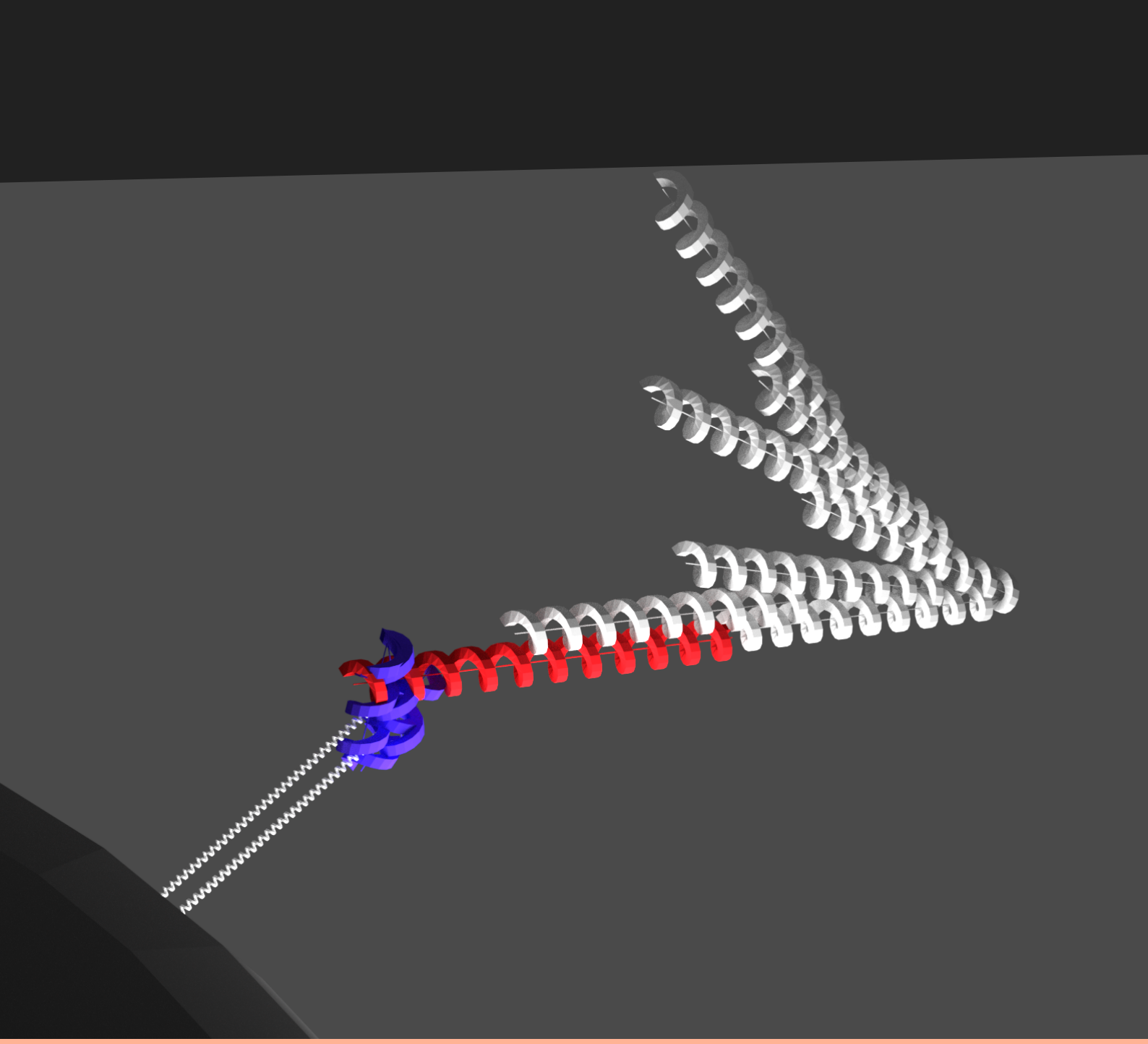

Effectors of RAB GTPases and Their Role in Plant Secretion

Ph.D. Thesis

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Effectors of RAB GTPases and Their Role in Plant Secretion

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ABSTRACT

Rab GTPases are small signaling molecules that play an important role in vesicle trafficking in eukaryotic cells. Correct signaling through small GTPases allows orchestration of vesicle transport among cellular organelles and also to the cell wall providing cell wall material for cell growth and elongation. Engagement of Rab GTPases in the regulation of endomembrane trafficking is one of the evolutionary conserved aspects of secretion regulation. The network of Rab GTPases interaction includes also various downstream effectors. One of them is the exocyst complex involved in vesicle docking at the plasma membrane. It is a complex composed of eight different subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84). Exocyst was discovered as Sec4p Rab GTPase effector in yeast and also data from animal models describe the Sec15 exocyst subunit as the Rab-interacting partner, but data from plants are missing. On the other hand, numerous studies identified exocyst role in tip growth of pollen tube and root hairs, seed coat formation, cell plate and cell wall formation, hypocotyl elongation, and importantly also PIN auxin efflux carriers recycling and polar auxin transport. There are two paralogues of SEC15 in the *Arabidopsis* genome, SEC15a and SEC15b, the previous one already shown to be important for polarized pollen tube growth.

In this thesis, we first test the hypothesis of conservation of RAB-exocyst interaction in *Arabidopis thaliana*. Using *in vitro* and *in vivo* techniques, we were able to show interaction of SEC15b with RAB GTPases from the RAB-A4 subgroup. Our experimental data suggest an intriguing possibility that RAB GTPases from the RAB-A4 subgroup are not redundant in respect to the interaction with exocyst.

The exocyst complex was proven to be important for hypocotyl elongation, thus we used etiolated *Arabidopsis* hypocotyl, a flexible connection between root and cotyledons, as a model system. Morphological, anatomical and cytological analyses of *Arabidopsis* mutants in several exocyst subunits, including SEC15, showed formation of a discrete region on the etiolated hypocotyl near the root-hypocotyl junction, overall morphology of which resembles the collet region. The collet region, root-hypocotyl junction, is an important transition zone between different environments. Despite its crucial importance for plant development, little is known about how this transition zone is specified. We also describe and discuss other aspects of the SEC15b mutation in *Arabidopsis* and redundancy of both SEC15 paralogues.

Homozygous *rgtb1-1* mutant plants that are defective in RAB GTPase geranylgeranylation are characteristic by short etiolated hypocotyls with irregular cell pattern and heavy starch accumulation. To address this phenomenon, we show that etiolated hypocotyls upon isoxaben treatment generally react on distortion of the cell wall expansion on saccharides-containing media by allocation of sugars in the form of starch accumulation. We also used different mutant lines that are defective in cellular transport showing very similar phenotype to wild-type plants treated with isoxaben. Moreover, we discovered that there is a switch mechanism redirecting the sink of internal sugars from the cell wall synthesis to starch accumulation.

At the end, we also shortly discuss potential of Rab GTPases as targets of biotechnologies.

Rab GTPázy jsou malé signální molekuly, které hrají důležitou roli ve váčkovém transportu. Jejich správné fungování umožňuje regulaci váčkového transportu mezi buněčnými organelami a také směrem do buněčné stěny, kdy je zdrojem materiálu pro růst a prodlužování buněk. Zapojení Rab GTPáz v regulaci andomembránového transportu je jeden z evolučně velmi konzervovaných aspektů řízení a kontroly sekrece. Mezi interaktory Rab GTPáz patří také různé 'downstream' efektory. Jedním z nich je komplex exocyst, který je nejvíce známý pro své zapojení do váčkového transportu na plazmatické membráně. Tento komplex je složen z osmi různých podjednotek (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) a byl objeven jako efektor Sec4p Rab GTPázy v kvasinkách. Dostupné informace z živočišných modelových organismů uvádějí SEC15 podjednotku jako podjednotku která interaguje s Rab GTPázami. Jaká je situace v rostlinách není dosud známo. Početné studie uvádějí důležitou funkci komplexu exocyst v 'tip growth' (vrcholový růst) pylových láček a kořenových vlásků, ve vytváření semenných obalů a také ve tvorbě buněčné přepážky, buněčné stěny a prodlužování hypokotylu. Také je známo zapojení komplexu exocyst v recyklaci auxinových přenašečů - PIN proteinů. V genomu *Arabidopsis* můžeme nalézt dva paralogy SEC15 podjednotky označované jako SEC15a a SEC15b z nichž, jak již bylo dříve ukázáno, SEC15a podjednotka je důležitá pro polární růst pylové láčky.

V předkládané práci, jsme se nejdříve soustředili na konzervovanost interakce RAB GTPáz s komplexem exocyst v rostlině *Arabidopsis thaliana*. Použitím *in vitro* a in *vivo* metod, jsme ukázali interakci SEC15b podjednotky s RAB GTPázou z RAB-A4 podskupiny. Výsledky našich experimentů ukazují na fascinující možnost, že RAB GTPázy z RAB-A4 podskupiny nejsou v kontextu interakce s exocystem redundantní.

Protože dřívější výsledky ukázaly, že komplex exocyst je důležitý pro prodlužování buněk hypokotylu, které tvoří flexibilní spojení mezi kořenem a kotyledonovými listy, použili jsme tuto část etiolovaných semenáčů *Arabidopsis* jako modelový systém. Morfologické, anatomické a buněčné analýzy mutantů *Arabidopsis* v několika podjednotkách komplexu exocyst, zahrnující také SEC15 podjednotku, odhalily vytvoření odlišitelné části etiolovaného hypokotylu blízko rozhraní podzemní a nadzemní části rostliny. Morfologicky se tento odlišný region podobal právě tomuto druhému rozhraní, které je důležitou přechodovou zónou mezi odlišnými prostředími a navzdory jeho klíčovému významu pro vývoj rostlin se málo ví o tom, jak je tato přechodová zóna determinována. Dále jsme také popsali a diskutovali další aspekty mutace SEC15b genu u *Arabidopsis* a redundanci obou SEC15 paralogů.

Homozygotní mutantní rostliny *rgtb1-1*, které jsou defektivní v enzymu RAB geranylgeranyláze jsou charakteristické krátkými etiolovanými hypokotyly s nepravidelným buněčným uspořádáním a akumulací škrobu. Pro uchopení tohoto jevu jsme použili etiolované hypokotyly *Arabidopsis* divokého typu (WT) opůsobené isoxabenem. Rostliny pěstované na médiu se sacharózou reagovaly na porušení buněčné stěny alokací cukrů a jejich uložením ve formě škrobu. Dále jsme také použili různé mutantní linie defektní v buněčném transportu, které vykazovaly stejný fenotyp jako WT rostliny opůsobené isoxabenem. Objevili jsme, že existuje kontrolní mechanismus který přepíná mezi využitím cukrů pro syntézu buněčné stěny a nebo jejich uložením ve formě škrobu.

Na konci celé práce také diskutujeme možnosti použití Rab GTPáz v biotechnologiích.

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At this place I would like to thank all members of the Laboratory of cell biology, Institute of Experimental Botany and the Laboratory of plant cell morphogenesis, Charles University for their help and support on my thesis.

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Závěrem, jedno velmi osobní a velmi speciální poděkování patří mémnu příteli Ondrovi, který můj vědecký i ten všední svět obohatil o LaTeX, přesné informatické myšlení, společenský tanec a dvě krásná města Edinburgh a Glasgow.

Dekuju.

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List of publications

Published review:

'Rab GTPases as potential targets in plant biotechnologies.'

Submitted publication:

'Developmental Plasticity of *Arabidopsis* Hypocotyl is Dependent on Exocyst Complex Function.'

Publications already prepared for the submission:

'Plant Exocyst Complex is an Effector of Small GTPases from RABA4 Class'

'Starch Accumulation in *Arabidopsis* Secretory Mutant Seedlings is a Result of the Cell Wall Biogenesis Inhibition.'

Following collaborators contributed to this work

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Declaration

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

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

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.

V Praze 31.3.2017

Contents

INTRODUCTION

1. Introduction

Complexity of eukaryotic cells demands for an efficient delivery of different cargoes from the point of their synthesis to the point of their consumption or storage. Delivery of protein cargoes to the plasma membrane and apoplast is called protein secretion and follows protein synthesis on the endoplasmic reticulum (ER). Because plants achieved multicellularity independently during their evolution, plant cells differ from other eukaryotes by different cellular organization (Umen, 2014). One of the main features of this independent evolution is largely diversified complex of membrane structures between the Golgi apparatus (GA) and the plasma membrane (PM) called Trans Golgi Network (TGN) that also serves as a recycling endosome in plants (Dettmer et al., 2006; Robinson et al., 2008; Woollard and Moore, 2008). Another structure analogous to TGN in the meaning of diversification, can be found between GA and a vacuole called Multivesicular body (MVB). Both of these structures sort exocytosed or endocytosed cargoes on their routes to PM or a vacuole. Despite the existence of these differences, common features of vesicle trafficking systems still can be found. These involve Rab GTPase (RAs related protein in Brain) signaling molecules and their effectors as key players of intracellular (vesicle) trafficking.

Integral proteins of the plasma membrane as well as secreted proteins are synthesized at the endoplasmic reticulum (ER) and are translocated across the ER membrane. All subsequent steps of the transport of molecules are done by vesicular trafficking. Most of the vesicles are moving through GA to TGN. In the plant cell, this is the main sorting station for different secreted cargoes. Recycling of the endocytosed PM proteins happens here too. The vesicles can be further directed to at least four main routes - to endosomes, vacuole, cell plate and plasma membrane (Figure 1.1).

The whole process of vesicular transport starts with vesicle budding driven by a coat assembly, which distorts a membrane of a donor organelle. There are at least three types of proteins that coat the vesicular surface by reversible polymerization called vesicular coatomers. First, vesicles that move from the plasma membrane and TGN compartment toward vacuole are coated with clathrin protein. Second, vesicles from ER to Golgi network have COPI coat. And third, vesicles that mediate retrograde transport from Golgi to ER are coated with COPII coat. The coat assembly is regulated with GTP-binding proteins (Arf, Sar and also Rab proteins).

The next step of vesicular transport is vesicle trafficking during which the created vesicle is

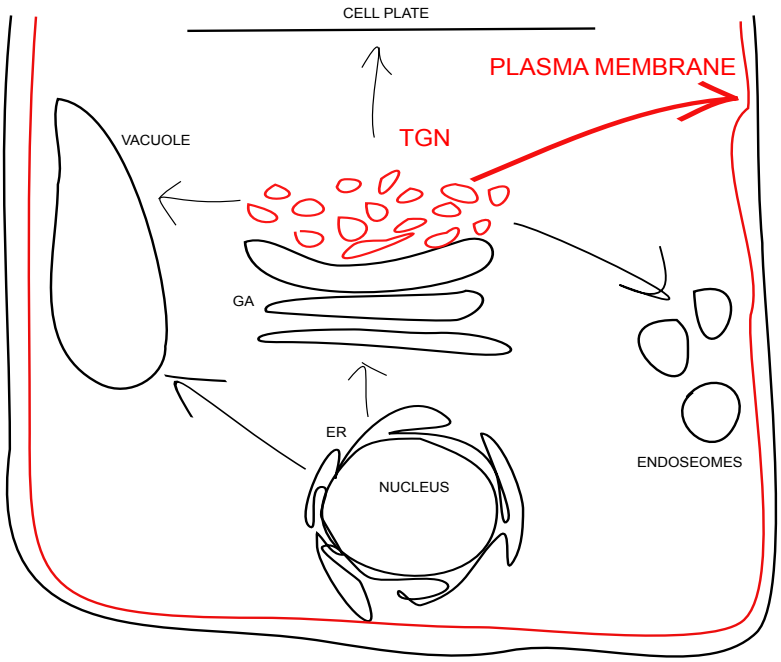

Figure 1.1: Four main routes of secretory vesicle trafficking from the TGN compartment. The proteins are synthesized at the ER. Packed in the vesicles, most of the proteins are transported through GA to TGN. TGN is issued as a main sorting station for the vesicle transport. Secretory vesicles can leave this compartment by four different routes – toward endosome, vacuole, plasma membrane and cell plate in the case of the plant cell division. TGN is also compartment where recycled and endocytosed PM proteins happen too.

transported toward the target membrane. The vesicle delivery is mediated by actin filaments and microtubules (cytoskelet), which facilitate vesicle transport. The necessity for the high specificity of different molecular motors attachments to the cytoskelet is assured by Rab GTPases (Seabra and Coudrier, 2004). Prior to the membrane fusion, the vesicle has to be uncoated, because vesicle coat complexes interfere with the membrane fusion process. In mammals, an example of the regulation of this process by Rab5 GTPase is known (Semerdjieva et al., 2008).

When the vesicle is approximately 40 - 50 nm distant from the target membrane, tethering complexes recruited by Rab GTPases interact with the vesicle and bring it near the target membrane. In the last step the vesicle is fused with the target membrane. This is mediated by N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex.

In this work we focused on the plant secretory pathway. In particular, we studied the penultimate step in this pathway that is the tethering of the secretory vesicle by the multiprotein tethering complex exocyst as a possible effector of Rab GTPases. Especially, we tried to answer the following questions:

1. What is the connection between tethering complex exocyst and secretory vesicle mediated?
2. How does the exocyst complex interact with the plasma membrane?
3. Is there a common secretory phenotype for mutants in different exocyst subunits?
4. Do the secretory mutants have a problem with the hypocotyl elongation and over-accumulation of starch when grown in the dark? Does the decision mechanism of plant ‘to grow or to store’ exist?
5. How do mutants in SEC15b subunit of the exocyst complex look like?

This thesis consists of three manuscripts and one review dealing with different aspects of

RAB GTPases involvement in plant secretion and also cell morphogenesis through the interaction with their effectors. In the first manuscript (chapter 8.1) we describe the exocyst complex as an effector of RAB GTPases. In the second and third manuscript (chapter 9.1 and 9.2) we summarize the phenotypic defects of mutant plant in particular exocyst subunits. In the chapters 8.2 and 9.3, we include yet unpublished results concerning interaction of the exocyst complex with the phospholipids of the plasmatic membrane and mutation in the SEC15b subunit of the exocyst complex. The published review is included in supplements (part IX).

2. Rab GTPases

Rab GTPases are small monomeric G proteins, whose role is regulation of vesicle transport inside a cell. Rab GTPases belong to the Ras protein superfamily, which also contains Ras, Ran, Rho and Arf protein families, that all have the same catalytic activity and structural features but differ in their function inside the cell. As molecular switches, Rab GTPases cycle between two states, GTP-bound and GDP-bound. GTP-bound is considered as active and GDP-bound as inactive (Stenmark et al., 1994). The Rab GTPase's transition from the GDP-bound to the GTP-bound form, requires regulatory proteins that are known as Guanine nucleotide Exchange Factors (GEFs). Once in the GTP-bound form, Rab GTPases are able to interact with their effector proteins (e.g. tethering factors). Even though Rab GTPases are able to hydrolyse GTP, they are not very effective in it (Pan et al., 2006). Therefore, they need another regulatory proteins, GTPase Activating proteins (GAPs), to make the hydrolysis more effective.

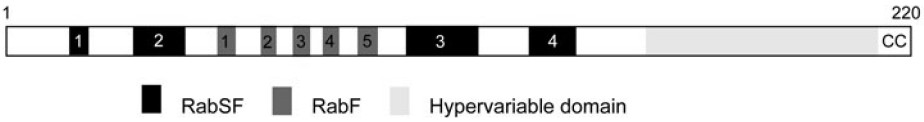

Figure 2.1: Conserved regions in the structure of Rab GTPase gene. RabF - family specific region, RabSF - subfamily specific region, hypervariable domain on the C terminus that is important for the targeting to the specific part of the cell and CC - two cystein residues that undergo posttranslational modification through which Rab GTPase is tethered in the membrane Brighouse et al. (2010).

2.1 Structure of Rab GTPases

Rab GTPases are monomeric proteins of molecular size between 20 to 35 kDa. There are two evolutionary conserved regions recognized in the structure of Rab GTPases (Figure 2.1). One of them is the G region of Rab GTPase that is responsible for binding of guanosine triphosphate (GTP) and hydrolysis of its gamma-phosphate. The mechanism of GTP/GDP binding is evolutionarily conserved - the GTP/GDP binding site creates a pocket with the Switch I and the Switch II domains

(RabF region). This pocket then undergoes conformational changes after GTP binding or hydrolysis (Dumas et al., 1999).

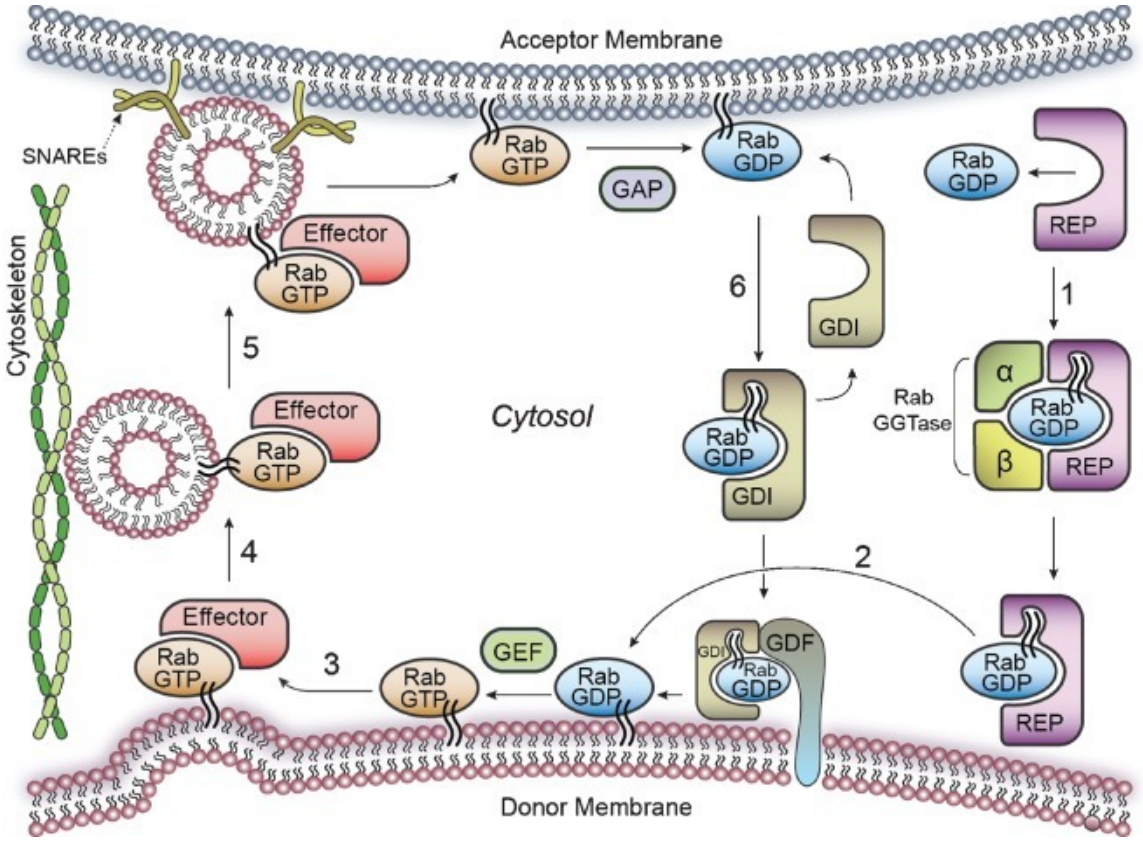

Figure 2.2: Rab GTPase cycle GTP-GDP cycle of Rab GTPases is composed from the subsequent steps. After the synthesis, Rab-GDP protein is bound to the REP protein (step 1.), which can bind the RabGGT enzyme that mediates posttranslational modifications of the C-terminus of Rab GTPase. With this modification, Rab GTPase is able to bind membranes. After this modification, REP protein delivers the Rab GTPase to the donor membrane (step 2.). In the cell, there are specific proteins (GEFs) that activate Rab GTPase and active GTPase can recruit effectors which are responsible for the budding (step 3.), trafficking (step 4.) and tethering (step 5.) of vesicles. During fusion of the vesicle with the acceptor membrane Rab GTPase is inactivated with the help of specific proteins (GAPs) that help to hydrolyze GTP (guanosine triphosphate). GDP-bound Rab is extracted from the acceptor membrane with GDI protein (step 6.), that recycles the Rab GTPase back to the donor membrane and finishes the whole Rab GDP-GTP cycle (Seixas et al., 2013).

The second region, which is conserved across all eukaryotes, is responsible for specific effector binding - RabSF region (Moore et al., 1995). This effector domain, that interacts with GAP proteins (Adari et al., 1988), is important in determining a functional specificity of small GTP-binding proteins (Becker et al., 1991). The conservation of these two regions was confirmed by several complementation studies (Bednarek et al., 1994; Fabry et al., 1995; Haubruck et al., 1990). Another Complementation studies showed that knock-out mutation in yeast Rab GTPase can be complemented by RAB GTPase from evolutionary distant plant *Arabidopsis thaliana* (Pereira-Leal and Seabra, 2001). These experiments together with conserved regions in the structure of Rab GTPases across eukaryotes strongly suggest highly conserved recognition mechanism of interactors, either effectors or general regulators.

Based on the GTP-binding conserved region dominant negative (DN) or constitutively active

(CA) Rab GTPase mutants are broadly used to reveal the actual contribution of each Rab protein toward cell development, differentiation and function in its native tissue (Dalla Via et al., 2017; Satoh et al., 1997; Tsutsui et al., 2015). DN mutants structurally mimic a situation where the GTPase binds the GDP (guanosine diphosphate). GTPase is in the GDP-bound state inactive, which means that is not able to interact with its effector molecules. CA mutants are mutants with structural change for their active state (GTP-bound state). These mutants differ in one concrete amino acid in the GTP/GDP binding pocket. In the case of DN mutant, the difference is that Serine (S) is replaced with Asparagine (N) and in the chase of CA mutant, Glutamine (Q) is replaced with Leucine (L).

The third structural feature of Rab GTPases is a C-terminal hypervariable region (Chavrier et al., 1991) that is important for localization of the Rab protein to the specific cellular compartment. Many eukaryotic proteins are post-translationally modified by lipids attachments. Rab proteins are not the exception. They contain one or two C-terminal cysteine residues that undergo post-translational modifications by covalent attachment of isoprenoid group (C20-geranylgeranyl) via thioether linkage. Geranylgeranylation facilitates Rab membrane association and in some cases, this modification also plays a major role in specific protein-protein interactions (Seabra, 1998). Generally, the geranylgeranylation reaction is mediated by the protein prenyltransferases family that includes: protein farnesyltransferase (FT), protein geranylgeranyl transferase type I (GGT-I) and Rab geranylgeranyl transferase (RabGGT or GGT-II) (Maurer-Stroh et al., 2003)

2.2 GTP-GDP Cycle of GTPases

All previously described conserved regions in the Rab GTPase structure are important for the proper cycling of Rab GTPases (Figure 2.2). The Rab GTPase cycling starts immediately after translation of the Rab protein. After synthesis, the translated Rab GTPase appears in cytosol, it is recognized by the REP protein and these two proteins form a tight complex (Alexandrov et al., 1994; Seabra, 1996; Shen and Seabra, 1996) that serves as a substrate for the Rab geranylgeranyl transferase enzyme (Rab GGT) in the next step.

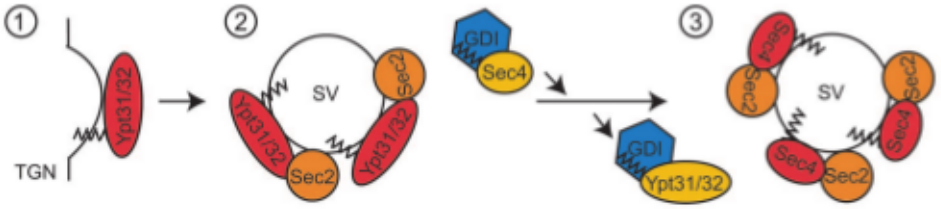

Figure 2.3: An example of the Rab-GEF cascade of Yeast Rab GTPases from TGN toward the plasma membrane (Grosshans et al., 2006).

This interaction results in post-translational modifications of Rab GTPase modifying two cysteine residues on the C-terminus of Rab GTPase by adding two geranylgeranyl moieties (Casey and Seabra, 1996). Then, the Rab-REP complex is separated from Rab GGT and delivered to the donor membrane where it dissociates by the mechanism similar to that of the Rab-GDI (GDP dissociation inhibitor) complex (Alexandrov et al., 1994). Peripheral binding of the Rab GTPase to the donor membrane is mediated by two geranylgeranyl moieties added in the post-translational modification. Thereafter, the REP protein from the complex is recycled for another nascent Rab GTPase.

Once delivered to the donor membrane, Rab GTPases enters the cycling between guanine nucleotide-bound states as well as among membrane compartments. First, the GDP-bound form of Rab GTPase interacts with GEF that catalyzes release of the bound GDP. This GDP is replaced by GTP, which is abundant in the cytosol of the cell. This exchange activates Rab GTPase by transition

to the GTP-bound form. Conformational changes of the Switch I and II domains of the active Rab protein allow Rab GTPase to interact with its effector proteins (for example tethering factors, myosins, proteins of lipid metabolism, etc). In parallel to this interaction, Rab GTPase is transported from the donor membrane to the acceptor membrane. Here, Rab GTPase has to be transformed back to the GDP-bound form, which is achieved by GAP protein action that catalyses hydrolysis of GTP. The Rab protein is then extracted from the acceptor membrane by GDP dissociation inhibitor protein (GDI) that transports Rab GTPase back to the donor membrane through the cytoplasm and completes the whole Rab GTPase cycle. Even though the GDI protein has very similar structure as the REP protein, GDI protein is not able to interact with a Rab GGT enzyme and its affinity is highly increased towards the prenylated Rab protein (Müller and Goody, 2017).

2.3 Rab-GEF and Rab-GAP Cascades

The identity of the membrane compartment and the transport vesicle is mostly determined by the population of proteins on its surface. Progression of the vesicle throughout the secretory pathway is connected with several exchanges of surface proteins. Rab GTPases are believed to be organizers of these exchanges, through functional sequences of Rab proteins, which are called Rab-GEF and Rab-GAP cascades (Rutherford and Moore, 2002). Both cascades initially allow an important step of recruiting a new Rab protein to the specific membrane that initially carried other Rab population.

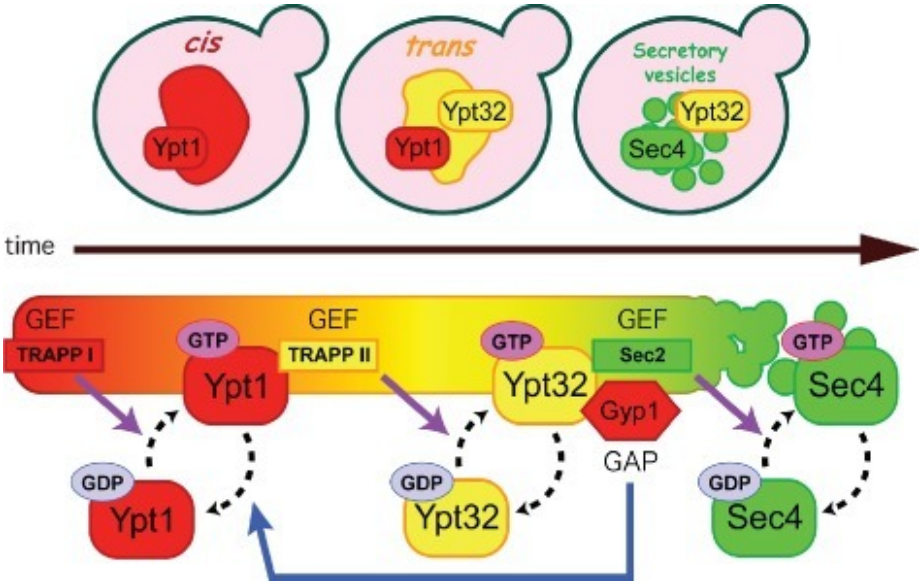

Figure 2.4: Rab GEF cascade with the Gyp1 GAP in budding yeasts. Gyp1 GAP for Ypt1 Rab GTPase is recruited to the Golgi compartment by Ypt32-GTP Rab GTPase. All these GEF and GAP cascades, which means changes of different Rab GTPases, contribute to maturation of specific compartments (Suda and Nakano, 2012).

First, a Rab protein residing on the membrane surface is activated by appropriate GEF protein. Once activated, it recruits other GEF protein to activate a next Rab in the cascade and simultaneously it is inactivated by specific GAP protein. It also triggers exchange of other membrane proteins through their effector interactions. The whole process continues until the secretory vesicles reach the target membrane.

Experimental evidence for such cascades was first brought in yeasts. The first active GTPase in the signaling pathway between TGN and PM membranes are Ypt31p/Ypt32p Rab GTPases. Ypt31p/Ypt32p are localized on the secretory vesicle and recruit the first GEF protein - Sec2p,

which is specific GEF for the subsequent Rab protein Sec4p, that is the second activated Rab protein in the pathway. Simultaneously with the activation of Sec4 Rab GTPase, the previous Rab Ypt31/Ypt32 is inactivated by its specific GAP protein - Gyp1p, which inactivates Ypt31p/Ypt32p by facilitating the GTP hydrolysis. The inactive form of Ypt31/Ypt32 Rab GTPase is than extracted from the membrane by the GDI protein, Sec19p that recycles the Rab GTPase back to the donor membrane. The secretory vesicle continues on its way toward the plasma membrane with the Sec4 Rab GTPase anchored in the membrane. The last step in the pathway is an interaction of the Sec4 Rab GTPase with its effector - the exocyst tethering complex that drags vesicle to the proximity of the PM and provides the vesicle to SNARE proteins, which are responsible for the last step of the vesicle trafficking by mediating a fusion of the vesicle with the PM.

In *Drosophila melanogaster*, there are three exocytic Rab GTPases. These are Rab3, Rab8 and Rab27. Wu et al. (2005) showed that Sec15 subunit of the exocyst complex is able to interact with all of them, although there was a higher preference for the interaction of the Sec15 subunit with the Rab11 GTPase, suggest that Rab11 is the main target. Wu et al. (2005) also located the interaction to C-terminus part of the Sec15 protein. Even more, they created a crystal structure of the C terminal domain of the Sec15 exocyst subunit and mapped Rab11 binding sites by mutagenesis of aminoacids. The situation in plants has not been published yet.

3. Exocyst Complex as Effector of Rab GTPases

Exocyst, a multiprotein tethering complex highly conserved across almost whole eukaryotic kingdom (Heider and Munson, 2012), was firstly discovered as an effector of Rab GTPases in yeasts (TerBush et al., 1996). It operates approximately 40 - 50nm from the plasma membrane. At this distance it is able to catch secretory vesicles and drag them to the closer proximity of the plasma membrane (around 10nm) and simultaneously to the vicinity of SNARE proteins, which mediate fusion of the vesicle with the plasma membrane (Chen and Scheller, 2001). To be able to exert the full role in the cell, exocyst has to perform subsequent steps. It has to create a functional complex in the place of its function and interact with the plasma membrane and with a secretory vesicle. The order of these events is not known yet.

3.1 Structure of the Exocyst Complex

The exocyst complex is a multiprotein complex that consists of eight subunits (Sec3, Sec5, Sec6 Sec8, Sec10, Sec15, Exo70 and Exo84) (Hála et al., 2008; TerBush et al., 1996). Every subunit is indispensable and has its unique place and function in the whole complex. The Exocyst complex belongs to the CATCHR protein family that is known to be conserved across the whole eukaryotic kingdom on the level of the secondary structure. This means that particular subunits are not conserved on the level of the primary structure but they all share very similar secondary and tertiary structure resulting in rod-like conformation. While the exocyst complex is conserved in eukaryotes, individual exocyst subunits underwent subsequent duplications in different species, especially inside the plant kingdom. As a result, there are different numbers of paralogues in different organisms (Figure 3.1).

Although the exocyst complex is considered conserved in the term of the general function, there is an increasing evidence that the interactions between individual exocyst subunits differ. For instance, Heider et al. (2016) created a model of yeast exocyst depicting the subunit connectivity within and between each exocyst module. Consequently, Vukašinovic and Ortmannová (unpublished data) created a similar model for plant exocyst that also shows interactive diversification (Figure 3.2). These results might reflect a different spectrum of interactors on the site of plasma membrane as well as on the site of secretory vesicle. One example of such diversification is the discovery of

Yeast/Human subunit	Plant subunit	Potential *A. thaliana* homolog
Exo70/EXO70	EXO70A1	At5g03540
-II-	EXO70A2	At5g52340
-II-	EXO70A3	At5g52350
-II-	EXO70B1	At5g58430
-II-	EXO70B2	At1g07000
-II-	EXO70C1	At5g13150
-II-	EXO70C2	At5g13990
-II-	EXO70D1	At1g72470
-II-	EXO70D2	At1g54090
-II-	EXO70D3	At3g14090
-II-	EXO70E1	At3g29400
-II-	EXO70E2	At5g61010
-II-	EXO70F1	At5g50380
-II-	EXO70G1	At4g31540
-II-	EXO70G2	At1g51640
-II-	EXO70H1	At3g55150
-II-	EXO70H2	At2g39380
-II-	EXO70H3	At3g09530
-II-	EXO70H4	At3g09520
-II-	EXO70H5	At2g28640
-II-	EXO70H6	At1g07725
-II-	EXO70H7	At5g59730
-II-	EXO70H8	At2g28650
Sec3/SEC3	SEC3a	At1g47550
-II-	SEC3b	At1g47560
Sec5/SEC5	SEC5a	At1g76850
-II-	SEC5b	At1g21170
Sec6/SEC6	SEC6	At1g71820
Sec8/SEC8	SEC8	At3g10380
Sec10/SEC10	SEC10a	At5g12370
-II-	SEC10b	NA
Sec15/SEC15	SEC15a	At3g56640
-II-	SEC15b	At4g02350
Exo84/EXO84	EXO84a	At1g10385
-II-	EXO84b	At5g49830
-II-	EXO84c	At1g10180

Figure 3.1: Exocyst subunits from yeast and human and their possible homologs in *Arabidopsis thaliana*(Vukašinović et al., 2016a).

ROP/RAC GTPase (Rop6) and its interaction with Sec3 subunit through ICR1 protein (Lavy et al., 2007).

Another example of the exocyst diversification is in its overall composition. Even though, Y-shaped structure of the exocyst complex was reported in mammals (Hsu et al., 1998), the structure of the exocyst complex in yeast *Saccharomyces cerevisiae* was shown to be more compact in the work of Heider et al. (2016). Bit, the recent publication of Picco et al. (2017) brought new view on the structure of the exocyst complex. In this publication, the authors used the CryoEM (cryo Electron Microscopy) method for 3D reconstruction of the yeast exocyst complex architecture *in vivo*. Based on this structure, they also proposed model of the exocyst function.

Even though conformation of the exocyst complex in plants is not known yet, we assume that it is different from yeasts and animals.

3.2 Function of the Exocyst Complex

The Exocyst complex plays an important role in the last steps of polar exocytosis and it was implied in the subsequent functions: determination of the site of exocytosis, tethering of the exocytic vesicles, cytokinesis and process of tubulation and mRNA splicing.

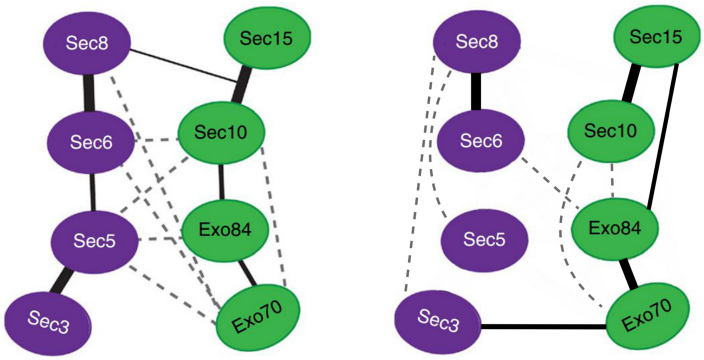

Figure 3.2: Model of connectivity between exocyst subunits and within exocyst module. On the left side is *Saccharomyces cerevisiae*, on the right side is *Arabidopsis thaliana* (Vukašinovič and Ortmannová, unpublished data).

