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PhD Thesis

Exocyst subunit AtSEC15b: its role in plant cell morphogenesis and characterization of its Rab interacting partner

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Michal Hála (Institute of Experimental Botany): prepared pBAR::AtRABA4a construct and recombinant protein AtRABA4a for mouse immunization (Chapters 4.5; 4.6).

Lukáš Synek, Martina Růžičková (Institute of Experimental Botany): participates in *in vitro* pollen germination experiment (Chapter 4.7.2).

Matyáš Fendrych (Institute of Experimental Botany): prepared transgenic plants expressing *AtEXO84b:GFP* (Chapter 4.3.3).

Declaration:

I hereby declare that I elaborated this thesis independently. All collaborators mentioned above were informed about the presentation of their results in this thesis and all the information sources and literature was mentioned. I also declare that this thesis has not been submitted in any form for another degree or diploma to any other university.

Prague, May 2011

Hana Toupalová

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Summary

Organization of endomembrane compartments in all eukaryotic cells is dependent on continuous transport of membrane vesicles. It is generally accepted that major part of the core regulators of intracellular membrane transport is represented by small GTPases from the Rab family. Rab GTPases cycle between the GTP-bound “active” and GDP-bound “inactive” forms. In their active form, they are able to interact with specific effectors and perform their functions. Exocyst is an octameric complex involved in regulation of secretion. It functions as an effector of Rab GTPases in yeast and mammals and tethers secretory vesicles to the plasma membrane prior to the actual membrane fusion. In yeast, Sec4 protein was identified to be an interacting partner of Sec15 subunit of the exocyst (Guo et al., 1999b). In mammals, Rab 11 GTPase was characterized as a protein interacting with Sec15 exocyst subunit (Zhang et al., 2004). In both, this interaction depends on the active form of Rab GTPase.

Using publicly available expression data, we have identified candidates from Rab GTPase family for the interaction with exocyst subunit AtSEC15b in plants and demonstrated that AtSEC15b specifically interacts with AtRABA4a GTPase, protein homologous to mammalian Rab11 GTPase. We also showed that, like in yeast and mammals, *Arabidopsis* Sec15b binds Rab GTPase also probably in GTP-dependent manner, implying that this interaction is well conserved throughout the eukaryotic kingdoms. This binding experiment was further confirmed by co-immunoprecipitation analysis.

We also successfully demonstrated the complementation of yeast thermo-sensitive mutant strain, *sec15-1*. We identified that expression of *AtSEC15b* in *sec15-1* fully restores the growing defect of this mutant strain under the restricitive temperature. Based on this observation we concluded that AtSEC15b is able to substitute the function of yeast SEC15 and restore the phenotype.

Co-localization experiment revealed the presence of endosome-like structure labeled by AtRABA4a protein. Our preliminary data hypothesized that it might define some novel compartment on secretory pathway, which is different from Golgi, PVC and recycling endosomes labeled by AtRABA5d.

Functional characterization of T-DNA insertional mutants in *AtSEC15b* gene suggested that AtSEC15b is essential for proper growth and development of *Arabidopsis thaliana*. Depletion of AtSEC15b leads to the generation of sterile dwarfed plants, with reduced apical dominance. Heterozygous plants are defective in transmission of the mutant allele through the male, which is accompanied by reduced growth of mutant pollen tubes. And additionally, heterozygous plants are also partially defective in producing of vital embryos.

Souhrn

Organizace vnitrobuněčných membránových kompartmentů ve všech eukaryotních buňkách závisí na nepřetržitém transportu membránových váčků. Je všeobecně známo, že velkou část klíčových regulátorů vnitrobuněčného membránového transportu představují malé GTPázy z rodiny Rab. Rab GTPázy cyklují mezi GTP-vazebnou „aktivní“ a GDP-vazebnou „neaktivní“ formou. Ve své aktivní formě mohou interagovat se specifickými efektory a vykonávat tak svou funkci. Exocyst je osmipodjednotkový komplex, který se podílí na regulaci sekrece. Funguje jako efektor Rab GTPáz u kvasinek a savců, kde poutá sekreční váčky k plazmatické membráně před samotným splynutím membrán. V kvasinkách byl jako interakční partner Sec15 podjednotky exocystu identifikován protein Sec4 (Guo et al., 1999b). U savců byla popsána GTPáza Rab11 jako protein interagující s podjednotkou Sec15 (Zhang et al., 2004). V obou případech tato interakce závisí na aktivní formě Rab GTPázy.

S pomocí veřejně dostupných expresních dat jsme nalezli několik kandidátů z rodiny Rab GTPáz, kteří by mohli interagovat s podjednotkou exocystu AtSEC15b u rostlin a dokázali jsme, že AtSEC15b specificky interaguje s AtRABA4a GTPázou, což je protein homologní k savčí GTPáze Rab11. Dále jsme ukázali, že stejně jako u kvasinek a savců tato interakci pravděpodobně probíhá v závislosti na GTP-vazebné formě Rab GTPázy, což naznačuje, že je tato interakce zachována v rámci všech Eukaryot. Tento vazebný experiment byl dále potvrzen koimunoprecipitační analýzou.

Dále jsme úspěšně předvedli komplementaci teplotně-senzitivního kvasinkového mutanta sec15-1. Zjistili jsme, že exprese *AtSEC15b* v těchto mutantních kvasinkách plně opraví růstový defekt mutantního kmene v restriktivní teplotě. Na základě tohoto pozorování jsme dospěli k závěru, že protein AtSEC15b je schopen funkčně nahradit kvasinkový protein Sec15 a tím obnovit normální fenotyp.

Kolokalizační analýza odhalila přítomnost endosomům podobného útvaru, značeného proteinem AtRABA4a. Předběžná data naznačují, že by se mohlo jednat o nějaký nový kompartment v sekreční dráze, který je odlišný od Golgiho aparátu, prevakuolárního kompartmentu i recyklačních endosomů značených proteinem AtRABA5d.

Funkční charakterizace T-DNA inzerčních mutantů v genu pro *AtSEC15b* předpokládá, že AtSEC15b protein je nezbytný pro správný růst a vývoj *Arabidopsis thaliana*. Ztráta AtSEC15b vede ke vzniku rostlin, které jsou sterilní, vykazují zakrslý růst a mají sníženou apikální dominanci. Heterozygotní rostliny jsou defektní v přenosu mutantní alely pylem, což je také doprovázeno sníženým růstem mutantních pylových láček. A navíc, heterozygotní rostliny jsou částečně defektní v produkci životaschopných embryí.

Contents

Chapter 1

AIMS OF THE THESIS

- Analyze evolution of Sec15 subunit of exocyst complex across eukaryots and compare primary sequence of SEC15 from various organisms.

- Find the interacting partners of the exocyst subunit AtSEC15b with the main focus on proteins from Rab GTPase family.

- Show the specificity of the interaction in respect to conformation of Rab GTPase.

- Determine a subcellular localization of AtSEC15b and an interacting Rab GTPase.

- Characterize *Arabidopsis thaliana* T-DNA insertional mutants in *AtSEC15b* gene with the emphasis on possible defects in polarized cell growth.

Chapter 2

CURRENT STATE OF KNOWLEDGE

2.1 Introduction

Cell morphogenesis is a fundamental process, which requires precise control of cell growth and division. Exocytosis plays a central role in this process being an essential membrane traffic event that mediates secretion of vesicular content as well as incorporation of proteins and lipids to the specific domains of the plasma membrane. Exocytosis takes place in all eukaryotic cells and is necessary for cell growth, cell polarity establishment, cell-to-cell communication, neurotransmission in animal cells, cell wall formation in plants and fungi, and many other processes that require delivery of vesicular cargo to the cell surface.

During exocytosis, secretory vesicles move along the cytoskeleton to the plasma membrane, where they fuse. This process is coordinated by specific recognition between the vesicle and its target, which is mediated by interaction of cognate proteins, called SNAREs (v-SNAREs and t-SNAREs, respectively; Rothman and Warren, 1994). Another ubiquitous components of vesicle trafficking are represented by Rab GTPases. They are involved in all transport steps from vesicle formation through vesicle transport to vesicle docking and fusion (reviewed in Stenmark, 2009).

In many cells, exocytosis is polarized. The establishment of polarized secretion is essential for development of many different cell types. The budding yeast *Saccharomyces cerevisie* uses polarized secretion for bud growth, cytokinesis, and response to mating factor (Govindan and Novick, 1995). In mammals, epithelial cell polarization and neuronal synaptogenesis are extensively studied examples requiring polarized exocytosis (Oztan et al., 2007; Murthy et al., 2003). In plants and fungi, there are several different examples of cells that exhibit polarized exocytosis resulting in polarized growth. These include pollen tubes, root hairs, moss protonemata, algal rhizoids, and fungal hyphae (reviewed in Hepler et al., 2001; Cole and Fowler, 2006).

The exocyst is a multiprotein complex, required for polarized exocytosis from yeast to plants. It is localized to the site of active secretion, where it facilitates the targeting and tethering of secretory vesicles to specific locations on the plasma membrane for subsequent membrane fusion mediated by SNARE proteins. Exocyst is composed of eight subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. It was first described in yeast (TerBush et al., 1996) and based on sequence homology, it was subsequently characterizied many other eukaryotic organisms, including

Schizosaccharomyces pombe (Wang et al., 2002), *Drosophila melanogaster* (Murthy et al., 2003), and mammals (Hsu et al., 1996; Kee et al., 1997).

Based on sequence homology, the presence of exocyst subunit in several plant genomes was identified. These include, *Arabidopsis thaliana* (Eliáš et al., 2003), rice, poplar and the moss *Physcomitrella patens* (Chong et al., 2009). Unlike in yeast and mammals, where each exocyst subunit is encoded usually by a single gene, plant exocyst subunits are mostly encoded by several paralogous genes, with extreme example of *EXO70*, represented by family of 23 genes in *Arabidopsis* (Eliáš et al., 2003) and 41 genes in rice (Chong et al., 2009). Recently it was demonstrated that all exocyst subunits function together *in vivo* in *Arabidopsis thaliana* and that they are essential for plant morphogenesis and development (Hála et al., 2008; Fendrych et al., 2010).

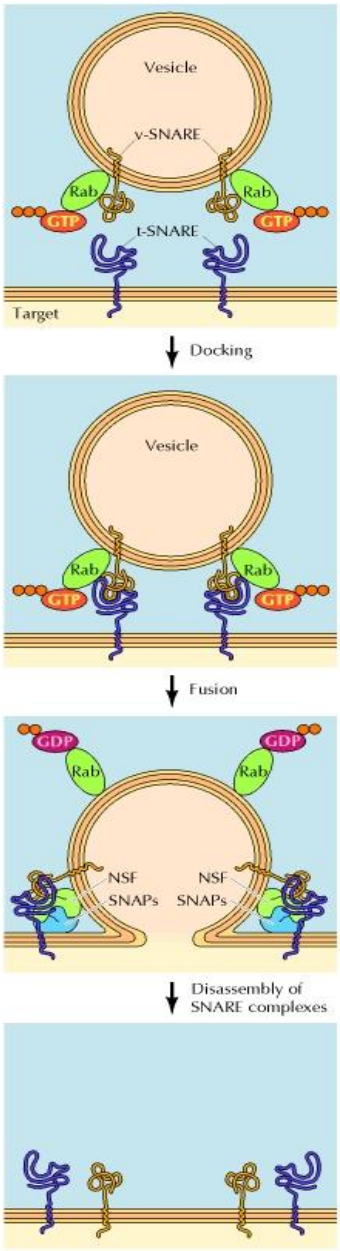
2.2 The conserved mechanism of vesicular transport

Membrane proteins and lipids as well as luminal contents are exchanged among the intracellular compartments of eukaryotic cell by vesicular transport. Because of rapid membrane turnover, this process must be highly coordinated to maintain cellular architecture. Numerous components of the molecular machinery that mediate vesicular transport are known. In the center of all, there are Rab GTPases, SNARE proteins, and tethering complexes.

Vesicular transport involves several distinct stages. First, transport vesicles are formed at a donor compartment and travel along cytoskeletal elements toward the target membrane. Rab GTPases have an essential role in this process, with different Rab proteins regulating traffic between different intracellular compartments. Second, transport vesicles are tethered to the target membrane by specific protein complexes, linking the vesicles to the acceptor membrane. It also occurs under regulation by several small GTP-binding proteins, including Rab GTPases. Finally, fusion between the phospholipids bilayers of the vesicle and the target membrane is facilitated by specific recognition of SNARE (soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment receptor) proteins (Figure 2.1; Tuvim et al., 2001).

2.2.1. The SNARE hypothesis

SNAREs are a large family of transmembrane proteins located both in a vesicle membrane (v-SNAREs) and in a target membrane (t-SNAREs). Their involvement in vesicle fusion was initially identified by James Rothman and his colleagues by biochemical analysis of reconstituted vesicular transport in mammalian cells. Analysis of these proteins led to formulation of “SNARE hypothesis”, in which it was postulated that specific pairs of v-SNAREs and t-SNAREs form a ternary complex, which drives actual membrane fusion (Figure 1.; Rothman and Warren, 1994). Individual SNARE proteins are unfolded, but they spontaneously assemble into a remarkably stable four-helix bundle that forms between membranes. Three helices anchored in one membrane (t-SNARE) assemble with the fourth helix anchored in the other membrane



(v-SNARE) to form a "trans-SNARE complex", which catalyzes fusion by forcing membranes closely together. In this process, Rab GTPases are required to facilitate formation of v-SNARE/t-SNARE complex. Moreover, two additionally proteins are needed to complete the process of vesicle transport. Once assembled, SNARE complex is recycled by the ATPase NSF and its adaptor eprotein SNAP. NSF is a hexamer that presumably uses three to six ATPs with each catalytic cycle to disrupt the SNARE complex allowing the SNAREs to be available for subsequent rounds of membrane fusion (reviewed in Tuvim et al., 2001; Südhof and Rothman, 2009). The schematic overview of vesicle fusion is shown in Figure 2.1.

Figure 2.1. Mechanism of vesicle fusion
Vesicle fusion is mediated by interaction between specific pairs of v- SNAREs and t-SNAREs on the vesicle and target membranes, respectively. Rab GTPases are required to facilitate formation of v-SNARE/t-SNARE complexes. Following membrane fusion, the NSF/SNAP proteins disassemble the SNARE complex (Cooper, 2000).

2.3. Tethering complexes

Despite the well-known mechanism of membrane fusion, there must be some other regulatory factors to achieve the specificity of this event. These are represented by tethering factors. They bridge newly formed transport vesicles with acceptor membranes to ensure correct docking and fusion. There are two classes of tethering factors. The first class consists of long coiled-coil proteins, and the second of large multisubunit complexes (Grosshans et al., 2006b).

Seven large conserved complexes have been proposed to have roles in vesicle tethering at distinct trafficking steps – COG, HOPS, TRAPPI, TRAPPII, DSL1, GARP and exocyst (Figure 2.2; Whyte and Munro, 2002). They are believed to tether transport

vesicles to their target membranes, acting there as effectors of Rab GTPases specific for a given transport step. Each of these tethering complexes resides on a specific cellular compartment where it mediates specific membrane fusion events. In anterograde transport, the TRAPPI (transport protein particle) complex mediates ER to Golgi traffic, COG complex participates in intra Golgi traffic, and TRAPPII functions in the exit from the Golgi. In budding yeast, the TRAPPII komplex has been shown to act also as a GEF for the two late Golgi Rab GTPases YPT31 and YPT32 (Morozova et al., 2006). From the TGN, secretion to the plasma membrane is mediated by the exocyst, whereas vesicles destined for the prevacuolar compartment and the vacuole are tetheres by HOPS/C-VPS complex. In retrograde transport, GARP complex tethers early or late endosomes to the TGN and Dsl1 complex mediates retrograde transport from the Golgi to the ER (reviewed in Koumandou et al., 2007).

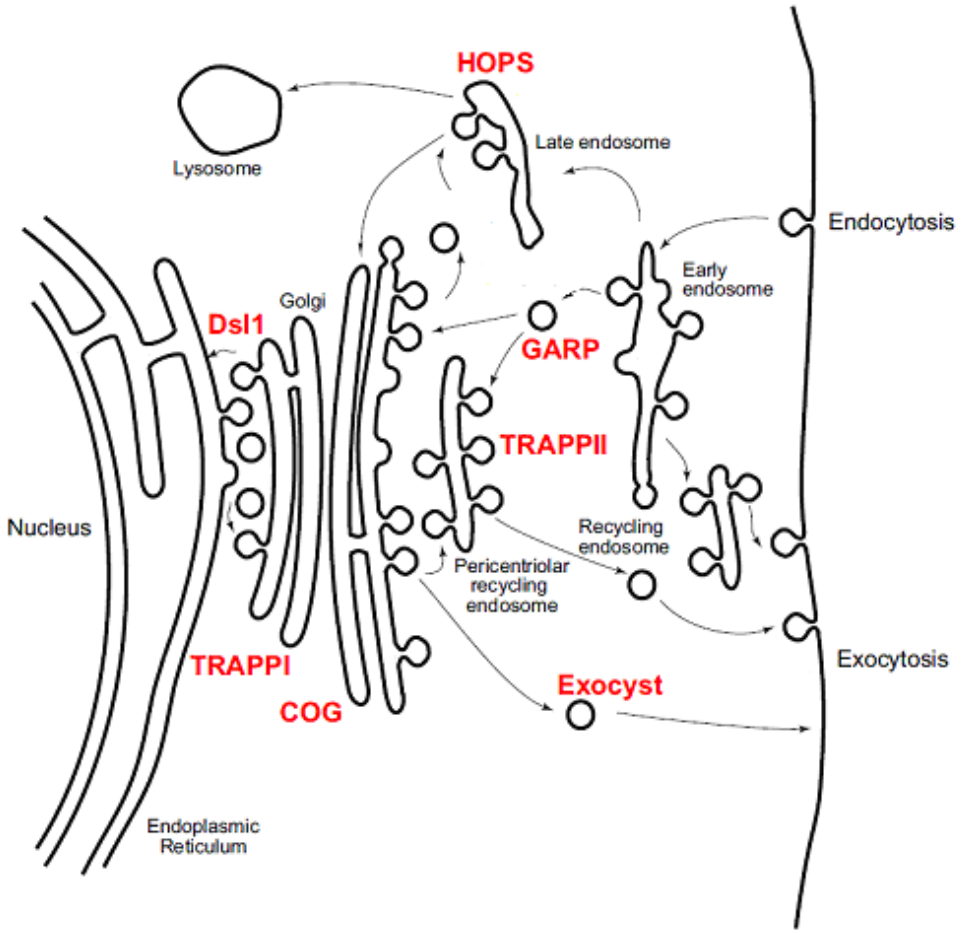


Figure 2.2. Location and functions of multi-subunit tethering complexes in eukaryotic cell. TRAPPI complex mediates ER-to-Golgi traffic, the COG complex mediates intra-Golgi traffic and the TRAPPII complex mediates exit from the Golgi as well as TGN functions. The GARP complex is also thought to reside on the TGN, where it tethers endocytotic vesicles. From the *trans*-Golgi, secretion to the plasma membrane is mediated by the exocyst complex. Finally, retrograde traffic from the Golgi to the ER is mediated by the Dsl1 complex on the ER membrane (according to Koumadou et al., 2007).

Some of these tethering complexes were also described in plant cells. In *Arabidopsis*, the HOPS/C-VPS complex resides on the vacuole and prevacuolar compartment and is required for vacuolar biogenesis. Mutants in VCL1 (VACUOLELESS1) subunit exhibit an extremely aberrant development that leads to embryo lethality at late torpedo stage. At the subcellular level, the most striking phenotype is the absence of vacuoles in the embryo and the accumulation of small vesicles and autophagosomes (Rojo et al., 2001; Rojo et al., 2003). Genetic analysis of some *Arabidopsis* GARP mutants showed a transmission defect through the male gametophyte associated with the altered pollen development (Lobstein et al., 2004; Guermonprez et al., 2008), as well as reduced resistance to heat and osmotic stress (Lee et al., 2006). The best studied tethering complex, and also the subject of this thesis, is the exocyst. Recently, a novel *Arabidopsis* gene, *CLUB/AtTRS130*, encoding a putative TRAPPII tethering factor was characterized. *club* mutants are seedling-lethal and fail to assemble a cell plate throughout the cytokinesis. Additionaly, KNOLLE-positive vesicles are formed and accumulated at the cell equator, but they fail to assemble into the cell plate in these mutants. These data provide an evidence that TRAPII is required for proper cell plate assembly during cytokinesis in *Arabidopsis* (Jaber et al., 2010).

Other complexes have not been extensively studied in plants but they are conserved among the eukaryotes and some of their subunits are encoded also by plant genomes (Koumandou et al., 2007).

2.4. Discovery of the exocyst complex

Exocyst was first identified in the yeast *Saccharomyces cerevisiae* and it was preceded by genetic screen for mutants defective in secretory pathway, initiated in the laboratory of Randy Schekman. These mutants are temperature-sensitive for cell growth, division and secretion. They normally grow at a permissive temperature (27°C), but their growth is arrested after shift to a restrictive temperature (37°C). Electron microscopy and invertase secretion assay showed that these mutants accumulate secretory vesicles and become dense during incubation at the restrictive temperature. They also block incorporation of plasma membrane permease and stop bud growth (Figure 2.3; Novick et al., 1980).

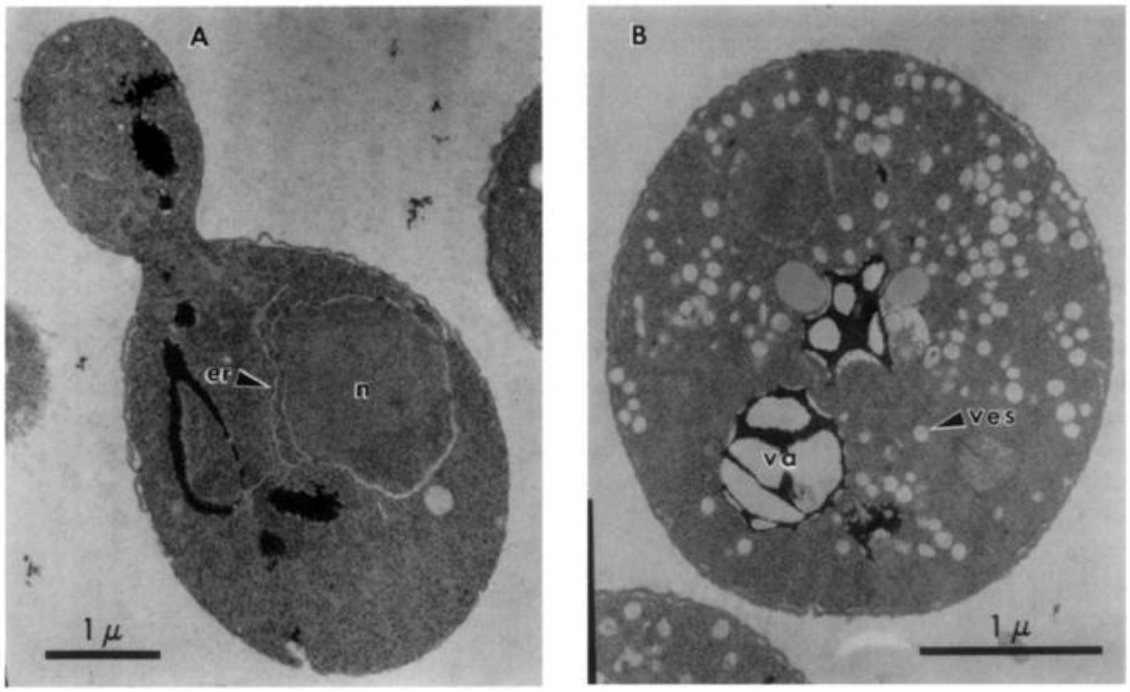


Figure 2.3. Thin section electron microscopy of yeast *Saccharomyces cerevisiae*.
a) *sec4-2* strain grown at permissive temperature resembles wild-type morphology.
b) *sec15-1* strain incubated at restrictive temperature exhibits mutant phenotype (Novick et al., 1980).
Abbreviations: (n) nucleus, (er) endoplasmic reticulum, (va) vacuole, (ves) vesicles.

This analysis led to identification of 23 genes, whose products are implicated in the process of delivering membrane and secretory proteins to the cell surface and which were classified into two groups. The first group is formed by 13 genes encoding proteins required for endoplasmic reticulum-to Golgi and intra-Golgi transport. Ten other genes (*SEC1, SEC2, SEC3, SEC4, SEC5, SEC6, SEC8, SEC9, SEC10, SEC15*) form the second group and their products are acting in the final stage of the secretory pathway, Golgi-to-plasma membrane trafficking (Novick et al., 1980).

These ten “late-acting” genes perform their roles at different steps of the secretory pathway. For example, the Sec1 protein has been found to play an important role in regulating SNARE proteins interaction during membrane fusion (Jahn, 2002). Sec4 is the founding member of the family of Rab GTPases functioning in post-Golgi events in yeast secretion (Salminen and Novick, 1987). Sec2 is a guanine nucleotide exchange factor for Sec4, which activates this GTPase (Walch-Solimena et al., 1997). Sec9 is the yeast homolog of SNAP-25, a neuronal t-SNARE (Brennwald et al., 1994).

Products of remaining six “late-acting” genes (*SEC3, SEC5, SEC6, SEC8, SEC10, SEC15*) function as a part of multisubunit complex, which localizes to the tip of the bud, the predominant site of exocytosis in *Saccharomyces cerevisiae* (TerBush et al., 1996). First data indicating that products of these genes may form a high molecular weight complex came from the analysis of the Sec15 protein in yeast. Sec15 was shown to associate with a 19.5S particle, which partially resides on the plasma

membrane, while the remainder is cytosolic. There was also the first evidence that Sec15 may act downstream of both Sec2 and Sec4, possibly to dock secretory vesicles to the plasma membrane before fusion (Bowser and Novick, 1991). Soon, Sec8 and Sec6 proteins were shown to co-segregate with Sec15 in a high molecular weight complex localized to sites of polarized exocytosis in yeast, indicating their possible role in vesicle targeting (Bowser et al., 1992; TerBush and Novick, 1995). Mutations in three other genes, *SEC3*, *SEC5* and *SEC10*, were found to disrupt the subunit integrity of the Sec6/Sec8/Sec15 complex, suggesting that these genes may encode additional components of the complex. This hypothesis was confirmed by microsequencing of components of purified complex and it was described that in addition to Sec6, Sec8 and Sec15, the complex contains also the proteins encoded by *SEC3*, *SEC5*, *SEC10*, and surprisingly a novel gene, *EXO70*. It was also the first time that this complex was named the exocyst, since it was required for exocytosis and not for other intacellular trafficking steps (TerBush et al., 1996). Finally, Guo et al. (1999a) identified the last component of exocyst in *Saccharomyces cerevisiae*, protein Exo84. Yeast cells depleted of Exo84 exhibit defects in secretory pathway similar to those in the late *sec* mutants. Exo84 co-immunoprecipitates with the exocyst components and it was furthermore demonstrated that the incorporation of Exo84 into the exocyst complex requires two other subunits, Sec5 and Sec10.

2.5. The structure of the exocyst complex

Understanding the structure of the exocyst may provide insights into the function of the exocyst at the molecular level. To date, crystal structures of four exocyst components have been determined (Figure 2.4). These include the C-terminal domain of *Drosophila* Sec15 (Wu et al., 2005), nearly full-length Exo70 from yeast (Dong et al., 2005; Hamburger et al., 2006) and mouse (Moore et al., 2007), the C-terminal domain of yeast Exo84 (Dong et al., 2005) and the C-terminal domain of yeast Sec6 (Sivaram et al., 2006). Although the sequence identity between the eight different exocyst subunits is less than 10%, they all are predicted to have highly helical compositions (40%-60%) yielding an overall similar shape – a tandem repeat of helical bundles, which are organized into a rod-like structure. Each bundle is a mixed antiparallel-parallel righthanded bundle and they pack against each other at their ends, with a slightly lefthanded superhelical twist. The structural conservation among exocyst components suggest that they evolved from a common ancestor. However, the exocyst proteins are not identical. They have many structural differences, consisting mainly of insertions and deletions of several residues and even small helices in their loop regions. Such variations probably result from the evolution of different functions for each subunit (Munson and Novick, 2006).

