ESCRT-related CHMP1A and CHMP1B (CHARGED MULTIVESICULAR BODY PROTEIN 1A/1B) proteins (Spitzer et al., 2009).

1.2.5 ICR1 and Rop GTPase in PIN polarization

ICR1 was originally described as an adaptor scaffolding the interaction between the active RHO GTPases and the SEC3 exocyst subunit in *Arabidopsis* (Lavy et al., 2007); its loss of function (LOF) mutation results in mislocalization and misexpression of PIN1 in *Arabidopsis* embryo and roots (Hazak et al., 2010). Another testimony showing importance of RopGTPase in PIN polarization comes from the study of lobes formation in *Arabidopsis* leave epidermal cells. Current model assumes

the effect of auxin activated Rop2-Ric4 pathways during lobe formation in epidermal pavement cells. Auxin induces rapid activation of Rop2 GTPase, leading to polar recruitment of RIC4 to the plasma membrane of the growing lobes, rearrangement of the actin cytoskeleton and consequently to the polarization of PIN1 in growing lobes (Xu et al., 2010). It is worth mentioning that depletion of ICR1 perturbed lobe formation on the pavement cell and results in square-shaped cells (Lavy et al., 2007). This fact points to possible involvement of adaptor protein ICR1 in the lobe formation.



(Gao et al., 2008)

Figure 4 A model of regulators and pathways contributing to polar PIN pocalization.

Schema showing PINs targeting to the apical, basal, lateral side of the root cell dependent on kinase PINOID (PID), protein phosphatase 2A (PP2A), retromer complex subunits SNX1, VPS29 and ARF GEFs GNOM and GNL1.

1.3 Defining the hypothesis

My PhD thesis is the extension of previous research in our laboratory on the characterization of the plant EXO70A1 exocyst subunit. *Arabidopsis* insertional *exo70A1* mutants exhibit some phenotypic features pointing to compromised auxin transport and/or signalling (Synek et al., 2006). First, the mutant exhibits delayed lateral root initiation that is dependent on auxin level (e.g. Hobbie and Estelle, 1995; Casimiro et al., 2001). Second, short root hairs of the mutant can be partly rescued by addition of external auxin to the growth medium. Root hair elongation is also known to be promoted by auxin (Leyser et al., 1996; Pitts et al., 1998). Third, the apical dominance is compromised in *exo70A1* plants, resulting in a bushy stature of flowering plants – a symptom well connected to the disturbance in polar auxin transport (PAT). Therefore the main aim of my PhD thesis was to elucidate whether exocyst is involved in auxin transport and if so than what is the mechanism of exocyst action in PAT.

Taking advantage of available transcriptomic data of *Arabidopsis thaliana* we hypothesize that EXO70A1 represents the main EXO70 exocyst subunit in sporophyte development, while other EXO70 isoforms may act in specific developmental stages, environmental situations or in different membrane domains. Therefore, the second aim of my project was to characterize along with EXO70A1 and SEC8 exocyst subunit other EXO70 isoforms with respect to PAT.

To achieve the goals of this study, I have defined two working hypotheses and tried to answer the following questions.

I. Hypothesis

Polar auxin transport is compromised in exocyst mutant *exo70A1*.

1) Is PAT compromised in *exo70A1* mutant plants?

a) measuring the auxin transport in *exo70A1* mutant and WT plants.

b) comparing the auxin distribution in *exo70A1* mutant and WT plants using DR5 reporter.

II.Hypothesis

EXO70A1 along with other exocyst subunits are involved in recycling and polarization of vesicles carrying PIN1 and PIN2 proteins.

2) Is Exo70A1 along with other exocyst subunits involved in PIN1 and PIN2 polarization?

a) monitoring PIN1 and PIN2 polarization in *exo70A1*, *sec8-m1* mutants and WT plants.

b) monitoring PIN1 and PIN2 repolarization after BFA treatment and its washing in *exo70A1*, *sec8-m1* mutants and WT plants.

c) monitoring PIN1 and PIN2 transcytosis provoked by prolonged BFA treatment in *exo70A1*, *sec8-m1* mutants and WT plants.

.

3) How specific is the action of EXO70A1 in PIN recycling? Does EXO70A1 function specifically in PIN recycling or is its role more general in protein recycling?

a) monitoring localization of some other recycling protein (BRI1:GFP) in *exo70A1* mutant and WT plants.

.

4) Does exocyst localization depend on the plasma membrane composition and BFA sensitive trafficking pathway?

a) treating GFP tagged exocyst subunits with BFA.

b) treating GFP tagged exocyst subunits with wortmannin.

5) Is there any adaptor protein linking EXO70A1 or other exocyst subunits to PIN proteins?

a) using co-immunoprecipitation to detect interactors of EXO70A1 in respect to PIN polarization.

2 Aims of the thesis

1) To measure and compare radioactively labeled IAA transport and activity in IAA maxima using DR5 reporter between WT and *exo70A1*.

2) To analyse possible involvement of EXO70A1 along with other exocyst subunits in auxin efflux carriers (PINs) localization and recycling.

3) To analyse specificity of EXO70A1 for PIN recycling.

4) To address the EXO70A1 and some other exocyst subunits localization in respect to membrane lipid composition.

5) To study direct or indirect interactions between EXO70A1 and other proteins using co-immunoprecipitation.

3 SUMMARY

The polarization of exocytosis in yeast and animals is assisted by the exocyst – an octameric vesicle tethering complex and an effector of Rab and Rho GTPases. Recently, the exocyst was described as a functional complex involved in morphogenesis also in plants. Hála et al. (2008) described involvement of exocyst complex in pollen tube growth and hypocotyls elongation in dark grown seedlings, Fendrych et al. (2010) uncovered key role of exocyst in cell plate formation, Kulich et al. (2010) emphasized the participation of exocyst in seed coat generation and Pečenková et al. (2011) described the contribution of exocyst subunits in plant defense towards the pathogens. All these processes are intimately linked to secretion. Here we show involvement of exocyst in auxin efflux carriers PINs recycling.

Using direct auxin transport measurement and GFP-tagged proteins, we showed that the exocyst is involved in recycling and polarization of PIN proteins and polar auxin transport regulation. Rootward polar auxin transport is compromised in loss-of-function mutants in exocyst subunits EXO70A1. On the cellular level we have detected accumulation of PIN2GFP in the enlarged BFA-like FM4-64 labelled compartments distinct from VHAa1 labeled endosomes. Moreover recycling of PIN1 and PIN2 is retarded in roots of *Arabidopsis* loss-of-function mutants in exocyst subunits EXO70A1 and SEC8 after brefeldin A treatment. Even more severe secretory defect is observed after prolonged BFA treatment. This approach normally provokes transcytosis – i.e. relocalization of PINs from BFA compartment to the apical PM in the WT plants. However in exocyst mutants *exo70A1* and *sec8* PINs remains internalized in the BFA compartment. We observed that also recycling of the brassinosteroid receptor BRI1 is disturbed in similar manner as PIN recycling indicating more general PM proteins recycling defect (see manuscript).

Plasma membrane localization of GFP-tagged EXO70A1 and other exocyst subunits studied (SEC8, SEC10) are resistant to brefeldin A treatment suggesting that studied exocyst subunits traffic BFA-insensitive pathway. On the contrary localization of these subunits are sensitive to wortmannin – an inhibitor that modifies membrane phospholipids. These findings indicate that binding of studied exocyst subunits to the PM might depend on the phospholipide membrane composition (see manuscript). Using co-immunoprecipitation we revealed that EXO70A1 is present in a complex

with ICR1 (see manuscript) – an adaptor protein mediating interaction of activated RHO/ROP GTPases with the SEC3 exocyst subunit (Lavy et al., 2007) Recently ICR1 was proved to contribute to the regulation of polar auxin transport through PIN1 polarization (Hazak et al., 2010).

Whereas EXO70A1 along with other exocyst subunits display uniform distribution on the PM (Fendrych et al., 2010; manuscript), EXO70G1 shows enrichment on the apical and basal cell sides in the root tip cells (see chapter Results). This localization pattern might point to the role in recycling of polarly localized protein such as PINs. Since *exo70G1* mutant did not show any discernible phenotype (Synek et al., 2006) we will have to prepare double or triple mutant of *exo70G1, exo70G2* and *exo70A1* to uncover its function.

Our data show that polar auxin flow mediated by PIN proteins in plants relies also on the proper function of vesicle tethering complex exocyst. Despite an independent origin of plant multicellularity, the exocyst conserved its role in cell polarization and significantly participates in the regulation of polarity and morphogenesis also in plants.

4 Manuscript and appended publications

5 Materials and methods

This chapter does not include materials and methods described in the appended manuscript and publications.

Plant material and growth conditions

Exo70G1 was cloned from the pCAT vector (Eliáš et al., 2003) to binary vector pBAR modified by insertion of GFP cassette using restriction sites BamHI. Construct was used for *Agrobacterium* – mediated transformation of Col-0.

Arabidopsis seeds were surface-sterilized (10 min in 20% commercial bleach and rinsed 3 times with sterile distilled water) and dispersed onto agar plates with growing medium: 1/2× MS-salts (Sigma) supplemented with 1% (w/v) sucrose (Fluka), vitamins, 1.6% (w/v) plant agar (Duchefa), buffered to pH 5.7. Stratification was performed at 4 °C for 3 days. Seedlings were grown vertically in a climate chamber at 22 °C under long-day conditions (16 h of light per day).

Microscopy

Seedlings expressing fluorescently labeled proteins were transferred on a block of agar into a chambered coverglass Lab-Tek II and observed with a Zeiss LSM 5 DUO confocal laser scanning microscope equipped with Zeiss C-Apochromat 40×/1.2 water corrected objective. Bleaching during the FRAP analysis was performed two times by 10 iterations using 75% of laser power on the cell; images were grabbed consequently every 20 s.

Drug treatments

Seedlings were transferred from agar plates into 6-well cell culture plates containing ½× MS liquid medium supplemented with 50 µM brefeldin A (Sigma, 50 mM stock in DMSO) and incubated in light conditions in a climate chamber for 2 h. DMSO in even concentration was added to negative controls. Washout of BFA was performed by transfer of seedlings to medium without the drug. Seedlings were labeled by FM 4-64 (Invitrogen) in 5 µM final concentration and by propidium iodide

(Invitrogen) in 10 μM final concentration 10 min before observation. Seedlings stained by propidium iodide was rinsed two times with water.

6 Results

6.1 Supporting information not included in the manuscript

We have revealed perturbed auxin canalization in *exo70A1* mutant plants after local application of IAA. Since our recent publication shows importance of exocyst in primary cell wall formation (Fendrych et al., 2010) we decided to test the impermeability of Casparian strips in *exo70A1* and *sec8* mutant plants to uncover whether the defect in auxin canalization cannot be explained by the escape of auxin through the apoplastic pathway. To test impermeability of Casparian strips we have used propidium iodide – compound that specifically stains cell walls and does not penetrate through the Casparian strips (Alassimone et al., 2010). This experiment confirmed complete impermeability of Casparian strips in mutants as in WT and therefore supported the hypothesis of perturbed active transport through auxin transporters. Moreover this experiment revealed pronounced staining of the cell walls in *exo70A1* and even more in *sec8* mutants in comparison to the WT. Increased staining indicates changes in cell wall composition which is in agreement with transcriptomic data for *exo70A1* (Genevestigator database; Zimmermann et al., 2004) and is the major subject of current exocyst project in our laboratory. Completely perturbed plasma membrane of dead cell enables internalization of propidium iodide and staining of nucleic acids (Jones and Senft, 1985). Therefore propidium iodide also used as a marker of dead cells. In our experiment propidium iodide have labelled a number of dead epidermal cells in *sec8* mutant, less in *exo70A1* mutant and none were detected in WT plant (Figure 1).