3.2.1 Determination of the Site of Exocytosis

The exocyst complex has to be properly localized on the membrane for execution of its function. There are two subunits, Sec3 and Exo70, that were shown to interact with PI(4,5)P2 phospholipid of the plasma membrane in yeasts (He et al., 2007; Zhang et al., 2008). In yeasts, the main plasma membrane landmark for the exocyst complex is the Sec3 subunit. After Sec3 subunit is bound to the target membrane, the rest of the exocyst complex can bind and create the complete protein complex (Boyd et al., 2004). A question whether the Sec3 subunit has the same plasma membrane landmark role in plants is still unanswered. But the interaction of Sec3 and/or Exo70 subunit with phospholipids is not the sole interaction which targets Sec3 and Exo70 to the plasma membrane. The interaction of N-terminal part of Sec3 with Rho1 and Cdc42 GTPases was shown in yeast (Zhang et al., 2001) and the indirect interaction of Sec3 subunit with ROP/RAC GTPase Rop6 through ICR1 protein was shown in plants (Lavy et al., 2007). These interactions might also contribute to the determination of the site of exocytosis.

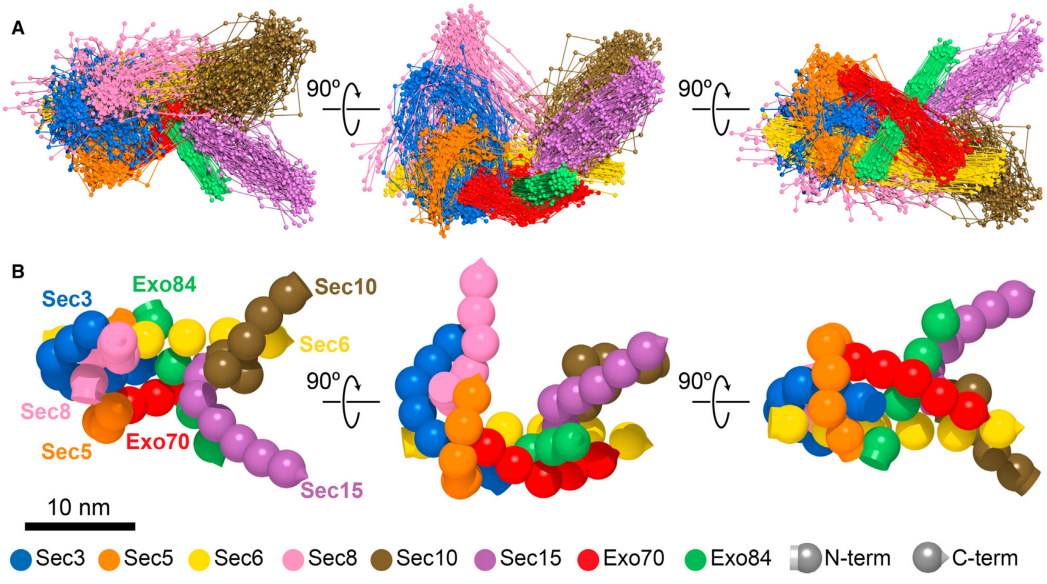

Figure 3.3: 3D structure of the yeast exocyst solved by CryoEM (Picco et al., 2017).

In plants, the Exo70 subunit underwent substantive diversification - there are 23 paralogues in the genome of *Arabidopsis thaliana* (Elias et al., 2003). The purpose of this diversification of Exo70 subunits in *Arabidopsis thaliana* is still not clear. One of the possibilities for different Exo70 paralogues might be their specific function in various plant tissues, where the Exo70 subunits might have different range of interactors (Žárský et al., 2009).

3.2.2 Tethering of the Secretory Vesicles

Tethering of the secretory vesicles is a process of the vesicle attachment to the distance of approximately 40 - 50 nm from the target membrane. The exocyst complex is already bound to the target membrane through the Sec3 and Exo70 subunits. On the other side toward the cytosol, the Sec15 subunit mediates the interaction with the secretory vesicle. This interaction was shown to be mediated by the Rab GTPases in yeasts, fruit flies and mammals (Salminen and Novick, 1989; Wu et al., 2005; Zhang et al., 2004).

It has not been published yet whether this interaction is mediated by Rab GTPases in plants. Also, a sequence of events of vesicle tethering and assembling of the exocyst complex is not known yet. There are two hypotheses. One possibility is that the exocyst subunits are coming together with the tethered vesicle, and then exocyst complex is assembled. The other possibility is that the exocyst complex is composed prior to the interaction with the secretory vesicle.

3.2.3 Cytokinesis and Process of Tubulation

The tubulation of the membranes is important during cytokinesis and cell plate formation, when exocytosis and endocytosis are the main fluxes. The homotypic fusion of vesicles is very important at the beginning of this process. According to new studies, two tethering complexes are necessary for the correct development of the cell plate. The exocyst complex is the first complex seen at the beginning and at the end of cytokinesis. Also the TRAPPII complex is present during this process. The TRAPPII complex is probably activated after the first emergence of the exocyst complex (Fendrych et al., 2010; Rybak et al., 2014).

3.2.4 mRNA Splicing

The exocyst complex has been also implicated in other cellular activities, for example in mRNA splicing. Awasthi et al. (2001) showed the physical association of the Exo84p subunit of the yeast exocyst with the spliceosome and also its involvement in pre-mRNA splicing. Another exocyst subunit that was proved to play a role in the pre-mRNA splicing is Exo70p. Dellago et al. (2011) published that Exo70p might be important for the regulation of alternative splicing, that is the motion force for diversity of a protein function.

3.3 Phenotype of the Plant Exocyst Mutants

The exocyst complex was shown to play a role in a broad range of cellular processes, namely polar cell expansion, cell division, autophagy and signalling between cells. Involvement of the exocyst complex in these processes led to the plenty of mutant phenotypes.

Drdova et al. (2013) and Tan et al. (2016) published results showing involvement of exocyst Exo70A1 and Sec6 subunits in the polar auxin transport via the recycling of PIN1 and PIN2 proteins (auxin efflux carriers). One form of the Exo70 *Arabidopsis* paralogues was implied in the vesicle trafficking required for light-induced stomatal opening (Hong et al., 2016) (Figure 3.4).

Hong et al. (2016) reported retarded light induced opening of stomata in the *exo70B1* mutant. This inhibition of light induced stomata opening is due to the interaction of EXO70B1 with RIC7 protein (ROP-interactive Cdc42- and Rac-interactive binding motif-containing proteins - RICs), which is the downstream factor of ROP2. The ROP2 is one of the 11 known ROP GTPases in

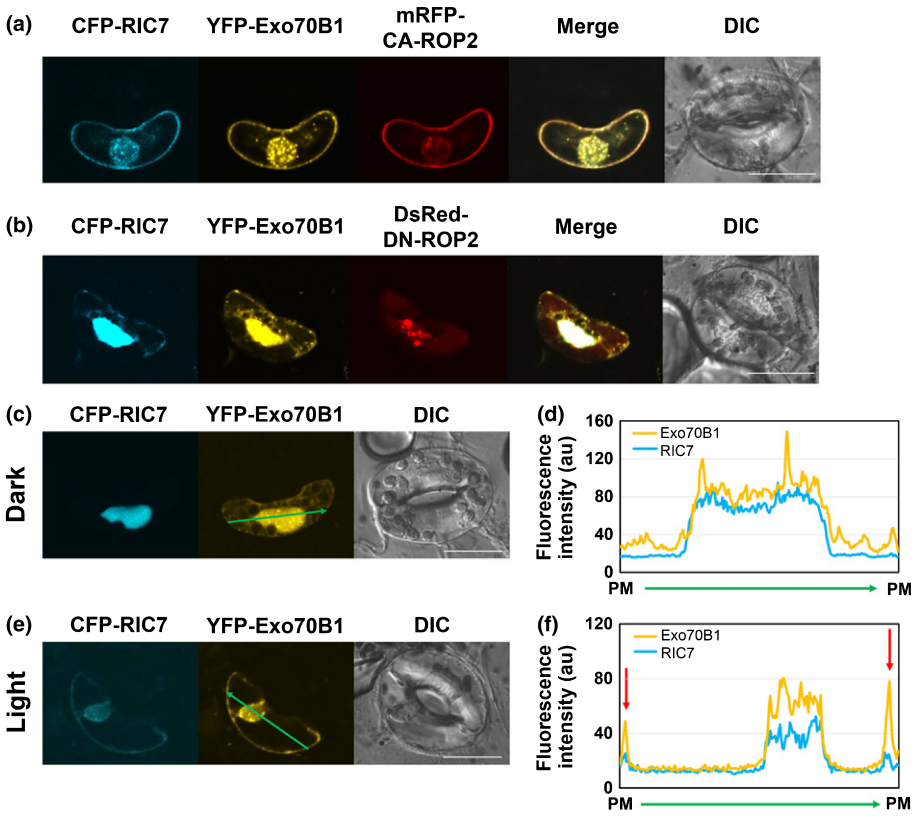

Figure 3.4: The light/dark induced change of the exocyst Exo70B1 subunit localization (Hong et al., 2016).

the *Arabidopsis* genome that were connected with the negative regulation of stomatal movements. Specifically, ROP2 inhibits light-induced stomatal opening and ABA (abscisic acid) induced stomatal closure (Hwang et al., 2011; Jeon et al., 2008).

Hong et al. (2016) also showed very interesting light dependent plasma membrane localization of transiently expressed EXO70B1 in the guard cells of *Vicia faba*. The same change of the localization in the stomata under dark or light condition was also shown for the other exocyst subunit - EXO84b (Figure 3.4).

The EXO70H4 exocyst subunit was linked with the trichomes maturation. In the trichomes of *Arabidopsis thaliana* there is created a boundary between the bottom and the upper part of the trichome. The layer of the secondary cell wall that is highly autofluorescent and callose rich is deposited only in the upper part of the trichome and the boundary is formed by the deposition of callose ring (named Ormannian ring – OR) that is EXO70H4 dependent (*exo70H4* mutant plants do not have this ring) (Figure 3.5).

The deposition of this callose ring as well as the expression of the EXO70H4 exocyst subunit is stimulated by UV radiation and inhibited by Methyl jasmonate (MeJa). The study of Kulich et al. (2015) of the mutants in EXO70H4 exocyst subunit also points to the possible exocyst cargo in the trichomes – callose from which the OR structure is made (Kulich et al., 2015).

The first report of the exocyst involvement in the cytokinesis and the cell plate maturation was shown in the work of Fendrych et al. (2010). This study of the exocyst EXO84b subunit showed disrupted cytokinesis that led to the severe growth retardation and sterility. Incomplete cytokinesis was especially very visible on the stomata cells that showed improper development. Different aberrant stomata phenotypes are shown on Figure 3.6.

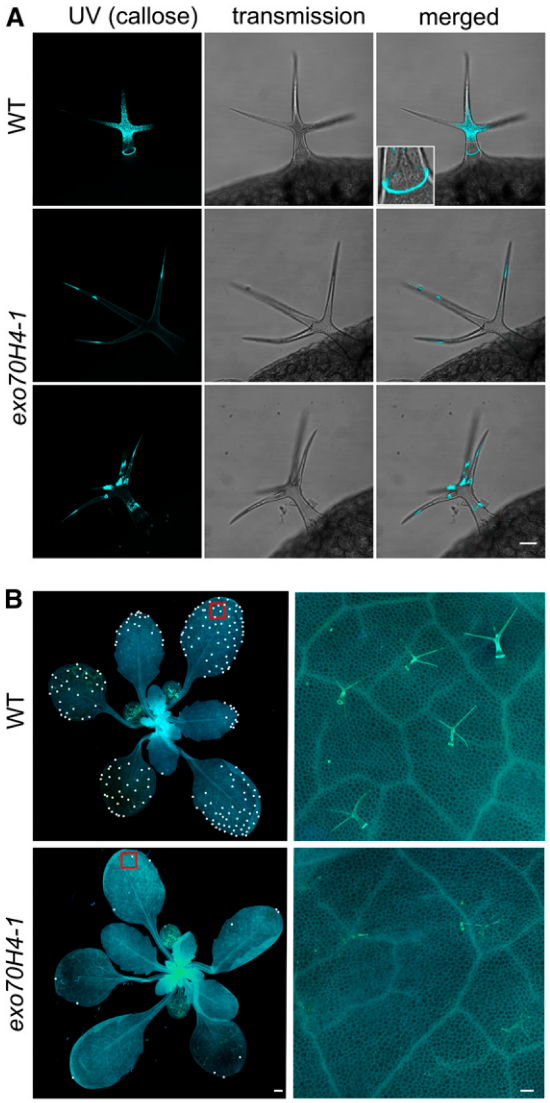

Figure 3.5: Highly auto-fluorescent structure of the Ortmannian ring in the WT *Arabidopsis* plant in comparison with the missing structure in the *exo70H4* mutant plant (Kulich et al., 2015).

Four years later Rybak et al. (2014) published a more detailed study of the involvement of exocyst complex in this process. According to the 'Relay Race' model, cytokinesis is a result of the sequential but overlapping work of the exocyst complex and the TRAPPII (TRAnsport Protein Particles II) complex. TRAPPII complex is a GEF for Rab GTPases and is required for intra- and post-Golgi traffic. In this model, cytokinesis is divided into four stages – initiation, biogenesis, expansion and maturation. Each of these parts is characteristic by different development stage of fragmoplast and was linked with exocyst or TRAPPII complex function, or both.

The exocyst complex plays an important role in the polarized deposition of the cell wall pectins, which is the key process in the seed coat development. Kulich et al. (2010) published a defect in generation of seed coat mucilage in the *sec8* and *exo70A1* exocyst mutants and linked this phenotype with the EXO70A1 interaction with ROH1 protein, which is the paralog of BYPASS (gene affecting root-shoot signaling by yet unknown mechanism).

The exocyst complex also plays an important role in the response to pathogens. Pečenková et al. (2011) showed the involvement of two EXO70 paralogs (EXO70B2 and EXO70H1) in the

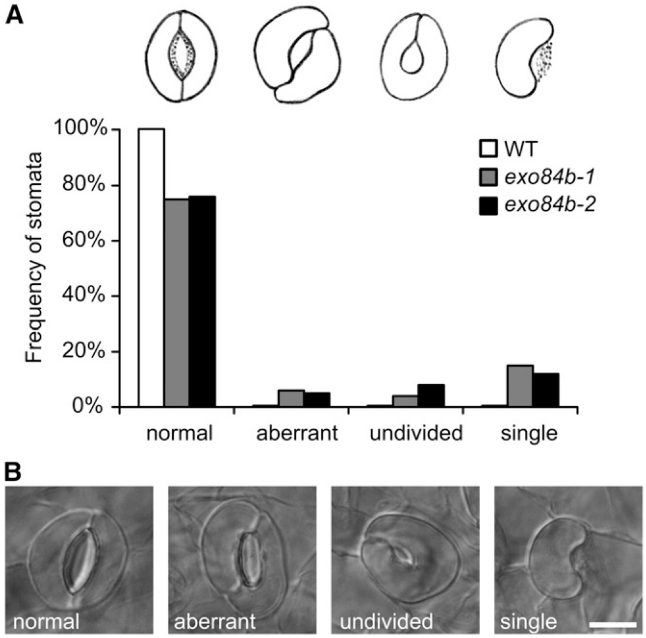

Figure 3.6: The mutation in the EXO84b subunit of the exocyst complex has an effect on the cytokinesis. The incomplete cytokinesis is visible also on the incorrectly developed guard cells of *exo84b* mutant plants (Fendrych et al., 2010).

cell wall apposition formation in response to pathogens. EXO70B1 subunit was connected with the *Arabidopsis* immune response in the work of Stegmann et al. (2013). In this study, authors showed that *exo70B1* mutants display lesion-mimic cell death and reduced responsiveness to PAMP signaling pathway (pathogen-associated molecular patterns).

EXO70B1 along with EXO84b and SEC5 subunit as a part of the exocyst complex were reported as regulators of autophagosome formation and autophagy-related Golgi-independent import into the vacuole. The *exo70B1* mutant plats showed the hypersensitive reaction and elevated levels of some acidic plant hormones - salicilic acid (SA), Jasmonic acid (JA), and Abscisic acid (ABA). The mutant seedlings were also hypersensitive to nitrogen starvation and had compromised vacuolar trafficking of anthocyanines (Kulich et al., 2013).

Another process in which exocyst was shown to play an important role is the cell wall deposition in developing tracheary elements (Oda et al., 2015; Vukašinović et al., 2016b). A phenotype defect of interrupted protoxylem vessels was shown in the *exo84b-1* mutants (Figure 3.7).

A phenotype of overexpressed exocyst EXO70 subunits in the plant pollen was also explored. Sekereš et al. (2017) published an analysis of the exocyst EXO70 subunits in tobacco pollen tubes. Besides a lot of localization studies of different EXO70 subunits and their co-localization with the phospholipid markers for Phosphatidic acid (PA) and Phosphatidylinositol 4,5-bisphosphate (PIP2) they also showed overexpression phenotype of chosen EXO70 subunits (Figure 3.8). Expression of SEC3a exocyst subunit showed the polar defect in the germinating pollen tubes (Bloch et al., 2016).

Moreover, in 2009, the EXO70A1 subunit was identified as an essential factor in the stigma for the pollen acceptance (Samuel et al., 2009). Using stigma-specific RNA-silencing constructs to suppress the expression of individual exocyst subunits, Safavian et al. (2015) showed the necessity of all used exocyst subunits in the stigma for acceptance of compatible pollen, which means that the whole complete exocyst complex is an important component in the compatible response pathway to promote pollen acceptance.

Even though exocyst complex has been studied intensively in the past, there are still many

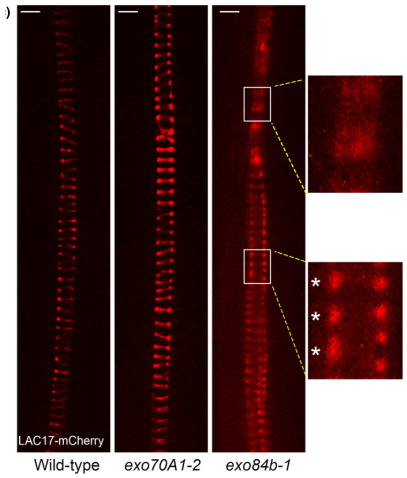

Figure 3.7: The tracheary elements of WT *Arabidopsis* plant and *exo84b-1* mutant plant.

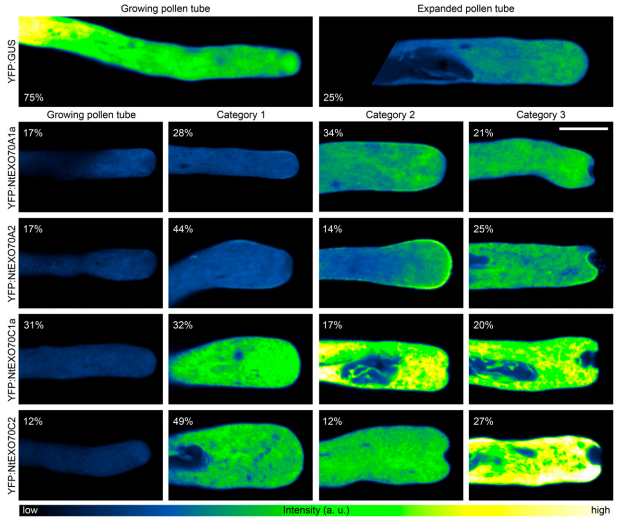

Figure 3.8: A phenotype of overexpressed EXO70 subunits in the pollen of *Nicotiana benthamiana* (Sekereš et al., 2017)

aspects that have to be further explored.

4. Proteins Moderating Rab GTPase Function

4.1 REP and GDI Proteins

These two proteins are an integral part of the Rab GTPase cycle and are structurally and functionally related (Seabra, 1998). The GDI and REP proteins are important during the Rab GTPase posttranslational modification that is mediated by RabGGT enzyme. These proteins also help the Rab GTPase to complete the whole GTP-GDP cycle.

REP protein is needed to carry Rab GTPase during the posttranslational modification, which is the double geranylation in the most of Rab GTPases. The geranylgeranylation of the Rab GTPase C-termini makes this part of the protein very hydrophobic. This means that this part of protein cannot exist in the cytosol in the free form and has to be bound to some membrane or carried by some protein (i.e. REP protein).

The REP protein, not the GDI protein, is the first one that is able to bind nascent unprenylated Rab GTPase. It is because, there is a previously mentioned Phe297 residue in the sequence of REP protein. This is a key residue not only for the binding of RabGGT enzyme to start posttranslational protein prenylation reaction, but it is also very important as a main distinguishing mark between REP protein and its cognate GDI protein, which doesn't have the Phe297 residue.

The GDI protein is a very important protein at the end of the whole Rab GTPase cycle. After the GTP hydrolysis, inactive (GDP-bound) Rab GTPase stays in the acceptor membrane and it has to be recycled back to the donor membrane. This inactive Rab GTPase is bound by GDI protein, which extracts it from the acceptor membrane. Subsequently, GDI protein finishes the Rab GTPase cycle by delivering the Rab GTPase back to the donor membrane (Novick and Zerialt, 1997).

4.2 Rab Geranylgeranyl Transferase

RabGGT is unique in the protein prenyl transferase family, because it modifies only members of a single protein subfamily - Ras-related Rab GTPases. Most of them possess a variable C-terminus with two cysteine residues arranged in the motifs such as -CC, -CXC, -CCX, -CCXX and RabGGT enzyme catalyzes transfer of two geranylgeranyl groups to these two cysteine residues (Farnsworth et al., 1994). The process of Rab GTPase geranylgeranylation requires presence of Rab escort

protein (REP), Anant et al. (1998) referred to this process as prenylation cascade. This cascade starts with REP protein that binds newly synthesized Rab protein and forms a stable Rab-REP complex. After that, a RabGGT enzyme is able to recognize a Rab-REP complex as its protein substrate and mediates a transfer of geranylgeranyl moieties to the relevant cysteine residues. Finally, geranylgeranylated Rab GTPase is able to bind biological membranes (Alexandrov et al., 1994).

4.2.1 Structure of RabGGT Enzyme

As well as the other prenyl transferases, RabGGT is a heterodimer composed of two subunits, alpha and beta. It differs from the other prenyltransferases by additional modules connected with the alpha subunit. The RabGGT alpha subunit is then composed from three compact domains: a helical domain, which is characteristic by tetratricopeptide repeat and is common to all prenyl transferases, Rab GGTase specific immunoglobulin like domain (Ig-like domain), and a leucin-rich repeat domain (LRR domain) (Zhang et al., 2000).

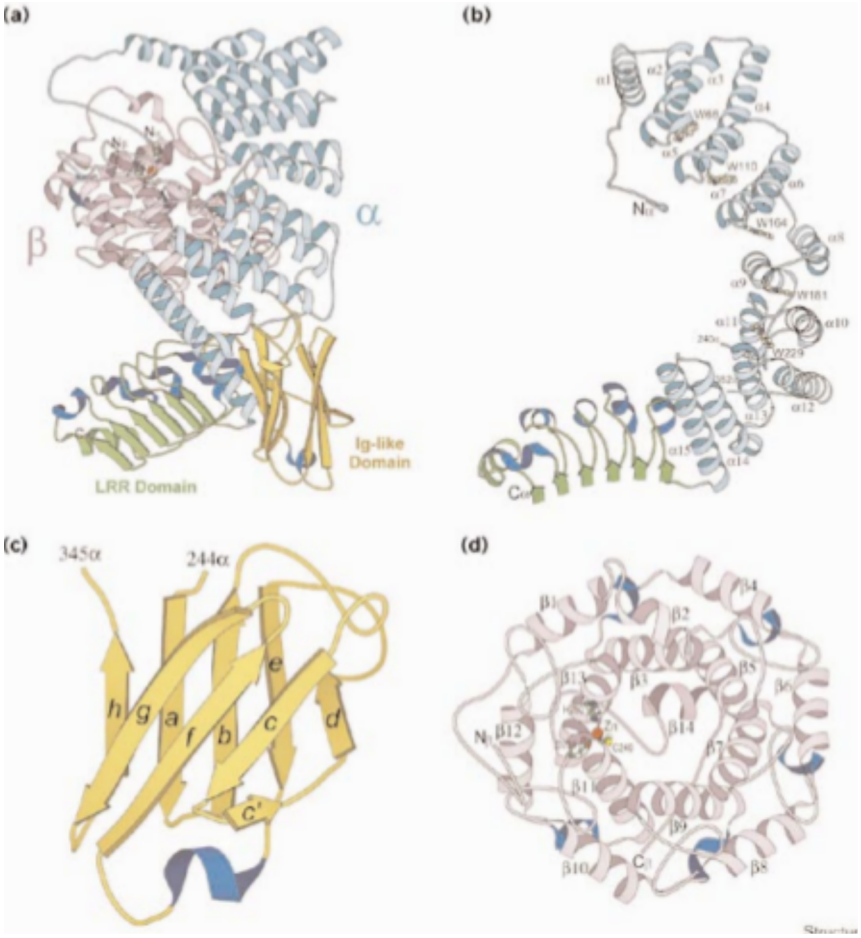

Figure 4.1: Domain structure of RabGGT enzyme (Zhang et al., 2000).

The beta subunit of RabGGT enzyme does not undergo any substantial changes and it is conserved in all prenyl transferases. It contains alpha-alpha barrel made up of 12 helices and it is in contact only with the conserved part of alpha subunit, the helical domain. There is also Zn2+ ion in the structure of RabGGT, which is classified as structural. It means that Zn2+stabilizes the quaternary structure of the RabGGT enzyme via coordination of the interaction of alpha and beta subunits.

4.2.2 Regulation of RabGGT activity

Based on structural data, Zhang et al. (2000) hypothesize the autoinhibitory effect of the N-terminus of alpha subunit. This part of the RabGGT enzyme is mobile and can bind the beta subunit and thus lock up existing interaction site. Even though we still do not know precise function of RabGGT inhibitors, a limited numbers of them are known. The first specific discovered inhibitor is a phosphono carboxylate called NE 10790 or 3-PEHPC, which was shown to function specifically *in vitro* and also *in vivo* in human bone cells - osteoclasts (Coxon et al., 2001). Between other inhibitors, there is also monoterpene perillyl alcohol, which affects not only RabGGTase but also GGT-I prenyltransferase (Ren et al., 1997).

4.2.3 Double Prenylation Mechanism

RabGGT enzyme possesses a single GGPP-binding site (geranylgeranyl pyrophosphate binding site), but the enzyme catalyzes the double GG transfer (Desnoyers and Seabra, 1998). A precise mechanism how geranylgeranyl (GG) moiety is added was described by Wu et al. (2009). The addition of geranylgeranyl chains starts from the cysteine residue more distant from the C-terminus. Monoprenylated product remains bound to the RabGGT enzyme and diphosphate head group of geranyl moiety dissociates from the active site. Then second GGPP can be able to bind to the enzyme. Although, Rab itself does not interact with RabGGT, its hypervariable C terminus is maintained in the RabGGT reaction centre by REP protein (Pylypenko et al., 2003).

Gomes et al. (2003) and Calero et al. (2003) shown that prenylation of Rab GTPases is important for their correct targeting and function. They observed that the mutants in Rab proteins with only one cysteine residue instead of two cysteine residues were mistargeted to the ER/GA compartment. Therefore there is a possibility that mono and di-prenylated Rab GTPases may be targeted by different routes and that different factors may be involved in the membrane recruitment. The dicysteine Rab GTPases are directly delivered to the target membrane but the monocysteine Rab proteins can transiently interact with the ER after prenylation (they undergo postprenylation processing in ER) before delivery to the target organelle (Leung et al., 2006).

5. Classification of RAB GTPases

Rab GTPases can be found in almost all eukaryotic organisms. In yeasts, Rab GTPases are named historically with regards to their discovery. In *Drosophila* and animals, they are classified numerically, but in *Arabidopsis thaliana* their classification is based on letters of the alphabet and they are denoted from A to H (Figure 5.2 (Rutherford and Moore, 2002).

For instance, 57 RAB GTPases have been identified in the *Arabidopsis* genome (Pereira-Leal and Seabra, 2001) that can be grouped into 8 groups (Bischoff et al., 1999), from which 6 are related to subgroups known in yeasts and animals. The remaining two groups are related to the mammalian Rab2 and Rab18 subgroups which cannot be found in yeasts (Lazar et al., 1997). Here we focus on RAB GTPases that play some role in vesicular trafficking towards the PM, i.e. those belonging to the first six groups.

5.1 RAB GTPases Involved in Trafficking between TGN and PM

The group A of *Arabidopsis* RAB GTPases is the most numerous group containing almost half of the RAB GTPases. According to Rutherford and Moore (2002), this group is homologous to yeast's Ypt31/Ypt32 and to mammalian's Rab11a, Rab11b and Rab25 proteins that have been all localized to the recycling endosome, which is an organelle where molecules from plasma membrane are recycled. The question is why this group of R GTPases is widely expanded in plants. Some authors speculate that there are some plant-specific functions of RAB GTPases from the group A, such as need for very dynamic membrane trafficking around TGN compartment, that have a broader function from the animal TGN (Chow et al., 2008; Preuss et al., 2006; Szumlanski and Nielsen, 2009). Another possible answer might be a changed cell wall composition of the *Arabidopsis* mutants deficient in particular RAB GTPases from class A, which points to a different cargo for vesicles marked by the presence of different RAB GTPases (Lunn et al., 2013).

Even though, the closest homologs of the A group of RAB GTPases in animals were shown to be localized to the recycling endosome, their localization is more diverse in plants but still restricted to the secretory pathways between TGN and PM. This localization of Rab-A group was shown by a treatment with a drug wortmanin. Rab-A proteins were not sensitive to the treatment with wortmanin, which is the inhibitor of PI3-Kinase and it inhibits endocytosis in plants. Because RAB-

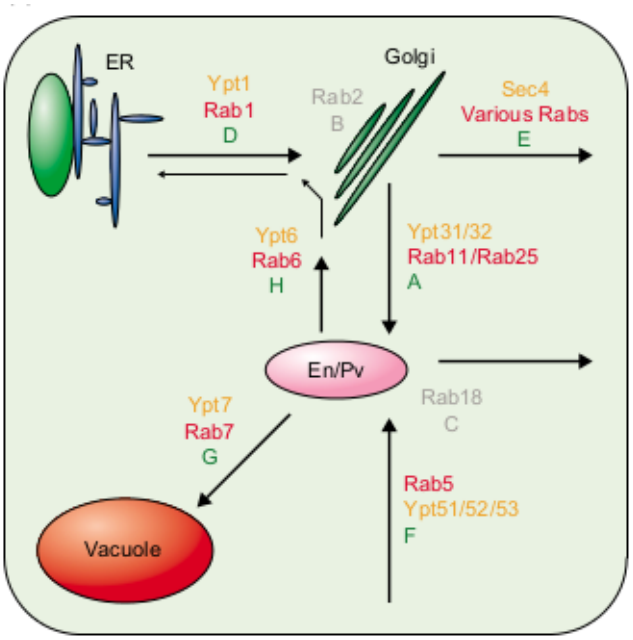

Figure 5.1: Trafficking pathway of a generic eukaryotic cell. The letters A to H represent 8 major clades of the *Arabidopsis* RAB GTPases. Six of these *Arabidopsis* clades are conserved in yeasts and animals. The 2 clades that has no yeasts homologs are shown in gray - clade B and clade C (Rutherford and Moore, 2002).

A proteins were not affected by this treatment, they are not supposed to be directly involved in either endocytosis or morphogenesis of other endomembrane compartment, such as MVB compartment (Qi and Zheng, 2013).

5.1.1 A1 Subgroup of RAB GTPases

The *Arabidopsis* RAB-A1subgroup resides in punctate structures adjacent to TGN and it co-localizes with VAMP721/VAMP722 R-SNARE proteins that are markers of a TGN compartment. This group contains five different RABs: RABA1a, RABA1b, RABA1c, RABA1d and RABA1e.

RABA1a was reported to be involved in auxin signaling (Koh et al., 2009) and RABA1b was reported in regulation of transport between TGN and plasma membrane (Feraru et al., 2012) by dynamic motion in actin-dependent fashion. RABA1b also co-localizes with another RAB GTPase from the A group - RABA2a, which was shown to create a specialized A2a/A3 compartment (Asaoka et al., 2013b). Another confirmation that RABA1b plays a role in the TGN PM signaling pathway is, that RABA1b is in a partial co-localization with RABF1 (Asaoka et al., 2013b), which mediates transport from the MVB towards the plasma membrane (Ebine et al., 2011). Moreover, expression of the dominant negative RABA1b mutant increased a size of RABF1 positive endosomes (Asaoka et al., 2013b), which shows some functional connection between these two RAB GTPases. Another finding from the same laboratory shows that RABA1a-d proteins are required for tolerance to salinity stress, which implies that these RAB GTPases might regulate the localization of integral plasma membrane-localized proteins, such as proton pumps and ion channels (Asaoka et al., 2013a).

Additionally, Qi and Zheng (2013) revealed that RABA1a-d are highly expressed in the root tip and they observed high functional redundancy between all four genes from the A1 subgroup. However, it seems that RABA1d is not fully redundant to the rest of RAB GTPases in the A1 subgroup. Moreover, the triple mutant in RABA1a, RABA1b and RABA1c had slightly retarded growth. In their work, they also showed co-localization of the RABA1c with other RABs from the

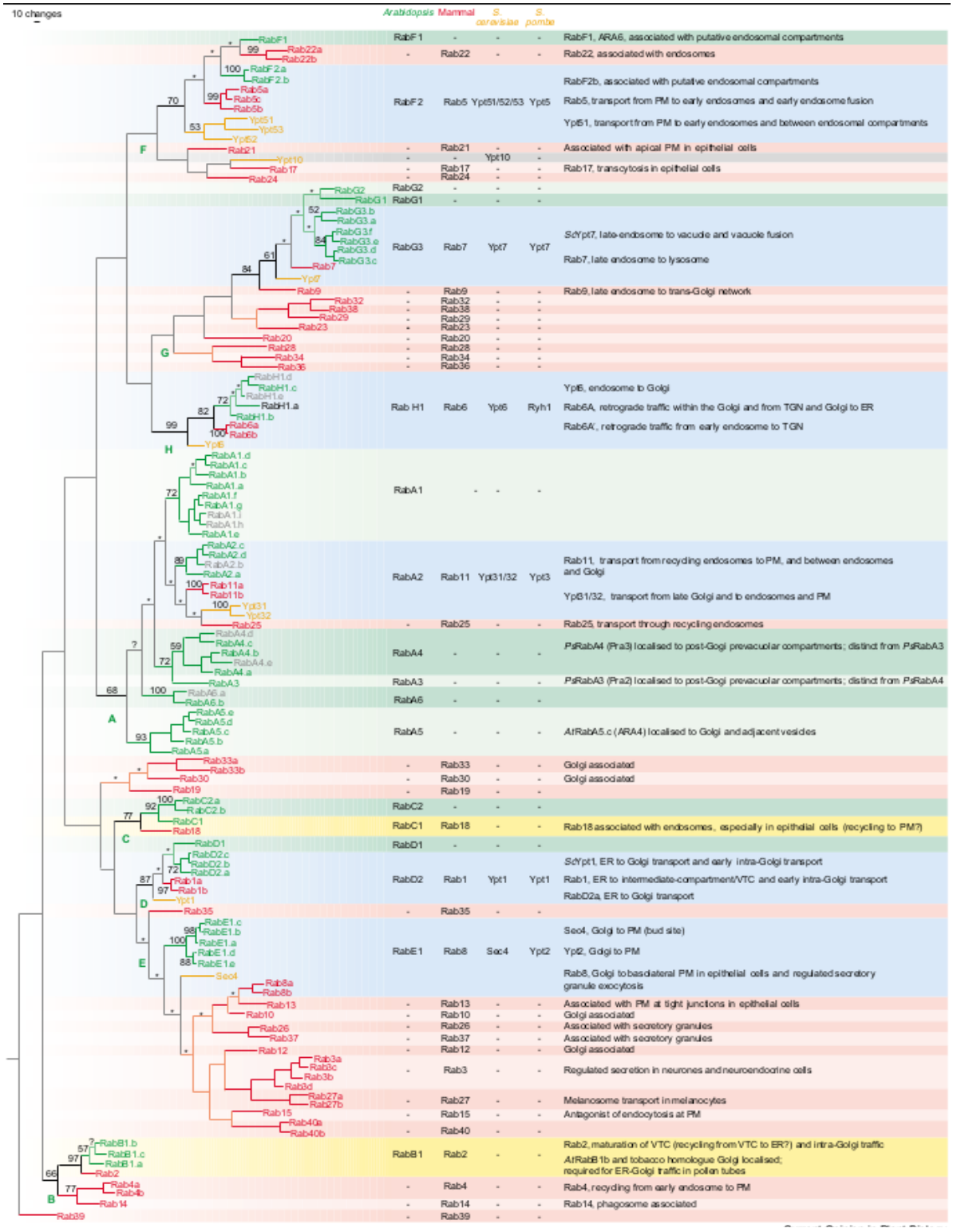

Figure 5.2: Phylogenetic analysis of the Rab GTPases families in *Arabidopsis*, mammals and yeasts (Rutherford and Moore, 2002).

A family, namely RABA2a and RABA3 that resides on a population of TGN that may contribute to cytokinensis and that creates the A2a/A3 compartment (Chow et al., 2008) and co-localization with

RABA4b GTPase that is involved in polar secretion of cell wall components (Kang et al., 2011; Preuss et al., 2006). RABA1a, RABA1b and RABA1c have distinct but overlapping expression in rapidly growing tissues where an active membrane trafficking is required. This is the case of a growing root hair where presence of RABA1d and RABA1e proteins was reported. The signal of RABA1d and RABA1e showed oscillations in a root hair tip, which nicely correlated with root hair growth (Berson et al., 2014). Another published plant RAB protein homologous to the RABA1 subgroup is the NtRAB11 GTPase from *Nicotiana tabacum*. This RAB GTPase has been shown to play an important role in the pollen tube growth (Graaf et al., 2005).

All described RAB GTPases from the A1 subgroup were shown to localize also to the growing cell plate (Berson et al., 2014; Davis et al., 2016; Kirchhelle et al., 2016). This localization is typical for RAB GTPases from the A group but the dynamics of their signal is different. Both, RABA1e and RABA2a have been shown to be localized to the cell plate but signals of these two Rab proteins do not co-localize together (Davis et al., 2016).

5.1.2 A2 and A3 Subgroup of RAB GTPases

Arabidopsis RABA2 and RABA3 have been reported to localize to a post-Golgi domain that partly overlaps with a TGN marker VHA-a1 in root tip cells. Blanco et al. (2009) reported *rabA2* common bean mutant with an altered root hair phenotype and a lack of nodulation that they explain as a consequence of compromised vesicle trafficking. In contrast with VHA-a1 marker, RABA2a and RABA3 localize also to the growing margins of the cell (Chow et al., 2008). Other confirmation that RABA2a and RABA3 play role in the trafficking between TGN and plasma membrane is that similarly to VHA-a1 TGN (Dettmer et al., 2006), the RAB A2/A3 compartment is an early site of FM4-64 labelling, the lipophylic dye that is internalized gradually to the cells of plant roots and may act as a tracer of endocytic pathway. The localization of RABA2a and RABA3 to the compartment between TGN (marked by VHA-a1) and EE also confirmed the treatment of the cells by brefeldin A (BFA).

5.1.3 TGN Compartment as the Main Station for RAB GTPases

After the treatment with BFA VHA-a1 marker, RABA2a and RABA3 co-localize in the BFA bodies, where proteins from TG (Trans Golgi) element, TGN and EE compartments, can be found. BFA is a fungal macrocyclic lactone substance that was proved as an inhibitor of protein trafficking in the endomembrane system, firstly in mammal cells (Sciaky et al., 1997). In all eukaryotic cells, BFA appears to have the same target, namely Sec7-type GEF that is necessary for activation of GTPase Arf1. The most studied effect of Arf1 GTPase is the recruitment of COPI (protein complex that coats vesicles transporting proteins between stacks of Golgi compartment) onto Golgi membranes. As a result, a majority of Golgi cisternae fuses directly with the ER to create an ER-Golgi hybrid compartment. A TG element and TGN compartment separate from the Golgi stack and merge with components of the endocytic pathway to form 'BFA-compartments'. In the structure of BFA bodies, we can find a core, which is enriched in RABA2/A3 membranes that are surrounded by a fraction with VHA-a1 and on the surface of BFA body, it is possible to find Golgi stacks and pre-vacuolar compartment (PVC) that are part of another pathway toward vacuole (Nebenführ et al., 2002).

