TNO1 & VPS45: SNARE-associated proteins required for plant growth

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TNO1 & VPS45: SNARE-associated proteins required for plant growth

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

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Ames, Iowa
2016

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DEDICATION

This thesis is dedicated to all the strong-willed women that I have met over the years, who have had an indelible impact on the kind of person I am in the process of becoming. To my Mom, for encouraging me to “conquer the world” and yet be compassionate. To my wife, for keeping me calm through tough times and loving me with all her heart. To my major professor, for teaching me the nuances of the plant cell and how to write. To my mother in law, for accepting and loving me as a son. To my aunts, for teaching me the power of resilience and hope. To my friends, for buying me coffee and letting me be myself. To my puppy, for teaching me to be patient. And lastly, to my daughter, for teaching me that all you need is love.
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ACKNOWLEDGMENTS

First, I would like to thank my committee chair and advisor, Dr. Diane C. Bassham, for her guidance and mentorship over the last six years. I am grateful for her patience and commitment towards my multifaceted development as a teacher, scientist and human. I am also thankful to my committee members, Dr. Yanhai Yin, Dr. Clark Coffmann, Dr. David Oliver and Dr. Linda Ambrosio for their continued guidance and essential feedback.

I would like to thank my parents, Pradip and Rita Roy for filling my life with love, learning, laughter, good food and music. I will forever be mindful and thankful for all of the struggles you have undergone to help me arrive at this point in my life. I am grateful for my wife and best friend, Amber Lynn Roy, who I met and married during my time here in Ames. She is a rock upon which I lean. Thank you for navigating me towards success and filling my life with so much happiness and warmth. I will also be forever indebted to her for being the mother to my daughter, Freya Karuna Roy. Thank you both of you for giving me the strength to carry on.

Words will never do justice to how much you mean to me. Thanks to Amber for also bringing a new pair of parents into my life. Thank you Siv and Laurie Wahlstrom for treating me like a son and being so much fun to hang out with. I owe this PhD as much to your support as to my biological parents’. I would also like to thank my cousins, uncles and aunts from the Roy, Chawla and Wahlstrom clans for your continued support and love. Also, a big thank you to my family in the US: Rama and Sridhar Ramaswami, Doug and Mary Millican, Sushma and Roli Saraf and the Mistry family for being there whenever I have needed the essence of home.

The list of friends and colleagues who have helped me through this journey is long. From scientific troubleshooting to life lessons, thank you for being there whenever I needed you.
Thank you Mike for all that you have done for me, especially for teaching me how to play the guitar. Thank you Divita for being such a true friend, a Pipi to my daughter and sharing your vision of the nanoworld with me. Thank you Pooja, Nikhil, Joshua, Sweta and Archit for understanding my REF so well and all the adventures we had. Thank you Gina, Mihir, Larry, Sarani and Nikita for the amazing grillouts and being such an awesome support system. Thank you to my old buddies, Basab, Priyo, Sudip, Ishani, Parama, Rahul, Dinesh and Adi for all the continued support over the phone and being awesome. Thank you Roykey buddies for helping me adapt here in Ames initially and all the amazing food. Thank you Beaudelaire buddies for the awesome times, music and memories we shared.

A special thanks to all the amazing colleagues I have had the pleasure of working with in the Bassham lab. Thank you Tony, Sang-Jin, Yimo, Brice, Xiaochen, Yunting, Yan, Yosia and Junmarie for making the lab feel like a family. I will miss the jokes and chats we shared. A special thank you to Xiaochen, Yunting and Junmarie for supporting my transition period to a dad and also keeping me sane during my thesis-writing phase. A special shout-out to Dr. Eric Henderson for sharing his vision and take on life with me. I am also indebted to Dr. Renu Srivastava for being like an elder sister and helping me out with personal and scientific challenges over the years. Thank You Dr. Manohar John and Dr. Dior Kelley for your insightful discussions and career guidance whenever I needed it the most. A big thank you to Linda, Constance, Deanne, Diane and Lynette for the long chats and helping me out with administrative work. Thank you Margie for teaching me the art of confocal microscopy. Thank you Dr. Barbara Krumhardt, Dr. Karri Haen Whitmer and Mary Basil Madsen for teaching me how to teach. Without your guidance and support I would never be the teacher I am today.

And a final thank you to the Universe for the way this journey panned out.
ABSTRACT

Cellular trafficking of cargo vesicles at the trans-Golgi network (TGN) is required for multiple processes such as cell expansion, stress responses and hormonal transport in plants. Activity of membrane proteins known as SNAREs drives membrane fusion events. Associated proteins such as tethering factors and Sec1/Munc18 proteins aid the fidelity and efficiency of these fusion events by interacting with SNAREs. The TGN-localized SYP41/SYP61/VTI12 SNARE complex is required for vacuolar and secretory cargo trafficking.

TNO1, a putative tethering factor, associates with SYP41 and is required for TGN membrane fusion dynamics and proper SYP61 localization. My dissertation research discovered a new role of TNO1 in auxin transport-related physiology. Mutants lacking TNO1 (tno1) display decreased gravitropic bending of plant organs, delayed lateral root emergence and increased sensitivity to natural auxin and a cell influx-specific synthetic auxin. Reduced auxin asymmetry at the tips of elongating lateral roots and gravistimulated primary root tips in the mutants confirms TNO1’s role in cellular auxin transport during these processes. Loss of TNO1 does not affect bulk endocytosis and arrival of membrane cargo at the TGN, suggesting a specific effect of TNO1 in auxin transport mechanisms by possibly affecting subcellular trafficking of auxin transporters.

The root gravitropic defects led me to hypothesize that root growth movements would be defective in the tno1 mutants. I discovered that tno1 mutant roots display exaggerated rightward deviation from the growth trajectory (skewing), correlated with an enhanced left-handed root epidermal cell file rotation, when grown on slanted impenetrable growth media. tno1 mutants also behave differently from wildtype in studies investigating the effect of microtubule-
disrupting drugs on root skewing and cell expansion show. This suggests that TNO1 might have a role in microtubule-associated mechanisms driving skewing and cell expansion, though a direct effect on MT array orientation was not observed. Altogether, this suggests TNO1’s role in both auxin transport and possibly MT-associated processes.

I also investigated the effect of a point mutation in VPS45, a SYP41-associated Sec1/Munc18 protein. The mutant (\textit{Atvps45-3}) displays dwarf phenotypes with highly reduced plant organ sizes and cell expansion. Mutant root hairs are short and thick compared to wildtype root hairs, suggesting defects in polarized cell expansion processes. The endocytic and secretory routes in \textit{Atvps45-3} plants seem unaffected suggesting a specific effect of the mutation on cell expansion.

Taken together, these results add to the knowledge of SNARE-associated proteins at the TGN and how post-Golgi traffic mediates lateral root emergence, gravitropism, root movement and root hair expansion.
CHAPTER I
INTRODUCTION

1. Plant Vesicular Transport

All eukaryotic cells contain membrane bound compartments that exchange diverse cargoes such as protein, lipids and polysaccharides. Major vesicular transport routes in eukaryotic cells include exocytic, endocytic and vacuolar/lysosomal trafficking (Alfonso et al., 2010). The exocytic pathway refers to the outward traffic of cargo synthesized inside the cell to the extracellular space, the endocytic pathway refers to the internalization of cargo from the exterior to the inside of a cell and vacuolar trafficking involves delivery of biosynthetic cargo and cargo targeted for degradation (Samaj et al., 2005; Saito and Ueda, 2009; Alfonso et al., 2010; Contenko and Bassham, 2012; Brandizzi and Barlowe, 2013; Drakakaki and Dandekar, 2013; Zhang et al., 2014; Gendre et al., 2015). These pathways help maintain a constant interaction of a cell with its environment while also maintaining functional compartmentalization inside the cell. The trafficking pathways utilize vesicular transport, which involves the budding of cargo-loaded vesicles, their transport and tethering followed by fusion with the target membrane. A diverse set of proteins aid in maintaining specificity, efficiency and fidelity of these events maintaining directionality and homeostasis in the cell (Cai et al., 2007; Alfonso et al., 2010; Chen et al., 2011; Contenko and Bassham, 2012; Cevher-Keskin, 2013). A defect in the traffic routes can lead to the destabilization of cellular homeostasis and compartmental integrity, leading to severe developmental & physiological defects. Transport of lipid and protein between compartments also creates challenges in maintenance of compartmental identity and
compartment size. This problem is addressed by a constantly operating retrograde transport step for each step of forward transport (anterograde transport), thus maintaining equilibrium.

The plant cell uses vesicle transport to direct cargo for important processes such as cell expansion and division while also responding to cues from the extracellular environment. Multiple processes such as hormonal transport and signaling (Friml, 2010), abiotic/biotic stress responses (Zhu et al., 2002; Takano et al., 2005), autophagy (Yang and Bassham, 2015), gravitropic responses (Strohm et al., 2012), lateral root emergence (Peret et al., 2013; Roy and Bassham, 2015) and development of seeds (Shimada et al., 2006) and flowers (Sohn et al., 2007) are dependent on cellular trafficking.

2. Organization of the Plant Endomembrane System

Understanding the components and types of vesicular trafficking involves reviewing the general organization of the endomembrane system in plants via which many proteins are routed for delivery or turnover. Major organelles involved in vesicular trafficking include the endoplasmic reticulum (ER), Golgi stacks, trans-Golgi network (TGN), early endosomes, late endosomes, multivesicular bodies and vacuoles (Figure 1). Peroxisomes have also been suggested to be a semi-autonomous part of the endomembrane system (Titorenko and Mullen, 2006; Titorenko and Rachubinski, 2009), though this will not be discussed here. Other components involved in vesicular trafficking will be discussed briefly in the sections below.

2.1. Endoplasmic reticulum (ER)

The ER consists of an interconnected network of sheets and tubules and is the site for protein synthesis, folding and quality control (Chen et al., 2012). Proteins and lipids bound for
the secretory and vacuolar routes as well as other endomembrane compartments are synthesized in the ER (Chen et al., 2012) (Figure 1). Ribosomes attached to the ER sheets biosynthesize the membrane and secretory proteins. The ER also associates with various organelles such as peroxisomes, chloroplasts and mitochondria (Chen et al., 2012). ER formation and movement in higher plant cells is dependent on actin, possibly via myosin action (Sparkes et al., 2009a). ER vesicles are generated at sites known as ERES (ER exit sites) (Langhans et al., 2012), which are then picked up by nearby Golgi stacks. A continuous flow of cargo from ER to Golgi has also been proposed because some regions of the ER display direct tubular connections with the Golgi cisternae (Sparkes et al., 2009b).

2.2. Golgi apparatus

The Golgi is functionally and physically associated with the ER and acts as a link between the ER and other sites such as the endosomes, PM and the vacuole (Sparkes et al., 2009a; Chen et al., 2012; Lerich et al., 2012). The Golgi apparatus is a single-membrane-bound organelle in eukaryotic cells, made up of stacks of cisternae (flattened membrane sacs) (Figure 1). The 5-20 cisternae in each stack are polarized from cis to trans sides, receiving cargo at the cis side from the ER and directing cargo from the trans side to post Golgi compartments. The Golgi is the major site for glycosylation of proteins and contains the enzymes required for N-glycan processing. Enzymes acting early in the N-glycan processing pathway reside on the cis compartments of the Golgi while enzymes acting later reside on the trans-cisternae and the trans Golgi network (Schoberer and Strasser, 2011). The Golgi complex also acts as a carbohydrate ‘factory’ supplying building blocks of the plant cell wall such as the non-cellulosic polysaccharides, hemicellulose and pectin (Schoberer and Strasser, 2011).
2.3. TGN/ early endosomes (EE)

The *trans* most cisternae of a Golgi stack faces the *trans*-Golgi network, a tubulo-vesicular cluster that exists as an independent organelle in plants (Dettmer et al., 2006; Gendre et al., 2015). The TGN can move rapidly in the plant cell, dissociating from Golgi stacks and re-associating with new Golgi stacks (Viotti et al., 2010) and acts as a station for cargo arriving from the Golgi that is targeted to the extracellular space or bound for the vacuole. It is also responsible for recycling and sorting endocytosed cargo arriving from the plasma membrane (Gendre et al., 2015) (Figure 1). Thus the TGN takes the role of an early endosome (EE) in plant cells. The discovery of different classes of cargo contained in the TGN structure and how mutations in certain TGN localized proteins affects the secretory pathway but not the endocytic route (Gendre et al., 2011; Van Damme et al., 2011), suggests that the TGN has distinct repertoires of proteins mediating the two different traffic routes. The TGN therefore may have different domains via which it receives or targets the different cargo types. Studies with immunogold electron microscopy of various TGN-localized proteins showed their distinct distribution on specific domains of the TGN, strengthening the hypothesis that the compartment might segregate different trafficking pathways (Bassham et al., 2000). Electron tomographic studies also suggest that the TGN consists of an early and a late compartment, although further studies are needed to confirm this (Staehelin and Kang, 2008; Kang et al., 2011).

2.4. The Late Endosome (LE)

From the TGN/EE, cargo can be recycled back to the PM or be transported onwards to a late endosome (LE) (Contento and Bassham, 2012). The LE sequesters ubiquitinated cargo
(tagging of cargo with ubiquitin molecules marks it for degradation) that is about to be trafficked to the vacuole for degradation. The exact mechanism of formation of the LE is still not clear though it is postulated that it forms by fusion of Golgi- and TGN- derived vesicles. A distinct morphological feature of the LE is that it contains small vesicles bound by an outer limiting membrane giving it its other name, the multivesicular body (MVB) (Figure 1). These endosomal intraluminal vesicles (ILVs) arise by inward budding of the outer membrane of the LE (Babst, 2011). This ensures that the ILVs containing membrane and membrane-bound cargo are delivered to the inside of the vacuole upon fusion of the MVB limiting membrane with the vacuolar membrane (tonoplast), where they can be degraded by hydrolases. The formation of ILVs and sequestration of cargo is mediated by a series of protein complexes called Endosomal Sorting Complex Required for Transport (ESCRTs) [reviewed in (Otegui et al., 2012)]. The MVBs carry cargo that is specific for vacuoles, including newly synthesized constituent vacuolar proteins and enzymes arriving from the Golgi complex. The MVBs also act as prevacuolar compartments (PVC), carrying storage proteins and proteases for delivery to the protein storage vacuoles (Otegui et al., 2006).

2.5. Vacuoles

The vacuole is the biggest organelle in plant cells and plays a role in diverse pathways involving storage and turnover of cellular components. The plant vacuole may occupy most of the cell’s volume and is responsible for maintaining turgor pressure and driving rapid cell expansion. Plant vacuoles are similar to lysosomes but have more specialized functions such as rapid volume changes during stomatal opening (Gao et al., 2005). Vesicular transport delivers proteins, lipids and even solutes such as anthocyanins to the vacuole (Uemura and Ueda,
Cargo usually arrives from the ER via the Golgi or from the plasma membrane to the vacuole via an intermediate prevacuolar compartment. Vacuoles help in ion homeostasis, sequestering toxins, storage and degradation of proteins and other compounds (Marty, 1999; Martinoia et al., 2012). Two types of vacuoles can be found in plants: lytic vacuole (LV) and protein storage vacuole (PSV) (Paris et al., 1996). Lytic vacuoles are found in most plant cells and are mainly required for protein and RNA degradation. Protein storage vacuoles (PSVs) are usually specific to tissues such as seeds and fruits since they accumulate storage proteins and processing proteases alongside globoids (phytic acid crystals) (Martinoia et al., 2012).

3. Cellular Trafficking Routes

The transport of cargo between the cellular organelles maintains cell homeostasis and also regulates physiological responses. Various trafficking routes exist in cells and are usually responsible for distinct functions, though plenty of overlap occurs amidst various traffic routes. The various trafficking routes are summarized in Figure 1.

3.1. Endocytosis

Endocytosis is the uptake of cargo vesicles formed from PM invaginations and is essential for plant physiology, development, signaling and communication with the environment. After uptake from the plasma membrane, endocytic vesicles arrive first at the TGN in plant cells. Studies in Arabidopsis lines expressing a TGN-specific protein, vacuolar ATPase subunit a1 (VHAa1) fused with a fluorescent GFP tag, showed that within minutes of exposure to FM4-64 (a fluorescent dye used as an endocytic trafficking marker) both the fluorescent signals co-
localized on the TGN compartment, suggesting it to be the first station for receipt of the endocytosed cargo (Dettmer et al., 2006).

Cargo selection for endocytosis is mostly under selective control but can be non-selective too. The post-translational modification (such as ubiquitination and phosphorylation) of membrane cargo or the presence of a linear amino acid motif within the cargo protein can signal endocytosis (Chen et al., 2011; Reyes et al., 2011). Specific modifications of the cargo can subsequently signal the cell to either recycle the protein back to the plasma membrane or target it for degradation. For example, phosphorylation status of the auxin transporters PIN regulates its uptake into the endocytic route while ubiquitination of BRI1, the brassinosteroid receptor leads to its endocytosis and targeting to the vacuole via MVBs (Bonifacino and Traub, 2003; Chen et al., 2011; Reyes et al., 2011; Habets and Offringa, 2014; Martins et al., 2015).

The most common form of endocytosis involves the coat protein called clathrin and is known as clathrin mediated endocytosis (CME). Clathrin assembles at the site of endocytosis and forms a curved lattice-like basket that brings about the characteristic inward budding of vesicles. CME in plants is important for several developmental processes such as determination of cell polarity, cytokinesis, cell elongation and gametogenesis (Chen et al., 2011). CME is discussed in more details in Section 4.1.

3.2. Vacuolar trafficking

A variety of cargo is trafficked to the vacuole by biosynthetic, endocytic and autophagic routes. The biosynthetic route (from the ER-Golgi) and endocytic routes (from the PM) converge at the TGN or in the MVB/PVC (Dettmer et al., 2006; Viotti et al., 2010; Reyes et al., 2011) with the cargo vesicles then fusing with the vacuole. The biosynthetic path maintains the biochemical
identity of the vacuole and transports enzymes, storage proteins and lipids to the vacuole and tonoplast. As part of this, the vacuolar proteins synthesized at the ER, arrive at the TGN via the Golgi and are sorted to the PVC/MVB by vacuolar sorting receptor (VSR). Seven VSR isoforms have been identified in *Arabidopsis thaliana* (Ahmed et al., 1997; Shimada et al., 2003; Zouhar et al., 2010). Once the cargo vesicle fuses with the PVC/MVB, the VSRs recycle back to the TGN for subsequent rounds of sorting, while the MVB fuses with the vacuole (Martinoia et al., 2012; Pereira et al., 2014). The VSRs recognize distinct vacuolar sorting signals/determinants (VSSs/VSDs) that are part of the protein’s peptide sequence. Three major groups of VSDs have been identified in plants: 1. C-terminal VSDs (ctVSDs) 2. Sequence specific VSDs (ssVSDs) and 3. Physical structure VSDs (psVSDs). The presence of these VSDs determines the sorting fate of the protein, targeting it to either storage or lytic vacuoles (Pereira et al., 2014). The existence of different VSDs suggests fine control of vacuolar targeting in different tissues as well as during different physiological stages. It should be noted that this specificity of VSD has been challenged by the discovery of proteins containing more than one VSD in their sequence. For example, the soybean beta-conglycinin protein has both, a ctVSD and ssVSD (Nishizawa et al., 2006).

The autophagic pathway also delivers cell components to the vacuole for degradation. These components either get invaginated by the vacuolar membrane directly (microautophagy) or get encased in specialized structures known as autophagosomes, which then fuse with the vacuole to be degraded (macroautophagy) (Yang and Bassham, 2015).
3.3. Secretory traffic

Conventional protein secretion involves ER-Golgi mediated secretion of proteins. After posttranslational modification of the proteins in the Golgi, secretory vesicles budding from the TGN deliver them to the extracellular space via fusion with the PM (Drakakaki and Dandekar, 2013). The TGN thus functions at the crossroads of endocytic and secretory routes (Gendre et al., 2014). Secretion of proteins and polysaccharides plays important roles in cell wall assembly. A combination of proteomics and immunoelectron microscopy has identified and confirmed secretory cargo such as cell wall modification enzymes, transporters, t-SNAREs and ion channels (Worden et al., 2012; Drakakaki and Dandekar, 2013). Hemicelluloses and pectins are synthesized in the Golgi and then secreted to the PM while cellulose is synthesized at the PM by secretion of the cellulose synthesis machinery (Kim and Brandizzi, 2014). Cellulose trafficking involves an intermediate compartment, known as microtubule-associated cellulose synthase compartment (MASC), which is involved in endocytosis and redistribution of the cellulose synthesis complex (Crowell et al., 2009).

Unconventional protein secretion refers to the process by which proteins (that are usually devoid of a signal peptide) are secreted in a Golgi independent manner (Robinson et al., 2016). This process is well studied in mammalian and yeast cells and is also reported in plants. Proteins such as celery mannitol dehydrogenase (MTD) and hygromycin phosphotransferase (HYG\textsuperscript{R}) are secreted in this manner (Cheng et al., 2009; Zhang et al., 2011). A novel organelle involved in unconventional secretion has been identified in Arabidopsis. This organelle, EXPO (exocyst positive organelle), is marked by EXO70E2 (an Arabidopsis exocyst subunit homologue) (Wang et al., 2010).
4. Steps in Cellular Trafficking

Vesicular trafficking involves four steps: vesicle budding, transport, tethering and fusion. Concerted action of these four steps maintains efficient routing and fusion fidelity of diverse cargo-laden vesicles in the cell. In the following sections these steps, along with examples of the players involved, will be explored in detail.

4.1. Vesicular budding

Transport of cargo is usually initiated by membrane deformation and cargo recruitment, which is facilitated by coat protein complexes (Figure 2). There are mainly three classes of coat protein complexes that are important for different trafficking steps/routes. Coat protein complex II (COPII) is involved in ER-to-Golgi trafficking, coat protein complex I (COPI) mediates intra-Golgi and Golgi-to-ER trafficking (Brandizzi and Barlowe, 2013) while the clathrin-based complexes are involved in multiple steps in post-Golgi trafficking (Chen et al., 2011). Each coat type is distinct though similarities in coating mechanisms exist. Once cargo is recruited to the membrane, a GTP cycle of a coat GTPase occurs aiding the coating process. The coat GTPase exists in an inactive cytosolic GDP-bound state, which then converts to its active, membrane-recruited GTP bound state by the action of the coating site localized GTP exchange factor (GEF) (Gillingham and Munro, 2007). The activated coat-GTPase then recruits effector proteins and subunits of the coat complexes from the cytoplasm onto the membrane budding site, deforming the membrane and releasing the vesicle. A GTPase activating protein (GAP) then causes the inactivation of the coat GTPase by activating its intrinsic GTPase activity, triggering uncoating of the vesicle, which is now ready for transport to its destination (Inoue and Randazzo, 2007). We discuss the example of clathrin mediated processes in the next paragraph.
During clathrin-mediated endocytosis, clathrin subunits assemble at the site of endocytosis in the form of hexameric, three legged triskelia with each triskelion being composed of three clathrin heavy chains (CHC) and three clathrin light chains (CLC) that can self-polymerize. With gradual polymerization of clathrin subunits, a curved lattice like structure starts forming causing negative membrane curvature (Doherty and McMahon, 2009). Finally, the invagination is completed and pinches off to form a clathrin-coated vesicle (CCV). Since clathrin cannot bind membrane or cargo by itself, it requires the function of scaffolding adaptor protein (AP) complexes (conserved amongst eukaryotes) that help the budding process. The *Arabidopsis* genome encodes five Assembly Polypeptide/Adaptor Protein (AP, AP1-5) complexes. Mutations in or loss of some of the identified plant adaptins (subunits of the AP complex) result in trafficking inhibition and developmental defects related to flower production and reproduction (Park et al., 2013; Wang et al., 2013; Yamaoka et al., 2013; Wang et al., 2014). There also exist plant-specific adaptin-like proteins such as TPLATE, which interacts with the clathrin machinery, aiding cell plate formation during cell division (Van Damme et al., 2011).

The ARF GTPases alongside ARF-GEFs and ARF-GAPs form a crucial regulatory cycle in endocytosis and vesicle trafficking pathways (Stenmark, 2009). The ADP Ribosylation factor (ARF) family is the coat GTPase for clathrin-type coat and COP-I formation. The *Arabidopsis* genome encodes 12 ARF isoforms, suggesting highly regulated trafficking (Cevher-Keskin, 2013). In plants, ARF1 localizes to the Golgi and endosomes and plays roles in regulating vesicular trafficking and cell proliferation as well as elongation (Park and Jurgens, 2011; Cevher-Keskin, 2013). The GEF for the ARF GTPase is defined by the presence of a Sec7 domain and can belong to any of the three classes i) The GEA/GNOM/GBF class, ii) The Sec7/BIG class and iii) The ARNO class (found in mammals, absent in plants). The *Arabidopsis* genome encodes 8...
ARF-GEFs with members belonging to either the GEA/GNOM/GBF or Sec7/BIG clade (Anders and Jurgens, 2008). The ARF-GEF, GNOM has been shown to partially co-localize with clathrin, suggesting its close association with early endocytosis events. The fungal toxin Brefeldin A can inactivate certain ARF-GEFs, a property that has aided the classification of different cargo trafficking steps as BFA sensitive or resistant. The ARF-GEFs GNOM (BFA sensitive) and GNOM-LIKE1 (GNL1)(BFA insensitive) have been shown to be important for endocytosis of PM-localized hormone transporters and ligand-receptor pairs. The ARF-GAP VASCULAR NETWORK DEFECTIVE 3 VAN3 localizes to the TGN (Koizumi et al., 2005) and is important for endocytosis of PM proteins.

Rabs are another set of key players in trafficking and are the largest family of small GTPases belonging to the Ras superfamily (Stenmark, 2009). They have a specific C terminal lipid modification (prenylation or myristoylation ) and can cycle on and off membranes following the same GTP cycle of activation/deactivation as described above. The Arabidopsis genome encodes for 57 Rab GTPases divided into 8 clades (RabA-H) (Park and Jurgens, 2011). Activated Rabs can recruit cargo, molecular motors and activate downstream effectors. Studies with the numerous Rabs in Arabidopsis is helping build a picture of how each Rab regulates trafficking and their biological functions (Saito and Ueda, 2009;Park and Jurgens, 2011)

During clathrin-mediated endocytosis, once the clathrin-coated pits have invaginated they are still attached to the PM by a narrow neck. Dynamin-related proteins (DRPs) are large GTPases that regulate membrane fission, fusion, and tubulation during cellular processes such as endocytosis, cytokinesis, peroxisome biogenesis, ER morphology maintenance and vacuolar sorting (Praefcke and McMahon, 2004;McNew et al., 2013). Dynamin, one of the best characterized DRPs (Ferguson and De Camilli, 2012) forms a helical structure around the neck
of the budding clathrin coated vesicle and, using GTP hydrolysis, causes an extension of the helix thus stretching the neck out and eventually causing membrane scission and release of the vesicle into the cytoplasm (Sweitzer and Hinshaw, 1998). Two of the six dynamin-related protein (DRP) families in plants, DRP1 and 2, have been shown to be important in plant endocytosis events and show clathrin-dynamin dynamics similar to that observed in animal cells as confirmed by live cell imaging studies (Fujimoto et al., 2010). Once the vesicle has completed the budding process, and lost its coat it proceeds to be transported to its destination.

