FUNCTION AND REGULATION OF SMALL RAB-A1 GTPASES IN *ARABIDOPSIS*

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ABSTRACT

In this study, the function and regulation of Rab-A1 proteins are investigated in the model plant species *Arabidopsis*.

Rab proteins are a family of small Ras-like GTPases that regulate the vesicle transport during membrane trafficking. The subfamily of Rab-A proteins, homologues of Ypt31/32 in yeast and Rab11 in animals, is greatly elaborated in plants. In *Arabidopsis,* 26 out of 57 putative Rab proteins are Rab-A members and they can be further categorized into 6 subclasses from Rab-A1 to Rab-A6. The unique radiation of the Rab-A subfamily in the plant lineage is thought to be a mechanism to meet the dynamic membrane trafficking around the *trans*-Golgi network (TGN) in plant cells. With fluorescence based imaging, I revealed that, RAB-A1c, a member of the Rab-A1 subclass, is strongly co-localized with members of the Rab-A2 and Rab-A4 subclasses in a population of TGN that only partially overlaps with VHA-a1-mRFP, a TGN marker. RAB-A1c is relocated to the growing cell plate in mitotic cells. Interestingly, this RAB-A1c positive compartment and the relocation of RAB-A1c to the cell plate in mitotic cells are sensitive to endosidin1 (ES1), a drug recently identified as an actin stabilizer that selectively interrupts recycling of several plasma membrane proteins from the TGN. Furthermore, although there is likely functional redundancy in Rab-A1 members, the root growth of *rab-a1a/b/c* triple mutant is slightly retarded and, importantly, it displays hypersensitivity to ES1. Histological staining revealed that enhanced defect in cytokinesis contributes to ES1 hypersensitivity in root growth of the triple mutant. Thus, I conclude that Rab-A1 proteins are involved in cytokinesis and they act in a membrane trafficking pathway(s) that is sensitive to ES1.

As the molecular switch of membrane trafficking, Rab proteins are under the regulation of the guanine exchange factor (GEF). In yeast, TRAPPII (transport protein particle II), a ten-subunit protein complex (seven subunits shared with TRAPPI including Trs20, Trs23, Trs31, Trs33, Trs85, Bet3 and Bet5, and three TRAPPII specific subunits including Trs65, Trs120 and Trs130) has been reported as a GEF for Ypt31/32. Using a combined approach of in-vivo imaging and genetics, we revealed that mutations in AtTrs120 and

AtTrs130, homologues of two TRAPPII-specific subunits in *Arabidopsis*, cause defect in cytokinesis as well as in cell polarity. In the background of *attrs120* and *attrs130*, vesicles and tubular-vesicular structures are abnormally accumulated, but ER-Golgi transport, trafficking route to the vacuole and endocytosis appear normal. Using secGFP as a secretion marker, I revealed that transport of secGFP can be inhibited at the TGN. In addition, recycling of PIN2 and AUX1, but not PIN1 is also impaired in *attrs120* or *attrs130*. I found that a functional YFP fused AtTrs130 co-localizes with GFP-RAB-A1c at a population of TGN that is sensitive to ES1. In *attrs130*, however, the majority of GFP-RAB-A1c is delocalized to the cytosol. Furthermore, the constitutive active RAB-A1c(Q72L), but not RAB-D2a(Q67L), could partially rescue *attrs130* in root growth. Taken together, I conclude that TRAPPII in *Arabidopsis* may function upstream of RAB-A1c, possibly as a GEF, in post-Golgi membrane trafficking.

RÉSUMÉ

Au cours de cette étude, je me suis intéressée à la fonction et à la régulation des protéines Rab-A1 au cours de la division cellulaire chez *Arabidopsis*.

Parmis la famille de petites GTPases Ras-like, la sous-famille des protéines Rab régulent le transport vésiculaire durant le trafic membranaire. La sous-famille Rab-A, homologue de Ypt31/32 chez la levure et de Rab11 chez les animaux, est considérablement sophistiquée chez les plantes. Chez *Arabidopsis*, 26 des 57 protéines Rab prédites sont des membres de la famille des protéines Rab-A et peuvent donc êtres organisés en 6 sous-classes, de Rab-A1 à Rab-A6. Cette arborisation unique des protéines Rab-A chez les plantes suggère un mécanisme spécifique lié au dynamisme du trafic membranaire autour du réseau Trans-golgien (Trans-Golgi Network ou TGN). RAB-A1c strictement avec certain membres des familles Rab-A2 et Rab-A4, mais ne co-localise que partiellement avec VHA-a1-mRFP, marqueur du TGN, suggérant une restriction de cette protéine à une sous-population du TGN. Dans les cellules mitotiques, RAB-A1c est relocalisée au niveau de la plaque cellulaire en croissance. De manière intéressante, ce compartiment ainsi que la relocalisation de RAB-A1c à la plaque cellulaire pendant la mitose sont sensibles à l'endosidine 1 (ES1), un stabilisateur d'actine récemment identifié qui interrompt de façon sélective le recyclage de différentes protéines de la membrane cellulaire du TGN. De plus, malgré la redondance fonctionnelle entre les membres de la famille Rab-A1, les racines présentent un défaut de croissance dans les triples mutants *rab-a1a/b/c*, démontrant une hypersensibilité à ES1. Ce retard de croissance, causé par un défaut de mitose, contribue à l'hypersensibilité à ES1 dans les racines en formation. Finalement, les protéines Rab-A1 seraient donc sensibles à ES1 durant la cytokinese et joueraient un rôle important dans la régulation du trafic membranaire.

Les protéines Rab, en tant que déclencheur moléculaire du trafic membranaire, sont régulées par les facteurs d'échange de guanine (GEFs). Chez la levure, TRAPII, un complexe protéique de 10 sous-unités (7 sous-unités partagées avec TRAPI incluant Trs20, Trs23, Trs31, Trs33, Trs85, Bet3 et Bet5, et 3 sous-unités spécifiques à TRAPII, appelées Trs65, Trs120 et Trs130) a été identifié comme étant le facteur GEF pour

YPT31/32. En utilisant une approche combinant l'imagerie *in vivo* et la génétique, nous avons révélé que les mutants *attrs120* et *attrs130*, codant deux des sous-unités spécifiques de TRAPII chez *Arabidopsis*, présentent des défauts de cytokinese semblable à une absence de polarité cellulaire. Dans ces mutants, les vésicules ainsi que les structures vésicule-tubules s'accumulent anormalement. Toutefois, le transport entre le Reticulum endoplasmique (RE) et le Golgi semble être normal. En utilisant secGFP comme marqueur de la sécrétion, j'ai démontré que le transport de cette protéine vers le TGN est inhibé dans les mutants *attrs120* et *attrs130*. De plus, le recyclage de PIN2 et AUX1, mais pas celui de PIN1 est ralenti dans ce contexte mutant. J'ai démontré que AtTrs130-YFP co-localise parfaitement avec GFP-RAB-A1c dans la sous-population de TGN sensible à ES1. Cependant, dans les mutants *attrs130*, la majorité des protéines GFP-RAB-A1c est délocalisée vers cytoplasme. Dans les mutants *attrs130*, la surexpression de RAB-A1c(Q72L), constitutionnellement active, compense partiellement le phénotype de défaut de croissance des racines, contrairement à celle de RAB-D2a(Q67L). L'ensemble de ces résultats montre que TRAPII, chez *Arabidopsis*, pourrait être le facteur GEF en amont des protéines Rab-A1 dans le trafic membranaire post-golgien.

ABBREVIATIONS

ABCB1	ATP-Binding Cassette, sub-family B (MDR/TAP), member 1
ABRC	Arabidopsis Biological Resource Center
ADL1	the Arabidopsis Dynamin-Like Gene Family member 1
AP	Adaptor Protein/Assembly Polypeptides
ARF	ADP-Ribosylation Factor
AT plate	Arabidopsis Theliana plate
AUX1	AUXin-resistant1
BFA	BreFeldin A
BY-2 cells	Bright Yellow 2 cells from tobacco
CCV	Clathrin-Coated Vesicle
CCZ-1	Calcium-Caffeine-Zinc sensitivity protein 1
CLIC/GEEC	CLathrin-Independent Carrier/ GPI- anchored-protein Enriched Endosomal Compartments
COP	COatomer Protein
DENN	Differentially Expressed Normal versus Neoplastic
Dex	DEXamethasone
DMSO	DiMethyl SulfOxide
DP1	DRTF1-polypeptide 1
DR5	a synthetic auxin response element
EE	Early Endosome
ER	Endoplasmic Reticulum
ERES	ER Exit Site
ERGIC	ER-Golgi Intermediate Compartment
ES1	Endosidin1
ESCRT	Endosomal Sorting Complexes Required for Transport
FM4-64	N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)-phenyl)hexatrienyl) pyridinium dibromide
GAP	GTPase Activating Protein
GDI	GDP Dissociation Inhibitor
GDP	Guanosine DiPhosphate
GEF	Guanine Exchange Factor
GFP	Green Fluorescence Protein

GFP-HDEL	a GFP version fused with the ER retrieval signal HDEL
GNL1	GNOM like 1
GNOM	allele of EMB30 (embryo defective 30)
GTP	Guanosine-5'-TriPhosphate
GUS	β-GlucUronidaSe
HPF	High Pressure Freezing
KEULE	"club" because the mutants are club-like in shape
KNOLLE	"tuber" because the mutants are tuber-like in shape
LE	Late Endosome
LhGR	transcription factor LhG4 Receptor
LV	Lytic Vacuole
Mon1	MONensin sensitivity protein 1/Vacuolar fusion protein MON1 homolog B
MVB	MultiVesicular Bodies
NSPN11	NanoSPaN11
Nuf	Nuclear fallout
PCR	Partially Coated Reticulum
PGP1	PhosphatidylGlycerolPhosphate synthase 1
PI	Propidium Iodide
PI4Kβ	Phosphatidylinositol 4-kinaseβ
PIN	PIN-formed auxin efflux carrier
PSC	Pearson correlation coefficient and the ranked Spearman's correlation coefficient
PSV	Protein Storage Vacuole
PVC	PreVacuolar Compartment
Rab	Ras-like protein in RAt Brain
RE	Recycling Endosome
REP	Rab Escort Protein
RGGT	Rab GeranylGeranyl Transferase
RHD3	Root Hair Defective3
RMR	the Receptor homology-transMembrane-RING H2 domain family
RT-PCR	Reverse Transcriptase-PCR
SAND-1	SAND endocytosis protein family 1
SAR1	Secretion-Associated RAS-Related protein 1

SCD1	Stomatal Cytokinesis Defective1
Sec4	temperature sensitive SECretory protein 4
SecGFP	SECretory GFP
Sey1	Synthetic Enhancement of YOP1
SNAP33	Soluble NSF Attachment Protein 33
SNARE	Soluble NSF Attachment protein Receptor
ST-YFP	SialylTransferase YFP
SYP41	Syntaxin of Plants 41
TAC	Tip Attachment Complex
T-DNA	Transfer DNA
TEM	Transmission Electron Microscopy
TGN	Trans-Golgi Network
TRAPP	TRAnsport Protein Particle
VHA-a1	Vacuolar-type proton ATPase complex, subunit a1
Vps9	Vacuolar Protein Sorting complex 9
VSD	Vacuolar Sorting Determinant
ssVSD	sequence-specific VSD
ctVSD	C terminal VSD
VSR	Vacuolar Sorting Receptor
VTI12	Vesical Transport v-SNARE 12
YFP	Yellow Fluorescence Protein
YOP1	YIP One Partner 1
Ypt	Yeast Protein Transport

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PREFACE

This thesis is presented in accordance with the manuscript-based thesis guidelines. It consists of an Introduction (Chapter I), which provides a detail literature review on membrane trafficking in plants and states the rationale and objectives of the study, three research chapters (Chapter II to IV) investigating the function and regulation of Rab-A1 proteins in *Arabidopsis*, and a conclusion chapter (Chapter V). Chapter II is a submitted manuscript, and Chapter III and IV are published papers, so these Chapters are simply re-formatted version of the manuscripts. Chapter II and III both include their own Abstract, Introduction, Results, Discussion and Materials and Methods. Chapter IV contains Abstract, Text, Conclusion and Methods. Each chapter is an independent paper, with its own Reference section.

The thesis has been prepared entirely by me. All the Chapters including the first version of the manuscripts are written by me. Contributions of co-authors in Chapter III have been list in <CONTRIBUTIONS OF AUTHORS> part, and contributions of people who are not authors have been mentioned in <ACKNOWLEDGEMENTS> part. Contributions of my supervisor are not specially listed as he is involved in all Chapters, contributed technical support, scientific ideas and interpretations. He also helped in preparation for all the manuscripts.

Contributions of authors

The results I obtained during my Ph.D study are presented in three manuscript-based chapters:

Chapter II is a submitted manuscript in Molecular Plant.

This chapter is a re-formatted version of:

Qi, X., and Zheng, H. (2012). RAB-A1c GTPase defines a population of trans-Golgi network that is sensitive to endosidin1 during cytokinesis in *Arabidopsis.* Mol. Plant.

Chapter III is an already published manuscript.

This chapter is a re-formatted version of:

Qi, X., Kaneda, M., Chen, J., Geitmann, A., and Zheng, H. (2011). A specific role for *Arabidopsis* TRAPPII in post-Golgi trafficking that is crucial for cytokinesis and cell polarity. Plant J. 68,234-248.

This paper was collaboration between me, Dr. Minako Kaneda and Dr. Jun Chen. I did all experiments except the TEM analysis on *attrs120* and *attrs130* done by Dr. Minako Kaneda and the construction of AtTrs120-YFP and AtTrs130-YFP made by Dr. Jun Chen.

Chapter IV is an already published manuscript.

This chapter is a re-formatted version of:

Qi, X., and Zheng, H. (2011). *Arabidopsis* TRAPPII is functionally linked to Rab-A, but not Rab-D in polar protein trafficking in trans-Golgi network. Plant Signal Behav. 6,1679-1683.

Claims for Originality

In this thesis, I reported the function and regulation of Rab-A1 proteins in *Arabidopsis*.

In Chapter II, we revealed that Rab-A1, A2/A3 and A4 subclasses highly co-localized on a population of TGN that only partially overlapped with the VHA-a1 defined TGN, which is the first report on the relationship between different Rab-A subclasses. This RAB-A1c positive compartment as well as the relocation of RAB-A1c to the cell plate is sensitive to ES1, a drug identified as an actin stabilizer acting in selective anterograde transport from the TGN. Genetic evidence on *rab-a1a/b/c* triple mutant indicates that Rab-A1 serves in membrane trafficking from the TGN to the cell plate in mitotic cells, and these Rab-A1 involved pathways is the target of ES1. To my knowledge, we are the first group that links ES1 with Rab-A proteins.

In Chapter III, we identified two mutants defective in the TRAPPII complex, *attrs120* and *attrs130*. Although TRAPPII have been reported in yeast and mammals before, we and the Assaad Lab at the Technical University of Munich simultaneously identified AtTrs120 and AtTrs130 in *Arabidopsis* for the first time. Furthermore, we tested various membrane trafficking using different markers in both mutants, and revealed that the secretory pathway from the TGN to the cell walls and to the cell plate in mitotic cells, as well as the recycling of the plasma membrane protein PIN2 and AUX1, but not PIN1 are impaired in *attrs120* and *attrs130*. Our findings provide novel knowledge in the function of Trs120 and Trs130 in plants, and offer opportunity in understanding the conservation of TRAPPII among species.

Chapter IV is a complementary study to Chapter III. As we noticed the auxin distribution is altered in *attrs120* and *attrs130*, whereas the detected defects in PIN2 are not sufficient to explain this impairment, we further tested the auxin influx carrier AUX1, and indeed, aberrant localization of AUX1was observed in both mutants. In addition, there is discrepancy about the TRAPPII function as a GEF for Rab11 in yeast and mammals: it is reported TRAPPII acts as a GEF for Rab1 instead of Rab11 in mammals. Curious about the target of TRAPPII in plants, we compared the rescue of constitutive

active RAB-A1c(Q72L) with RAB-D2a(Q67L) in *attrs130*, and concluded that in *Arabidopsis*, TRAPPII is a putative GEF for Rab-A proteins but not Rab-D, the homologue of Rab1 in animals. To my knowledge, no study has been reported before us to distinguish the GEF activity of TRAPPII on Rab-A and Rab-D in plants.

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CHAPTER I:
INTRODUCTION

Eukaryotic cells, unlike prokaryotic cells, have membrane encompassing organelles and therefore develop an elaborate endomembrane system, which is composed of the endoplasmic reticulum (ER), the Golgi apparatus, the *trans*-Golgi network (TGN), endosomes, lysosomes/vacuoles and the plasma membrane. With membrane enclosing a relatively independent compartment, each individual organelle executes distinct functions, including the biosynthesis of proteins and lipids in the ER, protein modification in the Golgi apparatus and protein degradation in the vacuoles. Membrane traffic, a process mediated by highly regulated vesicle transporting between these individual compartments, is essential for the integrity of the endomembrane system (Matheson et al., 2006; Woollard and Moore, 2008). Proper membrane traffic is also essential for cells to communicate with the environment surrounding them, thus is pivotal to cell development, especially critical to establish the cell polarity and to facilitate the successful cytokinesis during cell division. Within eukaryotes, plants are special sessile organisms, as they have rigid cell walls surrounding each cell to support their immobile bodies. Rapidly building and re-organizing the cell wall is therefore of great importance for plant development and for their adaptation to adverse circumstances (Bohlenius et al., 2010; Chow et al., 2008; Sukumar et al., 2009). Correspondingly, the endomembrane system and membrane trafficking molecular system have evolved independently from the animal lineage. In this chapter, I will summarize the current knowledge on the endomembrane system and the molecular mechanism of membrane trafficking with a focus on plant cells.

I) Endomembrane system

Endoplasmic Reticulum

The ER, a polygonal network of cisternae and tubules, is a continuous membrane organelle that includes the outer nuclear envelop and spreads throughout the cytoplasm into the cell periphery (English et al., 2009; Vedrenne and Hauri, 2006). Morphologically, the ER mainly has two distinct domains, sac-like flattened cisternae and long cylindrical tubules. The ER cisternae are often attached by membrane bound ribosomes, which is therefore named the rough ER (Waterman-Storer and Salmon, 1998) and are responsible for the biosynthesis of membrane and secretory proteins. The ER tubules, which are

mainly ribosome-free, are correspondingly called the smooth ER (Meusser et al., 2005), and are involved in the synthesis and delivery of lipids. The ER exit sites (ERES), where proteins and lipids are transported from the ER to Golgi, as well as regions where the ER contact with other membranes structures, such as mitochondria and plasma membrane are also found in the smooth ER (Leem and Koh, 2012; Levine and Rabouille, 2005; Voeltz et al., 2002). The function for association of the ER with these structures are protein delivery to the plasma membrane through non-conventional protein secretion routes (ER-plasma membrane), calcium transport and regulation (ER-mitochondria, ER-plasma membrane), transport of ER-synthesized lipids and steroids to other organelles (ER-Golgi, ER-plasma membrane) and potentially, transcellular traffic of viral proteins and transcription factors (ER-plasmodesmata) (Elbaz and Schuldiner, 2011). The ER thus acts as a trafficking network, delivering lipid, proteins, calcium and signalling molecules to different regions of the cell.

The ER is a highly complex and dynamic organelle. Although maintaining its characteristic morphology and functions, the ER is continuously remodelling itself. The continuous rearrangement of the ER is crucial for properly executing its function and thus is crucial for cell growth and cell function (Friedman and Voeltz, 2011; Ridge et al., 1999). The cytoskeleton is essential in remodelling the ER in living cells (Griffing, 2010). Studies in animal cells revealed that to guide ER remodelling, the microtubule cytoskeleton is primarily used in non-dividing cells, and during mitosis, cells may switch to actin-mediated movement of the ER (Poteryaev et al., 2005; Wollert et al., 2002). However, in yeast and most plants, non-dividing cells use the actin cytoskeleton to remodel the ER, whereas dividing plant cells, on the other hand, control the ER movement in a microtubule-dependent manner (Sparkes et al., 2009a). Several patterns of structure reorganization occur during the ER remodelling in both animals and plants: the cisternae and tubules undergo notable transition to each other (Anderson and Hetzer, 2008; Lu et al., 2009; Tolley et al., 2010; Voeltz et al., 2006); tubules are forming and retracting, fusing and breaking (Chen et al., 2011; Hu et al., 2009; Sparkes et al., 2009a; Wozniak et al., 2009); and vesicles are transported in and out of the ER (Chen et al., 2002; Donohoe et al., 2007; Miller et al., 2002).

Recent studies revealed that the transition from the cisternae to the tubules requires the generation of membrane curvature (McMahon and Gallop, 2005; Zimmerberg and Kozlov, 2006). The high membrane curvature of the tubules is generated by the selective insertion of reticulons and DP1/Yop1 into the outer monolayer of the membrane through "wedging" and "scaffolding" mechanisms, and their abundance may determine the ratio of the cisternal ER and the tubular ER (De Craene et al., 2006; Sparkes et al., 2010; Voeltz et al., 2006). For tubule formation and retraction, two distinct mechanisms: molecular motor model and tip attachment complex (TAC) model have been proposed. Motors such as kinesin and myosin, are believed to be able to pull out the tubules from a membrane reservoir along microtubules (mammalian cell) or actin filaments (plants and yeast cell) (Lee and Chen, 1988; Waterman-Storer and Salmon, 1998). However, in the TAC model, the tip of the ER tubule is attached to the tip of the microtubule plus end by TAC dynamics, and therefore grow and shrink with its microtubule partner. Another important trait occurring during ER remodelling is membrane fusion and fission. Recently, the dynamin-like integral membrane GTPases, including atlastins in human beings, RHD3 in plants and Sey1 in yeasts are identified as key factors in membrane fusion required for the generation and dynamics of the ER tubules in animals, plants and yeast, respectively (Chen et al., 2011; Hu et al., 2009; Orso et al., 2009; Rismanchi et al., 2008).

Golgi apparatus

The Golgi apparatus, due to its fairly large size, was discovered in 1897. It is composed of stacks of membrane-bound flat cisternae, which can be classified into three regions based on their position and function: the *cis*-Golgi, the *medial*-Golgi and the *trans*-Golgi cisternae. Each region contains specific groups of enzymes used to modify the cargoes going through it (Oka et al., 2004), and structural proteins used to maintain the morphology of each cisterna (Chiu et al., 2008).

The Golgi apparatus has been found in both plant and animal cells. In animal cells, the Golgi stacks cluster together and predominantly form a perinuclear ribbon around the

nucleus (Marra et al., 2007). A highly mobile sorting compartment, the ER-Golgi intermediate compartment (ERGIC), has been observed between the ER and the *cis*-Golgi cisterna (Appenzeller-Herzog and Hauri, 2006). In plant cells, the Golgi apparatus appears to be numerous individual cisternal stacks which are randomly scattered throughout the cytoplasm and show extensive mobility along the ER in vacuolated cells owing to their interaction with the actin/myosin cytoskeleton (Avisar et al., 2009; Boevink et al., 1998; Faso et al., 2009). No ERGIC-like compartment has been observed between the ER and the *cis*-Golgi cisterna in plant cells (Foresti and Denecke, 2008; Nebenfuhr et al., 1999).

Another obvious difference of the Golgi bodies between animals and plants is their behaviour during cell division. The plant Golgi stacks, unlike their counterparts in animal cells in which Golgi undergoes disassembly during mitosis and reassembly in cytokinesis, remain structurally and functionally intact during mitosis due to the requirement of active cargo trafficking from the Golgi bodies to the new plasma membrane and cell walls (Faso et al., 2009; Zaal et al., 1999). This discrepancy of Golgi existence in cell division between animal cells and plant cells brings great interest to the issue of Golgi biogenesis.

Contradictory to the traditional view that the Golgi stacks are autonomous and static structures, recent studies suggested that Golgi can be generated *de novo* from the ER (Langhans et al., 2007; Rossanese et al., 1999; Zaal et al., 1999). The first evidence came from a study using the fungal metabolite brefeldin A (BFA), a drug interrupting membrane trafficking between the ER and Golgi (Klausner et al., 1992). When mammalian cells were treated with BFA, the Golgi stacks disassembled with Golgi proteins redistributed into the ER, and when the drug was washed out, the Golgi complex reformed, suggesting the Golgi complex can be generated *de novo*. Further support is provided by the Lippincott-Schwartz lab. They found that when ER export is inhibited by interrupting the function of Sar1, a small GTPase required for ER-to-Golgi transport, Golgi proteins are redistributed into the ER and the Golgi structure disappears gradually in normal mammalian cells, whereas in mitotic cells, the Golgi reassembly is failed (Zaal et al., 1999). In addition, a comparison between two yeast strains, *Pichia pastoris* and

Saccharomyces cerevisiae revealed that the Golgi distribution matches the organization of the "transitional ER" (equivalent to ERES), suggesting that Golgi may also derive from the ER in yeast (Rossanese et al., 1999). In plants, studies in BY-2 cells revealed that the Golgi stacks were completely deconstructed by BFA and could be reformed after BFA washout, indicating that in plant cells, Golgi can be generated *de novo* (Langhans et al., 2007).

The primary function that the Golgi stacks serve is to process and package the macromolecules such as proteins and lipids that are synthesized in the ER, and place them in the corresponding membrane trafficking pathways for distribution. This is coupled with the trafficking of cargo molecules from the ER to, through and out of the Golgi stacks. Membrane trafficking has been detected between the ER and the Golgi apparatus. Two vesicular coat complexes, coatomer protein II (COPII) and coatomer protein I (COPI) are involved in this process. COPII mediates the anterograde transport of the secretory cargoes, whereas COPI recycles the ER resident materials back from post-ER membranes (Lee et al., 2004). Besides the well-established COPI mediated trafficking, a COPI-independent retrograde pathway, such as a retrograde network of tubules linking the periphery of cisternae or a direct flow of membrane backwards through the Golgi stack between cisternae, is also believed to exist (Chen et al., 2002; Johannes and Goud, 2000). In plants, it has been visualized that ER-associated Golgi continuously receives cargoes from the ER (Brandizzi et al., 2002b). After reaching the Golgi, the cargo proteins received from the ER are subjected to further modifications, mainly the modification of N-glycans by oligosaccharide-chain-processing enzymes residing at different subdomains of the Golgi (Nilsson et al., 2009). Once traversed through the Golgi, the cargoes are directed to the TGN for sorting with high fidelity.

Trans-Golgi network

In 1976, Novikoff and colleagues first discovered a membranous compartment associated with the *trans* face of the Golgi stack in mammalian cells (Novikoff, 1976). Ten years later, this structure was given a new acronym, the *trans*-Golgi network (TGN) (Griffiths and Simons, 1986). By definition, the TGN is a network of interconnected tubules and

cisternae at the *trans* face of the Golgi apparatus. It is, by electron microscopy studies on fixed cells, flattened cisternae that are peeling off from the *trans* face of the Golgi stack and further fragmented into small cisternae, tubules and vesicles (Kang et al., 2011; Rambourg et al., 1979). Researchers studying animals disagree on whether the TGN is an independent organelle from the Golgi. Ladinsky and colleagues built a three dimensional reconstructions of the Golgi complex based on their dual-axis, high-voltage EM tomography in normal rat kidney cells (Ladinsky et al., 1999). They suggested that clathrin-coated vesicles exclusively bud from the *trans*-most cisterna of the Golgi stack, whereas the other cisterna only produces non-clathrin coated buds. No TGN-like structure was observed in their study. These data raised the possibility that the TGN is part of the Golgi (Ladinsky et al., 1999). A large quantity of data supporting the view that the TGN is physically and functionally distinct from the Golgi apparatus is from the studies using BFA. When cells are treated with BFA, the Golgi is collapsed into the ER whereas the TGN is fusing with the endosomal system (Lippincott-Schwartz et al., 1991). In plants, data that supports the TGN is an organelle independent from the Golgi is also available. For example, when *Arabidopsis* root tip cells are treated with BFA, individual Golgi stacks reflected by γ-COP aggregate into large clusters whereas the distribution of TGN-localized AtTLG2a (SYP41) remains unchanged (Geldner et al., 2003). In root tip cells of *Arabidopsis,* the TGN displays rapid association and dissociation with individual Golgi stacks (Viotti et al., 2010). Studies in *Arabidopsis* root tip cells indicate that the TGN undergo a maturation process. After 'peeling off' from the *trans*-Golgi cisterna, the TGN matures from Golgi-associated TGN to free TGN, and finally fragments into vesicles and residual cisternae (Kang et al., 2011).

The TGN is the major sorting station of secretory pathway that targets the newly synthesized proteins and lipids to either the endosomal/lysosomal system or the plasma membrane for secretion. Cargo proteins and lipids, after undergoing modification through the Golgi stacks, are directed to the TGN, where their final step of modification would be made (Rockwell et al., 2002). The mature cargo molecules are then segregated into different tubular/vesicular carriers heading to their final destinations (Polishchuk et al., 2009). In plant cells, two types of vesicles: secretory vesicles carrying the newly

synthesized proteins to the plasma membrane or cell wall (Kang et al., 2011; Preuss et al., 2006; Szumlanski and Nielsen, 2009) and clathrin-coated vesicles (CCV) mediating transport to vacuoles/lysosomes (Dettmer et al., 2006) were found to be simultaneously released from the TGN (Kang et al., 2011). Multivesicular bodies (MVB) may also derive from the TGN in *Arabidopsis* root cells (Scheuring et al., 2011).

Endosomes

Eukaryotic cells contain various endosomes that differ in kinetics (early versus late), structure (tubular versus multivesicular), or function (sorting versus recycling) (Perret et al., 2005; Sachse et al., 2002). However, there is obviously overlap between these classifications. Generally, three types of endosomes are believed to co-exist in mammalian cells based on their main functions: early, recycling and late endosomes, though no clear definition has been demonstrated among them. Classically, when we trace an endocytosed cargo from the plasma membrane, the first endosomal compartment it reaches is defined as the early endosome (EE), a tubular-vesicular structure with clathrin-budding profiles (Gruenberg, 2001; Lam et al., 2007). Rab5, which is involved in early endocytic transport, is residing exclusively on EE in animal cells (Zerial and McBride, 2001). At the EE, proteins can be further sorted to the late endosomes (LE) or recycled back to the plasma membrane via recycling endosomes (RE) (Gan et al., 2002; Stoorvogel et al., 1996; Stoorvogel et al., 1991). In mammalian cells, Rab11, involved in protein recycling, for example, recycling of the transferring receptor and G-protein-coupled receptors, is located to the REs (Eggers et al., 2009; Li et al., 2008). Proteins that are not recycled back will move into LEs, and then to the lysosome for degradation (Silverman et al., 2011; Stoorvogel et al., 1991). The LEs can be exclusively marked by Rab7 and Rab9 in animal cells (Gruenberg and Maxfield, 1995; Seabra et al., 2002; Silverman et al., 2011).

In plant cells, the EE was initially identified as so-called partially coated reticulum (PCR) in the early days, based on nonspecific electron-dense markers in transmission electron microscopy (TEM) (Joachim and Robinson, 1984). This PCR, however, was found later on to be indistinguishable from the TGN in structure (Geldner, 2004). Thus, the TGN in

plant cells has been considered as EE. Further evidence supporting the TGN is equivalent to the EE in plants comes from the studies that the lipophilic styryl dye FM4-64, a dye commonly used to trace endocytosis, is co-localized with the TGN markers such as VHA-a1, soon after its internalization (Dettmer et al., 2006; Lam et al., 2007). In plant cells, no RE has been identified, but it is known that the TGN/EE can serve as a compartment for protein recycling (Ueda et al., 2004). Furthermore, a structure marked by GNOM, a putative Guanine nucleotide Exchange Factor (GEF) for ARF1 GTPases, may also function for recycling of PIN1, a plasma membrane localized auxin efflux transporter (Geldner et al., 2003). Multivescular bodies (MVB) or prevacuolar compartments (PVC) are equivalent to the LE in plants. Bearing ESCRT (the endosomal sorting complexes required for transport) at its surface, the MVB/PVC can selectively pick ubiquitinated membrane proteins to the vacuoles for degradation (Katzmann et al., 2001; Spitzer et al., 2009).

