Charles University in Prague
Faculty of science

Study programme: Special chemical-biological branches
Study branche: Molecular biology and biochemistry of organisms

Bachelor Thesis

Interplay of cytoskeleton and secretory pathway during exocytosis in plant cells

Souhra cytoskeletu a sekretorické dráhy v exostóze rostlinných buněk

Klára Aldorfová

Supervisor: Mgr. Juraj Sekereš

Prague, 2014

Acknowledgements:

I would like to express my very great appreciation to Mgr. Juraj Sekereš and everyone who provided me support and advices during writing of this thesis.

Prohlášení:

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

V Praze, 15.5.2014

Podpis

Abstract

Cytoskeleton is known to participate in exocytosis of yeast and animal cells. The role of plant cytoskeleton during exocytosis has not been fully understood yet. However, both actin and microtubules evidently contributes to the secretion of specific cargo proteins or cell wall components. Plant cytoskeleton influences the dynamics of exocytosis through various functions. First, secretory vesicles are delivered near the plasma membrane. Second, microtubules were shown to mark the place of exocytosis. Third, cytoskeleton is able to prevent membrane fusion by simple separation of compartments. Fourth, cytoskeleton potentially mediates the interaction between molecules of secretory apparatus. Secretion of certain cargo molecules appears to be dependent on different cytoskeleton types and the exocytosis seems to be specifically regulated in each tissue. This thesis aims to describe interplay of cytoskeleton and secretory pathway on the example of tip growth and to predict future direction of research on secretory pathway based on cellulose synthase secretory data.

Key words:

Plant exocytosis, secretory apparatus, actin, microtubules, tip growth, pollen tube, root hair, cellulose synthase containing compartments

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Abstrakt

Cytoskelet se v živočišných a kvasinkových buňkách významně podílí na exocytóse, avšak u rostlin dosud není přesný molekulární mechenismus interakce sekretorického aparátu s cytoskeletem znám. Novodobá pozorování však napovídají, že mikrotubuly i aktin ovlivňují dynamiku exocytosy určitých proteinů. Obecně se cytoskelet podílí na exocytóse dopravou sekretorických váčků k plasmatické membráně a je schopen označovat místa budoucí sekrece. Po překročení limitní koncentrace cytoskeletálních vláken může sloužit jako sterická zábrana a brání tak fúzi kompartmentů. Rostlinný cytoskelet také pravděpodobně přispívá k exocytóse přímou interakcí se sekretorickým aparátem. Sekrece různých rostlinných sekretorických váčků je závislá na aktinu, mikrotubulech, obou komponentách či nezávislá na cytoskeletu. Také se ukazuje, že exocytósa je řízena tkáňově specificky. Tato práce si klade za cíl uvést na příkladu vrcholového růstu souhru cytoskeletu při exocytóse jednotlivých proteinů a komponent buněčné stěny a na základě spolupráce aktinu s mikrotubuly při sekreci celulosa syntásy se snaží naznačit budoucí směr výzkumu dynamiky exocytósy v kontextu rostlinné buňky.

Klíčová slova:

Exocytósa rostlinných buněk, sekretorický aparát, aktin, mikrotubuly, vrcholový růst, pylová láčka, kořenové vlášení kompartmenty obsahující celulosa syntásu

Contents:

Abbreviations:

ACT	Actin
ADF	Actin depolymerizing factor
ADP	Adenosine-5´- diphosphate
Arp2/3	Actin- related protein- 2/3
ATP	Adenosine-5´- triphosphate
BY-2	Cultivar Bright Yellow- 2 of the tobacco plant
CalS	Calose synthase
CesA	Cellulose synthase
CSC	Complex of cellulose synthases
DUF	Domain of unknown function
F- actin	Filamentous actin
FRAP	Fluorescence recovery after photobleaching
GAP	GTPase activating protein
GDP	Guanosine-5´- diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine-5´- triphosphate
MASC	Microtubule- associated cellulose synthase compartment
PEN3	Penetration resistace 3
PI (4, 5)P2	Phosphatidylinositol 4, 5- bisphosphate
Rab GTPase	Rat brain GTPase
RIC	ROP- interactive CRIB- motif- containing protein
RIP	ROP interactive partner
ROP	Rho of plants
SmaCC	Small cellulose synthase- containing compartment
SNARE	Soluble N´ethyl maleimide sensitive factor adaptor Protein receptor
Suc	Sucrose synthase

1. Introduction

In eukaryotic cells, the secretory pathway is essential for morphogenesis, cell growth, the delivery of molecules to the plasma membrane, signalling and many other processes. The basic features of the process and the set of the molecules participating in exocytosis are evolutionary conserved, but at least some of the interactions between exocytic components appear to be unique for different eukaryotic groups including plants (Vaškovičová et al., 2013).

Plant cell is highly dependent on delivery of new cell wall material, lipids and membrane proteins to the plasma membrane during cell growth, morphogenesis and housekeeping processes. The dilivery must be spatiotemporally regulated. In general, the proteins are trafficked through the conventional protein secretion pathway. Proteins, which are cotranslationally inserted in endoplasmic reticulum, continue through the vesicle transport into the Golgi apparatus, where they undergo posttranslational modifications such as glycosylation. Then they are delivered to the vacule or to the plasma membrane. In contrast several evidences of unconventional protein secretion have appeared and it seems to be more common phenomenon than expected in the plant cells (Drakakaki and Dandekar, 2013).

Actin and microtubule cytoskeleton plays important roles in transport of donor compartments and secretory vesicles as well as exocytosis itself (Brandizzi and Wasteneys, 2013). Once created, the secretory vesicle is delivered to the cell periphery near the site of exocytosis.

Each type of compartment in the cell posseses a specific set of membrane identificators. Mainly, Rab GTPases (belong to the superfamily of small GTPases), SNARE (SNAP receptor, enables the final step of membrane fusion) proteins and specific membrane lipid composition. The Rab GTPases are able to switch between GTP and GDP binding states, but predominantly GTP-bound Rab GTPases orchestrate changes in lipid and membrane composition of the donor membrane and secretory vesicles during maturation via a number of effectors. The secretory vesicles vary in their size, morphological features, origin and content. Just those with the correct combination of SNARE proteins, Rab GTPases and lipids are delivered to the exocytic site and are able to fuse with the plasma membrane (Saito and Ueda, 2009).

The secretory vesicles are delivered near the plasma membrane. Docked vesicles are those which reached less than10-30 nm from the cell surface (Porat-Shliom et al., 2013) and are prepared for following step, vesicle tethering. Tethering results in targeting to the plasma membrane through multisubunit complex exocyst (Žárský et al., 2009). The final step, fusion of the secretory vesicle with plasma membrane, is called exocytosis and requires a coordination of a number of secretory proteins with cytoskeleton in animal and yeast cell and also potentially in the plant cell. The membrane fusion on its own is mediated by SNARE complex. Three SNARE proteins at the plasma membrane interact with one SNARE protein on the secretory vesicle, which leads to the physical contact and fusion of the

membranes (Saito and Ueda, 2009).

Since the late twentieth century, cytoskeleton disrupting drugs have been applied on plant cells as a powerful tool to reveal cytoskeleton dependent processes. Nowadays, combination of fluorescently labelled proteins, drug treatment and specialized microscopic techniques (such as fluorescent recovery after photobleaching and high resolution microscopy) helps to define dynamics and kinetics of specific cargoes during exocytosis and its interplay with cytoskeleton.

This thesis aims to summarize known interactions between secretory apparatus and cytoskeleton with emphasis on movement and exocytosis of secretory vesicles. The first part reviews basic knowledge about plant cytoskeleton and secretory apparatus mainly in the context of molecular interactions between these two components. Following part describes a general model of secretory vesicle generation, delivery and fusion with emphasis on interplay with cytoskeleton and associated motors. The third chapter is focused on the role of cytoskeleton during the delivery of secretory vesicles in tip growing cells. Tip growth is well described process characteristic for polarized elongation of a single cell dependent on a massive delivery of new cell wall material and involving massive exocytosis. Tip growing cells are widely studied and most experiments studying interplay of exocytosis and cytoskeleton in plant cells have been performed in this system. Both cytoskeleton assembly and disassembly are crucial regulatory steps, participating in positive and negative regulation of exocytosis in pollen tubes, a powerfull model system for plant secretory pathway and cell morphogenesis. The last chapter of thesis discusses exocytosis of special cargo- cellulose synthase complex. CesA (cellulose synthase) containing vesicles and their exocytosis with relation to cytoskeleton have been recently examined with high spatiotemporal resolution. High resolution observations of CesA containing compartments enable to describe details intangible by mere combination of drug treatment and laser scanning confocal microscopy. They allow precise observations of interplay between both cytoskeleton types during vesicle transport and exocytosis.

2. Cytoskeleton, secretory apparatus and interactions between them

This chapter introduces basic components of cytoskeleton and its regulators, as well as important protein sof secretory pathway. The key protein families involved in the exocytotic process appeal to be evolutionary conserved. Actomyosin systém, microtubules with associated kinesins, ROP and Rab GTPases, SNARE and exocyst participace in the final step of secretion among all eukaryotes (Vaškovičová et al., 2013). However, some of the interactions between secretory apparatus and cytoskeleton or thein regulations may differ among evolutionary distant eukaryotes. Known protein-protein interactions of importace for exocytosis- cytoskeleton interplay in plant cells are pointe dout and discussed.

2.1 Cytoskeleton, cytoskeleton associated molecular motors and cytoskeleton organization

Actin and tubulin polymerize into long fibers, which form highly dynamic cell skeleton. Architecture depends on cell type and conditions. Actin and microtubules enable the motility of secretory vesicles via associated molecular motors, myosins and kinesins (Romagnoli et al., 2007). However, the role of microtubules is less clear and appears to be associated only with certain types of cargo (Sampathkumar et al., 2013; Apostolakos et al., 2009). Cytoskeleton dynamics is orchestrated by ROP (Rho of Plants) GTPases and many associated regulatory proteins (Craddock et al., 2012).