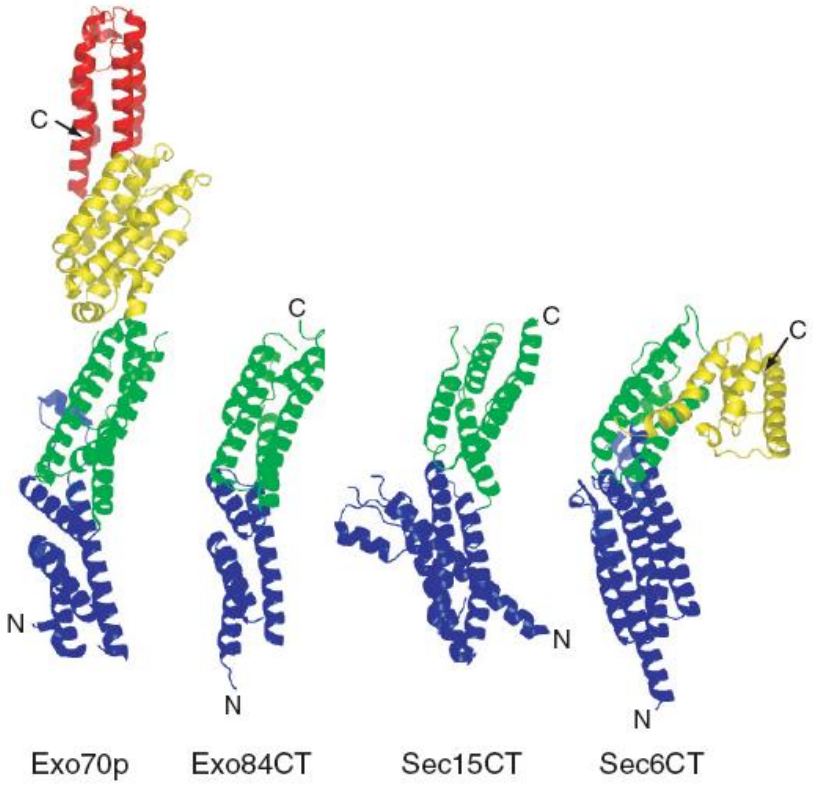


Figure 2.4. Exocyst subunit structures.
From left to right, Exo70, Exo84 C-terminal domain, Sec15 C-terminal domain, Sec6 C-terminal domain. Different exocyst proteins fold into a remarkably similar structure despite their very low sequence identity (Munson and Novick, 2006).

Progress for the other subunits has been arrested by lack of soluble proteins. Insolubility of proteins can occur for many different reasons – incorrect folding of recombinant proteins when overexpressed in *Escherichia coli*, incorrect posttranslational modifications or absence of some co-factors or binding partners. This problem can be alternatively bypassed by computational methods. Using hidden Markov model predictions it was showed that all of the exocyst subunits have similar helical bundle structures (Croteau et al., 2009).

2.6. The organization of the exocyst complex

The structure of individual exocyst components also provides a clue to how the exocyst complex is assembled. Initial mapping of the binding sites for Sec8 and Sec10 on the Exo70 structure indicated that the interactions are distributed along the length of the structure, suggesting that at least some of the subunits may pack together in an elongated side-to-side manner (Munson and Novick, 2006). Using quick-freeze/deep-etch cryoelectron microscopy of mammalian brain exocyst complex either unfixed or prefixed in glutaraldehyde, rod-like structures, predicted by protein crystallography studies, have been seen, suggesting that this is the predominant structure of exocyst.

The unfixed complex resembles a flower with four to six “petals” (4-6 nm wide x 10-30 nm long). In contrast, the prefixed complex appears as a wider structure (13 nm x 30 nm) with a couple of small appendages (Figure 2.5; Hsu et al., 1998). This leads to the suggestion that the structure represented by unfixed complex is in the process of disassembly into individual subunits upon adsorption to the mica substrate, while the structure of the fixed complex may more closely resemble the native exocyst structure, in which the rod-like subunits pack together along their length (Munson and Novick, 2006).

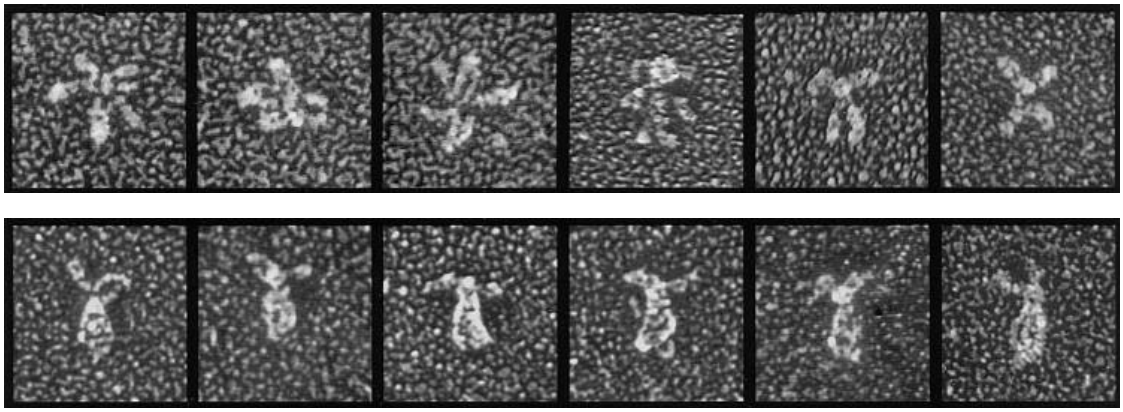


Figure 2.5. The structure of the mammalian brain exocyst complex.
The electron microscopic images shows the structure of purified exocyst complex in the unfixed state (a) or after fixation in glutaraldehyde (Hsu et al., 1998).

Using various approaches, including yeast two-hybrid assay, pull-down assay and co-imunoprecipitation experiments, multiple interactions between individual exocyst subunits were identified. Generally, in yeast, Sec10 is needed to link Sec15 to the remainder of the complex. The binding of Sec10/Sec15 to the rest of the exocyst is mediated by the interaction of Sec10 with Sec5. Sec5 also makes critical links to Exo70, Sec6 and Sec3, which marks the site of exocyst function. Thus, Sec5 appears to be at the core of the complex. Additionally, Sec5 and Sec10 interacts with Exo84 and Sec6 with Sec8 (Guo et al., 1999a; Guo et al., 1999b). Similar protein-protein interactions were found also in mammals and plants (Matern et al., 2001; Hála et al., 2008; Fendrych et al., 2010; Pečenková et al., 2011). These all are summarized in Figure 2.6.

Based on protein-protein interactions between exocyst subunits and the evidence that all of them resemble elongated rod-like structures, Munson and Novick (2006) proposed a hypothetical model, how these subunits pack together to form a structure similar to that seen by Hsu et al. (1998; Figure 2.7).

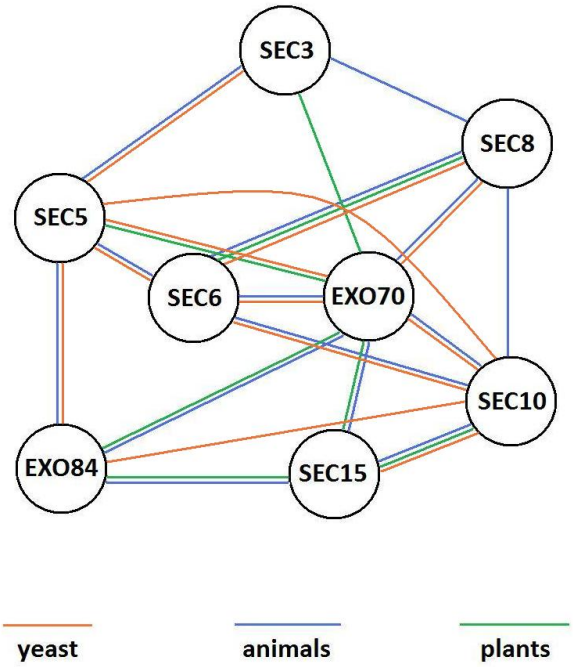


Figure 2.6. Interaction among different exocyst subunits.
A comparison between yeast, animals and plants. Interactions were detected by yeast two-hybrid assay (according to Munson and Novick, 2006; Hála et al., 2008; Fendrych et al., 2010; Pečenková et al., 2011).

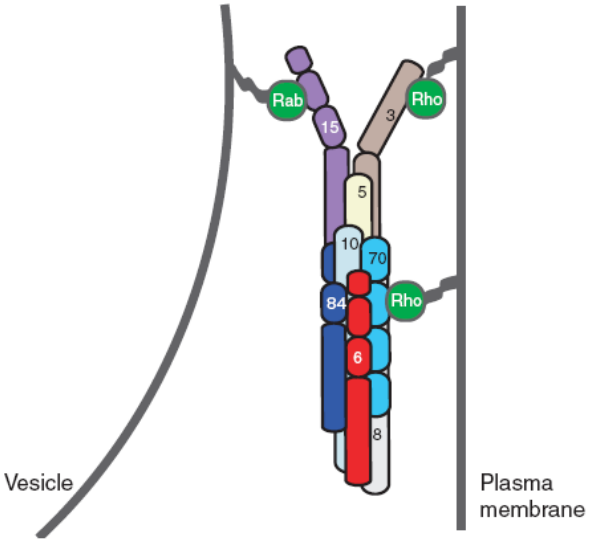


Figure 2.7. Hypothetical model of assembled exocyst complex
Schematic representation of the yeast exocyst complex, hypothesizing that each of the exocyst subunits has an elongated helical-bundle structure and that they pack together along their length (Munson and Novick, 2006).

2.7. Subcellular localization of the exocyst complex

Subcellular localization of the exocyst complex correlates with sites of polarized membrane growth. In order to understand exocyst function on the plasma membrane, it is important to know how the exocyst itself is targeted to the plasma membrane.

2.7.1. Localization and targeting of exocyst in yeast

In yeast, exocyst is localized to the growing bud, where it mediates secretion and cell surface expansion. The localization pattern is characteristic and cell cycle-dependent (Figure 2.8.). It begins with the appearance of a small cap at the site of bud formation and persists at the apical tip of the growing bud until the time of nuclear division when it shifts to an isotropic distribution over the surface of the growing bud. Finally, there is an abrupt shift in localization to the neck between the mother and daughter cells near the time of cytokinesis, where it remains until cell separation (TerBush and Novick, 1995; Finger et al, 1998).

It was also shown that Sec15 interacts with Bem1, a protein involved in polarity-establishment machinery. Sec15, which is defective in Bem1 binding, mislocalizes especially in the early stage of bud growth. It was then proposed that the interaction of Sec15 with Bem1 is important for Sec15 localization in yeast and through this interaction, Sec15 might play a crucial role in integrating the signals between Sec4 and the components of the early polarity-establishment machinery. This, in turn, helps to coordinate the secretory pathway and polarized bud growth (France et al., 2006).

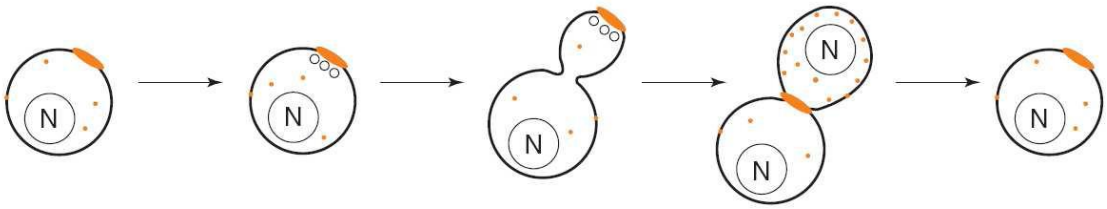


Figure 2.8. A model of the action of the exocyst complex in yeast.
Exocyst (shown in orange) is present at sites of secretion in the tip of the budding daughter cell, at sites of cytokinesis in dividing cells and in a patch at the bud scar that designates the site of exocytosis underlying future bud formation. Abbreviation: N, nucleus (Hsu et al., 1999).

Although all exocyst subunits share the same pattern of localization, their targeting to the plasma membrane involves different mechanisms. Sec3 is localized to the bud tip independently of actin cables. Exo70 uses two distinct pathways to arrive at the bud tip. A portion of Exo70 is transported on vesicles, possibly as a part of a partially assembled exocyst complex, whereas approximately half of Exo70 also localizes independently of vesicle traffic. The remaining exocyst subunits are

associated with exocytic vesicles and their delivery to sites of exocytosis depends on actin cytoskeleton (Figure 2.9; Boyd et al., 2004). But there is still question what recruits Sec3 and Exo70 to the plasma membrane. Besides the involvement of Rho GTPases, which is described in the Chapter 2.8.3., it was shown that both proteins interact with $PI(4,5)P_2$ and that this interaction is crucial to targeting of Sec3 and Exo70 to the plasma membrane. The disruption of the interaction of $PI(4,5)P_2$ with Sec3 and Exo70 blocks the plasma membrane association of the exocyst and results in severe secretion and growth defects or cell lethality (He et al., 2007; Zhang et al., 2008). Exo70 interact with $PI(4,5)P_2$ through conserved basic residues located at its C-terminus (He et al., 2007). Sec3 interacts with $PI(4,5)P_2$ through a polybasic region at its N-terminus (Zhang et al.,2008).

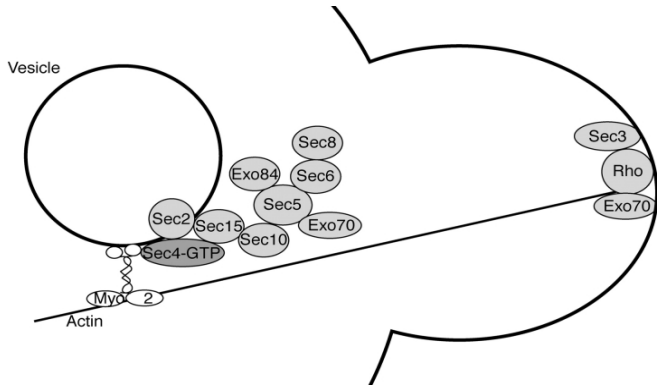


Figure 2.9. Model of targeting and tethering in *S. cerevisiae*.
The exocyst subunits (except for Sec3) associate with vesicles before movement to the bud tip. Once the vesicle has arrived at the bud tip, Sec3 and Exo70 bind to the rest of the exocyst to complete the formation of the tethering complex. Exo70 is also found to ride vesicles to sites of exocytosis in addition to localizing there by interacting with Rho3 (Boyd et al., 2004)

These data leads to the conclusion that Sec3 and Exo70 associate with the plasma membrane and interact with the rest of the exocyst components on arriving vesicles and that the assembly of the exocyst may tether the secretory vesicles to the plasma membrane for the subsequent fusion.

2.7.2. Localization and targeting of exocyst in mammals

In mammalian cells, the exocyst complex, sometimes also referred as the sec6/8 complex, is also present on plasma membrane at site of active secretion and membrane growth. Best characterized is the localization of exocyst in neurons and epithelial cells (Figure 2.10.).

Neurons undergo a characteristic sequence of developmental events culminating in the formation of synapses. Initially, neurons polarize, elaborating functionally and morphologically distinct axons and dendrites. This polarization arises also through the specific intracellular trafficking of lipid and protein components to appropriate locations in the cell. Vesicle trafficking is crucial for the membrane addition, which allows dynamic restructuring of axonal and dendritic plasma

membrane in the process of neurite outgrowth (Calakos and Scheller, 1996). Analysis of distribution of the exocyst complex in cultured hippocampal neurons during their development revealed that exocyst defines domains of membrane addition and synaptogenesis in growing axons. In young neurons, exocyst is present in the growing tips of neurites. Later, one neurite becomes the axon and here, exocyst is organized into periodic domains along the axon, and marks the site for synaptogenesis. As the neuron matures, vesicle clusters are found in some of these domains. After synapses are formed between the axon and dendrites, local clusters of synaptic vesicles are stabilized and the exocyst complex is downregulated (Hazuka et al., 1999).

In neuroendocrine PC12 cells, exocyst localization is dependent on the differentiation state of the cell. In undifferentiated PC12 cells, exocyst shows perinuclear localization. During differentiation, exocyst distributes from the perinuclear region into the growing neurite and becomes enriches in the growth cone (Vega and Hsu, 2001).

In Madin-Darby canine kidney (MDCK) epithelial cells, the exocyst complex relocalized during the development coincident with changes in polarity. In non-polarized cells, exocyst is localized mainly in cytosol. After initiation of calcium-dependent cell-cell adhesion, exocyst is rapidly recruited from the cytosol to cell-cell contacts. As cell polarity develops, the distribution of the exocyst complex becomes restricted to the apical-junctional complex on the lateral membrane domain, which includes tight junctions (Grindstaff et al., 1998; Lipschutz et al., 2000). Nevertheless, some exocyst complex still remain to be present in cytoplasm (Yeaman, 2003). It has been proposed that localization of exocyst to cell-cell junctions serves to direct trafficking of transport vesicles containing basal-lateral proteins to the developing lateral membrane domain (Oztan et al., 2007).

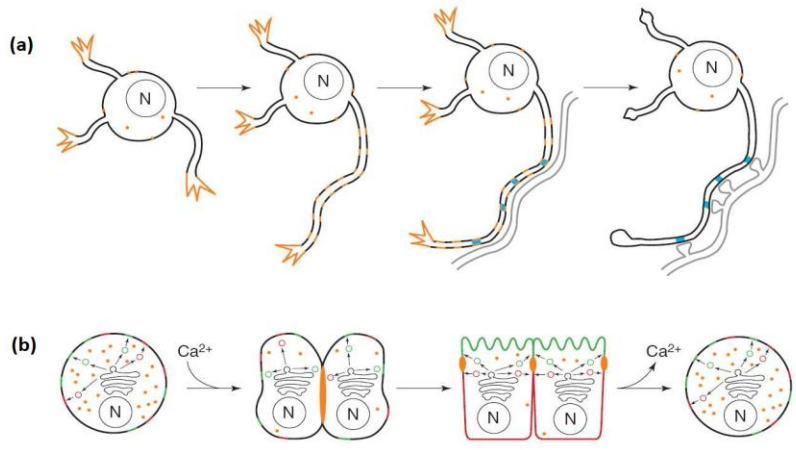


Figure 2.10. A model of the action of the exocyst complex in mammalian cells.
(a) In young neurons, the exocyst complex (shown in orange) is present in growth cones of neurites. Later, when one neurite becomes the axon, exocyst is organized into periodic domains along the axon and implicates synaptogenesis. As synapses are formed (shown in blue), exocyst is downregulated.
(b) In unpolarized MDCK epithelial cells, exocyst is dispersed throughout the cytosol. Upon the addition of Ca^{2+}, two cells contact each other and the exocyst is reorganized to the contacting plasma membrane. After cell polarization, exocyst is located along the membrane near the tight junctions. Abbreviation: N, nucleus (Hsu et al., 1999)

Once again, the question is, what is the mechanism, by which exocyst is recruited from cytosol to specific sites on plasma membrane. As the complex is assembled from eight cytosolic proteins subunits, it must associate with plasma membrane proteins that have restricted distribution in order to execute its function in localized vesicle delivery. Therefore, identification of membrane binding sites for exocyst complex is important for understanding the function of this essential complex. Yeaman et al. (2004) showed that in epithelial cells, exocyst complex is restricted to plasma membrane sites by cell-cell adhesion proteins specific to the apical-junctional complex. It results from observations that exocyst co-fractionates and co-immunoprecipitates with specific components of apical junctions. The two-hybrid screen revealed that the major binding partners of exocyst are E-cadherin and nectin, two major cell-cell adhesion molecules.

Nevertheless, there is evidence, that exocyst is recruited to plasma membrane in a similar way, as it was demonstrated in yeast. Several experiments proved that the localization of exocyst is also dependent on vesicle trafficking. For example, exocyst subunits Sec6 and Sec8 co-localize with vesicles carrying exocytic cargoes. Second, blockage of exocytosis inhibits recruitment of the exocyst complex to the plasma membrane and causes the accumulation of exocyst subunits at the perinuclear region. And finally, the introduction of various monoclonal antibodies against Sec6 and Sec8 into permeabilized MDCK cells results in protein cargo accumulation either at the perinuclear region or near the plasma membrane as a result of nonfunctional exocyst complex (Finger et al., 1998; Guo et al., 1999b).

Recently, it was proposed that similar to their yeast counterparts, mammalian Sec3 and Exo70 also interact with $PI(4,5)P_2$ through conserved regions (He and Guo, 2009) and thus function as landmarks at the plasma membrane for the remaining exocyst components, which are localized on post-Golgi vesicles arriving along the actin cables (Boyd et al., 2004). Exo70 directly interacts with $PI(4,5)P_2$ in plasma membrane via the positively charged residues at its C-terminus (Liu et al., 2007). It seems that this protein-lipid interaction is conserved from yeast to mammals and thus play critical role in recruiting Exo70, Sec3, and ultimately the other exocyst components to the plasma membrane. This association of exocyst with the plasma membrane is an important step in vesicle tethering. When and where this interaction takes place proceed in a precise regulation of the kinetics and location of exocytosis.

2.7.3. Localization of exocyst in plants

Unlike yeast and mammals, there are far less data revealing the localization of exocyst in plant cells. Similarly as in yeast and animal cells, exocyst is also localized in cytoplasm and weakly in nucleus (Fendrych et al., 2010; Pečenková et al., 2011). However, it is obvious, that as well as in other eukaryotes, exocyst is strongly localized to sites of extensive secretion and membrane growth. Using antibodies directed against SEC6, SEC8, and EXO70A1, Hála et al. (2008) demonstrated co-localization of these proteins at the apex of growing pollen tube, region of intensive polarized exocytosis. Exocyst is also localized to the growing cell plate during cytokinesis in

Arabidopsis thaliana. This is consistent with the understanding of plant cytokinesis as a highly dynamic and coordinated process of membrane fusion (Fendrych et al., 2010).

2.8. Regulation of the exocyst by small GTPases

To perform its functions properly, exocyst needs to be precisely regulated spatially as well as kinetically by other regulatory proteins. Small GTPases are thought to be the key regulators of vesicle docking and fusion events. Recent studies revealed that exocyst acts as a downstream effector of several small GTPases, which regulate both the localization and the function of the exocyst complex on the plasma membrane.

2.8.1. Small GTP-binding proteins

Small GTP-binding proteins (GTPases) are monomeric G proteins with molecular masses of 20-40 kDa, related to the α subunit of heterotrimeric G Proteins. They have been identified in all eukaryotes from yeast to plants. All these proteins belong to a superfamily, named the Ras superfamily, because the founding members are encoded by human *Ras* genes, initially discovered as cellular homologs of the viral *ras* oncogene. Based on a structure, sequence and function, the Ras superfamily is divided into five main families: Ras, Rab, Rho, Arf and Ran (reviewed in Van Dam et al., 2011).

The basic feature of these proteins is their ability to bind and hydrolyze GTP. Thus, they act in the cell as molecular switches that cycle between "active" (GTP-bound) and "inactive" (GDP-bound) state. This cycle is controlled by other regulatory proteins (Figure 2.11.). Guanine nucleotide exchange factors (GEFs) convert the inactive form of GTPases into the active form through GDP/GTP replacement. Inactivation of the active small GTPase is achieved through hydrolysis of the GTP. Because of weak intrinsic GTPase activity, efficient deactivation of small GTPases requires another class of regulatory proteins to accelerate this activity – GTPase activating proteins (GAPs). In addition, most small GTPases cycle between membrane and cytosol, which is under supervision of GDP dissociation inhibitors (GDIs). GDI extracts the inactive GTPases from the target membrane and deliver them back to the specific membrane compartment, where they can be re-activated by GEFs (reviewed in Takai et al., 2001; Yang, 2002; Van Dam et al., 2011).

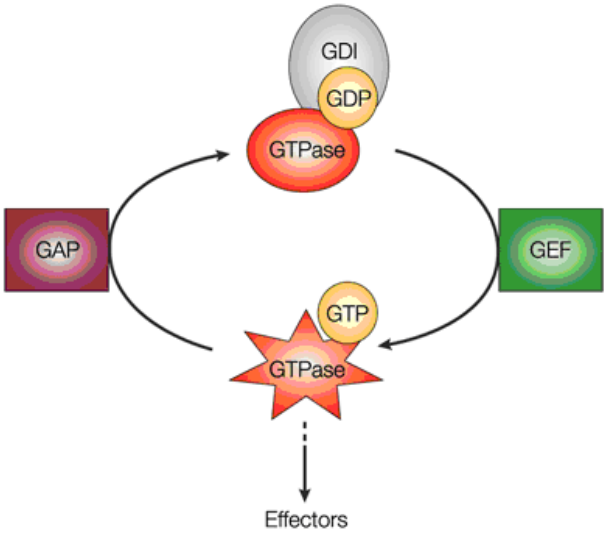


Figure 2.11. Functional cycle of small GTPases.
GEF catalyzes the conversion of the small GTPaseto the GTP-bound active form, in which it can interact with various downstream effectors. After that, GAP stimulates hydrolysis of GTP to GDP resulting in the inactivation of GTPase, which is furthermore removed from the membrane by GDI (Coleman et al., 2004).

The cycling between active and inactive state allows these proteins to modulate many signaling pathways in the cell. In their active conformation, small GTPases interact with multiple downstream effectors to perform diverse cellular functions, including differentiation, vesicle transport, cell division, nuclear assembly and control of the cytoskeleton. Exocyst complex represents important downstream effector of several small GTPases and the interaction between exocyst subunits and small GTPase appears to be critical for spatial and temporal regulation of polarized exocytosis (Lipschutz and Mostov, 2002; Wu et al., 2008). A model summary illustrating the interaction of exocyst with small GTP-binding proteins in yeast in animal is shown in Figure 2.13.

2.8.2. Rab GTPases

Rab GTPases comprise the most abundant family of small GTP-binding proteins. There are 11 different Rab GTPases in yeast and more than 60 in mammalian cells (Barrowman and Novick, 2003). In *Arabidopsis thaliana*, 57 Rab GTPases were identified and divided into 8 distinct subclasses based on sequence similarities (Rutherford and Moore, 2002; Woollard and Moore, 2008). They are present in all eukaryotic genomes and are well conserved from yeast to plants. Individual Rab GTPases are localized to the cytosolic face of intracellular membranes, where they function as regulators of distinct transport steps in membrane traffic pathways and are proposed to regulate targeting of transport vesicles to the appropriate cell compartment. In their active form, they are able to bind multiple effector proteins, such as sorting adaptors, tethering factors, kinases, phosphatases and cytoskeletal motors. Crosstalk between Rab GTPases and their effectors ensures the proper spatio-temporal regulation of vesicular traffic (reviewed in Stenmark and Olkkonen, 2001; Stenmark 2009).

In yeast and animals, the exocyst functions as an downstream effector of exocytosis-specific Rab GTPases and tethers secretory vesicles to the plasma membrane prior to the formation of the SNARE complex involved in the actual membrane fusion (Guo et al., 1999b; Zhang et al., 2004).

2.8.2.1. Exocyst as an effector of Rab GTPases in yeast

The first GTPase found to interact with the exocyst complex was Sec4, Rab GTPase from yeast *Saccharomyces cerevisiae* (Guo et al., 1999b). Sec4, initially identified in a screen for mutants with secretory defects, is associated with secretory vesicles and plays its role in regulation of post-Golgi secretion in yeast (Novick et al., 1981). Sec4 controls at least three different elements of the exocytic machinery in yeast. It may recruit myosin Myo2 to secretory vesicles to promote their active transport along polarized actin cables (Govindan et al., 1995; Walch-Solimena et al., 1997). In addition, Sec4 directly binds to Sro7, binding partner for the plasma membrane tSNARE Sec9 (Grosshans et al., 2006a). Finally, as it was mentioned above, Sec4 directly binds to exocyst via the interaction with Sec15 subunit (Guo et al., 1999b).

First indication that Sec15 might represent an effector of Sec4 comes from an observation that duplication of Sec4 supresses *sec15-1* mutation without altering the amount of the Sec15 in the cell. This rules out the increased synthesis or stability of the mutant protein as the mechanism of suppression and favors a hypothesis that Sec4 stimulate the residual Sec15 and so acts as an upstream activator of Sec15 function. This was, in addition, supported by disruption of polarized localization pattern of the Sec15 in *sec4* mutants (Salminen and Novick, 1989). Evidence that Sec4 and Sec15 directly interact in yeast came 10 years later. Using yeast two-hybrid assay, Guo et al. (1999b) showed that Sec4 physically interacts with Sec15. Importantly, this interaction is found to be GTP-dependent, while mutant form of Sec4 predicted to be in the GDP-locked conformation fails to interact with Sec15, whereas GTP-locked mutants show an increase in interaction with Sec15. In addition, they demonstrated that C-terminal geranylgeranylation, which is necessary for the attachment of Sec4 to the membranes, is not required for its interaction with Sec15. Sec15 protein interacts with other exocyst subunit, Sec10, leading to assembly of exocyst complex. It was also shown that functional Sec4 is required for efficient assembly of the exocyst proteins with Sec3, which marks the site of secretion. These results provide a network of molecular interactions that could function to target secretory vesicles to specific sites on the plasma membrane, which is required for polarized secretion.

Additionally, Medkova et al. (2006) showed that Sec2, the guanine nucleotide exchange factor (GEF) that activates the Sec4 GTPase also interacts with Sec15 in yeast. This interaction may help to maintain Sec4 in highly active state needed for vesicle transport and tethering. Sec2 is recruited to membrane by binding to another Rab protein, Ypt32 in its GTP-bound conformation, forming a Rab GEF cascade in which one Rab recruits the GEF that activates the next Rab along the secretory pathway. Ypt32 compete for binding to Sec2 with Sec15. After initial recruitment to membranes, Sec2 adopts a different conformation that allows Sec15 to replace Ypt32. This GEF-effector complex persists on the vesicle surface to promote transport and tethering. It was recently shown, that Sec15-Sec2 interaction is inhibited by PI4P (Mizuno-Yamasaki et al., 2010). It was demonstrated that Sec2 binds PI4P and that the production of PI4P within a Golgi is necessary, in combination with Ypt32, for Sec2 localization. Moreover the interaction of PI4P with Sec2 selectively inhibits Sec15 binding and so it may control recruitment of Sec2 during formation of secretory vesicles. PI4P levels decline

as vesicles mature, allowing Sec15 to replace Ypt32 (Figure 2.12.; Mizuno-Yamasaki et al., 2010).

