Charles University in Prague
Faculty of Science

Study program: Biology
Study branch: Experimental Plant Biology

Diploma Thesis
Diplomová práce

Characterization of the exocyst complex SEC15 subunit in *A. thaliana*

Charakterizace podjednotky SEC15 poutacího komplexu exocyst u *A. thaliana*

Bc. Klára Aldorfová

Supervisor: RNDr. Michal Hála, Ph.D.
Consultants: doc. RNDr. Viktor Žárský, CSc., Mgr. Martina Růžičková

Prague, 2016

Statement:

I state that I have completed this thesis by myself and that I have properly cited all used references and other sources. Neither this thesis nor its parts have been submitted to achieve any other academic title(s).

Prohlášení:

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

In Prague / V Praze, 29. 4. 2016

Signature / Podpis

Acknowledgements

I should like to express my gratitude to my supervisor RNDr. Michal Hála, Ph.D., and my consultants Mgr. Martina Růžičková and doc. RNDr. Viktor Žárský, CSc., for their kind support, meaningful comments and last but not least, for their great leadership. I want to thank to all members of Laboratory of Plant Morphogenesis and Laboratory of Cell Biology for their advices and inspiring and kind atmosphere during the experimental work. Special thanks go to Mgr. Hana Soukupová, Ph.D., for her comments on the manuscript. I am grateful to Marta Čadyová, Bc. Jana Šťovíčková, Ing. Andrea Potocká, Ph.D. for their technical assistance. I would like to acknowledge my family for their support.

Following people contributed by direct experimental work to the results included in this thesis:

Martina Růžičková (Institute of Experimental Botany) introduced me to the experimental work with proteins and she directly contributed to the optimalization of pull down assays.

Abstract

The final step of secretion termed exocytosis is mediated by the exocyst complex. The exocyst is an evolutionary conserved protein complex that tethers secretory vesicle to the target membrane and consists of eight subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo84, and Exo70. Sec15 exocyst subunit was previously shown to connect the rest of the exocyst complex with a secretory vesicle in yeast, mammals and fruit fly via interaction with Rab GTPase and GEF of Rab GTPase. Here, I show that plant SEC15B potentially functions in evolutionary conserved manner.

First, two mutant lines of *Arabidopsis thaliana sec15b* mutant were tested in characteristics typical for other exocyst mutants. Although some characteristics reach certain level of plasticity, both *sec15b-1* and *sec15b-2* show similar tendencies, which are mostly consistent with defects with other mutants in exocyst subunits. *sec15b-1* has been determined as a stronger allele that is defective in formation of seed coat, elongation of etiolated hypocotyl, growth of stem and primary root, establishment of axillary branches and lateral roots, diameter of rosette and, unexpectedly, growth of pollen tubes. Phenotype of *sec15b-1* was rescued by insertion of *SEC15B* gene under *SEC15B* promotor. Second, complementation test showed that SEC15B and SEC15A are functionally redundant in seed coat formation and development of etiolated hypocotyl. Third, SEC15B protein was successfully expressed, purified and tested in protein lipid interaction and protein-protein interaction with Rab GTPase. Using PIP Strip analysis, interaction of SEC15B with lipids was not confirmed. In contrast, RAB A4a GTPase was identified as a potential interactor of SEC15B in pull down assay. Therefore, plant SEC15B exocyst subunit potentially acts in evolutionary conserved manner.

Keywords:

SEC15A, SEC15B, exocyst complex, secretory pathway, secretory mutant, *Arabidopsis thaliana*, Rab GTPase, lipid binding assays

Abstrakt

Exocytóza je posledním krokem buněčné sekrece a zprostředkuje ji proteinový komplex nazývaný exocyst. Exocyst je evolučně konzervovaný poutací komplex, který slouží jako kotva, která přichycuje sekretorický váček k cílové membráně. Exocyst se skládá z osmi podjednotek: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo84 a Exo70, přičemž podjednotka Sec15 byla identifikována jako spoj mezi sekretorickým váčkem a zbytkem exocystu pomocí interakce s malou GTPázou a jejím výměnným faktorem (GEF). Tato práce si kladla za cíl ověřit, zda by podjednotka exocystu SEC15B mohla hrát podobnou úlohu i v rostlinné buňce.

Nejprve byly analyzovány dvě mutantní linie *sec15b Arabidopsis thaliana* ve znacích typických pro ostatní podjednotky exocystu. Přestože jsou některé fenotypové znaky plastické, obě linie vykazují stejné tendence v chování a zároveň jsou jejich fenotypové znaky zpravidla shodné s mutanty v jiných podjednotkách exocystu. Linie *sec15b-1* má výraznější projevy mutace, které zahrnují narušenou tvorbu semenného obalu, elongaci etiolovaného hypokotylu, růstu prýtu a primárního kořene, zakládání postranních větví a postranních kořenů, průměr růžice a nečekaně i růst pylových láček. Fenotyp linie *sec15b-1* byl úspěšně komplementován genem *SEC15B* pod vlastním promotorem. Komplementační test ukázal, že izoformy SEC15A a SEC15B jsou vzájemně redundantní na úrovni tvorby semenných obalů a vývoje etiolovaného hypokotylu. Nakonec byl protein SEC15B úspěšně exprimován v bakteriích, purifikován a poté byl využit pro studium protein-lipidových interakcí a protein-proteinové interakce s Rab GTPázu. Za pomoci PIP Strip metody bylo zjištěno, že protein SEC15B pravděpodobně neinteraguje s lipidy. Naproti tomu, SEC15B byl pomocí pull down testu určen jako kandidátní interaktor RAB A4a GTPáza. Proto je pravděpodobné, že rostlinná podjednotka exocystu SEC15B hraje podobnou roli jako u ostatních organizmů.

Klíčová slova:

SEC15A, SEC15B, komplex exocyst, sekretorická dráha, sekretorický mutant, *Arabidopsis thaliana*, Rab GTPáza, lipid vazebné eseje

CONTENT

List of abbreviations

A. thaliana	*Arabidopsis thaliana*
APS	ammonium persulfate
ARF	ADP- ribosylation factor
D. melanogaster	*Drosophila melanogaster*
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediamineteraacetic acid
GAP	GTPase activating proteins
GDP	guanosine 5´diphosphate
GEF	guanine nucleotide exchange factor
GST	glutathione S-tranferase
GTP	guanosine 5´triphosphate
IPTG	isopentenyl thiogalactoside
LOF	loss of function
LUV	large unilamelar vesicles
M	marker
OD	optical density
P	pellet
PA	phosphatidic acid
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
$PI(3,4,5)P_3$	phosphatidylinositol(3,4,5)trisphosphate
$PI(3,5)P_2$	phosphatidylinositol(3,5)bisphosphate
$PI(4,5)P_2$	phosphatidylinositol(4,5)bisphosphate
PI(5)P	phosphatidylinositol(5)phosphate
PI(4)P	phosphatidylinositol(4)phosphate
PMSF	phenyl methyl sulphonyl fluoride
PS	Phosphatidylserine
Rab GTPase	Rat brain GTPase
ROP GTPase	Rho of plants GTPase
SDS	sodium dodecyl sulfate
SN	supernatant
SNARE	Soluble N´ethyl maleimide sensitive factor adaptor protein receptor
TEMED	tetramethylethylenediamine
TGN	trans Golgi network
WT	wild type

Aims of the thesis

1) To find defects caused by *sec15b* loss of function (LOF) mutation in *Arabidopsis thaliana*
2) To analyse functional redundancy of SEC15A and SEC15B isoforms
3) To investigate the mechanism of *Arabidopsis* SEC15B action in relation to secretory vesicles

Introduction

Morphogenesis, cell growth, delivery of cargoes such as components of cell wall and transmembrane proteins to the plasma membrane, signaling and wide range of other processes are highly dependent on secretory pathway in all eukaryotic cells. In most instances, general model of secretion derived from opisthokonts appears to be evolutionary conserved in other organisms including plants. However, some molecular mechanisms are unique for specific eukaryotic groups, such as presence of turgor or specific signaling pathway (Vaškovičová et al., 2013).

In plants, secretory pathway ensures delivery of new cell wall material, lipids and proteins to the plasma membrane and enables spatiotemporal regulation of cell growth and morphogenesis. Conventional protein secretion includes cotranslational insertion into the endoplasmic reticulum with following maturation in the Golgi apparatus and transport to the vacuole or the plasma membrane. In addition, unconventional protein secretion based on leaderless secretory proteins, which do not host any signaling peptide, appears to be widely common phenomenon in plants (Drakakaki and Dandekar, 2013). Either way, an exocytotic vesicle is created, delivered to the proximity of the target compartment, and prepared for the final secretory step termed exocytosis.

During secretory event, the vesicle is formed, moved to the precise site of secretion, anchored to the target membrane, and finally, both target and vesicle membrane fuse. These steps involve similar key players in all eukaryotes including cytoskeleton, cytoskeletal motors, small GTPases and their regulators, tethering complexes and SNARE (SNAP receptor) proteins (Vaškovičová et al., 2013).

Cytoskeleton ensures movement of secretory vesicles to the site of secretion

Cytoskeleton is an inner scaffold of any cell, which polymerizes into long formations and serves as a highway for motility of all cell compartments. Both cytoskeleton types, actin and microtubules cooperate with appropriate cytoskeletal motors myosins and kinesins to enable regulated movement of particular organelles in plants (Romagnoli et al., 2007).

Small GTPases are master regulators of trafficking

Small GTPases are master regulators orchestrating wide range of processes including secretory vesicle formation and anchoring to the target membrane (see in Chapter 2). All small GTPases are able to bind GTP (guanosine 5´triphosphate) with high affinity, but hydrolyse this GTP with low

efficiency rate. In addition, some of the small GTPases are posttranslationaly modified by prenylation or S-acylation. In eukaryotes, Ras, Rab, Arf, Sar, Rho, and Ran GTPases are distinguished as subfamilies of small GTPases. However, only Rab, Rho, and Arf GTPases are directly involved in plant vesicular trafficking (Bischoff et al., 1999).

Activity of small GTPases is regulated by other proteins via hydrolysis of GTP and exchange of GDP for GTP. GEFs (Guanine nucleotide exchange factors) activate small GTPases through exchange of GDP for GTP. Once bound to GTP, the small GTPase switches its conformation into an active state and interacts with effector proteins. Active conformation of small GTPase is controlled by hydrolysis of GTP, which is regulated by GAPs (GTPase activating proteins). GDP-bound small GTPase becomes inactive and usually does not interact with related effectors anymore (Wu et al., 2008). In addition, activity of small GTPases is possibly influenced by direct interaction with lipids (Kost, 2008).

Tethering complexes attach vesicles to the target membrane

Tethering complexes serve as a physical link between two compartments before their fusion and thus attach vesicle at the precise site of secretion. Tethering complexes can be divided into two groups: group of long coiled-coil proteins that are able to form homodimers and multisubunit complexes, subunits of which act during distinct secretory steps in various combinations. In each step of trafficking that requires membrane fusion, appropriate tethering complexes anchor distinct vesicles to distinct target membranes (Whyte and Munro, 2002). During exocytosis, multisubunit complex called exocyst tethers secretory vesicles to the plasma membrane. Eight subunits of exocyst (SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO84 and EXO70) were shown to cooperate during secretion (Hála et al., 2008).

SNARE proteins define membrane identity and enable membrane fusion

SNARE (Soluble N´ethyl maleimide sensitive factor adaptor protein receptor) is a protein anchored in membranes and contains a coiled-coil SNARE motif, which forms a simple helical structure. SNARE proteins are divided into two groups depending on the central amino acid. First, Q-SNAREs carry glutamine and label mostly target membrane. Second, R-SNAREs contain conserved arginine and reside on membranes of vesicles (Lipka et al., 2007). During final step of exocytosis, compatible three Q-SNAREs and one R-SNARE associate to form a stable SNARE complex, which initiates membrane fusion (Saito and Ueda, 2009).

Interplay between cytoskeleton and secretory pathway enables spatiotemporally controlled secretion

Secretory vesicles are formed with assistance of small GTPases, delivered by components of cytoskeleton, attached to the target membrane through tethering complex, and membrane fusion is mediated by formation of the SNARE complex. However, distinct components of cytoskeleton and secretory pathway cooperate with and regulate each other making secretion more fluent cascade rather than isolated steps. Here I summarize state of knowledge corresponding to function of Sec15 as an exocyst subunit in all organisms, and I aim to describe known facts about involvement of plant SEC15 exocyst subunit in secretory pathway and known links between components of secretory pathway with exocyst complex in eukaryotes.

1. Current state of knowledge in Sec15 regulatory pathway and mechanism of function

1.1. The story from the Sec15 protein to the Sec15 as an exocyst subunit

The Sec15 protein was discovered relatively long time ago. In 1980, the Sec15 locus was found by Randy Schekman and his colleagues, who successfully prepared 23 yeast secretory mutants (Novick et al., 1980). Chemical mutagenesis was used for preparation of thermo-sensitive yeast colonies defective in various steps of protein secretion. This resulted in very simple but progressive genetic analysis with further medical applications. Therefore, Randy Schekman received the Nobel Prize in Physiology or Medicine in 2013.

In first electron microscopy characterizations, thermo-sensitive mutation in the Sec15 locus resulted in an accumulation of secretory vesicles (Novick et al., 1980). Eight years after sec15 mutant description, yeast Sec15 protein was observed to act in the process of exocytosis. Salminen and Novick (1987, 1989) showed that Sec15 overexpression causes clustering of secretory vesicles and postulated a genetic interaction between Sec4 gene, a gene for Rab GTPase (Salminen and Novick, 1989). Later on, Sec15p was suspected to play a role in more complicated net of interactions with other Sec proteins such as Sec2p (Rab GTP exchange factor) (Nair et al., 1990) or Sec10p (another exocyst subunit) (Salminen and Novick, 1987).

In 1991, Sec15 protein was found in a soluble 19.5S particle (Bowser and Novick, 1991). This was an important milestone in Sec15 research, because it brought the idea that Sec15 is a part of a multisubunit protein complex. Following cross-linking and co-immunoprecipitation analysis showed that Sec8p (Bowser et al., 1992), Sec6p (TerBush and Novick, 1995) and potentially five other proteins assemble during the 19.5S particle formation. Mutation in other three proteins,

Sec3p, Sec5p, and Sec10p disrupted the 19.5S particle and thus these were also suspected to be parts of the complex. Finally, the peptide microsequencing of the purified protein complex determined Sec6p, Sec8p, Sec15p, Sec3p, Sec5p, Sec10p and Exo70p as the complex members (TerBush et al., 1996). Exo84 is the last of the exocyst subunits described three years after the exocyst discovery (Guo et al., 1999a).

Role of Sec15 is predominantly associated with the exocyst complex. Wide range of studies show that alteration of Sec15 function results in abortion of multiple processes connected to secretion (cytokinesis; tracheal formation; neurotransmitter releases etc.). Therefore, it is important to understand the role of the whole exocyst complex and then we could elucidate the importance of Sec15 itself. Unfortunately, the role of Sec15 was examined mostly till 2000. Therefore, current state of knowledge benefits only from a little progress within several years.

1.2. The yeast Sec15 exocyst subunit

Exocyst is an evolutionary conserved protein complex that consists of eight long helical subunits. All of these proteins share tandem helical-bundle repeats and rod-like domains with unique hydrophobic and electrostatic interaction pattern (Sivaram et al., 2006). Sec15p, one of the exocyst subunits, was shown to mediate an important link between the secretory vesicle and the rest of the exocyst complex. Yeast genome possesses a single copy gene that is expressed into a protein of 116 kDa of molecular mass. Sec15 protein is soluble and negatively charged at neutral pH (Salminen and Novick, 1989), which enables the protein to attract a wide range of interactors during the final step of secretion.

1.3. Conventional interactions of Sec15 with other proteins

Vesicle formation, movement, tethering and fusion are the crucial steps during the vesicle trafficking (see Figure 1). Sec15p acts during vesicle movement and tethering through various important interactions with the other important secretory players, such as actin, myosin, Rab GEF, and Rab GTPase (see the conventional interactors of Sec15p in Table 1).

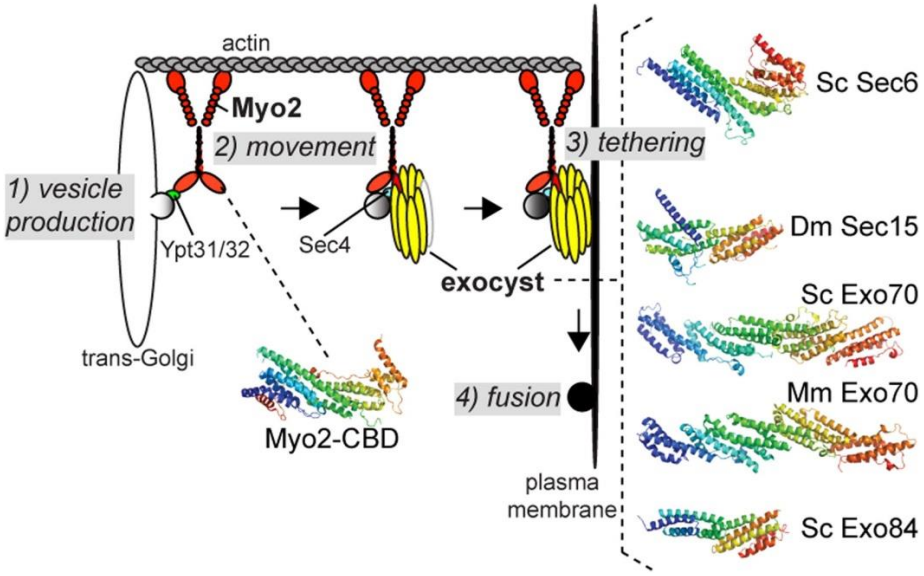

Figure 1: The proces of vesicle production, maturation, movement, tethering to the plasma membrane and finally fusion with the target membrane. Secretory vesicle is derived from trans-Golgi and undergoes maturation under the direction of Ypt31p/32p Rab GTPase. Movement of the vesicle is ensured by movement of Myo2p along actin cables. Rab GTPase Sec4p is bound to the vesicle and its activated form attracts the exocyst complex. Once the vesicle is tethered to the plasma membrane via the exocyst, vesicle and target membrane can fuse (Jin et al., 2011).

1.3.1. Vesicle formation

Vesicle formation is a process, in which a secretory organelle, mostly derived from the trans Golgi network (TGN), undergoes changes in specific lipid and surface protein composition and results in creation of a new compartment. Several steps in the precise order are required to reach the appropriate results and usually are orchestrated by Rab GTPases. First, two Rab GTPases Ypt31p/32p coordinate formation of the secretory vesicle (Jedd et al., 1997) and function in a concert with membrane lipid phosphatidylinositol(4)phosphate (PI(4)P). Second, Ypt32p enables recruitment of Sec2p (Ortiz et al., 2002), the Rab GEF for Sec4p Rab GTPase (Elkind et al., 2000); see below. Third, Sec2p is intensively phosphorylated, changes its conformation (Stalder et al., 2013; Stalder and Novick, 2016), and binds preferentially the Sec15p exocyst subunit (Medkova et al., 2006). This interaction is stimulated by decreasing concentration of PI4P (Mizuno-Yamasaki et al., 2010). Finally, Sec15p binds to Rab GTPase Sec4p (Guo et al., 1999b). This cascade leads to the active Sec2p/Sec4p/Sec15p GEF-effector complex (see Figure 2).

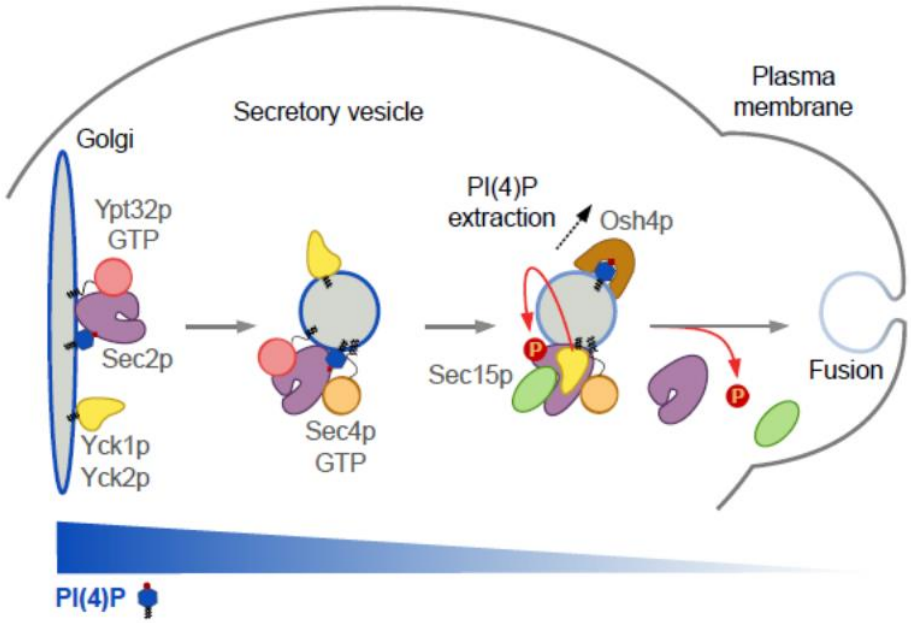

Figure 2: Vesicle formation and maturation is orchestrated by Rab GTPases. Rab Ypt32p recruits Sec2p to the Golgi. Sec2p is intensively phosphorylated by the casein kinase Yck1p and Yck2p and serves as GEF for Sec4p. Both, Sec2p and Sec4p attract Sec15p exocyst subunit to the secretory vesicle, which mediates tethering to the plasma membrane. During vesicle maturation, PI(4)P is extracted from the membrane, Sec2p is dephosphorylated and finally, secretory vesicle fuses with the plasma membrane (Stalder and Novick, 2016).

1.3.2. Vesicle movement

Secretory vesicles move along cytoskeletal cables, usually actin cables. Yeast Myosin V, Myo2 is an actin-related molecular motor that transports a wide range of compartments including secretory vesicles (Johnston et al., 1991). At first, a couple of Rab GTPases Ypt31p or Ypt32p recruit Myo2p to the secretory vesicle (Lipatova et al., 2008). Afterwards, Myo2p is recruited to Sec4p Rab GTPase and Sec15 exocyst subunit (Jin et al., 2011). Interestingly, the cargo binding domain of Myo2p shares structural similarity with the tethering complex. This possibly results in a strong interaction with the tethering complex and thus the myosin remains bound to the vesicle until its fusion.

1.3.3. Vesicle tethering

The most important role of the exocyst complex is the attachment of the secretory vesicle to a target organelle. This process is termed vesicle tethering and requires formation of the Sec2p/Sec4p/Sec15p complex (as described above). Once is the Sec15p with the exocyst core bound to the vesicle, landmark subunits determine appropriate site for exocytotic event. Sec15p itself serves as an adaptor protein between Sec4p bound to the secretory vesicle and the rest of the exocyst complex. In yeast cells, Sec15p was confirmed to interact predominantly with the

Sec10p exocyst subunit (Guo et al., 1999b). Both, Sec15p and Sec10p were found in a pool of free exocyst subunits and in a pool of pre-assembled exocyst complex.

1.3.4. Vesicle fusion

Once is the secretory vesicle tethered to the target membrane, both compartments can fuse together. Several SNARE proteins take part in this process (Saito and Ueda, 2009). Initially, R-SNARE located on the vesicle attaches Q-SNARE proteins on the target membrane forming the SNARE complex. The force created by the complex assembly is utilized for fusion pore formation. Once is the pore created, both compartments can easily fuse together (Chen and Scheller, 2001). Usually, cytoskeleton controls the membrane dynamics after fusion. During this step, the Sec15p exocyst subunit appears to play a passive role only.

1.3.5. Unconventional interactions of exocyst with other proteins

Some of the exocyst subunits were reported to participate in processes that are not directly related to the protein secretion. Several exocyst subunits can be involved in other functions. For instance, Exo84p was found to be directly involved in pre-mRNA splicing and thus links different steps of protein secretion (Awasthi et al., 2001). Interestingly, Sec15p is able to bind the translocon pore (Toikkanen et al., 2003) and thus possibly mediates a link between different steps of protein secretion.

Conventional Yeast Sec15p interactors		
Interactor	Process	Citation
Sec10p	The exocyst complex formation	(Guo et al., 1999b)
Sec2p	Vesicle formation, tethering	(Medkova et al., 2006)
Sec4p	Vesicle formation, tethering	(Guo et al., 1999b)
Myo2p	Vesicle movement	(Jin et al., 2011)

Table 1: Conventional interactors of yeast Sec15p.

1.4. The role of Sec15 exocyst subunit in multicellular organisms

As compared to yeast, the exocyst of multicellular organisms appears to be more dynamic. The animal individual exocyst subunits appear to be more interlinked compared to the yeast exocyst. For instance, yeast Sec15p interacts with Sec10p only, whereas mammalian Sec15 was shown to bind Sec10, Exo84 and Exo70 (Liu and Guo, 2012). In addition, most of the subunits expanded into several isoforms and splicing alternatives (at least three copies of Sec15 in nematodes, two copies in mammals, two to five copies in plants), which increases a number of possible exocyst variants (Dellago et al., 2011). This phenomenon is present in most multicellular organisms because of the

increased complexity in body architecture and need of spatialtemporally specialized secretion (see the increase in Sec15 interactors of mammals and fruit fly in Table 2). However, the fundamental scheme of trafficking appears to be similar in yeast and multicellular organisms (Jin et al., 2011; Feng et al., 2012; Takahashi et al., 2012) including mammals and plants (Vaškovičová et al., 2013).

1.4.1. Mammalian mode of secretion

The principals of Sec15 function derived from the yeast model appear to be applicable also for the mammalian trafficking. First, formation of secretory vesicles depends on similar factors including the regulatory role of Rab GTPases (Rab8, Rab11). GTP-bound Rab11 and phosphatidylserine recruit Rabin8 to the secretory vesicle (Chiba et al., 2013). Rabin8 mediates nucleotide exchange for mammalian Rab8 in accordance to Rabin8 phosphorylation during formation of primary cilium. Both, Rabin8 and Rab8 are able to bind Sec15 and the Rabin8-Rab8-Sec15 effector complex appears to promote the conformational switch of Rab8 and thus mediates activation of Rab8 dependent cascade (Feng et al., 2012). Additionally to the yeast model, Sec15 associates directly with Rab11, but not with Rab4, Rab6, and Rab7 (Zhang et al., 2004). Mammalian Rab11 was described to be regulated by Evi5, which is a GTPase activating protein that associates with Rab11 in GTP-dependent manner to regulate trafficking during cytokinesis (Westlake et al., 2007).

Movement of secretory vesicles is mediated by both microtubules and actin cytoskeleton. Microtubules usually enable the transport for long distances. In contrast, actin microfilaments mediate the short range transport and promote the exocytotic events (Porat-Shliom et al., 2013) in cooperation with the actin related motor protein myosin. Mammalian myosin Vb was shown to cooperate with several Rab GTPases: Rab8a, Rab10 and Rab11a (Roland et al., 2011). Myosin Vc function is closely connected with Rab8 and Rab10, and myosin Va was demonstrated to interact with Rab10, when each combination of Rab-myosin interaction mediates distinct secretory step (Roland et al., 2009). Similarly to the yeast model, mouse myosin Va contains conserved residues shared with yeast Myo2p and interacts with the exocyst complex (Jin et al., 2011).

Tethering of the secretory vesicle to the plasma membrane is provided by the exocyst complex in a similar manner compared to the yeast tethering event. Rab GTPases play the key regulatory role in concert with the exocyst complex during this secretory step (Takahashi et al., 2012). An effector complex including Sec15 attracts the rest of the exocyst to successfully anchor the secretory vesicle to the target membrane. Finally, membranes fuse together to release the content of secretory compartment.

1.4.2. Alternative regulatory pathway in fruit flies

In *Drosophila melanogaster*, maturation of secretory vesicles is directed by Rab11 and its GAP (GTPase activating protein) Evi5 (Laflamme et al., 2012). Evi5 catalyses GTPase activity of Rab and at the same time switches the Rab into inactive form. Modified function of Evi5 disrupted proper regulation of Rab11 and thus the cell polarization, which indicates that Evi5 participates in Rab11 - directed maturation of the secretory compartment. Similarly to the fundamental scheme, movement of secretory vesicle is ensured by myosin V (Li et al., 2007), which binds Rab11.

Sec15 was found to colocalize with Rab11 in the recycling endosomes, but does not bind Rab4, Rab6 and, Rab7, which reside on early endosomes, Golgi, and late endosomes or lysosomes. However, Sec15 was described to associate with Rab3, Rab8 and Rab27 (Wu et al., 2005) that regulate trafficking of synaptic vesicles and secretory vesicles in specialized secretory cells including granule release in T-lymphocytes. Interestingly, no sequence of Sec2p homolog, which is known to regulate homologue of Rab8, was found in *D. melanogaster* genome and therefore, regulation of this part of exocytosis remains uncovered in fruit flies (Elias, 2008).

The C terminal domain of *Drosophila* Sec15 protein was crystalized (see Figure 3) revealing that the whole structure is composed entirely of distinct α-helices (Wu et al., 2005) similarly to the other crystalized exocyst subunits.