2.1.1 Actin cytoskeleton

Actin is a globular protein that is able to create a highly dynamic filamentous polymer in dependence on its ADP/ATP binding state. Actin cytoskeleton serves multiple essential roles including establishment of the cell polarity, tip growth, determination of cellular shape, cytoplasmic streaming, organelle trafficking, responses to pathogen and many other important processes (Kandasamy et al., 2002). Several types of actin participate in different actions and appear to be developmentally and tissue specific (Ringli et al., 2002a). The actin dynamics is coordinated by a number of actin-binding proteins (Hussey et al., 2006).

Ten genes encoding actin are localized in the *Arabidopsis* genome, eight of them are functional. ACT1, 3 and 11, are expressed mainly in reproductive tissues (Meagher et al., 1999). The rest of the actin isovariants can be found in vegetative tissues. Both vegetative and reproductive actin types are very similar to each other on amino acid level. The divergence and different expression pattern of actin supports the idea that the actin isovariants participates in different processes (McDowell et al., 1996). Changes in cytoskeletal organization could be crucial for localization of exocytosis as shown during characterization of act2 mutant. ACT2 was shown to be essential for root hair tip growth, where defines exocytic site and participates in selection of the future tip position in trichoblast cell (Ringli et al., 2002).

Cytoskeleton organization is maintained by cytoskeleton binding proteins. Coordination of these proteins is mostly orchestrated by ROP GTPases (see below). Actin binding proteins could be also regulated by changes in lipid composition (Hussey et al., 2006). Actin nucleators polymerize the actin filaments in the correct localization in accordance to the cellular signals and include Arp2/3 complex and formins. Capping proteins, gelsolin, formin and profilin, were observed to bind to the barbed end of actin filaments. Fimbrins, villins and formins participate in actin bundling. ADF/cofilin exhibited severing of actin filaments (Hussey et al., 2006). Some of the actin interacting proteins are known to regulate actin assembly or disassembly in relation with exocytosis. Many of these proteins have multiple roles (Hepler et al., 2013).

2.1.2 Myosins, the actin- associated motors

Myosin is an F-actin associated molecular motor that converts chemical energy of ATP hydrolysis into kinetic energy. *Arabidopsis thaliana* genome encodes two classes of myosins (VIII, XI) and both groups share similar domain architecture with animal myosin V class (Madison and Nebenführ, 2013). Farquharson and Staiger speculate about broad plant myosin redundancy (Farquharson and Staiger, 2010). Myosins are known to facilitate cytoplasmic streaming, viral movement, plasmodesmata opening and trafficking of Golgi stacks, peroxisomes and mitochondria in the plant cells (Sparkes, 2011).

Myosin structure includes 4 domains. Amino terminus motor domain contains positively charched loopes involved in actin interaction and nucleotide binding site. Neck domain, which determines velocity and processivity, contains 6 IQ motifs that serve for calmodulin related protein interaction. Myosins dimerize through coiled coil rod regions. COOH terminus enables the myosin to bind the cargo vesicle (Tominaga and Nakano, 2012). Myosins switch between conformational states in calcium dependent manner (Tominaga and Nakano, 2012).

Myosin function appears to be redundant within the classes. Two redundant moss myosins myoXI accumulate at subcortical apex of protonemal tip where they participate in tip growth (Vidali et al., 2010). Arabidopsis XI-2 (also known as MYA2) and XI-K perform similar role in organelle trafficking during root hair tip growth (Peremyslov et al., 2008). Observation in tobacco leaf cells implies partial functional redundancy of myosin XI-2, XI-F and XI-K during trafficking. Myosin XI-K participates mainly in Golgi stack, peroxisome and mitochondrial organelle movements was confirmed by overexpression of headless tails (Avisar et al., 2008). Myosin proteins drive polarized trafficking. MYA2 appears to participate in polar growth in auxin dependent manner (Holweg and Nick, 2004). Myosin XI-K accumulates in growing root hair tip (Peremyslov et al., 2012). There is evidence that actin and actin related proteins affects behaviour of secretory vesicles and alter their dynamics of exocytosis (Cai et al., 2011; Sampathkumar et al., 2013). Myosins exhibit a number of interactions via

variable interactors as reviewed by Madison and Nebenführ (Madison and Nebenführ, 2013), however just several of them were found on secretory vesicles.

DUF 593 (domain of unknown function) was observed to bind myosin XI-K and XI-1. This interaction is important for formation of endosperm (Holding et al., 2007), although does not participate in exocytosis (Peremyslov et al., 2013). DUF 593 was found in transmembrane protein zein that is involved in the formation of endosperm (Holding et al., 2007)

RabC2 and RabD1 were shown to interact with Myo11B2 (MYA2) (Hashimoto et al., 2008) . RabD was observed to regulate trafficking from ER to Golgi apparathus (Woollard and Moore, 2008). In addition, myosin XI-K was co-fractionated with RabA4b, the regulator of exocytosis in root hairs, and Sec 6, the exocyst subunit and co-localized with secretory vesicle marker *in vivo* (Peremyslov et al., 2012).

2.1.3 Microtubules

Microtubules are long tubular polymers made of alpha and beta tubulin dimers. They participate in processes such as plant mitosis, where they mark preprophasic band followed by a formation of phragmoplast (Zhu and Dixit, 2012). In interphasic cells, clear cooperation between microtubules and cellulose synthesis was observed. Microtubules ensure proper organelle localization (Cai and Cresti, 2012) including Golgi stacks and are involved in in auxin polar transport (Brandizzi and Wasteneys, 2013). However, their role in trafficking is still unclear.

Interestingly, disassembly of microfilaments affects microtubule depolymerisation (Poulter et al., 2008), but depolymerisation of microtubules does not affect the actin organization. Microtubule organization could fine tune modulation of actin cytoskeleton and cytoplasmic streaming (Brandizzi and Wasteneys, 2013). Additionally, Golgi vesicles move along microtubules and microfilaments with different kinetics and are able to switch between both cytoskeleton types (Romagnoli et al., 2007), thus also microtubules regulate the organelle localization.

Microtubules appear to play a role during trafficking of several types of secretory vesicles. Microtubule organization affects synthesis of the cellulose microfibrils (Paredez et al., 2006), callose (Apostolakos et al., 2009) and potentially pectin delivery to the plasma membrane (McFarlane et al., 2008) (see below).

2.1.4 Kinesins, the microtubule associated motors

Kinesins are microtubules associated molecular motors that transfer energy from ATP to the force generation through conformational change. Eukaryotic kinesin proteins were distinguished into 14 families (Lawrence et al., 2004). Kinesins families 2, 3, 9, and 11 are not present in the plant genome,

families 7 and 14 diverged into plant specific functions (Zhu and Dixit, 2012) such as phragmoplast formation and transport of various organelles like mitochondria, chloroplasts and Golgi bodies. All *Arabidopsis* kinesins have not been fully characterized yet and their role might be underestimated (Cai and Cresti, 2012).

The kinesin protein consists of head part and filament stalk. Kinesin head includes catalytic core with ATP and microtubule binding site and neck- like domain which helps to amplify conformational change during catalytic core enzymatic activity and serves as direction determinant. Filament stalk is another part divided into coiled coil domain and tail domain that mediates binding of kinesin to cargo (Zhu and Dixit, 2012).

The kinesin family has further complex effect on plant physiology although the role during exocytosis has not been elucidated yet (Cai et al., 2011). Kinesins may participate in the interaction between microtubules and actin cytoskeleton. Cotton kinesin GhKCH2 contains additional domain that is believed to bind the actin filaments (Xu et al., 2009). Kinesins coordinate both cytoskeleton types and potentially affect the vesicle trafficking directly and indirectly via actin organization.In general, the kinesins are able to transport Golgi stacks (Wei et al., 2009), mediate slow directed movement along microtubules (Romagnoli et al., 2007) and enable stop and go movement of secretory vesicles (Nebenführ et al., 1999). Kinesin 13 motor proteins were shown to influence motility of Golgi bodies in interphasic cell (Cai and Cresti, 2012). Each kinesin appears to drive specialized cargo in contrast with redundancy observed among myosins. Kinesins have been shown to interact with Rab GTPases (Hála, pers. com., unpublished data). This may be possible link between exocytic vesicles and microtubules.

2.1.5 ROP GTPases, the ghosts in the machine

Small GTPases constitute a superfamily of proteins characterized by their ability to bind guanosine-5´triphosphate (GTP) with high affinity and very low efficiency of GTP hydrolysis. These soluble proteins are mostly modified with covalently attached lipid (prenyl and S-acetyl) (Yalovsky et al., 2008). Small GTPases were named “the ghost in the machine” with respect to their essential broad cellular functions (Wu et al., 2008). Ras, Rab, Arf, Sar, Rho and Ran subfamilies belong to the small GTPase superfamily (Bischoff et al., 1999).

The Rho superfamily orchestrates establishment of cell polarity. Plant genome encodes just ROP (Rho of plants) in contrast to animals and fungi containing also CDC42, Rho/Rac and RAL. 4 almost identical ROPs in basal plants seem have to undergone duplications and resulted in 11 ROPs in *Arabidopsis* genome, which evolved in diverse functions (Craddock et al., 2012; Christensen et al., 2003).

ROPs are relatively small proteins that consist of two domains. GTP binding domain is called G-domain and ensures binding and hydrolysis of GTP and interactions with effector proteins (Berken and Wittinghofer, 2008). G-domain is highly conserved in the plant kingdom (Christensen et al., 2003). The second hypervariable domain determinates subcellular targeting and can be prenylated (Berken and Wittinghofer, 2008).

ROP control requires a number of regulators as reviewed by Yalovsky et al. (2008). ROP GEFs (guanine nucleotide exchange factors) are ROP activators, ROP GAPs (GTPase activating proteins) acts as inhibitors. Several types of GAPs are known to participate in tip growth through GTP hydrolysis of active ROP in the pollen tube flank and balance between GAPs and GEFs enables spatiotemporal control of activated ROP. (Klahre and Kost, 2006). Non-protein regulation of ROP is possible through direct interactions with lipids. $PI4,5P_2$ may act as another ROP activator in up-stream and down- stream manner (Kost, 2008).