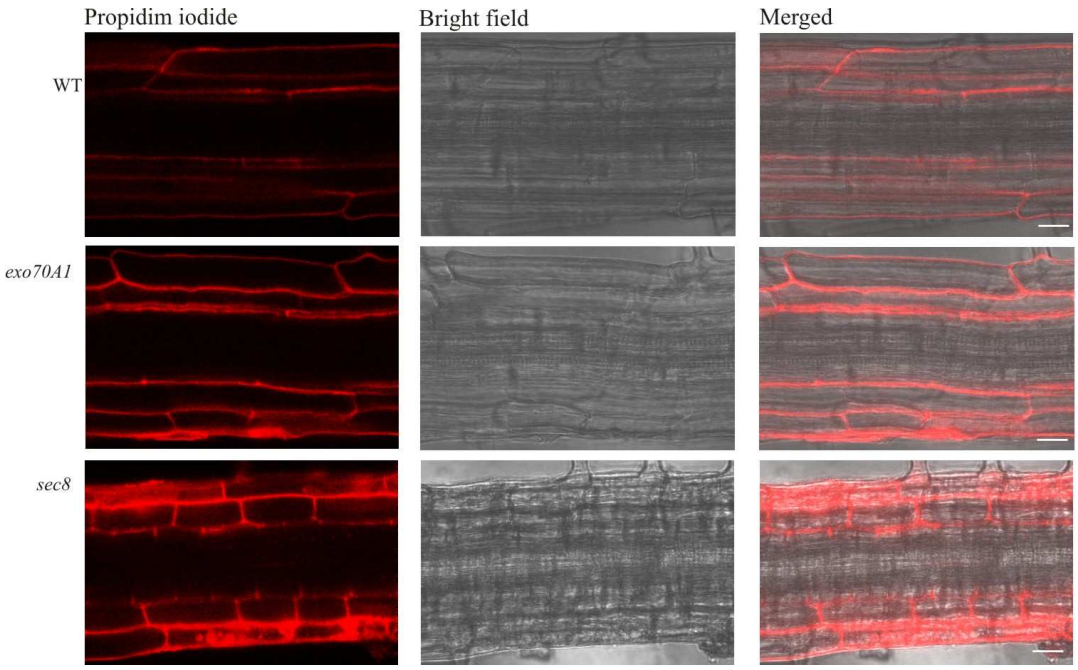


Figure 1 Roots of *exo70A1* and *sec8-m1* stained by propidium iodide

Casparian strips in *exo70A1* and *sec8-m1* mutants are impermeable for the propidium iodide.

Cell walls of epidermal and cortex cells in *exo70A1* and *sec8-m1* display pronounced staining in comparison to the WT plants. Bars = 20µm

6.2 Localization of GFP:EXO70G1

Using *Agrobacterium* mediated transformation, we have prepared plants expressing GFP:EXO70G1 under the control of 35S promoter. In these plants we have observed polar localization of GFP fused proteins preferentially to the transversal PM in the root tip cells. Significant portion of the fluorescent signal was also detected in the cytoplasm (Figure 2A). To distinguish whether the signal on the PM is apical, basal or both we utilized FRAP (Fluorescence Recovery After Photobleaching) analysis. Bleaching the signal from the whole cell revealed that the new signal on the apical and basal side is delivered from the neighboring cells with the same rate, this indicates enriched localization of EXO70G1 on both, apical and basal cell sides (Figure 2D). As in the work done by Fendrych et al. (2010) we monitored GFP:EXO70G1 during cell plate formation in root tip cells. In contrast to other studied exocyst subunits, the GFP:EXO70G1 was localized to the forming cell plate from the initiation phase till the maturation phase. Moreover the intensity of the signal

at the newly formed cell plate was weaker when compared to other non dividing adjacent cells (Figure 3). In contrast other studied exocyst subunits display enrichment in the maturing cell plate (Fendrych et al., 2010).

To get insight into the intracellular Exo70G1 recycling, we have treated the stable transformed plants expressing GFP:EXO70G1 with brefeldin A (BFA). The BFA treatment did not affect PM localization of the GFP:EXO70G1 (Figure 2B). On the contrary using wortmannin - the inhibitor of phosphoinositide 3-kinase (PI3K) and phosphoinositide 4-kinase (PI4K) - disrupted dynamic recycling of the EXO70G1 between the PM and cytoplasm and provoked the depletion of the signal from the PM and cytoplasmic pool enrichment (Figure 2C).

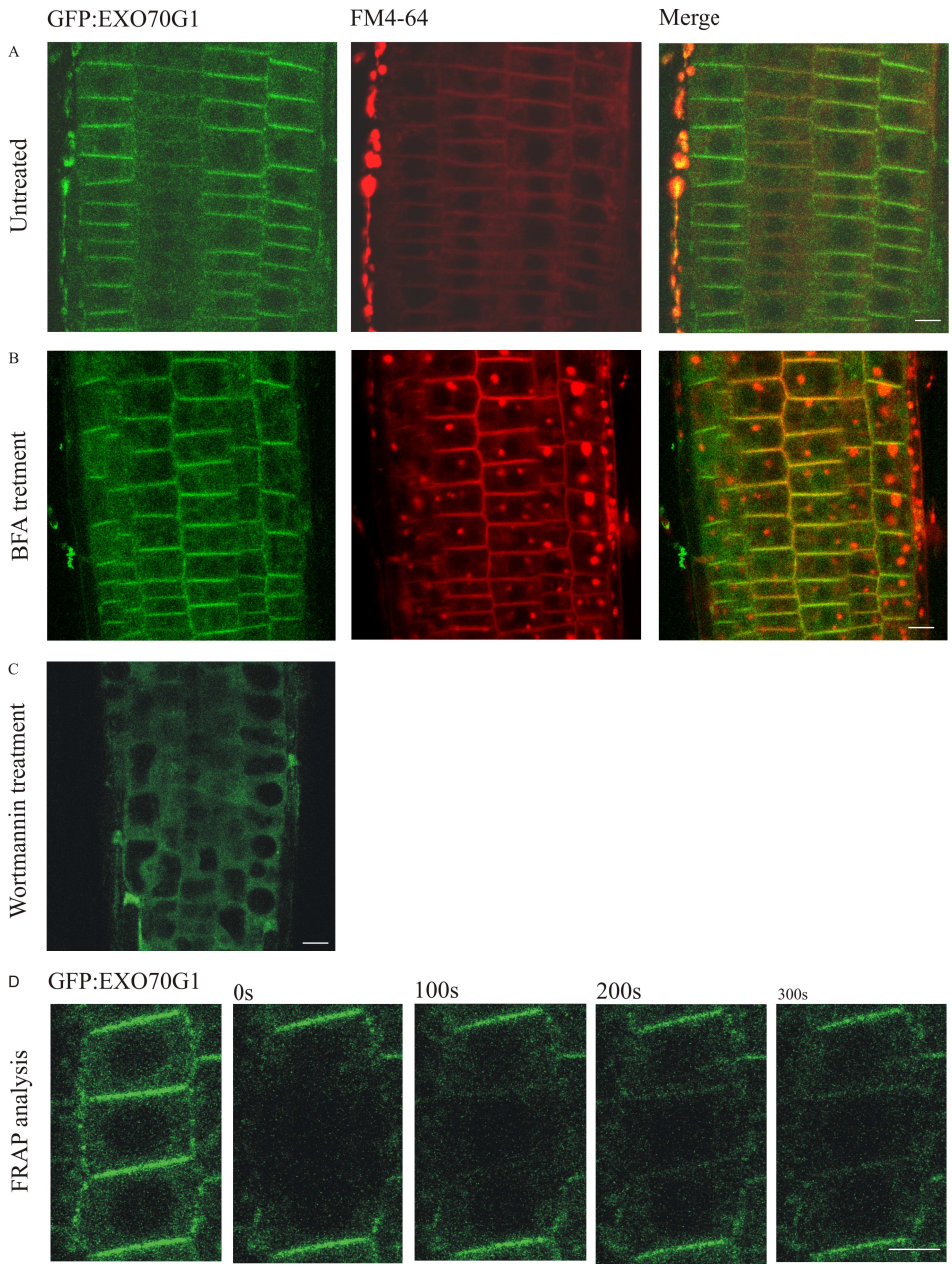


Figure 2 Localization of GFP:EXO70G1

A) Polar localization of GFP:EXO70G1 in the *Arabidopsis* root tip cells.

B) 50μM BFA treatment for 2 h do not perturbed GFP:EXO70G1 localization in the *Arabidopsis* root tip cells.

C) 66μM Wortmannin treatment for 2 h evoked the release of GFP:EXO70G1 from the PM of *Arabidopsis* root tip cells.

D) Monitoring GFP:EXO70G1 dynamic using FRAP analysis. Bleaching whole cell uncovered that the same time is needed to recover the signal on apical and basal transversal cell plate. Bars = 10μM

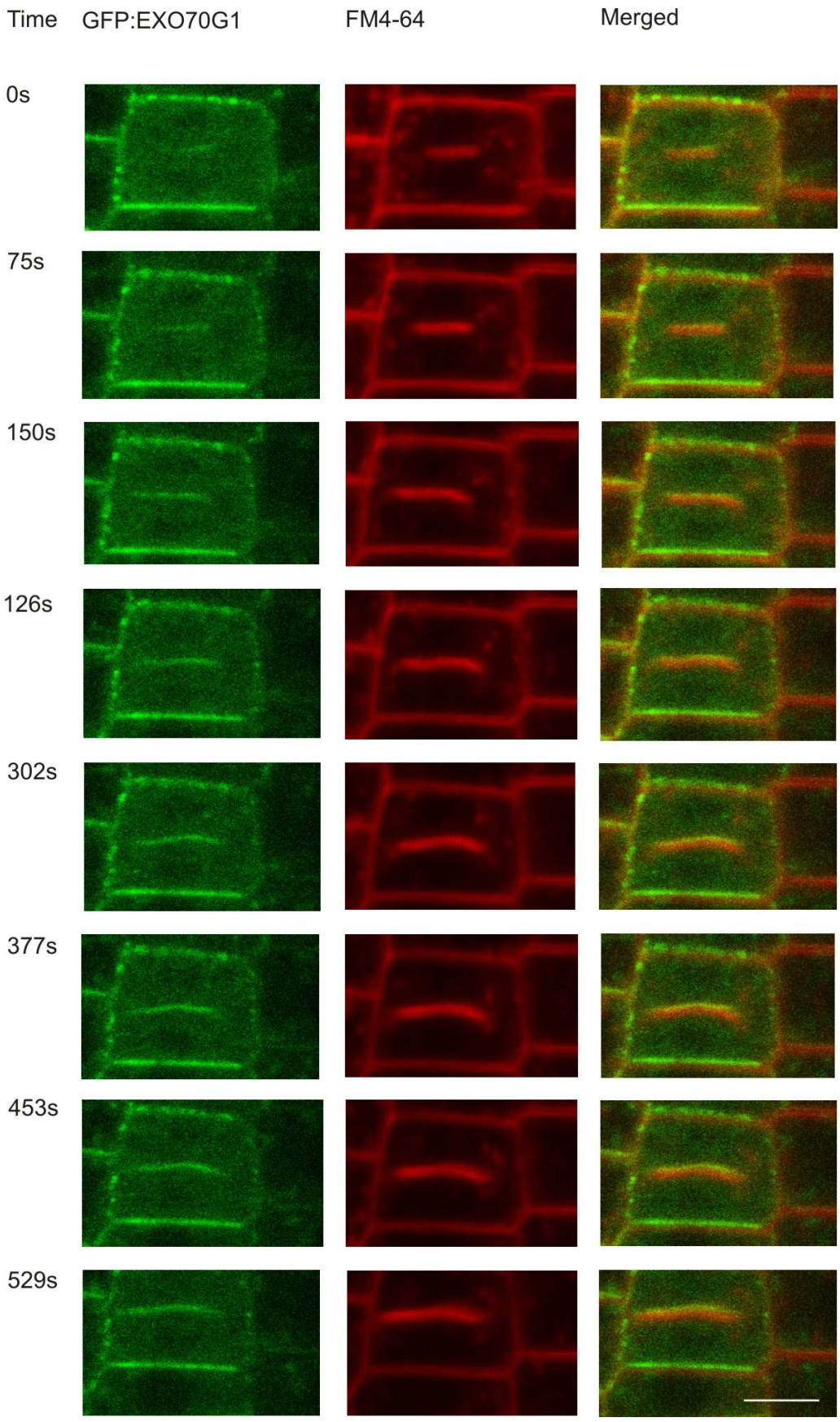


Figure 3 Localization of GFP:EXO70G1 during cell plate formation

GFP:EXO70G1 localize to the newly forming cell plate. Bar = 10µm

7 DISCUSSION

Auxin related phenotype of *exo70A1* mutant plants

The indirect proof for the connection of exocyst with the regulation of polar auxin transport and possible involvement of the EXO70A1 in the switching of the PINs destinations may be also found in the similarity of exocyst mutants *exo70A1* and *sec8-m1* with mutant in PIN polarization.