5.1.4 A4 Subgroup of RAB GTPases

There are four RAB GTPases in the RABA4 group (RABA4a, RABA4b, RABA4c and RABA4d) and one, which is considered a pseudogene (RABA4e). Three RABA4 GTPases (RABA4b, RABA4c, RABA4d) were published to play a role in the polar growth and in the defense response against pathogens. The function of RABA4a has not been published yet. The RABA4b was shown to function in polarized secretion in root hair cells in cooperation with its effector PI-4KB1 (Preuss et al., 2006; Preuss et al., 2004). RABA4c was connected to the defense against pathogens (Ellinger

et al., 2014) by showing an interaction with his effector PMR4. The PMR4 is a callose synthase that is enzymatically active after translocation to the place where the fungal pathogen penetrates the cell. Therefore, RABA4c can be important for translocation or for the activity of the callose synthase.

In contrast to plants, there is a callose synthase homolog in yeasts and its activation is mediated by RHO1 GTPase and not by RAB GTPase (Qadota et al., 1996). The last described RAB GTPase from the A4 group is RABA4d that is pollen specific and was shown to localize to the tip of growing pollen tube. Disruption of the gene revealed its necessity for the proper development of the pollen tube and showed a disturbed localization of a cell wall component - pectin (Szumlanski and Nielsen, 2009).

5.1.5 A5 Subgroup of RAB GTPases

From the RABA5 subgroup, localization of only RABA5c GTPase was published. It was shown to be localized to the TGN compartment in pollen grains (Ueda et al., 1996). In somatic cells, this RAB GTPase however labels the TGN compartment very faintly. Kirchhelle et al. (2016) published localization of RABA5c in the somatic cells of young seedlings of *Arabidopsis thaliana*. The RABA5c contributes to organogenesis of the cell and a disruption of this RAB GTPase leads to impairment of a cell geometry. This RABA5c GTPase GTP-dependently localized to the edges of the cell in the young lateral roots and to the shoot primordia. Thus, the authors identified very specific population of vesicles, probably exocytic, which was sensitive to a treatment with latrunculin as well as oryzalin. This observation implies an interaction of the RABA5c GTPase with the cytoskelet. Another RAB GTPase from the A5 subgroup, RABA5d, which was shown to label vesicles near the plasma membrane and in the secretory mutant background (mutant in *exo70A1* subunit of the exocyst tethering complex), is known to co-localize together with PIN2 auxin transporter on the vesicles (Drdova et al., 2013).

5.1.6 E Group of RAB GTPases

Based on the available data, RAB GTPases from the class A are the main regulators of the vesicular trafficking between TGN and PM. But there are also other subgroups of RAB GTPases that contribute to regulation of this signaling pathway.

In both *Nicotiana benthamiana* and *Arabidopsis thaliana*, RABE1 localizes to the TGN compartment and also to the plasma membrane (Ahn et al., 2013; Speth et al., 2009). Speth et al. (2009) also showed an interaction of RABE1d GTPase with AvrPto (patogene effector protein), which can indicate a potential role of RABE class of RAB GTPases in a response to pathogens. But other RAB GTPase from the same group, RABE1c, was connected with peroxisomes through an interaction with PEX7 in peroxisomes of *Arabidopsis thaliana*, rather than with the TGN-PM signalling pathway (Cui et al., 2013a; Cui et al., 2013b).

5.1.7 F Group of RAB GTPases

The last group of RAB GTPases considered to play a role in the vesicle transport between TGN and PM is the group F of RAB GTPases. In mammals, the closest group to RABF GTPases is the Rab5 group. This group in *Arabidopsis* is divided into two subgroups - F1 (RABF1 also known as Ara6) and F2 (RABF2a and RABF2b). In the literature, there are inconsistencies about a localization of RABF2b (also known as Ara7) to the cell plate of the dividing cell. Chow et al. (2008) showed that RABF2b does not localize to the cell plate but Dhonukshe et al. (2006) previously published appearance of this RAB GTPase in the cell plate. Moreover, Chow et al. (2008) presented data about disrupted cell plate formation in young *rabF2b* mutant seedlings that resulted in bi-nucleated cells. This data disparity could be due to use of different model cells - Chow et al. (2008) used more natural model of *Arabidopsis* root cells whereas Dhonukshe et al. (2006) used mainly tobacco

cells for RAB localization. Consequently, because of these controversial results from two different laboratories, the role of RABF2a and RABF2b in the pathway from TGN toward plasma membrane is not sufficiently confirmed. However, a role of RABF1 GTPase in the trafficking from MVB compartment toward plasma membrane is confirmed (Ebine et al., 2011). RABF1 GTPase is the only one from the whole family of 57 of RAB GTPases in the *Arabidopsis thaliana* that was shown to undergo N-terminal myristoylation instead of geranylation on the C-terminus.

The RABF1 protein was first shown to localize to the endosome compartment and was described as an endocytic GTPase (Ueda et al., 2001). Later, RABF1 was connected with the EE localization and sterol endocytosis which is important for establishing the cell polarity and the whole process is actin dependent (Grebe et al., 2003). Finally, the mutation in RABF1 was shown to affect sugar homeostasis (Tsutsui et al., 2015).

To conclude, there are three subgroups of RAB GTPases indicated to regulate vesicular trafficking from the TGN compartment toward PM (Chow et al., 2008). The most diversified is the class A, where 27 membres are divided into 6 subgroups in *Arabidopsis thaliana*. The other RAB GTPases possibly involved in this signaling pathway are the RAB proteins from the group E that were connected with peroxisomes and with the pathogene response (Speth et al., 2009). The last RAB GTPase is from the subgroup F1 containing only one member that was shown to act on the route from the MVB compartment to the PM (Ebine et al., 2011).

5.2 Regulation of Rab GTPases in the Membrane Trafficking

Even though Rab GTPases function in membrane traffic is mainly regulated by their regulatory proteins mentioned above, there are also other mechanisms that fine-tune Rab GTPases regulation. This thesis describes an example of two mechanisms that are most pronounced - phospholipids content and phosphorylation.

5.2.1 Regulation of Membrane Traffic by Phospholipids

An identity of compartments as well as transport between them is not regulated only by different populations of Rab proteins and their GEFs and GAPs, but also by phospholipids. An example of regulation of vesicular trafficking by phospholipids is known from budding yeasts. In particular, Sec2, which is an effector of Ypt32, is involved in binding of one of the effectors of Sec4 Rab GTPase - Sec15, subunit of the exocyst complex (Medkova et al., 2006). The same domain of Sec2 is important for Sec15 and also for Ypt32-GTP Rab GTPase binding, which means that there is competition between these two proteins. This competition is further regulated by PI(4)P phospholipid content. The strong inhibition of the interaction of Sec2 with Sec15 by addition of PI(4)P was documented (Mizuno-Yamasaki et al., 2010). When a secretory vesicle is formed and the concentration of PI(4)P is high, Sec2 is predominantly bound to Ypt32 and the interaction with Sec15 is blocked. Gradually on the route towards the membrane, where the secretory vesicle is transported to, concentration of PI(4)P content is reduced, which leads to a shift toward Sec2 - Sec15 interaction. That prepares the secretory vesicle for tethering by an exocyst complex and subsequent fusion with the plasma membrane (Figure 7.1).

5.2.2 Regulation of Membrane Traffic by Phosphorylation

Generally, phosphorylation plays an important role in a protein regulation. Proteins that are involved in the vesicle trafficking are not an exception.

In yeasts, Sec2p is phosphorylated under normal growth conditions (Elkind et al., 2000). Stalder et al. (2013) found the phosphorylation site in a structure of Sec2 protein. They demonstrated that phosphorylation regulates interaction of Sec2 with its binding partners, Ypt32, Sec15 and PI(4)P. The phosphorylated form of Sec2 binds Sec15 exocyst subunit more efficiently than non-

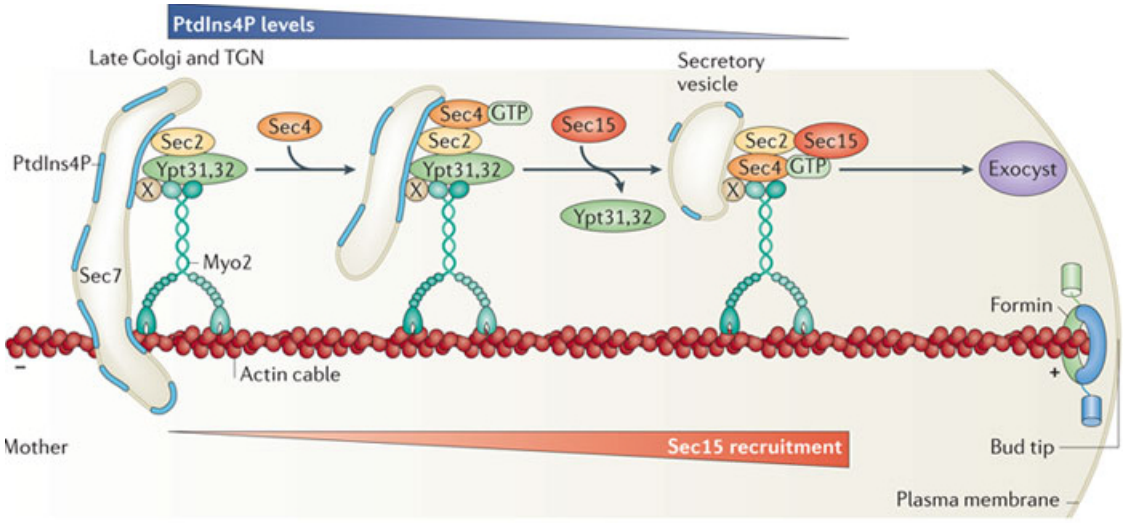

Figure 5.3: The involvement of PI4P phospholipid in the regulation of GEF and Rab GTPase localization and signaling (Hammer and Sellers, 2012). During the transport of the vesicle toward plasma membrane there are declining levels of the PI4P phospholipid and concurrently, the levels of Sec15 exocyst subunit are arising. These changing levels of exocyst subunit and phospholipid act as a dilution of the interaction, because they compete for the same interacting partner. And so, the closer to target membrane vesicle is, there is a higher probability for the interaction with the exocyst complex.

phosphorylated Sec2. On the other hand, the non-phosphorylated form of Sec2 binds preferentially Ypt32 Rab GTPase and PI(4)P phospholipid. Moreover, in their subsequent work, Stalder and Novick (2015) showed that phosphorylation of Sec2 is mediated by Yck1 and Yck2 protein kinases. To perform the phosphorylation, protein kinases bind same aminoacids of Sec2 as PI(4)P does (Mizuno-Yamasaki et al., 2010). After that, addition of PI(4)P can inhibit phosphorylation of Sec2 protein. Therefore, phosphorylation of Sec2 occurs probably after its association with a secretory vesicle and persists through vesicle transport. At the end of this process, dephosphorylation may facilitate recycling of Sec2 protein.

III

AIMS

6. Aims

Submitted thesis: 'Effectors of RAB GTPases and Their Role in Plant Secretion' has two main aims further described in this chapters. The first is focused on the exocyst on the molecular level and tried to answer questions about its functional characteristics. Second part is focused on the phenotypic effect of the mutation in various exocyst subunits. This task was solved on the physiological level, predominantly.

6.1 Functional Characteristics of the Exocyst Complex

1. **Interaction of the Exocyst complex with the secretory vesicles**
   What is the connection between tethering complex exocyst and secretory vesicle mediated?
   Paper: 'Plant Exocyst Complex is an Effector of Small GTPases from RABA4 Class.'
2. **Interaction of the Exocyst complex with the plasma membrane**
   How EXO70A1 exocyst subunit interacts with the phospholipids?

6.2 Phenotype of Exocyst Mutants

1. **Plasticity of exocyst mutant hypocotyls**
   Is there common secretory phenotype for mutants in different exocyst subunits?
   Paper:'Developmental Plasticity of *Arabidopsis* Hypocotyl is Dependent on Exocyst Complex Function.'
2. **Specific accumulation of starch in the hypocotyls of dark-grown exocyst mutants**
   Do the secretory mutants have a problem with the hypocotyl elongation and over-accumulation of starch when grown in the dark? Does the decision mechanism of plant 'to grow or to store' exist?
   Paper: 'Starch Accumulation in *Arabidopsis* Secretory Mutants Seedlings is a Result of the Cell Wall Biogenesis Inhibition.'
3. **Mutant in SEC15b subunit**
   How does the mutant in SEC15b subunit of the exocyst complex look like?

IV MATERIALS AND METHODS

7. Materials and Methods

7.1 Plant Material and Growth Conditions

Plants of *Nicotiana benthamiana*, for transient expression of fluorescent proteins, were grown under standard cultivation conditions to the stadium of four leaves and abaxial side of leaves was used for transient expression of studied proteins.

Arabidopsis seeds were surface-sterilized (4 min in 10% household bleach [Bochemie, www.savo.eu], 2x3 min in 70% EtOH and rinsed 5 times with sterile distilled water) and dispersed onto agar plates with growing medium: $^1/_2$-MS salts (Sigma, www.sigmaaldrich.com) supplemented with 1% (w/v) sucrose (Fluka, www.sigmaaldrich.com/Fluka) or mannitol (0,528%), vitamins, 1.6% (w/v) plant agar (Duchefa, www.duchefa.com), buffered to pH 5.7. Stratification was performed at 4 °C for 2 days in dark. Seedlings were grown for 7 days vertically in a climate chamber typically at 24 °C under continuous dark. *Arabidopsis* lines for breeding or seeds were grown on the $\frac{1}{2}$MS media prior to transfer to the sterile giffys tablets. *Arabidopsis* lines for the phenotypic analysis were grown in the complete dark, for 5-8 day, in 24 °C.

For the phenotypic and starch analysis we used following *Arabidopsis* mutant lines, that were previously described in cited publications. All used mutant plants are in Col0 background with exception of *det3*, which is in Landsberg background.

Used mutant lines: *exo70A1-1*,(Synek et al., 2006), *exo84b-1*, (Fendrych et al., 2010), *sec15b*, *rgtb1-1*, (Hála et al., 2010) and *det3* (Schumacher et al., 1999), *dwarf2*, (Wang et al., 2001), *pgm1*, (was kindly provided by C Wolverton, Ohio Wesleyan University, (Wolverton et al., 2011)).

7.2 Starch Detection

Starch was detected in the hypocotyls of seven days old dark grown seedlings and visualized by staining with the Lugol solution for 5 minutes followed by 10 minutes wash. Confirmation that Lugol solution stains starch was done by starch assay kit from abcam (ab83393) which was used for measurement of the amount of starch in the dark grown WT and *rgtb1-1* mutant plants. Starch accumulation was documented using the Olympus BX-51 microscope with the Olympus DP50 camera attached. The length of the hypocotyls and the amount of starch accumulation were

measured 7 days after germination. Rather than on the differences in the amount of the present starch, we focused on the presence or absence of the starch accumulation. Measurements were analyzed with ImageJ software.

7.3 Cloning and Protein Construct Preparation

7.3.1 Constructs for the Expression in *Escherichia Coli*:

RAB GTPases (AtRABA4a, AtRABA4b, AtRABA4c, AtRABA4d, AtRABA2a, AtRABE1d) used for pull-down experiments were cloned into pET30a+ expression plasmid with N-terminal His-tag. SEC15B subunit of exocyst complex (AtSEC15B) was cloned into pGEX-3-T1 with N-terminal GST-tag.

Point-mutated versions of AtEXO70A1 were generated by a series of PCR reactions, each step followed by cloning into the pJET vector (Thermo Fisher Scientific) and sequencing of the product. In the protein sequence, five lysin residues 339, 462, 549, 607, 611 or only two of them (607 and 611) were mutated to glutamate or alanine residues, respectively, to generate version indicated as EXO70A1-5xE, EXO70A1-5xA, EXO70A1-2xE, EXO70A1-2xA. Both point-mutated and truncated versions of AtEXO70A1 were cloned in the same way as the full-length (wild-type) version previously Drdova et al. (2013), Fendrych et al. (2013), and Rybak et al. (2014) to achieve compatible data.

7.3.2 Fluorescent Constructs for Microscopy:

For transient expression of fluorescently labelled proteins in *Nicotiana benthamiana* RAB GTPases under 35S promoter (AtRABA4a, AtRABA4b, AtRABA4c, AtRABA4d) cloned into pBAR plasmid with N-terminal mRFP and SEC15A and SEC15B proteins under 35S promoter in pBAR plasmid with N-terminal GFP were used.

7.4 Yeast Complementation

The *Saccharomyces cerevisiae* sec15-1 strain NY64 (MATa, ura3-52, sec15-1) was a kind gift from P. Novick (Yale University, New Haven, CT). Both, AtSEC15a and AtSEC15b cDNAs were cloned into pVT 103-U vector under the control of the ADH1 promoter with using of *BamHI* and *XhoI* restriction sites. Yeasts were transformed according to the LiAc transformation procedure and transformants were selected on -URA plates. Single colonies were resuspended in 100 μl of sterile water and several dilutions were prepared. 10 μl of each dilution was dropped on -URA selective plates and incubated at 28 °C or 35 °C for 5 days.

7.5 Yeast Two-hybrid Assay

The MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) was used for two-hybrid screening and all steps proceeded as described in the manufacturer's protocol. AtSEC15b, AtRAB-A2a, AtRAB-A4a, and AtRAB-E1d were cloned both into pGBKT7 and pGADT7 vectors. The yeast strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4, gal80, LYS2::GAL1UAS-GAL1TATA-HIS3, MEL1, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ) was co-transformed with AtSEC15b and RABs in both orientations (i.e. AtSEC15b with either GAL4-BD or GAL4-AD). Yeasts were grown on –LEU-TRP selective plates and then serial dilutions of single colonies in distiled water were made. 10 μl of each dilution were dropped on -ADE-HIS-LEU-TRP and –LEU-TRP selective plates. Results were observed after 4 days.

7.6 Co-immunoprecipitation

For co-immunoprecipitation 14 days old seedlings expressing EXO84b::EXO84b-GFP construct were used (Fendrych et al., 2010). Specific band that corresponds to A4 class of RAB GTPases was detected in the co-immunoprecipitate using co-immunoprecipitation kit (μMACS GFP Isolation Kit, o.n. 130-091-125, Milteny) and specific A4 antibody. Co-immunoprecipitation was simultaneously performed with other two control proteins free-GFP and plasma membrane protein SYP121::GFP-SYP121 (Kato et al., 2010).

7.7 Lipid Binding Assay

7.7.1 PIP/Lipid Membrane Strip

SEC15B with His/GST tag and EXO70A1 and mutated version of EXO70A1, EXO70A1 5xE with His/GST tag was purified under native conditions and used for PIP/Lipid membrane strip analysis. PIP/Lipid membrane strip analysis was done according to PIP/Lipid membrane strip echelon manual (`http://www.echelon-inc.com/content/EBI/product/files/PROTOCOL_Strip_Array.v9.pdf`) and for detection a recommended antibody against GST tag was used (SIGMa G1160, `http://www.sigmaaldrich.com/catalog/product/sigma/g1160?lang=en&region=CZ`) or His tag (`https://www.qiagen.com/fi/shop/protein-and-cell-assays/penta-his-antibody-bsa-free/#productdetails`) of our protein of interest.

7.7.2 Large Unilamelar Vesicle Method (LUV)

Purified SEC15b as well as purified EXO70A1 (WT, and mutated version) were used for LUV method.

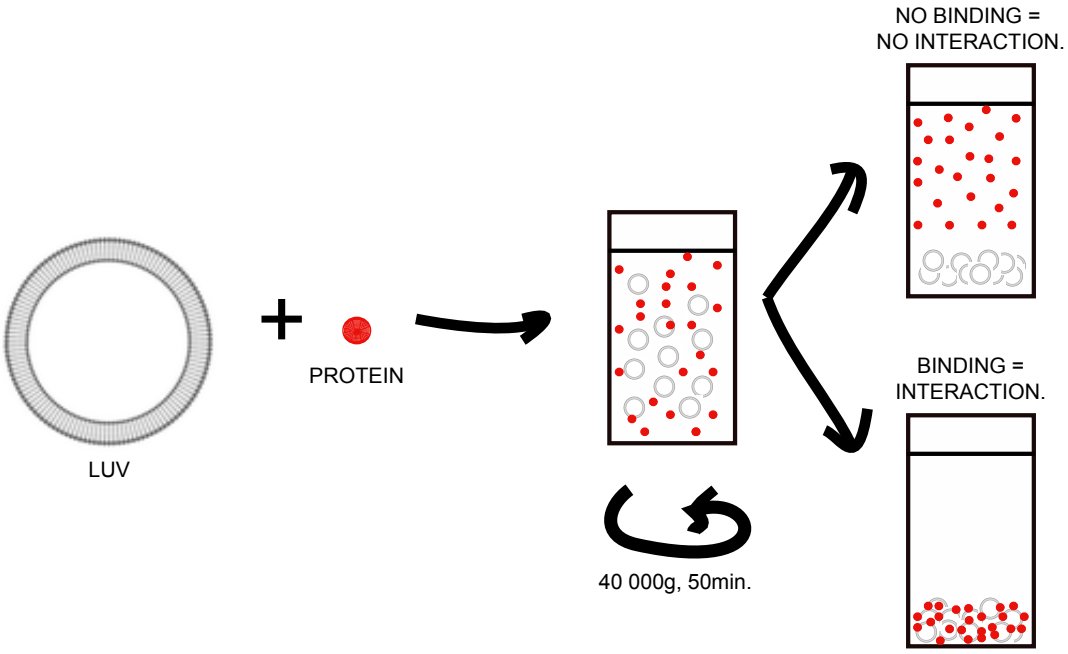

Figure 7.1: On the extruder large unilamelar vesicles consisting of the phospholipid of interest and filled with heavy sugar - rafinose, were made. Protein, that has to be tested is purified, mixed and co-incubated with the phospholipids vesicles. The mixture is centrifuged on high g. Because phospholipids vesicles are heavy they will go to the pellet. Protein of interest is light and will stay in supernatant. In the case of interaction between phospholipid and protein, the both will be found in the pellet.

For all experiments following phospholipids were used: DOPC, DOPE, DOPS, DOPA, DOPIP2, DOPI3P from Avanti or Sigma company. Different concentrations of phosphilipids were prepared. As a control for nonspecific interaction 100% DOPC and/or 50% DOPC with 50% DOPE were used. For the unraveling of possible interaction with phospholipids 20%PS in the mix with 80%PC, 20%PA in the mix with 80%PC and 5%PIP2 in the mix with 95%PC and 5%PI3P in the mix with 95%PC were used.

Mixed lipids were desiccated on desiccator (approximately 40 minutes on 35 °C). 1ml of extrusion buffer (250mM Raffinose pentahydrate, 25mM Tris-HCl pH=7.5, 1mM DTT) was added to every phospholipid mixture. Eppendorf tubes were vortex and left for 1hour in RT. After 2minutes of sonication, extruder was prepared and the same size of lipid droplets was prepared by the 20 times pass through the extruder membrane. 600 μl of Lipid binding buffer (LBB, 125/250/500/mM Kcl - according to stringency, 25mM Tris-Cl pH=7.5, 1mM DTT, 0.5mM EDTA) was added and the samples were centrifuged (40000g, 50min., 25 °C). The supernatant was discarded and 150 μl LBB was added to pellet and re-suspended. To the mixture maximum of 50 μl of protein of interest was added and incubated on RT with mild shaking for 30 minutes. The samples were centrifuged (40000g, 50 min., 25 °C). The supernatant was transferred to a new eppendorf tube where 1ml of cold aceton was added and it was let precipitate over-night on -20 °C. The pellet was re-suspended in 30μl of LBB or phosphate buffer().

The supernatants and pellets were visualized on the SDS gel stained with Comassie or on western blots with the use of specific antibody against the protein of interest.

7.8 Protein Expression and Purification

7.8.1 Protein Expression

RAB GTPases in pET30a+ plasmid were transformed into BL21 (RIPL) expression strain of *E.coli*. Expression was induced by 0.1mM IPTG, for 3 hours, 37 °C, 180rpm. After 3 hours of expression bacterial cells were collected and stored on -20 °C. SEC15B in pGEX-3-T1 plasmid and EXO70A1 and EXO70A1 5xE in pGEX-4-T2 were transformed into *E.coli* BL21 Arctica Express expression cells. Expression was induced by 0.1mM IPTG, for 24 hours, 12 °C, 180rpm. After expression, bacterial cells were collected and stored in -20 °C for one month maximally.

7.8.2 Protein Purification

All used proteins were purified under native conditions. Pelleted cells from 50ml of RAB GTPase bacterial culture were used and dissolved in 10ml of RAB lysis buffer (25mM TRIS pH=7,8, 250mM NaCl,10Mm imidazol, β-merkaptoethanol). Resuspended cells were sonicated, centrifuged for 1 hour, 20 000g, 4 °C and supernatant was used for purification through gravity column filled with 250 μl of NiNTA beads. Column was washed two times with the Rab lysis buffer with higher concentration of imidazol (50mM). RAB proteins were eluated with 0.5ml of RAB lysis buffer with 250mM imidazol. Purified RAB proteins were stored in the fridge on 4 °C for one week maximally.

For SEC15B and EXO70 A1 and EXO70A1 5xE purification, SEC15 lysis buffer was used (25mM TRIS pH=7.8, 250mM NaCl, β-merkaptoethanol). Pelleted cells from 0.05 - 4l of cell culture were re-suspended in the 30ml of SEC15 lysis buffer, sonicated and centrifuged for 1 hour, 20 000g, 4 °C and supernatant was used for gravity column purification filled with 30-60mg of glutathione-agarose beads. The column was washed two times with the SEC15 lysis buffer and SEC15b/EXO70A1/EXO70A1 5xE were eluted with 60μl of 30mM Glutathione - S transferase.

7.8.3 Pull-down Assay

RAB GTPases were purified according to a purification protocol and stored in the fridge. During SEC15b purification 60mg of rehydrated GST agarose beads were not loaded on the gravity column

but were added to the falcon with supernatant and incubated for 1 hour, 4 °C, mild shaking. Beads were pelleted and washed two times with the SEC15 lysis buffer, 20min, 4 °C, mild shaking. Washed GST agarose beads with bind SEC15B were divided into two falcons. To the first falcon, RAB GTPase pretreated with GTP analog (10mM γS) was added, and to the second falcon, RAB GTPase pretreated with GDP (10mM βS) analog was added. Falcons were incubated 1 hour, 4 °C, mild shaking. Beads were pelleted and washed two times with the SEC15 lysis buffer, 20min, 4 °C, mild shaking. Proteins bound to agarose beads were eluted by 30mM glutathione – S transferase in 1M TRIS pH=8.8. SEC15B and RAB GTPases were detected on western blot by commercial antibodies against GST-tag and His-tag.

7.9 Transient Expression in *Nicotiana benthamiana* and FLIM/FRET Microscopy

mRFP and GFP labelled constructs for FLIM/FRET analysis were transformed to *Agrobacterium* strains GV 3101::mp90 and used for transient expression in *Nicotiana benthamiana*. Co-transformation with viral p19 protein (TBSV) was used to overcome cellular mechanisms of post-transcriptional silencing and support high level of transient expression [10.1016/j.febslet.2013.01.036].

Abaxial side of leaf was investigated on the 2nd or 3rd day after transformation by a laser scanning confocal imaging Zeiss LSM 880 and Nikon spinning disc microscope for co-localization studies and Zeiss LSM 780 microscope system for FLIM/FRET microscopy. The FLIM/FRET system consists of a Zeiss LSM 780 inverted microscope with external In Tune Laser (488-640nm, <3nm width, pulsed) 1.5mW and GaAsP (32x) detector. Photon counting module Simple-Tau 150 (Compact TCSPC system based on SPC-150) with DCC-100 (Detector Controller Card) (Becker & Hickl GmbH) was coped to Zeiss LSM system to perform FLIM experiment.

Interaction of 35S::GFP-SEC15B and 35S::mRFP-A4a,b,c,d RAB GTPases were studied in selected regions of interest (ROI) at 256x256 pixels resolution. The leaf samples expressed labelled SEC15B protein and different mRFP labelled RAB GTPases were excited with donor excitation wavelength 490 nm with an appropriate laser power and PMT gain to obtain the confocal donor/acceptor image. The same ROI was excited with donor excitation wavelength 490 nm to generate intensity image and photon counting on B&H Simple-Tau module with original SPCM software.

To further analyse the 35S::GFP-SEC15B species distribution the B&H software SPCImage was used. For image analysis, we used pixel per pixel image analysis fitting model for two component system with fixed on 5 Chi-square value, binning parameter = 1.

We observe that the 35S::GFP-SEC15B species were noticeably more prone to plasma membrane and surroundings in their distribution. In an effort to demonstrate the variable degree of GFP lifetime in the region around the plasma membrane, we recorded the fluorescence lifetime for those membranes and close vicinity.

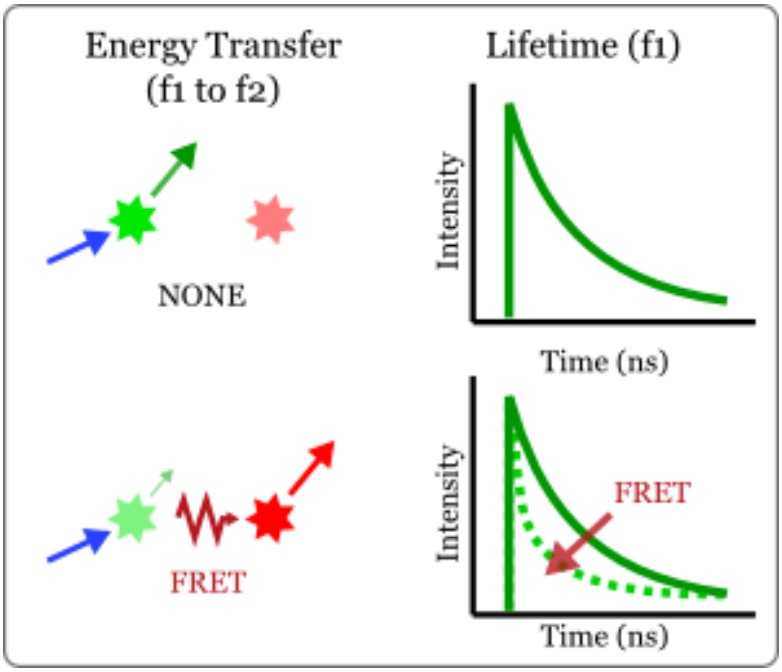

Figure 7.2: The mechanism of the energy transfer between donor and acceptor and change of the life time of the donor after the interaction with the acceptor.

V

RESULTS

8. Functional Characteristics of Exocyst Complex

Submitted thesis: ' Effectors of RAB GTPases and their role in plant secretion' has two main aims further described in this chapters. The first part is focused on the functional studies how exocyst complex interact with the secretory vesicle on one side and with the plasma membrane on the other side.

8.1 Interaction of the Exocyst Complex with the Secretory Vesicles

How is the connection between tethering complex exocyst and secretory vesicle mediated?

Paper: 'Plant Exocyst Complex is an Effector of small GTPases from RABA4 Class.'

Plant Exocyst Complex is an Effector of small GTPases from RABA4 Class

Martina Růžičková[123], Klára Aldorfová[12] Siarhei Dobrovolski[4], Viktor Žárský[12] Jan Hejátko[4] and Michal Hála[12]*

Abstract— **Engagement of RAB GTPases in the regulation of endomembrane trafficking is one of the evolutionary conserved aspects of secretion regulation. RAB GTPases are regulatory switches orchestrating vesicle transport among cellular endomembrane compartments and towards the plasma membrane via downstream effectors. One of them is the exocyst complex involved in vesicle docking at the plasma membrane. It is a complex composed of eight different subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) that occurs in almost all eukaryotes. Exocyst was discovered as a Sec4p RAB GTPase effector in yeast and data from animal models suggest SEC15 exocyst subunit as the RAB-interacting partner. However, the interaction of the exocyst with the RAB GTPases has not been shown in plants. Here we test the hypothesis of conservation of RAB-exocyst interaction in *Arabidopis thaliana*. We expressed the SEC15b subunit, one of two paralogous subunits of the exocyst complex as recombinant protein and transiently as GFP-SEC15b fusion protein in N*icotiana benthamiana*. Both in vitro and in planta, we were able to show interaction of SEC15b with RAB GTPases from the RAB-A4 subgroup. Interestingly, RAB-A4a and -A4b but not RAB-A4c and -A4d were shown to be interactors of the SEC15b subunit.**

I. INTRODUCTION

Exocytosis is an essential membrane traffic event by which a cell directs the content of secretory vesicles out of the cell. These vesicles contain soluble cargo as polysaccharides to be secreted as well as membrane proteins and lipids to become part of cell matrix and the plasma membrane. This process is crucial for cell division, growth, cell to cell communication and cell polarity establishment.

The key regulators of exocytosis, and in general intracellular vesicular transport, are RAB GTPases members of the RAS superfamily of GTP-binding proteins (Rutherford and Moore, 2002; Stenmark, 2009). These proteins cycle between active, GTP-bound and inactive, GDP-bound conformations (Olkkonen and Slenmark, 1997; Grosshans, Ortiz, and Novick, 2006). Different complexities of RAB GTPase families reflect different organization of vesicle transport in different organisms. In *Arabidopsis*, 56 RAB GTPases are organized in eight clades A-H. The A clade, homologous to Rab11, contains 26 members further divided into six subclades and its complexity raises possibly due to co-evolution with the post-golgi trafficking system in plants (Rutherford and Moore, 2002).

In the first step of exocytosis, which is the link between vesicle and target membranes is represented by tethering complexes. Several conserved complexes acting as tethering in endomembrane trafficking has been identified TRAPPI, TRAPPII, Dsl1, COG, EARP and the exocyst (Koumandou et al., 2007; Vukašinović et al., 2016).

The exocyst, an evolutionarily conserved protein complex, was first identified in yeast, where it participates in regulation of secretion and polarized growth (TerBush et al., 1996). Based on sequence homology, it was subsequently identified in mammalian cells and plants (Hsu et al., 1996; Kee et al., 1997; Elias et al., 2003). The plant exocyst, as in most other kingdoms, consists of 8 subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (Elias et al., 2003) localized to sites of active exocytosis, where they mediate the tethering of secretory vesicles to the plasma membrane prior to the formation of the SNARE complex involved in the actual membrane fusion (Hála et al., 2008; Fendrych et al., 2013).

In yeast and mammals, the exocyst complex functions as an effector of small GTPases from Rab, Rho and Ral families. In yeast, the exocyst associates with secretory vesicles via direct binding to Rab GTPase Sec4 (Guo et al., 1999). Medkova et al. (2006) showed that Sec2p, the guanine nucleotide exchange factor (GEF) that activates the Sec4 GTPase also interacts with Sec15p in yeast making it a part of secretory RAB cascade. It was recently shown, that the Sec15p-Sec2p interaction is selectively inhibited by phospholipid PI-4-P (Mizuno-Yamasaki et al., 2010).

Exocyst functions as a RAB effector also in mammalian cells (Guo et al., 1999). Zhang et al. (2004) showed that Sec15 colocalizes with Rab11 GTPase and exhibits GTP-dependent interaction with Rab11. Although Sec15 also weakly interacts with three other exocytic RAB proteins

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* Address correspondence to michal.hala@natur.cuni.cz The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors is: Michal Hála (michal.hala@natur.cuni.cz). M.R. and M.H. conceived the project, designed the experiments, analyzed the data, and wrote the article with contributions of all the authors; M.R. performed most of the experiments with contributions from K.A. and S.D; J.H and V.Z. initiated the research topic; M.H., J.H and V.Z. supervised the experiments.

(Rab3, Rab8 and Rab27) in *Drosophila*, Rab11 seems to be its major interaction partner (Zhang et al., 2004). It was also shown that only the C-terminal domain of Sec15 is responsible, and sufficient, for binding of a subset of RAB GTPases in GTP-dependent manner (Wu et al., 2005).

We tested the evolutionary conservation of RAB - exocyst interactions in angiosperm plants. Published data show that all of the exocyst subunits function together in vivo in *Arabidopsis thaliana* (Synek et al., 2006; Hála et al., 2008). In contrast to yeast and animal genomes, which contain single copies of each exocyst subunit, the plant exocyst subunits are mostly encoded by several paralogous genes, with the EXO70 subunit, represented by family of 23 paralogues in *Arabidopsis* (Elias et al., 2003; Cvrcková et al., 2012). Two paralogues of SEC15 can be found in the *Arabidopsis* genome but their deeper functional analysis is still missing. SEC15b was shown to take part in plant cell division together with other exocyst subunits (Fendrych et al., 2010). It was also shown to be important for stigma receptivity of compatible pollen in absence of its paralogue SEC15a (Safavian et al., 2015). SEC15a, on the other hand, was documented to be important for pollen tube growth (Hála et al., 2008). Here we show that AtSEC15b directly interacts with GTP-bound RAB GTPases from the A4 subclade confirming conservation of this regulatory module across eukaryotic kingdoms.

II. METHODS

A. Plant material and growth conditions

Nicotiana benthamiana plants were grown in the cultivation room on Jiffy pellets at 25C under the long day conditions.

B. Cloning and protein construct preparation

RAB GTPases (AtRABA4a, AtRABA4b, AtRABA4c, AtRABA4d, AtRABA2a, AtRABE1d) used for pull-down experiments were cloned into pET30a+ expression plasmid with the N-terminal His-tag. The AtSEC15B subunit was cloned into pGEX-3-T1 with the N-terminal GST-tag. For transient expression of fluorescently labelled proteins in *Nicotiana bentamiana* RAB GTPases under 35S promoter (AtRABA4a, AtRABA4b, AtRABA4c, AtRABA4d) were used and cloned into pBar plasmid with the N-terminal mRFP and SEC15A and SEC15B proteins under 35S promoter in pBAR plasmid with the N-terminal GFP fusion.

C. Co-immunoprecipitation

The μMACS Isolation Kit (Miltenyi Biotec) was used according to the manufacturer protocol. Fourteen- days-old *Arabidopsis* seedlings expressing different GFP-fused proteins were grown on $\frac{1}{2}$MS media supplemented with 1% sucrose under long-day conditions. Approximately 1g of fresh mass was used for isolation. The final pellet was eluted into 50 l of elution buffer. Of this, 20 μl was analyzed on 10% SDS gel followed by Western blotting on nitrocellulose membrane (Sigma). Presence of GFP was tested by polyclonal mouse anti-GFP antibody (1:3,000 in PBS with 5% non-fat dry milk) and secondary anti-mouse IgG antibody fused with HRP (Promega). The signal was visualized using ECL detection.

D. Lipid binding assay

SEC15B with GST tag was purified under native conditions and used for PIP strip analysis. PIP strip analysis was done according to PIP strip Echelon manual and for detection was used recommended antibody against GST (G1160, Sigma, recommended dilution for PIP strip assay 1:2000) tag of our protein of interest.

E. Yeast complementation

The *Saccharomyces cerevisiae* sec15-1 strain NY64 (MATa, ura3-52, sec15-1) was a kind gift from P. Novick (Yale University, New Haven, CT). Both, AtSEC15a and AtSEC15b cDNAs were cloned into pVT 103-U vector under the control of the ADH1 promoter with using of *BamHI* and *XhoI* restriction sites. Yeasts were transformed according to the LiAc transformation procedure and transformants were selected on -URA plates. Single colonies were resuspended in 100 μl of sterile water and several dilutions were prepared. 10 μl of each dilution was dropped on -URA selective plates and incubated at 28øC or 35øC for 5 days.