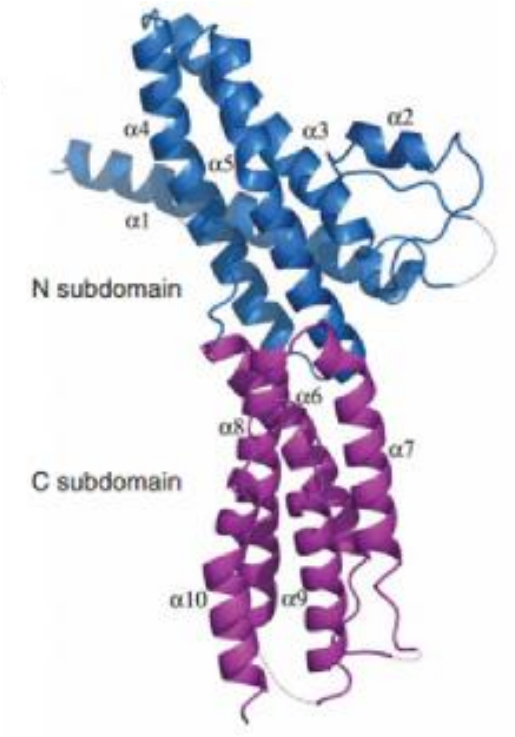

Figure 3: The C terminal part of fruit fly Sec15 was crystalized to identify two distinct subdomains, that consist of helical bundles in majority (Wu et al., 2005).

Conventional Sec15 interactors		
Interactor	Process	Citation
Rabin8	Mammalian vesicle maturation	(Feng et al., 2012)
Rab8	Mammalian vesicle maturation	(Feng et al., 2012)
Rab11	Mammalian vesicle maturation	(Zhang et al., 2004)
Myosin Va	Mammalian vesicle movement	(Jin et al., 2011)
Sec10	Mammalian vesicle tethering	(Liu and Guo, 2012)
Exo84	Mammalian vesicle tethering	(Liu and Guo, 2012)
Exo70	Mammalian vesicle tethering	(Liu and Guo, 2012)
Rab3	Fruit fly vesicle maturation	(Wu et al., 2005)
Rab8	Fruit fly vesicle maturation	(Wu et al., 2005)
Rab27	Fruit fly vesicle maturation	(Wu et al., 2005)

Table 2: Conventional interactors of mammalian and fruit fly Sec15 protein.

1.4.3. Unconventional function of exocyst subunit

Similarly to yeast Exo84p, human Exo70 was determined to participate in mRNA splicing to potentially couple several secretory steps (Dellago et al., 2011).

Using Protein Interaction Mining Tool, mammalian Sec15a was predicted to interact with cyclin-dependent kinase. This interaction can contribute to the cytokinesis via coupling secretion and cell cycle (http://liweilab.genetics.ac.cn/tm/search.php?st=gn&gn=cyclin-dependent%20kinase%20(%20cdk%20)%20-activating%20kinase%20complex&ti=9606&tn=2163&sot=nu&pg=67), which is in accordance with the hypothesis, that exocyst plays active role during cytokinesis (Neto et al., 2013).

2. Known links between exocyst and vesicle-based secretory pathway components

Sec15 was described to initiate tethering of secretory vesicles in wide range of nonrelated organisms and thus it is considered as a canonical trigger of exocyst binding to the secretory vesicle. However, it is necessary to ask, whether it is relevant to suppose that Sec15 is the only exocyst subunit able to bind secretory vesicles. Exocyst could be bound to the vesicle through wide range of interactions. In different organisms, nearly every exocyst subunit is able to specifically bind an active small GTPase, signaling lipid, or SNARE protein.

2.1. Sec5 and Exo84 interact with mammalian RalA GTPase

Ral (Ras-like) GTPase belongs to the Ras superfamily and links cytoskeleton regulation and trafficking. RalA was shown to associate with the membrane of transport vesicles and the plasma membrane during specialized exocytosis in endothelial cells, during filopodia formation and neuronal development (de Leeuw et al., 2001; Moskalenko et al., 2002; Das et al., 2014).

However, the active form of RalA preferentially resides on perinuclear recycling endosomes (Shipitsin and Feig, 2004). Exo84 and Sec5 are core exocyst subunits that directly interact with RalA GTPase in GTP-dependent manner (Fukai et al., 2003; Jin et al., 2005) and this interaction possibly integrates secretory pathway and cytoskeleton dynamics.

Precise coordination of RalA and Sec5 may regulate polarized secretion during specialized secretion events (Moskalenko et al., 2002). In contrast, Exo84 association with RalA is ensured in slightly different manner. The RalA binding motif of Exo84 (see Figure 4) completely overlaps with the lipid-binding PH (pleckstrin homology) domain. This suggests that Exo84 is able to bind RalA and phosphatidylinositol(3,4,5)trisphosphate (PI(3,4,5)P_3) and these two binding partners compete with each other (Moskalenko et al., 2003). Additionally, RalA is also important for regulation of direct link between Par6 (a component of the partitioning defective "PAR" complex) and Exo84 during development of neural progenitors and embryonic neurons to fine-tune cell polarization (Das et al., 2014).

Data from fractionated cells show that Sec5 and Exo84 appear to form several fractions of distinct exocyst sub-complexes, which might be dependent also on RalA (Guo et al., 1999a; Moskalenko et al., 2002; Moskalenko et al., 2003). RalA binding sites for Exo84 and Sec5 partially overlap and thus Sec5 competes for RalA interaction with Exo84 (Jin et al., 2005). Taken these data together, exocyst appears to be a very dynamic complex and small GTPases such as RalA appear to coordinate assembly of exocyst. In addition, RalA interactions with both Sec5 and Exo84 are required for exact control of actin microfilaments during cell migration and invasion through an extracellular matrix (Sugihara et al., 2002; Hazelett and Yeaman, 2012).

On the other hand, posttranslational modifications in yeast cells could inhibit formation of exocyst complex during special events like mitosis. Phosphorylation of Exo84 disables the protein to bind Sec10, which leads to disruption of exocyst assembly (Luo et al., 2013).

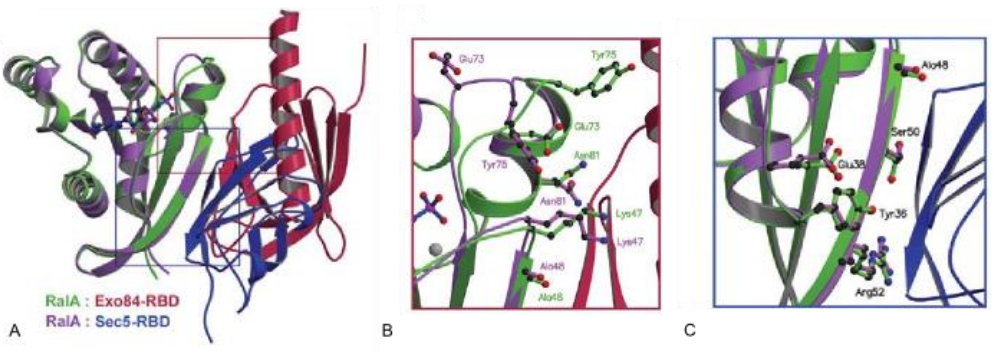

Figure 4: Crystal structure of RalA GTPase and the two exocyst subunits Exo84 and Sec5 helped to reveal the character of the interaction between small GTPase and the exocyst subunits. (A) RalA is coloured in green, while

interacts with Exo84 and purle, when creates a complex with Sec5. Exo84 is drawn in red, whereas Sec5 is highlighted in blue colour. The binding sites of Exo84 and Sec5 on the activated RalA overlap. (B) RalA changes its conformation around switch II, while interacts with Exo84. (C) RalA interacts with Sec5 via hydrogen bonds. The colours of proteins are unchanged in all three pictures. (Jin et al., 2005).

2.2. Sec8 was recently shown to interact with small GTPase *in vitro*

Recently, Sec8 was identified as an interactor of yeast Rho4 GTPase. Rho4 was shown to directly interact with Sec8 and Exo70 in GTP-dependent manner using pull down assay (Pérez et al., 2015). This interaction might be important during cytokinesis, when Rho-GEF Gef3 activates Rho4 (Wang et al., 2015). Interestingly, function of Rho4 GTPase is linked with Sec8, but Exo70 and Sec3 seem to be uninfluenced by Rho4 in *Saccharomyces pombe* (Pérez et al., 2015). This indicates that a single exocyst subunit or an exocyst subcomplex can provide a distinct role in the secretory pathway independent on the completely assembled complex.

Another link between Sec8 and small GTPase is Pob1 (pombe-boi-like) protein. It influences both actin dynamics and localization of Sec8 exocyst subunit in Rho3 dependent secretory pathway in *Saccharomyces pombe* (Nakano et al., 2011) and thus links secretory pathway with cytoskeleton dynamics.

2.3. Sec10 coordinates endosomal compartments via interaction with small GTPases

ARF6 (ADP-ribosylation factor 6) is a mammalian small GTPase, which is well known to participate in membrane recycling by regulation of endocytosis and its activity is required for regulated secretion in specialized tissues (Vitale et al., 2002). The Sec10 exocyst subunit co-localizes with ARF6 to recycling endosomes in GTP dependent manner (Prigent et al., 2003). Interestingly, FIP3 and FIP4 (family of Rab11-interacting protein) are able to interact simultaneously with Rab11 and Arf6. This could determine precise localization of FIP proteins. Moreover, FIP3 was shown to interact with Exo70p in pull down assays. Possibly, protein complex of FIP-Arf6-Exocyst could regulate tethering of recycling endosomes during cytokinesis (Fielding et al., 2005).

Rab10 was shown to regulate trafficking of endosomal tubules during basolateral recycling in *Caenorhabditis elegans* and possibly orchestrates endosomal compartments in concert with Sec10 and some other exocyst subunits, although interaction between the small GTPase and Sec10 appears to be indirect (Jiu et al., 2012; Chen et al., 2014).

3. The plant exocyst performs similar role in other organisms

3.2. Exocyst in multicellular organisms

Basic scheme of the plant secretory pathway seems to be evolutionary conserved including involvement of key players during secretion in comparison to the other organisms (Vaškovičová et al., 2013). The full complexity of the plant exocyst function has not been fully understood yet, however it is essential during a number of processes like autophagy, tip growth, cytokinesis, pathogen response, polar secretion and secretion in general (Kulich et al., 2013; Pečenková et al., 2011; Hála et al., 2008).

Plant genomes including angiosperms, lycophytes, and mosses contain homologous sequences of all eight subunits (SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70, and EXO84) of the exocyst complex (Cvrčková et al., 2012). Interestingly, some of the exocyst subunits largely expanded into gene families in contrast to yeast and animals, respectively (Table 3). For instance, EXO70 is the most diversified gene that resides in 8 to 47 paralogues in studied plants, which suggests a functional divergence of this family (Cvrčková et al., 2012; Synek et al., 2006; Kulich et al., 2013). In contrast, some of the other multiplied exocyst subunits SEC5 and SEC10 that are present in *Arabidopsis* genome in two paralogues only, are functionally redundant at least in some tissues (Vukašinović et al., 2014; Hála et al., 2008).

	SEC3	SEC5	SEC6	SEC8	SEC10	SEC15	EXO70	EXO84
Arabidopsis thaliana	2	2	1	1	2	2	23	3
Arabidopsis lyrata	2	2	1	1	1	2	23	3
Populus trichocarpa	2	2	2	2	2	5	29	8
Vitis vinifera	2^2	1^3	1^2	1^4	1^2	2^2	22^2	4^2
Solanum lycopersiocum	1	1	2	1	1	2	15	3
Oryza sativa	2	1	1	1	1	4	47	3
Sorghum bicolor	2	1	1	1	1	3	31	3
Brachypodium distachyon	2	1	1	1	1	3	27	3
Selaginella moellendorfii	2	1	2	2	2	1	8	2
Physcomitrella patens	3	3	1	3	3	2	13	7

Table 3: Number of exocyst subunits in plant genomes (Cvrčková et al., 2012; Vukašinović et al., 2014).

Nearly all of the plant exocyst subunits were shown to oligomerize into a complex *in vitro* and similar localization pattern of several subunits was demonstrated *in vivo* in *Arabidopsis* and tobacco (Hála et al., 2008; Chong et al., 2010). Analysis of mutants is another powerful tool to

study exocyst function in plants. Mutants of single copy exocyst genes are usually lethal, whereas mutants in subunits that reside in genome in more paralogues usually show characteristic phenotype. Typical defects of exocyst mutants were demonstrated mainly in *Arabidopsis thaliana* and occasionally observed in *Zea mais* and include altered embryo development, growth and germination of pollen tubes, aberrant cell plate, maturation of root hairs, altered etiolated phenotype, trichome development, architecture and length of mature sporophyte and some exocyst mutants show ectopic hypersensitive response (Kulich et al., 2013; Synek et al., 2006; Kulich et al., 2015; Hála et al., 2008; Cole et al., 2005; Zhang et al., 2013; Fendrych et al., 2010; Wen et al., 2005).

3.2. Interactions between plant exocyst with other components of secretory pathway

Up to date, no link between components of the plant secretory pathway residing on secretory vesicle and exocyst complex was found. However, it has been shown that several exocyst subunits interact with secretory components of the plasma membrane in conserved manner with respect to animals or yeast. In addition, novel plant-unique interactions with other tethering complexes were investigated, although there are some speculations about the relevance of these interactions in other organisms.

3.2.1. Plant SEC3, EXO70, and SEC6 act in a conserved manner

Sec3, Sec6, and Exo70 are known to participate in spatial land-marking for exocytosis in yeast and mammals via interaction with signaling molecules. Sec3p binds GTP-CDC42p (Zhang et al., 2001) and GTP-Rho1p (Guo et al., 2001), and Exo70 interacts with mammalian Rho protein TC10 (Inoue et al., 2003) and yeast Rho3 GTPase (Robinson et al., 1999). In addition, both Sec3 and Exo70 subunits interact with the plasma membrane directly (Zhang et al., 2008; He et al., 2007). In contrast, yeast Sec6 anchors exocyst at the plasma membrane through its interaction with the plasma membrane SNARE regulator Sec1 (Morgera et al., 2012) and the SNARE protein Sec9 (Songer and Munson, 2009). Interestingly, Sec9p-Sec6p complex does not appear to attract the rest of the exocyst complex, whereas Sec1p-Sec6p complex can interact with other exocyst subunits, which may support exact targeting to the site of secretion.

In plants, at least some of 23 isoforms of EXO70 and 2 isoforms of SEC3 appear to mediate similar land-marking function in comparison with yeast and animals (Synek et al., 2006). Plant EXO70A1 was described as a direct interactor with plasma membrane (Synek et al.; in preparation). Similarly, SEC3A was shown to bind plasma membrane directly (Fendrych et al., 2013; Potocký, personal communication). In addition, plant SEC3A cooperates with ROP1 (Rho of plants) small GTPase. This interaction is mediated indirectly by ICR1/RIP (ROP interactive partner1) protein

(Lavy et al., 2007; Bloch et al., 2008; Li et al., 2008). Plant SEC6 was shown to interact with KEULE, homologue of Sec1 SNARE regulator, during cytokinesis. Both proteins localize to the cell plate and thus mark the secretory site for the other exocyst subunits (Wu et al., 2013).

In conclusion, here I have shown that plant SEC3, EXO70, and SEC6 exocyst subunits act similarly compared to yeast and mammalian homologues. Therefore, it can be assumed that fundamental interactional pathway of the other exocyst subunit could be conserved in plants as well.

3.2.2. Plant unique exocyst interaction with other tethering complexes

Higher plants undergo plant-specific processes during development, which require unique regulations and evolutionary novel key players. For instance, process of plant cytokinesis is distinct from animals or yeast or xylem formation (xylogenesis) that requires highly regulated spatiotemporal secretion of secondary cell wall.

During xylogenesis, EXO70A1 cooperates with cortical microtubules (Li et al., 2013), conserved oligomeric Golgi complex 2 (COG2) protein and vesicle tethering 1 (VETH1) and VETH2 proteins (Oda et al., 2015) to define secondary cell wall patterns. First, VETH1 and VETH2 proteins are recruited to vesicles bound to cortical microtubules. Second, VETH1 and VETH2 bind COG2, which in turn leads to an interaction of the protein complex with EXO70A1 exocyst subunit.

COG complex belongs to a family of multisubunit complexes associated with tethering containing helical rods (CATCHR). Canonically, COG complex serves as tethering complex during intra-Golgi retrograde trafficking and interacts with all classes of molecules that are known to participate in intra-Golgi trafficking (SNARE, SNARE interacting factors, Rab GTPases, other tethers, vesicular coats and molecular motors) (Willett et al., 2013). In yeast two hybrid screens focusing on fruit fly proteom, potential interaction between COG2 and Exo70 and between COG6 and Sec6 has been identified, but has not been confirmed yet (Giot et al., 2003). In contrast, predicted interaction between *Arabidopsis* COG2 and SEC10 (Arabidopsis Interactome Mapping Consortium, 2011) has been verified *in vivo* (Vukašinovič; personal communication).

Interestingly, exocyst directly interacts with another multisubunit tethering complex termed transport protein particle II (TRAPPII) complex during formation of a cell plate. TRAPP operates during trafficking through the Golgi apparatus and coordinates other components of secretory pathway in several forms named TRAPI, TRAPII and TRAPIII in accordance to the secretory step and number of subunits involved (Chia and Gleeson, 2014). During intra-Golgi trafficking, TRAPII contains nine conserved subunits encoded by ten genes. Several subunits of TRAPII were shown

to co-localize with SEC6, EXO84B and EXO70A1 during cytokinesis and following pull downs confirmed physical interaction between exocyst and TRAPII.

Up to date, direct interaction between plant exocyst and TRAPPII as well as plant COG2 and SEC10 are the only confirmed and further studied associations between exocyst and other tethering complexes. However, there are some speculations about relevance of both interactions in other eukaryotes. I hypothesize that we can expect discovery of more crosslinks between components of secretory pathway, which may contribute to plant tissue specific spatiotemporally regulated secretion and that are not potentially unique for plants.

4. SEC15A and SEC15B exocyst subunits

SEC15 resides in the *Arabidopsis* genome in two paralogues. *SEC15A* and *SEC15B* show distinct localization on *Arabidopsis* chromosomes. Expression of SEC15 isoforms show distinct patterns throughout the plant tissues. Both, SEC15A and SEC15B potentially belong to the exocyst complex.

4.1. Both SEC15A and SEC15B are potentially exocyst subunits

Both, SEC15A and SEC15B appear to be full-fledged parts of the exocyst complex. Using yeast two hybrid analyses, SEC15B was identified as a strong interactor of SEC10 and EXO84B (Hála et al., 2008), which indicates that SEC15B potentially cooperates with the exocyst complex in evolutionary conserved manner. Similarly, SEC15A was co-purified with other exocyst subunits, which indicates that SEC15A physically interacts with the other exocyst subunits. Moreover, *sec15a* mutant displays synergistic defects with other exocyst subunits (Hála et al., 2008). Both GFP-tagged SEC15A and SEC15B are localized to globular structures, which reside near TGN/ early and late endosomes, similar to the localization pattern of the other exocyst subunits after transient expression in tobacco cell line BY-2. Interestingly, co-expression with EXO70E2 affects localization of both SEC15A and SEC15B (see Figure 5) (Chong et al., 2010). This suggests that both SEC15 isoforms are parts of the exocyst complex at least during specific events.

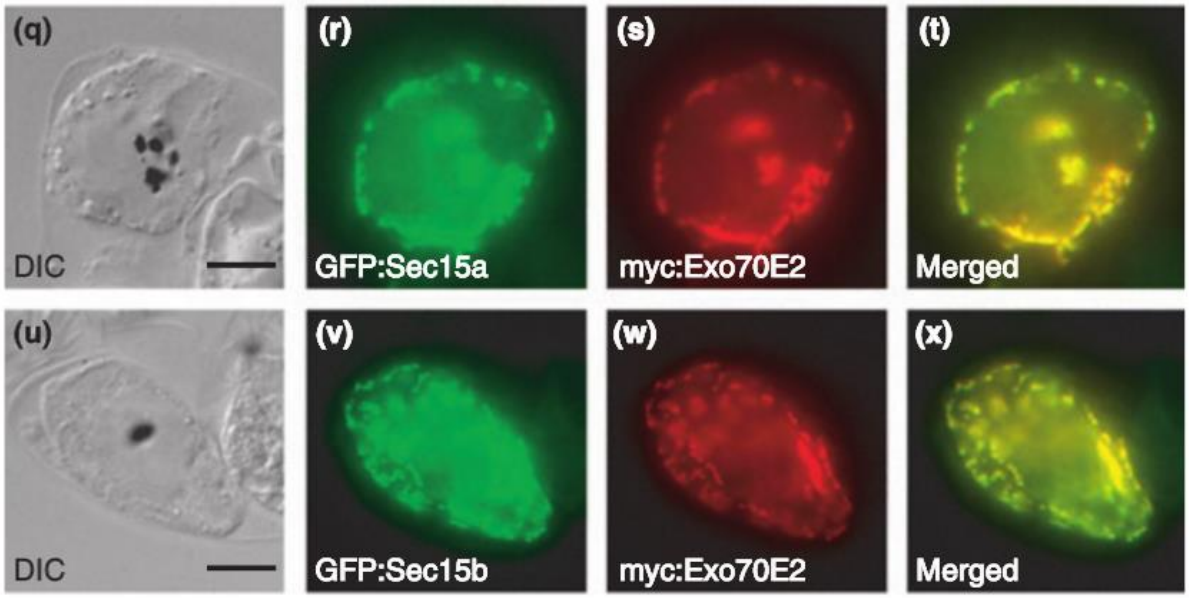

Figure 5: Co-expression (t and x) of Arabidopsis GFP:SEC15A (r) or GFP:SEC15B (v), respectively with myc:EXO70E2 (s and w) in BY2 cells (q and u) fixed in formaldehyde at 6 hours after biolistic bombardment (Chong et al., 2010).

4.2. The origin of Sec15 isoforms

Several exocyst subunits were multiplied in the plant genomes to increase complexity of cell responses (Table 3). In comparison with the other exocyst subunits, SEC15 is amplified moderately and the family size ranges between one to five paralogues, respectively. In general, plant *SEC15* might undergo two major duplications. First divergence seems to be dated around the origins of angiosperms and resulted into *SEC15A* and *SEC15B* subfamilies. Second, *SEC15A* was amplified in the first ancestors of monocots and gave rise to *SEC15A1* and *SEC15A2* isoforms (Cvrčková et al., 2012).

Interestingly, some of the rice SEC15 isoforms lack a part of C terminal domain and continue with non-homologous region instead (Cvrčková et al., 2012). In mammals and yeast, C terminal part of SEC15 mediates interaction with Rab GTPases and thus rice SEC15 potentially gained a novel function or localizes the whole exocyst complex in different manner.

Arabidopsis thaliana includes two paralogues of *SEC15*, *SEC15A* and *SEC15B* in its genome. Sequences of SEC15A and SEC15B share 48% amino acid similarity only (Cvrčková et al., 2012). Interestingly, *SEC15B* itself appears to be divergent among the *Arabidopsis* genomes. In the sequence, many single nucleotide substitutions were found compared with other *Arabidopsis thaliana* ecotypes. These changes influenced amino acid sequence in two cases in comparison with the consensus sequence (www.arabidopsis.org), but did not change the properties of peptide chain very much (Thr1843 into Ala; Val2176 into Ile). In addition, I found 17 bp-long insertion in terminator sequence (http://1001genomes.org/). This variability in *SEC15B* sequence

is not large, however may be meaningful in the ecological context and can influence processes such as RNAi or regulation of expression.

4.3. Documented roles of SEC15 in plants

As suggested by expression data, SEC15A is functionally coupled with the male gametophyte. In *sec15a* mutant, both processes, pollen germination and pollen tube growth are strongly reduced, which results in defective transmission of mutation through the male gametophyte (Hála et al., 2008). In addition, elimination of both, SEC15A and SEC15B affects secretion in stigma. RNA silencing of SEC15B in the *Arabidopsis* stigma induced in *sec15a1* mutant reduces acceptance of compatible pollen (Safavian et al., 2015). Surprisingly, any other visible defects of sporophyte typical for the other exocyst mutants are not present in *sec15a* mutants (Hála; personal communication).

Plant SEC15 potentially acts in evolutionary conserved manner (Hála et al., 2008). However, yeast temperature-sensitive mutation was restored by *Arabidopsis* RMA1 cDNA (growth and secretion defects were restored by RMA1). RMA1 is a membrane-bound ubiquitin E3 ligase (Matsuda et al., 2001) and contains a zinc finger motif with no structural or sequential similarity to yeast or plant Sec15 protein. It is possible, that RMA1 cooperates with SEC15 protein in some secretory step (Matsuda and Nakano, 1998). However, SEC15B itself does not complement yeast Sec15 temperature sensitive mutation (Růžičková, unpublished data) and no further biological relevance of plant RMA1 involvement in exocytosis or functional redundancy with Sec15 proteins was provided. Therefore, I hypothesize that there is no functional redundancy between RMA1 and SEC15B in *A. thaliana*.

Materials and methods

1. Characterization of *sec15b* mutant

1.1. Plant material

Two independent mutant lines were used to characterize phenotype of *Arabidopsis thaliana sec15b* mutant. *sec15b-1* is a line of Columbia-0 ecotype (signal.salk.edu/) with T-DNA insertion, whereas *sec15b-2* is a line of Nössen ecotype (No-0) (rarge.psc.riken.jp/) with Ds insertion.

Mutant line	AGI code	Line
sec15b-1	AT4G02350.1	SALK_130663
sec15b-2	AT4G02350.1	RAFL_15-1883-1
sec15a-1	AT3G56640	SALK_006302

Table 4: List of *Arabidopsis* mutants used in this thesis.

1.2. Cultivation of *Arabidopsis thaliana*

Cultivation conditions of non-sterile *Arabidopsis thaliana*

Arabidopsis thaliana seeds were sown on Jiffy tablets (Jiffy Products International AS, Norway) and grown in the growth chamber in 21±2°C under long day conditions (Photosyntetic photon flux 170 µmol $m^{-2}s^{-1}$).

Sterile cultivation

Sterilization of plant seeds

Dry seeds were surface-sterilized 4 minutes in 400 µl of 70% ethanol, shaked two times for 3 minutes with 400 µl of 10% SAVO and finally washed 6 times with 1 ml of distilled water. Seeds were stored for 3-7 days in 4°C and finally sowed on sterile half-strength Murashige and Skoog mineral salts (Duchefa) with 1.5% agar buffered to pH 5.7.

Sterile cultivation conditions of *Arabidopsis thaliana* seedlings

Plants were grown vertically on agar plates under constant conditions (21 ± 1°C; long day conditions: 16 hours of light, 8 hours of darkness).

1.3. Genotyping

Isolation of genomic DNA from plant material

Young leaf was mechanically homogenised and mixed well by vortexing with 200 µl of extraction buffer (200mM Tris-HCl, pH 7.5; 250mM NaCl; 25mM EDTA; 0.5% SDS). Mixture was centrifuged (2 minutes; room temperature; maximum speed) and supernatant was transferred into a new eppendorf tube. DNA was precipitated with 150 µl of isopropanol 2 minutes on ice and then spun down (5 minutes; room temperature; maximum speed). Supernatant was discarded and pellet

was washed by 1 ml of 70% ethanol, re-centrifuged and dried briefly. Isolated DNA was dissolved in 50 µl TE buffer (10mM Tris buffered to pH 8; 1mM EDTA) and stored at 4°C.

PCR identification of alleles

PCR reaction was performed in final volume of 20 µl (for 1 reaction: 17.3 µl of dH_2O; 2 µl of Dream Taq polymerase buffer (10x; Fermentas); 0.2 µl of each primer (100 µM); 0.25 µl of dNTPs (2.2 mM of each dNTP); 0.04 µl of Dream Taq polymerase; 1 µl of DNA template). In case of *sec15b* mutant, one forward and two reverse primers (left and right genomic primers with left T-DNA border primer) were used in one reaction for higher throughput.

Genotyping primers	
Left border primer of the T-DNA insertion	ATTTTGCCGATTTCGGAAC
SEC15B F	TTCACCAATAGCCAACTGACC
SEC15B R	ACTAAGGACATTTATACCTACCAACTG

Table 5: List of used primers.

104°C preheating
Initial denaturation: 94°C for 2 minutes
30 cycles of: denaturation: 94°C for 30 seconds, annealing: 59°C for 30 seconds, elongation: 72°C for 3 minutes, 20 seconds
Final extension: 72°C for 10 minutes

Table 6: PCR programme.

PCR fragments were visualized by 1% agarose electrophoresis (see below).

1.4. Size of seeds measurement

Dry seeds were hydrated for 20 minutes in distilled water and then immediately observed under the microscope (microscope Olympus BX51; Camera DP50-CV-Olympus optical CORTD; four times magnification; IPPlanApo adapted objectives) using Analysis® soft Imaging Systems software (www.soft-imaging.net). Images were processed in ImageJ (http://imagej.nih.gov/ij/; version 1.49) by measurement of transverse and longitudinal axes. Results were analysed by The R Project for Statistical Computation (www.r-project.org, version 3.1.2) using commander interface. Data set was visualized by boxplots and for statistical evaluation, Welch Two Sample t-test was used.

1.5. Visualization of pectin in seed coats and measurement of seed coat thickness

Dry seeds were hydrated (20 minutes in distilled water) and subsequently treated with water solution of ruthenium red (Sigma Aldrich; at final concentration 0.25 mg/ml) for 20 minutes in room temperature. Seeds were properly mixed with staining solution by inverting Eppendorf tube several times. After staining, seeds were washed with deionized water and directly observed

under microscope. Images were analysed in ImageJ (measurements of seed coat thickness on both sites of transverse axis of the seed). Obtained data were analysed in R software using Welch Two Sample t-test and box plot visualization.

