

ABSTRACT

RIVERA SERRANO, EFRAÍN EDUARDO. Chemical Genetic Approaches for the Study of Tonoplast Protein Trafficking in *Arabidopsis thaliana*. (Under the direction of Marcela Rojas-Pierce.)

The vacuole is an essential and dynamic organelle in plant cells, and its many functions depend on the deposition of membrane proteins. In our efforts to understand the targeting of membrane proteins from the endoplasmic reticulum (ER) to the vacuole membrane, or tonoplast, we used a chemical genetics approach. We examined protein localization by confocal scanning microscopy using an *Arabidopsis thaliana* transgenic line expressing both a 35S::GFP-TIP2;1, a tonoplast marker, and the ER marker UBQ10::mCherry-HDEL. Using a small chemical library of tobacco pollen growth inhibitors (Drakakaki et al., 2011), we successfully identified five chemicals that result in the mis-localization of the tonoplast marker at the ER. Intracellular localization of different markers in presence of the inhibitors suggests that the chemicals block the trafficking machinery at different steps. One of these chemicals, 6982834 (C834), specifically affects the intracellular localization of the tonoplast markers GFP-TIP2;1 and TIP3;1-YFP, but not that of TIP1;1-YFP; providing evidence for the presence of at least two pathways for tonoplast protein trafficking. Moreover, treatment with Brefeldin A (BFA), an inhibitor of the ER-to-Golgi vesicle trafficking, results in mis-localization of TIP1;1-YFP in an ER-like structure and “BFA compartments” but not the other two. This suggests that TIP2;1 and TIP3;1 travel via a Golgi-independent pathway, whereas TIP1;1-YFP passes through the Golgi before reaching the vacuole membrane. Interestingly, the effects of C834 in protein targeting are specific for a subset of tonoplast proteins, with the exception of the plasma membrane-localized auxin transporter PIN2-GFP. In the dark, C834-treated seedlings show decreased levels of PIN2

protein, suggesting an enhancement of the vacuole degradation pathway. C834-treated seedlings show less response to exogenous auxins that require polar transporters; but normal sensitivity to a diffusible auxin, suggesting a C834-induced defect in auxin transport. We also propose a novel link between protein accumulation at the ER and genetic regulation of the tryptophan biosynthetic pathway. Overall, these experiments show the first *in planta* evidence for the existence of multiple pathways for tonoplast trafficking in *Arabidopsis*. The fact that PIN2 levels are affected by C834 suggests a link between the Golgi-independent pathway and auxin transport. Finally, a putative link between ER stress and tryptophan biosynthesis was uncovered with the activity of the new inhibitors.

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Chemical Genetic Approaches for the Study of Tonoplast Protein
Trafficking in *Arabidopsis thaliana*

by
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DEDICATION

To my parents, Blanca Serrano Mendoza and Efraín Rivera Meléndez, for their support, love and motivation.

DEDICATORIA

A mis padres, Blanca Serrano Mendoza y Efraín Rivera Meléndez, por su apoyo, amor y motivación.

BIOGRAPHY

Efraín Rivera Serrano was born in December 23, 1987 in Ponce, a small town in the south coast of Puerto Rico. He is the only child of Blanca Serrano Mendoza and Efraín Rivera Meléndez. His passions outside the lab include exotic bird breeding techniques, farming and music. Efraín graduated Summa Cum Laude with a B.S. degree in Biology and a minor in Chemistry at the Pontificia Universidad Católica de Puerto Rico in May of 2009. During his undergraduate years, he became fascinated with science while working in a research project involving plant secondary metabolites and glucose metabolism in liver tissue cells under the direction of Dr. Alma Santiago. Efraín enrolled in the Plant Biology graduate program at North Carolina State University (NCSU) in August 2009 under the supervision of Dr. Marcela Rojas-Pierce.

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CHAPTER 1

The Plant Vacuole: Biogenesis, Functions and Trafficking

ABSTRACT

The vacuole is an essential and dynamic organelle in plant cells with an array of cellular and physiological functions required for plant growth and development. Evidence for the presence of protein storage vacuoles (PSVs) and lytic vacuoles (LVs) in plants suggests that dual presence and regulation of these multi-functional organelles is plant-specific. In this review, I present a summary of the major discoveries in vacuole biology, including membrane transporters and their regulation by hormonal and environmental cues. More importantly, classical and novel discoveries in trafficking of both soluble and membrane vacuolar proteins are discussed, as well as new perspectives in the identification and characterization of genes involved in vacuole protein targeting.

1.1 The plant vacuole as an essential organelle of the endomembrane system

The eukaryote cell is characterized not only by the presence of a nucleus, and other membrane-bound organelles, but also by a complex coordinated system of internal membranes inside the plasma membrane (PM). In plants, the endomembrane system consists of the endoplasmic reticulum (ER), the Golgi apparatus, the *trans*-Golgi network (TGN), prevacuolar compartments (PVCs), endosomal compartments, and the vacuole (Reviewed in Neumann et al., 2003). This evolved system is responsible for the folding, modification, packing, sorting and proper delivery of a great number of proteins throughout the cell. In addition, the Golgi and ER support the synthesis of polysaccharides and lipids, respectively. Although these organelles are highly dynamic, the communication between compartments occurs, mostly, via a series of vesicles that serve as cargo shuttles. These vesicles originate by budding from the membrane of the donor compartment and then they are transported by the cytoskeleton to the acceptor compartment, where tethering and fusion occurs. This mechanism releases the cargo into the new compartment. The molecular and cellular mechanisms that regulate and organize proper protein delivery has been of interest for years. In comparison with other eukaryotes, the plant proteome is enriched with proteins involved in the regulation of components of the endomembrane system (Lipka et al., 2007), and this repertoire and diversification is thought to reflect the necessity of specific proteins to be devoted to plant-specific processes such as plant-pathogen interactions and cell plate formation (Heese et al., 2001; Wick et al., 2003; Zhang et al., 2011). Moreover, phylogenetic analysis suggests that this phenomenon is highly associated with genome duplications after

the last common eukaryotic ancestor (Dacks et al., 2008), reinforcing the complexity of this system in the plant kingdom.

The plant vacuole is the largest and most prominent organelle, comprising more than 90% of the volume of a typical plant cell, and it is involved in maintaining homeostasis by acting in many cellular processes (Bassham & Raikhel, 2000). The vacuole is the storage site for inorganic ions, sugars, lipids, and soluble products of complex biosynthetic pathways. In addition, it stores proteins for later degradation, providing a source source of amino acids (reviewed in Martinoia et al., 2000). It also stores secondary metabolites like phenols, alkaloids, and proteins like chitinases and other proteases that are required for plant defense against herbivores. The vacuole plays major roles in sequestration of toxic compounds, maintenance of pH and the accumulation of flower pigments and metabolites required for fruit flavor, which have important impacts on the agricultural uses of plants (Yazaki, 2005; Poustka et al., 2007). Moreover, the hormone abscisic acid (ABA), which controls most aspects of abiotic stress, can be inactivated by sequestration in a sugar-conjugated form in the vacuole (Lee et al., 2006). It is due to this small molecule-storage function that vacuoles are tight regulators of turgor pressure, and therefore, they play major roles in the determination of cell size (Martinoia et al., 2007). Vacuolar functions are largely dependent not only on the transport of vacuole soluble proteins, but also on the proper deposition of membrane proteins to the vacuole membrane, also known as tonoplast. Membrane protein transporters are responsible for the mobilization of molecules in and out of the vacuole; therefore, these transporters are indispensable for vacuolar function. Not surprisingly, the mechanisms of protein transport to the vacuole lumen have been studied extensively, and many key players

involved in the vesicle traffic of vacuolar proteins have been identified and characterized (Fuji et al., 2007; Sohn et al., 2007, Tanaka et al., 2009; Teh and Moore, 2007).

The essential role of the vacuole in plants was shown with the discovery of the *vacuoless1* (*vcl1*) mutant (Rojo et al., 2001). The *vcl1* mutant was identified in a screen for *Arabidopsis thaliana* plants with defects in early embryo development, especially in suspensor cells, as these cells show a high number of vacuoles. This mutant is characterized by the lack of vacuoles and the accumulation of small vesicles that do not fuse together to form the large vacuole, while other organelles, such as the ER and Golgi appear to be normal. Moreover, the loss of *VCLI* activity seems to activate the formation of autophagosomes, which causes the engulfment of cytosolic compartments and loss of internal organization. *VCLI* encodes the Arabidopsis ortholog of the yeast Vps16p, which is 24% identical at the amino acid level. In contrast with yeast mutants that are viable, the loss of the plant vacuole results in aberrant embryo development and death at the torpedo stage (Rojo et al., 2001). Due to the lack of vacuoles in *vcl* mutants, proteins destined to the vacuole are instead mis-secreted to the apoplast, producing a cell wall that is up to four times thicker than a wild type embryo. In *Saccharomyces cerevisiae*, Vps16p belongs to a small family of proteins that are required for vacuole biogenesis and protein transport (Horazdovsky and Emr, 1993). In fact, Vps16p is part of a multi-protein complex known as HOPS (homotypic fusion and protein sorting), which has been shown to be involved in vacuole-vacuole fusion in budding yeasts (Seals et al., 2000). *VCL1* was later shown to interact with VACUOLAR PROTEIN SORTING 33 (*VPS33*) and *VPS11*, as well as with the membrane-fusion facilitators *SYP21* and *SYP22* at both the tonoplast and the prevacuolar compartment (Rojo

et al., 2003). The identification of the critical role of *VCL1* provides evidence for the indispensable role of the vacuole in plants.

1.2 One or two plant vacuoles?

Two vacuole types have been proposed to co-exist in plant cells, although this hypothesis has been controversial during the last years (Frigerio et al., 2008; Rogers, 2008). The protein storage vacuole (PSV), which has a neutral pH, is found in embryos and developing seeds of dicotyledonous species. A second type of vacuole, the lytic vacuole (LV), has an acidic pH and is reminiscent of the lysosomal compartment in animal cells. In pea root tips, the PSV is marked by the presence of barley lectin and the tonoplast protein α TIP (Paris et al., 1996). Furthermore, the vacuolar protease aleurain, which serves as a marker for acidified vacuoles, is exclusively contained within a compartment labeled with γ TIP; suggesting the existence of two different storage compartments in pea root tips that later fuse together during cell development (Paris et al., 1996). Evidence for this co-existence of different types of vacuoles has also been documented in barley and in the CAM (Crassulacean acid metabolism) plant *Mesembryanthemum crystallinum*. This last example is fascinating as, under salt stress, the mesophyll cells of *M. crystallinum* contain two separate vacuoles: one for salt storage and sequestration and another for night-time storage of malate via CAM photosynthesis (Epimashko et al., 2004). Recently, it has been shown also that two isoforms of the Two-Pore K^+ (TPK) family of ion channels in *Oryza sativa*, TPKa and TPKb, localize differently to the lytic vacuole and the protein storage vacuole, respectively (Isayenkov et al., 2011). In Arabidopsis, however, the spatial localization of tonoplast

intrinsic protein (TIP) markers is not as easy to study, as their expression is controlled by developmental stages. The PSV marker TIP3;1 is expressed exclusively in seeds and its expression decreases during development and, at the same time, the expression of the LV tonoplast marker TIP1;1 increases (Hunter et al., 2007). Thus, it is likely that, in Arabidopsis, the two types of vacuoles may be the result of a developmental switch where the PSV is transformed into a LV during the first days after germination, as it has been recently shown to happen in *Nicotiana tabacum* (Zheng and Staehelin, 2011). In fact, recent reports in Arabidopsis suggest the existence of a lytic vacuole embedded inside PSVs in dry seeds that later disappears after stratification, and is replaced by a new lytic vacuole (Bolte et al., 2011). Current literature suggests that the simultaneous presence of different types of vacuoles in plants is both tissue- and plant species-dependent, and further research is needed to obtain more insights about vacuole biogenesis.

1.3 Vacuolar transporters and their role in cell homeostasis

To adequately fulfill the functions of the vacuole, the tonoplast possess and array of membrane-spanning transporters. These transporters function as gates to selectively control the flux of ions and molecules in and out of the vacuole. Due to the relative large volume of the vacuole and its role in storing molecules, the concentration of a large number of compounds in the vacuole lumen is several folds higher than that of the corresponding concentrations in the cytosol (Rea and Sanders, 1987). In order for this accumulation of molecules to happen, vacuoles possess two types of proton pumps to create an electrochemical gradient: the vacuolar ATPase (V-ATPase) and a vacuolar pyrophosphatase

(V-PPase) (Rea et al., 1992; Kluge et al., 2003). The first is similar in structure to the F-ATP synthases and the H⁺-ATPases in archaea, and its expression is regulated both transcriptionally under salt stress and cell redox state (Kluge et al., 2003; Tavakoli et al., 2001), and post-translationally by phosphorylation (Hong-Hermesdorf et al., 2006). Interestingly, inhibition of V-ATPase with concanamycin A (ConcA) causes an endocytic traffic jam, resulting in inhibition of trafficking for proteins destined from endocytosis to the tonoplast (Dettmer et al., 2006). This directs them into the secretory pathway at the TGN (Dettmer et al., 2006), where the endocytic and secretory pathways seem to merge (Viotti et al., 2010). In contrast to the V-ATPase, the V-PPase use PP_i as energy source, and it is more active in young plant tissues (Rea et al., 1992). Experiments have shown that alterations in the levels of proton pumps cause interesting phenotypes. For example, the overexpression of *AVPI*, a vacuolar H⁺-PPase, results in increased tolerance to drought, salt and phosphate deficiency (Gaxiola et al., 2001; Gaxiola et al., 2012).

The family of the aquaporins constitutes a major group of transporters. These channel-like proteins are part of the Major Intrinsic Protein (MIP) family, which are found in membranes of most cells (Engel and Stahlberg, 2002). In animals, they are mostly restricted to secretory organs; however in plants, they abundantly accumulate in ubiquitous locations (Maurel et al., 2002). Biochemically, the quaternary structure of the protein is a homotetramer, with each monomer organized into six membrane-spanning helices connected by five loops (Murata et al. 2000; Savage et al., 2003). The subfamily of Tonoplast Intrinsic Proteins (TIPs) are localized to the vacuole membrane, where they facilitate the transport of water, glycerol, urea, boric acid, hydrogen peroxide, ammonia, and carbon dioxide in and out

of the vacuole (Reviewed in Keldenhoff and Fisher, 2006). The *Arabidopsis* genome encodes ten TIP isoforms, which have a long history as markers for vacuole identity and function (Gattolin et al., 2010a). Evidence has shown that TIP3;1 localizes to the membrane of the protein storage vacuole (PSV), whereas TIP1;1 to the lytic vacuole. Interestingly, the expression of TIPs is developmentally regulated, and their expression pattern is tissue specific (Hunter et al., 2007; Gattolin et al., 2009). The expression of TIP3;1 is restricted to developing embryos, and its expression decays during the first days after germination. Recent studies have shown that during this stage, both TIP3;1 and TIP3;2, are located both at the tonoplast and the plasma membrane (Gattolin et al., 2010b). Moreover, the water channel activity of TIP3;1 is post-translationally dependent on phosphorylation events (Maurel et al., 2005). In contrast, the expression of TIP1;1 and TIP2;3 (formerly δ TIP3) is activated during the decay of TIP3;1, leaving only a small window of time where all TIPs can be found in the same cell (Hunter et al., 2007). Biochemical studies using aquaporins from *Tulipa gesneriana*, TgTIP1;1 and TgTIP1;2, revealed the important role of the aromatic/arginine filter in aquaporins for substrate specificity (Azad et al., 2011). However, mutants in these genes do not show any obvious phenotype in *Arabidopsis* (Schüssler et al., 2008; Beebo et al., 2009). Mutants lacking both *TIP1;1* and *TIP1;2*, do not show major aberrant phenotypes, exhibiting only a slight but significant increase in anthocyanin content (Schüssler et al., 2008). In contrast, the single TIP1;1 mutant had a shorter root when grown in media containing glycerol (Beebo et al., 2009). Interestingly, the *TIP2;1* from *Glycine soja* is a stress-induced gene, and affects salt and dehydration tolerance when expressed in *Arabidopsis* (Wang et al., 2011).

Other transporters have been identified and characterized recently for the transport of plant primary metabolites. The vacuole represents the major storage of photosynthates, and although sucrose have been shown to be transported into the vacuole by facilitated diffusion in some plant species like celery and pear fruit, a mechanism involving an active proton antiport has been proposed for pea and sugar cane (Reviewed in Martinoia et al., 2000). Additionally, the identification of sucrose transporters (SUTs) has contributed to our knowledge in carbohydrate transport. In contrast with the aquaporin mutants, impaired function of tonoplast sucrose transporters has major consequences for plant growth (Eom et al., 2011). Other tonoplast monosaccharide-transporters have been shown to affect not only sugar content, but also sugar signaling in seeds when mutated or over-expressed (Poschet et al., 2011; Wingenter et al., 2010). The transport of secondary metabolites into the vacuole has been less explored, but identification of several anthocyanin transporters in both monocots and dicots has provided information regarding some of the mechanisms involved in this pathway. Anthocyanins are secondary metabolites that are responsible for most plant pigmentation and indispensable in some plants for attracting pollinators (Grotewold, 2004). Transporters of the multidrug and toxic compound extrusion (MATE) family have been implicated with the vacuolar transport of these compounds in species like grapevine (Gomez et al., 2009) and maize (Goodman et al., 2004). Interestingly, evidence also suggests that in some plants anthocyanins are transported via membrane-spanning transporters, as well as via vesicle trafficking (Gomez et al., 2011; Poustka et al., 2007).

The vacuole is also a reserve of nitrogen and phosphate, two essential nutrients in all organisms. The first is commonly stored in the form of nitrate (NO_3^-), an anion that shows a

high permeability through the tonoplast (Chrispeels et al, 1999). Nitrogen can also be stored in the form of amino acids and peptides, and proteolysis seems to be favorable during seed germination and senescence (Näsholm et al., 2009). Both nitrate and small peptides are, in part, transported into the vacuole by members of the PTR/NRT1 (peptide transporter/nitrate transporter 1) family (Komarova et al., 2008), although the substrate of some family members is still unknown (Weichert et al., 2011). Interestingly, the V-ATPase seems to be important for nitrate homeostasis, as the double mutant lacking the two tonoplast-localized isoforms, *vha-a2/vha-a3*, shows decreased levels of nitrate (Krebs et al., 2010). The story for phosphate (P_i) is a little bit different, with most of the characterized phosphate transporters being localized to the plasma membrane. The stored P_i from the vacuole is released in P_i starvation conditions, and the first tonoplast phosphate transporter was characterized more than 10 years ago in *Catharanthus* cells (Massonneau et al., 2000). Recent studies in *Acer pseudoplatanus* and *Arabidopsis* using methylphosphonate (MeP) as a P_i analog suggest that supply of phosphate prevents its efflux from the vacuole and a subsequent massive transfer from the cytoplasm into the vacuole (Pratt et al., 2009). Information is lacking in respect to other tonoplast phosphate transporters, and research is needed to fill these gaps.

An area with applications in phytoremediation is the study of heavy-metal transporters, providing evidence for novel vacuole roles in detoxification. Non-essential heavy metals usually interfere with the function of essential metals by competition of binding sites, affecting the plant physiology and biochemistry (Verbruggen et al., 2009). The most common mechanism for detoxification in fungi and plants involves phytochelatin (PCs). Although some PC transporters have been identified in yeast vacuole membranes (Mendoza-

Cózatl et al., 2010), such proteins have not yet being identified in plants. However, several tonoplast transporters of heavy metals in plants have been identified, including a cadmium/proton ($\text{Cd}^{2+}/\text{H}^+$) antiporter (Salt and Wagner, 1993) and an aluminum ATP-binding cassette (ABC) transporter in rice (Huang et al., 2011).

1.4 Intracellular protein transport – vesicles and regulators

Proper protein transport via vesicle trafficking is necessary for plant growth, development, and responses to environmental and hormonal cues (Surpin and Raikhel, 2004). After proper protein folding in the endoplasmic reticulum (ER), the majority of the proteins are packed and transported into organelle-derived vesicles for delivery to the correct compartment. This step requires a mechanism for cargo selection and accumulation in a specific site of the lumen of the donor compartment, and a signal to indicate a vesicle formation site. Once the vesicle is formed, coat proteins (COPs and clathrin) aid in the budding process and the scission of the newly formed vesicle. These processes are controlled by members from the ARF-family and the SAR1-family of coat-GTPases for COPI or COPII proteins, respectively (Bonifacino and Glick, 2004). The COPII machinery and its coat-GTPase, SAR1, are primarily responsible for the anterograde trafficking of vesicles from the ER to the Golgi (Tang et al., 2005). Expression of a dominant negative mutant of SAR1 results in inhibition of ER-to-Golgi trafficking (Takeuchi et al., 2000), and similar results are obtained when SEC12, the guanosine nucleotide exchange factor (GEF) of SAR1, is overexpressed transiently (Phillipson et al., 2001). In contrast, COPI coats are mostly involved in the retrograde trafficking from the Golgi apparatus, although there is evidence for

COPI-mediated vesicle formation at the ER and endosomes (Kirchhausen et al., 2000). The COPI coat is formed by two subunits, F-COP and B-COP, and its coat-GTPase is ARF1. F-COP is formed by four subunits (γ -COP, β -COP, δ -COP and ζ -COP) and controls the selection of the cargo, whereas B-COP is involved in forming the actual vesicle and consists of three subunits (α -COP, β' -COP and ε -COP) (Bassham et al., 2008). The last group of coat proteins consists of clathrin, and this group is mostly found in the *trans*-Golgi network and the plasma membrane (Robinson et al., 1998). Clathrin coats in plants are formed by two protein chains that form a three-legged shape that can be seen microscopically (Fotin et al., 2004). This structure has been shown to physically interact with some members of the adaptor protein (AP)-family (Lee et al., 2007; Bassham et al., 2008), proteins that have been shown to play major roles in vacuole biogenesis and trafficking of tonoplast proteins (Feraru et al., 2010; Zwiewka et al., 2011; Wolfenstetter et al., 2012).

Once the vesicle is excised and free in the cytosol it must find and distinguish the next corresponding organelle in the cell for cargo delivery. Members of the Rab/Ypt family are involved in cargo selection, the recruitment of motor proteins and promoting interactions with tethering factors (Saito and Ueda, 2009). The Arabidopsis genome encodes for 57 Rab proteins, and it is believed that each single step in protein trafficking pathways requires at least one Rab/Ypt GTPase. Rabs are small GTPases, meaning that their activity is dependent on the phosphorylation status of the guanine, cycling from inactive GDP to active GTP. Activation of Rabs is catalyzed by a guanine nucleotide exchange factor (GEF) that promotes the switch of GDP for GTP. In addition, there is a group of Rab effectors, which in most cases function in the tethering and docking between the transport vesicle and the acceptor

compartment. Many of these tethering factors have been identified in mammals and yeast, such as the TRAPPI and TRAPPII multiprotein complexes that function both in the Golgi and TGN (Markgraf et al., 2007). However, little is known about tethering factors in plants.

Once both the donor vesicle and the acceptor organelle are in close proximity, both lipid bilayers can fuse, releasing vesicle cargo in the process. This process demands high amounts of energy because it requires protein clearance from the fusion area, as well as overcoming the repulsive charges of the opposite membranes (Martens and McMahon, 2008). Members of a superfamily of proteins, called SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, provide the required energy and carry out these functions in eukaryotic cells. SNARE elements differ widely in size and structure, but they all share common motifs that contribute to the physical protein-protein interaction at the core of a SNARE complex. These proteins mediate membrane fusion, and they provide specificity in vesicle targeting by forming a complex made up of one SNARE (v-SNARE) localized in the donor vesicle and three SNAREs (t-SNAREs) localized in the acceptor organelle (Lipka et al., 2007). Even though all eukaryotic genomes encode for SNAREs, plant genomes encode a larger repertoire of SNAREs when compared to animals and yeasts (Lipka et al., 2007). This high number of SNAREs is due predominantly to increased number of isoforms within conserved SNARE families and not to evolution of new SNARE families (Sanderfoot et al., 2000). The many SNAREs in plant cells contributes to the formation of diverse and complex routes within the endomembrane system. Thus, the intracellular localization of SNAREs provides a roadmap for protein trafficking (Uemura et al., 2004).

The SNARE family of proteins is distinguished by having a so-called SNARE-motif, formed by stretches of 60-70 amino acids containing heptad repeats with a central '0' layer (Malsam et. al., 2008). Based on the amino acid located in the central '0' layers, SNAREs are classified into four classes: Q_a, Q_b, Q_c and R. Q SNAREs (Q_a, Q_b and Q_c) are characterized by the presence of a glutamine, whereas R SNAREs have an arginine in the central '0' layer. Most SNAREs possess a transmembrane domain that anchors the protein to the lipid bilayer and is required for complex assembly. The four-helix bundle is formed by protein-protein interactions between the SNARE motifs of the complementary SNAREs. Fully assembled SNARE complexes are extremely stable, and some complexes resist SDS-denaturation as well as thermal denaturation up to 80°C (Margittai et. al., 2001). Each SNARE complex releases about $35k_B T$ (~20 kcal/mol) of energy as it zippers up (Li et. al., 2007), fusing both membranes in the process.

Probably the most studied SNARE involved in vacuole biogenesis is VTI11, or ZIG1/SGR4. The ZIG1 name is derived from the characteristic "zig-zag" phenotype of the inflorescence stem seen in *vti11* mutants due to impairment in shoot gravitropism (Kato et al., 2002). The underlying mechanism causing the phenotype is impaired amyloplast mobility caused by the presence of abnormal and un-fused vacuoles in shoot epidermal cells. Shoot gravitropism depends on amyloplast sedimentation, including amyloplast travel through *trans*-vacuolar strands (Kato et al., 2002). In *vti11* plants, the amyloplasts are found outside the un-fused vacuoles and pushed towards the plasma membrane, resulting in inefficient sedimentation (Morita et al., 2002). Even though VTI11 localizes to the PVC and the vacuole, its homolog VTI12, which forms a SNARE complex at the TGN, can partially

substitute VTI11 functions in *vti11* mutants (Surpin et al., 2003). Surprisingly, these two homologs have been shown to act non-redundantly in different pathways. Mutant analysis demonstrated that *vti11* and *vti12* have defects in transporting proteins destined to the lytic or protein storage vacuole, respectively (Sanmartín et al., 2007).