Vacuoles

Vacuoles are membrane-bound compartments filled with water containing inorganic and organic molecules including enzymes in solution. They are diverse in shape, size, content and function according to the needs of the cell (Marty, 1999). The importance of vacuoles varies between cell types in different organisms, having much greater prominence in cells of plants and fungi than their counterparts in cells of animals and bacteria.

In plant cells, there are at least two types of vacuoles: lytic vacuoles (LVs) and protein-storage vacuoles (PSVs), which appear sequentially during embryogenesis and can co-exist within the same cell (Paris et al., 1996). The LV serves as the station where cells degrade and store waste products. It could be detected as early as in zygote cells, where it localizes to the basal part. The zygote then divides asymmetrically, with the large LV inherited to the basal cell and develops with the cell lineage. There is no direct evidence specifying the LV in zygote is generated *de novo* or developed from pre-existing small vacuoles in the egg cell or in the embryonic cell. It is postulated that vacuoles are formed through dilation and fusion of autophagosomes in a special type of autophagy (Dunn, 1990). Indeed, LVs, as well as PSVs, can be regenerated in evacuolated tobacco leaf cell

protoplast (Di Sansebastiano et al., 2001), indicating LVs could form *de novo* during normal cell development. The *de novo* formation of PSVs occurs at late stages of embryogenesis. The original PSV is a tubular structure surrounding a pre-existing LV. The tubular PSV further develops to incorporate the LV into its lumen (Frigerio et al., 2008). PSVs are the compartments where cells store proteins that are utilized as nutrients for the next generation during germination. A membrane bound organelle with lytic characteristics, namely the globoids, is detected inside of PSVs, and is believed to keep enzymes required for the digestion of the storage proteins (Jiang et al., 2001). The origin of the globoid is not clear, possibly the enclosed LV during the PSV generation. Previous study revealed that LVs and PSVs, despite their distinct functions, can fuse to form a large central vacuole (Paris et al., 1996), whereas recent findings believe the central vacuole is originally derived from the LV (Di Sansebastiano et al., 2001). The central vacuole, by accumulating osmotic active solutes such as potassium, promotes diffuse cell expansion (Barragan et al., 2012; Uchikawa et al., 2011). This cell expansion can be limited in certain direction by the resistive force from cell walls, thus develops turgor pressure. As a result, high mechanical strength is produced to support structures such as leaves and flowers (Barragan et al., 2012; Uchikawa et al., 2011).

Plasma membrane

The plasma membrane is the differentially permeable biological membrane that surrounds the cytoplasm of a cell and therefore separates the interior of the cell from the outside environment. It controls the exchange of ions and organic substances between the inside and the outside of the cell and protects the cell from outside forces. Bacteria, fungi and plants, different from animal cells, have rigid cell walls outside of the plasma membrane to mechanically support the cell.

The plasma membrane is very dynamic. Animal cells can change their shape to facilitate the cell movement (Anderson et al., 2006; Pollard and Cooper, 2009; Ridley et al., 2003). Due to the existence of the rigid cell wall, many plant cells cannot move. However, certain cell types such as the stomata cells can change their shapes based on the cell requirement (Liu and Luan, 1998; Szymanski and Cosgrove, 2009). In some polarized

plant cells, such as root hairs and pollen tubes, rapid growth of plasma membrane as well as the cell wall is required (de Graaf et al., 2005; Preuss et al., 2006; Szumlanski and Nielsen, 2009). Similarly, during cytokinesis in dividing cells, one-third plasma membrane and cell wall of the new daughter cell can be synthesized within half an hour (Chow et al., 2008; Mayer and Jurgens, 2004). Furthermore, in some plant cells, the plasma membrane may be defined into various subdomains, where particular proteins such as auxin efflux PIN1 and PIN2 have been reported to be selectively targeted (Jurgens and Geldner, 2002).

II) Membrane trafficking

1) Vesicle formation and fusion

Functional compartmentation enables the eukaryotic cell to dramatically increase its ability to survive and adapt to the diverse environment. However, it also necessitates the membrane trafficking between individual compartments in order to keep the integrity of the endomembrane system. Membrane trafficking is a process mediated by vesicle transport between compartments. In eukaryotic cells, three vesicles are well characterized based on their coat proteins: COPI, COPII and clathrin vesicles. COPI and COPII vesicles deliver proteins and lipids between the ER and the Golgi apparatus (Aridor et al., 1995; Barlowe et al., 1994; Scales et al., 1997; Storrie et al., 2000), whereas clathrin vesicles mediate endocytosis and trafficking from the TGN to vacuoles (Dhonukshe et al., 2007; Motley et al., 2003; Schroder and Ungewickell, 1991). A typical membrane trafficking process is defined as vesicle formation, targeting, tethering/docking and fusion with the acceptor membrane. The vesicle formation starts from the recruitment of two layers of coat proteins (inner and outer layers), which is stimulated by the active small GTPases like Sar1 (COPII) and Arf1 (COPI) (Lee et al., 2004; Orci et al., 1993). The recruitment of coat proteins leads to the localized curvature of the membrane that sculpts a vesicle out of the donor membrane. When coat components assemble on the vesicles, they specifically recognize cargo molecules and begin polymerization to drive the release of the vesicle from the donor membrane (Miller et al., 2002; Mossessova et al., 2003).

To fulfill their task as intracellular shuttles, vesicles are directed along a cytoskeletal track (microtubules or actin) to the target membrane (Luna et al., 2002; Presley et al., 1997; Wu et al., 2000). Motor proteins involved in the vesicle transport include kinesin, dynein and myosin (Hammer and Wu, 2002; Matanis et al., 2002; Short et al., 2002). A vesicle, once transported to the vicinity of the target membrane, is first tethered to ensure the accurate recognition of the vesicle by the target membrane. The process of tethering is mediated by Rab proteins (Allan et al., 2000; Guo et al., 1999; Moyer et al., 2001) and large tethering factors including coiled-coil proteins such as Uso1/p115, and protein complex such as Transport protein particles (TRAPP) (Gillingham and Munro, 2003; Sztul and Lupashin, 2006). Meanwhile, the active Rab proteins on the vesicle also recruit other factors to further strengthen the tethering, and help the assembly of SNAREs (soluble NSF attachment protein receptors), a protein complex required for membrane fusion (Grosshans et al., 2006; Hutagalung and Novick, 2011).

2) Trafficking pathways

As described in the previous sections, the organization of the endomembrane system is somewhat different between animals and plants, recent studies indicated that membrane trafficking also appears to be different between plant and animal cells. In the following sections, I will describe the major membrane trafficking within the endomembrane system, with a focus on the molecular mechanisms in plants.

2.1) ER-Golgi Trafficking

The first step in the transport of a newly synthesized protein made in the ER is its transfer from the ER to the Golgi apparatus. In animal cells, cargo molecules leave the ER from ERES, where they are selectively packaged into COPII vesicles (Aridor et al., 1995; Barlowe et al., 1994; Scales et al., 1997). The released COPII vesicles then fuse together to form a vesicular-tubular cluster named ERGIC. At ERGIC, many proteins are further transported to the Golgi apparatus along microtubules (Scales et al., 1997), but cargo receptors and the escaped ER-resident proteins are recycled back to the ER through the retrograde transport mediated by COPI vesicles (Cosson and Letourneur, 1994; Letourneur et al., 1994).

In plant cells, the Golgi stacks appear as scattered units that rapidly move along the ER (daSilva et al., 2004; Sparkes et al., 2009b). No ERGIC has been revealed in plant cells. Two models: 'vacuum cleaner' model and 'stop-and-go' model, have been proposed to explain the Golgi movement and the ER-derived vesicles targeting to the Golgi (Boevink et al., 1998; Nebenfuhr et al., 1999). The 'vacuum cleaner' model is derived from early studies in tobacco leaf epidermal cells, where the Golgi stacks move rapidly and extensively along the ER. In this model, vesicles are believed to be generated anywhere on the ER and released to the encountered Golgi stacks moving along the actin network. In the 'stop-and-go' model, which is based on the observations that the Golgi stacks often alternate between random slow "wiggling" motion reminiscent of Brownian motion and so-called fast directed movement along linear track in cultured tobacco BY-2 cells, vesicle production and exchange are assumed to occur at the ERES. When a stop signal is sent out from an ERES, the nearby Golgi stack would disassociate with the actin track and display a random "wiggling" motion around the ERES to pick up the vesicles. Once vesicles are received, the Golgi would resume its fast movement along the cytoskeleton to optimize the afterward membrane trafficking (Nebenfuhr et al., 1999). Given the different cell types used in the experiments, these two models are not mutually exclusive. Although the 'stop-and-go' model assumes that vesicles are produced at the ERES, it cannot account for the observation that the ERES moves together with the Golgi stacks, rather than being static as the model describes (Brandizzi et al., 2002a; daSilva et al., 2004). In addition, based on the observation that some regions of the ER are physically linked to the Golgi cisternae, it is also postulated that there is continuous flow of cargoes from the ER to the Golgi stacks through direct tubular connection (Hanton et al., 2005a; Sparkes et al., 2009b).

COPII complex is composed of the small GTPase Sar1, the internal structural heterodimer Sec23/24 and the outer cage Sec13/31 (Stagg et al., 2007). Research in mammalian and yeast cells revealed that COPII vesicles are derived from the ER. First of all, the ER-bound GEF Sec12 activates its downstream Sar1 GTPase by exchanging GDP for GTP (Barlowe and Schekman, 1993). The GTP-bound Sar1 then bind to the ER

membrane and recruit the internal coat heterodimer Sec23/24 by binding to the Sec23 protein (Reinke et al., 2004; Yoshihisa et al., 1993). Sec24, bearing multiple cargo-binding sites in its sequence, can selectively pick those cargo proteins with specific signals such as the cytoplasmically exposed di-acidic motif of transmembrane cargo proteins (Miller et al., 2003). The Sec23/24-Sar1 complex together with the recruited cargoes would form a "prebudding complex" (Kuehn et al., 1998). The subsequently recruited Sec13/31 form the outer cage of the coat and facilitate the pinch-off of the COPII vesicles (Lederkremer et al., 2001). In plants, multiple genes encoding COPII proteins exist, suggesting that COPII-mediated vesicle transport does occur in plant cells (Bassham et al., 2008). Various motifs such as di-acidic motif that guide the export of ER membrane proteins have been identified in plant cells (Hanton et al., 2005b; Yuasa et al., 2005), but it remains obscure whether these signals are recognized by COPII components.

Retrograde traffic from the Golgi to the ER requires COPI vesicles. COPI consists of the small GTPase Arf1 and two layers of coatomers, F-COP and B-COP (Stagg et al., 2007). F-COP contains four COP proteins (β, γ, δ, and ζ-COP) that when recruited, they interact with the cytoplasmic domain of cargo proteins or cargo receptors, whereas B-COP has three members (α, β- and ε-COP), which constitute the outer cage of the vesicle. Except for γ-COP and δ-COP, multiple isoforms of COP proteins have been found in plants (Bassham et al., 2008). Correspondingly, two populations of COPI vesicles different in size were observed in *Arabidopsis*: COPIa produced from *cis*-cisternae of the Golgi stack and COPIb derived from the medial and *trans*-cisternae (Donohoe et al., 2007). The restricted localization of COPI to the periphery of Golgi cisternae in plant cells (Ritzenthaler et al., 2002) is different from that in mammalian cells, where the COPI is recruited onto both the ERGIC and Golgi cisternae and is responsible for retrograde traffic both within the Golgi stacks and between the Golgi and the ER (Lee et al., 2004; Robinson et al., 2007). In plants, COPI vesicles are believed to act in the retrograde traffic from the Golgi apparatus to the ER, a process during which signals (e.g. -KSKIN and –YNNKL) within membrane proteins can be recognized (Contreras et al., 2004; McCartney et al., 2004).

2.2) Post-Golgi Trafficking

Trafficking to plasma membrane

After being transported and modified in the Golgi apparatus, cargo proteins, polysaccharides and lipids are further delivered to the TGN, where they are sorted into various vesicles. Secretory vesicles move to the plasma membrane whereas cargoes and lysosomal enzymes destined for degradation are transported to the vacuoles/lysosomes. In mammalian cells, protein secretion occurs constitutively where newly synthesized proteins or lipids destined to the plasma membrane appear to be transported by default (Vazquez-Martinez et al., 2012). In addition, regulated secretion also operates in some mammalian cells, which is the secretion of specialized products in demand (Beug et al., 2011; Sparks et al., 1996). In plant cells, secGFP is a secreted form of GFP which contains an N-terminal ER transit peptide. secGFP could be secreted from the cell to the apoplast (Batoko et al., 2000), suggesting that protein transport to the plasma membrane also occurs by default.

In mammalian and yeast cells, multiple TGN transport pathways to the plasma membrane have been suggested. Many proteins can be sent to the plasma membrane directly from the TGN, but some products are also found to be targeted to the plasma membrane via the endosomal system (Sheff et al., 1999; Vazquez-Martinez et al., 2012). In plant cells, accumulating evidence suggests that secretory cargoes may directly be transported from the TGN to the extra-cellular space (Kang et al., 2011; Keller et al., 2001; Preuss et al., 2006; Szumlanski and Nielsen, 2009). It has been demonstrated that the plant TGN could act as a sorting station and simultaneously release two types of vesicles: secretory vesicles heading to the plasma membrane and the cell wall, and clathrin coated vesicles mediating transport to vacuoles (Kang et al., 2011). Transport vesicles from the TGN are observed to directly fuse with the plasma membrane by live imaging (Keller et al., 2001). Two Rab-A4 members, RAB-A4b and RAB-A4d, residing in the TGN, are reported to mediate the transport of some cell-wall components from the TGN to the plasma membrane (Preuss et al., 2006; Szumlanski and Nielsen, 2009).

In plant cells, many integral plasma membrane proteins, extracellular cell wall proteins and secreted signaling peptides are distributed over the entire surface of the cell. However, some proteins display polarized plasma membrane localization. For instance, the auxin efflux carrier PIN1 preferably resides at the basal part of the plasma membrane in vascular cells (Steinmann et al., 1999). PIN2, another auxin efflux carrier is located apically in the plasma membrane of the root epidermis (Abas et al., 2006), and the auxin influx carrier AUX1 accumulates at the apical plasma membrane of protophloem cells (Kleine-Vehn et al., 2006). The mechanism for proper targeting and maintaining these proteins in specific plasma membrane domains is not unambiguously clear yet. It has been reported that the newly synthesized PIN1 and PIN2 are initially evenly delivered to the plasma membrane, but both PIN1 and PIN2 are constitutively internalized into the cell through the clathrin-mediated endocytosis (Dhonukshe et al., 2007). PIN1 can be recycled back to the proper domain of the plasma membrane through a BFA sensitive GNOM-dependent pathway (Geldner et al., 2003). Distinct from PIN1, GNOM is not required for the recycling of PIN2 (Teh and Moore, 2007). This suggests that proper targeting of different PIN proteins involves distinct plasma membrane recycling machineries. This notion is further supported by the data that the chemical endosidin1, based on a recent study in geminating pollen tubes, selectively affects the recycling of PIN2 but not PIN1 (Robert et al., 2008). It remains to be seen whether or not the pathway for plasma membrane recycling is equivalent of the biosynthetic transport pathway to the plasma membrane in plant cells.

Trafficking to cell plate in cytokinesis

Cytokinesis is a process that generates a large amount of plasma membrane to separate the genetic material and the cytoplasmic organelles into two new daughter cells. In animal cells, cytokinesis is facilitated by a complicated interplay between the cytoskeletal and membrane trafficking machineries (Albertson et al., 2005; Glotzer, 2005). The mitotic spindle, a structure based on a bipolar array of microtubules, specifies the position of the cleavage furrow (Rappaport, 1997), where an actin-based contractile ring, assembled at the cell cortex, further drives its ingression (Straight et al., 2003). During the ingression of the cleavage furrow, the additional membrane required is transported

through various membrane trafficking pathways. Increasing number of studies suggest that localized exocytosis (Lecuit and Wieschaus, 2000; Sisson et al., 2000; Skop et al., 2004) and endosome-mediated membrane recycling are critical to cytokinesis (Dornan et al., 1997; Pelissier et al., 2003; Rothwell et al., 1999).

In plant cells, cytokinesis requires the formation of a new structure called the cell plate in the phragmoplast, which is formed after mitosis to facilitate vesicle transport to the dividing plane (Otegui et al., 2001). Different from animal cells, plant cells contain multiple independent Golgi stacks during mitosis and both Golgi and the TGN are considered as the major source of the building materials to the cell plate (Baluska et al., 2006; Bednarek and Falbel, 2002; Nebenfuhr et al., 2000; Reichardt et al., 2007; Verma, 2001). Numerous secretory vesicles from Golgi/TGN are delivered to the phragmoplast and fuse with each other to generate a tubular network, which is then morphologically restructured into a disk-like membrane compartment called cell plate (Samuels et al., 1995; Staehelin and Hepler, 1996). Vesicles reaching the phragmoplast later on are relocated to the margin of the cell plate, allowing it to expand across the dividing plane, until it reaches the periphery of the cell and fuses with the pre-existing plasma membrane (Mayer and Jurgens, 2004).

A number of proteins have been implicated in the formation of the cell plate in cytokinesis. Recent studies suggest that Rab-A2/A3 are contributing to the cell plate formation, probably through its recruitment and relocation of the cytokinesis-specific syntaxin KNOLLE to the growing regions of the cell plate (Boutte et al., 2010; Chow et al., 2008; Woollard and Moore, 2008). In addition to the cytokinesis-specific t-SNARE, KNOLLE, its three interactors: the Sec1 family SNARE regulatory protein KEULE, the t-SNARE SNAP33 and the v-SNARE NSPN11 are also required for vesicle and tubule fusion during cytokinesis in plant cells (Assaad et al., 2001; Heese et al., 2001; Lauber et al., 1997; Muller et al., 2003; Zheng et al., 2002). Finally, SCD1 (Stomatal Cytokinesis Defective1), a putative Rab GEF, is also implicated in cell cytokinesis (Falbel et al., 2003). In *Arabidopsis*, there are 26 putative Rab-A proteins (see the section 'Function of Rab family' for detail), it remains to be seen if any other Rab-A proteins also act in cell

plate formation. It will also be interesting to determine whether SCD1 can actually activate Rab-A2/A3 or other Rab proteins or protein complex.

To balance the active transport to the cell plate, endocytosis and recycling are thought to exist in the interface of the developing cell plate, as 75% of the cell plate membrane is estimated to be removed during cell-plate maturation (Otegui et al., 2001). A critical factor involved in this process is the dynamin-related protein (Gu and Verma, 1996; Kang et al., 2003; Otegui et al., 2001; Park et al., 1997). Dynamin in mammalian cells is thought to play a role in the release of the clathrin-coated endocytic vesicles from the plasma membrane (van der Bliek, 1999). The plant specific dynamin-related protein phragmoplastin and ADL1 are found to act in formation/stabilization of fusion tubules that connect fusing cell-plate vesicles (Gu and Verma, 1996; Kang et al., 2003).

Trafficking to vacuoles

Vacuoles accept cargoes including proteins, lipids and even vesicles from biosynthetic and endocytic pathways. In plants, the successful separation of vacuole-targeted cargoes is depending on the sorting signals on the specific cargoes. Two main types of vacuolar sorting determinants (VSDs) have been identified, the sequence-specific VSD (ssVSD) and the C terminal VSD (ctVSD) (Nishizawa et al., 2006; Robinson et al., 2005). Two families of putative vacuolar sorting receptors, the Vacuolar Sorting Receptor (VSR) family residing on PVC/MVB, the TGN and the plasma membrane (Wang et al., 2011b), and the Receptor Homology-transmembrane-RING H2 domain (RMR) family residing on the PSV crystalloid (Jiang et al., 2000), have also been found in plants (Shimada et al., 2003; Wang et al., 2011a).

As described previously, two types of vacuoles exist in plant cells: LVs for protein degradation and PSVs for protein storage. The route to the LVs involves a multivesicular compartment, namely PVC/MVB. It is believed that cargoes destined for LVs are first transported to the Golgi stacks where they are recognized by the VSRs and then distributed into CCVs at the TGN (daSilva et al., 2006; Happel et al., 2004). Distinct from COPI and COPII vesicles, adaptors are needed to link clathrin with its cargo in

CCVs (Hirst and Carmichael, 2011; Lefkir et al., 2003). In animal cells, the best characterized adaptors are adaptor proteins/assembly polypeptides (APs) including AP1, AP2, AP3 and AP4 (Owen et al., 2004; Robinson, 2004). AP1 is found on the TGN and endosomes and participates in transport from/to the TGN and the endocytic compartments; AP2 resides at the plasma membrane and supports clathrin-mediated endocytosis; AP3 and AP4, which were discovered by homology analysis (Robinson and Bonifacino, 2001), are both localized on the TGN/endosomal membranes and appear to function independently of clathrin in membrane trafficking (Owen et al., 2004; Robinson, 2004; Robinson and Bonifacino, 2001). In plants, all four APs have been identified (Bassham et al., 2008), among which only AP1 has been well characterized. AP1 at the TGN can bind to the C-terminal cytosolic domain of the VSR, which interacts with the VSD-marked vacuolar cargoes (daSilva et al., 2006; Sanderfoot et al., 1998). Upon arrival at the PVC/MVB, VSR releases the cargo into the PVC/MVB and then is recycled back to the TGN for the next cycle of cargo transport (daSilva et al., 2005; Sanderfoot et al., 1998). However, a recent study in *Arabidopsis* roots also postulated an alternative model that the transport from the TGN to LVs could be independent of clathrin and that PVC/MVB is derived from the TGN through maturation (Scheuring et al., 2011).

Cargoes targeting to the PSVs are thought to be directed to the same PVC/MVB before a PSV is reached (Jiang and Sun, 2002). However, how cargoes might be segregated in the PVC/MVB before they are targeted to different vacuoles is largely unknown. Recently, two different SNARE proteins are shown to play divergent roles in trafficking to PSVs and LVs (Sanmartin et al., 2007).

Endocytosis

The anterograde transport of proteins and lipids from the Golgi apparatus to the plasma membrane are counterbalanced by various retrograde trafficking within the endomembrane system. Cargoes including receptor-ligand complexes are endocytosed from the plasma membrane to an intracellular compartment called the endosome, from which they are further sorted for recycling or degradation (Clague and Urbe, 2001; Shah et al., 2002).

Endocytosis is a crucial mechanism for eukaryotic cells to internalize plasma membrane components and extracellular materials, and therefore controls the composition of the plasma membrane and the interaction between the cells and the environment. It has been found that endocytosis fulfills many functions, including nutrient uptake, cell signaling and cell shape changes (Brown and Goldstein, 1983; Goldstein et al., 1985; Gruenberg and Maxfield, 1995; Smythe and Warren, 1991; Steinman et al., 1983). Thus, it is required for a multitude of cellular and developmental processes. In animal cells, the best defined endocytic pathway is mediated by CCVs (Motley et al., 2003; Vieira et al., 1996). Different from CCVs formed at the TGN, CCVs formed at the plasma membrane requires AP2 (Owen et al., 2004; Robinson, 2004; Robinson and Bonifacino, 2001). Some evidence suggested that, by selecting different adaptor proteins, different sub-types of CCVs may be formed to collect distinct cargoes (Schroder and Ungewickell, 1991). For the membrane scission for the final release of the CCV into the interior of the cell, a large GTPase dynamin is required (Carter et al., 1993; Doherty and McMahon, 2008; Praefcke and McMahon, 2004). However, though clathrin-mediated endocytosis is extremely crucial for animal cells, accounting for a large proportion of endocytic events, clathrin-independent endocytic pathways such as caveolae mediated endocytosis, the CLIC/GEEC endocytic pathway, Arf6-dependent endocytosis and flotillin dependent endocytosis are also found to coexist in animal cells (Mayor and Pagano, 2007; Sandvig et al., 2008).

In plant cells, endocytosis also plays critical roles in various cellular and developmental processes such as embryo differentiation (Geldner et al., 2003), gravitropism (Abas et al., 2006; Silady et al., 2004), guard cell movement (Shope et al., 2003), cell wall remodeling (Baluska et al., 2002), auxin transport (Geldner et al., 2003; Paciorek et al., 2005) and plant defence (Robatzek et al., 2006). Like animal cells, both clathrin-mediated endocytosis and clathrin-independent endocytosis occur in plant cells (Bandmann and Homann, 2011; Dhonukshe et al., 2007; Kitakura et al., 2011). The observation that some chemicals such as endosidin1 act in selective endocytic pathways for certain proteins also reflects the fact that endocytosis in plant cells is complicated (Robert et al., 2008; Teh and Moore, 2007). Likewise, in plant cells, multiple endocytic pathways exist.

Indeed, various endosomes with different identity have been found in plant cells. The ARF1-GEF GNOM, based on in-vivo imaging analysis in *Arabidopsis* root tip cells, is thought to reside on a population of endosomes distinct from the ER, the Golgi complex and the TGN (Geldner et al., 2003). Fluorescence-based localization analysis revealed that Rab-F1 labels a subpopulation of punctae that are only partially marked by the two Rab-F2 proteins, RAB-F2a and RAB-F2b (Ueda et al., 2004). In contrast to endosomes bearing RAB-F1, the Rab-F2-positive endosomes are sensitive to the *gnom* mutation, indicating these two types of endosomes are functionally differential (Ueda et al., 2004). How many structurally and functionally distinct endosomes co-exist in plant cells and how plant endosomes are organized are still largely unknown. Recently, the sequential uptake of the endocytosis tracer FM4-64 opened a window for a road map of the endocytic system in plant cells. When root cells are exposed to FM4-64, the TGN is labelled at an early stage and thereby is considered the EE in plant cells (Dettmer et al., 2006). Afterward, co-localization of the dye with Rab-F2, GNOM and RAB-F1 are detected (Geldner et al., 2003; Ueda et al., 2004). Vacuoles could be stained only after long exposure of cells to the dye. In mitotic cells, the nascent cell plate could also be labelled by FM4-64 (Dettmer et al., 2006).

III) Rab-A GTPases and the TRAPPII complex

Rab proteins, the largest family of monomeric small GTPases of the Ras superfamily, play important roles in the tethering/docking of vesicles to the specific target membrane in different membrane trafficking processes (Allan et al., 2000; Cao et al., 1998). There is also evidence suggesting that Rab proteins function in vesicle budding (Nuoffer et al., 1994), motility (Echard et al., 1998) and actual fusion (Rubino et al., 2000). Rab proteins are found in almost all membrane trafficking pathways. In fact, Rab proteins are generally used by eukaryotic cells as molecular markers to define the identity of various endomembrane compartments due to their restricted distribution (Zerial and McBride, 2001). In this section, I will discuss the function and regulation of Rab proteins with a focus on Rab-As in plant cells.

Function of Rab family

In *Saccharomyces cerevisiae*, 11 Rab proteins called Ypt (Yeast protein transport) are identified genetically or biochemically. The 11 Ypt proteins fall into eight subclasses and each of the subclasses functions in distinct major steps of membrane trafficking (Pereira-Leal and Seabra, 2001). For example, Ypt1 is isolated as a Rab involved in ER-Golgi and intro-Golgi trafficking (Jedd et al., 1995; Nuoffer et al., 1994), Sec4 directs secretory vesicles from the Golgi to plasma membrane (Goud et al., 1988), and Ypt31/32 pairs are required in late-Golgi to the cell surface trafficking (Benli et al., 1996; Jedd et al., 1997).

In higher eukaryotes, Rab gene families are greatly elaborated. In human beings, at least 60 different Rab proteins ascribed to about 40 different functional subclasses were identified (Pereira-Leal and Seabra, 2001). In the *Arabidopsis* genome, there are 57 Rab loci based on sequence similarity (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002), which are divided into eight subfamilies from Rab-A to Rab-H. Interestingly, detailed sequence analysis indicates that the Rab genes have been elaborated independently in animals and plants, which is speculated to be consistent with the advanced membrane trafficking system in each kingdom (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002). The striking feature of Rab proteins in *Arabidopsis* is that nearly half of the Rab members are Rab-As, the closest homologues of animal Rab11 and Rab25 known to act in the recycling endosomes (Emery et al., 2005; Gonzalez et al., 2007; Rutherford and Moore, 2002). Because the function of Rab-A proteins is the focus of this thesis, I will review what we know about Rab-As in detail in the following section.

In *Arabidopsis*, there are three Rab-B members, which are corresponding to mammalian Rab2, but has no counterpart in yeast (Rutherford and Moore, 2002; Vernoud et al., 2003). Rab2 localizes to the *cis*-Golgi and is essential for the maturation of ERGIC (Chavrier et al., 1991; Short et al., 2001; Tisdale and Balch, 1996). In plant cells, however, there is no intermediate compartment between the ER and Golgi (Foresti and Denecke, 2008; Nebenfuhr et al., 1999). A predominant Rab-B protein in tobacco pollen, NtRab2, is

reported to localize to Golgi bodies and regulates trafficking between the ER and Golgi (Cheung et al., 2002).

Also three Rab-C proteins have been identified in *Arabidopsis*. No Rab-C homologue is found in yeast cells (Rutherford and Moore, 2002; Vernoud et al., 2003). The most similar protein of Rab-C in mammals is Rab18, which is implicated in the endocytic transport (Segev, 2001; Zerial and McBride, 2001). Study on Rab-C in plant cells is so far limited. However, to predict its general function based on Rab18 information is not safe as the conserved domains of Rab18 and Rab-C substantially differ from each other (Rutherford and Moore, 2002).

Rab-D is related to Ypt1 and Rab1 in yeast and mammals, respectively (Rutherford and Moore, 2002; Vernoud et al., 2003). Yeast Ypt1 and Rab1 in animals act in ER-to-Golgi transport and early Golgi transport by regulating COPII vesicles tethering and fusing to *cis*-Golgi (Allan et al., 2000; Cooper et al., 2006; Jedd et al., 1995; Moyer et al., 2001; Nuoffer et al., 1994). In *Arabidopsis*, four Rab-D proteins are divided into two groups, single-member Rab-D1 subclass and Rab-D2 subclass with three members (Vernoud et al., 2003). In-vivo imaging analysis indicates that Rab-D proteins localize to the Golgi and the TGN (Geldner et al., 2009; Pinheiro et al., 2009). Expression of the dominant negative form of Rab-D1 and Rab-D2 revealed the function of Rab-D proteins in ER-to-Golgi anterograde transport (Batoko et al., 2000; Pinheiro et al., 2009; Saint-Jore et al., 2002). Genetic evidence revealed that the two Rab-D subclasses have distinct but overlapping functions in the early secretory pathway (Pinheiro et al., 2009).

Rab-E is homologous to Rab8 and Rab10 in mammals and to Sec4 in *S.cerevisiae* (Rutherford and Moore, 2002; Vernoud et al., 2003). However, despite the sequence similarity, the five *Arabidopsis* Rab-E proteins may not have conserved function as Rab8 and Rab10. The Rab8 group and their closely related Rab paralogues such as Rab10 and Rab13 are radiated spectacularly in animal cells, and they act in animal-specific post-Golgi transport (Chen et al., 2006; Peranen et al., 1996). In tobacco leaf epidermis, Rab-E is reported to act downstream of Rab-D proteins and the dominant-inhibitory mutant

RAB-E1d (NI) exhibits inhibitory effect on the transport of a secretory protein secGFP but not on the protein targeting to the Golgi or vacuoles (Zheng et al., 2005).

The Rab-F subfamily is corresponding to Rab5 and Rab22 in mammals and Ypt51/52/53 in yeast (Rutherford and Moore, 2002; Vernoud et al., 2003), all of which are implicated in endocytosis and endocytic-sorting pathways (Christoforidis et al., 1999; Kauppi et al., 2002; Segev, 2001; Zhu et al., 2009). Rab-F clade has three members in plants, RAB-F1, RAB-F2a and RAB-F2b. The two Rab-F2 members are thought to have identical function in the vacuolar trafficking from the Golgi to the PVC (Kotzer et al., 2004; Sohn et al., 2003), whereas Rab-F1 is acting downstream of Rab-F2 in the vacuolar transport pathway (Foresti et al., 2006; Goh et al., 2007; Ueda et al., 2004). Consistent with the function divergence, Rab-F1 and Rab-F2 only display partial co-localization on the PVC (Ueda et al., 2004). In addition, the Rab-F2 compartments are implicated to act as endosomes to mediate endocytosis of some plasma membrane proteins (Dhonukshe et al., 2006; Ueda et al., 2004).