exo70A1 and *sec8-m1* mutants display aberration in root meristem maintenance (Cole R. Ph.D. Thesis), in lateral root initiation and in auxin maxima maintenance, phenotypes linked to defect in polar auxin transport, typical for mutants in PIN polarization. Especially strong deviation in root meristem is seen in *gnom* (Steinmann et al., 1999), *icr1* (Lavy et al., 2007), mutants in RAB5 pathway (Dhonukshe et al., 2010), in mutants with enhanced expression of PID1, WAG1, WAG2 (Dhonukshe et al., 2010) and among the sterol biosynthetic mutants mutants *cpi1-1* (Man et al., 2008) and *smt1* (Willemsen et al., 2003). These mutants differently effect PINs polarization, mistargeting PINs to the apical, basal, lateral site or to the aberrant intracellular compartments.

Interestingly, all single *pin* mutants display various weak phenotypes in primary roots. Single mutant *pin1* and *pin2* have only slightly reduced root length and root meristem size, *pin3, pin4* and *pin7* have slight division defect in the quiescent centre and columela root cap cells (Blilou et al., 2005). As described Blilou et al. (2005) triple mutant *pin3 pin4 pin7* enhanced expression of PIN1 in lateral-basal membrane of the endodermis and PIN2 localized ectopically to the basal end of prevascular cells, the domains that originally express PIN3 and PIN7. These findings suggest partial redundancy between PIN proteins in the root meristem (Blilou et al., 2005). Furthermore Blilou et al. (2005) performed series of experiments utilizing double and triple *pin* mutants pointing to key role of PINs in root meristem size regulation and auxin maxima maintenance, which supports auxin related phenotypic of *exo70A1*.

However the most striking phenotype in comparison to *exo70A1* mutant display $gnom^{R5}$ and $gnom^{B/E}$ the weak *gnom* alleles. Whereas loose of function alleles of the *Arabidopsis GNOM* gene leads to severe defects in the embryonic stage, characterized by perturbed establishment of embryonic axis, lack the embryonic root and fused or

deleted cotyledons (Mayer et al., 1993), the weaker alleles except for rarely fused cotyledons are nearly indistinguishable from the WT up to the 5 days after germination. These weak *gnom* alleles as well as *exo70A1* and *sec8-m1* are defective in root meristem maintenance and lateral root formations (Getdner et al., 2004; Cole R. Ph.D.Thesis). To shed light on GNOM function Geldner et al. (2004) used similar approach as we did, they treated the $gnom^{R5}$ mutant seedlings carrying DR5:GUS reporter with synthetic auxin. They uncovered dramatic accumulation of DR5:GUS signal through the whole root tissue of the $gnom^{R5}$mutant, whereas the WT effectively canalized the auxin and probably provide the transport to the site of degradation (Geldner et al., 2004). This finding strongly resembles the defect observed in *exo70A1* plants and points to the common defect in effective auxin canalization and maintaining auxin gradients after external auxin application.

We have shown that *exo70A1* mutant displays the most severe defect on PIN recycling after blocking the function of GNOM by prolonged BFA treatment. There are other four BFA sensitive ARF GEFs functioning in the *Arabidopsis*, however expression of BFA resistant form of GNOM uncovered its major role in PIN trafficking from the endosomes to the PM (Geldner et al., 2004). These findings suggest that blocking GNOM function simultanously with depletion of *exo70A1* almost completely block trafficking to the PM and accumulate PINs and BRI1 in the enlarged BFA compartments.

We have shown that depletion of EXO70A1 resulted in completely perturbed root waving phenotype. It was proposed that waving reflects circumnutation, gravitropism and negative thigmotropism (Migliaccio and Piconese, 2001; Santer and Watson, 2006) and due to the impact of auxin on root meristem, it is expected that auxin transport and signaling is involved in this process as well (Santer and Watson, 2006). It is worth mentioning that even though there is an interplay between waving and gravitropism, the gravitropic response of *exo70A1* mutant seedling was indistinguishable from the WT. Despite the intensive study, precise involvement of auxin trasport in root waving are not elucidated. Until recently, WAG1 and WAG2, the proteins acting as the suppressor of waving (Santer and Watson, 2006) were shown to act redundantly to PINOID (PID) function (Dhonukshe et al., 2010). It was proved that WAG1 and WAG2 as well as PID are ACG kinases, that instruct basal to apical PIN polarity shifts (Dhonukshe et al., 2010; Kleine-Vehn et al., 2009).

The mutant seedlings *wag1*, *wag2*, *wag1 wag2* as well as *exo70A1* did not show any defect in gravitropic response. Unlikely to *exo70A1*, single *wag1*, *wag2* and double *wag1 wag2* mutants display pronounced waving phenotype. However triple mutant *wag1 wag2 pid* display gravitropic defect and completely lacked cotytedons, similarly to *pid pin1* mutant (Furutani et al., 2007; Dhonukshe et al., 2010). On the contrary overexpression of PID, WAG1 and WAG2 lead to the loose of gravity responses, depletion of auxin maxima and total collapse of root meristem. On the cellular level overexpression of PID, WAG1 and WAG2 induce apicalization of PIN1, PIN2 and PIN4 (Dhonukshe et al., 2010). These results indicate that even though the WAG1 and WAG2 play important role in PIN polarization, utilizing single *wag1*, *wag2* and double *wag1wag2* mutants uncover strong waving phenotype but mask their participation in gravitropic responses. Moreover these findings corroborate that root waving phenotype, root tip auxin maxima and root meristem size reflect auxin distribution and supports the fact that *exo70A1* phenotype mirrors the compromised auxin transport through PIN polarization.

Detail microscopic inspection of *Arabidopsis exo70A1* uncovered a unique unpublished root cap phenotype - we observed extreme accumulation of border-like cells (BLC) layers still attached to the root cap in *exo70A1*. In contrast WT plants release these cells from the root cap region as layers of connected cells (Driouich et al., 2006). Root cap architecture is under the control of auxin, however we speculate that this phenotype of *exo70A1* might reflect a defect in cell wall material or cell wall-degrading enzyme deposition, since BLCs are detached from the growing root cap by cell wall-degrading enzymes. Importance of cell wall composition in root cap organization was recently supported by characterization of *Arabidopsis* mutant *quasimodo* (Durand et al., 2009). This mutant has reduced biosynthesis of homogalacturonan and sheds isolated cells from the root cap (Durand et al., 2009).

Exocyst and PM protein recycling

Based on expression profile of EXO70s, EXO70A1 was predicted to function as the main EXO70 in sporophyte development. This expectation was strengthened by detailed analysis of T-DNA insertional mutant in EXO70A1 (Synek et al., 2006). Some phenotype deviations of *exo70A1* mutant resembled the defect in auxin transport or synthesis. Here we present data showing the involvement of EXO70A1

and other exocyst subunits in PINs and BRI1 recycling and therefore confirming evolutionary conserved role of exocyst in PM protein recycling also in plants

We have uncovered pronounced decrease (about 30 percent) in the rate of auxin transport for both mutant alleles *exo70A1-1* and *exo70A1-2*. Detailed monitoring of auxin transport through the tissue using DR5:GUS reporter line uncovered reduced auxin maxima in the root tip of *exo70A1*, confirmed compromised rootward auxin transport and additionally revealed perturbed canalization of auxin in *exo70A1* mutant plant. Since our recent publication showed importance of exocyst in primary cell wall formation (Fendrych et al., 2010) we tested the impermeability of Casparian strips in *exo70A1* and *sec8* mutant plants by propidium iodide staining. This experiment confirmed complete impermeability of Casparian strips and therefore the defect in auxin canalization cannot be explained by leakage of auxin from apoplastic pathway but rather by perturbed active transport through auxin transporters. The main auxin transporters are represented by efflux carriers PINs, influx carriers AUX1 and ABC transporter-like phosphoglycoprotein (PGP). We have shown that exocyst plays the important role in PIN1 and PIN2 recycling in root tip cells.

We have observed accumulation of PIN2 in the compartment distinct from VHAa1 labelled early endosomes. We did not uncover the origin of this compartment but we have suggested that it functions downstream of the early endosomes. Formation of aberrant compartments in plant exocyst mutant is in accordance with studies in animal cells. In *Drosophila* exocyst subunits Sec5, Sec6 and Sec15 localize to the recycling endosomes and regulate DE-Cadherin trafficking to the PM in epithelial cells and mutations in any these exocyst subunits provoke accumulation of DE-Cadherin in enlarged Rab11 recycling endosomes (Langevin et al., 2005). Another viable *Arabidopsis* exocyst mutant *exo84b* with pronounced secretory defect was characterized by Fendrych et al, (2010). Using transmission electron microscopy they observed accumulation of vesicles in the mutant leave epidermal cells, the aberration resembling the secretory defect in yeast exocyst mutants which is manifested by accumulation of secretory vesicles near the PM (Novick et al., 1980; Guo et al., 1999b).

Furthermore in *exo70A1* mutant as well as *sec8* mutant we have described perturbed repolarization of PIN1 and PIN2 after the washing BFA and even more severe defect was observed during transcytosis induced by prolonged BFA treatment. These results indicate that the transcytotic pathway for some transporters might be

exocyst dependent processes. In animals exocyst also participates during transcytosis as documented by Oztan et al. (2007). They observed localization of exocyst to the apical and basolateral endocytic compartments and described involvement of SEC15A in basolateral to apical transcytosis. In plants transcytotic re-localization of PIN occurs also under physiological conditions, as shown by a massive redirection of PIN2 from the apical and basal cell sides of the newly formed cell plate to strictly apical or basal polar localization (Kleine-Vehn et al., 2008). Since we have shown that exocyst plays the key role during maturation of newly formed cell plate (Fendrych et al., 2010) it is possible that also PIN polarization on the newly formed cell plate might depend on exocyst function, however we have not tested this possibility. Transcytotic relocalization of specific PINs to the lateral membrane is also the major factor regulating auxin flow during phototropic and gravitropic reaction (Friml et al., 2002).Considering the slower growth and skewing of the *exo70A1* homozygous root we did not measure any major difference in gravitropic response except for short term root growth disorientation during germination of the seedling which might or might not be the consequence of perturbed auxin transport. Another example of naturally occurring transcytosis of PINs in the cell represents repolarization of PIN1 from basal site to the outer lateral site during lateral roots initiation (Benková et al., 2003). Although *exo70A1* mutant displays delayed lateral root initiation, the brief inspection of PIN1 did not reveal any major defect in polarity switch (data not shown). These findings show that whereas drug induced transcytosis of PINs is almost completely blocked in exocyst mutants under physiological conditions, EXO70A1 is not required for lateral auxin redirection or alternatively there might be functional redundancy between EXO70 isoforms. In fact the functional redundancy between EXO70 isoforms is expected; it was previously reported that at least 16 out of 23 EXO70s are expressed in *Arabidopsis* roots (Genevestigator; Li et al., 2010).