The HOPS complex, which consists of six subunits, mediates homotypic fusion of vacuoles in *S. cerevisiae* (Seals et al., 2000). Interestingly, a similar complex also exists, CORVET; although it contains only four of the HOPS subunits, it can interconvert with HOPS by exchange of subunits (Peplowska et al., 2007). The yeast HOPS has been shown to interact with proteins that function in membrane fusion, as well as with phosphoinositides (Stroupe et al., 2006), but evidence for this mechanism in plants is lacking. By homology, the yeast orthologs for HOPS in Arabidopsis are VPS11, VPS18, VPS33, VPS39, VPS41 and VCL1. However, experimental data for the role of these proteins in homotypic vacuole fusion in plants is not yet available.

Vesicle trafficking is a complex process, and many proteins are involved to ensure that proteins get to the right compartment. A fundamental question is how does a cell know when to build a vesicle and where to send it? Coat proteins are required for vesicle formation, whereas Rabs, tethering factors, and SNAREs collectively collaborate to achieve fidelity by regulating trafficking at both temporal and spatial resolution. Although many proteins have been identified by sequence homology with yeast and classical genetics approaches, there are many questions unanswered regarding, for example, physical interactions between these proteins and both transcriptional and translational regulation.

Moreover, information regarding Rab proteins and SNAREs that specifically regulate trafficking to the vacuole is minimal.

1.5 Hormonal and environmental regulation of vacuole functions

Plants have the ability to sense and respond to endogenous and external stimulus to ensure optimal growth and development. Besides the essential genetics that regulates the molecular mechanisms underlying the integrity of the plant endomembrane system, evidence for both signaling and external factors controlling this machinery has also been reported. The hormone indole-3-acetic acid (IAA), the major form of auxin in plants, is well known for its ability to control and regulate almost every aspect of plant development and physiology (Chandler, 2009). In maize, both IAA and humic acids (HA) activate proton pumps localized at the plasma membrane and the tonoplast (Zandonadi et al., 2007). These authors propose that differential activation in vacuolar H^+ -ATPases and H^+ -pyrophosphatase by IAA induce the lateral root initiation. It has also been shown that the regulation of vacuolar proton pumps by endogenous signals is controlled at the transcriptional level. The synthetic auxin 2,4-D, or the stress hormone, abscisic acid (ABA), increases the expression of *HVPI*, a gene that encode for the V-PPase in barley (Fukuda and Tanaka, 2006). ABA also induced the expression of *HvVHA-A*, the gene encoding the catalytic subunit of the vacuolar H^+ -ATPase (Fukuda and Tanaka, 2006). The increases in transcripts abundance, along with the increased Na^+/H^+ antiport activity of *HvNHX1* and proton translocating activities of H^+ -PPase and H^+ -ATPase in barley tonoplast vesicles after 2,4-D and ABA treatment suggested a link between hormonal signals and vacuole function (Fukuda and Tanaka, 2006). A similar principle

seems to apply for vacuolar soluble proteins, as 2,4-D treatment in tobacco protoplasts results in a reduction in the levels of vacuole proteins, but not of proteins localized in other organelles (Meyer et al., 1987). Interestingly, application of exogenous IAA causes secretion of fluorescent vacuolar markers containing vacuolar sorting signals (Rosado et al., 2010). Hormonal regulation of transporter abundance may have other consequences, as data suggest an interplay among vacuolar transporters. For example, mutants in the tonoplast-localized cation transporter CAX1 have a 40% reduced activity for their vacuolar H⁺-ATPase, accompanied by an increase in the activity of the Ca²⁺-ATPases and increased expression of the putative cation transporters CAX3 and CAX4 (Cheng et al., 2003). Recently, mutants of a novel tonoplast protein involved in cell wall formation, known as WALLS ARE THIN1 (WAT1), were shown to have reduced levels of tryptophan, an auxin precursor, as well as auxin itself, suggesting a link between the tonoplast, auxin metabolism and secondary walls in fibers (Ranocha et al., 2010).

Light also acts as a critical environmental signal that controls plant growth and development. In the halophyte CAM plant *Mesembryanthemum crystallinum*, the transcript levels of tonoplast and plasma membrane aquaporins of the TIP family are regulated by the day and night cycle (Vera-Estrella et al., 2011). Interestingly, the mesophyll cells of *M. crystallinum* contain two separate vacuoles, one for storing the malate from the CAM cycle and a second for salt storage and sequestration (Epimashko et al., 2004). When the stomata are closed during the light period, malate is remobilized from the vacuole and its decarboxylation releases the CO₂ that is then reassimilated by Rubisco via the C₃ cycle (Cushman, 2001). This transcriptional regulation of aquaporins by diurnal changes has also

been reported in *Oriza sativa*, although so far only for aquaporins localized at the plasma membrane (Sakurai-Ishikawa et al., 2011).

The role of light has also been linked to other intracellular processes, such as distribution and recycling of plasma membrane proteins. In dark-grown *Arabidopsis* plants, members of the PIN FORMED (PIN) family of auxin transporters, which usually localize to the plasma membrane, undergo accumulation at the vacuole (Laxmi et al., 2008). In fact, the PIN2 protein is trafficked to the vacuole for degradation in light-grown seedlings after a short shift to darkness (Laxmi et al., 2008). It has also been reported that differences in light have major roles in regulating the transport and biosynthesis of auxin (Liu et al., 2011). When these proteins are constitutively transported to the vacuole, such as in the *vam3-4* mutant, the result is abnormal leaf vascular network due to differences in localization of auxin transporters (Shirakawa et al., 2009). Genetically speaking, the movement of PIN2 from the prevacuolar compartment back to the recycling pathway requires SORTIN NEXIN1 (SNX1) and VACUOLAR PROTEIN SORTING29 (VPS29), which are components of the retromer machinery and involved in the retrieving of proteins from the PVC back to the recycling pathway (Kleine-Vehn et al., 2008). Furthermore, inhibition of phosphatidylinositol-3-kinase (PI3K) by wortmannin abolishes the vacuolar accumulation of PIN2 (Kleine-Vehn et al., 2008), probably by inhibiting the recycling of vacuolar sorting receptors from the prevacuolar compartment and the *trans*-Golgi network, thus altering PVC identity (Matsuoka et al., 1995). This integration of external cues in the regulation of protein localization, and the fact that auxin itself regulates the abundance and localization of its transporters (Paciorek

et al., 2005) represents an example of a mechanism that drives the outstanding plasticity of plants.

1.6 Trafficking of vacuole lumen proteins

Proteins destined to the vacuole first enter the endomembrane system at the ER, where most of the protein folding and quality control mechanisms occur (Vitale and Boston, 2008). Proteins are then transported via vesicle trafficking to various compartments of the Golgi apparatus, the *trans*-Golgi network (TGN), the prevacuolar compartment, and intermediate endosomal compartments before they finally reach the vacuole (**Figure 1**). The default pathway for soluble proteins is secretion to the extracellular space (Chrispeels, 1991). Thus, it was hypothesized that vacuolar proteins must be sorted from secretory proteins at some point of the trafficking route by proteins that can recognize a vacuolar sorting signal.

The trafficking pathways for vacuolar lumen proteins have been extensively studied, including identification of vacuolar sorting signals. There is evidence for at least two different delivery routes for soluble vacuolar proteins, and these are distinguished by the location and peptide sequence of sorting signals. Proteins that are destined to the lytic vacuole are usually characterized by the presence of an NPIR (i.e., Asn-Pro-Ile-Arg) motif found within an amino-terminal propeptide (NTPP) signal, now referred as N-terminal vacuolar sorting signal (NT-VSS) (Matsuoka and Nakamura, 1991). Both the sweet potato sporamin and the barley aleurain contain the NTPP signal, and substitutions in the N-terminal sequence of sporamin result in the secretion of the protein (Nakamura et al., 1993). Once the proteins reach the TGN during the trafficking process, a cargo receptor recognizes the NTPP

signal and directs those proteins into clathrin-coated vesicles, that later deliver their cargo to the pre-vacuolar compartment before reaching the vacuole (Ahmed et al., 2000).

On the other hand, some vacuolar proteins have their signature-sorting signal at the carboxyl terminus, and these are usually targeted to the protein storage vacuole. The precursor to barley lectin and the tobacco chitinase contain a C-terminal propeptide (CTPP, or CT-VSS) signal that targets them to the vacuole, and deletion of this sequence results in secretion of these proteins (Wilkins et al., 1990; Neuhaus et al., 1991). In contrast with NTPP signals, CTPP signals do not seem to have a conserved domain, but are instead enriched in hydrophobic amino acids (Bednarek et al., 1990). Surprisingly, pharmacological data has shown that these two pathways employ different mechanisms (Matsuoka et al., 1995). However, an overlap of these pathways is possible during the development of central vacuoles after the fusion of lytic and protein storage vacuoles in some plant species (Di Sansebastiano et al., 2001). Recent data using mutant versions of Rab GTPases show evidence for a single route in tobacco leaf epidermal cells (Bottanelli et al., 2011).

1.7 Trafficking of vacuole membrane proteins

In contrast with the current stage of knowledge in the genetics for trafficking of vacuole soluble proteins, little is known about the molecular mechanisms for trafficking of proteins to the vacuole membrane (Rojo and Denecke, 2008). The tonoplast proteome contains a great number of membrane transporters and channels that are responsible for the transport of organic compounds and inorganic ions between the cytoplasm and the vacuolar lumen (Carter et al., 2004; Schmidt et al., 2007). The activity of some of these transporters

has been studied to some extent (de Angeli et al., 2006; Kawachi et al., 2008; Geisler et al., 2000), but the molecular mechanisms on how these transporters are trafficked to the tonoplast are still unclear.

Membrane proteins enter the endomembrane system at the ER, and the transmembrane domain is sufficient for translocation of the newly synthesized protein into the ER membrane. In contrast with soluble vacuolar proteins, tonoplast proteins do not seem to have a common sorting signal. Furthermore, there is no evidence for signal peptides within the cytoplasmic tails of membrane proteins like the ones identified in animal lysosomes (reviewed in Saftig and Kumperman, 2009). During the early '90s, the first evidence showing that vacuolar lumen proteins and vacuole membrane proteins are transported via different routes was reported. This work showed that the trafficking to the vacuole of phytohemagglutinin (PHA), the major lectin present in *Phaseolus* seeds, can be inhibited by the inhibitors Wortmannin and Brefeldin A (BFA) (Gomez and Chrispeels, 1993). In contrast, these chemicals have no effect in the delivery of TIP3;1, the most abundant tonoplast intrinsic protein in bean (Gomez and Chrispeels, 1993). Furthermore, incubation with Sortin1, a chemical that induces the secretion of vacuole soluble proteins, has no effect on the localization of tonoplast markers (Zouhar et al., 2004). These data suggest the existence of different sorting mechanisms for vacuolar and tonoplast proteins.

The vacuole membrane is not the default destination for membrane proteins, suggesting that specific signals and receptors are necessary for proper delivery of tonoplast proteins to the vacuole membrane (Brandizzi et al., 2002). To date, information about mechanisms for sorting integral vacuole membrane proteins is not clear. It has been shown

that the sequence motif PIEPPPHH in the cytoplasmic tail of *Phaseolus* TIP3;1 is required for proper delivery to the PSV tonoplast, and tobacco cells transformed with fluorescent protein fused to a truncated TIP3;1 lacking this motif (GFP-TIP3;1 Δ) localizes to vacuole-like structures as well as to an ER-like structure (Oufattole et al., 2005). Moreover, it has been shown that the cytoplasmic tail of TIP3;1 prevents the trafficking of the PVC-localized vacuolar sorting receptor BP-80 through the Golgi, and instead it is trafficked to the PVC directly (Jiang and Rogers, 1998). Interestingly, BP-80 traffics normally through the Golgi before reaching the PVC when the chimeric BP-80 has the cytoplasmic tail of TIP1;1. A different signal, the diacidic motif (DLE) at the C-terminus, has been identified as required for ER-export of the one member of the Arabidopsis tandem-pore potassium (TPK) family (Dunkel et al., 2008). Although this signal is not conserved among members of the TPK family and it is specific for TPK1, it is a common ER-export signal for other plasma membrane proteins of different plant species (Mikosch et al., 2006; Sorieul et al., 2011; Zelazny et al., 2009). A recent analysis of TPK proteins from *Oryza sativa* showed that members of this family are expressed in different types of vacuoles and that signals in the C-terminus also control vacuolar localization (Isayenkov et al., 2011). Not surprisingly, these proteins seem to carry specific signals in their C-termini that are essential for targeting to each type of vacuole. In fact, substitution of the C terminus of AtTPK4, a plasma membrane protein, with that of the tonoplast protein AtTPK1 is sufficient for delivery to the vacuole membrane (Maîtrejean et al., 2011). Moreover, a link between trafficking and vacuole identity was established in rice (Isayenkov et al., 2011). The targeting of TPKa in rice and Arabidopsis protoplasts, which localizes to the lytic vacuole, is BFA-sensitive whereas that

of its homolog and PSV-resident, TPKb, is BFA-insensitive. These data support the hypothesis that proteins destined to the PSV travel in a Golgi-independent manner, whereas lytic vacuole proteins require passage through the Golgi. Evidence showing conserved tonoplast sorting signals is lacking; and thus, the molecular mechanisms regulating vacuolar membrane protein trafficking are still a big question.

Members of the tonoplast intrinsic protein (TIP) family has been used extensively to study vacuole membrane biogenesis and function (Jauh et al., 1998; Jauh et al., 1999). It was using these vacuolar membrane proteins as markers that the putative existence of two separate pathways for the trafficking of tonoplast proteins was further supported (Jiang and Rogers, 1998). The transport of TIP3;1 to the tonoplast is BFA-insensitive and predicted to be Golgi-independent. In addition, Arabidopsis HA-TIP3;1 does not acquire *N*-glycans specifically added in the Golgi apparatus, and the protein was transported to the PSV membrane even when ER-to-Golgi vesicle traffic was abnormal in protoplasts expressing the dominant negative form of AtRab1/Ara5/RabD2 or overexpressing AtSec23 (Park et al., 2004). It is still unclear whether this is an ER-to-tonoplast direct pathway, or if TIP3;1-containing vesicles merge at some point with any intermediate compartments. Recently, it has been shown that transient co-expression of TIP3;1 and nucleotide-free mutants of the GTPases Rha1, Ara6 and Rab7 results in the accumulation of TIP3;1 in small punctate structures in tobacco (Bottanelli et al., 2011). The authors also demonstrated multiple routes for membrane cargo by showing impaired trafficking of the tonoplast-anchored protein Vam3/SYP22 only in the nucleotide-free Rab7 background, but not in the Rha1 or Ara6 mutant backgrounds (Bottanelli et al., 2011). This argument was further confirmed recently

when two different Golgi-dependent routes for mesophyll tonoplast proteins were suggested. It seems that although some tonoplast proteins are sensitive to Brefeldin A, an inhibitor of ER-to-Golgi traffic, some proteins may require the function of ADAPTOR PROTEIN-3 (AP-3) whereas others do not (Wolfenstetter et al., 2012). This suggests a divergence in the pathway after the Golgi, and the authors propose that some proteins travel directly from the Golgi to the tonoplast, whereas others require passage through the pre-vacuolar compartment. In this study, the authors also show that a di-leucine motif is responsible for targeting tonoplast proteins to the vacuolar membrane. When this signal is attached to plasma membrane or ER proteins, they are instead targeted to the tonoplast, while remaining functional (Wolfenstetter et al., 2012).

Despite the importance of membrane deposition to the vacuolar membrane via vesicle trafficking for proper vacuole function, the machinery that regulates this pathway is still largely uncharacterized. To date, only a subset of proteins have been implicated to play roles in the trafficking of tonoplast proteins. SYNTAXIN OF PLANTS21 (SYP21), also known as PEP12, was the first player linked to this pathway in tobacco plants (Foresti et al., 2006; Tyrrell et al., 2007). This protein is a member of the SNARE family and has been shown to localize to a post-Golgi compartment (da Silva Conceição et al., 1997). Overexpression of AtSYP21 causes the inhibition of trafficking from the PVC for both soluble and vacuolar membrane cargo, resulting in secretion of soluble proteins and the accumulation of the tonoplast-localized SYP22 at the PVC (Foresti et al., 2006). Likewise, expression of a truncated soluble version of AtSYP21 results in the accumulation of the lytic vacuole membrane marker TIP1;1 at the PVC (Tyrrell et al., 2007).

Because of the importance of maintenance of vacuolar membrane integrity, and lack of information regarding tonoplast protein trafficking (**Figure 2**), it is imperative to further characterize this pathway. Our hypothesis is that multiple pathways target membrane-spanning proteins to the tonoplast, with these pathways being regulated by developmental, environmental and hormonal pathways. Neither classical nor chemical genetic screens to identify components of the trafficking machinery have been performed; thus, there is a need to fill the informational gap. Genetic engineering of targeting of transporters to the vacuole membrane has potential applications in the improvement of stress and drought tolerance, as well as the nutritional value of crop plants. Similarly, the study of these pathways can also be used for developing plants enriched in secondary metabolites, as it has been documented that at least the pathway for trafficking of anthocyanins to the vacuole is Golgi-independent (Poustka et al., 2007), as has been proposed for a subset of tonoplast proteins.

1.8 Chemical genetics as a tool for dissecting trafficking to the vacuole

Although the plant genome is enriched with proteins involved in the endomembrane system that are the result of genome duplication, it seems that most of the proteins do not act redundantly (Sanderfoot et al., 2000; Bassham et al., 2008). This represents one of the challenges of studying protein trafficking in plants, as several knock-out mutations in the secretory pathway result in embryo lethality (Sanderfoot et al., 2001). This is particularly true for reverse genetics approaches using T-DNA insertion lines and phylogenetic comparisons of the *Arabidopsis* genome with that of *S. cerevisiae*. The yeast model has been a great source for identifying endomembrane system genes, but is not uncommon to obtain

differences in the localization of the proteins in Arabidopsis and yeast (Bassham and Raikhel, 2000). Thus, it is imperative to use alternative strategies to study tonoplast protein trafficking in a way that overcomes embryo lethality and redundancy.

The use of small bioactive compounds to perturb biological processes has evolved greatly during the past two decades, and important discoveries have been the result of this technology since then. Plant chemical genetics can address cellular and molecular questions that sometimes are impossible to obtain using mutational genetics (reviewed in Hicks and Raikhel, 2009). In principle, the use of chemical inhibitors allows rapid, conditional and reversible biological alterations by either inhibiting a specific component of a pathway (i.e. specific agonist) or by inhibiting multiple components of a single network (i.e. general agonist). The large number of libraries available, as well as the rapid evolution of technologies for chemical-target identification has led to important breakthroughs in cell and molecular biology (Tóth and van der Hoorn, 2009; Cong et al., 2011).

Probably the most recent elegant discovery using chemical genetics in plants is the identification of the abscisic acid (ABA) receptor PYRABACTIN RESISTANCE1 (PYR1) (Park et al., 2009). The ABA receptor has been a subject of controversy since the 1960s, with many previously reported putative receptors that were never globally accepted (Pennisi, 2009), and it was not until the use of chemical genetics that the identity of one major ABA receptor family became elucidated. Chemical genetics has been extensively used to identify novel components in hormone signaling pathways, such as in auxin (Armstrong et al., 2004; Zhao et al., 2003), ethylene (He et al., 2011), strigolactones (Tsuchiya et al., 2010), brassinosteroids (De Rybel et al., 2009) and jasmonic acid (Zheng et al., 2006). With the

advances in live-cell imaging, the use of small-molecule screens has been a powerful tool for dissecting endomembrane system pathways in plants (Drakakaki et al., 2009; Drakakaki et al., 2011; Robert et al., 2008; Rosado et al., 2011; Surpin et al., 2005; Zouhar et al., 2004). Along with this, several chemicals that affect vacuole morphology have been identified (Surpin et al., 2005). Therefore, chemical genetics is a valuable tool for dissecting tonoplast trafficking pathways and identifying genes that are also involved in hormonal and environmental-related processes.

1.9 Major research goals and outcomes

Because of the many agricultural applications of genetic manipulation of tonoplast transporters localization, it is imperative to obtain more information about this cellular process. For many years, the focus has been to study trafficking of soluble vacuole proteins, and this led to the identification of vacuolar sorting signals (VSS) that are necessary and essential for the proper delivery of proteins to the vacuole. In terms of tonoplast transporters, most efforts have been made to understand the activity of these integral proteins and their roles in plant growth and development. An area that has been less explored is the trafficking of tonoplast membrane proteins. With the rapid advances in live cell imaging, molecular biology techniques, and chemical genomics, the study of intracellular processes is in its best era. Knowledge on the trafficking of membrane transporters can be used to, for example, manipulate water uptake or develop drought-tolerant plants. The fact that the vacuole is the main storage location of many proteins, soluble carbohydrates, ions and fruit-flavor

compounds opens a gate for new avenues in the storage of compounds in seeds; as well as metabolomics engineering in crops.

In order to understand the molecular mechanisms for the targeting of integral proteins to the vacuole membrane, a chemical genetics approach was taken. A small library of bioactive molecules that inhibit pollen growth in tobacco was tested for inhibition of tonoplast proteins at the endoplasmic reticulum (ER) in the model plant *Arabidopsis thaliana*. We successfully identified novel chemicals in a confocal-based screen that induce the mis-localization of a tonoplast protein, GFP-TIP2;1, to the ER. One of the chemicals affects only a subset of tonoplast proteins, providing evidence for multiple trafficking pathways *in planta*. Because of the many roles of the hormone auxin in controlling cellular processes, we analyzed the effects of our inhibitors in different pathways of auxin homeostasis. Data suggesting an interaction between the plant endomembrane system and auxin transport, as well as with induction of tryptophan biosynthetic genes, will be discussed.

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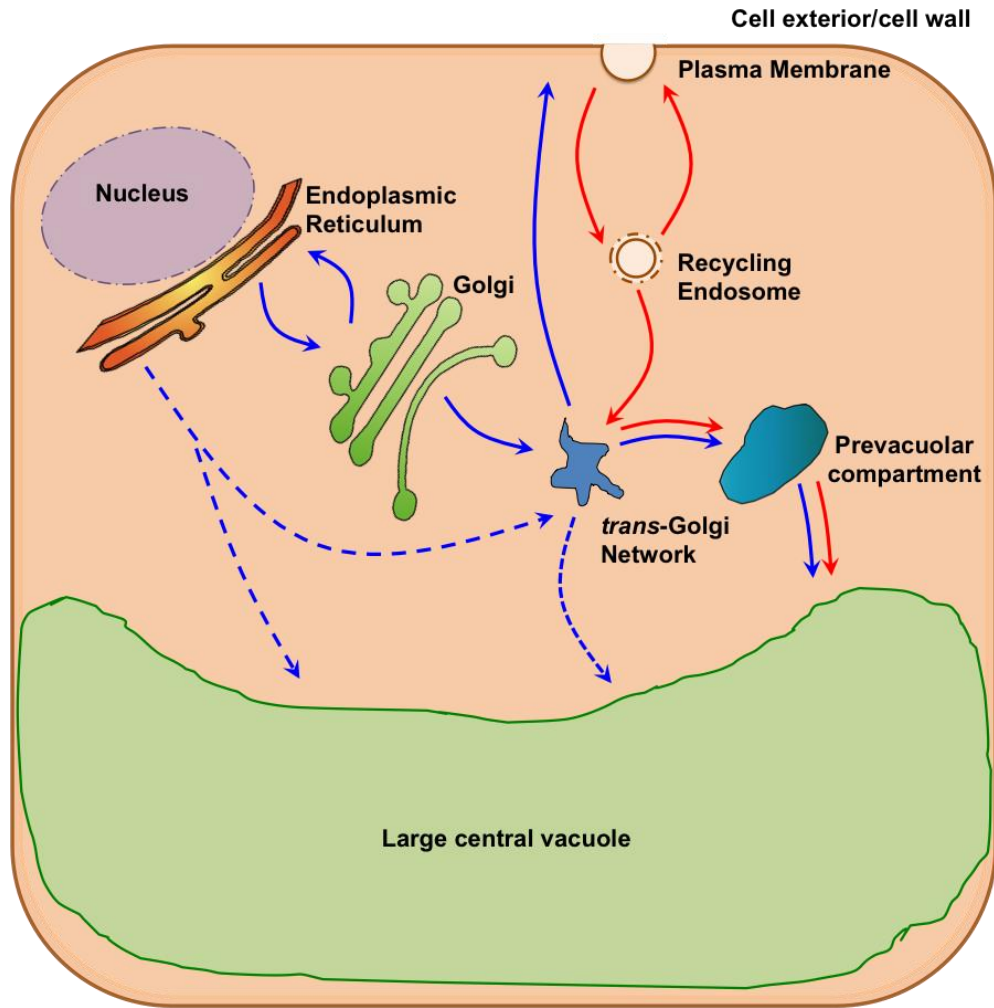


Figure 1. Hypothetical model for the secretory and the endocytic pathway in plants.

After entry into the rough endoplasmic reticulum (ER), proteins destined to the secretory pathway (blue arrows) are transported to different endomembrane system compartments, including the Golgi, the *trans*-Golgi network (TGN) and the prevacuolar compartment (PVC). Soluble vacuole proteins with either CTPP or NTPP signals follow a route of transport to the vacuole, whereas proteins lacking these signals are secreted to the cell exterior. Proteins can also enter the endomembrane system via endocytosis (red arrows) by recycling endosomes (RE).