4.2. Vesicular transport

The flow of the cytoplasm and intracellular components in plant cells is known as cytoplasmic streaming and causes transport of vesicles and organelles (Tominaga and Ito, 2015). There is an existing correlation between cytoplasmic streaming velocity and plant size (Tominaga et al., 2013). The cytoskeleton and motor proteins play crucial roles in cytoplasmic streaming and membrane transport in plant cells. Myosins are motor proteins that direct movement on actin filaments by using the energy liberated from ATP hydrolysis and hence are known as mechanoenzymes (Hartman and Spudich, 2012). Myosins localize to diverse intracellular compartments and are recruited to their target membranes by the action of specific myosin-binding proteins. Studies in yeast and mammalian cells suggest that myosins can associate with Rab GTPases, large protein complexes or lipids regulating directed vesicle trafficking (Hartman and Spudich, 2012). Organelle associated class XI myosins moving along actin filaments are required for the streaming movement in plant cells and transport of multiple organelles and vesicles. The small GTPases, AtRabD1 and AtRabC2a were identified as adaptors of the myosin XI MYA2 (Hashimoto et al., 2008) in plants. Localization studies in plant cells
demonstrate that the class XI myosins co-localize with secretory organelles and might influence their movement. Myosin XI-K also has roles in the dynamics and movement of the Golgi complex, ER and post-Golgi organelles (TGN, endosomes, PVC) (Avisar et al., 2008; Avisar et al., 2009; Ueda et al., 2010).

It was previously thought that, unlike other eukaryotic cells, the microtubule network in plant cells had little involvement in organelle movement and vesicle trafficking. Recent studies are unraveling and discovering novel roles of MTs in these processes (Brandizzi and Wasteneys, 2013). It has been suggested that MTs and their associated motors, kinesins might be responsible for anchoring or slowing down organelles for specific targeting to their final destinations (Cai and Cresti, 2012; Zhu and Dixit, 2012). Most plant kinesins are involved in cell division, affecting mitotic spindle assembly or phragmoplast formation (Cai and Cresti, 2012). The plant kinesin AtPAKRP2 is likely to be involved in delivery of Golgi vesicles to the phragmoplast and formation of the cell plate (Lee et al., 2001). Microtubules have been implicated in the process of cellulose deposition and orientation since endocytosed cellulose synthase complexes associate with MTs in specialized compartments referred to as small CesA-containing compartments (SMACs) (Gutierrez et al., 2009) or microtubule-associated cellulose synthase compartments (MASCs) (Crowell et al., 2009). It is possible that the mechanism of MT-cellulose synthase interaction involves kinesins. The FRAGILE FIBER 1 (FRA1) in Arabidopsis and BRITTLE CULM 12 (BC12) in rice are kinesins that are hypothesized to be involved in cellulose production (Zhang et al., 2010; Zhu et al., 2015). Further analysis is needed to better understand how kinesin regulates cellulose production and whether it actually occurs by tethering of the cellulose synthases to the microtubules. It has been suggested that other wall components such as wall modifying enzymes and matrix polysaccharides are trafficked in a MT dependent manner.
Another piece of the elusive puzzle of MT-membrane trafficking was solved when CLASP was shown to be a tether connecting a certain endosomal vesicle population to microtubules (Ambrose et al., 2007; Ambrose et al., 2013). Advances in live cell imaging of MT dynamics and cellular trafficking along with mathematical modeling/simulations (Eren et al., 2015; Celler et al., 2016) are adding to our understanding of how vesicle transport occurs in plant cells.

### 4.3. Tethering and fusion

Once the vesicle is transported to its destination, tethering factors bring the vesicle close to its target membrane and SNAREs drive the fusion process. Another set of proteins, the Sec1/Munc18 proteins are required for proper functioning of the SNARE machinery in subsequent fusion events. Each class of proteins has multiple members in eukaryotic cells, suggesting complex endomembrane system and trafficking routes. We discuss the tethering and fusion machinery in plant cells in the following sections.

#### 4.3.1. SNAREs

Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) are a class of transmembrane proteins that are required for membrane fusion. SNAREs are small polypeptides (~200-400 amino acids) characterized by the presence of a 60-70-residue SNARE motif made of heptad repeats and with a propensity to form a coiled coil structure (Jahn and Scheller, 2006). Formation of specific SNARE complexes drives membrane fusion and maintains trafficking fidelity inside the cell. SNAREs were initially classified as v-SNAREs or t-SNAREs for their location on vesicles or target membranes respectively (Figure 2).
Usually three t-SNAREs on the target compartment interact with an incoming cognate v-SNARE to form a heterotetrameric _trans_-SNARE complex, also known as a SNAREpin (Weber et al., 1998). This nomenclature of target and vesicles cannot be used for homotypic vesicle or organelle fusions. Thus, SNAREs are also classified as Q- or R-SNAREs based on whether the core amino acid of the hydrophobic heptad repeat in the SNARE motif is a glutamine (Q) or arginine (R) (Fasshauer et al., 1998). The Q SNAREs are further subdivided into the Qa-, Qb- and Qc-SNAREs (Bock et al., 2001).

Most Q-SNAREs act as t-SNAREs and R-SNAREs as v-SNAREs. Qa SNAREs harbor an autoregulatory N-terminal domain that interacts with its own SNARE motif resulting in a so-called “closed” conformation. This prevents the Qa SNARE from interacting with other SNARES. Assembly of the SNARE complex can only proceed if the Qa SNARE converts to the “open” conformation (Burgoyne and Morgan), brought about by Sec1/Munc18 family of proteins (discussed in section 4.3.3.).

There are around 65 SNAREs reported in _Arabidopsis_, involved in diverse plant cellular trafficking pathways. Studies have revealed redundancy as well as functional specificity. A total of 21 SNAREs localize to the ER and Golgi complex, another 21 SNAREs have been identified at the TGN, endosomes and vacuoles while 23 have been shown to localize at the PM (Kim and Brandizzi, 2012). Some SNAREs display dual localizations, such as AtSYP71 which localizes to the ER and PM (Suwastika et al., 2008). We discuss the functions of some TGN-land PVC-localized SNAREs here.

The TGN SNAREs function at the crossroads of secretory, endocytic and vacuolar traffic. The SYP4 group of Qa- SNAREs at the TGN has three members, SYP41, SYP42 and SYP43. SYP41 and SYP42 localize to separate subdomains of the TGN and form separate complexes,
suggesting the possibility of functional compartmentalization of the TGN (Bassham et al., 2000). The syp41syp42syp43 triple knockout mutants are lethal while the syp42syp43 double mutants exhibit severe pleitropic defects such as short roots and early senescence. The mutants also display enhanced susceptibility to the powdery mildew fungus, suggesting a role for the SNAREs in extracellular disease resistance (Uemura et al., 2012a). They also display increased sensitivity to salt and osmotic stress suggesting the SNAREs’ role in biotic and abiotic stress tolerance (Uemura et al., 2012b). SYP61, which associates with SYP41, is also required for abiotic stress tolerance. SYP61, a Qc-SNARE, is proposed to act as a t-SNARE at the TGN and also function in TGN-PM anterograde traffic. Proteomic analysis of SYP61 labeled vesicles identified subunits of the cellulose synthase complex, cell wall enzymes and a PM SNARE (Drakakaki et al., 2012). VTI12, a Qb-SNARE, associates with the SYP41 and SYP61 SNARE and is responsible for trafficking to the vacuole. The SYP2 family members localize on the PVC and cytoplasm. Overexpression of the PVC localized SYP21 mis-sorts vacuolar cargo and results in homotypic fusion of the PVC. This suggests a role for SYP21 in anterograde trafficking from the PVC to the vacuole (Foresti et al., 2006). VTI11, a SYP21-associated SNARE localizes to the vacuole and is required for maintenance of vacuolar morphology and plant responses to gravity (Yano et al., 2003). Thus, SNAREs in plants regulate multiple membrane trafficking events that then regulate growth and responses to the environment.

4.3.2. Tethering factors

SNAREs are not likely the sole determinant of the direction of vesicular trafficking since they are recycled after fusion events and thus will be present on both anterograde- and retrograde-bound vesicles. Moreover, SNARE interactions are known to be promiscuous (Tsui
and Banfield, 2000) and vesicular tethering can still occur if SNARE complex formation is disrupted (Hunt et al., 1994; Broadie et al., 1995). Thus SNAREs do not mediate the first contact between vesicles and target membranes. Rather, this seems to be mediated by a class of proteins known as tethering factors. Tethering factors act upstream of SNAREs, tethering and holding vesicles close to the target membrane and aiding the interaction between vesicle fusion components and membranes at specific membrane domains (Chia and Gleeson, 2014) (Figure 2). Recent studies with a tethering factor suggests that the simultaneous interaction of two of the tether’s coiled coil (CC) with two different SNAREs, brings the SNAREs closer and concentrates them at the membrane fusion sites (Grabski et al., 2012).

Tethering factors usually belong to one of two classes, long coiled-coil proteins or large multi-subunit complexes (Whyte and Munro, 2002; Gillingham and Munro, 2003; Chia and Gleeson, 2014). The coiled coil tethers are large, homodimeric proteins that can interact with vesicles over long distances of more than 200nm and help sequester vesicles before fusion occurs. The multi-subunit tethering complexes are a group of proteins with each member consisting of 3-10 subunits and can interact with vesicles over shorter distances, up to 30nm (Brocker et al., 2010; Chia and Gleeson, 2014). Coiled-coil tethers are often peripheral membrane proteins that usually associate with the Golgi-TGN complex (also known as golgins) (Munro, 2011) or endosomes (e.g., EEA1) (Mills et al., 1998). TGN golgins mediate TGN-PM anterograde transport and retrograde transport between endosomal system and the TGN (Lu et al., 2004; Derby et al., 2007; Lieu et al., 2007) and contribute to the retention and assembly of SNARE complexes. Interestingly golgins can interact with multiple Rabs family members and the cytoskeleton (Efimov et al., 2007) acting as membrane scaffolds. EEA1 (Early endosome
antigen-1) also acts as a scaffold to assemble various factors to ensure specific fusion of membranes and early endosomes (McBride et al., 1999).

Multisubunit tethering complexes can be split into two groups based on their function in either secretory pathways (e.g.- Dsl1p, COG, exocyst complexes) or endo-lysosomal pathways (e.g.- HOPS, CORVET, VPS complexes). CORVET and HOPS, act sequentially in fusion events at the early and late endosomes. The exocyst complex is composed of 8 subunits and functions in post Golgi secretion and. It has been proposed that the exocyst complex is involved in tethering cargo vesicles derived from recycling endosomes (Mills et al., 1998; McBride et al., 1999; Allan et al., 2000; Seals et al., 2000; Heider and Munson, 2012).

Most studies on tethers have been performed in the yeast or mammalian cell system while investigations in plants are scarce. In plants, Atp115 localizes to the Golgi and is important for anterograde ER-Golgi transport. Point mutant forms of Atp115 results in plants with dwarf-like phenotypes and accumulation of vacuole storage protein precursors in the ER (Kang and Staehelin, 2008). Interestingly no plant homologs of giantin and GMAP130 have been identified (Latijnhouwers et al., 2005). GRIP-related ARF-binding domain-containing Arabidopsis protein 1 (GDAP1) was identified as a plant homolog of GMAP-210, a golgin that binds microtubule and maintains the Golgi by recruiting microtubular machinery (Rios et al., 2004). GRIP domains are required for targeting golgins to the TGN. AtGRIP has been predicted to be involved in tethering events at the TGN. A GRIP-like protein was identified in Arabidopsis and shown to localize to TGN secretory vesicles (Chen et al., 2008).

Recently, TNO1 was identified as a putative tethering factor that localizes on the TGN in Arabidopsis. It interacts with the Qa-SNARE SYP41 and is required for proper localization of SYP61, vacuolar cargo sorting and endosomal aggregation upon Brefeldin A treatment (Kim and
Bassham, 2011). It remains to be seen if it is an actual functional tether and whether it interacts with any downstream effectors such as Rabs and the cytoskeleton. TNO1’s physiological role has been further elucidated in this dissertation in Chapter 2 and Chapter 3.

4.3.3. Sec1/Munc18 (SM) proteins

Once the v- and t-SNAREs assemble into a four-helix bundle they are ready to catalyze fusion by forcing the membranes together. This bundle is referred as the trans-SNARE complex or the “SNAREpin”. Once the fusion pore is formed, the fully zippered trans-SNARE complex now becomes a cis-SNARE complex (Bassham and Blatt, 2008; Carr and Rizo, 2010; Risselada and Grubmuller, 2012). Sec1/Munc18 (SM) proteins interact with the SNARE machinery and are as essential for membrane fusion as SNAREs; the absence of SM proteins causes a blockage in membrane fusion (Toonen and Verhage, 2007; Südhof and Rothman, 2009; Carr and Rizo, 2010). They are evolutionarily conserved peripheral membrane proteins in a size range of 60-90kDa that either bind individual SNAREs or the SNARE complex (Südhof and Rothman, 2009), regulating SNAREpin assembly and speed of fusion (Gerber et al., 2008). In vitro reconstitution studies suggest that SM proteins accelerate SNARE mediated fusion (Shen et al.; Scott et al., 2004; Rodkey et al., 2008). They are proposed to function in switching syntaxins from a closed to open conformation and thus facilitate heterotypic fusion (Südhof and Rothman, 2009). SM proteins also prevent the formation of non-physiological SNARE complexes (Peng and Gallwitz, 2002; McBride et al., 1999).

There are 4 major classes of SM proteins identified in the mammalian cells, Sly1 acting at the ER-Golgi, VPS45 acting at the endosome-TGN, VPS33 acting at the endocytic/lysosomal system and Munc18 acting at the PM (Rizo and Südhof, 2012). Structural studies on eight SM
proteins show the presence of three domains that fold into an arch-shaped structure. Studies with VPS33 suggest that SM proteins might facilitate opening of the fusion pore during vesicle fusion (Pieren et al., 2010). Interestingly VPS33 is part of the multisubunit tethering complex, HOPS. VPS45 regulates endosome to TGN retrograde trafficking in mammalian and yeast cells and can bind to its cognate SNAREs at different stages of the assembly/disassembly cycle as well as by different mechanisms (Carpp et al., 2006).

The Arabidopsis genome encodes 6 SM proteins, SLY1, VPS45, VPS33 and three Sec1p homologs (Sutter et al., 2006). VPS33 localizes to the PVC and has been suggested to be required for maintaining vacuolar morphology and its homotypic fusion (Rojo et al., 2003). AtVPS45 interacts with the SYP41/SYP61/VTI12 complex and is important for vacuole formation and cell expansion (Bassham et al., 2000;Zouhar et al., 2009). VPS45 knockouts were gametophyte lethal and RNAi lines that had reduced levels of the proteins displayed defects in sorting of vacuolar cargo and reduced protein levels of the SNARE SYP41. Decreased uptake of an endocytic tracer dye in ben2 roots, a point mutant of VPS45, suggested VPS45’s role and location in the endocytic pathway (Tanaka et al., 2013). KEULE is a Sec1 homolog that interacts with the syntaxin KNOLLE and localizes to the cell plate during cell division (Assaad et al., 1996;Waizenegger et al., 2000;Assaad et al., 2001). It is hypothesized that KEULE converts KNOLLE to its open state. Defects in keule mutants could be rescued by expressing a constitutively “open” form of KNOLLE. Recent studies on the SM protein KEULE have also revealed a link between the tethering complex TRAPPII and delivery of KEULE to the edges of the developing cell plate (Waizenegger et al., 2000; Steiner et al., 2015). KEULE also plays a central role in coordinating membrane fusion and microtubule dynamics during cell division (Steiner et al., 2015).
5. Role of Cellular Trafficking in Plant Physiology and Development

Altogether, the process of cargo trafficking and vesicle fusion is dependent on the concerted action of multiple players that work in a synchronized fashion. The efficient functioning of the cellular trafficking machinery utilizing these components leads to efficient control of plant growth. Various players acting along these trafficking routes have important roles in regulating processes such as cell division, root development, nutrient acquisition, disease resistance and hormonal responses. We now discuss the role of cellular trafficking in some key processes, which will be studied in the subsequent chapters of this dissertation.

5.1. Auxin transport related processes

Cellular trafficking is required for proper localization of auxin transporters in a cell, which then directs polar auxin transport and mediates multiple developmental processes. Auxin is a prominent plant hormone and has an intricate signaling mechanism affecting the spatiotemporal aspects of plant developmental programs (Esmon et al., 2006; Tanaka et al., 2006; Dubrovsky et al., 2011; Tanaka et al., 2014). Auxin undergoes a fast nondirectional phloem transport and a slower polar transport from cell to cell via specific PM localized transporters (Figure 3A). The former mode of transport delivers auxin from site of synthesis to various organs, while the polar transport fine-tunes auxin levels required for controlling developmental programs in developing tissues (Adamowski and Friml, 2015). Auxin transporters belong to four main classes, the auxin transporter 1 (AUX1) and auxin-transporter-like protein family (LAX) (referred together as AUX1/LAX), the pin-formed proteins (PIN), P-glycoproteins (PGP) of the ATP-binding cassette (ABC) transporter family and the pin-likes (PILS) (Grones and Friml,
Recently a tonoplast-localized auxin transporter, WAT1 (walls are thin 1) has also been shown to be important for intracellular auxin homeostasis (Ranocha et al., 2013).

The specific positioning of the efflux and influx transporters maintains the polar flow of auxin. Rearrangement and repositioning of the transporters can influence auxin-dependent patterning thus affecting plant development. To establish this polar localization, auxin transporters undergo subcellular trafficking to the PM via the TGN, constitutive recycling between endosomes and PM, transcytosis to different polar plasma membrane domains (Kleine-Vehn and Friml, 2008; Peer et al., 2011) or targeting to the vacuole for degradation (Kleine-Vehn et al., 2008b) (Figure 3C). PIN proteins are responsible for auxin efflux from cells (Petrasek et al., 2006) with different members that can have different localization such as basal, apical or lateral PM (Wisniewska et al., 2006). The distinct polar localization of different PINs suggests a polarity determinant in the protein sequence, which is aided by the actions of the Ser/Thr protein kinase PINOID (PID) and protein phosphatase 2A (PP2A) (Friml et al., 2004; Michniewicz et al., 2007). Phosphorylation of PINs leads to recruitment into the apical targeting path while dephosphorylation leads to recruitment onto the basal pathway. This resembles the mammalian epithelial cells where cargoes undergo phosphorylation for polar delivery (Kania et al., 2014).

PIN proteins are constitutively endocytosed and recycled back to the plasma membrane (Friml, 2010) for enabling rapid changes in polarity. Studies of fluorescently-tagged auxin transporter proteins with the endocytosed membrane dye FM 4-64 demonstrated that transporters such as PIN1 and PIN2 constantly cycle from the plasma membrane to endosomes and back to the plasma membrane. The clathrin-mediated endocytosis inhibitor tyrphostin A23 led to a loss of the internalization of PINs, suggesting that PIN endocytosis requires the clathrin machinery (Geldner et al., 2001; Dhonukshe et al., 2007). *Arabidopsis* mutants defective in clathrin structure...
and the dynamin related protein DRP1 also showed auxin transport defects. Studies have also shown that the dynamin related protein DRP1 interacts with PIN1 (Mravec et al., 2011). A study using chemical inhibitors that stall specific endocytic transport routes revealed that transport of certain auxin transporters was blocked while others remained unaffected. This suggests complex regulation of auxin transport utilizing multiple endocytic trafficking routes (Robert et al., 2008).

Multiple trafficking components have been identified for efficient subcellular trafficking of auxin transporters. The plant uses specific ARF-GEFs for targeting distinct auxin carriers along the apical-basal axis of the cell. ARF-GEFs such as GNOM, GNL1 and BEN1 are important for specific apical-basal targeting of the auxin carriers inside the cell. The endosomal regulatory ARF-GEF, GNOM, which mainly recycles from the endosomes to the PM, is required for basal targeting of auxin transporters such as PIN1 while apical targeting of PINs and AUX1 occurs independent of it, suggesting differences in endosomal trafficking dynamics between basal and apical routes. The fungal toxin Brefeldin A (BFA), a noncompetitive inhibitor that stabilizes ARF/ARF-GEF intermediates causing trafficking blockades, has been shown to affect GNOM (Kleine-Vehn et al., 2008a). Treatment with BFA leads to formation of endosomal aggregates and reversible accumulation of endocytosed cargo such as PIN1 in structures known as the BFA compartments though internalization is unaffected (Geldner et al., 2001; Geldner et al., 2003). BFA sensitive trafficking dynamics have also been demonstrated for other cargoes such as AUX1, the aquaporin PIP2, the plasma membrane H+-ATPase and the brassinosteroid receptor, BRI1 (Grebe et al., 2002) (Geldner 2001, Grebe 2002, 2003, Russinova 2004, Paciorek 2005).

ARF GAPs such as VASCULAR NETWORK DEFECTIVE 3 (VAN3) are also important regulators of endocytosis and internalization of auxin transporters (Naramoto et al.,
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... BEX1 (BFA VISUALIZED EXOCYTIC TRAFFICKING DEFECTIVE1) encodes ARF, ARF1A1C that localizes to the TGN/EE and the Golgi apparatus and is required for PIN recycling (Tanaka et al., 2014). VAN4 localizes to the TGN/EE and might be involved in recycling of PIN proteins thus affecting venation development. VAN4 encodes a putative subunit of the TRAPII complex functioning as a Rab-GEF effector and/or tethering factor (Jones et al., 2000; Sacher et al., 2008; Naramoto et al., 2014). Hence tethering is also required for proper auxin transport. Other factors such as the sterol composition of the plasma membrane (Willemsen et al., 2003) and cytoskeleton mediates auxin transporter trafficking too (Zhu and Geisler, 2015). Auxin transporters are also targeted for degradation via the multivesicular body. Arabidopsis mutants defective in the ESCRT-III subunits show an accumulation of PINs on the surface of the MVB as intraluminal vesicle formation is hampered (Spitzer et al., 2009). This highly dynamic sorting network for auxin carriers provides fine control over plant development, growth and polarity.

5.1.1. Lateral root emergence

The emergence of LRs form a primary root is dependent on auxin transport and subcellular dynamics of auxin transporters. Lateral roots initiate in the pericycle and cross the endodermis and subsequently the cortex and epidermis. Auxin flux is important for lateral root initiation steps and specification of the lateral root founder cells. AUX1 is expressed in the pericycle and mutants display 50% reduction in number of LRs (Hobbie and Estelle, 1995) since it is required for loading auxin into the vascular transport system (Marchant, 2002). The induction of PIN3 expression and trafficking in the endodermal cells adjacent to the lateral root primordium localizes the transporter to membrane facing the primordium. This burst of auxin
transport thus aids subsequent development of the lateral root primordium. The expression of
LAX3 in the cortical and epidermal cells situated in front of the LR primordial and the presence
of auxin maxima in the primordia led to the discovery that LAX3 is auxin inducible essential for
LR emergence (Swarup et al., 2008;Peret et al., 2013). Thus auxin from LR primordia enters the
cortex and induces LAX3 expression. This leads to increased uptake causing an increase in
auxin, which then results in an induction of cell wall remodeling enzymes. This can then cause
the progression of the primordia through the cortical cells by cell wall loosening.

5.1.2 Gravitropism

Gravitropism in plants is also dependent on the transport of auxin. Plant organs sense the
gravity vector and can orient their growth relative to it. Any deviation with respect to gravity is
sensed and transduced to a biochemical signal and physiological response leading to organ
curvature. Bending towards the gravity vector is referred to as positive gravitropism and vice
versa. Bending of roots towards gravity is an example of positive gravitropism, while stems
growing against gravity displays negative gravitropism (Figure 3B). The process of organ
curvature upon gravistimulation (change with respect to gravity vector) involves signal
transduction, auxin transport and cell expansion. Thus defects in cellular trafficking that regulate
these events can lead to gravitropic defects.

In vertically growing roots auxin flows down to vasculature to the columella in the root
tip. From here auxin flows out into the epidermal and cortical cell layers in a reverse fountain
flow model of auxin transport. The resorting of auxin transporters PIN3 and PIN7 in the
columella upon gravistimulation is required for root gravitropic bending (Friml et al.,
2002;Kleine-Vehn et al., 2010). PIN2 localizes to the shootward (facing the shoot/upwards)
faces in the lateral root cap and epidermal cells. Its relocalization to the lower side of the root upon gravistimulation helps build an auxin maxima required for the root bending response (Luschnig et al., 1998; Muller et al., 1998; Abas et al., 2006). The auxin importer AUX1 is also required for root gravitropic bending. It mediates auxin influx from the lateral root cap to the cells in the elongation zone in the lower side of the root post-gravistimulation hence driving differential growth. Degradative sorting of PIN2 to the vacuole also results loss of PIN2 activity in the upper side of the gravistimulated-root (Abas et al., 2006). Other trafficking components such as ALTERED RESPONSE TO GRAVITY 1 (ARG1/RHG) and ARG1-LIKE2 (ARL2/GPS4) localize to the ER, Golgi, adjacent to the PM and the cell plate in a BFA sensitive trafficking route. They are required for PIN3 relocalization in the columella cell post gravistimulation which then redirects auxin flow (Sedbrook et al., 1999; Guan et al., 2003; Harrison and Masson, 2008a; Harrison and Masson, 2008b) causing gravitropic bending.

5.2. Root growth: cell division, cell elongation and root hair growth

Root growth occurs due to a combination of cell division and cell elongation. The root apex consists of root initials that constantly divide to push cells shootward. Once these cells enter the elongation zone, they rapidly expand in an anisotropic fashion and finally stop expanding in the maturation zone (Petricka et al., 2012). Cell division in plants requires the formation of a cell plate that grows outwards from the center of the dividing cell. Cell plate formation is dependent on specialized secretory traffic from the TGN (Reichardt et al., 2007) and cell plate maturation coincides with clathrin mediated vesicle retrieval of the cell plate deposition machinery (Segui-Simarro et al., 2004). The proper secretion of wall constituents such as cellulose, pectin, hemicellulose and callose is required for cell plate formation (Samuels et al., 1995; Zuo et al.,
Three distinct cellulose synthase subunits also accumulate in the cell plate during early stages of cell plate assembly and undergo clathrin-mediated retrieval for relocalization to the periphery of the growing cell plate (Miart et al., 2014).