Rab-G subfamily, which contains eight members in *Arabidopsis*, is related to mammalian Rab7 and yeast Ypt7 (Rutherford and Moore, 2002; Vernoud et al., 2003), both of which are involved in transport to the vacuoles and Ypt7 has additional function for vacuole fusion (Segev, 2001; Zerial and McBride, 2001). Homologues of the five-member Rab-H subfamily are Rab6 in animals and Ypt6 in yeast (Rutherford and Moore, 2002; Vernoud et al., 2003). Mammalian Rab6 is reported to be required in retrograde transport through the Golgi stack and from Golgi to the ER (Storrie et al., 2000). However, studies on Rab-G and Rab-H are still scarce in plants.

Function of Rab-A subfamily in *Arabidopsis*

As described in the previous section, a striking feature of plant Rab proteins is that, Rab-A GTPases, the closest homologues of yeast Ypt31/32 and mammalian Rab11 and Rab25, are greatly elaborated (Woollard and Moore, 2008), but many mammalian Rab subclasses, notably those involved in regulating exocytosis (Rab 13, Rab10, Rab3 (Pereira-Leal and Seabra, 2001)) and endosomal recycling/polar secretion (Rab35, (Allaire et al., 2010)) are

missing. In *Arabidopsis*, 26 out of 57 Rab proteins are grouped in Rab-A subfamily, which is structurally categorized into 6 subclasses from Rab-A1 to -A6. In rice, there are 17 Rab-As within 52 family members. In the basal lineage of land plant *Physcomitrella patens*, Rab-As also have similar divergence (Ebine and Ueda, 2009). It is known that the counterpart of Rab-As in yeast, Ypt31/32 pair, regulate export from a late Golgi compartment to a pre-vacuolar/endosomal compartment and to the plasma membrane, whereas animal Rab11 acts at the recycling endosome and performs important functions in the delivery of receptors and transporters to cell surfaces (Emery et al., 2005; Gonzalez et al., 2007), and in the abscission of daughter cells in the late stages of cytokinesis (Prekeris and Gould, 2008; Wilson et al., 2005).

The substantial number of Rab-A proteins in plants has been thought to attribute to the plant-specific diversification of post-Golgi membrane trafficking in various stages of plant development (Rutherford and Moore, 2002). Indeed, in *Arabidopsis*, data has shown that Rab-A4s are involved in polar secretion of cell wall components in root hairs and pollen tubes (Preuss et al., 2006; Szumlanski and Nielsen, 2009). RAB-A2a is required for the root hair formation and nodulation (Blanco et al., 2009), but members of Rab-A2 and -A3 are also reported recently to act in post-Golgi membrane trafficking that contributes to cytokinesis in dividing cells (Chow et al., 2008). RAB-A1a is suggested in a recent paper to be involved in auxin-mediated responses, probably through the regulation on the proper targeting of auxin transporters such as PIN1 and PIN2 from endosomes to the plasma membrane (Koh et al., 2009). Besides the study in *Arabidopsis*, tobacco Rab11b is also reported to be crucial for tip-focused pollen tube growth (de Graaf et al., 2005). A tomato LeRab11a knock-down line keeps fruits firm for a much longer time than that of wild type at least partially due to the reduced level of two cell wall enzymes, polygalacturonase and pectinesterase (Lu et al., 2001), suggesting that Rab-A is involved in secretion of cell wall modification enzymes. The expression of the dominant negative mutant of this LeRab11a inhibits exocytosis of the secreted GFP in tobacco leaf protoplasts (Rehman et al., 2008), which confirmed the view. So far, all available data suggested that Rab-A proteins function in post-Golgi polar secretion, perhaps with functional diversification in cytokinesis, cell polarity and cell wall

biogenesis or modification between different subclasses. However, a full picture of how different Rab-A proteins may work in post-Golgi trafficking requires more work to be done.

Regulation of Rab proteins

Rab proteins serve as the molecular switches in membrane traffic by shuttling between their cytosolic GDP-bound inactive form and membrane associated GTP-bound active form (Seabra and Wasmeier, 2004). This cycle allows both spatial and temporal control of the Rab GTPase activity (Seabra and Wasmeier, 2004). A newly synthesized Rab GTPase needs to be modified by Rab geranylgeranyl transferase (RGGT), which adds two geranylgeranyl groups to the C-terminal of the Rab protein, before its association with membranes (Andres et al., 1993; Detter et al., 2000). The newly prenylated Rab is then delivered by a Rab escort protein (REP) to its target membrane, where a specific GEF activates the Rab protein by accelerating their intrinsic GDP release and GTP uptake reaction. The active Rab GTPase then recruits to the membrane various downstream effectors which facilitate the execution of its diverse functions. After a Rab protein performed its function, a GTPase activating protein (GAP) turns off the Rab protein to its inactive form by catalyzing the GTP hydrolysis. The thereafter GDP-bound Rab protein would be stabilized in the cytoplasm by forming a protein complex with a GDP dissociation inhibitor (GDI).

Clearly, of the factors involved in regulation of Rab proteins, the Rab GEFs are crucial for the successful execution of Rab functions. In yeast cells, Ypt1 controls ER-Golgi and intra-Golgi trafficking, and a protein complex TRAPPI is characterized as its GEF (Jones et al., 2000; Wang et al., 2000). Ypt6 involves in recycling from endosomes to the late-Golgi trafficking, and its GEF is Ric1p/Rgp1p (Siniossoglou et al., 2000). Two Rab proteins involved in late-Golgi to cell surface trafficking are Ypt31/32 and Sec4, which can be activated by TRAPPII and Sec2, respectively (Jones et al., 2000; Walch-Solimena et al., 1997). In multicellular eukaryotes, Vps9-domain (vacuolar protein sorting complex 9) proteins have been found to act as GEFs specific for the different members of the Rab5 proteins, which are recruited on early endosomes in humans (Delprato and Lambright,

2007; Mattera et al., 2006). The best characterized Vps9-domain GEF is Rabex-5, which activates Rab5 in the early endocytic pathway (Mattera et al., 2006). It is interesting to note that this Rabex-5 can act not only on Rab5 but also on the related Rab21 GTPase, another Rab5 family member that plays a role in the early endocytic pathway (Delprato and Lambright, 2007; Pellinen et al., 2006; Pellinen et al., 2008; Simpson et al., 2004). Rab7, the regulator of lysosome/vacuole related trafficking, is stimulated by the SAND-1/Mon1 and CCZ-1 complex in the maturation of early to late endosomes (Kinchen and Ravichandran, 2010; Poteryaev et al., 2010). The last family known to have Rab GEF activity is the DENN (differentially expressed normal versus neoplastic) domain family. Members in this family have been found to catalyze nucleotide exchange on various Rab proteins including Rab3, Rab8, Rab10, Rab27 and Rab35. It is therefore speculated that proteins with DENN domains may function as Rab GEFs in general (Brown and Howe, 1998; Denef et al., 2008; Figueiredo et al., 2008; Sato et al., 2008; Schuck et al., 2007; Wada et al., 1997).

Function of the TRAPP complex in yeast and mammals

As outlined above, in yeast cells, Ypt1, a Rab protein involved in ER-Golgi transport, and Ypt31/32, Rab proteins required for late-Golgi transport to cell surface, can be activated by TRAPPI and TRAPPII, respectively. TRAPPI is previously reported as a seven-subunit complex (Trs20, Trs23, Trs31, Trs33, Trs85, Bet3 and Bet5) that locates on the ER-to-Golgi COPII vesicles (Sacher et al., 2008), but a recent study revealed that Trs85 in yeast is associated with a large TRAPPIII protein complex that is distinct from TRAPPI and TRAPPII (Choi et al., 2011). Once recruited on the vesicle membrane, TRAPPI can activate Ypt1 and tethers the vesicle to its target membrane through the Ypt1 effector protein Uso1 (Cao et al., 1998). TRAPPII is a modified version of TRAPPI with three additional specific subunits Trs65, Trs120 and Trs130. Although many data collected from different species support the viewpoint that TRAPPII acts as a GEF for Ypt31/32 or their homologues (Jones et al., 2000; Morozova et al., 2006; Robinett et al., 2009; Zou et al., 2012), some data suggest that TRAPPII also activates Ypt1 (Barrowman et al., 2010; Cai et al., 2008).

Phylogenetic analysis revealed that while Trs65 is missing in higher eukaryotes, the two TRAPPII-specific subunits, Trs120 and Trs130, are conserved in all sequenced eukaryotic genomes (Cox et al., 2007). Consistent with yeast results from the Segev group, counterpart of TRAPPII component Trs120 in *Drosophila* named Bru interacts genetically with Rab11 and its effector PI4Kβ (Robinett et al., 2009). When *Bru* is mutated, the mutant shows defective cleavage furrow ingression in dividing male meiotic cells (Robinett et al., 2009). Based on the data that mammalian Trs130 (mTrs130) binds to the coatomer subunit γ-COP on COPI vesicles but not Golgi cisternae, and the RNAi lines of mTrs130 display more vesicles close to the Golgi and cargoes accumulate in an early Golgi compartment, Yamazaki et al. revealed that TRAPPII is a GEF for Rab1 in mammals (Yamasaki et al., 2009). The functional difference was thought to rise from the organization difference in the ER-Golgi interface between yeast and mammals (Yamasaki et al., 2009). However, a recent co-purification study revealed that the TRAPP complexes may have different organization in yeast and mammals (Choi et al., 2011). It is interesting to note that the TRAPP protein complexes, in addition to their role as GEF for Rab proteins, also function as tethering factors for vesicles (Sacher et al., 2008). Physical evidence for TRAPP complex serving as tethering factor is provided by the Ferro-Novick lab. They showed that TRAPP subunit Bet3 is required for the tethering and fusion of COPII vesicles using an in vitro assay (Yu et al., 2006). Later on, the direct interaction partner of Bet3 in vesicle tethering was identified as the coat protein Sec23 of COPII vesicles (Cai et al., 2007; Cai et al., 2005). Single particle electron microscopy of TRAPPI in yeast revealed that the protein complex has an elongated, flat architecture and such a structure could facilitate its function as tethering factor (Kim et al., 2006). The function of TRAPPII in membrane trafficking and its functional relationship with Rab GTPases during plant development remains uncharacterized.

IV) Rationale and objectives of the thesis

1) Developmental function of Rab-A1 proteins

As outlined above, Rab-A proteins in plant cells may regulate various membrane trafficking pathways from the TGN to the plasma membrane/cell wall and to the

developing cell plate in mitotic cells. However, as the TGN is a complex and dynamic compartment where the biosynthetic and endocytic pathways meet, how different Rab-A proteins, especially Rab-A1 proteins may work in the TGN is not yet well clarified. In the first part of this study, using a combined approach of in vivo imaging and genetics, I investigated the function of Rab-A1 proteins, with a focus on RAB-A1c in cytokinesis. The data revealed that RAB-A1c co-localizes with RAB-A2a and RAB-A4b on a population of TGN that is only partially marked by a previously identified TGN marker VHA-a1. The RAB-A1c mediated membrane trafficking(s), based on our imaging and genetic data, may be the target of endosidin1 during cytokinesis.

2) Function of TRAPPII in membrane trafficking and regulation of Rab-A1 proteins

Known as molecular switches for vesicle transport, Rab proteins rely largely on their upstream regulators to efficiently fulfill their function. Based on homology between Rab-A and Ypt31/32 proteins, the putative GEF of Rab-A proteins is TRAPPII. However, no functional data for TRAPPII has been obtained in the plant kingdom before my research. In the second part of my study, by analyzing two TRAPPII specific subunits, AtTrs120 and AtTrs130, I characterized the function of TRAPPII in membrane trafficking during plant development. My data demonstrate that TRAPPII acts in membrane trafficking from the TGN to the plasma membrane/cell wall and to the growing cell plate in mitotic cells. In addition, the recycling of PIN2 and AUX1 but not PIN1 is impaired in the background of both TRAPPII mutants. Genetic and imaging evidence indicates that TRAPPII acts upstream of Rab-A proteins, potentially as a GEF in *Arabidopsis.*

3) Function of TRAPPII is not linked to Rab-D in *Arabidopsis*

For the function of TRAPPII as a GEF for Rab proteins, there is a debate depending on data from different species. In yeast cells, TRAPPII can serve as a GEF for Ypt31/32 as well as Ypt1 in late Golgi trafficking. In *Drosophila*, TRAPPII interacts genetically with Rab11 (homologue of yeast Ypt31/32) and its effector PI4Kβ. In mammalian cells, however, TRAPPII acts as a GEF for Rab1 (homologue of yeast Ypt1) in early Golgi trafficking. Curious about the TRAPPII targets in plants, I examined the genetic

interaction between TRAPPII and Rab-A (homologue of Ypt31/32) and Rab-D (homologue of Ypt1) in *Arabidopsis* in the last part of the study. Our result indicates that TRAPPII functionally links with Rab-A but not Rab-D in post-Golgi membrane trafficking in *Arabidopsis*.

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CHAPTER II:

RAB-A1C GTPASE DEFINES A POPULATION OF TRANS-GOLGI NETWORK THAT IS SENSITIVE TO ENDOSIDIN1 DURING CYTOKINESIS IN *ARABIDOPSIS*

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Abstract

In plant cells, Rab-A proteins have been implicated to play important roles in membrane trafficking from the *trans*-Golgi network (TGN) to the plasma membrane/cell wall and to the newly formed cell plate in cytokinesis. But how different Rab-A proteins may work in the TGN is not well studied. We showed here that RAB-A1c defines a population of TGN that is partially overlapped with the VHA-a1 marked-TGN. Interestingly, the morphology of RAB-A1c defined-TGN is sensitive to endosidin 1 (ES1), but not to wortmannin. In mitotic cells, RAB-A1c is relocated to the cell plate. We revealed that this process could be interrupted by ES1, but not wortmannin. In addition, cytokinesis in root mitotic cells of *rab-a1a/b/c* triple mutant seedlings is hypersensitive to ES1, but not wortmannin. ES1 is known to selectively block the recycling of several plasma membrane auxin transporters, including PIN2 and AUX1. Together with the known facts that members of Rab-A1 proteins are involved in auxin-mediated responses in root growth and that mutations in TRAPPII, a protein complex that acts upstream of RAB-A1c, also selectively impairs the recycling of PIN2 and AUX1, we propose that the Rab-A1 mediated trafficking pathways around the TGN are the target of ES1.

Introduction

In plant cells, cytokinesis requires the formation of a structure called the cell plate in the center of the phragmoplast (Gu and Verma, 1996; Samuels et al., 1995; Segui-Simarro et al., 2004). The assembly of a cell plate requires transport and fusion of secretory vesicles (Jurgens, 2005; Reichardt et al., 2007). Recently, the *trans*-Golgi network (TGN) has been identified as a key organelle in vesicle transport to the cell plate (Boutte et al., 2010; Chow et al., 2008; Dhonukshe et al., 2006). The TGN is a tubular-vesicular network derived from the *trans* face of the Golgi apparatus (Zhang and Staehelin, 1992). In plants, the TGN is a highly dynamic organelle, displaying rapid association and dissociation with individual Golgi stacks (Viotti et al., 2010). In *Arabidopsis* root tip cells, the TGN undergoes a maturation process. After 'peeling off' from the *trans*-Golgi, the TGN matures from the Golgi-associated early TGN to the late TGN that is away from Golgi, and finally fragments into different vesicles for cargo delivery (Kang et al., 2011). In *Arabidopsis* root cells, the TGN may also mature into multivesicular bodies (MVBs) (Scheuring et al., 2011). In plant cells, the TGN has also been considered as an early endosome (EE) that receives endocytic materials from the plasma membrane (Dettmer et al., 2006; Viotti et al., 2010). At the TGN/EE, endocytic materials may be either transported to the vacuole via MVBs or recycled back to the plasma membrane (Spitzer et al., 2009). Thus the TGN in plant cells appears to serve as a major sorting station in both the secretory and endocytic pathways that target newly synthesized as well as endocytic proteins to the right destinations. The system therefore must operate with high fidelity and efficiency, but the molecular nature of TGN dynamics and how different membrane trafficking pathways from/to the TGN are regulated are not well defined. Several proteins have been implicated to regulate protein trafficking around the TGN in plant cells (Boutte et al., 2010; Chow et al., 2008; Dettmer et al., 2006; Drakakaki et al., 2011; Gendre et al., 2011; Preuss et al., 2006; Qi et al., 2011; Qi and Zheng, 2011; Synek et al., 2006; Szumlanski and Nielsen, 2009). For example, roles of small Rab-A GTPases, the counterparts of yeast Ypt31/32 and animal Rab11, in TGN trafficking to the cell plate and in polar secretion of cell wall components, have been demonstrated (Blanco et al., 2009; Boutte et al., 2010; Chow et al., 2008; de Graaf et al., 2005; Preuss et al., 2006; Szumlanski and Nielsen, 2009). Recently, a chemical screen also identified a chemical

compound called endosidin 1 (ES1), which selectively blocks the recycling of several plasma membrane-located auxin transporters including PIN2 and AUX1 (Robert et al., 2008). In *Arabidopsis* roots, treatment of ES1 leads to the formation of a TGN/EE aggregate (Robert et al., 2008). However, the molecular nature how ES1 inhibits the TGN related vesicle transport is still not clear.

In plant lineages, Rab-A proteins have been elaborated spectacularly in evolution. In the *Arabidopsis* genome, 26 putative Rab-A proteins can be identified, which have been structurally categorized into 6 subclasses from Rab-A1 to A6 (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002). The elaboration of Rab-A proteins is thought to be a mechanism for plant cells to meet the dynamic need of intracellular membrane trafficking around the TGN (Chow et al., 2008; Preuss et al., 2006; Qi et al., 2011; Szumlanski and Nielsen, 2009), but how different Rab-A proteins may work in the TGN is not well defined. In this paper, we focused on functional analysis of the Rab-A1 subclass. Using an *in vivo* imaging approach, we demonstrate that RAB-A1c, strongly co-localized with other Rab-A proteins, defines a population of TGN that is only partially overlapping with the TGN that is marked by VHA-a1, a subunit of the Vacuolar H^+ ATPase. Interestingly, the morphology of the RAB-A1c defined-TGN as well as the relocation of RAB-A1c to the cell plate is sensitive to ES1, but not to wortmannin, a PI3K inhibitor (Clague et al., 1995) that inhibits the formation of MVBs as well as endocytosis in plant cells (Emans et al., 2002; Wang et al., 2009). We revealed that *RAB-A1a*, *RAB-A1b*, and *RAB-A1c* are highly expressed in root tip cells. Analysis of T-DNA knock-out mutants of *rab-a1a*, *rab-a1b*, and *rab-a1c* suggests that there is a functional redundancy in root growth within the members of the Rab-A1 subclass; however, the root growth of the *rab-a1a/b/c* triple mutant is slightly retarded. Very interestingly, we found that the root growth of *rab-a1a/b/c* is hypersensitive to ES1, but not to wortmannin. Histological analysis indicated that an enhanced defect in cytokinesis contributes at least in part to the hypersensitivity of *rab-a1a/b/c* to ES1.

Results

RAB-A1c, strongly colocalized with other Rab-A proteins, defines a population of TGN that is partially overlapped with VHA-a1 marked-TGN

It has been reported previously that Rab-A2 and Rab-A3 proteins reside on a population of the TGN that may contribute to cytokinesis (Chow et al., 2008), while RAB-A4b marks a population of the TGN that may be involved in polar secretion of cell wall components (Kang et al., 2004; Preuss et al., 2006). We have recently demonstrated that RAB-A1c, a member of the Rab-A1 subclass, co-localizes with RAB-A2a in the TGN (Qi et al., 2011). Therefore in this study we asked whether RAB-A1c is co-localized with RAB-A4b. To do this, we crossed the transgenic plant expressing GFP-RAB-A1c (Qi et al., 2011) to the line expressing YFP-RAB-A4b (Preuss et al., 2004). When both proteins were co-expressed in the same cells, they co-localized in a population of punctate structures (Figure 1A, arrows). We also calculated the linear Pearson correlation coefficient ($\mathbf{r_p}$) and the ranked Spearman's correlation coefficient ($\mathbf{r_s}$) between GFP-RAB-A1c and YFP-RAB-A4b, using the PSC Colocalization plug-in in ImageJ (French et al., 2008). As indicated in the scatter plot in Figure 1A, the value of $\mathbf{r_p}$ and $\mathbf{r_s}$ was 0.868 and 0.798, respectively. The result confirmed that GFP-RAB-A1c and YFP-RAB-A4b are strongly colocalized in plant cells.

In the Rab-A1 subclass, there are nine members, namely RAB-A1a to RAB-A1i (Pereira- Leal and Seabra, 2001; Rutherford and Moore, 2002). We wondered if different members of the Rab-A1 subclass are also co-localized in plant cells. To test this, RAB-A1b was fused to YFP. When YFP-RAB-A1b and GFP-RAB-A1c were co-expressed in the same plant cell, they were co-localized to punctate structures (Figure 1B, arrows). The linear Pearson correlation coefficient $\mathbf{r_p}$ and the ranked Spearman's correlation coefficient $\mathbf{r_s}$ between YFP-RAB-A1b and GFP-RAB-A1c were 0.95 and 0.905, respectively (Figure 1B, scatter plot), suggesting that members of the Rab-A1 subclass are almost perfectly co-localized within cells. Markers for different Golgi and post-Golgi compartments were then used to define the subcellular localization of RAB-A1c. We found that GFP-RAB-A1c was strongly co-localized with VTI12-YFP ($\mathbf{r_p}$ =0.839, $\mathbf{r_s}$ =0.859) (Figure 1C, arrows), a SNARE protein residing on the TGN (Uemura et al., 2004). GFP-RAB-A1c was often associated but not highly overlapped with ST-YFP ($\mathbf{r_p}$

=0.563, $\mathbf{r_s}$ =0.571) (Figure 2A), a *trans*-Golgi marker. Furthermore, GFP-RAB-A1c (Figure 2B, yellow arrows) has no colocalization with YFP-RAB-F2a (Figure 2B, red arrows) ($\mathbf{r_p}$ =0.319, $\mathbf{r_s}$ =0.392), a prevacuolar compartment marker. This confirmed that RAB-A1c resides on the TGN (Chow et al., 2008). Consistently, although many GFP-RAB-A1c compartments were often associated with VHA-a1-mRFP, they only partially overlapped ($\mathbf{r_p}$ =0.552, $\mathbf{r_s}$ =0.525) (Figure 2C). Taken together, we conclude that RAB-A1c defines a population of the TGN that is associated but only partially overlapped with VHA-a1 marked-TGN.

RAB-A1c defined TGN is sensitive to ES1 but not wortmannin

ES1 is an inhibitory chemical compound that defines a population of TGN/EE, which is involved in selective recycling of several auxin transporters including PIN2 and AUX1 to the plasma membrane (Robert et al., 2008). How ES1 inhibits the recycling of these proteins from the TGN is not so clear. Interestingly, we found that, when treated with ES1, GFP-RAB-A1c-positive punctae tended to aggregate (compare Figure 3C to 3A). We previously showed that GFP-RAB-A1c is relocated to a disc-like flat structure resembling the growing cell plate in mitotic root tip cells (Qi et al., 2011) (Figure 3B). We noticed that, in root tips treated with ES1, the typical disc-like flat structure marked by GFP-RAB-A1c was barely found, instead, aggregates of punctae were routinely observed (Figure 3D). In contrast, wortmannin, an inhibitory drug that induces fusion of MVBs and also impairs endocytosis (Emans et al., 2002; Wang et al., 2009), had no effect on the subcellular distribution and the relocation of GFP-RAB-A1c to the cell plate (Figure 3E and 3F).

Members of the Rab-A1 subclass are strongly expressed in root and shoot meristematic zones

It has been shown that members of the *Rab-A2* and *-A3* subclasses display a differential yet overlapping expression in root tips (Chow et al., 2008), while *RAB-A4b* is ubiquitously expressed (Preuss et al., 2004). We therefore investigated the expression pattern of members of the *Rab-A1* subclass in different tissues in *Arabidopsis*. Despite the high level of sequence similarity, the nine members of the *Rab-A1* subclass had distinct

but overlapping transcription patterns based on the publicly available microarray expression data at http://www.bar.utoronto.ca/ (Schmid et al., 2005) and our RT-PCR result (Figure S1). The expression of *RAB-A1h* and *RAB-A1i* was pollen-specific, *RAB-A1e* was strongly expressed in roots, but other members were ubiquitously expressed. The high resolution technique, the *Rab-A1*-promoter::GUS staining revealed that three ubiquitously expressed members, *RAB-A1a*, *RAB-A1b* and *RAB-A1c* exhibited differential expression pattern (Figure 4). All three genes were expressed in cotyledons and hypocotyls (Figure 4A to 4C), root tips (Figure 4E, 4G and 4I), initiation sites of lateral roots (Figure 4F, 4H and 4J, red arrows) and mature pollen grains (Figure 4M to 4O and 4Q to 4S), but *RAB-A1b* and *RAB-A1c* were strongly expressed in root tips (Figure 4G and 4I) and shoot meristems (Figure 4B and 4C, red arrows). Interestingly, in root tips, *RAB-A1b* was expressed in both meristematic and elongating zones (Figure 4G, bracket), whereas the expression of *RAB-A1c* was restricted in a smaller region in the meristematic zone (Figure 4I, bracket). Consistent with the microarray and RT-PCR, the GUS activity of *pRAB-A1i::GUS* was detected only in mature pollen (Figure 4P and 4T). Taken together*, RAB-A1a*, *RAB-A1b*, *RAB-A1c* and *RAB-A1i* have distinct but overlapping expression in rapidly growing tissues where active membrane trafficking is required.

Root growth of *rab-a1a/b/c* triple mutant is only slightly retarded but hypersensitive to ES1 treatment

To study the developmental function of Rab-A1 proteins, we initiated a reverse genetic approach. T-DNA insertional mutants of *RAB-A1a, RAB-A1b and RAB-A1c* were identified from SALK lines generated in the Salk Institute in California and our RT-PCR result indicated that they were knock-out mutants in the respective genes (Figure S2B). Because of the strong expression of *RAB-A1a, RAB-A1b* and *RAB-A1c* in root tips, we therefore examined the root growth of these mutants. Comparing to wild type, the single *rab-a1a, rab-a1b,* and *rab-a1c* mutants, as well as the double *rab-a1a/b* mutant showed no statistically significant difference in the root length (Figure S2A and S2C). However, when *rab-a1a, rab-a1b* and *rab-a1c* were crossed together, the root growth of the *rab-a1a/b/c* triple mutant was inhibited (Figure 5A). The length of roots in the *rab-a1a/b/c* triple mutant was approximately 70% of wild type roots (Figure 5B, $P<0.01$). The result

indicates, although it is still subtle, the root growth in the *rab-a1a/b/c* triple mutant is affected.

Because the subcellular distribution of RAB-A1c can be affected by ES1, we wondered whether or not the root growth of the *rab-a1a/b/c* triple mutant is hypersensitive to ES1. The root development of both wild type and *rab-a1a/b/c* was then monitored under conditions in which three different concentrations of ES1 were used. As indicated in Figure 6A to 6D and 6I, the root growth of wild type seedlings was not significantly affected at 0.0835μM, but started to be affected at 0.167μM, with roughly 5% reduction in length (Figure 6I). When treated with 0.33μM ES1, the reduction in root length was approximately 17% (Figure 6I). But clearly, the root growth of *rab-a1a/b/c* was hypersensitive to ES1 treatments: e.g. when treated with 0.167μM ES1, there was approximately 40% reduction in root length when compared to roots of *rab-a1a/b/c* growing in the control AT media (Figure 6I). Interestingly, such hypersensitivity was not observed when *rab-a1a/b/c* seedlings were treated with wortmannin: as indicated in Figure 6E to 6H and 6J, both wild type and *rab-a1a/b/c* showed a similar reduction rate in root growth when treated with wortmannin. This result suggests that Rab-A1 proteins are functionally related to ES1, but not wortmannin.

Cytokinesis in *rab-a1a/b/c* triple mutant is hypersensitive to ES1 treatment

Rab-A1 proteins are relocated to the cell plate in mitotic root tip cells (Qi et al., 2011). This potentially implies that Rab-A1 proteins may be involved in cytokinesis. Because cell walls and nuclei can be stained with calcofluor and propidium iodide to study cytokinesis (Chow et al., 2008) (Figure 7A), we stained root tip cells of 7-day-old wild type and *rab-a1a/b/c* seedlings with calcofluor and propidium iodide. We found that, bi-nucleated cells cannot be routinely observed in wild type root tips (Figure 7B), but occasionally we found roughly 1-2 bi-nucleated cells (Figure 7C) in every 2 *rab-a1a/b/c* root tips examined. When seedlings of both wild type and *rab-a1a/b/c* were treated with 0.0835μM ES1, in wild type, consistent with the still normal root growth observed in Figure 6B, perfect cell files with one nucleus per cell were still observed (compare Figure 7D and 7A). In *rab-a1a/b/c,* strikingly, roughly 4-5 bi-nucleated cells per root tip can be

seen (Figure 7E to 7G), compared with only ccasional occurrence of bi-nucleated cells in non-treated *rab-a1a/b/c* (Figure 7B and 7C). This indicates that the ES1 treatment synthetically enhances the cytokinesis defect in *rab-a1a/ b/c*, suggesting that ES1 and RAB-A1a/b/c act in the same trafficking pathway important for cytokinesis.

Cytokinesis in plants expressing dominant-inhibitory mutants of RAB-A1c is also impaired

Dominant inhibitory approach has been routinely used to investigate the function of respective wild type Rab proteins (Chow et al., 2008; de Graaf et al., 2005; Olkkonen and Stenmark, 1997). To confirm the function of the Rab-A1 subclass in *Arabidopsis*, we generated two RAB-A1c mutants, namely RAB-A1c(S27N) and RAB-A1c(Q72L). RAB-A1c(S27N) has a serine to asparagine substitute in the conserved GxxxxGKS domain, which is predicted to be GDP-locked, whereas RAB-A1c(Q72L) has a glutamine to leucine substitute in the conserved WDTAGQ domain, predicted to be GTP-locked (Kotzer et al., 2004; Lee et al., 2004; Zheng et al., 2005a). Both mutated versions of RAB-A1c were transformed and expressed under the dexamethasone (dex)-inducible synthetic promoter pOP6 (Craft et al., 2005) in Col-0 *Arabidopsis*. As indicated in Figure 8, the root growth of transgenic seedlings expressing either RAB-A1c(S27N) (Figure 8B, 8E and 8J, $P<0.01$ compared with the WT seedlings treated with Dex) or RAB-A1c(Q72L) (Figure 8C, 8F and 8J, $P<0.01$ compared with the WT seedlings treated with Dex) was impaired, especially when treated with dex (Figure 8E and 8F). Quantification of root length indicated that, the reduction of root length in dex-treated seedlings expressing RAB-A1c(S27N) was more than 50% (Figure 8J, $P<0.01$ compared with the WT seedlings treated with Dex) [even in DMSO treated RAB-A1c(S27N), root growth was retarded (compare Figure 8B to 8A), likely due to the basal expression of RAB-A1c(S27N) under pOP6 (Craft et al., 2005)]. In seedlings expressing RAB-A1c(Q72L), the reduction was about 40% (Figure 8J, $P<0.01$ compared with the WT seedlings treated with Dex).

The cell file alignment in root tips of the transgenic plants were also disrupted when RAB-A1c(S27N) and RAB-A1c(Q72L) were induced by dex (Figure S3). When root tip

cells of seedlings expressing RAB-A1c(S27N) and RAB-A1c(Q72L) were stained with calcofluor and propidium iodide, in wild type cells, regular cell files were highlighted by calcofluor, with a single nucleus in each cell (Figure 8G). In seedlings expressing RAB-A1c(S27N) and RAB-A1c(Q72L), however, bi-nucleated cells were frequently observed (Figure 8H and 8I, arrows), indicating that cytokinesis in these plants is defective.

Discussion

How may Rab-A proteins act in the TGN in plant cells?