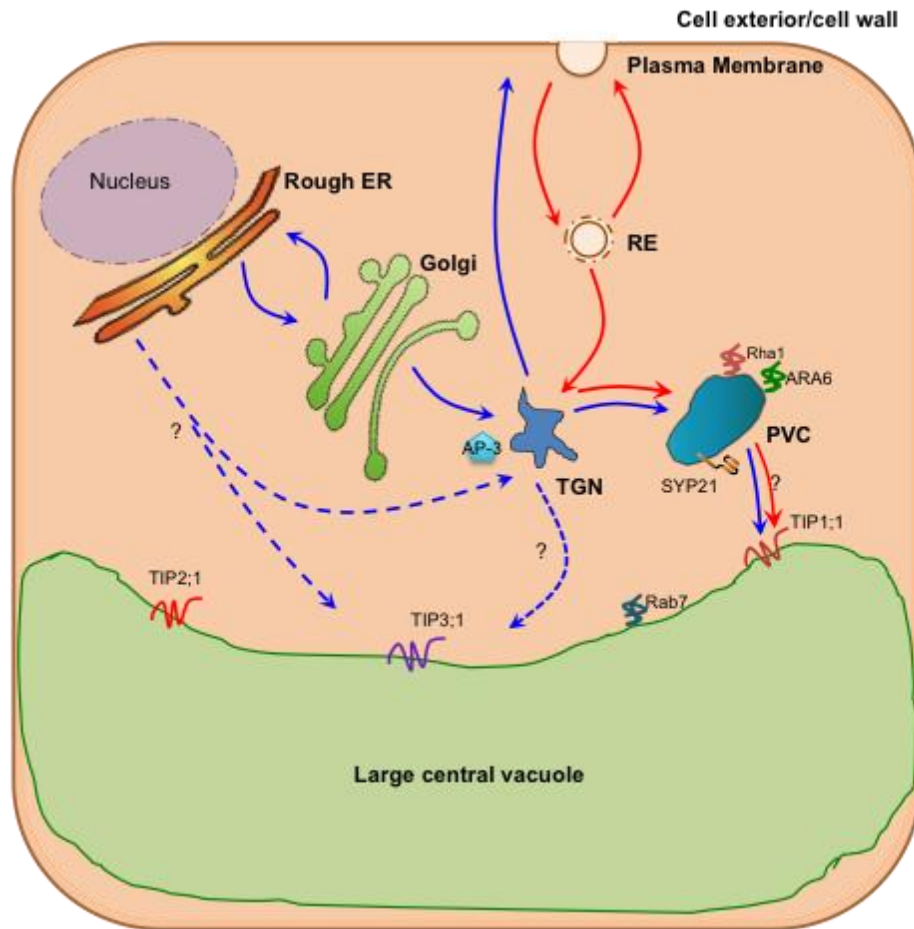


Figure 2. Regulators and putative pathways for the trafficking on TIPs. The trafficking of TIP3;1 has been shown to be Golgi-independent in *Arabidopsis* protoplasts. The localization of this protein is affected when nucleotide-free mutants of the GTPases Rha1, ARA6 and Rab7 are expressed in tobacco leaf epidermal tissue. TIP1;1 traffic to the tonoplast has been shown to be dependent on the activity of the SNARE protein SYP21. The ADAPTOR PROTEIN-3 (AP-3) complex has been shown to regulate trafficking of tonoplast proteins, however, evidence for the role of this complex in TIP traffic has not been reported. Blue and red arrows indicate routes for the secretory pathway and endocytic pathway, respectively.

CHAPTER 2

Tonoplast Proteins Travel Via Different Pathways in *Arabidopsis*

Part of the results shown in this chapter were included in a manuscript that is currently in preparation for publication under:

Efrain Rivera Serrano, María F. Rodríguez-Welsh, Glenn Hicks, Natasha V. Raikhel and Marcela Rojas-Pierce. (In preparation) A Small Chemical Inhibitor Partitions the Golgi-dependent and Golgi-independent Pathways for Tonoplast Proteins.

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ABSTRACT

The vacuole is an essential and dynamic organelle in plant cells with an array of multiple cellular and physiological functions required for plant growth and development. Many of the vacuole roles, such as the storage of proteins, carbohydrates, lipids and secondary metabolites, are of great value for human consumption. Vacuole biogenesis, integrity and function depend on constant deposition of membrane proteins via the endomembrane system, that starts at the endoplasmic reticulum (ER). Despite the essential role of the plant vacuole, the pathways that regulate trafficking of vacuolar membrane proteins are largely unknown. Using a small chemical library and a double-marker transgenic line expressing the vacuole membrane marker GFP-TIP2;1 and mCherry-HDEL labeling the ER, we identified five structurally-different chemicals that inhibit the trafficking of the vacuole membrane protein, resulting in the accumulation of the GFP-TIP2;1 marker at the ER. One chemical, referred to as C834, specifically affects the subcellular localization of the TIP2;1 and TIP3;1 markers, but not that of the TIP1;1 marker. Moreover, Brefeldin-A (BFA), a chemical that inhibits ER-to-Golgi traffic, induces the accumulation of the TIP1;1 marker in BFA-compartments, but does not affect TIP2;1 and TIP3;1. These results indicate the presence of multiple and independent trafficking pathways for tonoplast proteins in *Arabidopsis*, one that is Golgi-dependent and one that is Golgi-independent. C834 is a specific inhibitor of the Golgi-independent pathway and is being used to identify regulatory components of this trafficking pathway.

2.1 Introduction

Proteins and lipids stored in seeds represent the major food source for human consumption (Müntz, 1998). These compounds are synthesized during seed development and, at the cellular level, accumulation of seed storage proteins and other compounds occurs in storage compartments. In monocotyledonous species, these compartments are known as protein bodies (Larkins and Hurkman, 1978). However, in dicotyledonous species, such as legumes, proteins are stored in the protein storage vacuole (PSV) almost exclusively (Herman and Larkins, 1999). The vacuole represents the largest organelle in plant cells, occupying more than 90% of their volume. It is the main storage of ions, water, carbohydrates, proteins, secondary metabolites and other organic molecules; and it is also involved in protein recycling and cell detoxification (reviewed in Martinoia et al., 2010). In contrast with yeast, plants have two distinct and functional vacuoles: a protein storage vacuole (PSV) present in embryos and dry seeds and the counterpart of the animal lysosome, the lytic vacuole (Paris et al., 1996). The first is labeled with the vacuole membrane aquaporin TIP3;1, and barley lectin; whereas the lytic vacuole is marked by the presence of TIP1;1 and proteases such as aleurain (Jauh et al., 1999). The spatial and temporal presence of both vacuoles appears to be plant specific. In plants like tobacco, it has been shown that the PSV is transformed into a large lytic vacuole during germination (Zheng and Staehelin, 2011). In contrast, for example, in *Mesembryanthemum crystallinum* both types of vacuoles, with different biochemical and physiological roles, co-exist in a single cell (Epimashko et al., 2004). In *Arabidopsis*, however, vacuole identity seems to be a developmental process

involving gene regulation and two independent vacuoles have not been identified in a single cell. (Hunter et al., 2007; Frigerio et al., 2008)

The many functions of the vacuole depend on constant deposition of membrane transporters and other integral proteins into the vacuole membrane, also known as tonoplast. The transport of tonoplast and other membrane proteins occurs via vesicle trafficking from the endoplasmic reticulum (ER) (Jiang and Rogers 1998; Brandizzi et al., 2002; Dunkel et al., 2008). In contrast with the sorting signals present in vacuole soluble proteins (Matsuoka and Nakamura, 1991; Nakamura et al., 1993), common sorting signals have not been identified for vacuole membrane proteins, and the transmembrane domain may be sufficient for translocation to the ER. Two pathways have been proposed for the delivery of tonoplast proteins from the ER to the vacuole, a Golgi-dependent and a Golgi-independent route. Evidence for a Golgi-independent pathway involved in tonoplast targeting was first reported during the early '90s when it was shown that trafficking of TIP3;1 is insensitive to Brefeldin A (Gomez and Chrispeels, 1993), an inhibitor of the Golgi-to-ER trafficking. This data was further confirmed biochemically when it was reported that HA-TIP3;1 do not acquire N-glycans that are specific of passage through the Golgi (Park et al., 2004). It was later shown that the sequence motif PIEPPPHH in the cytoplasmic tail of α TIP from bean is required for proper delivery to the tonoplast (Oufattole et al., 2005). Moreover, this cytoplasmic tail prevents the Golgi-dependent trafficking of BP-80, a vacuolar sorting receptor that localizes to the prevacuolar compartment (PVC) (Jiang and Rogers, 1998). Instead, the BP-80 fusion to the bean cytoplasmic tail of α TIP is delivered to the PVC directly (Jiang and Rogers, 1998). Surprisingly, when the same experiment is carried out with the cytoplasmic tail of

TIP1;1, the BP-80 chimeric protein travels normally through the Golgi before reaching the PVC. Recently, it has been shown that isoforms of the Two-Pore K⁺ (TPK) channel family in rice travel via different mechanisms (Isayenkov et al., 2011). While TPKa localizes to the lytic vacuole and is sensitive to BFA, TPKb localizes to the PSV and its targeting appears to be Golgi-independent in rice protoplasts. In addition, the C-terminal domains of these proteins appear to have targeting signals for these different pathways (Isayenkov et al., 2011).

To date, only a subset of proteins has been implicated to the regulation of trafficking of tonoplast proteins. Overexpression of SYNTAXIN OF PLANTS21 (SYP21), a SNARE localized at a post-Golgi organelle (de Silva Conceição et al., 1997), causes the inhibition of trafficking from the prevacuolar compartment (PVC) for both soluble and vacuolar membrane cargo, resulting in secretion of soluble proteins and the arrest of the tonoplast protein SYP22 at the PVC (Foresti et al., 2006). Likewise, expression of a truncated soluble version of AtSYP21 results in the accumulation of the lytic vacuole membrane marker TIP1;1 at the PVC (Tyrrell et al., 2007). Recently, transient experiments in tobacco demonstrated that expression of mutated Rab GTPases Rha1, Ara6 and Rab7 result in mis-localization of TIP3;1 to punctate structures reminiscent of an intermediate compartment (Bottanelli et al., 2011). Evidence also suggests the existence of two Golgi-dependent pathways that diverge at some point after the Golgi and that is dependent on the activity of the adaptor protein (AP)-3 complex (Wolfenstetter et al., 2012). The fact that these proteins are involved in trafficking steps after the Golgi suggests that there are other proteins involved in the Golgi-independent pathway, whose identity remains unknown.

In our efforts to characterize the molecular mechanisms regulating protein targeting to the vacuole membrane, we designed a chemical genetics screen to identify inhibitors of this pathway. Here, we report the identification of five novel small drug-like molecules that result in the arrestment of tonoplast proteins at the ER. Localization analyses with markers that label different organelles suggest that the chemicals have different molecular targets. We report the identification of a chemical that specifically inhibits the trafficking of the Golgi-independent route, which inhibits the trafficking of TIP2;1 and TIP3;1 but not that of TIP1;1 in *Arabidopsis*. Using Brefeldin A, we confirmed that both TIP1;1 travels in a Golgi-dependent manner, whereas the other two bypass this organelle during their transport to the vacuole. This is the first evidence for the existence of both trafficking mechanisms *in planta*.

2.2 Results

2.2.1 Chemical genetics screen for inhibitors of tonoplast protein trafficking

In our efforts to characterize the molecular mechanisms that regulate protein transport to the tonoplast, we used a chemical genetics approach to identify inhibitors of the trafficking of membrane proteins. Our chemical library consisted of 360 compounds that were previously selected from a 56,000 chemical library shown to inhibit germination of tobacco pollen (Drakakaki et al., 2011). The important role of vacuole biogenesis and protein targeting in growth of pollen tubes and root hairs has been well documented (Hicks et al., 2004; Kost et al., 2008), and we expected to find compounds that affect this particular pathway in this chemical collection. In fact, chemicals that inhibit cellular processes such as

endocytosis, protein recycling and cell plate formation has been identified using this small sub-library (Robert et al., 2008; Drakakaki et al., 2009; Drakakaki et al., 2011).

Our hypothesis was that, in presence of compounds that block the transport of vacuolar proteins, these proteins should instead localize to an intermediate compartment. In order to test this, we designed a confocal microscope-based screen using a transgenic *Arabidopsis* line expressing two protein chimeras. Our double marker line carries a δ Tonoplast Intrinsic Protein (TIP2;1) tagged with GFP (Cutler et al., 2000) and a fusion of the mCherry fluorescent protein with an N-terminal signal peptide and an HDEL retention signal at the C-terminal (Nelson et al., 2007); labeling the vacuole membrane and the endoplasmic reticulum (ER), respectively. Under control conditions, the signal from the two fluorescent markers should not co-localize because they reside in separate organelles. We predicted that inhibition of protein targeting would cause accumulation of retained proteins at upstream compartments, including the ER. Measuring co-localization from both markers can identify chemicals that are able to inhibit trafficking processes. We performed a manual screen and identified 76 out of 360 putative compounds that induced mis-localization of GFP-TIP2;1 during the primary screen. As part of the secondary screen, we assayed the activity of the selected chemicals using three different concentrations, and only 36 compounds passed this selection round by showing consistent results. A third round of screen was performed as final confirmation criteria and 20 compounds were dropped from the list, as they did not show consistent results. Once we obtained new stocks from the supplier, only 5 compounds significantly inhibited trafficking of the tonoplast protein (**Figure 1**).

Under control conditions, a continuous and smooth membrane characterizes the tonoplast marker, whereas the ER marker looks discontinuous and patchy when looked from a cross section (Figure 1a). Out of these compounds, four induced the mis-localization of GFP-TIP2;1 to the ER in root cells, shown by appearance of the tonoplast marker and its co-localization with the ER marker (Figures 1b-1e). The last chemical, however, causes other aberrant phenotypes but does not inhibit trafficking at the ER. It was noticeable that some of the compounds also affected ER structure, as the aggregates that were induced by Compound 755 (C755) (Figure 1d). Interestingly, seedlings treated with C578 show smaller vacuoles, and both markers localize partially to their proper compartment as well as what it seems to be cytoplasm (Figure 1f). All five chemicals are substantially different in terms of chemical structures (**Table 1**), and their bioactivity has not been reported in the literature. It is interesting to note, however, that compound 410 is structurally similar to Gravacin, a compound that has been shown to be a target of the auxin transporter P-Glycoprotein19 (PGP19) and also to inhibit the delivery of TIP2;1 to the tonoplast by a different unknown mechanism (Rojas-Pierce et al., 2007; Surpin et al., 2005). C410 also inhibits gravitropism (data not shown) in a similar fashion as Gravacin and it has been shown to affect the auxin response using reporter genes (data not shown), so it is likely that these two compounds share an undiscovered cellular target.

In order to understand better the mechanisms of tonoplast trafficking, we used our set of chemical inhibitors and tested their effects on other members of the TIP family. We hypothesized that if these proteins are targeted by different mechanisms, then they should present different sensitivities to some chemicals. Thus, chemicals that inhibited targeting of

some but not all of the tonoplast markers would provide strong evidence for a multiplicity of pathways. All chemicals were tested for trafficking effects on two other members of the TIP family: TIP3;1-YFP and TIP1;1-YFP (**Figure 2**) (Hunter et al., 2007), that label the lytic vacuole and the protein storage vacuole (Paris et al., 2006), respectively. Under normal conditions, the localization of TIP3;1-YFP is very similar to that of TIP2;1 (compare Figure 2a with 2b). The pattern of TIP1;1 is a little bit different as this marker labels the tonoplast but also vacuolar bulbs (Figure 2c). In the presence of inhibitors C410, C755 and C103 the localization of all three tonoplast markers is inhibited and they are mis-localized to the ER (Figures 2d-2l). In contrast, treatment with C578 causes an apparent constriction of the vacuole, and also some cytoplasmic staining can be seen (Figures 2m-2o). One of the compounds, however, seemed to act different than the others and will be explained in detail further in this chapter.

We then tested the effects of the 4 compounds that affected all TIP proteins for effects on other components of the endomembrane system (**Figure 3**). For this, we used stably transformed seedlings expressing different fluorescent markers labeling the plasma membrane (Cutler et al., 2000; Figure 3a), the Golgi apparatus (Grebe et al., 2003; Figure 3f), the *trans*-Golgi network (Dettmer et al., 2006; Figure 3k) and the pre-vacuolar compartment (Jaillais et al., 2006; Figure 3p) and analyze their patterns under the confocal microscope. Compound C410, which is structurally similar as Gravacin, inhibits the trafficking of the plasma membrane marker PIP2a-GFP (Figure 3b) and causes its retention at the ER. In contrast, the Golgi marker NAG1-GFP appeared normal after C410 treatment. It also causes what it seems to be a partial “solubilization” of VHA-a1-GFP (Figure 3l) and of

SNX1-GFP (Figure 3q), that labels the TGN and the PVC, respectively, suggesting a general inhibition of the secretory pathway. In contrast, C755 seems to affect only tonoplast proteins, as no significant effect was detectable in the other markers tested (Figures 3c, 3h, 3m and 3r). In presence of compound C103, PIP2a-GFP localizes to the cortical ER (Figures 3d) while TGN proteins tend to form aggregates (Figure 3n). This last difference, and the fact that C103 does seem to affect SNX1 and NAG1 (Figures 3j, 3s), imply that C410 and C103 do not share the same cellular target although they seem to have broader effects on the endomembrane system. Interestingly, compound C578, which causes a shrinking effect on lytic vacuoles (Figure 1f), seems to cause aggregation of Golgi, TGN and PVC markers in cytoplasmic pools (Figures 3j, 3o and 3t), but the plasma membrane retains its integrity with the exception of localization in some small compartments (Figure 3e). Overall, we identified pharmacological tools for dissecting intracellular trafficking pathways at different steps and the molecular targets of our compounds are likely to be independent. Further characterization of these chemicals can reveal the identity of novel molecular players involved in the targeting of proteins to the vacuole membrane.

2.2.2 A novel inhibitor uncouples different pathways for tonoplast proteins

In contrast with the first four chemicals discussed above that showed trafficking inhibition of not only tonoplast proteins, but also to proteins in other organelles, the inhibitor C834 behaves in a different fashion. Surprisingly, compound C834 specifically inhibited the trafficking of a subset of tonoplast proteins (**Figure 4**). We first tested the localization of all three TIP markers in presence of C834. As expected from the results of the screen, C834

inhibites the localization of GFP-TIP2;1 to the tonoplast and causes its retention at the ER (Figure 4a-b). Similar results were obtained when we analyzed the localization of TIP3;1-YFP in presence of C834 (Figure 4c-d). Interestingly, the localization of the third family member, TIP1;1-YFP, was found to be insensitive to the effects of C834 and it normally localizes to the tonoplast in presence of the inhibitor (Figure 4e-f). These results suggest that while TIP2;1 and TIP3;1 are targeted to the tonoplast in a similar manner, the trafficking of TIP1;1 may be different. We then wanted to test if the inhibitory effects of C834 on the localization of a tonoplast protein from another family. For this, we analyzed the localization of the tonoplast protein TRANSPARENT TESTA12 fused to GFP (TT12-GFP), which has been shown to act as a vacuolar flavonoid/H⁺-antiporter at the vacuole membrane (Marinova et al., 2007). Under normal conditions, the localization of TT12-GFP was found at the tonoplast, as expected (Figure 4g). In presence of C834, localization of TT12-GFP was found to be normal (Figure 4h), suggesting that it travels in a C834-insensitive route. Overall, our results suggest that TIP2;1 and TIP3;1 are transported to the vacuole membrane by a similar molecular mechanisms, whereas TIP1;1 and TT12 travel in a different manner.

The genetic expression and protein accumulation of members of the TIP family is developmentally regulated, and these proteins are often used as markers of vacuole identity (Jauh et al., 1998; Gattolin et al., 2010). For instance, TIP3;1 can be found only labeling the membrane of the protein storage vacuole (PSV) found in mature embryos; whereas TIP1;1 is mostly found in lytic vacuoles of vegetative cells (Hunter et al., 2007). Our chemical inhibition analysis suggested the existence of different targeting pathways in lytic vacuoles, so we wanted to answer if the same pathway was present in cells containing PSVs. To this

end, we dissected mature embryos of dry seeds from transgenic lines expressing TIP3;1-YFP under its native promoter and incubated the embryos with C834 (**Figure 5**). To confirm that the vacuoles at that stage were indeed PSVs, we captured the autofluorescence from the lumen of these compartments (Shimada et al., 2003). Under control conditions, the localization of TIP3;1-YFP in embryos appears surrounding the membrane of PSVs, while the inside of the PSVs can be seen in the blue channel (Figure 5a). Just like in root cells, treatment with C834 resulted in mis-localization of TIP3;1-YFP to a compartment that resembles the ER (Figure 5b). We tried to visualize the TIP2;1 and TIP1;1 at this stage as well, but when these proteins are expressed ectopically in control embryos, they localize to both the tonoplast and an ER-like structure (data not shown). We conclude from this experiment that the targeting machinery for tonoplast proteins via the C834-sensitive pathway is present in both lytic and protein storage vacuoles.

It has been shown that, in tobacco leaf protoplasts, the delivery of TIP3;1 is independent of the Golgi compartment, as its trafficking is insensitive to the inhibitory effects of Brefeldin A (Gomez and Chirspeels, 1993). Moreover, the AtTIP3;1 protein itself does not acquire Golgi-specific modifications, and the cytoplasmic tail of *Pisum sativum* TIP3;1 is sufficient to prevent proteins from entering the Golgi (Park et al., 2004; Jiang and Rogers, 1998). Park et al. also provided evidence supporting this hypothesis by demonstrating that the trafficking of HA-TIP3;1 is not affected when the ER-to-Golgi pathway is impaired by expression of a dominant-negative of AtRab1 or overexpression of AtSec23 (Park et al., 2004). Because C834 affected TIP2;1 and TIP3;1 in a similar fashion, we hypothesized that the delivery of TIP2;1 is also Golgi-independent, different from the

delivery of TIP1;1. To test our hypothesis we analyzed the effects of Brefeldin A in *Arabidopsis* hypocotyl cells.

Brefeldin A (BFA) is a well-known fungal toxin that has been shown to inhibit ADP-ribosylation factor-guanine nucleotide exchange factors (ARF-GEFs) in mammals and plants; and, in *Arabidopsis*, GNOM has been identified as one of its targets (Steinmann et al., 1999). This effect on GNOM has been widely used to study protein recycling, as inhibition of GNOM by BFA inhibits the transport from endosomes to the plasma membrane during the endocytic pathway (Geldner et al., 2003). However, GNOM-LIKE1 (GNL1) is BFA-resistant and is involved in ER-to-Golgi traffic (Richter et al., 2007). In fact, the BFA-sensitive GEFs, GBF1 and Gea1/2p, are localized to the Golgi and implicated in the ER-to-Golgi traffic in mammals and budding yeast, respectively (Zhao et al., 2006; Peyroche et al., 2001). In non-root *Arabidopsis* tissues, BFA inhibits Golgi-localized ARF-GEFs that are essential for the formation of COPI and Golgi-to-ER retrograde traffic. In *Arabidopsis* hypocotyls and leaf cells, typical effects of BFA on Golgi have been demonstrated; indicating that a BFA-sensitive ARF-GEF is important for ER-to-Golgi traffic (Robinson et al., 2008). Moreover, inhibition by BFA was used to conclude that the *Arabidopsis* TPK1, a tonoplast-localized potassium channel, travels in a Golgi-dependent pathway, at least when expressed in onion epidermal cells (Dunkel et al., 2008).

We exposed transgenic lines expressing tonoplast proteins markers to BFA and analyzed protein localization by confocal microscopy (**Figure 6**). The signal from GFP-TIP2;1 appeared normally localized to the tonoplast in both control and after BFA treatment (Figures 6a-6b). Consistent with previous results using tobacco and *Arabidopsis* protoplasts,

the intracellular localization of TIP3;1-YFP was BFA-insensitive in *Arabidopsis* hypocotyls, thus, confirming that this protein was trafficked in a Golgi-independent route (Figures 6c and 6d). However, whereas TIP1;1-YFP localized to the vacuole membrane in the control, incubation with BFA resulted in both mis-localization to an ER-like network and to BFA-induced aggregates (Figures 6e and 6f). To address whether these aggregates were in fact BFA compartments, we visualized the localization of the Golgi marker NAG1-GFP (Grebe et al., 2003), and the TGN marker VHA-a1-GFP (Dettmer et al., 2006) in presence or absence of BFA. Under normal conditions, both the Golgi marker and the TGN accumulated in small punctate structures (Figure 6g, 6i). After BFA treatment, the Golgi marker localized to BFA compartments and to an ER-like structure (Figure 6h), whereas only BFA compartments were seen in the TGN marker (Figure 6j). This data confirms that inhibition of trafficking at the Golgi by BFA results in both an ER-like localization and BFA compartments in *Arabidopsis* hypocotyls. These results suggest the existence of different targeting pathways for tonoplast proteins of the same family, a Golgi-independent route that targets TIP2;1 and TIP3;1 and a Golgi-dependent pathway that targets TIP1;1 to the lytic vacuole. Interestingly, the tonoplast protein TT12 (Marinova et al., 2007), which travels in a C834-insensitive manner, is also insensitive to the effects of BFA (Figures 6K and 6L). This data suggest that TT12-GFP is likely to travel in a Golgi-independent route during its transport, but requires a different mechanism that does not involve the molecular target of C834. Overall, our results provide evidence *in planta* for multiple and independent pathways for tonoplast proteins in *Arabidopsis*.

2.2.3 C834 is not an unspecific inhibitor of the endomembrane system

In comparison with the other four inhibitors that were identified in this study, only C834 showed targeting specificity for tonoplast proteins. In order to gain insight of the trafficking pathways affected by C834, we analyzed the effect of this compound on the localization of multiple subcellular markers by means of confocal microscopy (**Figure 7**). We looked at the localization of three different Golgi markers, GOT1p-YFP, MEMB12-YFP and SYP32-YFP (Geldner et al., 2009) and could not detect any differences in root cells (Figures 7a to 7f). Similarly, NIP1;1-YFP and PIP1;4, which label the ER and the plasma membrane (PM), respectively, and are members of the Major Intrinsic Protein (MIP) family like the TIPs, were not affected (Figures 7g to 7j). This suggests that the effect of C834 is not specific to a particular family of proteins, but to a trafficking mechanism *per se*. NPSN1212-YFP was used as a PM marker from a different family and similar results were obtained (Figures 7k and 7l). We also looked at the localization of members of the RabD1 and RabD2 family, which has been localized to endosomal compartments associated to the Golgi and essentially implicated with ER-to-Golgi traffic during the early secretory pathway (Pinheiro et al., 2009). These markers (RabD1-, RabD2a- and RabD2b-YFP), along with other types of recycling and intermediate endosomal compartments labeled with RabA1e-, RabA5d-, RabC10 and RabE1d-YFP, were not affected (Figures 7m to 7z). We also looked at the subcellular localization of ARA7-YFP, RabG3f-YFP (Geldner et al., 2009) and SNX1-GFP (Jaillais et al., 2006), markers of the prevacuolar compartment, but no differences were found (Figures 7aa to 7ee). Similarly, the localization of the TGN marker VHA-a1-GFP and the vacuole marker VAMP711 is not affected by C834 either (Figures 7ff to 7ii). We could only

identify a phenotype in the localization of the auxin transporter PIN2-GFP (discussed in Chapter 3). Overall, our analysis shows that C834 inhibits the trafficking of a subset of tonoplast proteins specifically, and that the accumulation of these proteins at the ER is not the result of a canonical effect to the plant endomembrane system.