As the cells divide, they get pushed shootwards and enter the elongation zone. Rapid cell expansion in the root elongation zone is dependent on the cortical microtubulular arrangement and dynamics, vacuolar expansion and rapid cell wall deposition to maintain cell wall integrity (Crowell et al., 2010) (Figure 4). Therefore, defects in vacuolar trafficking and secretory trafficking of the cell wall are crucial for cell expansion. Microtubules (MTs) guide cellulose deposition and the transverse arrangement of MTs constrains cell elongation in the longitudinal axis due to a corresponding pattern of cellulose deposition (Li et al., 2014). Multiple mutants with defects in either MT dynamics or MT-associated proteins display defects in cell expansion (Ishida et al., 2007; Sedbrook and Kaloriti, 2008; Hamada, 2014). Mutants with defective secretory machinery at the TGN cause also cell expansion defects. Disruption of the TGN-localized H+-ATPase, VHA-a1 leads to defects in the cell elongation and cellulose content (Brux et al., 2008). The echidna mutant also displays a dwarf phenotype and cell expansion defects. ECHIDNA is a TGN-localized protein required for trafficking cell wall polysaccharides such as pectin (Gendre et al., 2011). Disruption of the AP1 adaptor protein complex, involved in clathrin mediated endocytosis, also causes cell elongation defects (Teh et al., 2013). The TGN SNARE SYP61 is required for secretion of multiple cellulose synthase complex subunits required for cellulose deposition, as discovered by proteomic analysis of SYP61 labeled vesicles (Drakakaki et al., 2012). The SYP61 proteome also houses pectin methylesterases that are enzymes required for cell wall extensibility. Thus, the secretory machinery at the TGN seems to be crucial for
depositing the cell wall as the cell expands. This works in conjunction with vacuolar trafficking and MT dynamics.

Root hair development from the primary root also requires rapid polarized cell expansion where the length of the cell is much larger than the width. Root hairs can grow to 1mm or more in length while maintaining a small diameter (~10um). Root hairs develop from the base of specialized root epidermal cells known as trichoblasts and function to increase the surface area of roots for water and nutrient acquisition while also anchoring plants (Grierson et al., 2014). Once root hair fate has been determined, the cells elongate in a tubular manner by a rapid mode of tip directed unidirectional requiring high volumes of exocytosis (Grierson et al., 2014). Root hairs of Arabidopsis exhibit almost 9000 exocytosis events per minute translating to a growth rate of 1 micron per minute (Ketelaar et al., 2008). There is rapid turnover of cargo vesicles, membrane and cytoskeletal elements during tip growth and SNARE mediated vesicle fusion is important for root hair expansion. The ARF-GEF GNOM is also required for root hair growth. Mutants lacking the Sec1/Munc18 protein KEULE have stunted and radially swollen root hairs leading to a proposed role for KEULE in facilitating vesicle fusion at the growing root hair tip (Lukowitz et al., 1996). The PM SNAREs SYP123 and SYP132 play crucial roles in root hair elongation too with the possibility of SYP132 mediating non-polar secretion while secretory vesicles are recycled and focally delivered to the root hair tip by the SYP123 machinery (Ichikawa et al., 2014). Vesicle secretion also depends on Rab GTPases and their activity at the root tip mediating tethering of vesicles to the growing membrane (Grierson et al., 2014).

In summary, we find that vesicle transport is dependent on close co-ordination of multiple molecular components. Plants show considerable complexity in their repertoire of
proteins regulating vesicle transport, with specialized mechanisms having evolved to regulate plant physiology and development.

6. Organization of the Thesis

This dissertation summarizes my findings on the role of two proteins, TNO1 and VPS45, that function at the TGN, in diverse physiological processes. Both, TNO1 and VPS45, associate with the TGN localized SYP41/SYP61/VTI12 SNARE complex and are known to function in vacuolar trafficking.

In Chapter 1, I discuss the plant endomembrane system and various steps of plant cellular trafficking with examples from the literature of the various components involved at each step and their physiological function.

Chapter 2 reports findings on the role of TNO1 in auxin transport related processes. My experiments showed the involvement of TNO1 in auxin dependent lateral root emergence from the primary root and gravitropic bending responses. Rescue of these physiological defects with exogenous auxin, sensitivity to natural and a cell influx-specific auxin and imaging of auxin promoter driven fluorescence in mutant roots confirmed TNO1’s role in auxin transport dependent processes. I conceived this project with extensive guidance from my major professor, Dr. Diane Bassham. I performed all the experiments associated with this chapter and wrote the manuscript with extensive feedback from my mentor, Dr. Diane C. Bassham and suggestions from Dr. Dior Kelley for publication in the peer reviewed journal *Frontiers in Plant science*.

In Chapter 3, I report the role of TNO1 in mediating a kind of root growth movement known as skewing. Loss of TNO1 causes exaggerated rightward movement from the expected growth trajectory when grown on a slanted, impenetrable media. The exaggerated skewing in the
mutants correlates with increased epidermal cell file rotation and mutant root skewing also displays altered sensitivities to microtubule-disrupting drugs. This suggests the role of TNO1 in microtubule-associated processes. These results, combined with the studies in Chapter 1, suggest that TNO1 might function at the TGN in auxin transport and microtubule dependent processes. This body of work was conceived and executed by me, with guidance from Dr. Bassham. I wrote the chapter with feedback from Dr. Bassham.

In Chapter 4, I report on the effect of a point mutation in the Sec1/Munc18 protein VPS45. The point mutants, Atvps45-3, display a dwarf phenotype with reduced cell expansion in roots and hypocotyls and short, thick root hairs. Bulk endocytic and secretory routes are unaffected in the mutants suggesting a specific effect on trafficking during cell expansion. I worked on this project with guidance from Dr. Bassham and wrote the chapter with her feedback.

Included in the appendix is an invited review article on the phenomenon of root growth movements, published in the journal *Plant Science*. I wrote this article with Dr. Bassham.

7. References


Figure 1: Schematic overview of the plant cellular trafficking routes: Cargo is trafficked from the Endoplasmic Reticulum to the Golgi and onto the trans-Golgi network via the anterograde trafficking route. Cargo can also be recycled back between the compartments by retrograde trafficking routes. The trans-Golgi network, which matures from the Golgi, acts as a sorting station driving 1. Vacuolar traffic via late endosomes/prevacuolar compartments/ multivesicular bodies (MVBs) 2. Secretory traffic or exocytosis to the plasma membrane and 3. Endocytic recycling of PM cargo, which can subsequently also be directed to vacuole (via MVBs).
Figure 2: Overview of cellular trafficking and membrane fusion steps: Soluble or membrane
bound cargo is recruited from donor sites and vesicular budding is initiated by coat proteins. The
cargo vesicle membrane consists of a v-SNARE that interacts with a specific t-SNARE complex
on the target site. After uncoating and transport of the cargo vesicle, approach and docking of the
vesicle to its donor site is facilitated by the action of tethering factors. Tethering factors increase
specificity of the v- and t-SNARE interaction and drive trans-SNARE complex formation along
with the aid of Sec1/Munc18 (SM) proteins. The trans-SNARE complex drives fusion of the
cargo vesicle with the target membrane releasing its cargo. The cis-SNARE complex is then
recycled to reassemble the t-SNARE complexes and v-SNAREs to drive subsequent cycles of
membrane fusion.
Figure 3: Auxin transport and cellular trafficking: A. The polar organization of auxin influx and efflux transporters determines polar flow of auxin in plant organs. B. Conditions such as gravistimulation (change in orientation with respect to gravity) result in the change of auxin flow leading to asymmetric flow and accumulation of auxin on the new physiological bottom. This then results in ether upward bending in shoots (negative gravitropism) or downward bending (positive gravitropism) in roots due to the varying auxin levels’ effect on cell expansion rates. C. At the cellular level this change in auxin flow occurs due to the subcellular trafficking of auxin transporter to different faces of the now gravistimulated organ or to the vacuole for degradation. These processes then change auxin transport dynamics resulting in a change in auxin flow patterns and change in expansion rates.
Figure 4: Cellular trafficking and cell expansion: Left panel- Elongating cells displaying anisotropic expansion possess a transverse array of microtubular arrays to constrain the elongation axis. This leads to a transverse deposition of cellulose microfibrils (which are guided by microtubules) and anisotropic expansion as the vacuole expands. Right panel- Vesicular trafficking routes at the TGN play crucial roles in anisotropic cell expansion. Trafficking to the vacuole leads to increase in vacuolar size, which results in cell expansion. The efficient trafficking of the cellulose synthase complexes (CSCs) and cell wall deposition machinery from the TGN is also required for maintaining cell wall integrity as the cell expands.
CHAPTER 2

GRAVITROPISM AND LATERAL ROOT EMERGENCE ARE DEPENDENT ON THE TRANS-GOLGI NETWORK PROTEIN TNO1

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a paper published in \textit{Frontiers in Plant Science}

1. Abstract

The trans-Golgi network (TGN) is a dynamic organelle that functions as a relay station for receiving endocytosed cargo, directing secretory cargo, and trafficking to the vacuole. TGN-localized SYP41-interacting protein (TNO1) is a large, TGN-localized, coiled-coil protein that associates with the membrane fusion protein SYP41, a target SNARE, and is required for efficient protein trafficking to the vacuole. Here, we show that a \textit{tno1} mutant has auxin transport-related defects. Mutant roots have delayed lateral root emergence, decreased gravitropic bending of plant organs and increased sensitivity to the auxin analog 2,4-dichlorophenoxyacetic acid and the natural auxin 3-indoleacetic acid. Auxin asymmetry at the tips of elongating stage II lateral roots was reduced in the \textit{tno1} mutant, suggesting a role for TNO1 in cellular auxin transport during lateral root emergence. During gravistimulation, \textit{tno1} roots exhibited delayed auxin transport from the columella to the basal epidermal cells. Endocytosis to the TGN was unaffected in the mutant, indicating that bulk endocytic defects are not responsible for the observed phenotypes. Together these studies demonstrate a role for TNO1 in mediating auxin responses during root development and gravistimulation, potentially through trafficking of auxin transport proteins.
2. Introduction

The trans-Golgi network (TGN) is a highly dynamic tubulo-vesicular organelle that matures from the two or three trans-most cisternae of the Golgi (Staehelin and Kang, 2008) and is crucial for endocytic, secretory and vacuolar trafficking routes in plant cells. TGN cisternae move rapidly inside the cell, dissociating from their associated Golgi and re-associating with a new Golgi stack (Staehelin and Kang, 2008; Kang, 2011; Uemura et al., 2014), and also contain distinct subdomains for various trafficking routes (Bassham et al., 2000; Chow et al., 2008; Gendre et al., 2011). The TGN functions as an early/recycling endosome (Dettmer et al., 2006; Viotti et al., 2010) that receives endocytosed cargo, including auxin transporters, plasma membrane receptors, and nutrient transporters (Russinova et al., 2004; Robatzek et al., 2006; Dhonukshe et al., 2007; Takano et al., 2010; Barberon et al., 2011), and recycles it back to the plasma membrane or to the vacuole for degradation. The TGN plays a crucial role in trafficking of biosynthetic traffic to the vacuole (Reyes et al., 2011). It also directs secretory cargo, including plasma membrane proteins and cell wall polysaccharides, to the cell surface, potentially via mobile secretory vesicle clusters that fuse with the plasma membrane (Toyooka et al., 2009; Gendre et al., 2015). The position of the TGN at the junction of the endocytic, vacuolar, and secretory pathways renders it important in regulating transport of key molecules and mediating cellular responses to the environment (Park and Jurgens, 2011; Reyes et al., 2011; Contento and Bassham, 2012).

High transport fidelity is needed to prevent mis-sorting of cargo during vesicle trafficking. This requires membrane fusion proteins termed soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs; Risselada and Grubmuller, 2012). SNAREs can be broadly classified as target SNARES (t-SNAREs) or vesicle SNAREs (v-SNAREs).
depending on their location, or as Q or R-SNAREs based on the core amino acid in the heptad repeat of the SNARE motif (Fasshauer et al., 1998). The interaction between a v-SNARE on a vesicle and t-SNAREs on its target membrane leads to membrane fusion via formation of a tetrameric trans-SNARE complex (McNew et al., 2000; Lipka et al., 2007; Kim and Brandizzi, 2012; Risselada and Grubmuller, 2012). Usually, three Q SNAREs (Qa, Qb, and Qc) form a t-SNARE complex and an R-SNARE acts as the v-SNARE. This interaction of SNAREs helps to overcome the thermodynamically unfavorable event of fusion of two hydrophobic lipid bilayers (Risselada and Grubmuller, 2012; Shi et al., 2012), enabling deposition of cargo into the target organelle. Proteins known as tethering factors aid in bringing membranes together and promoting SNARE interaction or actively stimulating trans-SNARE complex formation (Cai et al., 2007). Tethering factors thus increase vesicular trafficking efficiency (Sztul and Lupashin, 2009; Chia and Gleeson, 2011; Hong and Lev, 2014) and are either homodimeric long coiled-coil proteins or multisubunit tethering complexes (Markgraf et al., 2007; Hong and Lev, 2014). It was recently proposed that coiled-coil tethers simultaneously use their multiple coiled-coil domains to engage distinct SNAREs and promote SNARE complex assembly (Grabski et al., 2012). Spatiotemporal regulation of trafficking steps in the cell also requires a family of small GTPases called Rabs which then recruit downstream effectors (Stenmark, 2009). Tethers can act as Rab effectors by binding SNAREs or can function as GTP exchange factors (GEFs) for Rabs (Markgraf et al., 2007; Sztul and Lupashin, 2009; Hong and Lev, 2014).

Many SNAREs and tethering factors exist in Arabidopsis thaliana, with different localizations reflecting their specialized roles (Fujimoto and Ueda, 2012). The TGN-localized SYP4 (41/42/43) SNARE family (Bassham et al., 2000; Uemura et al., 2012) is responsible for maintaining Golgi/TGN morphology and regulating secretory and vacuolar trafficking (Uemura
et al., 2012). Another TGN SNARE, SYP61, interacts with SYP41 and helps to direct traffic to the plasma membrane (Drakakaki et al., 2012), with a role in mediating stress responses (Zhu et al., 2002). Recently, TGN-localized SYP41-interacting protein (TNO1), a large coiled-coil protein localized to the TGN, was identified as a SYP41 interactor (Kim and Bassham, 2011) and was hypothesized to be a tethering factor. Mutant plants lacking TNO1 partially mis-sort vacuolar cargo and mis-localize SYP61, suggesting decreased trafficking fidelity, while also showing hypersensitivity to salt and osmotic stress and displaying altered TGN dynamics (Kim and Bassham, 2011).

SYP42 and SYP43 play a role in root gravitropism, most likely via regulation of the localization of the auxin efflux transporters PIN-FORMED 1 (PIN1) and PIN2 (Uemura et al., 2012). Additionally, other proteins involved in auxin transport, such as the AUXIN1/LIKE-AUX1 (AUX1/LAX) family of auxin influx transporters and P-glycoprotein (PGP) proteins of the ATP-binding cassette transporter family, are localized via the SNAREs and TGN activity (Kleine-Vehn and Friml, 2008; Rakusová et al., 2015). Many of the auxin transporters undergo constitutive endocytosis, cycling between the recycling endosome/TGN and the plasma membrane, or are targeted for vacuolar degradation to maintain steady-state levels (Kleine-Vehn and Friml, 2008; Grunewald and Friml, 2010). Defects in TGN dynamics can, therefore, hamper recycling of these transporters and thus affect directional transport of auxin, which is critical for plant development (Grunewald and Friml, 2010).

Given the potential links between TGN-mediated protein trafficking and auxin transporters, we investigated auxin responses in the tno1 mutant during root development. Loss of TNO1 delayed lateral root (LR) emergence and decreased root and hypocotyl gravitropic bending. Additionally, tno1 roots failed to display characteristic asymmetry visualized with the
auxin response marker \textit{DR5rev:GFP} at the LR tips as well as after gravistimulation. Thus, TNO1 functions in auxin-mediated root development and response to gravity.

3. Materials and Methods

3.1. Plant material and growth conditions

The \textit{A. thaliana} seed stocks used in this study have been previously described: Col-0 (wild-type, WT), \textit{tno1} knockout mutant (SALK_112503; Kim and Bassham, 2011), complemented \textit{tno1} mutant (Kim and Bassham, 2011), and \textit{DR5rev:GFP} (Ottenschlager et al., 2003).

\textit{Arabidopsis} seeds were surface-sterilized in 33% bleach, 0.1% (v/v) Triton X-100 for 20 min, rinsed five times with sterile water and kept in the dark at 4°C for at least 2 days. \textit{Arabidopsis} plants were grown at 22°C in long-day conditions (16 h light) in soil or on 0.25× or 0.5× solid Murashige–Skoog (MS) medium (MS vitamin and salt mixture, Caisson, MSPA0910) with 1% sucrose, 2.4 mM MES (pH 5.7), and 0.6% (w/v) phytoblend agar (Caisson, PTP01).

3.2. Gravitropism assays

The hypocotyl gravitropism assay was modified from Stanga et al. (2009). Seeds were plated on 0.5× MS medium (Murashige and Skoog, 1962) containing 1% sucrose in square plates and kept vertically oriented in the dark. After 5 days, the plates were rotated 90° for gravistimulation. Pictures were acquired using a Canon Rebel XTS camera in a dark room with a green filter over the flash to prevent phototropic curvature of the hypocotyl toward the camera flash. Zero-hour images of each seedling were compared to later time point images of the same seedling using Image J (Schneider et al., 2012) to determine bending angles.
For root gravitropic assays, sterilized seeds were mixed with molten growth medium just before solidification and poured into square plates (Stanga et al., 2009). After 5 days of vertical growth the seedlings were gravistimulated by rotating the plate by 90°. Pictures were taken 6 and 24 h after gravistimulation and analyzed using Image J to assess gravitropic curvature.

For root and hypocotyl gravitropic rescue assays, the roots of 5-day-old vertically grown seedlings were overlaid with media containing either 100 nM 1-napthylacetic acid (1-NAA; Sigma–Aldrich, N0640), 30 nM 3-indoleacetic acid (IAA; Sigma–Aldrich, I2886), or 30 nM 2,4-dichlorophenoxyacetic acid (2,4-D; Gibco, 11215), followed by gravistimulation. Pictures of roots and hypocotyls after 12 h were compared to 0-h images using Image J. For all assays, at least three biological replicates were performed, with 15–20 seedlings per replicate.

3.3. LR density analysis

To determine the density of emerging LRs, sterilized Arabidopsis seeds were plated on 0.25× MS medium (Murashige and Skoog, 1962) containing 1% sucrose and grown vertically. After 10 days, the number of LRs emerging from the primary root were counted and divided by the root length to obtain LR density. For determination of LR primordia density, 7-day-old roots were cleared with 2.5% bleach for 10 min and visualized with an Olympus IX-71 inverted microscope. To assess rescue of LR emergence, 5-day-old seedlings were transferred to medium containing 100 nM 1-NAA or 1 μM IAA in the dark (to prevent photo-degradation). After 5 additional days, the number of emergent LRs was scored. For each analysis, three independent biological replicates were performed with 15–20 seedlings per replicate.
3.4. Root length inhibition assays

Seedlings were grown on 0.5× MS medium (Murashige and Skoog, 1962) with 1% sucrose for 5 days. They were then transferred to media containing either 2,4-D, IAA, 1-NAA, 1-N-naphthylphthalamic acid (NPA; Naptalam, Sigma–Aldrich, 33371), 1-napthoxyacetic acid (1-NOA; Sigma–Aldrich, 255416) at the concentrations indicated, or solvent controls [dimethyl sulfoxide (DMSO) or ethanol], and the position of the root tip was marked. Each plate contained all three genotypes (WT, tno1 mutant, and complemented lines) to compensate for possible effects of inter-plate variation. After 6 days, pictures were acquired and the length from the marked root tip position was measured using ImageJ (Schneider et al., 2012). Root elongation in the presence of the tested chemicals was compared with solvent controls and expressed as percent decrease in root length. At least three biological replicates with a minimum of 20 seedlings per replicate were conducted for each treatment.

3.5. Analysis of auxin response distribution

tno1 plants expressing DR5rev:GFP were generated by crossing tno1 (Kim and Bassham, 2011) with DR5rev:GFP lines (Ottenschlager et al., 2003). Homozygous tno1 plants carrying the DR5rev:GFP transgene were selected in subsequent generations by polymerase chain reaction (PCR)-based genotyping as previously described (Kim and Bassham, 2011) and green fluorescent protein (GFP) fluorescence visualization. For analysis of auxin redistribution, control and tno1 roots expressing DR5rev:GFP were gravistimulated and visualized with a Leica SP5 confocal laser scanning microscope (Leica Microsystems) at the Iowa State University Confocal and Multiphoton facility. A 40× oil immersion objective lens was used along with excitation and emission wavelengths of 488 and 507 nm for GFP visualization. Images were acquired under
identical conditions for both mutant and WT roots with equal exposure, scan frequency and line average settings. A total of 15 seedlings from at least three independent replicates were analyzed. GFP asymmetry was quantified using Image J by subjecting the confocal images to similar thresholding and post-processing. Equal volume boxes were drawn on the upper and lower flanks and total pixel intensity from the lower vs. upper box was expressed as a ratio. To analyze 

\textit{DR5rev:GFP} expression in LRs, a total of 30 stage II LRs from at least six different seedlings were imaged by confocal microscopy for each genotype. The number of root tips showing asymmetry of GFP expression was counted and expressed as a percentage of the total observed.

\textbf{3.6. FM4-64 staining and Brefeldin A (BFA) treatment}

FM4-64 staining was modified from Dettmer et al. (2006). To test bulk endocytosis, 4-day-old seedlings were transferred to 0.5× MS liquid medium containing 4 μM FM4-64 for 2 min and subsequently washed twice for 30 s each time in 0.5× MS liquid medium before microscopic analysis. For analyzing arrival of FM4-64 at Brefeldin A bodies, 4-day-old seedlings were transferred to 0.5× MS liquid medium containing 35 μM Brefeldin A (BFA) for an hour followed by a 10-min treatment with 4 μM FM4-64 plus 35 μM BFA and two subsequent washes of 30 s each. The root tips were visualized using a Leica SP5 confocal laser scanning microscope (Leica Microsystems) at the Iowa State University Confocal and Multiphoton facility, using a 63× oil immersion objective lens and excitation and emission wavelengths of 558 and 734 nm. Images were acquired under identical conditions for both genotypes with equal exposure, scan frequency and line average settings. A total of 15 seedlings from at least three independent replicates were analyzed for each treatment and genotype.
4. Results

4.1. TNO1 mediates LR emergence in an auxin-dependent manner

Auxin signaling pathways are integral to LR development during root architecture establishment (Lavenus et al., 2013). Polar auxin transport mediates emergence of LRs from the primary axis, and several auxin transport mutants display slow rates of emergence (Hobbie and Estelle, 1995; Ruegger et al., 1997). The SYP4 family of SNAREs is thought to be involved in directional auxin transport (Uemura et al., 2012), hence we tested whether the SYP41-interacting protein TNO1 may also be required for such processes. We hypothesized that if the tno1 mutant has defects in auxin transport or responses, visible phenotypes such as changes in LR emergence would be evident. Emergent LR densities were significantly lower ($P < 0.05$) in tno1 seedlings after 10 days of growth on 0.25× MS medium (Dubrovsky and Forde, 2012). Mutant lines expressing transgenic TNO1 under the control of its native promoter, termed complemented lines (Kim and Bassham, 2011), were similar to WT seedlings (Figures 1A, B). Since the major source of auxin in the root in the first 10 days after germination is transport from the leaves (Hobbie and Estelle, 1995; Ljung et al., 2001), one possible reason for this defect may be that auxin flux is reduced in tno1, leading to suboptimal auxin levels in the root and impairing LR emergence. To confirm that the delayed emergence was not due to an arrest or delay in LR initiation, the number of LR primordia was evaluated in the mutant roots. The LR primordia density of tno1 roots was equivalent to that of WT and complemented lines (Figure 1C), suggesting that LR initiation events were normal in tno1.

The lipophilic auxin 1-NAA has been shown to rescue LR defects in the auxin transport mutant aux1 (Marchant et al., 2002). Natural auxin (IAA) treatment also rescues LR emergence in dark conditions (Reed et al., 1998). To test whether the emergence defect may be due to
defects in auxin transport and hence reduced root auxin levels, LR emergence in the presence of 1-NAA (Murashige and Skoog, 1962; Marchant et al., 2002) or IAA in the dark (Reed et al., 1998) was assessed. Five-day-old seedlings were transferred to medium containing 100 nM NAA or 1 μM IAA, and LR density was analyzed after 5 days of growth. The LR density of tno1 resembled that of WT and complemented lines in the presence of either auxin (Figure 1D), indicating that exogenous auxins can rescue the emergent LR defect in tno1 roots. Therefore, TNO1 influences the temporal control of LR emergence from the primary root.

4.2. TNO1 is required for gravitropic bending

We further examined a possible role for TNO1 in plant auxin responses by investigating gravitropism. Gravitropism involves the bending of a plant organ in response to its change in orientation with respect to gravity (gravistimulation). Gravitropic bending of organs upon gravistimulation is aided by a readjustment of auxin flow. Since a number of proteins involved in gravitropic curvature are recycled and trafficked via the TGN (Strohm et al., 2012), this organelle is critical for regulating downstream events that influence gravitropic plant organ bending. We, therefore, hypothesized that loss of TNO1 might affect dynamics at the TGN and thus lead to a change in the gravitropic bending response.

To test this hypothesis, the gravitropic response of WT and tno1 mutant hypocotyls and roots was assessed. The tno1 mutant hypocotyls (Figure 2A) and roots (Figure 2B) showed delayed bending, and the angle of curvature at different time points was significantly lower ($P < 0.05$) than that of WT or complemented seedlings. This suggests that the loss of TNO1 causes a delay in the gravitropic bending of Arabidopsis roots and hypocotyls. Mutant roots and
hypocotyls have a similar length to WT under normal growth conditions (Kim and Bassham, 2011), suggesting that the defect in gravitropism is not due to defective growth.

The gravitropic bending response requires a reprogramming of auxin flow inside the shoot and root (Tanaka et al., 2006; Strohm et al., 2012), raising the possibility that the slower bending rate observed in tno1 could be due to changes in auxin flow. We hypothesized that if the gravitropic phenotype in tno1 mutants is due to defect(s) in auxin transport, it may be rescued by exogenous auxin application. To test this idea, we used the membrane-permeable auxin 1-NAA, which has been previously used to rescue gravitropic root bending defects in mutants defective in auxin transport (Marchant et al., 1999), the natural auxin IAA, and the influx-specific auxin 2,4-D. Following auxin treatments, the bending angles of tno1 roots were not significantly different ($P > 0.1$) from the bending angles of the WT and complemented lines (Figure 2C), indicating rescue of the gravitropic bending defect by exogenous auxin. The 2,4-D treatment led to an overall reduction of bending angles as has been previously reported (Surpin et al., 2005) but there were no significant differences between WT and mutant. Similarly, hypocotyl bending was also rescued after a 12-h treatment with either IAA or 1-NAA (Figure 2D), confirming that the gravitropic defect in tno1 mutants is most likely due to auxin-related defects. 2,4-D did not rescue the gravitropic defect in the mutant hypocotyls, with bending angles significantly lower than WT and complemented lines; this is consistent with previous reports demonstrating an effect of 2,4-D on gravitropism in roots but not in hypocotyls (Surpin et al., 2005). The tno1 mutant defects in gravitropic bending, therefore, may be due to defects in auxin transport or response.
4.3. *tno1* roots show increased sensitivity to 2,4-D and IAA

Auxin flux into plant cells is facilitated by influx carriers such as AUX1 and the LAX family of transporters, while exit from the cell involves efflux carriers belonging to the PIN and PGP families (Peer et al., 2011). Auxin analogs and transport inhibitors are important tools to uncover defects in auxin transport pathways. The auxin analog 2,4-D is an influx-specific substrate while 1-NAA is an efflux-specific substrate (Delbarre et al., 1996). The auxin transport inhibitor NPA interferes with auxin efflux while 1-NOA interferes with auxin influx and inhibits AUX1 (Thomson et al., 1973; Sussman and Goldsmith, 1981; Yang et al., 2006). To determine the effect of these inhibitors on the *tno1* mutant, their effects on root growth were assessed. We found that *tno1* roots were significantly (*P* < 0.05) more sensitive to 30 nM 2,4-D than WT or complemented roots (Figure 3A). This result is consistent with our observation of the failure of 2,4-D to rescue the gravitropic defect of mutant hypocotyls.