Rho GTPases regulate the cell polarity via a variety of effectors. The best known group of ROP interactors are RICs (Rop effectors containing CRIB). RIC 1 and RIC 4 control directional cell expansion under the leadership of ROP2 in developing pavement cells (Fu et al., 2005).RIC 3 helps to stimulate Ca2+ influx and F- actin depolymerisation (Gu et al., 2005).

ICR1/RIP1 (ROP interactive partner 1) is involved in positive feedback regulation of ROP1 and helps to establish polar sites during pollen germination (Li et al., 2008). ICR1 binds directly exocyst subunit Sec3 during exocytosis (Bloch et al., 2008), which suggests importance of ICR1 during tip growth. In addition, ICR 1 is required for recruitment of PIN proteins to polar domains at plasma membrane (Hazak et al., 2010). These findings indicate that ICR1 is an important marker of exocytic sites during a variety of processes (see below).

2.2 The secretory apparatus

Secretory apparatus ensures the process of exocytosis. Rab GTPases resides on the vesicle membrane and drives its maturation via Rab-conversion processes. GTP and GDP interaction with GTPase is characterized by rapid binding and nearly irreversible conformational change of the complex, which results in very low dissociation constant of both GTP and GDP. This implies that Rab GTPases require precise regulation of their enzymatic activity and dissociation of nucleotides (Simon et al., 1996).The Rab effectors ensure a new phospholipid composition of the membrane and binding of several down-stream effectors that prepare the vesicle for transport and membrane fusion. Exocyst, the tethering complex, mediates first contact between the vesicle and target membranes, which includes tethering, docking and possibly also facilitates formation of active fusiform SNARE proteins complex. The fusion step is mediated via SNARE proteins. Exocytosis plays a crucial role during cell

mormogenesis. The specificity and dynamics of exocytosis is highly spatiotemporally regulated via correct sequence of interactions between cytoskeleton and secretory apparatus.

2.2.1 SNARE and their regulators

SNARE (Soluble N´ethyl maleimide sensitive factor adaptor protein receptor) is a membrane protein containing coiled-coil SNARE motif. Correct combination of four SNARE helices initiates fusion of secretory vesicle with target membrane (Saito and Ueda, 2009). At least 30 different SNARE proteins are needed for proper trafficking in unicellular eukaryotic organisms (Sanderfoot, 2007). 60 loci encoding a SNARE protein were discovered in *Arabidopsis thaliana* genome (Lipka et al., 2007) and almost all of SNARE proteins are expressed through all tissues (Uemura et al., 2004).

SNARE protein forms a simple helical structure. Membrane attachment requires lipid anchor or COOH terminus transmembrane domain. Each SNARE protein contains one SNARE motif (excluding SNAP 25-like proteins with 2 heptad repeat domains). Functional domain harbours SNARE motif, which is able to fold into coiled-coil structure (Lipka et al., 2007). Four compatible SNARE motifs (Qa, Qb, Qc, R) are required to form a stable complex. SNARE complex can be dissociated via α-SNAP interaction. This process is regulated by NSF (N-ethylmaleimide- sensitive factor) and requires energy released from ATP hydrolysis (May et al., 2001).

SNARE proteins are distinguished due to the central amino acid in the SNARE domain. Q-SNAREs contain glutamine in their sequence and are mostly located on target membranes. This group includes Qa-SNARE (SYP=syntaxin of plant), Qb-SNARE, Qc- SNARE. In contrast R-SNAREs host conserved arginine in the SNARE R-domain. R-SNAREs usually label vesicle membrane (Lipka et al., 2007). Some of the Qa SNARE proteins contain autoregulatory domain at their amino terminus. This domain imitates already folded 4 helix bundle, so that SNARE can switch between “opened” and “closed” state (Saito and Ueda, 2009).

Regulators of SNARE proteins participate in trafficking machinery. Six members of Sec1/Munc18/KEULE family were found in the *Arabidopsis thaliana* genome and possibly regulate the final fusion of secretory vesicles by converting SNARE from closed into opened conformation and *vice versa* (Assaad et al., 2001). Interestingly, KEULE was observed to interact with Sec6 (Wu et al., 2013), the exocyst subunit, which supports the idea, that Sec1 family participates in coordination of secretory apparatus.

Arabidopsis Qa –SNAREs have been shown to interact with dozens of proteins, namely annexins, which participate in SNARE regulation, Band7 family, which plays essential role during establishment of microdomains, and enzymes involved in lignin biosynthesis. Interestingly, binding of myosins to specific SNARE was also observed during interactomic analysis (Fujiwara et al., 2014). However it is

not clear if the interaction is physiologically relevant. It could potentially serve for regulation of SNARE complex assembly by preventing its premature formation or potentially participate in polar distribution of Qa- SNARE, since some of the SNARE proteins are known to be focused in a specific membrane domain (Ichikawa et al., 2014).

2.2.2 Rab GTPases, membrane identificator

The Rab GTPase sub family belongs to small Ras-like GTPases superfamily. The term Rab is derived from the first eukaryotic Rab identification (screening cDNA of a RAt Brain). Each cellular compartment posseses at least one type of Rab on its cytosolic side of membrane (Saito and Ueda, 2009). Rab GTPases play crucial role as organelle determinants during vesicle trafficking and participate in vesicle formation, motility and vesicle fusion in dependence on the GTP/GDP bound state. There are 57 loci in *Arabidopsis thaliana* genome encoding Rab GTPase (Rutherford and Moore, 2002) with high sequence diversity, but conserved domain structure (Zhang et al., 2007). Rab subfamily nomenclature is based on 8 clades and 18 structural subclasses (Rutherford and Moore, 2002).

Molecular structure of eukaryotic Rab GTPases was widely characterized. Hypervariable region (35-40 amino acids), which appears to be important in targeting of Rab to specific compartment, is located at the COOH terminus of the protein. Next to the hypervariable region is situated CAAX box. Two cystein residues near this position are geranyl geranylated which is important for membrane attachment. GTP hydrolysis involves conformational change while the protein appears to be more disordered (Hutagalung and Novick, 2011).

Rab A (Animal Rab11 homologue) is crucial for the last step of exocytosis and plasma membrane recycling. AtRabA3 and AtRabA 4 are located on Golgi apparatus, prevacuolar and endosomal compartments (Rutherford and Moore, 2002). NtRabA4b in pollen tube drives a secretion of new cell wall material (Nielsen et al., 2008). RabA4b resides at the trans- Golgi network and initiates the formation of secretory vesicle. Diversification of Rab A clade could be caused by special requirements in polar exocytosis.

Regulation of Rab GTPases is very complex and several regulators are needed for proper function of Rab GTPases. GTPase activating protein (GAP) increase efficiency of GTP hydrolysis in Rab-GTP bound form. Guanine nucleotide exchange factor (GEF) helps GTPase to release GDP nucleotide and load GTP thus helps conversion into GTPase active state. Several proteins mediate posttranslational modifications of Rabs. Many downstream effectors of Rabs are thought to be involved in processes during vesicle formation, vesicle movement, tethering and membrane fusion (Saito and Ueda, 2009). AtRabA4b was observed to directly and specifically interact with phosphatidylinositol 4-OH kinase

PI-4Kβ1 in root hairs, which contributes to maturation of secretory vesicles (Preuss et al., 2006). RabC2 and RabD1 directly bind to the myosin Myo11B2 as previously described. (Hashimoto et al., 2008). This type of interaction should be investigated throughly.

2.2.3 Exocyst, the tethering complex

Exocyst is an octameric, evolutionary conserved protein complex that serves for preparation of exocytic fusion. Eight long helical subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) participate in interaction between vesicle and plasma membrane via small GTPases, lipid anchoring and possibly other interactors. Exocyst contributes to dozens of processes like autophagy, tip growth, cytokinesis, response to pathogen, polar secretion and exocytosis in general. However, many aspects of its precise function in plants have not been elucidated yet.

All of these proteins posses tandem helical- bundle repeats and rod-like domains performing characteristic unique hydrophobic and electrostatic interaction pattern (Sivaram et al., 2006). How the exocyst complex assembles and mediates tethering is still unclear, although it is generally accepted that in yeast and animals Sec3 and Exo70 serve as a landmark to target the complex at the plasma membrane. Each of the subunits is essential for proper exocyst assembly and assemply/docking mechanismus might be cell specific (Heider and Munson, 2012). Several interactions between exocyst subunits were identified and a number of them evidently differs within kingdoms (Liu and Guo, 2012).

Sec15 triggers interaction between exocyst and secretory vesicle in the yeasts, animals (Heider and Munson, 2012) and potentially in plants via Rab GTPases (Hála, pers. com., unpublished data).

Sec3 binds the plasma membrane through interactions with PI(4,5)P2 and small GTPases (Cdc42 and Rho1 in yeast model). Exo70 also binds directly PI(4, 5)P_2 and seems to define a spatial landmark for exocytosis in yeast (Zhang et al., 2008b) and possibly also the plant cell. *Arabidopsis* genome encodes 23 different Exo70 subunits, subdivided into main three subclasses, that are expressed in different tissues and participates in different exocytic processes (Synek et al., 2006).

Sec3 and Exo70 regulatory pathways are independent. However may partially overlap in function. Fission yeast double mutant of Sec3p and Exo70p is lethal in contrast with sec3p single mutants sensitive to restrictive conditions (Bendezú et al., 2012), however the regulatory paths mediated by Sec3 and Exo70 do not appear to be redundant (Roumanie et al., 2005). Interestingly, interaction between Arabidopsis Exo70A1 and Sec3a was proven by yeast two hybrid analysis in the plant cell (Hála et al., 2008). This behaviour was not observed in mammalian and yeast model and may imply interesting plant specific scenario of exocyst assembly.

2.3 Conclusions

Many celar examples of protein- protein interactions between component of secretory pathway and cytoskeleton or cytoskeleton- associated protein have been reported. Even more other data was acquired from interactomic screens (e. g. Fujiwara et al., 2014). However the physiological relevance of interactions was not always verified. Not much is known about molecular interactions between secretory apparatus and cytoskeleton in comparison to animal and yeast models. Sec3-ICR1-ROP1 (Bloch et al., 2008), myosin- Rab GTPase and other known adaptors (Li and Nebenführ, 2008), kinesin- Rab GTPase (Hála, pers. com.) are the only well described direct link between cytoskeleton and secretory apparathus to date. One would expect that many of the interactions known from opisthokonts are evolutionary conserved and will be discovered in plants. In my opinion, myosins may bind secretory Rab GTPases. Some of the interactions are clearly plant specific. For instance, the role of microtubules during exocytosis in plant cells is still underestimated (as is also noted and supported in following chapters) and probably differs in many aspects from situation in well investigated opisthokont models due to different global architecture of microtubule cytoskeleton.