F. Yeast two-hybrid assay

The MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) was used for two-hybrid screening and all steps proceeded as described in the manufacturers protocol. AtSEC15b, AtRAB-A2a, AtRAB-A4a, and AtRAB-E1d were cloned both into pGBKT7 and pGADT7 vectors. The yeast strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4, gal80, LYS2::GAL1UAS-GAL1TATA-HIS3, MEL1, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ) was co-transformed with AtSEC15b and RABs in both orientations (i.e. AtSEC15b with either GAL4-BD or GAL4-AD). Yeasts were grown on LEU-TRP selective plates and then serial dilutions of single colonies in distiled water were made. 10 μl of each dilution were dropped on -ADE-HIS-LEU-TRP and LEU-TRP selective plates. Results were observed after 4 days.

G. Pull-down assays

Rab GTPases in pET30a+ plasmid (Novagen) were transformed into BL21 (RIPL) expression strain of *E.coli*. Expression was induced by 0.1mM IPTG, for 3 hours, 37øC, 180rpm. Pelleted cells from 50ml of bacterial culture were dissolved in 10ml of RAB lysis buffer (25mM TRIS pH=7,8, 250mM NaCl,10mM imidazol, -merkaptoethanol). Resuspended cells were sonicated, centrifuged for 1 hour, 20 000g, 4øC and supernatant was used for purification through gravity column filled with 250 μl of NiNTA agarose (Qiagen). 15 column volumes of RAB lysis buffer with higher concentration of imidazole (20mM) were used for washing. Rab proteins were eluted with 0.5ml of RAB lysis buffer with 250mM imidazole.

AtSEC15B in pGEX-3-T1 plasmid was transformed into *E.coli* BL21 Arctica Express strain. An expression was

induced by 0.1mM IPTG, for 24 hours, 12øC, 180rpm. AtSEC15B pelleted cells from 4 liters of cell culture were re-suspended in the 30ml of SEC15 lysis buffer (25mM TRIS pH=7,8, 250mM NaCl, -merkaptoethanol), sonicated and centrifuged for 1 hour, 20 000g, 4øC. 60mg of rehydrated GST agarose beads were used for AtSEC15b isolation and incubated for 1 hour, 4øC, mild shaking. Beads were washed with 15 column volumes of the SEC15 lysis buffer and split into two aliquots.

The first aliquot was incubated with 20 μg of RAB GTPase pretreated with 10mM GTP S and the second aliquot with 20 μg of RAB GTPase pretreated with 10mM GDP S for 1 hour, 4øC, mild shaking. Beads were pelleted and washed two times with the SEC15 lysis buffer, 20min, 4øC, mild shaking. Proteins bound to agarose beads were eluted by 30mM glutathione S transferase in 1M TRIS pH=8,8. SEC15B and RAB GTPases were detected on a western blot by commercial antibodies against GST-tag and His-tag.

H. Transient expression in Nicotiana benthamiana and FLIM/FRET microscopy

Both, mRFP and GFP labeled constructs for FLIM/FRET analysis were transformed to *Agrobacterium* strain GV 3101::mp90 and used for transient expression in *Nicotiana benthamiana*. Co-transformation with viral p19 protein (TBSV) was used to overcome cellular mechanisms of posttranscriptional silencing and to support high level of a transient expression [10.1016/j.febslet.2013.01.036]. Abaxial side of a leaf was investigated on the 3rd day after transformation by a laser scanning confocal imaging Zeiss LSM 880 and Nikon spinning disc microscope for co-localization studies and Zeiss LSM 780 microscope system for FLIM/FRET microscopy. The FLIM/FRET system consists of a Zeiss LSM 780 inverted microscope with external In Tune Laser (488-640nm, ¡3nm width, pulsed) 1,5mW and GaAsP (32x) detector. Photon counting module Simple-Tau 150 (Compact TCSPC system based on SPC-150) with DCC-100 (Detector Controller Card) (Becker & Hickl GmbH) was coped to Zeiss LSM system to perform FLIM experiment.

Interaction of 35S::GFP-SEC15B and 35S::mRFP-A4a,b,c,d RAB GTPases were studied in selected regions of interest (ROI) at 256x256 pixels resolution. The leaf samples expressed labeled SEC15B protein and different mRFP labeled RAB GTPases were excited with donor excitation wavelength 490nm with an appropriate laser power and PMT gain to obtain the confocal donor/acceptor image. The same ROI was excited with donor excitation wavelength 490nm to generate intensity image and photon counting on B&H Simple-Tau module with original SPCM software. To further analyze the 35S::GFP-SEC15B species distribution the B&H software SPCImage was used. For image analysis we used pixel per pixel image analysis fitting model for two component system with fixed on 5 Chi-square value, binning parameter = 1.

III. RESULTS

A. AtRAB-A4 co-immunoprecipitates with the exocyst complex

To investigate possible interaction between the exocyst subunit SEC15b and RAB GTPases, we performed coimmunoprecipitation in *Arabidopsis* transgenic lines expressing GFP-tagged proteins. Unfortunately, the expression of N-terminally GFP-tagged AtSEC15b in *Arabidopsis* seedlings was silenced, therefore we employed *Arabidopsis* lines transformed with another exocyst GFP-fused subunit, namely AtEXO84b-GFP, which was previously shown to coimmunoprecipitate with AtSEC15b (Fendrych et al., 2010) and to interact with AtSEC15b in Y2H system (Hála et al., 2008). We supposed that if there was interaction between AtSEC15b and RAB GTPases, we could detect this GTPase also using AtEXO84b-GFP as a bait. As a negative control, we have used seedlings expressing free GFP and seedlings expressing membrane protein SYP121, a SNARE protein integral to the plasma membrane (Kato et al., 2010). Eluates were tested for presence of RAB GTPases using polyclonal mouse antibody prepared against full-length AtRAB-A4a. This antibody is group-specific recognizing members of the RAB-A4 subfamily only (supplementary data). We observed that this antibody detected the presence of AtRAB-A4 GTPase in the coimmunoprecipitation pellet with AtEXO84b-GFP, but not with those of control plants expressing GFP-SYP121 or free GFP (Fig. 1**A**).

B. SEC15b localizes to the plasma membrane in N. benthamiana

The instability of GFP-fused SEC15b in *Arabidopsis* let us use transient expression in *Nicotiana benthamiana* leaves. Although this is a heterologous system, it was already successfully used for studying *Arabidopsis* exocyst localization (Kulich et al., 2013). Expressing GFP-AtSEC15b under a strong 35S promoter we obtained a signal at the plasma membrane and in its close proximity (Fig. 1**C**). With prolonged time of the observation, 20 minutes and longer, larger fluorescent spots were formed suggesting aggregation of either fusion protein itself or SEC15b-positive compartments into larger structures(not shown). Only cells with the normal 35S::GFP-SEC15b localization were analyzed.

Infiltrated epidermal cells are filled mostly with vacuole and localization around cell margins, which may also mean a layer of cytoplasm. To confirm plasma membrane localization of SEC15b in *Nicotiana benthamiana*, we used 35S::RFP-PIN1 as a marker of the plasma membrane to co-localize the signals. Both signals co-localized on the plasma membrane. A densitogram constructed from merged pictures showed the colocalization in more detail (Fig. 1**B**). While the signal of 35S::RFP-PIN is fully concentrated on the plasma membrane, the signal of 35S::GFP-SEC15b is broader showing partial localization also into cytoplasm (Fig. 1**B,C**).

We further co-expressed RFP- AtRAB-A4a with GFP-AtSEC15b in *N. benthamiana* leaves. Again, we observed

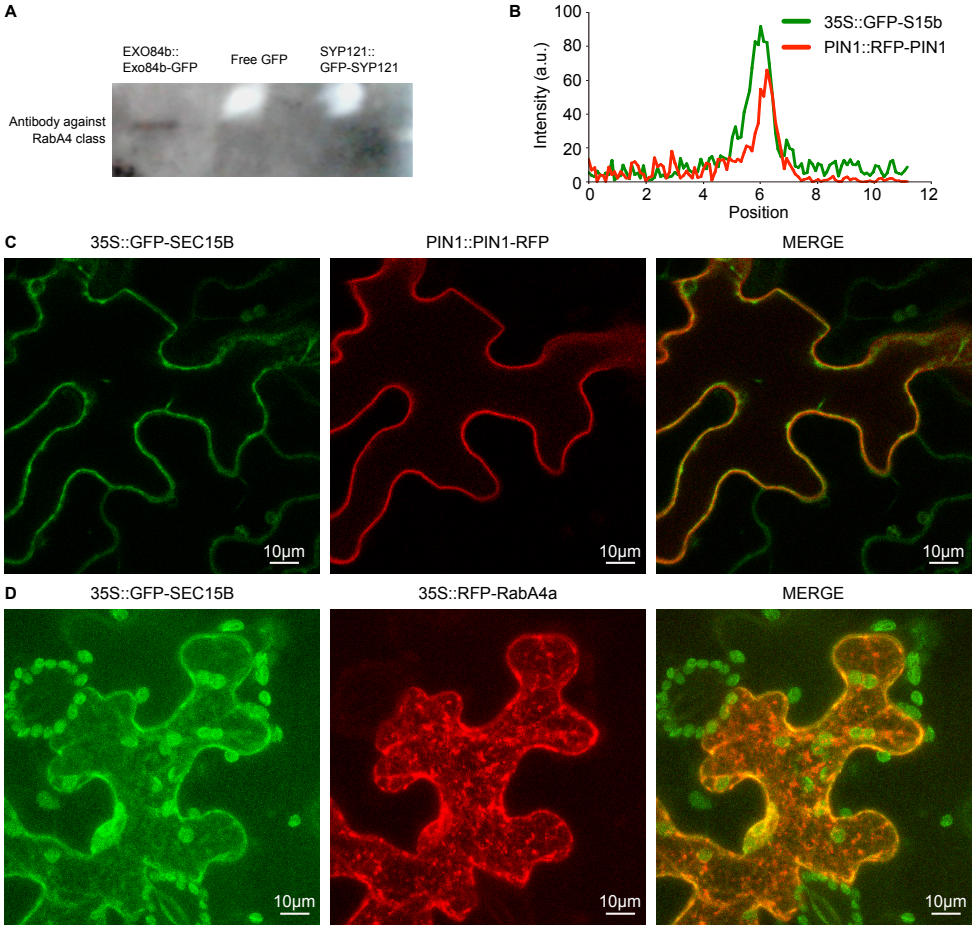

Fig. 1. Co-immunoprecipitation of RABA4 class and localization of transient expression of SEC15b and RABA4a in *Nicotiana bentamiana*. **(A)** Western blot RABA4 class coimmunoprecipitation through EXO84b::EXO84b-GFP subunit of the exocyst complex. As a controls were used free GFP and membrane protein SYP121::GFP-SYP121. In the fraction with EXO84::EXO84b-GFP we obtained RABA4 class specific signal. There was not signal in the coimmunoprecipitate with free GFP or with the SYP121::GFP-SYP121. **(B)** Comparison of the fluorescence intensity of 35S::GFP-SEC15b with plasma membrane marker PIN1::RFP-PIN1. There is overlap of signals but also noticeable shift of 35S::GFP-SEC15b signal from the plasma membrane to the cytoplasm. **(C)** Confocal images of 35S::GFP-SEC15b colocalized with PIN1::RFP-PIN1 plasma membrane marker transiently expressed in *N. bentamiana*. **(D)** Confocal images of 35S::GFP-SEC15b co-localized with 35S::RFP-RABA4a transiently expressed in *N. bentamiana*. 35S::RFP-RABA4a labels vesicular structure inside the cell and plasma membrane, where co-localize with SEC15b exocyst subunit.

co-localization up to 30% of total signal in proximity of the plasma membrane but the signal of RFP-RAB-A4a was mainly localized to spots deeper in the cytoplasm. These spots did not overlap with the GFP-AtSEC15b signal (Fig. 1**D**).

C. AtSEC15b does not interact directly with membranes

The interaction of GFP-AtSEC15b with the vesicular membrane can be caused either by direct interaction with the phospholipid bilayer or by interaction with proteins of the vesicule. To confirm or reject the first possibility, we prepared a recombinant His-tagged and GST-tagged AtSEC15b protein and tested it in lipid-binding assays. To perform phospholipid binding assay we incubated the recombinant GST-AtSEC15b with the PIP strip. We used a recombinant EXO70A1 protein known to bind specifically phosphatidic acid (**Synek˙in˙preparation**) as a positive control. There is clear signal which is phosphatidic acid specific in the case of control, while there is no binding of phospholipids by the recombinant GST-AtSEC15b (Fig. S1). This suggests that the interaction with and also association of AtSEC15b on membranes is probably indirect, mediated by protein-protein interactions.

To further corroborate our results from the previous experiment, we employed the method of Lipid Unilamelar Vesicles (LUV). This method detects binding of recombinant protein on artificial lipid vesicles with following separation by centrifugation. Unfortunately, the unstable recombinant GST-AtSEC15b sedimented in our experimental set-up even without presence of lipids forming probably large conjugates, which proved this method unsuitable (data not shown).

D. Recombinant GST-AtSEC15b interacts with members of RAB-A4 subfamily in vitro in a GTP-dependent manner

The yeast two-hybrid system (Y2H) is a powerful tool for finding interacting proteins. We tested interactions of

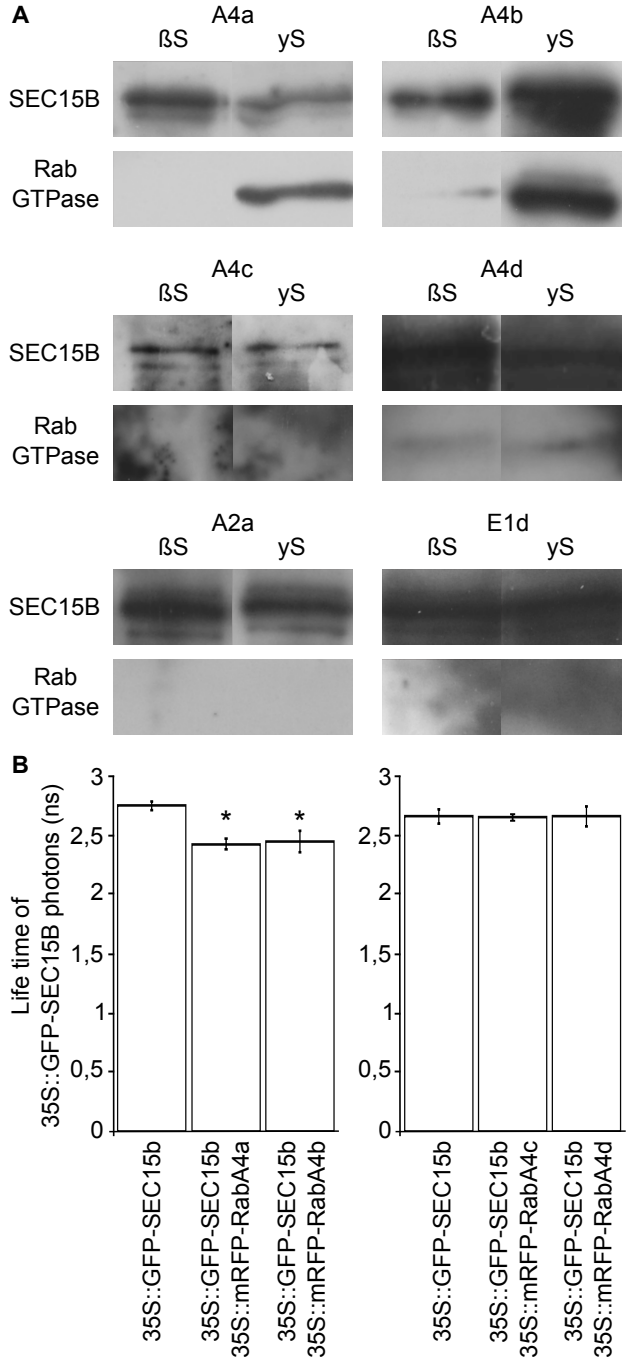

Fig. 2. *In vitro* and *in vivo* interaction of SEC15b with A4 class of RAB GTPases. **(A)** Rab GTPases exist in two forms, active and inactive. For *in vitro* interaction on pull down assay with purified GST-SEC15b, we used purified His-RAB GTPase activated by added γS (GTP analog) or inactivated by added βS (GDP analog). The GTP-dependent interaction between GST-SEC15b and RAB GTPase was confirmed in the case of His-RABA4a and His-RABA4b. We also obtained GTP/GDP independent interaction between GST-SEC15b and His-RABA4d. We did not observed GTP or GDP dependent interaction in the case of His-RABA4c and also in the case of other two Rab proteins from different classes, His-RABA2a and His-RABE1d. **(B)** For the FLIM/FRET analysis, we used the whole RABA4 class. Transiently expressed in *Nicotiana bentamiana*, we observed interaction between 35S::GFP-SEC15b and 35S::RFP-RABA4a and between 35S::GFP-SEC15b and 35S::RFP-RABA4b. We did not observed interaction between 35S::GFP-SEC15b and 35S::RFP-RABA4c and between 35S::GFP-SEC15b and 35S::RFP-RABA4d.

AtSEC15b with *Arabidopsis* RAB- A2a, RAB- E1d, RAB-A4a and RAB-A4b. All combinations were tested in both orientations, i.e. AtSEC15b with the binding domain and with the activation domain. We did not observe any interaction, moreover RAB-A4a fused with the activation domain showed autoactivation and was excluded from the experiment (Fig. S2). Furthermore we were interested in capacity of AtSEC15a and b to complement thermosensitive mutation of the yeast Sec15p exocyst subunit (Novick, Field, and Schekman, 1980). Expression of AtSEC15b under a strong ADH1 promoter did not restore growth of thermosensitive yeast strain under a non-permissive temperature 37øC indicating that AtSEC15b can not complement the yeast exocyst orthologue mutation (Fig. S2).

Based on the results from the coimmunoprecipitation analysis, we tested an interaction of recombinant proteins in a pull-down assay. Testing of the RAB GTPases interaction specificity in vitro benefits from existence of two nucleotide-bound states of RAB GTPases. Almost all true interactors known to-date prefer the GTP form of RAB GTPases. We have used recombinant RAB proteins pre-loaded with non-hydrolizable analogues of guanosine nucleotides GDP-βS or GTP-γS respectively. Different RAB GTPases prepared as His-tagged fusion recombinant proteins, either as GDP-βS- or GTP-γS-bound proteins, were used in the pull down assay with an immobilized recombinant GST-AtSEC15b. Presence of different RABs was detected in pellet using anti-His antibody.

Altogether, we used AtRAB-A4a, AtRAB-A4b, AtRAB-A4c, AtRAB-A4d, AtRAB-A2a and AtRAB-E1d for pull down assays with various results (Fig. 2). Recombinant His-AtRAB-A2a, His-AtRAB-A4c and His-AtRAB-E1d showed no interaction with recombinant GST-AtSEC15b under our experimental conditions independently on their form. Both forms of recombinant His-AtRAB-A4d were detected in pellets in comparable amounts. This observation suggests unspecific binding. Finally, His-AtRAB-A4a and His-AtRAB-A4b showed no interaction (or very weak) in the case of GDP-βS and a strong interaction in the case of GTP-γS formed conformations.

E. In vivo interaction of SEC15b and RAB A4 subfamily

In order to verify results obtained from *in vitro* experiments, we employed coexpression of GFP-SEC15b and RFP-RAB-A4 members under strong 35S promotor in *N. benthamiana*. The interaction was detected by the FLIM/FRET method. A lifetime of the excited state of every fluorophore depends strongly on its environment and energy transfers between neighboring molecules. In our experiments, we used GFP-SEC15b as a donor and RFP-RAB GTPases as acceptors. For analyses, we chose areas near the plasmatic membrane where signals of the two fusion proteins, GFP-SEC15b and RFP-RAB-A4 GTPases, overlap and where is no interference from chloroplasts. We have also chosen cells with strong enough signal to minimize influence of noise.

Fig. 2 shows that the lifetime of GFP-SEC15b dropped in the presence of RFP-RAB-A4a and b but not in the presence

of RFP-RAB-A4c and d. The interaction between AtSEC15b and both RAB-A1a and RAB-A4b was independently confirmed by two statistical significance tests, t-test and ANOVA test.

IV. DISCUSSION

The exocyst complex was originally described as an effector of Rab GTPases in yeasts. It was the interaction of Sec4p GTPase with the Sec15p subunit that opened the door for characterization of the exocytic signalling pathway and the step of exocytic vesicle tethering to plasma membrane (Salminen and Novick, 1989). Subsequently, other parts of the pathway were identified including other consecutive Rab GTPases as are Ypt31p/Ypt32p, and Ypt1p (Segev, 1991; Lai, Bard, and Kirsch, 1994; Benli et al., 1996; Jedd, Mulholland, and Segev, 1997) and guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) for Sec4p. Medkova et al. (2006) elucidate the involvement of Sec2p, the GEF that activates the Sec4 GTPase to this signalling pathway. Sec2p GEF is recruited to membrane by binding Ypt31p/Ypt32p Rab GTPase which competes for binding to Sec2p with Sec15p subunit of the exocyst complex. After initial recruitment to membranes, Sec2p adopts a different conformation that allows Sec15p to replace Ypt31p/Ypt32p in the interaction.

Unraveling of the exocyst-vesicle interaction in the *Drosophila* and mammals shows that although the secondary structure and function of the exocyst complex is conserved in eukaryotes, the interaction partners of the exocyst in the scope of Rab GTPases can differ. The Sec15 subunit preferentially interacts with Rab11 and Rab25 (Wu et al., 2005; Zhang et al., 2004) that are orthologous to yeast Ypt31/Ypt32 Rab GTPases. Moreover *Drosophila* is, along with plants, an example of a genome where direct homologue of Sec2p was not identified (Elias et al., 2003).

Interestingly little is known about mechanisms regulating subcellular localization of the Sec15 subunit. Mammalian GFP-Sec15 overexpressed in MDCK cells showed localization in punctate structures where it was later co-localized with endocytic marker (Zhang et al., 2004). A similar result was observed by Salminen and Novick (1989) when overexpression of Sec15p in yeasts caused post-golgi vesicle aggregation. These aggregates were positive on Sec15p. This is in accordance with our observation that formation of dotty structures was detectable at higher expression levels of GFP-SEC15b and might reflect general activity of SEC15 to aggregate post-golgi vesicles perhaps via interaction with RAB GTPases as suggested by Rossi et al. (2015) based on similar overexpression phenotype of Sro7p, another Sec4p effector in yeast.

An influence of the exocyst-Rab GTPase interaction by phospholipids was reported in yeasts. Mizuno-Yamasaki et al. (2010) shown that the Sec15-Sec2 interaction is inhibited by PI(4)P phospholipid. An interaction of PI(4)P with Sec2 selectively inhibits Sec15 binding and PI(4)P levels must decline, mostly by activity of Osh proteins, as vesicles reach secretory sites, allowing Sec15 to replace Ypt32 GTPase zcitepLing2014. Although PI(4)P plays important role in Sec15p function in yeasts, direct binding of Sec15p to phospholipids was never reported. Our results directly show inability of *Arabidopsis* SEC15b to bind phospholipids, which suggests that interaction with and localization of SEC15b on membranes is result of its role either as the exocyst complex component or its interaction with RAB GTPases.

The first attempts to address functional homology of the Sec15 exocyst subunits of yeasts and plants went through the complementation of yeast thermosensitive sec15 mutation with *Arabidopsis* c-DNA library but resulted only in isolation of RMA-1 protein, RING finger ubiquitin ligase, which was able to restore the temperature-sensitive growth and also secretory activity of sec15-1 mutant (Matsuda and Nakano, 1998). RMA-1, like other sec15 suppressors, was not able to suppress other exocyst subunit mutants tested. Moreover, direct complementation of thermosensitive sec15 by AtSEC15b or AtSEC15a also failed (Fig. S3).

RAB GTPases in *Arabidopsis* are divided into eight groups that contain together 18 structural subclasses (Rutherford and Moore, 2002; Vernoud et al., 2003). RAB-A group is the largest RAB group, comprising 26 members in *Arabidopsis* divided into six structural subclasses, and is homologous to two animal Rabs, Rab11 and Rab25, and yeast Ypt31/32 Rabs. Rab11 and Rab25 were reported to function on recycling endosome and they orchestrate vesicle transport from TGN to the specific regions of the plasma membrane (Welz, Wellbourne-Wood, and Kerkhoff, 2014). The plant Rab-A group underwent complicated evolution through multiplication and specialization in land plants. Rab-A6 subgroup, for example, occurs only in dicot Angiosperms (Zhang et al., 2007).

The RAB-A4 subgroup, together with RAB-A2 and A5 subgroups, is common for all land plants from moss to angiosperms (Elias et al., 2003), Purdue Genome Wiki,`http://wiki.genomics.purdue.edu/index.php/Ras_superfamily_GTPases`. Three RABA4 GTPases were published to play a role in the polar growth and in the defense response against pathogens. The RABA4b was shown to function in polarized secretion in root hair cells in cooperation with its effector PI-4KB1 and localized to the large structures derived from TGN in the tip of trichoblasts (Preuss et al., 2004; Preuss et al., 2006). RABA4c was connected with the defense against pathogens in the paper of Ellinger et al. (2014), where was shown the interaction with its effector PMR4. PMR4 is a callose synthase that is enzymatically active after translocation to the place where the fungus penetrates the cell. Thus, RABA4c can be important for localization and for activity of the callose synthase. In contrast, the yeasts homologue of callose synthase is activated through the action of RHO1 GTPase and not by RAB GTPases (Qadota et al., 1996). RABA4d is pollen specific and was shown to localize to the growing tip of the pollen tube. The disruption of the gene revealed its necessity for the proper development of the pollen tube and showed disturbed localization of cell wall components, especially pectins (Szumlanski and Nielsen,

2009).

Moreover, different changes in cell wall deposition were reported also for other insertional mutants in different RAB-A members (Lunn et al., 2013). Cellulose and hemicelluloses were deposited unevenly in mutants representing different RAB-A clades suggesting their non-redundant functions in the delivering of different cell wall components and/or cell wall important enzymes. Even more, RAB-A4b under control of the RAB-A4d promoter is unable to fully complement the RAB-A4d pollen defect (Szumlanski and Nielsen, 2009), which even more points toward non-redundant function even within the RAB-A4 clade.

Taken together, we have shown that the conserved interaction between the exocyst subunit SEC15 and RAB GTPases occurs also in plants and uncovers functional redundancy inside RAB clades but given the complexity of the secretory machinery we do not expect that presented interactions are the only ones.

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V. SUPPLEMENTS

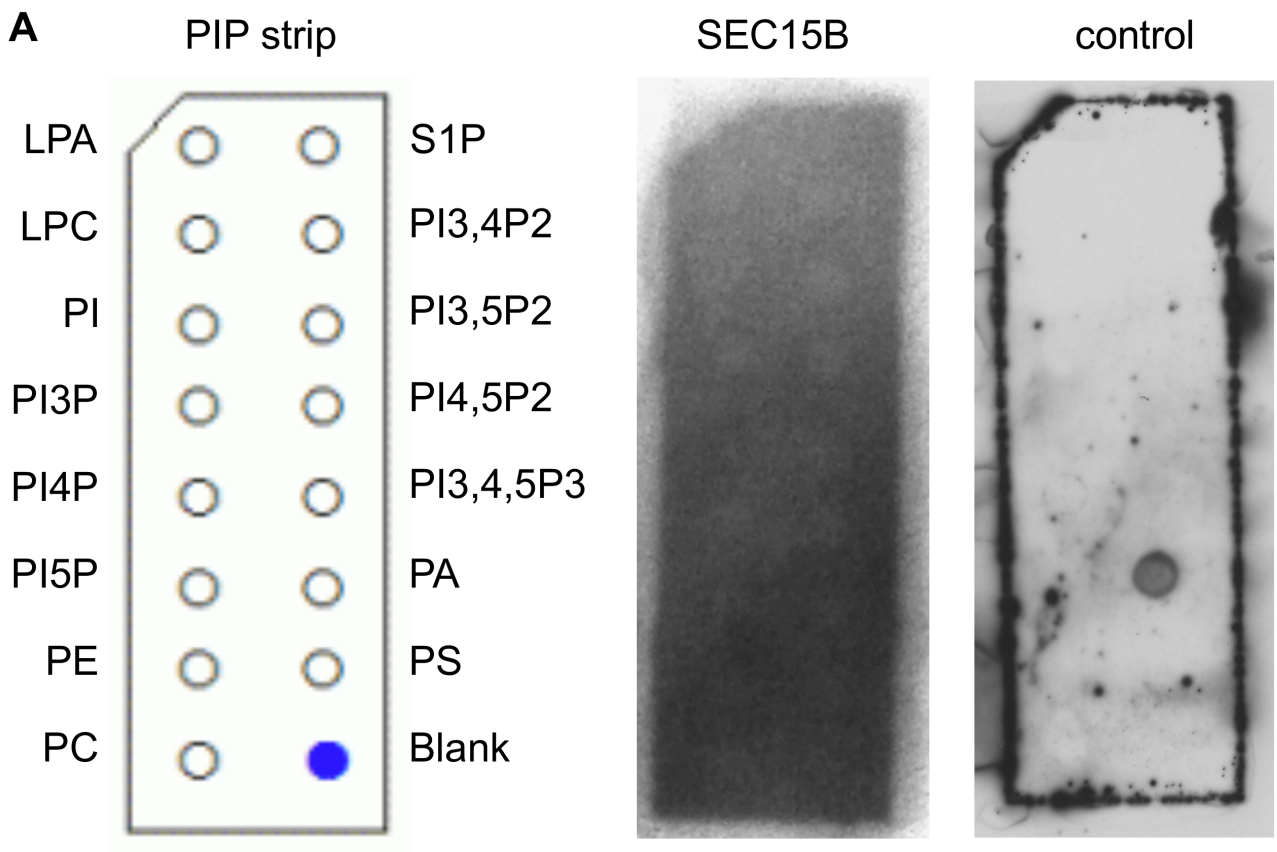

Fig. S1. The PIP strip binding assay. To test possible interaction of SEC15b exocyst subunit with phospholipids, we performed PIP strip binding assay. There was no intreaction of the SEC15b subunit with the phospholipids bound to the strip in comparison with our positive control. The higher background on the PIP strip with SEC15b protein is the result of the longer exposition.

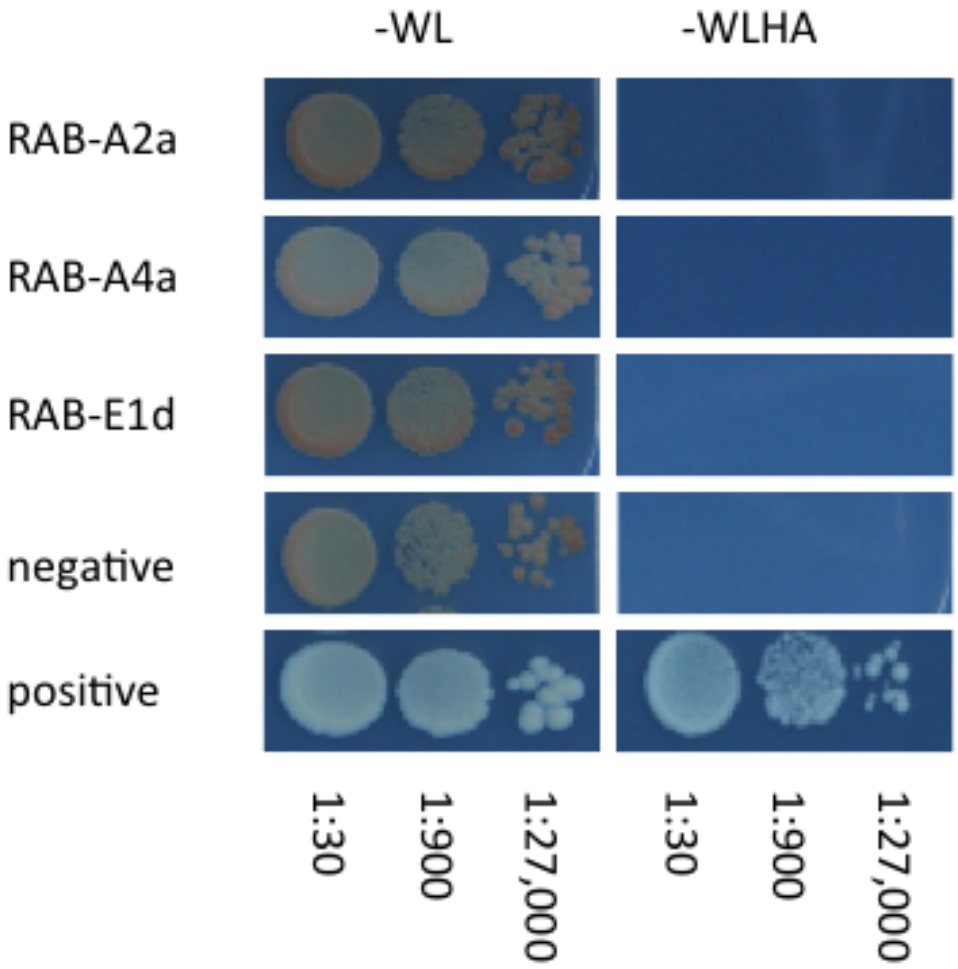

Fig. S2. Yeast two-hybrid analysis of interactions between RAB GTPases and SEC15b. The yeast strain AH109 was transformed with AtSEC15b fused with the GAL4 activation domain (AD) and different RAB GTPases fused with the GAL4 binding domain (BD). The left column shows serial dilutions of a single colony plated on a selective -LEU-TRP plate, the right column shows the same dilutions plated on a selective-LEU-TRP-HIS-ADE plate. In each case, 10 μl of yeast suspension was dropped on the plate. The negative control is represented by the yeast strain transformed with empty vectors, the yeast strain AH109 transformed with BD-AtSEC15b and AD-AtSEC10 was used as the positive control.

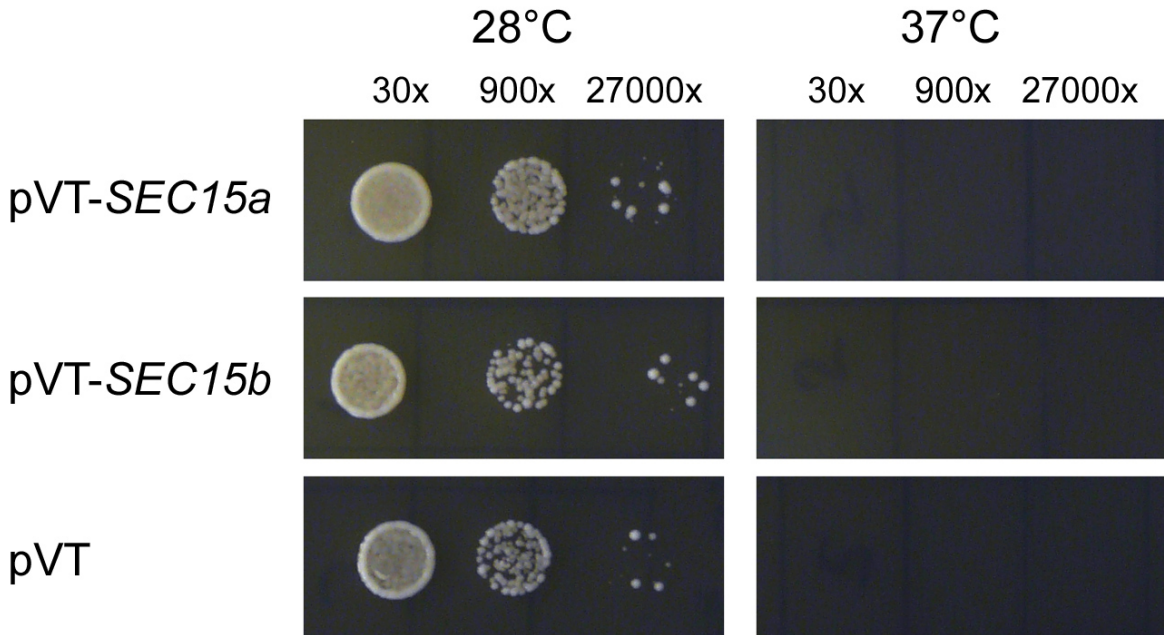

Fig. S3. The complementation of the yeast termo-sensitive mutation in SEC15 gene by plant SEC15a and SEC15b genes. For the complementation pVT vector with SEC15a or SEC15b plant gene was used. This vector was transformed to the yeast termosensitive strain with the mutation in yeast SEC15 gene. (There is the only one SEC15 gene in yeasts). In each case, 10 μl of yeast suspension was dropped on the plate. The negative control is represented by the yeast strain transformed with empty vectors. In the case of successful complementation of the termo-sensitive yeast strain, the yeasts should be able to growth in the restrictive temperature conditions of 37øC.

8.2 Interaction of the Exocyst complex with the plasma membrane

How EXO70A1 exocyst subunit interacts with the phospholipids?

The interaction of exocyst complex with the phospholipids of plasma membrane is a very up-to-date topic solved in our laboratory. The EXO70A1 subunit which was published as ubiquitously expressed in all tissues (Synek et al., 2006). The interaction between EXO70 subunit and phosphatidylinositol 4,5-bisphosphate (PI4,5P2) phospholipid on the plasma membrane is already known from the yeast model (He et al., 2007). My task was to express and purify EXO70A1 construct and point-mutated version - EXO70A1-5xE and test the ability and specificity of these proteins to bind phospholipids. The first analysis to confirm or reject possible linkage between EXO70A1 subunit and phospholipids was realized by PIP strip method. EXO70A1 construct with His-tag on the N-terminus was purified under native conditions and used for PIP strip analysis. Specific signal of our protein of interest was detected by commercial antibody against the His-tag. Fig. 8.1 shows the result of PIP strip assay, where is clear and specific interaction of EXO70A1 protein with PA phospholipid.

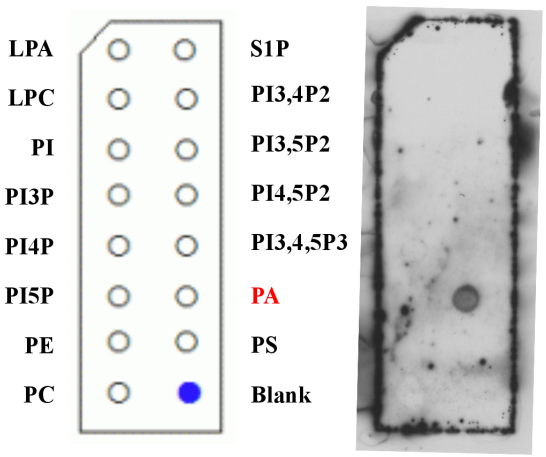

Figure 8.1: PIP strip assay with the His-EXO70A1 WT form. His-EXO70A1 WT form purified under native conditions interact specifically with the Phosphatidic Acid (PA) on the PIP strip.

To confirm PA binding by other method, the LUV method was performed. Purified EXO70A1 WT protein was incubated with different phospholipid mixtures. Phosphatidyl choline (PC) with Phosphatidyl ethanolamine (PE) represents non-specific binding and than other phospholipids (PA, PIP2, PI4P) were added in specific concentrations. LUV method (Fig. 8.2) confirmed interaction of GST-EXO70A1 with phospholipids. From the Fig. 8.2 is unmistakable the interaction of EXO70 with PA phospholipid, moreover this method showed also binding of EXO70A1 with PIP2 phospholipid.

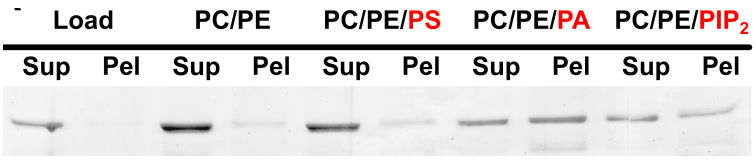

Figure 8.2: LUV assay with the GST-EXO70A1 WT form. GST-EXO70A1 WT form purified under native conditions showed interaction preference for the Phosphatidic Acid (PA) with use of LUV method. From the gel is also apparent interaction of EXO70A1 with PIP2 phospholipid that is the EXO70 interaction partner in yeasts.

Point-mutated version of EXO70A1 was created. Five aminoacids on the c-terminus were

deleted according to the accessible data from yeast EXO70 subunit that should be responsible for the phospholipid binding. This point-mutated version (EXO70A1-5xE) was expressed and purified using the same method as in the case of WT EXO70A1 form and was used for LUV method. Fig. 8.3 shows the comparison of these two EXO70A1 constructs and its interaction with phospholipids. In comparison with WT form, EXO70A1-5xE point-mutated form is not able to bind preferentially PA.