One possible explanation for this sensitivity is that influx routes are altered in the *tno1* mutant, which led us to test the effect of blocking auxin influx with 1-NOA. Mutant roots showed similar sensitivity to WT and complemented lines across a range of concentrations of 1-NOA (Figure 3B), suggesting that blocking auxin influx affects the *tno1* mutant to the same extent as wild-type. 1-NOA has a characteristic protective effect against root growth inhibition by 2,4-D (Parry et al., 2001); we, therefore, tested whether 1-NOA could rescue the root growth inhibition of mutant roots by 2,4-D to comparable levels as in the WT and complemented lines. Seedlings were transferred to medium containing 30 nM 2,4-D and 10 μM 1-NOA. The WT and complemented lines showed a significantly lower inhibition of root length than the mutants (*P* < 0.05), indicating that when auxin influx is blocked, *tno1* mutants still have enhanced sensitivity to 2,4-D (Figure 3C).
To determine whether the sensitivity to 2,4-D is also seen with other auxins, root growth inhibition on 40 nM IAA in the dark was assessed. *tno1* mutant roots are indeed more sensitive to IAA (*P* < 0.05) than WT or complemented lines (Figure 3D). By contrast, treatment with 0.1 μM of the membrane-permeable NAA inhibited the growth of *tno1* to a similar extent as WT and complemented plants (Figure 3E). Since 2,4-D and IAA are influx-specific substrates and NAA is not, the results are consistent with a possible defect in auxin influx pathways in *tno1*. *tno1*, WT, and complemented plants had similar responses to NPA treatment (Figure 3F), suggesting that the auxin efflux pathway is unaffected in *tno1*.

### 4.4. TNO1 helps mediate auxin responses during LR elongation

Lateral roots emerge perpendicular to the primary root and gradually start bending rootwards. Recently emerged LRs, which lack an elongation zone (stage I LR) gradually transition into stage II LRs, defined by an elongation zone and asymmetric growth of the upper and lower epidermal cell files (Rosquete et al., 2013). The differential growth peaks in the stage II LRs are due to asymmetric auxin distribution, which in turn is dependent on auxin transport mechanisms. Stage II LRs are thus defined by a characteristic asymmetric distribution of auxin response, which results in differential cellular elongation and downward root growth. This asymmetric auxin flux at the LR tip is caused by the specific activity and distribution of auxin transporters (Rosquete et al., 2013). The asymmetric auxin distribution pattern diminishes significantly as the LRs mature to Stage III and begin growing parallel to the primary root. It was reported that on average more than 75% of stage II LRs in WT plants display asymmetric distribution of auxin responses at LR tips (Rosquete et al., 2013), visualized by the pattern of *DR5rev:GFP* expression (Friml et al., 2003).
Since TNO1 is linked to LR emergence by a possible auxin-dependent mechanism, we hypothesized that asymmetric auxin transport or response is defective in tno1 LRs. Hence, the percentage of LRs showing asymmetric auxin responses in stage II tno1 LR tips would be lower compared to WT (or complemented) root tips. To test this, the percentage of stage II LRs with auxin response asymmetry in WT and tno1 background lines expressing DR5rev:GFP was determined by confocal microscopy. 40% of tno1 stage II LRs displayed auxin asymmetry at the LR tip compared to 80% in WT stage II LRs (Figures 4A, B). Thus, tno1 roots are defective in establishing the correct pattern of auxin response during LR development. This defect could explain the lower emergent LR density observed in tno1 compared to WT, since a disruption of auxin flow or establishment of asymmetry would prevent LR emergence.

4.5. TNO1 facilitates shootward auxin response during root bending

Normally, gravitropic bending of the primary root leads to an eventual return of the root tip toward the gravity vector. This occurs via a cascade of signals leading to asymmetric auxin flow from the columella cells to the lateral rootcap cells and eventually shootward through the epidermal and cortical cells (moving toward the root–shoot junction). During this process, auxin accumulates on the lower side of the root, leading to growth inhibition, while the top of the root continues to elongate, thus resulting in downward root bending (Ottenschlager et al., 2003; Kleine-Vehn et al., 2010; Baldwin et al., 2012). Since tno1 roots are defective in gravitropic bending (Figure 2) and the frequency of LR tips displaying auxin response asymmetry is lower than in WT LRs (Figure 4), we hypothesized that the defect in root bending could be due to a decrease in shootward transport of auxin from the columella to the lateral rootcap cells and on to the distal elongation zone.
To test this hypothesis, vertical plate-grown WT and *tno1* seedlings expressing *DR5rev:GFP* were gravistimulated by rotating the plates by 90°. Confocal images were acquired before gravistimulation and subsequently at 5, 8, and 12 h after gravistimulation for both WT and *tno1* root tips (Figures 5A–D). In the WT background, GFP fluorescence progressed shootward from the columella toward the lateral rootcap and epidermal cells of the lower side of the root. Conversely, the shootward appearance of fluorescence in *tno1* was severely inhibited and failed to reach WT levels (arrowheads in Figures 5B–D). The difference in GFP fluorescence intensity on the upper and lower flanks was expressed as a ratio for each genotype at different time points. This ratio was significantly higher for WT roots than for *tno1* mutants at all time-points, suggesting a stronger response in WT than in the mutants (Figure 5E). This in turn implies that the defect in gravitropic bending in *tno1* mutants may be due to a decrease in auxin transport, or a difference in auxin response, from the columella to the lateral rootcap and epidermal cells on the lower side of the gravistimulated root. Such a defect could explain the reduced bending observed in *tno1* roots. Since auxin asymmetry within LR tips has been suggested to occur by a similar mechanism (Rosquete et al., 2013), this could also explain the lower percentage of stage II LRs displaying auxin response asymmetry in *tno1*.

### 4.6. TNO1 is not required for bulk endocytosis

Auxin transporters such as the PIN proteins are continuously endocytosed to the TGN and recycled back to the plasma membrane to maintain an appropriate density, thus allowing steady auxin flux through plant cells (Kleine-Vehn and Friml, 2008; Friml, 2010). Defects in endocytosis and components involved in the endocytic route to the TGN can, therefore, cause a defect in auxin transport pathways. Since *tno1* mutants show defects in TGN dynamics (Kim and
Bassham, 2011) and display auxin-related defects, we hypothesized that loss of TNO1 might alter endocytic routes and/or arrival of cargo at the TGN. This in turn would manifest in altered auxin transporter trafficking dynamics, and subsequently alter auxin responses. To test this hypothesis, we analyzed endocytosis in root epidermal cells using the lipophilic styryl dye FM4-64, which is endocytosed and labels early endosomes. To capture early endocytic events, roots of 4-day-old seedlings were stained with 4 μM FM4-64 for 2 min and then washed twice for 30 s each time in MS medium. Both WT and tno1 root cells showed similar endosome labeling patterns (Figure 6A, yellow arrowheads), suggesting that bulk endocytic uptake is normal in tno1 roots.

To confirm that bulk endocytosis was unaffected and that endocytic vesicles are delivered normally to the TGN in the mutant, the incorporation of FM4-64 into BFA bodies was assessed. The fungal toxin BFA causes aggregation of the TGN and TGN-derived endosomes to form BFA bodies (Geldner et al., 2001; Dettmer et al., 2006; Lam et al., 2009). BFA body formation is delayed in tno1 cotyledons but occurs at similar rates in root cells of WT and tno1 mutants (Kim and Bassham, 2011). It was hypothesized that if tno1 has defects in the delivery of endocytosed material to the TGN, then FM4-64 labeling of BFA bodies in tno1 roots would be reduced compared with WT roots. To test this hypothesis, 5-day-old WT and mutant seedlings were incubated with 35 μM BFA for an hour to allow formation of BFA bodies. They were then stained with 4 μM FM4-64 for 10 min in the presence of BFA, followed by two washes in MS medium containing BFA. The BFA bodies in both WT and tno1 roots showed normal FM4-64 staining (Figure 6B, white arrowheads) after 10 min. Thus, loss of TNO1 does not affect bulk endocytosis and membrane flow from the plasma membrane to the TGN in root cells.
5. Discussion

Auxin flow in plants is an important determinant of root architecture and tropic bending such as gravitropism. The polar transport of auxin depends on the specific localization of auxin transporters, which in turn is dependent on the cellular trafficking machinery. The TGN is an important organelle controlling vesicle trafficking, with the presence of multiple proteins that aid the trafficking of cargoes such as auxin transporters. In this study we have demonstrated a role for a putative tethering factor localized at the TGN in efficient gravitropic bending and LR emergence, possibly by affecting auxin transport.

5.1. TNO1 may regulate gravitropic responses through trafficking of auxin transporters

Auxin flows in a polar manner from the shoot to the root tip, via the vasculature, and then back through the cortical and epidermal cell layers shootward. This polar transport is dependent on the polar localization of auxin transporters to plasma membranes, via trafficking pathways that achieve correct subcellular distribution (Kleine-Vehn and Friml, 2008). Auxin transporters undergo endocytic recycling from the plasma membrane as well as being targeted to new plasma membrane domains via the TGN (Kleine-Vehn and Friml, 2008). This enables a plant to respond to environmental cues and adjust its growth pattern accordingly. For example, during the bending of roots upon gravistimulation, an accumulation of auxin in the epidermal layer of the elongation zone in the lower half of the root is required to facilitate the bending process (Tanaka et al., 2006; Peer et al., 2011). This occurs by the relocalization of auxin transporters, AUX1 in the columella and lateral rootcap and AUX1 and PIN2 in the epidermal cells (Strohm et al., 2012). The resulting readjustment of auxin flow from the columella via the lateral rootcap cell to the epidermal cells in the root elongation zone leads to differential growth (Ottenschlager et al.,
Since *tno1* has defects in intracellular trafficking and TGN dynamics (Kim and Bassham, 2011), we hypothesize that these may hinder auxin transport pathways due to change(s) in trafficking of auxin transporter(s).

Phenotypic characterization of gravitropic responses in *tno1* roots and hypocotyls showed that these mutants have a decreased angle of bending upon gravistimulation. Pharmacological studies with auxin transport inhibitors and exogenous auxin analogs suggest that altered auxin pathways are likely to be the underlying cause of this defect. The gravitropic bending defect in *tno1* roots and hypocotyls can be rescued by exogenous application of 1-NAA and IAA, perhaps by overriding endogenous auxin levels and routes of polar auxin transport, thus restoring auxin responses. Growth of *tno1* roots is hypersensitive to 2,4-D and IAA but not to the efflux-specific substrate 1-NAA or the efflux blocker NPA, raising the possibility that the auxin influx pathway might be defective while auxin efflux is normal. Although blocking auxin influx with 1-NOA does not result in large differences in root elongation between the *tno1* mutant and WT, the sensitivity to 2,4-D in the presence of 1-NOA is still higher for the *tno1* mutant roots than the WT and complemented lines. This, coupled with the inability of 2,4-D to rescue the gravitropic bending defect of mutant hypocotyls, may suggest altered responses to the influx-specific substrate 2,4-D. Additionally, the delayed progression of *DR5rev:GFP* expression in epidermal cells of gravistimulated *tno1* roots compared to WT roots suggests that auxin flow from the columella to the epidermis (via the lateral rootcap cells) may be reduced in *tno1* roots. Based on these results, we hypothesize that altered auxin influx capacity underlies the gravitropic defects in *tno1*. Given the role of TNO1, this could occur through altered trafficking of auxin influx carrier(s). However, it is also possible that the sensitivity to 2,4-D in the *tno1* mutant is due to
differences in auxin signaling, feedback or response, since it is known that the auxin sensing mechanism is complex and varies for different kinds of auxins (Calderón Villalobos et al., 2012). Since auxin transporters are trafficked via endocytosis (Kleine-Vehn and Friml, 2008), we hypothesized that TNO1 may affect endocytosis from the plasma membrane to the TGN/early endosomes and hence alter transporter trafficking dynamics. Bulk uptake and arrival of a general endocytosis marker at the TGN was unaffected in the \textit{tno1} mutant and thus the auxin-related defects do not appear to be due to defects in bulk endocytosis. However, it is possible that TNO1 is required for the polar targeting of a particular subset of auxin transporter(s), either as they are newly synthesized or during the recycling process for membrane relocalization. Further detailed characterization of the behavior of such proteins in \textit{tno1} cells will help distinguish between these hypotheses.

The polar sorting of auxin transporters in plant cells is highly complex and our understanding of how the SNARE machinery might regulate this polarity is incomplete. Vacuolar SNAREs have been shown to play a role in maintaining auxin maxima and localization of the auxin efflux carrier PIN1 in the leaf primordium, thereby regulating leaf vasculature (Shirakawa et al., 2009). The SYP4 family of Qa SNAREs affects auxin distribution, root gravitropism, and the intracellular trafficking of the PIN2 transporter to the vacuole for degradation (Uemura et al., 2012). Since TNO1 associates with the SNARE machinery at the TGN, and its loss causes a mis-localization of SYP61 as well as vacuolar sorting defects (Kim and Bassham, 2011), the SYP41/SYP61/VTI12 complex in conjunction with TNO1 may regulate auxin transport by affecting auxin transporter trafficking and turnover.
5.2. TNO1 contributes to the temporal control of LR emergence

We have demonstrated that TNO1 is involved in LR emergence and generation of auxin response asymmetry in an emerging LR. LR development begins by division of LR founder cells (single or pairs of pericycle cells facing the xylem poles) in the primary root and subsequent formation of an auxin maximum and increased auxin responsiveness of the founder cells (De Smet et al., 2007; Dubrovsky et al., 2008; Peret et al., 2009). Auxin transport plays a crucial role in LR emergence, with both auxin influx and efflux carriers being involved. The auxin influx carrier AUX1 is important for loading auxin into the vascular system (Marchant et al., 2002) and aux1 mutants have an almost 50% reduction in LR number. Auxin in the endodermis and cortical cells induces another auxin influx carrier, LAX3, which also facilitates LR emergence (Swarup et al., 2008). The concerted action of AUX1 and LAX3, with subsequent induction of expression of PIN3 in the cortex, leads to emergence of the LR from the primary root (Swarup and Peret, 2012; Peret et al., 2013).

The LR emergence defects observed in tno1 are consistent with altered auxin transport due to disruption in the trafficking or targeting of auxin transporters. tno1 mutants have a significantly reduced percentage (40% vs. 80% in WT) of emergent Stage II LRs with asymmetric DR5rev:GFP expression, raising the possibility of auxin transport defect(s). Since the action of PIN transporters plays a crucial role in establishing this asymmetric pattern of auxin activity in emergent LRs (Rosquete et al., 2013), TNO1 might affect the trafficking of PIN transporters in the LR tip cells during this process.

LR emergence occurs within the pericycle and involves degradation of the pectin-rich middle lamella, with subsequent cell separation to allow growth of the LR through multiple cell layers (Vilches-Barro and Maizel, 2014). Focused auxin flow via transporters into the cortex and
the epidermis causes an induction of expression of cell wall remodeling enzymes (Swarup et al., 2008; Peret et al., 2013). Secretory cargo, including various cell wall remodeling enzymes, traffics via the Golgi and TGN toward the extracellular space (Foresti and Denecke, 2008; Toyooka et al., 2009; De Caroli et al., 2011), in a pathway potentially mediated by SYP61 (Drakakaki et al., 2012). Hence, it is possible that defects in the TGN, including the mis-localization of SYP61 seen in tno1, could also lead to disrupted secretion of cell wall remodeling enzymes, thus leading to slower LR emergence (Worden et al., 2012).

The identification of effectors directly associated with TNO1 may help elucidate its mechanism of action during these auxin-mediated processes. Since tethering factors can interact with Rabs (Markgraf et al., 2007), TNO1 could potentially indirectly affect auxin transporter sorting by affecting the Rab cycle or the function of Rab effectors. Selective targeting of auxin transporters between the plasma membrane and the TGN could be defective in tno1 independent of the constitutive pathways that constantly recycle them between these membranes. Turnover of auxin transporters could also be reduced due to defective targeting to the vacuole for degradation. Analysis of the trafficking of specific auxin transporters in the tno1 mutant would help to distinguish between these possibilities.

6. Acknowledgements

This work was supported by a grant from the National Aeronautics and Space Administration (grant no. NNX09 AK78G) to Dr. Diane Bassham. We thank Dr. Patrick Masson, Dr. Edgar Spalding, Dr. Renu Srivastava, Dr. Anindya Ganguly, Xiaochen Yang, and Divita Mathur for insights, Dr. Dior Kelley for critical review of the manuscript, Margaret Carter for help with the confocal imaging and Dr. Angus Murphy for providing the DR5rev:GFP seeds.
7. References


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8. Figures

Figure 1: Loss of TGN-localized SYP41-interacting protein (TNO1) function delays lateral root emergence in an auxin-dependent fashion. (A) Representative images showing emergent lateral roots (white arrowheads) in wild-type (WT), tno1 (KO), and tno1 complemented (COM) lines. (B) Emergent lateral root density of 10-day-old seedlings grown on 0.25× Murashige–Skoog (MS) medium (with 1% sucrose) was calculated by dividing the number of emerging lateral roots by root length. (C) Lateral root primordia density of 7-day-old seedlings was calculated by counting the number of primordia after microscopic analysis of cleared roots and dividing it by the root length. (D) Emergent LR density of 10-day-old seedlings, 5 days after transfer to medium containing 100 nM 1-napthylacetic acid (NAA) or 1 μM indoleacetic acid (IAA). All values represent analysis of three biological replicates with 15–20 seedlings for each set. Error bars indicate standard errors of the means. Asterisk indicates statistically significant difference ($P < 0.05$) by Student’s $t$-test.
Figure 2: TNO1 mediates gravitropic bending by an auxin dependent mechanism. (A) Hypocotyl bending of WT, KO and COM hypocotyls after gravistimulation. Dark-grown 5-day-old hypocotyls were gravistimulated in the dark and curvatures were calculated at 6 and 24 h after gravistimulation. (B) Root bending of WT, KO, and COM seedlings after gravistimulation. Light-grown, medium-embedded roots were gravistimulated and root curvatures were calculated at 6 and 24 h after gravistimulation. (C) Exogenous auxins can rescue the gravitropic bending defect in tno1 roots. Light-grown seedlings were overlaid with medium containing 100 mM 1-NAA, 30 nM IAA, or 30 nM 2,4-dichlorophenoxyacetic acid (2,4-D) and then gravistimulated. Root curvatures were calculated after 12 h. (D) Some exogenous auxins can rescue the gravitropic bending defect in tno1 hypocotyls. Roots of dark-grown seedlings were overlaid with medium containing 100 mM 1-NAA, 30 nM IAA, or 30 nM 2,4-D, followed by gravistimulation. Hypocotyl curvatures were calculated after 12 h. All values represent the means of three biological replicates with at least 20 seedlings for each set. Error bars indicate standard errors. Similar letters indicate no statistical difference while different letters indicate a statistically significant difference ($P < 0.05$) by Student’s $t$-test.
Figure 3: *tno1* roots show increased sensitivity to 2,4-D and IAA. Five-day-old seedlings were transferred to media with the indicated chemical or solvent as a control and root length was calculated after an additional 6 days of growth. Mean root growth of each genotype on the chemical treatment compared to the mean on the solvent control was expressed as percentage inhibition. Percent inhibition of root length on (A) 30 nM 2,4-D, (B) 1-1-napthoxyacetic acid (NOA; 30, 50, and 70 μM), (C) 30 nM 2,4-D + 10 μM 1-NOA, (D) 40 nM IAA, (E) 100 nM 1-NAA, and (F) 1-Naphthalphtalamic acid (NPA; 5 and 10 μM) for WT, KO, and COM lines are shown. Values represent analysis of three biological replicates with 20 seedlings for each set. Error bars indicate standard errors derived from means of the three replicates. Different letters indicate statistically significant differences ($P < 0.05$) by a Student’s *t*-test.
Figure 4: TNO1 helps mediate auxin response asymmetry at lateral root (LR) tips. (A) Representative confocal images of stage II LR tips of WT and tno1 expressing the auxin-responsive DR5rev:GFP reporter. Yellow arrowheads indicate asymmetry of the auxin reporter GFP expression. Scale bar = 50 μm. (B) The percentage of stage II LRs displaying an asymmetric auxin response pattern. A total of 30 stage II LRs from at least six seedlings were analyzed.
Figure 5: tno1 mutants have delayed auxin responses in epidermal cells in gravistimulated roots. Representative confocal images of mutant and WT primary root tips showing DR5rev:GFP expression under (A) vertical (non-gravistimulated condition), (B) 5 h gravistimulation, (C) 8 h gravistimulation, and (D) 12 h gravistimulation. Yellow arrowheads indicate auxin reporter expression expanding from columella to epidermal cells of the elongation zone. Scale bar = 50 μm. (E) Quantification of DR5rev:GFP asymmetry as a ratio of fluorescence intensity on the lower and upper flanks of gravistimulated roots at the indicated time points. Values are means from analysis of 10 roots for each time point and error bars indicate standard errors. Similar letters indicate no statistical difference while different letters indicate a statistically significant difference (P < 0.05) by Student’s t-test.
Figure 6: *tno1* mutants have normal bulk endocytosis and arrival of membrane cargo from plasma membrane to Brefeldin A (BFA) bodies. (A) Root cells showing uptake of FM4-64 after 2 min of treatment of 4-day-old seedlings with 4 μM FM4-64 in liquid 0.5× MS medium. Yellow arrowheads indicate early endosomes/TGN. (B) Root cells treated with 35 μM BFA for 1 h, followed by a 10 min incubation with 4 μM FM4-64, showing arrival of FM4-64 at BFA bodies. White arrowheads indicate BFA bodies.
1. Abstract

The movement of plant roots is key to their ability to interact with the environment and maximize anchorage and nutrient acquisition. Root movement is under the control of multiple endogenous and exogenous factors that signal amongst each other. The study of root movement in nature is difficult due to the challenge of tracking root trajectory in soil. This has necessitated study of root movement on the surface of growth media in the laboratory. Roots growing on slanted, impenetrable growth media display a characteristic waving and skewing movement due to an interplay of the root tips’ response to gravity and touch. Mutants with deviations in these phenotypes assist in identifying genes required for root movement. We have identified TNO1, a putative tethering factor localized at the trans-Golgi network, as being required for root movement. \textit{tno1} knockout mutants display enhanced rightward skewing responses and increased left handed epidermal cell file rotation. Skewing of the \textit{tno1} roots increases upon microtubule stabilization while destabilization has no significant effect on skewing when compared to wildtype roots that are treated similarly. The cell morphology in mature zones of roots and base of hypocotyls is also adversely affected in \textit{tno1} seedlings upon microtubule destabilization.

These observations suggest that TNO1 might be necessary for mediating the role of microtubules during these processes. Unexpectedly, the elongating cells of the mutant’s skewing roots do not contain an oblique orientation of the microtubule array, suggesting that the cell file rotation does not arise due to a direct effect of TNO1 loss on microtubule array arrangement. Thus TNO1
might affect microtubule dynamics indirectly or cell expansion that then affects root movement. Together these studies demonstrate a role for TNO1 in root movement in a possibly indirect microtubule-associated mechanism.

2. Introduction

The combined action of rapid cell division in the root meristem and elongation of cells in the elongation zone causes root growth. The stem cells in the root apical meristem divide to produce single cell files that are pushed shootwards via the transition zone into the elongation zone where they expand anisotropically in a direction parallel to the root’s growth axis (Hodge et al., 2009). Anisotropic cell expansion is mediated by the organization of almost inextensible cellulose microfibrils in the cell walls of these expanding cells (Szymanski, 2009). Their transverse orientation leads to a restriction of the plane of expansion to a longitudinal axis. Microtubules guide the direction of synthesis of cellulose microfibrils by cellulose synthases and hence microtubular orientation and dynamics play a role in directing cell expansion and root growth (Bringmann et al., 2012; Bashline et al., 2014a). This is supported by studies with mutants such as fragile fiber1 (fra1) and botero1 (bot1) that display defective cell expansion due to disorganized microtubules and cellulose microfibrils (Bichet et al., 2001; Zhu et al., 2015).

Root development and architecture are well studied but our understanding of root growth movement in the substrate is still not well understood since the actual process inside the substratum is difficult to study in real time. Root growth into the substratum presents multiple cues to the root tip such as mechanical obstacles, moisture and nutrient gradients. These cues are integrated and then signal with downstream processes involving hormonal pathways and cell expansion. This leads to a cumulative physiological response driving root movement and
establishing the root architecture. Understanding the basis of these movements is important since establishment of the root is crucial for anchorage and nutrient acquisition.

Studying root movement in the laboratory on synthetic growth media has helped uncover distinct movement types and discover crucial genes and pathways that control them (Roy and Bassham, 2014). Roots of Arabidopsis seedlings display various growth behaviors depending on external conditions. When embedded in a homogenous media (penetrable agar media) the roots grow downwards in response to gravity and show minimal deviation from the gravity vector. On the other hand when the roots are subjected to multiple directional cues, complicated growth patterns occur. For example Arabidopsis roots grown on slanted impenetrable media (1.5% agar) show a characteristic deviation from the vertical with a periodic wave-like pattern along their trajectory. This is referred to as skewing and waving respectively and arises due to a combination of touch, gravitropism, circumnutation, as well as physical interaction between the root tip and the growth media (Okada, 1990; Simmons et al., 1995; Rutherford and Masson, 1996; Migliaccio and Piconese, 2001; Buer et al., 2003). Skewing and waving roots also display a characteristic twisting of epidermal cell files along the root, referred to as cell file rotation (CFR). When roots of Arabidopsis seedlings skew and wave, the succession of sinusoidal waves alternate between left handed and right handed CFRs, which correlates with their rightward and leftward movement respectively (Okada, 1990; Rutherford and Masson, 1996). I describe skewing as rightward or leftward when viewing from the back of the plate according to convention (Rutherford and Masson, 1996). The handedness was defined as left handed or right handed using the right- and left-hand rule. One tries to curl their fingers around the twist of the root keeping the thumb facing up/shootwards. The handedness corresponds to the hand whose fingers can be curled around the twist, while keeping the thumb facing shootwards. Enhanced skewing
in roots of *Arabidopsis* seedlings exhibit a dominant left handed rotation around the growth axis resulting in a predominantly counterclockwise/left handed epidermal CFR. This usually corresponds to a rightward skewing when seen from the back of the plate (Rutherford and Masson, 1996; Marinelli et al., 1997; Furutani et al., 2000; Yuen et al., 2003).