As an organelle independent of Golgi, the TGN in plant cells sorts newly synthesized proteins received from Golgi and materials endocytosed from the plasma membrane to the proper destinations. The TGN in plant cells is a highly dynamic organelle (Viotti et al., 2010), yet little is known about the molecular nature of its dynamics. In this study, we showed that different members of Rab-A proteins, RAB-A1b, RAB-A1c, RAB-A2a and RAB-A4b reside on the same population of the TGN at the resolution of light microscopy. GFP-RAB-A1c marked-TGN is often associated with but only partially overlapped with VHA-a1-mRFP, a subunit of the V-ATPase (Dettmer et al., 2006). Previously, Chow et al. (2008) revealed that the TGN marked by Rab-A2/A3 proteins also only partially overlaps with the TGN marked by VHA-a1 (Chow et al., 2008). Therefore it is highly possible that members of Rab-A proteins largely reside on a subdomain of TGN that is distinct from but somewhat overlapped with VHA-a1. In *Arabidopsis* root tip cells, the early TGN that just buds off from *trans*-Golgi mature into the so called late TGN, which will eventually segregates into at least two major domains, one for transport/maturation to MVBs, another for secretion (Kang et al., 2011). RAB-A4b is found to be localized equally to both the early and late TGN, while VHA-a1 is preferentially localized to the late TGN (Kang et al., 2011). It is therefore possible that Rab-A proteins are involved in the membrane trafficking at both the early and late TGN, for example, the maturation of the early TGN to the late TGN and the protein sorting at the late TGN, but VHA-a1 is mainly involved in the function of the late TGN.

It is interesting to note that the subcellular distribution of RAB-A1c defined-TGN is sensitive to ES1, but not wortmannin. Wortmannin is a PI3K inhibitor (Clague et al.,

1995). In plant cells, it inhibits endocytosis (Emans et al., 2002) and also the formation and morphogenesis of MVBs (Wang et al., 2009). Our interpretation of the insensitivity of Rab-A to wortmannin is that Rab-A proteins are not directly involved in either endocytosis or the morphogenesis of MVBs. Indeed, we have previously shown that in the mutants defective in TRAPPII, a protein complex that acts upstream of RAB-A1c, endocytosis of FM4-64 as well as the transport of the dye via TGN/EE to the vacuole is not impaired (Qi et al., 2011). ES1 is a chemical compound first identified as a selective inhibitor of recycling of PIN2, AUX1 and BRI1, but not PIN1 (Robert et al., 2008). However, the molecular mechanism how these pathways are affected by ES1 is not yet clarified. Because 1) the subcellular distribution of RAB-A1c defined-TGN is sensitive to ES1; 2) Rab-A1 proteins have been implicated in auxin transport to root tips (Koh et al., 2009); 3) in *Arabidopsis* mutants defective in the formation of TRAPPII, recycling of PIN2 and AUX1, but not PIN1 is also affected (Qi et al., 2011; Qi and Zheng, 2011), we suggest that the TRAPPII/Rab-A defined pathway for selective protein recycling is the target of ES1.

A recent study on the circadian clock effectors in *Arabidopsis* revealed that ES1 impairs cellular actin dynamics (Toth et al., 2012). Interestingly, different from other actin drugs e.g. latrunculin B, ES1 does not stop dynamic actin rearrangement necessary for the endocytosis and the PVC-mediated vacuolar transport of FM4-64 (Toth et al., 2012). It was therefore suggested that ES1 may only affect the function of an actin-binding protein(s) that have an impact on cellular actin dynamics important for the polar transport of PIN2 from the TGN to the plasma membrane (Toth et al., 2012), but not necessary for endocytosis. In this regard, it is important to point out that, in animal cells, Rab11 remodels the local actin dynamics via its interacting proteins to regulate plasma membrane recycling (Hales et al., 2002). It is yet to know if Rab-A proteins or Rab-A effectors could affect cellular actin dynamics, but it is known that the function of the RAB-A4b positive endosomes is dependent on actin dynamics (Zhang et al., 2010). Therefore it is possible that ES1 may target to an actin binding protein(s) that may be functionally related to Rab-A proteins.

Function of Rab-A1 proteins in cytokinesis

RAB11b, a tobacco Rab-A1 homologue has been implicated to play a role in the delivery of secretory and cell wall proteins to the extracellular matrix of growing pollen tubes (de Graaf et al., 2005). Previously, we showed that GFP-RAB-A1c is relocated to the growing cell plate in mitotic cells (Qi et al., 2011). Here we show that the relocation of GFP-RAB-A1c to the cell plate can be disrupted by ES1. Furthermore, it is interesting to note that, although bi-nucleated cells could only be occasionally observed in roots of *rab-a1a/b/c*, cytokinesis in *rab-a1a/b/c* is hypersensitive to ES1. Expression of dominant inhibitory mutants of RAB-A1c(S27N) and RAB-A1c(Q72L) also leads to a severe defect in cytokinesis. The GDP- and GTP-locked Rab mutants potentially affects the function of the upstream GEFs (guanine nucleotide exchange factors) and downstream effectors respectively (Ang et al., 2003; Chen et al., 2003), therefore a severe defect is expected (Kotzer et al., 2004; Lee et al., 2004; Zheng et al., 2005a). Based on these results, we propose that Rab-A1 proteins, in addition to their roles in polar secretion and recycling of plasma membrane proteins, are also involved in cytokinesis. It is highly likely that Rab-A1 proteins are required for proper protein transport from the TGN to the newly formed cell plate in mitotic cells. Because cytokinesis in *rab-a1a/b/c* is hypersensitive to ES1, we think that, similar to the Rab-A defined pathway for selective protein recycling to the plasma membrane, the cell plate trafficking pathway regulated by Rab-A proteins may also be the target of ES1. In animal cells, during cytokinesis, Rab11 regulates the rate of local actin polymerization via Nuf, a Rab11 effector that interacts with the RhoGEF2-Rho1 pathway to maintain cytokinetic furrow integrity (Cao et al., 2008). It will be interesting to examine if homolog(s) of Nuf exists in *Arabidopsis*, and if it is a target of ES1.

Previously, members of the Rab-A2 and -A3 subclasses have been implicated in membrane trafficking to the cell plate (Chow et al., 2008), but RAB-A2a may be also involved in the formation of root hairs and nodulation (Blanco et al., 2009). A study in *Arabidopsis* root hairs also established a role for RAB-A4b in polarized secretion of cell wall components (Preuss et al., 2006). However, RAB-A4b is also relocated to the growing cell plate in root mitotic cells (Qi and Zheng, unpublished data). Likely there is a

functional overlap between Rab-A proteins, which explains why many Rab-A genes are highly expressed in root and shoot meristems, root hairs and mature pollen (Chow et al., 2008; Preuss et al., 2004; this study) and why plants lacking either RAB-A1a, or RAB-A1b, or RAB-A1c show no gross developmental phenotype. However, we do not think the functional redundancy among Rab-A proteins can explain why *Arabidopsis* has 26 Rab-A proteins. Indeed, expression of dominant inhibitory RAB-A2a (Chow et al., 2008) or RAB-A1c (this study), which is expected to sequester the function of the upstream GEF(s), only partially inhibits root growth, while expression of dominant inhibitory RAB-D1 is seedling lethal (Pinheiro et al., 2009). One of the reasons that plants expressing dominant inhibitory RAB-A2a or RAB-A1c can survive is that these proteins may be under the regulation of multiple GEFs, or that Rab-A proteins do have different functions in diversified protein trafficking around the TGN. The elaboration of Rab-A proteins has been thought to be a strategy for plants to meet the dynamic need of intracellular membrane trafficking around the TGN (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002). Under light microscopy, Rab-A proteins are localized to the same population of the TGN compartment, but we noted that the correlation coefficients between members of Rab-A1 proteins are higher than that between RAB-A1c and RAB-A4b. Therefore it would be interesting to examine the subcellular localization of each of them at the ultra-structural level with transmission electron microscopy (Kang et al., 2011). Recently, Ebine et al. (2011) demonstrated that RAB-F1, a plant specific radiation of Rab5, modulates the assembly of a distinct SNARE complex in an endosomal trafficking pathway in the salinity stress response in *Arabidopsis* (Ebine et al., 2011). Plants are immobile organisms growing under prevailing environmental conditions. Because cell reshaping is a way for plants to deal with stressful growth conditions, it will also be interesting to examine how plants defective in various Rab-A proteins respond to stressful environmental conditions and how cargoes being transported by different Rab-A proteins around the TGN.

Methods

Plant materials and growth conditions

Salk T-DNA insertional mutant lines were obtained from the *Arabidopsis* Biological Resource Center (http://www.*arabidopsis*.org). *Arabidopsis* plants co-expressing GFP-RAB-A1c with YFP-RAB-A4b (Preuss et al., 2004), VTI12-YFP (Uemura et al., 2004), VHA-a1-mRFP (Dettmer et al., 2006), ST-YFP (Zheng et al., 2004) and YFP-RAB-F2a (Haas et al., 2007) were made by crossing transgenic *Arabidopsis* expressing respective markers. Co-localization of GFP-RAB-A1c and YFP-RAB-A1b was done by infiltrating Agrobacteria containing GFP-RAB-A1c and YFP-RAB-A1b into tobacco leaves using method described in Zheng et al (2004). For the dexamethasone-inducible expression of RAB-A1c(S27N) and RAB-A1c(Q72L), RAB-A1c(S27N) and RAB-A1c(Q72L) vectors were transformed into the LhGR driver line 4C-S5/7 (Craft et al., 2005). Seedlings were germinated and grown on hygromycin and kanamycin AT (*Arabidopsis thaliana*) plates (Haughn and Somerville, 1986) supplemented with dexamethasone solution (20 μM) diluted from a 100 mM DMSO stock. Plants on AT plates or in soil (Sunshine#5; SunGro, http://www.sungro.com) were grown at 22–24 ^{o}C under continuous light (80–100 μE m-1s-1 photosynthetically active radiation).

Molecular biology and generation of constructs

To identify the homozygote of *rab-a1a, rab-a1b* and *rab-a1c*, genomic DNA was extracted from T3 seedlings of SALK_098941 (*rab-a1a*), SAIL_875_F08 (*rab-a1b*) and SALK_145363 (*rab-a1c*). Primers used to genotype the mutants are listed in supplementary table 1. To examine the expression of the nine members in the Rab-A1 subclass, total RNAs were extracted from roots, stems, rosette leaves and flowers of 3-week-old wild-type Columbia-0 and the whole seedlings of 10-day-old without roots (considered as shoot) as described by Zheng et al. (2004). To compare the expression of *RAB-A1a*, *RAB-A1b* and *RAB-A1c* in the wild type and mutants, total mRNAs were extracted from seedlings of the wild type and mutants. Reverse transcriptase (RT)-PCR was then performed using the Invitrogen SuperScript III System (Invitrogen, http://www.invitrogen.com). Primers used for RT-PCR are listed in supplementary table 1.

To make YFP-RAB-A1b construct, the cDNA of *RAB-A1b* was amplified using primers listed in supplementary table 1. The amplified cDNA was first subcloned into pCR8/GW/TOPO according to the manufacturer's instructions (Invitrogen) and sequenced. The sequenced clone was then subcloned into the pEarleyGate 104 vector (ABRC stock CD3-686) to make YFP-RAB- A1b. The cDNA of *RAB-A1c* was amplified using primers listed in supplementary table 1. The amplified cDNA was first cloned into pBluescript KS as the EcoRI-BamHI fragment and then sequenced. The S27N substitution and the Q72L substitution were effected into *RAB-A1c* by overlapping PCR using primers listed in supplementary table 1. The sequenced wild-type *RAB-A1c* cDNA was subcloned into pVKH-GFPN (Zheng et al., 2005b) as the SalI-SacI fragment to generate GFP-RAB-A1c, which was transformed into wild-type *Arabidopsis* ecotype Col-0. The modified RAB-A1c(S27N) and RAB-A1c(Q72L) was subcloned into the pV-TOP vector (Craft et al., 2005) and transformed into the LhGR driver line 4C-S5/7 (Craft et al., 2005). To make promoter::GUS constructs, the ~2kb upstream sequences of *RAB-A1a*, *RAB-A1b*, *RAB-A1c* and *RAB-A1i* were amplified using primers listed in supplementary table 1. The amplified promoters were first cloned into pBluescript KS as the EcoRI-BamHI fragment and then sequenced. The sequenced clones were then subcloned into PVKH-GUS (Zheng et al., 2005b) as the SalI-SacI fragment to generate pRAB-A1a::GUS, pRAB-A1b::GUS, pRAB-A1c::GUS and pRAB-A1i:: GUS. These vectors were then transformed into wild-type *Arabidopsis* ecotype Col-0.

Phenotyping and light microscopy

Images of GUS-staining were taken using a QImaging Micropublisher3.3 digital CCD color camera installed on a Leica MZ16F stereomicroscope. Seedlings for root length phenotype were imaged with a Nikon D80 digital camera (Nikon, http://www.nikon.com).

Inhibitor and dex treatment

For subcellular distribution of GFP-RAB-A1c, seedlings (7-days old) expressing GFP-RAB-A1c were incubated in 1 ml AT liquid medium containing 20 µM wortmannin (prepared from 1 mM stock solution dissolved in DMSO) or 33 µM ES1 (prepared from 1.67 mM stock solution dissolved in DMSO) at room temperature (23–25 ^{o}C) for 2 h.

Control treatments were performed with equal concentrations of DMSO. For hypersensitivity test, seeds of wild type and *rab-a1a/ b/c* were planted on AT medium plates containing 0 μM, 0.0835 μM, 0.167 μM and 0.33 μM ES1 or 0 μM, 0.1 μM, 1 μM and 10 μM wortmannin. To induce the expression of RAB-A1c(S27N) and RAB-A1c(Q72L), seeds of wild type, transgenic plants expressing RAB-A1c(S27N) and RAB-A1c(Q72L) were planted on AT medium plates containing 0 μM and 20 μM Dex. Length of roots was analyzed seven days after germination.

GUS, calcofluor and propidium iodide staining

Tissues of transgenic plants expressing *pRAB-A1a::GUS, pRAB-A1b::GUS, pRAB-A1c::GUS* and *pRAB-A1i::GUS* were immersed in the GUS substrate X-Gluc staining solution (Sigma #B6650), and incubated overnight at 37 ^{o}C. Then the staining solution was removed and tissues were washed with several changes of 50% ethanol until tissue clears. Photos were taken afterward. To stain cell walls and nuclei, seedlings were immersed in a staining solution containing 35 μg/mL calcofluor (diluted from a 3.5 mg/mL stock solution in 0.1 M Tris-HCl, pH 9), 200 μM $CaCl_2$, 10 μg/mL propidium iodide (diluted from a 1 mg/mL stock solution), 0.1% Triton, and 50mM Tris, pH 8.0, and incubated at room temperature for 60 min prior to imaging. The seedlings were then mounted in the staining solution for imaging.

Confocal microscopy

Confocal microscopy was carried out with an inverted Zeiss LSM 510 Meta confocal laser scanning microscope (Zeiss, http://www.zeiss.com), using a 10× objective for low magnification bright field imaging and a plan-Neofluar 40×/1.3 numerical aperture oil-immersion lens for higher magnification images. All images were acquired using a line-sequential configuration. Single-color images of GFP and multicolor images of GFP/YFP were acquired as described by Zheng et al. (Zheng et al., 2005a). For calcofluor/propidium iodide staining, signals were excited at 405nm and 543nm and simultaneously collected with a 420- to 480-nm emission filter and a LP 560-nm emission filter. The Zeiss LSM image browser, Image J and PHOTOSHOP CS2 (Adobe, http://www.adobe.com) were used for post-acquisition image processing. The PSC

colocalization plug-in in ImageJ was used for quantitative colocalization analysis on GFP-RAB-A1c and various markers (French et al., 2008).

Accession numbers

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative database under the following accession numbers: *RAB-A1a* (At1g06400), *RAB-A1b* (At1g16920), *RAB-A1c* (At5g45750), *RAB-A1d* (At4g18800), *RAB-A1e* (At4g18430), *RAB-A1f* (At5g60860), *RAB-A1g* (At3g15060), *RAB-A1h* (At2g33870), *RAB-A1i* (At1g28550).

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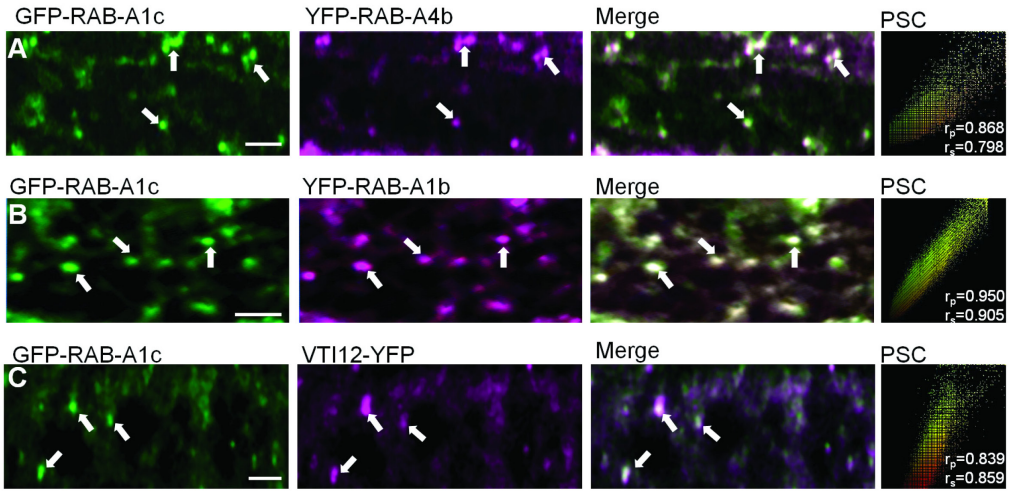


Figure 2.1. Rab-A proteins are co-localized to a population of TGN.

A) GFP-RAB-A1c is co-localized with YFP-RAB-A4b.

B) GFP-RAB-A1c and YFP-RAB-A1b co-localize at a population of punctae.

C) GFP-RAB-A1c is co-localized with VTI12-YFP.

White arrows indicate co-localized punctae. Pearson and Spearman correlation coefficients (PSCs) between GFP-RAB-A1c and each marker are indicated in the scatter plots on the right. Bars: 5μm for all

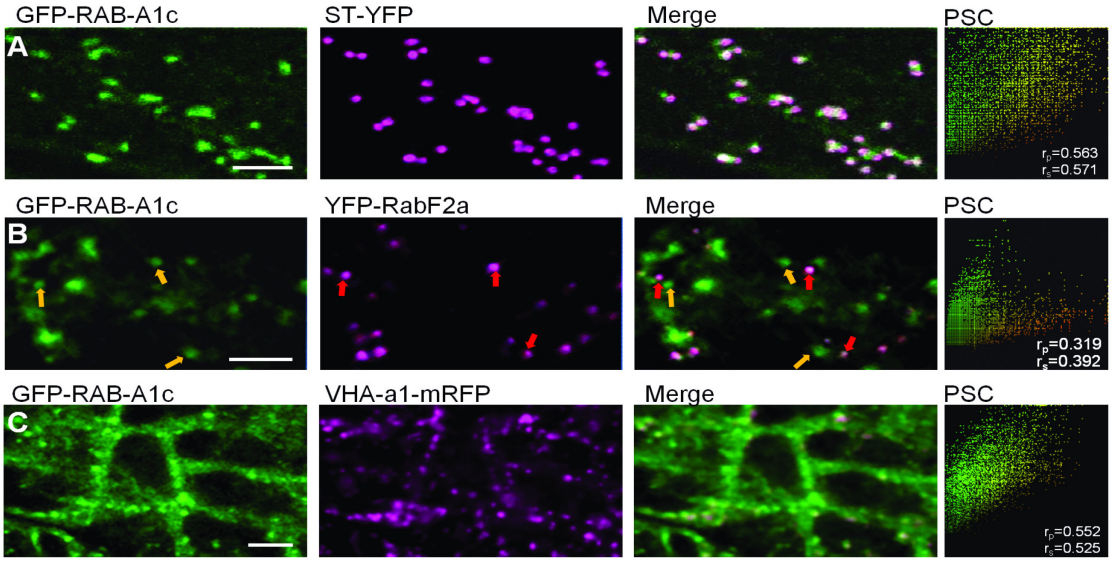


Figure 2.2. GFP-RAB-A1c defined punctae are distinct from Golgi and the pre-vacuolar compartment but partially co-localized with VHA-a1 marked TGN.

A) GFP-RAB-A1c punctae are associated but not completely co-localized with ST-YFP marked Golgi.

B) GFP-RAB-A1c and YFP-RAB-F2a label distinct punctae.

C) GFP-RAB-A1c only partially co-localize to VHA-a1-mRFP.

Yellow arrow indicates GFP-RAB-A1c only punctae, red arrow indicates YFP-RAB-F2a only punctae. Pearson and Spearman correlation coefficients between GFP-RAB-A1c and each marker are shown in the scatter plots on the right. Bars: 5μm for all

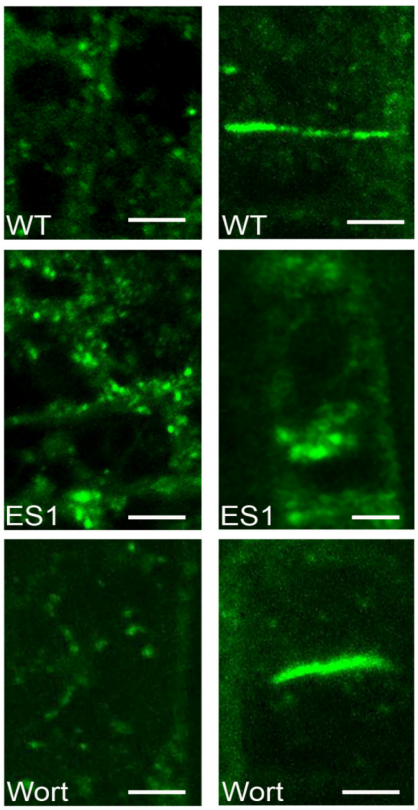


Figure 2.3. Effects of ES1 and wortmannin on the subcellular distribution of GFP-RAB-A1c.

A) Punctate structures labelled by GFP-RAB-A1c in a wild type cell.

B) GFP-RAB-A1c is relocated to a disc-like flat structure resembling the growing cell plate in mitotic cells in wild type.

C) Aggregation of GFP-RAB-A1c punctae in a cell treated with ES1.

D) Aggregation of GFP-RAB-A1c punctae close to a cell-plate-like structure after ES1 treatment.

E) Punctae distribution of GFP-RAB-A1c is not affected by wortmannin.

F) GFP-RAB-A1c is still relocated to cell-plate-like structure when treated with wortmannin. Bars: 5μm for all

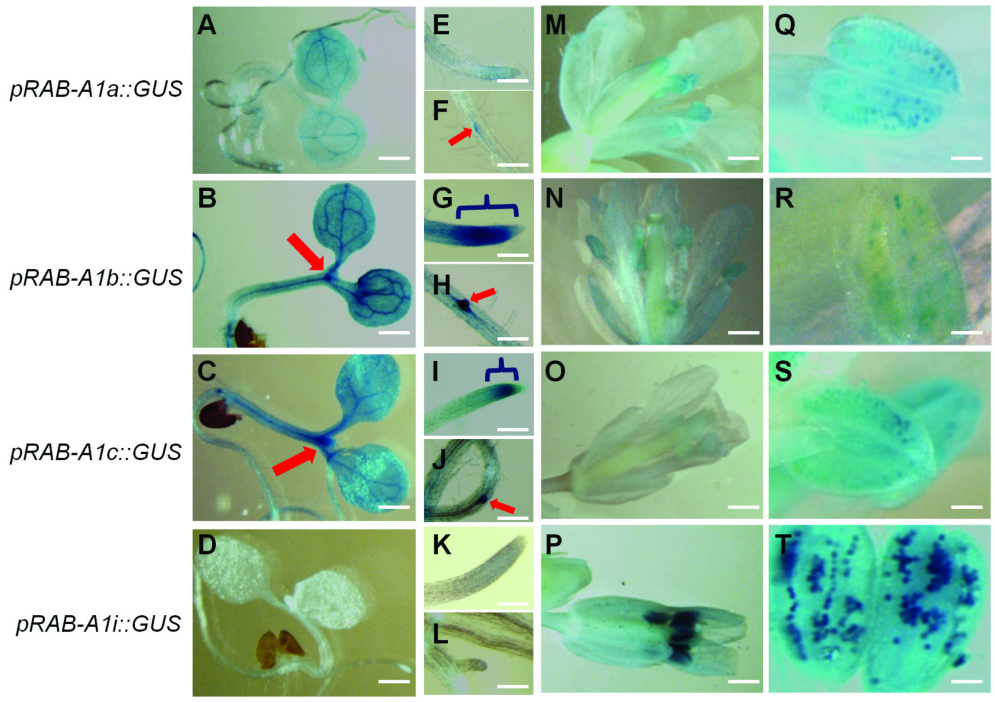


Figure 2.4. Promoter activity of *pRAB-A1a*, *pRAB-A1b*, *pRAB-A1c* and *pRAB-A1i* reported by GUS expression

Images of GUS staining of seedlings (A to D), roots (E to L), flowers (M to P) and pollen grains (Q to T) of transgenic plants expressing *pRAB-A1a::GUS* (A, E, F,M, Q), *pRAB-A1b::GUS* (B, G, H, N, R), *pRAB-A1c::GUS* (C, I, J, O, S) and *pRAB-A1i::GUS* (D, K, L, P, T). Note *pRAB-A1b::GUS* and *pRAB-A1c::GUS* have strong GUS activity in shoot meristems (B to C, red arrows), root tips (G and I, brackets) and at initiation of lateral roots (H and J, red arrows). *pRAB-A1a::GUS* also has GUS activity in root tips (E) and the initiation of lateral roots (F, red arrow). Bars: 1mm for (A-D, M-P); 0.5mm for (E-L); 0.2mm for (Q-T)

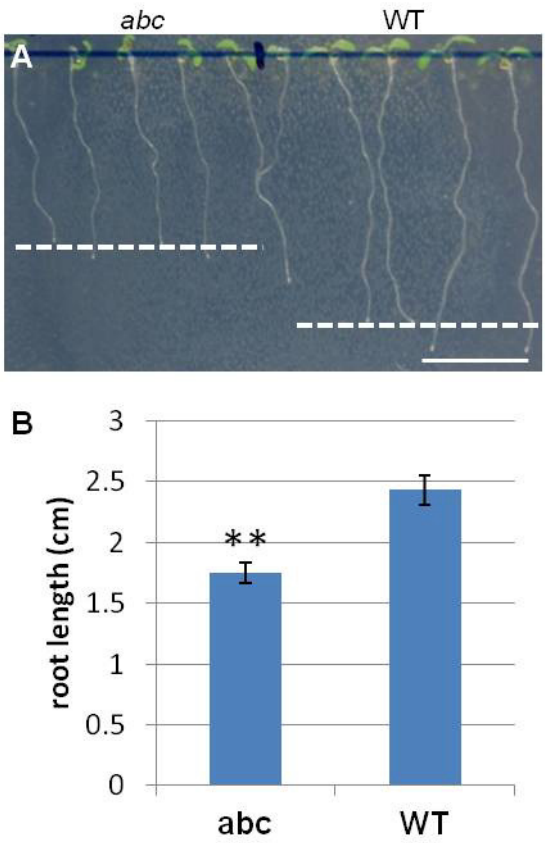


Figure 2.5. Root growth of *rab-a1a/b/c* triple mutant is slightly retarded

A) stereo-microscopic image of seven-day-old seedlings of *rab-a1a/b/c* (left) and wild type (right). (*abc* indicates *rab-a1a/b/c* triple mutant) Bar: 1cm

B) Quantification analysis of 50 seven-day-old seedlings of *rab-a1a/b/c* and wild type. (*abc* indicates *rab-a1a/b/c* triple mutant). Error bar represents standard error. **: $P<0.01$

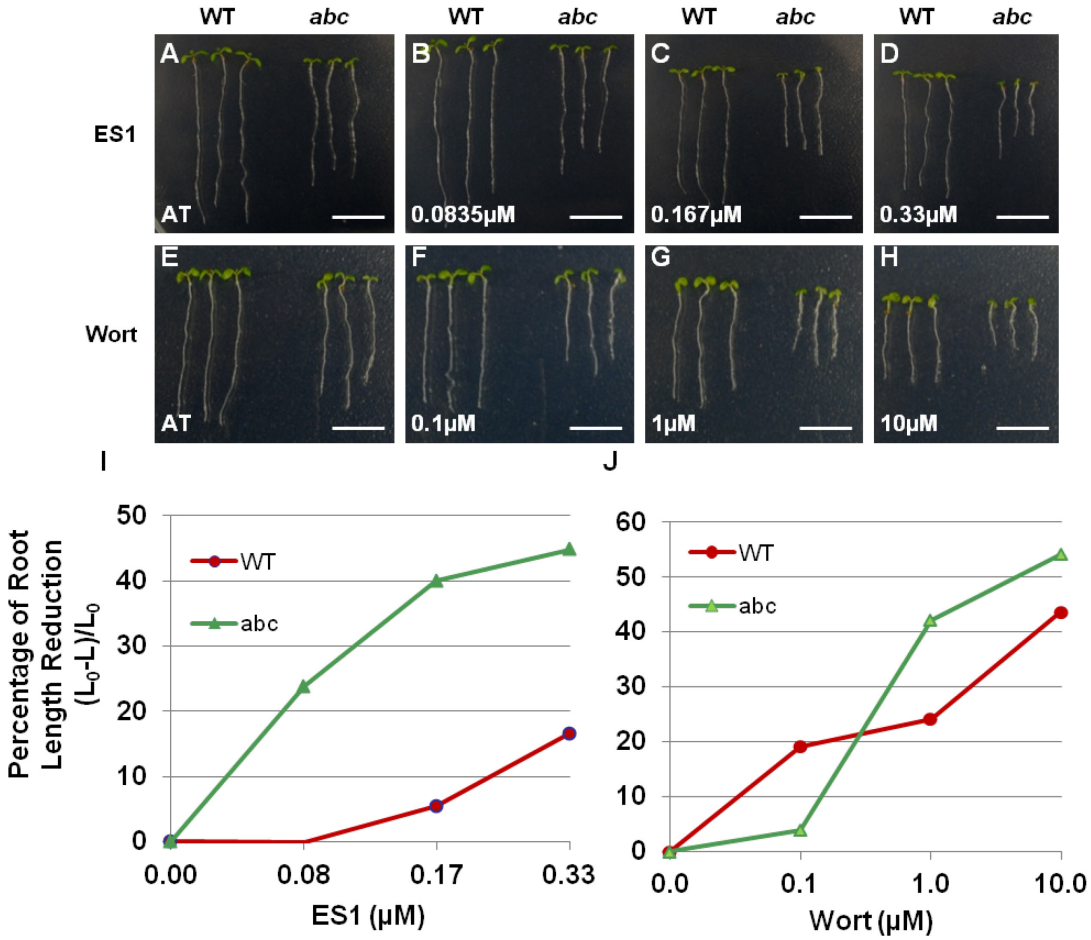


Figure 2.6. Different effects of ES1 and wortmannin on the root development of *rab-a1a/b/c* and wild type plants.

Seven-day-old seedlings of *rab-a1a/b/c* and wild type grown on AT plates with 0 (A), 0.0835μM (B), 0.167μM (C), 0.33μM (D) of ES1, and AT plates with 0 (E), 0.1μM (F), 1μM (G), 10μM (H) of wortmannin. Around 30 seedlings for each sample are used for quantification of reduction in root length in ES1 treated samples (I), and wortmannin treated samples (J). In (I) and (J), the x axis indicates concentration of drugs (μM), and the y axis indicates the percentage of root length reduction relative to the root length of seedlings grown on AT plate with no drugs. Bars: 1.5cm for all.