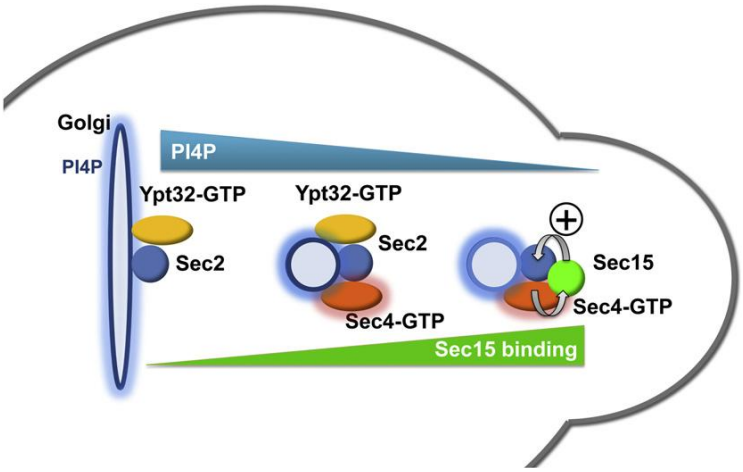


Figure 2.12. A model for recruitment and regulation of Sec2 by PI4P
From left to right: Sec2 is initially recruited to the Golgi membrane through the combined signals from PI4P and Ypt32-GTP. The interaction of PI4P with Sec2 also serves to block the binding of Sec15 with Sec2, thereby facilitating the interaction of Sec2 with Ypt32-GTP. Vesicles bud off of the Golgi carrying Sec2 and Ypt32-GTP, but containing reduced levels of PI4P. Sec2 activates Sec4, which then recruits its effector, Sec15. The reduced level of PI4P on the vesicle membrane allows Sec15 to competitively replace Ypt32-GTP on Sec2. This generates a positive feedback loop in which Sec2 activates Sec4, Sec4-GTP recruits Sec15, and Sec15 helps retains Sec2 on the vesicle. The elevated levels of Sec4-GTP and Sec15 prepare the vesicle to tether, dock, and fuse with the plasma membrane (Mizuno-Yamasaki et al., 2010).

2.8.2.2. Exocyst as an effector of Rab GTPases in animal cells

In mammalian cells, Sec15 protein was also described as an effector molecule for Rab GTPase. It was experimentally shown that mammalian Sec15 specifically interacts with Rab11 GTPase, which is a recycling endosome marker, also in a GTP-dependent manner. No interaction was detected with Rab4, Rab6 or Rab7, which are markers for early endosome, Golgi, late endosomes and lysosomes, respectively, suggesting that Sec15 doesn't function as an effector for all Rab proteins involved in plasma membrane-directed trafficking pathways. This was also supported by co-localization experiments. Overexpressed Sec15 associates with clusters of tubular-vesicular elements that are concentrated in the perinuclear region. Although some overlap between Rab4, Rab6, or Rab7 and Sec15 is apparent, it is clear that their localizations are predominantly distinct. In contrast, Rab11 exhibits a striking co-localization with Sec15-positive tubular-vesicular clusters (Zhang et al., 2004).

GTP-dependent interaction between Rab11 GTPase and Sec15 was observed also in *Drosophila melanogaster*. Furthermore, it was demonstrated that C-terminal domain of Sec15 is responsible for this interaction. Crystal structure of the C-terminal domain is composed of two subdomains – N-terminal subdomain (residues 382 – 563) and C-terminal subdomain (residues 564 – 699). The structure is comprised entirely of α-helices, which are equally distributed between these two subdomains. Compared to

the N subdomain, the C subdomain is more compact, because all of its antiparallel helices form a helix bundle and the loops connecting the helices are relatively short. It was demonstrated that only C subdomain harbors Rab-binding site and thus it is sufficient for the interaction with this GTPase. Binding studies have further showed that Sec15 lacks stringent substrate specificity *in vitro*. The C-terminal domain binds Rab11, but with reduces affinity also Rab3, Rab8 and Rab27. Nevertheless, Rab11 seems to be a major interacting partner of Sec15 (Wu et al., 2005).

2.8.2.3. What is the situation in plants?

To date, informations about interaction between exocyst subunit Sec15 and Rab GTPases in plants are missing. The only published attempt to identify the potential interactor of plant exocyst using a screen based on complementation of yeast *sec15-1* mutation with *Arabidopsis* cDNA library was unsuccessful. Only a non-related suppressor representing the RING finger motif-containing E3 ubiquitin ligase was found (Matsuda and Nakano, 1998; Matsuda et al., 2001).

2.8.3. Rho GTPases

Small GTP-binding proteins of the Rho family are present in all eukaryotic cells where they act as key regulators in signaling pathways that control multiple cellular processes, including actin dynamics, cell morphogenesis, cell cycle progression, gene transcription and the generation of superoxide anions. Rho GTPase family have evolved to diverge to Rho, Cdc42, and Rac subfamilies in animals and filamentous fungi and Cdc42 and Rho in yeast. Interestingly, plants possess a unique class of Rho GTPases, termed Rop (reviewed in Berken and Wittinghofer, 2008; Nagawa et al., 2010).

Rho GTPases represent the second class of small GTPases found to interact with exocyst (Guo et al., 2001; Zhang et al., 2001). In general, it is assumed that Rho GTPases locally activate the exocyst and have an important role in spatial regulation of exocystosis.

2.8.3.1. Exocyst as an effector of Rho GTPases in yeast

In a search for proteins that regulate the polarized localization of the exocyst in the budding yeast *Saccharomyces cerevisiae*, Guo et al. (2001) identified that certain *rho1* mutant alleles specifically affect the localization of the exocyst proteins. They found that Sec3, which is thought to be a spatial landmark defining sites of exocytosis (Finger et al., 1998), interacts directly with Rho1 in its GTP-bound conformation and that functional Rho1 is essential for establishment as well as maintenance of the polarized localization of Sec3 (Guo et al., 2001).

Another member of the Rho family, the Cdc42 protein, also directly interacts with the exocyst subunit Sec3 (Zhang et al., 2001). Cdc42 is important for the initial

establishment of polarity and functions very early in the signaling cascade that coordinates polarized morphogenesis (Chant and Stowers, 1995). In several *cdc42* mutants, the exocyst proteins are mislocalized and exocytosis runs in a random pattern. Cdc42 co-localizes with Sec3 protein at the bud emerging site or at the tips of the small buds suggesting that Sec3 needs Cdc42 for its initial targeting to the bud. A similar exocytotic defect was also observed in a *cdc24* mutant. Cdc24 is a guanine nucleotide exchange factor for Cdc42, which convert this GTPase to the GTP-bound form (Zheng et al., 1994). This leads to the suggestion that Sec3 binds Cdc42 in its active conformation (Zhang et al., 2001).

Both Rho1 and Cdc42, bind Sec3 on its amino acid terminus and it was found that they compete in this interaction. It was proposed that Cdc42 and Rho1 may function as exocyst regulators at different time during polarized growth. While Cdc42 is primarily involved in the early establishment of cell polarity, Rho1 may be mainly involved in the maintenance of polarized growth (Guo et al., 2001; Zhang et al., 2001).

Additionally, Rho3 was found as a direct interacting partner of exocyst in yeast (Robinson et al., 1999). Rho3 protein plays a critical role in *Saccharomyces cerevisiae* as it controls proper cell growth. It is responsible for directing the deposition of newly synthetized material to the bud by regulating polarized secretion and the actin cytoskeleton (Matsui et al., 1992). In yeast two-hybrid screen, two proteins were identified as Rho3 interactors, Exo70 and Myo2. Interaction with these proteins is dramatically reduced or abolished when mutations are introduced into the Rho3 effector domain and interaction between Exo70 and Rho3 *in vitro* depends on a presence of GTP. Moreover, these two proteins exhibit overlapping subcellular localization. It is obviously possible that the interaction between Exo70 and Rho3 is essential for proper localization of Exo70 (Robinson et al., 1999). The second interacting partner of Rho3, Myo2, is an essential myosin in yeast that plays an important role in organization of actin cytoskeleton and is also critical for the transport of secretory vesicles to the bud (Mulholand et al., 1994). Genetic analysis reveals the interaction between Myo2 and seven late-actin *sec* genes including four exocyst subunits (Govindan et al., 1995). These observations lead to the possibility that Myo2 and the exocyst work together to ensure polarized cell growth of the yeast by precise deposition of newly synthesized proteins at the bud site. Above all, Rho3 GTPase functions as an important protein in a regulation of this process (Robinson et al., 1999).

Finally, He et al. (2007) identified *exo70* mutant that accumulates post-Golgi vesicles during bud emergence. This phenotype is remarkably similar to that seen in *cdc42* mutant (Adamo et al., 2001) suggesting a role for Exo70 as a target for Cdc42 during exocytosis. However, they were not able to detect any physical interaction between Exo70 and Cdc42. Recently, it was demonstrated that Exo70 represent a direct effector for both Rho3 and Cdc42 function in spatial regulation of exocytosis and that C-terminal prenylation of these GTPases is essential for binding Exo70 protein (Wu et al., 2010).

In the fission yeast *Schizosaccharomyces pombe*, Rho3 regulates cell separation by modulating exocyst function (Wang et al., 2003). *S. pombe* divides using an actomyosin ring whose constriction is coordinated with the centripetal deposition of

new membranes and division septum. Exocyst is involved in the final stage of cytokinesis – digestion of the primary septum to liberate two daughter cells. It is localized to both cell tips as well as the site of cell division and it is interesting that this localization is dependent of F-actin cytoskeleton but independent on secretion. Exocyst is required for cell separation possibly by delivering primary septum-degrading enzymes at the division site (Wang et al., 2002). It was also shown that temperature-sensitive mutant *sec8* is defective in cell separation and vesicle fusion. Rho3 was found to be able to suppress the growth and the cell separation defect of *sec8* at restrictive temperature. But overexpression of Rho3 is unable to rescue the loss of Sec8 in *sec8* null mutant, indicating that Rho3 suppresses the *sec8* mutant by stimulating residual function of the mutant Sec8, rather than bypassing the requirement of Sec8. But in the case of another exocyst null mutant, *exo70*, Rho3 is able to fully restore the mutant phenotype and bypass the requirement for Exo70. Interestingly, the double mutant *rho3 sec8* is lethal and it was suggested that there is a genetic interaction between Rho3 and Sec8. Additionally, similar interaction was observed between Rho3 and Exo70. Rho3 is, similar to exocyst proteins, localized to the division site during cytokinesis. To investigate whether Rho3 regulates the localization of the exocyst complex at the division site, the localization of Sec8 and Sec6 was examined in the *rho3* null mutant. Both, Sec8 and Sec6, are clearly localized to the division site and growing tips independently of Rho3 function. Thus, Rho3 may modulate the exocyst function by mechanism probably independent of those involved in their proper localization (Wang et al., 2003).

2.8.3.2. Exocyst as an effector of Rho GTPases in animal cells

In mammalian cells, there is no evidence of direct interaction between Rho GTPases and Sec3 exocyst subunit. It is probably because mammalian Sec3 lacks the amino-terminal Rho binding domain found in yeast Sec3 (Matern et al., 2001). Nevertheless, Sec3 interacts with the polarity protein IQGAP1 to facilitate the targeted delivery of matrix metalloproteinases to tumor cell invadopodia, and this interaction seems to be regulated by the RhoA and Cdc42 GTPases. So it is possible that in multicellular eukaryots accessory proteins may represent a link between Sec3 and Rho GTPases (Sakurai-Yageta et al., 2008).

Nevertheless, there is an evidence of direct interaction between Rho GTPases and exocyst in mammalian cells. Inoue et al. (2003) showed that TC10, small GTPase from Rho family, interact with the amino terminus of Exo70 exocyst subunit. TC10 is essential for translocation of glucose transporter Glut4, required for insulin-stimulated glucose uptake (Chiang et al., 2001). Exo70 translocates to the plasma membrane in response to insulin through the activation of TC10, where it assembles a multiprotein complex and functions in the tethering of the Glut4 vesicles and so have a crucial role in the membrane targeting of Glut4 (Inoue et al., 2003).

2.8.3.3. What is the situation in plants?

Plants do not contain direct orthologs of Rho subfamilies found in yeast and animal. However, they have evolved a unique class of Rho GTPases named ROP – Rho of plants (Li et al., 1998; Zheng and Yang, 2000). They are implicated in the regulation of diverse signaling processes including cell polarity establishment, cell growth, morphogenesis, actin dynamics, H_2O_2 generation, hormone responses and probably many others (Zheng and Yang 2000).

To date there is no evidence of direct interaction between ROP GTPases and exocyst. Nevertheless, Lavy et al. (2007) identified a novel ROP effector proteins, designated ICR1 and ICR2 (interactor of constitutive active ROPs), that interact with GTP-bound ROPs. Function of ICR1 was examined in the T-DNA insertion mutants. ICR1 knockdown or silencing results in cell deformation in adaxial epidermis, collapse of root apical meristem resulting in short primary roots and development of numerous adventitious roots. Additionally, mutant plants are partially male sterile, indicating that pollen development is affected. To assess the interaction between ICR1 and ROPs, the *icr1* mutant was crossed with GFP-AtROP6(CA) overexpressing line. Crossed plants resemble the *icr1* single mutant phenotype leading to the hypothesis that ICR1 is required for AtROP6 gain of function. On the other hand, plants overexpressing GFP-ICR1 are virtually indistinguishable from plants that overexpress activated ROPs, suggesting that ICR1 is indeed a ROP effector. Yeast two-hybrid assay, pull-down assay, bimolecular fluorescence complementation (BiFC) and co-localization experiments revealed the physical interaction between ICR1 and ROP6, or ROP10, respectively, in *Arabidopsis thaliana*. This interaction likely takes place at or near the plasma membrane. ICR1 was then suspected as a scaffold, mediating interactions of ROPs with different proteins. To test this hypothesis, Lavy et al. (2007) performed yeast two-hybrid screens with ICR1 as a bait and surprisingly, one of the identified proteins was the subunit of the exocyst complex, protein SEC3a.

In yeast, Rho1 and Cdc42 directly interact with Sec3 to induce its recruitment to the plasma membrane (Guo et al., 2001; Zhang et al., 2001). Plant and animal homologs of yeast Sec3 lack the Rho interaction domain so these results suggest that in *Arabidopsis*, ICR1 provides the missing link between GTPases of the Rho family and the exocyst (Lavy et al., 2007).

2.8.4. Arf GTPases

Arfs (ADP-ribosylation factors) were initially identified due to their ability to stimulate the ADP-ribosylation activity of cholera toxin A (Moss and Vaughan, 1998). Arf GTPases are implicated in the regulation of membrane traffic and organization of cytoskeleton. They are mainly responsible for the formation of transport vesicles, including coat recruitment, cargo sorting, completion of fission, and uncoating transport vesicles (reviewed in Gillingham and Munro, 2007).

Using two-hybrid assay, an interaction between human proteins Arf6 and Sec10 was detected. For binding Sec10, GTP-bound conformation of Arf6 is preferred and the physicall interaction occurs on C-terminal domain of Sec10 (Prigent et al., 2003). Arf6 is involved in plasma membrane and cortical actin remodeling through the regulation of membrane recycling to specialized regions of the plasma membrane (Chavrier and Goud, 1999). In MDCK cells, localization of Sec10 is predominantly perinuclear. Sec10 is associated with tubulo-vesicular structures close to Golgi cisternae, corresponding presumably to the TGN, but extends also into recycling endosomes, where is the site of Arf6 function. However, recruitment of Sec10 to the RE doesn't require active Arf6, as Sec10 perinuclear localization is not affected by dominant-inhibitory Arf6. Rather, it is possible that GTP-Arf6, which accumulates at the plasma membrane, triggers the localization of Sec10, and so the exocyst complex, to dynamic regions of the plasma membrane (Prigent et al., 2003).

2.8.5. Ral GTPases

Ral (Ras-like) GTPases, consist of RalA and RalB proteins, are implicated in the regulation of a variety of dynamic cellular processes including proliferation, oncogenic transformation, actin dynamics, endocytosis and exocytosis. They thought to be animal specific because they are missing in yeast and plants (Moskalenko et al., 2003).

First insights into interaction between Ral GTPases and exocyst come from rat brain and RalA was the first mammalian protein shown to interact with the exocyst in a GTP-dependent manner (Brymora et al., 2001). In human cells, RalA GTPases regulate exocyst assembly through direct interaction with two subunits of the exocyst complex, Sec5 and Exo84. This interaction is selective for the GTP-bound, activated, conformation of Ral GTPase and seems to be critical for exocyst function because loss of RalA expression inhibits assembly of the exocyst complex (Moskalenko et al., 2002; Moskalenko et al., 2003). Later, a direct interaction also between RalB and Sec5 and its requirement for both assembly and localization of the exocyst was demonstrated (Rossé et al., 2006)

2.8.6. Ran GTPases

Ran GTPases participates mainly in regulation of nucleo-cytoplasmic transport of macromolecules through the nuclear pore complex, but they are also critical for other cellular processes, including mitotic spindle assembly and post-mitotic nuclear envelope assembly (Joseph, 2006). Because Ran GTPases are notsupposed to be involved in secretory pathway, one doesn't expect the direct link between these GTPases and the exocyst. Surprisingly, an interaction between DelGEF, a homologue of the nucleotide exchange factor for Ran GTPase (RanGEF), and exocyst subunit Sec5 in human HeLa cells was demonstrated. This interaction was found to be dependent on Mg^{2+} and stimulated by guanosine triphosphate (GTP) or deoxycytidine (dCTP). Downregulation of endogenous DelGEF in HeLa cells induced increased extracellular

secretion of proteoglycans indicating a possible role for DelGEF in the secretion process (Sjölinder et al., 2002).

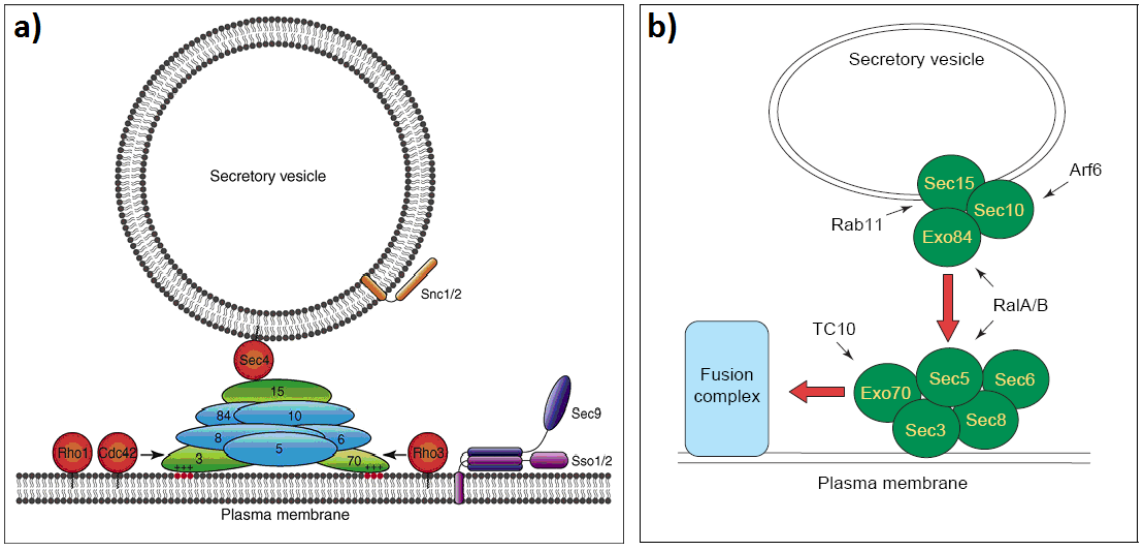


Figure 2.13. Summary of the interactions between the exocyst and small GTPases.
a) in yeast: Rab GTPase Sec4 interacts with the exocyst subunit Sec15 on the secretory vesicles. Rho1 and Cdc42 GTPases interact with exocyst subunit Sec3, whereas Rho3 interacts with Exo70 (He and Guo, 2009).
b) in animals: Rab11 GTPase interacts with exocyst subunit Sec15 to recruit subcomplexes to secretory vesicles. Ral GTPases interact with both Exo84 and Sec5 and regulate the assembly of the full octameric complex. Arf6 interacts with Sec10 and TC10, GTPase of the Rho family, interacts with Exo70 (Camonis and White, 2005).

2.9. Functions of the exocyst complex

As its name suggests, exocyst has a significant role in the regulation of exocytosis. But in general, it has been implicated in a variety of cellular processes including cytokinesis, cell migration, and tumor invasion. Additionally, in animals, exocyst plays an important role in a multiple developmental processes, such as the establishment of cell polarity, cell fate determination, neural and eye development, and oogenesis (He and Guo, 2009). In plants, exocyst regulates polarized cell growth such as tip growth of pollen tubes and root hairs (Cole et al., 2005; Synek et al., 2006) as well as process of cytokinesis (Fendrych et al., 2010). Taken together, exocyst has a various functions at the tissue and organ level.

2.9.1. Exocytosis

As already mentioned, most of the exocyst subunits was discovered in a genetic screen for yeast mutants defective in secretion (Novick et al., 1980). Later, the other two subunits, Exo70 and Exo84, were also shown to be part of the exocyst complex and function in the late secretory pathway (TerBush et al., 1996; Guo et al.,

1999a). Mutations is exocyst subunits lead to a common, temperature-sensitive, phenotype – yeast cells are defective in secretion and accumulate 80 to 100 nm secretory vesicles inside the cell as a result of defective tethering of these vesicles to the plasma membrane (Novick et al., 1980; Finger and Novick, 1997). Yeast cells depleted of individual exocyst subunits cannot survive. The only exception is Sec3 subunit, which is not essential because cells depleted of Sec3 are viable although they grow slowly. Moreover, with the respect to the secretory block, several *sec3* mutants accumulate ER and Golgi membranes in addition to exocytic vesicles, suggesting a possible role for Sec3 protein in earlier stages of the secretory pathway (Finger and Novick, 1997).

The exocyst in animal cells seems to have very similar role to the yeast complex. Its role in exocytosis and cell surface expansion has been demonstrated in variety of cell types. In polarized epithelial cells, the exocyst is required for transport to the lateral membrane, but apparently not to the apical membrane (Grindstaff et al., 1998). In neurons, it is required for neurite growth, branching and synaptogenesis (Hazuka et al,. 1999; Lalli and Hall, 2005) but doesn't seem to play a major role during neurotransmitter release at mature synapses (Murthy et al., 2003), which is also a specialized form of regulated exocytosis. This indicates that exocyst is not required for all types of exocytic events in animal cells. In addition, exocyst is required for dendritic delivery of NMDA (*N*-methyl-D-aspartate) receptors at postsynaptic membranes (Sans et al., 2003) or insulin-induced exocytosis of glucose transporter in adipocytes (Inoue et al., 2003).

2.9.1.1. Communication with SNAREs

Vesicle tethering occurs before the membrane fusion. In yeast and mammals, it was demonstrated that exocyst is required not only for tethering of secretory vesicles but also for facilitating the SNARE complex assembly (Grote et al., 2000).

In yeast, mutations in exocyst subunits Sec5, Sec6, Sec8 or Sec15 reduce binding of v-SNARE and t-SNARE and thereby prevent SNARE complex assembly (Grote et al., 2000). Similarly, mutation in Sec2, the nucleotide exchange factor for the Sec4 GTPase, which is required for transport of vesicles into the bud (Walch-Solimena et al., 1997), leads to a same defect. Taken together, mutations that block either secretory vesicle delivery or tethering prevent the assembly of the SNARE complex in yeast. These data indicate that the exocyst acts upstream of SNARE complex assembly, but do not provide a direct evidence for a physical interaction between the exocyst and SNARE proteins (Grote et al., 2000).

Later, physical interaction between Sec6 dimer and the plasma membrane t-SNARE Sec9 was revealed and it was shown that this interaction inhibits the association of Sec9 with the other t-SNARE protein Sso1 (Sivaram et al., 2005). Interaction between exocyst and SNARE regulators Sec1 (Wiederkehr et al., 2004), Sro7 and Sro77 (Zhang et al., 2005) was also demonstrated, suggesting that the exocyst complex actively direct SNARE complex assembly and SNARE-mediated membrane fusion.

In mammalian cells, Exo70 interacts with the t-SNARE binding protein Snapin, a ubiquitous protein known to associate with at least two t-SNAREs, SNAP23 and SNAP25. Exo70 competes with SNAP23 for Snapin binding, suggesting that Snapin doesn't provide a direct link between the exocyst and the SNARE complex but, rather, mediates cross-talk between the complexes by sequential interactions (Bao et al., 2008). Similar to the exocyst, the majority of tethering complexes in different stages of membrane traffic were shown to interact with t-SNAREs at the corresponding acceptor membranes. For example, the HOPS complex was shown to interact with the SNAREs and promote SNARE-mediated fusion *in vitro*. It may be a common theme that the tethering factors regulate SNARE assembly through a multitude of interactions to coordinate vesicle tethering and fusion (reviewed in He and Guo, 2009).

2.9.2. Cytokinesis

In several eukaryotic organisms, including plants, the exocyst has been shown to have important role during the cytokinesis. In *Saccharomyces cerevisiae*, exocyst localizes to the bud neck between the mother and the daughter cells (Finger et al., 1998). In mammalian cells, cytokinesis is thought to be driven by an actomyosin-containing conctractile ring that assembles around the equator of the cell and serves to bisect the daughter cells upon contraction. In this model, membrane traffic is not considered to be a significant feature. But in contrast, later studies bring new evidence that supports a role for membrane traffic also in mammalian cytokinesis. Especially membrane transport into the furrow and midbody during cytokinesis is of fundamental importance (Prekeris and Gould, 2008). In mammalian cells, Gromley et al. (2005) demonstrate the role of exocyst during terminal step of cytokinesis, called abscission. It was previously shown, that significant role during this process is performed by centriolin, protein localized to the midbody during cytokinesis (Gromley et al., 2003). Centriolin interacts with the exocyst subunit Sec15 and the SNARE-associated protein Snapin. It was described that exocyst and SNARE complexes require centriolin for localization to a unique midbody-ring structure to integrate membrane fusion with abscission. Moreover, disruption of exocyst function induces accumulation of intracellular vesicles containing v-SNAREs at the midbody ring, which corresponds to the concept that exocyst operate at the midbody ring to facilitate fusion of v-SNARE vesicles at the late stage of cytokinesis (Gromley et al., 2005).

In plants, cytokinesis depends on post-Golgi vesicular traffic to generate a unique structure, cell plate, which upon expansion by continued vesicle traffic ultimately divides the cell (Jürgens, 2005). It was recently shown by our team that the *Arabidopsis* exocyst complex is involved in cytokinesis and cell plate maturation. Fendrych et al. (2010) showed that exocyst subunit EXO84, SEC6, SEC8, SEC15 and EXO70 all localize to the nascent cell plate and later to the cell plate insertion site and along the postcytokinetic wall. This is in more detail desribed in the Chapter 2.10.3.

2.9.3. Membrane recycling

A number of data indicate that the exocyst is also required for membrane recycling. In general, recycling endosomes mediate the transport of the internalized plasma membrane receptors back to the cell surface and are major sources of cargos destined to the plasma membrane in many types of cells. Loss of exocyst function blocks the recycling of internalized cargos and results in their accumulation in recycling endosomes (He and Guo, 2009).

In animal cells, small GTPase Arf6 was found to regulate membrane recycling to regions of plasma membrane remodeling via the endocytic pathway (D'Souza-Schorey et al., 1998). Arf6 interacts with exocyst subunit Sec10 and it was shown that Sec10 localization in the perinuclear region is not restricted to the *trans*-Golgi network, but extends to recycling endosomes. In addition, the depletion of Sec5 exocyst subunit or dominant inhibition of Sec10 affects the function and the morphology of the recycling pathway. Arf6 specifies delivery and insertion of recycling membranes to regions of dynamic reorganization of the plasma membrane through interaction with the vesicle-tethering complex exocyst (Prigent et al., 2003).

In human COS7 cells, BIG2 protein, a brefeldin A-inhibited guanine nucleotide exchange factor for Arf GTPase, was found to localize with recycling endosomes during transferrin (Tfn) uptake and Tfn receptor recycling. BIG2 directly interacts with exocyst subunit Exo70, which is associated with Tfn and Tfn receptor-positive recycling endosomes. Thus, it is suggested that BIG2 functions in Tfn receptor trafficking from ER, Golgi and REs in the perinuclear region toward the cell surface via interaction with exocyst complex (Xu et al., 2005; Shen et al., 2006).

In epithelial MDCK cells, the exocyst is variably localized to the Golgi apparatus, the *trans*-Golgi network (TGN), recycling endosomes and the junctional complex and is proposed to regulate basolateral-to apical transcytosis as well as apical and basolateral recycling pathways (Oztan et al., 2007).

In *Drosophila*, exocyst functions in asymmetric division of sensory organ precursors leading to development of different cell types of the mature sensory organ. Mutation in exocyst subunit Sec15 results in generation of extra neurons at the expense of support cells, a phenotype consistent with loss of Notch signaling. Dynamic traffic of Sec15, its co-localization with the recycling endosomal marker Rab11, and aberrant distribution of Rab11 as well as accumulation of vesicular compartment containing Notch in basal areas of mutant sensory organ precursors indicates that Sec15 mediates a specific membrane recycling to ensure proper neuronal fate specification in *Drosophila melanogaster* (Jafar-Nejad et al., 2005).