Similar recycling defect in *exo70A1* mutant as described for PIN has been recorded also for brassinosteroid BRI1 receptor. Indeed, experiment revealing reduced sensitivity of *sec8* mutant to externally added brassinosteroids indicated involvement of the exocyst in brassinosteroid signaling (Cole R. Ph.D. Thesis). We cannot conclude that the defect in PINs polarization in exocyst mutants fully describes the dramatic reduction in rootward auxin transport and dwarfish phenotype of *exo70A1* mutant we have to consider the fact that also brassinosteroid signaling effects the auxin homeostasis and signalling (Hardtke et al., 2007) and therefore it might

significantly contributes to the phenotypic deviation in *exo70A1* plant. These findings supports the idea that exocyst function generally affecting several trafficking pathway, which is in agreement with our previous studies pointing to its key role in variety of secretory processes including cell plate formation (Fendrych et al., 2010), seed coat formation (Kulich et al., 2010), pollen tube growth and hypocotyl elongation (Hála et al., 2008).

As mentioned above ABC transporter-like phosphoglycoproteins (PGP) also operate as the auxin transporters. Two members from this family PGP1 and PGP19 significantly accelerate long distance auxin transport (Geisler et al., 2005; Noh et al., 2003), furthermore PGP19 is localized to the root endodermis and pericycle cells and is proposed to prevent escape of auxin from the stele (Blakeslee et al., 2007). Therefore the defect in PGP19 proteins resembles defect described in *exo70A1* mutants after application of IAA on the root/shoot junction. We cannot exclude possible involvement of EXO70A1 in PGP mediated auxin transport, but our result showing proportional reduction of auxin transport after application of PGP inhibitor gravacin in *exo70A1* mutant plants did not support this possibility. The same is true for auxin influx carrier AUX1/LAX, we have not tested the impact of depletion of exocyst subunits on localization of these transporters, but considering the result showing no less effect on IAA transport in *exo70A1* after application of AUX1/LAX auxin uptake inhibitor 1-NOA we might suggest that AUX/LAX are not the main target of exocyst function.

Exocyst - ICR1 interaction

We have shown that EXO70A1 coimmunoprecipitates with ICR1 protein. ICR1 was originally described as the adaptor protein scaffolding the interaction between SEC3 and RHO GTPase (Lavy et al., 2007). These findings support the hypothesis that the plant exocyst function is regulated by RHO GTPase likewise describe the yeast and mammalian models (Brennewald and Rossi, 2007). The exocytotic defects on the cellular level in *icr1* mutant such as accumulation of PINs inside the cell and delay in recruitment of PINs to the PM after BFA treatment resembles the defects observed in *exo701* mutant plants, however the aberrant PINs polarization in *icr1* mutant is much more pronounced and results in severe developmental defect which is notable from the triangular stage embryo onward. The root of *exo70A1* plant displays compromised auxin maxima in the root tip, reduced size of the root meristem (Cole R.

Ph.D. Thesis) and lower amount of starch. The similar deviations are known in the root tip of ICR1 knockdown, nevertheless the aberrations are much more severe during the time of post embryonic development leading to complete loose of auxin maxima and to the collapse of primary root 14 DAG (Hazak et al., 2010).

Whereas we have seen only slight intracellular accumulation of PIN2 in untreated *exo70A1* plant, *icr1* has pleotropic impact on PIN recycling. First, during embryogenesis *icr1* accumulated PIN1:GFP in enlarged compartment. Later during post embryonic development the basal to apical shift of PIN1 and PIN2 were observed in the root of *icr1*. Moreover this approach revealed misexpression of PIN1:GFP in epidermal cells and root hairs and its subsequent accumulation in intracellular aggregates (Hazak et al., 2010). These deviations were never observed in *exo70A1* background, suggesting that ICR1 might function as the adaptor for other regulatory proteins in the secretory pathway which contribute to the more severe defect in PIN polarization in *icr1* mutant. Alternatively losing one of the exocyst subunit does not completely impair the function of the remaining subcomplex, which can partly manage the tethering function in PIN recycling. Moreover we have to consider that large number of the EXO70s in the root might be partly redundant in their function.

The strength of the deviation in PIN polarization reflects the aberration on the organ level. Whereas in *exo70A1* plants we have discovered a delay in lateral root initiation, primary root of *icr1* collapsed before forming lateral roots and instead *icr1* forms the adventitious root with arrested primordia of new lateral roots. Considering the phenotypic deviations in aerial tissue, it is worth mentioning the square shape epidermal pavement cells of *icr1* mutant. This defect points to the possible role in lobe formation through ROP2-RIC4 and ROP6-RIC1 directed PIN1 polarization as pointed out Xu et al., (2010). In contrary *exo70A1* leaves carry properly shaped pavement cells similar to other studied exocyst mutant (*exo84b, sec8-m1*) suggesting that this process do not require EXO70A1 and might be exocyst independent.

GLUT4 trafficking versus PIN recycling

We have shown that in the plant exocyst regulate trafficking of auxin transporter PIN to the PM. This result correlates with studies in animal cell, describing involvement of exocyst in transport of several receptors and transporters to the PM. Best characterized is trafficking of vesicles carrying glucose transporter GLUT4 (Inoue et al, 2003; Bao et al., 2006; Wu et al., 2010). Comparing these two exocyst

dependent trafficking pathways in plant and animal cell reveals many similarities but also differences.

Comparing localization, the major pool of PIN proteins in the cell is polarly localized on the PM, to achieve the polar distribution, PIN have to recycle through clatrin mediated endocytosis, which require functional ARF GEFs, actin cytoskeleton and Rab5 endocytic pathway. Recycling of PINs to the PM runs through TGN and GNOM containing endosome. On the contrary in unstimulated animal cell GLUT4 reside mainly on the intracellular compartment, called GLUT4-specialized compartment (GSV). The process of sequestration of GLUT4 to the GSV is controled by IRAP and RabGAP AS160 (Jordens et al., 2010). Relocalization of GLUT4 transporters to the PM is induced by external hormonal signal - by insulin stimulation. Insulin provokes redirection of GLUT4 from the GSV via recycling endosomes to the PM. Accordingly PIN polarization is continually affected by the presence of plant hormone auxin. Increased auxin concentration inhibits endocytosis of PINs and promotes increase auxin transport from the cell (Paciorek et al., 2005). However the precise mechanism of auxin action on PINs remains elusive.

Localization of exocyst in these two pathways mirrors the distribution of the regulated transporters PIN and GLUT4. Whereas in animal cell EXO70 is predominantly locked in the endosomal compartments and the insulin stimulation induces relocalization to the PM, in the plant cell the EXO70A1 maintains rapid recycling to the PM. It is noteworthy that EXO70A1 recycling is much faster than PIN recycling.

In plants we have shown that EXO70A1 coimmunoprecipitate with ICR1, adaptor protein for ROP GTPase. The inherence of the link between exocyst and ICR1 was further supported by Hazak et al. (2010). They uncovered that ICR1 interacts directly with SEC3 and has a potential to recruit SEC3 to the PM (Lavy et al., 2007). Moreover ICR1 (Hazak et al., 2010) as well as EXO70A1 were shown to regulate PIN1 recycling. These findings document an essential role of exocyst and adaptor protein ICR1 in tethering of vesicles carrying PIN proteins. In mammals it is well documented that targeting of GLUT4 carrying vesicle requires direct interaction between EXO70 and RHO GTPase TC10 (Inoue et al., 2003).

Comparing Exo70A1GFP and Exo70G1GFP localization

Till now EXO70A1 and EXO70G1 were the only GFP fused EXO70s stably expressed in *Arabidopsis*. Comparing their localization in root tip cells uncovered several common features. First, GFP:EXO70G1 as well as GFP:EXO70A1 localizes on the PM and also significant portion of the GFP signal remains intracellular. Second, PM localization of both plant EXO70s are not affected by treatment of ARF GEF specific inhibitor brefeldin A. In mammalian studies certain discrepancy exists in relation to the sensitivity of exocyst subunits to the BFA. Whereas Vega and Hsu (2001) did not observe perturbed localization of Exo70 from Golgi structure after BFA treatment in PC12 cells, Xu et al. (2005) recorded BFA induced dispersion of Exo70:GFP from the Golgi in HepG2 cells. Third, in the plant root tip cell both EXO70s relocalize from the PM to the cytoplasm after treatment by wortmannin - the inhibitor of PI3K and PI4K. In *Arabidopsis* root tip cells wortmannin inhibits specifically SNX1 endosomes (PVC) and blocks retrograde transport from PVC to TGN (Jaillais et al., 2008). Due to the fact that PI4K resides also on the PM, we can speculate, that the high concentration of wortmannin treatment disturbs phospholipides composition of PM which can consequently block binding of EXO70s to the $PI(4,5)P_2$ and evokes the release of EXO70s to the cytoplasm. These results are consistent with the findings in yeast and mammals. Crystalografic analysis of EXO70 and detail characterization of C-terminus of the protein uncovered positively charged patch on the well conserved C-terminus of EXO70 that is directly liable for interaction with negatively charged phospholipides (Liu et al., 2007; He et al., 2007). As shown by Žárský et al, (2010) some of the plant EXO70s have well conserved this motif, suggesting that at least some plant EXO70 isoforms could bind directly the PM (Žárský et al., 2009). To confirm the direct binding of EXO70s to the PM lipids, it will be necessary to prepare point mutations in the conserved binding domain in the EXO70s and also test exocyst protein/domain interaction with liposomes.

However there are two important differences in localization of both EXO70 isoforms. First, GFP:EXO70A1 resides on the PM membrane homogenously, similar to other exocyst subunits (Fendrych et al., 2010), whereas EXO70G1 localizes preferentially on the apical and basal sides of the root cells. Second, as described by Fendrych at al. (2010) EXO70A1 along with other studied exocyst subunits localizes to the newly forming cell plate during the initiation phase, later during the growing phase the signals disappear and occur again during the final stage of cell plate maturation (Fendrych et al., 2010). On the contrary GFP:EXO70G1 persists in the

newly formed cell plate from the initiation till the maturation phase. Moreover, whereas EXO70A1 and other exocyst subunits display enrichment on the newly formed cell plate, the signal of GFP:EXO70G1 is weaker in comparison to the adjacent non dividing cells. Due to the pronounced polar distribution in the root cells, it seems that EXO70G1 might specifically function in vesicle tethering to the apical and basal domains of the PM. Such domains are extensively utilized by PINs and by the regulatory proteins that facilitate PINs recycling, such as ICR1 (Hazak et al., 2010). Unfortunately we did not find any discernible phenotype for *exo70G1* mutant plant which might be caused by redundancy between EXO70s. Therefore to shed light on EXO70G1 function it will be necessary to prepare double and/or triple mutant of *exo70G1*, *exo70G2* and *exo70A1*.

When we are discussing GFP:EXO70A1 and GFP:EXO70G1 localization pattern we have to consider the fact, that both GFP fused protein were expressed under the 35S promoter which might partly cause abnormal localization due to the overexpression. To avoid this, it will be necessary to express these GFP fused proteins under the native promoter.