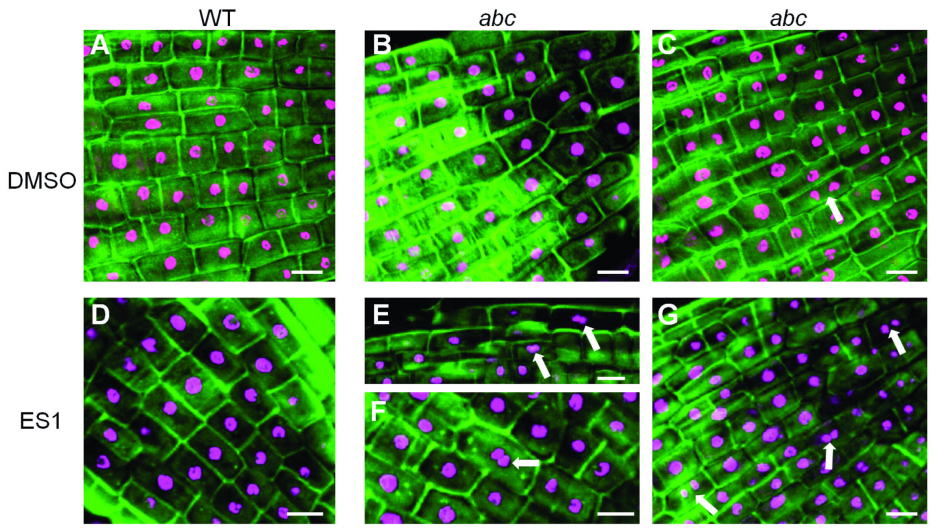


Figure 2.7. Cytokinesis in *rab-a1a/b/c* triple mutant is hypersensitive to ES1.

calcofluor and propidium iodide stained cells in root tips of wild type (A, D) and *rab-a1a/b/c* (B, C, E, F, G) growing on AT plates containing either DMSO (A to C) or 0.0835μM ES1 (D to G). Note in *rab-a1a/b/c*, bi-nucleated cells were frequently observed when treated with ES1 (E to G, arrows), whereas under the DMSO condition, bi-nucleated cells occur only occasionally (C, arrow). Bars: 5μm for all

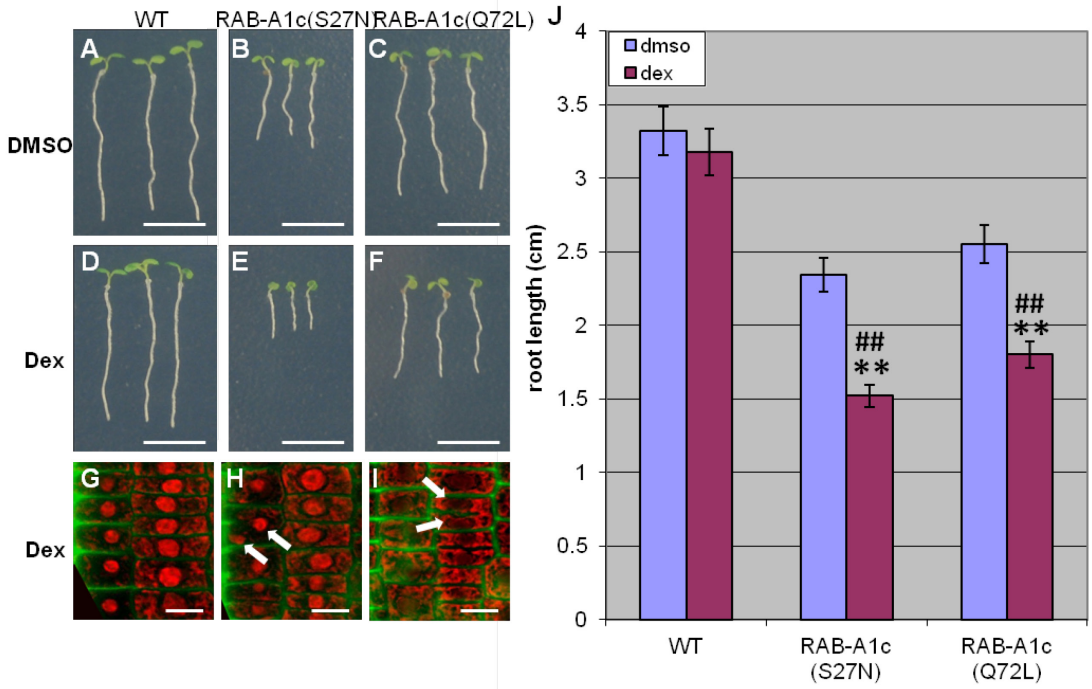
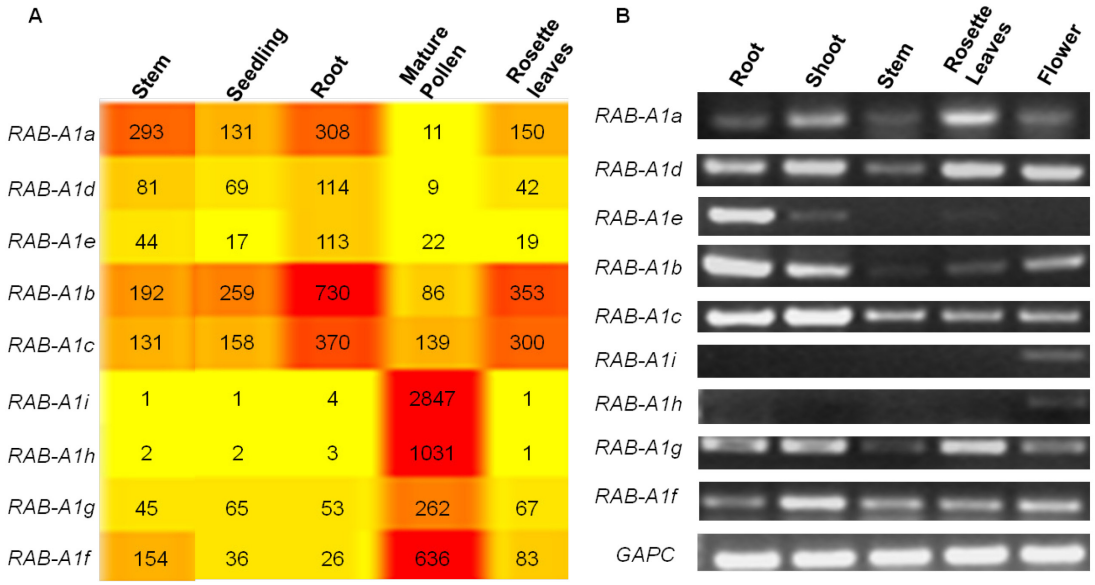


Figure 2.8. Cytokinesis in plants expressing dominant inhibitory RAB-A1c mutants is defective.

Seven-day old seedlings of wild type (A, D), transgenic plant expressing RAB-A1c(S27N) (B, E) and RAB-A1c(Q72L) (C, F) growing on AT plates containing either DMSO (A to C) or 20μM Dex (D to F). Around 30 seedlings for each sample are used for quantification analysis of root length (J). Calcofluor and propidium iodide staining of root tip cells of wild type (G), plants expressing RAB-A1c(S27N) (H) and RAB-A1C(Q72L) (I). Note bi-nucleated cells were frequently observed when the expression of dominant inhibitory RAB-A1c mutants is induced by Dex (H to I, arrows). Bars: 1.5cm for all. Error bar represents standard error. **: $P<0.01$ compared with the same seedlings treated with DMSO; ##: $P<0.01$ compared with the WT seedlings treated with Dex.



Supplementary data

Figure S2.1. Microarray and RT-PCR analyses of the transcription of *RAB-A1a-i*.

A) Microarray data (http://www.bar.utoronto.ca) reveals the differential but overlapping expression pattern of the nine members of the Rab-A1 subclass. Numbers in each box and the intensity of red color indicate the expression level (Red: high expression; orange: medial expression; yellow: low expression)

B) RT-PCR analyses of transcription of the nine Rab-A1 members. The housekeeping gene *GAPC* is used as an internal input control for the RNA amount used in each tissue.

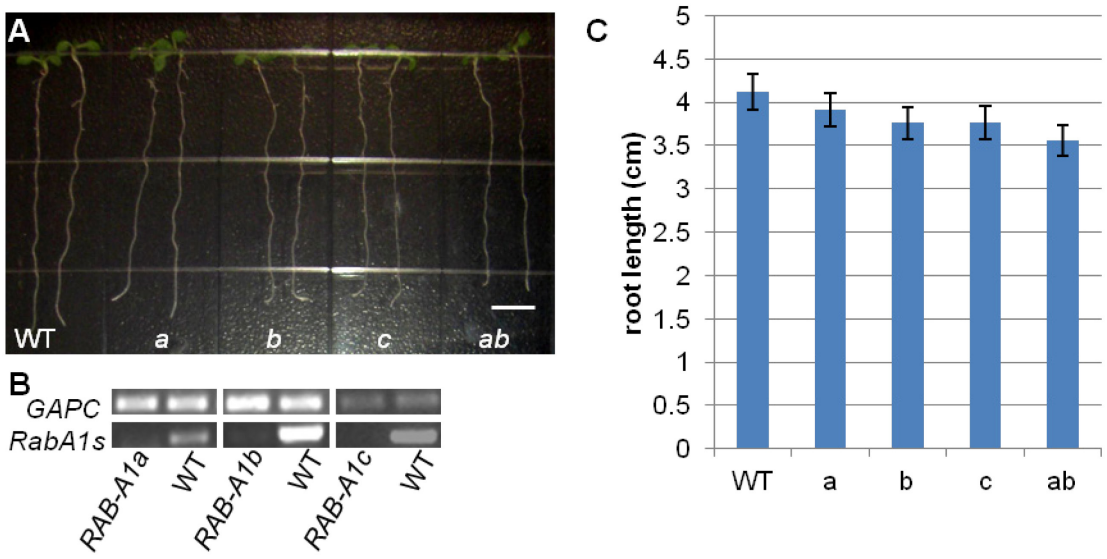


Figure S2.2. Root growth of knock-out mutants of *rab-a1a, rab-a1b, rab-a1c* and *rab-a1a/b*.

A) Stereo-microscopic image of ten-day old seedlings of wild type (WT), *rab-a1a, rab-a1b, rab-a1c* and *rab-a1a/b* (from the left to right, *a,b,c* mean *rab-a1a, rab-a1b, rab-a1c* single mutants respectively, and *ab* means *rab-a1a/b* double mutant). Bar: 1cm

B) RT-PCR of *RAB-A1*a*, RAB-A1b, RAB-A1c* in the respective mutants used in (A). The housekeeping gene *GAPC* is used as an internal input control for the RNA amount used in each sample.

C) Quantification of the root length of ten-day-old seedlings of wild type (WT), *rab-a1a, rab-a1b, rab-a1c* and *rab-a1a/b.* Error bar represents standard error.

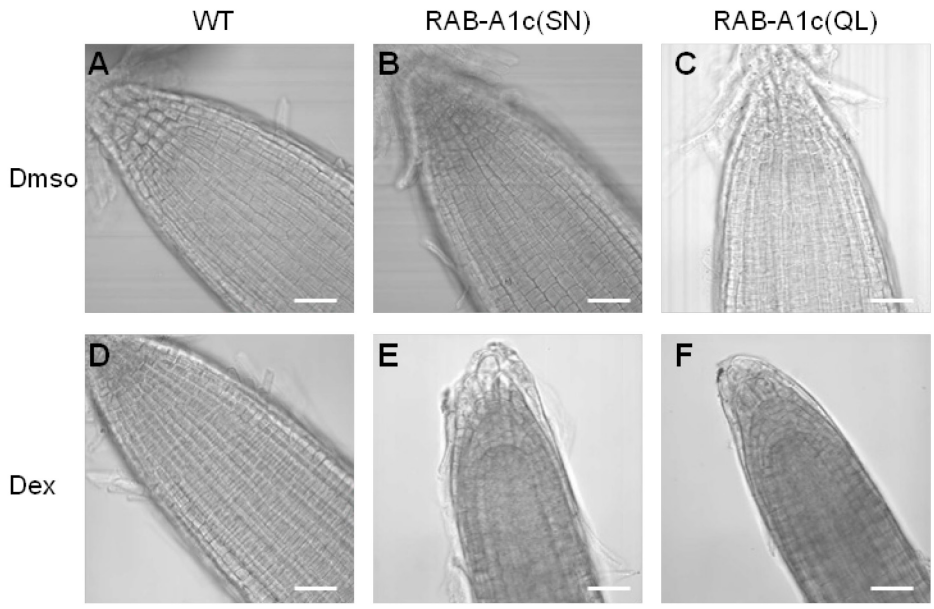


Figure S2.3. Morphology of root tips of wild type (WT), transgenic plant expressing *RAB-A1c(S27N)* and *RAB-A1c(Q72L).*

Bright field images for root tips of seven-day old seedlings of wild type (A, D), transgenic plant expressing *RAB-A1c(S27N)* (B, E) and *RAB-A1c(Q72L)* (C, F) growing on AT plates containing either DMSO (A to C) or 20µM Dex (D to F). Bars: 20µm for all

Table S2.1. List of primers used in the paper

Primer name	**Primer sequence (5'---3')**
SALK_098941 forward	ACGGGAATTGACCTAGACAGG
SALK_098941 reverse	TCCAGGTTGCTAGCTAAGGAG
SAIL_875_F08 forward	AATGTATGTCATGGCGTGAGTC
SAIL_875_F08 reverse	CTCCCATTCACTCCAAAACTG
SALK_145363 forward	ATTGCCGTAGCCATAACTGTG
SALK_145363 reverse	CGATTTCTTCTGTTGTTTTTGC
RAB-A1a-RT forward	CGAAGGCAAAGTCGTCAAAGC
RAB-A1a-RT reverse	CTCTTCTGTTTTGACTGCCACC
RAB-A1b-RT forward	TATAGGCGATTCTGGAGTTGG
RAB-A1b-RT reverse	GCTTCAAGGGCAGATGTTTCC
RAB-A1c-RT forward	CACGATTCACGAAGAACGAGT
RAB-A1c-RT reverse	CCAAATGGGCATTACTCAACC
RAB-A1d-RT forward	ATCGGTGATTCAGGTGTTGG
RAB-A1d-RT reverse	ACTGCGGAAACATCAACATCG
RAB-A1e-RT forward	GGACACCGCTGGTCAAGAAAG
RAB-A1e-RT reverse	CGGACCAAGTCTTGTTCAATTC
RAB-A1f-RT forward	ATTCGCCACTCGTAGCATCC
RAB-A1f-RT reverse	TCTCAGCGAAAGCCTTAGCG
RAB-A1g-RT forward	TCAAGGCTCAGATTTGGGAC
RAB-A1g-RT reverse	GATCATCACCAATATCCAACG
RAB-A1h-RT forward	ATCTGCTATCCCGATTCACC
RAB-A1h-RT reverse	AGCAACCAGATTTCTTGACGG
RAB-A1i-RT forward	AAATGATTTCAGCCACGACTC
RAB-A1i-RT reverse	TATGCAGAGCAGCAACCAGG
RAB-A1b-cDNA forward	GGGAATTCGTCGACATGGCAGGGTACAGAGTG
RAB-A1b-cDNA reverse	GCGGATCCTCAATTTGAGCAGCACCCGAG
RAB-A1c-cDNA forward	GGGAATTCGTCGACATGGCGGGTTACAGAGC
RAB-A1c-cDNA reverse	GCGGATCCGAGCTCTTAGTTCGAGCAGCATCC
RAB-A1c-SN forward	CAGGTGTGGGCAAAAACAATTTGCTTTCACG
RAB-A1c-SN reverse	CGTGAAAGCAAATTGTTTTTGCCCACACCTG

RAB-A1c-QL forward	GGGATACTGCTGGTCTAGAAAGGTACCGAGCC
RAB-A1c-QL reverse	GGCTCGGTACCTTTCTAGACCAGCAGTATCCC
RAB-A1a-promoter forward	GGTCTAGAAGCTTAATAGCACTTCCCTTCC
RAB-A1a-promoter reverse	CCGGATCCTTGGCTCTAAATCACCC
RAB-A1b-promoter forward	GGTCTAGAAGCTTACGCGGTAGAGCTGTAAG
RAB-A1b-promoter reverse	CCGGATCCGTGGTTGCTTCTTCTCAG
RAB-A1c-promoter forward	GGGGATCCAAGCTTACAGTATAAGGCAGAACG
RAB-A1c-promoter reverse	CGTCTAGATACTCCTTCAGATTCGAG
RAB-A1i-promoter forward	GGGGATCCAAGCTTGGACGTCAAATGTTGACTGAG
RAB-A1i-promoter reverse	CGTCTAGATTATTTCCGAAATAAAC

CONNECTING STATEMENT: BRIDGING CHAPTERS II AND III

In the previous chapter, I reported the localization and function of Rab-A1 proteins in *Arabidopsis*. As molecular switch of membrane trafficking pathways, Rab proteins need precise regulation in order to successfully execute functions. Therefore, I investigated the regulation of Rab-A1 proteins, with a focus on the function of their upstream regulator TRAPPII complex, in this chapter.

CHAPTER III:

A SPECIFIC ROLE FOR ARABIDOPSIS TRAPPII IN POST-GOLGI TRAFFICKING THAT IS CRUCIAL FOR CYTOKINESIS AND CELL POLARITY

Xingyun Qi, Minako Kaneda, Jun Chen, Anja Geitmann, Hugo Zheng

The Plant Journal, vol.68:234-248 (2011)

Abstract

Cytokinesis and cell polarity are supported by membrane trafficking from the *trans*-Golgi network (TGN), but the molecular mechanisms that promote membrane trafficking from the TGN is poorly defined in plant cells. Here we show that TRAPPII in *Arabidopsis* regulates post-Golgi trafficking that is crucial for assembly of the cell plate and cell polarity. Disruptions of *AtTRS120* or *AtTRS130*, two genes encoding two key subunits of TRAPPII, result in a defective cytokinesis and cell polarity in embryogenesis and seedling development. In *attrs120* and *attrs130*, the organization and trafficking in the ER-Golgi interface is normal. However, post-Golgi trafficking to the cell plate and to the cell wall, but not to the vacuole, is impaired. Furthermore, TRAPPII is required for selective transport of PIN2, but not PIN1 to the plasma membrane. We revealed that AtTRS130 is colocalized with RAB-A1c. Expression of constitutively active RAB-A1c partially rescues *attrs130*. RAB-A1c, which resides at the TGN, is delocalized to the cytosol in *attrs130*. We propose that TRAPPII in *Arabidopsis* acts upstream of Rab-A GTPases in post-Golgi membrane trafficking in plant cells.

Introduction

Plant membrane trafficking, especially post-Golgi membrane trafficking exhibits several unique features with respect to polar secretion, endocytic cycling, cytokinesis and cell polarity (Dhonukshe et al., 2008; Ebine & Ueda, 2009; Lee et al., 2008; Teh & Moore, 2007; Uemura et al., 2004). Circumstantial evidence suggests that, in plant cells, secretory, vacuolar, endocytic membrane trafficking and trafficking to the cell plate in mitotic cells converge in the *trans*-Golgi network (TGN)/early endosome (EE) (Boutte et al., 2010; Chow et al., 2008; Dettmer et al., 2006; Viotti et al., 2010). The TGN/EE in plant cells has been regarded as a highly dynamic organelle. However, the molecular mechanisms that promote membrane trafficking around the TGN/EE are still poorly defined.

Rab proteins, a family of small monomeric GTPases, have well established roles in regulating tethering/docking of vesicles to a specific target membrane (Zerial and McBride, 2001). In *Arabidopsis*, members of several Rab-A subclasses (the closest homologues of Rab11) have been shown to perform important regulatory roles in exocytic trafficking (Blanco et al., 2009; de Graaf et al., 2005; Preuss et al., 2004; Preuss et al., 2006; Szumlanski and Nielsen, 2009) and targeted delivery of materials to the growing cell plate (Chow et al., 2008; Boutte et al., 2010). However, the regulation of Rab-A proteins in plant membrane trafficking remains elusive. In yeast, trafficking between the *trans*-Golgi, early endosome and plasma membrane also requires a pair of Rab11 proteins, the Ypt31/32 pair (Benli et al., 1996; Chen et al., 2005). In animal cells, Rab11 acts at the recycling endosome in the delivery of receptors and transporters to cell surfaces (Emery et al., 2005; Gonzalez et al., 2007; Ren et al., 1998) and performs important functions in the abscission of daughter cells in the late stages of cytokinesis (Prekeris & Gould, 2008; Wilson et al., 2005). Genetic evidence suggested that in yeast, TRAPPII, a modified version of TRAPPI (a seven-subunit TRAnsport Protein Particle - Trs20, Trs23, Trs31, Trs33, Trs85, Bet3 and Bet5) with addition of three specific subunits Trs65, Trs120 and Trs130, acts upstream of Ypt31/32 (Sciorra et al., 2005; Yamamoto & Jigami, 2002). In yeast cells, TRAPPII can serve as a guanine nucleotide

exchange factor (GEF) for Ypt31/32 proteins (Jones et al., 2000; Morozova et al., 2006; Sacher et al., 2008). In higher eukaryotic cells, Trs65 is missing, but the two TRAPPII-specific subunits, Trs120 and Trs130, are conserved in all sequenced eukaryotic genomes (Cox et al., 2007). In *Drosophila*, the Trs120 homolog Bru is required for proper localization of Rab11 and genetically interacts with Rab11 and Rab11 effector PI4Kβ1 during cleavage furrow ingression in dividing male meiotic cells (Robinett et al., 2009). However, recent data suggest that, in mammalian cells, Trs130 colocalizes with early Golgi markers and can serve as a GEF for Rab1 in early Golgi trafficking (Yamasaki et al., 2009).

The role of TRAPPII in plant membrane trafficking has not been investigated. In *Arabidopsis*, there are single homologues of yeast Trs120 and Trs130, At5g11040 and At5g54440, respectively. In this paper, we refer to them as AtTRS120 and AtTRS130, respectively. Using a combined approach of genetics, molecular biology, optical and transmission electron microscopy, we provide evidence that TRAPPII in *Arabidopsis* acts upstream of Rab-A GTPases. The function of TRAPPII is required for proper transport of proteins in post-Golgi trafficking pathways to the growing cell plate in mitotic active cells and selective transport of proteins to the plasma membrane in polarized plant cells, but not for ER-to-Golgi as well as biosynthetic and endocytic vacuolar transport. The function of TRAPPII is crucial for normal plant development.

Results

Disruption of either *AtTRS120* or *AtTRS130* leads to similar defects in embryogenesis and post-embryonic organ development

Based on a survey on the Expression Atlas of *Arabidopsis* Development (Schmid et al., 2005) and our RT-PCR analyses, we noted that *AtTRS120* and *AtTRS130* were expressed ubiquitously in almost all plant tissues (Supplemental Figure S1). We therefore identified four T-DNA insertion mutant lines for *AtTRS120* (designated as *attrs120-1*, *attrs120-2, attrs120-3*, and *attrs120-4*) and one for *AtTRS130* (designated as *attrs130*) (Figure 1a) for further functional analyses. Semi-quantitative RT-PCR analyses indicated that *attrs120-1*,

attrs120-2, attrs120-3 and *attrs130* are transcriptional knockout mutants in the respective genes and *attrs120-4* is a transcriptional knockdown mutant (Figure 1b, only *attrs120-2*, *attrs130* and *attrs120-4* were shown).

We noted that knockout alleles of *attrs120* and *attrs130* exhibited very similar developmental defects. Embryos that were homozygous for these alleles were able to survive early embryogenesis, but in the late stage of embryogenesis, mutant hypocotyls did not elongate properly and bent abnormally. This was paired with distorted cotyledons (Figure 1c, only *attrs120-2* and *attrs130* were shown), which often resulted in a curled mature seed (Figure 1c). These mutant embryos were able to germinate, but with no differentiation of true leaves, and root growth was arrested at very early seedling stage (Figure 1d). In siliques (n=42) of heterozygous *attrs120*-2 or *attrs130*, no aborted ovules or aborted embryos were found. However, only 5-10% of embryos developed waved hypocotyls, distorted cotyledons and were seedling lethal (Figure 1c, 1d). These phenotypes co-segregated with the T-DNA insertions in *AtTRS120* or *AtTRS130*. The result indicates that the observed defects in embryogenesis and seedling development are genetically linked to the T-DNA insertions in the respective genes. Male transmission is likely defective in the mutants.

The weak allele *attrs120-4* was able to complete embryogenesis with no detectable defect. However, the growth of the primary root was reduced in *attrs120-4* (Figure 1e). Under our growth conditions, the average root length of *attrs120-4* 10 days after germination was approximately 2/3 of the length of wild-type roots (2.7±0.25cm for *attrs120-4* roots vs. 4.0±0.3cm for wild type roots). Adult *attrs120-4* plants were also dwarfed (Figure 1f). When AtTRS120-YFP was introduced into *attrs120-2* and *attrs120-4*, and AtTRS130-YFP was introduced into *attrs130*, the developmental phenotypes of *attrs120-2* (Figure 1g, compare seedling #3 to #2), *attrs130* (Figure 1h, compare seedling #3 to #2) and *attrs120-4* were complemented, confirming that the phenotypes were caused by the loss of the respective proteins. The molecular complementation was further supported by genetic complementation tests between *attrs120* alleles. When *attrs120-1*, *attrs120-2* and *attrs120-3* were crossed with each other, and when *attrs130* was crossed with *attrs120-2*,

F1 plants heterozygous for either allele of *attrs120* still presented typical mutant phenotypes (Supplemental Figure S2b, S2d), F1 plants heterozygous for *attrs120-2* and *attrs130* had a wild type phenotype (Supplemental Figure S2c). We therefore used *attrs130* and *attrs120-2* plus *attrs120-4* for subsequent analyses.

Mutations in *AtTRS120* and *AtTRS130* affect cytokinesis and cell polarity

To explore the cellular basis of developmental defects revealed in *attrs120-2* and *attrs130*, we analyzed the cellular morphology of hypocotyls and cotyledons in late embryos using propidium iodide. Strikingly, in both *attrs120-2* and *attrs130*, a considerable number of cells had unfinished cell plates (Figures 2a, 2b arrows) and were multi-nucleated (Figures 2a, 2b arrowheads; Supplemental Movies 1-3, data not shown). This observation was further confirmed by toluidine blue staining of thin sections of cotyledons (Supplemental Figure S3). In wild type cotyledons, pavement cells had many interlocked, jigsaw puzzle shaped lobe structures (Figures 2a, 2b), but in both *attrs120-2* and *attrs130*, pavement cells were largely rectangular or round with very few concave bends (Figures 2a, 2b). We also analyzed cellular morphology of root tip cells of young seedlings after germination. In root tip cells of *attrs120-2* and *attrs130*, incomplete cell plates were also observed (Figure 2c red arrows). Furthermore, compared to well patterned cell files in wild type root tips (Figure 2d; Supplemental Movie 4, data not shown), the cell file alignment in root tips of *attrs120-2* and *attrs130* was disordered and many cells were abnormally shaped (Figure 2d, Supplemental Movies 5-6, data not shown). Together, these results indicate that cytokinesis as well as the subsequent cell shaping/growth that are crucial for generating a basic body plan (e.g. cotyledons) and post-embryonic organ formation (e.g. roots), are compromised in both *attrs120-2* and *attrs130*.

Abnormal vesicular-tubular structures accumulate in the cytoplasm of *attrs120-2* and *attrs130*

In plants, both cell division and cell polarity are supported by membrane trafficking from the TGN to deliver membrane and cell wall materials to the cell plate in mitotic active cells (Boutte et al., 2010; Chow et al., 2008) and to selected regions of the cell wall/plasma membrane in polarized plant cells (Lee et al., 2008; Preuss et al., 2004;

Preuss et al., 2006; Szumlanski & Nielsen, 2009). To understand the subcellular basis of the cytokinesis and cell polarity defects observed in *attrs120-2* and *attrs130*, we examined the endomembrane system of seedling cells of *attrs120-2* and *attrs130* at the ultrastructural level by transmission electron microscopy (TEM). We found that the structure of the endoplasmic reticulum (ER) (compare the ER in Figure 3b (*attrs130*) to 3a (wild type); also see Figure 4a-b for overall ER morphology) and Golgi stacks (compare Golgi bodies in Figure 3b-c (*attrs130*) to 3a (wild type), also see Figure 4c-d for overall Golgi distribution) appeared normal in both *attrs120-2* and *attrs130* (here and hereafter we will only show data from either *attrs120-2* or *attrs130* as identical results were always obtained for *attrs120-2* and *attrs130* in our analysis). However, TEM revealed that there was abnormal accumulation of vesicles in the cytoplasm of the mutant cells (compare Figure 3b-d to 3a, arrows). In wild type cells, roughly 1.7 vesicles/μm^2 (Figure 3e, $P<0.01$) were found in the cytoplasmic regions on transmission electron micrographs (a total of 66 μm^2 cytoplasm not occupied by organelles was examined), but in *attrs130* mutant cells, more than twice the number of vesicles (approximately 3.7 vesicles/μm^2, Figure 3e, $P<0.01$) were observed (a total of 147 μm^2 cytoplasm was examined). There was no significant enlargement of vesicles in the mutants as vesicles in both wild type (Figure 3a) and the mutants (Figure 3b-d) had diameters ranging from 60 to 110 nm. However, we found that in wild type cells, most of the vesicles (~85%, Figure 3f, $P<0.01$) were within a 0.5 μm radius from the center of Golgi apparatus and many of them were located at the *trans*- side of Golgi (Figure 3a), probably representing those vesicles just budded off the TGN. In *attrs130* mutant cells, however, the majority of the vesicles (~80% of vesicles, Figure 3f, $P<0.01$) was located away from the Golgi and accumulated elsewhere in the cytoplasm (Figure 3b-d, arrows). Some vesicles were found to be associated with tubular structures (Figure 3b-d, arrowheads), but structures resembling typical TGN (Figure 3a) were rarely seen in mutant cells (Figure 3b-d).

Secretion of secretory GFP, but not vacuolar transport is affected in *attrs120-2* and *attrs130*

TRAPPII in yeast cells acts in post-Golgi trafficking pathways (Jones et al., 2000; Morozova et al., 2006), but in mammalian cells TRAPPII acts in early Golgi trafficking

(Yamasaki et al., 2009). The TEM results described above motivated us to use an *in vivo* imaging approach to investigate which step of the vesicle trafficking pathways was impaired in mutant cells. When the ER marker GFP-HDEL and the *trans*-Golgi marker ST-YFP were crossed into both *attrs130* and *attrs120-2*, we noted that the morphology of the ER and Golgi was not affected in the mutants. GFP-HDEL revealed a polygonal ER network in the wild type (Figure 4a) and *attrs120-2* (Figure 4b). ST-YFP was still targeted to the Golgi with no retention in the ER in *attrs120-2*, resulting in punctate structures (compare Figure 4d to 4c). The motility of Golgi in the mutants also appeared normal [Compare Supplemental movie 8 (*attrs120-2*) to 7 (wild type) , data not shown]. Furthermore, the biosynthetic vacuolar transport of secN-R_m-2A, an RFP-based vacuolar transport marker (Samalova et al., 2006) was not inhibited in *attrs130* either (Figure 4e-h). FM4-64 is a lipophilic styryl fluorescent dye used to track endocytic transport from the plasma membrane to the tonoplast. In root tip cells of *Arabidopsis*, FM4-64 (5 μM) is first internalized into the TGN/EE positive for Rab-A2/A3 and VHA-a1 (a subunit of the vacuolar V-ATPase complex). Roughly one hour after internalization, the dye is transported to the late endosome (LE) positive for RAB-F GTPases. The dye reaches the tonoplast roughly in two hours (Chow et al., 2008; Dettmer et al., 2006; Figure 4i). Interestingly, the internalization and transport of FM4-64 to tonoplast membranes in *attrs120-2* or *attrs130* were not delayed (Figure 4i-j). This indicates that, similar to biosynthetic vacuolar transport of secN-R_m-2A, endocytic transport of FM4-64 from the plasma membrane to the tonoplast was not impaired in the mutants.

secGFP is a secretory variant of GFP that is synthesized in the ER but is transported via Golgi to the cell wall where the condition is suboptimal for GFP fluorescence (Zheng et al., 2004; Zheng et al., 2005a). Fluorescence of secGFP in wild type *Arabidopsis* seedlings was dim but could sometimes be observed in the cell wall (Figure 5a) and a few cells (~4%, n=323) showed intracellular secGFP signal (Figure 5c). However, when secGFP was introgressed into *attrs130* and *attrs120-2* and imaged using identical confocal microscope settings, relatively strong GFP signal was detected in mutant cells of *attrs120-2* (Figure 5d, $P<0.01$) and *attrs130* with ~24% of cells (n=358) exhibiting intracellular secGFP signal (Figure 5c, $P<0.01$). Higher magnification confocal analysis

revealed that the intracellular secGFP marked punctate organelles (Figure 5b). When *attrs120-2* expressing secGFP was stained with FM4-64, we noted that most secGFP punctae (~95%, n=132) were stained by early FM4-64 (Figure 5e, white arrows) within 60 minutes of administration of the dye. The secGFP marked compartment was also partially co-localized and/or closely associated with the VHA-a1-mRFP compartment (Figure 5f, white arrows). The results indicates that secGFP in the mutants is retained in TGN/EE. Based on these data we conclude that in *attrs120-2* and *attrs130*, ER-to-Golgi as well as biosynthetic and endocytic vacuolar transport is not affected, but Golgi transport to the cell wall is inhibited at the level of TGN.