In plants, the exocyst complex is involved in the recycling of PIN auxin efflux carriers (Drdová et al., in preparation). PIN proteins are associated with differential localization at the plasma membrane controlling the direction of auxin transport. Polar localization of PINs is connected to their constitutive cycling between the plasma membrane and endosomal compartments. This recycling is sensitive to brefeldin-A (BFA; Dhonukshe et al., 2007). In *Arabidopsis exo70a1* and *sec8* exocyst subunit mutants, auxin transport is compromised along with recycling of PIN1 and PIN2

proteins from BFA-induced compartments back to the polarized PM domain. The fact that also BRI1 recyclinig is similarly disturbed in *exo70a1* mutant after BFA washout indicates that EXO70A1 and possibly whole exocyst is involved in general PM recycling in plant cells (Drdová et al., in preparation).

2.9.4. Secretory and membrane proteins synthesis

In MDCK cells, the exocyst complex also significantly affects the delivery as well as the synthesis of secretory and basolateral membrane proteins. The synthesis of secreted proteins is increased when the human Sec10 protein is overexpressed, despite the fact that mRNA levels are unchanged, suggesting the implication of exocyst in post-transcriptional regulation (Lipschutz et al., 2000).

Moreover, in yeast and animal cells, exocyst was found to associate with the Sec61β component of the translocon, which is furthermore increased by overexpression of Sec10. The translocon is a protein-conducting pore in the ER through which newly synthesized proteins are translocated into or across the ER membrane. This association might be one mechanism by which exocyst overexpression leads to an increase in secretory and basolateral protein synthesis. This leads to the hypothesis that exocyst is involved in the regulation of traffic through the secretory pathway, especially in coordination of events at the plasma membrane with events earlier in the secretory pathway, specifically those at the translocon. These observations represent an important link between the cellular membrane trafficking and protein synthesis machinery (Lipschutz et al., 2003; Toikkanen et al., 2003).

2.9.5. Modulating of the cytoskeleton dynamics

It was already mentioned that the exocyst functions in vesicle targeting to specific areas of the plasma membrane during neuronal differentiation, promoting neurite outgrowth and synaptogenesis (Hazuka et al., 1999). To uncover the role of the exocyst in this process, subcellular localization and molecular associations were monitored. It was shown that during neuronal differentiation, subcellular localization of exocyst changes in association with microtubules. In addition, the exocyst complex co-immunoprecipitates with microtubules from total rat brain lysate. Moreover, microtubule-disrupting drugs such as nocodazole or colchicine, affect the subcellular localization of the exocyst complex. These results indicate that exocyst complex targets vesicles toward specific plasma membrane domains through its association with microtubules, promoting neurite outgrowth and, consequently, neuronal differentiation (Vega and Hsu, 2001).

In further studies, Wang et al. (2004) tested the hypothesis that exocyst function may involve modulating of microtubules. They examined co-localization of exocyst subunits with microtubules and investigated the effect of exocyst on microtubule polymerization dynamics. They uncovered the role of intact exocyst complex in the inhibition of tubulin polymerization *in vitro*. And surprisingly, exocyst

subunit Exo70 itself is also capable of inhibiting tubulin polymerization. Additionally, deletion of exocyst subunits Sec5, Sec6, Sec15, or Exo70 diminishes their tubulin polymerization inhibition activity. Taken together, these results indicate that exocyst may coordinate vesicle trafficking and cytoskeletal organization to promote cell growth and cell differentiation.

Immunolocalization studies of exocyst complex in mammalian epithelial cells revealed that this complex is present at desmosomes, adhesive junctions that link intermediate filament networks to sites of strong intercellular adhesion. Sec3 targets exocyst to desmosomes, where it is possibly required for assembly and maintenance of functional desmosomes. Alternativelly, another possibility for exocyst function at the desmosome is that it may organize microtubule tracks required for the efficient delivery of membrane components to desmosomes (Andersen and Yeaman, 2010). This is consistent with previous suggestions of a role for the exocyst in regulating microtubule dynamics (Vega and Hsu, 2001; Wang et al., 2004).

In addition, the exocyst has been shown to modulate actin cytoskeleton. Through the interaction with Arp2/3 complex, exocyst affects invadopodia formation of tumor cells (Liu et al., 2009). Invadopodia are actin-rich membrane prostrusions formed by tumor cells that degrade the extracellular matrix for invasion. Invadopodia formation involves Arp2/3-mediated actin polymerization (Yamaguchi et al., 2005) and recently, the function of exocyst complex in this process was revealed. Inhibition of the exocyst component Exo70 or Sec8 by RNAi results in the failure of invadopodia formation. On the other hand, overexpression of Exo70 promotes invadopodia formation. It was also found that Exo70 interacts with the Arp2/3 complex in cells with high invasion potential and blocking of this interaction inhibits Arp2/3-mediated actin polymerization and invadopodia formation. Additionally, exocyst is required for secretion of matrix metalloproteinases (MMPs) at the focal degrading sites. Together, these results suggest that the exocyst plays important roles in cell invasion by mediating secretion of MMPs and regulating Arp2/3-mediated actin dynamics (Liu et al., 2009). It was then evident that Exo70, in addition to its function in exocytosis, also regulates actin dynamics and therefore coordinates both, cytoskeleton and membrane traffic, during tumor invasion.

2.10. Exocyst in plant morphogenesis and development

Plants develop an extensive range of cell shapes and sizes reflecting the specific functions they adopt. Because plant cells are surrounded by cell walls and cannot migrate, plant morphology is generated by coordinated regulation of directions in which cells divide and expand. In this process, exocytosis plays a major and irreplaceable role.

2.10.1. Polarized exocytosis and tip growth

Recent studies have implicated exocyst in polarized cell growth in plants, including such processes as root hair elongation, hypocotyls elongation, and pollen tube growth.

Unicellular root hairs elongate via localized tip growth, a process mediated by polar exocytosis of secretory vesicles. In maize, three root hair mutants, *roothairless1*, *2*, and *3* (*rth1*, *rth2*, *rth3*) are impaired in root hair elongation (Wen and Schnable, 1994). Later it was revealed that *RTH1* gene encodes a homolog of exocyst subunit SEC3. This was the first hint that exocyst is involved in exocytotic developmental process in plants – elongation of root hairs (Wen et al., 2005).

In *Arabidopsis thaliana*, disruption of the *EXO70A1* gene leads to a similar phenotype. Mutant seedlings germinate normally and their cotyledons reach the same size and shape as wild-type plants. However, *exo70A1* seedlings have shorter roots, retarded initiation of lateral roots and defects in root hairs elongation. Similarly, mutations in EXO70A1 significantly reduce the number and length of hypocotyl cells in etiolated seedlings, which result in shortened hypocotyls (Synek et al., 2005). Furthermore, combination of this mutation with mutations in other two exocyst subunits (SEC8 or SEC5a) that normally do not display vegetative phenotypic defects, results in more severe reduction of hypocotyl elongation in etiolated seedlings than the *exo70A1* mutation alone. This synergistic effect indicates a functional relationship between exocyst subunits (Hála et al., 2008).

In contrast, growth of pollen tube, another example of tip-growing cell, harboring the *exo70A1* mutation is probably unaffected. In spite of this, *exo70A1* mutants are almost completely sterile. But this is rather due to combination of underdeveloped stigmatic papillae and aberrant pollen development caused by sporophytic defects in anther function. Anthesis is delayed and all anthers contain fewer pollen grains compared with the wild-type. Pollen is also unable to germinate *in vitro* and to pollinate wild-type flowers. (Synek et al., 2005). On the other hand, exocyst subunits SEC5, SEC6, SEC8, and SEC15a were shown to be involved in pollen germination and pollen tube growth in *Arabidopsis* (Cole et al., 2005; Hála et al., 2008). Mutations of any of these four exocyst components dramatically reduce pollen germination. However, mutant grains can occasionally produce pollen tube. The mutant pollen tubes are significantly shorter and thicker than the wild-type pollen tubes. It was also shown that exocyst subunits co-localize at the tip of growing pollen tubes, reflecting its role in the regulation of the intensive secretion at that site (Hála et al., 2008).

Defects in pollen tube germination and growth in the mutants mentioned above, result in reduced transmission of the mutant alleles through the male. T-DNA insertions in *SEC6*, *SEC8*, and *SEC15a* cause an absolute, male-specific transmission defect. Similarly, the *sec5a* and *sec5b* alleles were never transmitted through the pollen together, although neither single mutation demonstrated a transmission defect, reflecting a redundant function for these genes in pollen (Cole et al., 2005; Hála et al., 2008).

2.10.2. Shoot meristem function

Other dramatic phenotypic alterations in *exo70A1* mutants occur in the inflorescence architecture and lifespan. In spite of normal bolting time, secondary inflorescences are initiated earlier. Furthermore, instead of initiating floral meristems at the flanks of the apical meristem, *exo70A1* mutants develop lateral secondary inflorescence meristems, resulting in an indeterminate highly branched inflorescence with cauline leaves. This repetitive pattern of secondary inflorescences correlates with a significantly delayed senescence. The lifespan of *exo70A1* plants is approximately 5 months, which is more than twice the lifespan of wild-type plants (Synek et al., 2005).

2.10.3. Cytokinetic defects

While *exo70A1* seedlings resemble the wild-type phenotype immediately after germination on light, upon the formation of true leaves, the shoot phenotype changes to semidwarf plants with much smaller rosette leaves. Comparison of the size and number of epidermal cells of rosette leaves shows that the cell size is not changed, indicating that the smaller leaves are a result of fewer cell divisions (Synek et al., 2005). Similar observation has been reported after disruption of exocyst subunit EXO84b. Mutants defective in EXO84b exhibit a dwarf phenotype with cytokinetic defects. Their leaves are angular with small and irregularly developed trichomes. On the cellular level, the mutant leaf epidermis contains cell wall stubs, indicating a failure in cytokinesis. Cytokinetic defects occur also during stomata development, resulting in some morphological abnormalities. They are ranging from aberrant highly asymmetric stomata and stomata with imperfectly divided guard cells with incomplete ventral walls to single guard cells lacking any viable counterparts (Fendrych et al., 2010).

It was also shown that EXO84b, SEC6, SEC8, SEC15b, and EXO70A1 all localize distinctly to the nascent cell plate and later to the cell plate insertion site after the cell plate attached to the mother wall. To further support the involvement of exocyst in plant cytokinesis, it was shown that mutation in EXO70A1 affects the cell plate morphology, especially in the moment of its appearance. In the *exo70A1* mutants, the cell plate emerges by fusion of visible spots, forming a donut-shaped or horseshoe-shaped structure with an empty space in its center. Mutant cell plates further expand not only centrifugally but also centripetally to fill the central gap. This gap is probably caused by the inability of vesicles to fuse efficiently. Based on these observations, it was proposed that exocyst is involved in cytokinesis in plant cells. Exocyst may trigger the initial cell plate vesicle fusion as well as facilitating the delivery of specific cargoes necessary for cell wall maturation (Fendrych et al., 2010).

2.10.4. Seed coat development

Consistent with its role in vesicle trafficking, exocyst functions in polarized pectin delivery during seed coat development in *Arabidopsis thaliana*. T-DNA insertions in SEC8 and EXO70A1 results in considerable deviations from normal seed coat development, especially reduced pectin deposition and defects in the formation

of the central columella of seed epidermal cells. In addition, an interacting partner of EXO70A1 was identified, a protein named ROH1, which is a member of a previously uncharacterized, plant-specific family. A mutational misregulation of ROH1 results in a seed coat developmental defect reminiscent of that seen in the exocyst subunit mutants. Participation of exocyst subunits in mucilage deposition provides direct evidence for the role of the exocyst in polarized cell wall morphogenesis (Kulich et al., 2010).

2.10.5. Plant-pathogen interaction

Plants have evolved multiple defense strategies for combating invading pathogens. Based on the involvement of exocyst in the regulation of secretory pathway and the cell polarity, it is expected that exocyst might influence the capability of the plant cell to react defensively against pathogen attack. It was demonstrated that EXO70B2 and EXO70H1 are strongly up-regulated by treatment with an elicitor peptide, elf18, derived from the bacterial elongation factor. The insertional mutation in these genes enhances the susceptibility of plants to *Pseudomonas syringae* attack. It is speculated that the exocytosis defects in *exo70B2* and *exo70H1* mutants render the apoplastic environment more permissive for bacterial growth (Pečenková et al., 2011).

Beneath the attempted pathogen penetration sites, the cell wall defensive appositions called papillae are formed. And it was revealed that exocyst is required for proper formation of papillae after infection by *Blumeria graminis*. Mutants in exocyst subunit EXO70B2 shows apparent deviations in the cell wall structure. An increase in the proportion of abnormal papilla formation, with and over-accumulation of vesicle-like structures around *B. graminis* attacking site, was found in *exo70B2* mutants (Pečenková et al., 2011)

Chapter 3

MATERIAL AND METHODS

3.1. Phylogenetic analysis of SEC15

Eukaryotic Sec15 coding sequences were identified by PSI-BLAST (Altschul et al., 1997) with default settings searching against the non-redundant protein database with the yeast Sec15 as a query. The MAFFT algorithm in E-INSI mode was used to construct a multiple alignment of C-terminal domain (corresponding to Drosophila Sec15 crystal structure, PDB code 2a2f) of selected Sec15 proteins. The final figure and secondary structure assignment was produced using the Jalview alignment editor (Waterhouse et al., 2009). For phylogenetic tree production, maximum-likelihood analysis was performed with PhymL 3 software (Guindon and Gascuel 2003) using the following parameters: WAG+Γ+I substitution model, equilibrium amino-acid frequencies and proportion of invariable sites estimated from the dataset, four relative substitution rate categories with gamma distribution shape parameter estimated from the dataset.

Al sequences are based on genomic data in the GenBank database (https://www.ncbi.nlm.nih.gov/GenBank), except for *Populus trichocarpa* and *Chlamydomonas reinhardtii*, which were retrieved from Phytozome (https://www.phytozome.net).

3.2. Expression data analysis

Public expression data from experiments using the Affymetrix ATH1 *Arabidopsis* genome array were retrieved from the Genevestigator database (https://www.genevestigator.com; Zimmermann et al., 2004). For our purpose, we selected only arrays from wild-type plants grown under physiological conditions. List of experiments used for the analysis is listed in Supplemental Data 2. Average values of normalized signal intensities were determined. For representational purposes, expression values were grouped into six categories (0-400, 401-800, 801-1200, 1201-1600, 1601-2000, > 2000).

To search for potential interacting partners and determine whether the corresponding genes showed coordinated expression patterns, we used the Expression

Angler program of the Botany Array Resource (BAR) (http://bar.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi; Toufighi et al., 2005). The software calculates the similarity of expression patterns to the marker genes for the other genes in the database using a Pearson correlation coefficient (*r* value). To this purpose we filtered the dataset with correlation coefficients larger than a threshold of 0.5. Expression Angler finds co-expressed genes in data sets from the AtGenExpress Consortium (Schmid et al., 2005), from The Botany Array Resource (Toufighi et al., 2005), or from NASCArrays (Craigon et al., 2004). The expression values in all cases were measured using Affymetrix's GeneChip microarray technology.

3.3. Biological material

Bacterial strains were used as follows: *Escherichia coli* DH5α, *Escherichia coli* XL1 – Blue, *Escherichia coli* BL21 Codon+, *Escherichia coli* Rosetta, *Agrobacterium tumefaciens* GV3101. All were grown on LB (Luria Broth) medium.

Yeast strains were used as follows: *Saccharomyces cerevisiae* AH109 (*MATa*, *trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, MEL1, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2, URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ*), *Saccharomyces cerevisiae* NY64 (*MATa, ura3-52, sec15-1*). All were grown on YPD (yeast extract peptone dextrose) medium.

Suspension culture of *Nicotiana tabacum* Bright Yellow-2 (BY-2) was cultivated in LS medium (Linsmaier-Skoog), *Arabidopsis thaliana* suspension culture was cultured in MS medium (Murashige-Skoog, Sigma) containing Gamborg B5 vitamins (Duchefa). Both were grown in a dark place with shaking. Cells for transformation were harvested at the late exponential phase (4-6 day).

3.4. Plant material and growth conditions

Arabidopsis thaliana (ecotype Columbia-0) seeds were surface sterilized (1 min in 70% ethanol, 10 min in 10% bleach and washed three times with sterile distilled water), stratified at 4°C for 3-5 days and planted onto Petri dish with growth medium (½ Murashige and Skoog medium supplemented with 1% (w/v) sucrose, 0.01% (w/v) myoinositol, 0.05% (w/v) MES, 0.0001% [w/v] nicotinic acid, 0.0001% [w/v] thiamine-HCl, 0.0001% [w/v] pyroxidine-HCl and 1.6% agar) supplemented with additional required reagents – Basta (PPT) in concentration 20 mg/l, or claforan antibiotic (Sephotac) in concentration 100 mg/l. Plants were grown in a climate chamber at 22°C under long day conditions (16h light/8h dark) until the development of true leaves (cca 10 days). After that, seedlings were transferred from plates onto the Jiffy pellets (www.jiffypot.com) and further cultivated under the same conditions.

Nicotiana benthamiana plants were sown directly into the soil and cultivated under the same conditions as *Arabidopsis thaliana* plants.

3.5. Genotype analysis of T-DNA insertional mutants

T-DNA insertions in *AtSEC15b* gene (SALK_042723) were examined by PCR genotyping of individual plants. Total DNA for genotyping was extracted from young seedlings according to DNazol Reagent protocol (GibcoBRL, Invitrogen). Briefly, 50 mg of plant tissue was pulverized in liquid nitrogen to obtain homogenous powder. The frozen powder was transferred to a microcentrifuge tube containing DNazol Reagent (usually 150 μl of DNazol for 50 mg of plant tissue). The solution was mixed by gentle inversion and incubated at 25°C with shaking for 5 min. After that, 150 μl of chloroform was added, mixed vigorously, and further incubated at 25°C for another 5 min. Extract was centrifuged at 12 000 x g for 10 min and resulting supernatant was transferred into a fresh tube. DNA was then precipitated with 112 μl of 100% ethanol.

PCR was performed with DreamTaq DNA polymerase (Fermentas) using the following cycle: 95°C for 3 minutes, then 30 cycles of 95°C for 30 seconds, 59°C for 30 seconds and 72°C for 70 seconds, and finally 72°C for 10 minutes. The sequences of primers used for genotypic analysis are shown in Supplemental Data 1.

3.6. Crossing of *Arabidopsis* plants

On the recipient plant, all young flower buds, opened self-fertilized flowers and developing siliques were cut off. In a chosen flower with immature anthers, all flower parts except the ovary were removed with using the clean tweezers. From the donor plant, mature stamens were removed and brushed onto the prepared stigma at the recipient plant to release the pollen. When plenty of pollen was at the tip of the ovary, it was wrapped with the foil for 24h to avoid stigma dessication and undesired pollen contamination.

3.7. RNA isolation and RT-PCR

The total RNA from 100 mg of plant material was isolated using RNeasy Plant Mini Kit (Qiagen) according to manufacturer's protocol. RNA concentration was measured spectroscopically and optimal amount of RNA was used for reverse transcription. The first strand cDNA was synthesized by PCR using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to manufacturer's instructions with oligo(dT) primer.

3.8. Cloning of constructs for yeast two-hybrid assay

Standard molecular cloning methods were used to generate constructs of interest. Full-length *AtSEC15b* and selected *AtRAB* genes were PCR amplified using cDNA from 7-day-old *Arabidopsis* seedlings, specific set of primers (listed in Supplemental data 1) and Phusion high-fidelity DNA polymerase (Finnzymes). *AtSEC15b* was cloned in frame, using BamHI and XhoI restriction sites, into pGADT7

vector, designed to express a protein of interest fused to a GAL4 activation domain (AD) under the ADH1 promoter. Individual *AtRAB* genes were cloned in frame via BamHI and SalI restriction sites into pGBKT7 vector, expressing proteins fused to GAL4 DNA binding domain (DNA-BD) under the ADH1 promoter. All final constructs were sequenced.

Constitutively active (GTP-bound) and dominant-negative (GDP-bound) forms of AtRABA4a GTPase were made using PCR-based techniques. Point mutations to generate the amino acid substitutions S(31)N or Q(76)L in the AtRABA4a sequence were created by two step PCR using pGBKT7::AtRABA4a as a template. In the first round, mutated fragments were obtained by amplifying the segment of AtRABA4a wild-type gene with one flanking primer and one mutagenized primer. Then this fragment was used in combination with another flanking primer to obtain the full-length mutagenized gene (primer sequences are listed in Supplemental data 1). Resulting mutagenized fragments were cloned into the pGBKT7 expression vector using BamHI and SalI restriction sites and sequenced.

3.9. Yeast two-hybrid assay

The MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech Laboratories, Inc.) was used for two hybrid screening and all steps proceed as described in the manufacturer's protocol. The yeast strain AH109 was transformed with AtSEC15b fused with GAL4 activation domain and selected AtRABs fused with GAL4 DNA binding domain. Yeast were grown on –LEU–TRP selective media at 30°C and then transferred to –ADE–HIS–LEU–TRP selective media and simultaneously to filter paper for β-galactosidase assay. A murine p53 fused with the DNA binding domain and SV40 large T-antigen fused with the activation domain were used as a positive control. A combination of empty pGADT7 and pGBKT7 vectors was used as a negative control. All was provided by manufacturer's kit. Strength of interaction was determined by serial dilutions prepared in sterile distiled water and dropped on highly selective media (–ADE–HIS–LEU–TRP).

3.10. Yeast complementation

The *Saccharomyces cerevisiae sec15-1* strain NY64 was a kind gift from Peter Novick (University of California, San Diego; Salminen and Novick, 1987). Coding sequence of *AtSEC15b* was cloned via BamHI and XhoI restriction sites into the yeast expression vector pVT-103U (Vernet et al., 1987). This plasmid contains the URA3 selectable marker and the yeast alcohol dehydrogenase (ADH1) promoter assuring a high level of gene expression on media containing glucose as a carbon source. Yeasts were transformed according to LiAc transformation procedure and transformants were selected on -URA plates. Single colonies were resuspended in 100 μl of sterile water and several dilutions were prepared. 10 μl of each dilution was dropped on –URA selective plates, which were then incubated for 5 days at 28°C or 37°C.

3.11. Protein electrophoresis and immunoblotting

Protein samples were incubated in SDS-PAGE sample buffer at 95°C for 10 min before separation in 8%, 10% or 12.5% polyacrylamide gels. Protein samples were transferred from gels to nitrocellulose membranes (Optitran BA-S 83, Whatman) using semi-dry blot (Biometra, 1 mA per 1 cm^2 gel area). The membrane was blocked in 5% non-fat dry milk in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, with pH odjusted to 7.4) supplemented with 0.1% Tween-20 at 4°C overnight. This solution was also used for further steps. Primary antibodies were incubated with membrane at room temperature for 1 h. After 3 washes, 10 min each, the membrane was incubated with secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Promega) at room temperature for another 1 h and then washed as above. Proteins detected by antibodies were visualized using ECL Western blotting detection reagents (Amersham Pharmacia Biotech) according to manufacturer's instructions. Resulting chemiluminescence was detected by exposure to X-ray film (Kodak).

3.12. Antibody preparation

N-terminal half of *AtSEC15b* (1 – 380 aa) and *AtRABA4a* full-length were PCR amplified using pGADT7::*AtSEC15b* and pGBKT7::*AtRABA4a*, respectively, as templates. Specific set of primers introducing BamHI and SalI restriction sites (listed in Supplemental Data 1) were used and PCR fragments were cloned in frame into expression vectors. N-terminal *AtSEC15b* was cloned into pGEX-4T2 vector (Amersham Pharmacia Biotech), and *AtRABA4a* was cloned into pQE32 vector (Qiagen). Each of resulting constructs was transformed into *Escherichia coli* BL21 Codon+ competent cells and the expression of recombinant protein was induced with 2μM IPTG. Because of insolubility of N-terminal AtSEC15b, cells expressing this protein were boiled in SDS-PAGE sample buffer and proteins were resolved by the preparative SDS-PAGE. Stripes containing the GST:N-AtSEC15b were excised from several preparative gels and homogenized in a buffer containing 150 mM NaCl, 1 mM KCl abd 1 mM Na,K-phosphate, with pH adjusted to 7.0, using the Dounce homogeniser. The yielded suspension was directly used for mouse immunization.

Cells expressing His(6x)-tagged AtRABA4a were resuspended in PBS buffer supplemented with 1% Triton X-100, incubated 30 min on ice and sonicated 5 times for 30 sec using ultrasonic homogeniser SONOPULS (Bandelin). Protein was purified from cell lysate using Ni-NTA agarose (Qiagen) and subsequently eluted with immidazol. Purified protein was dialysed against PBS overnight at 4°C, concentrated by ultracentrifugation using Centricon tubes (Millipore) and used for mouse immunization and preparation of polyclonal antibodies (Biopharm Research Institute of Biopharmacy and Veterinary Drugs, Jílové u Prahy). The resulting antibodies were used at a 1:1000 dilutions and it was verified that both obtained antisera recognize a desired antigen in plant extracts.

3.13. Co-immunoprecipitation

Protein complexes with GFP-tagged proteins were isolated using μMACS GFP Tagged Protein Isolation Kit (Miltenyi Biotec) with some modifications of manufacturer's protocol. Approximately 1 g of 10-day-old *Arabidopsis* seedlings were homogenized in liquid nitrogen resuspended in 2 ml of provided lysis buffer supplemented with Protease Inhibitor Coctail (Sigma) and centrifuged at 10 000 x g at 4°C for 15 min. The supernatants were equilibrated to the same protein concentration (approximately 50 μg/ml) using the Bradford assay (Sigma). 100 μl of anti-GFP microbeads were added and incubated for 30 min on ice with shaking. The samples were washed on the column four times with the lysis buffer and once with provided Wash buffer 2. Immunoprecipitates were eluted with 70 μl of the elution buffer. Isolated proteins were separated on 10% polyacrylamide gel, blotted onto a nitrocellulose membrane and immunodetected. Primary antibodies, anti-AtRABA4a, anti-AtSEC15b, and anti-GFP (Roche), were diluted 1 : 1000. Anti-mouse secondary antibody was diluted 1 : 10 000.

3.14. Light microscopy

Microscopic pictures were acquired with the DP50 camera (Olympus) attached to Olympus BX51 microscope. For pollen germination experiments, *Arabidopsis* pollen was transferred from the mature anthers into the liquid pollen germination medium (18% sucrose, 0.01% H_3BO_3, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, with pH adjusted to 6.5). A small droplet (70 μl) of germination medium was placed onto microscopic glass slide and drop surface was touched by the extracted anthers several times to release the pollen. Each slide was incubated in a humid chamber at 22°C in dark for 12-16 h and subsequently observed. To achieve better germination efficiency, pistil was added into germination medium and co-cultivated with the pollen. For silique observation, mature siliques were cut off and then decolorized by incubation in a mixture of ethanol and acetic acid (3:1) supplemented with 10% household bleach at room temperature overnight. Siliques were then washed with 30% ethanol for 30 min and finally submerged in water. They were subsequently observed using light microscope.

3.15. Fluorescent protein fusions

Full length *AtSEC15b* was PCR amplified using genomic DNA of wild-type *Arabidopsis thaliana* Col-0 by Phusion high-fidelity DNA polymerase (Finnzymes). Primers used for the amplification are listed in Supplemental Data 1. PCR fragment was cloned in frame into the modified binary vector pBAR (kindly provided by Ben Holt, University of North Carolina) using BamHI and XbaI restriction sites. In this case, pBAR was modified by inserting a GFP cassette for N-terminal fusions driven by 35S promoter (prepared by Marek Eliáš). The sequence of coding region in the resulting construct was verified by sequencing.

Full length *AtRABA4a* was PCR amplified using cDNA from 7-day-old *Arabidopsis* seedlings also by Phusion polymerase. Primers used for the amplification are listed in Supplemental Data 1. PCR fragment was cloned in frame into the pBAR vector modified by inserting an mRFP cassette for N-terminal fusions driven by 35S promoter (prepared by Michal Hála). Finally, resulting construct was checked by sequencing.

Plants expressing GFP:BP-80 (Kotzer et al., 2004) were a gift from Ian Moore (University of Oxford). Construct of ST:YFP (Grebe et al., 2003) and VHA-a1:GFP (Dettmer et al., 2006) was kindly given by Karin Schumacher (University of Heidelberg). Plants expressing YFP:AtRABE1d or YFP:AtRABA5d (Geldner et al., 2009) were ordered from The Nottingham Arabidopsis Stock Centre (NASC; http://arabidopsis.info).