Comparing localization of exocyst in plant, yeast and animal cell

We have previously published that GFP:EXO70G1 is localized to the distinct patches near or on the PM and to the nucleus in unpolarized *Nicotiana tabacum* protoplasts (Eliáš et al., 2003). Similar localization pattern was observed also for GFP:EXO70A1 (Synek L. Ph.D. Thesis). Wang et al. (2010) utilized the same method and overexpressed transiently 8 out of 23 *Arabidopsis* EXO70 isoforms. However only EXO70A1, EXO70B1 and EXO70E2 localized to the patches in the PM and in the cytoplasm, which might partly resembles localization pattern for EXO70G1 and EXO70A1 in protoplasts prepared in our laboratory. The remaining studied EXO70s showed strictly cytoplasmic localization (Wang et al., 2010). Wang and coworkers focused the interest on EXO70E2, trying to elucidate the origin of the patches. Using immunolocalization in *Arabidopsis* roots, transient expression of EXO70:GFPs in BY2 cells and in protoplasts, they described novel plant specific compartment called EXPO, which carry EXO70E2, EXO70A1 along with other exocyst subunits, but did not colocalize with any standard endomembrane markers neither the FM4-64 dye. This compartment was not sensitive to BFA or wortmannin treatment. Surprisingly our detailed study of GFP:EXO70A1, GFP:EXO70G1 and other exocyst subunits

(SEC8:GFP, EXO84b:GFP, SEC10:YFP) in roots of stable transformed *Arabidopsis* plants did not reveal any structure resembling the EXPO compartment. Using confocal scanning microscopy we have detected strong uniform PM signal for all exocyst subunits, except for GFP:EXO70G1 which kept predominantly polar PM distribution. It is noteworthy that whereas expression of EXO7084b:GFP under the native promoter led to the PM localized of fused protein, overexpression of EXO7084b:GFP provoked accumulation in the intracellular compartments (Fendrych et al., 2010). This finding confirms that overexpression of same fused proteins might lead to the mislocalization. Our results stand in contradiction to Wang's results and therefore further study is needed to clear up the discrepancy. Based on our findings I suggest that loosing polarity in protoplast evokes relocalization of EXO70s to the patches near/on the PM and to the nucleus. It is evident that cell polarization and proper exocyst localization and function are intimately linked. For example in non polarized MDCK epithelial cells exocyst is localized on TGN at the perinuclear region (Yeaman et al., 2001, 2004) whereas in polarized MDCK cells, SEC8 and SEC6 reside on the TGN and the PM of tight junction (Grindstaff et al., 1998; Yeaman et al., 2001, 2004). These findings indicate that exocyst localization might change in relation to the differentiation state of the cells.

PM localization of exocyst complex in the plant correlates with exocyst localization in animals and yeast. As described Hála et al. (2008) exocyst subunits colocalize in the tip of tobacco pollen tube and participate probably in secretion of cell wall material, fundamental process for pollen tube growth. Similar function delivering material for polar or apical growth is attributed to the exocyst in yeast and mammals. In yeast *Saccharomyces cerevisiae* exocyst localizes to the tip of growing bud (Finger et al., 1998), in neuronal cell exocyst resides in the tip of growing neurit ensuring vesicle targeting during neurit outgrowth and synaptogenesis (Vega and Hsu, 2001). This finding is further supported by Murthy et al. (2003) showing key role of exocyst subunit SEC5 in secretion of membrane proteins and material essential for neurit growth. Moreover in the plant cell Fendrych et al. (2010) have shown that exocyst participates in cytokinesis and localizes to the cell plate during initiation phase and later during post-cytokinetic cell plate maturation. This result is consistent with the findings in yeast and mammals, showing localization of exocyst to neck between daughter and mother cell in dividing *Saccharomyces cerevisiae* (Finger et al., 1998),

to the abscission site in *Saccharomyces pombe* (Wang et al., 2002) and to the midbody in dividing mammalian cell (Gromlea et al., 2005).

In addition to huge impact of exocyst on tethering vesicles to the PM in yeast, animals and plants, there are several lines of evidence pointing to exocyst function on TGN or endosomes in cargo selection or vesicle trafficking in mammals. As reported Grindstaff et al. (1998) and Yeman et al. (2001) blocking SEC6/SEC8 complex inhibits exit of cargo from the TGN. In mammalian kidney cells (COS7 cell lines) EXO70 functions in trafficking of transferrin receptor (TfnR) through interaction with endosomal localized ARF GEF BIG2 (Shin et al., 2004; Shen et al., 2006). In the root tissue we have detected GFP fused to the exocyst subunits mainly polarly or non-polarly in the PM. The significan portion of the signal manifest intracellular localization, however no structures resembling endosomes/TGN were detected. Therefore the localization of plant exocyst to the endosomes remains elusive.

The assembly of the exocyst complex in plants and Opisthokonts

We have shown that plant exocyst coordinates various processes connected to polarized secretion such as root hair growth (Synek et al., 2006), pollen tube growth and hypocotyl elongation in etiolated seedlings (Hála et al., 2008), formation of seed coat (Kulich et al., 2010) and initiation and maturation of cell plate (Fendrych et al., 2010). All of these functions involve the establishment and maintenance of the cell polarity, which is a well described role for exocyst in Opisthokonts. Our work on the composition of the plant exocyst complex revealed conserved pairwise interactions SEC3a-EXO70A1, SEC15b-SEC10 and SEC6-SEC8 known from yeast and animals, however it is possible that plant exocyst subunits might assemble in a slightly different way utilizing multiplied subunits (Hála et al., 2008). Additionally Fendrych et al. (2010) uncovered direct interaction pairs EXO84b-EXO70A1 and EXO84b-SEC15, the interactions described in animals, but not in yeast (reviewed in Munson and Novick, 2006).

All these plant studies describing the role of the plant exocyst in polarized secretion, analysed the role for EXO70A1, the closest homologue of yeast and mammalian EXO70 subunit (Eliáš et al., 2003; Synek et al., 2006). Extreme mutiplication in EXO70 family rises the question, whether other EXO70 subunits act individually or as a part of the exocyst complex. For two other EXO70 isoforms, EXO70B2 and EXO70H1, interactions with SEC5a, SEC15b and for EXO70H1 also

SEC3a were found using Y2H system. Moreover the unexpected interaction of EXO70B2 with EXO70H1 was also uncovered. On the other hand the interaction of EXO70B2 and EXO70H1with the putative core exocyst subunits SEC6 and SEC8 was not detected by Y2H system (Pečenková et al., 2011). Based on these results it is suggested that different population of exocyst complexes or subcomplexes might exist in plants (Pečenková et al., 2011). Since the Y2H system may not always reflect the true physiological interaction, the verification of these results will be matter of further experiments.

Vesicle tethering independent of exocyst function

In mammals exocyst is involved in many but not in all secretory pathways. For example fusion of synaptic vesicles to the PM is exocyst-independent process. Instead Munc13 protein is required for tethering vesicles and neurotransmitter release. Munc13 protein contains MUN domain that can bind to the syntaxin1 and stabilizes it in the open conformation that is necessary for SNARE complex formation. Interestingly detail sequence analysis uncovered similarity of Munc13 to known tethering complexes exocyst, COG, GARP and Dsl1. Munc13 originated probably from duplication of one exocyst or GARP subunit (Pei et al., 2009). Interestingly genome of *Arabidopsis thaliana* contains 5 orthologues containing MUN domain/DUF810 (At5g06970, At2g33420, At2g20010, At2g25800, At1g04470) (Pei et al., 2009). In plants till now, the only identified tethering complex acting on the PM was the exocyst. Our results suggest that exocyst participates in various exocytic events at the PM, but it is possible, that some processes might not require exocyst or might not require the whole exocyst complex. Plant MUN domain containing proteins are not experimentally characterized in plants. However transcriptomic data suggest that four of these genes are highly expressed in the whole sporophyte tissue or specifically in the mature pollen (*Arabidopsis* eFP Browser). Intensive study will be required to uncover whether the plant MUN domain containing proteins regulate tethering of vesicles to the PM alternatively to the exocyst complex also in the plant cells.

It is clear, that plant exocyst possess many characteristic common to yeast and mammalian exocyst, however many features are unique to the plant exocyst and further study is needed to shed light on regulation of exocytosis mediated by plant exocyst.

8 CONCLUSION

1) Using direct auxin transport measurement we have shown that rootward polar auxin transport is compromised in T-DNA insertional mutant in exocyst subunits EXO70A1. Using DR5:GUS reporter line we uncovered reduced auxin maxima in the root tip of *exo70A1* mutant plants. Moreover we detected canalization defect after local application of IAA in *exo70A1* mutant plants.

2) On the cellular level we have detected accumulation of small portion of PIN2:GFP in the "BFA-like" compartments, that are different from early endosomes marked by VHAa1. Moreover recycling of PIN1:GFP and PIN2:GFP to the plasma membrane is retarded in roots of *exo70A1* and *sec8-m1* mutants after washing out the brefeldin A. Even more severe secretory defect is observed after prolonged BFA treatment. This approach normally provokes transcytosis – i.e. relocalization of PINs from BFA compartment to the apical PM in the WT plants. However in exocyst mutants *exo70A1* and *sec8-m1* PIN1:GFP and PIN2:GFP remain internalized in the BFA compartments.

3) We observed that recycling of the brassinosteroid receptor BRI1 is also disturbed in similar manner as PINs recycling indicating more general PM proteins recycling defect.

4) Plasma membrane localization of studied GFP-tagged exocyst subunits (EXO70A1, SEC8, SEC10) are resistant to brefeldin A treatment, which indicates that these subunits traffic BFA-insensitive pathway. On the contrary localization of these subunits is sensitive to wortmannin – an inhibitor that modifies membrane phospholipids. These findings indicate that binding of studied exocyst subunits to the PM might depend on the phospholipide membrane composition.

5) Using co-immunoprecipitation we revealed that EXO70A1 is present in a complex with ICR1, indicating that PIN recycling might be regulated by interplay between exocyst, adaptor protein ICR1 and RHO/ROP GTPases.

SOUHRN

1) Přímým měřením auxinového transportu jsme ukázali, že transport auxinu ke špičce kořene mutanta *exo70A1* je výrazně zpomalen. S využitím reportérové linie DR5::GUS jsme odhalili zmenšené auxinové maximum v kořenové špičce mutanta *exo70A1* a navíc lokální aplikací IAA se nám podařilo odhalit kanalizační defekt v kořeni mutanta *exo70A1*.

2) Na buněčné úrovni jsme detekovali akumulaci malého množství fúzního proteinu PIN2:GFP do kompartmentů odlišných od endosomů nesoucích VHAa1. Navíc byla po působení brefeldinu A silně narušena zpětná recyklace fúzních proteinů PIN1:GFP a PIN2:GFP na plazmatickou membránu v kořenech mutantů *exo70A1* a *sec8-m1*. Ještě výraznější recyklační defekt byl patrný po prodloužení působení BFA. Tento přístup vyvolává transcytózu – tj. relokalizaci PIN proteinů z BFA kompartmentů do apikální PM v buňkách kořene WT. Avšak v případě mutantů *exo70A1* a *sec8-m1* zůstávají fúzní proteiny PIN1:GFP a PIN2:GFP internalizovány v BFA kompartmentech.

3) Zjistili jsme, že mutant *exo70A1* vykazuje defekt v recyklaci brasinosteroidového receptoru BRI1, obdobný jako v případě proteinů PIN1 a PIN2, což poukazuje na obecnější defekt při recyklaci bílkovin plasmatické membrány.

4) Lokalizace GFP značených podjednotek exocystu (EXO70A1, SEC8, SEC10) v plazmatické membráně je rezistentní k působení brefeldinu A, což indikuje, že studované podjednotky exocystu putují dráhou, která není citlivá k BFA. Naopak lokalizace těchto podjednotek je senzitivní k působení wortmanninu – inhibitoru modifikujícího membránové fosfolipidy. Tyto poznatky naznačují, že vazba podjednotek exocystu na plazmatickou membránu by mohla záviset na jejím fosfolipidovém složení.