The basis for CFR formation is a controversial topic and its cellular basis is still not completely understood. Roots embedded in a homogenous agar media still display CFR, suggesting that it is an endogenous phenomenon caused by the circumnutating root tip (Sedbrook et al., 2002). Nutatory movements such as circumnutation, are controlled by an internal mechanism that results in an elliptical or circular trajectory around an imaginary axis of growth (Migliaccio et al., 2013). Helical patterns of cell division at the root tip and lagging cell expansion rates between inner and outer cell layers of the root are also proposed to be the basis for its formation (Okada, 1990; Rutherford et al., 1998; Yuen et al., 2005). The characteristic spiral cell division patterns of the outer circle of meristematic cells in the root apex causes a spiraling of the cell files, thus resulting in CFR formation (Wasteneys, 2004) although this fails to explain why CFR initiates at the base of the elongation zone away from the site of initial cell divisions. A lag in anisotropic expansion rates between the epidermal and the inner cell layers can also cause a twisting of the cell files to compensate for stresses and strains that might otherwise cause tissue breakage (Furutani et al., 2000). CFR also shows a correlation with MT array orientation in the twisting cell files though exceptions do exist (Vaughn et al., 2011).

Analysis of mutants with altered waving or skewing has led to identification of multiple genes and factors important for root movements such as external cues (e.g- moisture, light, gravity), hormonal pathways, and cytoskeletal and cell wall dynamics (Roy and Bassham, 2014). External cues signal changes in hormonal signaling pathways including auxin, ethylene,
cytokinin and brassinosteroid pathways. Downstream of hormonal and environmental perception, changes in the cytoskeleton and cell wall deposition patterns modulate cell division and cell expansion dynamics thus mediating root movements. Defects in tubulin structure or activity and microtubule-associated proteins result in altered MT dynamics/array orientation. This then leads to changes in anisotropic expansion and affects cell file rotation and skewing (Ishida et al., 2007; Sedbrook and Kaloriti, 2008). Cell wall properties and the trafficking dynamics of cell wall components to the plasma membrane is key to root elongation and movement as inferred from the altered root movements observed in mutants defective in cellulose deposition, wall-anchored proteins or crosslinking of cell wall components (Sedbrook et al., 2002; Roudier et al., 2005; Wolf et al., 2012).

Sorting of cell wall components occurs at the trans-Golgi network (TGN), a tubulo-vesicular cluster maturing from the two or three trans- most cisternae of the Golgi (Staehelin and Kang, 2008). It acts as an early endosome, receiving endocytosed cargo as well as directing secretory traffic, with distinct subdomains predicted for various trafficking routes (Reyes et al., 2011; Contento and Bassham, 2012; Gendre et al., 2014). Specific membrane fusion factors called SNAREs (soluble N-ethylmaleimide–sensitive factor attachment protein receptors), maintain trafficking fidelity and cargo sorting (Kim and Brandizzi, 2012) in cells. The TGN-localized SYP4 (41/42/43) SNARE family is required for multiple transport pathways that in turn regulate auxin homeostasis and disease resistance (Uemura et al., 2012). SYP61, another TGN localized SNARE that interacts with SYP41, affects vacuolar trafficking and secretion of cell wall machineries such as CESAs and pectin-polysaccharide-modifying protein, suggesting a role for SYP61 in cell wall trafficking (Bassham et al., 2000; Zhu et al., 2002; Drakakaki et al., 2012). TNO1 (TGN-localized SYP41-interacting protein) is a TGN localized coiled-coil protein that
interacts with the SYP41 SNARE machinery. Mutant plants lacking TNO1 (*tno1*) partially mis-sort vacuolar cargo, mis-localize SYP61 and display altered TGN dynamics and slower gravitropic responses (Kim and Bassham, 2011; Roy and Bassham, 2015).

We investigated the function of TNO1 in root movement, since gravitropic bending, which is a key component of root growth movements, is defective in the *tno1* roots. We report here that TNO1 is a negative regulator of root skewing, as *tno1* mutant roots have enhanced skewing which also correlates with an enhanced CFR.

### 3. Methods

#### 3.1. Plant material and growth conditions

The *Arabidopsis thaliana* seed stocks used in this study have been previously described: Col-0 (wild-type, WT), *tno1* knockout mutant (SALK_112503) and complemented *tno1* mutant (Kim and Bassham, 2011).

For sterilizing seeds, *Arabidopsis* seeds were surface-sterilized in 33% bleach, 0.1% (v/v) Triton X-100 for 20 minutes, rinsed 5 times with sterile water and kept in the dark at 4°C for at least 2 days. *Arabidopsis* hypocotyls were grown in a similar manner at 22°C in darkness by wrapping the plates with aluminum foil sheets.

#### 3.2. Root skewing and waving assays

Skewing and waving assays were performed as described previously (Rutherford and Masson, 1996; Rutherford et al., 1998). *Arabidopsis* plants were grown at 22°C in long-day conditions (16 hours light) on 0.5X solid Murashige-Skoog (MS) media [(Murashige-Skoog vitamin and salt mixture, Caisson, MSPA0910] with 1% sucrose, 2.4 mM MES (pH 5.7), and
1.5% (w/v) Phytoblend agar (Caisson, PTP01). Seedlings were grown vertically for 3 days after which the root tip position was marked. The plates were then slanted backwards, 30° to the vertical and grown for another 4 days. Images of the roots were acquired from the back of the plate using a Nikon SMZ1000 light microscope equipped with a Nikon S10 CoolPix camera. Analysis of root parameters was performed using Image J. Root tip deviations to the right from the back of the plate were considered positive while deviations to the left were considered negative.

Root morphometric analyses were performed as described (Grabov et al., 2005). Images of skewing/waving roots were analyzed for root tip abscissa (Lx) and length of the root (L) followed by calculation of horizontal growth indices (Lx/L).

3.3. Drug sensitivity assays

Seedlings were grown vertically on plates containing either taxol (Paclitaxel, Sigma Cat#T7191) or propyzamide (Sigma-Aldrich, Cat#45645) at the indicated concentrations for 5 days and then analyzed for skewing angles of roots. Root cell phenotypes were analyzed by staining with propidium iodide and confocal imaging with a Leica confocal laser scanning microscope (Leica SP5; Leica Microsystems) at the Iowa State University Confocal and Multiphoton Facility. Hypocotyl cell phenotypes were analyzed by placing 7 day old hypocotyls on the surface of warm 3% low melting point agarose on a slide and then analyzing the imprints (Mathur and Koncz, 1997) with light microscopy using a Zeiss Axioplan II microscope.
3.4. Cell File rotation analysis

*Arabidopsis* seedlings were grown for the skewing assay or grown vertically on growth media with taxol or propyzamide as described above. The root surface was then visualized using a Zeiss macro-zoom microscope at the Bessey Microscopy and NanoImaging facility, Iowa State University or stained with propidium iodide (ThermoFisher Scientific Cat# P3566) and visualized using a Leica confocal laser scanning microscope (Leica SP5; Leica Microsystems) at the Iowa State University Confocal and Multiphoton Facility. Excitation and emission wavelengths were 488 nm and 617 nm. Laser power, scan frequency and line averaging were optimized and kept constant between samples and replicates. Cell file rotation angles of the roots were calculated as described (Rutherford and Masson, 1996; Mochizuki et al., 2005) using ImageJ. Left handed root-tip rotation was considered positive (Rutherford and Masson, 1996).

3.5. Immunostaining of microtubules

For visualization of microtubules within the elongation zone of the roots, 5-day-old *Arabidopsis* seedlings growing on slanted media were fixed and immunostained as described earlier (Sugimoto et al., 2000). Mouse anti-α- tubulin antibodies (Sigma-Aldrich, Cat#T6074), diluted 1:100, were used for immunolabeling of microtubules followed by AlexaFluor 488-conjugated goat anti-mouse IgG (ThermoFisher Scientific, Cat#A11029) as a secondary antibody, diluted 1:500. Samples were imaged by confocal microscopy with a Leica confocal laser scanning microscope (Leica SP5; Leica Microsystems) at the Iowa State University Confocal and Multiphoton Facility, using a 40X or 63X Leica oil immersion objective. Excitation and emission wavelengths were 488 nm and 507 nm. Laser power, scan frequency and
line averaging were optimized and kept constant between samples and replicates for discerning the microtubules clearly.

3.6. MT array analyses

For analyses of MT arrays of root elongation zones, confocal images of immunostained roots were analyzed with the freely available software package MicroFilament Analyzer (MFA) (Jacques et al., 2013), and data was displayed as circular plots.

4. Results

4.1. Loss of TNO1 results in exaggerated root skewing

Mutants lacking TNO1 have normal root elongation, but show a lag in root gravitropic bending upon gravistimulation (Roy and Bassham, 2015). Positive gravitropism is an important force driving root movements, and we hypothesized that TNO1 might play a role in regulating root movement.

To test this hypothesis, 3-day-old seedlings grown vertically on the surface of hard agar media (1.5% agar) were subjected to 4 more days of growth after tipping the plates over by 30° (Figure 1A). The slant forces the root tip to press against the impenetrable media due to positive gravitropism. As the root fails to penetrate the media, cell expansion and circumnutation drive the root to the side and this alternates between rightward and leftward corrective deviations resulting in a characteristic waving and skewing responses (Rutherford and Masson, 1996; Roy and Bassham, 2014). \textit{tno1} mutant roots (KO) formed sinusoidal wave-like growth patterns similar to WT (Columbia) roots but had an exaggerated rightward skew compared to the WT roots when seen from the back of the plate (Figure 1B). \textit{tno1} mutants complemented with
transgenic TNO1 under the control of its own promoter (COM) had reduced skewing and were similar to the WT roots (Figure 1B). Root images were analyzed using ImageJ and quantified for deviation of the root tips from their position at the time of slant initiation (β) (Figure 1C) (Rutherford and Masson, 1996; Grabov et al., 2005). tno1 mutants had a higher angle of deviation than the WT or complemented roots (Figure 1D; P<0.05). To confirm the difference in phenotype, another morphometric parameter, the horizontal growth index (HGI), was calculated for the skewing roots. HGI is the ratio of the root tip abscissa (Lx) and length of the root (L) (Figure 1C) and is a sensitive and robust parameter to quantify lateral directions of growth (Grabov et al., 2005). Root tips skewed to the right yield positive Lx values and negative values when roots skew left. A positive HGI value suggests a rightward deviation while a negative value suggests leftward deviation. tno1 roots had a significantly higher HGI than the WT and complemented lines (Figure 1E; P<0.05), confirming that the observed rightward deviation from the vertical in the tno1 mutants is indeed higher than the WT and complemented lines.

4.2. The skewing tno1 roots display enhanced root epidermal cell file rotation

CFR, usually preceding root bending and skewing (Mochizuki et al., 2005), originates at the base of the elongation zone of skewing roots. Rightward skewing mutants usually display a dominant left handed CFR (Vaughn et al., 2011; Roy and Bassham, 2014). We hypothesized that the enhanced rightward skewing in the tno1 mutants would also correlate with an increase in the left-handed CFR. Microscopic analysis of mutant slanting roots showed a marked left handed epidermal CFR initiating at the basal region of the root elongation zone (black arrow, Figure 2A) while WT and complemented roots mainly seem to have a vertical arrangement of cell files with a lack of distinct CFR in this region (Figure 2A). Confocal imaging of propidium iodide-stained
roots revealed a distinctive left handed CFR with a higher twist in the elongation zone of tno1 lines compared to the WT and complemented lines (Figure 2B). Images were analyzed to quantify the left-handed twist of the cell files relative to the longitudinal axis of the root. The tno1 CFRs were significantly higher (P<0.05) than the WT and complemented root CFRs (Figure 2C). This possibly explains the enhanced skew of the mutant roots, since the increased left-handed twist might cause larger deviations from the axis of growth.

4.3. Skewing of tno1 roots is enhanced upon MT stabilization by taxol

CFR can be induced directly by cortical microtubule dynamics, which influences anisotropic expansion by constraining the microtubule alignment which then alters cellulose deposition (Fisher and Cyr, 1998; Wasteneys, 2004). Hence, we hypothesize that the enhanced CFR in the tno1 mutants might be due to changes in the cortical microtubular array.

Several mutants defective in MT dynamics have enhanced right- or leftward skewing with a dominant left or right-handed CFR respectively. They also display an opposite handed orientation of cortical MT arrays with respect to the CFR handedness in the elongation zone of the roots. Mutants defective in microtubule interacting proteins spr1, spr2 and wvd2-1 show right handed CFR, skew to the left and display left handed cortical MT arrays in the elongation zone (Buschmann et al., 2004; Sedbrook et al., 2004). We hypothesized that altered skewing and CFR of tno1 roots in presence of taxol, when compared to WT and complemented lines, would suggest defects in MT arrays or dynamics.

The MT-stabilizing chemical taxol is known to cause MT bundling and enhances rightward skewing in Columbia (WT) seedlings (Furutani et al., 2000; Field et al., 2013). The MT-destabilizing chemical propyzamide inhibits a straight conformation of microtubules and
also enhances rightward skewing in Columbia (WT) seedlings (Akashi et al., 1988; Furutani et al., 2000). When grown vertically on increasing concentrations of taxol (Figure 3A), tno1 roots show significantly higher rightward skewing than the WT and complemented lines. The increase in skew for the mutant roots in the presence of taxol is higher than the other genotypes (Figures 3A, B). The percentage increase in skewing angles of the mutant roots from 0μM to 0.2μM taxol treatments is significantly higher than (P<0.05) the WT and complemented line roots (Figure 3C). We hypothesized that this increase in skewing was due to an increase in the left handed twist of the roots. Analysis of confocal images of propidium iodide stained roots in the presence of 0.2μM taxol reveals a larger left handed CFR in the tno1 roots compared to WT and complemented lines (Figure 3D). Since the seedlings are grown vertically on taxol-containing media, the CFR is originating mainly due to the effect of taxol on the roots and not slanted growth. These results suggest that MT stabilization exaggerates the root skewing and CFR phenotype of the tno1 mutants.

4.4. Effect of MT destabilization by propyzamide on root skewing in tno1 mutants

The microtubule-destabilizing drug propyzamide (PPZ) induces enhanced rightward root skewing (Furutani et al., 2000). We tested the effect of PPZ on root skewing in the tno1 mutants. The WT and complemented lines showed a slight increase in rightward skew at 1μM PPZ and a marked increase at 3μM PPZ. Surprisingly the skewing of tno1 roots did not increase (Figures 4A, B). The percentage increases in skewing observed for WT and complemented roots in the presence of PPZ was significantly higher than the mutants (Figure 4C). The percentage increase in skewing for the complemented lines was also significantly higher than the WT suggesting that either levels of the TNO1 protein in the complemented lines were different from WT levels or
the difference could be due to an artifact of the statistical analysis. We hypothesized that reduced skewing of \textit{tnol} roots in the presence of PPZ would correlate to a reduced CFR when compared with CFRs of PPZ treated-WT and complemented roots. Confocal microscopic analysis of the surface of roots growing on 3μM PPZ reveals a twisting of WT and complemented epidermal cell files. Unexpectedly, the mutant root epidermal cell files also display considerable left handed CFR (Figure 4D). This seems to contradict the observation that mutant roots do not show an increase in rightward skewing upon treatment with 3μM PPZ. Hence skewing is not changing in the mutants even though CFR formation occurs normally.

To test whether PPZ treatment affected other regions of the root and hence root growth, we visualized the mature regions of PPZ treated roots by confocal imaging of propidium iodide stained-roots. Surprisingly, mature regions of \textit{tnol} roots grown on 3μM PPZ had more severe defects in cell expansion when compared to the PPZ treated WT and complemented lines with the mutants forming thicker/malformed cells (Figure 5A). This defect in cell expansion would interfere with the ability of the \textit{tnol} roots to elongate and skew in the presence of PPZ even as the CFR in the elongation zone forms normally.

The observed cell expansion defects in the mutant roots led us to hypothesize that other organs in the mutant might also be sensitive to PPZ induced MT disruption. We analyzed the agarose imprints of control and PPZ treated hypocotyls and found mild disruption in cell morphology (slightly thicker compared to the non drug treated) in the WT and complemented lines compared to their untreated counterparts. \textit{tnol} hypocotyls displayed hypersensitivity to PPZ with much more severe defects in cell expansion when compared to the untreated \textit{tnol} seedlings as well as the PPZ treated WT and complemented seedlings (Figure 5B). This suggests that the
loss of TNO1 leads to an increased susceptibility of cell expansion processes to microtubule destabilization by PPZ.

4.5. The orientation of MT arrays in the elongating cells of skewing mutant roots is not affected

MT arrays in elongation zones of roots are usually arranged in a transverse orientation to facilitate anisotropic expansion. Skewing roots of many mutants with marked CFRs have been shown to have distinct oblique arrays of MTs. The handedness of a MT array is observed to be opposite that of the CFR. Strong correlations have also been suggested between angle of skewing and pitch of the MT arrays (Vaughn et al., 2011). Many mutants defective in MT associated processes display change in the pitch of MT arrays associated with a corresponding CFR. Surprisingly, some mutants with enhanced CFR show no obliqueness of the MT array, but rather have transverse arrangements similar to the central elongation zone cells of the WT roots.

We hypothesized that the dominant left handed CFR in the *tno1* roots would correspond to an right handed MT array in the epidermal cells of the elongation zone compared to WT roots. Immunostaining of tubulin in four-day-old chemically fixed roots of WT and *tno1* was carried out with as described by (Sugimoto et al., 2000) and imaged by confocal microscopy. Confocal images of the epidermal cells of the mutant root did not show any distinct handedness or change in the pitch of the MT arrays compared to the WT epidermal MT arrays (Figure 6A). Quantitative analyses on collected images was performed utilizing the MATLAB software package, MicroFilament Analyzer (Jacques et al., 2013). This aided detection of MT orientation in multiple images from different biological replicates in an objective manner. The representative circular output graphs from the analyses suggest a mainly transverse orientation of the MTs in
the elongation zone of both WT and tno1 roots (blue line along the 0-180 degree axis) (Figure 6B). We hypothesized that a difference in the percentage of oblique MTs between the WT and mutant’s might explain the basis of a higher CFR in the mutants. MT angles of at least 10 root cells of were classified into three classes, 0 to 10, 11 to 30 and 31-90 degrees and analyzed. We found that there was no significant difference in the percentage of MTs in various angle classes between the WT and mutants.

Thus we conclude that tno1 mutants have normal transverse MT arrays in the skewing roots suggesting an alternative mechanism of CFR origination.

5. Discussion

TNO1 is known to be required for root gravitropism, which is a primary component of the root growth movement response. We hypothesized that the tno1 mutants would have defective root growth movements due to the gravitropic bending defects. We discovered that tno1 mutants had exaggerated rightward skew on the surface of slanted impenetrable media, which correlated with an increase in the left-handed CFR of epidermal cells in the root’s central elongation zone. We also Thus this study reports a novel role for a TGN localized, SNARE-associated protein in root skewing and adds to the repertoire of proteins required for regulating root growth movements.

5.1. TNO1 might affect MT dynamics and MT associated proteins

MT arrays in roots transition from a disordered state in the meristematic zone, to a transverse orientation in the elongation zone and finally parallel to the axis of growth in the mature zone of the roots (Sugimoto et al., 2000). The transverse cortical MT array in the
elongation zone is important for facilitating rapid anisotropic expansion. Defects in tubulin structure/activity and microtubule associated proteins (MAPs) can result in oblique MT arrays in the central elongation zone of roots which then results to an epidermal CFR and skewing of roots (Ishida et al., 2007; Oliva and Dunand, 2007; Sedbrook and Kaloriti, 2008; Roy and Bassham, 2014). Oblique MT orientation in elongating cells causes cells to expand at an angle to the vertical leading to cell file rotation. The correlation between CFR and MT arrays thus reflects a connection between the MT arrays and wall deposition. Treatments with either MT stabilizing (taxol) or destabilizing (propyzamide) drugs result in biased left-handed cell file rotation and rightward skewing of roots (Sedbrook et al., 2004; Yuen et al., 2005) and are used to uncover defects in MT dynamics in mutants.

We found that skewing of tno1 roots increased by a greater extent upon MT stabilization by taxol when compared to WT roots. Rightward skewing of the tno1 roots did not increase with MT destabilization by propyzamide unlike the WT roots. This led us to postulate a role of TNO1 in MT dependent processes. We also found that the mature root cells and hypocotyls of tno1 seedlings treated with propyzamide showed marked disruption of cell expansion, suggesting a role of TNO1 in the cell expansion processes.

Unexpectedly, the skewing tno1 roots did not show a distinct obliqueness of the MT array in the basal region of the central elongation zones and had transverse arrays. This suggests that CFR in tno1 mutants arises independent of the organization of MT arrays in the root elongation zone. Other mutants such as sku6/spr1 also display a transverse MT array in the elongation zone of roots but have a distinct left-handed CFR (Sedbrook et al., 2004). According to an alternative model of CFR formation, a lag in the expansion rates of epidermal cells and more internal tissue causes roots to skew to prevent mechanical shearing (Furutani et al.,
This would explain the transverse MT arrays in twisting roots of the tno1 mutants even as they twist and skew. Thus TNO1 might be required for regulating cell expansion only during deviation of roots from their growth trajectory, since vertically grown tno1 seedlings have similar root lengths as WT seedlings suggesting normal cell expansion during vertical growth. We speculate that loss of TNO1 causes a lag in expansion rates of the underlying tissue and epidermal layers leading to CFR formation.

The defect in cell expansion could be due to the possible role of TNO1 in proper localization of SYP61. SYP61 is a TGN localized Q-SNARE, which interacts with the Q-SNAREs SYP41 and VTI12 (Bassham et al., 2000; Kim and Brandizzi, 2012). Proteomic studies of SYP61-labeled vesicles have revealed the presence of cellulose synthase complex (CSC) subunits suggesting its role in trafficking of cellulose synthase subunits, which then drives cellulose deposition. MT array dynamics modulate the deposition of cellulose microfibrils in expanding cells since the (CSC) runs parallel to the MT array and CELLULOSE SYNTHASE INTERACTIVE1 acts as a link between the cellulose synthase machinery and the cortical microtubules (Lei et al., 2013; Bashline et al., 2014a; Bashline et al., 2014b; Lei et al., 2015). Disrupting MT arrays affects cellulose deposition while inhibiting cellulose synthase activity causes defects in MT arrays (Paredez et al., 2006) which can then affect root movement. During cell expansion and cellulose deposition, the cellulose synthase complex traffics via the secretory route at the TGN and can also be recycled via endocytosis. Upon endocytosis it localizes to small subcellular compartments known as microtubule associated cellulose synthase compartments (MASCs) and are speculated to be involved in delivering the CSCs to the plasma membrane (Crowell et al., 2009; Gutierrez et al., 2009). tno1 mutants display trafficking defects and
mislocalize SYP61 (Kim and Bassham, 2011) which might result in defects in cellulose deposition and MT arrays, thus causing defective root movements.

Trafficking of other proteins required for cell expansion and root movement might also be defective in the tno1 mutants. For example, proteins such as COBRA and SKU5 are required for enzymatic reactions at the cell wall and cell wall microfibril patterning respectively and mutants lacking these proteins show enhanced root skewing (Sedbrook et al., 2002; Roudier et al., 2005).

Further experiments, such as will be necessary to test the exact role of TNO1 in regulating root skewing and CFR. Future studies on investigating TNO1’s interaction with MTs or/and MAPs and studying expansion dynamics in skewing roots will be required to better understand the role of TNO1. Identifying interacting partners of TNO1 can help elucidate the function of TNO1. Since TNO1 affects auxin transport (Roy and Bassham, 2015), our study now places TNO1 at the crossroad of auxin transport and possibly cytoskeletal dynamics. Whether TNO1 only affects transport of components in these pathways to the plasma membrane or directly interacts with the cytoskeletal nexus would be a key discovery in uncovering the roles of the TGN SNARE machinery in plant growth and morphogenesis.

6. Acknowledgements

This work was supported by a grant from the National Aeronautics and Space Administration (grant no. NNX09 AK78G) to Dr. Diane Bassham. We would like to thank Dr. Patrick Masson and Dr. Kate Baldwin for advice on the skewing assays, Colton McNinch for helping setup and analyze the effects of taxol and propyzamide on root skewing and Xiaochen Yang for helping optimize the root immunostaining protocol. I would also like to thank Bin
Dong and Jennifer Johnson for assistance with the MFA software, Margaret Carter for expert guidance on confocal imaging and Divita Mathur for conceptual insights.