1.6. Length of etiolated hypocotyls

Plants were cultivated on vertically laid agar plates in complete darkness. Pictures of one week old seedlings were taken (camera LUMIX Panasonic DE-A44 DMC-FZ18), images were analysed in ImageJ (Segmented line tool) and data were evaluated by R software (Welch Two Sample t-test and box plot tool).

1.7. Primary root length measurement

Plants were cultivated under standard aseptic conditions for one week and then its images were taken. Primary rooth length was measured using ImageJ (Segmented line tool) and results were analysed in R software (Welch Two Sample t-test and box plot).

1.8. Number of lateral roots

One week old seedlings were directly observed under binocular (Leica S6D) and the number of lateral roots was manually counted. Data were evaluated by chi quadrat test in Microsoft Excel and visualized by line chart.

1.9. Diameter of rosette and length of stem

Three week old plants were directly measured and results were analysed in R software (Welch Two sample t-test and box plot visualization).

1.10. Pollen tube length

Pollen was harvested from five-week-old *Arabidopsis* plants and added to a droplet of germination solution (water solution of sucrose (1 g/l); boric acid (1 mg/l); magnesium sulphate (3 mg/l); potassium nitrate (2 mg/l)) on a glass slide. Pollen germinated 16 hours under stable wet conditions (21 ± 1°C) and then was immediately observed under microscope. Images were analysed by ImageJ (Segmented line tool) and data set was tested using R software (chi quadrat test and histogram tool).

2. Complementation test of *sec15b*

2.1. PCR of DNA fragments from the plant genomic DNA

Genomic DNA was isolated from wild type three-week-old *Arabidopsis thaliana* (Columbia-0) according to protocol of Plant DNAzol Reagent (Thermo Fisher Scientific). Polymerase chain reaction with Phusion High-Fidelity DNA polymerase was used for DNA fragments amplification in

final volume of 50 µl (37.5 µl dH_2O; 10 µl Phusion HF reaction buffer (5x); 1 µl dNTPs (2.5mM of each dNTP); 0.5 µl of each primer (100 µM); 0.5 µl of Phusion High-Fidelity polymerase (New England Biolabs); 1 µl of template genomic DNA).

104°C preheating
Initial denaturation: 98°C for 1 minute
30 cycles of: denaturation: 98°C for 20 seconds, annealing: 63°C for 20 seconds, elongation: 72°C for 50 seconds
Final extension: 72°C for 5 minutes

Table 7: PCR programme.

PCR products were visualized by agarose gel electrophoresis and immediately isolated.

Primers used for PCR	
SEC15A promotor	
Sec15Apromotor F	TTGTCGACGAGGGTCACATCGCATTTC
Sec15Apromotor RC	TCTGAGAAAAGCTTATCAATGG
SEC15A gene	
Sec15AgenF	ATGATGGGAGGCCAAACC
Sec15AgenRC	TTGCGGCCGCTCCTGAGCCTGTGAGCCATA
SEC15B promotor	
Sec15Bpromotor F	TTGTCGACGCGATTGCTTTTTGCCATGC
Sec15Bpromotor RC	TGTTTTATCTCCGATTAGGTAAAAG
SEC15B gene	
Sec15BgenF	ATGCAATCGTCGAAAGGAC
Sec15BgenRC	TTGCGGCCGCGAGAGACCAACGAATCATCAACA

Table 8: List of used primers.

2.2. Agarose gel electrophoresis

Agarose gel was prepared by mixing 1% (w/v) agarose (Invitrogen) in TBE buffer (10mM Tris; 20mM boric acid, 1mM EDTA buffered to pH 8) with addition of 0.01% (v/v) GelRed (Biotium). DNA sample was mixed with loading Dye (Fermentas) in ratio 1:5 and GeneRuler DNA Ladder Mix (Fermentas) was used as a reference.

DNA fragments were separated in agarose gel submersed in TBE buffer under constant voltage of 4-10 V/cm for 5-90 minutes depending on length of fragment using Consort EV 231 power supply. DNA fragments were visualized by UV light and, when needed, fragment of interest was cut using a sharp scalpel and isolated with the High Pure PCR Product Purification Kit (Roche).

2.3. Ligation of PCR product into pJET plasmid with following cloning into pENTR

DNA fragments containing both promotor and gene sequence were directly cloned into pJET plasmid due to the CloneJET PCR Cloning Kit (Thermo Fisher Scientific).

Electroporation and cultivation of *Escherichia coli* strains

1 µl of pJET clones were transformed into 50 µl of *E.coli* (DH5α of TOP10 strains) by electroporation (2500 V for approximately 6 ms; Eporator (Eppendorf)). Transformed bacteria were cultivated 1 hour in liquid media (MPA; 37°C) and then transferred on agar plates containing LB medium (1% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl; for agar medium 1.75% (w/v) agar) of MPA (1.5 g/l meat extract; agar; 6 g/l peptone from meat; 3 g/l yeast extract; for agar medium 15 g/l agar) with antibiotics depending on used plasmid.

In other steps, bacteria were cultivated on agar plates in termostat (at 37°C) or liquid (MPB or LB) medium on shaker (180 rpm; 37°C) with appropriate antibiotics in media.

DNA isolation by alcaline lysis

2 ml of cell suspension was spun down (5 minutes; room temperature; maximum speed) and properly mixed in 100 µl of GTE (50mM glucose; 25mM Tris-HCl, pH 8; 10mM EDTA) by vortexing. 180 µl of NaOH/SDS (100 mM/1%) was added to the mixture and incubated 3 minutes in room temperature. 135 µl of KAc/HAc (3 M/2 M) was added, mixed well and the suspension was cooled down 3 minutes on ice. Precipitate was removed by centrifugation (5 minutes; 4°C; maximum speed) and supernatant was mixed with 1 ml of EtOH (96%) by vortexing. DNA was precipitated 5 minutes on ice and centrifuged (5 minutes; 4°C; maximum speed). Pellet was dissolved in 40 µl of TE.

Restriction of pJET plasmid with following ligation into pENTER plasmid

Isolated pJET clone and pENTER1a plasmid were cut by restriction endonuclease (SalI, NotI; 10 U/µl; New England Biolabs). After 2 hours, DNA fragments from all reactions were separated using agar gel electrophoresis and purified by High Pure PCR Product Purification Kit (Roche). Fragments of promotor and gene (*SEC15A* promotor and *SEC15A* gene; *SEC15B* promotor and *SEC15B* gene) were ligated with pENTR1a in two separate reactions (1 µl of T4 DNA ligase buffer; 0.5 µl of T4 DNA ligase (New England Biolabs); approximately 25 ng of plasmid DNA; approximately 19 ng of insertion fragment (*SEC15A* promotor and *SEC15A* gene or *SEC15B* promotor and *SEC15B* gene); dH_2O till 10 µl), reaction was incubated at 16°C overnight. 0.5 µl of ligation was directly porated into bacteria cells.

2.4. Ligation of two PCR product into pENTR plasmid

DNA fragments of promotors (of both *SEC15A* and *SEC15B*) and genes (of both *SEC15A* and *SEC15B*) only were cleaved by restriction endonucleases (SalI: both promotors; NotI: both genes)

in a final volume of 30 µl (3 µl of restriction buffer (10x); 0.5 µl of restriction enzyme (New England Biolabs); 13.5 µl of DNA fragments).

Subsequently, pENTR1a plasmid was restricted by SalI and NotI restriction endonucleases.

After 2 hours, restriction was terminated by separation of DNA fragments on agarose gel electrophoresis. Fragments of promotors, genes and vector were isolated using High Pure PCR Product Purification Kit (Roche). Three DNA fragments were ligated in two separate reactions (1 µl of T4 DNA ligase buffer; 0.5 µl of T4 DNA ligase (New England Biolabs); approximately 25 ng of plasmid DNA; approximately 9.5 ng of each insertion fragment (*SEC15A* promotor and *SEC15B* gene; *SEC15A* promotor and *SEC15B* gene); dH_2O till 10 µl) at 16°C overnight. Next day, 0.5 µl of reaction was used for bacterial transformation.

2.5. Isolation of plasmid DNA with commercial kits and sequence verification

High Pure Plasmid Isolation Kit (Roche) was used for plasmid isolation in accordance with protocol. Plasmid DNA was then analysed by control restriction by restriction endonucleases (BglII, EcoRI, BamHI, NotI; 10 U/µl; Fermentas). Candidate clones were sequenced (Faculty of Science core facility DNA sequencing lab) and finally, the sequences was controlled by comparison

Sequencing primers	
SEC15A promotor	
SEC15ApromotorF	TTGTCGACGCAGGGTCACATCGCATTTC
SEC15A1S	ATCGGGCGACTTCGTCTTCT
SEC15A2S	TGGCTCATTCCTTGTCATAA
SEC15A gene	
SEC15AgenR	TTGCGGCCGCTCCTGAGCCTGTGAGCCATA
SEC15ANR	TTTGTCGACTCAACCGTACTGTCTAAGGG
SEC15ACF	TTGGATCCAAATGGGTCCAGTTCTTGATGCC
SEC15B promotor	
SEC15BpromotorF	TTGTCGACGCGATTGCTTTTTGCCATGC
SEC15B1S	AGCACGTACCTTTTCTACAA
SEC15B2S	ACCAAATCAGTTTCGTTGTC
SEC15B gene	
SEC15BgenR	TTGCGGCCGCGAGAGACCAACGAATCATCAACA
SEC15BNR	TAACAACTCATGATAC
SEC15BCF	TACAAAAAAGCAGGT

Table 9: List of sequence primers.

with reference sequence (found on www.arabidopsis.org) in Geneious 6.0 software (Biomatters). Divergences in reference sequence were consulted with a Catalog of *Arabidopsis thaliana* Genetic Variation (http://1001genomes.org/).

2.6. LR reaction

LR reaction was used for gene transfer from entry clone to destination vector (pBGW; source: Ghent University; www.psb.ugent.be). A mix of entry clone (50-150 ng of DNA per one reaction), destination vector (300 ng of DNA per one reaction; Invitrogen), LR Clonase™ II enzyme (Clonetech; 1 µl) and TE buffer (pH 8; up to 5 µl in total) was incubated overnight at room temperature. 1µl of proteinase K was used to stop LR reaction by incubation for 10 minutes at 37°C.

2.7. Transformation and cultivation of *Agrobacterium tumefaciens*

Approximately 0.5 µl of plasmid DNA was electroporated (2,000V; approximately 6 ms; Eporator, (Eppendorf)) into *Agrobacterium tumefaciens* (strain GV 3101) cells. Transformants were cultivated in liquid medium (MPB) without antibiotics and after 1 hour were transferred on agar medium (MPA) in termostat (28°C) with antibiotics in respect to the plasmid.

2.8. Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*

A single colony of *Agrobacterium* was picked by a toothpick and transferred into 2 ml of medium (with appropriate antibiotics). Cell culture was incubated overnight on shaker (180 rpm; 28°C). 1 ml of this culture was inoculated into 300 ml of fresh medium (with appropriate antibiotics) and cultivated overnight on shaker (180 rpm; 28°C). Afterwards, cells were pelleted (15 minutes; 22°C; maximum speed) and mixed well in medium containing 5% (w/v) sucrose and 0.05% (v/v) silwet by vortexing.

All matured siliques were removed and immature buds with meristematic cells were dipped in *Agrobacterium tumefaciens* suspension for 0.5-1 minutes. Inoculated plants were covered by a plastic foil and incubated in darkness overnight. This procedure was applied twice on same plants in a week interval.

Transformed seedlings were selected in sterile conditions by treatment with claforan (protect against bacterial overgrowth; 25 µg/ml) and phosphinotricin (BASTA active coumpound; 150 ng/ml). One week old candidate seedlings were transferred into soil medium and harvested seeds of this generation were used for further characterisation.

3. Protein preparation, lipid binding assays and pull down

3.1.1. Transformation of chemocompetent bacteria

Chemocompetent cell aliquots were thawed on ice and 5-10 µl of plasmid were added immediately to the stock of the cells. Mixture was incubated 30 minutes on ice and then was replaced in 42°C for 20-45 seconds. After the heat shock, the cells were cooled down in ice for 2 minutes. 0.5 ml of LB medium was added to the cells and the cells were cultivated 1 hour in 37°C for 180 rpm. Finally, the cells were replaced on solid agar medium with a selection antibiotic and stored, grown overnight and stored in the fridge.

3.1.2. Preparation of cell glycerol stocks:

1 ml of overnight culture of cells was spelleted and resuspended in 1 ml of 20% (v/v) solution of glycerol. This stock was covered by liquid medium and then stored at -80°C.

3.1.3. Cultivation of BL21 (RIPL) cells, protein expression

Bacteria (taken from agar plate or glycerol stock) were cultivated in liquid LB in thermostat (37°C) on shaker (180 rpm; 37°C; overnight). Selected antibiotic was used in proper concentration in dependence of used plasmid. Next day, 1 ml of culture was inoculated into 500 ml of fresh LB medium. When OD 600 reached 1, IPTG (isopentenyl thiogalactoside) was added to the culture medium in 0.1% concentration for induction of expression. Protein was expressed approximately 4 hours. After all, cell culture was centrifuged 10 minutes at 4°C at 5,000 g and the pellet was stored at -20°C.

3.1.4. Cultivation of BL21 Arctica (RIL) cells, protein expression

Overnight culture was prepared from a glycerol stock with proper concentration of appropriate antibiotic (180 rpm; 37°C; overnight). Following day, 1 ml of overnight culture was added into 500 ml of LB media with appropriate concentration of antibiotics. When OD reached 0.6, IPTG in total concentration of 0.1 mM was added to the culture to induce protein expression. Protein was expressed for 24 hours at 12°C (180 rpm). After 24 hours, cells were pelleted (5,000 g; 4°C; 10 minutes).

3.1.5. Purification of proteins with His-tag

Pellet from 50 ml of cell culture containing pET30a plasmid (kanamycin resistance; antibiotic used in a final concentration of 50 µg/ml) was properly dissolved in 5 ml of lysis buffer (25mM TRIS (pH=7.9); 0.25M NaCl; 1mM inhibitor of proteases PMSF (phenylmethyl sulphonyl fluoride); with 5mM imidazole) and then sonicated 3x 35 seconds on ice. Mixture was spun (15,000 g; 4°C; 60 minutes). Supernatant was gently shaked with 250 µl of washed His-tag resin in a purification

column at 4°C. After 1 hour, flow-through was replaced and resin linked to the protein of interest were twice washed with lysis buffer supplemented with 80mM imidazole. Finally, protein was eluted with 500 µl of elution buffer (lysis buffer containing 300mM imidazole) and stored at -20°C.

3.1.6. Purification of proteins with GST- tag

Pellet from 4 l of cell culture transformed by pGEX-4T-1 (ampicillin resistance; antibiotic used in a final concentration of 100 µg/ml) was properly resuspended in 30 ml of lysis buffer (25mM TRIS (pH=7.9); 0.25 M NaCl; 1mM inhibitor of proteases PMSF) and then sonicated 3x 35 seconds on ice. Mixture was centrifuged (15,000 g; 4 °C; 60 minutes). Supernatant was gently shaken with 0.075 g of washed hydrated and washed GST-tag resin in a purification column at 4 °C. After 1 hour, flow-through was replaced and resin linked to the protein of interest were washed twice with lysis buffer. Finally, protein was eluted with 500 µl of elution buffer (15 µl of 1M reduced glutathion in 0.5 ml TRIS-HCl; pH 8.8) and stored at -20°C.

3.2. Polyacrylamide gel electrophoresis

A) 10% thin separating polyacrylamide gel (2.5 ml of dH_2O; 1.25 ml of 40% Bisakrylamide; 1.25 ml of Tris, pH 8.8; 33 µl of 10% APS; 6 µl of TEMED) or 12.5% thick separating polyacrylamide gel (2.2 ml of dH_2O; 1.55 ml of 40% Bisakrylamide; 1.25 ml of Tris, pH 8.8; 33 µl of 10% APS; 6 µl of TEMED) or 15% thick separating polyacrylamide (1.84 ml of dH_2O; 1.875 ml of 40% Bisakrylamide; 1.25 ml of Tris, pH 8,8; 33 µl of 10% APS; 6 µl of TEMED): components were mixed, loaded into polyacrylamide gel aparature and the gel was polymerized approximately 1 hour under water layer.
B) Stacking gel was prepared by mixing chemicals (0.75 ml of dH_2O; 0.375 µl of TRIS (pH 6.8); 0.17 ml of 40% bisacrylamide; 10 µl of APS; 3 µl of TEMED), loading on the top of separating gel and polymerizing about 30 minutes under electrophoretic comb.

10 µl of a protein sample was mixed with 5 µl of loading dye (7 ml TRIS-Cl/SDS (pH 6.8); 3 ml glycerol; 1 g SDS; 0.93 g DTT) and boiled for 5 minutes. Loading mixture was loaded into a separate hole on a gel with 3-5 µl of Page Ruler Prest Protein Ladder (Biogen). Proteins were separated in running buffer (25mM TRIS (pH 8.3), 250mM glycine, 0.1% SDS) under constant voltage of 150 V in stacking gel and of 180 V in separating gel for approximately 1 hour and 30 minutes depending on the molecular weight of proteins of interest.

Once the proteins in polyacrylamide gel were optimally separated, the gel was stained by Coomassie Briliant Blue R (Sigma Aldrich). Solution A (25% (v/v) isopropanol; 10% (v/v) acetic acid; 0.05% (w/v) Coomassie Briliant Blue R) was boiled with the gel several seconds in microwave oven

and incubated another 10 minutes on shaker. Cooled gel was covered by solution B (10% (v/v) isopropanol; 10% (v/v) acetic acid; 0.005% (w/v) Coomassie Briliant Blue R and boiled and cooled for 10 minutes one more time. Excessive Coomassie was removed by solution C (10% (v/v) acetic acid).

3.3. Western blot

Proteins separated in polyacrylamide gel were transferred to nitrocellulose membrane (GE Healthcare Life Science Whatman™; 0.2 μm thick). Gel and nitrocellulose membrane were embedded into three layers of filter paper rinsed with transfer buffer (5.8 g TRIS; 2.9 g glycin; 0.37 g SDS; 200 ml methanol) on each side. Current was used in dependence on gel size (approximately 1.5 mA per cm^2) for 1 hour 42 minutes.

Efficiency of protein transfer was tested by Ponceau S staining (Fluka; 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid). After protein visualization, dye was washed out with distilled water.

Nitrocellulose membrane was blocked with blocking solution (5% milk; 0.5% Tween in Phosphate-buffered saline (PBS)) at 4°C overnight with gentle shaking. Washed membrane was incubated with primary antibody (diluted 1:1,000 in 5% milk and 0.5% Tween in PBS) 2 hours at room temperature. Excess of primary antibody was washed out 3 times by 0.5% Tween in PBS (10 minutes for each wash). Membrane was incubated with conjugated secondary antibody (diluted 1:10,000 in 5% milk with 0.5% Tween in PBS) for 55 minutes at room temperature. Afterwards, membrane was washed 3 times by 0.5% Tween in PBS (10 minutes for each wash). Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) was used for visualization of results.

Antibody	Type	Epitope	Dilution	Company
Primary	Polyclonal	Anti-SEC15B	1:1,000	Sigma-Aldrich
Primary	Monoclonal	Anti- His tag	1:1,000	Sigma-Aldrich
Primary	Monoclonal	Anti- GST tag	1:1,000	Sigma-Aldrich
Secondary	Monoclonal	Anti-Mouse antibody	1:10,000	Sigma-Aldrich
Secondary	Monoclonal	Anti-Rabit antibody	1:10,000	Sigma-Aldrich

Table 10: List of used antibodies.

3.4. Lipid binding assay: PIP Strip

PIP Strip (Echelon; P-6001) was blocked overnight in 2% milk in PBS in 4°C. After blocking, 3 ml of newly prepared 2% milk in PBS with protein of interest (in final concentration 0.5 μg/μl) was added and reaction was incubated 60 minutes at room temperature with gentle shaking. Strip was washed 3 times in PBS solution (each wash 5-10 minutes; room temperature). 1.5 μl of

primary antibody was added 3 ml of 2% milk in PBS and incubated with PIP Strip 60 minutes at room temperature with gentle shaking. Primary antibody was washed out 3 times with PBS (5-10 minutes for each wash; room temperature). 1.5 µl of secondary antibody was incubated with PIP Strip in 3 ml of 2% milk in PBS 60 minutes with gentle shaking. Finally, PIP Strip was washed with PBS three times (5-10 minutes for each wash; room temperature). Results were visualized by using Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences).

Antibody	Type	Epitope	Dilution	Company
Primary	Polyclonal	Anti-GST tag	1:1,000	Sigma-Aldrich
Secondary	Monoclonal	Anti-Mouse antibody	1:10,000	Sigma-Aldrich

Table 11: List of used antibodies.

3.5. Lipid binding assay: LUV

Lipids were mixed in appropriate ratios (PC vesicles: 100% PC; PA vesicles: 20% PA, 80% PC; PIP_2 vesicles: 5% PIP_2, 95% PC, PI_3P vesicles: 5% PI_3P, 95% PC) in total volume 200 µl and mixed well by vortexing. Solvent was evaporated in vacuum trap (40 minutes; 35°C). Lipids were hydrated with 1 ml of extrusion buffer (3.72 g of raffinose; 25mM TRIS-HCl buffered to pH 7.8; 1mM DTT) 1 hour at room temperature and then sonicated for 2 minutes. Size of vesicles was unified using Mini-extruder (Avanti Polar Lipids, Inc). Lipid solution was pushed through a filter (Whatman®; 0.1 µm) from one syringe to another syringe at least 20 times. Sample was divided into two halves and 600 µl of lipid binding buffer was added into each half. All samples were spun down (ultracentrifuge Sorvall M120SE; 40,000 rpm; 50 minutes; 22°C). Pellet was mixed with 150 µl of lipid binding buffer (125mM KCl; 25mM TRIS-HCl buffered to pH 7.5; 1mM DTT; 0.5mM EDTA) and 50 µl of purified protein (at the concentration 0.5 µg/µl). Proteins were incubated with lipids for 30 minutes with gentle shaking at room temperature. Solution was centrifuged (40,000 rpm; 50 minutes; 22°C).

Pellet was resuspended in 20 µl of lipid binding buffer properly and the solution was transferred into the new Eppendorf tube. Cuvette was washed with additional 10 µl of lipid binding buffer, so the total volume of pellet fraction was 30 µl.

Supernatant was mixed with 1 ml of precooled acetone and incubated at -20°C overnight. Next day, soluble fraction was centrifuged (18,000 g; 10 minutes; 4°C). Pellet was re-suspended in 30 µl of lipid binding buffer. All samples were transferred on 10% polyacrylamide gel.

3.6. Pull down assay

4 l of cell culture expressing GST-SEC15B protein was centrifuged (5,000 g; 10 minutes; 4°C). Pellet was precisely resuspended in lysis buffer in total volume of 30 ml and then sonicated 6x

35 seconds on ice. Lysate was cleared by centrifugation (14,000 rpm; 1 hour; 4°C). Supernatant was divided into two halves and each half was incubated 1 hour with 500 µl of washed GST-resin at 4°C by shaking on an orbital shaker. After incubation, mixture was centrifuged (500 rpm, 2 min, 4°C) and pellet was washed by 10 ml of lysis buffer (25mM TRIS (pH=7.9); 0.25M NaCl; 1mM inhibitor of proteases PMSF) twice (each wash wash was incubated on orbital shaker 20 minutes at 4°C).

50 ml of cell culture expressing GST only was centrifuged (5,000 g; 4°C; 10 minutes) and pellet was mixed with 5 ml of lysis buffer. Afterwards, mixture was sonicated 3x 35 seconds on ice and centrifuged (14,000 rpm; 1 hour; 4°C). Supernatant was treated 1 hour with 500 µl of washed GST-resin at 4°C by shaking on an orbital shaker. Subsequently, sample was centrifuged (500 rpm; 2 min; 4°C) and pellet was washed by 10 ml of lysis buffer twice.

500 µl of GST resin was washed and prepared for pull down assay.

25 µl of purified Rab GTPase was added into each sample (SEC15b protein, GST protein and resin sample) and lysis buffer was added up to 5 ml in each treatment. Mixtures were incubated 1 hour on orbital shaker at 4°C and then centrifuged (500 rpm; 2 min; 4°C). Pellets were washed twice by addition of approximately 8 ml lysis buffer (each wash was treated 20 minutes on an orbital shaker at 4°C). Finally, 60 µl of elution buffer (15 µl of 1M glutathione in 500 µl of TRIS-HCl buffered to pH 8.8) was added to all samples and incubated 30 minutes on an orbital shaker at 4°C and centrifuged (500 rpm; 2 min; 4°C).

30 µl of solution from all samples was mixed with 5 µl of loading dye, boiled 5 minutes and loaded on polyacrylamide gel (GST-SEC15B treated proteins: 12.5% gel; GST protein and empty GST resin: 15% gel).

Results

1. Transcription and expression patterns of SEC15A and SEC15B

Both, *SEC15A* and *SEC15B* are very similar in size, although SEC15A and SEC15B proteins show 48% similarity only. Isoforms of *SEC15* gene reside on different chromosomes (see table 12), which enables independent assortment of *SEC15A* and *SEC15B*. Interestingly, both *Arabidopsis SEC15* exocyst subunits do not contain any intron sequence. In TAIR web sites (https://www.arabidopsis.org/), small intron is annotated in *SEC15B* sequence, but several studies of cDNA indicate no splicing sites in the sequence (Hála, personal communication).

		SEC15A	*SEC15B*
Gene	Chromosome	3	4
	Length [bp]	2370	2361
Protein	Length [AA]	790	787
	Molecular weight	89999,4	88370,1
	Isoelectric point	5,5249	6,3947

Table 12: Properties of *SEC15A* and *SEC15B* genes, and SEC15A and SEC15B proteins.

SEC15A and SEC15B seem to be expressed to different extent in all tissues. However, the ratio between them varies during specialized developmental processes (growth of the root, seed maturation, leaf growth, embryo development, floral development; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). SEC15A is expressed predominantly in male gametophyte and during stamen development, whereas SEC15B expression is increased in sporophyte in comparison with Sec15A (see Figure 6 and 7).

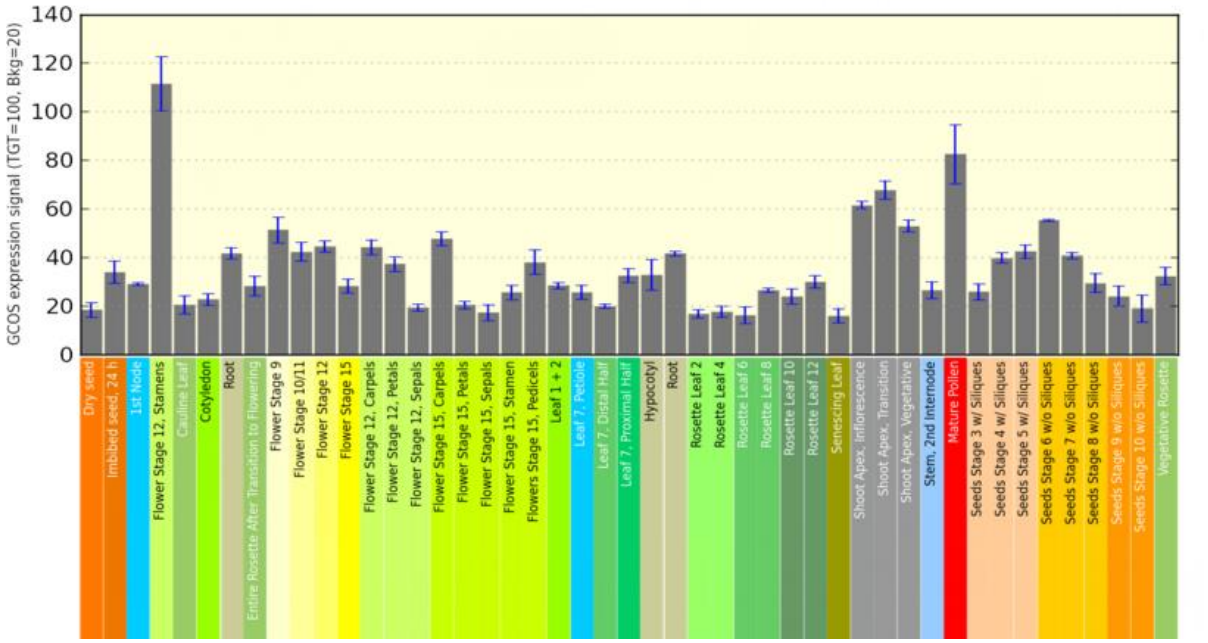

Figure 6: Tissue specific expression of SEC15A (Schmid et al., 2005).