5) Pomocí koimunoprecipitace jsme ukázali, že EXO70A1 je přítomna v komplexu s ICR1, což naznačuje, že by recyklaci PIN proteinů mohla být regulována společným působením exocystu, adaptorového proteinu ICR1 a ROP GTPáz.

9 List of Publications

Eliáš, M., Drdová, E., Žiak, D., Bavlnka, B., Hála, M., Cvrčková, F., Soukupová, H., Žárský, V. (2003) The exocyst complex in plants. Cell Biol. Int. 27: 199–201.

Hála, M., Cole, R., Synek, L., Drdová, E., Pečenková, T., Nordheim, A., Lamkemeyer, T., Madlung, J., Hochholdinger, F., Fowler, J.E., Žárský V. (2008) An exocyst complex functions in plant cell growth in *Arabidopsis* and tobacco. Plant Cell 20: 1330–1345.

Fendrych, M., Synek, L., Pečenková, T., Toupalová, H., Cole, R., Drdová, E., Nebesárová, J., Sedinová, M., Hála, M., Fowler, J.E., Žárský, V. (2010) The Arabidopsis exocyst complex is involved in cytokinesis and cell plate maturation. Plant Cell 22: 3053-3065.

Kulich, I., Cole, R., Drdová, E., Cvrčková, F., Soukup, A., Fowler, J., Žárský, V. (2010) Arabidopsis exocyst subunits SEC8 and EXO70A1 and exocyst interactor ROH1 are involved in the localized deposition of seed coat pectin. New Phytol. PMID: 20618910.

Pečenková, T., Hála, M., Kulich, I., Kocourková, D., Drdová, E., Fendrych, M., Toupalová, H., Žárský, V. (2011) The role for the exocyst complex subunits Exo70B2 and Exo70H1 in the plant-pathogen interaction. J Exp Bot.

My contribution to the publications

I prepared construct pCAT GFP Exo70G1, transformed the tobacco protoplast by this construct and recorded the localization of GFP:EXO70G1 (Eliáš et al., 2003).

In cooperation with Rex Cole I optimized the pollen germination protocol, looked for pollen tube growth defect and measured pollen tubes (Hála et al., 2008).

I prepared stable transformed *Arabidopsis* plants expressing GFP:EXO70G1 and SEC10:YFP. Using confocal microscopy I recorded localization of these exocyst subunits during formation of new cell plates. These results were not included in the final version of the manuscript (Fendrych et al., 2010).

I crossed *exo70A1* into Landsberg background. These plants retain dwarfish phenotype similar to Col-0 mutant plants, but on the contrary to Col-0 counterpart,

these mutants are not sterile and produce homozygous seeds. These seeds were further analyzed and the defect in seed coat formation was observed (Kulich et al., 2010).

I prepared double mutant *exo70A1 exo70B2* plants. Those plants were used for experiments with pathogens, but the results were not included in the final version of the manuscript (Pečenková et al., 2011).

Contribution of coautors to the manuscript

Lukáš Synek prepared *exo70A1* carrying DR5::GUS reporter, prepared stable transformed *Arabidopsis* overexpressing GFP:EXO70A1, performed complementation of *exo70A1* plants by GFP:EXO70A1 construct, described the waving phenotype in *exo70A1*, and measured the gravitropic response during seedling germination. Lukáš Synek and Edita Janková Drdová prepared mouse anti-Sec6 and anti-Exo70A1 antibody.

Tamara Pečenková prepared *exo70A1* plants carrying VHAa1:GFP reporter.

Michal Hála and Edita Janková Drdová performed membrane separation in Percoll gradient.

Ivan Kulich described the skewing phenotype in *exo70A1* mutant.

Angus Murphy repeated my measurement of auxin transport and in addition he included inhibitors (gravacin, 1-NOA) in his experiment.

Marek Eliáš prepared binary construct for expression SEC10:YFP.

Matyáš Fendrych, Steffen Vanneste, Jiří Friml, John E. Fowler, Markus Geisler contributed to the writing of the manuscript.

Viktor Žárský and Edita Janková Drdová designed the experiments and wrote the manuscript.

Edita Janková Drdová performed all the other experiments:

- measurement of auxin (*exo70A1-1*, *exo70A1-2*, complemented plants),
- monitoring DR5:GUS activity after local application of IAA,
- crossing *exo70A1-2* with PIN1GFP, PIN2:GFP, BRI1:GFP, ICR1:GFP expressing plants and crossing GFP:EXO70A1 expressing plants with *icr1* mutant
- drugs and dye treatments (BFA, wortmannin, FM4-64), starch staining
- co-imunoprecipitation (PIN1:GFP, PIN2:GFP, BRI1:GFP, ICR1:GFP)
- preparing SEC10:YFP expressing plants.
- measurement of gravitropic response
- all the microscopic inspection of plants.

10 References

Alassimone J, Naseer S, Geldner N: A developmental framework for endodermal differentiation and polarity. Proceedings of the National Academy of Sciences 2010, 107:5214 -5219.

Baek K, Knödler A, Lee SH, Zhang X, Orlando K, Zhang J, Foskett TJ, Guo W, Dominguez R: Structure-function study of the N-terminal domain of exocyst subunit Sec3. Journal of Biological Chemistry 2010, 285:10424-10433.

Bao Y, Lopez JA, James DE, Hunziker W: Snapin interacts with the Exo70 subunit of the exocyst and modulates GLUT4 trafficking. Journal of Biological Chemistry 2008, 283:324-331.

Bassham DC, Blatt MR: SNAREs: cogs and coordinators in signaling and development. Plant Physiology 2008, 147:1504-1515.

Behnia R, Munro S: Organelle identity and the signposts for membrane traffic. Nature 2005, 438:597–604.

Bendezu FO, Martin SG: Actin cables and the exocyst form two independent morphogenesis pathways in the fission yeast. Molecular Biology of the Cell 2011, 22:44-53.

Benjamins R, Scheres B: Auxin: the looping star in plant development. Annu. Rev. Plant Biol. 2008, 59:443–465.

Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J acute accent]: Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation. Cell 2003, 115:591-602.

Blakeslee JJ, Bandyopadhyay A, Lee OR, Mravec J, Titapiwatanakun B, Sauer M, Makam SN, Cheng Y, Bouchard R, Adamec J, Geisler M, Nagashima A, Sakai T, Martinoia E, Friml J, Peer WA, Murphy AS: Interactions among PIN-FORMED and P-Glycoprotein Auxin Transporters in Arabidopsis. The Plant Cell Online 2007, 19:131 -147.

Bonifacino JS, Hierro A: Transport according to GARP: receiving retrograde cargo at the trans-Golgi network. Trends in Cell Biology 2011, 21:159-167.

Boyd C, Hughes T, Pypaert M, Novick P: Vesicles carry most exocyst subunits to exocytic sites marked by the remaining two subunits, Sec3p and Exo70p. The Journal of cell biology 2004, 167:889-901.

Brembu T, Winge P, Bones AM, Yang Z: A RHOse by any other name: a comparative analysis of animal and plant Rho GTPases. Cell Research 2006, 16:435–445.

Brennwald P, Rossi G: Spatial regulation of exocytosis and cell polarity: Yeast as a model for animal cells. FEBS Letters 2007, 581:2119-2124.

Bröcker C, Engelbrecht-Vandré S, Ungermann C: Multisubunit Tethering Complexes and Their Role in Membrane Fusion. Current Biology 2010, 20:R943–R952.

Bryant DM, Datta A, Rodriguez-Fraticelli AE, Peranen J, Martin-Belmonte F, Mostov KE: A molecular network for de novo generation of the apical surface and lumen. Nat Cell Biol 2010, 12:1035-1045.

Casimiro I, Marchant A, Bhalerao RP, Beeckman T, Dhooge S, Swarup R, Graham N, Inze D, Sandberg G, Casero PJ, Bennett M: Auxin transport promotes Arabidopsis lateral root initiation. The Plant Cell Online 2001, 13:843-852.

Chong YT, Gidda SK, Sanford C, Parkinson J, Mullen RT, Goring DR: Characterization of the Arabidopsis thaliana exocyst complex gene families by phylogenetic, expression profiling, and subcellular localization studies. New Phytologist 2010, 185:401–419.

Cole RA, Synek L, Zarsky V, Fowler JE: SEC8, a subunit of the putative Arabidopsis exocyst complex, facilitates pollen germination and competitive pollen tube growth. Plant Physiology 2005, 138:34-45.

Cvrckova F, Elias M, Hala M, Obermeyer G, Zarsky V: Small GTPases and conserved signalling pathways in plant cell morphogenesis: From exocytosis to Exocyst. Cell Biology of Plant and Fungal Tip Growth 2001:105–122.

Dhonukshe P, Aniento F, Hwang I, Robinson DG, Mravec J, Stierhof YD, Friml J: Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in Arabidopsis. Current biology 2007, 17:520–527.

Dhonukshe P, Huang F, Galvan-Ampudia CS, M\äh\önen AP, Kleine-Vehn J, Xu J, Quint A, Prasad K, Friml J, Scheres B, Offringa R: Plasma membrane-bound AGC3 kinases phosphorylate PIN auxin carriers at TPRXS (N/S) motifs to direct apical PIN recycling. Development 2010, 137:3245-3255.

Dhonukshe P, Tanaka H, Goh T, Ebine K, Mahonen P, Prasad K, Blilou I, Geldner N, Xu J, Uemura T, Chory J, Ueda T, Nakano A, Scheres B, Friml J: Generation of cell polarity in plants links endocytosis, auxin distribution and cell fate decisions. Nature 2008, 456:962–966.

Dhonukshe P: Cell polarity in plants. Commun Integr Biol 2009, 2:184-190.

Dong G, Hutagalung AH, Fu C, Novick P, Reinisch KM: The structures of exocyst subunit Exo70p and the Exo84p C-terminal domains reveal a common motif. Nature structural & molecular biology 2005, 12:1094–1100.

Driouich A, Durand C, Vicré-Gibouin M: Formation and separation of root border cells. Trends in Plant Science 2007, 12:14-19.

Durand C, Vicré-Gibouin M, Follet-Gueye ML, Duponchel L, Moreau M, Lerouge P, Driouich A: The Organization Pattern of Root Border-Like Cells of Arabidopsis Is Dependent on Cell Wall Homogalacturonan. Plant Physiology 2009, 150:1411 -1421.

Eliáš M, Drdová E, Žiak D, Bavlnka B, Hála M, Cvrčková F, Soukupová H, Žárskỳ V: The exocyst complex in plants. Cell Biology International 2003, 27:199–201.

Fendrych M, Synek L, Pečenková T, Toupalová H, Cole R, Drdová E, Nebesářová J, Šedinová M, Hála M, Fowler JE, Žárský V: The Arabidopsis Exocyst Complex Is Involved in Cytokinesis and Cell Plate Maturation. The Plant Cell Online 2010, 22:3053 -3065.

Fielding AB, Schonteich E, Matheson J, Wilson G, Yu X, Hickson GRX, Srivastava S, Baldwin SA, Prekeris R, Gould GW: Rab11-FIP3 and FIP4 interact with Arf6 and the exocyst to control membrane traffic in cytokinesis. The EMBO journal 2005, 24:3389–3399.

Finger FP, Hughes TE, Novick P: Sec3p Is a Spatial Landmark for Polarized Secretion in Budding Yeast. Cell 1998, 92:559-571.

Finger FP, Novick P: Sec3p is involved in secretion and morphogenesis in Saccharomyces cerevisiae. Mol Biol Cell 1997, 8:647-662.