7. References


8. Figures

**Figure 1**: Loss of TGN-localized SYP41-interacting (TNO1) protein function causes exaggerated rightward skewing. A) Schematic showing side view of the setup for the root movement (waving and skewing) assay on growth media solidified with 1.5% agar. Seedlings were grown vertically for 3 days in long day (LD) conditions, after which the root tip position was marked. The plates were then slanted backwards, 30° to the vertical and grown for another 4 days. B) Representative images of WT, tno1 mutant (KO) and complemented tno1 mutants (COM) displaying waving and skewing of roots when grown for the skewing and waving assay. C) Schematic of image analysis of a root for analyzing the angular deviation of the root tip (β), the horizontal root-tip deviation (Lx) and the length of the root (L). D) Angular deviation of root tips of WT, KO and COM seedlings subjected to the movement assay. E) Horizontal growth index (HGI) of roots of WT, KO and COM seedlings subjected to the movement assay. HGI is the ratio of the horizontal root tip deviation (Lx) and root length (L). All values represent the means of three biological replicates with at least 20 seedlings for each set. Error bars indicate standard errors. Asterisks indicate a statistically significant difference ($P < 0.05$) by Student’s $t$-test.
Figure 2: The skewing *tno1* roots display enhanced root epidermal cell file rotation. A) Representative image of the surface of root tips of WT, KO and COM lines subjected to skewing assay, imaged under a macro zoom microscope. Black arrow points to a forming epidermal cell file rotation. Scale bar = 100 μm. B) Representative image of propidium iodide-stained elongation zone of WT, KO and COM root subjected to skewing assay, imaged with a confocal microscope. Scale bar = 100 μm. C) Cell file rotation angles of skewing roots of WT, KO and COM lines. All values represent the means of three biological replicates with at least 15 cell files from 5 seedlings analyzed for each set. Error bars indicate standard errors. Asterisks indicate a statistically significant difference ($P < 0.05$) by Student’s *t*-test.
Figure 3: Skewing of *tnol* roots is enhanced upon MT stabilization by taxol. A) Angle of skew with respect to horizontal for WT, KO and COM roots grown on two different concentrations of taxol. Seedlings were grown vertically in LD conditions for 5 days on growth media solidified with 1.5% agarose and either containing taxol or solvent control. All values represent the means of three biological replicates with at least 15 seedlings for each set. Error bars indicate standard errors. Asterisks indicate a statistically significant difference (*P* < 0.05) by Student’s *t*-test. B) Representative light microscopic image of roots of WT, KO and COM lines grown vertically in the presence of 0.2 μM taxol for 5 days. C) Mean root skew of each genotype on taxol compared to the mean on the solvent control was expressed as percentage inhibition. Values represent analysis of three biological replicates with 15 seedlings for each set. Error bars indicate standard errors derived from means of the three replicates. Different letters indicate statistically significant differences (*P* < 0.05) by a Student’s *t*-test. D) Representative image of propidium iodide-stained elongation zone of WT, KO and COM root grown on 0.2 μM for 5 days, imaged with a confocal microscope. Scale bar= 100 μm.
Figure 4: Effect of MT destabilization by propyzamide on root skewing in tno1 mutants. A) Angle of skew with respect to horizontal for WT, KO and COM roots grown on two different concentrations of propyzamide. Seedlings were grown vertically in LD conditions for 5 days on growth media solidified with 1.5% and either containing propyzamide or solvent control. All values represent the means of three biological replicates with at least 15 seedlings for each set. Error bars indicate standard errors. Asterisks indicate a statistically significant difference ($P < 0.05$) by Student’s $t$-test. B) Representative light microscopic image of roots of WT, KO and COM lines grown vertically in the presence of 3μM propyzamide for 5 days. C) Mean root skew of each genotype on propyzamide compared to the mean on the solvent control was expressed as percentage inhibition. Values represent analysis of three biological replicates with 15 seedlings for each set. Error bars indicate standard errors derived from means of the three replicates. Different letters indicate statistically significant differences ($P < 0.05$) by a Student’s $t$-test. D) Representative image of propidium iodide-stained elongation zone of WT, KO and COM root grown on 3μM propyzamide for 5 days, imaged with a confocal microscope. Scale bar= 100 μm.
Figure 5: Effect of 3μM propyzamide on cell morphology of mature region of the root and the base of the hypocotyl. A) Representative image of propidium iodide-stained mature zone of WT, KO and COM root grown on solvent control or 3μM propyzamide for 5 days, imaged with a confocal microscope. Scale bar = 100 μm. B) Representative agarose imprints of WT, KO and COM hypocotyl bases grown on solvent control or 3μM propyzamide for 7 days, imaged with a macro zoom microscope. Scale bar = 100 μm
Figure 6: The orientation of MT arrays in the elongating cells of skewing mutant roots is not affected. A) Representative confocal images of anti-α-tubulin immunostained root elongation cells of 5-day-old Arabidopsis seedlings growing on slanted media displaying the cortical microtubular array. B) Representative circular plot derived from the analysis of the confocal image (described in A) with the MicroFilament Analyzer software (MFA). Blue line denotes the dominant orientation of the MT array. C) Microtubule angle distribution in WT and KO elongation cells. Values represent analysis of three biological replicates with at least 500 MTs analyzed from 3 cells of three different roots for each replicate. Error bars indicate standard errors derived from means of the three replicates.
CHAPTER 4

A POINT MUTATION IN THE SEC1/MUNC18 PROTEIN AtVPS45 LEADS TO DWARF PHENOTYPE AND DEFECTIVE ROOT HAIR GROWTH IN *ARABIDOPSIS THALIANA*

1. Abstract

Sec1/Munc18 proteins are peripheral membrane proteins that interact with Qa-SNAREs and regulate membrane fusion. AtVPS45, one of the six Sec1/Munc18 proteins found in *Arabidopsis*, associates with the SYP41/SYP61/VTI12 SNARE complex located at the trans-Golgi network. It is required for SYP41 stability, and it functions in cargo trafficking to the vacuole and cell expansion. It also resides on the endocytic route and is required for recycling of auxin transporters. We have investigated *Atvps45-3* plants recovered via a TILLING approach, containing a novel point mutation in the AtVPS45 gene. The mutant harbors a serine-to-phenylalanine substitution at the 284th amino acid position in the polypeptide chain. *Atvps45-3* plants display severe growth defects with significantly reduced organ size similar to the previously reported *Atvps45-2* RNAi transgenics that have reduced AtVPS45 protein levels. Root hair elongation is also highly compromised in *Atvps45-3*, with the development of short and thick root hairs. Exogenous auxin (1-NAA) treatment causes root hair expansion in the *Atvps45-3* suggesting that the competency to respond to auxin and undergo cell expansion is not compromised in the mutants. The bulk endocytic and secretory routes seem to be unaffected in *Atvps45-3* plants, pointing to specific effects of the point mutation on the vesicle fusion machinery required for cell expansion in *Arabidopsis*. 

2. Introduction

Membrane trafficking in cells requires the coordinated action of vesicle fusion machineries involving SNAREs, Rab GTPases, tethers and regulatory Sec1/Munc18 (SM) proteins (Kim and Brandizzi, 2012; Rizo and Sudhof, 2012; Hong and Lev, 2014). Incoming cargo vesicles are tethered to their target membrane by tethering factors, and subsequently SNAREs drive vesicle fusion by forming a trans-SNARE complex (Fasshauer et al., 1998). The complex involves three target SNAREs (t-SNAREs) and a vesicle-SNARE (v-SNARE), resident on the target membrane and incoming cargo vesicle, respectively. SNAREs are also classified as Q- or R-SNAREs depending on the presence of a central glutamine or arginine residue in the SNARE motif (Fasshauer et al., 1998). SNARE mediated membrane fusion also depends on Sec1/Munc18 proteins, which are peripheral membrane proteins that interact with Qa-SNAREs (Rizo and Sudhof, 2012). Structural information on eight SM proteins suggests conserved topologies with three domains that fold into an arch or U-shaped conformation (Archbold et al., 2014). Multiple models have been proposed to explain their mode of function. According to the models, they can either: 1. Assist formation of SNARE complexes (Shen et al., 2007; Schollmeier et al., 2011), 2. Stabilize the trans-SNARE complex (Collins et al., 2005; Starai et al., 2008), 3. Catalyze membrane fusion by lipid binding (Xu et al., 2011) or 4. Bind the forming four-helix SNARE bundle and exert force on the fusing membranes (Dulubova et al., 2007). In vitro binding studies also suggest that SM proteins prevent formation of non-physiological SNARE complexes by stimulating specific SNARE complex formation (Peng and Gallwitz, 2002).

There are 6 SM proteins in Arabidopsis thaliana (Sanderfoot et al., 2000; Bassham et al., 2008). The SM protein SEC11 (KEULE) binds the Qa-SNARE SYP111 (KNOLLE) during cell division and cell plate formation (Waizenegger et al., 2000; Park et al., 2012). It also binds the
plasma membrane SNARE SYP121 selectively and affects secretory traffic driven by SYP121 (Karnik et al., 2015). VPS33 localizes to the late endosomes and tonoplast and is involved in vacuolar traffic (Rojo et al., 2003). AtVPS45, functions at the trans-Golgi network (TGN) and is required for trafficking of vacuolar cargo (Bassham et al., 2000; Zouhar et al., 2009). The SM proteins SEC12/SEC1a and SEC13/SEC1b are expressed at very low levels or not at all (Sanderfoot et al., 2000; Bassham and Blatt, 2008) while the sixth SM protein shows close homology to the yeast SM protein Sly1p suggesting its function in trafficking between the endoplasmic reticulum and the Golgi (Blatt, 2003).

AtVPS45 is associated with SYP41/SYP61/VTI12 SNARE machinery at the TGN. The TGN is a sorting station, maturing from the Golgi network, that functions in directing secretory traffic, recycling endocytosed cargo and driving biosynthetic cargo to the vacuole (Gendre et al., 2014). The SYP41/SYP61/VTI12 SNAREs regulate trafficking at the TGN for vacuolar-cargo sorting, secretion of cell wall components, auxin homeostasis and abiotic/biotic stress responses (Bassham et al., 2000; Zhu et al., 2002; Surpin et al., 2003; Uemura et al., 2012). Normal VPS45 levels are crucial for SYP41 stability and essential for plant growth since AtVPS45 knockouts are male gametophyte lethal (Zouhar et al., 2009). RNA interference lines with reduced AtVPS45 protein levels have reduced SYP41 protein levels and mis-sort vacuolar sorting receptors (VSRs) (Zouhar et al., 2009), which are membrane bound receptors that drive transport to vacuoles. VSRs bind to vacuolar sorting determinants (VSDs), which are specific amino acid sequences on vacuole bound protein cargo. After binding, the VSRs deliver the cargo to the prevacuolar compartment (PVC) to be sorted onto vacuoles, while the VSRs recycle back to the TGN (daSilva et al., 2005). VTI12 functions in trafficking cargo containing C-terminal VSDs (ctVSDs) while SYP61 is required for recycling of the VSRs form the PVC to the TGN.
(Sanmartin et al., 2007; Niemes et al., 2010). AtVPS45 silencing lead to recycling defects of VSRs from the PVC to the TGN and also interfered with the sorting of ctVSD containing cargo.

Analysis of the ben2 mutant, a point mutant form of AtVPS45 with an aspartate-to-asparagine substitution at the 129th amino acid position, also reveals a role for AtVPS45 in auxin transporter recycling and endocytic uptake of membrane cargo from the plasma membrane (Tanaka et al., 2013). Together, these results suggest a critical role for AtVPS45 along with the SYP41/SYP61/VTI12 complex at the TGN in endocytic and vacuolar cargo sorting.

In this study, a new point mutant of VPS45, recovered via a TILLING (Targeting Induced Local Lesions in Genomes) approach, was analyzed for understanding the SM protein’s role at the cellular and physiological level. This point mutant, designated as Atvps45-3, harbors a serine-to-phenylalanine substitution at the 284th position of the polypeptide. Atvps45-3 plants display a dwarfed phenotype, reduced organ size and cell expansion defects similar to the previously reported RNAi lines (Zouhar et al., 2009). The mutants also display severe effect on polarized cell expansion as observed in highly defective root hair growth. The root hairs still expand in the presence of a membrane permeable auxin suggesting that the mutation does not affect auxin responses. Bulk endocytic uptake and secretory routes to the apoplastic space are unaffected in the Atvps45-3 cells. Further investigations with this mutant form will aid the analysis of AtVPS45’s exact role in trafficking at the TGN and interaction with the TGN SNARE machinery.
3. Methods

3.1. *Arabidopsis thaliana* genotypes and plant growth conditions

All genotypes were grown at 22°C either on soil in growth chambers or sterile nutrient media under light racks. Soil-grown plants were kept in long day (16hr light/8hr dark) conditions. For growth on nutrient media, seeds were surface sterilized in 33% (v/v) bleach and 1% Triton solution for 10 minutes and washed with sterile water at least five times. After two days of cold treatment in the dark the seeds were plated on solid Murashige-Skoog medium with vitamins (MS)(MSP09; Caisson Labs), 1% sucrose, 2.4mM MES pH5.7, and 0.8% (w/v) phytoagar (PTP01; Caisson Labs). To test the effect of exogenous auxin on root hair growth, seedlings were grown on MS media containing 100nM of 1-naphthalene acetic acid (1-NAA) (Sigma-N0640) for seven days.

The Columbia-0 accession was used as a wild type control and the *Atvps45-3* TILLING mutants were a gift from Dr. Diane Bassham. Complementation of *Atvps45-3* mutants was performed by transforming them with a binary vector containing a partial VPS45 genomic sequence fused with a VPS45 cDNA fragment and driven by the VPS45 promoter [described in (Zouhar et al., 2009)] The floral dip method of *Agrobacterium tumefaciens*-mediated transformation was utilized (Clough and Bent, 1998). Complemented lines were recovered and screened by resistance to hygromycin (30 mg L⁻¹) and restriction digestion of an amplified 587bp region of the cDNA with MseI. The point mutation introduces a novel restriction site in the amplified 587bp region of the *Atvps45-3* cDNA, resulting in two bands of 328bp and 259bp upon MseI digest while the WT cDNA lacks this site and thus results in a single 587bp band upon digestion. Homozygous transformant lines were screened for by appearance of all three
bands in the restriction profile and resistance to hygromycin in subsequent progenies of the primary transformants.

### 3.2. FM4-64 Staining and Brefeldin A (BFA) Treatment

FM4-64 staining was modified from Dettmer et al. (2006). To test bulk endocytosis, 4-day-old seedlings were transferred to 0.5× MS liquid medium containing 4 μM FM4-64 for 2 min and subsequently washed twice for 30 s each time in 0.5× MS liquid medium before microscopic analysis. For analyzing arrival of FM4-64 at Brefeldin A bodies, 4-day-old seedlings were transferred to 0.5× MS liquid medium containing 35 μM Brefeldin A (BFA) for an hour followed by a 10-min treatment with 4 μM FM4-64 plus 35 μM BFA and two subsequent washes of 30 s each. The root tips were visualized using a Leica SP5 confocal laser scanning microscope (Leica Microsystems) at the Iowa State University Confocal and Multiphoton facility, using a 63× oil immersion objective lens and excitation and emission wavelengths of 558 and 734 nm. Images were acquired under identical conditions for both genotypes with equal exposure, scan frequency and line average settings. A total of 15 seedlings from at least three independent replicates were analyzed for each treatment and genotype.

### 3.3. Transient secretory marker assays

Protoplasts isolated from long day grown plants were transformed (Yoo et al., 2007) with 20 micrograms of the sec-RFP construct (Faso et al., 2009) to test secretory defects. Transformed protoplasts were incubated at room temperature in darkness for 40 hours with gentle shaking (50rpm), followed by confocal microscopic analysis as described in (Faso et al., 2009). The leaf epidermal transient assays method was adapted from (Kim et al., 2009). The abaxial side of
leaves of 30-day-old plants grown in long day conditions was syringe infiltrated with overnight
grown Agrobacterium GV3010 strains (O.D adjusted to 0.6) containing the sec-RFP construct
(Faso et al., 2009). Similar developmental stages of leaves were used for confocal analysis, 40
hours post-infiltration.

3.4. Agarose imprinting

Hypocotyl and root cell phenotypes were analyzed by placing 7 day old seedlings on the
surface of warm 3% low melting point agarose layered on a slide and then analyzing the imprints
post-cooling (Mathur and Koncz, 1997) with a Zeiss Axioplan II light microscope.

4. Results

4.1. Atvps45-3 mutants display severe phenotypic defects

A VPS45 point mutant was recovered by a TILLING approach, which we designated as
Atvps45-3 (because the knockout lines were designated as Atvps45-1 and the RNAi transgenics
as Atvps45-2 in (Zouhar et al., 2009)). The point mutation is caused by a C-to-T substitution at
the 851st nucleotide of the AtVPS45 coding sequence, leading to a serine-to-phenylanaline
substitution at the 284th amino acid position (Figure1A). Atvps45-3 displayed dwarfed
phenotypes with highly reduced root, hypocotyls, siliques and rosette sizes similar to the
previously reported mutants (Zouhar et al., 2009), though not as severe (Figure 1B). Analysis of
the hypocotyl and root cells using an agar imprinting method (Mathur and Koncz, 1997) also
shows reduced size of cells in the mutant organs, similar to the previously reported mutants
(Zouhar et al., 2009) (Figure 1C). To test whether the defects were indeed caused by the point
mutation, complementation of the mutant plants was carried out by introducing floral dipping
transformation and transformation was confirmed with a restriction digest with MSeI (Figure 1D) and resistance to hygromycin. There was a significant increase in plant size, root, hypocotyl and silique length and root hair morphology in the complemented lines compared to the mutants (Figures 1B, C) suggests that the substitution of the Serine to a Phenylalanine, i.e. a polar to non-polar substitution affects VPS45 function. Since binding of a SM protein to its cognate SNARE is required for SNARE complex function, the defects in the mutant might be caused by defective binding of VPS45 to SYP41 and altered stability of both the SM protein and the SNARE.

4.2. _Atvps45-3_ mutants display defective root hair growth

Since cell expansion seems to be defective in the _Atvps45-3_ mutants, we also looked at root hair growth, a polar cell expansion process, in the mutants. Polar cell expansion in root hairs involves development of apical-basal polarity of the cell endomembrane system with rapid secretion at the tip of the developing root hairs (Grierson et al., 2014). We observed that the mutant root hairs were significantly shorter and wider than the wild type root hairs (Figure 2A). Analysis of the mutant root showed failure of the root hairs to elongate when compared to the WT (Figure 2B, left panel). Complementation of the mutant rescues the root hair elongation defects (Figure 2B) suggesting that the point mutation causes defects in the polarized cell expansion machinery in root hairs.

The _ben2_ mutant has defects in auxin transporter cycling. The phytohormone auxin determines root hair initiation and growth (Jones et al., 2009; Grierson et al., 2014) with auxin transport in the neighboring non root hair cells being responsible for expansion of the root hair cell (Jones et al., 2009). Mutants lacking the auxin influx transporter AUX1 or the auxin signaling protein AXR1 have short root hairs (Grierson et al., 2014) suggesting important roles
for auxin transport and signaling. We tested the effect of exogenous 1-NAA, a membrane permeable auxin, on root hair growth in the *Atvps45-3* mutants and found that root hair length increased suggesting that the mutants still retain the competency to expand in response to auxin (Figure 2D). This observation suggests that auxin signaling is still functional in the *Atvps45-3* mutants, but other processes downstream of it might be affected.

### 4.3. Endocytosis and cargo arrival at the TGN is unaffected in the mutants

Studies with *ben2*, a point mutant form of AtVPS45, suggest that VPS45 functions on the endocytic route because the mutation blocked endocytic uptake of the marker dye FM4-64 (Tanaka et al., 2013). We hypothesized that endocytosis and recycling might be affected in the Atvps45-3 plants. This could explain the cell expansion phenotype owing to slower trafficking at the TGN during cell expansion (Gendre et al., 2014), particularly during root hair growth. To test this we carried out FM4-64 uptake assays in roots and found that uptake efficiency of the dye was similar to WT cells (Figure 3A). This suggests that the serine-to-phenylalanine substitution at the 284th position does not affect VPS45’s role in endocytosis in contrast to the aspartate-to-asparagine substitution at the 129th position of the BEN2 mutant form (Tanaka et al., 2013). We also tested whether membrane cargo arrival at the TGN was affected in the mutants. We stimulated formation of TGN-endosomal aggregates by Brefeldin A treatment and subsequently treated with the FM4-64 dye. Labeling of the BFA compartments with the dye occurred at similar times suggesting that BFA body formation and arrival of membrane cargo at the surface of the TGN is not affected in the mutant. Thus, defects in endocytosis and membrane cargo trafficking from the PM to the TGN seem to be unaffected in *Atvps45-3* mutants.
4.4. Secretion of the sec-RFP marker occurs normally in the mutants

Since cell expansion also involves efficient secretory trafficking to the extracellular space in synchrony with vacuolar expansion, we tested whether the cell expansion defects might be due to defective secretion and exocytosis at the TGN. We hypothesized that changes in secretory trafficking at the TGN would cause a decreased arrival of cell wall cargo as the cell expands thus decreasing efficiency of the process. To test this hypothesis we used the sec-RFP marker (Faso et al., 2009) that is transported from the ER to the apoplastic space. In wildtype cells, sec-RFP is synthesized in the endoplasmic reticulum and secreted to the apoplast normally while any accumulation of red fluorescence signal inside mutant cells would suggest defects in the secretory pathway. We transformed protoplasts with the sec-RFP construct and imaged them under a confocal microscope. Appearance of majority of the red fluorescence on the exterior surface of the protoplasts was observed in both mutant and WT protoplasts, 40 hours post-transformation, suggesting no observable defects in secretory trafficking in the Atvps45-3 mutants (Figure 4A). To confirm this observation in vivo, we conducted a transient assay in epidermal cells of Arabidopsis leaves (Kim et al., 2009). Mutant and WT leaves grown in short day condition were infiltrated with Agrobacterium containing the sec-RFP construct and imaged under a confocal microscope. The red fluorescence appeared on the edges of both WT and Atvps45-3 pavement cells with almost no detectable fluorescence in the endomembrane system (Figure 4B), suggesting that bulk secretory routes were indeed unaffected in the Atvps45-3 mutants.
5. Discussion

5.1. The *Atvps45-3* phenotype: Vacuolar trafficking

*AtVPS45* is a SM protein associated with the SYP41/SYP61/VTI12 SNARE complex at the TGN, where it regulates SNARE complex activity and trafficking to the vacuole (Bassham and Raikhel, 1998; Bassham et al., 2000; Zouhar et al., 2009). RNAi lines with reduced *AtVPS45* protein levels display cell expansion defects correlated with reduced vacuolar size, altered localization of VSRs and defective trafficking of ct-VSD containing vacuolar cargo (Zouhar et al., 2009). VTI12 drives vacuolar traffic and SYP61 is required for retrograde trafficking of VSRs from the prevacuolar compartment (Sanmartin et al., 2007; Niemes et al., 2010). Together, these results suggest the requirement of the SYP41/SYP61/VTI12/AtVPS45 complex for efficient vacuolar trafficking that then plays central role in turgor driven cell expansion.

*Atvps45-3* harbors a serine to phenylalanine substitution at the 284\(^{th}\) position of the polypeptide chain. We hypothesized that *Atvps45-3* would also have cell expansion defects due to defects in vacuolar trafficking. We found marked reduction in size of the *Atvps45-3* plants, which seems to arise due to diminished cell expansion. Root hair growth is also stunted and suggests defects in the polarized cell expansion process. We speculate that the point mutation affects AtVPS45 function at the TGN in regulating trafficking by altering its mechanism of action on the SNARE machinery. This might lead to defects in VSR recycling and sorting of ctVSD-containing cargo thus affecting vacuolar morphology and subsequently cell expansion. This would also explain the root hair growth defect in the *Atvps45-3* mutants since vacuole enlargement is critical during root hair expansion (Galway et al., 1997; Grierson et al., 2014). The SNARE, VTI13 (belonging to the same family as VTI12) localizes to the vacuole and the TGN and is speculated to play roles in trafficking to the vacuole. Interestingly the *vti13* mutant
displays mislocalization of SYP41 and defective root hair growth (Larson et al., 2014). This lends support to the connection between vesicle fusion machineries at the TGN and vacuole and root hair growth. Further analysis of vacuolar morphology and cargo sorting to the vacuole in the Atvps45-3 mutants would reveal the basis for the defects.

5.2. The Atvps45-3 phenotype: Auxin transport and signaling

The TGN acts as a sorting station receiving endocytosed cargo and recycling it back to the PM or targeting it to the vacuole for degradation (Gendre et al., 2014). These processes are key for fine-tuning cell growth and expansion by affecting key processes such as auxin-mediated cell elongation. Cargoes such as auxin transporters utilize this strategy of recycling via the TGN for maintaining polar auxin flow in plant organs and adjusting the flow based on physiological demands (Habets and Offringa, 2014; Grones and Friml, 2015). The SYP61 compartment at the TGN is important for auxin transporter traffic (Robert et al., 2008) and mutants lacking SYP4 SNARE family members show defects in auxin homeostasis (Uemura et al., 2012). Analysis of ben2 mutants, harboring a point mutation in AtVPS45, suggests a role for VPS45 in endocytosis and auxin transporter recycling (Tanaka et al., 2013). Thus the SYP41/SYP61/VTI12 SNARE complex along with the SM protein AtVPS45 seems to play important roles in auxin transport/responses by mediating transporter recycling and sorting.

We hypothesized that the Atvps45-3 mutant would also have defects in endocytosis, which might then affect auxin transporter trafficking, and hence auxin mediated cell elongation. Analysis of FM4-64 uptake in Atvps45-3 mutants suggests that endocytosis is unaltered and studies with the fungal toxin Brefeldin A (Lippincott-Schwartz et al., 1991) suggests that membrane cargo arrival at the TGN-endosomal aggregates is also unaffected. This indicates that
the mutation (S284F) probably doesn’t affect AtVPS45 function in the same manner as the ben2
(D129N) mutation and bulk endocytosis remains functional. Analysis of trafficking of individual
auxin transporters such as AUX1 and PINs (Habets and Offringa, 2014) in the Atvps45-3
background might reveal distinct sorting defects.

We also hypothesized that root hair growth defects might be due to altered auxin
transport/response in Atvps45-3. Auxin transport and signaling is important for root hair
expansion since mutants lacking the auxin influx transporter AUX1 or the auxin signaling
protein AXR1 have short root hairs (Grierson et al., 2014). Interestingly, auxin transport in the
neighboring non root hair cells is also important for expansion of the root hair cell (Jones et al.,
2009). Exogenous treatment with the membrane permeable auxin 1-NAA causes root hair
expansion in the Atvps45-3 mutants suggesting that competency to respond to auxin is unaltered
in the mutants. It should be noted that the root hairs increased in length but showed a distinctive
waving along their length. The basis for this is open to interpretation, but could point towards
downstream effects on the cytoskeleton since perturbing actin and microtubule dynamics affects
the direction of root hair growth (Sieberer et al., 2005; Grierson et al., 2014). Further studies
elucidating auxin transporter recycling dynamics and auxin responses in the Atvps45-3 mutant
roots and root hairs are needed to better understand the basis for the defect.

5.3. The Atvps45-3 phenotype: secretory traffic to the plasma membrane

Sorting and secretion (exocytosis) of extracellular cargo, via the TGN, is imperative for
efficient cell expansion as the plant cell wall is synthesized and deposited (Worden et al., 2012).
Polarized cell expansion in root hairs requires high volumes of exocytosis (Ketelaar et al.,
2008; Grierson et al., 2014). Mutants with defective secretory machinery at the TGN, such as
echidna, usually display cell expansion phenotypes (Gendre et al., 2011). The TGN SNARE SYP61 is crucial for secretion of multiple cellulose synthase complex subunits (CESAs) required for cellulose deposition, as revealed by proteomic analysis of SYP61 labeled vesicles (Drakakaki et al., 2012). Defects in the SYP41/SYP61/VTI12 machinery in the Atvps45-3 might thus cause a change in secretory dynamics of the cell wall deposition machinery and hence cell expansion. Analysis of secretion of a generic secretory marker did not reveal any defects in appearance of the marker in the apoplastic space of the Atvps45-3 mutant protoplasts and leaf epidermal cells. Thus the secretory defect, if any, could be due to the effect on specific PM bound cargo such as CESAs and root tip specific CSLD3 (Park et al., 2011) that are required for cell wall deposition.

Our study reveals a novel mutant to investigate VPS45 function in Arabidopsis thaliana and places a TGN-localized SM protein and SNARE machinery at the polarized tip growth process in root hairs. Further investigations are required to delineate its exact mode of action.