Overexpression of the GTP-locked form of RAB-A1c partially suppresses *attrs130*

In *Arabidopsis*, RAB-A2a is localized to a population of TGN/EE (Chow et al*.,* 2008). In our functional analysis of RAB-A1c, we found that GFP-RAB-A1c was co-localized (Supplemental Figure S4a) and moved together with YFP-RAB-A2a (Supplemental Movie 9, data not shown). Furthermore, GFP-RAB-A1c was also extensively [93% of GFP-RAB-A1c (n=475)] co-localized with FM4-64 at one hour of staining (Supplemental Figure S4b). Remarkably, AtTRS130-YFP, a functional AtTRS130 fusion (Figure 1h) was extensively co-localized with GFP-RAB-A1c (Figure 6a, white arrows). Furthermore, when constitutively active RAB-A1c(Q72L) was expressed under the dexamethasone (dex) inducible synthetic promoter pOP6 (Craft et al*.,* 2005) in *attrs130*, we found that RAB-A1c(Q72L) was able to partially rescue *attrs130* (Figure 6b-i and 6j, $P<0.01$). Roots of *attrs130*::RAB-A1c(Q72L) were 3-4 times longer (Figure 6e and 6j, $P<0.01$) than those of un-induced *attrs130*::RAB-A1c(Q72L) (Figure 6d and 6j, $P<0.01$), un-induced (Figure 6b and 6j) or induced (Figure 6c and 6j) non-transformed *attrs130* seedlings, but still shorter than those in the wild type (Figure 6f-g and 6j, $P<0.01$). Expression of RAB-A1c(Q72L) in wild type seedlings did not enhance the root growth of wild type plants (Figure 6h-i, 6j, $P<0.05$). Chow et al. (2008) reported that an inactive form of RAB-A2a is largely delocalized to the cytoplasm. In wild type cells (n = 114), GFP-RAB-A1c was mainly seen in TGN as numerous small punctate structures (Figure 7a), but when GFP-RAB-A1c in *attrs130* cells (n = 83) was imaged with the same confocal microscope settings [40x/1.3 oil objective, pinhole 66 μm, excitation with 488

nm (13.8% of argon laser power)], GFP-RAB-A1c showed a diffuse localization in the cytoplasm in *attrs130* (Figure 7b, Supplemental Movie 10, data not shown) cells, suggesting that RAB-A1c was not activated properly in the mutant. Taken together, these results suggest that TRAPPII in *Arabidopsis* acts upstream of RAB-A1c, probably as a GEF.

In *attrs130*, GFP-RAB-A1c-positive punctate organelles, some of which appeared to be larger or agglomerated (Figure 7b, arrow), were still visible. We also noted in our FM4-64 endocytic transport study that, in root epidermal cells of *attrs120-2* and *attrs130* at early time points, structures labeled by FM4-64 appeared larger than those in wild type cells (compare Figure 9f to 9e). When *attrs130* expressing GFP-RAB-A1c was stained with FM4-64, we noted an extensive co-localization (~92%, n=156) between remnant punctate organelles marked by GFP-RAB-A1c and features labeled by internalized FM4-64 within 60 minutes (Figure 7c, white arrows). This suggests that the remnant organelle marked by GFP-RAB-A1c in the mutant is TGN/EE in nature and that TGN/EE is disorganized in the mutants.

GFP-RAB-A1c as well as FM4-64 is abnormally accumulated in mitotic cells of *attrs120* and *attrs130*

It has been increasingly clear that, in plants, Rab-A proteins play important roles not only in post-Golgi trafficking to the cell wall, but also to the cell plate in mitotic active cells (Boutte et al., 2010; Chow et al., 2008). Similar to YFP-RAB-A2a and FM4-64, GFP-RAB-A1c was also relocated to a disc-like flat structure resembling the cell plate in mitotic active cells in root tips (Supplemental Figure S4c) and shoot meristems (Supplemental Figure S4d). Therefore, we examined the assembly of the cell plate using GFP-RAB-A1c and FM4-64 in root tip cells of *attrs120-4* and *attrs130*. In wild type root tips (n = 73 seedlings), disc-like flat structures that resemble growing cell plates were frequently observed in mitotic active cells with GFP-RAB-A1c (Figure 8a, 8c arrows) and FM4-64 (Figure 8e arrows). In root tips of *attrs120-4* seedlings (n = 89 seedlings), cell plates were often patchy (Figure 8b, arrow). In *attrs130* (n = 67 seedlings), irregular aggregation of GFP-RAB-A1c, as well as FM4-64 punctates (Figure 8d, 8f, arrowheads)

was often seen around a cell plate-like structure. Our results were consistent with Thellmann et al. (2010) who showed that in *attrs120* mutants, assembly of KNOLLE, a cytokinesis-specific SNARE into the cell plate is defective. These results indicate that, in mitotically active root tip cells of TRAPPII mutants, materials destined to cell plates were abnormally accumulated, and the assembly of the cell plate was affected.

The establishment or maintenance of polar localization of PIN2, but not PIN1, is significantly affected in *attrs130* and *attrs120-2*

In *Arabidopsis* root tips, PIN1, an auxin transport carrier, is localized to the basal plasma membrane in root stele cells; PIN2, another auxin transport carrier, is localized to the apical plasma membrane in root epidermal cells (Grieneisen et al., 2007). Recent data suggest that PIN1 and PIN2 may take different pathways to achieve polar distribution (Jaillais et al., 2007; Teh & Moore, 2007; Robert et al., 2008). To examine if the TRAPPII-regulated trafficking pathway plays a role in PIN1 and PIN2 trafficking, we investigated the localization of PIN1-GFP and PIN2-GFP in root tip cells of the *attrs130* and *attrs120-2* mutants. In wild type, PIN1 was mainly localized on the basal part of the plasma membrane in root stele cells (Figure 9a) and PIN2 was mainly located on the apical part of the plasma membrane in root epidermal cells (Figure 9c, arrow). In both *attrs120-2* (Figure 9b) and *attrs130*, although PIN1-GFP highlighted a disorganized cell file, the fusion protein was targeted to the basal part of the plasma membrane in root stele cells. On the other hand, PIN2-GFP was distributed with less polarity in root epidermal cells of the mutants as a strong signal was found on other plasma membrane domains (Figure 9d, arrowheads) in addition to the apical plasma membrane (Figure 9d arrow). Furthermore, intracellular punctate structures (Figure 9d inset, arrowhead), larger than those occasionally seen in wild type cells (Figure 9c), were also observed in our mutants. When *attrs120-2* expressing PIN2-GFP was stained with FM4-64, we found that in root epidermal cells intracellular punctae of PIN2-GFP (91%, n=342) were marked by early internalized FM4-64 (Figure 9f, white arrows). Furthermore, the intracellular PIN2 compartment was also partially co-localized and/or closely associated with VHA-a1-mRFP (Figure 9g, white arrows), suggesting that PIN2-GFP is retained in TGN/EE in the mutants. According to Robert et al. (2008), trafficking of PIN2 is sensitive to Endosidin1,

a drug that affects the morphology of a population of TGN. We observed that in cells treated with Endosidin1, but not with Wortmannin (an inhibitor that impairs endocytosis and also induces swelling of multi-vesicular bodies (MVBs) (Emans et al., 2002; Wang et al., 2009), AtTRS130 tended to aggregate (Figure 10).

In plants, auxin flows downward from the shoot apical region to the root tip and a stable auxin maximum is established and maintained at the root tip (Grieneisen et al., 2007). The expression of GFP under the control of the auxin-responsive DR5 promoter has been used to monitor the auxin maxima (Grieneisen et al., 2007). When DR5::GFP was expressed in *attrs120-2* or *attrs130*, we found that the auxin maximum displayed an altered pattern in terms of cells involved. In wild type, the auxin response peak was seen only in several layers of root cap cells in front of the quiescent center (Supplemental Figure S5a and S5e), whereas in root tips of the mutants, GFP signal was observed in a wider region of the root tip (Supplemental Figure S5b and S5f), indicating that the auxin distribution is impaired in the mutant.

Discussion

Role of TRAPPII in post-Golgi trafficking

The function of TRAPPII and its mode of the action in membrane trafficking have been debated in yeast, *Drosophila*, and mammalian cells (Jones et al., 2000; Morozova et al., 2006; Robinett et al., 2009; Sacher et al., 2008; Yamasaki et al., 2009). In this study, we provide evidence that TRAPPII in *Arabidopsis* acts in post-Golgi trafficking pathways. It appears that the function of TRAPPII is not required for biosynthetic vacuolar transport of SecN-Rm-2A and endocytic transport of FM4-64 to the tonoplast, but required for secretion of secGFP, polar targeting of PIN2 to the plasma membrane, and the assembly of the cell plate. The rescue of *attrs130* by RAB-A1c(Q72L) and delocalization of GFP-RAB-A1c in the mutants suggest that TRAPPII in *Arabidopsis* acts upstream of Rab-A GTPases, potentially as a GEF. In mammalian cells, Golgi is linked to the ER with a highly mobile ER-Golgi intermediate compartment ERGIC (Appenzeller-Herzof and Hauri, 2006). However, in yeast, plants, and *Drosophila*, Golgi stacks exhibit a close

spatial association with transitional ER sites (Brandizzi et al., 2002; Kondylis and Rabouille 2003; Matsuura-Tokita et al., 2006). Yamasaki et al. (2009) proposed that the difference in the architecture of the ER-Golgi pathway may be the underlying reason for different action of TRAPPII in budding yeast and mammalian cells that the ERGIC in mammalian cells may be equivalent to Golgi in yeast. If this is true, the action of TRAPPII in post-Golgi trafficking in yeast (Jones et al., 2000; Morozova et al., 2006), plant (this study) and *Drosophila* (Robinett et al., 2009) may be expected.

Membrane trafficking in the *trans*-Golgi network (TGN) in plant cells is highly complex. It is known that Rab-A proteins play important roles in post-Golgi trafficking to the cell plate in mitotic active cells (Boutte et al., 2010; Chow et al., 2008) and to the cell wall in polarized plant cells (Blanco et al., 2009; de Graaf et al., 2005; Lee et al., 2008; Preuss et al., 2004; Preuss et al., 2006; Szumlanski & Nielsen, 2009). Chow et al. (2008) show that Rab-A2/A3 marks a population of TGN/EE that is distinct but largely overlapping with VHA-a1, a subunit of the vacuolar V-ATPase complex (Chow et al., 2008; Dettmer et al., 2006; Kang *et al,* 2011). Concanamycin A, a V-ATPase inhibitor can inhibit membrane trafficking to the vacuole (Dettmer et al., 2006). Perhaps at the TGN, the TRAPPII-Rab-A1/A2/A3 pathway may be largely involved in membrane trafficking from the TGN/EE to the cell wall/plasma membrane and to the cell plate in mitotic cells, the VHA-a1 related pathway may be mainly involved in trafficking from the TGN to the LE and the vacuole.

We noted that when GTP-locked RAB-A1c(Q72L) was overexpressed in *attrs130*, the developmental phenotype of *attrs130* could only be partially rescued. One possible explanation is that TRAPPII might have an additional function e.g. serve as a tethering factor for secretory vesicles (Sacher et al., 2008) that cannot be suppressed by overexpression of RAB-A1c(Q72L). In addition, unlike their animal counterparts, Rab-A proteins in plants were proliferated spectacularly during the evolution of the plant kingdom (Woollard & Moore, 2008). It is therefore likely that, in the absence of TRAPPII, multiple members of the Rab-A subfamily are improperly activated, so RAB-A1c(Q72L) could only partially complement the defect of *attrs130.* In yeast, the

expression of either Ypt31 or Ypt32 suppresses a growth defect of *trs130*Δ mutant cells but the extent of suppression is different between Ypt31 and Ypt32 as the two proteins could act at distinct but overlapping pathways (Yamamoto & Jigami, 2002). It will be interesting to test whether members of other Rab-A subclasses, especially Rab-A2/A3, which represents the ancestral Rab-A in the plant lineage (Woollard & Moore, 2008), can rescue *attrs130* at the gross and cellular levels. Recently, Cai et al. (2008) revealed that TRAPPII in yeast cells may also act as a GEF for Ypt1. In *Arabidopsis*, Rab-D proteins, homologues of Ypt1 have been shown to localize to a population of TGN/EE (Pinheiro et al., 2009), but the functional significance of this localization is not known. It will be interesting to examine if TRAPPII is also functionally linked to Rab-D proteins in post-Golgi trafficking in *Arabidopsis*.

Role of TRAPPII in plant cytokinesis

In plant cells, cytokinesis is accompanied by the formation of a cell plate in the center of the phragmoplast (Verma and Gu, 1996). The assembly of a cell plate generally consists of the following stages: 1) transport and fusion of Golgi-derived vesicles; 2) formation of tubular-vesicular network; 3) formation of an interwoven tubular network; and 4) maturation of a new cell plate (Samuels et al., 1995; Segui-Simarro et al., 2004). However, little is known about the molecular regulation of these processes. KNOLLE, a syntaxin-related protein has been implicated in vesicle fusion in cytokinesis (Lauber et al., 1997). Phragmoplastin, a cell plate localized dynamin-like protein (Gu and Verma, 1996) is proposed to be involved in the fusion or squeezing of the vesicles into the tubular structure at the cell plate (Verma and Gu, 1996). In *attrs120* and *attrs130* mutants, while the structure of ER-Golgi interface is not altered, there is abnormal accumulation of vesicles and tubular-vesicular structures in the cytoplasm of the mutants. We suspect that in the mutants, fusion of Golgi-derived vesicles necessary for the formation of tubule-vesicular network and/or an interwoven tubular network (Samuels et al., 1995; Segui-Simarro et al., 2004) is defective. Such a defect may be arisen from improper activity of Rab-A proteins, which are proposed to regulate vesicle fusion in cytokinesis (Chow et al., 2008).

In the absence of TRAPPII, *Arabidopsis* plants can still survive early embryogenesis, but later embryogenesis is affected. Our interpretation for this is that TRAPPII has no detectable effect in cell division in early embryogenesis but may have a pronounced effect on actively dividing cells in the later embryogenesis. In yeast, Ypt31/32 is important for cell viability (Benli et al., 1996). Rab11 in the model animal systems is crucial for embryogenesis (Cao et al., 2008; Zhang et al., 2008). If the function of Rab-A proteins in plants is indispensable, it is possible that TRAPPII is not the sole GEF for plant Rab-A proteins. In this regard, we noted that dominant negative RAB-A2a (Chow et al., 2008) and RAB-A1c (Qi and Zheng, unpublished data), which presumably stabilizes their interaction with their GEFs, do not produce developmental defects to the same extent as described in this study for *attrs120-2* or *attrs130*. Rab-A proteins may preferentially interact with and titrate different GEFs. A candidate protein that may serve as a GEF for Rab-A proteins is SCD1, a DENN domain protein that is involved in cytokinesis and polarized cell expansion (Falbel et al., 2003).

Role of TRAPPII in polar targeting of PIN2

It has been recently reported that, to achieve polar distribution, PIN proteins are first evenly distributed to the plasma membrane and then undergo endocytosis, before being redirected to the proper domains of the plasma membrane in a polar distribution via endosomal recycling mechanisms (Dhonukshe et al., 2008). The subcellular detail of the transport and recycling of different PIN proteins, however, remains elusive. It is interesting to note that the polar targeting of PIN2, but not PIN1, was significantly affected in both *attrs130* and *attrs120-2*. It seems that the TRAPPII-Rab-A mediated trafficking pathway acts selectively in the polar transport of PIN2. At the moment, we do not know how polar targeting of PIN2 is affected in the absence of TRAPPII. It is possible that there is a delayed traffic of the newly synthesized PIN2 molecules to the plasma membrane. PIN2 is seen in the plasma membrane domains other than the apical plasma membrane in *attrs120-2* or *attrs130* mutants. Furthermore, the endocytic transport of FM4-64 appears normal in the absence of TRAPPII. Thus it is possible that polar recycling of PIN2 may not operate properly. Different from PIN1 (Jaillais et al., 2007), PIN2 is largely internalized and recycled through a GNL1-positive pathway to a

population of endosomes that is sensitive to Endosidin1 (Robert et al., 2008; Teh & Moore, 2007). We show here that the distribution of AtTRS130 is sensitive to Endosidin1. Therefore it is possible that TRAPPII-Rab-A1/A2/A3 defines a population of endosomes that is identical to the endosome defined by Endosidin1 (Robert et al., 2008).

In addition to aberrant targeting of PIN2, we noted that the auxin distribution also altered in a wider region in root tips of either *attrs130* or *attrs120-2*. In *pin2*, the change in DR5::GFP is relatively minor (Shin et al., 2005). It is therefore likely that in *attrs120-2* or *attrs130*, the polar distribution of other auxin transporters is also affected. Indeed, the observed DR5::GFP pattern in *attrs120-2* mutants is somewhat similar to what is seen in *pin2 pgp1/abcb1* double mutant (Blakeslee et al., 2007). It will be interesting to test whether the transport of ABCB1 is impaired in our mutants.

Materials and methods

Plant materials and growth conditions

Salk T-DNA insertional mutant lines were obtained from the *Arabidopsis* Biological Resource Center (www.*arabidopsis*.org). *attrs120-2*, *attrs120-4* and *attrs130* expressing GFP-RAB-A1c, secGFP, GFP-HDEL, ST-YFP (Zheng et al., 2004), SecN-R_m-2A-GH (Samalova et al., 2006), PIN1-GFP, PIN2-GFP (Grieneisen et al., 2007) and DR5::GFP (ABRC stock CS9361) were made by crossing transgenic *Arabidopsis* lines (pollen donors) to heterozygous *attrs120-2* and *attrs130* and homozygous *attrs120-4* (egg donors). *Arabidopsis* plants expressing GFP-RAB-A1c and YFP-RAB-A2a were made by crossing the transgenic YFP-RAB-A2a line (Chow et al., 2008) to transgenic *Arabidopsis* expressing GFP-RAB-A1c. To co-localize VHA-a1-mRFP with secGFP or PIN2-GFP in *attrs120-2* and *attrs130*, transgenic VHA-a1-mRFP line (Dettmer et al., 2006) was crossed to heterozygous *attrs120-2* and *attrs130* expressing secGFP or PIN2-GFP, respectively. For dexamethasone-inducible expression of RAB-A1c(Q72L) in *attrs120-2* and *attrs130*, heterozygous *attrs120-2* and *attrs130* (egg donor) was crossed with the LhGR driver line 4C-S5/7 (Craft et al., 2005) expressing RAB-A1c(Q72L). Seedlings were germinated and grown on hygromycin and kanamycin AT plates

(Somerville & Ogren, 1982) supplemented with dexamethasone solution (20μM) diluted from a 100 mM DMSO stock. Plants on AT plates or on soil (Sunshine#5, SunGro, Montreal, QC, Canada) were grown at 22 to 24 ^{o}C under continuous light (80-100 μE $m^{-1}s^{-1}$ photosynthetically active radiation).

Molecular biology and generation of constructions

To examine the expression of *AtTRS120 and AtTRS130*, total RNAs were extracted from roots, stems, leaves, and flowers of 3-week old wild type Columbia *Arabidopsis* plants and siliques of mature plants as described (Zheng et al., 2004). To compare the expression of *AtTRS120 and AtTRS130* in wild type and mutants, total mRNAs were extracted from seedlings of wild type and mutants. Reverse transcriptase-PCR was then performed using the Invitrogen SuperScript III System (Invitrogen, Carlsbad, CA, USA).

To rescue *attrs120-2* and *attrs120-4*, the cDNA of *AtTRS120* was amplified using primers AtTRS120 forward (5′-CCGTCGACATGGAACCTGACGTC-3′) and AtTRS120 reverse (5′-CAGTGCACCTCCAGCTACACAG-3′). The amplified cDNA was first subcloned into pCR8/GW/TOPO according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA) and sequenced. The sequenced clone was then subcloned into the pEarleyGate 101 vector (ABRC stock CD3-683) to make AtTRS120-YFP. To rescue *attrs130*, the cDNA of *AtTRS130* was amplified using primers AtTRS130 forward (5′-CCGGATCCATGGCGAACTACTTG-3′) and AtTRS130 reverse (5′-CTTGACAGGTAAGCAGTAGGAAG-3′) and cloned and sequenced in pCR8/GW/TOPO. The sequenced clone was then subcloned into pEarleyGate101 to generate AtTRS130-YFP. The cDNA of *RAB-A1c* was amplified using primers RAB-A1c forward (5′-GGGAATTCGTCGACATGGCGGGTTACAGAGC-3′) and RAB-A1c reverse′ (5′-GCGGATCCGAGCTCTTAGTTCGAGCAGCATCC-3′). The amplified cDNA was first cloned into pBluescript KS as the EcoRI-BamHI fragment and sequenced. The Q72L substitution was effected into RAB-A1c by overlapping PCR using primers QL forward (5′-GGGATACTGCTGGTCTAGAAAGGTACCGAGCC-3′) and QL reverse (5′-GGCTCGGTACCTTTCTAGACCAGCAGTATCCC-3′). The sequenced wild type *RAB-A1c* was subcloned into pVKH-GFPN (Zheng et al., 2005b) as the SalI-SacI fragment to generate GFP-RAB-A1c, which was transformed into wild type *Arabidopsis* ecotype Col-

0 and crossed to *attrs130*. The modified *RAB-A1c(Q72L)* was subcloned into the pV-TOP vector (Craft et al., 2005) and transformed into the LhGR driver line 4C-S5/7 (Craft et al., 2005) and crossed to *attrs130*.

Phenotyping and light microscopy

For embryo development, developing embryos of heterozygous *attrs120* and *attrs130* mutants were removed and cleared in the Hoyers solution as described (Liu and Meinke, 1998). They were analyzed with a Leica DMI6000B microscope (Richmond Hill, ON, Canada) and images were recorded with a QImaging Retiga EXi digital color camera (Burnaby, BC, Canada). Images of seeds and seedlings were taken using a QImaging Micropublisher3.3 digital CCD color camera (Burnaby, BC, Canada) installed on a Leica MZ16F stereomicroscope. Adult plants were pictured with a Nikon D80 digital camera (Mississauga, ON, Canada).

Inhibitor treatment

7-day old seedlings of *attrs130* expressing AtTRS130-YFP were incubated in 1 ml AT liquid medium containing 20μM Wortmannin (prepared from 1 mM stock solution dissolved in DMSO) or 33μM Endosidin1 (prepared from 1.67 mM stock solution dissolved in DMSO) at the room temperature for two hours. Control treatments were performed with equal amounts of DMSO.

Propidium iodide (PI) and FM4-64 staining, fluorescence microscopy and confocal microscopy

To visualize cell morphology, embryos and seedlings were mounted in 1mg/mL propidium iodide (P3566, Invitrogen, Carlsbad, CA, USA) on slides for 1min. To study the localization and dynamics of FM4-64 as well as to visualize early endosomes and cell plates in wild type and mutant cells, seedlings were stained with 5μM FM4-64 (T13320, Invitrogen, Carlsbad, CA, USA, diluted from a 5 mM stock in water) on microscropy slides for 5, 10, 20, 40, 60, 80, 100, 120 and 140 min. At each given time point, 4-6 seedlings were stained. Each experiment was repeated at least three times. Visualization of FM4-64 was done in root epidermal cells. For fluorescence microscopy of GFP, YFP,

PI and FM4-64, seedlings were analyzed with a Leica DMI6000B microscope (Richmond Hill, ON, Canada). Images were recorded with a QImaging Retiga EXi digital color camera (Burnaby, BC, Canada). Confocal microscopy was carried out with an inverted Zeiss LSM 510 Meta confocal laser scanning microscope (Toronto, ON, Canada). Single color of GFP, PI, FM4-64 and multicolor images of GFP/YFP, GFP/PI, GFP/FM4-64 and YFP/FM4-64 were acquired as described (Zheng et al., 2005a). Zeiss LSM image browser (www.zeiss.com), Volocity (PerkinElmer, Woodbridge, ON, Canada), and Adobe PhotoshopCS2 (www.adobe.com) were used for post-acquisition image processing.

High pressure freezing (HPF) and freeze substitution

Cotyledons and root tips of seedlings were high-pressure frozen in 1-hexadecene using an EM PACT2 (Leica Microsystems, Wetzlar, Germany). Frozen samples were transferred to a frozen freeze-substitution medium containing 2% osumium tetroxide and 8% 2,2-dimethoxypropane in glass-distilled acetone in cryo-vials. The vials with frozen samples were transferred to a pre-cooled (-95 ^{o}C) temperature control chamber FreasySub (Cryotech, Schagen, The Netherlands). The freeze-substitution starts at -90 ^{o}C. The samples were remained at -90 ^{o}C for 48 hrs, then warmed to -60 ^{o}C (8hrs), to -20 ^{o}C (8hrs) and to 4 ^{o}C (2hrs). The rate of temperature change was 5 ^{o}C per hour. The samples were left at room temperature for 2 hrs, rinsed in pure acetone, and processed for embedding in Spurr resin. The resin was gradually added over 4 days. Polymerization was performed at 65 ^{o}C for 12 hours.

For light microscopy, 300 nm sections were mounted on glass slides and stained with toluidine blue. Observation was done with a Zeiss light microscope AXIO (Carl Zeiss AG, Germany). Images were captured using an AxioCam MRm digital black/white camera (Zeiss, Germany). For electron microscopy, 50 to 70 nm thick sections were mounted on formvar-coated grids, stained with uranyl acetate for 30 min and lead citrate for 15 min. Observation, quantification and imaging was done with a FEI Tecnai electron microscope (FEI, The Netherlands) operated at 120 kV.

Accession numbers

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative database under the following accession numbers: *AtTRS120* (At5g11040); *AtTRS130* (At5g54440) and *RAB-A1c* (At5g45750).

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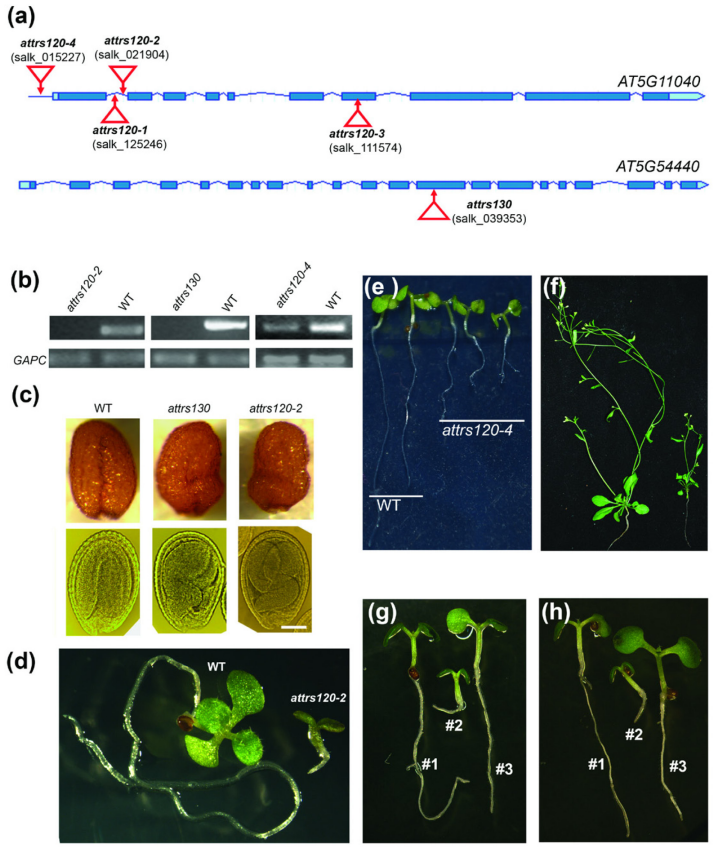


Figure 3.1. Disruption of either *AtTRS120* or *AtTRS130* leads to similar developmental phenotypes.

(a) The schematic structure of the *AtTRS120* and *AtTRS130* genes and the positions of T-DNA insertions. Light blue boxes indicate the UTR regions, deep blue boxes indicate exons and lines connecting boxes indicate introns.

(b) The expression of *AtTRS120* in *attrs120-2*, *attrs120-4*, *AtTRS130* in *attrs130* and wild type seedlings. *GAPC* is the RNA loading control.

(c) The morphology of the mature seed (top row) and the late embryo (bottom row) of wild type, *attrs130*, and *attrs120-2*. Scale bar = 100 μm for all.

(d) The morphology of 11-day old seedlings of wild type and *attrs120-2*.

(e) The morphology of roots of 7-day old seedlings of wild type (the left 2 seedlings) and *attrs120-4* (the right 3 seedlings).

(f) 4-week old adult plants of wild type (left) and *attrs120-4* (right).

(g-h) Molecular complementation on *attrs120-2* (g) and *attrs130* (h). #1 - wild type; #2 - mutant; #3 – mutant expressing the respective *AtTRS120* or *AtTRS130*.

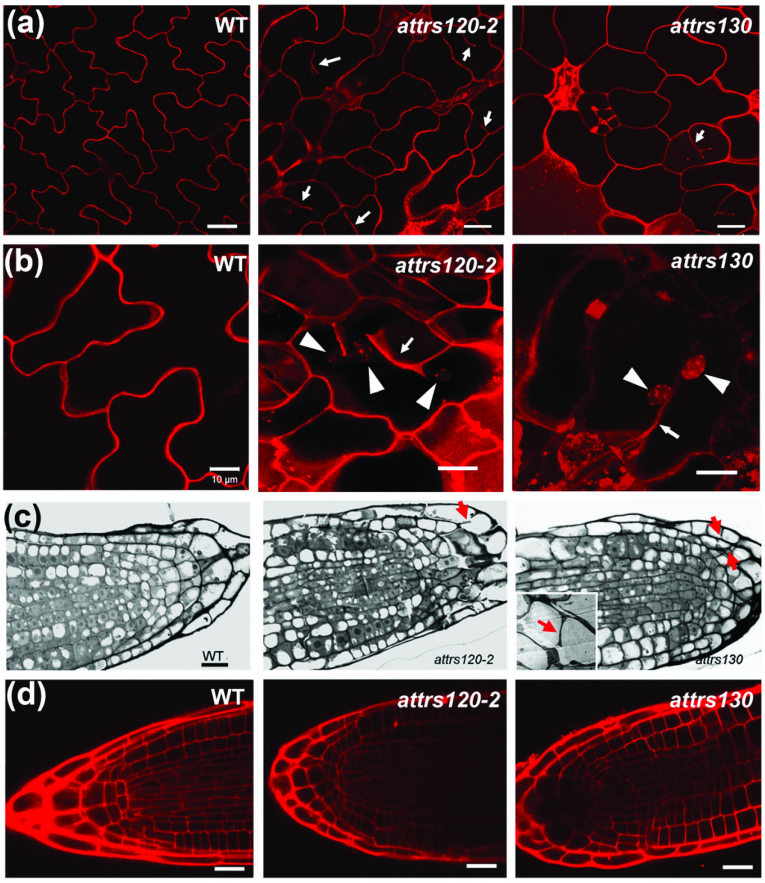


Figure 3.2. Mutations in both *AtTRS120* and *AtTRS130* affect cytokinesis and polarized cell growth.