3 From the donor compartment to the plasma membrane

The secretory vesicle originates from a donor compartment, undergoes a process of maturation, is delivered toward cell surface, tethered by tethering complex and finaly fuses with plasma membrane. General scheme of these processes is well known in animal and yeast cells, and most of the features were also observed in plant models. Many detailes of the exocytosis are believed to be conserved among all eukaryotes. However, some of them have not yet been investigated in plant cell in sufficient details. Next part summarizes known facts about several steps of secretory pathway from secretory vesicle biogenesis to exocytosis and accompanying molecular mechanism. In most instances, state of knowledge in plant cell is introduced. In case of exocytosis steps not yet investigated in plant cells in detail, general model derived from opisthokont research is introduced and the rare relevant details known from plants are discussed afterwards.

Exocytosis is a highly coordinated process which results in the delivery of proteins, lipids and components of cell wall to the plasma membrane and/or extracellular space. The secretory vesicles are delivered into the cell cortex and then fuse with the plasma membrane.

3.1 Secretory vesicle origin, biogenesis and maturation

Plant secretory vesicles could be distinguished from each other in size, morphological features, origin and content. The size of average exocytic vesicle ranges between 60 and 180 nm in dependence on secretory tissue. The origin of secretory vesicles is variable and depends on cell type and actual conditions (Žárský et al., 2009). Based on SNARE protein analysis, Uemura speculates about three different paths that result in exocytosis (Uemura et al., 2004). The RabA GTPase family participate in

trafficking to the pre-vacuolar compartment, recycling endosomes, Golgi and also helps do define exocytic compartments. Multiplication of RabA/Rab11 subfamily possibly corresponds to special requirements during plant exocytosis (Asaoka et al., 2012). In last decade, many examples of plant unconventional protein secretion were investigated (Ding et al., 2012; Drakakaki and Dandekar, 2013). Unconventional secretion appears to be more common than it was supposed.

Not many examples of protein cargo for Golgi apparatus- independent exocytotic paths were examined, although several cases were described. Mannitol dehydrogenase from celery and hygromycin phosphotransferase from *Arabidopsis* were never observed to reach the Golgi or Golgi associated compartments (Drakakaki and Dandekar, 2013). Another example of non-typical trafficked compartment is exocyst positive organelle; however our knowledge about this newly described compartment is very limited (Ding et al., 2012). Unconventional protein secretion should not be underestimated, although I will focus this thesis on classical Golgi apparatus- dependent pathway, mainly due to scarity of relevant research focused on trafficking by alternative pathways. Prevacuolar compartments are another important plant source of exocytic vesicles in form of exosomes (Žárský et al., 2009).

Processes resulting later in formation of secretory vesicles begin in endoplasmic reticulum. Major components of the cell wall matrix are created in the lumen of Gogli apparathus. These compounds are trafficked through the Golgi apparatus and trans-Golgi network toward the plasma membrane. The cargo proteins undergo maturation in each compartment. Precise mechanism of protein sorting in plant cells requires further investigation (Žárský et al., 2009). Trans- Golgi network was determined as early (rececling) endosome (Woollard and Moore, 2008)and contains several overlapping domains that serve as contact intermediary steps between other compartments.

Each domain posseses a different set of small GTPases, which implies a different sorting and regulatory mechanisms (Rutherford and Moore, 2002). Rab GTPases drive the maturation of post-Golgi compartments through interaction with phosphatidyl inositol 4-OH kinase. This kinase responds on Ca^{2+} elevations and produces PI-4P in active state. Once generated, phosphatidyl inositol initiates the secretory vesicle formation (Preuss et al., 2006). When the secretory vesicle is prepared for exocytosis, it is transported by cytoskeleton near the place of secretion.

3.2 Cytoskeleton drives the transport of secretory vesicles towards the plasma membrane

Many experiments showed that the vesicular trafficking requires actin and/or microtubules (e.g. Cai et al., 2011). Both microtubule and actin based movement requires molecular motors. Additionally actin filaments participate in cytoplasmic streaming that also affects vesicular movement.

It is believed that microtubules participate in long range transport, whereas actin cytoskeleton mediates short range movement and stimulates the fusion of secretory vesicle with the plasma membrane in animals (Porat-Shliom et al., 2013). The secretory vesicles could switch from the actin to microtubules and vice versa (Schroeder et al., 2010). Manneville examined organelle movement and precise role of the human cytoskeleton at the cell periphery. Long- range transport of vesicles, dependent primarily on the microtubules, was interrupted by pauses in which the vesicles exhibited rotations and oscillations. The breaks in the movement were mostly registered near the plasma membrane, where the actin cytoskeleton is denser. Short- range transport that is dependent on actin and microtubules showed diffusive-like movement. Most of the observed vesicles performed short-range transport (Manneville et al., 2003).

In tobacco pollen tube shank the motion is more vectorial, in contrast with apex where the movement appeared to be more chaotic (de Win et al., 1999), which implies that the organelle motion is nearly independent on cytoplasmic streaming in the apex region. This is consistent with the fact that in the pollen tube apex the actin cytoskeleton is much denser and the vesicles are transported mainly along the actin cytoskeleton (Lovy-Wheeler et al., 2005). In contrast, microtubules participate in organelle motion in the tube shank more often (Cai et al., 2011). Golgi vesicles isolated from tobacco pollen tubes were tested in actin- and tubulin- binding assay. Organelles moved fast and switched between actin filaments often. Vesicles suddenly stopped, while interacting with microtubules, and then continued to move slower and more vectorially in kinesin dependent manner (Romagnoli et al., 2007). Golgi stacks were observed to perform “stop- and go” movements characterized by fluctuations between random-like movement, which is possibly caused by switches of the vesicles between cytoskeletal filaments, and directed motion mediated by cytoplasmic streaming (Nebenführ et al., 1999).

Trafficking is mediated by the cytoskeleton; in plants the acto- myosin system plays a central role in the vesicular delivery to the plasma membrane. This movement appears to be diffusive and fast in accordance to the myosin motor speed and procesivity (Tominaga and Nakano, 2012). It is possible that microtubules affect the trafficking through both interaction with actin cytoskeleton and marking the exocytotic sites.

It is possible that the pause is an important step for cargo sorting or localizing to distinct sites or simply, it could be caused by a steric hindrance of the filaments with or without any functional meaning. The data implies that the cytoskeleton serves as a highway for vesicles to a treshold density (Lee et al., 2008). From this threshold filament concentration the cytoskeleton serves as the steric hindrance and local destabilization is required for implementation of exocytosis. Taken these data together, the cytoskeleton ensures polarized delivery of secretory vesicles and both assembly and disassembly is crucial for trafficking.

3.3 Myosins drive the secretory vesicles via adaptors

Myosins are associated with the vesicles through adaptor proteins. Although many adaptor proteins of myosins were not identified yet, several myosin interactors were revealed. Myosin V protein was also observed to associate directly with membrane adaptors in animal and fungal cells. Neither myosin interacting motif nor any typical folds do appear to be homologous or similar to each other, which complicates the identification of adaptor proteins (Li and Nebenführ, 2008).

Plant myosin XI is homologous to animal myosin V and yeast myo2p and several similarities were observed. Interestingly, two interaction events of the two different globular tail subdomains could influence proper docking of myosin on the vesicle (Li and Nebenführ, 2007). These subdomains are highly conserved among the myosin V group. Pashkova described five conserved residues that are responsible for secretory vesicle trafficking and another five highly conserved residues required for vacuole movement in the myosin V structure. This suggests that the regulation of vesicular trafficking is more complex and every type of organelle could be regulated independently on each other, although the organelles share the same myosin motor (Pashkova et al., 2006). The plant myosin XI motor was analyzed and 42 amino acid residues were found to be homologous to the yeast myo2p tail. Transient expression of this subdomain resulted into the inhibition of mitochondria and Golgi movement (Sattarzadeh et al., 2013). Myosin molecules were shown to dimerize. The proper anchoring to the cargo potentially requires dimerization of adaptors on the organelle surface (Li and Nebenführ, 2008).

Intracellular trafficking could be regulated on different levels. Alternative splicing of myosins broadly described in the plant cells is one of the possibilities (Tominaga and Nakano, 2012). Second, myosin XI is known to bind acidic phospholipids (Nunokawa et al., 2007). It is believed that this a general feature of myosins XI, but not myosins V (Li and Nebenführ, 2008). Regulation of the phospholipid composition in the membrane cytosolic leaflet is an important regulatory step since lipid turnover can cause association or release of myosins from vesicle. Third, regulation can be affected by conformational change of myosin or myosin interacting protein (Ishikawa et al., 2003). Dissociation and association constant affects the binding properties of different myosins and the regulatory system of the cell affects these constants on the different levels (Li and Nebenführ, 2008). Different myosins are active in different organs during a number of processes. The pool of specifically expressed myosins can be another possibility how to regulate the function of the molecular motors (Sparkes, 2011).

Myosins were shown to interact with several organelles through adaptors. Although many adaptor proteins are still unknown, the majority of animal and fungal identified interactors are Rab GTPases (Li and Nebenführ, 2008). Yeast and animal myosins transport a number of particles and organelles including secretory vesicles and interaction of myosin and Rab was shown to be a key regulatory step during exocytosis (Donovan and Bretscher, 2012). In non-plant models many myosin isoforms

transport a specific organelle. However the plant myosins were found to be redundant (Farquharson and Staiger, 2010). Mutation in one myosin gene results in pleiotropic effect including altered secretory pathway (Peremyslov et al., 2008). This suggests that one myosin binds is able to bind several adaptor proteins.