Fölsch H, Pypaert M, Maday S, Pelletier L, Mellman I: The AP-1A and AP-1B clathrin adaptor complexes define biochemically and functionally distinct membrane domains. The Journal of Cell Biology 2003, 163:351 -362.

Friml J, Yang X, Michniewicz M, Weijers D, Quint A, Tietz O, Benjamins R, Ouwerkerk PBF, Ljung K, Sandberg G, Hooykaas PJJ, Palme K, Offringa R: A PINOID-Dependent Binary Switch in Apical-Basal PIN Polar Targeting Directs Auxin Efflux. Science 2004, 306:862 -865.

Fukai S, Matern HT, Jagath JR, Scheller RH, Brunger AT: Structural basis of the interaction between RalA and Sec5, a subunit of the sec6/8 complex. The EMBO Journal 2003, 22:3267–3278.

Furutani M, Kajiwara T, Kato T, Treml BS, Stockum C, Torres-Ruiz RA, Tasaka M: The gene MACCHI-BOU 4/ENHANCER OF PINOID encodes a NPH3-like protein and reveals similarities between organogenesis and phototropism at the molecular level. Development 2007, 134:3849 -3859.

Gao X, Nagawa S, Wang G, Yang Z: Cell polarity signaling: focus on polar auxin transport. Mol Plant. 2008, 6:899-909.

Gälweiler L, Guan C, Müller A, Wisman E, Mendgen K, Yephremov A, Palme K: Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. Science 1998, 282:2226-2230.

Geisler M, Blakeslee JJ, Bouchard R, Lee OR, Vincenzetti V, Bandyopadhyay A, Titapiwatanakun B, Peer WA, Bailly A, Richards EL, Ejendal KFK, Smith AP,

Baroux C, Grossniklaus U, Müller A, Hrycyna CA, Dudler R, Murphy AS, Martinoia E: Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AtPGP1. The Plant Journal 2005, 44:179-194.

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jürgens G: The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell 2003, 112:219–230.

Geldner N, Friml J, Stierhof YD, Jurgens G, Palme K: Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature 2001, 413:425–427.

Geldner N, Richter S, Vieten A, Marquardt S, Torres-Ruiz RA, Mayer U, Jürgens G: Partial loss-of-function alleles reveal a role for GNOM in auxin transport-related, post-embryonic development of Arabidopsis. Development 2004, 131:389 -400.

Gerges NZ, Backos DS, Rupasinghe CN, Spaller MR, Esteban JA: Dual role of the exocyst in AMPA receptor targeting and insertion into the postsynaptic membrane. The EMBO journal 2006, 25:1623–1634.

Grindstaff KK, Yeaman C, Anandasabapathy N, Hsu S-C, Rodriguez-Boulan E, Scheller RH, Nelson WJ: Sec6/8 Complex Is Recruited to Cell-Cell Contacts and Specifies Transport Vesicle Delivery to the Basal-Lateral Membrane in Epithelial Cells. Cell 1998, 93:731-740.

Gromley A, Yeaman C, Rosa J, Redick S, Chen C-T, Mirabelle S, Guha M, Sillibourne J, Doxsey SJ: Centriolin Anchoring of Exocyst and SNARE Complexes at the Midbody Is Required for Secretory-Vesicle-Mediated Abscission. Cell 2005, 123:75-87.

Grosshans BL, Andreeva A, Gangar A, Niessen S, Yates JR, Brennwald P, Novick P: The yeast lgl family member Sro7p is an effector of the secretory Rab GTPase Sec4p. The Journal of cell biology 2006, 172:55-66.

Grosshans BL, Ortiz D, Novick P: Rabs and their effectors: Achieving specificity in membrane traffic. Proceedings of the National Academy of Sciences 2006, 103:11821 -11827.

Guo W, Grant A, Novick P: Exo84p is an exocyst protein essential for secretion. Journal of Biological Chemistry 1999, 274:23558-23564.

Guo W, Roth D, Walch-Solimena C, Novick P: The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. The EMBO journal 1999, 18:1071–1080.

Guo W, Tamanoi F, Novick P: Spatial regulation of the exocyst complex by Rho1 GTPase. Nature cell biology 2001, 3:353–360.

Hála M, Cole R, Synek L, Drdová E, Pecenková T, Nordheim A, Lamkemeyer T, Madlung J, Hochholdinger F, Fowler JE, Zársky V: An Exocyst Complex Functions

in Plant Cell Growth in Arabidopsis and Tobacco. The Plant Cell Online 2008, 20:1330 -1345.

Hamburger ZA, Hamburger AE, West Jr AP, Weis WI: Crystal structure of the S. cerevisiae exocyst component Exo70p. Journal of molecular biology 2006, 356:9–21.

Hardtke CS, Dorcey E, Osmont KS, Sibout R: Phytohormone collaboration: zooming in on auxin-brassinosteroid interactions. Trends in Cell Biology 2007, 17:485-492.

Hazak O, Bloch D, Poraty L, Sternberg H, Zhang J, Friml J, Yalovsky S: A rho scaffold integrates the secretory system with feedback mechanisms in regulation of auxin distribution. PLoS biology 2010, 8:e1000282.

Hazuka CD, Foletti DL, Hsu SC, Kee Y, Hopf FW, Scheller RH: The sec6/8 complex is located at neurite outgrowth and axonal synapse-assembly domains. J. Neurosci 1999, 19:1324-1334.

He B, Xi F, Zhang X, Zhang J, Guo W: Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. The EMBO Journal 2007, 26:4053–4065.

Hertzog M, Chavrier P: Cell polarity during motile processes: keeping on track with the exocyst complex. Biochem. J 2011, 433:403–409.

Hobbie L, Estelle M: The axr4 auxin-resistant mutants of Arabidopsis thaliana define root initiation. The Plant Journal 1995, 7:211–220.

Inoue M, Chang L, Hwang J, Chiang SH, Saltiel AR: The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. Nature 2003, 422:629–633.

Inoue M, Chiang SH, Chang L, Chen XW, Saltiel AR: Compartmentalization of the exocyst complex in lipid rafts controls Glut4 vesicle tethering. Molecular biology of the cell 2006, 17:2303-2311.

Jaillais Y, Fobis-Loisy I, Miège C, Gaude T: Evidence for a sorting endosome in Arabidopsis root cells. The Plant Journal 2008, 53:237–247.

Jaillais Y, Gaude T: Sorting Out the Sorting Functions of Endosomes in Arabidopsis. Plant Signal Behav 2007, 2:556-558.

Jaillais Y, Santambrogio M, Rozier F, Fobis-Loisy I, Miège C, Gaude T: The retromer protein VPS29 links cell polarity and organ initiation in plants. Cell 2007, 130:1057–1070.

Jin R, Junutula JR, Matern HT, Ervin KE, Scheller RH, Brunger AT: Exo84 and Sec5 are competitive regulatory Sec6/8 effectors to the RalA GTPase. The EMBO Journal 2005, 24:2064–2074.

Jordens I, Molle D, Xiong W, Keller SR, McGraw TE: Insulin-regulated Aminopeptidase Is a Key Regulator of GLUT4 Trafficking by Controlling the Sorting

of GLUT4 from Endosomes to Specialized Insulin-regulated Vesicles. Mol. Biol. Cell 2010, 21:2034-2044.

Kleine-Vehn J, Dhonukshe P, Sauer M, Brewer PB, Wisniewska J, Paciorek T, Benkova E, Friml J: ARF GEF-dependent transcytosis and polar delivery of PIN auxin carriers in Arabidopsis. Current biology 2008, 18:526–531.

Kleine-Vehn J, Huang F, Naramoto S, Zhang J, Michniewicz M, Offringa R, Friml J: PIN Auxin Efflux Carrier Polarity Is Regulated by PINOID Kinase-Mediated Recruitment into GNOM-Independent Trafficking in Arabidopsis. The Plant Cell Online 2009, 21:3839 -3849.

Kleine-Vehn J, Leitner J, Zwiewka M, Sauer M, Abas L, Luschnig C, Friml J: Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. Proceedings of the National Academy of Sciences 2008, 105:17812 -17817.

Koumandou VL, Dacks JB, Coulson RMR, Field MC: Control systems for membrane fusion in the ancestral eukaryote; evolution of tethering complexes and SM proteins. BMC evolutionary biology 2007, 7:29.

Langevin J, Morgan MJ, Rossé C, Racine V, Sibarita JB, Aresta S, Murthy M, Schwarz T, Camonis J, Bella\ïche Y: Drosophila exocyst components Sec5, Sec6, and Sec15 regulate DE-Cadherin trafficking from recycling endosomes to the plasma membrane. Developmental cell 2005, 9:365–376.

Lavy M, Bloch D, Hazak O, Gutman I, Poraty L, Sorek N, Sternberg H, Yalovsky S: A novel ROP/RAC effector links cell polarity, root-meristem maintenance, and vesicle trafficking. Current biology 2007, 17:947–952.

Laxmi A, Pan J, Morsy M, Chen R: Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in Arabidopsis thaliana. PLoS One 2008, 3:e1510.

Leyser HMO, Pickett FB, Dharmasiri S, Estelle M: Mutations in the AXR3 gene of Arabidopsis result in altered auxin response including ectopic expression from the SAUR-AC1 promoter. Plant Journal 1996, 10:403–414.

Li S, van Os G, Ren S, Yu D, Ketelaar T, Emons AMC, Liu CM: Expression and functional analyses of EXO70 genes in Arabidopsis implicate their roles in regulating cell type-specific exocytosis. Plant physiology 2010, 154:1819-1830.

Liu J, Zuo X, Yue P, Guo W: Phosphatidylinositol 4, 5-bisphosphate mediates the targeting of the exocyst to the plasma membrane for exocytosis in mammalian cells. Molecular biology of the cell 2007, 18:4483-4492.

Mayer U, Buttner G, Jurgens G: Apical-basal pattern formation in the Arabidopsis embryo: studies on the role of the gnom gene. Development 1993, 117:149 -162.

McLaughlin S, Wang J, Gambhir A,Murray D: PIP2 AND PROTEINS: Interactions, Organization, and Information Flow. Annu. Rev. Biophys. Biomol. Struct. 2002, 31: 151-175

Medkova M, France YE, Coleman J, Novick P: The rab exchange factor Sec2p reversibly associates with the exocyst. Molecular biology of the cell 2006, 17:2757.

Michniewicz M, Zago MK, Abas L, Weijers D, Schweighofer A, Meskiene I, Heisler MG, Ohno C, Zhang J, Huang F, Schwab R: Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. Cell 2007, 130:1044–1056.

Migliaccio F, Piconese S: Spiralizations and tropisms in Arabidopsis roots. Trends in Plant Science 2001, 6:561-565.

Molendijk AJ, Ruperti B, Palme K: Small GTPases in vesicle trafficking. Current opinion in plant biology 2004, 7:694–700.

Moore BA, Robinson HH, Xu Z: The crystal structure of mouse Exo70 reveals unique features of the mammalian exocyst. Journal of molecular biology 2007, 371:410–421.

Mucha E, Fricke I, Schaefer A, Wittinghofer A, Berken A: Rho proteins of plants - Functional cycle and regulation of cytoskeletal dynamics. J Cell Biol. 2011,

Müller A, Guan C, Gälweiler L, Tänzler P, Huijser P, Marchant A, Parry G, Bennett M, Wisman E, Palme K: AtPIN2 defines a locus of Arabidopsis for root gravitropism control. The EMBO Journal 1998, 17:6903–6911.

Munson M, Novick P: The exocyst defrocked, a framework of rods revealed. Nature structural & molecular biology 2006, 13:577–581.

Murthy M, Garza D, Scheller RH, Schwarz TL: Mutations in the Exocyst Component Sec5 Disrupt Neuronal Membrane Traffic, but Neurotransmitter Release Persists. Neuron 2003, 37:433-447.