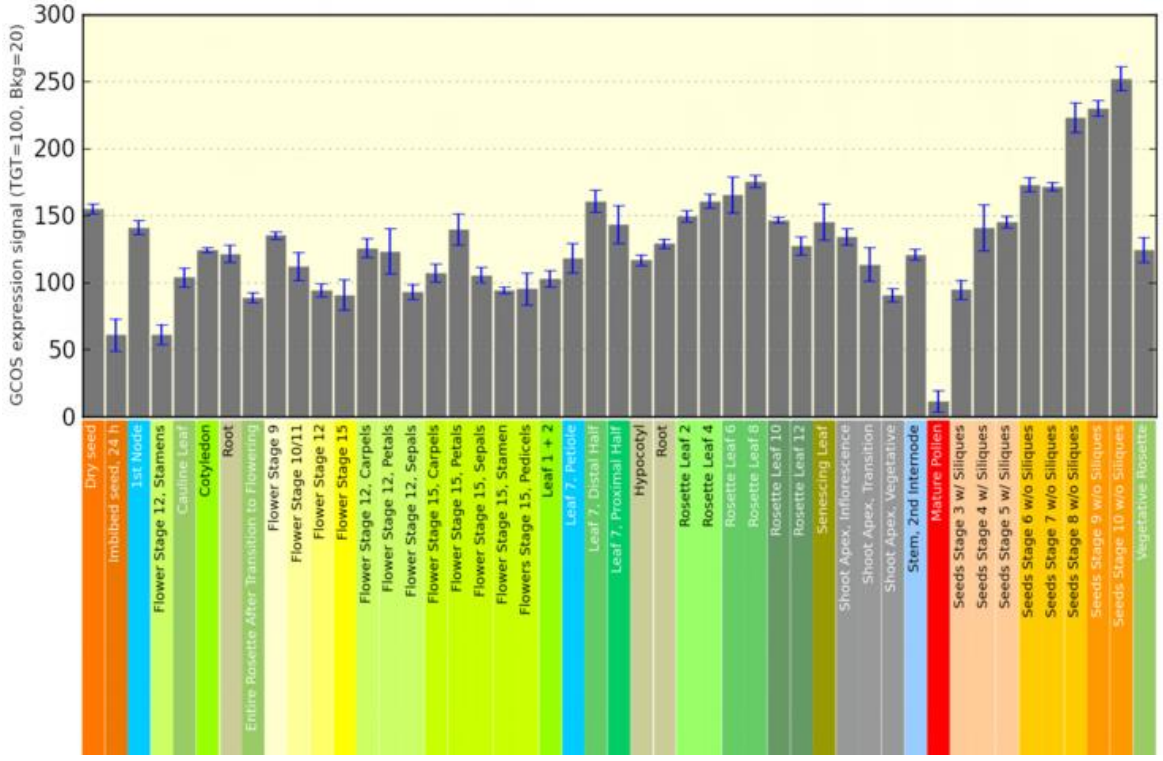

Figure 7: Tissue specific expression pattern of SEC15B (Schmid et al., 2005).

2. Characterization of *sec15b* mutant

Two *Arabidopsis thaliana* mutant lines in *sec15b* subunits were characterized with a focus on characteristics typical for the other exocyst subunits including secretion of pectin in seed coat,

length of etiolated hypocotyls, length of primary root, number of lateral roots, size of mature plant and pollen tube growth.

2.1. Verification of *sec15b-1* and *sec15b-2* mutant lines

Seven-day-old mutant seedlings were tested for a presence of *SEC15B* cDNA by PCR. The presence of full length transcript was disproved in both, *sec15b-1* and *sec15b-2* using *ACT7* as a control (see Figure 8).

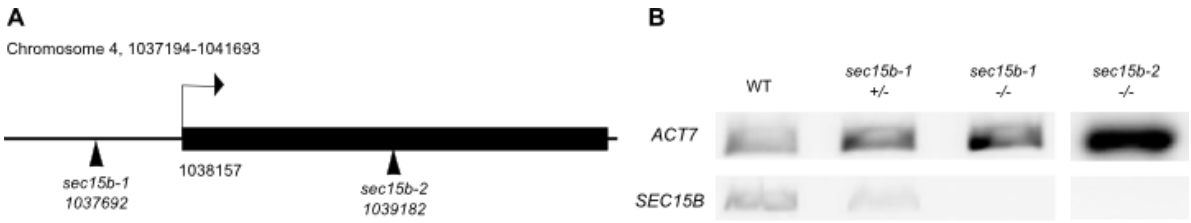

Figure 8: (A) The locus of *SEC15B* on chromosome 4 with marked places of insertions in *sec15b-1* and *sec15b-2* (B) PCR products of *ACT7* and *SEC15B* using cDNA of wild type plants, *sec15b-1* heterozygous and homozygous plants and *sec15b-2* mutants.

2.2. Seed characteristics

It is well known that seed characteristics are very variable within several generations, especially when plants are cultivated under different conditions. Therefore, it is neccessarry to analyze more generations to postulate mutant phenotype. Here, two generations of *sec15b-1* and *sec15b-2* mutants were tested in comparison with appropriate wild types. First, size of mutant and wild type seeds were measured. Second, seed coat thickness and structure were characterized.

2.2.1. Seed size

Difference in seed size is typically not a common feature of exocyst mutants. However, some of the *sec15b* mutant generations drew my attention by obviously larger seeds. Therefore, both *sec15b* mutant lines were tested in a size of a seed (see Figure 9 and 10). Seeds of *sec15b-1* are longer and wider than appropriate Columbia-0 (Length *sec15b-1*: 1.05±0.075 mm, wild type: 0.92±0.074 mm, p-value: 0.004; Width: *sec15b-1*: 0.55±0.04 mm, wild type: 0.527±0.04 mm, p-value: 3.21 e -11). In contrast, *sec15b*-2 seeds varied in size more, but are significantly longer and not wider as compared to appropriate wild type (Length *sec15b-2*: 1.036±0.08 mm, wild type: 1±0.063 mm, p-value: 8.84 e-11; Width: *sec15b-2*: 0.536±0.06 mm, wild type: 0.54±0.05 mm, p-value: 0.27). Nevertheless, this feature will be further studied, because seed characteristics naturally vary in this respect.

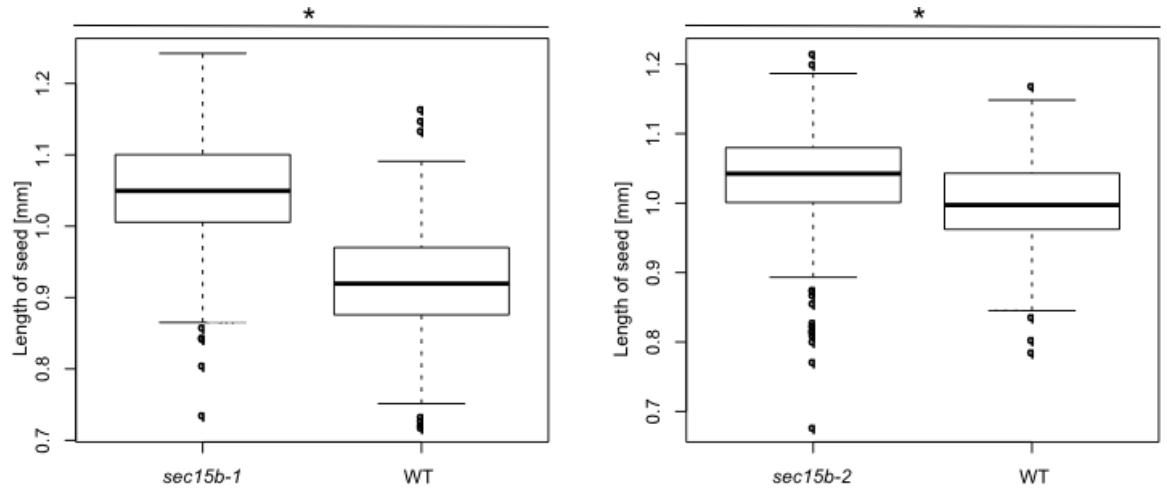

Figure 9: Length of seeds of *sec15b-1* and *sec15b-2* mutant lines in comparison with appropriate wild types. *=significant, n.s.=non-significant

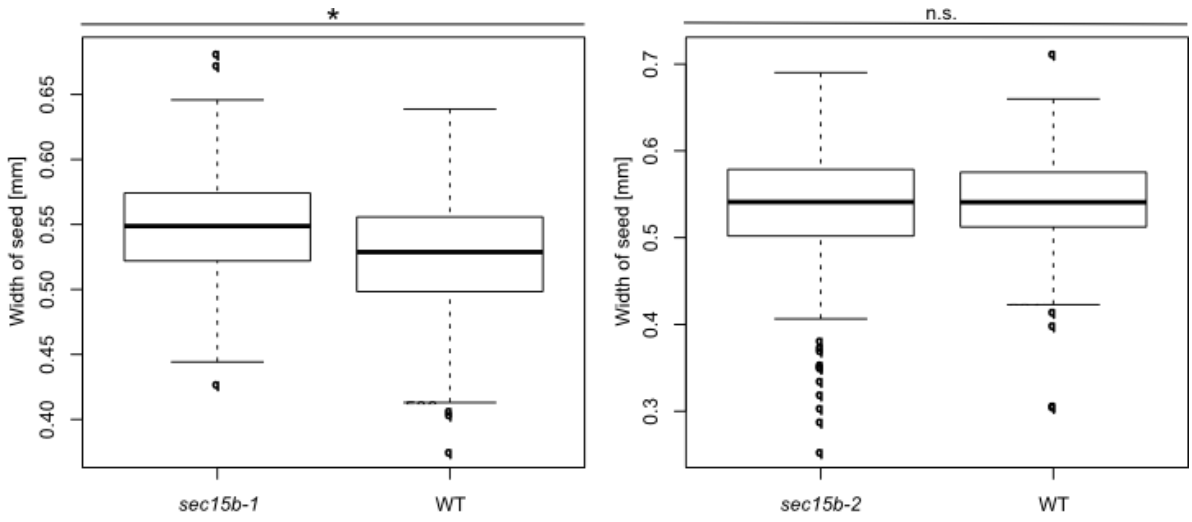

Figure 10: Width of seeds of *sec15b-1* and *sec15b-2* mutant lines in comparison with appropriate wild types. *=significant, n.s.=non-significant

Seeds of *sec15b-1* are longer and wider than wild type. In contrast, *sec15b-2* seeds varied in size more, but are significantly longer and not wider compared to appropriate wild type. Nevertheless, this feature will be further tested, because seed characteristics naturally vary in this respect.

2.2.2. Thickness of seed coats

Defects in seed coat deposition are a characteristic feature for some exocyst mutants (*exo70a1* and *sec8;* Kulich et al., 2010). During manipulation with seeds, *sec15b* mutant behaved in similar manner in comparison with these exocyst mutants. For instance, kinetics of sedimentation of hydrated seeds in water solutions is strongly affected. Therefore, seed coats of *sec15b* mutants were visualized using ruthenium red staining. As seen in the Figure 11, architecture of seed coat in case of both *sec15b* mutants is altered. Seed coats of *sec15b-1* are thinner, whereas *sec15b-2*

seed coats are thicker in comparison with appropriate wild type (*sec15b-1*: 0.05±0.05 mm, wild type: 0.14±0.05 mm, p-value: 2.2 e-16; *sec15b-2*: 0.19±0.04 mm, wild type: 0.16±0.04 mm, p-value: 1.52 e -10). s*ec15b-1* seed coats are nearly separated from the seed and almost disrupted, but in case of *sec15b-2* is the structure modified and less dense rather than disrupted.

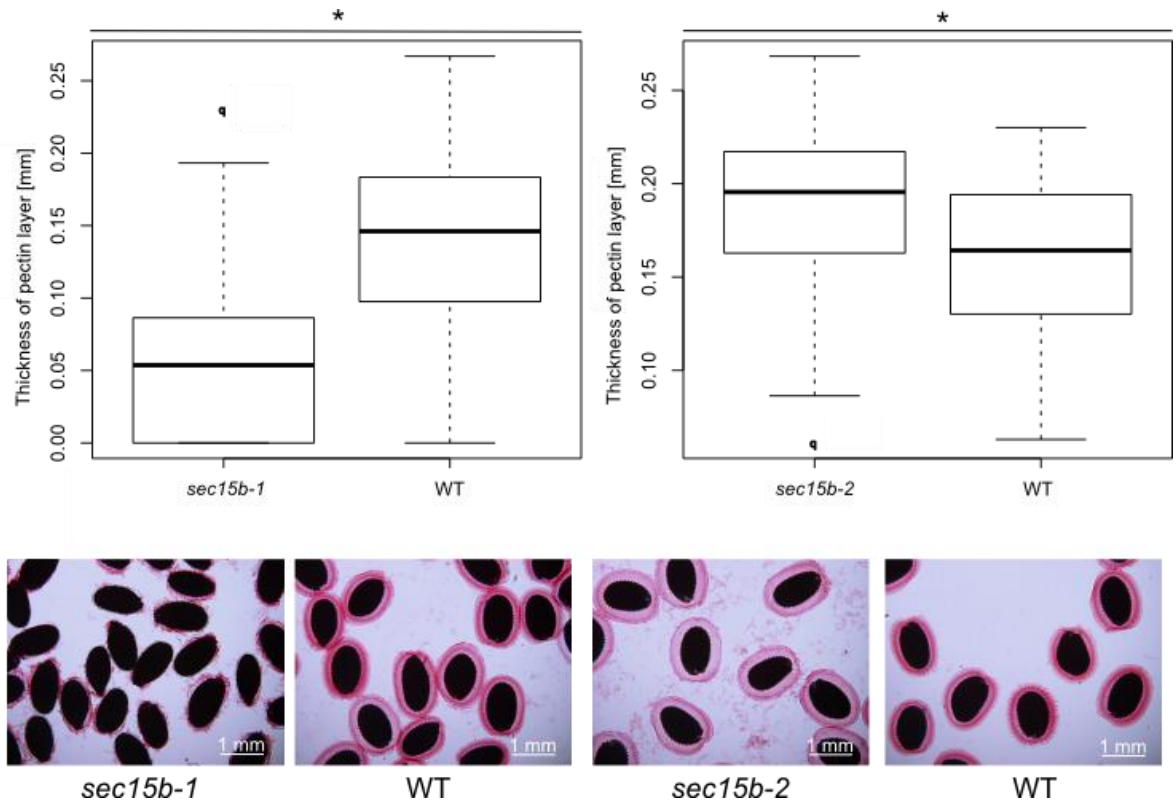

Figure 11: Thickness of pectin layers of *sec15b-1* and *sec15b-2* mutants (upper panels) stained by ruthenium red (lower panels). More than 300 seeds were measured in each experiment. *=significant, n.s.=non-significant

2.3. Length of etiolated hypocotyl

Normally, etiolated plants elongate its hypocotyls to reach light. However, some other exocyst subunit mutants (*exo70a1* especially in *sec8-4* and *sec5a-1* backgrounds) are defective in the elongation process (Hála et al., 2008). Similarly to the other exocyst mutants, *sec15b-1* and *sec15b-2* show defective hypocotyl growth in darkness (see Figure 12) (*sec15b-1*: 0.75±0.34 cm, wild type: 1.75±0.22 cm, p-value: 2.2 e-16; *sec15b-2*: 1.07±0.57 cm, wild type: 1.78±0.35 cm, p-value: 2.2 e -16). In addition, etiolated mutant seedlings define shifted interface between root and shoot, which results in formation of a double collet (Janková Drdová; in preparation) and exhibit agravitropic reaction of shoot (see the direction of growth in the case of mutant seedlings).

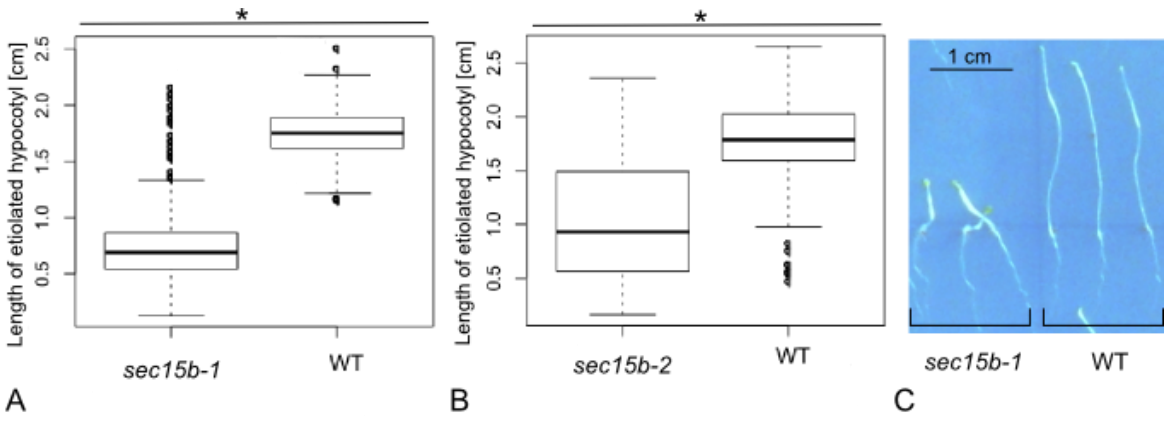

Figure 12: Length of etiolated hypocotyls of *sec15b-1* (A) and *sec15b-2* (B) lines. Panel (C) shows 7 days old etiolated seedlings of *sec15b-1* mutant in comparison with appropriate wild type. *=significant, n.s.=non-significant

2.4. Primary root length

Seven-day-old *Arabidopsis* seedlings were cultivated under sterile conditions at 21±1°C and tested in length of primary root. In case of *sec15b-2* mutant, no significant change in length was observed. In contrast, primary roots of *sec15b-1* mutant were slightly shorter in comparison to the wild type (see Figure 13) (*sec15b-1*: 2.25±2.43 cm, wild type: 2.73±0.45 cm, p-value: 6.05 e-13; *sec15b-2*: 2.34±0.52 cm, wild type: 2.35±0.48 cm, p-value: 0.76). However, in other observations primary root size of both mutants obviously elongated the primary root more compared to wild type (data not shown). *sec15b-1* allways shows stronger phenotype, but *sec15b-2* follows the mutant characteristics in the same manner in each generation.

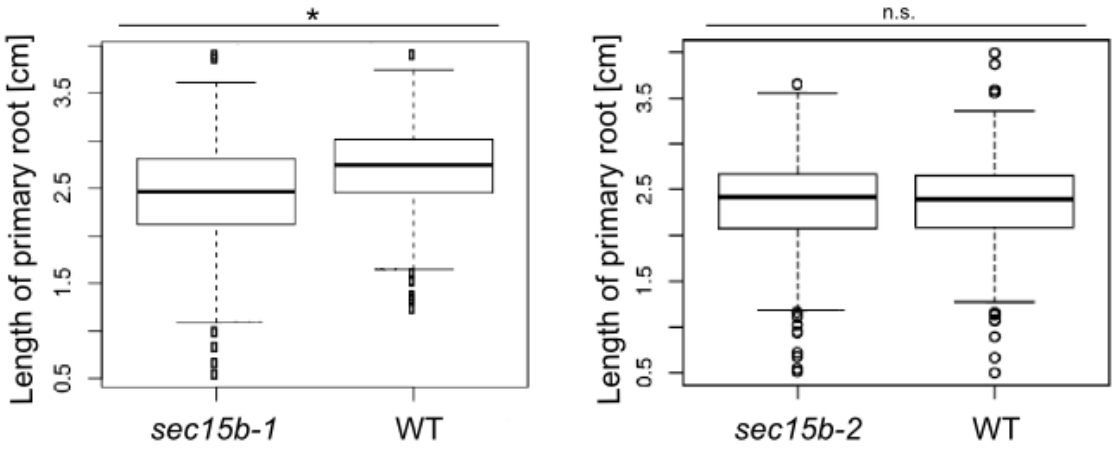

Figure 13: Analysis of length of primary roots *sec15b-1* (left) and *sec15b-2* (right). *=significant, n.s.=non-significant

2.5. Number of lateral roots

Quantification of lateral roots was investigated on seven-day-old seedlings. In case of wild type plants, most abundant population did not create any lateral roots and the count of plants were decreased with increasing number of lateral roots. Both, *sec15b-1* and *sec15b-2* mutants revealed

a global maximum at one lateral root and the populations with two, three and four lateral roots were also more abundant in comparison with wild type (see Figure 14). In average, mutant plants create more lateral roots (*sec15b-1*: 2.33 of lateral roots in average, wild type: 1.42 of lateral roots in average, p-value: 5.18 e-24; *sec15b-2*: 1.9 of lateral roots in average, wild type: 1.8 of lateral roots in average, p-value: 5.6 e-7). This phenotype appears to be stable across more generations in contrast to the length of primary roots.

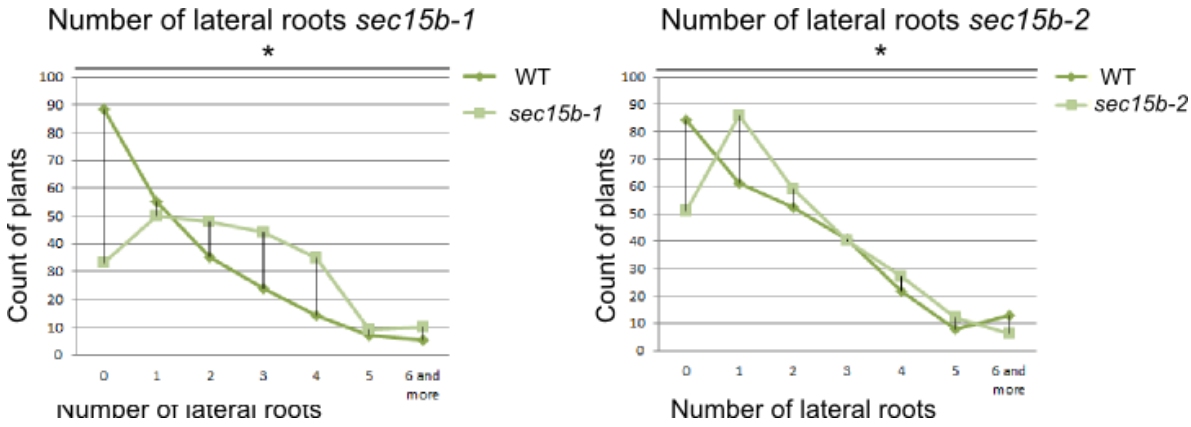

Figure 14: Number of lateral roots of *sec15b-1* (left) and *sec15b-2* (right). *=significant, n.s.=non-significant

2.6. Mature plants

Seven-week-old plants were analysed in several characteristics including diameter of rosette, length of stem and number of lateral shoots.

2.6.1. Diameter of rosette

Size of the rosette is an important feature of *exo70a1*. As shown in the plots, *sec15b-1* diameter of rosette is shorter and the leaves are smaller in total (see Figure 15) (*sec15b-1*: 3.61±0.82 cm, wild type: 4.22±0.71 cm, p-value: 0.000313). Wild type leaves show redish colour, whereas *sec15b-1* leaves do not turn red. This might correspond to disruption of the accumulation of anthocyanins in case of mutant plants. In addition, yellowish to brownish spots on three-week-old mutant plants can be observed (see Figure 16). Based on the phenotype of other exocyst mutants, these spots might be described as autophagic lesions, which is consistent with phenotype of altered accumulation of anthocyanins. In contrast, *sec15b-2* does not show significant change in rosette size (*sec15b-2*: 5.34±0.97 cm, wild type: 5.39±0.81 cm, p-value: 0.79) as well as any other features for *sec15b-1* rosette leaves including formation of lesions or variation from the colour (Figure 16).

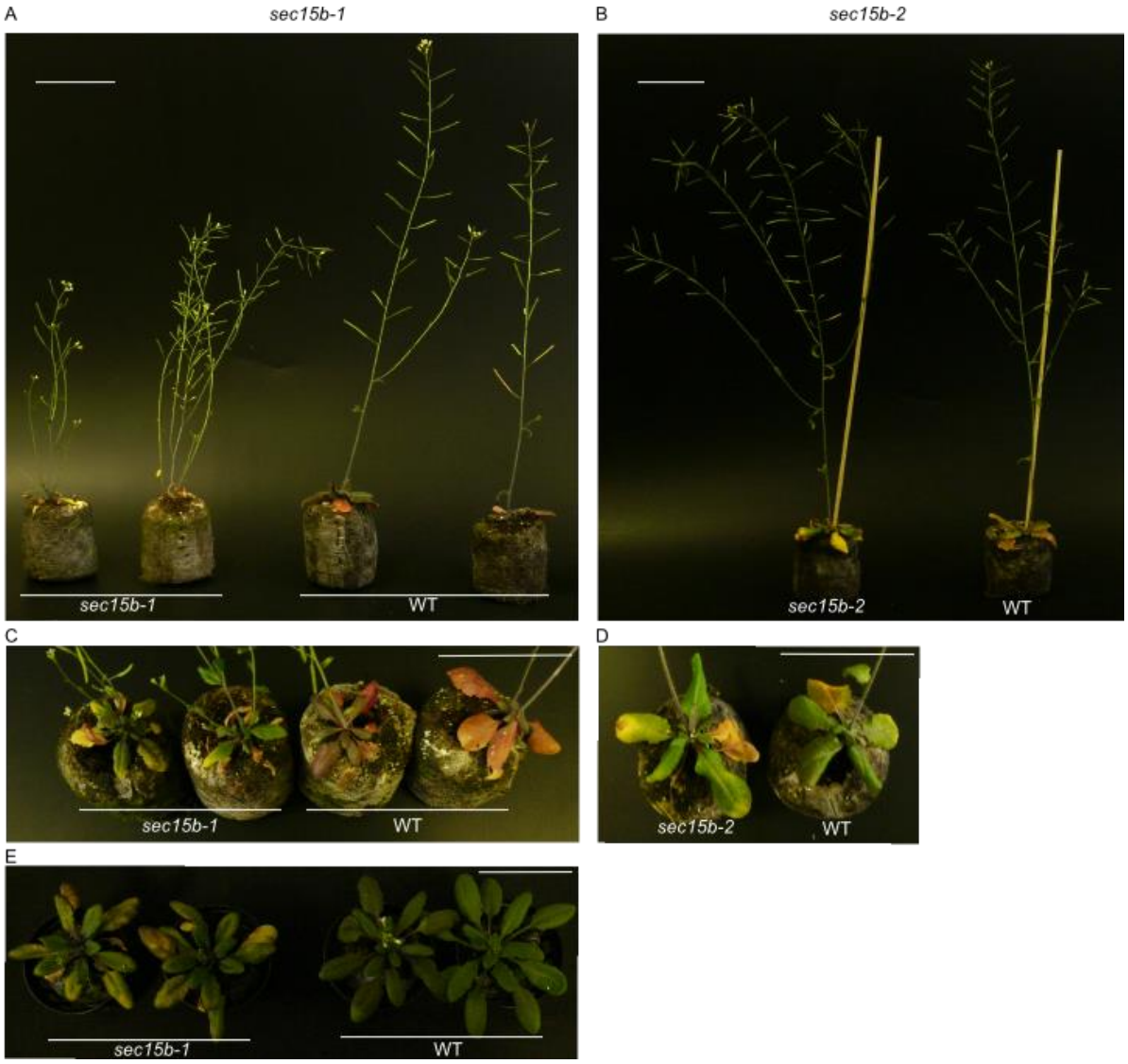

Figure 15: Comparison of *sec15b* phenotype with wild type (A) *sec15b-1* plants are shorter, create more axiliary buds and exhibit defects in apical dominance. (B) *sec15b-2* mutant does not observably differ from wild type plants, although, some of the axiliary branches are longer in comparison to the wild type, but rarely overgrow the main stem. (C) Rosettes of *sec15b-1* are smaller and does not change their colour. (D) *sec15b-2* rosettes do not vary in the phenotype or rosettes. (E) *sec15b-1* mutants display lesions on leaves, which might correspond to autophagic lesions. Scale bar=10 cm

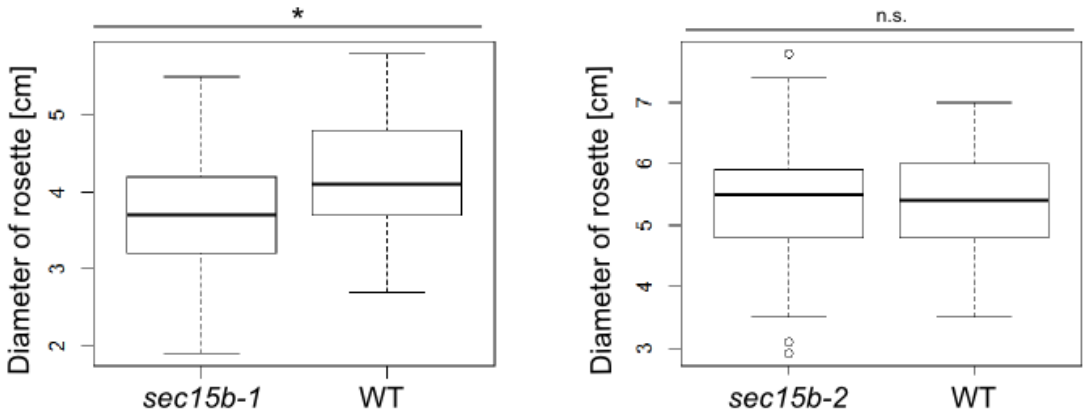

Figure 16: Diameter of rosette of 7-week-old *sec15b-1*, and *sec15b-2* mutants (around 50 plants in each group measured). *=significant, n.s.=non-significant

2.6.2. Length of stem

Total length of stem varies in case of *sec15b-1* mutants (Figure 17). Stem length of *sec15b-1* is dramatically shorter in accordance to appropriate wild type (s*ec15b-1*: 13.58±2.9 cm, wild type: 20.35±4.9 cm, p-value: 7.2 e-12). This is consistent with observation of other exocyst mutants, which usually show dwarf phenotype (*exo70a1, exo84b*). *sec15b-2* created longer stems than wild type in average, nevertheless did not differ significantly compared to the wild type (*sec15b-2*: 29.88±4.4 cm, wild type: 28.65±4.3 cm, p-value: 0.16), which is caused by a small number of observation (approximately 50 plants in each group).