3.4 Cargo delivery to the plasma membrane

Many models of plant secretion take into account general scheme of protein secretion with all steps. Several model systems were investigated and all of the results are generalized. However this approach is not successfully appliable in every case. For example, the role of the microtubules during the tip growth in pollen tube and trichomes appears to be different (Cai and Cresti, 2010; Folkers et al., 2002). Pattern of delivered CesA to the plasma membrane seems to be similar with localization of pectin, but does not completely match with other components of the cell wall (Cai et al., 2011; Szumlanski and Nielsen, 2009). These observations indicate that the regulation of trafficking can be specialized for each cargo.

Establishment of a jigsaw puzzle-like shaped pattern of pavement cells is highly coordinated process driven by small GTPases and their effectors. Both, microtubule (Microtubule organization 1, Microtubule associated protein 18 and Angustifulia) and actin (Arp2/3 and its homologs, SCAR/ WAVE complex) organizers are required for proter characteristic pattern formation. Microtubules participate in formation of necks and actin is involved in organizing of lobes (Qian et al., 2009). Exocytosis is directed to the lobes, where the ROP1 links interaction between cytoskeleton assembly and exocyst through interaction of ICR1-Sec3 (Lavy et al., 2007).

Details about exocytosis of specific cargo proteins have been less investigated in comparison with endocytosis. For instance, PIN, auxin transporter, trafficking has been widely studied in context of endocytosis. However not much is known about PIN exocytosis and its regulation. PINs are localized in specific non-mobile domains. Although the exocytosis of PINs is polar, it is not sufficient to maintain polar distribution and endocytosis at the edge of the domains is required to prevent lateral diffusion (Kleine-Vehn et al., 2011). Microtubules seem to have indirect long-term effect on PIN1 positioning (Boutté et al., 2006) and contribute to the PIN targeting in basal region (Kleine-Vehn et al., 2008). In contrast actin microfilaments were directly observed to drive PIN recycling (Geldner et al., 2001) and targeting to the apical domain (Kleine-Vehn et al., 2008).

Majority of other cargo proteins were shown to be exocytosed in cytoskeleton dependent manner. Penetration resistance 3 (PEN3), protein associated with resistance to pathogens, accumulates at the plasma membrane in dependence on actin cytoskeleton during papillae formation (Underwood and Somerville, 2013). In contrast, neither actin nor microtubules contribute to the PEN3 delivery to the plasma membrane and exocytosis in epidermal root cells (Langowski et al., 2010). Similarly, no type

of cytoskeleton appears to drive the plasma membrane polarization or exocytosis inendodermis since actin and microtubule disruption by Latrunculin B and oryzalin did not alter exocytosis of polarly localized proteins (Alassimone et al., 2010).

Secondary cell wall is deposited along dense cortical microtubules, which are coordinated by ROP GTPases (Oda and Fukuda, 2013). The molecular mechanism of exocytosis has not been elucidated to date and deposition of primary cell wall was examined more (Cai et al., 2011). For instance, calose sysnthase (CalS) cellular distribution is regulated by acto-myosin system, although the microtubules participate in trafficking of CalS compartments in distal region. The data suggest that the microtubule dynamics, not the presence on its own, is required for proper compartment organization (Cai et al., 2011). Depositionof CalS is dependent on microtubules, with oryzalin completely disrupting the pattern of callose microfibrils in fern stomata (Apostolakos et al., 2009).

Oryzalin, inhibitor of microtubule association, alters significantly the pattern of pectin deposition, in unicellular algae. Microtubule disruption altered strength and thickness of the cell wall, thus affected the cell morphology during elongation. The authors hypothesize that the oryzalin treatment influenced cellulose synthesis which in turn affected pectin deposition (Domozych et al., 2014). Alteration of the microtubule organization resulted in defect in pectin deposition during the maturing of seed coat. The site of pectin cargo fusion with the plasma membrane coincidences the microtubule and CesA localization (McFarlane et al., 2008). Similarly, defects in cellulose synthesis caused by 2,6-dichlorobenzonitrile resulted in reorganization of microtubules and reduction of pectin deposition (Peng et al., 2013). It is not known if the defect in pectin deposition is caused primarily by altered vesicular trafficking to the plasma membrane or if disruption of cellulose deposition is secondary followed by altered pectin trafficking or both effects are involved.

3.5 Docking and tethering the secretory vesicles

Once delivered to the cell cortex near the site of exocytosis, the secretory vesicles docks under the plasma membrane up till a signal triggers the exocytosis. Calcium ions, phosphatidyl inositol-4, 5-bisphosphate, D-myo-inositol-1, 4, 5-trisphosphate and phosphatidic acid are the direct or indirect decisive signal during tip growth (Monteiro et al., 2005). Elevation of Ca^{2+} results in different states of actin polymerization. Proteins polymerize actin cables in high level of calcium, which enables the replacement of secretory vesicles to the cell cortex. In contrast decrease in calcium is a signal for actin disassembly. High concentration of actin filament serves as a steric hindrance and the destruction of actin results in ability of secretory vesicles to contact the plasma membrane and fuse.

Cytoskeleton, especially actin, is known to participate in exocytosis in yeast and animal cells. Specificity of exocytosis is mediated by exocyst, while secretory vesicles are brought closer to the plasma membrane (Heider and Munson, 2012). Rab GTPase binds yeast myosin V, Myo2p, which

enables to deliver the secretory vesicle toward the plasma membrane. Near the plasma membrane, Myo2p interacts with Sec15, the exocyst subunits that triggers vesicle tethering (Jin et al., 2011). It is likely, that tethering of secretory vesicles is at least kinetically dependent on actin cytoskeleton in plants (Fendrych et al., 2013). However, similar molecular mechanism is believed to be found in the plant model.

Interactions between exocyst and small GTPases appear to be crucial in mammalian and yeast cells. This binding enables specific and polarized exocytosis of vesicles. The animal exocyst has been shown to bind RalA/B, Arf6 and Rab11, in contrast yeast exocyst directly binds Rho1p, Cdc42p, Sec4p and Rho3p (Munson and Novick, 2006; Heider and Munson, 2012). Plant ROP GTPases potentially interact with exocyst through adaptor proteins (such as ICR1). Polarization of exocyst is also possibly enabled by interaction with specific phospholipids.

Tethering of yeast and animal secretory vesicle is initiated by interaction between homolos RabA GTPase with homologous exocyst subunit Sec15 (Guo et al., 1999; Wu et al., 2005). Hypothetically, the plant exocyst also binds the exocytic compartments through Rabs.

Sec3 and Exo70 exocyst subunits are bound to the plasma membrane and facilitate the interaction between exocyst core and plasma membrane. Exo70 is able to link exocyst with actin polymerization through direct docking of Arp2/3 complex at the plasma membrane during lamelopodia formation. (Liu et al., 2012).

Sec3 exocyst subunit performs is link of secretory aparathus with cytoskeleton through its indirect interaction with ROP GTPase in plant cell (Bloch et al., 2008). In addition, yeast Sec3 interacts with formin For3, the actin nucleator (Jourdain et al., 2012). This interaction ensures presence of actin filaments in the exocytic site and enables direct track for exocytic vesicle. Disruption of actin cytoskeleton leads to altered pattern of exocyst distribution in one hour after Latrunculin B application The dynamic and density of exocyst attached to the plasma membrane supports the theory, that tethering complex marks the future site of exocytosis at the plasma membrane, rather that the exocyst core is delivered to the plasma membrane with secretory vesicle and potentially cooperates with actin cytoskeleton (Fendrych et al., 2013).

Exocyst, and also Exo70 subunit alone, is able to inhibit tubulin polymerization in animal cells (Wang and Hsu, 2006). Kinesins may be involved in exocytosis of Callose synthase (Cai, 2011; Apostolakos et al., 2009) since the interaction between Rab GTPases and kinesin proteins has been observed (Hála, pers. com). However no details about possible link between kinesins and exocyst have been investigated. If this is proven, another plant unique interaction will be postulated (Wang and Hsu, 2006).

3.6 What happens after the vesicle tethering?

Once delivered close to the cell surface, SNARE proteins of vesicle interact with SNARE proteins at the plasma membrane and the fusion pore is created. Bandmann measured capacitance of BY-2 protoplasts during exocytosis. Kinetics of opening and closing of the fusion pore appears to be periodic and secretory vesicles oscillate between both states quite long time in some of the observed exocytotic events. Non- fused state lasts approximety 200 ms and fused state persists about 50 ms during the periodic changes. In the same experiment, two types of compound exocytosis were described. First, one exocytotic event follows the previous one after complete internalization of the previously fused vesicle. Second, newly delivered vesicle can interact with another secretory vesicle before the exocytic event (Bandmann et al., 2011). It is believed that the molecular mechanism may be similar in plant and animal cells due to the similar fusion and fission oscillations (Thiel et al., 2009).

Not much is known about creation of a fusion pore, however in plant cell were observed two possible scenarios. In the first one, fusion pore expands and the vesicle fully incorporates into the plasma membrane. The second, the cargo releases from the vesicle into the extracellular space, however the pore closes in several seconds and the vesicle is separated back to the cytoplasmic area. This type of vesicles is termed as “kiss and run” and was widely observed in mammal exocrine tissues, but was also observed in plant cells (Weise et al., 2000; Bandmann et al., 2011).The cytoskeleton is able to control the membrane dynamics after fusion. In animal model the actomyosin system was observed to regulate the fusion pore opening and closure and posttranslational modifications of myosin II plays an essential role. Activation by phosphorylation of myosin leads to the following expansion of exocytic pore and the vesicle is fully integrated in the plasma membrane (Chan et al., 2010). In animals, actomyosin system is characterized by its contractile activity and mediates the pressure force on secretory vesicles. First, myosin delivers the vesicles along the actin cables to the cell cortex. Second, actin is able to generate force parallel to the cell surface and supports the opening of fusion pore. Third, actin prevents the inadvertent fusion of intracellular compartments including secretory vesicles (Porat-Shliom et al., 2013). This effect in the plant cell is possibly increased due to the cytoplasmic streaming.