2.2.4 Chemistry of C834 and induced phenotypes by the drug

As a result of our chemical genetics screen, we identified C834; a molecule that selectively inhibits the delivery of a subset of proteins to the vacuole membrane. In order to gain a better understanding about the kinetics of this compound, we determined the minimum concentration and incubation time for inhibition of trafficking. By performing time course and concentration curve assays, we determined that C834 induces the mis-localization of GFP-TIP2;1 *in planta* after 8 hours of incubation, and requires a minimum concentration of 55 μM (data not shown). Interestingly, no effects were observed in hypocotyls and cotyledons even at higher concentrations, suggesting that either the compound is not being transported to these tissues or that the molecular target is not present in these cells. Another characteristic of C834 is that its effect on trafficking can be fully reversed (**Figure 8**). After inducing the mis-localization of GFP-TIP2;1 by C834 to the ER (Figures 8a-b), we can recover the tonoplast localization of this marker by transferring the seedlings to C834-free media for 24 h (Figure 8c), although recovery can be seen as early as 6 h (data not shown).

The chemical structure of compound C834 is quite complex, having two major parts. We decided to test four different analogs that are structurally similar to C834 to understand its activity at the chemical level (**Table 2**). The effects of these compounds were assayed by

means of inhibition of GFP-TIP2;1 trafficking in root cells (**Figure 9**). According to the ChemBridge database (www.hit2lead.com), chemical C388 is 99% identical to C834, lacking only the ethyl group at the 10th carbon of the bulkiest side of the molecule, and this change does not seem to drastically change the inhibition activity in comparison to C834 (Figure 9b, 9c). Compound C195 contains a hydroxyphenyl group attached to the acridinedione side of the molecule, instead of the 5-bromo-2-propoxyphenyl group found in C834. Compound C780 shows differences at both sides of the molecule; this compound lacks the ethyl group (just like C388) and carries some modifications that make the molecule bulkier in mass and space. Interestingly, both these compounds induce the mis-localization of TIP2;1 to the ER (Figures 9d, 9e), suggesting that these groups and the specific size of C834 are not essential for C834 activity. In contrast with the first three analogs, C957 showed lack of activity in terms of trafficking inhibition and we refer to this compound as aC834 (Figure 9f). The chemical structure of C957 is very similar to that of C834, lacking only the bromide and having a change of propoxyphenyl to a methoxyphenyl group. These results do not show a particular functional group that contributes to C834 specific activity.

We later noticed that C834 requires “light activation” for its inhibitory activity on protein trafficking (**Figure 10**). Seedlings that are exposed to C834-containing media that has not been light activated do not show trafficking inhibition (Figure 10a, 10b). However, when the same assay is performed using C834-containing media that has been incubated in light, C834 is bioactive for trafficking inhibition (Figure 10c). To further confirm these results, we analyzed the absorption of C834 and “light-activated” C834 by means of UV-vis spectroscopy (**Figure 11**). In fact, light exposure causes a shift in the absorption spectra of

C834 (Figure 11b), which normally shows a characteristic peak within the 370 and 390 nm range (Figure 11a). Notably, all the experiments presented here were done with light activated plates. This molecular change caused by light explains the unanswered results from the analog assay. It is likely that the analogs are also undergoing structural changes catalyzed by light, which explains the difficulty in defining structure-activity relationships.

In order to gain more insight regarding C834 activity, we determined the phenotypes induced by this compound in *Arabidopsis* seedlings (**Figure 12**). *Arabidopsis* plants grown in C834-containing media do not show any morphological phenotype (compare Figure 12a and 12b). In contrast with primary root development which was not affected, C834-treated seedlings show a clear reduction in root hair length. This last phenotype is not surprising because of the nature of the chemical library from where C834 was identified. Root hair elongation and pollen growth share mechanisms for tip growth, which requires an active endomembrane system. Because C834 inhibits both pollen and root hair elongation, it is likely that this phenotype is caused by a defect in tip growth probably due to lack of endomembrane system integrity. Interestingly, C834 causes a reduction of anthocyanin accumulation in hypocotyls and cotyledons of young seedlings (Figure 12c, 12e). Moreover, we cannot recover this phenotype by adding naringenin, a precursor in the anthocyanin biosynthetic pathway (Figure 12d, 12f). These compounds are synthesized from the phenylpropanoid pathway by enzyme complexes that are localized to the cytoplasmic face of the ER (Winkel-Shirley, 1999), and are eventually transported and stored in the plant vacuole to prevent oxidation (Grotewold, 2004). Recent evidence has shown that vesicle trafficking plays major roles for the transport of these compounds (Poustka et al., 2007). In *Arabidopsis*,

vesicle trafficking pathway for anthocyanins occurs in an ER-to-vacuole direct pathway, bypassing the Golgi apparatus (Poustka et al., 2007). It is likely that the trafficking inhibition by C834 may result a block of anthocyanin delivery to the vacuole by a direct target that also sends tonoplast proteins via this pathway.

2.2.4 Genetic screen for C834-resistant mutants

Chemical genetics represent a powerful new technology in plant sciences; however, a common obstacle for this approach is compound-target identification. Some techniques used to serve this purpose include affinity chromatography, yeast-3-hybrid screens, phage display, protein microarray and chemical proteomics (Cong et al., 2011; Tóth and van der Hoorn, 2009). These techniques are usually expensive, time consuming and require a vast experience in organic chemistry techniques. Among plant biologists, a resistance screen is currently the most widely used strategy for target identification. This approach is based on identification of mutants that show resistance to the inhibitor and mapping the corresponding gene.

In order to characterize the Golgi-independent pathway for tonoplast proteins, a resistance screen was performed to identify *C834-resistant (C834-r)* mutants in Arabidopsis. Our strategy was to use ethyl-methylsulfonate (EMS) –based mutagenesis in the GFP-TIP2;1 background and look for plants with normal development of root hairs and proper delivery of the chimeric protein in presence of C834, which normally inhibits root hair growth and induces accumulation of GFP-TIP2;1 to the ER (**Figure 13**). With this in mind, we used a mutagenized population of transgenic Arabidopsis (M_0) dual marker line that was available in the lab. Around 15 small pools have been successfully screened in presence of 55 μM , a

concentration that completely inhibits the elongation of root hairs in wild type plants. Mutants with elongated root hairs were selected for protein localization analysis under the confocal microscope. We have screened ~9,000 EMS-mutagenized M2 GFP-TIP2;1 seeds and selected ~1,080 mutants that show resistance for root hair inhibition. We identified 108 putative mutants (~10%) during this small screen, but ~40% died during development. We screened the progeny (M₃) of some of the mutants that survived and were able to produce seeds, and four mutants were confirmed (**Figure 14**). These mutants are characterized by proper delivery of GFP-TIP2;1 to the tonoplast in presence of C834. Further characterization of these and novel mutants in the pathway will contribute to the understanding of tonoplast trafficking pathways, as well as underscore the potential of chemical genetic approaches to address questions that would be intractable using classical forward genetics.

2.3 Discussion

Despite the major importance for plants to properly traffic proteins to the proper compartment, little is known about the molecular mechanisms involved in the regulation of these cellular mechanisms. Understanding trafficking pathways for membrane vacuole proteins has been difficult because of the differences among vacuole-types and protein localization in different plant species. Taking advantage of chemical genetics as a tool for dissecting endomembrane system pathways and confocal microscopy, we present here a set of novel inhibitors that disrupt the pathway that targets proteins to the vacuole membrane. Using these small molecules, we uncovered an inhibitor of the Golgi-independent pathway for tonoplast proteins and propose a novel trafficking route for the trafficking of two proteins

of the TIP family. This pathway has been described for proteins targeted to the protein storage vacuole, but not to the lytic vacuole (Jiang and Rogers, 1998; Park et al., 2004; Isayenkov et al., 2011).

Evidence for a so-called “Golgi-independent” pathway for the trafficking of proteins has been previously reported. The tonoplast-localized aquaporin TIP3;1 bypasses the Golgi apparatus during its traffic to the protein storage vacuole, as it is BFA-insensitive and does not acquire N-glycans that are specific of passage through this organelle in leaf protoplasts (Park et al., 2004). More recent studies have shown that members of the OsTPK family of potassium transporters are also targeted to the vacuole membrane via different routes in rice protoplasts (Isayenkov et al., 2011). The fact that OsTPKa localizes to the lytic vacuole and is BFA-sensitive, and OsTPKb to the protein storage vacuole and appears to travel in a Golgi-independent manner suggests different targeting mechanisms for different types of vacuoles. Our results demonstrate that whereas the PSV marker, TIP3;1, does not require passage through the Golgi, the lytic vacuole aquaporin TIP1;1 travels in a Golgi-dependent manner. This multiple trafficking pathways have been reported for vacuole soluble proteins as well. For example, the tobacco chitinase contains a vacuolar sorting signal (VSS) that, when fused to GFP (GFP-Chi), targets the protein chimera to the PSV, whereas barley aleurain (ALEU-GFP) is targeted to the lytic vacuole (Di Sansebastiano et al., 2001; Maruyama et al., 2006). It has been proposed that vacuole proteins targeted to the lytic vacuole are sorted at the Golgi by vacuolar sorting receptors (VSRs) and a mechanism that requires clathrin-coated vesicles (Ahmed et al., 2005; Song et al., 2006). In contrast, trafficking of proteins to the PSV can be sorted at the Golgi into plant-specific vesicles

surrounded by an unknown type of coat, or travel directly from the ER to the PSV (Herman and Schmidt, 2004). Moreover, the ER-to-vacuole direct traffic has also been reported for the sequestration of secondary metabolites in the plant vacuole and soluble proteins in maize aleurone cells (Poustka et al., 2007; Reyes et al., 2011). The fact that seedlings treated with C834 do not show accumulation of anthocyanins suggests a putative link between this phenotype and ER-to-vacuole trafficking.

The plant Golgi apparatus is responsible for receiving most of the proteins that have been exported from the rough endoplasmic reticulum (ER) via COPII vesicles and, at the exit face of the Golgi, secretory cargoes must be sorted into an array of vesicles for delivery of proteins to their final destination (Glick and Nakano, 2009; Hawes, 2004). It is also greatly involved in the processing of newly synthesized glycoproteins and glycolipids. The fact that proteins that use this “bypass” mechanism are usually those found in PSVs suggest a link between developmental stage and sorting machineries. PSVs are common in embryonic cells, where there is an almost complete dependence on seed-stored nutrients. It is likely that this mechanism is favored because of quick and energy-efficient transport of proteins for the developing embryo.

In some eukaryotes, the existence of an ER-Golgi Intermediate Compartment (ERGIC) has been shown to be involved in the targeting of plasma membrane proteins via a Golgi-independent route (Sannerud et al., 2006; Schotman et al., 2008). However, presence of a similar compartment has not been described in plant cells (Foresti et al., 2008; Nakano and Luini, 2010). Interestingly, an intermediate compartment known as Precursor-Accumulating (PAC) vesicles have been shown to mediate trafficking directly from the ER

to the vacuole in some plant species (Hara-Nishimura et al., 1998). These PAC vesicles can be induced in Arabidopsis by ectopic expression of proteins that normally localize to PAC vesicles in species like pumpkin (Hayashi et al., 1999). Similar vesicles have been discovered in wheat and rice, and have been shown to be derived directly from the ER and mediate the traffic of vacuolar storage proteins directly, bypassing the Golgi (Levanony et al., 1992; Takahashi et al., 2005). These data provide further evidence of a Golgi-independent pathway for plant vacuole proteins, and the fact that some plants have independent vesicles to serve this purpose open new doors for cell biology research.

Evaluation of the mechanisms that regulate the targeting of membrane proteins to the tonoplast via a Golgi-independent pathway is essential to reveal the plasticity of plants at the molecular level. The recent findings on the presence of at least two Golgi-dependent pathways suggest a new level of complexity in tonoplast protein targeting (Wolfenstetter et al., 2012). Our small-compounds represent new tools for dissecting the tonoplast protein trafficking machinery. These compounds are commercially available and can be used to uncover links and phenotypes that can sometimes be untraceable using classical genetics. Further identification of the molecular targets of the inhibitors will provide genetic evidence of important players in this pathway. Using C834, we propose a model for trafficking of members of the TIP family (**Figure 15**). There are still many questions unanswered, including why some proteins take a “shortcut” and bypass passage through organelles. It is likely that this mechanism may represent an ancestral targeting mechanism that still remains for the quick transport of proteins to the storage organelle.

2.4 Materials and methods

2.4.1 Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used in all experiments. Seeds were sterilized and sown on half-strength (0.5X) Murashige and Skoog (MS) media (RPI) containing 3 g/L GelRite (RPI) and 1% sucrose. Seeds were then stratified in darkness for 72 h at 4°C. Plates were incubated in the light (16 h per day) at 22°C. Vacuolar membranes were visualized using transgenic lines containing either a 35S::GFP-TIP2;1 (Cutler et al., 2000), TIP3;1::TIP3;1-YFP, 35S::TIP3;1-YFP or 35S::TIP1;1-YFP (Hunter et al., 2007). The ER marker was generated by substituting the 35S promoter of the ER-rk (Nelson et al., 2007) construct with a *UBQ10* promoter from the pNIGEL vector (Geldner et al., 2009) by traditional cloning. Stably-transformed plants were crossed with the 35S::GFP-TIP2;1 to generate the double marker line used in the screen. The lines NAG1-GFP (Grebe et al., 2003), SNX1-GFP (Jaillais et al., 2006), PIP2a-GFP (Cutler et al., 2000), TT12-GFP (Marinova et al., 2007), and VHA-a1-GFP (Dettmer et al., 2006) have been previously described. Other markers used for this research include YFP fusions to GOT1p, MEMB12, SYP32, NIP1;1, PIP1;4, NPSN12, RabD2a, RabD2b, RabA1e, RabA5d, RabC1, RabD1, RabE1d, ARA7, RabG3f, and VAMP711 (Geldner et al., 2009).

2.4.2 Chemical genetics screen

A small chemical library of 360 organic molecules (ChemBridge, San Diego) containing pre-selected inhibitors of tobacco pollen tube germination and growth (Drakakaki et al., 2011) were used for the screen. For the chemical stock solutions, 0.1 mg of each

compound was dissolved in 20 μL of 100% DMSO (Sigma), for a final concentration of 5 mg/mL. For screening, 2 μL of each chemical was added to 398 μL of MS media with GelRite (RPI) to a final concentration of 25 $\mu\text{g}/\text{mL}$, corresponding to 50-100 μM based on the molecular mass of each compound. Dual marker Arabidopsis transgenic lines were germinated in 0.5X MS for 72 h and then transferred to each chemical (3-4 seedlings per well) for 48 h before analysis of protein localization.

For the secondary screen, seedlings were incubated for 48 h on 0-, 12.5-, 25-, and 50- $\mu\text{g}/\text{mL}$ concentrations of relevant chemicals and analyzed for tonoplast marker localization. Potential tonoplast protein trafficking inhibitors were subjected to a tertiary screen. The tertiary screen included chemical incubation of transgenic plants expressing different endomembrane system markers. For the purpose of this paper, compounds 6982834, 7703755, 5556103, 5838410 and 5262578 were purchased from ChemBridge (San Diego) for further characterization.

2.4.3 Confocal and light microscopy

A Zeiss LSM 710 confocal microscope from the Cellular and Molecular Imaging Facility (CMIF) at NCSU was used for all experiments. To image the double marker line, line switch sequential acquisition using both the GFP and the mCherry signals from each seedling was carried out using a 40X water objective (1.1 NA) and two laser lines (488 and 561 nm). PSV autofluorescence was acquired with a 405 nm laser excitation and emission was collected between 660-700 nm. Root morphology, root hairs and anthocyanins of

seedlings were imaged in a Leica M205C stereomicroscope equipped with a Leica DFC425C digital camera. Images were processed using Photoshop CS4.

2.4.4 UV-visible spectroscopic studies

Optical spectra were recorded using quartz microcuvettes (1 cm path length) on a Cary 50 UV-visible spectrophotometer equipped with thermostated cell holders at 25°C. C834 samples were dissolved in DMSO to a final concentration of 11 mM. For the concentration curve, sample was dissolved and stored at -20°C. For light treatments, control represents a sample taken directly from the -20°C; for dark, the dissolved C834 was wrapped in aluminum foil and stored for 72 h in the growth chamber. Light treatments were done in the same way as dark, but without the aluminum foil cover.

2.4.5 Chemical treatments

For tonoplast trafficking inhibition, 3-day old seedlings expressing the indicated constructs were transferred to 25 µg/mL of each compound in 0.5X MS containing 3g/L GelRite and 1% sucrose. Tonoplast chemical inhibitors were used at a final concentration of 55 µM C834, 62 µM C410, 88 µM C755, 79 µM C103 and 80 µM C578. Chemical analogs of C834 (6950388, 6947195, 6983780, 6979957) were purchased from ChemBridge (San Diego). Naringenin was dissolved in ethanol and supplemented to the media at a final concentration of 25 ng/mL. For BFA, 5-day old seedlings were transferred to liquid 0.5X Murashige and Skoog (MS) supplemented with 1% sucrose containing 75 µM BFA (Sigma) and imaged after 3 h.

2.4.6 Embryo dissection

Dry seeds from transgenic lines expressing TIP3;1::TIP3;1-YFP (Hunter et al., 2007) were dissected by removing the seed coat and the aleurone layer under a dissecting scope. Embryos were immediately submerged into 0.5X MS containing 1% sucrose and either DMSO or 27 μ M C834, and imaged under the confocal microscope after 18 h of incubation.

2.4.7 Screen for C834-r mutants

Ethyl methanesulfonate- (EMS-) mutagenized M2 seeds of Arabidopsis transgenic lines expressing 35S::GFP:TIP2;1 and 35S::mCherry-HDEL were available in the Rojas-Pierce lab. Around 15 pools of M2 seeds were germinated and grown vertically on MS media containing 55 μ M C834 for 5 days. Seedlings with presence of root hairs were pre-selected as candidates and analyzed under a Zeiss LSM 710 confocal microscope. Seedlings showing proper tonoplast localization of the GFP:TIP2;1 marker were transplanted to soil. Resulting M3 seeds from the putative mutants were confirmed using confocal microscopy.

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TABLE 1. Chemical structure of tonoplast trafficking inhibitors.

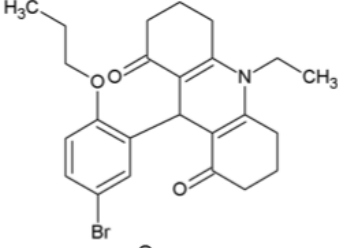
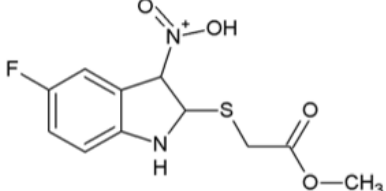
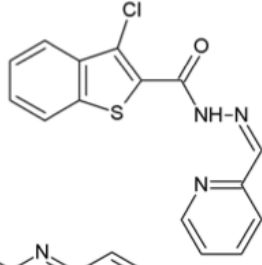
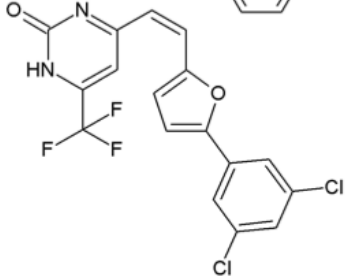
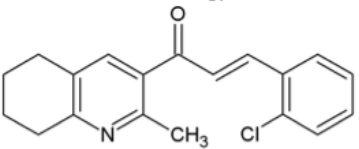
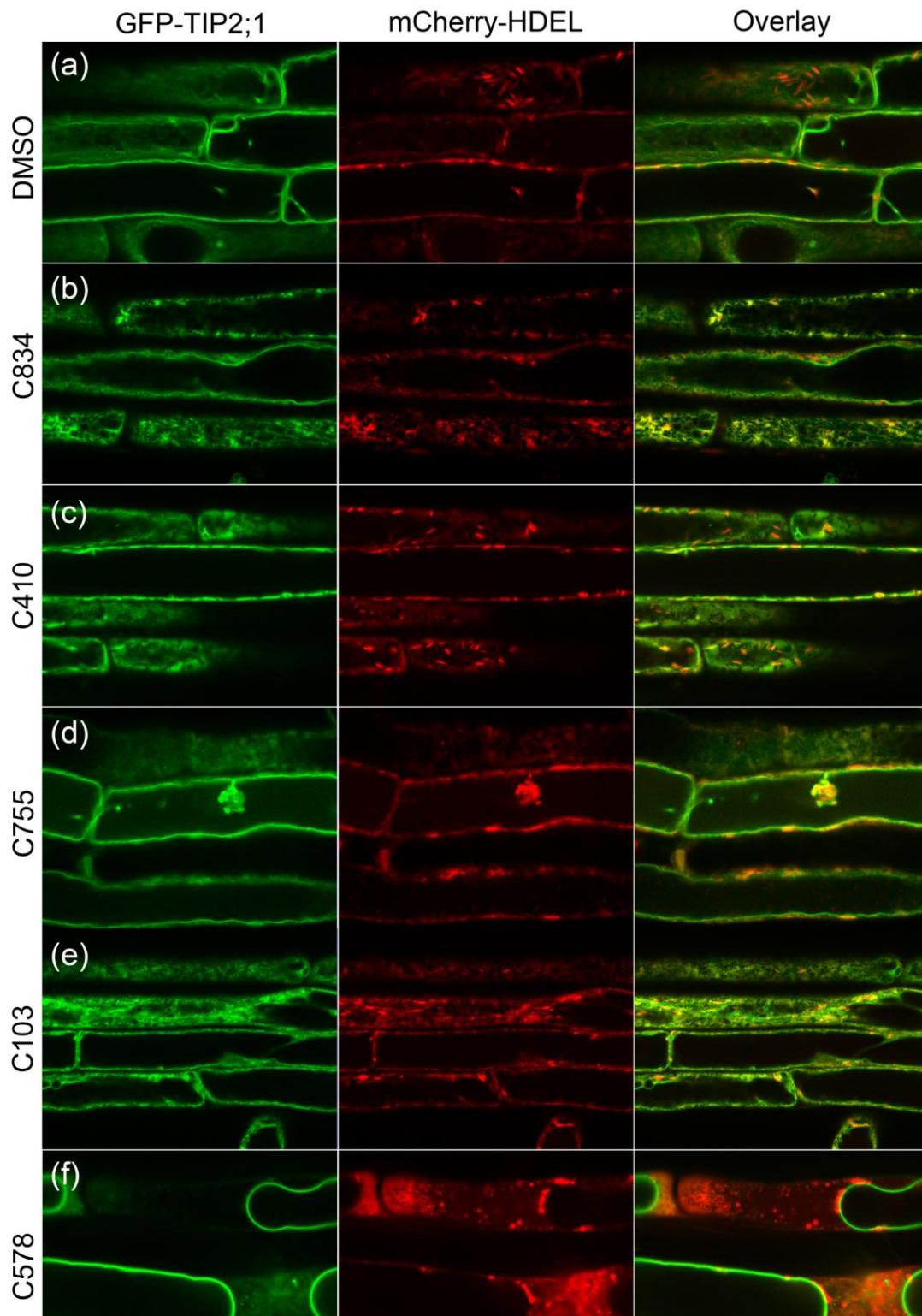
Chemical Structure	PubChem ID (name in text)	IUPAC Name
	2295811 (C834)	9-(5-bromo-2-propoxyphenyl)-10-ethyl-3,4,6,7,9,10-hexahydro-1,8(2H,5H)-acridinedione
	750417 (C755)	methyl [(5-fluoro-3-nitro-1H-indol-2-yl)thio]acetate
	6883924 (C103)	3-chloro-N'-(2-pyridinylmethylene)-1-benzothiophene-2-carbohydrazide
	5337111 (C410)	4-{2-[5-(3,5-dichlorophenyl)-2-furyl]vinyl}-6-(trifluoromethyl)-2(1H)-pyrimidinone
	5720071 (C578)	3-(2-chlorophenyl)-1-(2-methyl-5,6,7,8-tetrahydro-3-quinolinyl)-2-propen-1-one

TABLE 2. Chemical structure of C834 and its analogs.