6. Acknowledgements

I would like to thank Dr. Diane Bassham for providing with the Atvps45-3 seeds and the construct for complementation of the mutant plants, Margie Carter for assisting with the confocal imaging and Dr. Federica Brandizzi for providing the sec-RFP construct for the transient secretory assays

7. References


8. Figures

**Figure 1:** Atvps45-3 mutants display severe phenotypic defects. A) Schematic for protein sequence of VPS45 displaying sites of amino acid substitution for *Atvps45-3* and the previously reported *ben2* mutants. *Atvps45-3* harbors a serine to phenylalanine substitution at the 284th position of the polypeptide while *ben2* displays an aspartate-to-asparagine substitution at the 129th position. B) Phenotypes of WT, Atvps45-3 and complemented Atvps45-3. In clockwise order: long day grown 40 day-old plants, 10-day-old roots grown on growth media under long day conditions, 5-day-old dark grown hypocotyls, siliques from 40-day-old long day grown plants, rosettes from 20-day- long day grown plants. C) Representative agarose imprints of hypocotyls and roots from 5-day-old dark grown seedlings D) MseI restriction digest profiles of an amplified 587 bp region (where the point mutation occurs and introduces a MseI cut site) of the cDNA of WT, Atvps45-3 and complemented mutant plants.
Figure 2: *Atvps45-3* mutants display defective root hair growth but can still expand upon exogenous auxin treatment (1-NAA). **A)** Left panel- Color inverted images of 5-day-old roots of WT and *Atvps45-3* displaying root hair silhouettes. Right panel- Light microscopic images of WT and *Atvps45-3* root hairs. **B)** Representative light microscopic images of WT, *Atvps45-3* and complemented *Atvps45-3* (COM) root hairs. **C)** Representative light microscopic images of WT, *Atvps45-3* and complemented *Atvps45-3* (COM) root hairs grown in the absence (-NAA) or presence (+NAA) of 100nM 1-NAA, an exogenous membrane permeable auxin.
**Figure 3:** *Atyps45-3* mutants have normal bulk endocytosis and arrival of membrane cargo from plasma membrane to Brefeldin A (BFA) bodies. A) Root cells showing uptake of FM4-64 after 2 min of treatment of 4-day-old seedlings with 4 μM FM4-64 in liquid 0.5× MS medium. White arrows indicate early endosomes/TGN. B) Root cells treated with 35 μM BFA for 1 h, followed by a 10 min incubation with 4 μM FM4-64, showing arrival of FM4-64 at BFA bodies. Yellow arrows indicate BFA bodies. Scale bar=10 μm.
Figure 4: *Atvps45-3* mutants display normal secretion of the sec-RFP marker in protoplasts and leaf epidermal cells. (A) Z-stacked projections of protoplasts showing the appearance of the sec-RFP marker at the extracellular space. Protoplasts were transformed with 30 μg of the sec-RFP plasmid and incubated for 40 hours in the dark. Scale bar=7.5 μm. (B) Confocal images of leaf epidermal cells transiently expressing sec-RFP at the extracellular space. The abaxial side of leaves of 30-day-old plants (grown under long day conditions) were syringe infiltrated with overnight grown *Agrobacterium* GV3010 strains (O.D = 0.6) containing the sec-RFP construct and imaged 40 hours post infiltration. Scale bar=10 μm.
CHAPTER 5
DISSERTATION DISCUSSION

1. Introduction

In the previous chapters of this thesis I have presented the research conducted during the course of my PhD. My work focused on the role of two proteins that localize to the trans-Golgi network and associate with the SYP4/SYP61/VTI12 SNARE machinery. TNO1, the first protein investigated, is a putative tethering factor with multiple coiled coil domains while the second, VPS45, is a Sec1/Munc18 protein. Both proteins are required for proper functioning of the SYP41/SYP61/VTI12 SNARE complex. I investigated three research directions: (1) TNO1’s involvement in auxin transport dependent processes (2) TNO1’s involvement in root growth movements and (3) Effect of a point mutant form of VPS45 on plant development and physiology. These investigations have led me to key discoveries in the role of TNO1 and VPS45 in plant physiological processes such as lateral root emergence, gravitropic organ bending, root skewing and root hair expansion.

The TGN is a key structure at the crossroad of endocytic and secretory transport playing important roles in hormonal signaling, plant response to biotic and abiotic stresses and deposition of the cell wall (Viotti et al., 2010; Gendre et al., 2015). Multiple SNAREs involved in various trafficking routes decorate the TGN (Kim and Brandizzi, 2012). The members of the SYP41/SYP61/VTI12 complex on the TGN are required for efficient cellular trafficking and multiple processes such as auxin homeostasis, abiotic and biotic stress responses and secretion of the cell wall biosynthetic machinery. The SYP4 family has three members, SYP41, SYP42 and SYP43, which have partly redundant functions. The SYP4 SNARE family is important for
vacuolar and secretory trafficking, maintenance of Golgi-TGN morphology, root gravitropism and extracellular defense (Uemura et al., 2012). VTI12 is required for trafficking to the vacuole (Sanmartin et al., 2007). SYP61 functions in osmotic stress tolerance and is also involved in secretory traffic to the extracellular space (Zhu et al., 2002). A proteomic study of SYP61 labeled vesicles has led to the identification of cellulose synthase and other wall machinery as cargo that is trafficked via the TGN (Drakakaki et al., 2012).

Defects in the SNARE-associated machinery also result in defective cellular trafficking and altered physiological processes. TNO1 is a SYP41-interacting, putative tethering factor that is involved in vacuolar trafficking and membrane fusion at the TGN. It is also required for abiotic stress tolerance and proper localization of SYP61 (Kim and Bassham, 2011). VPS45 is a known Sec1/Munc18 protein that associates with the SYP41/SYP61/VTI12 SNARE complex and is required for cell expansion, trafficking of vacuolar cargo and auxin homeostasis (Bassham et al., 2000; Zouhar et al., 2009; Tanaka et al., 2013). My research has led to the discovery of novel roles of TNO1 and VPS45 in various plant physiological processes. I discovered that TNO1 is involved in the auxin transport dependent processes of lateral root emergence and gravitropic bending of plant organs (Roy and Bassham, 2015). Mutants lacking TNO1 also display defects in root movements, with an enhanced deviation from the growth trajectory (skewing) and a correlative twisting of root epidermal cell files (CFR). Studies with drugs that affect microtubule (MT) dynamics and thus root movement, suggest that loss of TNO1 might affect MT-associated processes. These discoveries put TNO1 at the crossroads of auxin transport and cytoskeletal dynamics. Phenotypic analysis of a point mutant form of VPS45 also adds a SM protein to the list of proteins required for growth of root hairs in Arabidopsis and uncovers a novel role of the SYP4/SYP61/VTI12 SNARE machinery in rapid tip growth.
2. TNO1: A TGN-localized, Putative Tethering Factor at the Crossroad of Auxin Transport, the Cytoskeleton and Cell Expansion

This dissertation suggests a role for TNO1 in the function of TGN-resident SNARE machineries, auxin transport and microtubule (MT) dependent processes that affect cell expansion.

2.1. TNO1 and auxin transport

TNO1’s role in auxin transport adds another molecular player to the complex process of auxin transport from cell to cell. Polar transport of auxin in plants is crucial to plant development and physiology and occurs due to polar localization of specific auxin influx and efflux transporters. The polar localization and relocalization of auxin transporters are dependent on the cellular trafficking machinery that regulates endocytosis, recycling and vacuolar trafficking (Friml, 2010; Grunewald and Friml, 2010; Habets and Offringa, 2014; Grones and Friml, 2015). This helps in maintaining auxin homeostasis and redirecting auxin flow in plant organs during processes such as lateral root emergence and gravitropic organ bending (Strohm et al., 2012).

The trafficking of various auxin transporters has been elucidated in detail, with various factors such as phosphorylation/ dephosphorylation status of the transporters, ARF-GTPases, ARF-GEFs and actin playing key roles (Friml, 2010; Habets and Offringa, 2014). The role of SNAREs in auxin-mediated processes is still not well understood with only a few studies connecting the two. The vacuolar SNARE VAM3 is required for polar localization of the auxin efflux transporter PIN1 in leaf cells and the complex containing VAM3 is required for formation of auxin dependent venation pattern in leaves (Shirakawa et al., 2009). Another recent study shows
how auxin might modulate vacuolar SNAREs to affect vacuolar morphology and hence cell
growth (Lofke et al., 2015). The TGN localized SYP4 SNARE family was shown to be required
for degradative sorting of the auxin efflux transporter, PIN2 to the vacuole and auxin distribution
in the root (Uemura et al., 2012). Since TNO1 associates with SYP41, TNO1 was hypothesized
to be involved in auxin-mediated processes.

My research supports this hypothesis because the auxin transport dependent processes of
lateral root emergence and gravitropic bending of organs are defective in the tno1 mutants and
can be rescued by exogenous addition of natural (IAA) and synthetic membrane permeable
auxins (1-NAA). The increased sensitivity of mutant root elongation to an influx specific auxin
(2,4-D) and normal response to blocking auxin efflux (with NPA) compared with WT roots
suggests that tno1 might have defective auxin influx pathways. Studies with the auxin response
marker DR5rev:GFP reveal reduced fluorescence asymmetry at the tips of emergent LRs and
gravistimulated roots in tno1 mutants, suggesting a defect in auxin transport. Bulk endocytosis
and arrival of membrane cargo at the TGN was found to be unaffected in tno1 roots, suggesting
that TNO1 possibly affects auxin transport by acting specifically on sorting of auxin transporters
or components driving their sorting (Figure 1A).

2.2. TNO1 and MT-associated processes

My research also shows that TNO1 affects the skewing movement of roots and root
epidermal cell file rotation suggesting a role in MT-dependent processes. Root movement studies
are a good model to investigate defects in root growth processes. A combinatorial response
involving gravitropism, touch and endogenous circumnutatory pathways results in specialized
root movements known as waving and skewing on the surface of slanted, hard impenetrable
media (Oliva and Dunand, 2007; Roy and Bassham, 2014). Roots move by integrating multiple cues at the root tip and transducing the signal to regulate changes in cell expansion. As a root grows, cell division in the root apical meristem pushes cells shootward into the elongation zone where anisotropic expansion causes cells to elongate rapidly resulting in root growth and elongation.

Disordered MT arrays in the meristematic region give way to transverse arrays in the elongation zone, facilitating rapid anisotropic expansion and finally arranging longitudinally in mature root cells (Sugimoto et al., 2000). MT patterning and dynamics driven by the MT’s self-organizing nature and the action of several microtubule-associated proteins (MAPs) can regulate root movements by affecting cell wall properties and directional cell elongation (Sedbrook and Kaloriti, 2008; Shaw, 2013; Roy and Bassham, 2014). Mutants defective in MT structure or several MAPs display distinctive cell elongation and root movement phenotypes (Struk and Dhonukshe, 2014). My studies reveal that the tno1 roots display an exaggerated rightward skew (as seen from back of the plate) compared to the WT roots and also displayed higher angles of left handed cell file rotation. Skewing of the tno1 roots increased upon MT stabilization, while destabilization had no significant effect on skewing when compared to wildtype roots after similar treatments. Cell morphology in the mature zones of the roots and the base of the hypocotyl was also adversely affected in the tno1 seedlings when MTs were destabilized.

These results suggest TNO1 plays a role in MT-associated mechanisms of root movement and cell expansion. MT arrays display distinct oblique orientations in twisting cell files of other mutants, yet the MT array in the cell files of skewing tno1 roots did not show an oblique orientation. This suggests TNO1 is involved in a MT dependent cell expansion process that does not affect the MT array orientation and that the exaggerated CFR of the skewing mutant roots
might originate by an alternative mechanism. Possibilities of an indirect effect on MTs by interactions with MAPs also need to be explored (Figure 1B).

Alternatively, TNO1 might be affecting deposition of the cell wall and cell expansion thus driving CFR formation and root skewing. MT arrays co-align with cellulose microfibrils during cell elongation and guide cellulose deposition (Ledbetter and Porter, 1963; Heath, 1974) (Figure 1B). Mutants such as *fragile fiber1* (*fra1*) and *botero1* (*bot1*) display defective cell expansion due to disorganized microtubules and cellulose microfibrils (Bichet et al., 2001; Zhu et al., 2015). Cellulose is deposited by cellulose synthase complexes (CSCs), which are composed of catalytic units known as cellulose synthases (CESAs) (Li et al., 2014). CESAs assemble into CSCs in the Golgi and traffic to the PM via the TGN as confirmed by co-localization with the TGN SNAREs SYP41, SYP42 and SYP61. Proteomic analysis of SYP61 labeled vesicles also reveals the presence of CESAs and other wall components such as KORRIGAN1 (KOR1) suggesting SYP61’s role in cell wall deposition (Crowell et al., 2009; Gutierrez et al., 2009; Drakakaki et al., 2012) (Figure 1B).

CESAs also interact with the clathrin-mediated endocytic machinery and are endocytosed to control their density at the PM (Bashline et al., 2013). After endocytosis, CESAs localize to specialized MT- associated trafficking compartments that are also hypothesized to contain CESAs derived from the de novo secretory pathway (Lei et al., 2015). These compartments, known as microtubule associated cellulose synthase compartment (MASCs) or small cellulose synthase compartments (*SmaCCs*), are hypothesized to be fast recycling endosomes (Crowell et al., 2009; Gutierrez et al., 2009; Lei et al., 2015). MTs guide cellulose deposition and the peripheral protein CELLULOSE SYNTHASE INTERACTING PROTEIN1 (CS11) links MTs and cellulose synthase complexes and is also required for formation of MASCs/SmaCCs (Gu et
al., 2010; Bringmann et al., 2012; Lei et al., 2012; Li et al., 2012; Lei et al., 2015). MTs affect cellulose biosynthesis by regulating exocytosis of CSC containing vesicles or CSC-associated proteins such as KOR1 and CSI1 (Robert et al., 2005; Li et al., 2014; Worden et al., 2015). The small molecule CESA TRAFFICKING INHIBITOR (CESTRIN) affects trafficking of CSCs, KOR1 and CSI1, causes MT instability and reduces cellulose content leading to alterations in anisotropic growth of *Arabidopsis* hypocotyls. CESTRIN treatment also increases co-localization of SYP61 with CESAs in CESTRIN bodies, while not affecting bulk endocytic and secretory routes (Worden et al., 2015).

Thus there is close interplay of trafficking at the TGN, MTs and the cell wall deposition machinery. Loss of TNO1 mis-localizes SYP61, therefore *tno1* mutants might mis-sort cell wall components and have altered cellulose deposition (Figure 1B). Changes in the cell wall deposition machinery can alter the cell expansion pattern from an anisotropic to an isotropic one. *spr1*, a mutant with enhanced CFR, displays strong isotropic expansion of the cortical layers compared to the anisotropic expansion of the epidermal cells. This forces the cell file to twist to prevent shearing due to disproportionate expansion rates (Furutani et al., 2000; Ishida et al., 2007). Thus one explanation for the enhanced CFR in the skewing *tno1* roots might be the alteration of expansion rates in various cell layers caused by differential effects of the mislocalized cell wall deposition machineries in the interior and exterior cells. Mislocalization of the cell wall machinery might also offer an alternative explanation for the delayed LR emergence seen in the *tno1* roots. If cell wall remodeling enzymes were not efficiently sorted to the extracellular space during LR emergence, the overlying cortical layer would loosen at a slower rate, delaying the LR’s emergence.
2.3. **TNO1 and other plausible mechanisms of action**

Auxin transport defects might also affect cell expansion dynamics in *tno1* roots during gravitropic bending and root skewing. Auxin is responsible for cell expansion, the basis for which is currently explained by the acid growth hypothesis (Perrot-Rechenmann, 2010). Extracellular auxin perceived by an auxin receptor leads to activation of PM proton pumps that pump protons into the extracellular space. The acidic extracellular pH leads to the activation of cell wall loosening proteins such as expansins and xyloglucan endotransglycosylase/hydrolases (XTH). The proton pump activity also activates voltage dependent potassium inward channels causing osmotic uptake of water and driving cell expansion (Wang and Ruan, 2013). Thus the slower auxin transport in mutants might cause lags in cell wall acidification and subsequent cell expansion.

TNO1 might also physically tether the TGN or endosomes to MTs directly or indirectly and hence affect the recycling and turnover of auxin transporters or cell wall components. This would be similar to the microtubule associated protein CLASP’s mode of action (Ruan and Wasteneys, 2014). CLASP can tether specialized SNX1 endosomes to MTs and modulate PIN2 levels by affecting its recycling and vacuolar targeting (Ambrose et al., 2013; Kakar et al., 2013; Ruan and Wasteneys, 2014). Since *tno1* has vacuolar trafficking defects and auxin transport defects with MT dynamics based phenotypes, it would be interesting to test whether TNO1 traffics auxin transporters to the vacuole or tethers endosomes to MTs.
3. VPS45: A Sec1/Munc18 (SM) Protein Required for General and Polarized Cell Expansion

This dissertation also reports a novel role for the regulatory Sec1/Munc18 protein VPS45 in polarized cell expansion in plants. SM proteins are involved in SNARE mediated membrane fusion. Multiple models have been proposed to explain their mode of function by either: 1. Assisting formation of SNARE complexes (Shen et al., 2007; Schollmeier et al., 2011) 2. Stabilizing the trans-SNARE complex (Collins et al., 2005; Starai et al., 2008) 3. Catalyzing membrane fusion by lipid binding (Xu et al., 2011) or 4. Binding the forming four-helix SNARE bundle and exerting force on the fusing membranes (Dulubova et al., 2007). VPS45 in yeast is required for delivery of proteins from the TGN into the endosomal system (Cowles et al., 1994; Piper et al., 1994). Studies in yeast also suggest that Vps45 regulates the cellular levels of its binding SNARES Tlg2 and Snc2 (Shanks et al., 2012).

The Arabidopsis VPS45 (AtVPS45) is known to localized at the TGN and interact with the SYP41/SYP61/VTI12 SNARE machinery (Bassham et al., 2000; Zouhar et al., 2009). It is required for cell expansion, maintenance of vacuolar morphology and sorting of vacuolar cargo (Zouhar et al., 2009). Studies with ben2, a VPS45 point mutant reveals its importance for endocytosis and recycling of auxin transporters (Tanaka et al., 2013). Our study with Atvps45-3, another point mutant form of VPS45 recovered via a TILLING approach (Henikoff and Comai, 2003; Henikoff et al., 2004; Chen et al., 2014), confirms the importance of VPS45 in overall cell expansion. The Atvps45-3 mutants have a severely dwarfed phenotype with small leaves, short plant organs and display delayed bolting of the inflorescence. The mutants also display short and thick root hairs that fail to expand to wildtype lengths. A VPS45 promoter driven expression of a fusion of partial VPS45 genomic DNA sequence with a partial VPS45 cDNA sequence
complements the mutant phenotypes, confirming that the defects are indeed caused by the point mutation.

Root hair expansion occurs by finely orchestrated tip growth, which depends on efficient exocytosis of cell wall material at the tip and other factors such as the cytoskeleton and calcium ions (Grierson et al., 2014). AtVPS45 interacts with the TGN localized SYP4/SYP61/VTI12 SNARE machinery which has roles in recycling and sorting of vacuolar and PM cargo (Zouhar et al., 2009). I hypothesized that the mutation affects these routes of membrane trafficking required for cell expansion and rapid tip growth in growing root hairs. Confocal microscopic studies on uptake of the lipophilic styryl dye FM4-64 in root cells and transient expression studies of the secretory marker sec-RFP (Faso et al., 2009) in protoplasts and leaf epidermal cells suggests that the bulk endocytic and secretory routes are not affected in the mutants. It remains to be seen if those routes are specifically affected in the root hair of the mutants. The mutant form of VPS45 might also affect the cellular levels of its interacting SNAREs or be defective in its binding with the SNARE(s), thus hampering membrane fusion processes at the TGN. Defects in the SYP41/SYP61/VTI12 machinery in the \textit{Atvps45-3} mutants might cause a change in secretory dynamics of the cell wall deposition machinery and hence cell expansion (Figure 1B).

The \textit{ben2} point mutant displays defective auxin transporter recycling suggesting AtVPS45’s role in auxin transport and responses. Auxin is required for root hair initiation and growth. Non hair cells in roots show a high level of expression of the auxin influx transporter AUX1 and canalize auxin into hair cells driving root hair growth (Jones et al., 2009). We hypothesized that the root hairs might be defective in \textit{Atvps45-3} plants due to defective auxin signaling and transport. To test this, I observed the effect of exogenous membrane permeable auxin on root hair elongation in mutant and WT root hairs. Exogenous auxin (1-NAA) treatment
caused a drastic increase in the length of mutant root hairs similar to the effect on wildtype root hairs. This suggested that the competency to respond to auxin and expand is still present in the mutant root hairs. Altogether, this led me to speculate that the root hair expansion phenotype might be due to reduced auxin responses or levels in the \textit{Atvps45-3} mutants, though auxin-signaling pathways during root hair expansion seem unaffected.

4. Future Studies

4.1. What is the mode of action of TNO1 and VPS45?

TNO1 and VPS45 seem to function at the intersection of anterograde and retrograde traffic routes at the TGN. TNO1’s exact role in the vesicle fusion machinery is still unclear. The presence of multiple coiled-coil domains, partial homology with other tethers and association with a SNARE (SYP41) suggests that TNO1 may function as a coiled-coil tether at the TGN (Kim and Bassham, 2011) facilitating cargo vesicle arrival and fusion at the TGN. TNO1 co-immunoprecipitates with SYP41 and is required for proper localization of SYP61 (Kim and Bassham, 2011), but direct binding between TNO1 and the individual SNAREs or the SNARE complex has not been tested. The mislocalization of vacuolar cargo suggests TNO1 has a role in retrograde trafficking, while mislocalization of SYP61 suggests TNO1 plays a role in anterograde trafficking to the PM, driving secretory traffic (Kim and Bassham, 2011). Normal VPS45 protein levels are required for SYP41 stability, trafficking cargo to the vacuole as well as retrograde trafficking of vacuolar sorting receptors. Bulk endocytic and secretory routes seem unaffected in the \textit{Atvps45-3} mutants suggesting specific effects on the SNARE machinery and cargo trafficking for cell expansion.
Future studies to ascertain the mode of action of TNO1 will involve testing whether TNO1 is an actual tethering factor by answering questions such as: 1. Does TNO1 physically link a cargo vesicle to its target membrane? 2. Does TNO1 bind to monomeric SNARE motifs 3. Is TNO1 required for SNARE complex assembly/disassembly and is it part of the final SNARE complex? Tethering and docking capacity of TNO1 will be tested utilizing in vitro single vesicular assays where membrane aggregation and SNARE-mediated vesicular docking will be assayed (Diao, 2015). This will also involve in vitro studies testing binding of purified fragments of TNO1, containing various combinations of the coiled coil domains, with the SYP41/SYP61/VTI12 machinery (individual members as well as the entire SNARE complex). Techniques such as bead binding assays and gel filtration binding studies will be utilized as described in (Wang et al., 2015) to answer the above mentioned questions.

Tethering factors are known Rab effectors. Rabs are small GTPases that cycle between active GTP-bound and inactive GDP-bound states, activating downstream effectors and affecting various cellular trafficking steps (Cai et al., 2007). If TNO1 were indeed a functional tethering factor, it would be interesting to test whether it is also a Rab effector. This would involve testing potential candidate Rabs via pull down assays, yeast two hybrid screens, and genetic studies. Strong candidates are expected to be from the RABA1 subclass, which localizes at the TGN, are involved in trafficking to the PM and mediate auxin responses (Koh et al., 2009).

Studies with VPS45 in Arabidopsis suggest its importance for stability of the SNARE SYP41 (Zouhar et al., 2009). It remains to be seen if cellular levels and localization of members of the SYP41/SYP61/VTI12 complex are changed in the Atvps45-3 mutants. This will involve studies utilizing subcellular fractionation followed by western blotting to ascertain SNARE levels. Co-immunoprecipitation studies will also be conducted to see whether binding of the
mutant AtVPS45 to the SNAREs is affected. In vitro liposome fusion studies with mutant and wildtype VPS45 forms and the SNAREs will confirm whether fusion events are affected or not by the point mutation. Protein structure analysis and modeling simulations can complement these studies by predicting how the point mutation affects VPS45’s interaction with SYP41.

4.2. Does TNO1 affect trafficking of auxin transporters?

How TNO1 mediates auxin transport remains to be explained. Future studies investigating candidate auxin transporters in tno1 background will help identify TNO1’s exact role. The reprogramming of auxin influx and efflux by shuffling transporters to different PM faces is required for gravitropic organ bending and lateral root emergence. AUX1 is required for LR initiation (De Smet et al., 2007) while LAX3 promotes LR emergence (Swarup et al., 2008). Subcellular trafficking studies of AUX1-YFP and LAX3-YFP during LR initiation and emergence in the tno1 background can help uncover whether TNO1 is required for their sorting or not. Relocalization of PIN3 in the emergent stage II LR tips is required for the asymmetric auxin response (Rosquete et al., 2013) that mediates LR emergence. Since emerging tno1 LRs display defects in auxin asymmetry formation, analysis of fluorescently tagged PIN3 in the LR tips can elucidate whether the defect occurs due to its altered sorting dynamics.

Trafficking of PIN transporters to different faces of the plasma membrane is crucial to the gravitropic response (Chapter 1, Figure 3). Root gravistimulation causes PIN3 and PIN7 to shift to lateral faces of the columella, elevating auxin flow to the lower side of the gravistimulated root. Root gravistimulation also causes a shootward shift of the PIN2 auxin efflux carrier in epidermal cells of the lower part of the root drives gravitropic bending (Strohm et al., 2012). Sorting of PIN2 to the vacuole in the upper half of gravistimulated roots is also required for the
bending response (Kleine-Vehn et al., 2008). In gravistimulated hypocotyls, PIN3 moves from the upper endodermal to the lower endodermal cells, causing increased auxin response in the lower hypocotyl side. This results in an upward bending response (Rakusova et al., 2011). Future studies on subcellular re-localization of various GFP-tagged auxin transporters in response to gravistimulation in the tno1 plants will confirm whether TNO1 has a role in their sorting during gravitropic bending. TNO1 may function by guiding cargo vesicles carrying these transporters and position them for SNARE mediated fusion with their destination membranes. TNO1 might also be involved in vacuolar transport and rapid turnover of these transporters during lateral root emergence and gravitropic organ bending, thus affecting auxin flux in organs.

4.3. Does TNO1 affect MT dynamics and cell wall deposition?

Studies with taxol and propyzamide suggest the possibility of TNO1 being required in a MT dependent process. Future studies would investigate whether the link between TNO1 and MTs is direct or indirect. Analysis of TNO1’s ability to bind and bundle MTs will be ascertained via MT cosedimentation assays and in vitro MT-bundling assays utilizing either recombinant protein (full length or fragments) or cell extracts. If TNO1 fails to bind MTs directly, studies targeting identification of microtubule-associated proteins that bind TNO1 could be carried out utilizing in vitro binding assays, co-immunoprecipitaions and genetic studies.

Real time confocal imaging studies of cell wall markers can help elucidate the role of TNO1 in dynamics of secretion to the extracellular space for cell wall deposition. Analysis of secretory markers and specific cargoes such as the CESA subunits of the cellulose synthase complex will help elucidate the role of TNO1 in secretion and specifically in cellulose deposition. Analysis of MT dynamics following cellulose synthesis inhibition would also
confirm TNO1’s localization or action at the interface of MT and cellulose deposition. This would involve investigations of MT array organization and dynamics upon isoxaben (cellulose biosynthesis inhibitor) treatment in the tno1 mutants. The spatiotemporal dynamics of appearance of MASCs/SmaCCs due to isoxaben treatment in the mutants would also elucidate whether TNO1 does indeed act at the interface of secretory traffic, MTs and cellulose deposition.

4.4. How does VPS45 regulate root hair expansion?

VPS45 might regulate tip growth by affecting cell wall cargo secretion at the root hair tip or by changing auxin transporter recycling or cytoskeletal dynamics. Future studies will involve teasing these possibilities apart by using the Atvps45-3 point mutant. Studies testing auxin transporter recycling and turnover will also be carried out to analyze changes in expression or localization of these proteins in the Atvps45-3 mutants. Effect of cellulose synthesis inhibitors on mutant root hairs might point at possible connections between VPS45 and cell wall secretion at the tip. Analysis of candidate tip-enriched protein markers involved in cellulose deposition such as an eYFP-CSLD3 (Park et al., 2011) might also support the possible connection.