3.16. Transient transformation of suspension culture by particle bombardment (Gene Gun)

Arabidopsis and tobacco suspension cultures were transformed using Helios Gene Gun System (BioRad). Prior to transformation, the plasmid DNA was attached to the gold particles according to manufacturer's protocol. In general, this was accomplished by precipitation of the DNA (plasmids expressing mRFP:AtRABA4a or GFP:AtSEC15b, separately or together) from the solution in the presence of gold microcarriers (0.6 μm in diameter) and the spermidin by the addition of $CaCl_2$. The particles were then washed extensively with ethanol to remove the water and resuspended in ethanol. Using the Tubing Prep Station, the DNA/gold solution was coated onto the inner wall of the plastic tube and dried. The tube was then cut to approximately 1 cm length cartridges, which were inserted into the cartridge holder of the Helios Gene Gun and gold particles with the DNA were introduced into the suspension culture by the helium discharge. The expression of fusion proteins was observed after 12 h.

3.17. Transient transformation of tobacco leaves by *Agrobacterium* – mediated infiltration

The *Agrobacterium tumefaciens* strain GV3101 was transformed with plasmids of interest by electroporation. Transformed cells were grown overnight at 28°C in LB medium supplemented with appropriate antibiotics, sedimented, washed twice with an infiltration buffer (50 mM MES pH 5.6, 2 mM Na_3PO_4, 0.5% glucose, 20 mM $MgSO_4$) and finally resuspended in the infiltration buffer supplemented with 200 μM acetosyringone (Sigma) to adjust the cell density to $O.D._{600}$ 0.2. Additionally, suspension of *Agrobacterium* transformed with a plasmid expressing the p19 protein from *bushy stunt tomato virus* (Voinnet et al., 2003) to reduce silencing of delivered genes, was similarly prepared. The same volumes of both suspensions were mixed and infiltrated into young leaves of *Nicotiana bentramiana* through stomata of the lower epidermis. Leaves were observed 1-3 days after infiltration.

3.18. *Agrobacterium* – mediated stable transformation of *Arabidopsis*

For stable transformation of *Arabidopsis thaliana*, we performed the most widely used floral dip method (Clough and Bent, 1998). *Arabidopsis* plants were grown as a lawn at a density of approximately 100 plants/12 cm pot until they were flowering. All mature flowers and fertilized siliques were clipped and only immature floral buds were retained. *Agrobacterium* cells were grown in LB medium containing appropriate antibiotics, harvested by centrigufation and then resuspended in 10% sucrose supplemented with 0.05% surfactant Silwet L-77 (Lehle Seeds). Aboveground parts of plant, especially inflorescence, were dipped into *Agrobacterium* solution for a few seconds with gentle agitation. Plants were covered with a foil for 16-24 h to maintain high humidity and placed in a dark place. Plants were then returned to normal growing environment and mature seeds were collected for selection.

3.19. Confocal microscopy

For confocal microscopy analysis, plant material, mounted in a drop of water under a coverslip or in a half-strength MS agar inside a chambered coverglass Lab-Tek II (Lab-Tek), respectively, was observed with Zeiss LSM 5 DUO confocal laser scanning microscope using the C-apochromat 40x/1.2 water-immersion lens. GFP, YFP and mRFP were imaged using single track detection. GFP and YFP were excited by 488 nm laser line and detected with a 505-550 nm band pass filter. mRFP and FM4-64 were excited by a 561 nm laser and detected with a 575-615 nm band pass filter. The excited light was splitted by a NFT 565 beam splitter. LSM software (Zeiss) was used for postacquisition image processing.

To visualize a cytokinesis, *Arabidopsis* seedlings were labeled with endocytic marker FM4-64 (Invitrogen). The dye was resuspended in water to a final concentration of 5 μm and added into the chambered cover glass and incubated for 10 min prior to observation.

Chapter 4

RESULTS

4.1. Phylogenetic analysis of SEC15 genes in eukaryotes

To gain closer insight into the distribution and evolution of Sec15 subunit of exocyst complex, we performed initial *in silico* analysis of its distribution and evolution across eukaryotes. Although the experimentally characterized Sec15 orthologs from yeast and mammals show very low level of sequence conservation (human and yeast Sec15 share only 14% identical and 34% similar residues), genes clearly homogous to Sec15 were identified in all major eukaryotic kingdoms with completely sequenced genomes, i.e. in Ophistokonta, Amebozoa, Plantae, Chromalveolata and Excavata (Figure 4.1 and data not shown). Sec15 is typically represented as single-copy gene or as small gene family with two to four paralogs per genome. This is true also for plants where green algae posses one Sec15 gene and higher plant genomes contain 2-4 copies. To resolve further the evolutionary history of Sec15 genes we performed maximum likelihood and distance analysis of 34 genes from 9 completely sequenced plant genomes and 11 non-plant model organisms (Figure 4.1). Our results suggest that eukaryotic common ancestor had one Sec15 gene which further diversified after separation of plants and other eukaryotic lineages. Within plant kingdom, Chlorophyta retained single copy of Sec15 while one major duplication occurred during evolution of vascular plants as documented by clearly separated Sec15a and Sec15b subfamilies. Figure 4.1 suggests that the diversification of Sec15a and Sec15b occurred after separation of mosses and ferns from the path leading to seed plants. In addition, several independent duplication events led to further duplication of Sec15 in Physcomitrella, Sec15a in monocots and both Sec15a and Sec15b in some dicots (e.g. poplar).

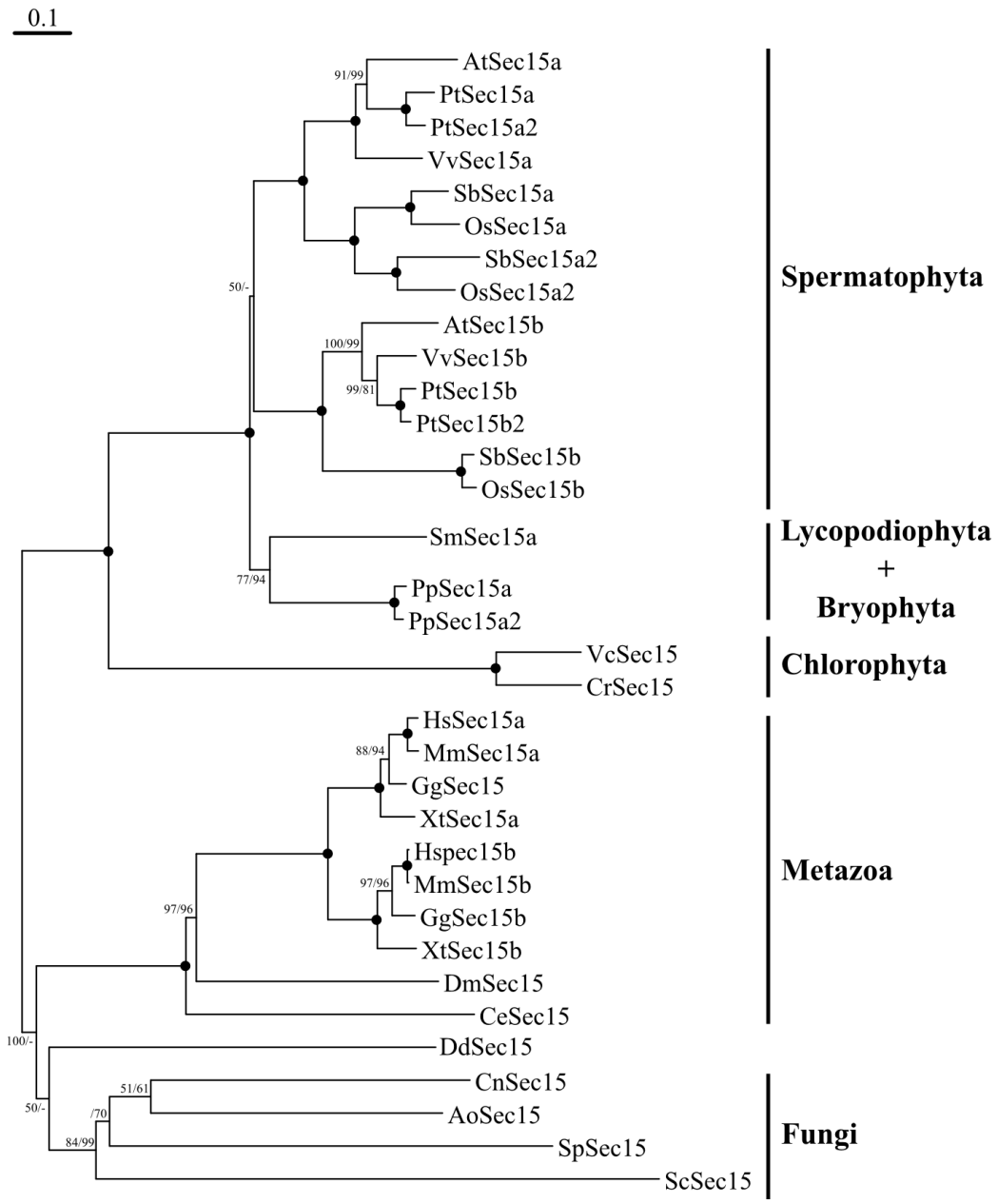
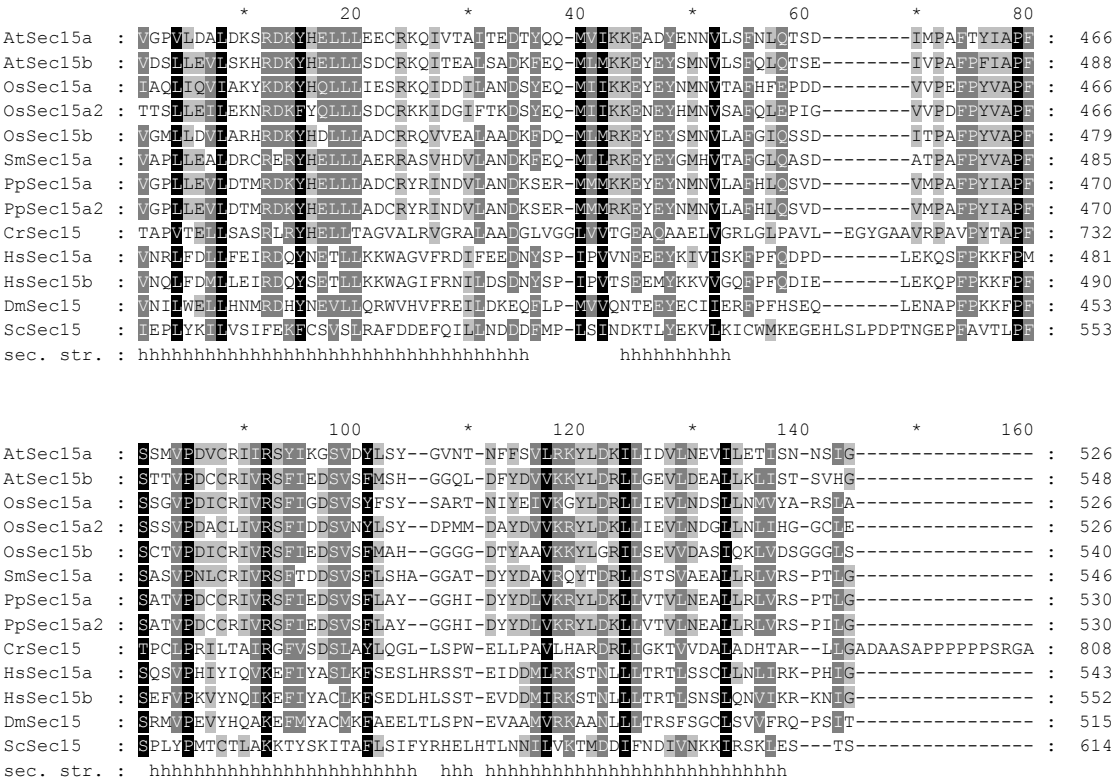


Figure 4.1. Phylogenetic analysis of Sec15 family

Evolutionary tree of 34 Sec15 protein sequences obtained from nine plant and eleven non-plant species. The branch lengths indicate the distances between operational taxonomic units (OTUs); approximate likelihood ratio test support values (aLRT) and bootstrap values from neighbor-joining (BioNJ) analysis are shown for nodes that received support over 50% with black circles representing 100% aLRT and bootstrap support; dash indicates that different topology was suggested by BioNJ method. The scale bar represents 0.1 substitutions/site. Species abbreviations: Ao, *Aspergillus oryzae*, At, *Arabidopsis thaliana*; Cn, *Cryptococcus neoformans*; Cr, *Chlamydomonas reinhardtii*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Pt, *Populus trichocarpa*; Sb, *Sorghum bicolor*; Sc, *Saccharomyces cerevisiae*; Sm, *Selaginella moellendorffii*; Sp, *Schizosaccharomyces pombe*; Vc, *Volvox carteri*; Vv, *Vitis vinifera*; Xt, *Xenopus tropicalis*.

Closer inspection of Sec15 multiple alignment suggests that in contrast to some other exocyst subuntits (e.g. Sec3 or Exo84), all Sec15 homologs retained similar primary structure of approximately 800 aminoacids consisting of alternating alpha-helices and loops creating a rod-like structure (Wu et al., 2005; Croteau et al., 2009). As noted above the level of sequence identity among differnt eukaryotic lineages is very low with the helical parts being more conserved than loop regions. In higher plants, Sec15a and Sec15b typically share approximately 45% identical residues (47% betwen *Arabidopsis* Sec15a and ec15b) while orthologous genes are more conserved (75% between *Arabidopsis* and poplar Sec15a, 77% between *Arabidopsis* and poplar Sec15b).

Wu et al. (2005) described the interaction site for Rab11 on Sec15 from *Drosophila*. Sec15 binds Rab11 mainly through single alpha helix located at the C terminus. These aminoacids are located at the exposed side of protein are flanked by highly conserved residues. Interestingly, although the Rab-interacting helix is generally well conserved, residues revealed by site-directed mutagenesis in *Drosophila* Sec15 exhibit rather high variability (Figure 4.2), even between Sec15a an Sec15b.



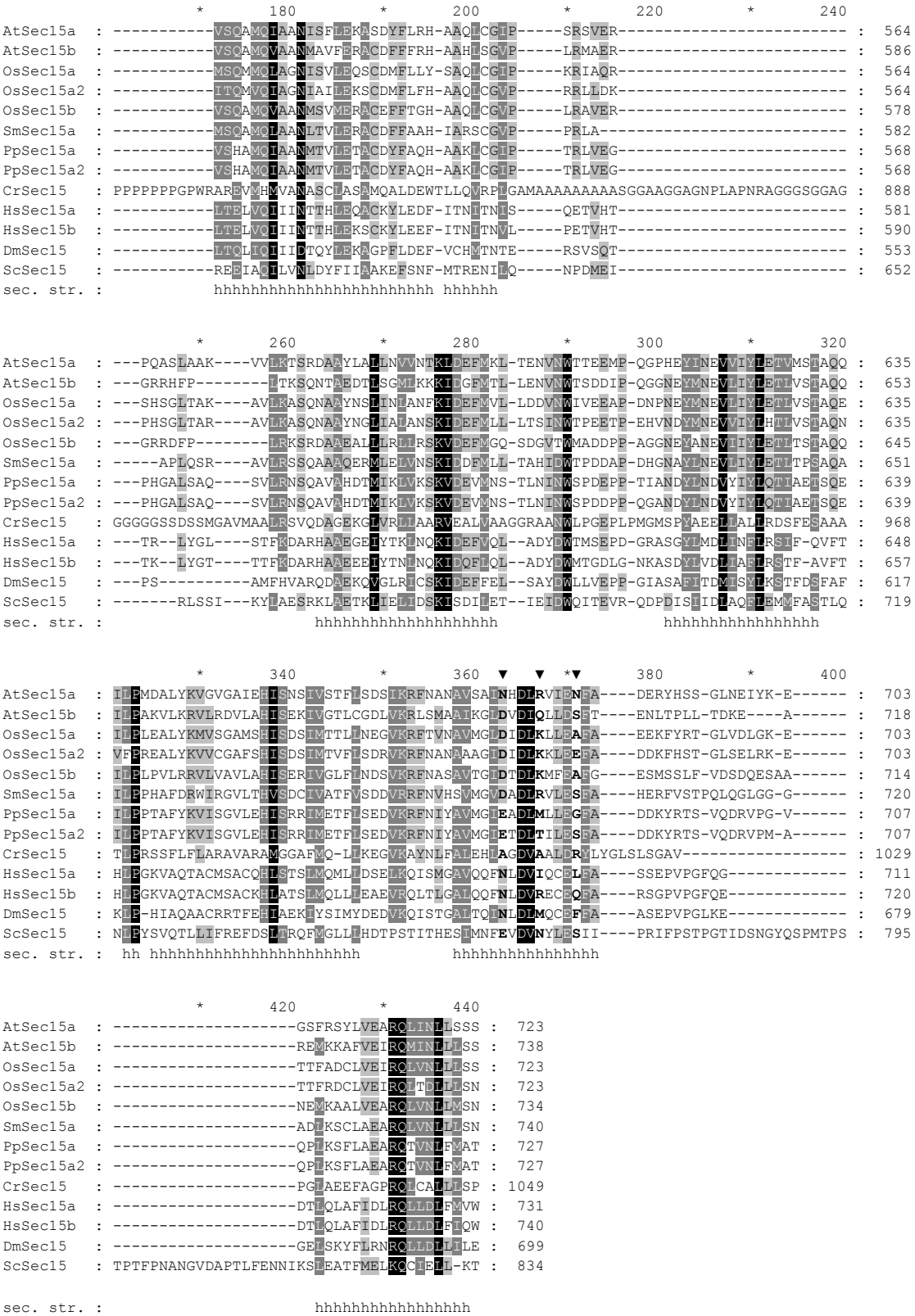


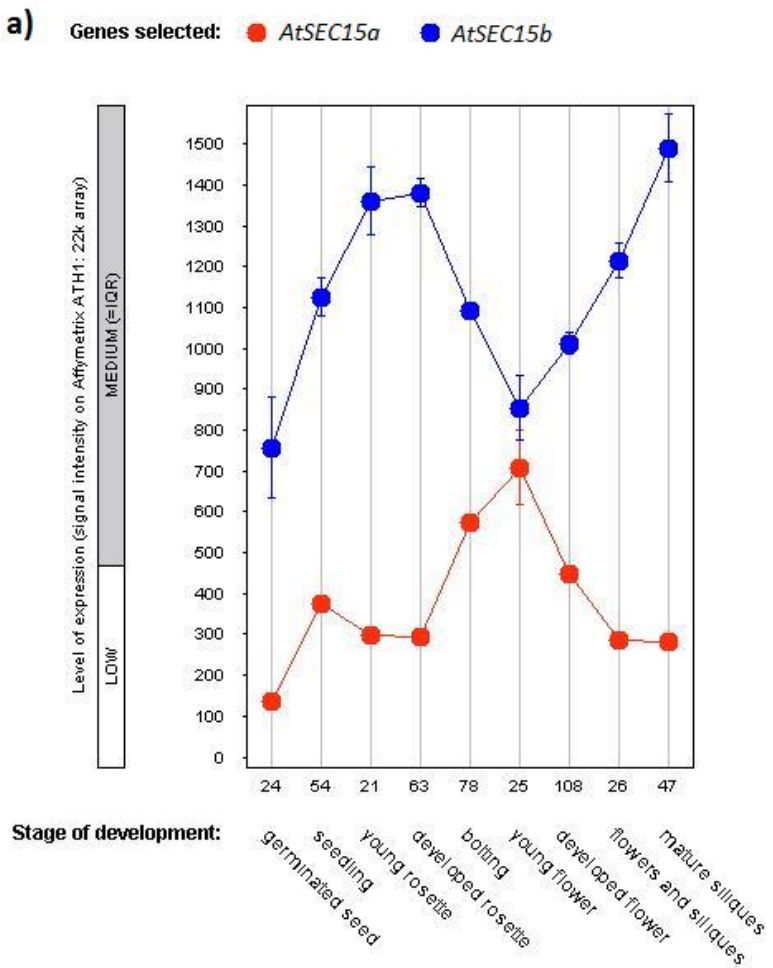
Figure 4.2. Sequence analysis C-terminal region in Sec15.

Multiple alignment of C-terminal domain of selected Sec15 proteins. The inverted black triangles indicate candidate residues involved in binding to Rab GTPase. Species abbreviations: At, *Arabidopsis thaliana*; Cr, *Chlamydomonas reinhardtii*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Sc, *Saccharomyces cerevisiae*; Sm, *Selaginella moellendorffii*.

4.2. Expression databases based analysis of expression pattern for the exocyst subunits SEC15 in *Arabidopsis thaliana*

To gain insights into the developmental and organ-specific regulation of the expression of SEC15 in *Arabidopsis thaliana*, we analysed the publicly available expression data for selected genes from the Genevestigator database (Zimmermann et al., 2004). For this purpose we analysed only expression data from wild-type plants grown under the physiological conditions.

SEC15 is present as a two-paralogous gene in *Arabidopsis* genome (Eliáš et al., 2003). We examined the expression of both paralogues, *AtSEC15a* and *AtSEC15b,* in *Arabidopsis thaliana*. Comparison between the expression level of these genes showed that both are expressed throughout the *Arabidopsis* development with *AtSEC15b* being much more abundant (Figure 4.3a). In more detailed analysis of expression data from individual *Arabidopsis* organs, more significant increase in *AtSEC15a* expression was found only in pollen indicating that AtSEC15a seems to be pollen-specific whereas AtSEC15b is probably a major SEC15 protein expressed in *Arabidopsis thaliana* (Figure 4.3b). This is also the reason why we focused on characterization of AtSEC15b subunit in this thesis.



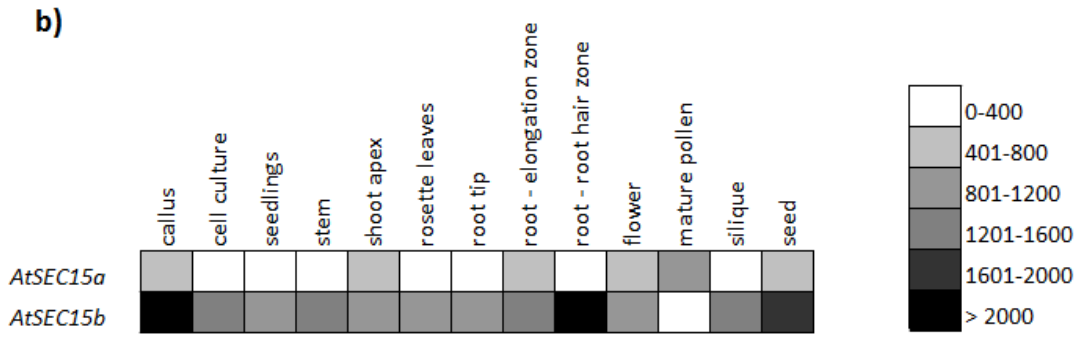


Figure 4.3. Expression analysis of *Arabidopsis SEC15* genes.
a) Level of *AtSEC15a* (in red) and *AtSEC15b* (in blue) expression during *Arabidopsis* development.
b) Normalized expression data extracted from the Genevestigator database. Particular microarray experiments (chip) included in this analysis are listed in Supplemental data 2.

4.3. Interaction of exocyst with Rab GTPases

In yeast and animals, the interaction between exocyst and small GTPases from Rab family was well described (Guo et al., 1999b; Zhang et al., 2004; Wu et al., 2005). Here, we were trying to determine if the mechanism of the post-Golgi to membrane targeting of exocytic cargo is conserved during evolution via examination of possible interaction between exocyst and Rab GTPases in plant cells.

4.3.1. Searching for potential AtSEC15b interactors via co-expression analysis

In order to identify candidate proteins for the interaction with AtSEC15b we were initially searching for genes co-expressed with *AtSEC15b* using the Expression Angler program on the Botany Array Resource (BAR) data set (https://bar.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi; Toufighi et al., 2005). We found a significantly similar expression pattern between AtSEC15b and two proteins from Rab GTPase family – AtRabH1c and AtRABA4a. This observation might suggest related functions of these proteins. AtRABH1c is homologue of the yeast Ypt6, protein, which takes part in a retrograde protein transport (Bednarek et al., 1994; Johansen et al., 2009), whereas AtRABA4a is homologous with Rab11 GTPase, protein interacting with the exocyst subunit Sec15 in mammalian cells in a GTP-dependent manner (Zhang et al., 2004). Thus AtRABA4a became a most interesting candidate for studying possible interaction with the exocyst.

4.3.2. Yeast two-hybrid assay revealed direct interaction between AtSEC15b and AtRABA4a

The interactions between exocyst subunit AtSEC15b and several Rab GTPases were tested using the yeast two-hybrid assay. We used the GAL4 system, based on the split GAL4 transcription factor controlling expression of reporter genes. Among possible candidates on the interaction with AtSEC15b, we have focused on Rab GTPases obtained from co-expression data analysis (AtRABH1c and AtRABA4a) as well as other Rab GTPases from RabA clade, which is homologous to mammalian Rab11. In addition to wild-type AtRABA4a GTPase, we prepared two mutant forms of AtRABA4a protein. The mutation S(31)N is believed to stabilize the GDP-bound state resulting in a dominant-negative (DN) isoform of AtRABA4a, while Q(76)L mutation is believed to reduce intrinsic GTPase activity of AtRABA4a causing constitutively-active (CA) isoform (Olkkonen and Stenmark, 1997; Ueda et al., 2001; Sohn et al., 2003; Kotzer et al., 2004; Zheng et al., 2005; Chow et al., 2008). All *RAB* genes tested were N-terminally fused with the DNA-binding domain of GAL4, leaving its C terminus available for prenylation, which is necessary for Rab association with membranes. *AtSEC15b* was fused with the GAL4 activation domain also on its N-terminus to prevent eventual incorrect folding of C-terminus, which is believed to be supposed for the interaction with Rab GTPases in animals. Yeast cells were transformed with pairs of plasmids and grown on selective plates.

We observed strong interaction between AtSEC15b and AtRABA4a in wild-type form as well as in CA form. Moreover, we did not observe any direct interaction between AtSEC15b and dominant-negative AtRABA4a. These results were also confirmed by β-galactosidase assay (Figure 4.4). On the other hand, we were not been able to detect any interaction of AtSEC15b with AtRABH1c as well as with other selected exocytotic Rabs. Beside AtRABH1c, we tested the interaction of AtSEC15b with wild-type form of other Rabs from *Arabidopsis* RabA family (A4b, A4c, A4d, A2a, and A5c), bul also with two Rab GTPases in a constitutively-active conformation – A4b(QL) and E1d(QL). Neither of these GTPases interacted with AtSEC15b in our two-hybrid assay (data not shown) indicating that AtRABA4a might be a specific interaction partner for AtSEC15b.

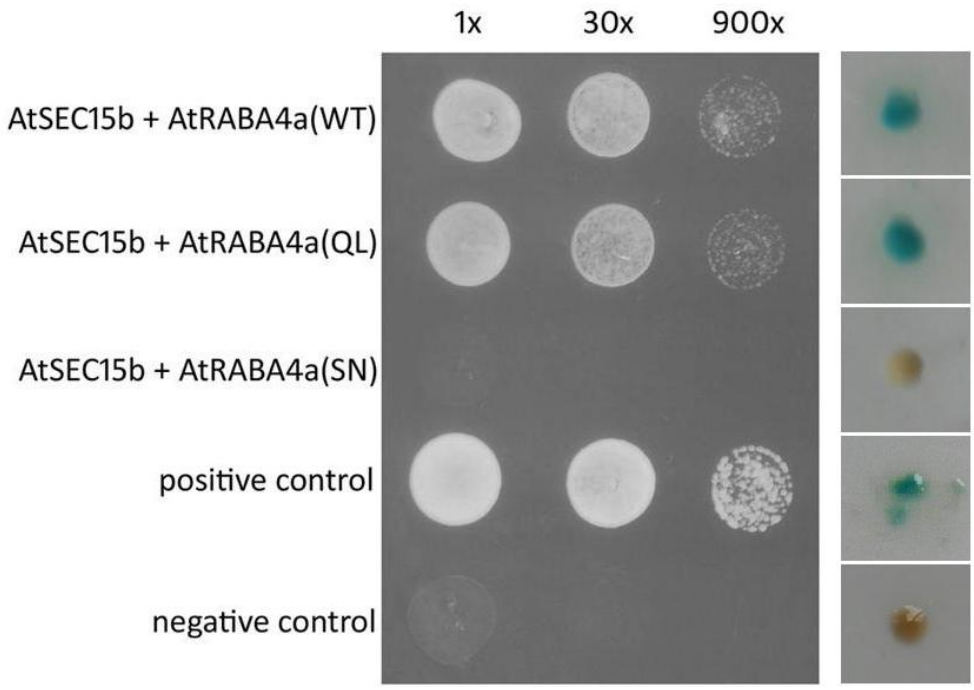


Figure 4.4. Yeast two-hybrid analysis of pairwise interactions between AtSEC15b and selected Rab GTPases.
Pairwise interactions of AtSEC15b and various forms of AtRABA4a. The strength of interactions is demonstrated by a dilution series and compared with a positive control. Yeast were grown on –ADE–HIS–LEU–TRP plates at 28°C and interactions were further confirmed by β-galactosidase assay.

4.3.3. AtRABA4a co-immunoprecipitates with the exocyst complex in a total soluble *Arabidopsis* protein extract

To test whether the AtRABA4a GTPase associates with the exocyst complex *in vivo*, we conducted co-immunoprecipitation experiments in extracts from *Arabidopsis* transgenic lines expressing GFP-tagged proteins. As a first step, mouse polyclonal antibodies were raised against the recombinant AtRABA4a and AtSEC15b (N-terminal half) proteins (see Matherials and Methods, Chapter 3.11.). These antibodies, but not the corresponding pre-immune sera, detected bands of approximately 25 kDa, or 87 kDa, respectively, on Western blots of *Arabidopsis* protein extracts as expected (data not shown).