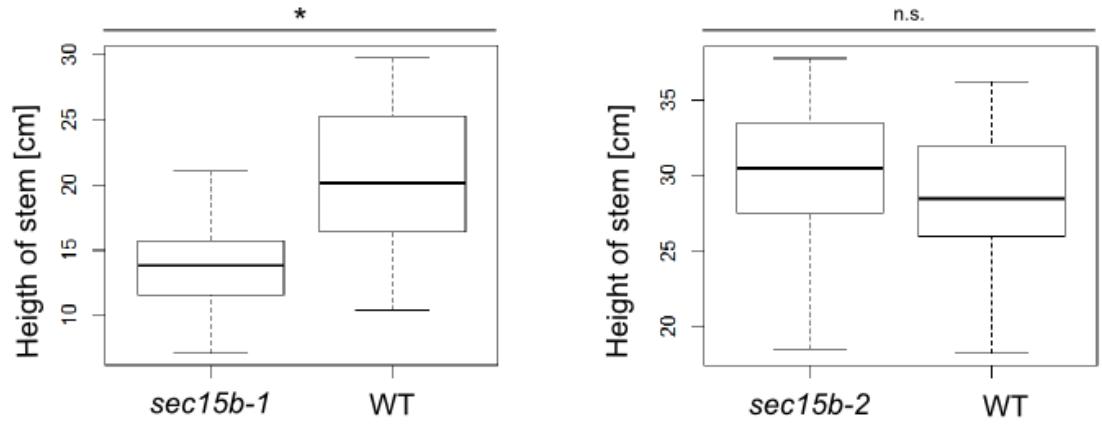

Figure 17: Height of stems of *sec15b-1* (left) and *sec15b-2* (right) in comparison to the appropriate wild types. *=significant, n.s.=non-significant

2.6.3. Number of axillary buds

Establishment of axillary buds appears to be altered in *sec15b-1* mutant plants (see Figure 18). In comparison to wild type, *sec15b-1* creates more lateral shoots (*sec15b-1*: 2.95 of axillary buds in average, wild type: 0.52 of axillary buds in average, p-value: 1.15 e-156). Additionally, lateral shoots occasionally overgrow shoot apical stem. In contrast, both *sec15b-2* and appropriate wild type of *sec15b-2* create more lateral shoots in comparison to wild type of *sec15b-1*. However, difference between *sec15b-2* and wild type of *sec15b-2* is not significant (*sec15b-2*: 2.06 of axillary buds in average, wild type: 2.31 of axillary buds in average, p-value: 0.33). Moreover, shoot apical stem of *sec15b-2* is usually not overgrown by lateral shoots. This difference might be caused by a different ecotype background of both wild types.

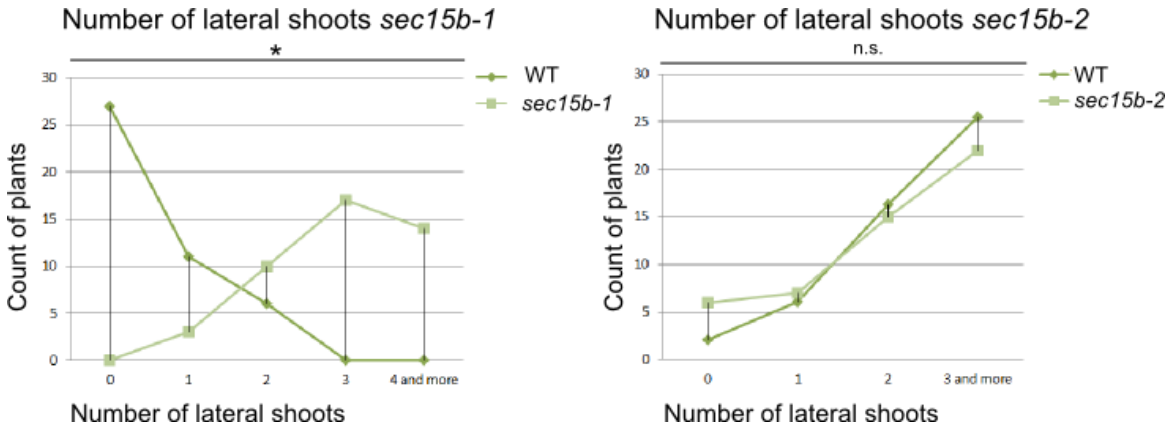

Figure 18: Number of lateral roots of *sec15b-1* (left) and *sec15b-2* (right). *=significant, n.s.=non-significant

2.7. Pollen tube length

Pollen tube is highly polarized and the most rapidly growing plant cell type and its growth puts high demand on secretory system. Exocyst was already shown to assist during the process of pollen tube growth. Transcripts of several exocyst subunits including *SEC15A* are enriched in pollen tubes. As expected, *sec15a* mutant exhibited delayed growth of the pollen tubes (Hála et al., 2008). Therefore, it was questionable whether *sec15b* suffer from similar defect. In both *sec15b* mutants, high number of pollens grain germinated, but the growth appeared arrested prematurely (see Figure19). Moreover, pollen tubes that continued their maturation were much shorter than wild type (*sec15b-1*: 0.24±0.3 cm, wild type: 0.56±0.49 cm, p-value: 2.2 e-16; *sec15b-2*: 0.5±0.53 cm, wild type: 0.91±0.69 cm, p-value: 1.86 e -12) and revealed swollen tip phenotype in much more cases compared to the wild type.

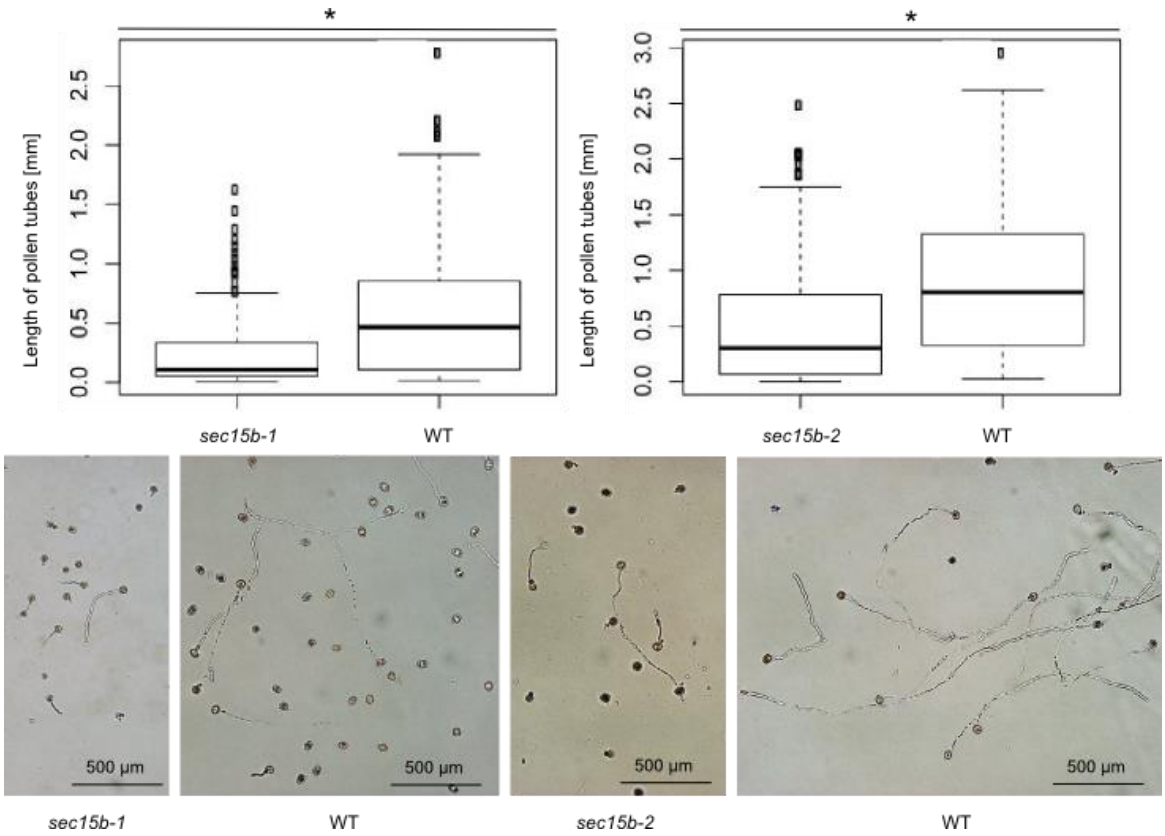

Figure 19: Length of pollen tubes *sec15b-1* (left) and *sec15b-2* (right) plotted in the upper panels and compared in the representative pictures of each group (lower panels). *=significant, n.s.=non-significant

3. Complementation of *sec15a* and *sec15b*

To further understand the role of *SEC15A* and *SEC15B* genes, redundancy between SEC15A and SEC15B was tested by complementation. For complementation test, mutant plants (*sec15b-1*) were transformed by four different constructs (plasmids pBGW containing combinations of promotor *SEC15A* or *SEC15B* and genes of *SEC15A* or *SEC15B*) carried by *Agrobacterium tumefaciens* using floral dip method. Nine to eighteen complemented lines of each combination were analysed in three features typical for *sec15b-1* mutant. Structure of seed coat, formation of double collet and elongation of hypocotyl were investigated (see Figures 20, 21, 22 and 23). From each complemented group, three best complemented lines are documented here.

Most of the complemented lines exhibited at least a slight structural divergence and full recovery of the seed coat phenotype is rare in comparison to the complementation of etiolated hypocotyl length. The closest similarity to the wild type seed coat architecture revealed lines complemented by construct carrying *SEC15B* gene under *SEC15B* promotor. However, other complemented lines exhibited a significant improvement of mutant phenotype and at least one line from each group was indistinguishable from wild type. Seed coat thickness is a widely variable characteristic, which is recommended to be analysed in several generations. Therefore, another analysis of phenotype recovery should be done.

sec15b-1 mutant is reliably identified by shortened etiolated hypocotyls and double collet formation. Therefore, the complemented lines were tested in this manifestation of phenotype. In all complemented groups, proper elongation of hypocotyl and suppression of double collet formation could be found, which indicates that SEC15A and SEC15B act redundantly in the seedling hypocotyl and potentially other sporophytic tissues.

Taken these data together, it appears that SEC15A and SEC15B expressed under both, *SEC15A* and *SEC15B* promotors are able to complement *sec15b-1* mutant phenotype. It appears that proper amount of any SEC15 protein is necessary for the proper development rather than expression of specific isoform. Therefore, I conclude that the role of SEC15A and SEC15B might be very similar and both subunits possibly share their interactors at least partially.

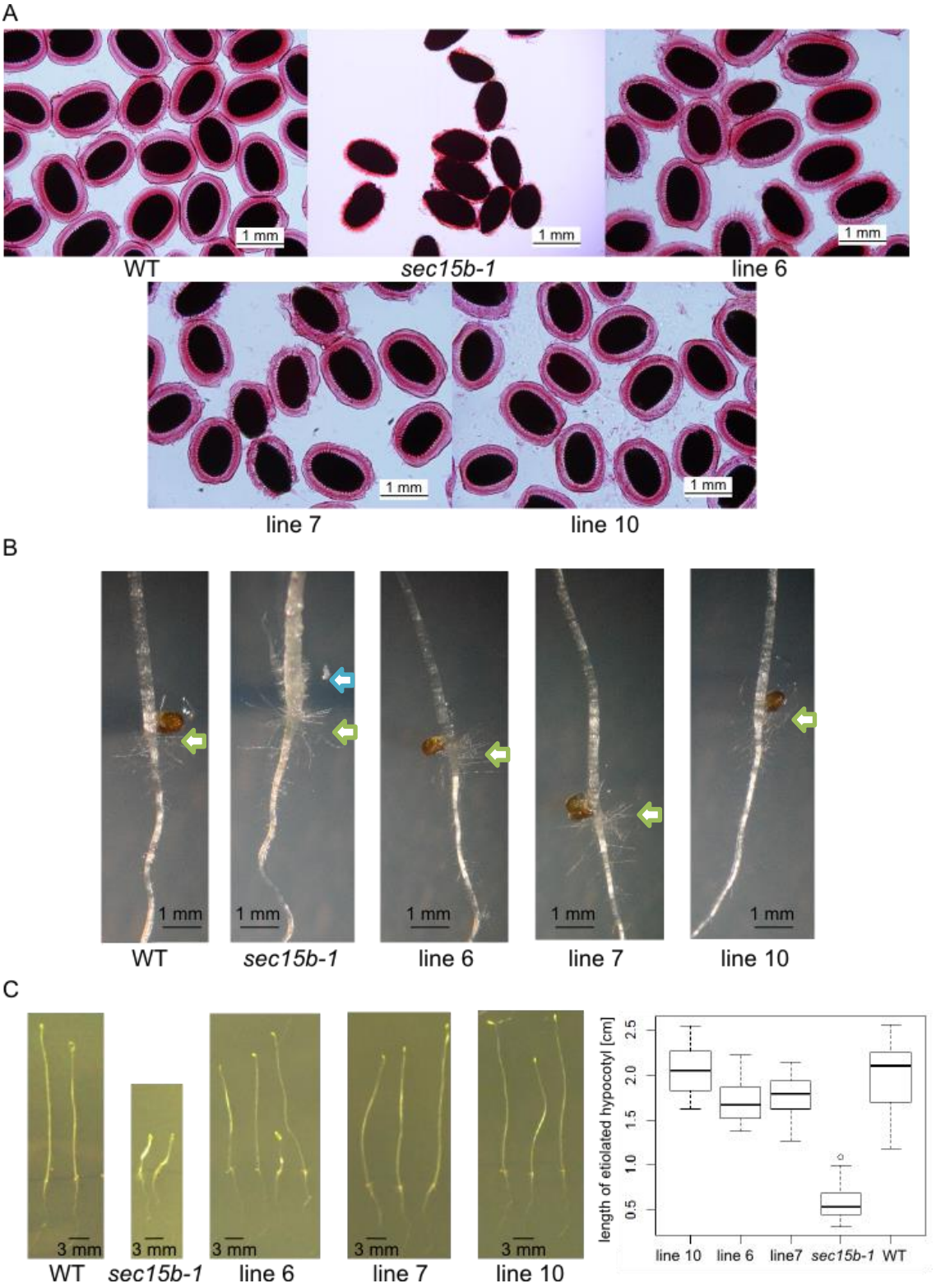

Figure 20: Complementation of *sec15b*-1 mutation by *SEC15A* under *SEC15A* promotor (A): ruthenium red-stained seed coats of wild type, *sec15b-1* and three independent complemented lines (6, 7 and 10) (B) double collet phenotype of 7-day-old etiolated seedlings of wild type, *sec15b-1* mutants and three independent complemented lines (6, 7 and 10). Green arrows show true collet region, blue arrow points to double collet (C) 7-day-old etiolated hypocotyls of WT, *sec15b-1* mutant, and three independent complemented lines (6, 7 and 10). Right panel summarizes lengths of these hypocotyls (5 to 30 complemented seedlings measured in each line).

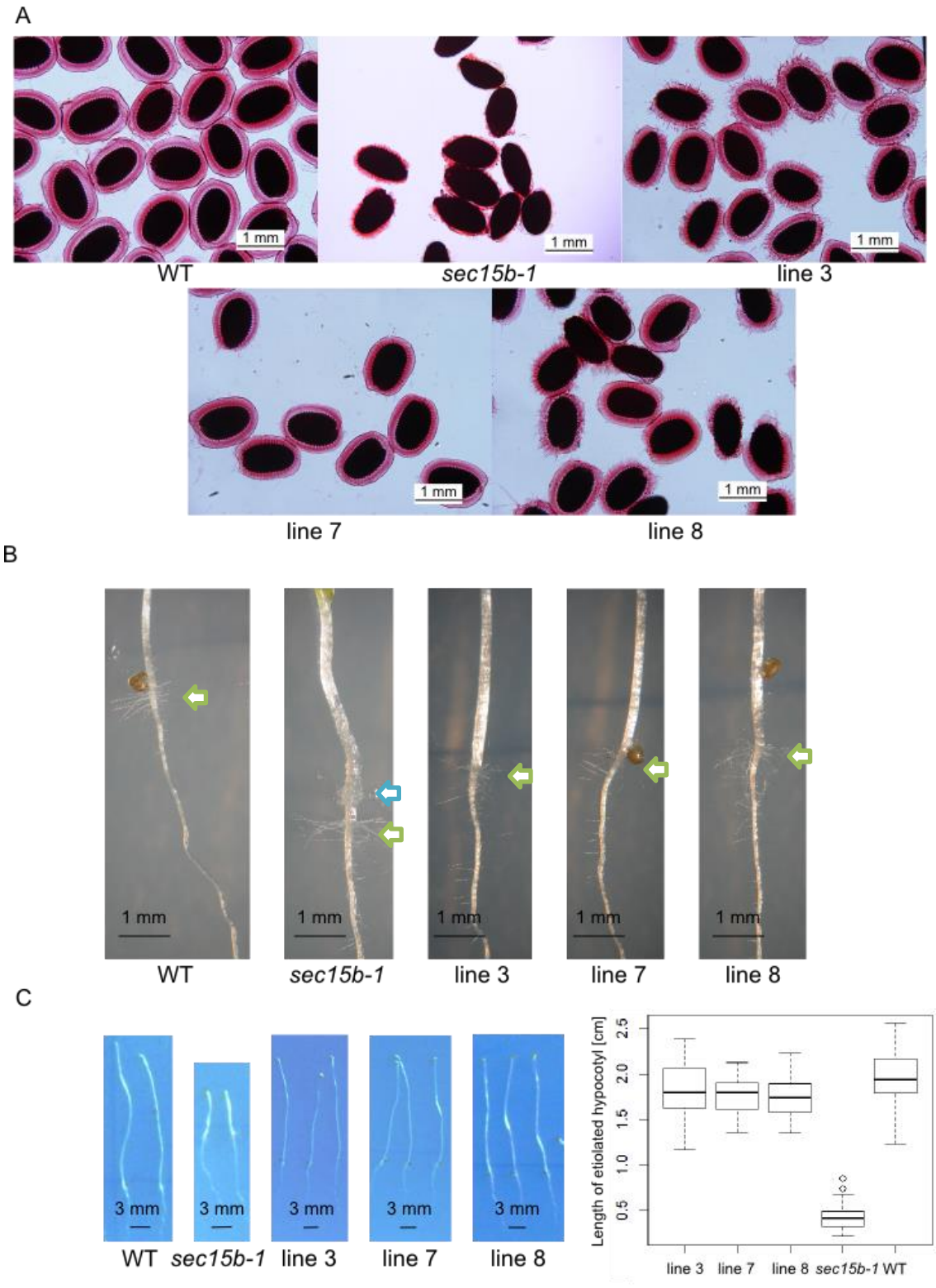

Figure 21: Complementation of *sec15b-1* mutation by *SEC15B* under *SEC15A* promotor (A): ruthenium red-stained seed coats of wild type, *sec15b-1* and three independent complemented lines (3, 7 and 8) (B) double collet phenotype of 7-day-old etiolated seedlings of wild type, *sec15b-1* mutants and three independent complemented lines (3, 7 and 8). Green arrows show true collet region, blue arrow points to double collet (C) 7-day-old etiolated hypocotyls of WT, *sec15b-1* mutant, and three independent complemented lines (3, 7 and 8). Right panel summarizes lengths of these hypocotyls (5 to 30 complemented seedlings measured in each line).

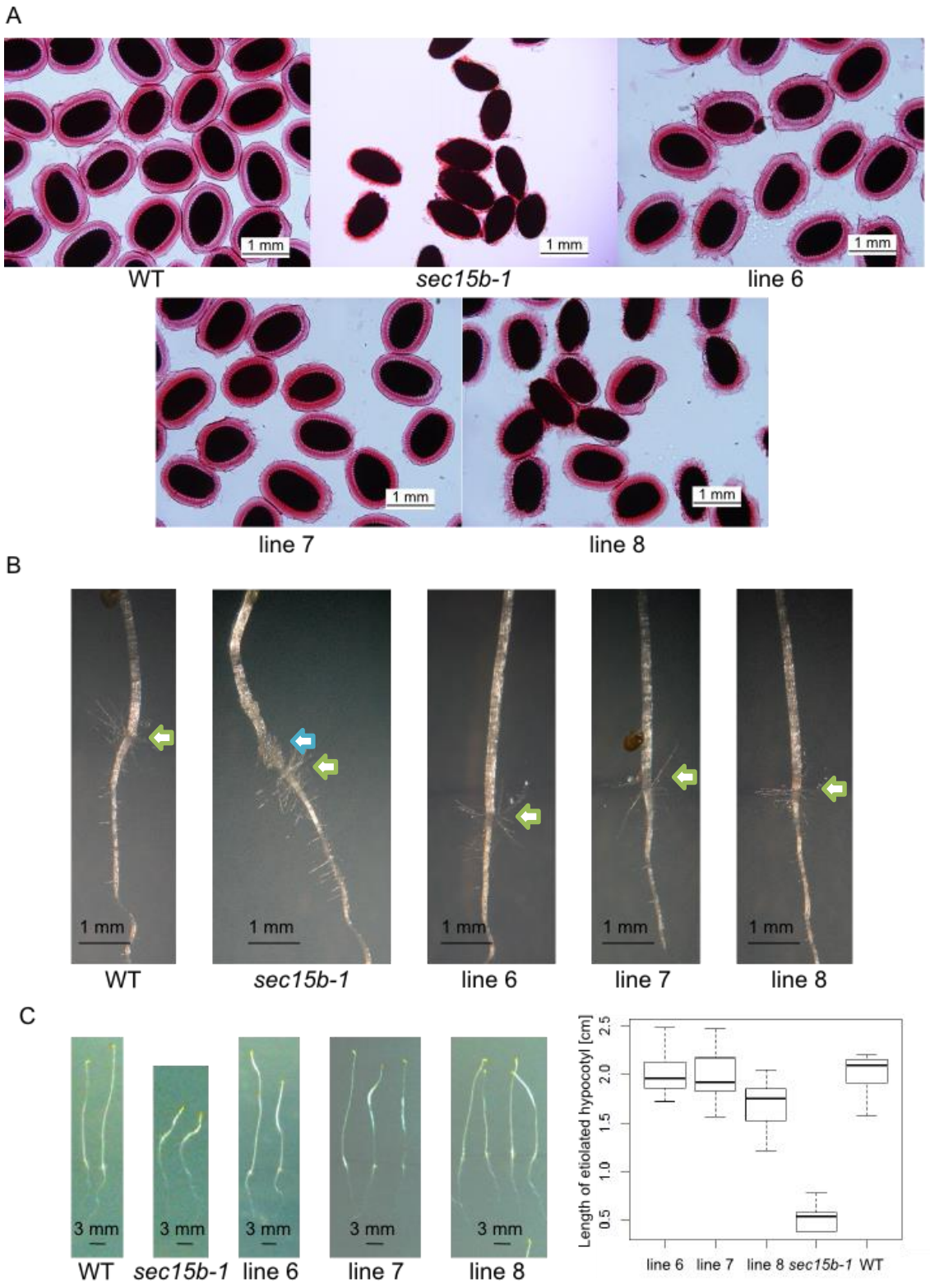

Figure 22: Complementation of *sec15b-1* mutation by *SEC15A* under *SEC15B* promotor (A): ruthenium red-stained seed coats of wild type, sec15b-1 and three independent complemented lines (6, 7 and 8) (B) double collet phenotype of 7-day-old etiolated seedlings of wild type, sec15b-1 mutants and three independent complemented lines (6, 7 and 8). Green arrows show true collet region, blue arrow point to double collet (C) 7-day-old etiolated hypocotyls of WT, *sec15b-1* mutant, and three independent complemented lines (6, 7 and 8). Right panel summarizes lengths of these hypocotyls (5 to 30 complemented seedlings measured in each line).

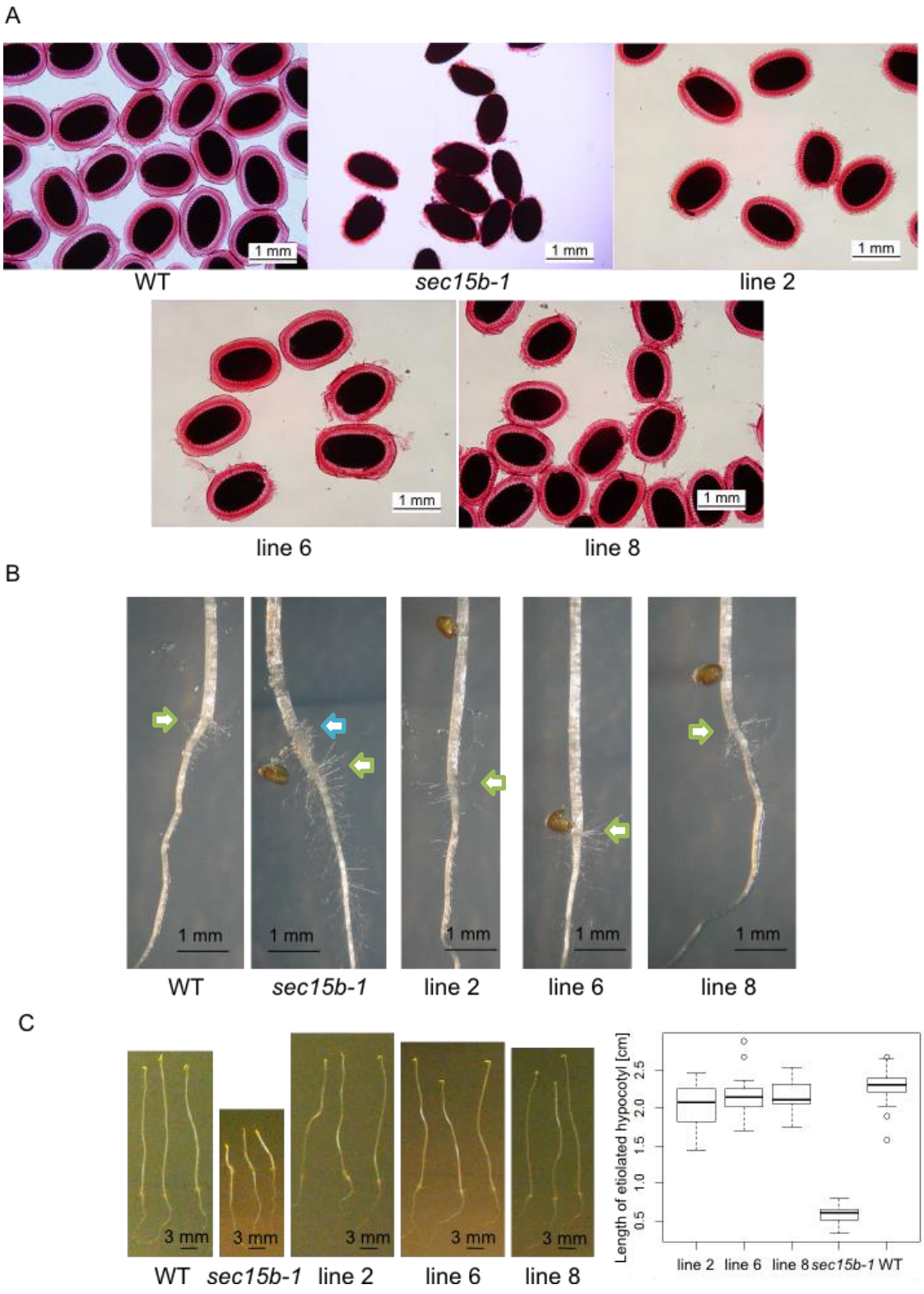

Figure 23: Complementation of *sec15b-1* mutation by *SEC15B* under *SEC15B* promotor (A): ruthenium red-stained seed coats of wild type, *sec15b-1* and three independent complemented lines (2, 6 and 8) (B) double collet phenotype of 7-day-old etiolated seedlings of wild type, *sec15b-1* mutants and three independent complemented lines (2, 6 and 8). Green arrows show true collet region, blue arrow points to double collet (C) 7-day-old etiolated hypocotyls of WT, *sec15b-1* mutant, and three independent complemented lines (2, 6 and 8). Right panel summarizes lengths of these hypocotyls (5 to 30 complemented seedlings measured in each line).

4. The role of SEC15B protein on a molecular level

In the last part of my diploma thesis, I focused my interest on molecular mechanism of SEC15B function. I hypothesized that SEC15B can potentially provide a link between secretory vesicles and the rest of the exocyst complex directly through an interaction between SEC15B and lipids or in evolutionary conserved manner via interaction with Rab GTPase. First, link between SEC15B and lipids was analysed using PIP Strip and LUV (large unilamelar vesicles). Second, protein-protein interaction between SEC15b and RAB A4a was tested by pull down assay. RAB A4a was chosen in accordance to the high level of co-expression with SEC15B throughout plant tissues and due to the previous co-immunoprecipitation analysis, where SEC8 exocyst subunit was shown to bind RAB A4a indirectly (Fendrych, unpublished data).

4.1. Expression of SEC15B protein

Expression of SEC15B protein was tested in two different expression cell strains BL21 (RIPL) and BL21 Arctica (RIL). Both bacterial strains failed to produce enough of soluble protein under standard conditions (37 °C; 180 rpm; 4 hour induction of expression).