(a-b) Confocal laser scanning micrograph of propidium iodide staining of epidermal cells of a cotyledon of wild type, *attrs120-2* and *attrs130* at low magnification (a) and high magnification (b, 3-D projection of 20 optical sections acquired at 0.5 µm intervals). Incomplete cell plates are indicated by arrows, nuclei are indicated by arrowheads. Bars in (a) =20 µm, Bars in (b) = 10 µm.

(c-d) Brightfield micrograph of toluidine blue staining (c) and confocal micrograph of propidium iodide staining (d) of the root tip cells of wild type, *attrs120-2* and *attrs130*. Incomplete cell plates are indicated by red arrows. Bars = 20 µm for all.

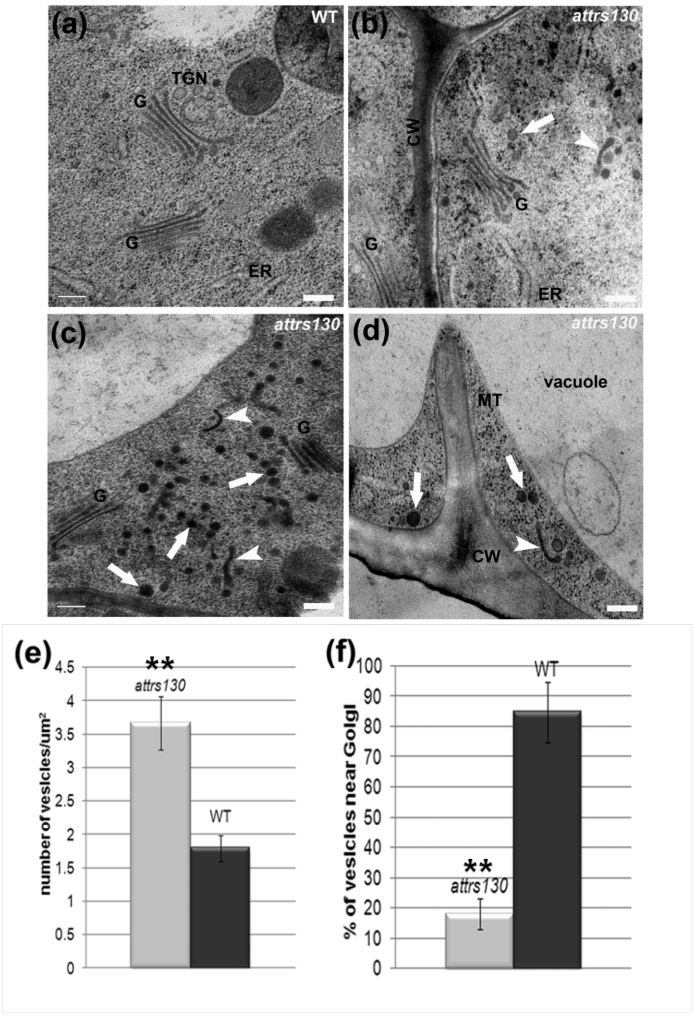


Figure 3.3. Abnormal accumulation of vesicular-tubular structures in the cytoplasm of *attrs130.*

(a-d) Transmission electron micrographs of cotyledon cells of wild type (a) and *attrs130* (b-d). ER = endoplasmic reticulum; G = Golgi; TGN = *trans*-Golgi network; CW = cell wall; MT = microtubules. Arrows indicate vesicles and arrowheads indicate abnormal tubular structures. Bars = 0.2 μm.

(e) Quantitative analysis of vesicle accumulation in wild type and *attrs130* mutant cells. Error bar represents standard error. **: $P<0.01$

(f) Distribution of vesicles in wild type and *attrs130* mutant cells. Vesicles within a 0.5 μm radius from the center of Golgi apparatus were defined as vesicles near Golgi. Error bar represents standard error. **: $P<0.01$

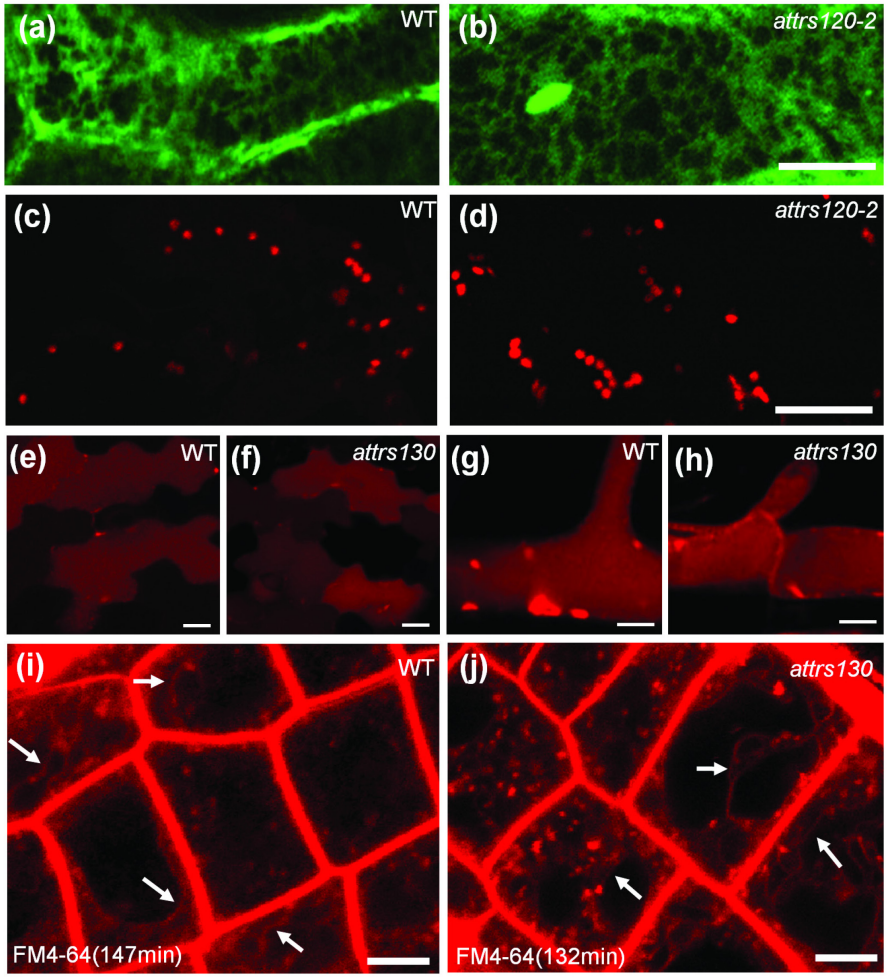


Figure 3.4. Organization and trafficking in the interface between the endoplasmic reticulum (ER) and Golgi, and vacuolar transport, are not affected in *attrs120*-2 and *attrs130* mutants.

(a-b) The morphology of the ER highlighted by GFP-HDEL in wild type (a) and *attrs120-2* (b). Bar = 10 µm for (a) and (b).

(c-d) The targeting of ST-YFP and the distribution of Golgi bodies in wild type (c) and *attrs120-2* (d). Bar = 10 µm for (c) and (d).

(e-h) The biosynthetic vacuolar transport of SecN-R_m-2A in cotyledon (e-f) and root cells (g-h) of wild type (e and g) and *attrs130* (f and h). Bars = 5 µm.

(i-j) Endocytic trafficking of FM4-64 to the vacuole in root tip cells of wild type (i) and *attrs130* (j). Bars = 5 µm.

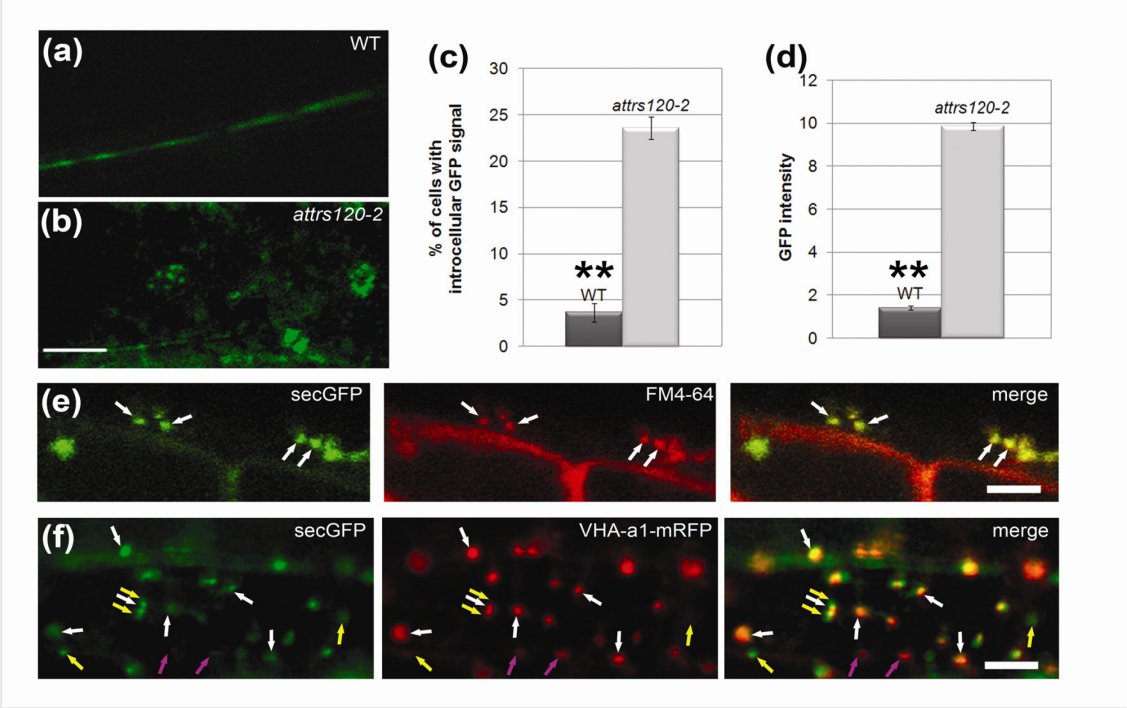


Figure 3.5. Secretion of secretory GFP is affected in *attrs120-2*.

(a-b) The subcellular distribution of secGFP in wild type (a) and *attrs120-2* (b) in root cells. Note the intracellular punctae marked by secGFP in *attrs120-2*. Bar = 10 µm for (a) and (b).

(c) Percentage of cells showing intracellular secGFP in wild type and *attrs120-2* seedlings when viewed under the same confocal microscope settings. Error bar represents standard error. **: $P<0.01$

(d) Relative fluorescence intensity of secGFP in wild type and *attrs120-2* seedlings. Error bar represents standard error. **: $P<0.01$

(e) Co-localization between secGFP and internalized FM4-64 in *attrs120-2*. Arrows indicate co-localization of secGFP and FM4-64. Bar = 5 µm.

(f) Partial co-localization and/or close association between secGFP punctae and VHA-a1-mRFP. White arrows indicate co-localization of secGFP and VHA-a1-mRFP; purple arrows indicate VHA-a1-mRFP only; Yellow arrows indicate secGFP only. Bar = 5µm.

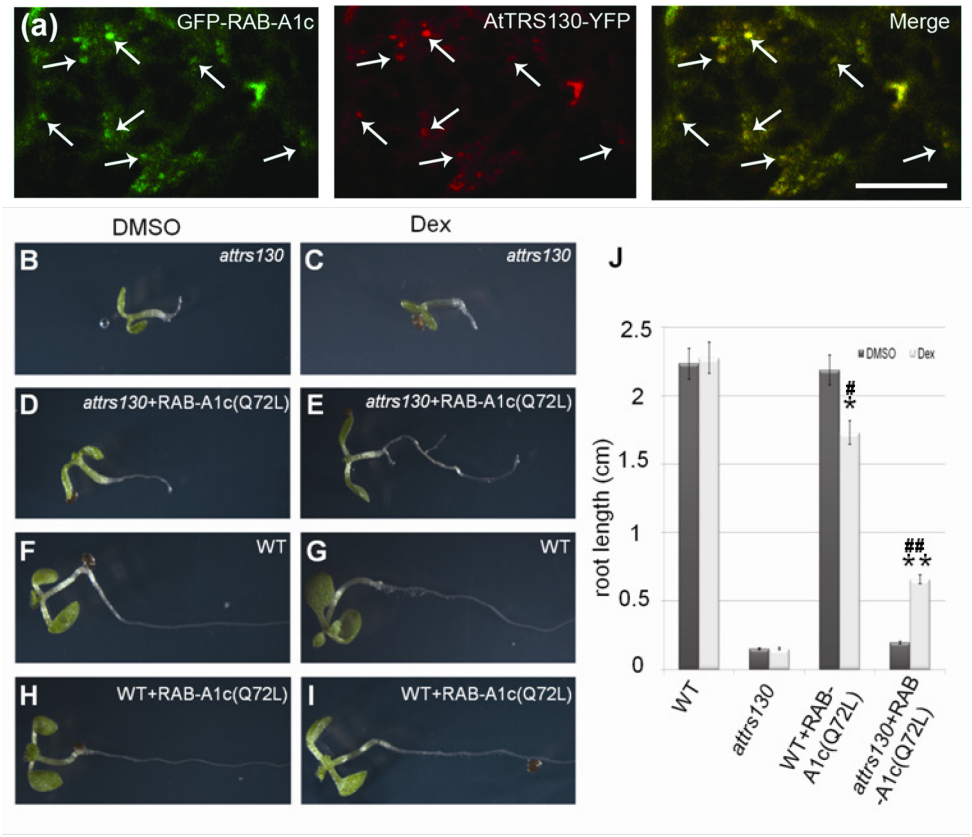


Figure 3.6. Overexpression of RAB-A1c(Q72L) partially rescues *attrs130*.

(a) Co-localization of GFP-RAB-A1c and AtTRS130-YFP. White arrows indicate co-localized punctae. Bar =10 µm.

(b-i) 7-day-old seedlings of *attrs130* (b-c), *attrs130*::RAB-A1c(Q72L) (d-e), wild type (f-g) and wild type::RAB-A1c(Q72L) (h-i) un-induced (b, d, f and h) and induced (c, e, g and i) with dexamethasone.

(j) Quantification of root length after complementation of *attrs130* by RAB-A1c(Q72L). Error bar represents standard error. *: $P<0.05$ compared with the same seedlings treated with DMSO; **: $P<0.01$ compared with the same seedlings treated with DMSO; #: $P<0.05$ compared with WT treated with Dex; ##: $P<0.01$ compared with WT treated with Dex.

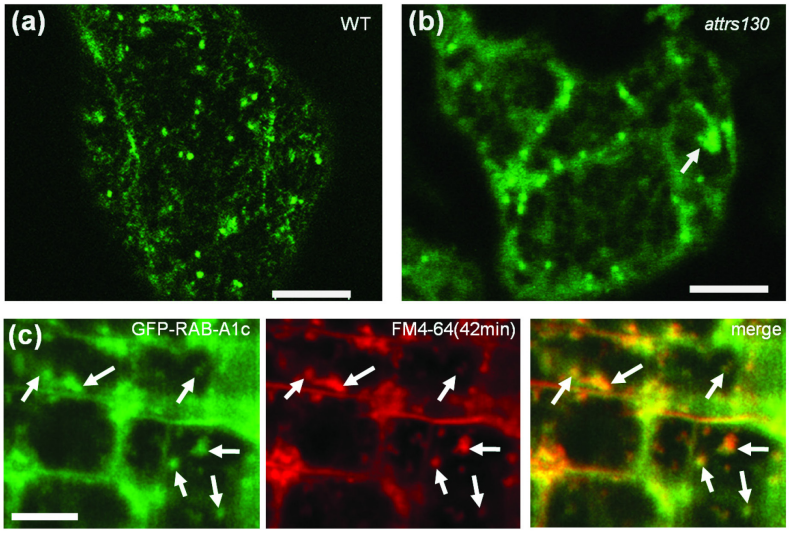


Figure 3.7. Mutation in *AtTRS130* affects subcellular distribution of GFP-RAB-A1c.

(a-b) Subcellular distribution of GFP-RAB-A1c in a cotyledon cell of wild type (a) and *attrs130* (b) viewed under the same confocal settings. Note the delocalization of GFP-RAB-A1c in the cytoplasm and agglomeration of GFP-RAB-A1c punctae in *attrs130* (arrow). Bars = 10 μm.

(c) Extensive co-localization of the GFP-RAB-A1c punctae with internalized FM4-64 within 60 min after dye administration in *attrs130*. Arrows indicate co-localization of GFP-RAB-A1c and FM4-64. Bar = 10 μm.

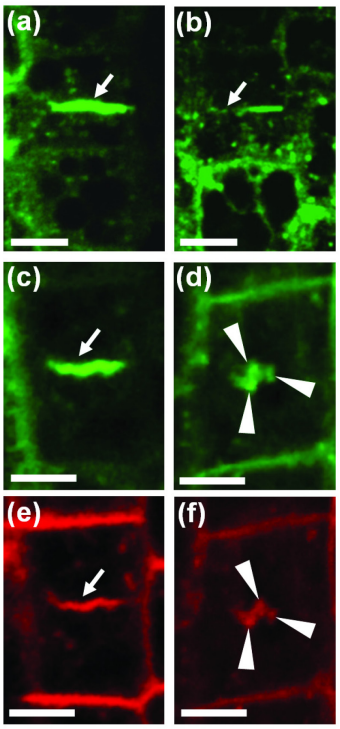


Figure 3.8. Assembly of membrane materials positive to GFP-RAB-A1c and FM4-64 into the cell plate is impaired in *attrs120* and *attrs130*.

(a-d) Assembly of GFP-RAB-A1c into the cell plate in mitotic root tip cells of wild type (a and c), *attrs120-4* (b) and *attrs130* (d). Arrows indicate growing cell plates; Arrowheads in (d) indicate irregular aggregation of GFP-RAB-A1c. Bars = 5 μm.

(e-f) Assembly of FM4-64 into the cell plate in a root tip cell of wild type (e) and *attrs130* (f). Arrow in (e) indicates a growing cell plate; Arrowheads in (f) indicate irregular aggregation of FM4-64. Bars = 5μm.

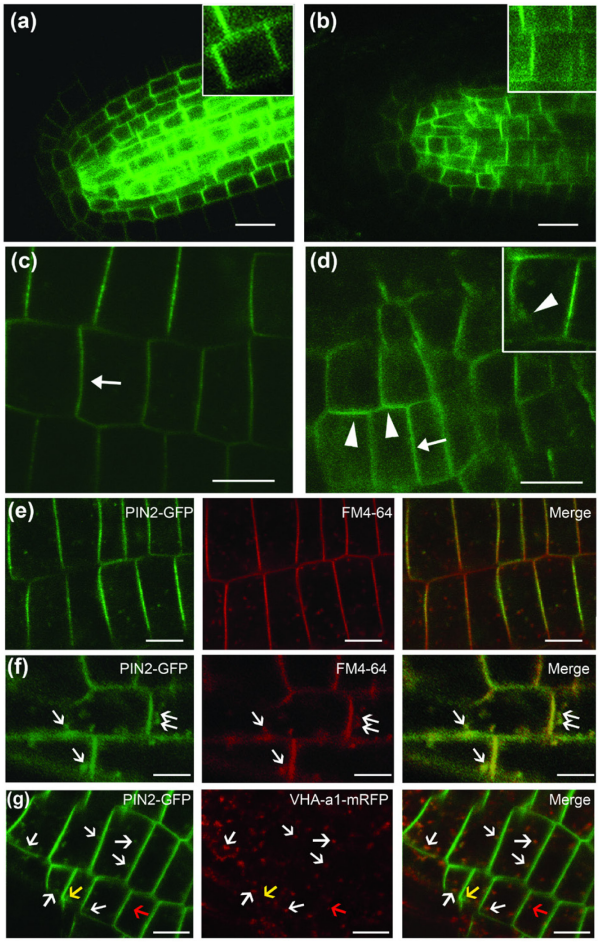


Figure 3.9. Polar localization of PIN1-GFP and PIN2-GFP in *attrs120-2*.

(a-d) PIN1-GFP (a-b) and PIN2-GFP (c-d) in root tips of wild type (a and c) and *attrs120-2* (b and d). Note the enhanced signals of PIN2-GFP on intracellular punctae (arrowhead in d inset) and other plasma membrane domains (arrowheads in d) in addition to the apical plasma membranes (arrows). Bars = 10 µm.

(e-f) Faint and small intracellular punctae of PIN2-GFP in wild type (e) and large intracellular punctae of PIN2-GFP in *attrs120-2* (f) are extensively stained by internalized FM4-64 within 60 min (white arrows). Bars = 5 µm.

(g) Partial co-localization and/or close association between PIN2-GFP punctae and VHA-a1-mRFP compartments in *attrs120-2*. White arrows indicate co-localization of PIN2-GFP and VHA-a1-mRFP; purple arrows indicate VHA-a1-mRFP only; Yellow arrows indicate PIN2-GFP only. Bars = 4 µm.

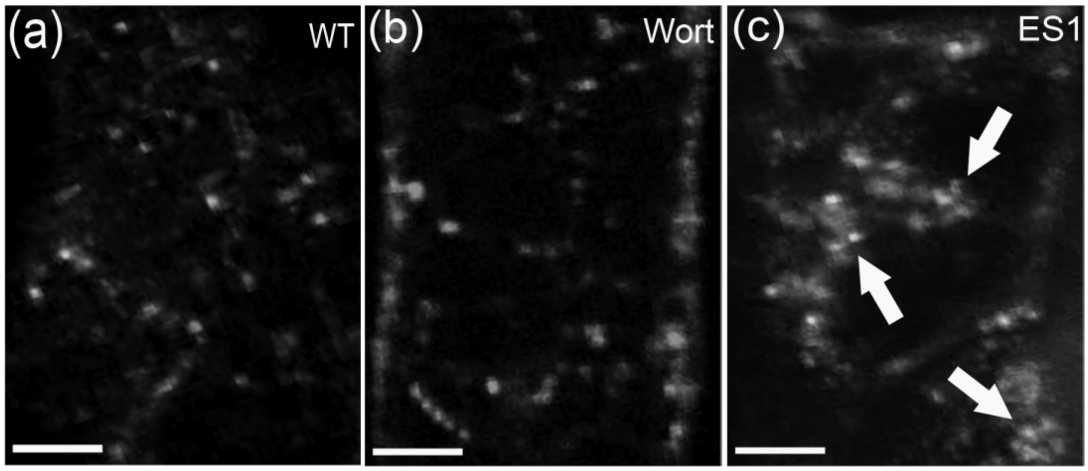
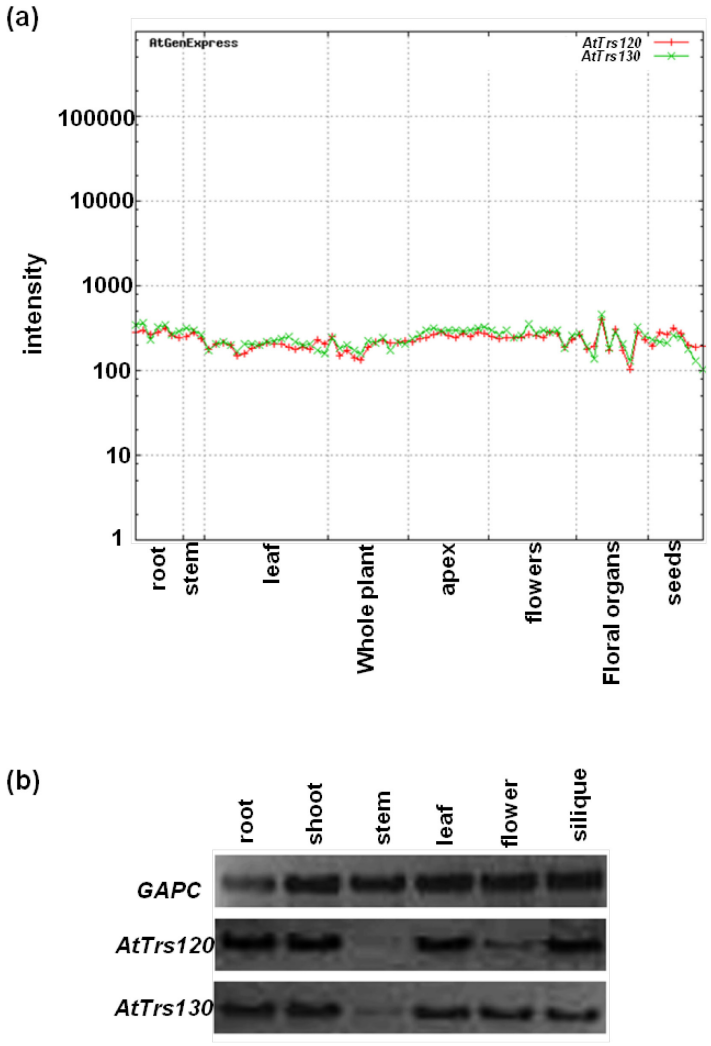


Figure 3.10. Endosidin1 but not Wortmannin induces the aggregation of AtTRS130-YFP. (a-c) 7-day-old *attrs130* seedlings expressing AtTRS130-YFP were treated for two hours with DMSO only (a), 20 μM Wortmannin (b) and 33 μM Endosidin1(c). Note aggregates in cells treated with Endosidin1 (arrows in c). Bars = 5 μm.

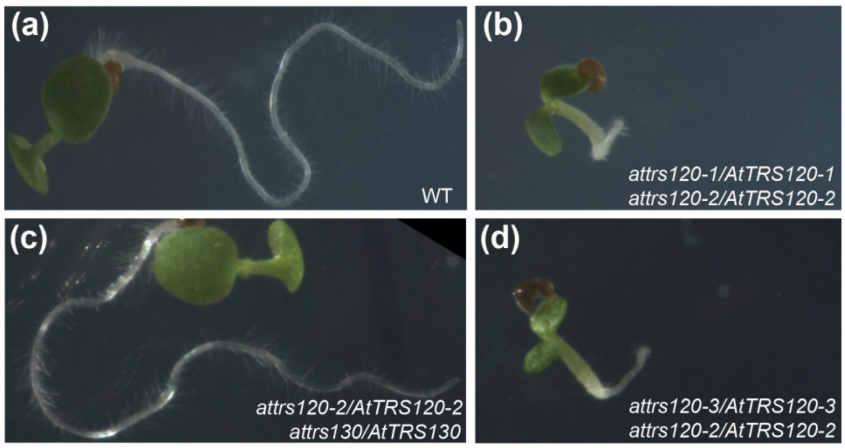


Supplementary material

Supplemental Figure S3.1. *AtTRS120* and *AtTRS130* are co-expressed at similar levels in *Arabidopsis*.

(a) Microarray of *AtTRS120* (*At5G11040*, -|-) and *AtTRS130* (*At5G54440*, -x-) on the Expression Atlas of *Arabidopsis* Development (Schmid *et al*, 2005). The X axis indicates the tissues tested while the Y axis indicates the intensity of the signal.

(b) RT-PCR of *AtTRS120* and *AtTRS130* in different tissues. *GAPC* coding for glyceraldehyde-3-P dehydrogenase is the RNA loading control.



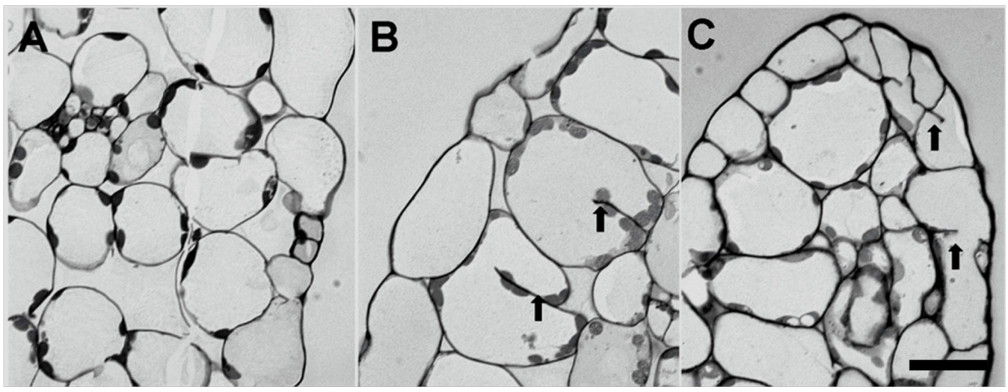
Supplemental Figure S3.2. Genetic complementation tests between *attrs120* alleles.

(a) A 6-day old seedling of wild type Col-0.

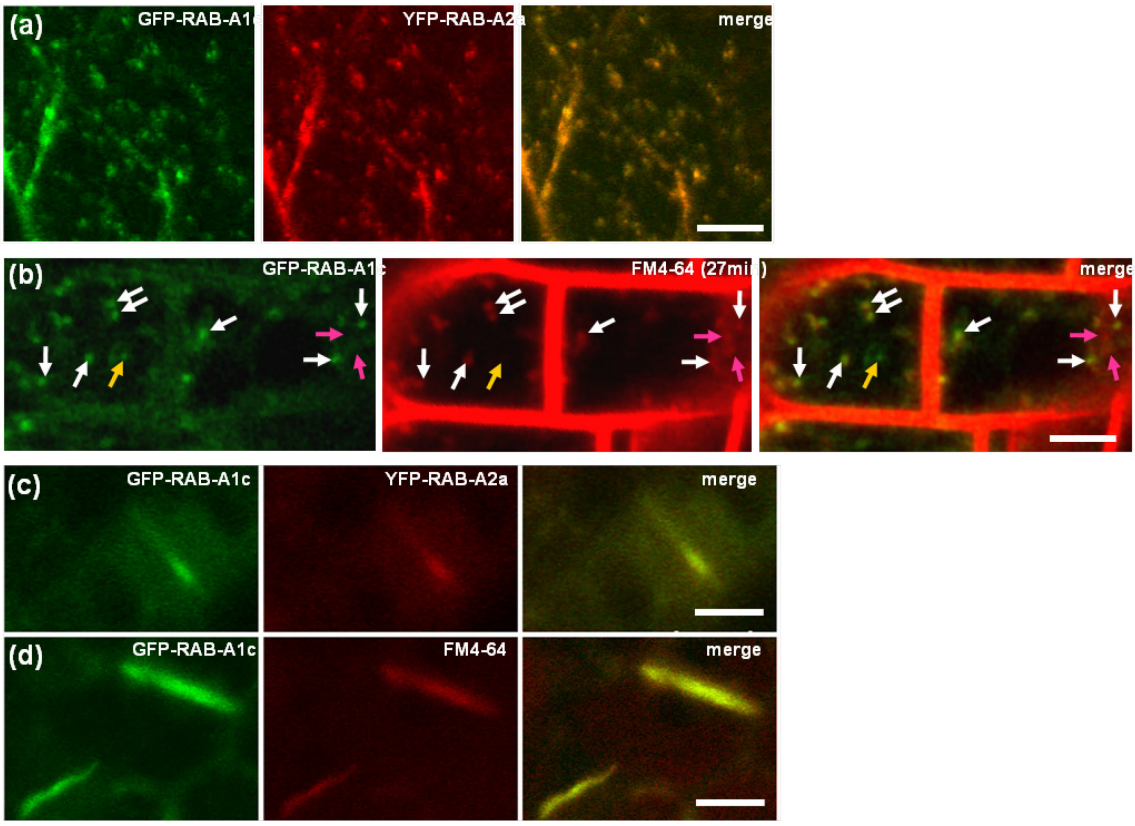
(b) A 6-day old seedling heterozygous for both *AtTRS120-1* and *AtTRS120-2*.

(c) A 6-day old seedling heterozygous for both *AtTRS130* and *AtTRS120-2*.

(d) A 6-day old seedling heterozygous for both *AtTRS120-3* and *AtTRS120-2*.



Supplemental Figure S3.3. Bright-field micrograph of toluidine blue staining of cotyledonal cells of wild type (a), *attrs120*-2 (b) and *attrs130* (c). Arrows indicate incomplete cell plates. Bar in (c) = 10μm for all.



Supplemental Figure S3.4. GFP-RAB-A1c localizes to TGN/EE and is relocated to the cell plate in mitotic active cells of Col-0.