Further we transformed wild-type *Arabidopsis thaliana* with 35S::GFP:*AtSEC15b* to use this transgenic line for co-immunoprecipitation experiments. Unfortunatelly, the expression of N-terminally GFP-tagged *AtSEC15b* in *Arabidopsis* seedlings was too weak thus we were unable to detect the presence of AtRABA4a GTPase in the GFP:AtSEC15b co-immunoprecipitate. However, from previous data from our laboratory we know that AtSEC15b co-immunoprecipitates with another exocyst subunit, AtEXO84b, in *Arabidopsis* (Fendrych et al., 2010) and we supposed that if there exists an interaction between AtSEC15b and AtRABA4a, we will be able to detect

this Rab GTPase in co-immunoprecipitate from transgenic lines constitutively expressing GFP-tagged AtEXO84b (GFP tagged on the C-terminus of AtEXO84b; Fendrych et al., 2010). As a control, we used seedlings expressing free GFP. Using specific polyclonal antibodies against AtRABA4a and AtSEC15b we detected the presence of these proteins in the AtEXO84b:GFP co-immunoprecipitate. We observed only very weak signal corresponding to AtRABA4a protein and no signal corresponding to AtSEC15b protein in immunoprecipitate from control, GFP expressing plants, indicating the specificity of co-immunoprecipitation experiments (Figure 4.5). From these results, we concluded that AtRABA4a GTPase associates with the exocyst complex probably through its interaction with AtSEC15b subunit *in vivo* in *Arabidopsis thaliana*.

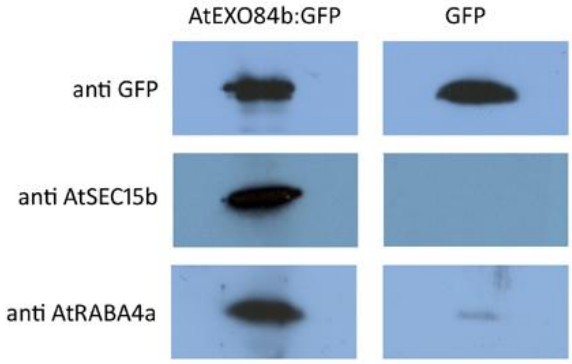


Figure 4.5. Co-immunoprecipitation of AtSEC15b and AtRABA4a with GFP-tagged AtEXO84b. AtSEC15b and AtRABA4a were identified in the immunoprecipitate from plant expressing AtEXO84b:GFP by immunoblotting. Samples were loaded in the same volumes.

4.4. Expression of AtSEC15b complements the growth defect of *Saccharomyces cerevisiae sec15-1* mutation

The yeast *Saccharomyces cerevisiae* genome contains single gene encoding exocyst subunit Sec15. It is well documented that mutation in this gene (*sec15-1*) causes a thermosensitive growth defect accompanied by accumulation of secretory vesicles inside the cell (Novick et al., 1980). First we tested the ability of these mutants to grow at the permissive temperature (28°C) but not at the restrictive temperature (37°C; Figure 4.6a). We transformed the *sec15-1* mutant strain with a pVT-103U plasmid containing *AtSEC15b* gene under the control of the ADH1 promoter to express high levels of AtSEC15b. As a control, we transformed this mutant strain also with the empty pVT-103U vector. We examined the ability of transformed yeast to grow at the non-permissive temperature. Yeast were grown simultaneously at 28°C, and at 37°C on selective plates (–URA) containing glucose as a carbon source to induce high level of gene expression.

We observed that while at 28°C, yeast transformed with pVT-103U::*AtSEC15b* as well as with an empty vector grew normally, at 37°C only yeast transformed with pVT-103U::*AtSEC15b* were able to grow (Figure 4.6b). It is obvious that expression of

AtSEC15b fully restores the growth defect of the *sec15-1* budding yeast at the restrictive temperature. As it is known that yeast Sec15 directly interacts with Rab GTPase Sec4 (Guo et al., 1999b), we concluded that *Arabidopsis* SEC15b can restore the mutant phenotype of *sec15-1* by functional substitution of yeast Sec15 protein also possibly via the interaction with the Rab GTPase Sec4.

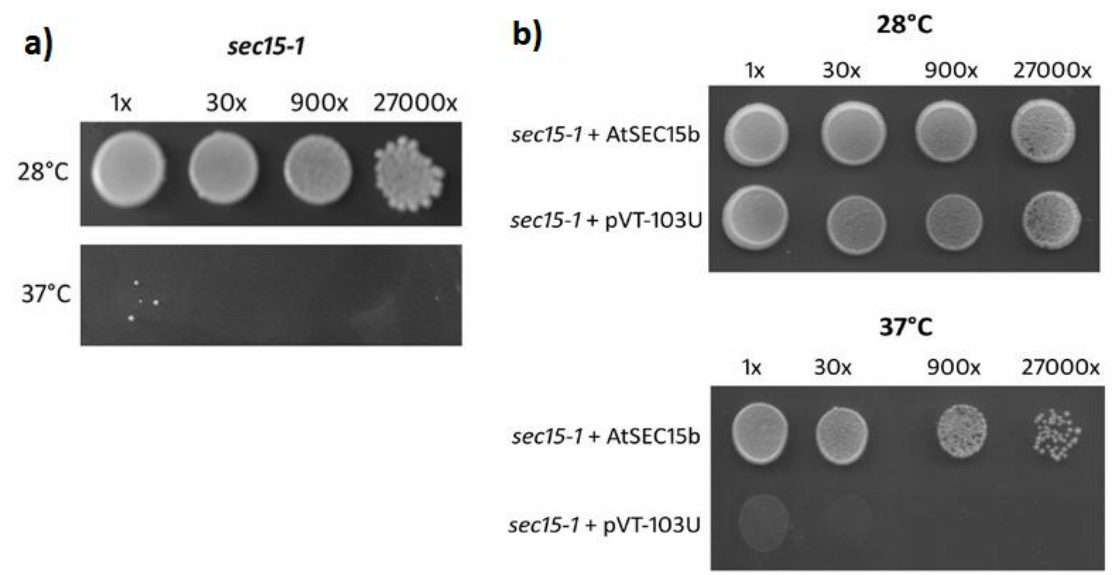


Figure 4.6. Functional complementation of *sec15-1* phenotype.
a) Thermosensitive growth defect of *sec15-1* mutant strain.
b) *sec15-1* mutant strain transformed with pVT-103U expression plasmid containing *AtSEC15b* or pVT-103U alone. Single colonies were resuspended in 100 μl of sterile water and serial dilutions were prepared. Yeast were dropped on –URA plates containing glucose as a carbon source and grown at 28°C or at 37°C.

4.5. Subcellular localization of AtSEC15b and AtRABA4a

To examine the intracellular distribution of AtSEC15b and AtRABA4a *in vivo*, we constructed N-terminally GFP- or mRFP-tagged versions of these proteins. The constructs were placed under the 35S promoter and used to transiently transform tobacco leaves and suspension culture as well as *Arabidopsis* suspension culture by *Agrobacterium*-mediated transformation or by the biolistic method. Based on a demonstrated interaction between AtSEC15b and AtRABA4a, we focused on a possible co-localization of these proteins in plant cell. In all of the transformed tissues, GFP:AtSEC15b and mRFP:AtRABA4a exhibited similar localization pattern. We observed a diffuse signal in cytoplasm and cytoplasmic strands, which was stronger in patches adjacent to the plasma membrane and a nucleus. But surprisingly, we observed that mRFP:AtRABA4a labeled also numerous punctuated, endosome-like structures located preferentially in the cortical cytoplasm, which are GFP:AtSEC15b negative (Figure 4.7a,b,c).

We further studied a localization of these fusion proteins in stably transformed *Arabidopsis* plants. Observing the cells in the meristem and elongation zone of 4-day-

old seedling roots we have seen similar results showing that mRFP:AtRABA4a also appeared in this highly mobile punctuated structures of approximately 0.6 μm in diameter against a faint cytosolic background (Figure 4.7e) whereas GFP:AtSEC15b exhibited mainly an uniform cytosolic localization (Figure 4.7d). Both proteins were excluded from a nucleus and were also strongly visible at the cell periphery.

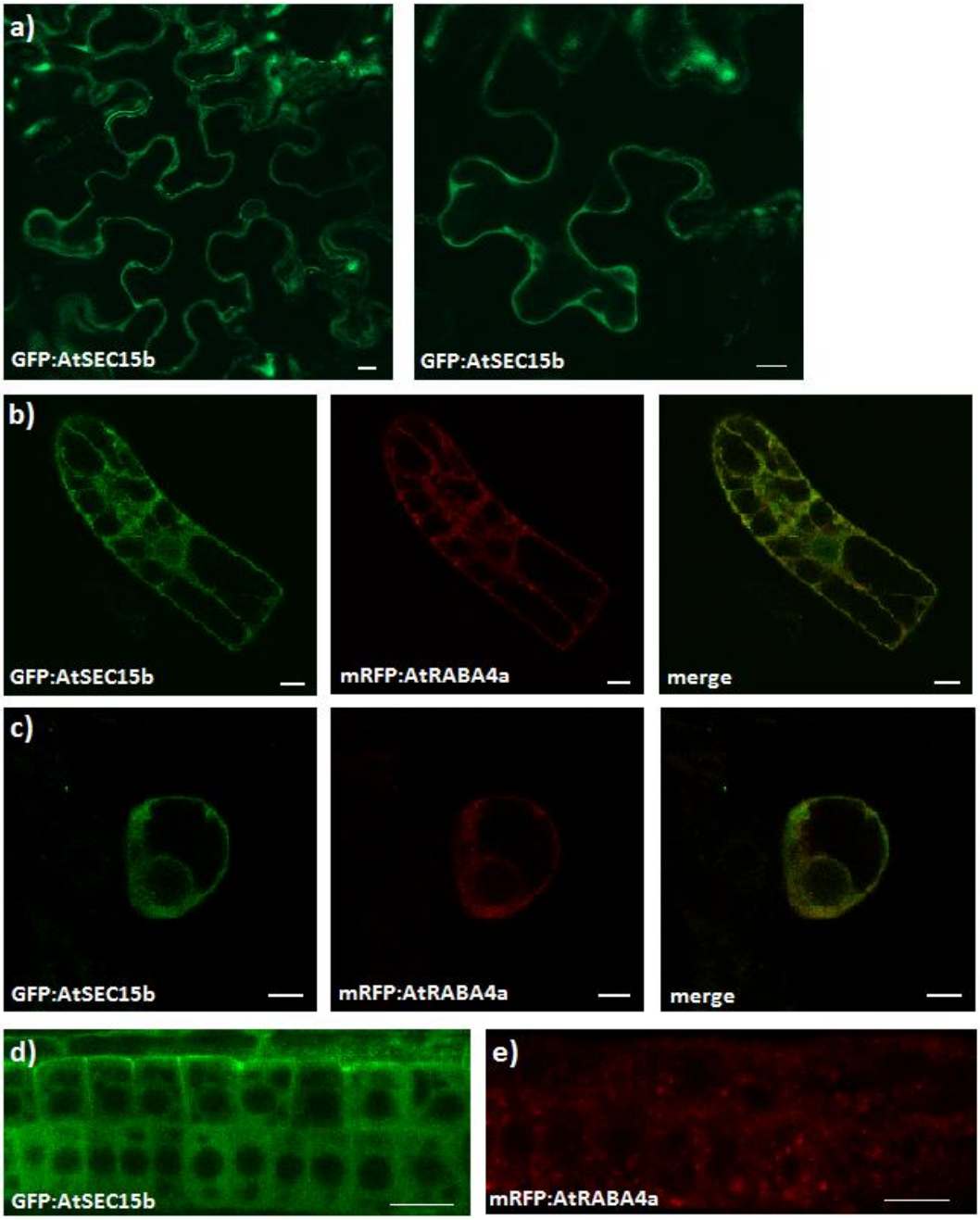


Figure 4.7. An overview of localization pattern of AtSEC15b and AtRABA4a in plant cells.
a) A localization of transiently expressed GFP:AtSEC15b in *Nicotiana benthamiana* epidermal cells after agroinfiltration. Bars = 10 μm.
b) and **c)** Co-localization experiments of GFP:AtSEC15b with mRFP:AtRABA4a in tobacco (b) and *Arabidopsis* (c) suspension cultures. GFP:AtSEC15b was co-expressed together with mRFP:AtRABA4a after biolistic transformation. Bars = 10 μm.
d) and **e)** *Arabidopsis* seedlings stably expressing either GFP:AtSEC15b (d) or mRFP:AtRABA4a (e). Bars = 10 μm.

4.5.1. AtSEC15b and AtRABA4a locate to the cell plate during cytokinesis

In addition, observations of *Arabidopsis* seedling roots expressing GFP:AtSEC15b or mRFP:AtRABA4a revealed localization of these fusion proteins to the cell plate during cytokinesis. Unfortunatelly, no cell expressed both GFP:AtSEC15b and mRFP:AtRABA4a suggesting a mutual silencing of transgenes probably caused by presence of double 35S promoter. Thus we were no able to compare the localizations of these proteins into the same cell plate. On the other hand, it is evident from the individual either GFP:AtSEC15b or mRFP:AtRABA4a localization observations that these proteins were characterized by some differences in their localization to the cell plate.

Firstly, we identified that another exocyst subunit, AtEXO84b:GFP, localized at the early and postcytokinetic cell plate during *Arabidopsis* cytokinesis and we noticed that also other exocyst subunits, including GFP:AtSEC15b shared this cytokinetic localization with AtEXO84b:GFP (Fendrych et al., 2010). We examined root meristem of 4- to 7-day-old *Arabidopsis* seedlings, a tissue with high cytokinetic activity. Detailed study of GFP:AtSEC15b localization during plant cytokinesis showed that during the initiation of cytokinesis GFP:AtSEC15b signal strongly associated with the newly formed cell plate at the moment of its emergence. This signal persisted for 30 to 60 seconds and then gradually disappeared. At the moment of cell plate insertion, when the cell plate attached the mother cell wall, the signal reappeared at the insertion site and then spread into the rest of cell plate (Figure 4.8c,d). After finishing the cytokinesis, GFP:AtSEC15b remained associated with the maturating postcytokinetic cell wall for approximately 1 hour and during that time the intensity of GFP signal decreased.

In some meristematic cells of the root tip, mRFP:AtRABA4a protein was also seen to label cell plate during cytokinesis (Figure 4.8a). Compared to what we saw in the case of GFP:AtSEC15b, the localization pattern of mRFP:AtRABA4a was slightly different. At the beginning of cytokinesis, mRFP:AtRABA4a strongly localized to the newly formed cell plate. But in contrast to GFP:AtSEC15b, as the plate expanded, signal didn’t disappear but was increasingly restricted to the growing margins. After the cell plate insertion, AtRABA4a fusion protein was subsequently distributed along the maturating cell wall in a uniform intensity, which further decreased during the time (Figure 4.8b).

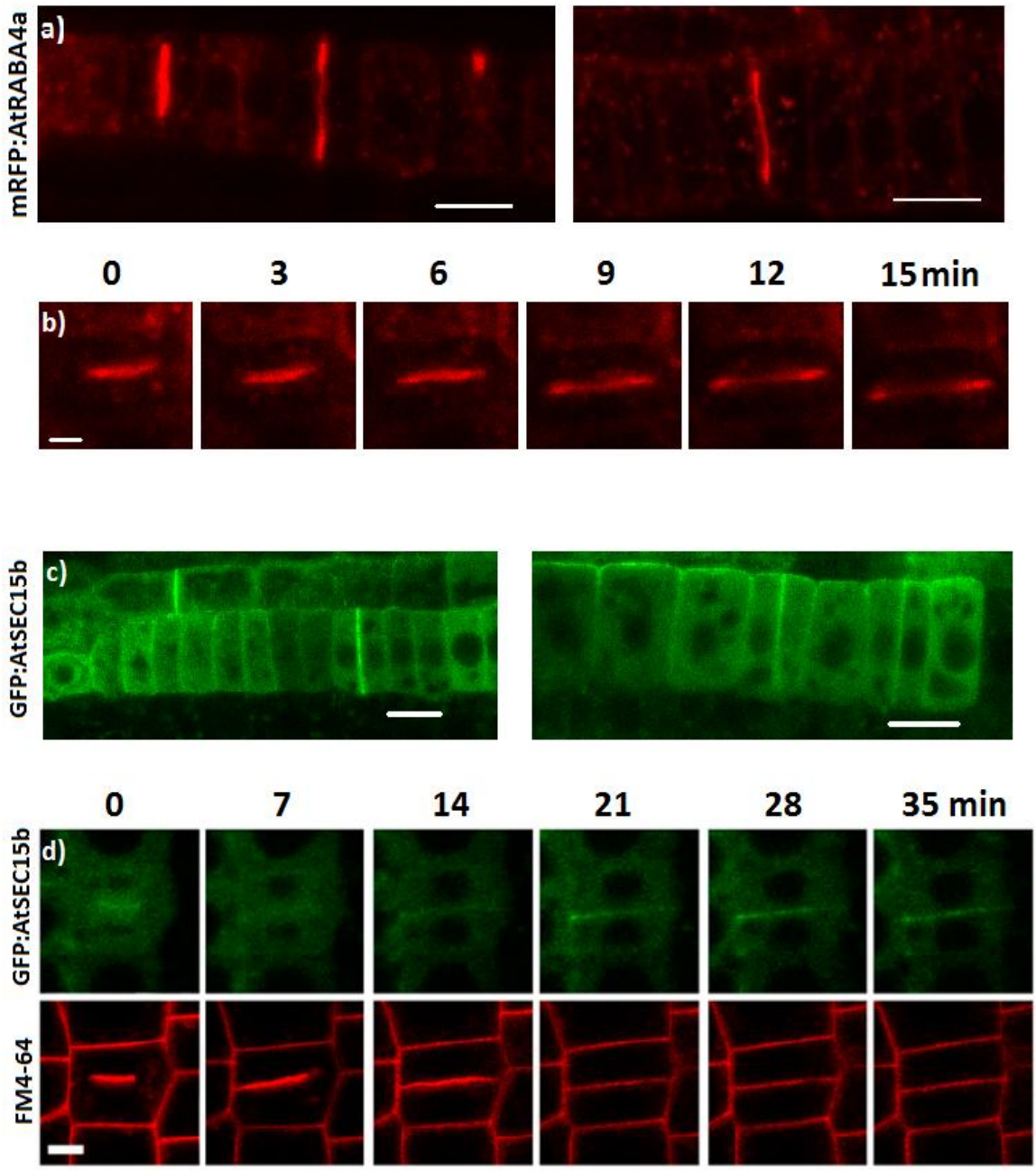


Figure 4.8. Localization of AtRABA4a and AtSEC15b during cytokinesis.
a) mRFP:AtRABA4a labels growing cell plate in the *Arabidopsis* root. Bar = 10 μm.
b) Time series showing localization dynamics of mRFP:AtRABA4a during cytokinesis. Firstly, signal appears at the newly emerged cell plate and then, at later stages of cytokinesis, is preferentially concentrated at the growing margins of the cell plate. Bar = 5 μm.
c) Post-cytokinetic cell walls in the *Arabidopsis* root labeled by GFP:AtSEC15b. Bar = 10 μm.
d) Time series showing localization of GFP:AtSEC15b during cytokinesis of root meristem cells stained with FM4-64. GFP:AtSEC15b strongly localizes to the nascent cell plate and then during the final stages of cytokinesis, to the site of cell plate insertion with the expansion to the whole post-cytokinetic cell wall. Bar = 5 μm.

4.5.2. Identification of AtRABA4a punctuated structures

We also started to investigate the identity of punctuated structures observed in plants expressing mRFP:AtRABA4a. For this purpose we crossed these transgenic plants with plants expressing different GFP- or YFP-tagged endomembrane markers. So far, we have examined a potential co-expression of mRFP:AtRABA4a with Golgi marker sialyl transferase (ST):YFP (Grebe et al., 2003), prevacuolar compartment (PVC) marker GFP:BP-80 (Kotzer et al., 2004), and two Rab fusion proteins – YFP:AtRABE1d, which is also targeted to the Golgi apparatus, and YFP:AtRABA5d, labeling recycling endosome.

Golgi stacks, labeled by ST:YFP marker, were visible as large flattened discs with dark areas at their centers and were physically different from mRFP:AtRABA4a structures, which appeared as smaller punctae. Although both structures were often in close proximity, they move independently in a streaming cytoplasm and their partial overlapping localization was only transient (Figure 4.9a).

The multivesicular PVCs, marked by GFP:BP-80, were visible as smaller structures, similar to those identified by mRFP:AtRABA4a, but we didn't observed any major co-localization between these structures. Rarely the prevacuolar compartment partially associated with mRFP:AtRABA4a speckles, but also only transiently and we concluded that mRFP:AtRABA4a labeled compartment of different origin (Figure 4.9b).

Additionaly, we didn't observed any co-localization between mRFP:AtRABA4a and YFP:AtRABE1d (Figure 4.9c) nor YFP:AtRABA5d (Figure 4.9d). These preliminary data raise the question whether mRFP:AtRABA4a might define a novel "compartment" different from PVC, Golgi and recycling endosomes or if our observations are only an artifact of mRFP:AtRABA4a overexpression.

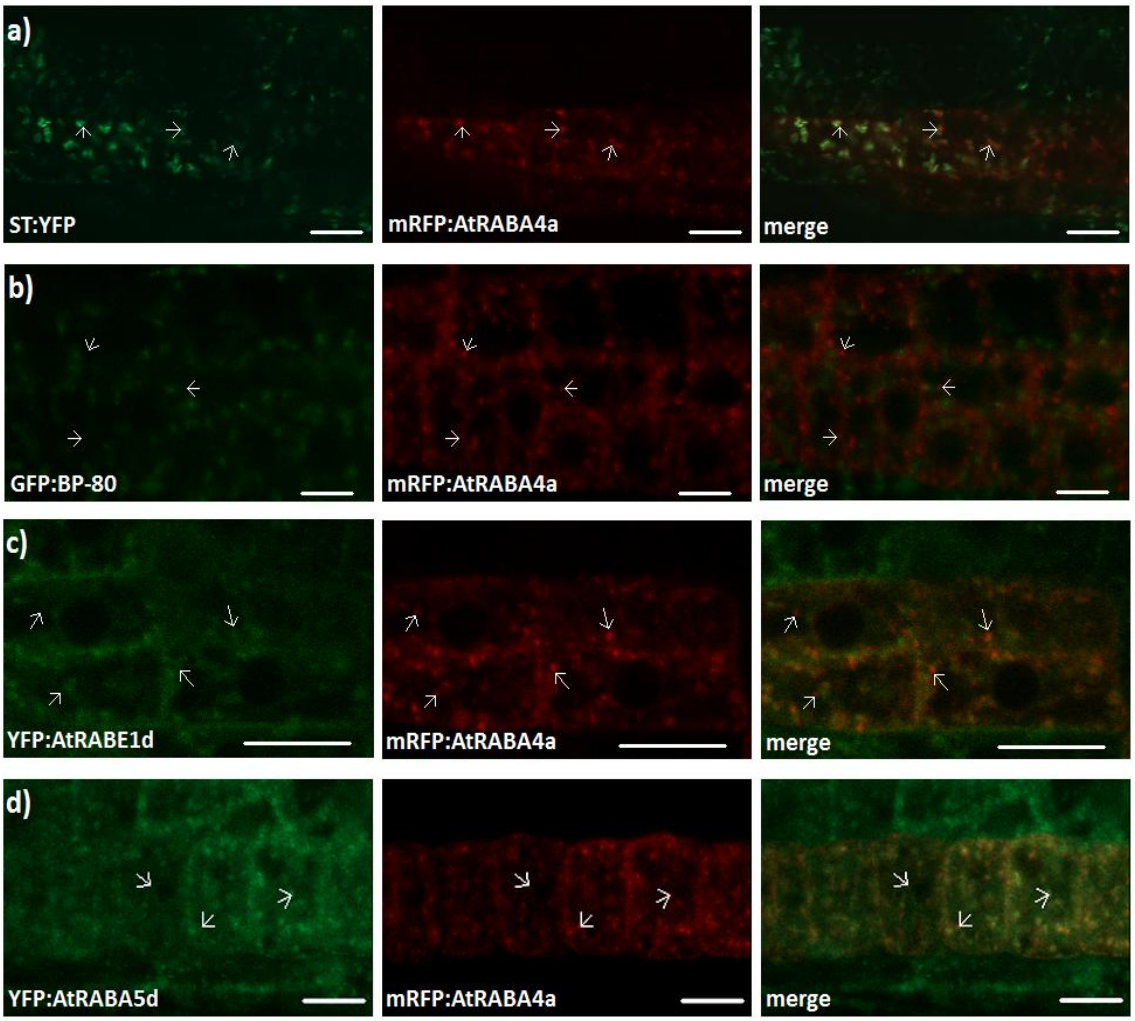


Figure 4.9. AtRABA4a labels compartment distinct from the Golgi, PVC and recycling endosomes.
Confocal analysis of *Arabidopsis* seedling root tips coexpressing mRFP:AtRABA4a with ST:YFP (a), GFP:BP-80 (b), YFP:AtRABE1d (c), or YFP:AtRABA5d (d). White arrows indicate mRFP:AtRABA4a-labeled structures. Bars = 10 μm.

4.6. Functional characterization of AtSEC15b

Genome of *Arabidopsis thaliana* contains two copies of *SEC15* gene – *AtSEC15a* and *AtSEC15b* (Eliáš et al., 2003). The function of AtSEC15a has already been described. It was identified that T-DNA insertion in *AtSEC15a* gene results in a male-specific transmission defect and dramatically affects both pollen germination and pollen tube growth (Hála et al., 2008). On the other hand, nothing is known about the function of AtSEC15b in plant cells.

In order to examine this, we ordered publicly available *Arabidopsis* T-DNA insertional mutant line, *atsec15b-1*, containing a T-DNA insertion within an exon (Figure 4.10), from SALK collection (The SALK Institute; Alonso et al., 2003). The presence of T-DNA insertion in the mutant plants was verified by PCR-based genotyping with using of T-DNA left border (LB) and gene-specific primers (listed in Supplemental data 1).

Figure 4.10. The position of T-DNA insertion in *atsec15b-1* mutant line.
A single exon gene *AtSEC15b* (At4g02350) interrupted with the indicated T-DNA insertion.

4.6.1. Mutation in *AtSEC15b* gene results in a male-specific transmission defect

At the sporophyte level, heterozygous plants *AtSEC15b/atsec15b-1* resembled the wild-type phenotype. But genetic studies revealed that the mutant alleles were not transmitted to progeny in the expected Mendelian ratio (1:2:1). Self-crossed heterozygots produced the homozygous progeny in significantly reduced frequencies. Compared to expected 25% of homozygous plants, we detected only 5% of homozygots in *AtSEC15b/atsec15b-1* progeny (Table 4.1; Figure 4.11)).

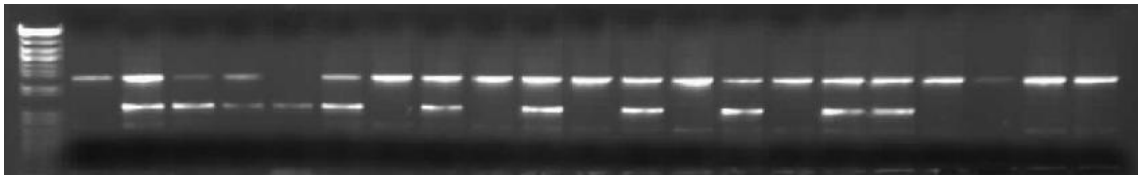


Figure 4.11. Genotype analysis of heterozygous self-crossed progeny.
Upper bands represents WT allele, lower bands correspondes to the mutant allele.

Table 4.1. Segregation ratio of the *AtSEC15b/atsec15b-1* heterozygous progeny indicates a reduced inheritance of *sec15b-1* mutant allele. n (number of tested plants), +/+ (wild-type plants), +/m (heterozygous plants), m/m (plants homozygous for *atsec15b-1* mutation).

allele	n	+/+	+/m	m/m
expected	244	25%	50%	25%
atsec15b		40%	55%	5%

To determine whether this was caused by defect in male or female gametogenesis, we performed reciprocal outcrosses between plants heterozygous for *atsec15b-1* allele and wild-type Columbia-0 plants. When wild-type plant was used as a pollen donor to pollinate a stigma of heterozygous *AtSEC15b/atsec15b-1* plant, the mutant allele was transmitted through the female at expected frequency (1:1

Medelian ratio of *AtSEC15b/atsec15b-1* heterozygots to wild-type plants). On the other hand, when *AtSEC15b/atsec15b-1* heterozygous plants were used to pollinate wild-type plants, we observed a strong reduction of mutant allele transmission through the male gametophyte. The frequency of heterozygots in the progeny was significantly reduced from expected 50% to 19% (Table 4.2). These data strongly argue for an important function of AtSEC15b in the male gametophyte function.