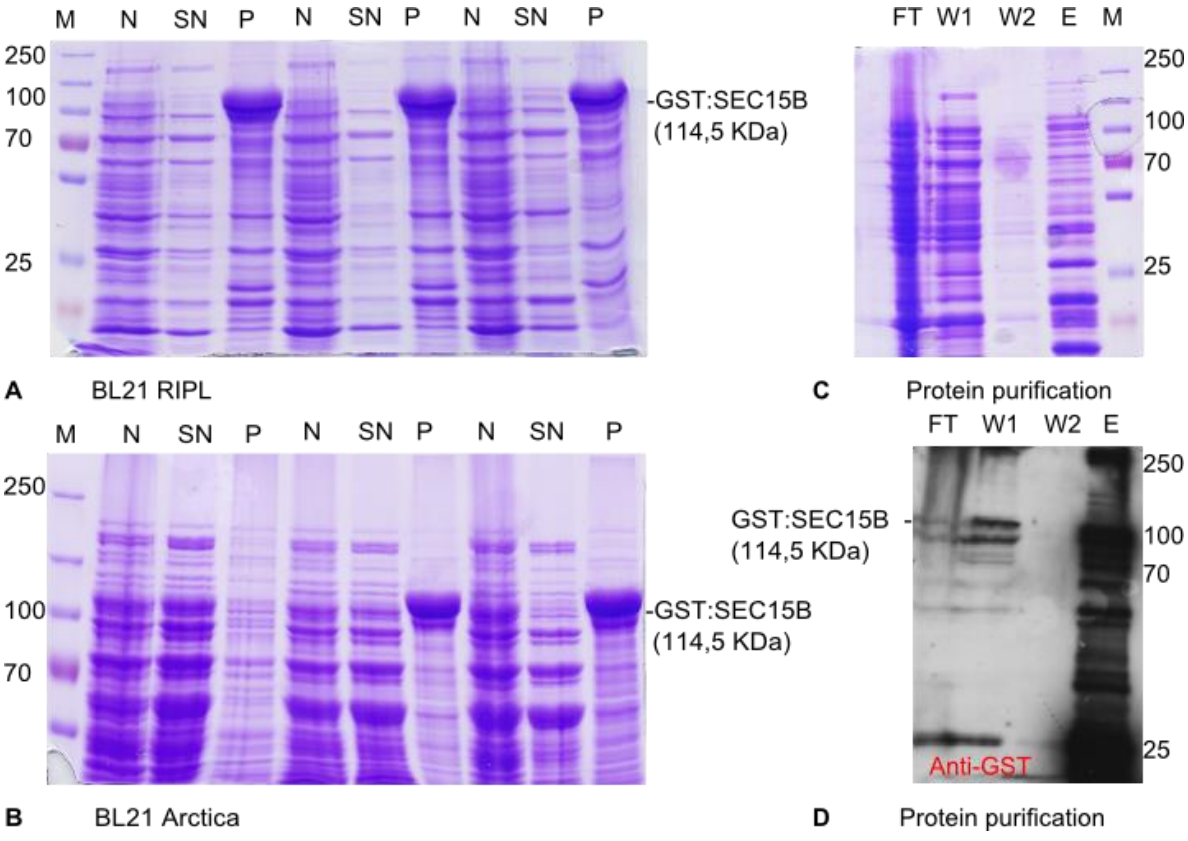

Figure 24: Purfication of recombinant SEC15B (A) BL21 (RIPL) and (B) BL21 Arctica (RIL) cells (M=marker; N= non-induced control cells; SN= supernatant; P=pellet) (C) Expression of protein in BL21 Arctica (RIL) cells in a volume of 2 l at 12°C for 24 hours with following purification (FT=flow-through; W1= wash 1; W2= wash 2; M=marker) (D) Western blot analysis of SEC15B in purified fraction.

Sufficient fraction of SEC15B protein was isolated from BL21 Arctica (RIL) cells expressing at 12°C for 24 hours in total volume of 2 l. Presence of the SEC15B protein in fraction of elution was successfully verified by western blot analysis (Figure 24) using anti-GST antibody. Nevertheless, recombinant SEC15B protein is very unstable and requires immediate processing.

4.2. Lipid binding assays

It was already shown that several plant exocyst subunits interact directly with lipid surface of membranes (Růžičková, Potocký, Rawat, Ortmannová; personal communication). Therefore, SEC15B subunit was tested in several lipid binding assays to investigate the potential plant unique molecular role of SEC15B.

4.2.1. PIP Strip

PIP Strip is an artificial membrane containing specific lipid composition used for analysis of interactions between proteins and lipids. Once the protein is let to bind some lipid specifically, antibodies are used to visualize the signal on the membrane. First, protein SEC15B purified in buffer with supplement of sarcosyl revealed specific strong interaction with several signaling phospholipid types (Phosphatic acid (PA), Phosphatidylinositol(5)phosphate (PI(5)P), Phosphatidylinositol(4)phosphate (PI(4)P), Phosphatidylinositol(4, 5)bisphosphate (PI(3,4)P_2), Phosphatidylinositol(4,5)bisphosphate (PI(4,5)P_2) and Phosphatidylinositol(3,5)bisphosphate (PI(3,5)P_2)) and not with lipids that are enriched in all membranes such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE) (see Figure 25, panel A). This very promising result was not confirmed by LUV analysis and SEC15B showed only non-specific interaction (see bellow) with all tested lipids (PC, PA, PI(3,4,5)P_3 and PI(3)P).

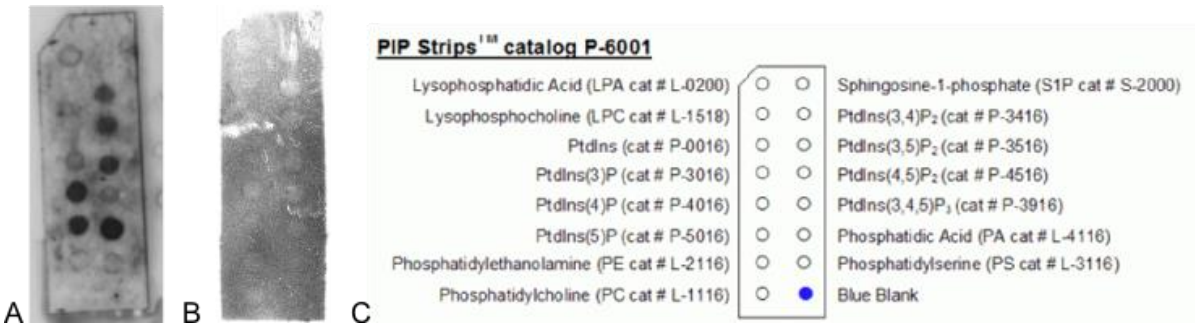

Figure 25: Western blott analysis of PIP Strip using anti-GST antibody against SEC15B purified using 0.5% sarcosylResult from PIP Strip revealing that SEC15B purified using 0,5 % sarcosyl (A) and with no supplement of sarcosyl (B). (C) Membrane PIP Strip diagram.

Therefore, SEC15B was purified using buffer without addition of sarcosyl and such treated protein did not interact with any phospholipid using PIP Strip method (Figure 25, panel B).

4.2.2. LUV

LUV method, which includes formation of large unilamelar vesicles, is the second analysis used to investigate protein-lipid interactions. In this experiment, vesicles containing PC, PA, PIP_2 and PI(3)P were incubated with SEC15B. In case of interaction between SEC15B and vesicles, SEC15B should be present in a fraction of pellets after ultracentrifugation. Once there is no interaction between protein and lipids, SEC15B should remain solubilized in supernatant. As a control sample, purified SEC15B protein was treated with lipid binding buffer only and ultracentrifuged in the same manner as SEC15B treated with artificial vesicles.

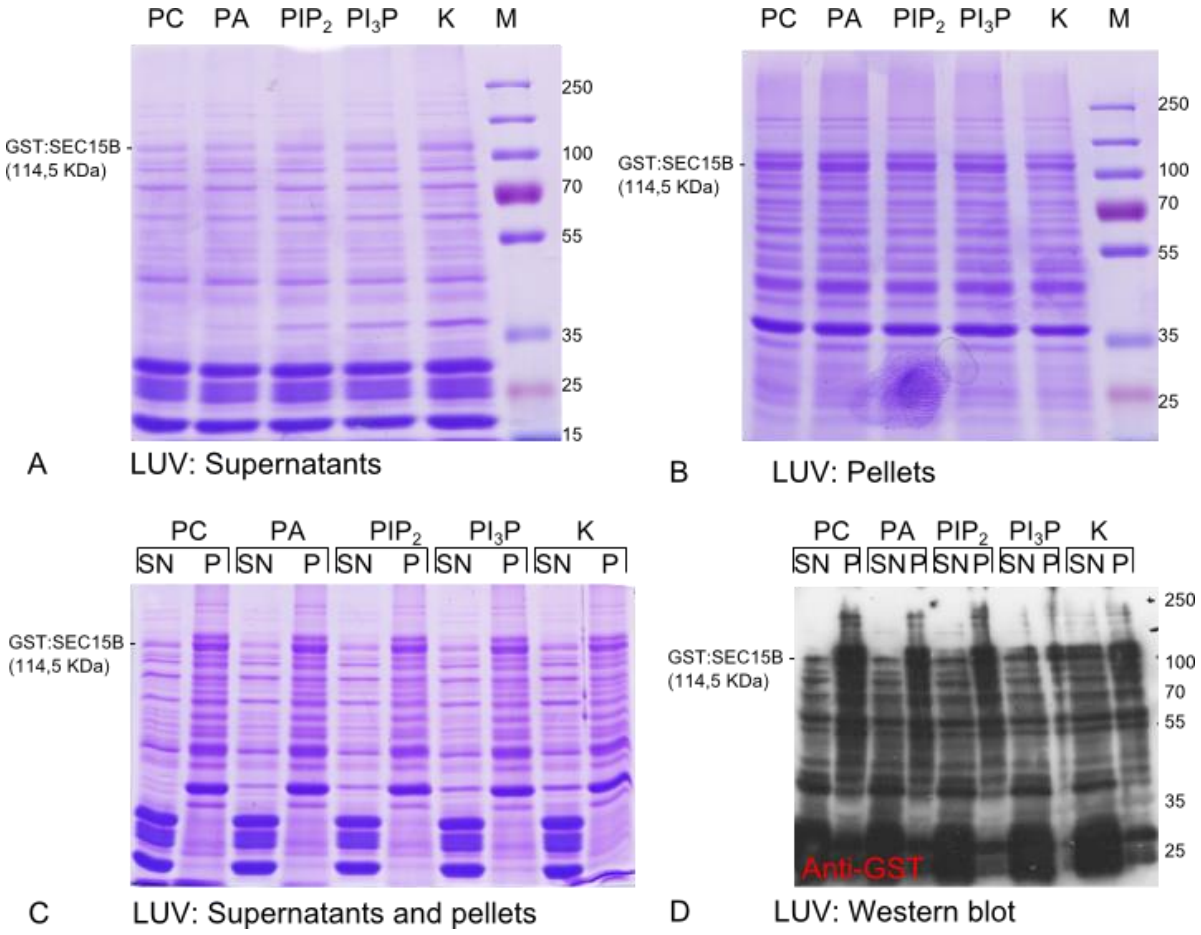

Figure 26: LUV analysis shows that SEC15B is present in all fraction of Supernatants (A) and Pellets (B) comparison of supernatants (C). (D) Western blot analysis using anti-GST antibody.

In this analysis, SEC15B can be found in the pellet fraction with PC, PA, PIP_2 and PI(3)P. Compared to the other pellets, the amount of SEC15B is slightly higher in case of PA (Figure 26, panel B). However, SEC15B itself tends to precipitate and thus SEC15B is enriched in pellet. With decreasing concentration of salts (ranges between 125 mM and 500 mM KCl), fraction of precipitated SEC15B is higher (data not shown). Therefore, this method cannot be used to verify or disprove

interaction between SEC15B and specific phospholipid and SEC15B is postulated to do not bind any phospholipid in accordance to the result obtained by PIP Strip analysis.

4.3. Pull down of SEC15B and RAB A4a GTPase

Pull down is a powerful tool for testing protein-protein interactions *in vitro*. Here, three distinct experiments were done. First, RAB A4a was incubated with empty affinity resin only. Second, RAB A4a was tested in interaction with free GST. Third, SEC15B was attached to the resin and incubated with RAB A4a. To present the exact molecular weight of RAB A4a, a load of protein was added (the last column in the Figure 27, panel A, B and C), which represents a fraction of purified RAB. There is no RAB A4a in the elution fraction using empty resin (Figure 27, panel A) and in the elution fraction of the experiment with free GST (Figure 27, panel B), only a band of size about 26 KDa can be observed that corresponds to free GST In contrast, third experiment demonstrates that RAB A4a is able to bind SEC15B, which is present in the fraction of elution (see Figure 27, panel C). Therefore, RAB A4a interacts with SEC15B and not with free GST or resin *in vitro*. This result was verified by western blot analysis (Figure 27, panel D), which confirms the presence of SEC15B by two antibodies (anti-SEC15B and anti-His tag antibodies) and RAB A4a by a single antibody (anti-GST antibody) proteins of correct size in a fraction of elution. In conclusion, plant SEC15B potentially acts in evolutionary conserved manner and serves as an effector protein of RAB A4a GTPase and does not bind the secretory vesicles directly through interaction with lipids.

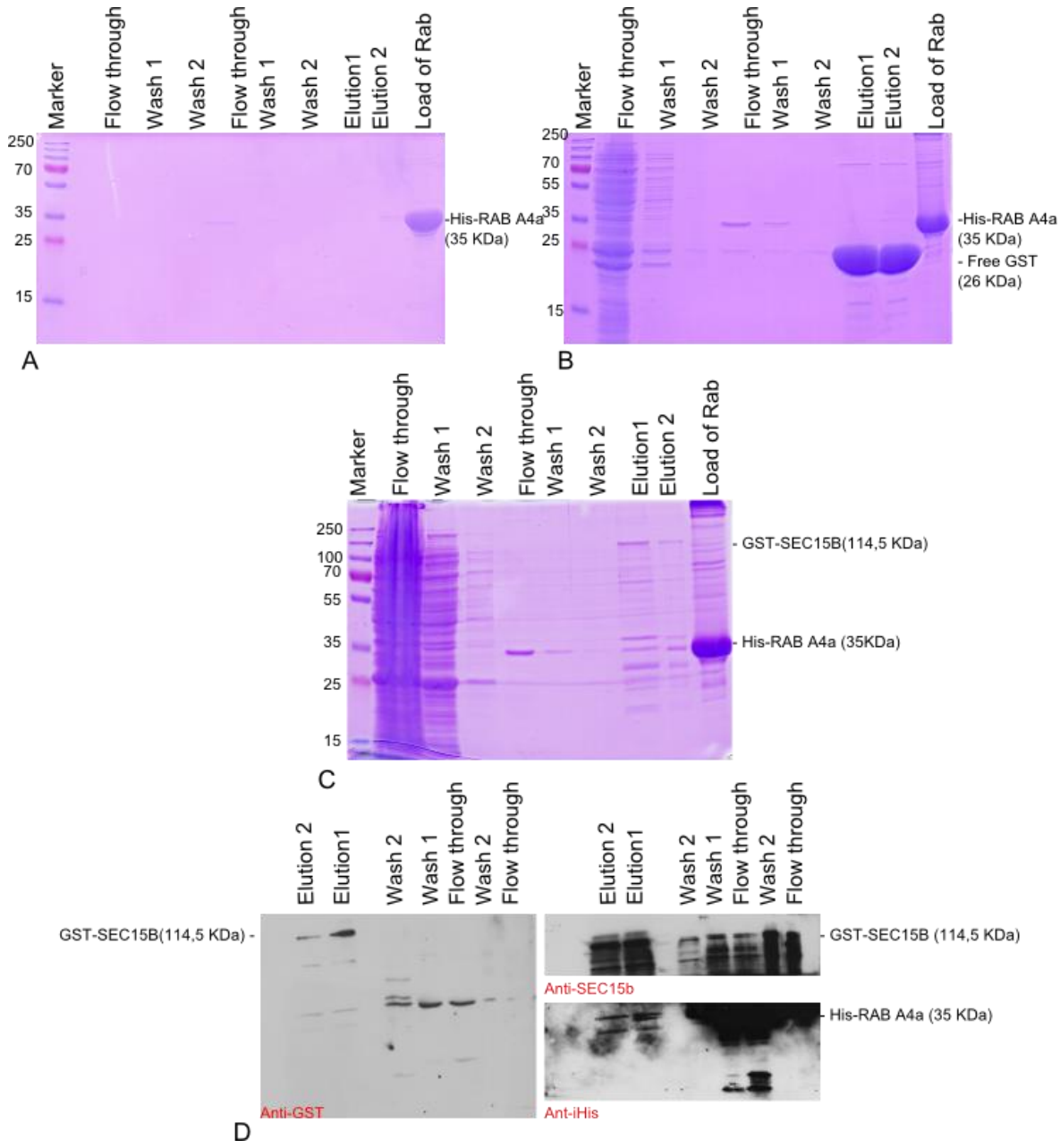

Figure 27: Analysis of protein-protein interaction between SEC15B and RAB A4a using pull down assay (A) Control pull down of RAB A4a with empty resin. (B) Control pull down of RAB A4a with free GST. In the elution fraction, only a band of size about 26 KDa can be observed and corresponds to free GST. (C) Pull down of SEC15B with RAB A4a. Upper band of 114,5 KDa corresponds to GST-SEC15B, lower strong signal refers to RAB A4a. (D) Western blot analysis of fractions from the panel (C) used anti-GST and Anti-SEC15B antibody against GST-SEC15B and Anti-His antibody against His-RAB A4a

Discussion

1. *sec15b* mutant phenotype shares characteristics with the other exocyst mutants

1.1. Plant homologues of SEC15 interactors and their LOF mutants

Mutant analysis helps us understand a role of the mutated gene and enables study of genetic interactions in most cases. Sec15 was shown to physically interact with Rab GTPases, GEFs of Rab GTPases and myosin in yeast and animals. Mutants in these components usually show similar or more extensive defects in comparison to sec15 mutants. However, mutation of most plant secretory components does not reveal obvious phenotype due to the functional redundancy of homologues (Rojo and Denecke, 2008).

Arabidopsis myosins XI-2/MYA2 and XI-K participate in polarized secretion and thus are candidates for interaction with exocyst. However, both myosins coordinate vesicular movement simultaneously (Peremyslov et al., 2008). Myosins XI-2, XI-F and XI-K were shown to act partially redundantly during tobacco leaf trafficking (Avisar et al., 2008). Moreover, direct interaction between plant SEC15 and myosins was reported only in the yeast two hybrid system (Hashimoto et al., 2008) and remains to be verified *in vivo*.

Arabidopsis class RAB A gene family is homologous to the animal Rab11. In plants as in animals, this clade operates during exocytosis, on plasma membrane recycling, prevacuolar and endosomal compartments (Rutherford and Moore, 2002). Due to the co-expression data, function of RAB A4d might be connected predominantly with SEC15A and less with SEC15B. This is consistent with the phenotype of *sec15a* mutant, which is predominantly defective in pollen tube growth (Szumlanski and Nielsen, 2009). Changes in the composition of the cell wall were also observed in single mutants of RAB GTPase from different RAB A sub classes in *Arabidopsis* (Lunn et al., 2013).

Similarly to *Drosophila* secretory pathway, any direct sequential homologue of yeast Sec2p, the GEF for Sec4p Rab GTPase, was not found in plants (Elias, 2008). Therefore, there must be a different mechanism of secretory pathway regulation. Potentially, secretory cascade is orchestrated mainly by GAPs, as was shown in fruit fly. However, the mutants of plant exocytic GAP have not been characterized up to date (Metlička; unpublished data).

In contrast, mutants of several exocyst subunit isoforms suffer from a number of defects and thus contribute to understanding of the real function of the whole complex. Exocyst subunits were found to contribute to a number of processes including embryo formation, pollen tube

development, tip growth, and proper growth of shoot and root (Kulich et al., 2013; Synek et al., 2006; Zhang et al., 2013; Hála et al., 2008; Cole et al., 2005). Therefore, I will compare phenotypes of *sec15b* mutant lines with other exocyst subunits and not with other mutants in components of secretory path.

Multicellular organisms require more specific pathways leading to more complex spatial-temporary regulation of secretion. Multiple exocyst subunits are involved in regulation of exocyst assembly at specific secretory sites during distinct processes. Therefore, I speculate that every core subunit can be an important link between secretory vesicles and the rest of the exocyst complex in plants.

1.2. *sec15b-1* and *sec15b-2* show similar phenotypic deviations, although some characteristics are plastic

Maternal wild type plants of *sec15b-1* and *sec15b-2* insertional lines belong to different ecotypes (Columbia-0 and Nössen-0) and display distinct phenotypes. Therefore, some features of mutant plants were not visible to the same degree in both mutant lines or the behaviour was distinct in specific conditions. Seed coat phenotype, length of etiolated hypocotyl, number of lateral roots, diameter of rosettes, length of stem and number of lateral shoots appear to be stably defective in any *sec15b-1* mutant. Length of etiolated hypocotyls, number of lateral roots and architecture of seed coat were strongly influenced in *sec15b-2* mutant. Both *sec15b-1* and *sec15b-2* showed similar tendencies in a number of characteristics including defects in seed coat, length of etiolated hypocotyls and number of lateral roots.

The distinct phenotype of *sec15b-1* and *sec15b-2* might be related to differential expression levels of *SEC15A* and *SEC15B*, which cooperate during secretion. It was already shown above in the results that there is very variable tissue specific ratio of expression between *SEC15A* and *SEC15B* in different *Arabidopsis* ecotypes. For instance, *Arabidopsis* found in accession Goettingen (Germany) expresses *SEC15B* in majority, whereas in plants harvested from accession Ovelgoenne (Germany) cDNA of *SEC15A* is more abundant, although the ratios between their expressions are not higher than two fold (Figure 28). The observed difference in basal expression of *SEC15* genes might be influenced by distinct ecological conditions in different habitats, which is supported by distinct ratios of *SEC15A* and *SEC15B* expression during biotic and abiotic stresses.

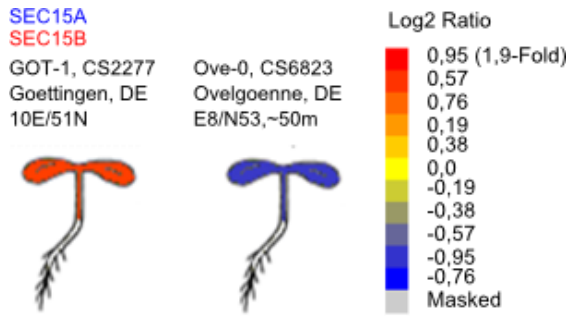

Figure 28: Natural variation in expression of Sec15A and Sec15B (Lempe et al., 2005)

Some features are observed to be very variable. For instance, length of primary root of *sec15b-1* was first evaluated as shorter in comparison to appropriate wild type (in summer), but in some other experiments were the mutant roots longer than wild type (in winter). However, both mutant lines behaved in a similar manner under the same cultivation conditions, which implies broad plasticity of both *sec15b* mutants. Variability of some characteristics may be caused by differential activity of *SEC15A* and *SEC15B* in different conditions including biotic and abiotic stresses. Temperature is a factor that strongly influences ratios of *SEC15A* and *SEC15B* mRNA expression. Heat stress causes upregulation of *SEC15A*, whereas cold induces first transient fast upregulation of *SEC15A* followed by the upregulation of *SEC15B* (efp browser data from affymetrix).

1.3. Characteristic features of *sec15b* phenotype

1.3.1. Seed characteristics

Seed coat phenotype is known to be variable and comparison of more seed generations is necessary. Thus, data presented here must be taken as preliminary, but they are supported by the seed coat phenotype deviations observed in mutants of other exocyst subunits (Kulich et al., 2010).

Seed coat formation of *sec8* and *exo70a1* mutants is aborted and results in thinner layer of mucilage around seeds (Kulich et al., 2010). Seeds of *sec15b-1* nearly lack seed coat. In contrast, seeds of *sec15b-2* create a thicker coat, which is less dense compared to wild type. In addition, a lot of cell wall material is washed out from the seed sample during staining. Based on these observations I hypothesize that the amount of secreted material to the seed coat in exocyst mutants is similar compared to the wild type, but the architecture of the coat is changed. This might be caused by the lack of some other component of cell wall in the seed coat or by differential modification of secreted material.

Seed size is influenced by the embryo growth, endosperm formation, maternal tissues and other external conditions (Fang et al., 2012). Mechanisms that coordinate these factors are poorly understood and therefore it is challenging to connect this question to secretion proper. In addition, no other exocyst mutants were observed to significantly vary in this feature. Therefore, it is surprising that both *sec15b-1* and *sec15b-2* seeds are significantly longer and in addition, *sec15b-1* seeds are wider compared to the wild type plants.

1.3.2. Etiolated hypocotyl

Shortened length of etiolated hypocotyl is also already described phenotype of exocyst mutants. *exo70a1* mutant was shown to suffer from defects in elongation of hypocotyl during development in darkness (Synek et al., 2006). Combining *exo70a1* with weak partially functional alleles *sec8-4* or *sec5a-1*, which do not show any phenotype of hypocotyl growth itself, enhanced the phenotype deviation from the WT (Hála et al., 2008). Other features of triple response are maintained and ethylene signaling might not be altered (Synek et al., 2006). Both *sec15b-1* and *sec15b-2* mutants develop shorten hypocotyls during growth in darkness and no other defects in triple response were observed. Interestingly, hypocotyls of mutant plants appear to be agravitropic. This might be caused by the hyperaccumulation of starch in this region (Růžičková, unpublished data).

1.3.3. Primary root length and number of lateral roots

Primary root length is also affected in *exo70a1* and *sec8* mutants (Synek et al., 2006; Cole et al., 2014). Mutation in this exocyst subunit results in shorter primary root (Synek et al., 2006). This is consistent with the observation of *sec15b-1* mutant in summer, whereas the *sec15b-2* mutation did not display any difference in comparison to the wild type. In winter, primary roots of both *sec15b-1* and *sec15b-2* seem to be obviously longer in comparison with both wild types. This variability might be caused by differential expression of SEC15A and SEC15B in different conditions (see above), but this hypothesis needs to be further tested.

Initiation and progression of lateral roots in *exo70a1* is defective and results in a low number of lateral roots (Drdová et al., 2013). This is explained by lower sensitivity of the mutant to auxin, because addition of either 10nM or 100nM naphtyl acetic acid did not rescue the phenotype (Synek et al., 2006). In contrast, *sec15b-1* and *sec15b-2* mutants stably initiated a significantly higher number of lateral roots and thus *exo70a1* and *sec15b* exocyst mutants behave surprisingly in this relation in a quite opposite manner. This might indicate that auxin transport or signaling (or both) is altered in both exocyst mutants, but possibly whith different consequences.

1.3.4. Characteristics of shoot

Size of shoot organs is shortened in *sec15b-1* mutant in comparison to wild type. Both, length of stem and diameter of a rosette are significantly diminished. Phenotype of *sec15b-1* appears to be milder compared to the changes in *exo70a1*. Synek et al. (2006) explain small organ size of *exo70a1* by defects of cell plate formation or inappropriate nutrition caused by disruption of root hair development (Synek et al., 2006). However, stem growth in some of the mutants with root hair phenotype is not altered and therefore, *exo70a1* and *sec15b-1* phenotypes might be a result of compromised formation of the cell plate or of general cell expansion. Both EXO70A1 and SEC15B GFP tagged proteins were shown to localize to the cell plate (Fendrych et al., 2010).

Defect in the establishment or specification and growth of lateral inflorescence stems has been previously described in *exo70a1* mutant (Synek et al., 2006) and *sec15b-1* creates more axillary branches, which sometimes overgrow the main stem. Similarly to the formation of lateral roots, this might be partially explained by modified sensitivity of both mutants to different growth regulators and their transport.

Interestingly, *sec15b-1* mutant forms spontaneous necrotic lesions on leaves, which might indicate defects in autophagy. Similar phenotype was already observed in the case of *exo70b1-2* mutant. In addition, *exo70b1* has altered trafficking of anthocyanins into vacuoles, which results in different pigmentation of mutant rosette (Kulich et al., 2013). The colour of *sec15b-1* rosette leaves differs from wild type in later sporophytic development. Therefore, I hypothesize that EXO70B1 and SEC15B cooperate during vesicular trafficking directed to vacuole. Next, *sec15b-1* mutant will be crossed into autophagic marker line expressing ATG8f-RFP and dynamics of autophagosomal bodies will be observed to confirm defects of secretory pathway related to the autophagy. Subsequently, nitrogen and carbon starvation of *sec15b* will be analysed, because *exo70b1* is known to be hypersensitive to the lack of nitrogen, which might be a consequence of progress in hypersensitive reaction.

1.3.5. Tip growth

Characteristic phenotype of several knocked-out (LOF) mutants in exocyst subunits is a defective tip growth (Synek et al., 2006; Hála et al., 2008; Cole et al., 2005; Wen et al., 2005). In plants, tip growth is an important type of cell expansion linked to the extremely polarized secretion expecially in root hairs (defective in *Arabidopsis exo70a1* and maize *sec3*) and pollen tubes (altered in *sec8, sec5, sec6* and *sec15a*). Based on expression data, I hypothesized that pollen tube maturation and growth might not be affected in *sec15b* LOF due to the abundance of SEC15A in the pollen, whereas initiation and growth of root hairs might be affected in *sec15b* LOF mutant

due to low expression of SEC15A. I have observed slightly affected elongation of root hairs, but the data set is not complete yet and observations are not shown in this thesis. Unexpectedly, *sec15b* pollen tube growth was negatively affected in comparison to the wild type phenotype indicating that despite abundance of SEC15A also SEC15B is important.