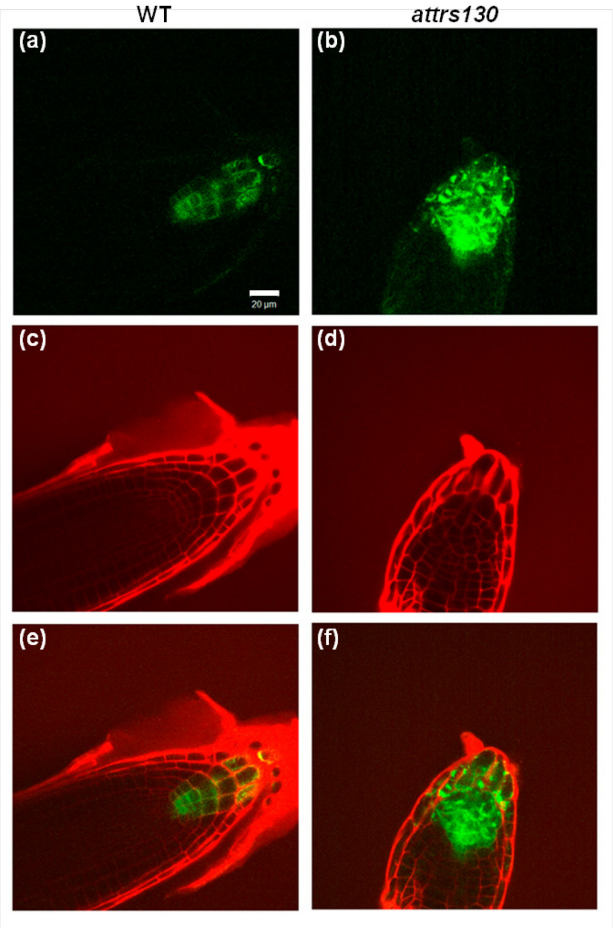
(**a**) Extensive co-localization of GFP-RAB-A1c and YFP-RAB-A2a on TGN.

(**b**) Extensive co-localization of GFP-RAB-A1c and FM4-64 in 27 minutes.

(**c**) Co-localization of GFP-RAB-A1c and YFP-RAB-A2a to a cell plate in a root tip cell.

(**d**) Co-localization of GFP-RAB-A1c and FM4-64 to cell plates in shoot meristematic cells.

In (a), co-localized punctae are indicated by white arrows, GFP-RAB-A1c only punctae are indicated by yellow arrows, and FM4-64 only punctae are indicated by red arrows. Bars =5μm for all.



Supplemental Figure S3.5. Auxin flow reflected by DR5::GFP is affected in *attrs130*. **(a-f)** The cellular distribution of DR5::GFP in root tips of wild type (a, c and e and *attrs130* (b, d and f). (c) and (d) are PI staining of (a) and (b) respectively. (e) and (f) are merged images of (a and c) and (b and d) respectively. Bar in (a) = 20µm for all.

Additional Supporting information may be found in the online version of this article in The Plant Journal.

Supplemental Movies 1-3. 3D view of propidium iodide stained cotyledonal cells of wild type (Movie 1), *attrs120-2* (Movie 2) and *attrs130* (Movie 3).
Supplemental Movies 4-6. 3D view of propidium iodide stained root tip cells of wild type (Movie 4), *attrs120-2* (Movie 5) and *attrs130* (Movie 6).
Supplemental Movies 7-8. Golgi motility in hypocotyl cells of wild type (Movie 7) and *attrs120-2* (Movie 8).
Supplemental Movie 9. Co-localization and motility of GFP-RAB-A1c and YFP-RAB-A2a.
Supplemental Movie 10. Delocalization of GFP-RAB-A1c and agglomeration of TGN/EE in the cytosol of a cotyledonal cell of *attrs130.*

CONNECTING STATEMENT: BRIDGING CHAPTERS III AND IV

In the previous chapter, I reported the characterization of TRAPPII as putative GEF for Rab-A1 proteins in *Arabidopsis*. This chapter is a complementary study on chapter III. As auxin distribution is altered in mutants defective in TRAPPII, yet the detected impairment in auxin efflux carrier PIN2 is not sufficient to explain the aberrant auxin distribution, we wondered if the auxin influx carrier AUX1 is affected or not. In addition, there are controversial reports in yeast and mammals indicating TRAPPII acts as GEF for Rab1 but not Rab11. We are curious about the relationship between Rab-D (Rab1 homologue) and TRAPPII in plants. In this chapter, these two questions have been addressed.

CHAPTER IV:

ARABIDOPSIS TRAPPII IS FUNCTIONALLY LINKED TO RAB-A, BUT NOT RAB-D IN POLAR PROTEIN TRAFFICKING IN TRANS-GOLGI NETWORK

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Abstract

The *trans*-Golgi network (TGN) in plant cells is an independent organelle, displaying rapid association and dissociation with Golgi bodies. In plant cells, the TGN is the site where secretory and endocytic membrane trafficking meet. Cell wall components, signaling molecules and auxin transporters have been found to undergo intracellular trafficking around the TGN. However, how different trafficking pathways are regulated and how different cargoes are sorted in the TGN is poorly defined in plant cells. Using a combined approach of genetic and in vivo imaging, we recently demonstrated that *Arabidopsis* TRAPPII acts in the TGN and is required for polar targeting of PIN2, but not PIN1, auxin efflux carrier in root tip cells. Here, we report that, TRAPPII in *Arabidopsis* is required for polar distribution of AUX1, an auxin influx carrier in protophloem cells and epidermal cells of *Arabidopsis* root tips. In yeast cells, TRAPPII serves as a guanine-nucleotide exchange factor (GEF) for Ypt1 and Ypt31/32 in late Golgi trafficking, while in mammalian cells, TRAPPII acts as a GEF for Rab1 (homolog of yeast Ypt1) in early Golgi trafficking. We show here that TRAPPII in *Arabidopsis* is functionally linked to Rab-A proteins, homologues of yeast Ypt31/32, but not Rab-D proteins, homologues of yeast Ypt1 and animal Rab1 proteins.

Text

Plants are sessile organisms. To establish a proper architecture in response to prevailing environmental conditions, they rely purely on cell division and anisotropic cell growth in both roots and shoots. Different from animals, immobile plants developed a specific extracellular structure called cell wall that provides the mechanic strength they need. Proper plant cell division and growth thus require continually remodeling of the cell wall structure, which involves targeted delivery and/or recycling of cell wall polysaccharides, enzymatic and structural proteins, hormone transporters and signaling molecules from the inside of cells to the plasma membrane and cell walls (Hofte, 2010; Matheson et al., 2006).

Recently, the *trans*-Golgi network (TGN) has been identified as a key organelle in polar protein transport to the plasma membrane and cell walls in plant cells (Kang et al., 2011; Viotti et al., 2010). Morphologically, the TGN is a tubular-reticular network derived from the *trans*-Golgi (Zhang and Staehelin, 1992). In plants, the TGN has been considered as an independent organelle, displaying rapid association and dissociation with Golgi bodies (Viotti et al., 2010). In *Arabidopsis* root tip cells, after separation from the Golgi apparatus, the TGN may undergo maturation from the early TGN that is associated with Golgi to the late TGN that is away from Golgi, and finally fragment into vesicles for cargo delivery (Kang et al., 2011). TGN could simultaneously release secretory vesicles that carry different cargoes destined for the plasma membrane or for the cell wall (Kang et al., 2011; Preuss et al., 2006; Szumlanski and Nielsen, 2009), and clathrin-coated vesicles (Kang et al., 2011) presumably for vacuolar transport (Dettmer et al., 2006). The TGN has also been considered as the early endosome (EE) that receives endocytosed materials from the plasma membrane (Dettmer et al., 2006; Viotti et al., 2010). At the TGN/EE, endocytosed materials will be either transported to the vacuole via the prevacuolar compartment (PVC)/multi-vesicular body (MVB), or recycled back to the plasma membrane (Spitzer et al., 2009). How different trafficking pathways are regulated and how different cargoes are sorted in the TGN is, however, poorly defined in plant cells.

Several proteins have been implicated to regulate protein trafficking around the TGN (Chow et al., 2008; Dettmer et al., 2006; Drakakaki et al., 2012; Gendre et al., 2011; Preuss et al., 2006; Rehman et al., 2008; Synek et al., 2006; Szumlanski and Nielsen, 2009). For example, roles of small Rab-A GTPases, homologues of yeast Ypt31/32 and animal Rab11 in TGN trafficking to the cell plate in cytokinesis (Chow et al., 2008) and in polar secretion of cell wall components (Preuss et al., 2006; Rehman et al., 2008; Szumlanski and Nielsen, 2009a) have been demonstrated. Using a combined approach of genetic and *in vivo* imaging, we recently showed that *Arabidopsis* TRAPPII acts in intracellular trafficking in the TGN in root tip cells (Qi et al., 2011). TRAPPII serves as a positive regulator of Rab-A proteins, probably as a guanine-nucleotide exchange factor (GEF) of Rab-A proteins in plant cells (Qi et al., 2011). Here, we extend our functional analysis of TRAPPII and report that, TRAPPII in *Arabidopsis* is required for polar distribution of AUX1, an auxin influx carrier in protophloem cells and epidermal cells in *Arabidopsis* root tips. In yeast cells, TRAPPII has been implicated to serve as a GEF for the activation of both Ypt1 and Ypt31/32 (Cai et al., 2005; Cai et al., 2008; Morozova et al., 2006; Yip et al., 2010) while in mammalian cells, TRAPPII acts as a GEF for Rab1 (homolog of yeast Ypt1) (Yamasaki et al., 2009). We show here that TRAPPII in *Arabidopsis* is not functionally linked the Rab-D2a protein, a homolog of yeast Ypt1 and animal Rab1 proteins.

Arabidopsis TRAPPII is involved in polar distribution of AUX1 in protophloem cells and epidermal cells of root tips

We recently demonstrated that TRAPPII in *Arabidopsis* is required for polar localization of PIN2, but not PIN1 auxin efflux carrier (Qi et al., 2011). Therefore we further examined the polar localization of AUX1 in *attrs130* and *attrs120-2* (Qi et al., 2011). As shown previously (Kleine-Vehn et al., 2006; Swarup et al., 2001), YFP-tagged AUX1 signal is enriched at the apical plasma membrane of protophloem cells (Fig. 1A and 1B, arrows), at both the apical and basal sides of epidermis and lateral root cap (Fig. 1A and 1C, arrows), but without pronounced polar distribution in the columella (Fig. 1A). We found that in *attrs130* and *attrs120-2* (Fig. 1D-1F), strong AUX1-YFP signal was seen at the lateral plasma membrane in protophloem cells (Fig. 1D and 1E, arrowhead) and in the epidermis

and lateral root cap (Fig. 1D and 1F, arrowhead), indicating that polar distribution of AUX1 is affected in the mutants.

How could the polar distribution of AUX1 be affected in the absence of TRAPPII? We know that TRAPPII is not involved in pre-Golgi trafficking, but transport from the TGN to the plasma membrane (Qi et al., 2011), it is therefore likely that TGN trafficking of AUX1 to the plasma membrane is affected in *attrs130* and *attrs120-2*. AUX1 is known, after being transported to the plasma membrane in a non-polar manner, to cycle between the plasma membrane and a population of TGN/endosome (Kleine-Vehn et al., 2006; Swarup et al., 2001). Robert et al. (Robert et al., 2008) show that, different from PIN1, but similar to PIN2, AUX1 uses an endosidin1-sensitive TGN recycling pathway to achieve its polarity in the plasma membrane. Since the TRAPPII marked TGN is sensitive to endosidin1 (Qi et al., 2011), we suspect that, in the absence of TRAPPII, the recycling of AUX1 from the TGN back to the plasma membrane that is necessary for its polarity is defective. In this regard, it is interesting to note that AUX1-YFP signal was dim in cells away from the root apex in *attrs130* and *attrs120-2* (Fig. 1D). It is known that that, once it is endocytosed, AUX1 could be sorted to the vacuole by an MVB sorting pathway (Spitzer et al., 2009). Since protein transport from the TGN to the vacuole is not affected in *attrs130* and *attrs120-2* (Qi et al., 2011), it is possible that AUX1 in the mutants is not correctly recycled back to the plasma membrane but mis-sorted to the vacuole where it is degraded.

How could the action of TRAPPII in plant cells be different from that in mammalian cells? One possible explanation proposed was the different organization of the Golgi apparatus between plants and mammals (Qi et al., 2011; Yamasaki et al., 2009). In plant cells, Golgi stacks are simply distributed throughout the cytoplasm, they are motile and closely associated with the ER (Boevink et al., 1998), while in mammalian cells, Golgi stacks are clustered near the nuclear envelope and there is a compartment called ER-Golgi intermediate compartment (ERGIC) links the ER with Golgi (Martinez-Menarguez et al., 1999). Indeed, TRAPPII was also found to act in the late Golgi in yeast and Drosophila (Cai et al., 2005; Morozova et al., 2006; Robinett et al., 2009), where the organization of

Golgi is similar to that in plant cells (Kondylis and Rabouille, 2003; Matsuura-Tokita et al., 2006). However, the organization and assembly of TRAPPII between yeast and mammalian cells have been recently shown to be different (Choi et al., 2011), therefore it is possible that the TRAPPII complex in plant cells is also organized differently from that in mammalian cells.

Expression of RAB-A1c, but not RAB-D2a partially rescues the root growth of *attrs130*

We recently demonstrated that the growth of *attrs130* could be partially rescued by expression of constitutively active RAB-A1c (Q72L) (Qi et al., 2011). In yeast cells, expression of either constitutively active or wild type Ypt31/32 rescues the yeast *trs130Δ* mutants (Zhang et al., 2002). Therefore we asked if expression of wild type RAB-A1c could at least partially rescue *attrs130*. As Figure 2 indicates, similar to expression of constitutively active RAB-A1c (Q72L) (Qi et al., 2011), when wild type RAB-A1c fused with GFP was crossed to *attrs130*, the length of *attrs130* roots was doubled (Fig. 2A-B, $P<0.01$ compared with *attrs130*), indicating that the growth of *attrs130* is partially rescued.

In yeast and mammalian cells, biochemical evidence suggests that TRAPPII can serve as a GEF for Ypt1 and Rab1, respectively (Cai et al., 2008; Yamasaki et al., 2009; Yip et al., 2010). *Arabidopsis* RAB-D2a, a homolog of yeast Ypt1 and animal Rab1, has been localized to a population of TGN (Pinheiro et al., 2009). So we tested if expression of either wild type or constitutively active RAB-D2a (Q67L) could rescue the growth of *attrs130*. Of 8 *attrs130* lines expressing wild type RAB-D2a and 11 *attrs130* lines expressing RAB-D2a (Q67L), none of them showed better root growth (Fig. 2A-D). These results suggest that, unlike expression of RAB-A1c, expression of either wild type or constitutively active RAB-D2a (Q67L) is unable to rescue the growth of *attrs130*. Our interpretation of these genetic results is that, in *Arabidopsis*, TRAPPII can act upstream of Rab-A, but not Rab-D proteins in TGN trafficking. If Rab-D proteins also function in the late Golgi trafficking in plant cells, in addition to its role in ER-to-Golgi trafficking

(Batoko et al., 2000; Pinheiro et al., 2009; Zheng et al., 2005), it may require an upstream regulator other than TRAPPII.

How could TRAPPII work together with Rab-A proteins in TGN-related polar protein targeting in plant cells? Based on the partial rescue of *attrs130* by RAB-A1c and mislocalization of RAB-A1c in *attrs120-2*, we proposed that TRAPPII can act as a GEF for Rab-A proteins in TGN (Qi et al., 2011). In yeast cells, it was proposed that TRAPPI can only activate Ypt1 in a late stage after COPII vesicles already been tethered with the Golgi membrane to further strengthen the tethering or help the subsequent SNARE complex assembly (Miller, 2007). It will be interesting to know when TRAPPII is required to activate Rab proteins, in case of plant cells, Rab-A proteins. Since dominant negative RAB-A2a is localized to Golgi stacks, Moore and his colleagues proposed that Rab-A proteins can be activated early in Golgi (Chow et al., 2008). It is unlikely that this early activation of Rab-A protein in Golgi is carried out by TRAPPII. A candidate protein that can activate Rab-A early in Golgi would be SCD1, a DENN domain protein (a GEF for Rab35 in mammalian cells (Allaire et al., 2010)) which is required for polar secretion in cell expansion and cytokinesis (Falbel et al., 2003). We propose that TRAPPII in plant cells, similar to the action of TRAPPI on Ypt1 in yeast cells (Miller, 2007), may only act on Rab-A proteins in a late stage in the TGN.

In yeast cells, both TRAPPI and TRAPPII also serve as a tethering factor involved in the initial vesicle tethering (Cai et al., 2007; Kim et al., 2006; Sacher et al., 2008; Yip et al., 2010). In the case of TRAPPI-mediated COPII tethering, it is believed that the initial tethering requires an interaction between TRAPPI and Sec23 (Cai et al., 2007), perhaps also an interaction between TRAPPI and Ypt1 (Kim et al., 2006). It is likely that TRAPPII in plant cells can also serve as a tethering factor for vesicle trafficking in the TGN. It will be interesting to search for proteins that work as tethering partners of TRAPPII in vesicle trafficking in the TGN.

Conclusions

In plant cells, intracellular membrane trafficking around the TGN, which plays a vital role in plant-specific cell proliferation and morphogenesis, exhibits many unique features with respect to polar secretion and endocytic recycling. How different trafficking pathways are regulated and how different cargoes are correctly sorted in the TGN remain unclear. Our functional characterization of the plant TRAPPII complex and its involvement in different cargo transport opened a door for further studies on how protein transport around the TGN could be regulated in plant cells. Consistent with the unique feature of TGN trafficking in plant cells, Small Rab-A proteins are elaborated spectacularly in the plant lineage (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002). Circumstantial evidence suggests that, Rab-A play important roles in intracellular membrane trafficking around the TGN, but their functional diversity has not been revealed. The availability of plant mutants defective in the key components of the TRAPPII complex offers new opportunities not only for investigating the in vivo function and formation of the complex in plant cells, but also for the regulation and functional diversification of Rab-A proteins in plants.

Methods

To express RAB-A1c in *attrs130*, GFP-RAB-A1c was crossed into a heterozygous *attrs130* line (Qi et al., 2011); to express RAB-D2a and RAB-D2a(Q67L) in *attrs130*, a heterozygous *attrs130* line (Qi et al., 2011) was transformed with an agrobacterium strain containing pVKH18-RAB-D2a (Zheng et al., 2005) and pEarleyGate103-RAB-D2a(Q67L), respectively. The Q67L mutation in RAB-D2a was made using site-directed mutagenesis in full length *RAB-D2a* cloned in pCR8/GW/TOPO, and was subcloned into pEarleyGate103.

Acknowledgements

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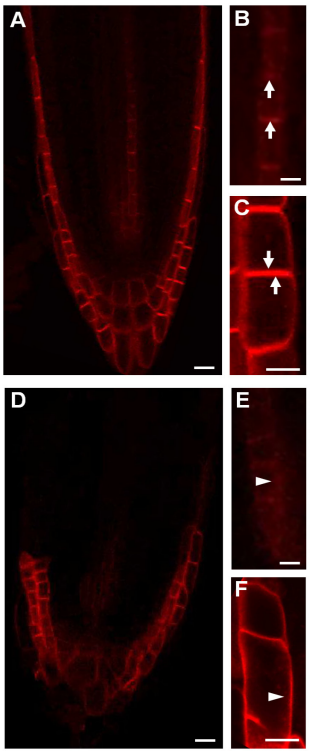

Figure 4.1. Polar distribution of AUX1-YFP in root tips of *attrs120-2* is altered.

(A)-(C) Distribution of AUX1-YFP in the root tip (A), protophloem cells (B), and epidermal cells (C) of a root of a wild type *Arabidopsis* seedling. Note the apical enrichment of AUX1-YFP in protophloem cells (B, arrows) and apical and basal enrichment of AUX1-YFP in epidermal cells (C, arrows). Bar in (A) =10μm, Bars in (B) and (C) = 5μm.

(D)-(F) Distribution of AUX1-YFP in the root tip (D), protophloem cells (E), and epidermal cells (F) of a root of *attrs120-2*. Note a strong signal in the lateral plasma membrane in protophloem cells (E, arrowhead) and in epidermis cells (F, arrowhead). Bar in (D) =10μm, Bars in (E) and (F) = 5μm.

To visualize AUX1, an *Arabidopsis* line expressing AUX1-YFP under the *AUX1* promoter (Swarup et al., 2001) was crossed to a heterozygous *attrs120-2* line (Qi et al., 2011). AUX1-YFP in roots of 7-day old seedlings of wild type and *attrs120-2*, obtained from a segregated population of the AUX1-YFP crossed heterozygous *attrs120-2* was imaged with an inverted Zeiss 510meta system as described in Qi et al (Qi et al., 2011).

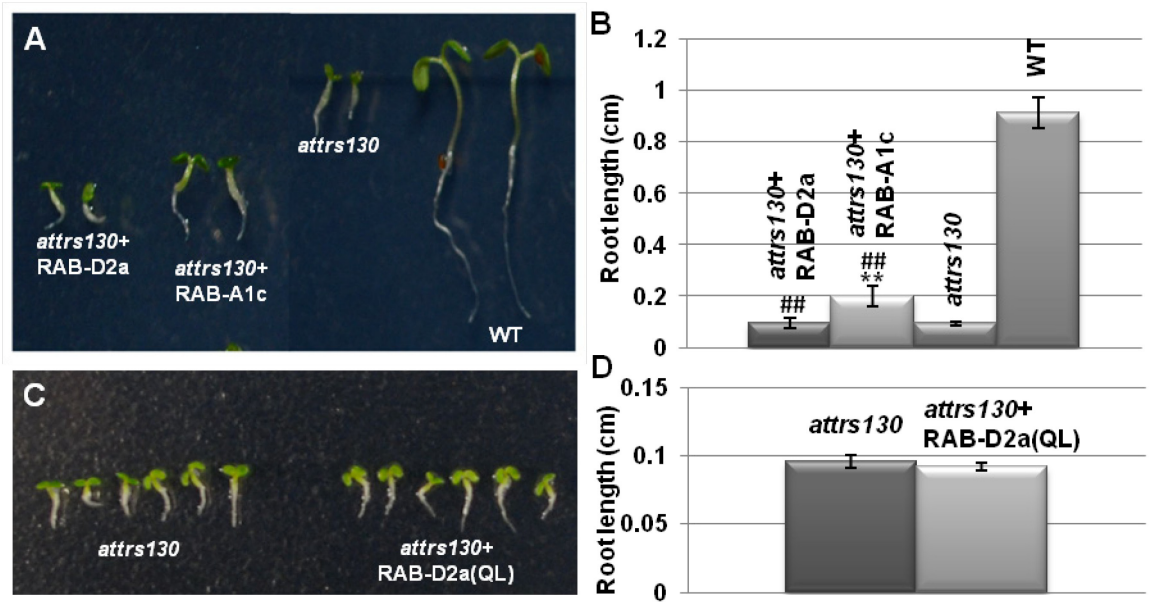

Figure 4.2. Root growth of *attrs130* is partially rescued by RAB-A1c, but not RAB-D2a. (A)-(B) image (A) and quantification (B) of 6-day old seedlings of *attrs130* expressing RAB-D2a, *attrs130* expressing RAB-A1c, *attrs130* and wild type. Error bar represents standard error. **: $P<0.01$ compared with *attrs130*; ##: $P<0.01$ compared with WT. (C)-(D) image (C) and quantification (D) of 6-day old seedlings of *attrs130* and *attrs130* expressing RAB-D2a(Q67L). Error bar represents standard error.

To express RAB-A1c in *attrs130*, GFP-RAB-A1c was crossed into a heterozygous *attrs130* line (Qi et al., 2011); to express RAB-D2a and RAB-D2a(Q67L) in *attrs130*, a heterozygous *attrs130* line (Qi et al., 2011) was transformed with an agrobacterium strain containing pVKH18-RAB-D2a (Zheng et al., 2005) and pEarleyGate103-RAB-D2a(Q67L), respectively. The Q67L mutation in RAB-D2a was made using site-directed mutagenesis in full length *RAB-D2a* cloned in pCR8/GW/TOPO, and was subcloned into pEarleyGate103.

CHAPTER V:
CONCLUSION

In this thesis, I examined the function and regulation of Rab-A1 proteins in the model plant species *Arabidopsis*.

Rab-A1 proteins define a subdomain of TGN that only partially overlaps with VHA-a1 marked TGN

In my study, using a combined approach of in-vivo imaging and genetics, I reported that RAB-A1c defines a population of TGN that only shows partial overlap with VHA-a1 marked TGN, consistent with the previous report that Rab-A2/A3 partially co-localizes with VHA-a1 (Chow et al., 2008). Our data also revealed that Rab-A1, A2/A3 and A4 highly co-localize to the same population of TGN. We thus propose that Rab-A proteins reside on a subdomain of TGN that is different from VHA-a1. In plants, the TGN is a dynamic organelle that undergoes maturation from early TGN just peeling off from the Golgi to the late TGN that eventually is fragmented into vesicles and cisternae (Kang et al., 2011; Viotti et al., 2010). RAB-A4b is detected evenly on both early and late TGN, whereas VHA-a1 is preferentially located on the late TGN (Kang et al., 2011). It is proposed that VHA-a1 mainly functions in the late TGN to PVC/MVB (Dettmer et al., 2006). We believe that the Rab-A proteins act in both the maturation process and the sorting in the late TGN. Base on evidence provided in this thesis, together with the other published evidence (Blanco et al., 2009; Chow et al., 2008; Preuss et al., 2006; Szumlanski and Nielsen, 2009), it is likely that Rab-A proteins are involved in membrane trafficking from the TGN to the plasma membrane/cell walls and to the growing cell plate in dividing cells.

Rab-A1 proteins act together with ES1 in plasma membrane recycling and cytokinesis in mitotic cells

We revealed in this study that, the TGN localization of GFP-RAB-A1c and the relocation of GFP-RAB-A1c to the cell plate are sensitive to ES1 but not Wortmannin. Furthermore, the root growth of *rab-a1a/b/c* triple mutant is also hypersensitive to ES1 but not Wortmannin. Wortmannin is a PI3K inhibitor (Clague et al., 1995) that affects endocytosis (Emans et al., 2002) and also the formation of PVC/MVB (Wang et al., 2009) in plant cells. The insensitivity of Rab-A1s to Wortmannin may be a reflection that Rab-

A1s are not directly involved in endocytosis nor transport to the vacuole. This conclusion is also supported by our study in TRAPPII. When TRAPPII subunits *AtTrs120* and *AtTrs130* are depleted, endocytosis of FM4-64 and vacuolar transport of SecN-Rm-2A appear normal.

ES1 is a compound known to selectively interrupt recycling of several plasma membrane proteins including PIN2 and AUX1, but not PIN1 (Robert et al., 2008). A similar defect is also observed in TRAPPII mutants in this thesis. RAB-A1a has been implicated in auxin transport (Koh et al., 2009). Therefore it is interesting to test the localization of these auxin transporters in the background of *rab-a1a/b/c* triple mutant we generated in this study. Because the relocation of RAB-A1c to the disc-like structure resembling the cell plate is sensitive to ES1, and bi-nucleated cells are frequently observed in the triple mutant in the presence of ES1, we thus propose that RAB-A1c-mediated TGN transport to the cell plate is also a target of ES1. Toth and colleagues recently revealed that ES1 affects the cellular actin dynamics that is necessary for selective membrane trafficking pathways, possibly by interacting with an actin associated protein(s) (Toth et al., 2012). In plant cells, RAB11b (a tobacco Rab-A1 homologue) is known to mediate the actin organization in pollen tube growth (de Graaf et al., 2005). Rab11 in animal cells regulates actin polymerization in plasma membrane recycling (Hales et al., 2002) and during furrow ingression in cytokinesis through its effectors (Cao et al., 2008). Therefore, it will be interesting to examine if any effectors of Rab-A1 is the molecular target of ES1.

Rab-A proteins have overlapping but distinct functions in plants

Rab-A proteins in *Arabidopsis* are greatly elaborated. We show here that Rab-A1 proteins are involved in cytokinesis. RAB11b (a tobacco Rab-A1 homologue) is implicated in secretion of cell wall proteins in the pollen tubes (de Graaf et al., 2005). Members of Rab-A2/A3 have previously been implicated to contribute to cytokinesis in mitotic cells (Chow et al., 2008), but it is also required for the root hair formation (Blanco et al., 2009). Rab-A4 members are involved in secretion of cell wall materials in root hairs (Preuss et al., 2006; Szumlanski and Nielsen, 2009). However, RAB-A4b is also detected in growing cell plates in root mitotic cells (our preliminary data). It is highly likely that the

function of Rab-A proteins in TGN is overlapped. However, in the *rab-a1a/b/c* triple mutant, root growth is slightly retarded, suggesting that the function of RAB-A1a/b/c could not be totally substituted by other Rab-A members. In yeast, Ypt31/32 is important for cell viability (Benli et al., 1996). Rab11 in the model animal systems is crucial for embryogenesis (Cao et al., 2008; Zhang et al., 2008), but the transgenic plants expressing either RAB-A1c(S27N) or RAB-A1c(Q72L), the GTP-binding and hydrolysis RAB-A1c mutants that are expected to inhibit the upstream GEF(s) and downstream effectors respectively (Ang et al., 2003; Chen et al., 2003), can still survive, with an inhibition in root growth. The subtle defect caused by the expression of the two RAB-A1c mutants suggests that Rab-A proteins do have diverse functions via different effectors, and may under the regulation of different GEFs. Indeed, RAB-A1c(Q72L) could only partially rescue *attrs130* in root growth, which also point to the possibility that, besides RAB-A1c, TRAPPII also affects the function of other Rab-A proteins. It is worth pointing out that though members of different Rab-A subclasses largely co-localize to the TGN under light microscopy, the correlation coefficients between Rab-A1 members are much higher than those between Rab-A subclasses. Therefore it would be interesting to examine the subcellular localization of different Rab-A members at the ultra-structural level with transmission electron microscopy. It is also interesting to test whether other Rab-As could rescue *attrs130*, and if so, to what extent. Last but not least, cargo identification for different Rab-A subclasses could also provide valuable information on their possible functional diversifications.

TRAPPII acts upstream of RAB-A1c possibly as a GEF

TRAPPII functions as a GEF for Ypt31/32 and Ypt1 in yeast (Cai et al., 2005; Cai et al., 2008; Jones et al., 2000; Morozova et al., 2006; Yip et al., 2010). However, in mammalian cells, TRAPPII serves as a GEF for Rab1 but not for Rab11 in pre-Golgi trafficking recently emerges (Yamasaki et al., 2009). In our study, we revealed that TRAPPII acts as an upstream regulator, potentially as a GEF for RAB-A1c but not RAB-D2a in post-Golgi trafficking in *Arabidopsis*. The discrepancy of the TRAPPII function between species can be explained by the difference in the architecture of the ER-Golgi pathway (Qi et al., 2011; Qi and Zheng., 2011; Yamasaki et al., 2009). However, it is also possible

that the organization and assembly of TRAPPII is different between mammalian cells, yeast and plants. For example, trs85, an essential component of TRAPPI, is recently revealed to be associated with TRAPPII in mammalian cells but not in yeast (Choi et al., 2011). In *Arabidopsis*, except Trs65, homologues of each TRAPP subunit are present (Cox et al., 2007), it will be interesting to examine the function of each subunit in *Arabidopsis* and to determine the composition of TRAPPI and TRAPPII.

An interesting phenomenon is that both *attrs120* and *attrs130* could still survive early embryogenesis. Our interpretation is that TRAPPII does not have profound functions during early embryogenesis, e.g. it is not expressed at early embryogenesis or there are alternative back-up resolutions. If during early embryogenesis, Rab-A proteins are essential in *Arabidopsis* as Ypt31/32 in yeast and Rab11 in animals (Benli et al., 1996; Cao et al., 2008; Zhang et al., 2008), there may be GEFs other than TRAPPII that can activate Rab-A proteins at this stage. A candidate protein is SCD1, a DENN domain protein that is involved in cytokinesis and polarized cell expansion (Falbel et al., 2003). It would be interesting to test the subcellular localization of Rab-A proteins in the background of *scd1*, and to examine how the constitutive active Rab-A mutants could rescue *scd1*.

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