Table 4.2. Data from reciprocal crosses between *AtSEC15b/atsec15b-1* heterozygots and wild-type plants indicate that mutation in *AtSEC15b* gene results in a male-specific transmission defect. n (number of testes plants), +/+ (wild-type plants), +/m (heterozygous plants), m/m (plants homozygous for *atsec15b-1* mutation).

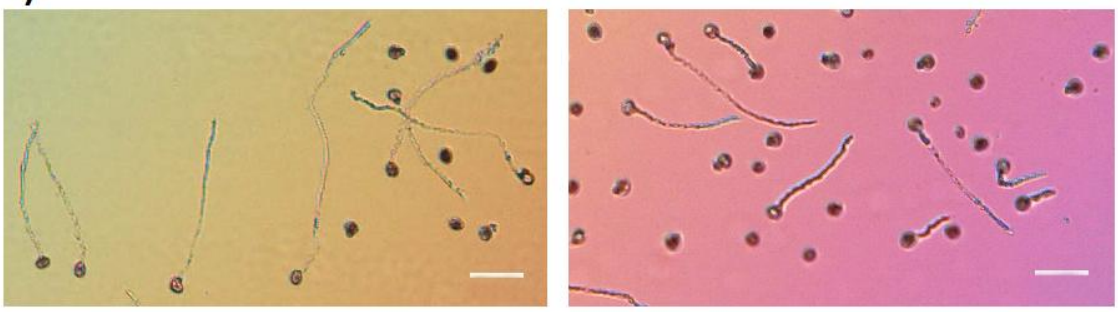
		Pollen donor: +/m Pollen recipient +/+		Pollen donor: +/+ Pollen recipient +/m	
Allele	n	+/+	+/m	+/+	+/m
expected	60	50%	50%	50%	50%
Sec15b		81%	19%	51%	49%

4.6.2. Mutation in *AtSEC15b* affects pollen tube tip growth

We were interested to know whether the reduced transmission of mutant allele through the male gametophyte is associated with impaired growth of pollen tube. We hypothesized that if AtSEC15b is important for pollen tube growth, then pollen with disrupted expression of AtSEC15b may display tip growth defects.

Because of the probable absence of mature pollen in homozygous plants, to determine this, pollen from heterozygous plants was germinated *in vitro* and compared with the pollen of the wild-type. Both, wild-type and mutant pollen grains seemed to germinate normally and they also formed pollen tubes with no morphological abnormalities. But in contrast, while wild-type pollen formed relatively long pollen tubes, pollen grains from *AtSEC15b/atsec15b-1* heterozygotes developed, beside the pollen tubes, which were indistinguishable from those of wild-type, pollen tubes, which were significantly shorter (Figure 4.12). Heterozygotes also had a much lower frequency of long pollen tubes, which well documents the assumption that heterozygous pollen is a mixture of mutant and wild-type pollen in a ratio 1:1. Taken together, we proposed that the mutation in *AtSEC15b* gene results in an impaired polar growth of pollen tube.

a)



wild-type *AtSEC15b/atsec15b-1*

b)

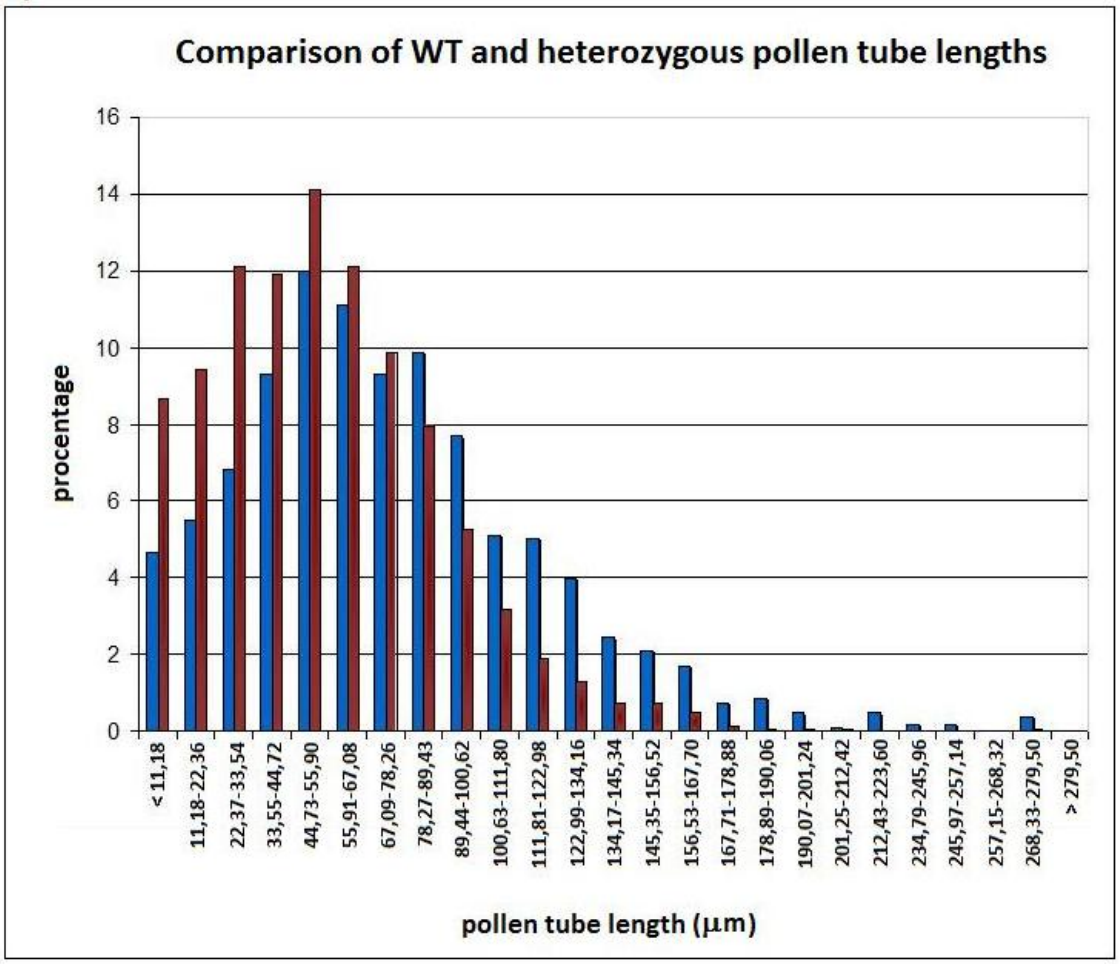


Figure 4.12. The *atsec15b-1* mutation decreases the length of pollen tubes *in vitro*.
a) An illustrative picture of germinated pollen from WT and heterozygous plants. Bars = 100 µm.
b) Histogram comparing the length of pollen tubes from two different WT plants (blue bars) and three different heterozygous plants (red bars) shows a different distribution of pollen tube lengths between different genotypes. Tubes were measured 17 h after germination; n(WT) = 1054, n(*AtSEC15b/atsec15b-1*) = 1707.

4.6.3. Disruption of AtSEC15b gene results in a dwarf phenotype

To gain insight into the function of AtSEC15b in *Arabidopsis thaliana* we performed a phenotype analysis of *atsec15b-1* homozygous plants and we found that a mutation in *AtSEC15b* gene dramatically affects the development of *Arabidopsis* plants resulting in a dwarf phenotype. To verify a disruption of *AtSEC15b* allele, we performed RT-PCR analysis of cDNA from *atsec15b-1* homozygots and wild-type Columbia-0 plants. No full-length *AtSEC15b* transcript was detected in 7-day-old *atsec15b-1* seedlings (Figure 4.13a), indicating that expression of the *AtSEC15b* gene was disrupted in this T-DNA insertional mutant.

Although it seems that *atsec15b-1* mutant seedlings germinate normally, it is evident that their sporophytic development is largely impaired resulting in a highly reduced growth of mutant plants, which have also much smaller organs and reduced apical dominance (Figure 4.13b). Additionally, the mutation affects also silique development as we didn't see any siliques on *atsec15b-1* homozygous plants, indicating that these plants are sterile (Figure 4.13c). This is also supported by preliminary observations that homozygous plants are impaired in pollen development, since we didn't observed any pollen in mature flowers. These observations are only our preliminary data and a mutant phenotype needs to be further examined in more detail, especially on a cellular level. It is also important to confirm that these phenotype alterations are indeed caused by mutation in *AtSEC15b* gene by complementation assay. Despite these additional experiments, we concluded that AtSEC15b is crucial for a normal development of *Arabidopsis thaliana*.

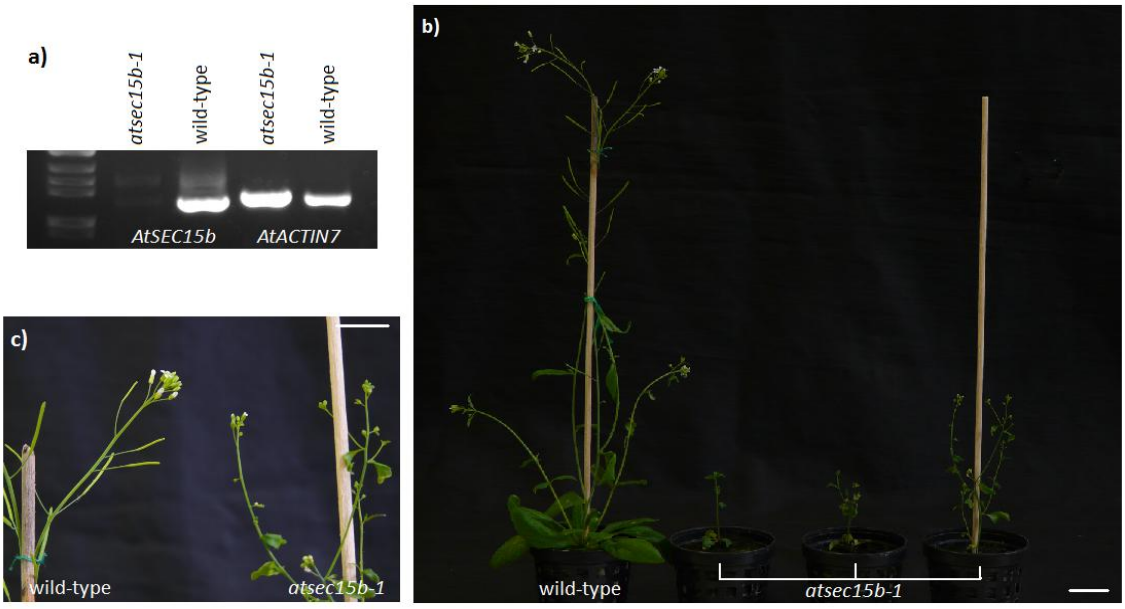
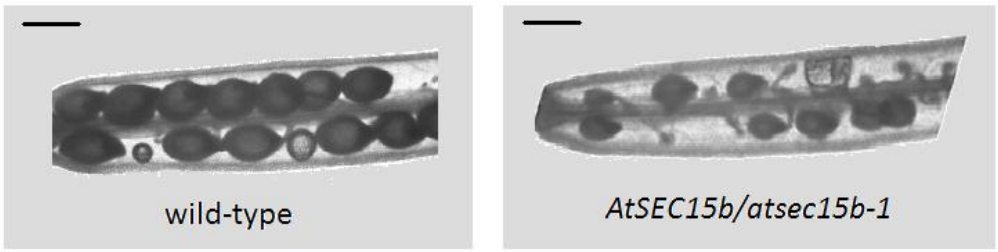


Figure 4.13. Disruption of AtSEC15b leads to a mutant growth phenotype.
a) Total RNA extracted from *atsec15b-1* homozygous and wild-type Col-0 seedlings was reverse-transcribed and PCR amplified using primer specific for *AtSEC15b* full-length. Primers specific for *AtACTIN7* (constitutively transcribed gene) amplification from the same cDNA were used as a loading control.
b) Seven-week-old wild-type and *atsec15b-1* homozygous plants in Jiffy pellets. Bar = 1 cm.
c) Generative organs of seven-week-old wild-type and *atsec15b-1* homozygous plants. Bar = 1 cm.

4.6.4. Mutation in *AtSEC15b* results in a partial embryonic lethality

Based on the observations that plants homozygous for *atsec15b-1* allele were not able to develop any normal siliques and thus failed to produce a next generation, we examined silique and embryo development after self-pollination of plants heterozygous for *atsec15b-1* allele compared to wild-type plants. Siliques of heterozygous plants develop normally and morphologically resemble the wild-type siliques. On the contrary, when we looked inside the developing siliques (approximately 7 day after fertilization), we observed that in heterozygous siliques there are significantly more aborted embryos then in siliques from wild-type plants. We analysed the percentage of non-developing embryos and we found that compared to average 5% of aborted embryos in wild-type siliques, in siliques from *AtSEC15b/atsec15b-1* heterozygous plants there are an average of 21% embryos which are not further developing. In few cases, more than 50% of aborted embryos were found in some heterozygous siliques (Figure 4.14, Table 4.3).

a)



b)

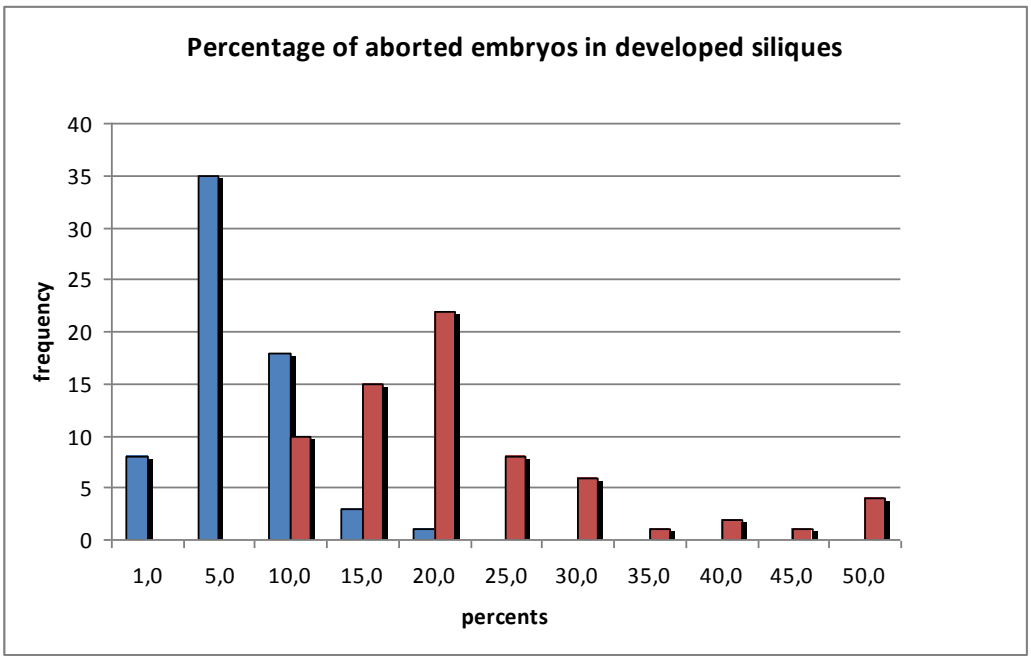


Figure 4.14. Mutation in AtSEC15b affects embryonal viability.
a) 7-day-old siliques from wild-type and *AtSEC15b/atsec15b-1* heterozygous plants. In *AtSEC15b/atsec15b-1* siliques some embryos are not developing.
b) Histogram comparing the percentage of non-developing embryos inside the growing siliques. Wild-type siliques are in blue, *AtSEC15b/atsec15b-1* siliques are in red.

Table 4.3. Percentage of aborted embryos in siliques from wild-type and AtSEC15b*/atsec15b-1* heterozygous siliques.

	number of analysed siliques	total number of embryos	aborted embryos	percentage of aborted embryos
wild-type	65	1925	91	5%
AtSEC15b/atsec15b	69	1938	402	21%

Chapter 5

DISCUSSION

This thesis intends to further improve our understanding of the function of plant exocyst, a tethering complex involved in the final step of exocytosis, with the main emphasis on one of its subunits – AtSEC15b. In yeast and mammals, the exocyst complex is well characterized in respect to the function of Sec15 protein in these organisms. In non-plant eukaryotic cells studied to date, exocyst is thought to facilitate the tethering of secretory vesicles to the plasma membrane and thus participates in many vital processes requiring polarized exocytosis. In yeast, Sec15 is essential for vesicular traffic from the Golgi apparatus to the cell surface and its mutation leads to the accumulation of secretory vesicles inside the cell. Moreover, *sec15* null mutants are non-viable (Novick et al., 1980; TerBush et al., 1996). Yeast Sec15 also exhibits strong genetic interaction with Sec4 protein, a member of Rab GTPase family (Guo et al., 1999b), reflecting its role in membrane trafficking. In multicellular organisms, the exocyst proteins were shown to be implicated in the neurite outgrowth, cell polarity and cell viability. In *Drosophila melanogaster* neurons, loss of *sec15* causes a targeting defect of specific cell adhesion and signaling molecules required for the establishment of synaptic specificity, leading to the formation of synapses between inappropriate partners (Mehta et al., 2005). Furthermore, Sec15 promotes Notch signaling during the asymmetric division of *Drosophila* sensory organ precursors, by which different cell types of the mature sensory organ are generated. The *sec15* mutation leads to the formation of extra neurons at the expense of other cells and based on the observations that vesicular compartments containing Notch accumulate in basal areas of sensory organ precursors with mutated *sec15* subunit, it was supposed that Sec15 mediates a specific vesicle trafficking event to ensure proper neuronal fate specification in *Drosophila melanogaster* (Jafar-Nejad et al., 2005). In mammalian cells, Sec15 was shown to be involved in rapid transfferin receptor recycling and thus in iron metabolism. A mutation in the mouse Sec15 causes anemia in hemoglobin deficient mice. Sec15 is linked to the transferrin cycle through its interaction with Rab11, a GTPase involved in vesicular trafficking (White et al., 2005; Lim et al., 2005). All these data show that Sec15 plays a critical role in developmental processes of eukaryotic organisms connected with the precise regulation of vesicular transport.

5.1. *In silico* insight into the Sec15 evolution and its interaction with Rab GTPases

Exocyst is extensively studied in many eukaryotic organisms and several phylogenetic studies revealing the evolutionary relationship among predicted exocyst complex subunits in yeast, animal and plant genomes were described (Eliáš et al., 2003; Synek et al., 2006; Chong et al., 2010). Our analysis contributes to the deeper examination of SEC15 gene family from all major completely sequenced eukaryotic genomes covering all important lineages. Plant exocyst is characterized by the presence of several gene copies for almost all individual subunits with SEC15 being present as a two-paralogous gene in *Arabidopsis* genome (Eliáš et al., 2003; Synek et al., 2006). Our results suggest that ancestral eukaryotic organism had only one SEC15 gene and during the evolution, probably after separation of plants and other eukaryotic lineages, several SEC15 paralogs have evolved. Diversification of plant SEC15 genes is not surprising due to the finding that even the smallest of plant genomes has incurred multiple duplication events (Paterson, 2005). Our phylogenetic analysis also showed that Sec15a and Sec15b subfamilies are clearly distinguished in higher plants. Interestingly, the same seems to be true for Metazoan Sec15, although these two events occurred independently. The possibility, how to explain gene duplication is that individual SEC15 paralogs may be involved in different types of exocytosis or they may function at different time or in different tissues during plant development. Given the distinct expression pattern for *Arabidopsis* Sec15a and Sec15b, one can assume that the cellular roles and regulation of Sec15a and Sec15b subunits are conserved in higher plants. Nevertheless, our further data showed that i.e. in pollen, where AtSEC15a is characterized by higher level of expression, both AtSEC15 paralogs are essential.

In yeast and animals, Sec15 performs its function via interaction with Rab GTPases (Guo et al., 1999b; Zhang et al., 2004; Wu et al., 2005). We were further interested to know if Rab binding motif in SEC15 sequence remained conserved during the evolution. Wu et al. (2005) determined C-terminal domain of *Drosophila* Sec15, which is sufficient for Rab binding. The crystal structure of this domain is comprised entirely of in total ten α-helices, which are equally distributed between two subdomains, only one of which (C subdomain) harbors the Rab binding site. By performing site-directed mutagenesis, the residues required for Rab binding was revealed to be part of α9 helix. They are located at the exposed side of the C subdomain and flanked by highly conserved residues. It was found that single mutation in one of the three residues (Asn659, Met663 or Phe667) essentially abolished binding to Rab11 GTPase (Wu et al., 2005). *Drosophila* C subdomain contains mostly hydrophobic residues. The participation of hydrophobic residues in binding is a common feature observed in several crystal structures of complexes of effectors with

their cognate small GTPases (Panic et al., 2003). Our multiple alignment revealed that all Sec15 homologs retained similar structure consisting of alternating α-helices and loops, with the helical part being more conserved. Rather unexpectedly, putative Rab interaction sites located near the C-terminus of the exocyst molecule are not conserved. Wu et al (2005) have proposed that Sec15-Rab binding mediated through these residues is driven mainly due to sterical complementarity and not due to electrostatic binding. Variability in Rab-binding region could therefore provide strategy for specific Sec15-Rab pairs.

5.2. *AtSEC15b* full-length complements the growth defect of yeast *sec15-1* mutation

In yeast, *sec15* null mutation is lethal but it was possible to isolate a conditionally lethal mutation. Temperature-sensitive *sec15-1* mutant blocks exocytosis and accumulate secretory vesicles in the cytoplasm (Novick et al., 1980). This phenotype can be suppressed by overexpression of several other yeast late secretory genes. For example, duplication of yeast SEC4 was found to suppress the growth defect, the secretion defect, and the accumulation of vesicles in the *sec15-1* strain (Salminen and Novick, 1987). Suppression of *sec15-1* phenotype was also reported upon expression of several other proteins, including yeast syntaxins Sso1 and Sso2 (Aalto et al., 1993), or β subunit of the Sec61p ER translocation complex (Toikkanen et al., 2003). Concerning genes from other eukaryots, rat synaptotagmin was shown to be able to partially rescue the growth defect exclusively in *sec15-1* thermosensitive mutants but not in other exocyst mutants (Damer and Creutz, 1996) and also overexpression of 14-3-3 protein from *Trichoderma reesei* was efficient in *sec15-1* suppression (Vasara et al., 2002). The only attempt to suppress the s*ec15-1* mutation with *Arabidopsis* c-DNA library resulted in the isolation of RMA1 protein, a RING finger motif-containing E3 ubiquitin ligase, which was able to restore the temperature-sensitive growth and also secretory activity of the yeast *sec15-1* mutant (Matsuda and Nakano, 1998). Surprisingly, no experimental data are available on the complementation of yeast *sec15-1* mutantion with SEC15 from other organism.

We were interested to know if functional domains of SEC15 protein remained conserved through the evolution. In our complementation assay, we were able to successfully complement yeast *sec15-1* mutation by overproduction of AtSEC15b and thus restore the temperature-sensitive growth defect of the yeast mutant strain. This led us to conclude that functional domains vital for the function of AtSEC15b in yeast remain conserved during the evolution and we can also speculate that full-length protein is necessary for the successful complementation and was underrepresented in the libraries used for complementation screens earlier.

5.3. Exocyst subunit AtSEC15b interacts with AtRABA4a in a GTP-dependent manner

Work over the past decade has shown small GTPases to be crucial regulators of vesicular transport in all eukaryotic organisms. An important step in the vesicular transport mechanism is the tethering of vesicles to their target membranes. Sec15 subunit participates in the initial step of this machinery by interacting with vesicle-associated Rab GTPases, assisting in targeting vesicles to the exocytotic sites.

In yeast, Sec15 associates with secretory vesicles via direct binding to the active Rab GTPase Sec4 (Guo et al., 1999b). In mammals and *Drosophila*, an interaction between Sec15 and Rab11 GTPase was observed and as in yeast, it was demonstrated that the interaction depends on GTP-bound form of Rab GTPase (Zhang et al., 2004; Wu et al., 2005). Our *in vitro* and *in vivo* studies have shed light on a possible interaction between these proteins in plants.

The first indication that exocyst and Rab GTPases might represent binding partners in plants comes from analysis of expression data from the Expression Angler tool (BAR; Toufighi et al., 2005). Since we found significant correlation between the expression of AtSEC15b and two proteins from the Rab family, AtRabA4a and AtRABH1c, we focused on these proteins as potential interacting partners with a main preference for AtRABA4a as a homologue of mammalian Rab11 and also as a protein possibly involved in exocytosis. Using yeast two hybrid assay, we proved that WT and CA forms of AtRABA4a, but not WT AtRABH1c, directly binds AtSEC15b. As we have essentially observed no binding of the GDP-locked AtRABA4a to the AtSEC15b, we proposed that this interaction occurs in a GTP-dependent manner. Our binding studies were additionally supported by co-immunoprecipitation experiment revealing that Rab GTPase, AtRABA4a, was found in a complex with exocyst *in vivo* in *Arabidopsis thaliana*.

In yeast, Sec15 interaction with Sec2 protein was described (Medkova et al., 2006). Sec2 is an exchange factor for Sec4, ensuring the highly active state of Sec4, which is necessary for proper running of vesicular transport (Walch-Solimena et al., 1997). Further it was demonstrated that this interaction is inhibited by phosphoinositide PI4P. Based on these findings a working model, in which communication between Sec15 and Sec4 is coordinated by recruitment and regulation of Sec2 by PI4P, was proposed (Mizuno-Yamasaki et al., 2010). There is not a direct Sec2 homologue in plants (Eliáš, 2008). Nevertheless it is possible that some other protein evolved convergently and substitutes function of Sec2. However, it is not known whether there is a Rab cascade in animal or plant late secretory pathway.

It is interesting that unlike in yeast and animals, where Sec15 exhibit a GTP-dependent interaction Rab GTPases, we detected strong positive interaction not only with CA AtRABA4a, but also with wild-type AtRABA4a. Rab GTPases possess a highly evolutionary conserved sequence. While many differences are found in their C-terminus, which is necessary for intracellular localization, the most conserved region is the site of the interaction with nucleotides. This “switch” regions are responsible for the transition between the GDP- and GTP- bound conformations and Rab proteins use

them, in addition to other regions, to determine the specificity of binding to protein partners (Paduch et al., 2001; Pereira-Leal and Seabra, 2001). It is generally assumed that GTP-bound conformation of Rab GTPases is responsible for interaction with multiple effectors including exocyst complex. *In vitro* binding assay in yeast and animals showed that Sec15 also interact with wild-type Rab GTPases, but the strenght of the interaction was reduced in comparison to the constitutively-active protein (Guo et al., 1999b; Zhang et al., 2004; Wu et al., 2005). Our results demonstrate the similar strength of interaction for both isoforms and thus raised the question, to what extent is this conformation required for this interaction in plants. Despite of this, we still have to think about the possibility that the interaction between AtSEC15b and WT AtRABA4a might be a false positive result, which is still the big problem of yeast two-hybrid screens. Nevertheless, we did not see any interaction between AtSEC15b and DN AtRABA4a, which is consistent with the observations from yeast and animals, indicating that also in plants, interaction between the exocyst and Rab GTPase occurs in GTP-dependent manner. This implies that the interaction is probably well conserved throughout the eukaryotic kingdoms.

We were not further able to detect any interaction between AtSEC15b and other putative exocytotic Rabs neither in their wild-type form nor in the constitutively-active form. Nevertheless, it will be necessary to further test this interaction with other CA Rabs from RabA family as well as with CA AtRABH1c. Rab GTPases in *Arabidopsis* are divided into eight groups that contain together 18 structural subclasses (Rutherford and Moore, 2002; Vernoud et al., 2003). RabA group is the most abundant group, comprising 26 members in *Arabidopsis* divided into six structural subclasses, and is homologous to two animal Rabs, Rab11 and Rab25. These two Rabs were reported to function on recycling endosome and they orchestrate vesicle transport from TGN to the specific regions of the plasma membrane (Casanova et al., 1999; van IJzendoorn 2006). RabA group underwent complicated evolution through multiplication and specialization in land plants. RabA6 subclass, for example, occurs only in dicot Angiosperms (Zhang et al., 2007). RabA4 subclass, together with RabA2 and RabA5 subclasses, is common for all land plants from moss to angiosperms (Eliáš et al., 2003, Purdue Genome Wiki, http://wiki.genomics.purdue.edu/index.php/Ras_superfamily_GTPases). Its member AtRABA4b was shown to be localized to the tip of trichoblasts where it labels large structures derived from TGN (Preuss et al., 2004). It was also shown that its active form interacts with PI-4Kβ, plant phosphatidylinositol 4-OH kinase producing PI4P, connecting Rab GTPases with the lipid metabolism (Preuss et al., 2006). Phosphoinositides (PIs) are currently being shown to be involved, in addition to their classical role in cell signalling, in regulation of membrane trafficking events in plants (Thole and Nielsen, 2008). It will be fascinating to examine the connection between Rab GTPases and formation of PIs through specific PI-kinases in plant membrane trafficking system. Another member of RabA4 subclass, RabA4d, which was shown to be pollen-specific, localizes to the tip of the pollen tube, and plays an important role in its germination. Mutant pollen tubes are defective, bulging, with observed altered deposition of cell wall components (Szumlanski and Nielsen, 2009). Surprisingly, neither of these two Rabs interacted in our two-hybrid assay with AtSEC15b. It will be interesting to test whether AtRABA4a could also interact with PI-4Kβ kinase or if there exist some substrate specificity for these GTPases, which might indicate functional

specialization of RabA4 GTPases during evolution. It may also be that the interaction is sensitive to the orientation of the fused domain. This has yet to be studied in more detail in a future.