Naramoto S, Kleine-Vehn J, Robert S, Fujimoto M, Dainobu T, Paciorek T, Ueda T, Nakano A, Van Montagu MCE, Fukuda H, Friml J: ADP-ribosylation factor machinery mediates endocytosis in plant cells. Proceedings of the National Academy of Sciences 2010, 107:21890 -21895.

Niemes S, Langhans M, Viotti C, Scheuring D, San Wan Yan M, Jiang L, Hillmer S, Robinson DG, Pimpl P: Retromer recycles vacuolar sorting receptors from the trans-Golgi network. The Plant Journal 2010, 61:107–121.

Noh B, Bandyopadhyay A, Peer WA, Spalding EP, Murphy AS: Enhanced gravi- and phototropism in plant mdr mutants mislocalizing the auxin efflux protein PIN1. Nature 2003, 423:999-1002.

Novick P, Field C, Schekman R: Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 1980, 21:205-215.

Oztan A, Silvis M, Weisz OA, Bradbury NA, Hsu SC, Goldenring JR, Yeaman C, Apodaca G: Exocyst requirement for endocytic traffic directed toward the apical and basolateral poles of polarized MDCK cells. Molecular biology of the cell 2007, 18:3978-3992.

Paciorek T, Zazımalová E, Ruthardt N, Petrásek J, Stierhof YD, Kleine-Vehn J, Morris DA, Emans N: Auxin inhibits endocytosis and promotes its own efflux from cells. Nature , 435:1251–1256.

Pečenková T, Hála M, Kulich I, Kocourková D, Drdová E, Fendrych M, Toupalová H, Žárský V: The role for the exocyst complex subunits Exo70B2 and Exo70H1 in the plant–pathogen interaction. Journal of Experimental Botany 2011, 62:2107 -2116.

Pei J, Ma C, Rizo J, Grishin NV: Remote Homology between Munc13 MUN Domain and Vesicle Tethering Complexes. Journal of Molecular Biology 2009, 391:509-517.

Perera IY, Davis AJ, Galanopoulou D, Im YJ, Boss WF: Characterization and comparative analysis of Arabidopsis phosphatidylinositol phosphate 5-kinase 10 reveals differences in Arabidopsis and human phosphatidylinositol phosphate kinases. FEBS letters 2005, 579:3427–3432.

Pitts RJ, Cernac A, Estelle M: Auxin and ethylene promote root hair elongation inArabidopsis. The Plant Journal 1998, 16:553–560.

Prigent M, Dubois T, Raposo G, Derrien V, Tenza D, Rossé C, Camonis J, Chavrier P: ARF6 controls post-endocytic recycling through its downstream exocyst complex effector. The Journal of cell biology 2003, 163:1111-1121.

Robert S, Chary SN, Drakakaki G, Li S, Yang Z, Raikhel NV, Hicks GR: Endosidin1 defines a compartment involved in endocytosis of the brassinosteroid receptor BRI1 and the auxin transporters PIN2 and AUX1. Proceedings of the National Academy of Sciences 2008, 105:8464 -8469.

Robinson DG, Jiang L, Schumacher K: The endosomal system of plants: charting new and familiar territories. Plant Physiology 2008, 147:1482.

Robinson NGG, Guo L, Imai J, Toh-e A, Matsui Y, Tamanoi F: Rho3 of Saccharomyces cerevisiae, which regulates the actin cytoskeleton and exocytosis, is a GTPase which interacts with Myo2 and Exo70. Molecular and cellular biology 1999, 19:3580-3587.

Roumanie O, Wu H, Molk JN, Rossi G, Bloom K, Brennwald P: Rho GTPase regulation of exocytosis in yeast is independent of GTP hydrolysis and polarization of the exocyst complex. The Journal of cell biology 2005, 170:583-594.

Saito T, Shibasaki T, Seino S: Involvement of Exoc3l, a protein structurally related to the exocyst subunit Sec6, in insulin secretion. Biomedical Research 2008, 29:85–91.

Shen X, Xu KF, Fan Q, Pacheco-Rodriguez G, Moss J, Vaughan M: Association of brefeldin A-inhibited guanine nucleotide-exchange protein 2 (BIG2) with recycling endosomes during transferrin uptake. Proceedings of the National Academy of Sciences of the United States of America 2006, 103:2635-2640.

Sivaram MVS, Furgason MLM, Brewer DN, Munson M: The structure of the exocyst subunit Sec6p defines a conserved architecture with diverse roles. Nature structural & molecular biology 2006, 13:555–556.

Spitzer C, Reyes FC, Buono R, Sliwinski MK, Haas TJ, Otegui MS: The ESCRT-related CHMP1A and B proteins mediate multivesicular body sorting of auxin carriers in Arabidopsis and are required for plant development. The Plant Cell Online 2009, 21:749-766.

Steinmann T, Geldner N, Grebe M, Mangold S, Jackson CL, Paris S, Gälweiler L, Palme K, Jürgens G: Coordinated Polar Localization of Auxin Efflux Carrier PIN1 by GNOM ARF GEF. Science 1999, 286:316 -318.

Südhof TC, Rothman JE: Membrane fusion: grappling with SNARE and SM proteins. Science 2009, 323:474-477.

Synek L, Schlager N, Eliáš M, Quentin M, Hauser MT, Žárskỳ V: AtEXO70A1, a member of a family of putative exocyst subunits specifically expanded in land plants, is important for polar growth and plant development. The Plant Journal 2006, 48:54–72.

Tanaka H, Kitakura S, De Rycke R, De Groodt R, Friml J: Fluorescence Imaging-Based Screen Identifies ARF GEF Component of Early Endosomal Trafficking. Current Biology 2009, 19:391-397.

Teh O, Moore I: An ARF-GEF acting at the Golgi and in selective endocytosis in polarized plant cells. Nature 2007, 448:493-496.

TerBush DR, Maurice T, Roth D, Novick P: The Exocyst is a multiprotein complex required for exocytosis in Saccharomyces cerevisiae. The EMBO journal 1996, 15:6483.

Thole JM, Nielsen E: Phosphoinositides in plants: novel functions in membrane trafficking. Current opinion in plant biology 2008, 11:620–631.

van Leeuwen W, Okrész L, Bogre L, Munnik T: Learning the lipid language of plant signalling. Trends in plant science 2004, 9:378–384.

Vanneste S, Friml J: Auxin: a trigger for change in plant development. Cell 2009, 136:1005–1016.

Vega IE, Hsu S-C: The Exocyst Complex Associates with Microtubules to Mediate Vesicle Targeting and Neurite Outgrowth. The Journal of Neuroscience 2001, 21:3839 -3848.

Vernoud V, Horton AC, Yang Z, Nielsen E: Analysis of the Small GTPase Gene Superfamily of Arabidopsis. Plant Physiology 2003, 131:1191 -1208.

Wang H, Tang X, Balasubramanian MK: Rho3p Regulates Cell Separation by Modulating Exocyst Function in Schizosaccharomyces pombe. Genetics 2003, 164:1323 -1331.

Wang H, Tang X, Liu J, Trautmann S, Balasundaram D, McCollum D, Balasubramanian MK: The Multiprotein Exocyst Complex Is Essential for Cell Separation in Schizosaccharomyces pombe. Mol. Biol. Cell 2002, 13:515-529.

Wang J, Ding Y, Wang J, Hillmer S, Miao Y, Lo SW, Wang X, Robinson DG, Jiang L: EXPO, an Exocyst-Positive Organelle Distinct from Multivesicular Endosomes and Autophagosomes, Mediates Cytosol to Cell Wall Exocytosis in Arabidopsis and Tobacco Cells. The Plant Cell Online 2010, 22:4009 -4030.

Wiederkehr A, De Craene J-O, Ferro-Novick S, Novick P: Functional specialization within a vesicle tethering complex. The Journal of Cell Biology 2004, 167:875 -887.

Willemsen V, Friml J, Grebe M, van den Toorn A, Palme K, Scheres B: Cell Polarity and PIN Protein Positioning in Arabidopsis Require STEROL METHYLTRANSFERASE1 Function. The Plant Cell Online 2003, 15:612 -625.

Wiśniewska J, Xu J, Seifertová D, Brewer PB, Růžička K, Blilou I, Rouquié D, Benková E, Scheres B, Friml J: Polar PIN Localization Directs Auxin Flow in Plants. Science 2006, 312:883.

Woollard AAD, Moore I: The functions of Rab GTPases in plant membrane traffic. Curr. Opin. Plant Biol 2008, 11:610-619.

Wu H, Rossi G, Brennwald P: The ghost in the machine: small GTPases as spatial regulators of exocytosis. Trends in Cell Biology 2008, 18:397-404.

Wu H, Turner C, Gardner J, Temple B, Brennwald P: The Exo70 subunit of the exocyst is an effector for both Cdc42 and Rho3 function in polarized exocytosis. Molecular biology of the cell 2010, 21:430-442.

Wu S, Mehta SQ, Pichaud F, Bellen HJ, Quiocho FA: Sec15 interacts with Rab11 via a novel domain and affects Rab11 localization in vivo. Nature structural & molecular biology 2005, 12:879–885.

Xu K-F, Shen X, Li H, Pacheco-Rodriguez G, Moss J, Vaughan M: Interaction of BIG2, a brefeldin A-inhibited guanine nucleotide-exchange protein, with exocyst protein Exo70. Proceedings of the National Academy of Sciences of the United States of America 2005, 102:2784 -2789.

Xu T, Wen M, Nagawa S, Fu Y, Chen JG, Wu MJ, Perrot-Rechenmann C, Friml J, Jones AM, Yang Z: Cell surface-and Rho GTPase-based auxin signaling controls cellular interdigitation in Arabidopsis. Cell 2010, 143:99–110.

Yamashita M, Kurokawa K, Sato Y, Yamagata A, Mimura H, Yoshikawa A, Sato K, Nakano A, Fukai S: Structural basis for the Rho-and phosphoinositide-dependent localization of the exocyst subunit Sec3. Nature structural & molecular biology 2010, 17:180–186.

Yang Z, Fu Y: ROP/Rac GTPase signaling. Current opinion in plant biology 2007, 10:490–494.

Yeaman C, Grindstaff KK, Wright JR, Nelson WJ: Sec6/8 complexes on trans-Golgi network and plasma membrane regulate late stages of exocytosis in mammalian cells. The Journal of cell biology 2001, 155:593-604.

Žárskỳ V, Cvrčková F, Potockỳ M, Hála M: Exocytosis and cell polarity in plants–exocyst and recycling domains. New Phytol 2009, 183:255–272.

Zhang J, Nodzyński T, Pěnčík A, Rolčík J, Friml J: PIN phosphorylation is sufficient to mediate PIN polarity and direct auxin transport. Proceedings of the National Academy of Sciences 2010, 107:918-922.

Zhang X, Bi E, Novick P, Du L, Kozminski KG, Lipschutz JH, Guo W: Cdc42 Interacts with the Exocyst and Regulates Polarized Secretion. Journal of Biological Chemistry 2001, 276:46745 -46750.

Zhang X, Orlando K, He B, Xi F, Zhang J, Zajac A, Guo W: Membrane association and functional regulation of Sec3 by phospholipids and Cdc42. The Journal of Cell Biology 2008, 180:145-158.

Zhang X, Zajac A, Zhang J, Wang P, Li M, Murray J, TerBush D, Guo W: The critical role of Exo84p in the organization and polarized localization of the exocyst complex. Journal of Biological Chemistry 2005, 280:20356-20364.

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W: GENEVESTIGATOR. Arabidopsis Microarray Database and Analysis Toolbox. Plant Physiology 2004, 136:2621 -2632.