Reduction of *sec15b* pollen tube growth and altered morphology is a very interesting aspect, which potentially indicates partial non-redundancy of *SEC15A* and *SEC15B* genes in male gametophyte. Pollen tubes are known to be the fastest growing cell type in plants highly dependent on effectiveness of vesicular trafficking. Therefore, every deviation from the optimal balance potentially results in slower kinetics of growth. Growth of pollen tube might be negatively influenced by decreased activity of any of both SEC15 exocyst subunits. In addition, SEC15A and SEC15B potentially interact with distinct partners or SEC15A and SEC15B differ in strength of these interactions. This hypothesis will be further investigated using complementation test. LOF mutant in the RAB A4d GTPase has been shown to affect pollen tube growth in similar negative manner (Szumlanski and Nielsen, 2009) as SEC15A proteins do and thus this or related RAB GTPases might be a potential interactor of one of the subunits or both.

1.3.6. Some of the mutant characteristics are similar to the defective auxin action phenotype

A lot of characteristics remind mutants in phytohormonal signaling. Induction of axillary branches, apical dominance, and length of primary root and establishment of lateral roots are typically connected with altered levels of auxin, gibberellic acid and strigolactones. Recently, it has been shown that exocyst is able to influence localization of PIN1 and PIN2 via proper recycling (Drdová et al., 2013) and thus auxin (and possibly some other phytohormones) is potentially functionally linked with exocyst function. Exocyst potentially directs polarized secretion of other membrane carriers and thus affects indirectly transport of several phytohormones.

Feature	*sec15b1* phenotype	WT	Significance of difference	*sec15b2* phenotype	WT	Significance of difference	The other exocyst mutants with similar defect	Citation
Seed size	Length: 1.05±0.08 mm Width: 0.055±0.038 mm	Length: 0.92±0.07 mm Width: 0.527±0.04 mm	* *	Length: 1.036±0.08 mm Width: 0.536±0.06mm	Length:1±0.64 mm Width: 0.54±0.05 mm	* n.s.	- -	- -
Thickness of seed coat	0.054±0.05 µm	0.14±0.05 µm	*	0.19±0.04 µm	0.16±0.04 µm	*	*exo70a1, sec8*	Kulich et al., 2010
Length of etiolated hypocotyl	0.752±0.34 cm	1.750±0.22 cm	*	1.067±0.57 cm	1.778±0.35 cm	*	*exo70a1*	Hála et al., 2008; Synek et al., 2006
Primary root length	2.432±0.53 cm	2.735±0.45 cm	*	2.339±0.52 cm	2.351±0.48 cm	n.s.	*exo70a1*	Synek et al., 2006
Number of lateral roots	2.33 of lateral roots	1.42 of lateral roots	*	1.9 of lateral roots	1.8 of lateral roots	*	*exo70a1*	Synek et al., 2006
Diameter of rosette	3.613±0.82 cm	4.218±0.71 cm	*	5.343±0.97 cm	5.39±0.81 cm	n.s.	*exo70a1*	Synek et al., 2006
Length of stem	13.583±2.94 cm	20.351±4.93 cm	*	29.882±4.1 cm	28.65±4.3 cm	n.s.	*exo70a1*	Synek et al., 2006
Number of lateral shoots	2.89 of axillary buds	0.522 of axillary buds	*	2.06 of axillary buds	2.31 of axillary buds	n.s.	*exo70a1*	Synek et al., 2006
Pollen tube length	0.25±0.3 cm	0.56±0.49 cm	*	0.5±0.53 cm	0.9±0.69 cm	*	*sec 5, sec6, sec8, sec15a*	Synek et al., 2006; Hála et al., 2008; Cole et al., 2005

Table 13: Characteristics of *sec15b-1* and *sec15b-2* mutant lines in comparison with other exocyst subunits, the number expresses average and standard deviation (*=significant difference, n.s.=non-significant)

2. Complementation test indicates functional redundancy between SEC15A and SEC15B

In general, high copy exocyst subunits are not redundant in all aspects (Kulich et al., 2015; Pečenková et al., 2011; Kulich et al., 2013), whereas majority of low copy subunits can be complemented by other isoforms resulting in no phenotypic changes of e. g. single *sec5a* and *sec5b* mutants (Hála et al., 2008). Additionally, mutants in single copy subunits were not recovered suggesting lethality of mutated lines (Cole et al., 2005; Hála et al., 2008).

EXO70 subunit of exocyst started to evolve new paralogues concomitantly with the plant terrestrialization (Cvrčková et al., 2012), which diverged from original three basal paralogues (Synek et al., 2006) into a large family of proteins with unique functions in new developmental and stress contexts. EXO70A1 was determined as the most related paralogue of EXO70 clade to other eukaryots and its mutation affects all sporophytic tissues (Synek et al., 2006). In contrast, *exo70b2* and *exo70h1* do not show any phenotypic deviation under standard conditions, but both mutants are more sensitive to bacterial pathogen attack (Pečenková et al., 2011). Additionally, EXO70B1 paralogue is functionally specialized to mediate autophagy pathway and *exo70b1* mutant exhibits ectopic hypersensitive response resulting in spontaneous necrotic leaves lesions (Kulich et al., 2013). Interestingly, EXO70H4 plays an important role during cell wall maturation of trichomes (Kulich et al., 2015).

Two SEC10 and two SEC5 paralogues in *Arabidopsis thaliana* have been shown to be redundant (Vukašinović et al., 2014; Hála et al., 2008). SEC10 isoforms differ in substitution of five amino acid residues and insertion/deletion of four amino acid residues only. In addition, *sec10a* and *sec10b* mutants do not differ from wild type plants, which indicates complete functional redundancy of SEC10A and SEC10B (Vukašinović et al., 2014). SEC5A and SEC5B were suggested to be functionally redundant due to no transmission defects of single mutants (Hála et al., 2008). SEC15 is present in Arabidopsis genome in two copies only and therefore, this subunit was expected to show functional redundancy.

As expected, *sec15b-1* mutant line was complemented by all of the constructs carrying any combination of *SEC15A* and *SEC15B* promotor and gene. This was observed in case of seed coats, elongation of etiolated hypocotyls and formation of double collet, which supports the hypothesis of SEC15 functional redundancy in sporophyte. However, it might be interesting to analyse redundancy in pollen tubes, the male gametophyte, because of defects in both *sec15a* and *sec15b* mutant, which is a topic for future research.

SEC15A and *SEC15B* transcripts are differentially expressed throughout plant tissues (Figure 29). Generally, *SEC15A* is predominantly expressed in stamen and pollen and shoot apex, whereas *SEC15B* dominates in other sporophytic tissues. Interestingly, expression pattern strongly differs during biotic and abiotic stresses and thus it seems that SEC15A and SEC15B are not redundant under all conditions. Suprisingly, expression very much differs also between different accessions (see above). This again might be interpreted as an indication of large functional redundancy.

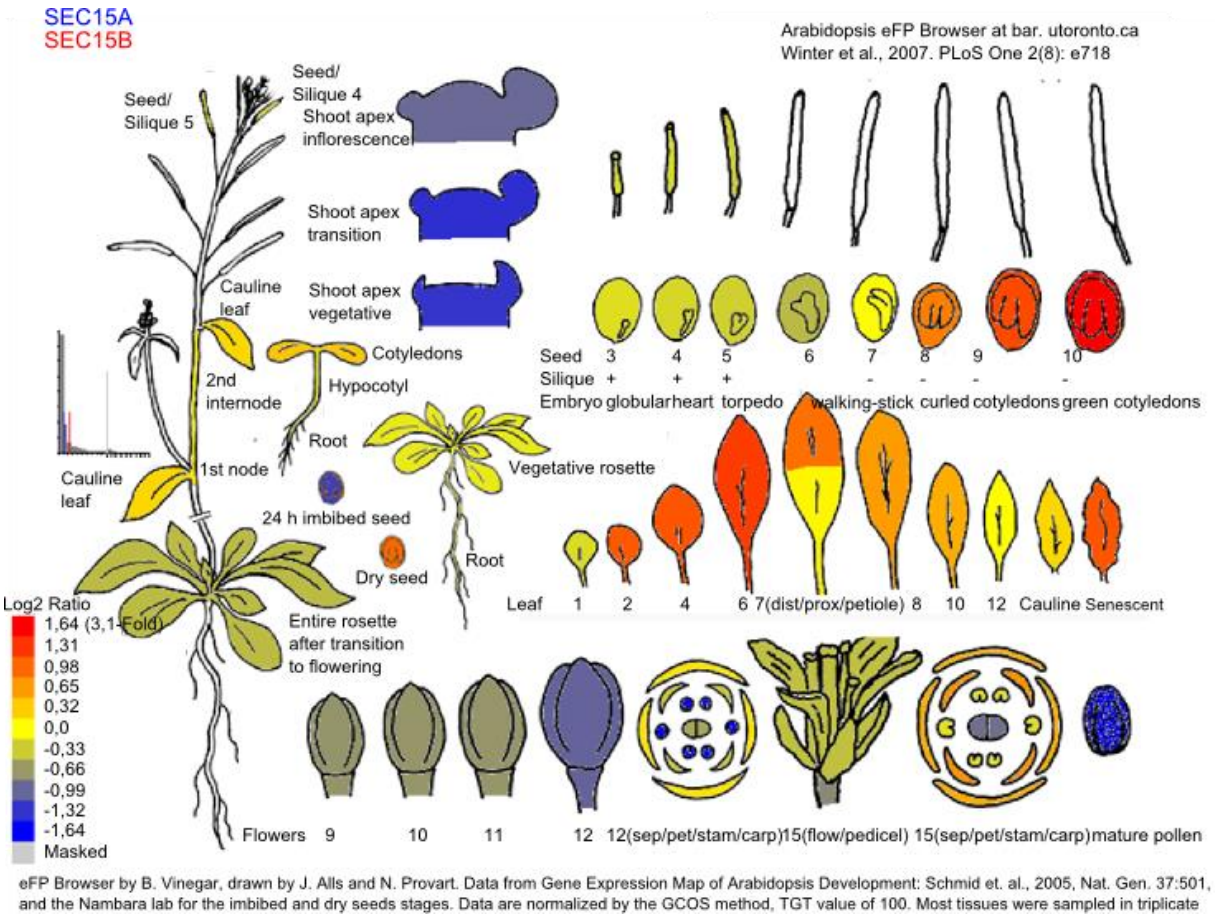

Figure 29: Expression pattern of *SEC15B* throughout sporophytic tissues during distinct developmental processes and pollen tube (Schmid et al., 2005; Winter et al., 2007).

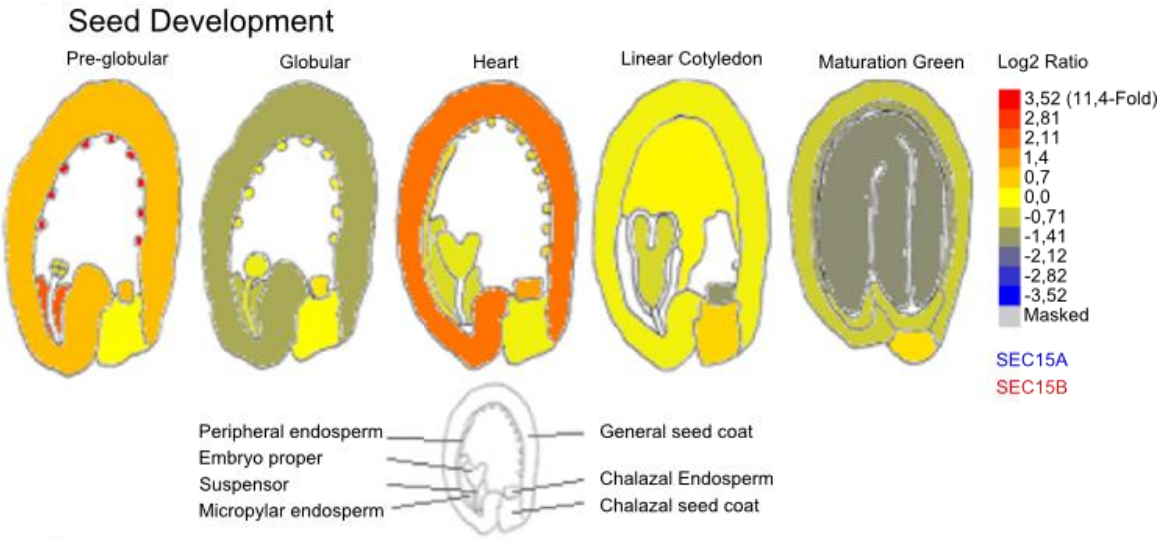

Figure 30: Transcriptional activity of *SEC15A* and *SEC15B* during seed development (Le et al., 2010).

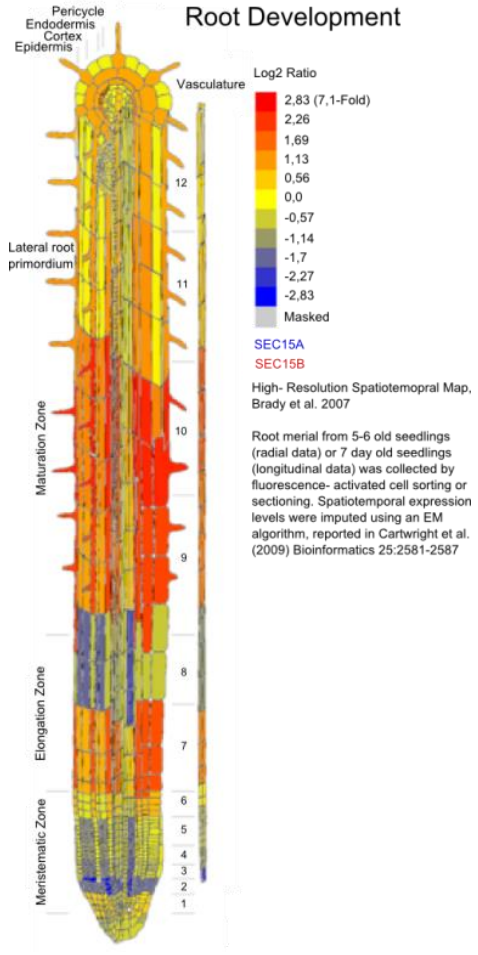

Figure 31: Spatiotemporal resolution of *SEC15A* and *SEC15B* expression patterns in *Arabidopsis* root (Brady et al., 2007).

It is obvious that levels of SEC15A and SEC15B transcripts show differential expression during embryogenesis (Figure 30) and root development (Figure 31). This might indicate that distinct isoforms are preferred in different tissues or processes. For instance, expression of SEC15A is slightly enriched in meristematic zone of root and shoot apex, which suggests possible specific role of SEC15A during cytokinesis, although SEC15B was already shown to localize into cell plate (Fendrych et al., 2010). On the other hand, these data concern only mRNA level and nothing is known about levels of both corresponding proteins. This is also another topic for the future research.

3. The role of SEC15B on a molecular level

Yeast and mammalian Sec15 mediates protein secretion via interaction with different parts of secretory machinery. Plant SEC15B most likely participates in protein secretion, but the exact role remains unclear.

Previously, SEC15B was unsuccessfully tested in interaction with Rab GTPase using yeast two hybrid method (Hála; unpublished data). Therefore, the goal was to confirm or disprove this result by pull down assay and to investigate whether SEC15B interacts directly with membrane lipids, although this interaction was not observed in any organism.

Possible models of the plant SEC15B functions:

- Binds through conserved interaction with Rab GTPase (and potentially other interactors)
- Binds directly to signaling lipids typical for secretory vesicle as some other exocyst subunits
- Binds through an adaptor protein to a signaling molecule typical for secretory vesicle
- The mechanism of SEC15B function is different

Above, I show that SEC15B exocyst subunit does not interact with membranes, but most likely mediates secretion in a conserved manner via interaction with Rab GTPase.

3.1. SEC15B might not interact with membrane lipids

Yeast or mammalian Sec15 was not shown to directly interact with specific membrane lipids. However, study of yeast Sec15p showed that the protein is hardly soluble and has a tendency to occur in pellet fraction. Therefore, Salminen et al. even speculated about a possible direct interaction of Sec15p with lipids during the first experiments (Salminen and Novick, 1989), but this hypothesis was never confirmed. With this, plant SEC15B protein expressed in bacterial cells is nearly insoluble and shows high level of instability. We hypothesized that the protein might interact with membranes.

SEC15B protein was purified using 0.5% sarcosyl, which is commonly used detergent (Frankel et al., 1991), and subsequently analysed by PIP Strip binding assay. In this test, SEC15B showed a specific interaction with signaling lipids. However, following more natural LUV method did not confirmed this result. Both, PIP Strip and LUV are widely used to investigate interaction between proteins and lipids, but LUV analysis is considered to be more reliable due to the creation of whole vesicles instead of flat lipid surface as used in PIP Strips. Commonly, PIP Strips are used as an indication of interaction between proteins and lipids with following confirmation and refinement of the interaction with candidate lipids. Therefore, positive result obtained by PIP Strip with negative result revealed by LUVs is confusing and the interaction cannot be considered as real. However, it was previously shown that C terminal domain of mammalian Exo70 exocyst subunit interacts with lipids specifically, whereas N terminal domain interacts with all tested lipids (He et al., 2007b). Therefore, we hypothesized that this might be a case of SEC15B as well. Unfortunately, N terminal part of SEC15B is extremely unstable and cannot be used for further analysis. C terminal part of SEC15B appears to be stable, but it behaves in different manner from full length in pull down assays (data not shown).

SEC15B full-length purified without sarcosyl did not show any interaction with phospholipids using PIP Strip and LUV analysis showed non-specific interaction with all tested lipids and additionally, the protein itself without any addition of lipids was found in the pellet fraction. In future, other methods of interaction analysis between proteins and lipids could be applied on SEC15B. But based on preliminary results presented here and due to the evolutionary conserved function of Sec15, which seem not to interact with lipids in other eukaryots, SEC15B most probably does not interact with phospholipids.

3.2. SEC15B might interact with RAB A4a

In the introduction, I have shown that interaction between Rab GTPases and Sec15 exocyst subunit is highly conserved from yeast and fruit fly to mammals. In several multicellular organisms, Sec15 is able to bind certain Rab GTPases related to the exocytosis and membrane recycling. In accordance with the conservation of this ancient pathway present possibly in LECA (Elias, 2008), there is every reason to believe that plant SEC15 conserves the fundamental interactors connections.

Fruit fly C terminal domain of Sec15 protein was crystalized to further understand mechanism of Rab-Sec15 interaction. Sec15 reveals no structural homology to the other studied helical domains that serves as effectors for Rab GTPases. A single helix α9 from Sec15 C terminal domain was shown to be involved in binding of Rab11. Using site- direct mutagenesis, Phe667, Gln664, Met663 and Asn659 were shown to mediate the link between Sec15 and Rab11 (Wu et al., 2005).

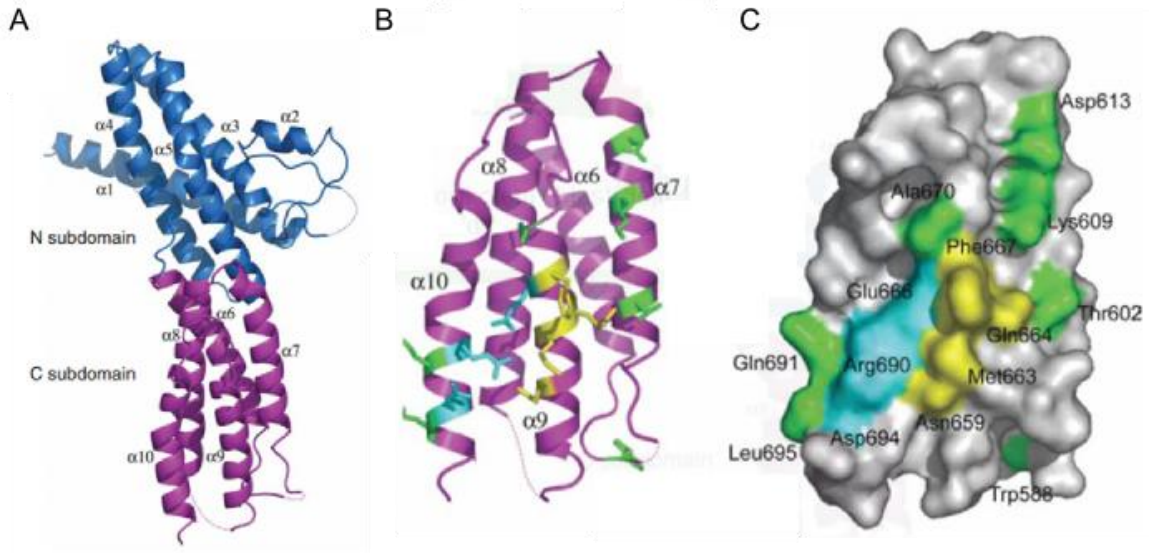

Figure 32: (A) Crystal structure of C terminal part of fruit fly Sec15 protein (B) C subdomain of C terminal part with highlighted residues. Residues participating in interaction with Rab11 are marked yellow, green colour labels tested residues that are not involved in interaction with Rab11, cyan colour represents residues creating a network of salt links (C) (Wu et al., 2005).

Neither *Arabidopsis* SEC15B nor SEC15A complemented yeast temperature-sensitive *sec15* mutant (Růžičková; unpublished data). This indicates that both *Arabidopsis* SEC15 isoforms potentially bind the other interactors in slightly different manner as compared to yeast. Bioinformatic analysis of the RAB-SEC15 interaction shows that the key amino acids of SEC15B binding Rab GTPase are not conserved (Potocký, unpublished data). Therefore, interaction between Sec15 and Rab GTPase itself is highly conserved, but the mechanism of binding differs depending on the evolution context.

In animals, Sec15 was shown to interact with Rab11 and Rab8 (Feng et al., 2012; Zhang et al., 2004) and fruit fly Sec15 C terminal domain binds four exocytic Rabs, Rab11, Rab3, Rab 8 and Rab27 (Wu et al., 2005). This indicates that plant SEC15B protein might also interact with more than one secretory RAB GTPase. Secretory RabA1 to RabA6 subclades are homologues of mammalian Rab11 and Rab25 and therefore, SEC15B potentially interacts with any GTPase of RAB A clade (Rutherford and Moore, 2002). It was already shown that in *exo70a1* PIN2 is enriched in abnormally enlarged compartments, which are RAB A5d positive (Drdová et al., 2013) and therefore I hypothesize that RAB A5d might physically bind SEC15B. Similarly, RabE clade is most related to mammalian Rab8 and Rab10 and sustains post-Golgi trafficking (Rutherford and Moore, 2002), which might imply another potential link with the exocyst. Once SEC15B mediates autophagy, RAB F1, RAB F2 and RAB G3, which control traffic to the vacuole, can be other potential interactional partners of SEC15B.

Single mutation of most plant RAB GTPases does not cause any visible phenotype on a level of a whole plant. Interestingly, FT-IR analysis of single gene knockouts of several RAB A GTPases revealed changes in composition of cell wall. Mutants belonging to the RAB A1, RAB A2 and RAB A4 subclades show altered ratio between secretion of pectin, cellulose and hemicellulose (Lunn et al., 2013). Therefore, *sec15a* or *sec15b* mutants might exhibit changes in cell wall composition as well and this speculation is supported by altered composition of seed coat.

As SEC15A complements phenotype of *sec15b* mutant, SEC15A should bind Rab GTPase as well. Due to the redundancy of plant secretory RAB GTPases, SEC15A potentially binds a slightly different spectrum of RABs. This hypothesis needs to be further tested. However, this could explain distinct expression patterns of SEC15A and SEC15B during different biotic or abiotic stresses.

Interaction between SEC15B and RAB A4a was identified by a single method and might be artificial. SEC15B must be tested whether it binds RAB A4a in GTP-dependent manner. This could be investigated using GTP- and GDP-locked form of RAB A4a or loading wild type RAB A4a with GDPβS and GTPγS, which are non-hydrolysable analogues of GTP and GDP, respectively. Some other analyses must be used to confirm this interaction. The best method to prove the physical link might be bimolecular fluorescence complementation (BiFC) or Förster resonance energy transfer (FRET), which are methods that determine interactions *in planta.*

Conclusions

Most of the aims of this thesis were fullfiled and based on the obtained data, I conclude:

- Two mutant lines of *sec15b* mutant were tested in characteristics typical for the other exocyst mutants. Although some mutant features exhibit high level of plasticity, both, *sec15b-1* and *sec15b-2* behave in similar manner, which is mostly consistent with phenotypic characteristics of the other exocyst mutants. *sec15b-1* has been determined as a stronger allele and reveals defects in formation of seed coat, elongation of etiolated hypocotyl, growth of stem and primary root, establishment of axillary buds and lateral roots, diameter of rosette and unexpectedly, growth of pollen tube.
- *sec15b-1* mutant was successfully complemented by construct carrying *SEC15B* gene under *SEC15B* promotor.
- Functional redundancy of SEC15A and SEC15B was confirmed using complementation test.
- SEC15B protein was expressed and followingly tested in interaction with lipids and a Rab GTPase protein. Using PIP Strip analysis, SEC15B was shown that it interacts with lipids. In contrast, RAB A4a was identified as a potential interactor of SEC15B.

In the close future, following experiments should be done for further understanding the role of the plant SEC15B:

- Analyse seed characteristics of more generations; investigate, in which conditions the root phenotype is stable; further measure length and study morphology of pollen tubes, explain relation of mutant with autophagy defects, connect mutant phenotype with phytohormonal signaling, focus on root hair analysis.
- Prepare fluorescent protein tagged SEC15B and complement *sec15b-1* mutant phenotype with this construct.
- Test functional relation between SEC15A and SEC15B in pollen tubes using complementation approach.
- Further test binding of SEC15B and RAB A4a in GTP-dependent manner and use another methods to confirm this interaction.
- Test other Rab GTPases in interaction with SEC15B.

References

Arabidopsis Interactome Mapping Consortium, 2011. Evidence for network evolution in an Arabidopsis interactome map. Science 333, 601–607. doi:10.1126/science.1203877

Avisar, D., Prokhnevsky, A.I., Makarova, K.S., Koonin, E.V., Dolja, V.V., 2008. Myosin XI-K Is Required for Rapid Trafficking of Golgi Stacks, Peroxisomes, and Mitochondria in Leaf Cells of Nicotiana benthamiana. Plant Physiol. 146, 1098–1108. doi:10.1104/pp.107.113647

Awasthi, S., Palmer, R., Castro, M., Mobarak, C.D., Ruby, S.W., 2001. New roles for the Snp1 and Exo84 proteins in yeast pre-mRNA splicing. J. Biol. Chem. 276, 31004–31015. doi:10.1074/jbc.M100022200

Bischoff, F., Molendijk, A., Rajendrakumar, C.S., Palme, K., 1999. GTP-binding proteins in plants. Cell. Mol. Life Sci. CMLS 55, 233–256.

Bloch, D., Hazak, O., Lavy, M., Yalovsky, S., 2008. A novel ROP/RAC GTPase effector integrates plant cell form and pattern formation. Plant Signal. Behav. 3, 41–43.

Bowser, R., Müller, H., Govindan, B., Novick, P., 1992. Sec8p and Sec15p are components of a plasma membrane-associated 19.5S particle that may function downstream of Sec4p to control exocytosis. J. Cell Biol. 118, 1041–1056. doi:10.1083/jcb.118.5.1041

Bowser, R., Novick, P., 1991. Sec15 protein, an essential component of the exocytotic apparatus, is associated with the plasma membrane and with a soluble 19.5S particle. J. Cell Biol. 112, 1117–1131. doi:10.1083/jcb.112.6.1117

Brady, S.M., Orlando, D.A., Lee, J.-Y., Wang, J.Y., Koch, J., Dinneny, J.R., Mace, D., Ohler, U., Benfey, P.N., 2007. A High-Resolution Root Spatiotemporal Map Reveals Dominant Expression Patterns. Science 318, 801–806. doi:10.1126/science.1146265

Chen, S., Li, L., Li, J., Liu, B., Zhu, X., Zheng, L., Zhang, R., Xu, T., 2014. SEC-10 and RAB-10 coordinate basolateral recycling of clathrin-independent cargo through endosomal tubules in Caenorhabditis elegans. Proc. Natl. Acad. Sci. 111, 15432–15437. doi:10.1073/pnas.1408327111

Chen, Y.A., Scheller, R.H., 2001. SNARE-mediated membrane fusion. Nat. Rev. Mol. Cell Biol. 2, 98–106. doi:10.1038/35052017

Chia, P.Z.C., Gleeson, P.A., 2014. Membrane tethering. F1000Prime Rep. 6. doi:10.12703/P6-74

Chiba, S., Amagai, Y., Homma, Y., Fukuda, M., Mizuno, K., 2013. NDR2-mediated Rabin8 phosphorylation is crucial for ciliogenesis by switching binding specificity from phosphatidylserine to Sec15. EMBO J. 32, 874–885. doi:10.1038/emboj.2013.32

Chong, Y.T., Gidda, S.K., Sanford, C., Parkinson, J., Mullen, R.T., Goring, D.R., 2010. Characterization of the Arabidopsis thaliana exocyst complex gene families by phylogenetic, expression profiling, and subcellular localization studies. New Phytol. 185, 401–419. doi:10.1111/j.1469-8137.2009.03070.x

Cole, R.A., McInally, S.A., Fowler, J.E., 2014. Developmentally distinct activities of the exocyst enable rapid cell elongation and determine meristem size during primary root growth in Arabidopsis. BMC Plant Biol. 14, 386. doi:10.1186/s12870-014-0386-0

Cole, R.A., Synek, L., Zarsky, V., Fowler, J.E., 2005. SEC8, a subunit of the putative Arabidopsis exocyst complex, facilitates pollen germination and competitive pollen tube growth. Plant Physiol. 138, 2005–2018. doi:10.1104/pp.105.062273

Cvrčková, F., Grunt, M., Bezvoda, R., Hála, M., Kulich, I., Rawat, A., Žárský, V., 2012. Evolution of the Land Plant Exocyst Complexes. Front. Plant Sci. 3. doi:10.3389/fpls.2012.00159

Das, A., Gajendra, S., Falenta, K., Oudin, M.J., Peschard, P., Feng, S., Wu, B., Marshall, C.J., Doherty, P., Guo, W., Lalli, G., 2014. RalA promotes a direct exocyst–Par6 interaction to regulate polarity in neuronal development. J. Cell Sci. 127, 686–699. doi:10.1242/jcs.145037

de Leeuw, H.P., Fernandez-Borja, M., Reits, E.A., Romani de Wit, T., Wijers-Koster, P.M., Hordijk, P.L., Neefjes, J., van Mourik, J.A., Voorberg, J., 2001. Small GTP-binding protein Ral modulates regulated exocytosis of von Willebrand factor by endothelial cells. Arterioscler. Thromb. Vasc. Biol. 21, 899–904.