5.4. Subcellular localization of interacting proteins revealed the endosome-like compartment labelled uniquely by AtRABA4a

We were also looking into subcellular localization pattern of fluorescent protein-tagged versions of AtSEC15b and AtRabA4a in transiently transformed cells as well as in stably transformed *Arabidopsis* plants. Images from confocal microscopy showed partially overlapping localization of GFP:AtSEC15b and mRFP:AtRABA4a in cytoplasm. Further confocal microscopy analysis revealed that mRFP:AtRABA4a is accumulated to the multiple mobile structures whose origin and function remains to be clarified. Interestingly, these structures are not labeled by GFP:AtSEC15b. Our preliminary data led us to hypothesize that it should be a novel compartment distinct from Golgi, PVC or recycling endosome targeted by AtRABA5d.

In animal cells, Rab11 GTPase resides on recycling endosome, which is thought to be central trafficking intermediate in both exocytic and endocytic pathways and control the traffic of cargo from the perinuclear recycling endosomal compartment to the plasma membrane (Jafar-Nejad et al., 2005). They are also crucial for delivery of new membrane from the recycling endosome to the cleavage furrow during cytokinesis (van IJzendoorn, 2006; Yu et al., 2007). It was shown that Sec15 co-localizes with Rab11 in the perinuclear region and through its interaction with Rab11 it may regulate the traffic from recycling endosome to the membrane (Zhang et al., 2004; Jafar-Nejad et al., 2005). Similarly, Chow et al. (2008) characterized a domain of the TGN/EE defined by AtRABA2 and AtRABA3 GTPases, plant GTPases homologous to Rab11, and so called RabA2/A3 compartment, which lies on the secretory as well as endocytotic pathway. Another member of RabA family, AtRABA4b, labels a unique TGN compartment functioning in trafficking of cargo from *trans*-Golgi to the plasma membrane (Preuss et al., 2004). In transiently transformed BY-2 cells, AtSec15b was also seen to be localized to punctuate structures throughout the cell, which corresponded with TGN/EE markers (Chong et al., 2010). However, we have not seen anything similar in our observation of AtSEC15b localization. Nevertheless, AtEXO70H1, one of the *Arabidopsis* EXO70, which interacts with AtSEC15b, was shown to label multiple punctuate structures in the cortical cytoplasm (Pečenková et al., 2011), indicating the presence of exocyst at some of these similar structures.

It was currently proposed that in a single plant cell several subtypes of TGN/EEs and other endosomes are present. And despite the known mobility of endosomes, this also demands spatio-temporal”subcompartmentalizaton” of endosomes within the cell (Žárský et al., 2009). Unfortunately, we were unsuccesful in generating plants expressing mRFP:AtRABA4a together with *trans*-Golgi network marker, VHA-a1:GFP, which might give us at least some elucidation of AtRABA4a structures origin. But consistent with all these findings we can speculate that our observations might also indicate an existence of a novel endosomal compartment, participating in secretory

pathway, which is defined by AtRABA4a GTPase. But these are only assumptions, which have to be further examined as well as possibility that some artificial compartment is induced by overexpression of mRFP-fused Rab protein. For this case, we also plan to visualize the localization of AtRABA4a by indirect immunofluorescence using a polyclonal mouse anti-AtRABA4a antibody.

5.5. AtSEC15b and AtRABA4a participate in cytokinesis

Unlike yeast and animals, which form cleavage furrows from the plasma membrane to separate the dividing cells, the cytokinesis of plant cells is viewed as a generation of a new membrane independently of the plasma membrane by fusion of Golgi-derived vesicles. In somatic cells, a plant-specific cytoskeletal array, called the phragmoplast, is thought to deliver vesicles to the plane of division. Vesicle fusion generates a membranous network, called the cell plate, which, by fusion of later-arriving vesicles with its margins, centrifugally expands towards the cell periphery and fuses with the plasma membrane (Jürgens, 2005). In *Arabidopsis*, numerous SNAREs and some of their interactors, such as the syntaxin KNOLLE/AtSYP111, have been shown to localize to the cell plate. They have been proposed to mediate the fusion of individual vesicles (Otegui et al., 2005). We have now identified that AtSEC15b, as well as other exocyst subunits, associates with the cell plate in *Arabidopsis* (Fendrych et al., 2010).

In dividing meristematic root cells, AtSEC15b intensively localized to the cell plate at the moment of its emergence. This is consistent with the fact that exocyst functions as a tethering complex. It is therefore supposed that exocyst is involved at initial phase of cytokinesis by tethering of vesicles to the phragmoplast midline and thus facilitating the cell plate assembly. This observation corresponds to the electron tomographic analysis of cell plate formation in *Arabidopsis* meristematic cells, where the structures tethering the membrane vesicles and resembling the images of purified mammalian exocyst complex (Hsu et al., 1998) were observed. These structures were similar in both morphology and length (Otegui and Staehelin, 2004; Seguí-Simarro et al., 2004). If the structure observed in plant cells is really the exocyst, one could suppose that exocyst in plant cells has a similar rod-like structure as the yeast exocyst (reviewed in Munson and Novick, 2006) and that this conserved structure is required for its function. Later in the cytokinesis, exocyst partially disappears from the cell plate until the moment, when the growing cell plate reaches the plasma membrane. At the final stage of the cytokinesis, exocyst strongly labels the insertion site and then expands to the entire postcytokinetic wall remaining there throughout the cell wall maturation. From the mechanistic viewpoint, the cell plate inserts in the mother wall through a multitude of finger-like fusion tubes that contact the parental plasma membrane in the zone of adhesion (Samuels et al., 1995). Because the cell plate membrane and the plasma membrane are at first independent structures, it was proposed that their unification rely on a membrane fusion process that involves membrane fusion machinery different from the KNOLLE/KEULE/SNAP33 SNARE complex that is required for cell plate formation and expansion (Jürgens, 2005). It was established that TPLATE protein, contributes to the anchoring of the cell plate to the

mother wall (Van Damme et al., 2006). Our observations indicate that exocyst probably also participates in the cell plate to plasma membrane fusion.

A slightly different localization pattern during plant cytokinesis was determined for AtRABA4a GTPase. AtRABA4a labels the cell plate throughout the whole cytokinesis. At the beginning of cytokinesis, AtRABA4a is uniformly present at the cell plate at the time of its emergence similarly as AtSEC15b. But the main difference is that later in the cytokinesis, when AtSEC15b only weakly labels the growing cell plate, AtRABA4a remains localized in the whole cell plate with the more intensive location to its growing margins. This was observed also for other *Arabidopsis* Rab GTPases. Chow et al. (2008) identified that all members of the AtRABA2 and AtRABA3 GTPases are intensively localized to the cell plate in dividing cells of *Arabidopsis* root tip and that they are also concentrated at the growing regions of the cell plate. It was mentioned above that these GTPases define the novel TGN/EE membrane domain called AtRABA2/A3 compartment and it was also proposed that this compartment provides membrane to the cell plate. As this compartment may be involved in both endocytic and secretory traffic, it is responsible for delivery of recycling molecules as well as Golgi-derived biosynthetic cargo to the cell plate (Chow et al., 2008; Woollard and Moore, 2008). This is a parallel to the situation in animal cytokinesis. The final stage of cytokinesis in animal cells requires membrane trafficking events to deliver new membrane to the cleavage furrow and for abscission. New membrane is derived from the TGN compartment and Golgi-derived vesicles are targeted to the furrow where they fuse with the plasma membrane. However, Golgi is not the only source for the membrane addition during animal cytokinesis. It was demonstrated that endocytic recycling is also important for the delivery of membrane to site of division and this process is largely dependent on the function of endosomal Rab11 GTPase (Yu et al., 2007; Ai and Skop, 2009) paralogous to plant RabA GTPases (Rutherford and Moore, 2002). Rab11 is associated with REs and in complex with other accessory proteins regulates the targeting of REs to the cleavage furrow during late cytokinesis (Wilson et al., 2005; van IJzendoorn, 2006). From our observations we suppose that another member of RabA GTPase family, protein AtRABA4a, participates in the plant cytokinesis and contributes to the cell plate formation, progression and maturation by delivering vesicles for the subsequent fusion. It is consistent with requirement of a new cell wall material and plasma membrane for intensive transport of secretory vesicles to the growing cell plate.

5.6. AtSEC15b is vital for plant development

In our laboratory, the role of the exocyst in plant cell morphogenesis and development is intensively studied. Homologs of all eight exocyst subunits have been identified in the genome of *Arabidopsis thaliana* (Eliáš et al., 2003) and it was shown that all of them operate together *in vivo* to form a functional complex (Hála et al., 2008; Fendrych et al., 2010). Genetic analysis of the function of different *Arabidopsis* exocyst subunits was examined. It was demonstrated that they have a vital function in the plant development and that exocyst is involved in the physiological processes depending on proper polar exocytosis. AtEXO70A1, the dominant EXO70 isoform in

Arabidopsis, is important for hypocotyls elongation, tip growth of root hairs and initiation of lateral root indicating its dominant role in polar growth. *atexo70A1* mutants also display impaired flower development and are nearly sterile (Synek et al., 2006). AtEXO70A1 together with AtSEC8 also participate in polarized pectin delivery during seed coat development in *Arabidopsis* providing an evidence for the role of the exocyst in polarized cell wall morphogenesis (Kulich et al., 2010). Mutation in the AtEXO84b leads to a dwarf phenotype and also results in polar growth abnormalities (Fendrych et al., 2010). Involvement of SEC3 in polar exocytosis was reported by Wen et al. (2005). They studied maize *ROOTHAIRLESS1* gene (*RTH1*), homolog of *SEC3*, and showed that *rth1* mutants are impaired in root hair elongation and exhibit other growth abnormalities. Mutations in any of AtSEC5, AtSEC6, AtSEC8, and AtSEC15a subunits result in a similar pollen-specific transmission defect caused by reduction of pollen tube germination and defective polarized growth of pollen tubes resulting in aberrant short and wide pollen tubes (Cole et al., 2005; Hála et al., 2008). These data strongly support the indispensable requirement of the exocyst in the regulation of polar exocytosis in *Arabidopsis*.

Generally, although the exocyst complex seems to consist invariantly of eight subunits in all eukaryotes, the number of alleles for each subunit differs among kingdoms. In contrast to yeast and animals, plant exocyst subunits are encoded by multiple genes, indicating the possible redundant function of individual subunits. In *Arabidopsis*, only *SEC6* and *SEC8* are single-copy genes, *SEC3*, *SEC5* and *SEC15* are encoded by two alleles, and *SEC10* and *EXO84* by three alleles. Huge multiplication occurs in the case of *EXO70* represented by 23 alleles (Eliáš et al., 2003, Synek et al., 2006). A broad phylogenetic analysis revealed that all *EXO70* genes in plants can be grouped into three major families, which are further subdivided into nine subfamilies designated EXO70A-EXO70I. The multiplication appears to be generated through ancient duplication in a single ancestral gene. This exocyst subunit genes expansion is common for all land plants, including nonvascular mosses (Eliáš et al., 2003; Synek et al., 2006; Chong et al., 2010) and importance of such multiplication is a subject of research and is discussed elsewhere (Žárský et al., 2009; Zhang et al., 2010). One explanation is that different *EXO70* genes are expressed in different time during development or in different tissues. This was supported by expression analysis revealing that several *EXO70* genes are ubiquitously expressed in most organs, while most of them exhibits organ-specific expression pattern (Synek et al., 2006; Chong et al., 2010). Another possibility is that different EXO70 subunits may function in different membrane trafficking steps and interact with different partners in plants (Chong et al., 2010; Zhang et al., 2010). As the remaining *Arabidopsis* exocyst subunit genes are also duplicated, with the exception of SEC6 and SEC8, these assumptions can be generalized also for other subunits.

From the two-allelic genes encoding exocyst subunits in *Arabidopsis*, SEC5 was studied from the genetic point of view. It was shown that both alleles are redundant and *atsec5a* and *atsec5b* single mutants have no obvious morphological changes compared to wild-type plants. Also the transmission through the pollen was not affected in any of single mutants. However, some morphological defects were observed in the case of *atsec5a/atsec5b* double mutant (Hála et al., 2008). The *Arabidopsis* SEC15 subunit is also encoded by two alleles, *AtSEC15a* and *AtSEC15b*, out

of which *AtSEC15a* was already studied. *atsec15a-1* mutant allele is not transmitted through the pollen in the described experimental set-up but homozygous mutant plants are produced rarely from heterozygous plants. Our observations presented in this thesis show that *atsec15b-1* mutant exhibits similar phenotype, although the transmission of the mutant allele through the pollen was possible, however at lowered rate when compared to the wild type. Additionally, *atsec15a-1* mutation is accompanied by reduced pollen germination and defective polarized growth of the pollen tube as exemplified by aberrant short and wide pollen tubes (Hála et al., 2008). Pollen from *AtSEC15b/atsec15b-1* germinates to form pollen tubes morphologically undistinguishable from wild-type pollen tubes. However, when we measured the length of these pollen tubes, we uncovered that pollen from heterozygous plants formed pollen tubes that were generally shorter, reflecting probably the defect in polar growth as a consequence of mutation in *AtSEC15b*. Heterozygotes also had much lower frequency of long pollen tubes compared to wild-type. This might be due to the fact that heterozygous pollen is a mixture of mutant and wild-type pollen in a ratio 1:1. But this applies only if wild-type and mutant pollen germinates with the same efficiency. These findings suggest that while SEC5 alleles are largely redundant, SEC15 might adopt certain specialization during pollen germination in *Arabidopsis*. Nevertheless, it will be necessary to complete these findings by direct observations of possible affected germination or morphological changes of mutant pollen tubes. For this purpose we plan to cross *AtSEC15b/atsec15b-1* heterozygous plants with plants homozygous for the *qrt1* (*quartet1*) mutation. In the *qrt1* background, all products of a single meiosis are always released in tetrads consisting of two wild-type and two mutant pollen grains allowing us to examine pollen phenotypes resulting from a gametophytic mutation (Preuss et al., 1994).

Moreover, we have noticed significant increase of aborted embryos in *AtSEC15b/atsec15b-1* heterozygous siliques, implicating the requirement of functional AtSEC15b for proper embryo development. In *Drosophila*, the exocyst component Sec5 is essential during the oogenesis, a process, which requires the establishment and maintenance of cellular asymmetry within the developing oocyte as a result of directed membrane traffic. Sec5 localization undergoes dynamic changes during *Drosophila* oogenesis, correlating with the sites at which it is required for the traffic of membrane proteins. Mutants in this gene are characterized by defects in membrane trafficking and the posterior positioning of the oocyte leading to embryonic lethality. It was concluded that Sec5 is required for directed membrane traffic and consequently for the establishment of polarity within the developing oocyte (Murthy and Schwarz, 2004). Later, isolation of new allele of *sec5* with temperature-sensitive phenotype allowed further investigation of exocyst function in *Drosophila* embryo development and it was found that *Drosophila* Sec5 mediates embryo cellularization. During this process a syncytium of ~6000 nuclei is subdivided into discrete cells by specialized form of cytokinesis, during which Sec5 is concentrated at the site of membrane addition. In mutant embryo, the cleavage furrows do not invaginate between nuclei and consequently cells do not form. Additionaly, post-Golgi trafficking is blocked and newly synthesized membrane protein accumulates in cytoplasmic vesicular compartments instead of the plasma membrane (Murthy et al., 2010). This process is similar to budding yeast, where exocyst is also essential for the separation of the daughter and mother cells (TerBush et al., 1996). In fission yeast, the exocyst is also

required for septum growth and separation during cytokinesis (Wang et al., 2002). In higher eukaryots, exocyst *sec8* knockout mice display very early embryonic lethality probably also as a consequence of impaired cell division in the absence of proper secretion (Friedrich et al., 1997). As we demonstrated that all *Arabidopsis* exocyst subunits also participates in cytokinesis, which was further supported by observations that *atexo70A1* mutant is defective in initial cell plate morphology (Fendrych et al., 2010), we can suppose that the arrest of embryo development resulting from *atsec15b-1* mutation may be also accompanied by defective plant cytokinesis. Another possibility is that functional AtSec15b as a part of the tethering complex is involved in the secretion of specific proteins during *Arabidopsis* embryogenesis, which is in mutant embryos compromised.

Finally, complete depletion of AtSEC15b affects the growth of sporophyte resulting in a dwarfed phenotype of *atsec15b-1* homozygous plants with reduced size of organs and loss of apical dominance. These mutants are in addition sterile as these plants probably lack mature pollen and are not able to produce any siliques. In future, we would like to characterize also *atsec15b-1/atraba4a* double mutant plants, which we have already started to generate, to investigate, whether a disruption of both interacting partners leads to more dramatic changes during *Arabidopsis* development. We have previously characterized T-DNA insertional mutants in other exocyst subunits. On a sporophytic level, *atexo70a1* mutation results in a discernible phenotype, with semi-dwarf stature, smaller organs, reduced apical dominance, shortened hypocotyls in etiolated seedlings and disruption of root hair growth (Synek et al., 2006). At certain points this phenotype is very similar to *atsec15b-1* mutant phenotype (Figure 5.1). On the other hand, *atexo70A1* plants are able to sporadically generate homozygous progeny, although they produce much fewer seeds in siliques comparing to wild-type indicating their partial sterility (Synek et al., 2006). This may reflect a huge multiplication of EXO70 family and possibly redundant functions of individual members.

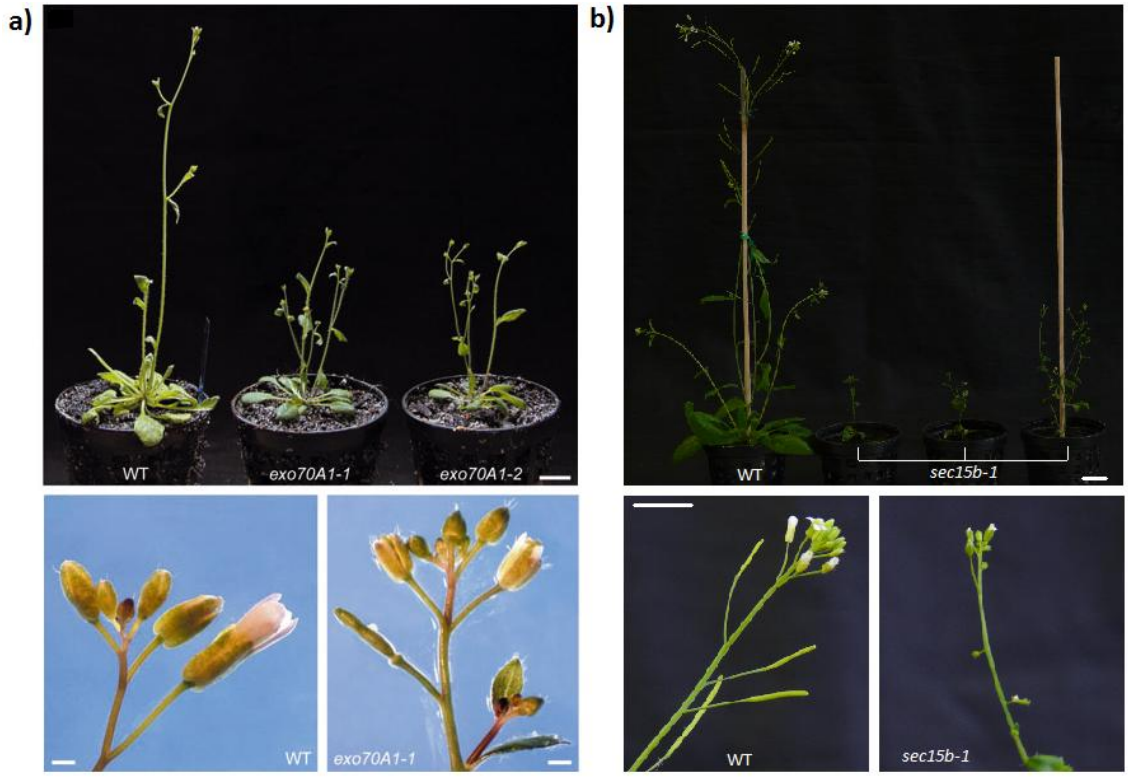


Figure 5.1. Comparison of phenotypic defects in *Arabidopsis exo70A1* and *sec15b-1* mutants.
a) *atexo70A1* phenotype compared to wild-type plants. Six-week-old plants in soil, bar = 1 cm (upper row) and detailed inflorescence, bar = 1 mm.
b) *atsec15b-1* phenotype compared to wild-type plants. Seven-week-old plants in soil, bar = 1 cm (upper row) and detailed inflorescence, bar = 1 cm.

Mutation in another exocyst subunit, AtEXO84b, leads to more severe growth retardation compared to other viable plant exocyst mutants. *Atexo84b* homozygotes have compromised leaf epidermal cell and guard cell division and are also sterile (Fendrych et al., 2010). These data indicate that each exocyst subunit performs its unique and irreplaceable role and that for the proper function of the whole exocyst complex in plant morphogenesis and development, each individual subunit must be functional.

These all results, together with expression analysis retrieved from Genevestigator databases, led us to propose that AtSEC15b represents the more abundant SEC15 allele in *Arabidopsis*, affecting developmental processes in both, gametophyte and sporophyte, while AtSEC15a seems to functionally specialize during evolution to participate predominantly in pollen development. Since we expected that in *atsec15b-1* homozygous plants, AtSEC15a subunit is functional, these data also indicate that the loss of function of the AtSEC15b gene is not fully compensated by AtSEC15a in *atsec15b-1* plants, and thus we concluded that AtSEC15b is essential for proper plant growth and development.

Chapter 6

CONCLUSIONS

I. Phylogenetic analysis revealed that SEC15 diversified from eukaryotic common ancestor with one major gene duplication occurring during the evolution. Multiple sequence alignment concerning on Rab binding site suggest that all Sec15 homologs retained generally conserved structure, nevertheless residues responsible for Rab binding in *Drosophila* Sec15 exhibit rather high variability.

II. Expression analysis revealed that *Arabidopsis* SEC15 genes exhibit tissue specific expression with *AtSEC15a* being expressed especially in pollen, whereas expression of *AtSEC15b* is generally stronger and more ubiquitous.

III. Using two-hybrid assay in *Saccharomyces cerevisiae*, we identified the interaction between AtSEC15b and AtRABA4a and showed the specificity of the interaction in respect to nucleotide bound state of Rab GTPase. These results imply that the interaction is well conserved throughout the eukaryotic evolution.

IV. We prepared fusion proteins to obtain specific antibodies against AtSEC15b and AtRABA4a and used them for verification that Rab GTPase AtRABA4a associates with exocyst *in vivo* by co-immunoprecipitation experiment.

V. Overexpression of *AtSEC15b* in yeast thermosensitive mutant *sec15-1* was able to fully complement the growing defect of the *sec15-1*, indicating that AtSEC15b is able to rescue the mutant phenotype possibly via the substitution of the yeast Sec15 protein function, which further demonstrates a conservation of Sec15-Rab interaction throughout the evolution.

VI. Subcellular localization studies uncovered the existence of AtRABA4a-positive compartments, which are not labelled either by AtSEC15b or by selected endomembrane markers. Additionally, AtSEC15b and AtRABA4a localize to the cell plate demonstrating that they participates in plant cytokinesis.

VII. Functional analysis of insertional mutants revealed that mutation in *AtSEC15b* gene results in male-specific transmission defect caused by reduced growth of pollen tubes reflecting probable defect in polar exocytosis. Moreover, functional AtSEC15b is required for proper embryo development. And finally, *atsec15b-1* mutant plants exhibit dramatic phenotypic changes in plant development. Mutants are dwarfed with smaller organs, accompanied by loss of apical dominance and an absence of siliques indicating that they are not able to produce progeny.

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Abbreviations

ADH1	alcohol dygedrogenase 1
BP-80	binding protein of 80 kDa
CA	constitutively-active
DN	dominant-negative
ECL	enhanced chemiluminescence
GAL	galactose
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
GST	glutathione-S-transferase
IPTG	isopropyl-beta-D-thiogalactopyranoside
MDCK	Madin Darby canine kidney
mRFP	monomeric red fluorescent protein
Ni-NTA	nickel-nitrilotriacetic acid
PBS	phosphate buffered saline
PI4P	phosphatidylinositol 4 phosphate
PC12	pheochromocytoma cells
PPT	ammonium 2-amino-4-(hydroxymethylphosphinyle) butyrate
RT-PCR	reverse transcriptase-polymerase chain reaction
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ST	sialyltransferase
T-DNA	transferred DNA
VHA-a1	vacuolar proton ATPase, subunit a1
WT	wild-type
YFP	yellow fluorescent protein

List of Publications

Carol, R.J., Takeda, S., Linstead, P., Durrant, M.C., Kakešová, H., Derbyshire, P., Drea, S., Žárský, V., Dolan, L. (2005). A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. Nature **438**: 1013-1016.

For this paper, I performed pull-down assay to test the interaction between WT or mutant RhoGDI and Rop GTPases and participated in writing of the corresponding part of the manuscript.

Fendrych, M., Synek, L., Pečenková, T., Toupalová, H., Cole, R., Drdová, E., Nebesářová, J., Šedinová, 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.

For this paper, I prepared *AtSEC15b:GFP* stably transformed *Arabidopsis* plants and visualized the localization of AtSEC15b in meristematic cells during cytokinesis.

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 **62**: 2107-2116.

For this paper, I prepared two constructs for yeast two-hybrid assay.

Manuscript in preparation:

Toupalová, H., Hála, M., Potocký, M., Synek, L., Žárský, V. Exocyst subunit AtSEC15b is important for polarized growth and intermediates interaction with small GTPase AtRABA4a

For this paper, I did a majority of experimental work and currently I am writing the manuscript.

Supplemental data

Supplemental data 1: List of primers

Primer	Sequence
AtRABA4a pQE32 RP	5´-GACGTGGATCCAGATGACTAGTGGAGGAGG-3´
AtRABA4a pQE32 LP	5´-GAGTCGACTTATCTAAGAGTTACAACACATG-3´
N-AtSEC15b pGEX4T2 RP	5´-TCGGATCCAAAACAATGCAATCGTCGAAAGG-3´
N-AtSEC15b pGEX4T2 LP	5´-CATAATCGTCGACCAGTTAGAGATGATTAGC-3´
AtRABA4a pGBKT7 RP	5´-TTGGATCCAGATGACTAGTGGAGGAGGAT-3´
AtRABA4a pGBKT7 LP	5´-TTGTCGACCTAAGAGTTACAACACATGTT-3´
AtRABA4b pGBKT7 RP	5´-TTGGATCCAGATGGCCGGAGGAGGCGGAT-3´
AtRABA4b pGBKT7 LP	5´-TTTGTCGACTCAAGAAGAAGTACAACAAGT-3´
AtRABA4c pGBKT7 RP	5´-TTGGATCCCGATGTCAAAATTTCAGAGCA-3´
AtRABA4c pGBKT7 LP	5´-TTGTCGACCTATGATGTTCCACAACAACC-3´
AtRABA4d pGBKT7 RP	5´-TTGGATCCAGATGTCTAATTTGTATGGAGAT-3´
AtRABA4d pGBKT7 LP	5´-TTGTCGACTTACGATTTGCCGCAACATCC-3´
AtRABH1c pGBKT7 RP	5´-GGAAGGATCCATATGGCTTCGGTTTCACC-3´
AtRABH1c pGBKT7 LP	5´-TTATGTCGACTTGAATCAACAAGAACAGCC-3´
AtSEC15b pGADT7 RP	5´-GGAGGGATCCCAATGCAATCGTCGAAAGG-3´
AtSEC15b pGADT7 LP	5´-GTGCCTCGAGCCATCATCAGCTCACATC-3´
AtSEC15b pBAR1 RP	5´-TCGGATCCAAAACAATGCAATCGTCG-3´
AtSEC15b pBAR1 LP	5´-GTTCTAGAATCCATCATCAGCTCAC-3´
AtRABA4a pBAR1 RP	5´-GAGACGGGATCCAAGATGACTAGTGG-3´
AtRABA4a pBAR1 LP	5´-GAATTGATCTAGATTATCTAAGAGTTAC-3´
AtRABA4a SN	5´-GCCAGTATCTGGTTTTTCCCAACCGCGG-3´
AtRABA4a QL	5´-GGGATACCGCCGGCCTAGAACGATACAGAGC-3´
AtSEC15b RP	5´-TTGGATCCATATGCAATCGTCGAAAGGA-3´
AtSEC15b LP	5´-CAGTAAGAGATGATTAGCCGTC-3´
LBb1	5´-CTGGGAGAACATAAGAACGTGAAG-3´
ACT7 RP	5´-GAAACTCACCACCACGAACCA-3´
ACT7 LP	5´-GCCGATGGTGAGGATATTCAGC-3´

Supplemental data 2: Experiments included in the expression analysis

Experiment IDs		
AT-00019	AT-00094	AT-00283
AT-00022	AT-00096	AT-00298
AT-00042	AT-00117	AT-00317
AT-00087	AT-00133	AT-00322
AT-00088	AT-00136	AT-00327
AT-00089	AT-00172	AT-00331
AT-00090	AT-00178	AT-00332
AT-00091	AT-00189	AT-00410
AT-00092	AT-00191	
AT-00093	AT-00218	