Chemical Structure	ChemBridge ID Number	IUPAC Name
	6982834 (C834)	9-(5-bromo-2-propoxyphenyl)-10-ethyl-3,4,6,7,9,10-hexahydro-1,8(2H,5H)-acridinedione
	6950388 (C388)	9-(5-bromo-2-propoxyphenyl)-3,4,6,7,9,10-hexahydro-1,8(2H,5H)-acridinedione
	6947195 (C195)	10-ethyl-9-(4-hydroxyphenyl)-3,4,6,7,9,10-hexahydro-1,8(2H,5H)-acridinedione
	6983780 (C780)	9-{3-bromo-4-[(4-methylbenzyl)oxy]phenyl}-3,4,6,7,9,10-hexahydro-1,8(2H,5H)-acridinedione
	6979957 (C957)	10-ethyl-9-(3-methoxyphenyl)-3,4,6,7,9,10-hexahydro-1,8(2H,5H)-acridinedione

Figure 1. GFP-TIP2;1 is mis-localized to the ER in presence of the inhibitors. 3-day old Arabidopsis seedlings expressing GFP-TIP2;1 (green) and mCherry-HDEL (red) were transferred to media containing the indicated chemical treatment and incubated for 48 h before confocal imaging. DMSO was used as the control. Compounds were added at a final concentration of: 55 μ M C834, 62 μ M C410, 88 μ M C755, 79 μ M C103 and 80 μ M C578. Co-localization of the two markers can be visualized in the overlay channel by the yellow signal. Scale bar = 10 μ m



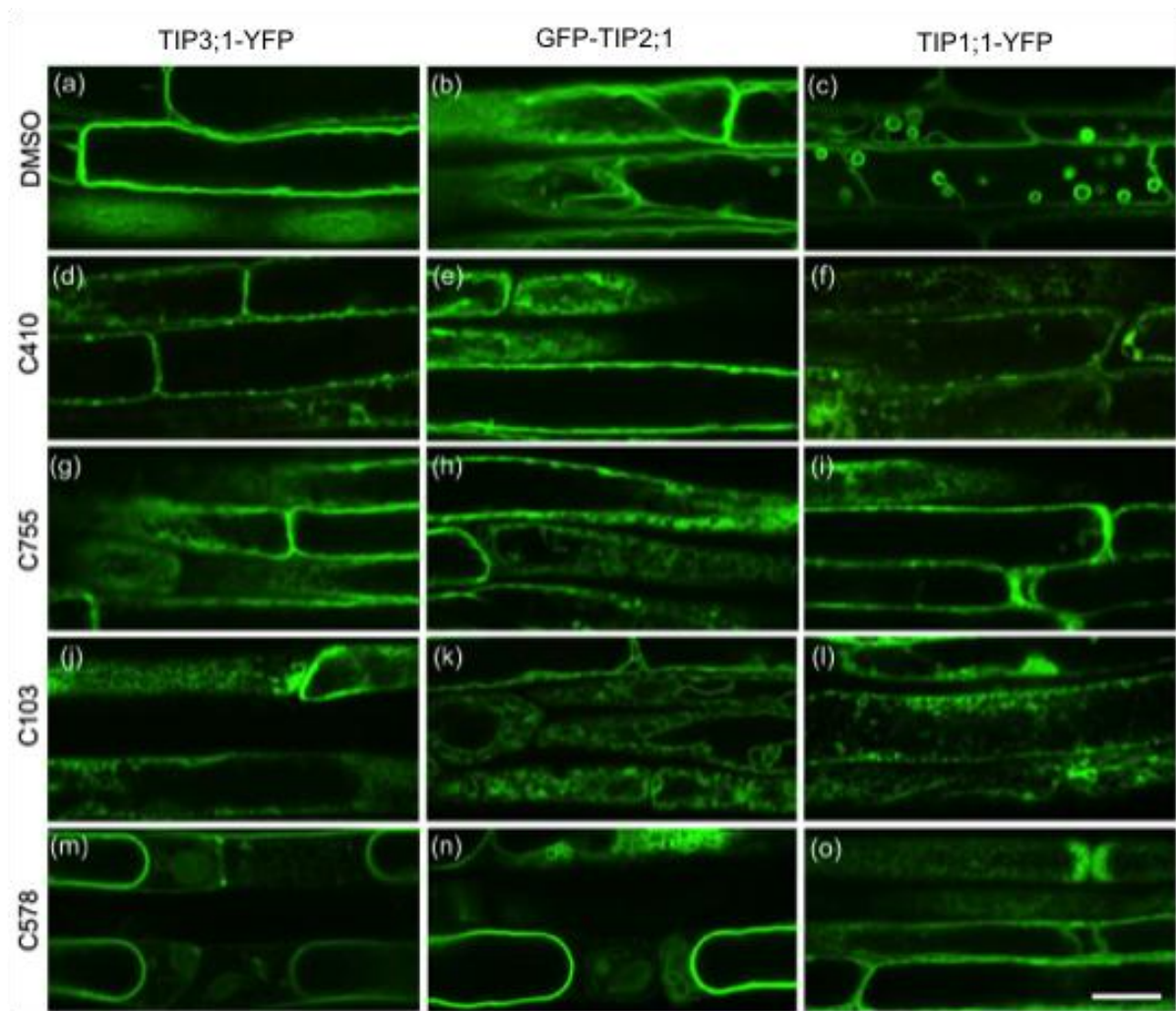


Figure 2. Four compounds inhibit targeting of TIP proteins indiscriminately. 3-day old Arabidopsis seedlings expressing either TIP3;1-YFP, GFP-TIP2;1 or TIP1;1-YFP were transferred to media containing the indicated chemical treatment and incubated for 48 h before confocal imaging. . Compounds were added at a final concentration of: 62 μ M C410, 88 μ M C755, 79 μ M C103 and 80 μ M C578. Note the network pattern of the tonoplast markers in presence of the inhibitors. Scale bar = 10 μ m

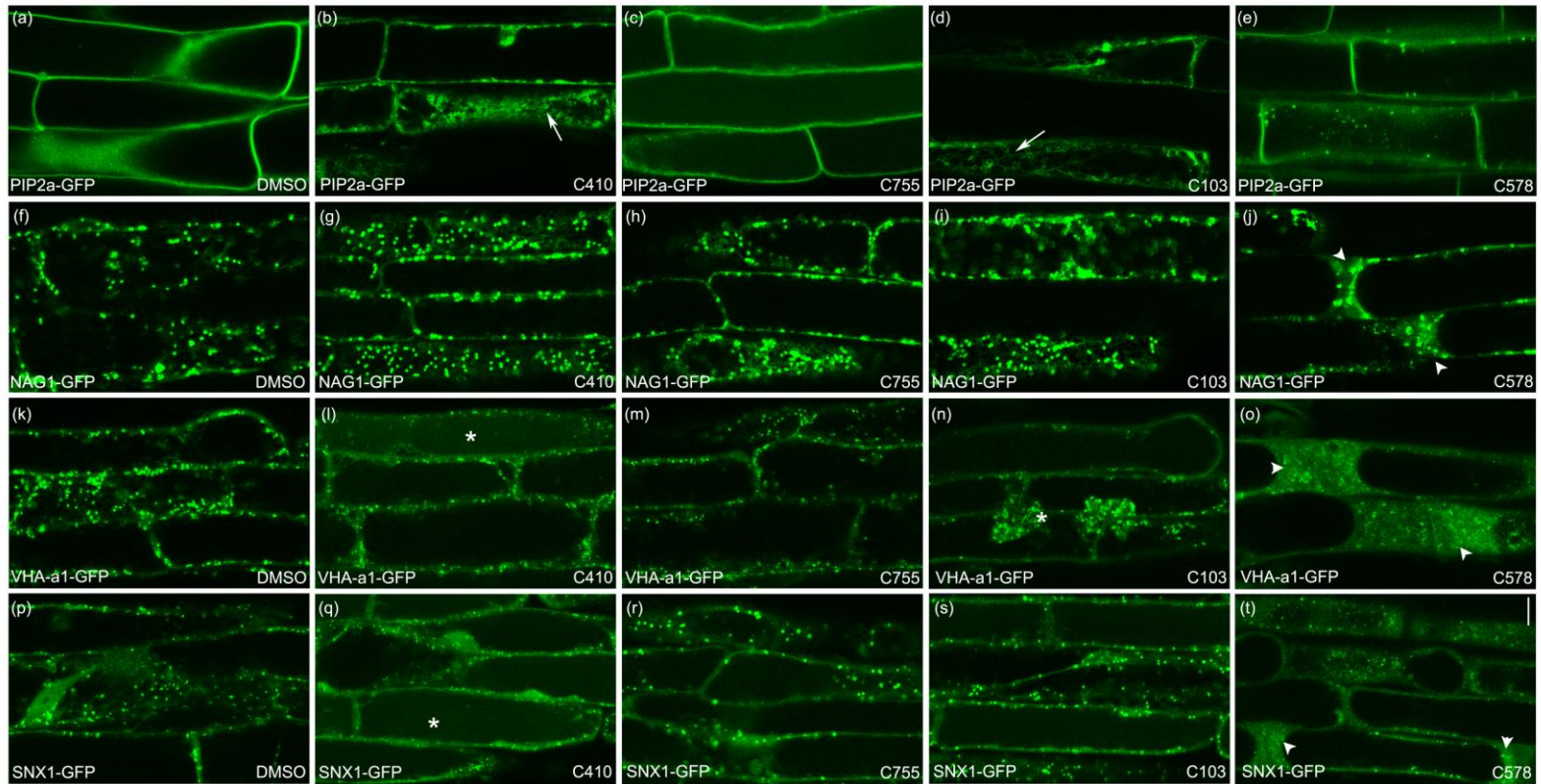


Figure 3. Effect of tonoplast inhibitors on the endomembrane system. 3-day-old *Arabidopsis* seedlings expressing either PIP2a-GFP (plasma membrane), NAG1-GFP (Golgi), VHA-a1-GFP (TGN) or SNX1-GFP (PVC) were transferred to either DMSO- or inhibitor-containing media and incubated for 48 h before confocal imaging. Arrows represent ER localization of the marker after treatment; asterisks (*) represent “solubilization” or aggregates of the chimeric protein and arrowheads represent cytoplasmic staining. Scale bar = 10 μ m

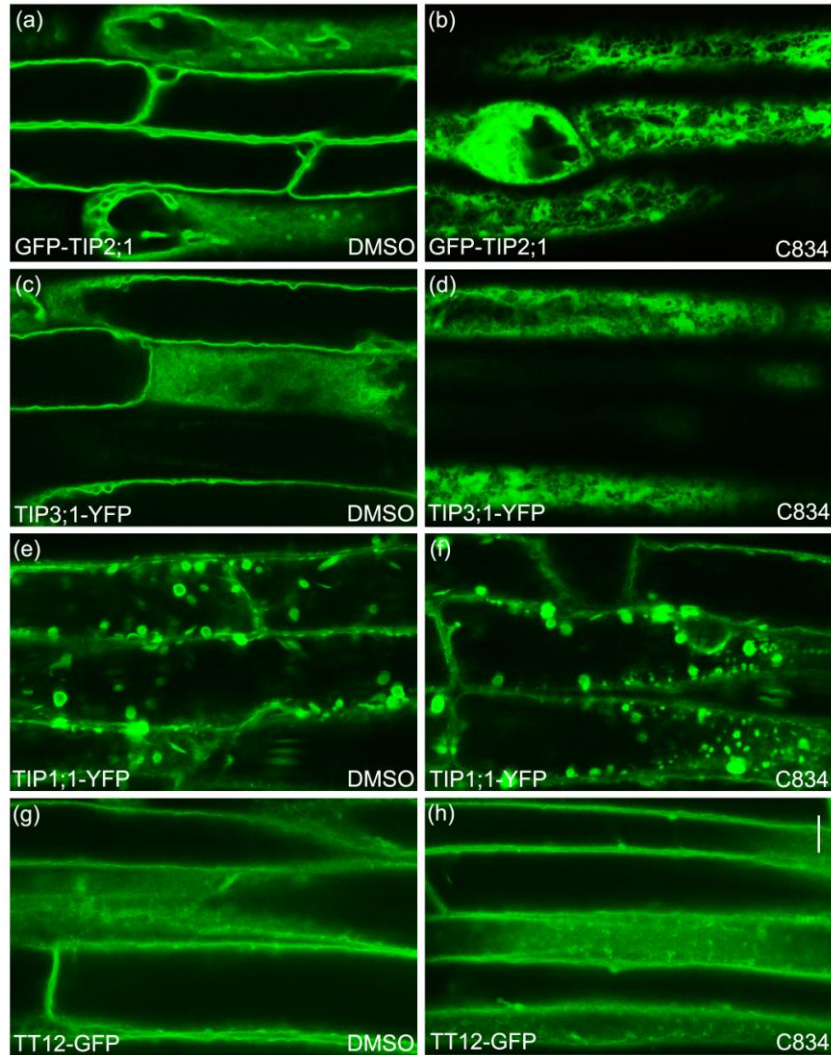


Figure 4. C834 differentiates tonoplast trafficking pathways. 3-day old Arabidopsis seedlings expressing either TIP3;1-YFP, GFP-TIP2;1, TIP1;1-YFP or TT12-GFP were transferred media containing either DMSO or 55 μ M C834 for 48 h and visualized using confocal microscopy. Both GFP-TIP2;1 and TIP3;1-YFP localize to the ER after C834 treatment (compare 4a with 4b, and 4c with 4d), whereas TIP1;1-YFP and TT12-GFP localize normally to the tonoplast in both control and chemical treatment (compare 4e with 4f, and 4g with 4h). Scale bar = 10 μ m

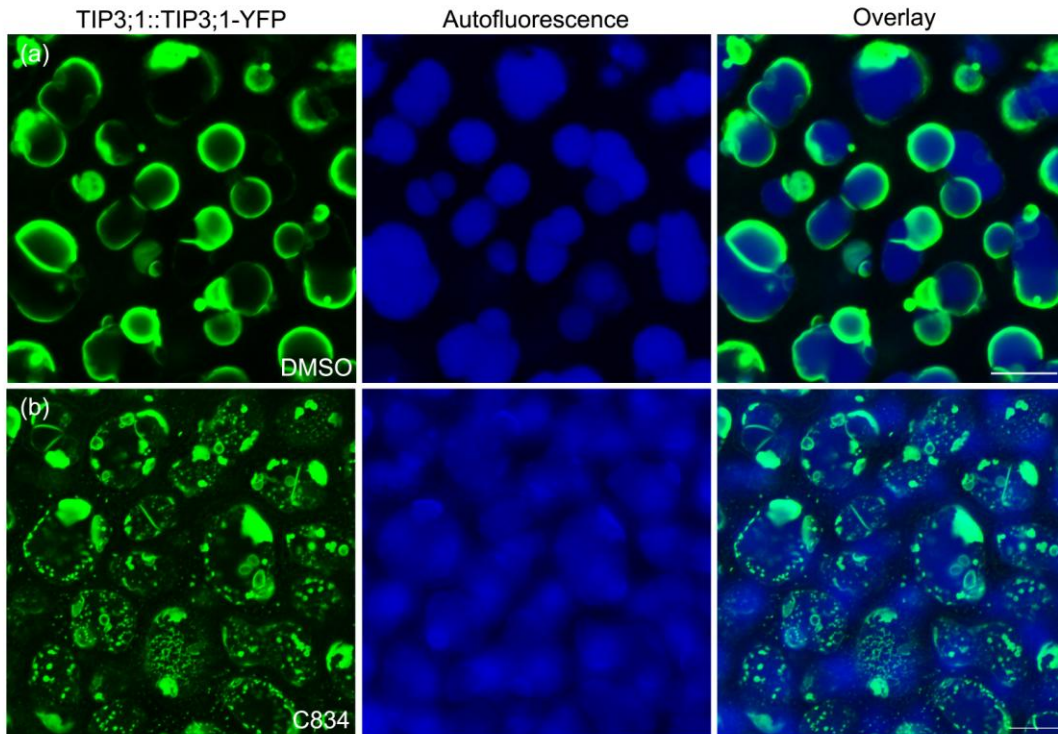


Figure 5. The Golgi-independent pathway is present in PSVs. Mature embryos were obtained by dissecting dry seeds of Arabidopsis transgenic lines expressing TIP3;1::TIP3;1-YFP. Embryos were then submerged in liquid media containing either DMSO or 27 μ M C834 for 18 h. Tonoplast localization was visualized by GFP (green) and vacuole lumen can be seen as a result of anthocyanin autofluorescence (blue). Note the network-like localization of TIP3;1-YFP after C834 treatment in comparison with the control. Scale bars = 10 μ m

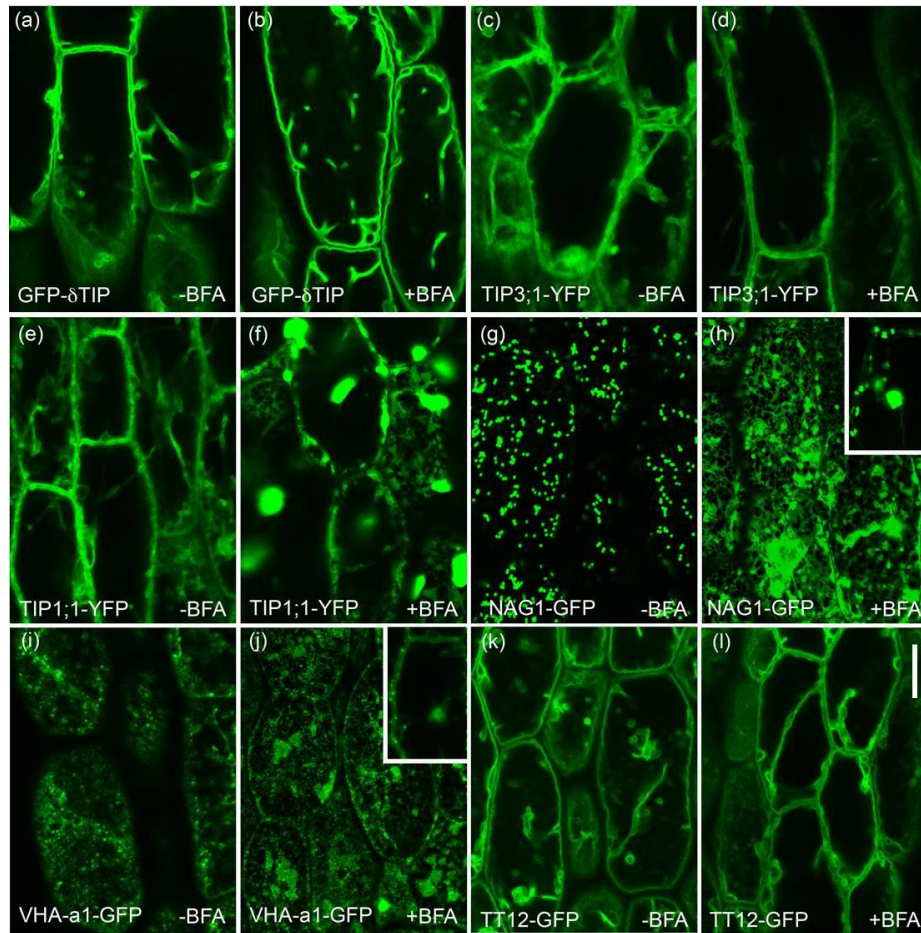


Figure 6. BFA affects the localization of a TIP1;1 but not that of the other tonoplast markers. 3-day old *Arabidopsis* seedlings expressing the indicated constructs were transferred media containing either DMSO (-BFA) or 75 μ M BFA (+BFA) for 3 h and visualized using confocal microscopy. The tonoplast markers GFP- δ TIP (GFP-TIP2;1), TIP3;1-YFP and TT12-GFP appear normally localized to the tonoplast before and after treatment (6a-6d, 6k-6l). The tonoplast protein TIP1;1-YFP localizes to both an ER-like structure and BFA compartments after BFA treatment (6e and 6f), similar to those seen in the Golgi and TGN markers after BFA treatment (6g-6h, 6i-6j, insets, respectively). Scale bar = 20 μ m

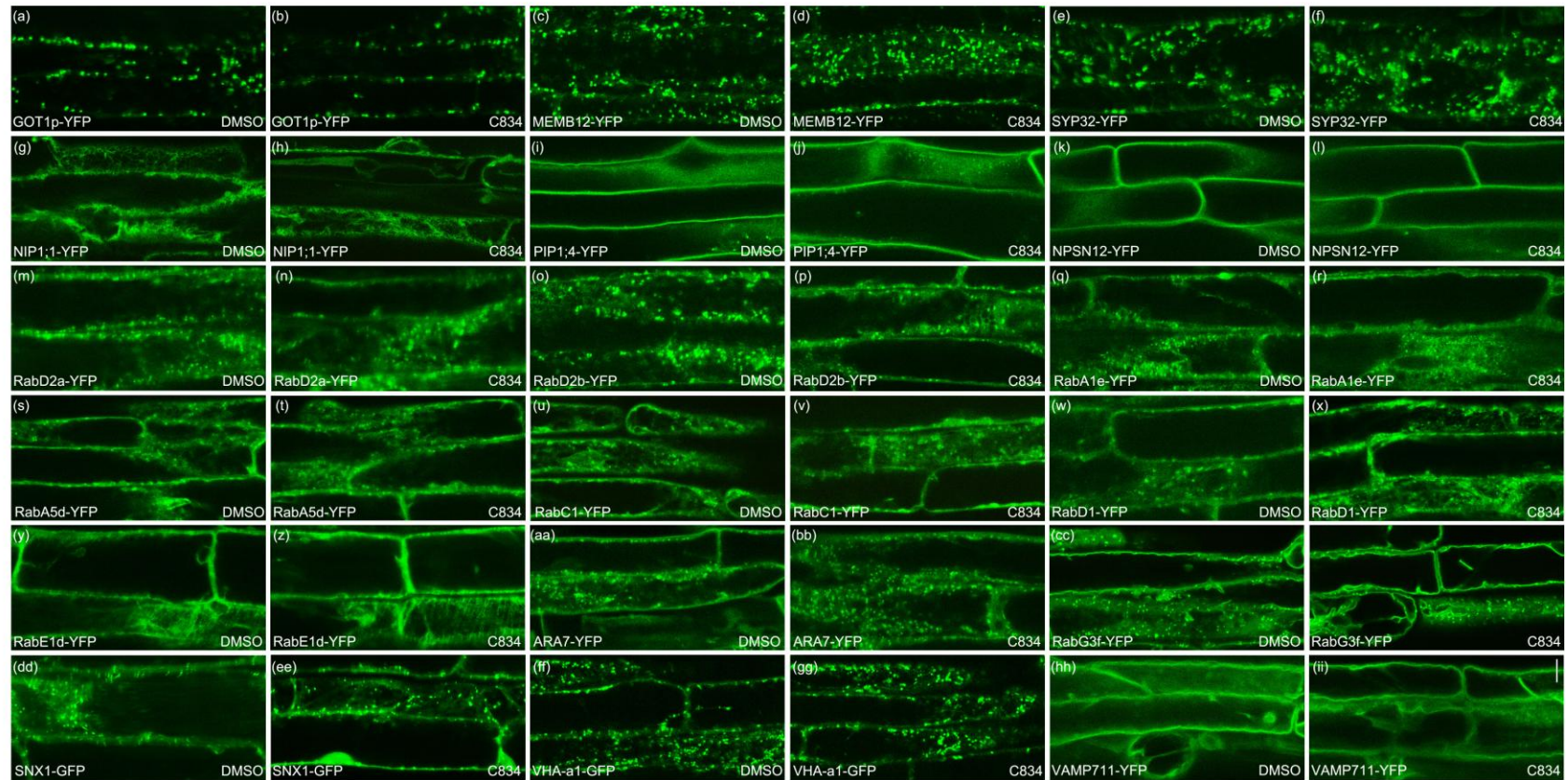


Figure 7. C834 is not an unspecific inhibitor of the endomembrane system. 3-day old seedlings expressing the indicated fluorescent chimeras were transferred to either DMSO- or C834-containing media for 48 h. Neither Golgi (7a-7f), ER (7g-7h), plasma membrane (7i-l), endosomes (7m-7z), PVC (7aa-7ee), TGN (7ff-7gg) or vacuole/tonoplast marker (7hh-7ii) was found to be affected by C834. Scale bar = 10 μ m

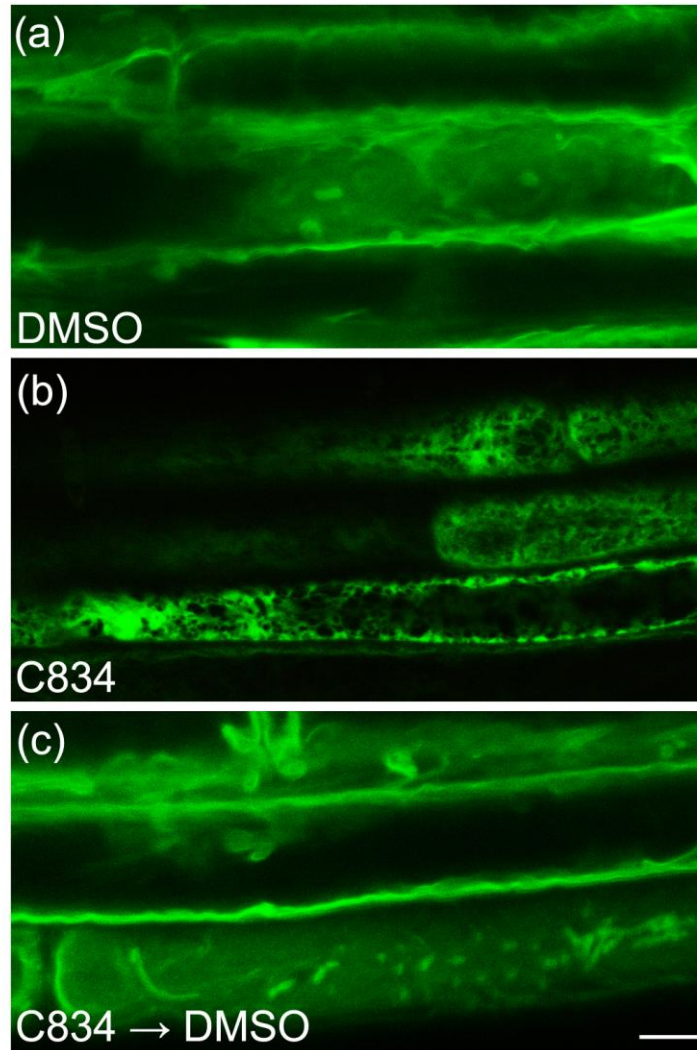


Figure 8. Inhibition of trafficking by C834 is reversible. 3-day old seedlings expressing GFP-TIP2;1 were transferred to either DMSO- (a), or C834-containing media (b) for 48 h to confirm the induced phenotype. Note the ER localization of the tonoplast marker after incubation with C834 (similar effects were seen in seedlings after 24 h of treatment). For reversibility, seedlings were treated with C834 for 24 h and then transferred to chemical-free media for 24 h (c). Note that tonoplast localization is recovered after wash-out.