3.7 Conclusions

Studies focusing on roles of cytoskeleton in different steps of plant secretory pathway are gradually accumulating. Many observations fir the prevalent model based on investigation yeast and animal models. The “general concept” known from other organisms is an excellent hint to study plant exocytosis, but it may not be correct in every detail. For example, the actin based tip growth of pollen tubes and animal invadopodia appears share many features (Vaškovičová et al., 2013). However, trafficking of callose appears to undergo plant specific regulations. Actin is major regulator of animal and yeast exocytosis. On the other hand, plant microtubules should not be underestimated, since both actin and microtubules contribute to plant exocytosis (Brandizzi and Wasteneys, 2013). Specific cargo

proteins appear to require their own regulated pathways, which interact with cytoskeleton in different manner. Interestingly, these regulations seem to be tissue and context specific like in the case of PEN3 exocytosis (Underwood and Somerville, 2013; Langowski et al., 2010). One should keep this fact in mind, although it is often useful to think in terms of a general model of plant exocytosis. In many cases, properties and behaviour of specific cell types will have to be examined to complenet the general scheme derived from well- accessible system. Currently, the only systematically examined cells in plant biology are tip growing cells. For this reason, I will focus on cooperation of cytoskeleton and secretory apparatus in pollen tube and root hairs in the next chapter.

4 Tip growth

Tip growth is a process in which the cell expands in polar manner at the tip. Root hairs and pollen tubes perform this type of morphogenesis. Both pollen tubes and root hairs are amenable for easy treatment with drugs and accessible with microscopy. Cells exhibiting tip growth tend to be very sensitive for genetic and pharmacological disruptions (Libault et al., 2010; Qin and Yang, 2011). As such, they are good model system to uncover the critical components of growth/exocytosis- related processes. Tip growth is well characterized in many aspects and considerably contributes to our knowledge about plant exocytosis.

Tip growth is accompanied by characteristic polarization and oscillatory dynamics of cytoskeleton orchestrated by ROP GTPases. Small GTPases drive via their effectors tip localized production of specific membrane lipid compounds and ROS in root hairs together with tip focused Ca^{2+} influx (Qin and Yang, 2011; Guan et al., 2013; Carol and Dolan, 2006), which results in periodic actin assembly and disassembly. A number of positive and negative feedback loops with periodic oscillation of various components that are shifted in phase to each other, enables regulated trafficking to the plasma membrane (Gu et al., 2005; Li et al., 2008; Yang et al., 2007). System of feedback loops appears to be self- sustaining and was defined as LENS (for localization enhancing network, self-sustaining) (Cole and Fowler, 2006). This chapter aims to characterize cytoskeleton dynamics and its interactions with secretory vesicles in pollen tubes and root hairs.

Pollen tube and root hair can be divided into 3 distinct zones (Cole and Fowler, 2006; Shaw et al., 2000). Three distinct pools of vesicles were identified during pulse chase labelling with FM1-43 and FM4-64 (Zonia and Munnik, 2008). The very apex is a zone of constitutive clathrin independent endocytosis. The “clear zone” (so called inverted cone) harbours secretory vesicles. Following part, subapical organelle rich zone adjacent to the apical dome (3-5 µm), displays massive exocytosis and highest curvature (Parton et al., 2001). The shank begins 6-10 microns distal to apex and its membrane performs predominant clathrin dependent endocytotic activity (Zonia and Munnik, 2008). The organelle movement is more vectorial near the exocytic sites and their exocytosis oscillates with a period of 35-45 seconds. Fluctuations were observed 5-10 µm distal to the apex, in the subapical organelle rich region (Parton et al., 2001).

Pollen tube is guided by a number of pistil signals including phytohormones and ligand peptides for receptor like kinases. Auxins, gibberillins and brassinosteroids are the main phytohormonal regulators of pollen tube development (Guan et al., 2013). Root hair growth initiation is dependent on auxin level and receptor like kinases for various ligands such as nutrients maintain the activity of ROP GTPases (Shaw et al., 2000). Both pollen tube and root hair regulation of ROP cascade requires protein kinase that phosphorylates ROP GEF, which in turns activates ROP (Zhang et al., 2008a); Duan et al., 2010). Localized signal input is the first possible way of active ROP polarization. Alternatively, ROP is

specifically delivered to the plasma membrane irrespective of its activation (Lavy et al., 2007). ICR1 is an example of a protein stabilizing polar localization of ROP1 at the plasma membrane and overexpression of this protein affects pollen tube polarization. Before germination, ICR1 is situated in the nucleus of pollen grain. During pollen tube growth initiation, ICR1 moves from nucleus to the future site of exocytosis and marks positioning for ROP1 (Li et al., 2008). In addition, ICR1 mediates localized exocytosis via interaction with Sec3 exocyst subunit, thus links the actin cytoskeleton with secretory apparatus in polarized manner (Bloch et al., 2008).

ROPs are known to determine the germination time, total length, localization and balanced ratio between endocytosis and exocytosis in pollen tubes and root hairs on morphological level (Venus and Oelmüller, 2013). On the molecular level, ROPs control actin assembly, disassembly and polarization. Overexpression of ROP caused stabilization of F-actin and tip swelling in pollen tube (Fu et al., 2001), and formation of multiple tips in root hairs (Jones et al., 2002). Dominant negative form of rop2 resulted in reduced number of shorter and wavy root hairs (Jones et al., 2002) in comparison with dominant negative rop1, which decreases the rate of exocytosis in pollen tubes (Lee et al., 2008).

ROP1 is the activator of two antagonistic pathways through RIC3 and RIC4 as seen in Figure1 (Gu et al., 2005). RIC4 regulates assembly of collar-like F-actin structure in the apical dome, whereas RIC3 helps to create Ca^{2+} gradient, possibly via NADPH oxidase, which leads to actin disassembly. Ratio of RIC3 and RIC4, which is in physiological conditions about ½, appeared to orchestrate the actin dynamics; however the total concentration is not important (Lee, 2005). Overexpression of RIC4 induces enormous polymerization of F-actin that inhibits exocytosis. Similar effects were also detected in pollen tubes treated with jasplakinolide, the actin stabilizing drug. 5nM latrunculin B, the actin disrupting drug, suppresses the defects of RIC4 overexpression, which is consistent with the theory, that accumulation of F-actin near the membrane potentially blocks exocytosis (Lee et al., 2008).

The gradients of protons, Ca2+ and ROS affect cell wall formation (Hepler et al., 2013) and cytoskeletal regulators. A number of actin interacting proteins are influenced by elevation of ion concentration. ADF and AIP, actin interacting proteins, are pH sensitive. In contrast villin/gelsoin and profilin participate in response to Ca2+ elevations. Phospholipids are other key players that affect actin dynamics. PIP_2 directly binds to AtCP, profilin and ADF (Hussey et al., 2006; Fu, 2010). In pollen tube model, profilin and villin/gelsolin are known to participate in degradation of actin fringe (Hepler et al., 2013), in contrast *Arabidopsis* formins have been shown to initiate microfilament formation (Fu, 2010; Ye et al., 2009). ROP GEF directly interacts with WAVE complex, which recruits ARP 2/3 complex to the plasma membrane and mediates the actin nucleation and assembly in trichomes and potentially in other tissues (Basu et al., 2008). Most mutants of actin remodelling proteins are characterized by twists and turns of root hair or pollen tube and altered cytoplasmic streaming (Qin and Yang, 2011; Miller et al., 1997).

Thousands of secretory vesicles are formed from Golgi apparatus per minute and moved behind the tip in reverse fountain like pattern (Bove et al., 2008).The polymerized cytoskeleton functions as a traffic road for secretory vesicles in the shank; however fusion of the exocytotic compartments with the plasma membrane is prevented by high density of actin filaments in the subapex. Free access to the plasma membrane is enabled by actin depolymerisation. This suggests that the oscillatory manner of actin dynamics is necessary for the delivery of new secretory vesicles and following exocytosis (Hepler et al., 2013). The precise function of actin cytoskeleton during exocytosis is not known to date, although each type of cytoskeleton appear to participate in specific cargo trafficking in distinct parts of tip growing cell (see above and below).

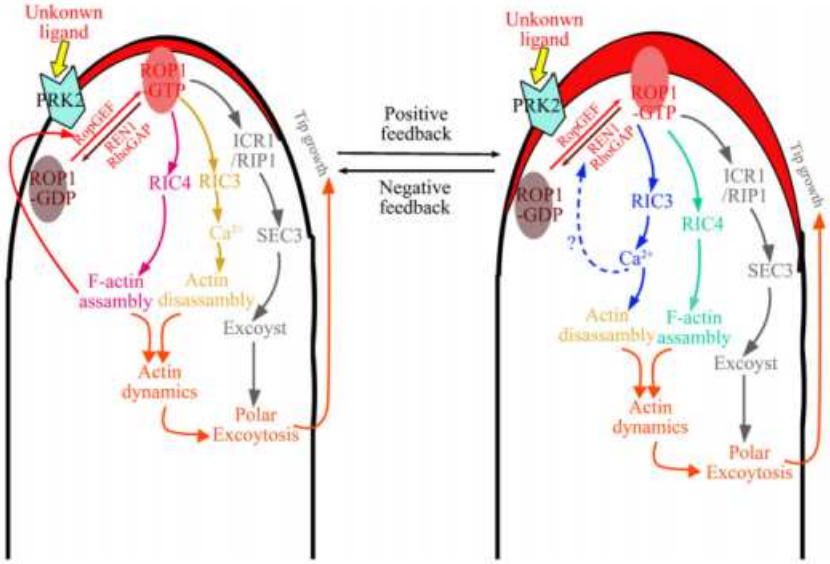

Figure 1: Pollen tube ROP1 regulated by RopGEFs and RhoGAPs orchestrates the actin assembly and disassmebly via its downstream effectors RIC4 and RIC3. Interaction between ROP1 and Sec3 through RIP1/ICR1 facilitates the polarized exocytosis

ROP effectors mediate formation of cortical fringe (Lovy-Wheeler et al., 2005; Ketelaar et al., 2003), which is essential for trafficking of secretory vesicles. Major role of actin cytoskeleton in pollen tube trafficking was investigated for many years. Low (5 nM) concentration of latrunculin B, the inhibitor of actin polymerization, primarily disrupts clathrin dependent endocytosis that affects proper recycling of membrane material back to the secretory pathway and affects the oscillatory pattern of pollen tube growth, but not the cell expansion (Moscatelli et al., 2012). Treatment in 20 nM latrunculin B results in affected morphology of pine pollen tubes and different pattern of exocytotic sites. Deposition of pectin and cellulose was massively reduced and concentrated to the extreme apex (Chen et al., 2007). At 25 nM concentration of latrunculin B, the cytoplasmic streaming was completely inhibited. In addition, at 250 nM concentration most of the pollen tubes exploded. Cytochalasin D is another drug, which results in inhibitory effect on actin polymerization, and similarly performs stronger inhibition of tip growth rather than cytoplasmic streaming, although the morphology of treated pollen tube appears to be more swollen (Vidali et al., 2001).