Dellago, H., Löscher, M., Ajuh, P., Ryder, U., Kaisermayer, C., Grillari-Voglauer, R., Fortschegger, K., Gross, S., Gstraunthaler, A., Borth, N., Eisenhaber, F., Lamond, A.I., Grillari, J., 2011. Exo70, a subunit of the exocyst complex, interacts with SNEVhPrp19/hPso4 and is involved in pre-mRNA splicing. Biochem. J. 438, 81–91. doi:10.1042/BJ20110183

Drakakaki, G., Dandekar, A., 2013. Protein secretion: how many secretory routes does a plant cell have? Plant Sci. Int. J. Exp. Plant Biol. 203-204, 74–78. doi:10.1016/j.plantsci.2012.12.017

Drdová, E.J., Synek, L., Pečenková, T., Hála, M., Kulich, I., Fowler, J.E., Murphy, A.S., Žárský, V., 2013. The exocyst complex contributes to PIN auxin efflux carrier recycling and polar auxin transport in Arabidopsis. Plant J. 73, 709–719. doi:10.1111/tpj.12074

Elias, M., 2008. The guanine nucleotide exchange factors Sec2 and PRONE: candidate synapomorphies for the Opisthokonta and the Archaeplastida. Mol. Biol. Evol. 25, 1526–1529. doi:10.1093/molbev/msn113

Elkind, N.B., Walch-Solimena, C., Novick, P.J., 2000. The role of the COOH terminus of Sec2p in the transport of post-Golgi vesicles. J. Cell Biol. 149, 95–110.

Fang, W., Wang, Z., Cui, R., Li, J., Li, Y., 2012. Maternal control of seed size by EOD3/CYP78A6 in Arabidopsis thaliana. Plant J. 70, 929–939. doi:10.1111/j.1365-313X.2012.04907.x

Fendrych, M., Synek, L., Pečenková, T., Drdová, E.J., Sekeres, J., de Rycke, R., Nowack, M.K., Zársky, V., 2013. Visualization of the exocyst complex dynamics at the plasma membrane of Arabidopsis thaliana. Mol. Biol. Cell 24, 510–520. doi:10.1091/mbc.E12-06-0492

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. doi:10.1105/tpc.110.074351

Feng, S., Knödler, A., Ren, J., Zhang, J., Zhang, X., Hong, Y., Huang, S., Peränen, J., Guo, W., 2012. A Rab8 Guanine Nucleotide Exchange Factor-Effector Interaction Network Regulates Primary Ciliogenesis. J. Biol. Chem. 287, 15602–15609. doi:10.1074/jbc.M111.333245

Fielding, A.B., Schonteich, E., Matheson, J., Wilson, G., Yu, X., Hickson, G.R.X., Srivastava, S., Baldwin, S.A., Prekeris, R., Gould, G.W., 2005. Rab11-FIP3 and FIP4 interact with Arf6 and the exocyst to control membrane traffic in cytokinesis. EMBO J. 24, 3389–3399. doi:10.1038/sj.emboj.7600803

Frankel, S., Sohn, R., Leinwand, L., 1991. The use of sarkosyl in generating soluble protein after bacterial expression. Proc. Natl. Acad. Sci. 88, 1192–1196. doi:10.1073/pnas.88.4.1192

Fukai, S., Matern, H.T., Jagath, J.R., Scheller, R.H., Brunger, A.T., 2003. Structural basis of the interaction between RalA and Sec5, a subunit of the sec6/8 complex. EMBO J. 22, 3267–3278. doi:10.1093/emboj/cdg329

Giot, L., Bader, J.S., Brouwer, C., Chaudhuri, A., Kuang, B., Li, Y., Hao, Y.L., Ooi, C.E., Godwin, B., Vitols, E., Vijayadamodar, G., Pochart, P., Machineni, H., Welsh, M., Kong, Y., Zerhusen, B., Malcolm, R., Varrone, Z., Collis, A., Minto, M., Burgess, S., McDaniel, L., Stimpson, E., Spriggs, F., Williams, J., Neurath, K., Ioime, N., Agee, M., Voss, E., Furtak, K., Renzulli, R., Aanensen, N., Carrolla, S., Bickelhaupt, E., Lazovatsky, Y., DaSilva, A., Zhong, J., Stanyon, C.A., Finley, R.L., White, K.P., Braverman, M., Jarvie, T., Gold, S., Leach, M., Knight, J., Shimkets, R.A., McKenna, M.P., Chant, J., Rothberg, J.M., 2003. A protein interaction map of Drosophila melanogaster. Science 302, 1727–1736. doi:10.1126/science.1090289

Guo, W., Grant, A., Novick, P., 1999a. Exo84p Is an Exocyst Protein Essential for Secretion. J. Biol. Chem. 274, 23558–23564. doi:10.1074/jbc.274.33.23558

Guo, W., Roth, D., Walch-Solimena, C., Novick, P., 1999b. The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. EMBO J. 18, 1071–1080. doi:10.1093/emboj/18.4.1071

Guo, W., Tamanoi, F., Novick, P., 2001. Spatial regulation of the exocyst complex by Rho1 GTPase. Nat. Cell Biol. 3, 353–360. doi:10.1038/35070029

Hála, M., Cole, R., Synek, L., Drdová, E., Pečenková, T., Nordheim, A., Lamkemeyer, T., Madlung, J., Hochholdinger, F., Fowler, J.E., Zárský, V., 2008. An exocyst complex functions in plant cell growth in Arabidopsis and tobacco. Plant Cell 20, 1330–1345. doi:10.1105/tpc.108.059105

Hashimoto, K., Igarashi, H., Mano, S., Takenaka, C., Shiina, T., Yamaguchi, M., Demura, T., Nishimura, M., Shimmen, T., Yokota, E., 2008. An isoform of Arabidopsis myosin XI interacts with small GTPases in its C-terminal tail region. J. Exp. Bot. 59, 3523–3531. doi:10.1093/jxb/ern202

Hazelett, C.C., Yeaman, C., 2012. Sec5 and Exo84 Mediate Distinct Aspects of RalA-Dependent Cell Polarization. PLoS ONE 7. doi:10.1371/journal.pone.0039602

He, B., Xi, F., Zhang, X., Zhang, J., Guo, W., 2007a. Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. EMBO J. 26, 4053–4065. doi:10.1038/sj.emboj.7601834

Inoue, M., Chang, L., Hwang, J., Chiang, S.-H., Saltiel, A.R., 2003. The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. Nature 422, 629–633. doi:10.1038/nature01533

Jedd, G., Mulholland, J., Segev, N., 1997. Two New Ypt GTPases Are Required for Exit From the Yeast trans-Golgi Compartment. J. Cell Biol. 137, 563–580.

Jin, R., Junutula, J.R., Matern, H.T., Ervin, K.E., Scheller, R.H., Brunger, A.T., 2005. Exo84 and Sec5 are competitive regulatory Sec6/8 effectors to the RalA GTPase. EMBO J. 24, 2064–2074. doi:10.1038/sj.emboj.7600699

Jin, Y., Sultana, A., Gandhi, P., Franklin, E., Hamamoto, S., Khan, A.R., Munson, M., Schekman, R., Weisman, L.S., 2011. Myosin V transports secretory vesicles via a Rab GTPase cascade and interaction with the exocyst complex. Dev. Cell 21, 1156–1170. doi:10.1016/j.devcel.2011.10.009

Jiu, Y., Jin, C., Liu, Y., Holmberg, C.I., Jäntti, J., 2012. Exocyst Subunits Exo70 and Exo84 Cooperate with Small GTPases to Regulate Behavior and Endocytic Trafficking in C. elegans. PLoS ONE 7, e32077. doi:10.1371/journal.pone.0032077

Johnston, G.C., Prendergast, J.A., Singer, R.A., 1991. The Saccharomyces cerevisiae MYO2 gene encodes an essential myosin for vectorial transport of vesicles. J. Cell Biol. 113, 539–551.

Kost, B., 2008. Spatial control of Rho (Rac-Rop) signaling in tip-growing plant cells. Trends Cell Biol. 18, 119–127. doi:10.1016/j.tcb.2008.01.003

Kulich, I., Cole, R., Drdová, E., Cvrcková, F., Soukup, A., Fowler, J., Zárský, V., 2010. Arabidopsis exocyst subunits SEC8 and EXO70A1 and exocyst interactor ROH1 are involved in the localized deposition of seed coat pectin. New Phytol. 188, 615–625. doi:10.1111/j.1469-8137.2010.03372.x

Kulich, I., Pečenková, T., Sekereš, J., Smetana, O., Fendrych, M., Foissner, I., Höftberger, M., Zárský, V., 2013. Arabidopsis exocyst subcomplex containing subunit EXO70B1 is involved in autophagy-related transport to the vacuole. Traffic Cph. Den. 14, 1155–1165. doi:10.1111/tra.12101

Kulich, I., Vojtíková, Z., Glanc, M., Ortmannová, J., Rasmann, S., Žárský, V., 2015. Cell wall maturation of Arabidopsis trichomes is dependent on exocyst subunit EXO70H4 and involves callose deposition. Plant Physiol. 168, 120–131. doi:10.1104/pp.15.00112

Laflamme, C., Assaker, G., Ramel, D., Dorn, J.F., She, D., Maddox, P.S., Emery, G., 2012. Evi5 promotes collective cell migration through its Rab-GAP activity. J. Cell Biol. 198, 57–67. doi:10.1083/jcb.201112114

Lavy, M., Bloch, D., Hazak, O., Gutman, I., Poraty, L., Sorek, N., Sternberg, H., Yalovsky, S., 2007. A Novel ROP/RAC effector links cell polarity, root-meristem maintenance, and vesicle trafficking. Curr. Biol. CB 17, 947–952. doi:10.1016/j.cub.2007.04.038

Le, B.H., Cheng, C., Bui, A.Q., Wagmaister, J.A., Henry, K.F., Pelletier, J., Kwong, L., Belmonte, M., Kirkbride, R., Horvath, S., Drews, G.N., Fischer, R.L., Okamuro, J.K., Harada, J.J., Goldberg, R.B., 2010. Global analysis of gene activity during Arabidopsis seed development and identification of seed-specific transcription factors. Proc. Natl. Acad. Sci. 107, 8063–8070. doi:10.1073/pnas.1003530107

Lempe, J., Balasubramanian, S., Sureshkumar, S., Singh, A., Schmid, M., Weigel, D., 2005. Diversity of Flowering Responses in Wild Arabidopsis thaliana Strains. PLOS Genet 1, e6. doi:10.1371/journal.pgen.0010006

Li, B.X., Satoh, A.K., Ready, D.F., 2007. Myosin V, Rab11, and dRip11 direct apical secretion and cellular morphogenesis in developing Drosophila photoreceptors. J. Cell Biol. 177, 659–669. doi:10.1083/jcb.200610157

Lipatova, Z., Tokarev, A.A., Jin, Y., Mulholland, J., Weisman, L.S., Segev, N., 2008. Direct Interaction between a Myosin V Motor and the Rab GTPases Ypt31/32 Is Required for Polarized Secretion. Mol. Biol. Cell 19, 4177–4187. doi:10.1091/mbc.E08-02-0220

Lipka, V., Kwon, C., Panstruga, R., 2007. SNARE-ware: the role of SNARE-domain proteins in plant biology. Annu. Rev. Cell Dev. Biol. 23, 147–174. doi:10.1146/annurev.cellbio.23.090506.123529

Li, S., Chen, M., Yu, D., Ren, S., Sun, S., Liu, L., Ketelaar, T., Emons, A.-M.C., Liu, C.-M., 2013. EXO70A1-mediated vesicle trafficking is critical for tracheary element development in Arabidopsis. Plant Cell 25, 1774–1786. doi:10.1105/tpc.113.112144

Li, S., Gu, Y., Yan, A., Lord, E., Yang, Z.-B., 2008. RIP1 (ROP Interactive Partner 1)/ICR1 marks pollen germination sites and may act in the ROP1 pathway in the control of polarized pollen growth. Mol. Plant 1, 1021–1035. doi:10.1093/mp/ssn051

Liu, J., Guo, W., 2012. The exocyst complex in exocytosis and cell migration. Protoplasma 249, 587–597. doi:10.1007/s00709-011-0330-1

Lunn, D., Gaddipati, S.R., Tucker, G.A., Lycett, G.W., 2013. Null Mutants of Individual RABA Genes Impact the Proportion of Different Cell Wall Components in Stem Tissue of Arabidopsis thaliana. PLOS ONE 8, e75724. doi:10.1371/journal.pone.0075724

Luo, G., Zhang, J., Luca, F.C., Guo, W., 2013. Mitotic phosphorylation of Exo84 disrupts exocyst assembly and arrests cell growth. J. Cell Biol. 202, 97–111. doi:10.1083/jcb.201211093

Matsuda, N., Nakano, A., 1998. RMA1, an Arabidopsis thaliana gene whose cDNA suppresses the yeast sec15 mutation, encodes a novel protein with a RING finger motif and a membrane anchor. Plant Cell Physiol. 39, 545–554.

Matsuda, N., Suzuki, T., Tanaka, K., Nakano, A., 2001. Rma1, a novel type of RING finger protein conserved from Arabidopsis to human, is a membrane-bound ubiquitin ligase. J. Cell Sci. 114, 1949–1957.

Medkova, M., France, Y.E., Coleman, J., Novick, P., 2006. The rab exchange factor Sec2p reversibly associates with the exocyst. Mol. Biol. Cell 17, 2757–2769. doi:10.1091/mbc.E05-10-0917

Mizuno-Yamasaki, E., Medkova, M., Coleman, J., Novick, P., 2010. Phosphatidylinositol 4-Phosphate Controls Both Membrane Recruitment and a Regulatory Switch of the Rab GEF Sec2p. Dev. Cell 18, 828–840. doi:10.1016/j.devcel.2010.03.016

Morgera, F., Sallah, M.R., Dubuke, M.L., Gandhi, P., Brewer, D.N., Carr, C.M., Munson, M., 2012. Regulation of exocytosis by the exocyst subunit Sec6 and the SM protein Sec1. Mol. Biol. Cell 23, 337–346. doi:10.1091/mbc.E11-08-0670

Moskalenko, S., Henry, D.O., Rosse, C., Mirey, G., Camonis, J.H., White, M.A., 2002. The exocyst is a Ral effector complex. Nat. Cell Biol. 4, 66–72. doi:10.1038/ncb728

Moskalenko, S., Tong, C., Rosse, C., Mirey, G., Formstecher, E., Daviet, L., Camonis, J., White, M.A., 2003. Ral GTPases Regulate Exocyst Assembly through Dual Subunit Interactions. J. Biol. Chem. 278, 51743–51748. doi:10.1074/jbc.M308702200

Nair, J., Müller, H., Peterson, M., Novick, P., 1990. Sec2 protein contains a coiled-coil domain essential for vesicular transport and a dispensable carboxy terminal domain. J. Cell Biol. 110, 1897–1909. doi:10.1083/jcb.110.6.1897

Nakano, K., Toya, M., Yoneda, A., Asami, Y., Yamashita, A., Kamasawa, N., Osumi, M., Yamamoto, M., 2011. Pob1 Ensures Cylindrical Cell Shape by Coupling Two Distinct Rho Signaling Events During Secretory Vesicle Targeting. Traffic 12, 726–739. doi:10.1111/j.1600-0854.2011.01190.x

Neto, H., Balmer, G., Gould, G., 2013. Exocyst proteins in cytokinesis. Commun. Integr. Biol. 6. doi:10.4161/cib.27635

Novick, P., Field, C., Schekman, R., 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 21, 205–215. doi:10.1016/0092-8674(80)90128-2

Oda, Y., Iida, Y., Nagashima, Y., Sugiyama, Y., Fukuda, H., 2015. Novel coiled-coil proteins regulate exocyst association with cortical microtubules in xylem cells via the conserved oligomeric golgi-complex 2 protein. Plant Cell Physiol. 56, 277–286. doi:10.1093/pcp/pcu197

Ortiz, D., Medkova, M., Walch-Solimena, C., Novick, P., 2002. Ypt32 recruits the Sec4p guanine nucleotide exchange factor, Sec2p, to secretory vesicles; evidence for a Rab cascade in yeast. J. Cell Biol. 157, 1005–1015. doi:10.1083/jcb.200201003

Pečenková, T., Hála, M., Kulich, I., Kocourková, D., Drdová, E., Fendrych, M., Toupalová, H., Zársky, V., 2011. The role for the exocyst complex subunits Exo70B2 and Exo70H1 in the plant-pathogen interaction. J. Exp. Bot. 62, 2107–2116. doi:10.1093/jxb/erq402

Peremyslov, V.V., Prokhnevsky, A.I., Avisar, D., Dolja, V.V., 2008. Two Class XI Myosins Function in Organelle Trafficking and Root Hair Development in Arabidopsis. Plant Physiol. 146, 1109–1116. doi:10.1104/pp.107.113654

Pérez, P., Portales, E., Santos, B., 2015. Rho4 interaction with exocyst and septins regulates cell separation in fission yeast. Microbiol. Read. Engl. 161, 948–959. doi:10.1099/mic.0.000062

Porat-Shliom, N., Milberg, O., Masedunskas, A., Weigert, R., 2013. Multiple roles for the actin cytoskeleton during regulated exocytosis. Cell. Mol. Life Sci. CMLS 70, 2099–2121. doi:10.1007/s00018-012-1156-5

Prigent, M., Dubois, T., Raposo, G., Derrien, V., Tenza, D., Rossé, C., Camonis, J., Chavrier, P., 2003. ARF6 controls post-endocytic recycling through its downstream exocyst complex effector. J. Cell Biol. 163, 1111–1121. doi:10.1083/jcb.200305029

Robinson, N.G., Guo, L., Imai, J., Toh-E, A., Matsui, Y., Tamanoi, F., 1999. Rho3 of Saccharomyces cerevisiae, which regulates the actin cytoskeleton and exocytosis, is a GTPase which interacts with Myo2 and Exo70. Mol. Cell. Biol. 19, 3580–3587.

Rojo, E., Denecke, J., 2008. What Is Moving in the Secretory Pathway of Plants? Plant Physiol. 147, 1493–1503. doi:10.1104/pp.108.124552

Roland, J.T., Bryant, D.M., Datta, A., Itzen, A., Mostov, K.E., Goldenring, J.R., 2011. Rab GTPase–Myo5B complexes control membrane recycling and epithelial polarization. Proc. Natl. Acad. Sci. 108, 2789–2794. doi:10.1073/pnas.1010754108

Roland, J.T., Lapierre, L.A., Goldenring, J.R., 2009. Alternative Splicing in Class V Myosins Determines Association with Rab10. J. Biol. Chem. 284, 1213–1223. doi:10.1074/jbc.M805957200

Romagnoli, S., Cai, G., Faleri, C., Yokota, E., Shimmen, T., Cresti, M., 2007. Microtubule- and actin filament-dependent motors are distributed on pollen tube mitochondria and contribute differently to their movement. Plant Cell Physiol. 48, 345–361. doi:10.1093/pcp/pcm001

Rutherford, S., Moore, I., 2002. The Arabidopsis Rab GTPase family: another enigma variation. Curr. Opin. Plant Biol. 5, 518–528.

Safavian, D., Zayed, Y., Indriolo, E., Chapman, L., Ahmed, A., Goring, D., 2015. RNA silencing of exocyst genes in the stigma impairs the acceptance of compatible pollen in Arabidopsis. Plant Physiol. doi:10.1104/pp.15.00635

Saito, C., Ueda, T., 2009. Chapter 4: functions of RAB and SNARE proteins in plant life. Int. Rev. Cell Mol. Biol. 274, 183–233. doi:10.1016/S1937-6448(08)02004-2

Salminen, A., Novick, P.J., 1989. The Sec15 protein responds to the function of the GTP binding protein, Sec4, to control vesicular traffic in yeast. J. Cell Biol. 109, 1023–1036. doi:10.1083/jcb.109.3.1023

Salminen, A., Novick, P.J., 1987. A ras-like protein is required for a post-Golgi event in yeast secretion. Cell 49, 527–538. doi:10.1016/0092-8674(87)90455-7

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D., Lohmann, J.U., 2005. A gene expression map of Arabidopsis thaliana development. Nat. Genet. 37, 501–506. doi:10.1038/ng1543

Shipitsin, M., Feig, L.A., 2004. RalA but Not RalB Enhances Polarized Delivery of Membrane Proteins to the Basolateral Surface of Epithelial Cells. Mol. Cell. Biol. 24, 5746–5756. doi:10.1128/MCB.24.13.5746-5756.2004

Sivaram, M.V.S., Furgason, M.L.M., Brewer, D.N., Munson, M., 2006. The structure of the exocyst subunit Sec6p defines a conserved architecture with diverse roles. Nat. Struct. Mol. Biol. 13, 555–556. doi:10.1038/nsmb1096

Songer, J.A., Munson, M., 2009. Sec6p Anchors the Assembled Exocyst Complex at Sites of Secretion. Mol. Biol. Cell 20, 973–982. doi:10.1091/mbc.E08-09-0968

Stalder, D., Mizuno-Yamasaki, E., Ghassemian, M., Novick, P.J., 2013. Phosphorylation of the Rab exchange factor Sec2p directs a switch in regulatory binding partners. Proc. Natl. Acad. Sci. U. S. A. 110, 19995–20002. doi:10.1073/pnas.1320029110

Stalder, D., Novick, P.J., 2016. The casein kinases Yck1p and Yck2p act in the secretory pathway, in part, by regulating the Rab exchange factor Sec2p. Mol. Biol. Cell 27, 686–701. doi:10.1091/mbc.E15-09-0651

Sugihara, K., Asano, S., Tanaka, K., Iwamatsu, A., Okawa, K., Ohta, Y., 2002. The exocyst complex binds the small GTPase RalA to mediate filopodia formation. Nat. Cell Biol. 4, 73–78. doi:10.1038/ncb720

Synek, L., Schlager, N., Eliáš, M., Quentin, M., Hauser, M.-T., Žárský, V., 2006. AtEXO70A1, a member of a family of putative exocyst subunits specifically expanded in land plants, is important for polar growth and plant development. Plant J. 48, 54–72. doi:10.1111/j.1365-313X.2006.02854.x

Szumlanski, A.L., Nielsen, E., 2009. The Rab GTPase RabA4d Regulates Pollen Tube Tip Growth in Arabidopsis thaliana. Plant Cell 21, 526–544. doi:10.1105/tpc.108.060277

Takahashi, K., Hayashi, K., Kinoshita, T., 2012. Auxin activates the plasma membrane H+-ATPase by phosphorylation during hypocotyl elongation in Arabidopsis. Plant Physiol. 159, 632–641. doi:10.1104/pp.112.196428

TerBush, D.R., Maurice, T., Roth, D., Novick, P., 1996. The Exocyst is a multiprotein complex required for exocytosis in Saccharomyces cerevisiae. EMBO J. 15, 6483–6494.

TerBush, D.R., Novick, P., 1995. Sec6, Sec8, and Sec15 are components of a multisubunit complex which localizes to small bud tips in Saccharomyces cerevisiae. J. Cell Biol. 130, 299–312.

Toikkanen, J.H., Miller, K.J., Söderlund, H., Jäntti, J., Keränen, S., 2003. The beta subunit of the Sec61p endoplasmic reticulum translocon interacts with the exocyst complex in Saccharomyces cerevisiae. J. Biol. Chem. 278, 20946–20953. doi:10.1074/jbc.M213111200

Vaškovičová, K., Žárský, V., Rösel, D., Nikolič, M., Buccione, R., Cvrčková, F., Brábek, J., 2013. Invasive cells in animals and plants: searching for LECA machineries in later eukaryotic life. Biol. Direct 8, 8. doi:10.1186/1745-6150-8-8

Vitale, N., Chasserot-Golaz, S., Bailly, Y., Morinaga, N., Frohman, M.A., Bader, M.-F., 2002. Calcium-regulated exocytosis of dense-core vesicles requires the activation of ADP-ribosylation factor (ARF)6 by ARF nucleotide binding site opener at the plasma membrane. J. Cell Biol. 159, 79–89. doi:10.1083/jcb.200203027

Vukašinović, N., Cvrčková, F., Eliáš, M., Cole, R., Fowler, J.E., Žárský, V., Synek, L., 2014. Dissecting a hidden gene duplication: the Arabidopsis thaliana SEC10 locus. PloS One 9, e94077. doi:10.1371/journal.pone.0094077

Wang, N., Wang, M., Zhu, Y.-H., Grosel, T.W., Sun, D., Kudryashov, D.S., Wu, J.-Q., 2015. The Rho-GEF Gef3 interacts with the septin complex and activates the GTPase Rho4 during fission yeast cytokinesis. Mol. Biol. Cell 26, 238–255. doi:10.1091/mbc.E14-07-1196

Wen, T.-J., Hochholdinger, F., Sauer, M., Bruce, W., Schnable, P.S., 2005. The roothairless1 Gene of Maize Encodes a Homolog of sec3, Which Is Involved in Polar Exocytosis. Plant Physiol. 138, 1637–1643. doi:10.1104/pp.105.062174

Westlake, C.J., Junutula, J.R., Simon, G.C., Pilli, M., Prekeris, R., Scheller, R.H., Jackson, P.K., Eldridge, A.G., 2007. Identification of Rab11 as a small GTPase binding protein for the Evi5 oncogene. Proc. Natl. Acad. Sci. 104, 1236–1241. doi:10.1073/pnas.0610500104

Whyte, J.R.C., Munro, S., 2002. Vesicle tethering complexes in membrane traffic. J. Cell Sci. 115, 2627–2637.

Willett, R., Ungar, D., Lupashin, V., 2013. The Golgi puppet master – COG complex at center stage of membrane trafficking interactions. Histochem. Cell Biol. 140, 271–283. doi:10.1007/s00418-013-1117-6

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., Provart, N.J., 2007. An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets. PLOS ONE 2, e718. doi:10.1371/journal.pone.0000718

Wu, H., Rossi, G., Brennwald, P., 2008. The ghost in the machine: small GTPases as spatial regulators of exocytosis. Trends Cell Biol. 18, 397–404. doi:10.1016/j.tcb.2008.06.007

Wu, J., Tan, X., Wu, C., Cao, K., Li, Y., Bao, Y., 2013. Regulation of Cytokinesis by Exocyst Subunit SEC6 and KEULE in Arabidopsis thaliana. Mol. Plant 6, 1863–1876. doi:10.1093/mp/sst082

Wu, S., Mehta, S.Q., Pichaud, F., Bellen, H.J., Quiocho, F.A., 2005. Sec15 interacts with Rab11 via a novel domain and affects Rab11 localization in vivo. Nat. Struct. Mol. Biol. 12, 879–885. doi:10.1038/nsmb987

Zhang, X., Bi, E., Novick, P., Du, L., Kozminski, K.G., Lipschutz, J.H., Guo, W., 2001. Cdc42 Interacts with the Exocyst and Regulates Polarized Secretion. J. Biol. Chem. 276, 46745–46750. doi:10.1074/jbc.M107464200

Zhang, X.-M., Ellis, S., Sriratana, A., Mitchell, C.A., Rowe, T., 2004. Sec15 Is an Effector for the Rab11 GTPase in Mammalian Cells. J. Biol. Chem. 279, 43027–43034. doi:10.1074/jbc.M402264200

Zhang, X., Orlando, K., He, B., Xi, F., Zhang, J., Zajac, A., Guo, W., 2008. Membrane association and functional regulation of Sec3 by phospholipids and Cdc42. J. Cell Biol. 180, 145–158. doi:10.1083/jcb.200704128

Zhang, Y., Immink, R., Liu, C.-M., Emons, A.M., Ketelaar, T., 2013. The Arabidopsis exocyst subunit SEC3A is essential for embryo development and accumulates in transient puncta at the plasma membrane. New Phytol. 199, 74–88. doi:10.1111/nph.12236