Scale bar = 10 μ m

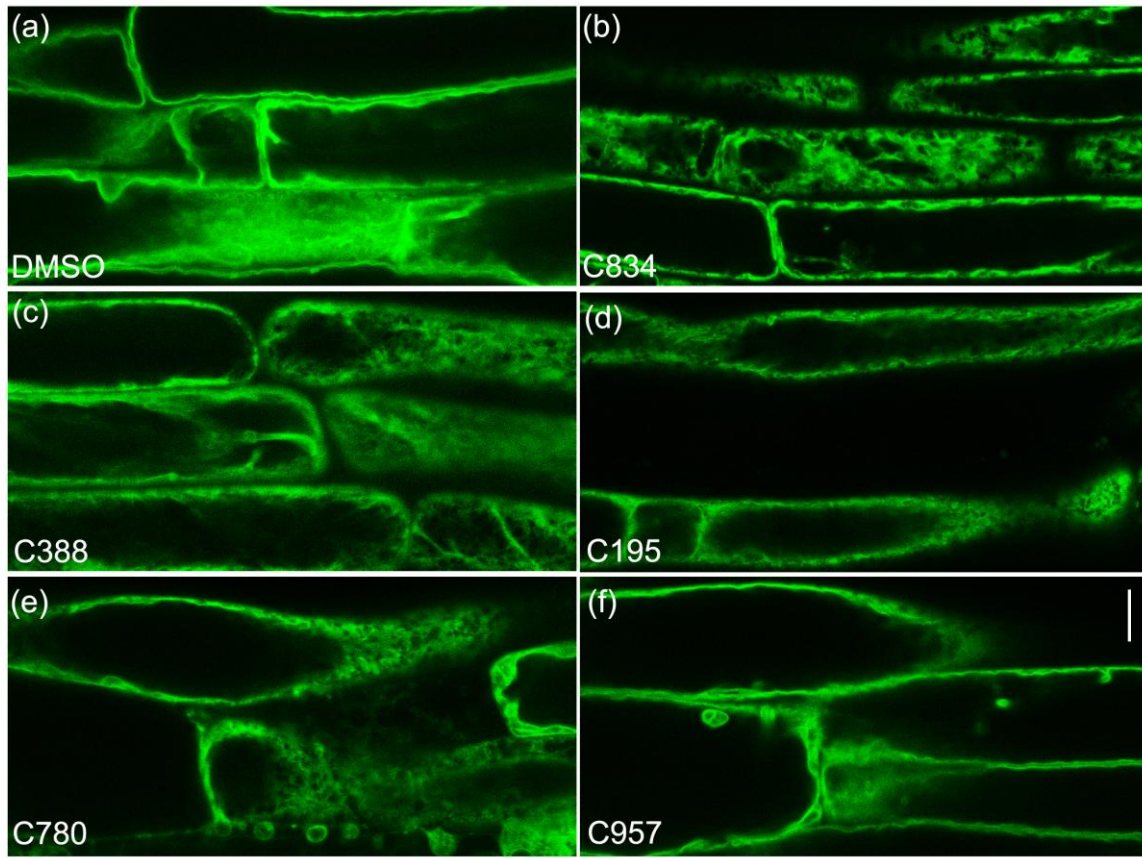


Figure 9. Effects of C834 chemical analogs on GFP-TIP2;1 localization. 3-day old seedlings expressing GFP-TIP2;1 were transferred to media containing the indicated chemicals for 48 h. (a) DMSO was used as the control. Compounds were added at a final concentration of: (b) 55 μ M C834, (c) 58 μ M C388, 74 μ M C195, 51 μ M C780 and 71 μ M C957. The normally localized tonoplast protein (a) accumulates at the ER in presence of C834 and analogs C388, C195 and C780 (c-e). Analog C957 does not affect the localization of GFP-TIP2;1 and, thus, it is not bioactive for inhibition of trafficking. Scale bar = 10 μ m

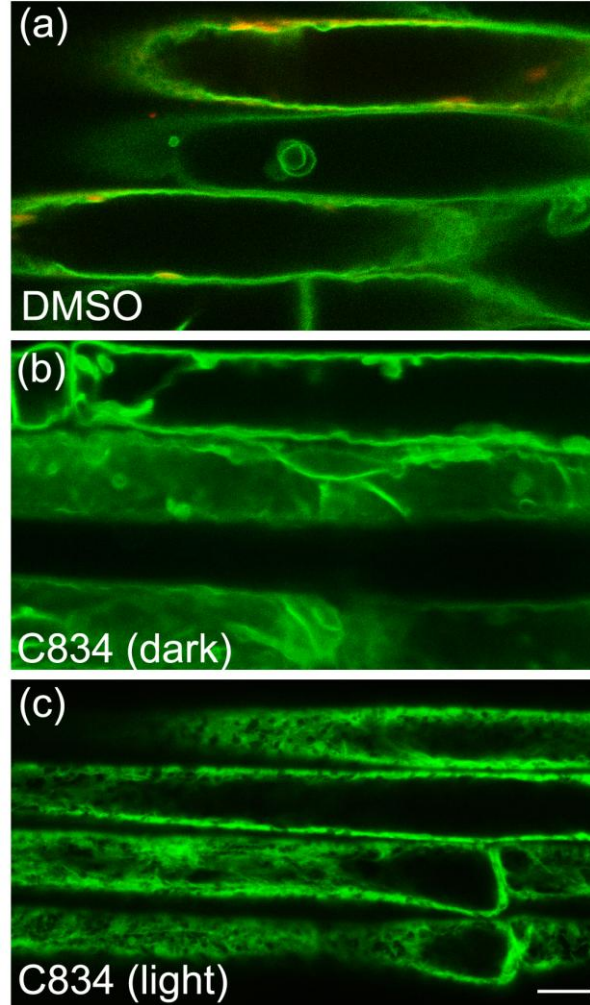


Figure 10. C834 requires light activation for its activity on trafficking. 3-day old seedlings expressing GFP-TIP2;1 were transferred to media containing either (a) DMSO (a), (b) C834 without exposure to light or (c) C834 that was previously incubated for 24 h in light. Seedlings were incubated for 48 h in the dark and analyzed under the confocal microscope. In both control and C834 that has not being exposed to light, the GFP-TIP2;1 marker localizes correctly to the vacuole membrane. In contrast, seedlings treated with “light activated” C834-containing media show mis-localization of the marker. Scale bar = 10 μ m

Figure 11. UV-vis spectra of dark- and light-activated C834. (a) Absorption spectra of C834 at different concentrations. A characteristic peak appears in the 370-390 nm range. (b) Absorption spectra of C834 after different time exposures to growth-chamber light at 22°C. C834 was dissolved in DMSO and treated accordingly to a concentration of 137.5×10^{-6} M before light treatments. Note that the characteristic peak of C834 decreases after a short exposure to light, and disappears after a long period exposure.

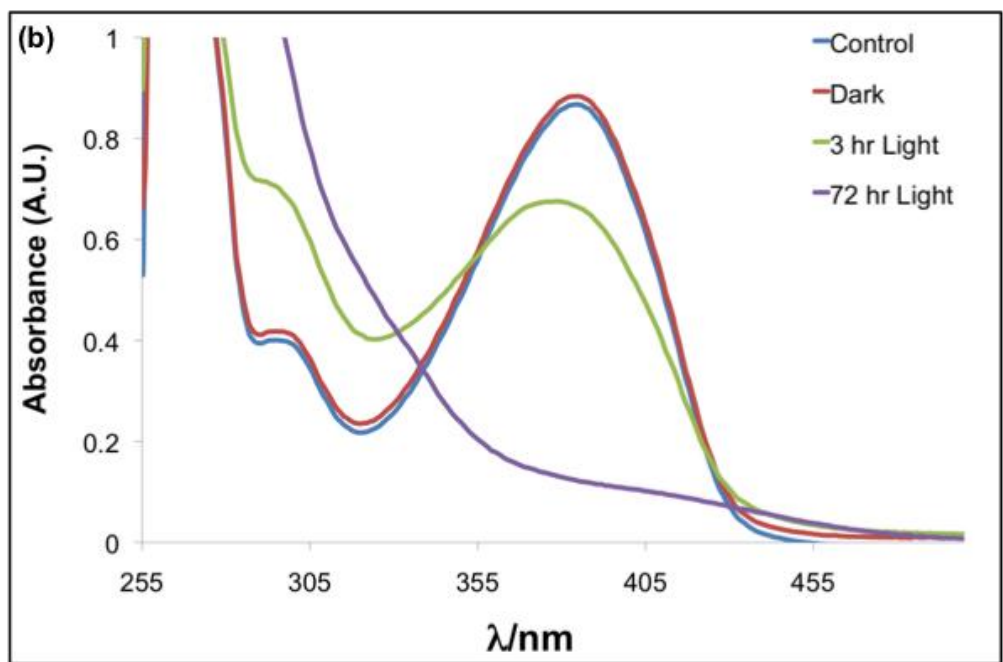
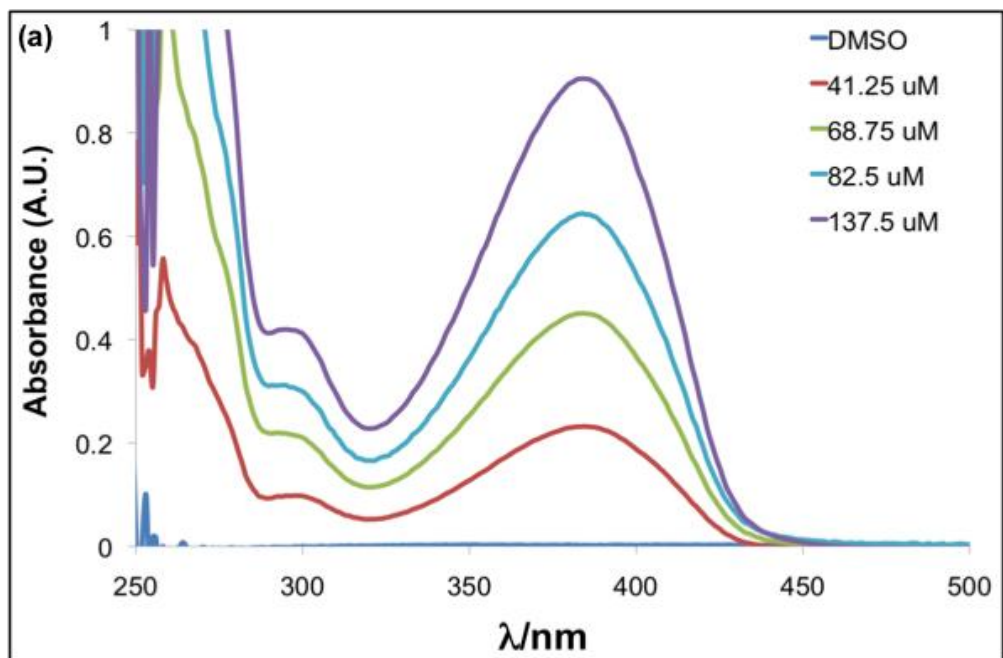
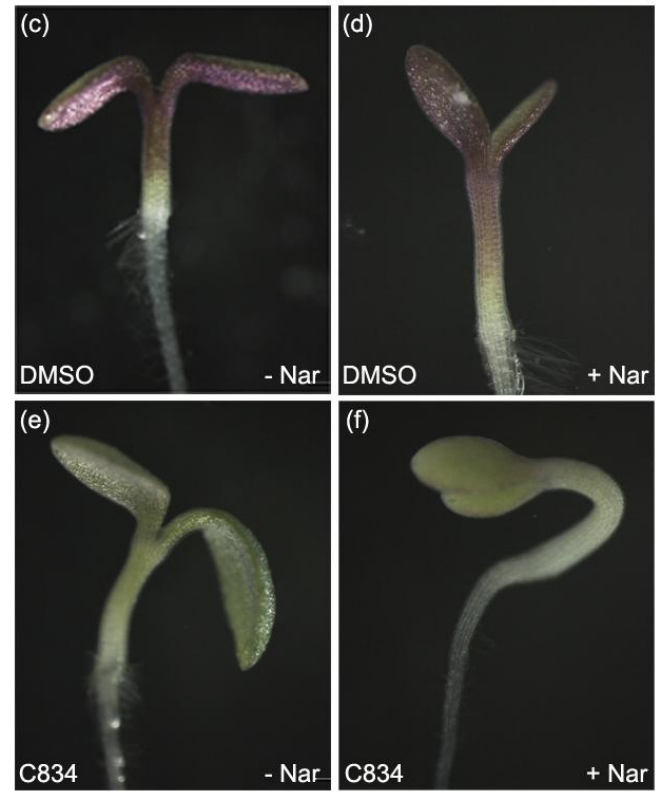
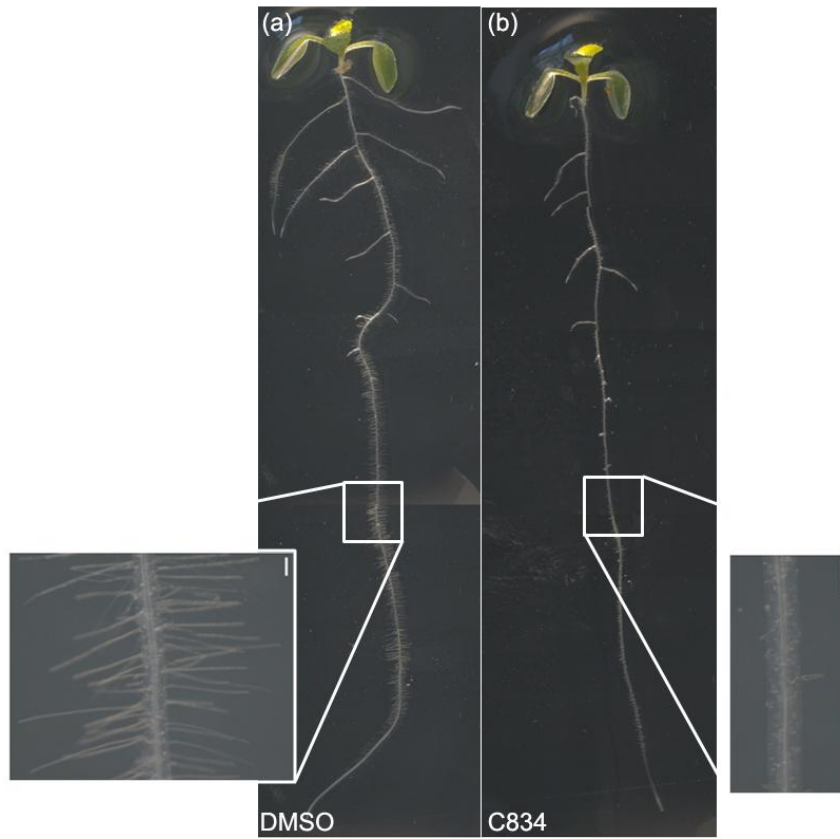


Figure 12. Phenotypes of light-grown C834-treated seedlings. For root hair visualization, Arabidopsis Col-0 seeds were grown for 8 days in media containing either (a) DMSO or (b) 55 μ M C834 (b). Note the lack of root hairs in seedlings treated with C834 (b, magnification) in comparison to control (a, magnification). For anthocyanin accumulation, Col-0 seeds were grown in media containing either (c) DMSO, (d) DMSO and 25 ng/mL naringenin, (e) 55 μ M C834 or (f) 55 μ M C834 and 25 ng/mL naringenin and visualized after 5 days. (a) Control seedlings show presence of anthocyanins in hypocotyls and cotyledons. (b) Addition of a precursor of anthocyanins, naringenin, to the media increases the levels of anthocyanins and creates epinastic cotyledons. (c) Seedlings grown in C834 do not show signs of anthocyanin levels, a phenotype that can not be rescued by supplementing naringenin to the media (d). Scale bar = 500 μ m



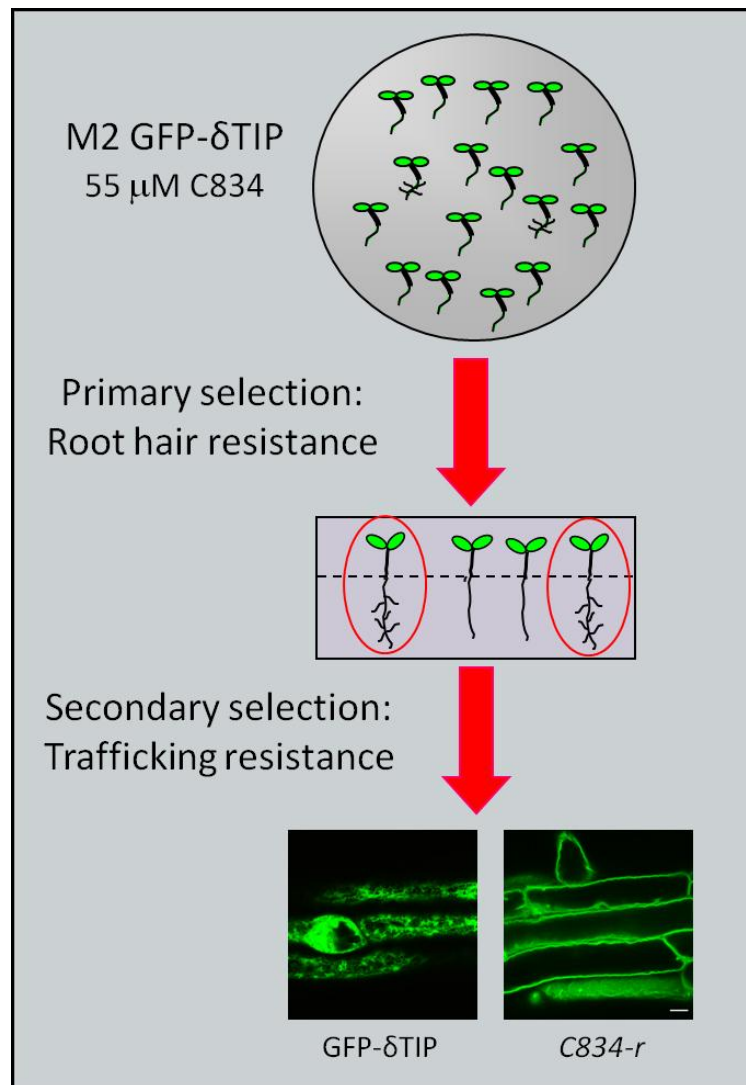


Figure 13. Strategy for identification of *C834-resistant* mutants. M2 seeds from an EMS-mutagenized population of GFP-TIP2;1 transgenic plants are grown vertically in media containing 55 μ M C834 for 5 days. Putative candidates are pre-selected if they show resistance to the inhibitory effects of root hair development by C834. These mutants are then analyzed under the confocal microscope for protein localization. Mutants that show normal tonoplast localization of GFP-TIP2;1 are transplanted to soil for confirmation and are considered putative *C834-res* mutants.

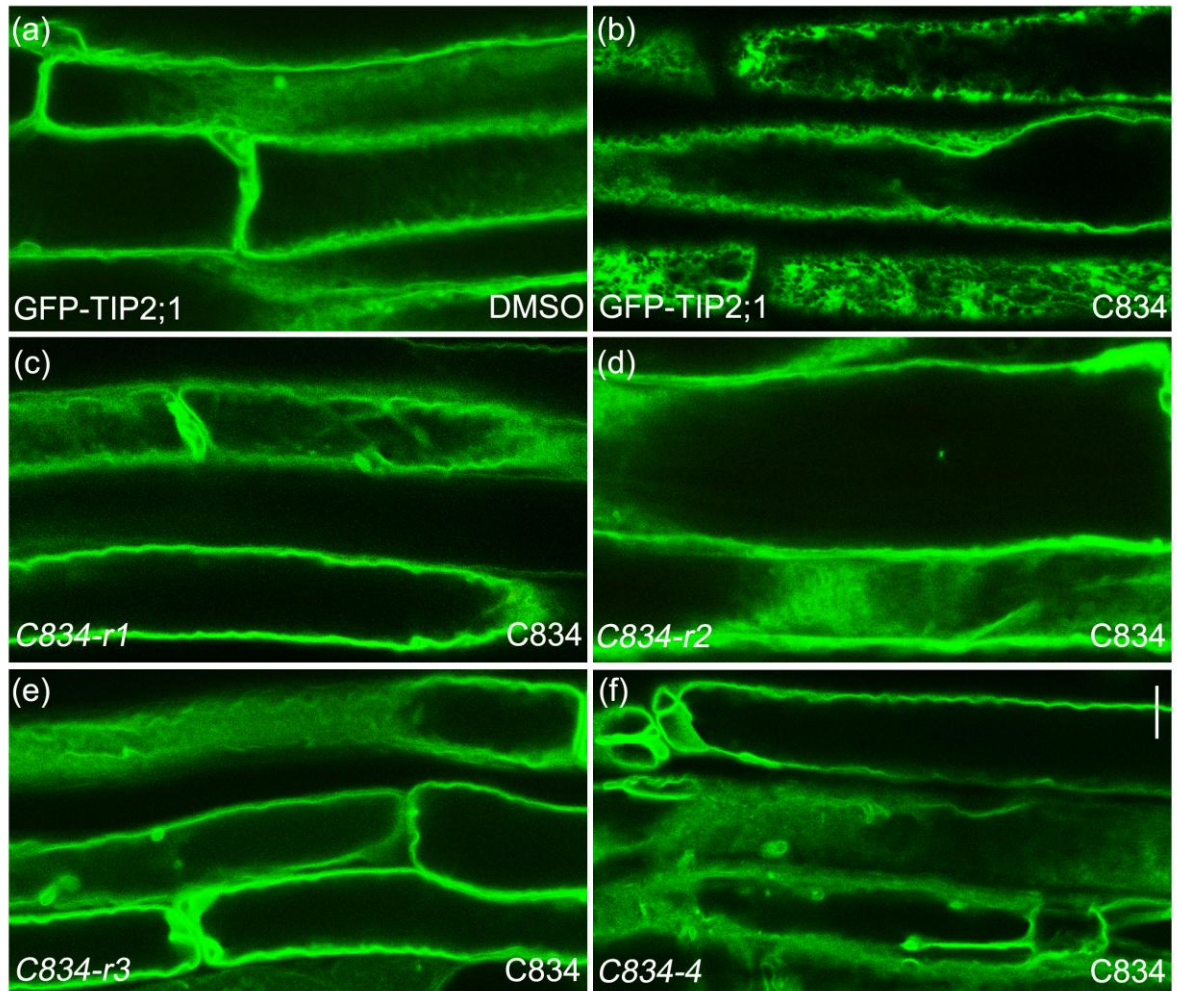


Figure 14. GFP-TIP2;1 is not mis-localized after C834 treatment in *C834-r* mutants. M3 seeds that were obtained from progeny of the primary screen were sown and grown in C834-containing media for 5 days. (a) In control, GFP-TIP2;1 localizes normally to the tonoplast, whereas in C834-treated seedlings the tonoplast marker localizes to the ER (b). (c-f) In *C834-resistant* mutants, GFP-TIP2;1 localizes to the tonoplast in presence of C834.

Scale bar = 10 μ m

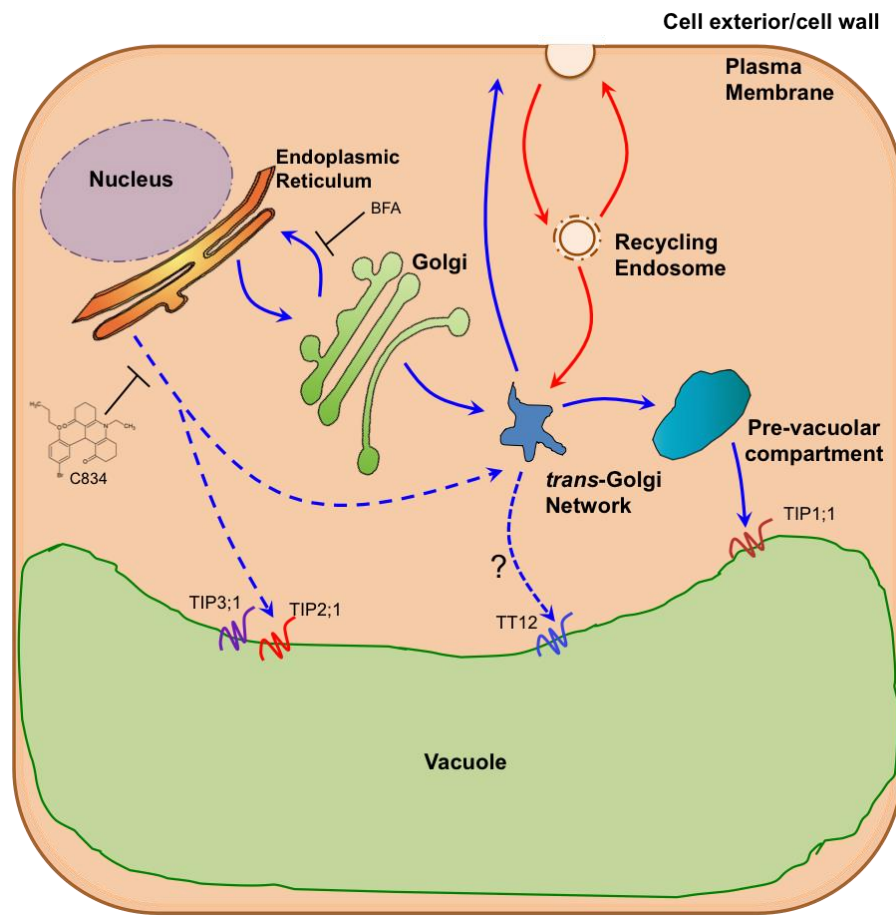


Figure 15. Proposed model for C834 trafficking inhibition. Members of the TIP family of tonoplast proteins start their trafficking journey at the endoplasmic reticulum (ER). The lytic vacuole marker, TIP1;1, is targeted in a Golgi-dependent pathway that is insensitive to C834, but sensitive to Brefeldin A (BFA). In contrast, TIP2;1 and the protein storage vacuole marker TIP3;1 travel in a Golgi-independent pathway that is sensitive to C834, but insensitive to BFA. TT12 is insensitive to both C834 and BFA, suggesting a bypass of the Golgi that perhaps requires a different target than that for TIP proteins. It remains unknown whether there is a merge of the two pathways or if proteins require an intermediate compartment after bypassing the Golgi. Blue arrows represent the secretory pathway and red arrows represent the endocytic pathway.

CHAPTER 3

A Link Between Vacuole Trafficking and Auxin Transport and Biosynthesis

Note: Special thanks to Wanda M. Figueroa-Cuilan for performing some of the experiments presented here.

ABSTRACT

Plants, as sessile organisms, rely on different hormonal and signaling mechanisms to overcome environmental challenges. These signaling mechanisms contribute to their plasticity and ability to adapt. The hormone auxin, which is synthesized from tryptophan, regulates a great number of cellular and physiological processes, and its homeostasis can be regulated by changes in its intra- and intercellular transport. Using pharmacological tools, we used a set of drug-like compounds that induce accumulation of vacuole membrane proteins at the endoplasmic reticulum (ER) and tested their effects in auxin transport and response. In here, we report that C834, an inhibitor of the Golgi-independent pathway for vacuole membrane proteins, enhances the vacuolar degradation of PIN2, an auxin transporter, in the absence of light. We also show that inducing protein accumulation at the ER with protein trafficking inhibitors, as well as inducing the unfolded protein response (UPR) with tunicamycin, induce a transcriptional activation of *ASAI* and *PATI*, two genes involved in tryptophan biosynthesis. We propose that an unfolded protein response (UPR) leads to an induction of the tryptophan biosynthetic pathway as a stress signal to modulate trafficking, possibly via a jasmonate-like mechanism.

3.1 Introduction

The plant hormone indole-3-acetic acid (IAA), the most common naturally occurring auxin, acts as a versatile signaling molecule that regulates almost every aspect of plant growth and development. Most auxin biosynthesis is dependent on the amino acid tryptophan (Trp), although a Trp-independent pathway in *Arabidopsis* has been proposed (Ljung et al., 2002). Tryptophan is synthesized via the shikimate pathway, also known as the chorismate biosynthetic pathway (**Figure 1**). This pathway is responsible for the conversion of phosphoenolpyruvate and erythrose 4-phosphate into chorismate, which can later be used for the biosynthesis of aromatic amino acids, vitamin B9, vitamin K1, salicylic acid and plant pigments (Tzin and Galili, 2010). The pathway includes seven enzymatic reactions; the first four being necessary for the biosynthesis of shikimate, and the last three for the conversion of shikimate into chorismate.