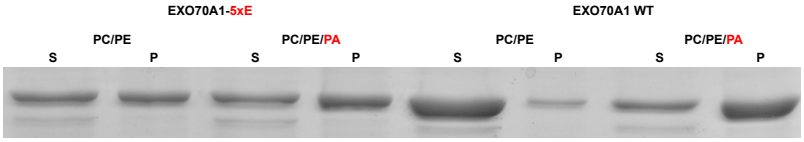

Figure 8.3: LUV assay with the GST-EXO70A1 WT form as a control and point-mutated version - GST-EXO70A1-5xE. For these LUV assay were used only PC/PE phospholipids as a negative control and PC/PE with 5%PA phospholipid as a main EXO70A1 interacting phospholipid *in vitro*. GST-EXO70A1 WT form purified under native conditions showed interaction with PC/PE/PA phospholipids. On the other hand, point-mutated version of EXO70A1 showed very low or no interaction specificity in the case of PC/PE/PA phospholipids.

9. Phenotype of Exocyst Mutants

The second part is focus on the phenotypic effect of the mutation in various exocyst subunits. This task was solved on the physiological level, predominantly.

9.1 Plasticity of Exocyst Mutant Hypocotyls

Is there common secretory phenotype for mutants in different exocyst subunits?

Paper:'Developmental Plasticity of *Arabidopsis* Hypocotyl is Dependent on Exocyst Complex Function.'

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Developmental plasticity of Arabidopsis hypocotyl is dependent on exocyst complex function

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Key Words:	Arabidopsis thaliana, exocyst, root-hypocotyl junction, auxin, etiolated hypocotyl, starch accumulation

Title: Developmental plasticity of *Arabidopsis* hypocotyl is dependent on exocyst complex function

Running title: Exocyst in hypocotyl plasticity

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SIGNIFICANT STATEMENT

Etiolated hypocotyls of exocyst mutants form an ectopic collet-like structure above regular collet that is related to deviations in auxin regulation and starch metabolism indicating an important role of exocyst in the hypocotyl plasticity regulation.

SUMMARY

The collet region, root-hypocotyl junction, is an important transition zone between different environments. Despite its crucial importance for plant development, little is known about how this transition zone is specified. Here we describe the involvement of the exocyst complex in this process. Exocyst is an octameric vesicles tethering complex involved in the secretion and membrane recycling, in plants participating in tip growth of pollen tube and root hairs, seed coat formation, cell plate and cell wall formation, hypocotyl elongation, defense and importantly also PIN auxin efflux carriers recycling and polar auxin transport. In this study morphological, anatomical, and cytological analyses of *Arabidopsis* mutants in several exocyst subunits showed the formation of a discrete region on the etiolated hypocotyl above the regular collet whose overall morphology resembles the true collet region. Penetration of this phenotypic defect is significantly influenced by cultivation temperature and carbon supply, and is related to the defect in auxin regulation. Adventitious roots induction after the dark pretreatment was compromised in the exocyst mutant hypocotyls. These observations open new insights into the regulation of developmental plasticity of the hypocotyl.

Keywords

Arabidopsis thaliana, exocyst, root-hypocotyl junction, auxin, etiolated hypocotyl, starch accumulation

INTRODUCTION

Germinating seedlings of many plant species require mechanical support and stabilization for proper development provided by their roots. Before the primary root fully develops, such stabilizing factors are the collet hairs growing from the collet region (root-hypocotyl junction). Detailed clonal analyses of *Arabidopsis* embryogenesis (Scheres et al., 1994, 1996) showed that the collet region develops during the embryogenesis concomitantly with the root and hypocotyl but mechanisms behind its establishment are not understood. The collet region is a transition zone between two structures that strikingly differ in their external and internal anatomy, i.e. the root, which has one cortical cell layer, and hypocotyl, characterized by two cortical layers (Lin and Schiefelbein, 2001). Accompanying aspects are different stages of plastids differentiation with prevailing chloroplasts in hypocotyl and amyloplasts in roots, as well as the presence of cuticle on the hypocotyl surface. Mechanisms related to collet hair growth and development were recently studied especially in respect to endoreduplication status (Sliwinska et al., 2012, 2015). The collet region in *Arabidopsis* is also characterized by the competence to form adventitious root primordia.

The plant morphogen auxin and in particular its polar transport, metabolism, and specific signaling are important for embryonic establishment of root as well as of the collet region (reviewed e.g. in ten Hove et al., 2015). In germinating seeds, auxin also plays a role in hypocotyl elongation, influences morphogenic processes involved in the collet region development, and takes part in adventitious roots establishment and development. The effect of etiolation on the hypocotyl competence to produce adventitious roots clearly indicates a post-germination process of environment dependent rooting optimization (reviewed in Verstraeten et al., 2014). While molecular pathways regulating the root formation are relatively well-known, the mechanisms controlling the formation of the collet region remain elusive.

Here we report that the plasticity of hypocotyl, specifically the formation of ectopic collet-like structures and the establishment of adventitious roots is affected by the modulation of auxin transport through the knock-down mutations in the exocyst complex. The exocyst complex participates in tethering and docking of secretory vesicles to the plasma membrane along with the big machinery of other regulatory proteins. Exocyst is an evolutionary conserved octameric complex consisting of the subunits Sec3, Sec5, Sec6, Sec8,

Sec10, Sec15, Exo70, and Exo84. In yeast and mammals, assembly of the exocyst complex enables tethering of secretory vesicles to the discrete domains of the plasma membrane (PM). This process is regulated in concert with different small GTPases and membrane lipids prior to SNARE complex formation (TerBush et al., 1996; Heider and Munson, 2012; Pleskot et al., 2015; Wu and Guo, 2015). The exocyst is ancient and ubiquitous among eukaryotes and the presence of all eight exocyst subunits has been predicted and demonstrated also in plant genomes (Hála et al., 2008; Koumandou et al., 2007; Synek et al., 2006). Comparative analyses of genomes uncovered a peculiar feature of plant exocyst - the extreme multiplication of EXO70 paralogs, which is unique to land plants. The EXO70 gene family comprises three major clades further subdivided into eight clusters (A-H) containing total number of 23 members in *Arabidopsis* while in rice there are even 47 EXO70 paralogs (Eliáš et al., 2003; Synek et al., 2006; Cvrčková et al., 2012). The highest similarity to the yeast and mammalian Exo70 is displayed by the cluster A paralogs with EXO70A1 as the most related and also expressed ortholog of this group (Synek et al., 2006).

As in animals and yeasts, the plant exocyst has been demonstrated to function as a protein complex that regulates vesicle transport to the PM (Fendrych et al., 2013; Hála et al., 2008; Kulich et al., 2010). In mutants lacking EXO70A1 or EXO84b subunits the decreased polarized trafficking of v-SNARE VAMP721 to the PM was observed (Fendrych et al., 2013). Furthermore we found that exocyst subunit EXO70A1 and SEC8 are involved in recycling of auxin transporters PIN1, PIN2 and the brassinosteroid receptor BRI1 to the PM - indicating that plant exocyst is more general regulator of PM proteins recycling (Drdová et al., 2013). As expected, exocyst knock-down mutants display various morphological or cell polarity defects, such as compromised growth of pollen tube and root hairs, defective seed coat deposition, cell plate formation during cytokinesis, and inhibited hypocotyl elongation - a consequence of perturbed vesicular trafficking (Cole et al., 2005; Fendrych et al., 2010; Hála et al., 2008; Kulich et al., 2010; Synek et al., 2006; Wen et al., 2005).

Synek et al. (2006) characterized mutants in two *exo70A1* alleles (i.e. *exo70A1-1* and *exo70A1-2*) with the same phenotypic defects. While 7-day-old light-grown mutant seedlings are still phenotypically almost indistinguishable from the WT, older plants are characterized by stunted growth, perturbed apical dominance and almost sterile inflorescence (Drdová et al., 2013; Synek et al., 2006). Moreover, 7-day-old etiolated seedlings depleted of the

exocyst subunit EXO70A1 have shorter hypocotyl as a consequence of significant reduction in the number and length of the cells (Synek et al., 2006).

Here we report that etiolated hypocotyls of different exocyst mutants (*exo70A1-1*, *exo70A1-2*, *sec15b-1*, *sec15b-2*, *exo84b-1*, and *sec8m3/LAT52::SEC8*) not only generally show reduced cell and organ elongation as reported before (Synek et al., 2006; Hála et al., 2008; Fendrych et al., 2013) but also conditionally forms previously undescribed ectopic collet hair-like structure above the normal collet region. We propose for this new class of mutants a name "twin-skirt" phenotype. These deviations are correlated with the changes in auxin response, impaired PIN3-GFP localization and ectopic starch accumulation in the affected region. Formation of adventitious roots in etiolated hypocotyl of some exocyst mutants is in parallel compromised. These observations suggest an important role of exocyst in the environmentally regulated hypocotyl developmental plasticity related to polar auxin transport and signaling.

RESULTS

Etiolated hypocotyls of exocyst mutants produce ectopic collet-like region

Exocyst mutants (i.e. mutant alleles of *exo70A1-1*, *exo70A1-2*, *sec15b-1*, *sec15b-2*, *exo84b-1*, and *sec8m3/LAT52::SEC8*) grown 5 days in dark on media containing 1% sucrose as the carbon source display developmental defects on etiolated hypocotyls (Figure 1). In order to quantify the defects in etiolated hypocotyls of *exo70A1* and *sec15b* mutants, we classified the phenotype deviation into three categories. First category encompassed simple shortening of hypocotyl (hereafter referred to as 'phenotypic deviation 1') and was reported earlier for some exocyst mutants (Synek et al., 2006; Hála et al., 2008). Newly, we uncovered a discrete defective region on the hypocotyl above the regular collet, which we call ectopic collet-like region (due to the morphological similarity to the collet), where epidermal cells were irregularly shaped (hereafter 'phenotypic deviation 2') or the irregularly shaped epidermal cells were able to form ectopic collet-like hairs (hereafter 'phenotypic deviation 3'). This ectopic collet-like region was always formed on the hypocotyl in the close proximity above the real collet region (Figure 1a, b), which was undisturbed. In the hypocotyls of *exo84b-1* and *sec8m3/LAT52::SEC8*, we distinguished only two categories of the phenotypic

defects, namely hypocotyl shortening (phenotypic deviation 1) or ectopic collet-like structure with developed hairs (phenotypic deviation 3), since extremely short hypocotyls of these mutants did not allow clear-cut detection of ectopic collet-like structure formed by irregularly shaped cells (Figure 1a, b). All these pronounced phenotypic deviations did not occur earlier than in 3-day-old etiolated seedlings (data not shown) and were never observed in the WT.

In the *exo70A1-1*, *exo70A1-2*, *sec15b-1*, and *sec15b-2* mutants, the ectopic collet-like region was separated from the regular collet by a region of apparently normal hypocotyl cells. In remaining mutants, such a separation was not always apparent due to their extremely shortened hypocotyls (Figure 1). Since the hairs formed at the ectopic collet-like region are much shorter than those at normal collet region in *exo84b-1* we could clearly identify defective region of the hypocotyl even in this extremely dwarfed mutant (Figure 1a). However, regular collet hairs of *sec8m3/LAT52::SEC8* are short and therefore the defective region is not so prominent in this mutant. Thus, we focused on relatively weak *exo70A1-2* (hereafter *exo70A1*) and *sec15b-1* (hereafter *sec15b*) mutants with relatively longer hypocotyls in our further work.

Temperature affects the formation of ectopic collet-like region in *sec15b* mutant

We noted that the penetration of the phenotypic defect (i.e. proportions of phenotypic classes described above) varied among experiments. Such variation did not depend on the seed stock used or on the time of stratification (1, 2, 5 days; data not shown). In order to test the effect of cultivation temperature, we let the seedlings grow at 28°C, 22°C, and 18°C. While in the *exo70A1* mutant the hypocotyl defect was not notably affected by cultivation temperature, the proportion of phenotypic classes significantly changed with increasing cultivation temperature in the *sec15b* mutant (χ^2 test $p<<0.001$). At 18°C, most of *sec15b* seedlings possessed only shortened hypocotyl (phenotypic deviation 1) but at 28°C most *sec15b* mutants had ectopic collet-like structures with developed hairs (phenotypic deviation 3). The cultivation at 22°C resulted in equal proportion of all phenotypic deviations (Figure 1d).

Altered internal anatomy of the ectopic collet-like region in *exo70A1* mutant

Two approaches were adopted to study the internal anatomy of the hypocotyl, i.e. transversal section using vibratome or propidium iodide (PI) staining followed by optical sectioning by confocal microscopy. Transversal sections through the ectopic collet-like region (phenotypic deviation 3) of *exo70A1* hypocotyl revealed that mutants possess irregular cell organization, enlarged diameter of the cells in the epidermis, cortex and endodermis. Collapsed cells and incomplete two layers of cortex were observed (Figure 2b, c). The number of cells in the endodermis and inner layer of cortex is in some cases also altered. Ectopic collet-like structure (phenotypic deviation 3) usually formed many hairs and, importantly, the hair-producing cells were adjacent to each other as in normal WT collet region (Figure 3c). The transversal sections of the region between the ectopic collet-like structure and true collet region displayed typical characteristics of hypocotyl, i.e. two complete layers of cortex, however the diameter of the cells in this region was also enlarged and the number of cells in the cortex and endodermis was in some cases higher than in the WT (Figure 2d).

In contrast, the longitudinal section through the ectopic collet-like region of *exo70A1* revealed extremely shortened and irregularly shaped cells in the endodermis, cortex, collapsed cells in this region and bulbous epidermis cells with ectopic hairs (Figure 2f). The improved PI staining (Truernit et al., 2008) further revealed accumulation of starch in the ectopic collet-like region of *exo70A1* mutant. This was subsequently confirmed by regular starch staining also for other exocyst mutants studied. Whereas *exo70A1* and *sec15b* mutants accumulated starch only in the affected region of the hypocotyl, *exo84b-1* and *sec8m3/LAT52::SEC8* mutants accumulated the starch in the whole above-ground tissue. Such starch accumulation was not visible in WT hypocotyls (Figure S1).

Ectopic collet-like regions formation in *exo70A1* and *sec15b* mutants is coupled with the altered auxin response

We described previously the involvement of EXO70A1 in the root rootward auxin transport and in PIN1 and PIN2 recycling (Drdová et al., 2013). To explore whether the ectopic collet-like structure on the hypocotyl of exocyst mutants can also be related to changes in auxin activity, we employed two auxin responsive reporters DR5::GUS (Ulmasov et al., 1997) and

BA3::GUS (Oono et al., 1998) in *exo70A1* and WT plants. While the DR5::GUS signal was not visible in the hypocotyl of the WT plants, the cells in the stele of ectopic collet-like region of *exo70A1* mutants showed distinct GUS activity, reporting auxin accumulation or increased auxin response (Figure 3a). There were also clear differences between WT and mutant with respect to signal of BA3::GUS (Figure 3b), which was generally more pronounced than in the case of DR5::GUS reporter. While the signal was confined to the central part of the hypocotyl in the WT plants, it occurred intensively throughout whole tissue in the defective region of *exo70A1*. Both experiments demonstrated increased auxin response or accumulation in the ectopic collet-like region of the etiolated *exo70A1* hypocotyl.

Furthermore we compared localization of PIN3-GFP in the middle and lower region of hypocotyl between the WT and *sec15b* mutant. Whereas we observed membrane localization of PIN3-GFP along the entire stele of the WT and in the middle region of mutant's hypocotyl, lower part of mutant's hypocotyl carrying the ectopic collet-like structure accumulated PIN3-GFP signal preferentially in the vacuoles (Figure 3c). We also detected weakening of PIN3-GFP signal in endodermal cells in the direction from upper to lower part of the hypocotyl in both WT and mutant seedlings (Figure 3c).

To further test the hypothesis of auxin involvement, we have grown the seedlings on the media with inhibitor of auxin transport NPA (5 µM) or with auxin itself (20nM IAA). NPA treatment partially rescued the phenotypic deviation of *exo70A1* and *sec15b* mutant seedlings. In comparison to non-treated plants, the NPA-grown mutant seedlings had high incidence of phenotypic deviation 1 and 2, while the incidence of phenotypic deviation 3 was significantly diminished (Figure 4a). The NPA-grown mutant seedlings also had significantly elongated hypocotyl when compared to non-treated parallels (Figure 4b). On the contrary, NPA had opposite effect on WT seedlings leading to the slight reduction in the hypocotyl elongation as reported previously by Jensen et al., 1998. The growth on the media supplemented with 20nM IAA had no effect on the hypocotyl elongation and frequency of phenotypic deviations 2 and 3 in *exo70A1* or *sec15b* mutants (Figure 4).

Ectopic collet-like region formation in *exo70A1* and *sec15b* mutants is coupled with the altered starch accumulation

Due to the accumulation of starch in the ectopic collet-like region of *exo70A1* and *sec15b* mutants and due to the close correlation between starch accumulation and auxin response maxima distribution, we designed experiments modulating sugar metabolism and auxin transport and observed the effect on ectopic collet-like structure formation and hypocotyl elongation. We grew seedlings on the media with reduced metabolic carbon supply by substituting sucrose with mannitol (referred to as mannitol medium). We also let them grow on the mannitol medium with additional NPA treatment (referred to as mannitol+NPA medium). The growth of mutant *exo70A1* and *sec15b* seedlings on mannitol plates significantly suppressed phenotypic deviations of the hypocotyls and significantly prolonged their hypocotyl lengths (Figure 4). Similar results were achieved on the mannitol+NPA media; moreover the effect of mannitol+NPA seemed to be most effective in restoring the hypocotyl growth and allowing proper development of the *sec15b* mutant plants (Figure 4). Slight elongation of WT hypocotyls was recorded on the mannitol media compared to control plates (Figure 4b) as also reported previously (Poupart et al., 2005).

We also compared the influence of NPA and reduced carbon supply in the media on starch accumulation in *exo70A1* and *sec15b* seedlings. Seedlings grown on NPA supplemented medium with the regular sucrose level (1%) accumulated less starch in the ectopic collet-like region (Figure 5a) and it appeared significantly closer to the regular root-hypocotyl junction than in non-treated plants (Figure 5b). In some cases, the starch accumulated right above the root-hypocotyl junction, which was never observed in non-treated mutants (Figure 5). Mutant *exo70A1* and *sec15b* seedlings grown on mannitol and mannitol+NPA media did not accumulate starch in the hypocotyl at all (Figure 5a).

The identity of cells in ectopic collet-like structure in *exo70A1* and *sec15b* mutants

Transcription factor GL2 (GLABRA2) is known to be preferentially expressed in cell files of hypocotyl or root epidermis where it prevents the differentiation of stomatal cells and root hair cells, respectively. Its expression was not observed in WT collet hair (reviewed in Qing and Aoyama, 2012). To investigate regulation of cell identity in the ectopic collet-like region, we introduced the GL2::GFP marker to *exo70A1* plants. For better orientation in anatomy of ectopic collet-like region we stained seedlings with PI. Surprisingly, hairs were formed both by cells with and without the GFP signal (Figure 6a) in the ectopic collet-like region

suggesting that GL2::GFP absence is not correlated with the presence of ectopic collet- like structures. The expression pattern of GL2::GFP in other parts of the mutant epidermis, such as hypocotyl above the defective region, regular root-hypocotyl junction, and root epidermis, was the same as in the WT (data not shown).

Next we took advantage of the fact that hypocotyl cells are known to form cuticle, while root cells do not. We used the toluidin blue dye that stains cells only in the absence of the cuticle (Tanaka et al., 2004). Short term staining of *exo70A1* mutants did not stain the ectopic collet-like structure without hairs (phenotypic deviation 2). However, protruding hairs from ectopic collet hair-like structure (phenotypic deviation 3) were strongly labelled by the dye although the body of these cells remained unlabeled (Figure 6b). The hypocotyl cells between regular root-hypocotyl junction and ectopic collet-like region were covered with the cuticle as expected (monitored by the absence of staining).

Last, we transferred 5-day-old dark grown *sec15b* seedlings to long day conditions for one week and studied the distribution of chloroplast based on the red autofluorescence of the chlorophyll in the plant tissues. Mutant plants strikingly differed from WT (Figure 6c) in this experiment. In the WT seedlings, the root-hypocotyl junction represents sharp border between chlorophyll-rich hypocotyl and chlorophyll-poor root (Scheres et al., 1994). In mutants, the hypocotyl cells above and below the ectopic collet-like structure produced high amount of chlorophyll, however the cells in the ectopic collet-like region obviously produced less chlorophyll.

Hypocotyl adventitious roots initiation after the dark pre-treatment is compromised in exocyst mutants.

To further investigate overall hypocotyl plasticity in exocyst mutants we compared WT and exocyst mutants in terms of adventitious roots induction along the hypocotyls upon the transition to light after 5 days germination and cultivation in dark (Correa et al., 2012; Verstraeten et al., 2014). As expected (Correa et al., 2012), WT plants formed adventitious roots along entire hypocotyls after etiolation, especially in the upper part (Correa et al., 2012). On the other hand, *exo70A1* and *sec15b* mutants with the ectopic collet-like structure formed adventitious roots mostly in the true collet region but formed few, if any, adventitious roots along the rest of hypocotyl (see supplementary material Table S1).

Analysis with the generalized linear mixed effect model GLMM showed that the distribution of adventitious roots is significantly affected by the plant type (for WT vs. sec15b the test z-values/p-values equal -2.28/0.027, for WT vs. exo70A1 the z-values/p-values equal -2.69/0.007), as well as by plant region (z-values/p-values for collet vs. hypocotyl region equal -4.28/1.85e-5 in *sec15b* dataset and -3.27/0.001 in *exo70A1* dataset). Importantly, GLMM also showed significant interaction between the plant type and region (z-values/p-values equaled 6.01/1.92e-09 in *sec15b* dataset and 4.11/3.9e-05 in *exo70A1*). This altogether suggests that WT and mutant plants prefer the establishment of adventitious roots in different regions along the hypocotyl. This observation indicates that post-embryonic developmental plasticity of *Arabidopsis* exocyst mutants hypocotyls is altered resulting in the formation of ectopic collet-like region.

DISCUSSION

Developmental plasticity of *Arabidopsis* hypocotyl comprises stomata formation (Qing and Aoyama, 2012), cell elongation in etiolated hypocotyl (Gendreau et al., 1997), and the ability to initiate adventitious roots along the whole etiolated hypocotyl (Verstraeten et al., 2014), the latter indicating the tendency to optimize rooting based on the seed position within the soil. Some of these processes are obviously compromised in the exocyst subunit mutants we studied.

This study documents a novel type of phenotypic deviations in etiolated hypocotyl of exocyst knock-down mutants characterized by the formation of ectopic collet-like structures localized in the discrete region closely adjacent above the regular collet. We propose to call this phenotypic deviation, which has never been observed on WT hypocotyls, „twin-skirt“phenotype. Apart from a seminal clonal analysis of *Arabidopsis* embryogenesis, which demonstrated existence of a specific root-hypocotyl junction region early in the heart and post-heart stage embryos (Scheres et al., 1994, 1996), there are very few data available for the comparison with our observations. True collet hairs at the root-hypocotyl junction are regularly produced by many plant species to fix the seedling in substrate and facilitate the water uptake before the emergence of the primary root with root hairs (Parsons, 2009). Hair-like structures were reported also in mutants constitutively overexpressing *MIF1* gene

(Hu and Ma, 2006) and in mutant with modified GL2 function (Ohashi et al., 2003). However, contrary to both these mutants characterized by hair-like structures covering large parts of their bodies (Ohashi et al., 2003; Hu and Ma, 2006), the exocyst mutants formed ectopic collet-like hairs exclusively in the discrete region on the etiolated hypocotyl close above the true collet region.

The overall morphology of the mutant aberrant hypocotyl region was generally similar to the true collect region, which lead us to call it 'ectopic collet-like region'. In particular, this region is narrowly restricted and the hairs on the ectopic collet-like region are formed on cells often adjacent to each other, similar to the hairs in the true collet region. However, the decision whether cells of the ectopic collet-like region have the identity of true root-hypocotyl junction is complicated as currently no positive developmental markers are available for this region in *Arabidopsis*.

Arabidopsis epidermal cell patterning of root and hypocotyl is well established model system to study cell fate decisions. In WT plants, the root rhizodermis differentiates periodically in root hair cell files (H cells) and non-hair cell files (N cells) and analogously hypocotyl epidermis differentiate stomata cell files and non-stomata cell files (e.g. Qing and Aoyama, 2012). The only region where this pattern is not preserved is the root-hypocotyl junction where collet hairs are formed by all epidermal cells (Qing and Aoyama, 2012). One of the crucial players in this patterning regulation is the transcription factor GL2, which is specifically expressed in N cells and represses root hair and stomata formation. GL2 is not expressed in the root-hypocotyl junction, allowing collet hair formation from all epidermal cells (Qing and Aoyama, 2012). Therefore, GL2 might be considered as a negative marker for the true collet region. We proved that GL2::GFP is continuously expressed through the entire hypocotyl of *exo70A1* mutant and that also in the hair carrying cells of the ectopic collet-like region in *exo70A1* mutants are expressing GL2. This might indicate that hairs emerging from the ectopic collet-like structure are initiated from epidermal cell with original hypocotyl identity and that GL2 repression in true collet is not substantial for the formation of ectopic collet-like hairs. The developmental plasticity of hypocotyl epidermal cells thus appears to be compromised in exocyst mutants resulting in the formation of the ectopic collet-like structure.

Disturbed identity of cells in the ectopic collet-like structure of the hypocotyl is further indicated by the non-homogenous distribution of the cuticle on the surface of the

ectopic collet hair-like structure as demonstrated by toluidin blue staining of emerged hairs (Figure 6) and by the reduction of chlorophyll in the mutants recovered from the dark on the light, which was precisely restricted to the ectopic collet-like region but not above or below this region. This indicates that the alteration of cell fate is confined to the ectopic collet-like structure and that the region of hypocotyl that is located between this one and the true root-hypocotyl junction has the identity of normal etiolated hypocotyl.

Anatomical analyses showed that the defect in *exo70A1* mutant extends into the organization of inner tissues (cortex, endodermis), where irregular cell organization and incomplete cell layers in the cortex were observed. The hypocotyl of the WT plant is known to contain two layers of properly organized cortical cells (outer layer containing 14.6±0.9 cells and inner layer containing 8.0±0.0 cells) (Lin and Schiefelbein, 2001). The true collet region thus represents a transition zone from such a two-layer cortex structure to the one-layer cortex structure of the root (Lin and Schiefelbein, 2001). Two incomplete cortex layers of the ectopic collet-like region may thus resemble the transitory state of the true collet region.

At this stage, we are not unambiguously able to decide, at what ontogenetic stage this aberrant developmental deviation is established. Indeed, the hypocotyl cell numbers are determined already during the embryonic phase (Gendreau et al., 1997). Given that Synek et al. (2006) described reduced cell number in epidermis of etiolated *exo70A1* hypocotyls, it may be possible that the competence to form ectopic collet-like structure is established already during the embryonic development correlated with a lower number of cells in mutant's hypocotyls. However, it is important to note that the observed phenotypic deviations occur only in dark-grown seedlings, while light-grown mutant seedlings do not show any similar defect in the hypocotyl. This rather indicates that the defect of cell layers in the ectopic collet-like region is the result of postembryonic development, possibly accompanied by the cell death in the dark grown seedlings. Such an interpretation is consistent with confocal microscopy of PI stained mutant hypocotyls showing collapsed cells in the ectopic collet-like region. Moreover, we have also shown that the cultivation temperature significantly affects the frequency of phenotypic classes in *sec15b*, which is another strong indication that the ectopic collet-like structures evolve during postembryonic development. Another strong evidence for a postembryonic process in initiation of the ectopic collet-like region is the presence of GL2 marker in this region.

Our data clearly indicate that the formation of the ectopic collet-like region and the defect in the hypocotyl adventitious root formation are both correlated with defective distribution of auxin, which is well established morphogen directing whole plant patterning including the control of the adventitious root formation (Verstraeten et al., 2014) . Compared to other parts of hypocotyl, the ectopic collet-like region of *exo70A1* mutants shows increased auxin response using both DR5::GUS and BA3::GUS reporters and our data further suggest that auxin accumulates in the stele (DR5::GUS) of the defective region and also penetrates the whole tissue (BA3::GUS; see Figure 3). Crucially, the treatment with NPA, the inhibitor of polar auxin transport, partially, yet distinctly, rescues the phenotype of *exo70A1* and *sec15b* mutant, reduces the frequency of phenotypic deviations 2 and 3, and reduces the amount of starch in the hypocotyl. We assume that NPA treatment inhibits auxin transport from the apex (Lehman et al., 1996) and hence restores the balance in the auxin distribution in the hypocotyls of *exo70A1* and *sec15b* mutants resulting in the normalization of phenotypes.

Our previous study showed that EXO70A1 is involved in the rootward polar auxin transport and has an impact on PIN1 and PIN2 recycling (Drdová et al., 2013). Therefore, known role of auxin in tissue patterning and adventitious roots formation along with our data in this report, suggest that perturbed auxin distribution plays an important role in the development of phenotypic deviations on hypocotyls of *exo70A1* and *sec15b* mutants. This is also supported by the fact that PIN3-GFP is localized preferentially to the vacuoles in the ectopic collet-like region of *sec15b* mutant. However, it seems unlikely that the formation of the ectopic collet-like structure is induced solely by the elevation of auxin level in etiolated hypocotyls since the elevation of auxin levels by gene manipulation or external application of IAA in *Arabidopsis* seedlings does not induce any ectopic collets and results in the induction of adventitious roots along the hypocotyl (Boerjan et al., 1995; King et al., 1995).

Interestingly, apart from NPA treatment, the phenotype of mutant plants could also be rescued by the growth on the media with no additional sucrose, which resulted in reduced formation of ectopic collet-like structures. Mere substitution of sucrose with mannitol also resulted in reduced starch accumulation in mutant plants, which is consistent with the fact that sucrose is a starch precursor, and may suggest that phenotypic deviations are linked with carbon metabolism. However, sucrose is not only starch precursor, but also an important morphological signal in the etiolated hypocotyls (Rolland et al., 2006) and its

elevation in the growth media affects the expression of auxin related signaling compounds, promotes auxin accumulation in etiolated seedlings (Stokes et al., 2013) and induces expression on PIN7 and promotes acropetal auxin transport from the cotyledons to the root (Lilley et al., 2012). Since the defects in hypocotyls of *sec15b* were most strongly suppressed by simultaneous treatment of mutant plants with both the mannitol and NPA, it is also possible that effects of sucrose as a carbon supply and signaling molecule are additive with the auxin transport and signaling and might be at least partly independent.

Since hypocotyl defects depended strongly on the cultivation conditions (dark grown plants, temperature) and GL2 expression pattern along with cuticle distribution might indicate hypocotyl-related identity of ectopic collet-like region, we interpret observed deviations in exocyst mutants as a result of post-embryonic process. Obviously, the root-hypocotyl junction is correctly established in exocyst mutants, but the cells in the basal part of the hypocotyl near the root-hypocotyl junction did not maintain this original identity during dark germination and subsequent cell elongation, and started to differentiate as the ectopic collet-like structure. Our data indicate that disturbed auxin transport and sugar metabolism are crucial components in the explanation of these phenotypic defects in the exocyst mutants.

Root-hypocotyl junction is an important transition zone in the Angiosperm plants and its correct organization together with hypocotyl developmental plasticity allowing optimal adventitious root positioning along the hypocotyl is crucial for plant fitness. Here we show new role of the exocyst complex in these processes and indicate an important contribution of auxin transport and signaling as expected. After the years of negligence fascinating collet region of root-hypocotyl junction deserves lot of experimental attention in the future.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Mutant *Arabidopsis* lines *exo70A1-1, exo70A1-2* (Synek et al., 2006), *sec8m3/LAT52::SEC8* (Cole et al., 2005) and *exo84b-1* (Fendrych et al., 2010) have been described previously as well as lines expressing DR5::GUS (Ulmasov et al., 1997), BA3::GUS (Oono et al., 1998), *PIN3::PIN3-GFP* (Žádníková et al., 2010) and GL2::GFP (Lin and Schiefelbein, 2001). Mutant

line *sec15b-1*, SALK_130663 of the Columbia-0 ecotype of *Arabidopsis thaliana* L. Heynh with T-DNA insertion was obtained from the SALK Institute (Alonso et al., 2003); transposonal insertional line of the Nossen-0 ecotype *sec15b-2,* RATM15-1183-1_H was obtained from Riken BRC (Ito et al., 2002) and backcrossed to Columbia-0 ecotype. The location of each T-DNA insertion within the *SEC15b* gene (At4g02350) was verified by PCR with specific primers (see supplementary material Table S2).

Arabidopsis seeds were surface-sterilized 10 min in 20% household bleach (Bochemie, www.savo.eu), rinsed 3 times with sterile distilled water, and sowed onto agar plates with growing medium: ½MS-salts (Sigma, www.sigmaaldrich.com) supplemented with 1% (w/v) sucrose (Fluka, www.sigmaaldrich.com/Fluka) or mannitol (0,528%), vitamins, 1.6% (w/v) plant agar (all Duchefa, www.duchefa.com), buffered to pH 5.7. Stratification was performed at 4°C for 2 days in dark. Seedlings were grown vertically in a climate chamber typically at 22°C or other temperatures as mentioned in the text under continual dark or under long day (16h/8h) conditions.

mRNA level analysis by RT-PCR

Samples of 2-week-old seedlings were harvested and immediately frozen in liquid nitrogen. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. RNA (1 µg) was converted to cDNA by the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer's recommendations using an oligo-dT primer. The cDNA was amplified by PCR using a set of primers specific to the *SEC15B* (At4g02350) gene (see supplementary material Table S2, Figure S2). An equal quantity of PCR product was loaded on 0.8% agarose gel. PCR amplification of the constitutively expressed actin gene *ACT7* (At5g09810) was used as an internal amplification and template control. The graphical view of insertion positions and confirmation of null alleles of *sec15b-1* and *sec15b-2* by sqRT-PCR see in supplementary material (Figure S2).

Staining

For GUS staining, 5-day-old etiolated seedlings were vacuum-infiltrated with staining solution (50mM sodium phosphate buffer [pH 7.2], 250µM $K_3Fe(CN)_6$, 250µM $K_4Fe(CN)_6$, 2% Triton-X, and 1mM X-GlcA (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, Duchefa, www.duchefa.com) for 1 h and then incubated at 37°C till proper colour development.

Five-day-old etiolated seedlings were stained for 5 min with the Lugol's solution, followed by 10 min washing.

Improved propidium iodide staining was done as described in (Truernit et al., 2008) with modifications (80°C prewarmed ethanol was applied for 1 min and Hoyer's solution was not used).

Nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) diluted in ½ MS (1 µg ml^{-1}). Solution was applied for 15 min, followed by washing.

Cuticular defect was detected using an aqueous solution of 0,05% toluidin blue (Sigma-Aldrich) for 10 min, followed by washing with water.

Microscopy

Morphology of seedlings was documented by binocular Leica S6D with camera attached. Seedlings expressing DR5::GUS, BA3::GUS, toluidin or Lugol's solution (Sigma, www.sigmaaldrich.com) staining were documented using an Olympus BX-51 microscope with an Olympus DP50 camera attached.

Seedlings expressing GL2::GFP and propidium iodide stained seedlings were observed using a Spinning Disc confocal microscope (Yokogawa CSU-X1 on Nikon Ti-E platform, laser box Agilent MLC400, Zyla sCMOS camera by Andor). Full z-stack confocal images were 3D-projected using the Fiji software.

Seedlings expressing PIN3-GFP were observed using a Zeiss LSM 880 confocal scanning microscope equipped with Zeiss C-Apochromat 40x/1.2 water-corrected lens.

Vibratome

Seedlings fixed in 5% agar were cut using vibratome to 150 µm sections and observed using Olympus AX70 microscope.

Statistical methods

We used generalized linear mixed effect model with poisson error distribution (GLMM implemented in the R library lme4; Bates et al., 2014) to test for differences in the distribution of numbers of adventitious roots between mutant and WT plants in respective regions (root-hypocotyl junction and hypocotyl). Specifically, we tested whether the number of adventitious roots, as the dependent variable, significantly depends on three explanatory variables, i.e. plant type (WT or mutant), plant region and the interaction between both. The fitted models incorporated a nested group effect of individual plants being nested within plant types. The models were fitted separately to the dataset incorporating *sec15b* mutant with its WT counterpart and to the dataset incorporating *exo70A1* mutant with the respective WT counterpart. The significance of individual terms was tested with the lmerTest library of R.

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Author contribution

E.J.D.- planning of experiments, main body of experimental work, interpretation of data and writing of the manuscript, M.R. and M.H.- characterization of *sec15b* mutant, starch accumulation experiments, K.J.- statistic analyses of data and writing, H.S.- preparation of and analysis of *Arabidopsis* lines with markers. V.Ž.- planning of experiments, managing of the project, interpretation of data and writing. All authors contributed to manuscript preparation in their respective parts.

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LEGENDS TO FIGURES

Figure 1. Phenotypic defects of etiolated hypocotyls in exocyst mutants (*exo70A1*, *sec15b*, *exo84b and sec8m3/LAT52::SEC8*).

(a) Ectopic collet-like structure with developed hairs (phenotypic deviation 3) on etiolated hypocotyls of 5-day-old *exo70A1*, *sec15b*, *exo84b* and *sec8m3/LAT52::SEC8* mutants. (b) Ectopic collet-like structure formed by irregularly shaped cells (phenotypic deviation 2) in a discrete hypocotyl zone of *exo70A1* and *sec15b* mutants. Irregularly shaped cells in the discrete region near the root-hypocotyl region are not distinguishable in the dwarfish mutants *exo84b* and *sec8m3/LAT52::SEC8* (c) Shortened hypocotyls without additional structures (phenotypic deviation 1) in *exo70A1*, *sec15b*, *exo84b* and *sec8m3/LAT52::SEC8* mutants; collet hairs pointed by black arrows, ectopic collet-like structure (phenotypic deviation 2 and 3) pointed by red arrows. (d) Effect of cultivation temperature on the proportion of three phenotypic categories in mutants *exo70A1* and *sec15b*. Mosaic diagrams display percentage of individual phenotypic categories (phenotypic deviation 1, 2, 3) of mutant *exo70A1* and *sec15b* developed under different cultivation temperature (18°C, 22°C, 28°C). In total 233 *exo70A1* and 452 *sec15b* mutant plants were analysed and proportion of phenotypic categories significantly changed with increasing cultivation temperature in the *sec15b* mutant (χ^2 test $p<<0.001$). The width of the column reflects the amount of counted seedlings for certain cultivation temperature. Displayed mosaic graph represents one experiment. The experiment was repeated 3 times with the similar result.

Figure 2. Anatomy of the ectopic collet-like region with developed hairs (phenotypic deviation 3) in *exo70A1* mutant.

(a-d) Transversal sections through etiolated hypocotyl of 5-day-old seedlings. (a) WT with two complete layers of the cortex, 8 cells in endodermis and 8 cells in the inner layer of cortex; (b,c) Irregular organization of epidermis and cortex of the *exo70A1* mutants in the ectopic collet-like region (phenotypic deviation 3). Note the adjacent position of ectopic-hair carrying cells. Collapsed cells pointed by white arrows, incomplete two layers of cortical cells pointed by black arrow (d) Section through the *exo70A1* mutant hypocotyl below the defective region, 9 cells in endodermis and 9 cells in the inner layer of cortex (sections from 2 mutant hypocotyls out of 5 showed this type of deviation). ep - epidermis; c1, c2 - two layers of cortex; en - endodermis. (e-f) Longitudinal sections through etiolated hypocotyl of

PI stained 5-day-old seedlings. (e) WT seedling. (f) Section through the ectopic collet-like region of *exo70A1* hypocotyl, displayed irregular cell organization, collapsed cells and starch accumulation. Starch granules pointed by red arrows.

Figure 3. Etiolated hypocotyls of exocyst mutants with introduced auxin response reporters DR5::GUS, BA3::GUS and auxin transporter PIN3-GFP.