Actomyosin system drives cytoplasmic streaming that is formed in reverse fountain like pattern in both pollen tube and root hairs. Cycling of the cytoplasmic particles is able to move organelles including secretory vesicles. It is very debatable how to distinguish between the effect of cytoplasmic streaming and direct interactions between myosins and secretory vesicles. Both effects are required for tip growth, although the portion of action appears to be hypothetical. Actin altering drugs may affect trafficking rather than cytoplasmic streaming as described above, but the real effect may be more complex. Observation of single organelle movement investigated unique tracks for each compartment that are independent on other vesicles. In addition, motion of secretory vesicles is more chaotic in the pollen tube apex in comparison with pollen tube shank (de Win et al., 1999). This implies the importance of molecular motors. Secretory vesicles were shown to bind myosins and kinesins, although myosins appear to play major role during secretory trafficking (Romagnoli et al., 2007).

Myosins in pollen tube and root hairs appear to be at least partially redundant (Peremyslov et al., 2008) and mutants are reduced in organelle velocities (Prokhnevsky et al., 2008), growth rate and length and display hooked phenotype similar to arp 2/3, profilin and adf1 mutants (Ojangu et al., 2007). Myosins are believed to be activated by Ca^{2+}.This supports the idea that secretion is complexly orchestrated by ROP GTPases. Interestingly, root hair myosin XI- K participates in ROP GTPase positioning to the plasma membrane, thus may affect the dynamics of actin filaments, however this finding requires further investigation. Myosin XI- K is localized near the tip in actin dependent manner and root hair mutant exhibits reduced growth rate and stop its elongation earlier. However the architecture of actin cytoskeleton is not altered (Park and Nebenführ, 2013).

Secretory vesicles from pollen tube were tested in *in vitro* motility assay. Motion of Golgi vesicles was slower and directed along microtubules, in contrast with movement along actin microfilaments, which appeared to be faster (Romagnoli et al., 2007). This is consistent with observation of “stop and go” movements of plant Golgi stacks described previously (Nebenführ et al., 1999) and reveals a potential role of microtubules during trafficking.

Microtubules serve diverse functions and participate in forming of the pollen tube and root hair morphology. Destabilization of *Arabidopsis* microtubules results in depolarization and creation of multiple tips in a single root hair (Bibikova et al., 1999) and the role of this cytoskeleton type is similar among common root hair models (Sieberer et al., 2005). In contrast, gymnosperm and angiosperm pollen tubes differ in cytoskeletal architecture and the effect of microtubule destabilization on growth (Cai and Cresti, 2010), with gymnosperm tubes being more sensitive to the microtubule depolymerisation (Anderhag et al., 2000).

Microtubules appear to participate in exocytosis in several regions due to their cellular distribution. Two types of microtubules are formed during root hair initiation. In contrast, endoplasmic microtubules are present just in elongating root hairs and assemble close to the apex region, where

they possibly participate in exocytosis (Sieberer et al., 2002). Endoplasmic microtubules were not described during pollen tube elongation, although tobacco pollen tube pollen tube microtubules localize in the shank along plasma membrane and in the central domain of the apex (Idilli et al., 2013). Microtubules are absent in the pollen tube very tip (Cai and Cresti, 2010).

Distinct effects of microtubule destabilizing drugs were observed. Taxol did not affect the root hair morphology. 1 µM oryzalin completely disrupted endoplasmic microtubules, however cortical microtubules appeared to be unaffected (Sieberer et al., 2002). Papaver pollen tube revealed no deffect during treatment with oryzalin (Poulter et al., 2008) (Gossot and Geitmann, 2007), 5µM concentration of nocodazol altered both endocytosis and exocytosis in tobacco pollen tube. Nocodazol did not change architecture of actin, nevertheless largely affected recycling of plasma membrane material and exocytotic events in central region (Idilli et al., 2013). The differences between the effects of microtubule depolymerising drug are potentially caused by different tested species (tobacco and papaver).

Root hair undergoes reorganization of cytoskeleton after initiation of growth. This organization is dynamic and can be restored after microtubule architecture damage (Bruaene et al., 2004). Microtubules form the exocytosis organizing centre (Qin and Yang, 2011) considering that 5 nM latrunculin B, actin destabilizing drug, is able to disturb microtubule organization in tobacco pollen tube (Idilli et al., 2012). It is likely that microtubule cytoskeleton affects cytoplasmic streaming (Cai and Cresti, 2010), helps in organization of actin via interaction with kinesins (Yang et al., 2007), slows down the organelles in general (Romagnoli et al., 2007) and determines the direction of elongation (Bibikova et al., 1999). In accordance with current knowledge, the precise role of the microtubules has not been elucidated yet (Cai and Cresti, 2010). Microtubules appear to participate in the local exocytosis of specific cargo, such as callose synthase(Cai, 2011).

Kinesin- like protein participates in transport of secretory vesicles. Firstly, pollen tube ATP- binding 90 kDa kinesin was co-fractionated with microtubules (Romagnoli et al., 2003). Transglutaminases, calcium dependent enzymes, have been shown to affect the affinity of interaction between kinesins and tubulin in apple pollen tube (Del Duca et al., 2009) indicating importance of this interaction in calcium oscillations dependent manner. Armadillo repeat kinesin 1 and 2 influence root hair morphogenesis by regulation of endoplasmic microtubules (Sakai et al., 2008) and the mutant displays similar phenotype as class ARF GAP mutant (Yoo and Blancaflor, 2013), and leads to disruption of RabA4b and ROP2 GTPase signalling. Another kinesin, the conifer calmodulin binding kinesin, participates in organelle streaming. Interestingly, microinjection of affinity purified antibodies regulating calmodulin resulted in reorganization of vacuoles and altered organelle motility in the tip region (Lazzaro et al., 2013).

Pectin methyltransferase (NtPPME1) is a soluble enzyme changing rigidity properties of cell wall via demethylesterefication of pectin. This enzyme is delivered to the cell wall space of the tip region by F-actin in oscillatory dependent manner. Long- term treatment with 2 nM latrunculin B, wortmannin and 0,15 nM jasplakinolide, the drugs affecting actin behaviour, results in altered the targeting of NtPPME1 vesicles (Wang et al., 2013).

Sucrose synthase (Suc) undergoes transport through Golgi to the plasma membrane or extracellular space (Persia et al., 2008). Suc vesicles are believed to be driven by actin and not microtubules in pollen tubes. Internalization of membrane containing Suc is potentially actin dependent. Another enzyme participating in cell wall synthesis, cellulose synthase, is also delivered to the plasma membrane via secretory vesicles. CesA appears to be organized along microtubules, but this cytoskeleton type is not essential for CesA internalization in pollen tubes (see bellow) in comparison with the actin skeleton.

Callose forms a genotype specific plug and the pattern of callose deposition is heritable (Qin et al., 2012). Deposition of CalS is dependent on microtubules. However, no specific accumulation of any kinesin-like protein near callose plug was observed. Interrestingly, direct interaction between CalS and tubulin has been observed, but the physiological relevance has not been elucidated to date (Cai, 2011). In addition, maize Pti1-like kinase has been shown to colocalize with callose, which implicates possible signalling involved in deposition of other cell wall components (Herrmann et al., 2006).

Pollen tube is an excellent model to study a periodic oscillator that performs spatiotemporally regulated exocytosis. Subapical region is “exocytic hotspot” with exocytosis likely dependent on actin due to no presence of microtubules (Hepler et al., 2013). Cytoplasmic streaming in this region potentially supports trafficking to this region. Microtubule cytoskeleton drives exocytosis mainly distal to the cell apex (Cai et al., 2011). Connetion between cellular components, such as cytoskeletar fibers and cell wall polymers patterns, should be examined in the future research. Different components of cell wall could also mutually regulate their trafficking (Domozych et al., 2014; (Herrmann et al., 2006). Many interesting facts about exocytosis should be elucidated from relations between delivery of different cell wall components.

5 Interpaly of actin and microtubules during cellulose synthase exocytosis

Cellulose is an important component of plant cell wall and participates in proper cell wall, architecture and physical properties, direction of cell expansion and cell morphogenesis. Trafficking and regulations of cellulose synthase (CesA) have been studied and several details about the exocytosis were described. Recently, several key point studies of CesA trafficking have been performed. Relations between the pattern of cytoskeleton and distribution of CesA comparrments near the plasma membrane have been observed and functional aspects of cytoskeleton during CesA trafficking have been analyzed. Considering the level of details provided, CesA provides large insight in role of cytoskeleton in exocytosis, including mutual interplay of microfilaments and microtubules.

Cellulose microfibrils are formed at the plasma membrane by transmembrane enzymes of the CesA family. The complex of cellulose synthases (CSC) catalyzes reaction between UDP- glucose and 1, 4-beta-D-glucosyl chain forming a cellulose polymer. Cellulose microfibrils were largely described to be co-aligned with microtubules (Somerville, 2006). Microtubules potentially act as mechano elastic sensors via kinesins (Preuss et al., 2004), mark the exocytotic sites (Nebenführ et al., 1999), modulate crystalline and morphous content of cellulose microfibrils (Fujita et al., 2011), guide the trajectories of CSC and regulate the balance between insertion and internalization of CSC (Crowell et al., 2009). Actin orchestrates distribution of CSCs on cellular level (Cai et al., 2011) and potentially mediates exocytosis (Sampathkumar et al., 2013). The precise role of cytoskeleton during exocytosis is widely discussed (Bashline et al., 2014). However the molecular mechanism remains unclear.