Once chorismate is synthesized, the enzyme ANTHRANILATE SYNTHASE (AS) acts as the rate-limiting step for favoring the biosynthesis of tryptophan. Two genes, *ASA1* and *ASB1*, encoding the two subunits of the enzyme have been identified in plants, and their expression have been shown to be induced by biotic stress, ethylene and jasmonic acid (Niyogi and Fink, 1992; Niyogi et al., 1993; Stepanova et al., 2005; Sun et al., 2009). After the production of anthranilate by AS, five more enzymatic reactions are needed to produce tryptophan. This involves the enzymes phosphorybosylanthranilate transferase (PAT1), the phosphorybosylanthranilate isomerase (PAI), the indole-3-glycerol phosphate synthase (IGPS), and the two subunits of the tryptophan synthase (TS) (Tzin and Galili, 2010). Similar to AS, the chorismate mutase (CM) catalyzes the first committed step for the biosynthesis of

the other two aromatic amino acids and it is also induced by pathogens (Mobley et al., 1999). The prephenate aminotransferase (PAT) follows in the pathway, producing aroenate, a compound that can be used to synthesize either phenylalanine or tyrosine by the activity of aroenate dehydrase (ADT) or aroenate dehydrogenase (TyrA), respectively (Tzin and Galili, 2010).

The fate of tryptophan towards the production of auxin has been well studied, and recently new changes have been added to the pathway (**Figure 2**). The simplified new model suggests that TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) converts Trp into indole-3-pyruvic acid (IPA) (Stepanova et al., 2008; Tao et al., 2008), which is in turn converted into IAA via the YUCCA family of flavin-monooxygenases (Mashigushi et al., 2011, Stepanova et al., 2011; Won et al., 2011). It has been postulated that local auxin biosynthesis may control developmental processes via positive and negative feedback on its own distribution (Chandler, 2009). Auxin is a unique plant hormone as it is being actively transported around the plant by a series of transmembrane pumps of the PIN FORMED (PIN) and the ABC-type family (Petrášek and Friml, 2009). This directional process is crucial in building up the spatial and temporal auxin gradients, which are required for plant growth and development (Galtwailer et al., 1998; Friml et al., 2003, Benková et al., 2003). Most PIN proteins have a polar plasma membrane localization that determines the direction of intercellular auxin flow (Wisniewska et al., 2006). The abundance and localization of these proteins is essential for proper auxin flow, and factors such as light, gravity and auxin itself have been shown to control this machinery (Laxmi et al., 2008; Kleine-Vehn et al., 2010; Paciorek et al., 2005).

Plasma membrane-localized PIN transporters undergo constant and dynamic recycling via endocytosis, and failure in this process causes major defects in plants at early stages of development (Geldner et al., 2003; Geldner et al., 2004). Early studies of the *GNOM* gene in *Arabidopsis* revealed the link between auxin homeostasis and regulation of intracellular PIN localization (Steinmann et al., 1999). *GNOM* encodes a small GTPase of the ARF-GEF family that regulates membrane trafficking processes from recycling endosomes to the PM (Geldner et al., 2003). Pharmacological experiments using Brefeldin A, an inhibitor of GNOM and other ARF-GEFs, demonstrate that mis-localization of auxin transporters result in classical auxin mutant phenotypes (Geldner et al., 2001).

The PIN family of proteins represents a major link between hormonal pathways and the endomembrane system (Dhonukshe et al., 2008). Pharmacological data provides evidence for multiple recycling pathways for PIN proteins (Robert et al., 2008; Kleine-Vehn et al., 2008), suggesting the existence of complex endocytic machinery. The role of the vesicle coat protein clathrin has been shown to mediate both endocytosis and PM distribution of PIN transporters (Kitakura et al., 2011). Novel players in the pathway have been identified, including BEN1, an ARF-GEF that mediates trafficking of cycling PM proteins such as PIN1 and PIN2 (Tanaka et al., 2009). Interestingly, these proteins are targeted to the plant lytic vacuole for degradation under specific environmental signals, including phototropism, which is accompanied by an increase in both auxin transport and biosynthesis (Laxmi et al., 2008; Liu et al., 2011). The retromer components SORTIN NEXIN1 (SNX1) and VACUOLAR PROTEIN SORTING29 (VPS29) have been proposed to contribute in retrieving PIN2 from the pre-vacuolar compartment back to the recycling pathways (Kleine-Vehn et al., 2008).

In this report, we show that incubation with C834, a specific inhibitor of trafficking of tonoplast proteins, results in auxin transport defects. This inhibitor seems to enhance the vacuolar degradation pathway of PIN2 in the dark, but not the other PIN proteins. Furthermore, pharmacological analysis using a set of small-molecules that inhibit the targeting of tonoplast proteins at the endoplasmic reticulum (ER) provide evidence for a novel link between protein mis-targeting and transcriptional regulation of the tryptophan biosynthetic pathway. We propose that accumulation of tonoplast proteins at the ER results in induction of genes favoring the Trp biosynthetic pathway, a stress signal similar to that caused by jasmonic acid.

3.2 Results

3.2.1 *PIN2 levels are greatly reduced in C834-treated seedlings in the dark*

It has been well established that members of the PIN family of auxin transporters require the endomembrane system for endocytic pathways (Grunewald and Friml, 2010). In a chemical genetics screen for inhibitors of vacuole membrane protein trafficking, we identified a series of drug-like compounds that cause the accumulation of a subset of tonoplast proteins at the ER (Rivera-Serrano et al., in preparation). In an effort to further characterize these compounds, we tested their effects on the intracellular localization of PINs. This small collection included chemicals that inhibit the trafficking of vacuole membrane proteins specifically, and others that affect the endomembrane system in a larger scale. We decided to focus first on the effects of C834, as this compound was shown to act in a very specific pathway. We performed C834-incubation assays in both light and dark (**Figure**

3), as it has been shown that light controls abundance of PIN proteins at the plasma membrane and, in absence of light, these proteins are targeted to the vacuole (Laxmi et al., 2008). In the light control treatment, all PIN proteins localize to the plasma membrane and, in PIN1 and PIN2, also in endosomal compartments (Figure 3a, 3c, 3i, 3k and 3m). In the dark, PIN levels at the plasma membrane decrease in comparison to light, and some vacuole localization can be seen for PIN2 (Figure 3e, 3g, 3o, 3q and 3s). In the light assay, neither of the PIN chimeric GFP fusions showed any signs of mis-localization or changes in protein levels due to bioactivity C834 (Figures 3a-3d, 3i-3n). Interestingly, the levels of PIN2 in seedlings treated with C834 in the dark were greatly reduced when compared to the control (Figure 3e-3f), whereas the levels of PIN1-, PIN3-, PIN4- and PIN7-GFP appeared to be normal compared to the control (Figure 3g-3h, 3o-3t). These data suggest an enhancement of the vacuole degradation pathway for PIN2 in C834-treated seedlings. The fact that other PIN proteins were not affected was not surprising as previous reports suggest the existence of multiple targeting pathways for PIN proteins (Robert et al., 2008). We then tested the effect of C410, C103, C755 and C578 inhibitors on the localization of PIN2-GFP in both light and dark (**Figure 4**). PIN2 localization was consistent with the previous experiment, localizing to the plasma membrane and endosomes in presence of light (Figure 4a). A similar localization pattern was observed in chemical-treated seedlings when in light (Figures 4c, 4e, 4g, and 4i). None of the other four inhibitors of tonoplast protein trafficking showed any major effect on PIN2 in the dark when compared to control (Figures 4d, 4f, 4h and 4j). The apparent effect of C103 on PIN2 levels (Figure 4f) may be due to cell death, as the treated seedlings were very weak. The role of light on PIN2 localization has been shown to involve the

photomorphogenic machinery, and this pathway controls, at least in part, the distribution of PIN2 between the plasma membrane and the vacuole (Laxmi et al., 2008). Recent evidence demonstrated the essential role of the phototropin1 (PHOT1) photosensor and the signal transducer NONPHOTOTROPIC HYPOCOTYL3 (NPH3) in vacuole targeting of PIN2 (Wan et al., 2012). The fact that PIN2 levels are drastically reduced after a short incubation with C834, suggest an enhancement of the vacuole degradation pathway for this protein in the dark. These results suggest the existence of a putative link between trafficking of proteins in a Golgi-independent pathway and the targeting of PIN2 to the vacuole.

3.2.2 C834 inhibits auxin transport in *Arabidopsis* roots

Multiple lines of evidence indicate crosstalk between auxin transport, signaling and protein trafficking mechanisms (Geldner et al., 2001; Kitakura et al., 2011; Paciorek et al., 2005). To test if C834 had an effect on auxin sensitivity, we used a root growth assay using different types of auxins in presence or absence of C834 (**Figure 5**). Indole-3-acetic acid (IAA) is the most common natural auxin in plants, and one of its precursors is indole-3-butyric acid (IBA), which is synthesized in the plant peroxisome (Strader and Bartel, 2011). In contrast with the first two, both naphthalene-1-acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) are considered synthetic analogues of auxin, and whereas NAA does not require polar transport for its activity, 2,4-D inhibits auxin transport (Marchant et al., 1999; Yamamoto and Yamamoto, 1998). *Arabidopsis* seedlings were germinated in media containing DMSO or C834 and supplemented with either different concentrations of IAA, or with IBA, 2,4-D or NAA. We noticed that in contrast with the

control, where IAA inhibits root growth even at low concentrations, seedlings treated with both IAA and C834 did not show the classic inhibition of root growth (Figure 5a). To test if this effect was specific to IAA, plants were exposed to the presence of IBA or 2,4-D in the presence of C834 (Figure 5b). C834 prevented the root growth-inhibition activity of these two compounds similar to that seen when treated with IAA. However, when plants were grown in the presence of NAA, a diffusible auxin, and C834, the plants showed the typical auxin-induced inhibition of root growth. These results show that whereas C834 reduces the sensitivity of seedlings to exogenous auxins that require some type of transport, it does not inhibit the effects caused by NAA. These suggest that C834 is an inhibitor of auxin transport in roots, causing reduction in sensitivity to external auxins.

In order to analyze auxin response at the tissue level, we used transgenic *Arabidopsis* lines expressing the auxin reporter gene *DR5-GUS* to monitor auxin levels in roots. The activity of this promoter has been shown to correlate with auxin levels, and an increase in the levels of this promoter can be visualized by the activity of β -*GLUCURONIDASE* (*GUS*) (Sabatini et al., 1999). For this, 4-day-old seedlings were transferred to media containing either DMSO or C834 in presence of ethanol (solvent control for auxins), IAA or NAA (**Figure 6**). In the control, the expression of *DR5-GUS* was restricted to the quiescent center and some cells of the vasculature (Figure 6a; Sabatini et al., 1999). Seedlings treated with C834 showed similar *GUS* patterns, suggesting that C834 does not induce changes in auxin levels (Figure 6d). We then looked at the response of the *DR5* promoter to IAA or NAA in either presence or absence of C834. As expected, both auxins strongly induced the expression of the reporter gene in control conditions, as shown by the increase in levels of

the blue precipitate (Figure 6b-c). In contrast, when C834 is applied in combination with IAA, the induction of the promoter by IAA is reduced, compared to that of the control (compare Figure 6b with 6e). These results are consistent with the reduction in IAA sensitivity observed in seedlings (Figure 5). Because seedlings showed normal sensitivity to NAA in terms of root length (Figure 5b), we hypothesized that C834 would not inhibit the induction of *DR5* promoter by NAA. Indeed, NAA induced the activity of *DR5* at similar levels, regardless of the presence of C834 (compare Figure 6c with 6f). Overall, these results suggest that the reduction in sensitivity for IAA in C834-treated seedlings is most likely due to auxin transport inhibition by the drug. Moreover, the root growth measurements are consistent with auxin response levels in chemical treated plants.

3.2.3 Accumulation of proteins at the ER causes induction of ASA1

Given the fact that C834 induces a reduction in sensitivity to exogenous auxins, probably by inhibiting auxin transport, we then wanted to test if a feedback mechanism on auxin biosynthesis was activated by C834. In plants, IAA is synthesized using tryptophan (Trp) as a precursor. The enzyme ANTHRANILATE SYNTHASE (AS) is responsible for the conversion of chorismate into anthranilate, and its activity marks the rate-limiting step for Trp biosynthesis. The gene encoding the α -subunit of AS, *ASA1*, has been shown to act as a hub for several hormone signaling processes, including ethylene and jasmonic acid (Stepanova et al., 2005; Sun et al., 2009). Moreover, *asa1* mutants show enhanced reduction of PIN2 accumulation after methyl jasmonate (MeJA) treatment when compared to wild type (Sun et al., 2009; Sun et al., 2011). To determine if the activity of the *ASA1* promoter was

being affected by C834, we used a transcriptional fusion of the promoter of *ASAI* and *GUS* (**Figure 7**). Under normal conditions, *ASAI-GUS* expression is mostly restricted to the root apical meristem (Stepanova et al., 2005; Figure 7a). Interestingly, treatment with C834 strongly induced the expression of *ASAI-GUS* (Figure 7b). In contrast, treatment with an inactive analog of C834 (aC834, Appendix Table 1A) that does not inhibit trafficking did not activate *ASAI* expression (Figure 7c), indicating a tight link between inhibition of protein trafficking and transcriptional control of Trp biosynthesis. To test whether the effect of C834 on *ASAI* expression was a result of trafficking inhibition at the endoplasmic reticulum (ER), we tested the effect of other tonoplast traffic inhibitors: C410, C103, C755 and C578 (Rivera-Serrano et al., in preparation). As shown in Figure 7, all these inhibitors induced the activation of the *ASAI* promoter in this reporter line (Figure 7d-g). Activity of both C834 and C410 results in *ASAI* expression throughout the root (Figures 7b, d). The effect of C755 was interesting as the *ASAI* expression was found opposite of the control, lacking the signal in the apical meristem and increasing the levels in upper regions of the root (Figure 7e). Both C103 and C578 increase promoter activity to some extent in roots, but mostly in the vasculature (Figures 7f-g). Moreover, none of these chemicals induce the *DR5-GUS* reporter (Appendix Figure 1A), suggesting that the activation of the *ASAI* promoter is not due to an unspecific transcriptional regulation.

Differences in *GUS* gene expression may be caused by a general inhibition of trafficking in the endomembrane system, or a specific inhibition of traffic at the ER. In order to differentiate between these two possibilities, we used Brefeldin A (BFA) and Wortmannin (Wm), two well characterized trafficking inhibitors that block protein targeting at different

endomembrane compartments. BFA inhibits protein recycling at the plasma membrane, also inhibits Golgi-to-ER retrograde traffic, and induces the fusion of the ER with the Golgi in most cells (Robinson et al., 2008). In contrast, Wm inhibits traffic from the prevacuolar compartment (PVC) to the *trans*-Golgi network and induces the fusion of PVCs, a later step in the secretory pathway (Kundra and Kornfeld, 1998; Wang et al., 2009). Incubation with BFA, but not with Wm, also induced *ASAI* in a similar way as with the other inhibitors tested (Figures 7h, i). These results suggest that inhibition of trafficking by BFA may generate a signaling mechanism to initiate the transcriptional activation of *ASAI* in a similar fashion as with inhibitors of trafficking at the ER. Moreover, it suggests that the effects seen by our chemicals are not due to non-specific effects on general transcriptional regulation mechanisms. The unfolded protein response (UPR) is a well-characterized stress response in eukaryotes that perceives the accumulation of unfolded proteins at the ER (Walter and Ron, 2011). This mechanism is sensed as a stress signal caused by accumulation of defective proteins that are not being folded properly, and results in an increase in the biosynthesis of chaperones and other proteins to help with folding (Vitale and Boston, 2008). We hypothesized that perhaps the accumulation of proteins at the ER in C834-treated seedlings results in an UPR-like response that lead to activation of *ASAI*. In order to test this, we tested whether tunicamycin (Tun), a chemical that induces the UPR response by inhibiting *N*-glycosylation (reviewed in Liu and Howell, 2010), had similar effects on *ASAI:GUS* expression. Indeed, tunicamycin treatment induces *ASAI* expression (Figure 7j), providing supporting evidence for our hypothesis. We propose that protein accumulation at the ER may

be a molecular mechanism that initiates a signaling cascade and results in induction of *ASAI*, the gene that encodes the rate-limiting enzyme in Trp biosynthesis.

3.2.3 Induction of genes in the Trp biosynthetic pathway by C834

Aromatic amino acids are synthesized via the shikimate pathway, which is then followed by a branched metabolic pathway to produce tryptophan, tyrosine or phenylalanine (Figure 1). These amino acids are important not only for protein synthesis, but they also serve as precursors for phytohormones and other secondary metabolites that are essential for human health and plant defense (Tzin and Galili, 2010). So far, we have identified a putative link between protein accumulation at the ER and *ASAI* induction, which encodes the first enzyme in the Trp biosynthetic pathway. We then wanted to test if this activation was only for *ASAI*, or if there were other genes in the Trp biosynthetic pathway that were been up-regulated. To address this question, we looked at available lines expressing chimeric enzymes in the pathway that were tagged with yellow fluorescent proteins (YFP) and driven by their native promoters (Zhou et al., 2011) (**Figure 8**). We first analyzed protein levels of CUE1 and SK2 in either presence or absence of C834 (Figure 8a-d). CUE1 is a phosphoenolpyruvate (PEP)/phosphate translocator from the plastids, providing the starting PEP for the shikimate pathway (Fischer et al., 1997). SK2 catalyzes the phosphorylation of shikimate into shikimate-3-phosphate (Figure 1). In control seedlings, both markers localize to plastids throughout the root (Figures 8a, c). Neither CUE1 nor SK2 showed any sign of up-regulation after C834 treatment (Figures 8b, d), suggesting that the induction of *ASAI* is not the result of an early induction of genes in the shikimate pathway. Once chorismate is

produced, both *ASA1* and the chorismate mutase (*CM*) are essential for favoring the biosynthesis of either Trp or phenylalanine and tyrosine, respectively (Figure 1). We knew that inhibitors of protein trafficking, at least transcriptionally, were inducing *ASA1*, so we tested enzymes downstream in the Trp biosynthetic pathway and downstream of *CM*. The localization of the phosphoribosylanthranilate transferase (*PAT1*), the enzyme that follows *ASA1* in the pathway, was seen only in cells near the quiescent center in control seedlings (Figure 8e), and no signal was seen in the upper root or the hypocotyl (Figure 8f, inset). Interestingly, the levels of *PAT1* were greatly increased after C834 treatment, and fluorescence could be seen even in chloroplasts of the hypocotyl (Figures 8g, h, inset), where it is not normally found. The tryptophan synthase (*TSA*) was also tested, and its plastid localization in both control and C834 were very similar (compare 8i with 8j). This suggests that accumulation of proteins at the ER results in an increase in the expression and protein levels of genes in the early Trp biosynthetic pathway. We could not test all the enzymes in this pathway because some of them are not yet available or because the available lines show a high degree of variation in fluorescence levels. Nevertheless, we tested *TyrA2* levels as an enzyme in the non-Trp aromatic amino acid biosynthetic pathway. Similar to the other enzymes, *TyrA2*-YFP localizes to plastids in roots in control seedlings (Figure 8k). We could not detect any major differences in the levels of *TyrA2* between control and C834 treatment, suggesting a specific link between trafficking and tryptophan biosynthesis, but not for the other two aromatic amino acids. Overall, our results suggests that *ASA1* and *PAT1* expression is induced when proteins accumulate at the ER, probably to favor the production of Trp or other intermediate that serves as a signaling molecule. Since most of the biosynthesis of

auxin comes from a pathway that requires tryptophan as a substrate (Figure 2), we also looked at the levels of TAA1-GFP protein, which is involved in auxin biosynthesis. However, no significant differences were observed between control and C834 treatment (data not shown), suggesting a specific upregulation in genes favoring biosynthesis of Trp. In addition, it is possible that other genes from a different branch of the IAA biosynthetic pathway are also upregulated. Overall, we report a putative novel link between protein trafficking and Trp biosynthesis.

3.3 Discussion

3.3.1 C834 inhibits auxin transport in roots

Protein trafficking pathways are essential for protein delivery throughout the cell. In order to further characterize a novel set of inhibitors of vacuole membrane trafficking, we analyzed their effects on auxin homeostasis. We identified C834 as a chemical that specifically enhances the trafficking of PIN2 from the plasma membrane to the vacuole, as this chemical does not affect any other PIN proteins. This phenomenon seems to be dependent on light, an environmental signal that has been shown to control the localization of these proteins from the plasma membrane to the vacuole for degradation (Laxmi et al., 2008; Liu et al., 2011). None of the other chemicals tested had similar effects on PIN2, suggesting a link between the target(s) of C834 and PIN2 trafficking. Because C834 was previously shown to inhibit the Golgi-independent pathway that directs membrane proteins to the vacuole (Rivera-Serrano et al., in preparation), this is perhaps a new link between PIN2 traffic to the vacuole and the Golgi-independent machinery.

DR5-GUS levels are reduced after IAA application in C834-treated seedlings compared to the control. This indicates that C834 is somehow affecting the auxin transport. Moreover, this is consistent with the fact that application of C834 reduces the response of plants to exogenous auxins that require polar transport. We propose that C834 is affecting the intercellular import of auxin to the plant cell, as these seedlings react normally to NAA. Overall, we propose that inhibition of the Golgi-independent pathway for tonoplast proteins induces an enhancement of the vacuole degradation pathway for PIN2 in dark, and also blocks the incorporation of transport of exogenous auxins into the plant cell. Characterization of *C834-resistant* mutants will provide genetic information that would test our hypothesis.

3.3.2 Protein accumulation at the ER and induction of genes in the Trp biosynthetic pathway

Because of the different effects induced by C834 on auxin transport, we tested the effects of this chemical on auxin biosynthesis. *ASA1* has been reported for acting as a merging point for several hormonal pathways (Stepanova et al., 2005; Sun et al., 2009), and is the first enzyme in the biosynthesis of Trp, the precursor of auxin. All of our inhibitors of protein trafficking resulted in induction of the *ASA1* promoter in a similar way as it has been shown for jasmonic acid (Sun et al., 2009). Using tunicamycin, a chemical that activates the UPR response, we further confirmed that this up-regulation might be due to increased accumulation of proteins at the ER as a result of chemical treatment. The hormone ethylene has been shown to induce *ASA1* to some degree to favor auxin biosynthesis (Stepanova et al., 2005). However this specific pathway does not seem to be the target of our chemicals, as the

ASAI induction occurs even in *ein2-5* (data not shown), a mutant background that is insensitive to ethylene (Alonso and Stepanova, 2004). Methyl jasmonate (MeJa) is a strong inducer of *ASAI*, and it also modulates PIN2 levels at the plasma membrane (Sun et al., 2009; Sun et al., 2011). This may be due to the fact that jasmonate controls many aspects of plant defense, a major trigger of genes in the Trp biosynthetic pathway (Niyogi and Fink, 1992). Because of the similarities between MeJa and our chemicals, we tested if MeJa or the oxypilin precursor ODPA had any effect on tonoplast protein trafficking. Using the same treatment as C834, neither MeJa nor ODPA had any effect on the localization of the tonoplast protein GFP-TIP2;1 (Appendix Figure 2A), although we did observe an increase in the abundance of ER bodies labeled with a Cherry-HDEL marker after MeJa treatment, as previously reported (Matsushima et al., 2002; Matsushima et al., 2003; Hara-Nishimura and Matsuhima, 2003). This is interesting because ER bodies have been implicated with the Golgi-independent delivery of proteins from the ER to the vacuole in the Brassicaceae (Herman and Schmidt, 2004). Due to difficulties in the propagation of jasmonate-insensitive mutants, we could not address whether the effect of C834 and the other chemicals was due to inhibition of the jasmonate-signaling pathway. The fact that only *ASAI* and *PAT1*, but not *CUE1*, *SK2*, *TSA* nor *TyrA2*, levels were shown to increase drastically after C834 treatment suggest a very specific link between tryptophan biosynthesis and protein stress at the ER. We propose a pathway in which accumulation of proteins at the ER results in stress-mediated induction of Trp biosynthetic genes to possibly further regulate protein delivery via ER bodies. Because jasmonate and different types of stresses induce these organelles, it would be necessary to test the effects of these chemicals on gene expression in jasmonate-insensitive

mutants backgrounds. Further experimental data can support this hypothesis and a putative novel link between ER stress and Trp biosynthesis.

3.4 Materials and methods

3.3.1 Plant Material

Arabidopsis thaliana ecotype Columbia (Col-0) was used in all experiments. Seeds were sterilized and sown on half-strength AGM (0.5X MS, 3 g/L GelRite, 1% sucrose, pH 5.7) and stratified in darkness for 72 h at 4°C. Plates were incubated in the light (16 h per day) at 22°C. Analysis of auxin response was monitored using transgenic lines expressing *DR5::GUS* (Ulmasov et al., 1997). Seeds of *ASA1::GUS* and *ASB1::GUS*, in both Col-0 and *ein2-5* backgrounds, (Stepanova et al., 2005) were kindly provided by Dr. Anna Stepanova (NCSU). The lines *PIN1::PIN1:GFP* (Benková et al., 2003; Friml et al., 2003), *PIN2::PIN2:GFP* (Abas et al., 2006), *PIN3::PIN3:GFP* (Žádníková et al., 2010), *PIN4::PIN4:GFP* (Vieten et al., 2005) and *PIN7::PIN7:GFP* (Blilou et al., 2005) have been previously described. Transgenic lines expressing YFP fusions to genes in the tryptophan biosynthetic pathway (*CUE1::CUE1-YFP*, *SK2::SK2-YFP*, *PAT1::PAT1-YFP*, *TyrA2::TyrA2-YFP* and *TSA1::TSA1-YFP*) were obtained from Dr. Jose Alonso (Alonso and Stepanova, unpublished data).

3.3.2 Chemicals and hormones

The tonoplast trafficking inhibitors were obtained from ChemBridge (CB, San Diego), and their CB ID numbers are included: C834 (6982834), aC834 (6979957), C410 (5838410),

C755 (7703755), C103 (5556103) and C578 (5262578). DMSO, indole-3-acetic acid, indole-3-butyric acid, 2,4-dichlorophenoxyacetic acid, naphthalene-1-acetic acid, Wortmannin, Brefeldin A and tunicamycin, propidium iodide were purchased from Sigma. For experiments performed in dark, a “light activated” form of C834 was used. The “light activated” plates are made by preparing 55 μM C834 in 0.5X MS and incubating the plates at 22°C in the growth chamber for a period of 18-24 h. Inhibition of tonoplast protein trafficking using a GFP-TIP2;1 construct is used as a control for dark experiments.

3.3.3 Root growth analysis for auxin transport

For auxin sensitivity assays, Col-0 seeds were germinated in 0.5X MS containing either DMSO or 55 μM C834 (ID 6982834; ChemBridge, San Diego) in addition to either 1 or 0.1 μM IAA, 1 μM IBA, 80 nm 2,4-D or 1 μM NAA, previously dissolved in ethanol. Plates were scanned after 5 days and root length was quantified using the ImageJ software.