(a) Distribution of activity of DR5::GUS auxin response reporter shows the accumulation of auxin in the stele of ectopic collet-like structure of *exo70A1* mutant. (b) Distribution of activity of BA3::GUS auxin response reporter shows the penetration of the BA3::GUS signal to the whole tissue of irregularly shaped cells of *exo70A1* compared to its localization to the stele in the WT. (c) Localization of PIN3-GFP in the middle and lower region of the hypocotyl in the WT and *sec15b* mutant. Membrane localization of PIN3-GFP is disturbed and preferentially detected in the vacuoles in the ectopic collet-like region (lower region of hypocotyl) of *sec15b* mutant, collet hairs pointed by black arrows, ectopic collet hair-like structure pointed by red arrows, endodermal cells pointed by white arrowhead, stele cells pointed by red arrowheads.

Figure 4. Effect of growth media on the phenotypic defect in the mutant plants.

(a) Incidence of hypocotyl defect (phenotypic deviation 1, 2, 3) depending on the type of treatment in *exo70A1* and *sec15b*. The effects of medium type on the proportion of observed phenotype classes were highly significant in all cases (χ^2 test, $p<<0.0001$). Both experiments were repeated twice with similar results. In each experiment, more than 800 mutants were analysed and the width of the column reflects the amount of counted seedlings grown on certain cultivation media. Displayed mosaic graph represents one experiment. No WT seedlings were counted because no such hypocotyl defects were ever observed (b) Hypocotyl length depending on the type of treatment (NPA, mannitol, mannitol+NPA, IAA) in WT, *exo70A1* and *sec15b*. For each cultivation media the number of counted seedlings were n>70.

Figure 5. Influence of NPA treatment, reduced carbon supply, and the combination of both on the accumulation of starch in the etiolated hypocotyls of *exo70A1* and *sec15b*.

(a) 5-day-old etiolated seedlings of *exo70A1* and *sec15b* grown on the NPA (5 µM) plates had milder hypocotyl defect and reduced starch accumulation compared to non-treated mutants. Mutant seedlings grown on the mannitol and mannitol+NPA media displayed reduced hypocotyl defect without starch granules compared to non-treated mutants. No starch accumulation was observed in the hypocotyl of WT seedlings. (b) Influence of NPA treatment on the position of ectopic collet-like structure in the hypocotyl of *exo70A1*. Collet hairs pointed by black arrows, ectopic collet-like structure pointed by red arrows. The number of counted seedlings for each group was n>10.

Figure 6. Disturbed cell identity of ectopic collet-like structure in exocyst mutants.

(a) Expression of GL2::GFP in the cell files of WT hypocotyl and in ectopic collet-like structure of *exo70A1* mutant, cell walls were stained for 10 min in PI (red signal). 3D-projection of z-stack confocal images. (b) Incorporation of toluidin blue into 5-day-old etiolated seedlings of *exo70A1* indicates the absence of the cuticle in the emerging hairs but not in the irregularly shaped cells of ectopic collet-like structure. Higher magnification bottom line. (c) Red autofluorescence of chlorophyll in the ectopic collet-like region of *sec15b* seedlings grown 5 days in dark and 7 days in long day conditions is reduced (GFP filter, bright field). Collet region pointed by white arrows, ectopic collet-like structure pointed by red arrows.

Figure S1. The starch accumulation in exocyst mutants.

The starch was accumulated in the collet-like region of *exo70A1* and *sec15b* mutants and whole above-ground tissue of *exo84b* and *sec8m3/LAT52::SEC8* mutants. No starch was observed in the WT hypocotyl.

Table S1. The number of adventitious roots emerged on regenerating seedlings after dark pre-treatment.

Exocyst mutants mostly intiated adventitious roots in the true collet and less along the hypocotyl. WT formed adventitious roots preferentially along the hypocotyl.

Table S2. Primers used for genotyping and mRNA level analysis of *sec15b-1* and *sec15b-2* mutant lines.

Figure S2. (A) Graphical visualization of *sec15b-1* and *sec15b-2* insertions in *SEC15B* gene and promoter region. (B) Confirmation of knockout mutation of *SEC15B* gene in *sec15b-1* and *sec15b-2* mutant lines.

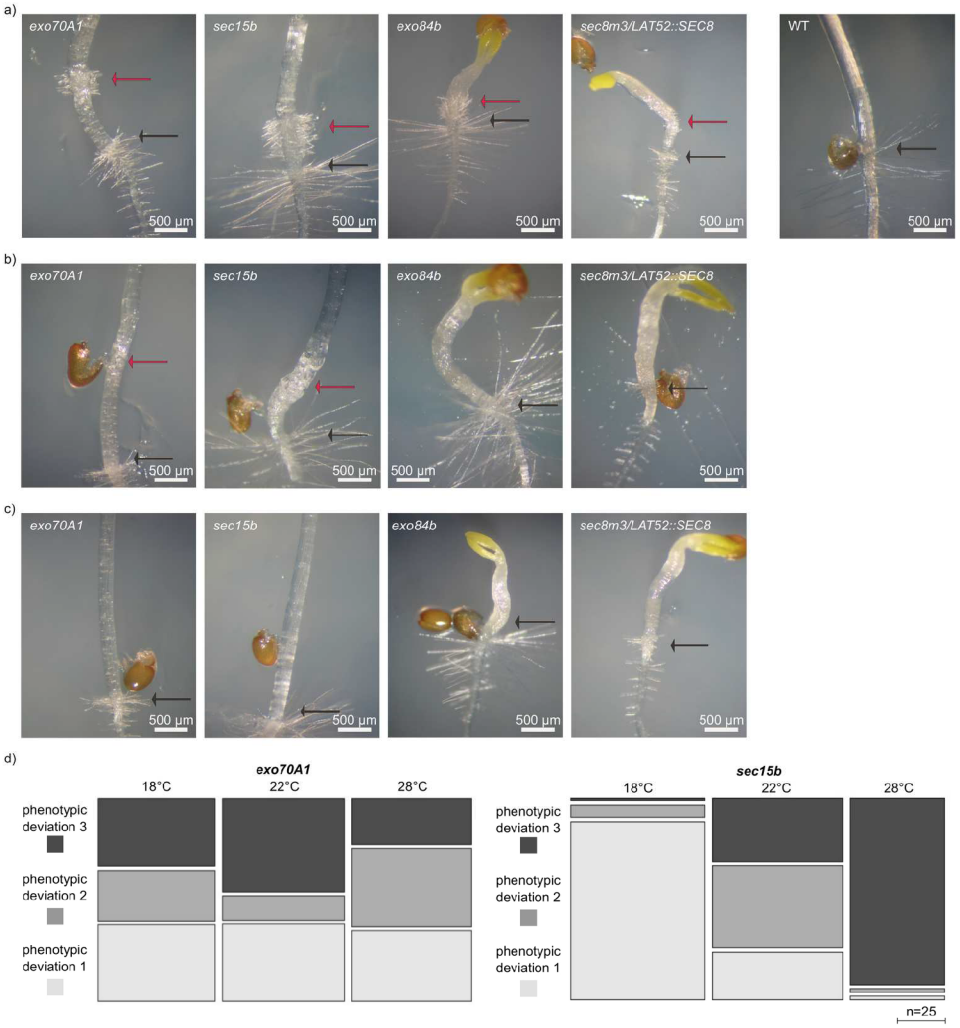

Figure 1. Phenotypic defects of etiolated hypocotyls in exocyst mutants (exo70A1, sec15b, exo84b and sec8m3/LAT52::SEC8).

(a) Ectopic collet-like structure with developed hairs (phenotypic deviation 3) on etiolated hypocotyls of 5-day-old exo70A1, sec15b, exo84b and sec8m3/LAT52::SEC8 mutants. (b) Ectopic collet-like structure formed by irregularly shaped cells (phenotypic deviation 2) in a discrete hypocotyl zone of exo70A1 and sec15b mutants. Irregularly shaped cells in the discrete region near the root-hypocotyl region are not distinguishable in the dwarfish mutants exo84b and sec8m3/LAT52::SEC8 (c) Shortened hypocotyls without additional structures (phenotypic deviation 1) in exo70A1, sec15b, exo84b and sec8m3/LAT52::SEC8 mutants; collet hairs pointed by black arrows, ectopic collet-like structure (phenotypic deviation 2 and 3) pointed by red arrows. (d) Effect of cultivation temperature on the proportion of three phenotypic categories in mutants exo70A1 and sec15b. Mosaic diagrams display percentage of individual phenotypic categories (phenotypic deviation 1, 2, 3) of mutant exo70A1 and sec15b developed under different cultivation temperature (18°C, 22°C, 28°C). In total 233 exo70A1 and 452 sec15b mutant plants were analysed and proportion of phenotypic categories significantly changed with increasing cultivation temperature in the sec15b mutant ($\chi 2$ test $p<<0.001$). The width of the column reflects the amount of counted seedlings for certain cultivation temperature. Displayed mosaic graph represents one experiment. The experiment was repeated 3 times with the similar result.

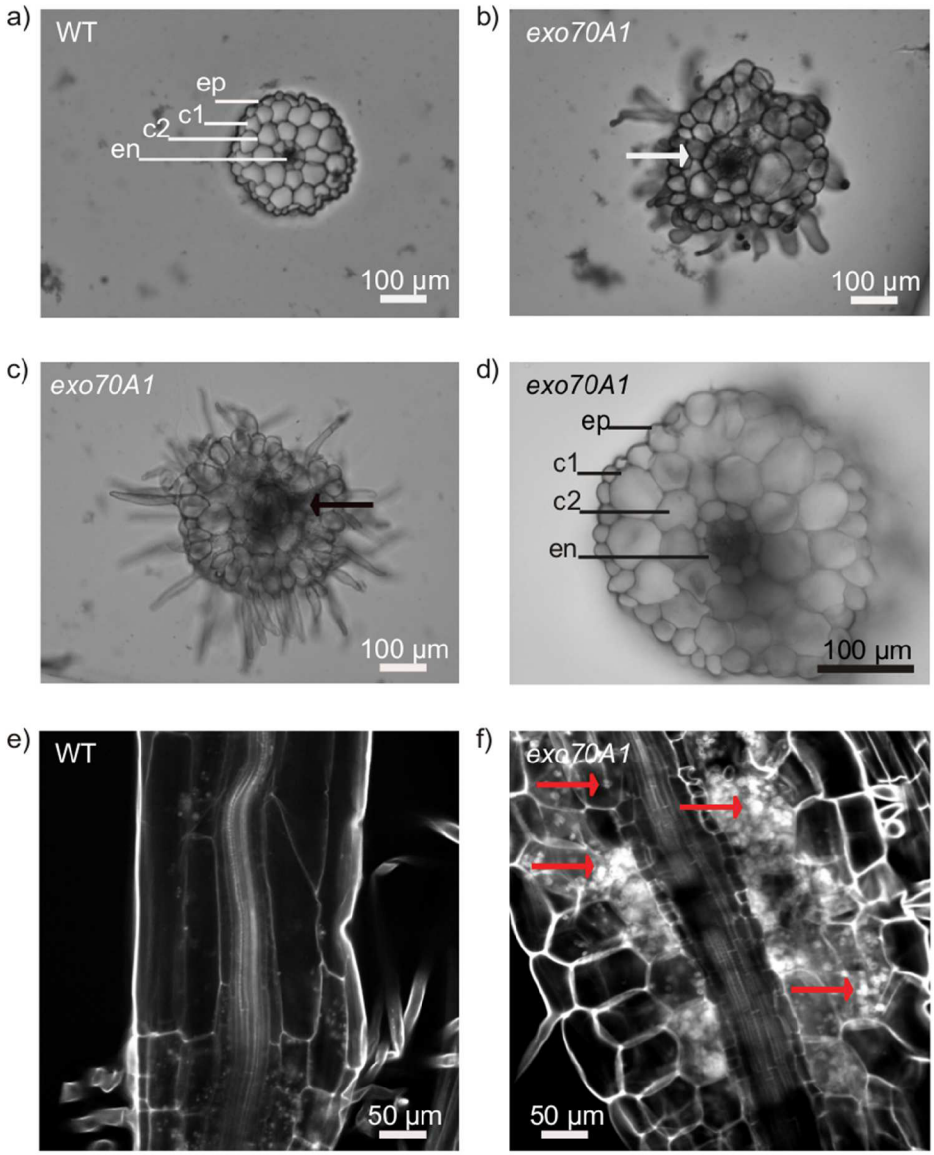

Figure 2. Anatomy of the ectopic collet-like region with developed hairs (phenotypic deviation 3) in exo70A1 mutant.
(a-d) Transversal sections through etiolated hypocotyl of 5-day-old seedlings. (a) WT with two complete layers of the cortex, 8 cells in endodermis and 8 cells in the inner layer of cortex; (b,c) Irregular organization of epidermis and cortex of the exo70A1 mutants in the ectopic collet-like region (phenotypic deviation 3). Note the adjacent position of ectopic-hair carrying cells. Collapsed cells pointed by white arrows, incomplete two layers of cortical cells pointed by black arrow (d) Section through the exo70A1 mutant hypocotyl below the defective region, 9 cells in endodermis and 9 cells in the inner layer of cortex (sections from 2 mutant hypocotyls out of 5 showed this type of deviation). ep - epidermis; c1, c2 - two layers of cortex; en - endodermis. (e-f) Longitudinal sections through etiolated hypocotyl of PI stained 5-day-old seedlings. (e) WT seedling. (f) Section through the ectopic collet-like region of exo70A1 hypocotyl, displayed irregular cell organization, collapsed cells and starch accumulation. Starch granules pointed by red arrows.

79x98mm (300 x 300 DPI)

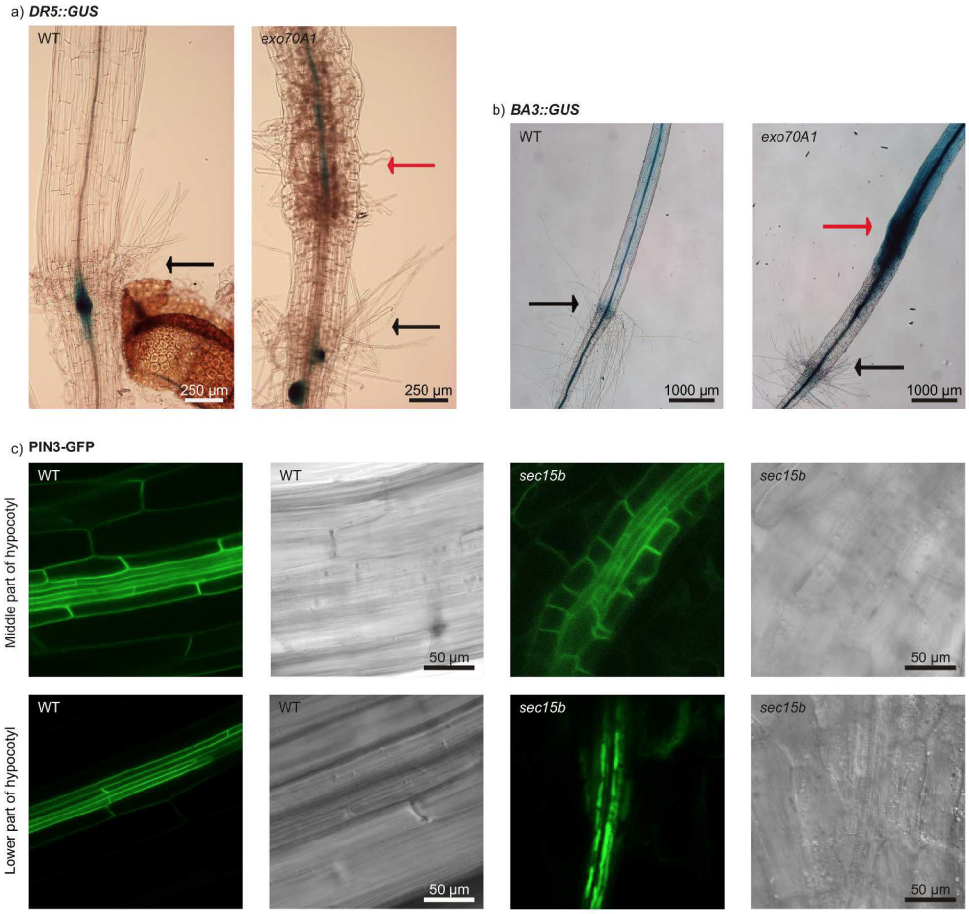

Figure 3. Etiolated hypocotyls of exocyst mutants with introduced auxin response reporters DR5::GUS, BA3::GUS and auxin transporter PIN3-GFP.
(a) Distribution of activity of DR5::GUS auxin response reporter shows the accumulation of auxin in the stele of ectopic collet-like structure of exo70A1 mutant. (b) Distribution of activity of BA3::GUS auxin response reporter shows the penetration of the BA3::GUS signal to the whole tissue of irregularly shaped cells of exo70A1 compared to its localization to the stele in the WT. (c) Localization of PIN3-GFP in the middle and lower region of the hypocotyl in the WT and sec15b mutant. Membrane localization of PIN3-GFP is disturbed and preferentially detected in the vacuoles in the ectopic collet-like region (lower region of hypocotyl) of sec15b mutant, collet hairs pointed by black arrows, ectopic collet hair-like structure pointed by red arrows, endodermal cells pointed by white arrowhead, stele cells pointed by red arrowheads.

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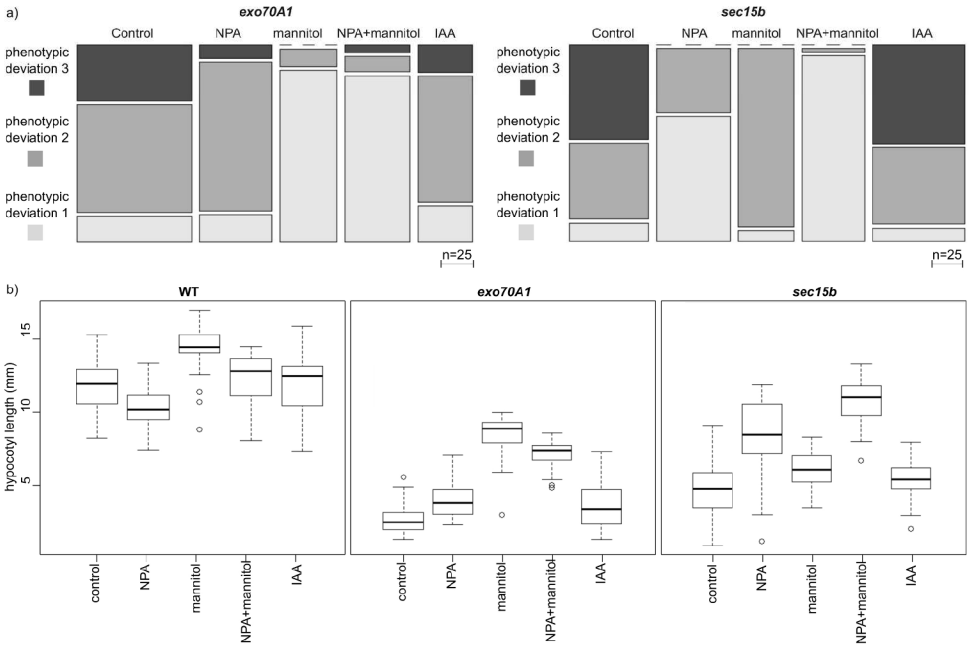

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111x74mm (600 x 600 DPI)

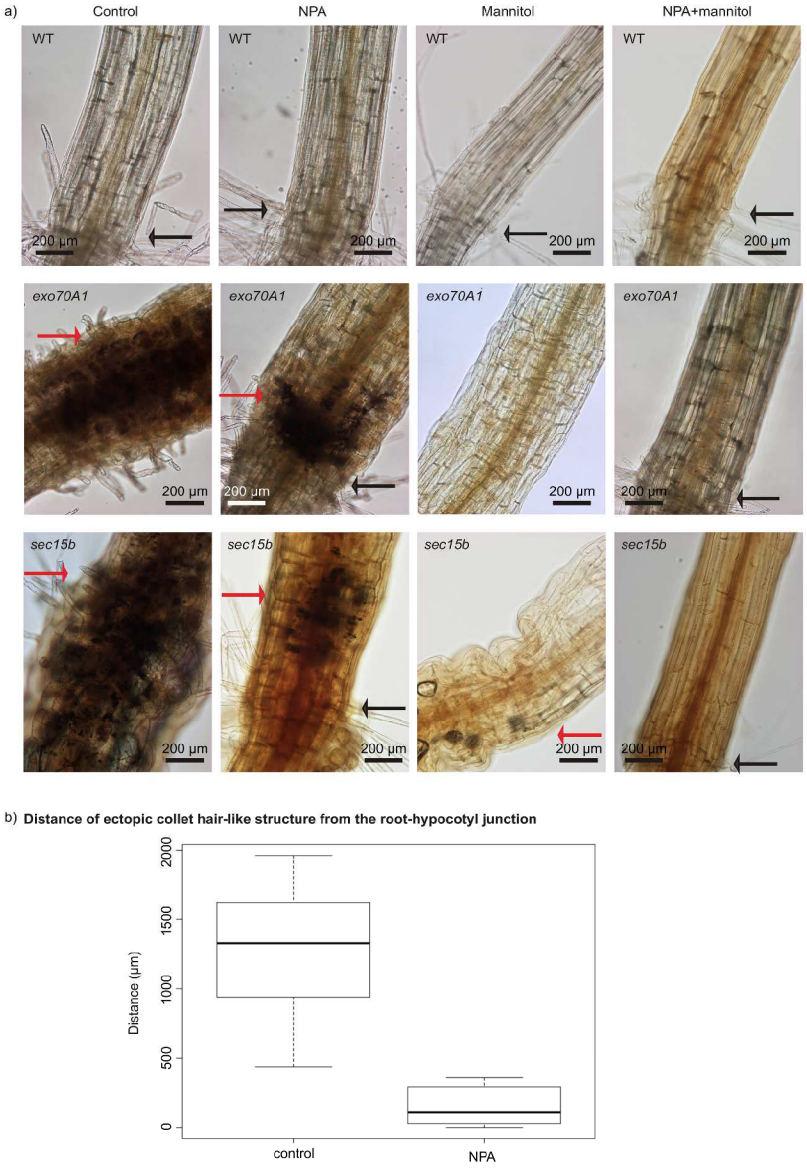
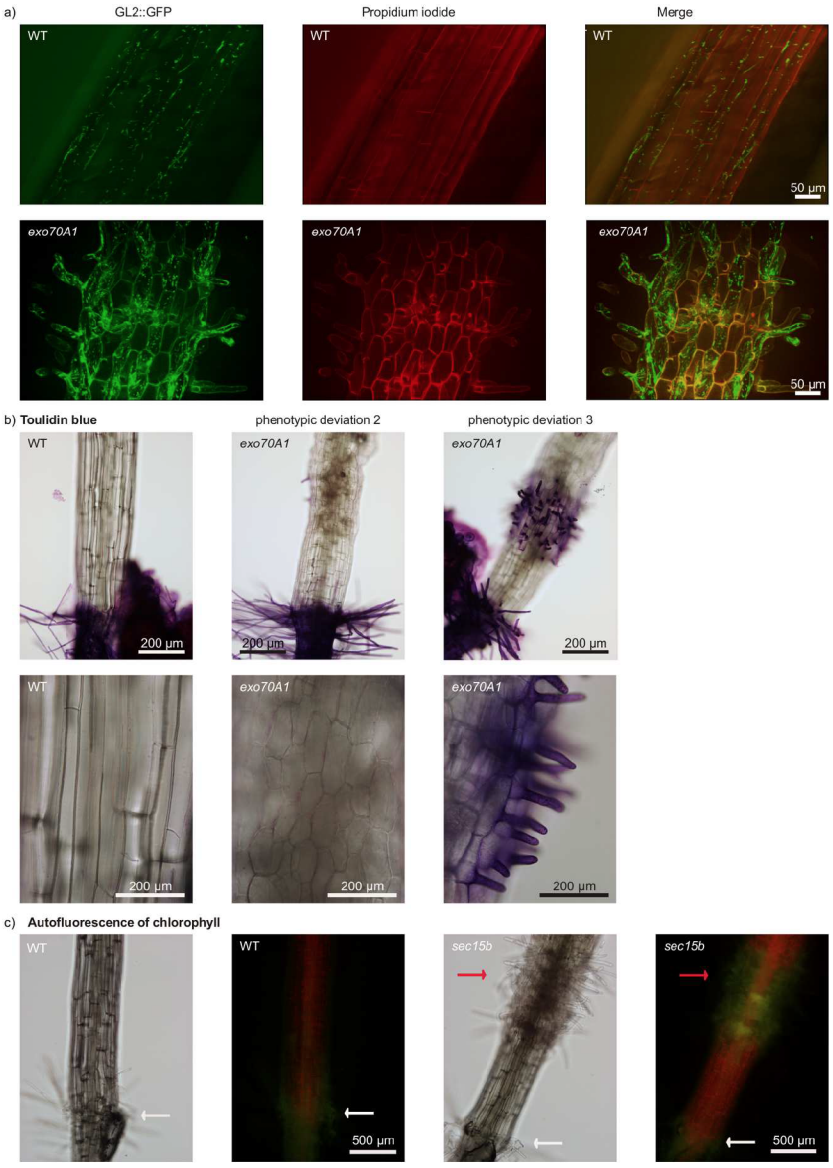


Figure 5. Influence of NPA treatment, reduced carbon supply, and the combination of both on the accumulation of starch in the etiolated hypocotyls of exo70A1 and sec15b.
(a) 5-day-old etiolated seedlings of exo70A1 and sec15b grown on the NPA (5 µM) plates had milder hypocotyl defect and reduced starch accumulation compared to non-treated mutants. Mutant seedlings grown on the mannitol and mannitol+NPA media displayed reduced hypocotyl defect without starch granules compared to non-treated mutants. No starch accumulation was observed in the hypocotyl of WT seedlings.
(b) Influence of NPA treatment on the position of ectopic collet-like structure in the hypocotyl of exo70A1. Collet hairs pointed by black arrows, ectopic collet-like structure pointed by red arrows. The number of counted seedlings for each group was n>10.

192x276mm (600 x 600 DPI)



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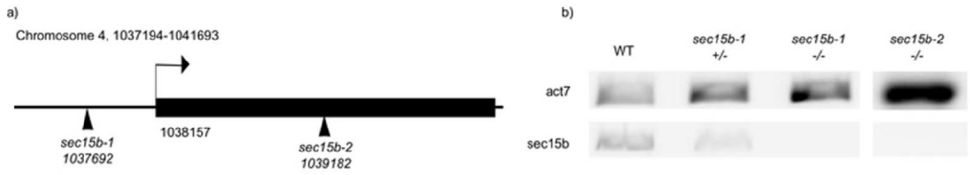

Figure S1. The starch accumulation in exocyst mutants.
The starch was accumulated in the collet-like region of exo70A1 and sec15b mutants and whole above-ground tissue of exo84b and sec8m3/LAT52::SEC8 mutants. No starch was observed in the WT hypocotyl.

32x6mm (600 x 600 DPI)

Number of adventitious roots on regenerating seedlings after dark pretreatment			
Plant lines	hypocotyl	root-hypocotyl junction	number of plants
WT (from the sec15 dataset)	51	11	n=42
sec15b	6	40	n=56
WT (from the exo70A1 dataset)	14	1	n=21
exo70A1	1	28	n=39

Table S1. Number of adventitious roots on regenerating seedlings after dark pretreatment

Primer	Sequence (5′ – 3′)	Purpose
SEC15BPROM-RP	GTTGAATTCCTATGGTTTC TAGAAAAC	genotyping of SALK_130663 line
SEC15BPROM-LP	AAGCATCCGTTTCAGCGTT GAC	genotyping of SALK_130663 line
LBNEW	GAACAACACTCAACCCTAT CTCGGGC	genotyping of SALK_130663 line
SEC15BRIK-RPBF	TTGGATCCATATGCAATCG TCGAAAGGA	genotyping of RATM15-1183-1_H line
SEC15BRIK-LPBIR	CAGTAAGAGATGATTAGCC GTC	genotyping of RATM15-1183-1_H line
DS52A	TCCGTTCCGTTTTCGTTTT TTAC	genotyping of RATM15-1183-1_H line
NPT_LP	TCCGAGTACGTGCTCGCTC GATGC	primers specific to kanamycin resistance gene
NPT_RP	GCTTGGGTGGAGAGGCTAT TCGGC	primers specific to kanamycin resistance gene
SEC15BPROM-RP	GTTGAATTCCTATGGTTTC TAGAAAAC	mRNA level analysis
SEC15BPROM-LP	AAGCATCCGTTTCAGCGTT GAC	mRNA level analysis
SEC15BNBF	TTGGATCCCAATGCAATCG TCGAAAGGACGG	mRNA level analysis
SEC15BNRV	TTGAATTCTCAGCCATACC TGCGTAGAC	mRNA level analysis
SEC15BCFW	TTGAATTCATGGTTGATTC CCTTCTCGAACTAT	mRNA level analysis
SEC15BCRV	TTGTCGACTCAGCTCACAT CTTTCAATCTC	mRNA level analysis
SEC15BRIK-RPBF	TTGGATCCATATGCAATCG TCGAAAGGA	mRNA level analysis
SEC15BRIK-LPBIR	CAGTAAGAGATGATTAGCC GTC	mRNA level analysis

SEC15BNTERMREVEXP	ATCATCCTCTTCTTCGTTTAGCGCATAAAC	mRNA level analysis
ACT7-FWD	GCCGATGGTGAGGATATTCAGC	mRNA level analysis
ACT7-REV	GAAACTCACCACCACGAACCAG	mRNA level analysis

Table S2. Primers used for genotyping and mRNA level analysis of *sec15b-1* and *sec15b-2* mutant lines.

Figure S2. (A) Graphical visualization of sec15b-1 and sec15b-2 insertions in SEC15B gene and promoter region. (B) Confirmation of knockout mutation of SEC15B gene in sec15b-1 and sec15b-2 mutant lines.

43x11mm (600 x 600 DPI)

9.2 Starch Accumulation in the Hypocotyls of Dark-grown Mutants

Do the secretory mutants have a problem with the hypocotyl elongation and over-accumulation of starch when grown in the dark? Does the decision mechanism of plant ‘to grow or to store’ exist?

Paper: ’Starch Accumulation in *Arabidopsis* Secretory Mutants Seedlings is a Result of the Cell Wall Biogenesis Inhibition.’

Starch Accumulation in *Arabidopsis* Secretory Mutants Seedlings is a Result of the Cell Wall Biogenesis Inhibition

Martina Růžičková[123], Edita Drdová[1], Hana Soukupová[1], Viktor Žárský[12] and Michal Hála[12]*

***Abstract*— Cell wall biogenesis in plants is very complex process. It also represents important sink for sugars in form of cell wall polysaccharides. For the proper cell wall biogenesis and cell elongation in general, functional secretion is needed. In this study, we were asked the question how plant with the disrupted secretion deal with the unused energy the polysacharides.**

To address this question, we use etiolated *Arabidopsis* hypocotyl, a flexible connection between roots and cotyledons, as a model system. The cells of the hypocotyl undergo rapid elongation in the dark and intensive transport to the cell wall takes place during this process. Secretory mutants, used in this work are characterized by short etiolated hypocotyls with irregular cell pattern and ectopic starch accumulation. To address this phenomenon, we measured the length of hypocotyls of dark grown secretory mutants in comparison with WT plants using normal growing conditions. Subsequently, we correlated the length of the hypocotyls with the starch accumulation. We conducted the same measurement for the plants grown on the media supplemented with the drug isoxaben. We show that etiolated WT hypocotyls upon isoxaben treatment generally react on distortion of the cell wall expansion on saccharides-containing media by allocation of sugars in the form of starch accumulation, which is the effect very similar to the starch accumulation of the secretory mutants.

Moreover, we discovered that in plant seedlings, there is a switch mechanism redirecting the sink of internal sugars from the cell wall synthesis to starch accumulation.

I. INTRODUCTION

In every moment, plants have to decide whether to invest energy to its growth or to store it. This decision is based on a plenty of external and internal factors. Among the main external factors, there are the length of photoperiod, daytime, physical conditions (i.e. temperature, water status, etc.), and availability of external nutrients, especially nitrogen and phosphorus. The internal factors determine the capacity of the cell to grow, depending on its developmental, biochemical and physiological status. These factors are predominantly growth regulators, internal nutrients signaling and efficiency of the secretory machinery.

Photosyntates partitioning is the main process affecting how the energy is distributed. Its regulation was investigated in soybean plants grown in different photoperiods. In case of the very short photoperiod (7h light/17 h dark), 90% of photosynthetic production was allocated into starch during the light period. Conversely, only about 60% of assimilates were allocated into starch during the longer photoperiod (14 h light/10 h dark) (Smith and Stitt, 2007).

Developmental dependence was observed using 14CO2 pulse chase labeling in *Arabidopsis* rosette leaves. Mature source leaves stored about 14% of assimilated 14CO2 in starch and only about 6% as cell wall components at the beginning of the light period. This ratio was even more significant at the end of light period when about 31% of assimilated 14CO2 was stored as starch and only about 5% as cell wall components. Opposite ratio was observed, on the other hand, in young growing sink leaves when about 9% of assimilated and transported 14CO2 was stored as starch compared to 12% stored as cell wall components in the beginning of the light period. At the end of the light period, the ratio remained stable with about 8% of assimilated and transported 14CO2 stored as starch and about 10% as cell wall components (Kölling et al., 2015).

Published data suggest that there are two major sinks for hexose phosphate pool inside plant cells starch synthesis and cell wall component synthesis. Starch is the most significant form of carbon reserves in plants. Synthesis of starch starts with sucrose synthase and includes conversion of hexoses pool - fructose-6-phosphate to Glc-1-phosphate by the sequential action of phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM). As was shown in *Arabidopsis* mutants, reduced activity of PGI, PGM and AGPase enzymes results in reduced levels of starch accumulated in leaves (about 10-15% of the WT level in the case of the *pgi* mutant and up to 3% of the WT level in case of the *pgm-1* mutant) (Caspar, Huber, and Somerville, 1985; Kofler et al., 2000; Gibon et al., 2004; Niittylä et al., 2004; Streb et al., 2009; Bahaji et al., 2015). However, higher levels of starch, as high as 15% of the corresponding WT levels, were achieved by increasing an amount of an external sugar (Muñoz et al., 2006).

Saccharide-based cell wall material consists of cellulose, hemicelluloses, pectins and a range of glycoproteins. Cellulose, β-1,4-glucan, is formed on large membrane complex cellulose synthase. This large protein complex uses cytoplasmic UDP-Glc for production of cellulose microfibrils.

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Connection between CesA and sucrose synthase was also reported although it is probably not general feature (Carpita and McCann, 2015). Hemicelluloses, mainly xyloglucans and xylans, are synthesized also in the GA. Glukosidic backbone of xyloglucans is formed by activity of protein complex cellulose synthaselike family C. Catalytic domain of this complex faces cytoplasm and utilizes cytoplasmic UDP-Glc. Synthesis of the glucan chain is connected with its translocation to the GA lumen. Several glycosyl transferases then modify its hydroxyl groups. Xylans are synthesized directly in the lumen of GA by the activity of xylan synthase but sugar building blocks are again imported from the cytoplasm. The xylan backbone then undergoes similar modifications as xyloglucans. (Pauly et al., 2013). Although pectins are formed in Golgi cisternae all building blocks must be imported from the cytoplasm. Then they are converted by action of glycosyl transferases into different pectins (Anderson, 2016). Hemicelluloses and pectins are subjects of vesicle transport to the cell surface.

In this paper we focus on internal factors that have impact on starch accumulation inside etiolated hypocotyls of certain secretory mutants under conditions of external carbon supply. Supposing that one hexose phosphate pool is used to feed both cell wall material synthesis and starch synthesis, we demonstrate that compromising of cell wall biosynthesis accelerates starch accumulation in secretory mutants.

II. MATERIAL AND METHODS

A. Plant material and growth conditions

We used following *Arabidopsis* mutant lines, that were previously described in cited publications. All used mutant plants are in Col0 background with exception of *det3*, which is in Landsberg background.

Secretory mutants: *exo70A1-1*,(Synek et al., 2006), *exo84b-1*, (Fendrych et al., 2010), *sec15b*, *rgtb1-1*, (Hála et al., 2010) and *det3* (Schumacher et al., 1999). Non-secretory mutant: *dwarf2*, (Wang et al., 2001) Starch metabolic mutant: *pgm1*, (was kindly provided by C Wolverton, Ohio Wesleyan University, (Wolverton, Paya, and Toska, 2011)

Arabidopsis seeds were surface-sterilized (4 min in 10% household bleach (Biochemie), 2x3 min in 70% EtOH and rinsed 5 times with sterile distilled water) and dispersed onto agar plates with growing medium: $\frac{1}{2}$MS-salts (Sigma) supplemented with 1% (w/v) sucrose (Fluka) or mannitol (0,528%), vitamins, 1.6% (w/v) plant agar (Duchefa), buffered to pH 5.7. Stratification was performed at 4øC for 2 days in dark. Seedlings were grown for 7 days vertically in a climate chamber typically at 24øC under continuous dark.

B. Starch detection

Starch was detected in the hypocotyls of eight days old dark grown seedlings and visualized by staining by the Lugol solution for 5 minutes and followed by 10 minutes wash. The whole staining process was done with the respect to the dark grown phenotype of the seedlings, which means that the samples were avoid of the contact with the light as much as possible. The confirmation of starch staining by Lugol solution was done by starch assay kit from abcam (ab83393). The amount of starch in the dark grown WT and rgtb1-1 mutant plants was measured using this kit.

C. Microscopy

Starch accumulation was documented using the Olympus BX-51 microscope with the Olympus DP50 camera attached. The different objectives were used for the proper detection of the starch accumulation. The whole seedlings were observed and searched for the starch accumulation. But the starch phenotype manifest itself only in the seedlings hypocotyls as the place of the extreme cell elongation.

D. Software

The length of the hypocotyls and the amount of the starch granules accumulated in the hypocotyls of 7 days old dark-grown plants were measured. For this measurement the ImageJ software was used. Using cell counter ImageJ program we counted the amount of visible starch granules accumulated in the seedlings hypocotyls and related it to the measured area. In the final analysis, we therefore focused on the presence or absence of the starch accumulation rather on the differences in the amount of the starch.

III. RESULTS

A. Detection of starch accumulation in the dark-grown 7-days old seedlings

The Cultivation of *rgtb1-1* secretory mutants *in vitro* on media with supplemented 1% sucrose revealed strong ectopic starch accumulation. In etiolated *rgtb1-1* seedlings, we observed accumulation of starch in cells of hypocotyls but not roots. The *rgtb1-1* mutant was previously shown to be defective in etiolated hypocotyls elongation (Hála et al., 2010) and we decided to corroborate the observation using different mutants defective in hypocotyl elongation. We used secretory mutants mutants in exocyst subunits EXO70A1, SEC15b-1 and EXO84b, mutant in V-ATPase subunit DET3 (VHA-C), non-secretory DWARF2 (DWF2) mutant, and starch metabolic mutant -PHOSPHOGLUCOMUTASE1 (PGM1). The exocyst complex is a tethering complex regulating delivery of secretory vesicles to the plasma membrane while the VHA complex resides on TGN and regulates trafficking through this compartment. The DWF2 gene is allelic to BRI1 the plasma membrane-localized receptor for brassinosteroid growth regulators that undergoes endosomal cycling and represents other group than secretory mutants in our experiment.

Under the same conditions used previously for *rgtb1-1*, an ectopic starch accumulation in hypocotyls was visible not only in *rgtb1-1* but also in *sec15b*, *exo84b-1*, *det3*, and sub-population of *exo70A1* mutants. However, some of *exo70A1* mutants as well as all of *dwf2* and *pgm1* mutants did not accumulate starch in cells along the whole length of etiolated hypocotyl. The hypocotyl length analysis showed that those mutants accumulating starch (including the sub-population of *exo70A1* mutants) have shorter hypocotyls than the rest where the starch accumulation is not observed (Fig: 1**B**).