CesA was found to reside at the plasma membrane, Golgi vesicles and special CesA compartments. CesA containing vesicles were termed MASC (microtubule- associated cellulose synthase compartments), which are defined by VHA-a1 marker and CesA3 (Crowell et al., 2009), and SmaCCs (Small CesA- containing compartments) that are smaller compartments containing CesA6 (Gutierrez et al., 2009). SmaCCs appear to be heterogenous and a single marker was not determided (Gutierrez et al., 2009), thus the SmaCCs category might be superior to the MASCs. Both CesA containing vesicles are distinguished form Golgi by size and more directed movements (Gutierrez et al., 2009). Cellulose synthesis is regulated by equilibrium between exocytosis and endocytosis. MASCs possibly act as both recycling endosomes and intermediary compartment between Golgi apparatus and plasma membrane (Crowell et al., 2009).

Gutierezz observed triphasic behaviour of individual SmaCCs tracks. First, vesicles moved rapidly and exhibited random like movement. Second, the vesicles stayed fixed at the immovable position and third, the vesicles followed a linear track (Gutierrez et al., 2009). MASCs are possibly those compartments moving along linear tracks aligned with microtubules in accordance with the fact that destabilization of microtubules by oryzalin affected pattern of MASCs in the cortical region. These tracks are independent on actin cytoskeleton (Crowell et al., 2009). Pausing behaviour of vesicles

could be interpret as "stop and go" movemet (Nebenführ et al., 1999), as described previously. In general, the SmaCCs were shown to associate with microtubule depolymerising ends and microtubule bundles (Gutierrez et al., 2009). This movement is possibly enabled by a kinesin like protein, which is able to depolymerise microtubules. Such examples of kinesin- driven motion were widely observed (Lombillo et al., 1995). Kinesin like protein FRA1 (fragile fiber1), a microtubule binding protein, localizes to the cell periphery and possibly controls order of cellulose microfibrils. Mutation in fra1 causes reduction in mechanical properties of the fibers and results in altered orientation of microfibrils. However no direct interaction between AtCesA7 and FRA1 was described and the primary and secondary effect of this mutation could be discussed (Zhong et al., 2002). Interestingly, microtubules participate in SmaCC trafficking, however they are not essential for this process (Gutierrez et al., 2009). This suggests, that microtubules serve potentially as landmark for targeting secretory vesicles, but do not act during the act of exocytosis and they are not essential for any step of vesicle delivery to the plasma membrane.

In general, directed movement along actin filaments, along microtubules and cytoplasmic streaming potentially participate and cooperate in movement of CesA containing compartments (Romagnoli et al., 2007; Gutierrez et al., 2009; Crowell et al., 2009). Stabilization of actin filaments influences mictotubule organization, thus the actin stabilization can cause a change in CesA trajectories (Sampathkumar et al., 2011). Actin also appears to orchestrate positioning of the SmaCCs at the cellular level. Actin cytoskeleton is required for the distribution of cellulose synthase complexes during primary and secondary cell wall synthesis of cellulose (Gutierrez et al., 2009). Destabilization of actin affected the distribution of SmaCCs in the subcortical region (Sampathkumar et al., 2013). Treatment of tobacco pollen tubes in LatB resulted in massive redistribution of CesA compartments. BDM, the myosin inhibitor, caused a decrease in delivery of SmaCC to the plasma membrane (Cai et al., 2011).

Single mutants of actins act2, act7 and act8 show only weak phenotypes, in contrast the double mutants exhibit compromised phenotype with compromised cell growth (Kandasamy et al., 2009). The double mutant act2act7 results in altered actin organization and cortical actin microfibrils perform more fragmented ends. Golgi tends to make aggregates in these mutants and higher percentage of SmaCCs remained in fixed position.

Based on FRAP analysis, it is believed that cellulose containing compartments are not exocytosed randomly. MASCs appear to be inserted along linear paths potentially guided by microtubules. Newly inserted cellulose synthase complexes into the plasma membrane lost the common pattern after destabilization of microtubules by oryzalin, while the stabilization of microtubules by taxol caused more aligned pattern in comparison to control (Crowell et al., 2009). This is consistent with the fact

that inhibition of microtubule polymerization altered precise, fine- scale pattern of YFP-CESA6 in the plasma membrane (Paredez et al., 2006).

Actin also appears to participate in insertion events of SmaCCs into the plasma membrane. The analysis of SmaCCs exocytosis distinguished between wild type cells and act2act7 mutants indicating that the actin organization plays a role in efficient exocytosis of SmaCCs (Sampathkumar et al., 2013). Disruption of actin cytoskeleton also causes changes in cell wall thickness and defects in cellulose deposition. Actin cytoskeleton regulates the rate of the SmaCC delivery and life time of cellulose synthase complexes at the plasma membrane. This implies that the actin cytoskeleton contributes to exocytosis.

Interestingly, relation between exocyst and CSC exocytosis is questionable. Co-localization test of Sec3 exocyst subunit with newly inserted CesA6 was negative (Zhang et al., 2013). However, Exo70 exocyst subunit should be also examined.

Once inserted in the plasma membrane, CSC synthesizes cellulose microfibrils in both microtubule dependent and independent manner. Cellulose synthase interactive, which guides the cellulose synthase along microtubules is functionally important for optimal velocity of cellulose (Lei et al., 2013). However, cellulose microfibrils continue to be oriented in the absence of well organized microtubules (Sugimoto et al., 2003). In addition, application of 2, 6- dichlorobenzonitrile affects CesA mobility at the plasma membrane and possibly affects both the microtubule organization and Golgi trafficking (Peng et al., 2013), which implies tight connection between CSC and tubulin cytoskeleton. In addition, recent observations link cellulose synthesis and CSC delivery. Fluorescent signal recovery of CSCs (labelled by YFP-CesA6) displayed dependence on sustaining cellulose synthesis (Fujita et al., 2013).

The signal cascade that regulates exocytosis and endocytosis of CSC is not known, albeit is influenced by osmotic stress and a number of functional CSC in the plasma membrane (Gutierrez et al., 2009). Several other kinesins, cotton kinesin KCH1 and KCH2, facilitate interactions between actin and tubulin (Brandizzi and Wasteneys, 2013). The data implicate that kinesins serve as sensors of mechanical stresses and therefore control the microtubule organization and cellulose synthesis at multiple levels.

SmaCC motility is facilitated by both microtubules and actin cytoskeleton. Disassembly of actin microfilaments affects distribution of SmaCCs in subcortical region (Sampathkumar et al., 2013), destabilization of microtubules by oryzalin affected pattern of secretory vesicles in the cortical region (Crowell et al., 2009). This implies, that actin organizes SmaCCs distribution on cellular level, whereas microtubules fine tune SmaCC distribution near the plasma membrane. Exocytosis events are regulated by both microtubules and actin cytoskeleton. Microtubules help to stabilize the SmaCCs

under the plasma membrane (Nebenführ et al., 1999); in contrast actomyosin system may trigger the exocytotic process (Sampathkumar et al., 2013). The effect of cytoplasmic streaming was not examined and possibly participates in exocytosis. Neither the proper molecular mechanism is known, nor are the components mediating exocytosis or triggering the responses in the cellular and intercellular context. Future research on CesA compartments may help to investigate further relations between cytoskeleton and secretory apparatus and cooperation between actin and microtubules during exocytosis. Moreover, current knowledge about CesA delivery provides excellent platform for general detailed examination of exocytosis. Microscopic observations with high spatial and temporal resolution will bring many yet unknown details about interplay of cytoskeleton and delivery of various proteins to plasma membrane. Such observations also allow to uncover actin and microtubule interplay during exocytosis, a well known phenomenon not yet characterized in details.

6 Conclusions

The role of cytoskeleton during plant exocytosis isindisputable but many details of cytoskeleton-secretory pathway still provide many questions. In general, actin, microtubules and combination of both, cytoplasmic streaming together with molecular mechanisms independent on cytoskeleton contribute to spatiotemporal regulation of plant exocytosis. Current most widespread methodology involving drug treatment enables to study dependence of exocytosis on cytoskeleton, although it is very hard to distinguish between primary and secondary effects. Actin disruption affects motility of secretory vesicles via disrupting interactions with myosins and alters cytoplasmic streaming. Interactions between both cytoskeleton types also appear to play their role.

Most interactions between palnt cytoskeleton and secretory apparatus probably still await to be uncovered. In contrast to the case in opisthokont systems, plants Rab GTPases were not shown to bind myosin yet. On the other hand, Rab GTPases were detected to interact with kinesins and this fact should not be underestimated. Taken together with many *in vivo* data, microtubules significantly contribute to exocytosis of at least some cargoes.

In one cell, different cargoes are delivered and fused with the plasma membrane in different manner. For example, delivery of pectin, a major component of cell wall, is dependent on actin. In contrast, callose appears to be inserted into the plasma membrane in dependance on microtubules. This can be potentially caused by different requirements for cell wall composition in different regions of pollen tubes and root hairs. On the other hand, exocytosis of one cargo molecule, for example PEN3, can be driven by actin cytoskeleton in one instance (papillae formation) or fully independent on both actin and microtubules in cells in other case root epidermis.

Cellulose synthase (CesA) synthesizes an important component of cell wall and its delivery to the plasma membrane requires precise regulation. Actin drives trafficking of CesA compartments near the plasma membrane.The vesicles are slowed down by microtubules and prevented from the untimely exocytosis. Insertional events into the plasma membrane are also influenced by actin. This implies that both actin and microtubules contribute to trafficking of CesA compartments. Future research should focus on cooperation between both cytoskeleton types during docking and exocytosis, because microtubules and microfilaments evidently influence each other. Hopefully, the trend of CesA compartment exocytosis research involving high-resolution measurements will serve as example for other investigations of cytoskeleton-exocytosis interplay in plant cells.

Both actin and microtubules were shown to mediate exocytosis. Secretion of certain cargoes requires different cytoskeleton type, organization, dynamics and localization. However, regulation of secretion appears to be tissue and context specific. The precise molecular mechanisms of interaction between cytoskeleton and secretory apparathus need to be elucidated in future research.

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