Overexpression of the SNAREs SYP41 and SYP61 in the mutant background will be tested to see if it rescues the growth phenotypes. This will suggest that availability of more functional SNARE complexes might bypass the trafficking defects due to the inability of the mutant VPS45 to aid SNARE complex function.

Overall endomembrane organization will also be analyzed by visualizing various marker Rabs that localize to distinct compartments in root hairs. This might help identify any gross morphological changes in the endomembrane organization of the mutant root hairs as a basis for
the root hair defect. For example RabG3c can be used to inspect vacuolar morphology since it labels the tonoplast (Preuss et al., 2004; Zhang et al., 2014).

5. Impact of dissertation

I have investigated roles of two proteins associated with the SYP41/SYP61/VTI12 machinery at the TGN. This has led to the discovery of new roles for this vesicle fusion machinery at the TGN, in lateral root emergence, root growth movements and root hair expansion. This adds to the field’s understanding of the role of the TGN in plants and how the vesicle fusion machineries involved in post-Golgi traffic regulate plant physiology and growth.

A better understanding of these mechanisms at the TGN can lead to information that can be used to manipulate lateral root emergence rates, root movement in various soil conditions and improve root hair mediated nutrient uptake. This potential to improve root architecture and dynamics could result in engineering superior crops with better soil penetration and nutrient assimilation traits.

6. References


Figure 1: Model for TNO1’s mode of action: A) TNO1 may regulate auxin transport by affecting recycling or sorting of auxin transporters to different faces of the cell or to the vacuole for degradation. B) TNO1 may regulate cell expansion and thus root movement secretion of SYP61 vesicles carrying cellulose synthase complexes or other cell wall components. TNO1 might also directly or indirectly associate with microtubules (MTs) or microtubule-associated proteins (MAPs) leading to downstream effects on trafficking events required for root movement (skewing).
APPENDIX

ROOT GROWTH MOVEMENTS: WAVING AND SKEWING

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a review article published in \textit{Plant Science}

1. Abstract

Roots anchor a plant in the soil, acquire nutrition and respond to environmental cues.

Roots perform these functions using intricate movements and a variety of pathways have been implicated in mediating their growth patterns. These include endogenous genetic factors, perception of multiple environmental stimuli, signaling pathways interacting with hormonal dynamics and cellular processes of rapid cell elongation. In this review we attempt to consolidate our understanding of two specific types of root movements, waving and skewing, that arise on the surface of growth media, and how they are regulated by various genes and factors. These include crucial factors that are part of a complex nexus of processes including polar auxin transport and cytoskeletal dynamics. This knowledge can be extrapolated in the future for engineering plants with root architecture better suited for different soil and growth conditions such as abiotic stresses or even extended spaceflight. Technological innovations and interdisciplinary approaches promise to allow the tracking of root movements on a much finer scale, thus helping to expedite the discovery of more nodes in the regulation of root waving and skewing and movement in general.
2. Introduction

Roots are hidden parts of plants usually growing downward and branching inside the soil in an intricate and dynamic fashion. They help anchor the plant and acquire nutrients while also sensing the soil milieu, which in turn mediates overall plant physiological responses to its environment. As a seed germinates, the movement of roots into the substratum is controlled by both genetic and environmental cues. This seemingly simple process is actually a complex phenomenon as the root integrates cues from gravity, touch, light, nutrition and other environmental conditions that regulate its movement and architecture.

The development of roots has been investigated for many decades and substantial progress has been made in understanding root structure and developmental physiology (Petricka et al., 2012). Roots grow by the concerted action of rapid cell division and subsequent rapid elongation of the newly formed cells in the elongation zone. These processes are under the control of hormones, structural proteins and a dynamic cellular trafficking machinery to provide new materials for the elongating root. The root also has complex cross-talking signaling pathways that perceive and respond to diverse environmental cues such as light, gravity, mechanical obstacles, moisture and nutrient gradients, allowing an optimal growth trajectory (Petricka et al., 2012; Boonsirichai et al., 2002; Overvoorde et al., 2010; Vanstraelen and Benkova, 2012; Bisgrove, 2008; Baluška and Mancuso, 2013).

Charles Darwin was a pioneer in systematically documenting plant movements in his book, *The Power of Movement in Plants* (Darwin and Darwin, 1880). Before beginning a specific discussion on root waving and skewing, it is imperative to understand the types of movement in plants. Though sedentary, terrestrial plants show different classes of movement, arising due to unequal expansion in different regions of a plant organ (Darwin and Darwin,
Tropic movements occur in response to a directional cue. For example, roots are usually negatively phototropic (move away from light) and positively gravitropic (grow toward gravity). Nastic movements also occur in response to external factors but are independent of their position, e.g. the closing of leaves at night. Nutations refer to movement mainly controlled by an internal mechanism, and circumnutation specifically refers to an autonomous circular nutation that results in an elliptical, circular or irregular motion of the plant organ around an imaginary axis in the direction of growth (Figure 1A). For an in-depth historical perspective and understanding on tropisms and circumnutation, see Refs. (Migliaccio et al., 2013; Migliaccio and Piconese, 2001; Edwards and Moles, 2009; Masson and Gilroy, 2008).

These various forms of movements usually occur together; for example, it has been shown that gravity amplifies the circumnutatory response in Arabidopsis thaliana (Johnsson and Solheim, 2009). Hence the movement of roots comes about by a combination of tropistic responses to gravity, touch, moisture and light and circumnutation, though further studies are needed across different species to build a better model of how these processes interact. It should also be noted that even though plants with delicate roots like Arabidopsis show a slight circumnutation of the tip as they grow, other plant species with considerably thicker roots do not show this characteristic circumnutation. In this review we will discuss specifically two classes of movements, waving and skewing, that are essentially surface-dependent movements of roots growing on slanted impenetrable (high agar concentration) growth media. Most of the studies have been performed with the model plant A. thaliana, which has helped shape our understanding of how roots move. However, the movement of the delicate roots of Arabidopsis and how they react to mechanical obstacles and other cues could be very different.
from more robust roots of other bigger plants. Hence, though helpful as a model organism for uncovering basic mechanisms of root movement, true understanding of movement in other species is an urgent need. In addition, although analysis of waving and skewing is a good model to understand the dynamics of root growth and movement, the actual movement of a root in the soil is very different and any parallels drawn need confirmation in the latter system.

3. Waving and Skewing: “Root” Cause

The cultivation of Arabidopsis seedlings on a slanted impenetrable medium (high agar density of 1.5–2%) leads to the appearance of waving and skewing. Though they have been studied for quite some time now, a universally accepted model does not exist. Two models have been put forward to explain the presence of waving and skewing of roots of plants grown on agar plates.

3.1. Model 1

One model explains waving and skewing as a result of touch, gravity and circumnutation (Migliaccio and Piconese, 2001). According to this model, the growing root tip is forced down due to gravity on a slanted impenetrable medium, causing it to respond to touch (thigmotropism). The interaction of touch and gravity, coupled with the inherent tendency of the root tip to circumnutate with a fixed handedness, leads to a characteristic deviation from the vertical (skewing) and formation of sinusoidal wave-like patterns (waving) (Figure 1B). The waves arise due to spiral growth of the circumnutating root tip with alternating reversals in its handedness midway into each wave due to the effect of thigmotropism. The skewing can be explained based on the inherent handedness with which the root grows. If the roots are left-
handed, the left-handed turns are larger compared to the right-handed ones and the root gradually skews to the right.

3.2. Model 2

Alternatively, another interesting model explains skewing and waving as a result of physical interaction of the root tip with the growth media (Thompson and Holbrook, 2004). According to this model, the slanted growth medium frictionally impedes the root tip's movement, while the elongation zone keeps growing. This causes a distinct nontropic curved deflection of the root to one direction until the root tip is impeded again. The process repeats to give rise to waves. This model for waving along with the inherent circumnutating tendency might explain the skewing of the roots.

Similar root trajectory observations were made by Darwin with roots of various plant species (Darwin & Darwin, 1880). Hence these two phenomena are mechanistically explained by an interaction between thigmotropism, gravitropism, circumnutation and physical root-media interactions (Migliaccio et al., 2013; Thompson and Holbrook, 2004; and (Oliva and Dunand, 2007). It should be noted that waving and skewing are surface dependent phenomenon that do not appear when a root grows embedded in the growth medium or in liquid medium since uniform touch/pressure exists around the growing root.

Skewing is defined as rightward or leftward when looking through the medium (from the back of the plate) and waves are usually quantified by their frequency and amplitude. An endogenous chirality can explain why the roots tend to slant in a certain direction. This endogenous nature of root slanting is clear from studies in microgravity where in the absence of gravity, roots have an exaggerated skewing (Millar et al., 2011). It is also interesting to note that
the *Arabidopsis* accession Cvi shows a naturally exaggerated root skew compared to other ecotypes, possibly because of its habitat of growth on rocky walls (Vaughn and Masson, 2011).

Skewing is directly proportional to the slant angle of the medium, presumably due to increase in the force of contact between the root tip and the medium during growth (Rutherford and Masson, 1996). During these movements, epidermal cell files of the roots also show a twist referred to as cell file rotation, which may be due to the effect of mechanical impedance on elongation of the root. This rotation occurs due to differential rates of anisotropic expansion between outer and inner cell layers of an elongating root, with the outer epidermal cell files twisting thus offsetting the elongation lag between the two layers (Ishida et al., 2007). Cell file rotation becomes more pronounced when the roots are cultivated on a horizontal impenetrable medium, resulting in the root tip forming coils (Figure 1C). Cell file rotation usually correlates with skewing, a rightward skewing root showing left handed cell file rotation and vice versa. A good correlation also exists between the degree of cell file rotation and skewing, although exceptions exist. For example, *spr2* mutant roots show strong helical growth but cell file rotation is similar to that of wild-type roots (Vaughn and Masson, 2011; Furutani et al., 2000). More studies are needed to elucidate the basis for these exceptions. It should also be noted that according to one study, cell file rotation is not coupled to root waving and skewing (Buer et al., 2003).

4. Factors Implicated in Waving and Skewing: a Complex Nexus

4.1. Hormones and environmental cues

A plethora of factors have been implicated in regulating root movement (Migliaccio et al., 2013). Studies to elucidate key elements began with isolation of the *wavy* mutants (*wav1-6*)
that are defective in the root gravitropic response and waving dynamics such as amplitude and wavelength of the waves (Oliva and Dunand, 2007). Some of these mutations were further identified as disrupting genes required for mediating influx (WAV5/AUX1) and efflux (WAV6/PIN2) of the plant growth hormone auxin (Migliaccio et al., 2013; Oliva and Dunand, 2007) establishing auxin as a major factor in movement. The differential accumulation of auxin on lateral faces of roots, mediated by shuffling of the cellular localization of auxin importers and exporters, can result in differences in cellular elongation rates across the organ, thus promoting root curvature (Vanneste and Friml, 2009; Kleine-Vehn and Friml, 2008). Auxin transport inhibitors block root waving, confirming a role for auxin transport in waving (Rashotte et al., 2000). The positioning of lateral roots along the primary root axis follows a similar alternating pattern as waving, mediated by fluctuating auxin levels at the lateral root initiation sites (De Smet et al., 2007). This observation takes on a very interesting angle considering that the bending force on a root is a cue for development of lateral roots, suggesting that there is crosstalk of touch responses with auxin dynamics (Richter et al., 2009).

It is still not completely understood how the different cues perceived by the root tip as it grows translate to a change in auxin flux within the root cells and how the transfer of auxin transporters to different faces of the root cells is controlled, although reports of signaling components that are also important for root movement are emerging. The CLE-like peptides (involved in root meristem activity and growth) are involved in both lateral root formation and control of the waving movement of roots (Meng et al., 2012). Other genes have been implicated in modulating the root waving phenotype by affecting auxin transport dynamics and auxin signaling pathways. These include gene products that either are signaling molecules or affect auxin transport dynamics by regulating the trafficking and turnover machinery (Santner and
Watson, 2006; Mochizuki et al., 2005; Sakai et al., 2012; Whitford et al., 2012). KNAT1 (belonging to the KNOTTED1 homeobox family of transcription factors) negatively modulates root skewing via regulation of auxin transport, increasing auxin accumulation in the roots (Qi and Zheng, 2013). Some members of a family of extra large G proteins have been shown to positively regulate root waving and skewing patterns, possibly also by altering auxin transport dynamics (Pandey et al., 2008). Two G protein-coupled receptors belonging to the MLO (Mildew Resistance) locus also regulate root movements, since mlo mutants have curled roots and other abnormal phenotypes related to auxin function (Chen et al., 2009). Heterotrimeric G proteins seem to mediate crosstalk between nutrient (glucose) levels and auxin dynamics, affecting root growth and movement and thus suggesting a key role for nutrient status around a root (Booker et al., 2010).

Other hormones also affect root movement, mostly through their interaction with auxin pathways. Sucrose and ethylene concentrations seem to have a combined effect on root movement (Buer et al., 2003). Ethylene suppresses root skewing and ethylene perception and auxin transport together facilitate tomato root penetrability into the substratum (Santisree et al., 2012). As it is well established that ethylene stimulates auxin biosynthesis and its basipetal transport to the elongation zone in the root (Vanstraelen and Benkova, 2012), the exact role of ethylene and auxin in mediating root waving and skewing is of great interest. Cytokinin also regulates root waving and coiling, with an inverse correlation existing between cytokinin-induced root growth and the coiling-waving response (Kushwah and Jones, 2011). This observation can be explained by the antagonistic relation between cytokinin and auxin signaling pathways. There is also extensive overlap of glucose- and cytokinin-regulated gene expression, possibly utilizing auxin signaling pathways as a common node (Kushwah and Laxmi, 2014). The
auxin–cytokinin interaction circuit is modulated by brassinosteroids, which with auxin synergistically regulate expression of auxin-responsive genes and also enhance polar auxin transport in roots (Vanstraelen and Benkova, 2012). Interestingly, a recent study has shown that exogenous brassinosteroid application increases waving frequency and torsion in *Arabidopsis* roots (Lanza et al., 2012). The potential role of other hormones such as strigolactones, jasmonic acid and gibberelins in root waving and skewing remains to be seen.

Light also regulates root movements, again at least partially through an interaction with auxin signaling. Though roots are usually negatively phototropic, red light specifically induces positive phototropic curvature in roots (Kiss et al., 2003), indicating that root growth responses to light are likely to be complex. The *HY5* gene, encoding a bZIP protein that functions as a transcription factor in light-induced signaling, is required for root waving since a mutant failed to wave on slanted medium. *HY5* has been proposed to affect gravitropism and touch responses by regulating the auxin-signaling pathway (Oliva and Dunand, 2007). The induction of waving of seminal roots of rice has also been demonstrated to be brought about by a light-mediated change in polar auxin transport (Wang et al., 2011). The *Arabidopsis wav1-1* mutant had decreased root waving and the mutated gene is allelic to *phototropin1*, a gene that encodes a blue light receptor required for phototropism (Mochizuki et al., 2005). A recent study aboard the International Space Station suggests that negative phototropism plays a crucial role in mediating waving and skewing. Since skewing and waving still took place during spaceflight (zero gravity conditions), gravity can be ruled out to be a driver for these movements and rather acts in directional cueing (on Earth), which in this study was provided by the light source (Paul et al., 2012; Kiss et al., 2012).
Hydrotropism is another key factor that mediates root movement. The miz1 mutant, defective in a yet uncharacterized gene required for hydrotropism, has a modified waving response, with roots forming waves of a smaller amplitude (Kobayashi et al., 2007). It will be interesting to see how hydrotropism modulates root movement since it has been shown to function independently of gravitropism but is regulated by light and the hormone abscisic acid. For a comprehensive review of hydrotropism and its molecular mechanism see (Moriwaki et al., 2013).

The above studies indicate that the various tropistic movements that regulate the trajectory of a root seem to converge on common signaling pathways, the output of which determines the resultant direction of growth. The most common pathway targeted for regulation is polar auxin transport in root cells, whose regulation by other hormones determines root elongation and bending. Hormonal control is also intertwined with nutrient sensing and signaling pathways, providing robust crosstalk and control in guiding root growth movement in the soil while also maximizing absorption of nutrients.

4.2. Cytoskeleton and cell wall dynamics

Downstream of hormonal and environmental regulation, root movements are facilitated by changes in the cytoskeleton and by cell wall deposition patterns. Microtubules are transversely oriented in spirals with respect to the longitudinal axis of the cell in elongating roots cells (Wasteneys and Fujita, 2006). These transverse microtubules guide the positioning of cellulose synthase, which, guided by the microtubules, synthesizes cellulose microfibrils, thus determining the direction of cell expansion (Bringmann et al., 2012). Hence, defects in microtubule patterning can cause aberrant anisotropic expansion, which in turn causes the
epidermal and cortical layers to lag in expansion rates such that cell file rotation and skew
increases (Ishida et al., 2007). Stabilization and destabilization of microtubules by drugs directly
correlated microtubule orientation with root skewing and cell file rotation (Oliva and Dunand,
2007). Roots having a left-handed cell file rotation usually have a right-handed oblique array of
microtubules and vice versa. Arabidopsis mutants with enhanced rightward or leftward skew
have been identified and a number of factors regulating microtubule dynamics and root
movement have emerged (Oliva and Dunand, 2007; Sedbrook and Kaloriti, 2008). Defects in
tubulin structure/activity and microtubule-associated proteins result in a change in the
obliqueness or handedness of microtubules and subsequently cell file rotation and
skewing (Sedbrook and Kaloriti, 2008). Real-time imaging of microtubule dynamics has shown
that destabilization of cortical microtubules correlates with leftward skewing and left handed
twisting of roots while stabilization correlates with the opposite phenotypes (Sedbrook and
Kaloriti, 2008). Dynamic instability may therefore be a link in mediating differential cell
expansion and the handedness of axial twisting.

A collection of microtubule-associated proteins called plus end tracking proteins are
important for regulating root movement. Plus-end tracking proteins localize at the plus end of
microtubules and manipulate multiple microtubular dynamics, interact with plasma membrane-
bound proteins, and also might link the actin-microtubule network since specific capture sites by
these proteins often contain F-actin (Akhmanova and Steinmetz, 2010; Goode et al., 2000).
Some putative plus-end tracking proteins, for example Arabidopsis END BINDING 1, affect root
skewing and coiling by repressing thigmotropism and enhancing gravitropism but do not directly
affect microtubule integrity, suggesting alternate pathways of regulating root movements in a
microtubule orientation-independent way. Studies in animals hint at possible explanations for
how END BINDING 1 might regulate this network by interacting with ion channels, actin cytoskeleton and other cellular signaling components (Bisgrove et al., 2008; Gleeson et al., 2012). The nucleation (initiation) of microtubules is also implicated in mediating root movement since abnormal nucleation can lead to depletion and change in the angle of microtubule arrays and also change their chirality (Nakamura and Hashimoto, 2009).

Actin is also important in root movement and growth and is an important regulator of the gravitropic response (Blancaflor, 2013). Actin is a negative regulator of root skewing, and actin mutants during spaceflight have exaggerated root skew and cell wall deposition defects (Nakashima et al., 2013). Actin interacts with the hormonal networks as actin reorganization activates brassinosteroid signaling and thereby increases auxin responsiveness, thus mediating the waving response (Lanza et al., 2012).

The cytoskeleton affects cell wall deposition and therefore root movement as mentioned earlier, but in addition, cell wall structure and biochemistry can have a more direct effect on root skewing. For example, *Arabidopsis* mutants defective in the glycosyl-phosphatidylinositol anchored cell wall proteins COBRA and SKU5 have aberrant skewing (Roudier et al., 2005; Sedbrook et al., 2002). The activity of pectin methylesterases is important for regulating root waving. Loss of cell wall extensibility due to reduction in pectin methylesterase activity can initiate crosstalk with the brassinosteroid signaling pathway (Wolf et al., 2012). These studies suggest that biochemical control of cell wall structure is another crucial factor in root expansion and movement and might also interact with hormonal pathways.

The cytoskeleton thus mediates root movement by integrating signals from cues such as touch and by regulating cellular expansion and axial twisting in roots. Proteins associated with microtubules help fine tune the dynamics and in turn interact with other cellular pathways and
hormonal networks. The regulation of cell wall deposition is another key way by which the cytoskeleton might regulate the patterns of root growth.

4.3. A working model: an urgent need

It is difficult to explain the process of root movement in a simple model, considering the multitude of factors involved. A comprehensive model for the mechanism of root movement incorporating the many cellular and physiological aspects that are being uncovered is still lacking. Here we provide a schematic (Figure 2) of the steps and factors that mediate the root movements discussed above, although additional information connecting environmental cues, signaling pathways and execution of movement is greatly needed.

It appears that the mechanism for root movement includes crosstalk between multiple perception and signaling pathways for the manipulation of root growth. The root has an initial perception step for sensing its local environment, which is then transduced as a signal to modulate root growth. The interplay of signaling arising from light, touch, moisture, nutrition and gravity leads to the net growth trajectory of the root in order to take an optimal path through the soil. An endogenous genetic predisposition of the root tip to circumnutation is also a key to the directionality and pattern of root movement. Studies are gradually emerging on how these different pathways can inhibit or induce each other. The actual pattern of root growth primarily depends on how auxin is transported within the root and on the polar localization of the transport apparatus via cellular trafficking. Changes in auxin flux lead to differential growth of the root and therefore tropistic movements. Interaction with other major hormonal pathways and nutrient sensing networks modulates final root trajectory. The pushing of the root tip against an obstacle also puts mechanical constraints, which is possibly perceived by the cytoskeletal apparatus via
intracellular signals, affecting handedness of root twisting and overall skewing of the root. The role of cellular trafficking in cell wall deposition and its interaction with the cytoskeletal apparatus is also key to the movement of roots as it grows. Root movement may be under constant feedback control of cues perceived at the root tip, allowing the plant to regulate the movement process as needs arise in a spatiotemporal manner.

Well-planned studies eliminating various cues one at a time along with high throughput technologies like transcriptomics and metabolomics can help uncover how different stimuli regulate root waving and skewing. Hopefully a much clearer picture of the different players and their place in the complex genetic and biochemical network of root growth will emerge as the field progresses.

5. Future Directions and Concluding Remarks

Just as constructing a building requires a firm understanding of the foundation, it is imperative to understand how roots of terrestrial plants have adopted such a complicated growth pattern and movement as they anchor themselves. Much of our understanding of the factors required for movement has come from studies of mutants with movement defects, some of which are subtle or occur under a certain set of condition. Hence it is of prime importance to detect aberrant movement in these mutants and quantify them in a uniform manner in order to compare the phenotypes and decipher the precise role of the affected genes. This requires the development of innovative imaging platforms and image analysis software for tracking root parameters such as skew intensity, waving dynamics and cell file rotation. Novel root morphometric parameters such as horizontal and vertical growth indices have been proposed and shown to be good tools for quantifying root movement (Grabov et al., 2005). Ingenious setups (Mullen et al., 2000) can
help maintain a constant stimulus on the root tip while collecting reliable data for measuring root parameters. Real-time tracking of root morphology is also becoming easier due to advances in the use of automated high throughput imaging systems utilizing robotic setups. This approach, in concert with the field of kinematic and morphometric analysis, has the potential to revolutionize the ability to gather high precision, real-time data from a large sample size with minimal labor (Spalding and Miller, 2013). Advanced techniques such as multi-angle image acquisition and three-dimensional reconstruction at cellular resolution promise to better track root phenotypes (Fernandez et al., 2010).

It is expected that further studies will aim at understanding root movements in three-dimensional space. Waving and skewing are phenotypes that are presently induced in an artificial setup but offer a tool for understanding the roles of various factors in modulating root movements. Translation of knowledge from this artificial system to understanding root movement in the soil milieu is essential. Since the soil presents a much more complex substrate than the surface of growth media, the development of techniques to track and quantify root movements within the soil is required. Recent research promises the ability to quantify three-dimensional root architecture (Topp et al., 2013). This could be utilized in conjunction with growth of roots under various conditions that regulate root movement, giving a much more accurate picture of what goes on inside the soil under different conditions. The utilization of computer simulations and modeling can also be an important tool in predicting root movement once parameters controlling various aspects of the phenomenon are better understood, allowing the further generation of hypotheses for experimental testing. Approaches such as finite element modeling reliably predicted tip-induced growth for other systems and similar studies with roots can help uncover as well as predict movement on a finer scale (Fayant et al., 2010). For a
comprehensive review of modeling of root growth and architecture, see Ref. (Dupuy et al., 2010).

At the cellular level, novel sensors are used in the mapping of auxin dynamics at increased spatio-temporal resolution (Brunoud et al., 2012). This, coupled with advances in super-resolution microscopy, promises to shed light on the internal workings of the cell and allow visualization of the dynamics of proteins and cytoskeleton elements. Together with transcriptomics and proteomics data gathered from different phases of cue perception and root elongation, these approaches can help uncover novel networks and build a comprehensive picture of what goes on in roots as they move (Lewis et al., 2013).

The interest in root movements and how they define final plant architecture and physiology is justified since the soil milieu and its interaction with roots are critical factors for crop improvement. Since Arabidopsis has been the reference organism of choice, most of our insight into root movements comes from this organism, with sporadic reports in other species. With the need for translating research to economically important plants and for better understanding the ecological effects of root movement (Inoue et al., 1999), a much more thorough study into other plants’ root movements and architecture will be required in the future. This, along with the co-operation of different scientific fields and techniques, will help paint a more comprehensive picture of root movement during the developmental process. We hope this review will trigger renewed interest in the way the hidden half of the plant moves, and how it can be applied to engineering plants for stress avoidance, better nutrient utilization, and growth in extreme environments such as microgravity during space travel.
6. Acknowledgements

This work was supported by a grant from the National Aeronautics and Space Administration (grant no. NNX09AK78G) to DCB. We thank Brice Floyd for critical reading of the manuscript and Divita Mathur for helping with the figures.

7. References


8. Figures

Figure 1: Helical motion of roots, root waving and skewing. (A) Diagram depicting the helical circumnutation of a root. The spiral motion around an imaginary axis can be clockwise (right-handed) or counterclockwise (left-handed) when looking down toward the direction of root growth. (B) Left panel – Cartoon depicting the waving and skewing of the root as it grows on a slanted hard growth medium. Right panel – Root of Arabidopsis thaliana grown on a slanted hard growth medium showing skewing and waving. (C) Confocal image of a propidium iodide-stained root coil showing marked epidermal cell file rotation.
**Figure 2:** Schematic diagram of the general factors that regulate root movement. Gravity, light, moisture and touch cues are perceived by the root tip, which then initiates signaling cascades to integrate hormonal pathways and cytoskeletal dynamics to bring about a change in auxin flux in the root. This, coupled with a modification in cell elongation patterns and cell wall deposition, regulates root movements. Other key regulators including nutrient sensing pathways, physical interaction between the root and media and endogenous circumnutatory tendencies contribute to the final trajectory and physiology of the root as it moves.