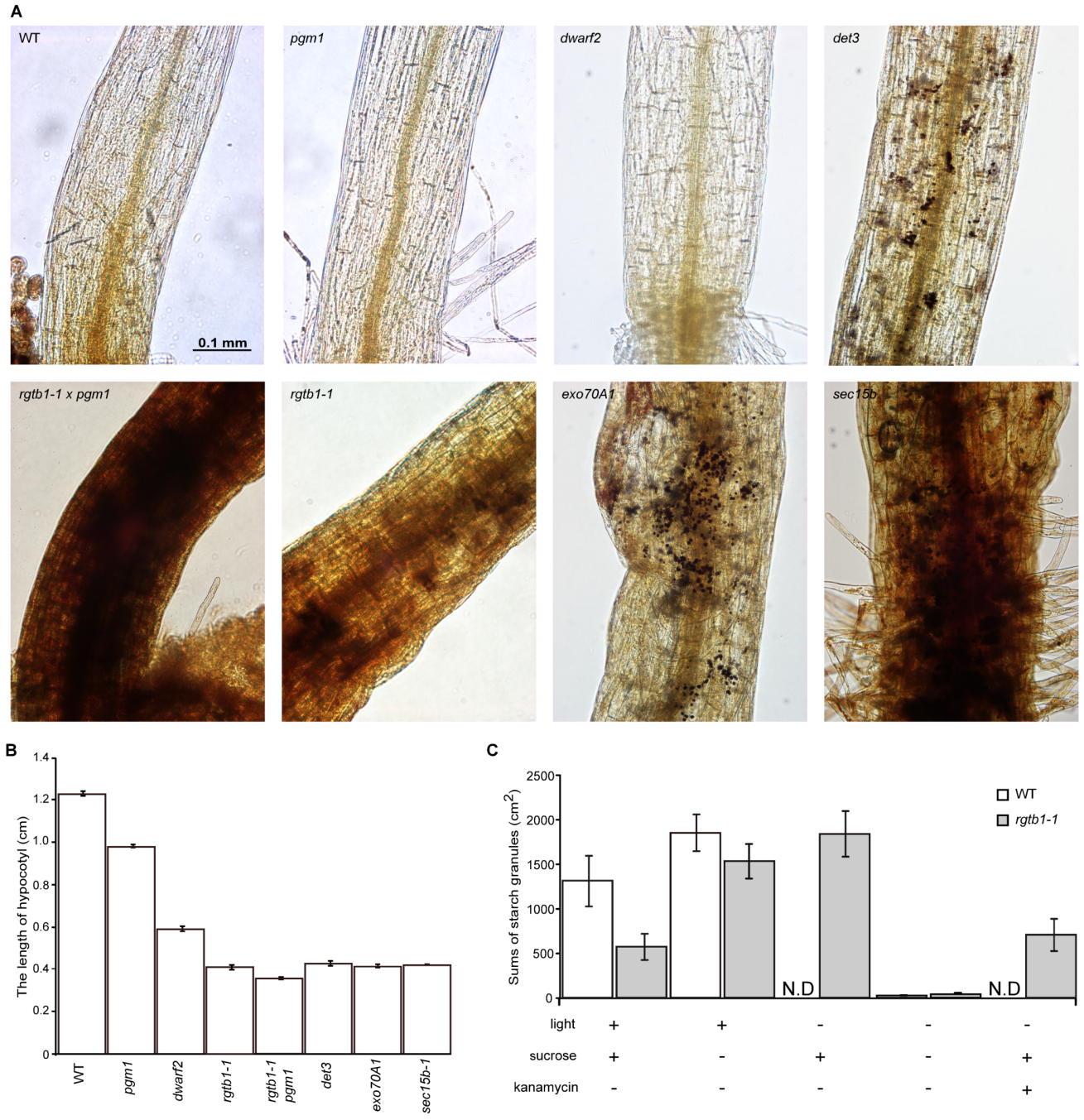

Fig. 1. **(A)** The hypocotyls of chosen mutants stained for starch. The different secretory non-secretory and metabolic mutants we grown in the complete dark for 7 days. The accumulation of starch was visualized by Lugol solution staining. The boundary between root and hypocotyl (the region above the collet-hair region) was documented. **(B)** The hypocotyl length of chosen mutants after 7 days of growth in the dark. **(C)** Starch accumulation in the *rgtb1-1* mutant in different light- and dark-grown conditions in combination with the media supplemented with the drug kanamycin. Scale bar = 0.1mm At least 15 plants from each group was analyzed. Data represents means of 15 or more seedlings +- SE.

B. The dependence of the starch accumulation on the external media

We were further interested how the starch accumulation is dependent on presence of sucrose in the growing media. We grew the etiolated seedlings on plates without external carbon source (we used only 1% mannitol to maintain the osmotic strength of the medium) in dark. As expected, none of mutant or WT plants, included in our experiments did accumulate starch granules.

In order to quantify our previous observations, we decided to work with well established *rgtb1-1* mutant. The *rgtb1-1* mutation results in pronounced growth retardation. Working with *rgtb1-1* mutant we always used media with external sucrose (Hála et al., 2010). Our observations on plates without external sucrose revealed that starch accumulation is not directly connected with the phenotype as *rgtb1-1* hypocotyls were short, malformed hypocotyls, and opened cotyledons as published in Hála et al. (2010) although no starch accumulation was detected. Further, we decided to count visible starch granules in the hypocotyl region rather than measure total starch content in seedlings. Fig. 1**C** shows that both WT and *rgtb1-1* plants accumulate starch granules when grown on long day light conditions independently on the presence of sucrose in the medium. In dark grown seedlings, only visible accumulation of starch granules was detected in *rgtb1-1* seedlings grown on media with sucrose. The number of granules reached the number observed in WT grown on light without sucrose. WT plants did not accumulate starch granules in dark to any countable number regardless on the presence of sucrose in the medium.

Kanamycin is an antibiotics interfering with prokaryotic translation on ribosomes. It was reported to decrease the number of starch granules in dark grown *det3* mutants. We have grown *rgtb1-1* seedlings on media supplemented with kanamycin for all the time of seedlings growth. The effect is described on Fig. 1**C** resembling that reported for *det3* mutant plants. Kanamycin decreased the number of starch granules accumulated *rgtb1-1* mutants in dark on media containing sucrose to 40% of the level observed without kanamycin. On the other hand, WT did not accumulate starch granules neither with nor without kanamycin.

C. The correlation of the starch accumulation with the hypocotyl length of dark-grown seedings

Our observation that starch does not accumulate in etiolated hypocotyls of all dwarfed mutants but only in those with rather short hypocotyls inspired us to test whether the extent of cell elongation during the hypocotyl growth in dark can be correlated with starch accumulation. We applied isoxaben, a drug that selectively blocks transport and function of CES-A subunit of the cellulose synthase complex. This drug is very efficient in wide range of concentrations but with fine tittering, we were able to find rather narrow interval of isoxaben concentration where the effect on starch accumulation can be observed (Fig. 2).

Cultivating WT seedlings on plates with isoxaben in pM concentrations, we were able to observe gradual shortening of etiolated hypocotyls in the interval 0-8 pM of isoxaben (Fig. 2**B**). Than the effect of the isoxaben reached a plateau and further increase in this drug concentration did not lead to further hypocotyl shortening. The maximum of hypocotyl's length inhibition corresponds to 25% of untreated WT hypocotyl length. The average of untreated WT hypocotyl length reached 1.2cm while the average length of isoxaben-treated hypocotyls was only 0.4cm in the maximum of inhibition. Seeds germinated well in all the range between 0-40 pM isoxaben concentration.

D. Isoxaben treatment of the WT dark-grown seedlings

We also observed accumulation of starch granules in hypocotyls in correlation with hypocotyl shortening due to isoxaben treatment (Fig. 2**C**. Surprisingly, we did not observe any gradual increase of starch granules numbers with gradual hypocotyl shortening. Instead, we observed an abrupt increase between 8pM and 12pM isoxaben concentrations. This abrupt formation of starch granules correlates well with reaching the maximal inhibition of hypocotyl length at the same time.

We then tested whether the effect of isoxaben will be similar with different sugars added to the growing media. We exchanged sucrose for glucose keeping the same range of isoxaben concentrations. The glucose alone had effect on hypocotyl length increasing the average length up to 2.2 cm. On the other hand, even the smallest isoxaben concentration reduced dramatically length of hypocotyls down to 0.6 cm (i.e. 28% of original length) (data not shown). With the higher concentrations of the isoxaben the length of the isoxaben further gradually decreased to 0.2 cm, which is similar effect as was seen in the case of seedlings grown on the media with sucrose.

IV. DISCUSSION

Starch accumulation is a common physiological phenomenon connected with energy storage and carbon partitioning. In a plant cell, two main sinks are competing for hexose phosphates starch biosynthesis and cell wall material biosynthesis. The existence of a partitioning mechanism that distributes carbon from the hexose phosphate pool was described in Kölling et al. (2015). In this work, authors fed the *Arabidopsis* matured leaf with 14CO2 and analyzed its partitioning not only there but also in a young developing sink leaf. Results shown that in matured leaves, where cells do not undergo extensive elongation, major part (31%) of 14C was stored as starch and only 8% was stored as cell wall material. On the other hand, in developing leaves, where cell elongation takes place, more 14C was embedded into cell wall components (10%) than into starch (8%).

In our work, we used etiolated hypocotyls as a model system because these cells undergo massive elongation growth that is tightly organized. The elongation of the hypocotyl starts from the cells close to root-shoot junction and gradually continues toward cotyledon leaves (Refrégier et al., 2004). In first few hours during germination, right after the hypocotyl emergence, the thickness of the outer epidermal

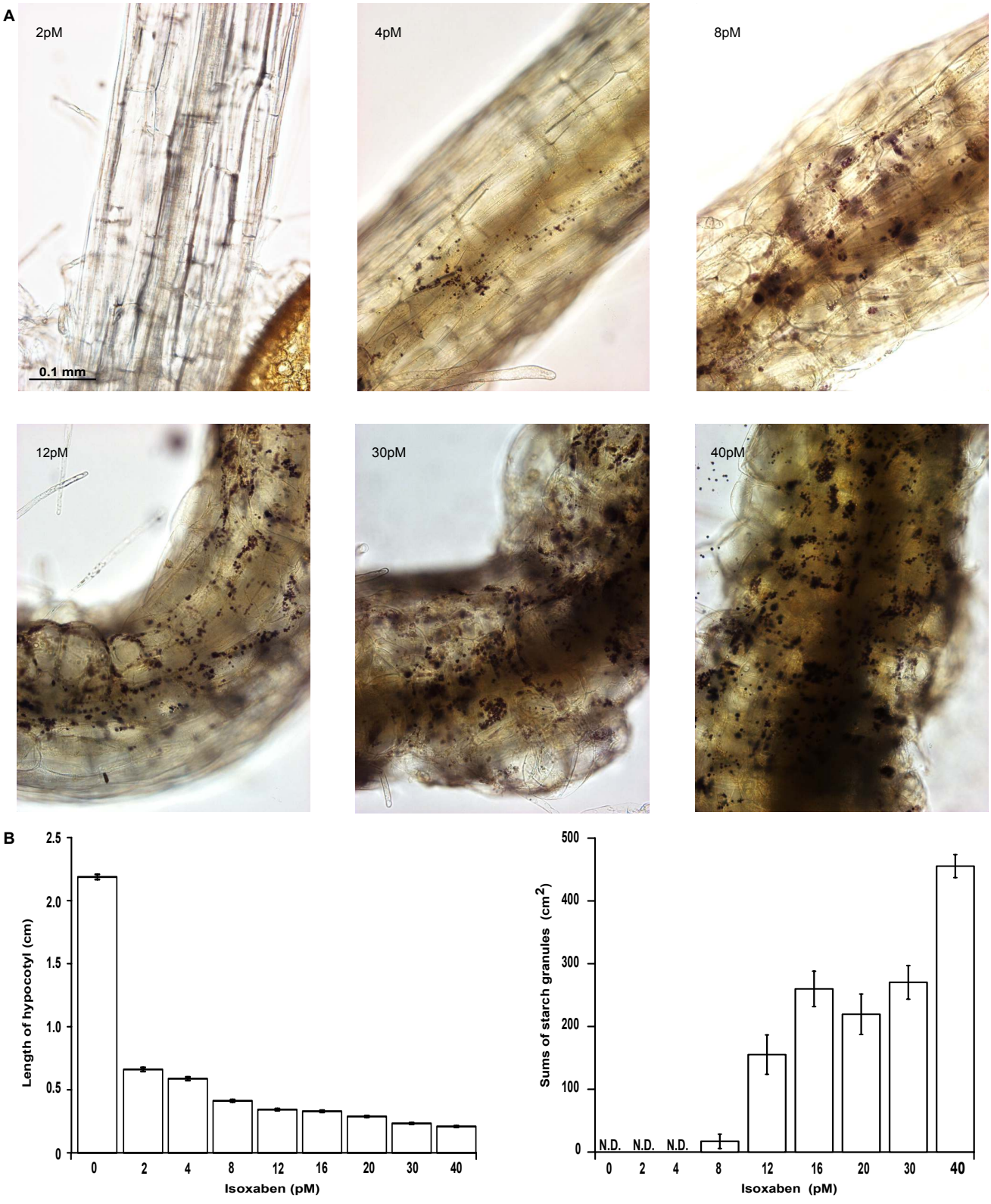

Fig. 2. **(A)** The WT plants grown on the different concentrations of the isoxaben drug. The WT plants were grown in the complete dark on the plates supplemented with the different concentrations of the isoxaben drug for 7 days. Afetr that, the accumulation of starch was visualized by Lugol solution staining. The boundary between root and hypocotyl (the region above the collet-hair) was documented. **(B)** The hypocotyl length of WT plants after 7 days of growth in the dark on the media with different concentrations of isoxaben. **(C)** Starch accumulation in the hypocotyls WT plants grown on the media with different concentrations of isoxaben. Scaale barr = 0.1mm. At least 15 plants from each group was analyzed. Data represents means of 15 or more seedlings +- SE.

cell wall reaches its maximum. After this point, the cell wall thickness decreases with increasing cell elongation, and remains steady for the rest of the hypocotyl development (Derbyshire et al., 2007). This process requires, among others, intensive cell wall biosynthesis. The cell wall biosynthesis is a complex process consuming vast amount of plant energy which is nin the plant deposited in the carbon pool of the cell. To support its needs of energy (energy of the carbon bonds), seedlings were grown on the media with 1% sucrose.

In several monocots, external supply of sucrose induces fructan polysaccharide accumulation (Loreti et al., 2005; Solfanelli et al., 2006) but this accumulation was never observed in dicots. The adding of external sucrose was also shown to activate SnRK1 kinase in potato leading also to increased starch synthesis (McKibbin et al., 2006). Our experiments with the sucrose and glucose as external saccharide show similar results. Based on the application of the different sucrose levels to secretory, non-secretory and metabolic mutants we can nevertheless conclude that observed starch accumulation is not result of specific sucrose signaling. On the other hand, presence of external saccharide, which can be metabolized by plant, in cultivation medium turned out to be a vital condition to observe starch accumulation.

Sucrose was shown to affect phenotypic deviation of the *petit1* (*pet1*) *Arabidopsis* mutant. This mutant defective in cellulose synthesis accumulated more starch than WT in light-grown hypocotyls when cultivated on media containing 1% sucrose. Phenotypic deviation of dark-grown *pet1* plants also depended on presence of sucrose in cultivation media. Sucrose in the medium resulted in more pronounced cell elongation arrest connected with irregular cell shapes similar to *rgtb1-1* plants. On the other hand, this phenotypic deviation was much weaker on media without sucrose, unlike in *rgtb1-1* plants. Yeats and Somerville (2016) reported similar phenomenon on *shv3svl1* cellulose synthesis mutant growing on 60mM (ca 2%) sucrose. Etiolated seedlings showed short hypocotyls with starch accumulation. This observation was sucrose-specific and no other saccharide in similar concentration in growth media was able to induce such phenotypic deviation. SHV3 is a GPI- modified protein that interacts with the cellulose synthase complex. The mutation causes decrease of cellulose content in the cell wall and hyperpolarization of plasmatic membrane and increased accumulation of sucrose inside the cell.

Our observations made on different mutants imply that starch accumulation depends more on the hypocotyl elongation arrest (or the arrest of individual cell elongation) than on mutation type. This hypothesis is further supported by segregating *exo70a1* homozygous population. In the exo70A1 homozygous plants we can find two fractions with the different length of hypocotyls. The fraction with shorter hypocotyls accumulates starch granules and the fraction with longer hypocotyls does not.

This observation led us to the idea, that observed accumulation of starch granules is connected with the compromised secretion to the cell wall. Therefore we tested general importance of observed starch accumulation in correlation with decreased cell elongation and a possible link with the compromised secretion. For that purpose, we used drug isoxaben which is the inhibitor of cellulose synthesis (Scheible et al., 2001).

Using a fine-tuning of isoxaben concentration, we observed an existence of the switch decision mechanism. In the WT plants treated with isoxaben, there is no transition range where the number of starch granules would start to rise fluently. Instead, there is a threshold concentration of isoxaben that separates states with no starch granules detectable and states with relatively high number of starch granules present. As an example of starch metabolic mutant, we also used mutant in plastidial PHOSPHOGLUCOMUTASE1 1 (PGM1) to further elucidate mechanism of starch accumulation in secretory mutants. *Arabidopsis pgm1* mutants similarly to mutants lacking plastidial PGM in tobacco show strongly decreased growth, while *pgm* mutants of pea and *Lotus japonicus* are reported normal in their growth (Vriet et al., 2010). The reason of observed variability lies in varying capacity of different species to accumulate other storage products in their leaves, thus compensating for the inability to make starch. Interestingly *gwd1* mutant of *Lotus japonicus* has a strong starch-excess phenotype and severe growth defect showing that starch accumulation is not preferred way of carbon and energy storage (Vriet et al., 2010).

Despite the fact that *pgm1* mutants contain only about 3% of WT starch, analysis of metabolites from the starch synthesis pathway revealed presence of almost normal levels of ADP-glucose (ADPG) in leaves of pgm1 plants. This surprising observation was explained by altered activity of cytoplasmic enzyme sucrose synthase that upon elevated concentration of cytoplasmic sucrose can produce ADPG. Cytoplasmic ADPG can be then transported via unknown yet mechanism into chloroplasts and serve as precursor for limited starch synthesis.

Taking all together, we showed here that secretory mutants that cannot elongate their hypocotyls in dark properly accumulate starch. This starch accumulation is part of the general switch mechanism that we propose here. The redistribution of sugar sources between cell wall synthesis and starch accumulation in correlation with cell wall biosynthesis, which is highly dependent on the secretion, is the main message.

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9.3 Mutant in SEC15b Subunit

How does the mutant in SEC15b subunit of the exocyst complex look like?

The subsequent mutant lines were ordered from SALK collection : SALK_130663, SALK_042723 and RIKEN_RATHM15-1183-1_H. The *Arabidopsis* mutant lines were back-crossed to Col0 WT plants and genotyped for T-DNA or transposomal insertion. Insertion in the SEC15b gene and in the SEC15b promoter was confirmed in two *Arabidopsis* lines (SALK_130663 and RATHM15-1_H).

This two confirmed lines were further studied. The first genotyping of the RATHM-1183-1_H line showed existence of *sec15b* homozygous plants that did not display any apparent phenotypic effect. Later, the phenotypic effect was visible after the first back-cross to the background Col0 (data not shown).

The T-DNA insertional line SALK_130663 was genotyped and in the population of 320 plants was found 51 homozygous plants that displayed visible phenotype. Phenotype was noticeable in the 4-weeks old light grow plants, when the plants started to switch from vegetative to generative phase. There was disturbed apical dominance and plants were half size of the WT plant. Also some spots were visible on the leaves, that might be of autophagic origin. In the dark, phenotype of *sec15b* was visible immediately. Dark-grown seedlings were not able to elongate their hypocotyls properly and created second ectopic collet-hair like region. The phenotype of ectopic collet-hair like region was also observed in other exocyst mutant and is described to more detail in the publication 'Developmental Plasticity of *Arabidopsis* Hypocotyl is Dependent on Exocyst Complex Function', that is included in this disertation. More over, *sec15b* mutant plants show heavy accumulation of starch granules in this part of hypocotyl. Starch accumulation is dedicated in the manuscript 'Starch Accumulation in *Arabidopsis* Secretory Mutants Seedlings is a Result of the Cell Wall Biogenesis Inhibition' that is also part of this thesis.

Sec15b hmozygous plants SALK and RIKEN lines were confirmed to be knock-outs (9.2).

Figure 9.1: (A) The phenotype of *sec15b* adult homozygous plant in comparison with WT. (B) The five-days-old dark grown *sec15b* mutant in comparison with WT..

Figure 9.2: (A)Graphical ilustration of the T-DNA insertion in promotor region and transposomal insertion in the exone of SEC15b gene. (B) Confirmation that both insertional mutant *Arabidopsis* lines are knock-outs. SEC15b protein is not translated.

VI DISCUSSION

10. Discussion

In this chapter I tried to summarize and discuss our experiments that are parts of the included manuscripts. For the first time in plants, we proved that the exocyst complex was the real effector of RAB GTPases and we focused on the phenotypic aspects of the mutation in SEC15, which is the exocyst subunit interacting with RAB GTPases. Next, I discuss starch accumulation observed in *rgtb1-1* plants, mutant in the RAB geranylgeranyl transferase beta subunit, which also can be generalized to other secretory mutants.

10.1 What Is the Connection between Tethering Complex Exocyst and Secretory Vesicle Mediated?

The connection between the exocyst complex and the secretory vesicle is known to be mediated by Rab GTPases in yeast, *Drosophila* and mammals. To date, the mechanism of the interaction between vesicle and exocyst was not known. There were two main possibilities how it works. The first possibility was the interaction of the exocyst complex directly with the phospholipids of the tethered vesicle. The second option was the interaction of the exocyst complex indirectly via RAB GTPases. We tested both eventualities.

Preliminary data from co-immunoprecipitation led us toward the indirect interaction via RAB GTPases, because we obtained a specific signal for the RAB GTPase from the A4 subclass in a co-immunoprecipitation analysis through EXO84b-GFP exocyst subunit. It is the Rab11 class of Rab GTPases, that is considered as the main exocyst interacting partner. But also Rab GTPases from other classes, such are Rab3, Rab27 and Rab8, were shown to interact with the exocyst complex (Wu et al., 2005). In plants, the most related to Rab11 and Rab27 are RAB GTPases from the class A. This plant class of RAB GTPases is interesting by its huge diversification. The RAB GTPases from the class A has 6 subclasses from which only RAB-A2 subclass has homologues in yeast, *Drosophila* and animals. Other five subclasses, namely RAB-A1, RAB-A3, RAB-A4, RAB-A5 and RAB-A6 are plants specific. The mammals Rab8 is closely related to the plant RAB-E class. Therefore RAB-A and RAB-E were the most likely candidates for the interacting partners of the exocyst complex. Besides, other RAB GTPases were shown to localize to plasma membrane, e.g. RAB-C and RAB-F1 (Chow et al., 2008; Tsutsui et al., 2015).

We continued using the classical approach of screening for the interaction in yeasts using yeast two-hybrid assay (Y2H). Unfortunately, this method did not provide positive result. Therefore, we started to consider the direct interaction of the exocyst complex with the phospholipids of the exocytic vesicle. To confirm or reject this possibility, we tested a purified AtSEC15b subunit on this interaction on PIP strip and membrane strip as well as using the LUV method. We obtained negative results from these methods which show that there is no interaction of AtSEC15b subunit with phospholipids. Therefore, we returned back to the indirect interaction hypothesis.

There are various reasons why Y2H assay may not be successful. One of them is that in yeasts, there are not established proper post-translational modifications of plant proteins and the whole interaction is forced to be done in the yeast nucleus which is not optimal for the interactions that are normally performed on the membrane. Moreover, the conformational changes in the structure of AtSEC15b protein of RAB GTPases induced by the fusion of tested proteins with parts of the yeast GAL4 transcription factor may also contribute to a false negative result (Brückner et al., 2009). At last, an "induced fit" mechanism, which can be dependent on the interaction with the rest of the exocyst complex, might play an important role in the interaction with other proteins.

Finally, a pull-down assay revealed the GTP-dependent interaction of the RAB-A4a and RAB-A4b GTPase with the AtSEC15b subunit of the exocyst complex. By using GTP and GDP analogs we confirmed that only the active form of RAB GTPase interacts with the SEC15b subunit. On the other hand, this feature was a disadvantage in *in vivo* studies. There are two approaches to study Rab GTPases. The first is the isolation of knock-out mutants and their crossing with the other knock-out mutants to create double or triple mutants in RAB GTPases. This approach was shown not so effective because the single knock-outs did not have phenotypic defects frequently (Rutherford and Moore, 2002). The seconds approach consists in overexpression of a single RAB GTPases, although its activation and intracellular localization is tightly controlled.

Under normal conditions, the majority of the RAB GTPase in the inactive GDP-bound form and only very small portion exists in the active GTP-bound form in the cell. This approach then rather exploits the potential of point mutated RAB GTPases, in which the binding capacity towards the guanine nucleotide is changed. These mutants include the constitutively active (CA) form that has compromised the hydrolytic activity leading to accumulation of GTP-bound forms. On the other hand, the dominant negative form (DN) is unable to stabilize binding of the third phosphate group in the nucleotide-binding pocket and is considered to be GDP-bound. It was experimentally shown that some Rab GTPases tend to lose the guanine nucleotide and become nucleotide-less (Olkkonen and Slenmark, 1997). Over-expression of these forms in living cells is often lethal and the only possibility is to use transient expression or to use inducible promoters.

For the *in vivo* studies, we used transient overexpression of RAB GTPases in *Nicotiana benthamiana* in their WT, CA and DN forms. Although they were transiently expressed, we observed different localization of either DN and CA RAB GTPases based on the signal intensity. Therefore, we decided to use only WT RAB GTPases for the interaction studies. To be able to capture this feature and based on the knowledge that GTP-bound RAB GTPases appear in a very small amount in the living cell, we performed FLIM/FRET microscopy. The FLIM/FRET microscopy confirmed the previous results from the *in vitro* pull-down assay.

So far we were not able to answer the question why only two RAB GTPases from the A4 subclass interact with the AtSEC15b subunit of the exocyst complex. We have two hypotheses. The first assumes that there is the SEC15a subunit, a AtSEC15b paralogue, that was previously shown to play role predominantly in the male gametophyte, being critical for pollen tube germination (Hála et al., 2008). On the other hand, our expression data analysis shows a possible role of the AtSEC15a subunit also in the sporophyte. Therefore, RAB GTPases RAB-A4c and RAB-A4d that do not interact with AtSEC15b in our experiments might interact with the AtSEC15a subunit of the exocyst complex. RAB-A4d is pollen specific and was shown to localize to the growing

tip of pollen tube. The disruption of the gene revealed its necessity for the proper development of the pollen tube and showed disturbed localization of cell wall components, especially pectins (Szumlanski and Nielsen, 2009). Moreover, RAB-A4b under control of RAB-A4d promoter is unable to fully complement RAB-A4d pollen defect (Szumlanski and Nielsen, 2009), which even more suggests non-redundant function even within the RAB-A4 clade and nicely corresponds with our experimental data.

The second hypothesis assumes that we were not able to catch other potential interacting RAB GTPases because of a probability of some specific conditions during pull-down assay and also because of the limited options of the transient expression in *Nicotiana benthamiana*. This hypothesis is very probable in respect to other RAB subclasses then RAB-A4 (see above).

Other highly probable exocyst interactors are RAB GTPases from the A class as well as from the E subclass (Woollard and Moore, 2008). The A subclass of RAB GTPases underwent the differentiation in the six subclasses from which five are plant specific. Chow et al. (2008) suggests that this diversification of RAB GTPases mirrors a different organization of the plant TGN compartment. Various transport routes meet in these different TGN compartments and each has to be regulated by specific RAB GTPases. To support this theory, there are various parts of TGN compartment marked by distinct markers and also distinct RAB GTPases in *Arabidopsis thaliana*. The RAB GTPases in this compartment overlap partially but do not co-localize (Chow et al., 2008). The different organization of TGN and broad spectrum of plant specific RAB GTPases might also implicate different functions of various TGN parts. Thus, RAB GTPases from the different RAB A subclasses might deliver plant specific cargos to the plasma membrane/periplasmic space. Albeit, different RAB GTPases were shown to mark diverse TGN compartments, experimental data suggest high redundancy inside the RAB-A subclasses (Chow et al., 2008). Very similar situation was observed inside the RAB-D class of *Arabidopsis* RAB GTPases. RAB-D1 and RAB-D2 subclasses were shown to play similar but not completely redundant role in the transport from the ER (Pinheiro et al., 2009). Similarly, Lunn et al. (2013) described in his work mutants in various RAB GTPases. *Arabidopsis* plants lacking single RAB GTPase had no visible phenotypic defect but the authors were able to prove a change in the cell wall composition of these mutants. The work of Lunn et al. (2013) not only further corroborates the non-redundancy of RAB GTPases, but also brings the piece of evidence that specialized RAB GTPases regulate trafficking of vesicles with specific cargoes. This puts extra importance on studying RAB GTPases and their regulation in plants.

10.2 How Does the Exocyst Complex Interact with the Plasma Membrane?

There are two exocyst subunits that are known to interact with the plasma membrane. These are SEC3 and EXO70 (He et al., 2007; Zhang et al., 2008). In the genome of *Arabidopsis thaliana* there are two paralogues of SEC3 subunit, SEC3a and SEC3b, and 23 paralogues of EXO70 subunit (Elias et al., 2003). The SEC3 subunit contains a PH-domain (pleckstrin homology domain) that is evolutionarily conserved from yeast to mammals and plants (Baek et al., 2010) and was previously predicted to be involved in the interaction with phospholipid PI(4,5)P2 and RHO GTPases (Guo et al., 2001; Zhang et al., 2001). Plant SEC3a was recently shown to interact with phosphoinositides *in vitro*. In the pollen tube, SEC3a colocalized with PIP2 marker and also molecular dynamics simulation showed the interaction with the membrane through the PIP2 phospholipid (Bloch et al., 2016). Although we may expect interesting discoveries in this topic in the future, our data suggest that the SEC15 exocyst subunit is not involved in connecting exocyst and membrane directly but only as an effector of RAB GTPases.

10.3 Is There Common Secretory Phenotype for Mutants in Different Exocyst Subunits?

We proved that the exocyst complex is the effector of RAB GTPases in plants. Therefore, we were interested in some specific features of the phenotype of the exocyst mutants. The dark-grown exocyst mutants were not able to elongate their hypocotyl properly which is the sign that was previously shown also in other mutants, that were named *cop/det/fus* mutants. These mutants mimic a phenotype of light-grown seedlings when grown in the dark, which also cannot elongate their hypocotyls, they have opened the apical hook with the cotyledons and they are green when grown in the dark (Schwechheimer and Deng, 2000).

Moreover, in the used dark-grown exocyst mutants, we observed a phenotype that has not been published yet. It was the creation of the ectopic collet hair-like region. Collet hairs in *Arabidopsis* are produced by files of root cells adjacent to the root-hypocotyl junction in the region of double cortex cell layers and serve to stabilize germinating seedling in the substrate. This region originates from the heart stage of embryo and does not regularly undergo further cell division instead it enters endoreplication cycle (Gendreau et al., 1997; Sliwinska et al., 2012). Our unpublished experimental data underline the importance of endoreduplication for collet hair production, and root hairs in general, as hydroxyurea treatment abolished its formation.

The creation of the ectopic collet-like region is a conditional mutation. The conditions differ slightly in the case of the core subunits as are SEC8 and EXO84b, and other used subunits - EXO70A1 subunit and SEC15b subunit. This differences can mirror that SEC15b subunit as well as EXO70A1 subunit are the main exocyst subunits that interact with the other signaling molecules (e.g. phospholipids or RAB GTPases). In the case of EXO7A1, it is mainly the combination of temperature and plant hormone auxin that caused this conditional phenotype. In the case of SEC15b mutant, it is the temperature that has the biggest phenotypic effect.

We did not observe this phenotype in other exocyst mutants available in our laboratory. This might be because these subunits have other paralogues that have higher expression in the seedlings and are redundant. For example, this phenotype was reported for the knock-out mutation of the SEC15b subunit, but it was not observed for the knock-out mutation of SEC15b paralogue - the SEC15a subunit.

We were not able to solve what causes this interesting phenotypic deviation on the cellular level yet. However, we think that it is a higher plasticity of the hypocotyl cells that plays important role on the physiological level of the phenotype of dark-grown exocyst mutants. We showed that the additional collet-like region does not have the attributes of the legitimate collet. In the root and also in the shoot of plant rhizodermis (epidermis) differentiate on the cells with hair (stomata) and without hair (without stomata). This patterning is disrupted only in the region of the true collet region where all rhizodermis cells are able to create hairs. Establishing of the marker GLABRA2 helped Qing and Aoyama (2012) to show that in true collet region there is no expression of this transcription factor which allowed all cells of the true collet to create collet hairs. We observed that the cells of the collet-like region do not have this feature. In the collet-like region GLABRA2 is expressed normally and we can find there two types of the cells - cells that are able to create hairs and cells that are not able to create them.

We don't know whether observed defect in hypocotyl may be explained by above mentioned mechanisms but we have also observed the defect in the secretory pathway using RAB GTPases as GFP markers introduced into *exo70A1* mutant plant. In the defective region of the *exo70A1* hypocotyl we found the impaired localization of YFP:RAB-A5d and YFP:RAB-A2a, markers of recycling endosome (or TGN) acting on post-Golgi trafficking route to the plasma membrane. In the previous study, it was described that approximately 7% of *exo70A1* root tip epidermal and cortical cells carry YFP:RAB-A5d positive abnormal enlarged compartment (Drdova et al., 2013). These observations again raise the possibility that RAB-A5 class members might be direct exocyst

interactors in plants.

The occurrence of ectopic hairs on hypocotyl has been described after modulation of expression in several genes but contrary to presented exocyst mutants these ectopic hairs have never been localized in the discrete region and were not selectively formed only on the etiolated hypocotyl. One example is mutant constitutively overexpressing MIF1, which causes dramatic developmental defect and formation of ectopic hairs, described as ectopic root hairs by the authors, on cotyledons and hypocotyl (Hu and Ma, 2006). The exact function of this protein is not known, however based on the expression profiling and physiological test this protein function on the cross talk between hormones - auxins, brassinosteroids, cytokinins, gibberellins and ABA (Hu and Ma, 2006). Another example of mutant forming ectopic root hairs on the hypocotyl was created by modification of GL2 function so that it acts as stronger activator of gene expression (Ohashi et al., 2003).

The hypocotyl part of the plant is extremely interesting developmental model. The hypocotyl cells of the seedling undergo extreme elongation to deliver cotyledons above ground when grown in the dark. Once on the light, elongation of the hypocotyl is stopped and adventitious roots and root hairs start to grow from the hypocotyl. Therefore, the part of the plant that is shoot when grown on the light became root when elongated and grown in the dark. The correct organization and function of the hypocotyl is important for the transport of nutrition and water and the plant fitness, generally.

10.4 Do the Secretory Mutants Have a Problem with the Hypocotyl Elongation and Over-accumulation of Starch when Grown in the Dark?

The healthy plants have to balance constantly the growth with the energy gain. In our work, we used the model of dark-grown secretory mutants to show that if the secretion to the cell wall is compromised, plant has to use the accepted energy the different way, for example to store. The common physiological phenomenon connected with the energy storage is the accumulation of starch, which was reported in the hypocotyls of some dark-grown secretory mutants (Schumacher et al., 1999).

In our study, we used secretory mutants in the exocyst subunits (SEC15b, EXO70A1), mutant in the VHA-a1 subunit of the ATPase (*det3*) and mutant in the RabGGTase that mediates posttranslational modifications of Rab GTPases (*rgtb1-1*). As a non-secretory mutant we used mutant in the BRI1 plasma membrane localized brassinosteroid receptor (bri1) and as a sort of other negative control we used mutant in enzyme phosphoglucomutase (*pgm1*), that is not able to accumulate starch.

The observation that the starch accumulation depends on the hypocotyl cell elongation more than on the type of the mutation was further supported by the segregation population of the *exo70A1* mutant seedlings. The all secretory mutants showed shorter hypocotyls and accumulation of starch. The very similar phenotype was published in the case of the mutant in the SHV3 gene. It is GPI modifying enzyme that interacts with cellulose synthase complex and so the mutation in this enzyme decrease the cellulose content in the cell wall (Yeats and Somerville, 2016). Our and also all other reported starch phenotypes were dependent on the addition of the external source of the sugar. This led us to formulate hypothesis ‘to grow or to store’. It is a very basic idea that the obtained energy plants invest to growth. But if the growth is not possible (disrupted transport of the material from the TGN compartment toward the plasma membrane and the cell wall) plant cannot leave the energy of the sugar bonds unused. Therefore has to deposit it in some well accessible form i.e. starch granules.

To further support the hypothesis, that plants has to make a decision ‘to grow or to store’ we used commonly used cell wall drug, isoxaben. The isoxaben is a drug that inhibits the growth of the plant by the alteration of cellulose synthesis and transport. Effect of isoxaben was observed on isoxaben- habituated *Arabidopsis* cell culture. Cell culture treated with isoxaben created large aggregates in comparison with the untreated cells. Distribution of cellulose in these aggregates was

more random and less organized than in untreated controls. The same was true also for xyloglucans. On the other hand, localization of pectins remains unaffected. Transcriptomic analysis confirmed simultaneous enhanced expression of various isoforms of glucan transferases but, at the same time, expression of other isoforms was decreased (Manfield et al., 2004).

We added isoxaben drug in the pM amounts to the growing media and let the WT plants grow on it in the dark. By the using a range of the concentrations we were able to observe shortening of the WT hypocotyls and in some point when the hypocotyls of the plants were shortened under some value, the seedlings start to accumulate starch. The length of the hypocotyls of the WT dark-grown plants that start to accumulate starch was approximately the same as in our secretory mutants. Based on this observation, we postulate the existence of a decision/switch mechanism, which determines the predominant energy flow in the seedling i.e. either the investment of the energy to the growth or the energy storage.

10.5 How Do Mutants in SEC15b Subunit of the Exocyst Complex Look Like?

To this date, only viable knoc-kout mutants in the exocyst subunits were those that have other paralogues in the genome (Fendrych et al., 2010; Kulich et al., 2015; Synek et al., 2006). On the contrary, knock-out mutants in the subunits that are encoded by single genes (SEC6 and SEC8 in case of the *Arabidopsis* genome) were shown to be lethal and only weaker alleles of the mutation causing only partial loss of function were viable (Cole et al., 2005). This led us to the idea that all exocyst subunits are needed for the proper function of the complex.

In other kingdoms however, exocyst subunits are usually encoded by single genes. In *Drosophila*, SEC15 knock-out leads to developmental defect caused by affected vesicle trafficking, which results in non-regular division of sensory organ precursors and appearance of extra neurons at the expense of support cells (Jafar-Nejad et al., 2005). Mutation in mouse Sec15 affects rapid transferrin receptor recycling due to extensively slowed exocytosis (Garrick and Garrick, 2007). In yeast, Sec15 null mutation is lethal but it was possible to isolate a conditionally lethal mutation. The temperature-sensitive (ts) *sec15-1* mutant blocks exocytosis and accumulate secretory vesicles in the cytoplasm (Novick et al., 1980). This phenotype can be suppressed by overexpression of other yeast late secretory genes (eg. SEC1, SEC4, SSO1, or SSO2, (Aalto et al., 1993; Salminen and Novick, 1989)) or genes from different organisms. Rat synaptotagmin was shown to suppress exclusively *sec15* ts mutants but not other exocyst mutations (Damer and Creutz, 1996) and also overexpression of 14-3-3 protein from *Trychoderma reesei* was successful in Sec15 suppression (Vasara et al., 2002). Because there are two paralogs of SEC15 subunit in the genome of *Arabidopsis thaliana*, we expected the existence of a viable *sec15b* mutant. We used three different mutant lines to find knock-out mutation. Luckily, we were able to find two mutant lines using genotyping approach that were lately by RT-PCR confirmed as knock-out lines. The major feature of its phenotype is the presence of the collet-like region when the plants are grown in the dark and disturbed apical dominance of the shoot in the case of light grown plants. The two knock-out *sec15b* lines are not, however, in the same ecotype background. The T-DNA insertional line carries the insertion in the promoter region of the SEC15b gene and is in the Col0 background. The other mutant line is a transposon-derived mutant line, where the insertion is localized in the middle of the single exone of the SEC15 gene and is in the Nossen background. We think that this feature can play an important role in the expression of the mutant phenotype.

In the *Arabidopsis thaliana* genome, there are two SEC15 paralogues - the SEC15a and the SEC15b genes. The SEC15a subunit has gametophytic expression and its function was confirmed in the study of Hála et al. (2008) who showed the pollen transmission defect in *sec15a* mutant plants. The homozygous *sec15a* plants are observed only exceptionally in the progeny of heterozygous parent. However, these plants are fertile and show no obvious phenotypic deviation. According to the available data from expression databases such as Genevestigator (`https://genevestigator.`

com/gv) or eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), the division of SEC15 subunits to gametophytic and sporophytic is not so clear. There are apparent changes of expression levels of SEC15a gene vs. SEC15b gene (Figure 10.2 10.3). Depending on the developmental stage and the ecotype background, there are higher levels either SEC15a or SEC15b (Figure 10.1). This various expression of the SEC15a and SEC15b proteins might explain why we were not able to see any visible phenotypic defect in the case of transposon-derived insertional mutant from the RIKEN collection (ecotype Nossen). Moreover, visible phenotypic defect was observed immediately after first back-cross of this line to the Col0 ecotype background of *Arabidopsis thaliana*. This observations might serve as preliminary results that are pointing to the possibility of the redundancy of SEC15 paralogues. However, other experiments have to be done to confirm this hypothesis; for example studying SEC15b and/or SEC15a mutations in other ecotypes, or complementation study of SEC15b mutants by expressing SEC15a and vice versa. Moreover, the feature of two SEC15 subunits is unique to plants and two SEC15 subunits are not commonly present in other eukaryotes. It will be very interesting to further study the relationship between these two plant paralogues.

VII

CONCLUSIONS

The whole Ph.D. thesis is focused on the exocyst complex as an effector of RAB GTPases mainly. In more detail, we raveled and discussed the aspects of mutation of different exocyst subunits and mutations of RAB GTPases and what connects them together. We also correlated the phenotypic defect of exocyst mutants with the starch accumulation and therefore we linked it with the sugar metabolism.

1. In the first part, we focused on the functional characteristics of the exocyst complex. In particular, we described how the interaction of the tethering exocyst complex with the secretory vesicle is mediated. Even though the interaction of the exocyst complex with Rab GTPases is known from the other organisms, the situation in plants has not been observed yet. Because of the previous unsuccessful attempts to find an exocyst interacting RAB GTPase, the direct interaction with the phospholipids of the vesicle and also indirect interaction via RAB GTPases was considered. Using co-immunoprecipitation and pull-down assays, we were able to identify two exocyst interacting - RAB GTPases from the class A in our model plant *Arabidopsis thaliana*. Moreover, we were able to conclude that SEC15b subunit of the exocyst complex is not able to interact with the phospholipids of the membranes. This was achieved by using lipid binding assay. This result shows the conservation of the exocyst complex, where the interaction with the transported vesicle occurs through RAB GTPases signaling molecules in plants as was shown also in other eukaryotes.

2. We also tested other exocyst subunit for the interaction with phospholipids. We chose EXO70A1 subunit that together with SEC3 subunit mediates the interaction of the exocyst with the plasma membrane in other eukaryotes. Our results show that EXO70A1 subunit interacts with phospholipids. Moreover, there is a preferential interaction of EXO70A1 with the Phosphatidic Acid (PA) *in vitro*. This observation is included in this thesis and will be part of the publication about the interaction of the exocyst complex with the membranes. Obviously, we are aware, that all results that are performed *in vitro* has to be shown *in vivo* and/or *in planta* to cover and unravel the correct mechanism. Therefore, it is necessary to compare and confirm our *in vitro* results with *in vivo* studies.

3. The second part of this thesis raveled and discussed the aspects of mutation of different exocyst subunits. There are two manuscripts, which resulted from this work. The first manuscript focuses on the yet unseen phenotypic defect, which is a very specific conditional phenotype localized in the hypocotyl/collet-hair region of dark-grown exocyst mutants.

4. The second manuscript follows the previous observations and focuses on the aspect of the starch accumulation in the hypocotyl of dark-grown secretory mutants and shows that there is a decision mechanism in plants which we call 'to store or to grow'.

5. The last section of the work shows the *Arabidopsis* mutant in the SEC15b subunit of the exocyst complex. This result will be part of the other publication.

VIII

LITERATURE

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IX

Supplements

Rab GTPases as potential targets in plant biotechnologies

Potenciál Rab GTPáz v rostlinných biotechnologiích

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Introduction

From very ancient times, plants have been a source for food, textile and construction materials for mankind. With the onset of modern technologies, plants have become a source for chemicals in developing industry. Plants are current object of fast developing biotechnologies.

Although plants naturally produce a broad range of useful compounds, this can be further expanded through appropriate genetic manipulations of plant species. Additionally, the number of plant species that can be used genetically engineered continuously increases providing an ever-expanding genetic engineering toolbox. Transgenic plants are now used for the production of nutritional components (e.g. vitamins, fatty acids, amino acids), therapeutic products (e.g. edible vaccines, antibodies, enzymes, growth factors, drugs), biodegradable plastics (e.g. starch-based polymers), and other industrial products. For the production and storage of such produced materials, different plant parts are being used. For example, crop grains are ideal for the production and storage of different proteins because there is no pressure to process them immediately after harvest, which is often a limiting factor for proteins produced in leaves or other vegetative parts of plants. In ripe seeds, proteins are protected against proteolysis and can be stored for a long time before processing[1].

There are several *in vitro* techniques that can be used to efficiently produce useful biological compounds in plant model systems. Calli or suspension cultures containing non-differentiated plant cells are widely used for production of different compounds that are secreted to the medium from the transgenic plant cells, and the system can be used for the continuous production of the desired compounds. In this way, mosses with a humanized glycosylation pattern of proteins are used to produce important glycoproteins[1].

Very interesting modification of a transgenic approach is the conversion of plastids, especially chloroplasts, into bioreactors. This approach is beneficial in many ways. Chloroplasts possess relatively small genomes that can be easily regulated and exhibit a very low level of gene silencing allowing for higher accumulation of the product of interest. Finally, the maternal inheritance of chloroplasts in most angiosperm plants limits unwanted spread of the transgene into the nature[2].

Utilizing plants in biotechnology is not only focused on compound production but also on the introduction of favourable traits including the plant resistance against biotic and abiotic stress factors which are frequently encountered by the plant. Plants as sessile organisms have to deal with different forms of external abiotic and biotic factors. When the level of external factor reaches certain level it becomes stressor for the plant causing internal stress.

Although plant responses vary based on stress type, common aspects of these stress responses are lower biomass production, reduced growth, and premature senescence. Stressors can cause serious losses in agricultural production. Some studies estimate that various stresses are responsible for up to 50% decline in agricultural production of major crops[3].

Two basic mechanisms provide plants with resistance against stress - avoidance and tolerance. The avoidance mechanism is the ability of plants to avoid transmission of an external stress into an internal stress by forming protective barriers (e.g. the cuticle on the surface of plant body protect the plant against water loss), movements (e.g. thigmonasty), or by simply avoiding the impact of periodic stress conditions by acceleration or postponing of their important ontogenetic stages (usually germination or flowering). The tolerance mechanism, on the other hand, involves the active recruitment of defense and adaptation mechanisms at the cellular level by adjusting the metabolism level, modifying the internal structures (e.g. membrane composition), and production of protective compounds, which can be either proteins or low molecular chemicals[4].

Any of the mentioned aspects of plant biotechnologies are connected with the vesicle transport inside the cell. Production of transgenic proteins is often connected to its transport to storage compartments or secretion outside the cell, which requires functional vesicle transport. Similarly, stress responses are connected with membrane system reconstitution, transport of different cargoes inside the cell, and cargo secretion out of the cell. In all these cases, vesicle transport is organized and regulated by very complicated protein machinery as well as other factors. Our research topic is RAB (Ras of Brain) GTPases, which are small GTP-hydrolysing proteins that work as organizers of vesicle transport, thus we focus this mini-review on their potential for employment in plant biotechnologies. The authors remind readers from different scientific areas that rules for writing protein names in plant literature require full name in capital letters (RAB), while literature concerning other organisms only capitalize the first letter (Rab). We follow these rules throughout the text.

Rab GTPases

Rab GTPases are small monomeric G-proteins that belong to the Ras superfamily, which also includes Ras, Ran, Rho and Arf families. All Ras-related GTPases are characterized by conserved G regions that are responsible for binding of guanosine triphosphate (GTP) and harbor the GTPase activity. The mechanism of GTP/GDP binding is very conserved. The GTP/GDP binding site creates a pocket that is associated with two domains, the Switch I domain and the Switch II domain, which undergo conformational changes upon GTP binding or hydrolysis[5].

Other region responsible for effectors binding specificity is also conserved in Rab GTPases across eukaryotes. The Rab GTPase effector domain interacts with GTPase-activating proteins (GAP) and is important in determining the functional specificity of small GTP-binding proteins[6]. Complementation studies have shown that the Rab GTPase from *Arabidopsis thaliana* is able to complement a mutation in the yeast Rab GTPase providing strong evidence for conserved effector and regulator recognition mechanisms across eukaryotic phyla[7].

The third structural feature of Rab GTPases is a hypervariable C-terminal region that is important for the localization of Rab proteins to the specific membrane compartments. Rab GTPases mostly occur as peripheral membrane proteins, although their amino acid composition implies a hydrophilic nature. This localization is achieved by their post-translational modification of attachments with lipids moieties. Rab proteins typically contain two C-terminal cysteine residues that undergo post-translational modifications by the covalent attachment of geranylgeranyl (C20 isoprenoid) groups via thioether linkage. The geranylgeranylation facilitates membrane association and in some cases also plays a major role in specific protein-protein interactions[8].

The geranylgeranylation reaction is mediated by the protein prenyl transferase family that includes protein farnesyl transferase (FT), protein geranylgeranyl transferase type I

(GGT-I), and Rab geranylgeranyl transferase (RabGGT or GGT-II). All prenyl transferases are heterodimers consisting of α- and β-subunits. Rab GTPases are unique substrates of RabGGT because the process of Rab GTPase geranylgeranylation requires the presence of the Rab escort protein (REP). First, REP binds a newly synthesized Rab protein and forms a stable Rab-REP complex. Second, RabGGT is able to recognize the Rab-REP complex as its protein substrate and catalyses the transfer of geranylgeranyl moieties to the relevant cysteine residues. After geranylgeranylation, Rab GTPase is delivered to a donor membrane compartment in the same manner as during its regular cycling[9].

Cycling of Rab GTPases

Rab GTPases enter cycling immediately after translation. The first cycle is represented by the switching between GDP- and GTP-bound forms while the second cycle includes the translocation of the RAB GTPase from a donor membrane compartment to a target membrane compartment (Fig.1). Both RAB cycles are tightly connected.

First, the GDP-bound form of Rab GTPase interacts with a Guanine nucleotide exchange factor (GEF) that facilitates release of the bound GDP and replaces it with GTP, changing the conformation of Switch I and II domains. This allows the GTP-bound Rab protein to interact with the effector proteins. Upon this interaction, Rab GTPase is transported from the donor membrane compartment to a target membrane compartment via vesicle transport. To be able to continue in the cycle, the Rab GTPase has to be converted back to the GDP-bound form as its internal hydrolytic activity is very weak. This is achieved by an interaction with a Rab GTPase activating protein (RabGAP) that catalyzes hydrolysis of GTP. The now GDP-bound Rab GTPase is then extracted from the target membrane by a Rab GDP dissociation inhibitor protein (RabGDI) that transports Rab GTPase through the cytoplasm and back to the donor membrane compartment to complete the cycle. A GDI Displacement Factor (GDF) facilitates release of Rab GTPase from RabGDI by a yet unknown mechanism. Generally, substrate specificity of GEFs, GAPs and GDFs is usually limited to few Rab GTPases, while GDIs are promiscuous[5].

The function of Rab GTPases as vesicle trafficking regulators is based on exchange of one type of Rab GTPases on the vesicle surface for another type, which is achieved through so called RabGEF and RabGAP cascades. First, a Rab GTPase in the regulatory pathway is activated by an appropriate RabGEF. Once activated, it recruits other RabGEFs that activate a next Rab GTPase in the cascade and simultaneously is inactivated by a corresponding RabGAP. The whole process continues until the vesicle reaches the target membrane[10].

Classification of RAB GTPases in plants

Although it seems that Rab GTPases are present in all eukaryotes, the number of paralogues significantly differs among organisms. Generally, the number is lower in unicellular organisms and it increases with higher organism complexity. In yeast *Saccharomyces cerevisiae*, there are eleven paralogues while mammalian genomes encode about 60 Rab paralogues. In these organisms, Rab GTPases are classified numerically (e.g.Rab1) in order of when they were discovered.

In plants, the number of paralogues is similar to that in animals but the overall structure of the group differs. For instance, 57 RAB GTPases have been identified in the *Arabidopsis thaliana* genome and they can be grouped into eight subgroups related to only eight Rab GTPases from animals (RAB-A to RAB-H subgroups), each containing several paralogues[7]. Similar number of Rab GTPases is encoded in other Angiosperm plant genomes. On the other hand, there are only about ten to twenty Rab GTPases in Bryophytes and green algae.

Structural homology also corresponds to functional classification. The subgroup RAB-A (Rab11 homologue) functions in the trans-Golgi network and in the vesicle transport to the plasma membrane (PM), the RAB-B subgroup (Rab2 homologue) organizes vesicular

transport between the Golgi apparatus (GA) and the endoplasmic reticulum (ER), the RAB-C subgroup (Rab18 homologue) associates with peroxisomes, the RAB-D subgroup (Rab1 homologue) regulates vesicle transport between ER and GA, the RAB-E subgroup (Rab8 homologue) participates in TGN to PM transport, the subgroup RAB-F (Rab5 homologue) regulates endocytosis, the subgroup RAB-G (Rab7 homologue) affects vesicle transport to the vacuole, and the subgroup RAB-H (Rab6 homologue) is localized in GA[11]. Further text will focus on those subgroups involved in plant stress responses and cell wall component deposition.

RAB GTPases and plant stress tolerance

Stress conditions put more demand on membrane recycling. Maintaining integrity of the biological membranes is the first priority during stress onset. At the same time, a spectrum of membrane proteins changes and endocytosis is the most effective way of recycling them.

One of the endocytotic RAB GTPases, RAB-G3e, is transcribed under oxidative stress. At the same time, its transcription can be induced by salicylic acid treatment. This implies its involvement in biotic stresses. Overexpression of this RAB results in acceleration of endocytosis and the increased resistance of transgenic plants to osmotic stresses. Transgenic plants grown on high salinity media accumulate significantly more sodium in shoots than WT (wild type) plants, indicating an enhanced accumulation in vacuoles. Similarly, increased tolerance occurs when these plants are cultivated on media containing high sorbitol concentration, indicating that the primary effect is in osmotic tolerance and not ion toxicity due to the salt stress[12].

The subgroup RAB-F is also involved in the regulation of endocytosis. Moreover, it includes a plant-specific RAB GTPase RAB-F1 (known also as Ara6), which is not geranylgeranylated in a canonical manner but instead it is palmitoylated[13]. The structural difference is accompanied by a functional difference. Unlike the rest of the family, RAB-F1 plays a role in recycling from endosomes to the plasma membrane[13]. In the same study, it was shown to be involved in the proper response to elevated salinity conditions. Overexpression of RAB-F1 significantly helped plants overcome root growth arrest both on media with high sodium chloride and sorbitol concentrations. Surprisingly, even such specialization does not make it unique in the plant cell. Knock-out mutants in this gene showed no phenotypic deviation from WT[13]. This illustrates how redundant RAB GTPases are across subfamilies, making such research more difficult. RAB-F1 was recently shown to be a part of the extrahaustorial membrane, the membrane that surrounds fungal haustorium, and protects plant cell against invading fungus[14].

The RAB-F subgroup is also important for salt stress tolerance in rice (*Oryza sativa*). Transformed lines over-expressing OsRAB7 have significantly higher tolerance to salt stress represented by faster shoot growth and higher lateral root number. This effect was connected to a 3.5 fold higher accumulation of proline, which is known to play role in osmotic adjustment as well as an antioxidant. However, the direct mechanism connecting RAB GTPases and proline accumulation remains unknown[15].

Recent finding shows that some members of the secretion-involved RAB-A1 subgroup are required for tolerance to salinity stress, which implies that these RAB GTPases might regulate the localization of proteins integral to the plasmatic membrane, such as proton pumps and ion channels[16].

RAB GTPases involved in cell wall biogenesis

Plant cell wall components are important materials for many industrial branches and are therefore subjects of research. The plant cell wall is a complex structure composed typically of cellulose (1,4-β-D-glucan), hemicelluloses (xyloglucans, gluconoarabinoxylans, glucomannans), and pectins (homogalacturonans, xylogalacturonans, rhamnogalacturonans I and rhamnogalacturonans II). Cellulose combines with different types of hemicelluloses and pectins depending on the plant species and the tissue type. In special cases, cellulose is

substituted or supported by callose (1,3-β-D-glucan). Synthesis of cellulose or callose occurs directly on the plasma membrane where functional complexes of cellulose synthase or callose synthase reside having been delivered as cargoes of vesicle transport. Synthesis of other cell wall components is generally localized to the GA where products are then transported to the apoplast via vesicle trafficking. There are a number of proteins in the cell wall too (e.g. expansins, extensins, pectin-modifying enzymes etc.) that are delivered to the apoplast via vesicle transport. Biogenesis of the cell wall is rather complex process that requires the orchestration of many steps that are surprisingly plastic in respects to external manipulation. Experiments focused on investigating the affects of specific cell wall properties through knock-down of pectin modifying enzymes only had very limited success. Only combining the knock-down of multiple genes causes that the phenotypic deviation can be observed, which shows how dynamic cell wall structure and organization is[17].

RAB GTPases as organizers of vesicle trafficking are also potential targets for biotechnology manipulations. For years, the role of RAB GTPases in fruit ripening has started to be studied. Fruit ripening is an economically important process that is accompanied by significant cell wall modification. The RAB GTPases in the two subgroups, RAB-A and RAB-D, were shown to play a role in tomato (*Lycopersicon esculentum*) fruit ripening. Three paralogues of LeRAB1 (RAB-D subgroup) were detected depending on the stage of fruit development. While LeRAB1a was expressed in green growing fruits, the other two paralogues were highly expressed during ripening[18]. Moreover, they seemed to not be fully redundant in their GTPase function. More interesting are data obtained for LeRAB11a (RAB-A subgroup). Transgenic plants expressing antisense mRNA for *LeRAB11a* under a constitutive promoter produced fruits that changed colour properly upon ripening but remained firm for a long time. The authors also observed reduced activity of the pectinesterase and polygalacturonase in the apoplast[19].

Intensively growing pollen tube is an interesting model for studying the relationship between secretory events and cell wall biogenesis. Compromising Rab11b (homologous to the RAB-A subgroup) function led to the arrest of secretion and accumulation of soluble cell wall protein, invertase, in the cytoplasm[20]. Moreover, this paper showed the potential of point mutations in RAB GTPases that led to changes in the binding capacity of the guanine nucleotide. These mutants include the constitutively active (CA) form that has compromised hydrolytic activity, which leads to accumulation of GTP-bound forms of the RABs. On the other hand, the dominant negative form (DN) is unable to stabilize binding of the third phosphate group in the nucleotide-binding pocket and is considered to be GDP-bound. Some DN RAB GTPases tend to lose the guanine nucleotide and become nucleotideless[5]. Over-expression of these forms in living cells is often lethal, requiring the use of transient expression or inducible promoters to express these GTPases in plants.

Another pollen-specific RAB GTPase from the RAB-A subgroup is RAB-A4d, which was shown to localize to the growing tip of *Arabidopsis* pollen tubes. Knock-out mutations of the gene revealed it is required for the proper development of the pollen tube and showed a disturbed localization pectin in the cell wall[21].

Direct involvement of RAB GTPases in cell wall biogenesis was tested in the pioneering work of Lunn et al[22]. They analyzed the proportion of basic cell wall components in numerous *Arabidopsis* single knock-out RAB GTPases mutants belonging to different RAB-A subgroups. Their results show that cell cell walls of RAB mutants in the RAB-A1 clade had a lower amount of pectin, in mutants from the RAB-A2 subgroup had a lower amount of cellulose, and mutants from the RAB-A4 subgroup had a lower amount of hemicelluloses. This simple experiment suggests a specialization of RAB GTPases for different vesicular cargoes.

Finally, RAB-A4c was connected to the defense against fungal pathogens by showing an interaction with its effector, PMR4. PMR4 is a callose synthase and its enzymatic activity is activated after translocation to a location on the plasma membrane where the fungal pathogen forms an appressorium to penetrate the cell[23]. The role of RAB-A4c is then seen in the translocation of the callose synthase. This observation nicely connects cell wall modification and biotic stress tolerance.

Conclusion

RAB GTPases are undoubtedly important regulators of vesicle trafficking. On the other hand, their potential for biotechnologies is limited. To improve plant responses to stress, the most promising subgroups are RAB-F and RAB-G, which regulate the endocytotic routes. Recycling proteins from the plasma membrane and decision of whether these proteins should be degraded in vacuole or stored, is obviously very important to the plant stress responses.

On the other hand, cell wall material travels through the transport route controlled by the RAB-A subgroup. Moreover, it seems that different components of the cell wall use different routes to the cell surface. Experimental data implies there are ways to change or affect composition of the cell wall by simply changing the equilibrium among various RAB-A paralogues. Cell wall biogenesis regulation is very complex and has high plasticity to genetic manipulation. In this respect, RAB GTPases offer very interesting tools represented in their CA and DN mutant forms, and the expression of these forms can shift the process out of equilibrium.

Not commented in this text but an important part of RAB GTPase potential is their participation in the interaction between plants and surrounding biosphere. Roles of RAB GTPases in pathogen resistance were already mentioned in the text, but as they become focus of many researchers, interesting discoveries can be expected in the near future.

Moreover, RAB-A2 GTPase is crucial for legumes nodulation in the presence of symbiotic bacteria (e.g.genus *Rhizobium*), which is important for fixing atmospheric nitrogen and its utilization by plant. As the first step of the nodulation process, reorientation of the root hair axis and induction of nodulation genes can be observed. In bean plants with knocked-down levels of a RAB-A homologue, the nodulation was disabled in the first step of interaction[24].

Although we present many examples of how RAB GTPases are involved in plant responses to stress or during cell wall biogenesis, the main question is still if or how RAB GTPases can be employed for plant biotechnologies and it remains the topic for further research.

Abstract

Plants are not only a food resource but are also a valuable source of different materials. Plant biotechnologies help improve food or material yield and increase the number of compounds obtained from plants. Affecting plant secretory machinery is one of the ways we can modify plant bio-production. Rab GTPases are important organizers of vesicle transport through their cycling between GDP- and GTP-bound forms as well as between membrane compartments. Here, we discuss whether RAB GTPases can be targets for biotechnology research. We focus on the involvement of RAB GTPases in plant stress responses and cell wall biogenesis, which are two aspects that highly influence plant development. We conclude is that the complexity of RAB GTPase families and their regulation make these proteins difficult targets for biotechnologies.

Keywords: Plant biotechnology, RAB GTPases, plant stress, plant cell wall biogenesis

Shrnutí

Rostliny poskytují lidstvu nejenom potravu, ale jsou i cenným zdrojem surovin pro různá odvětví průmyslu. Rostlinné biotechnologie pomáhají zlepšovat výnosy jak potravin, tak surovin získávaných z rostlin a také rozšiřovat jejich spektrum. Jedním z nástrojů je ovlivňování buněčného sekrečního aparátu. Rab GTPázy mají důležitou funkci právě při organizaci váčkového transportu, důležité je zejména jejich cyklování mezi různými formami s navázaným GDP či GTP a také mezi různými membránovými kompartmenty. Jak název

naší práce napovídá, chceme zvážit možné využití RAB GTPáz v biotechnologiích. V naší práci ukazujeme na vybraných příkladech z literatury úlohu RAB GTPáz v odpovědích rostlin na stres a ve tvorbě buněčné stěny u rostlin, což jsou procesy zásadně ovlivňující rostlinnou produkci. Naším závěrem je, že využití RAB GTPáz v biotechnologiích by bylo možné, ale, vzhledem k dosud ne zcela probádané a velmi komplexní síti regulací, vyžaduje další výzkum.

Klíčová slova: Rostlinné biotechnologie, RAB GTPázy, stresová odpověď rostlin, tvorba buněčné stěny.

Legend to figure:

Fig.1 – Schematic view of Rab GTPase cycling between GTP- and GDP-bound forms and among different membrane compartments. Rab GTPase is first activated by exchange of GDP for GTP through the action of GEF in a donor compartment (e.g. secretory vesicle). It allows binding of effectors and membrane fusion with a acceptor compartment (e.g. plasma membrane). Upon fusion, Rab GTPase is inactivated though the action of GAP and retrieved from the membrane by GDI. Back at the donor compartment, GDF releases GDI from the complex with Rab GTPase and the Rab GTPase is localized to the membrane again.

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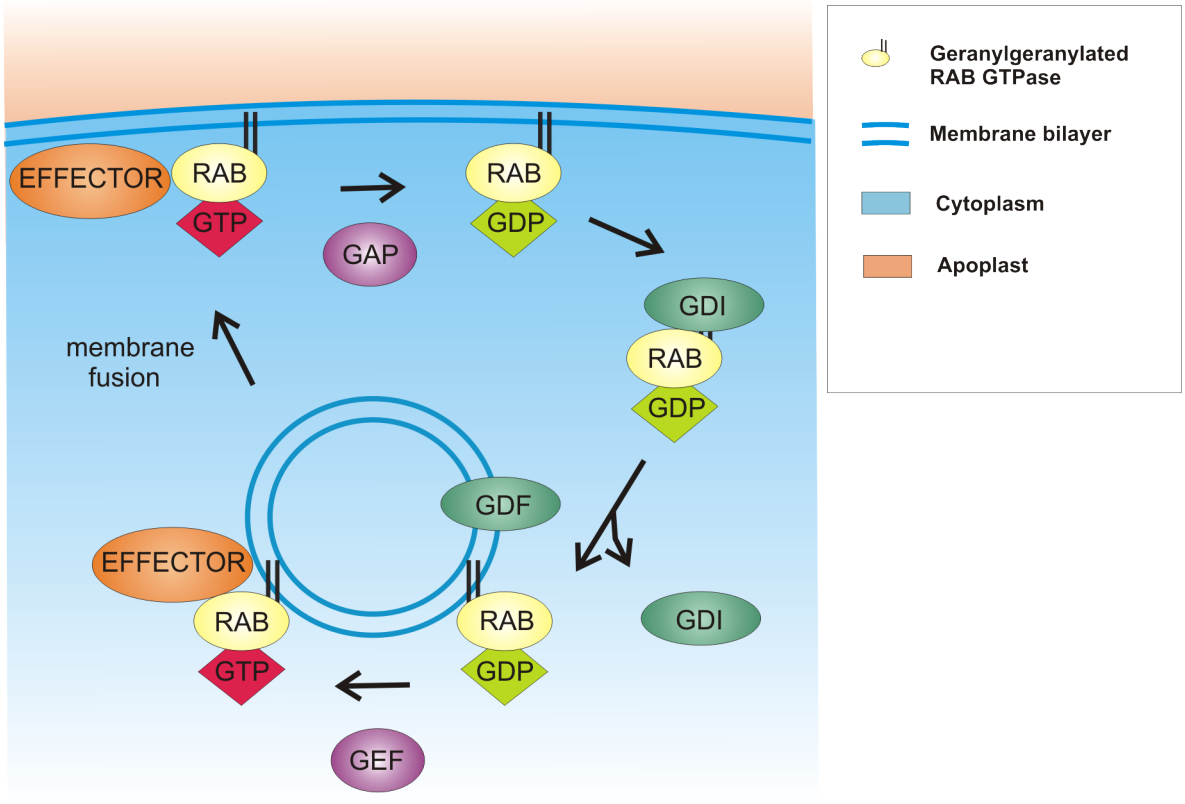
EFFECTOR
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RAB
GDP
GDI
GEF
Geranylgeranylated
RAB GTPase
Membrane bilayer
Cytoplasm
Apoplast

Fig.1

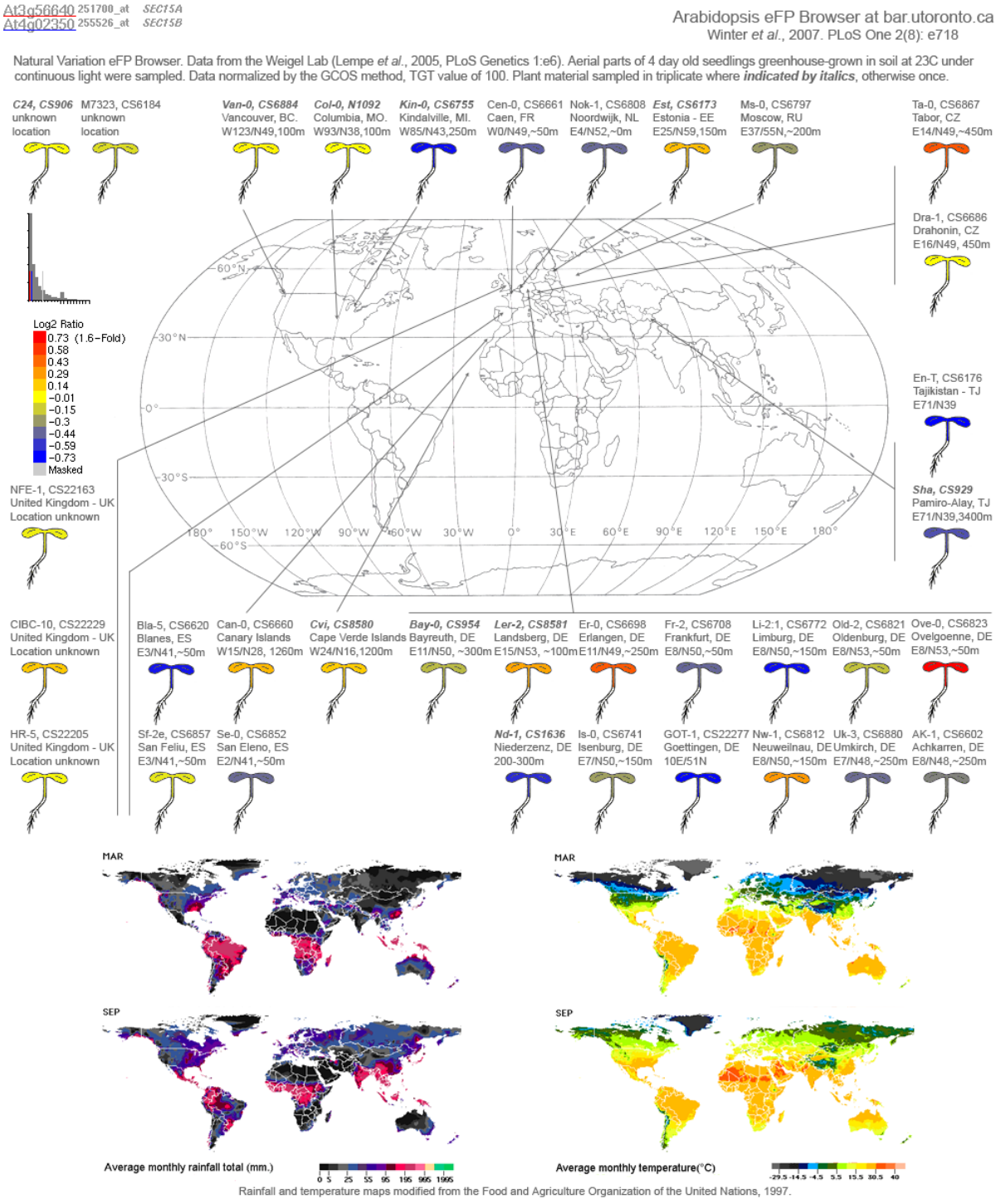

Figure 10.1: The natural variation in the level of SEC15a and SEC15b expression in different *Arabidopsis thaliana* ecotypes. SEC15A is depicted in red color and SEC15b in blue color.

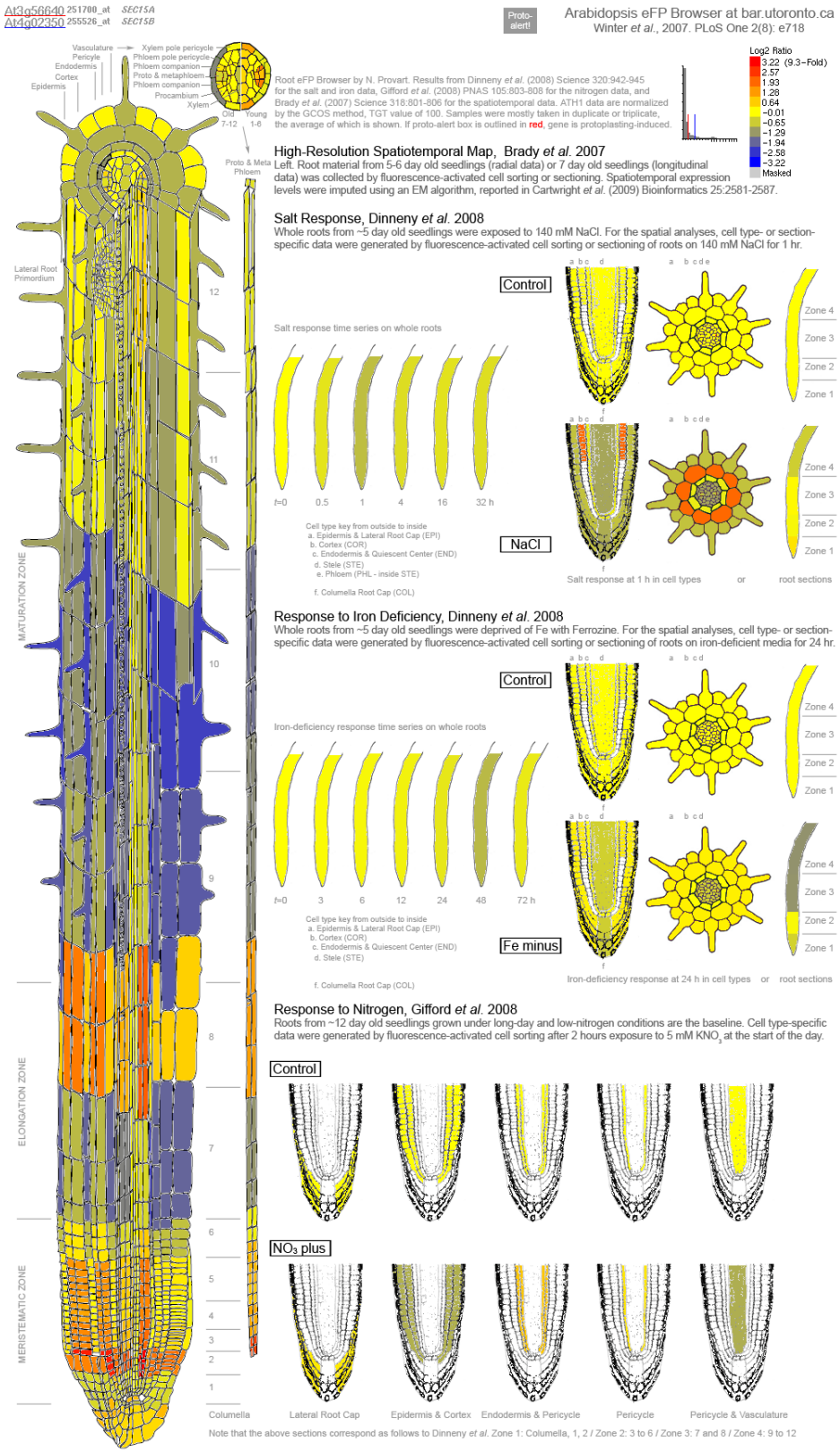

Figure 10.2: The comparison of SEC15A and SEC15B expression in the *Arabidopsis* root. SEC15A is depicted in red color and SEC15b in blue color.

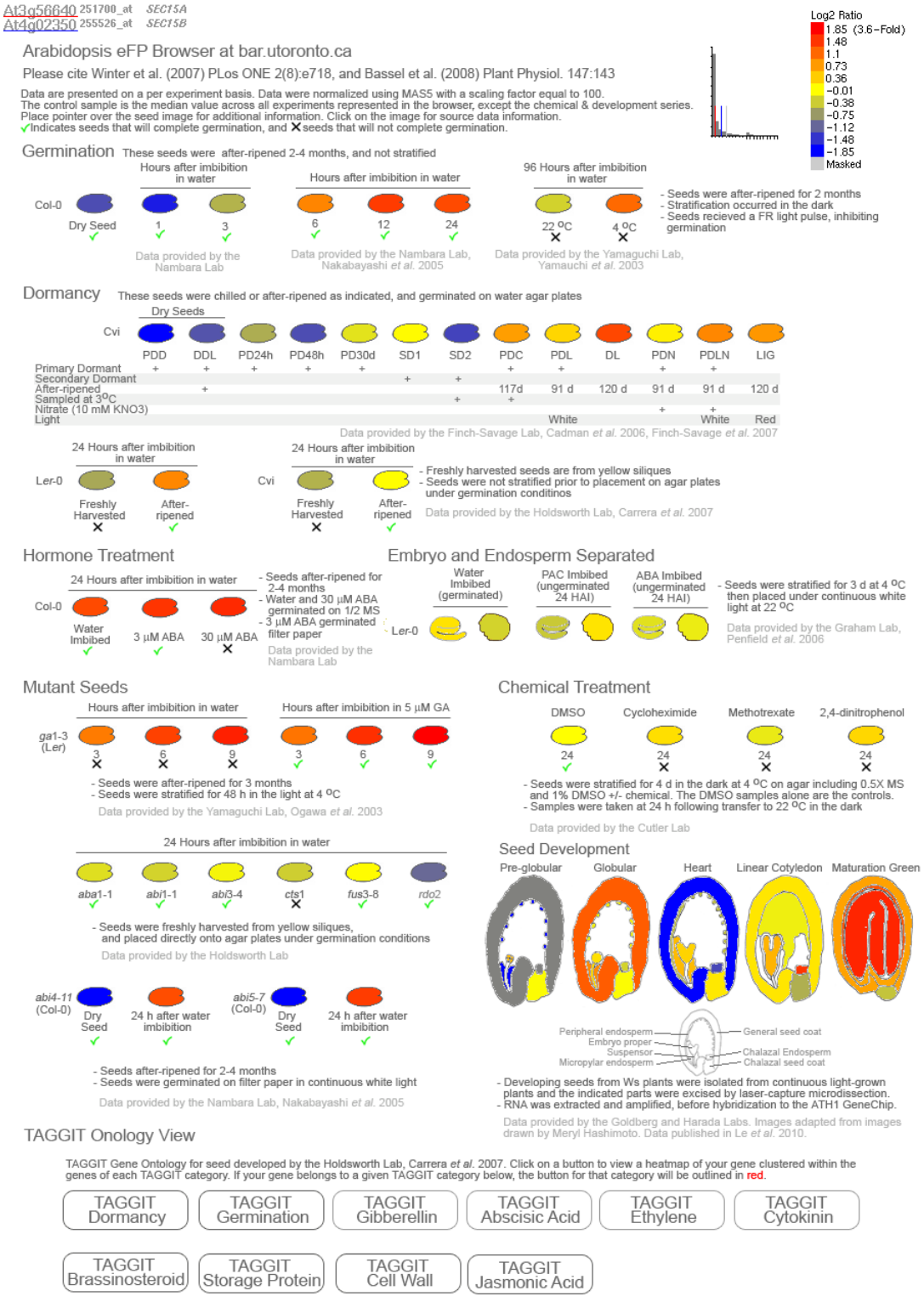

Figure 10.3: The comparison of SEC15A and SEC15B expression in the *Arabidopsis* seed. SEC15A is depicted in red color and SEC15b in blue color.