3.3.4 Histochemical analysis of GUS expression

For auxin response experiments, four-day old *DR5::GUS* seedlings were transferred to media supplemented with either DMSO or 55 μM C834 for 4 hr, and then transferred to C834 +/- media supplemented with 1 μM IAA or 1 μM NAA for 24 h. For analysis of *ASAI::GUS* expression, 3-day-old seedlings expressing the construct were transferred to either DMSO or the indicated chemical (all were dissolved in DMSO) treatment for 48 h. Plant tissue was submerged into the GUS stain solution containing 100 mM sodium phosphate buffer, pH 7.0 (from a 1 M stock: 1 M Na_2HPO_4 , 1 M NaH_2PO_4 in a 57:43 ratio), 10 mM ethylene diamine

tetraacetic acid (EDTA), 0.1% Triton X-100, 1 mg/mL 5-bromo-4-chloro-3-inolyl- β -D-glucuronic acid (X-Gluc; Sigma), 100 μ g/mL chloramphenicol, 2 mM potassium ferricyanide, and 2 mM potassium ferrocyanide; and placed in a vacuum for 15 min. Samples were then incubated for 24 h at 37°C. To clear the tissue, sample was incubated in 70% ethanol for 48 h, replacing the ethanol every 6-8 h.

3.3.5 Confocal and light microscopy

A Zeiss LSM 710 confocal microscope from the Cellular and Molecular Imaging Facility (CMIF) at NCSU was used for all experiments. The imaging of the yellow fluorescent proteins (YFP) and GFP from each seedling was carried out using a 20X air objective (0.8 NA) or a 40X water objective (1.1 NA), and a 488 nm laser line. Cell wall was stained using propidium iodide (PI) at a concentration of 10 μ g/mL for 2 min and rinsed with water before imaging. GUS stainings were imaged in a Leica M205C stereomicroscope equipped with a Leica DFC425C digital camera. Images were processed using Photoshop CS4.

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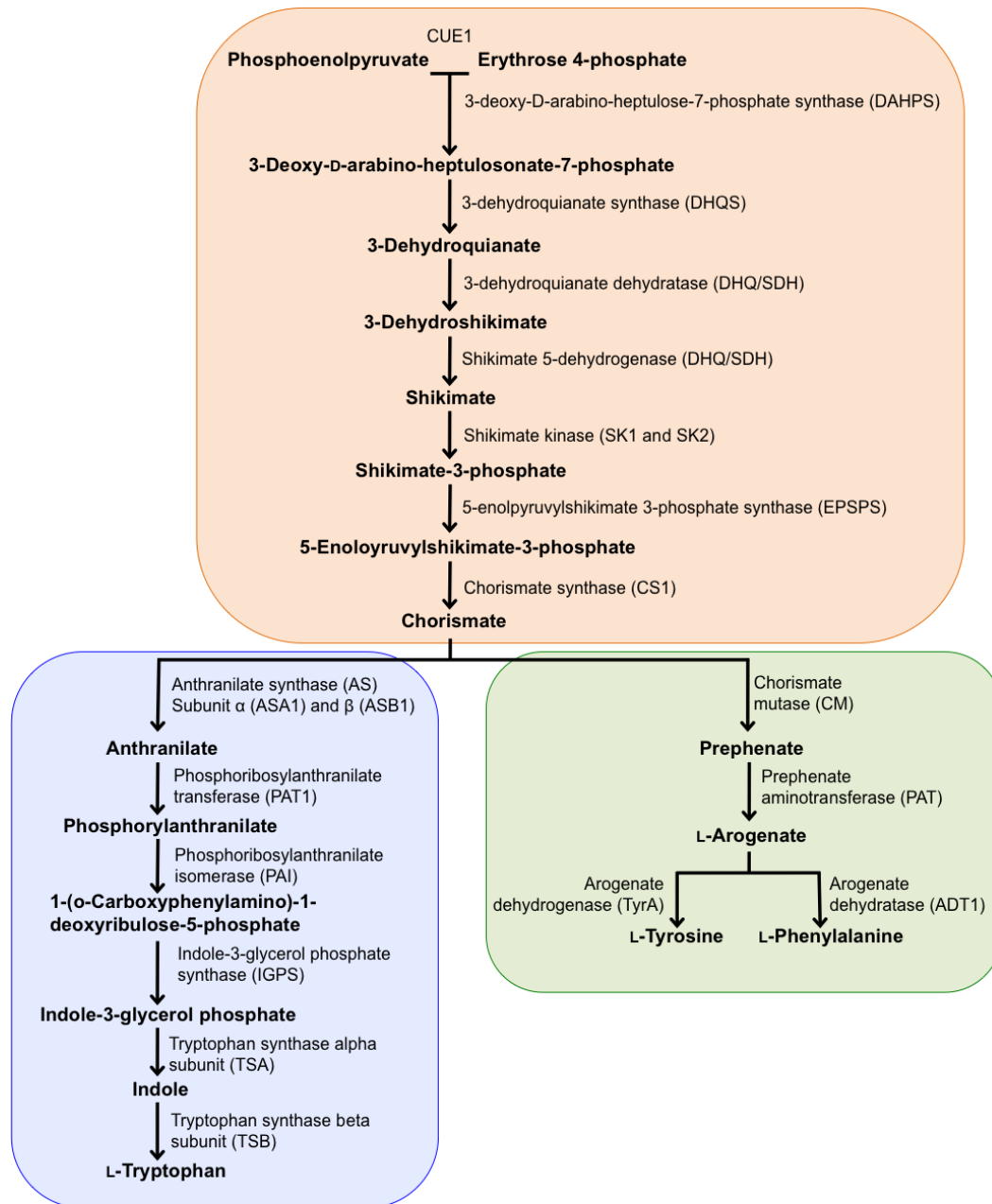


Figure 1. Shikimate and aromatic amino acid biosynthetic pathways in plants. The biosynthetic pathway of shikimate (pale orange box), tryptophan (blue box) and phenylalanine and tyrosine (green box) are shown. (Modified from Tzin and Galili, 2010)

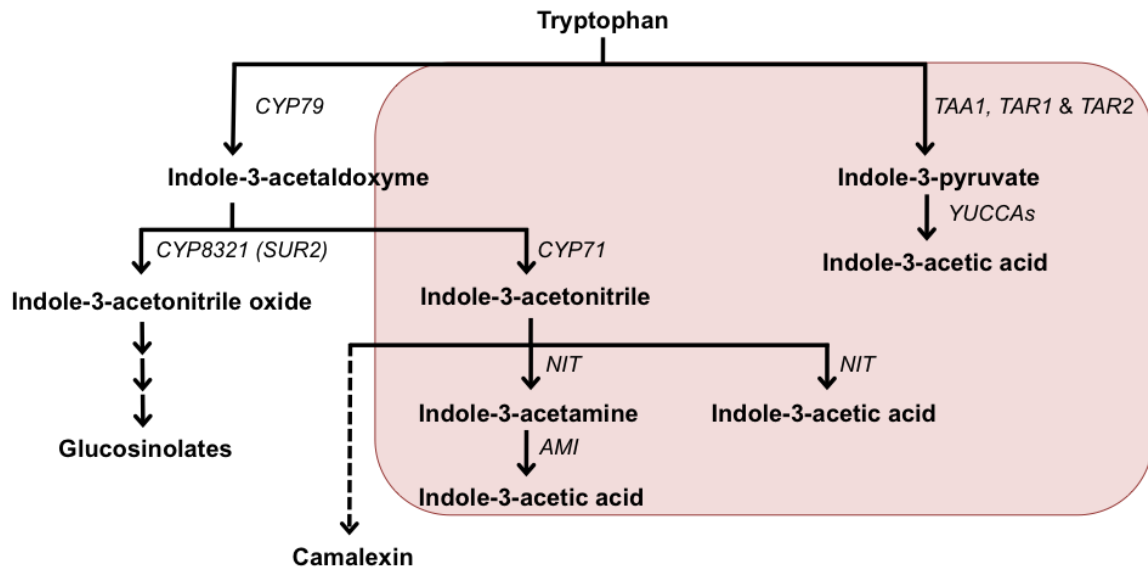
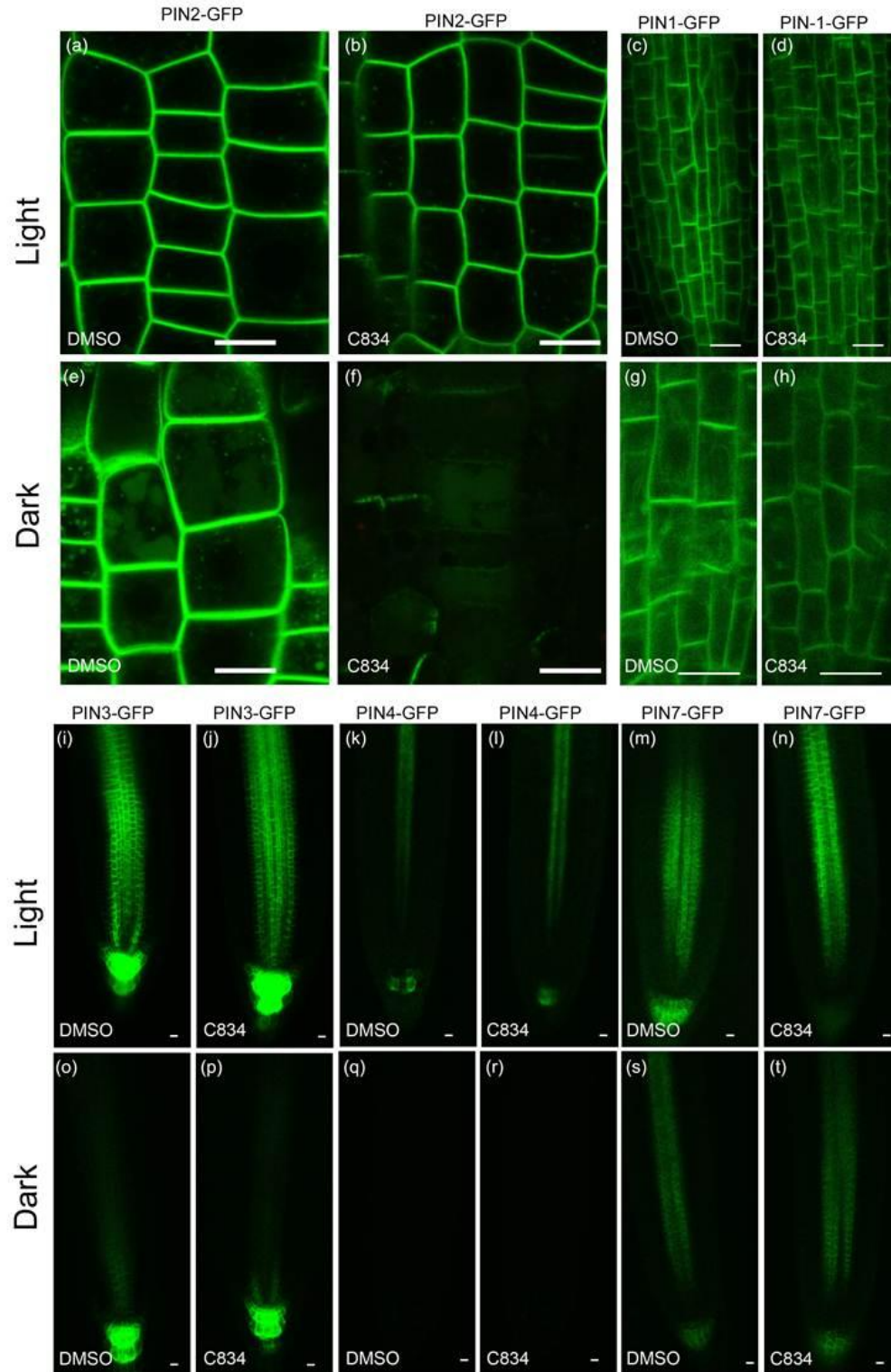


Figure 2. Catabolic fates of tryptophan after its biosynthesis in Brassicaceae. Once synthesized, tryptophan serves as substrate to produce either glucosinolates, camalexin or indole-3-acetic acid (IAA). The routes for IAA biosynthesis are highlighted in pink.

(Modified from Tzin and Galili, 2010)

Figure 3. PIN2 levels are greatly reduced in C834 treatment in dark. Three-day-old seedlings expressing PIN2-GFP, PIN1-GFP, PIN3-GFP, PIN4-GFP or PIN7-GFP were transferred to media containing either DMSO or 55 μ M in the light (a-d, i-n) or in the dark (e-h, o-t) for 18 h. In presence of light, none of the PIN markers showed any drastic differences between the DMSO (control) and C834. C834-treated seedlings in the dark show decreased levels of PIN2 at the plasma membrane (f), compared to control seedlings (e). Protein levels for the other PIN markers did not show major differences between control and treatment with C834. Scale bar = 10 μ m



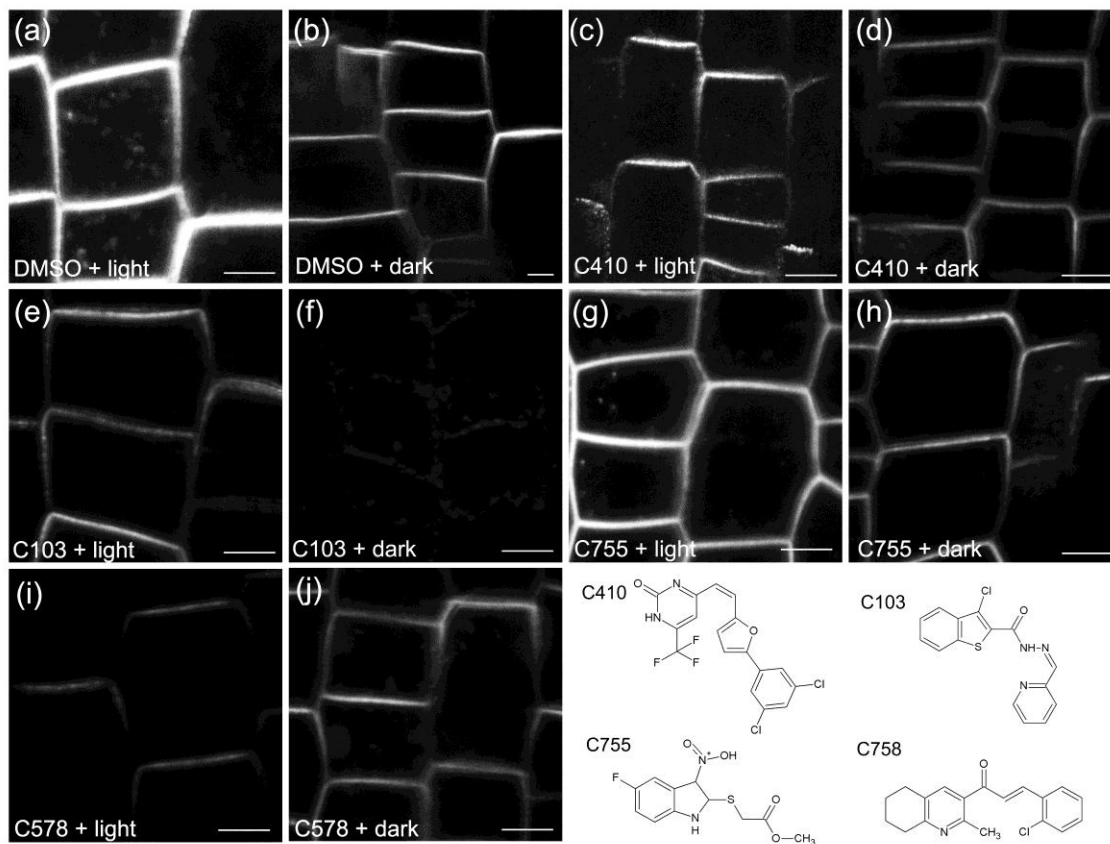
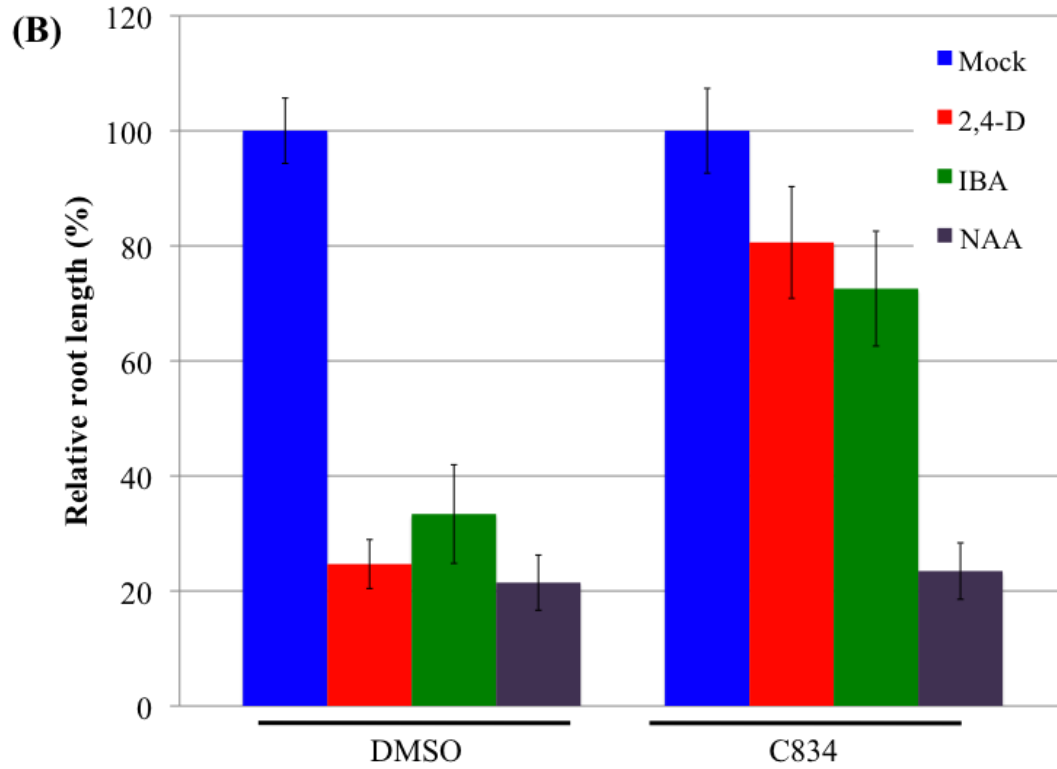
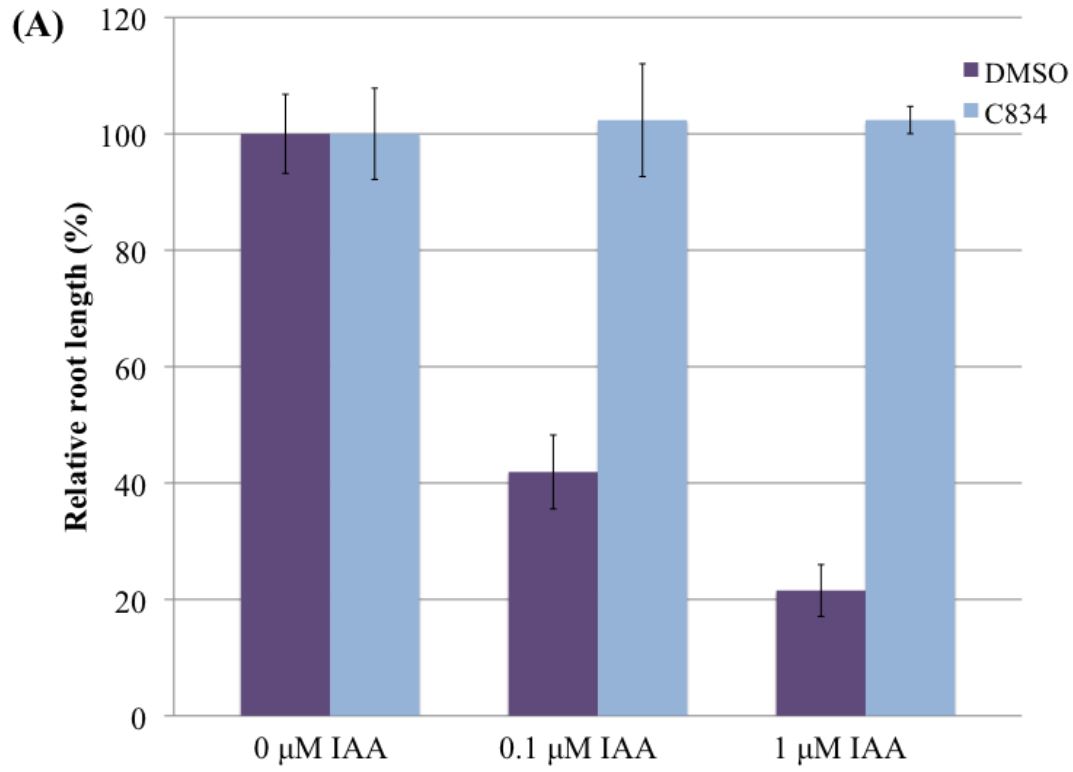


Figure 4. Effect of trafficking inhibitors on PIN2 localization. Three-day-old seedlings expressing PIN2-GFP were transferred to media containing either: (a, b) DMSO, (c, d) 62 μM C410, (e, f) 79 μM C103, (g, h) 88 μM C755, or (i, j) 40 μM C578, in the light or in the dark for 18 h. Only C103 seemed to decrease PIN2 levels in the dark (compare e with f), however, seedlings treated with this compound in the dark are usually sick, suggesting that the decrease in fluorescence may be due to cell death. Chemical structures of the inhibitors are shown. Scale bar = 10 μm

Figure 5. C834-treated seedlings show reduced sensitivity to exogenous auxins. Col-0 seeds were germinated and grown in media containing either DMSO or 55 μ M C834, and in the presence of auxins for 5 days. (A) Relative root length (expressed as a percent root length compared to the IAA-free control for either DMSO or C834) is plotted on the y-axis, and IAA concentration on the x-axis. Note that whereas IAA induces inhibition of primary root length in the DMSO control, seedlings grown in presence of both IAA and C834 do not show this classic phenotype. (B) Relative root length (expressed as a percent root length compared to the mock for either DMSO or C834) is plotted on the y-axis, and presence or absence of C834 for each auxin tested on the x-axis. Note that all auxins inhibit root length in control (DMSO) seedlings. However when C834 is present, the seedlings show a decrease in sensitivity to 80 nM 2,4-D and 1 μ M IBA, but not to 1 μ M NAA. Error bars represent standard error (SE). $15 > n < 25$.



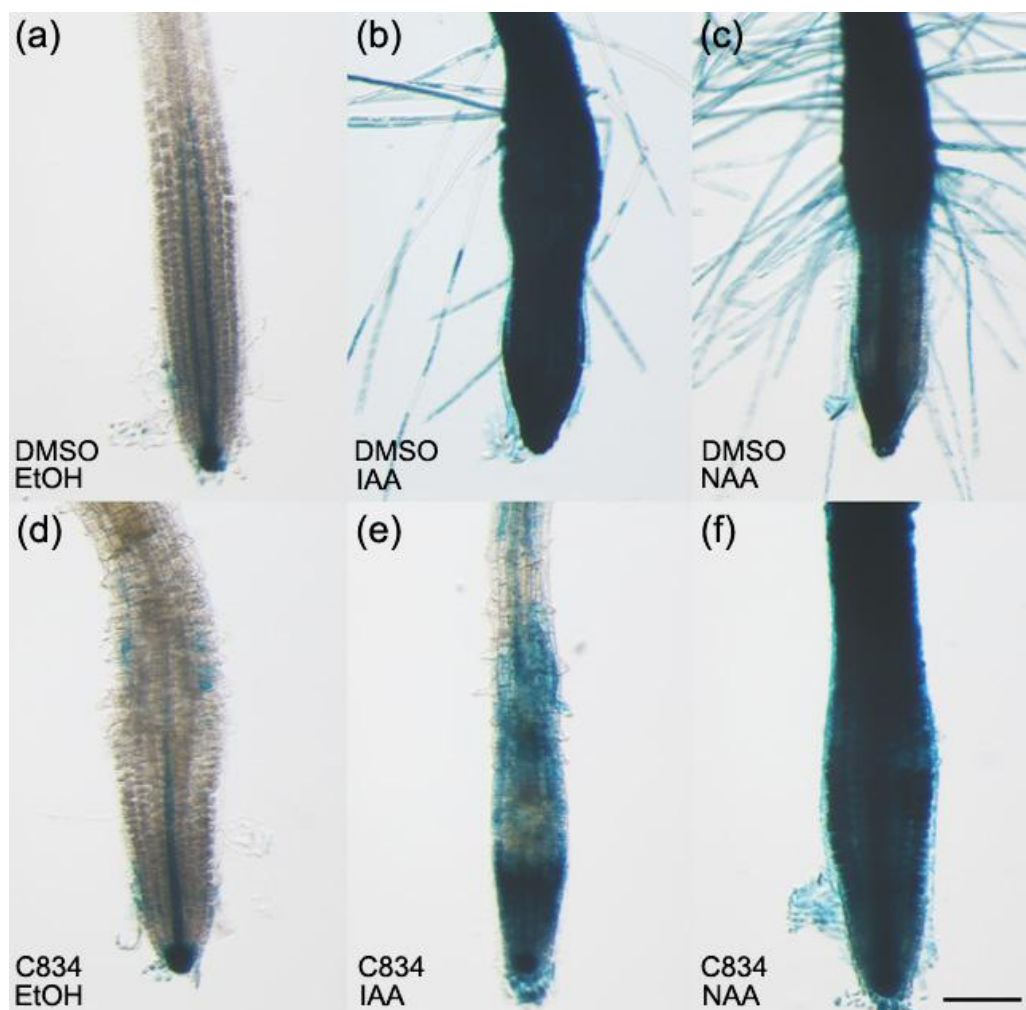


Figure 6. Histochemical analysis of *DR5::GUS* in C834-treated seedlings. Four-day-old seedlings expressing *DR5::GUS* were transferred to media supplemented with either DMSO or 55 μ M C834 for 4 hr. Seedlings pre-treated with DMSO were then transferred to media containing DMSO and either ethanol (a), 1 μ M IAA (b) or 1 μ M NAA (c). Seedlings pre-treated with C834 were transferred media to media containing 55 μ M C834 and either ethanol (d), 1 μ M IAA (e) or 1 μ M NAA (f). Scale bar = 200 μ m

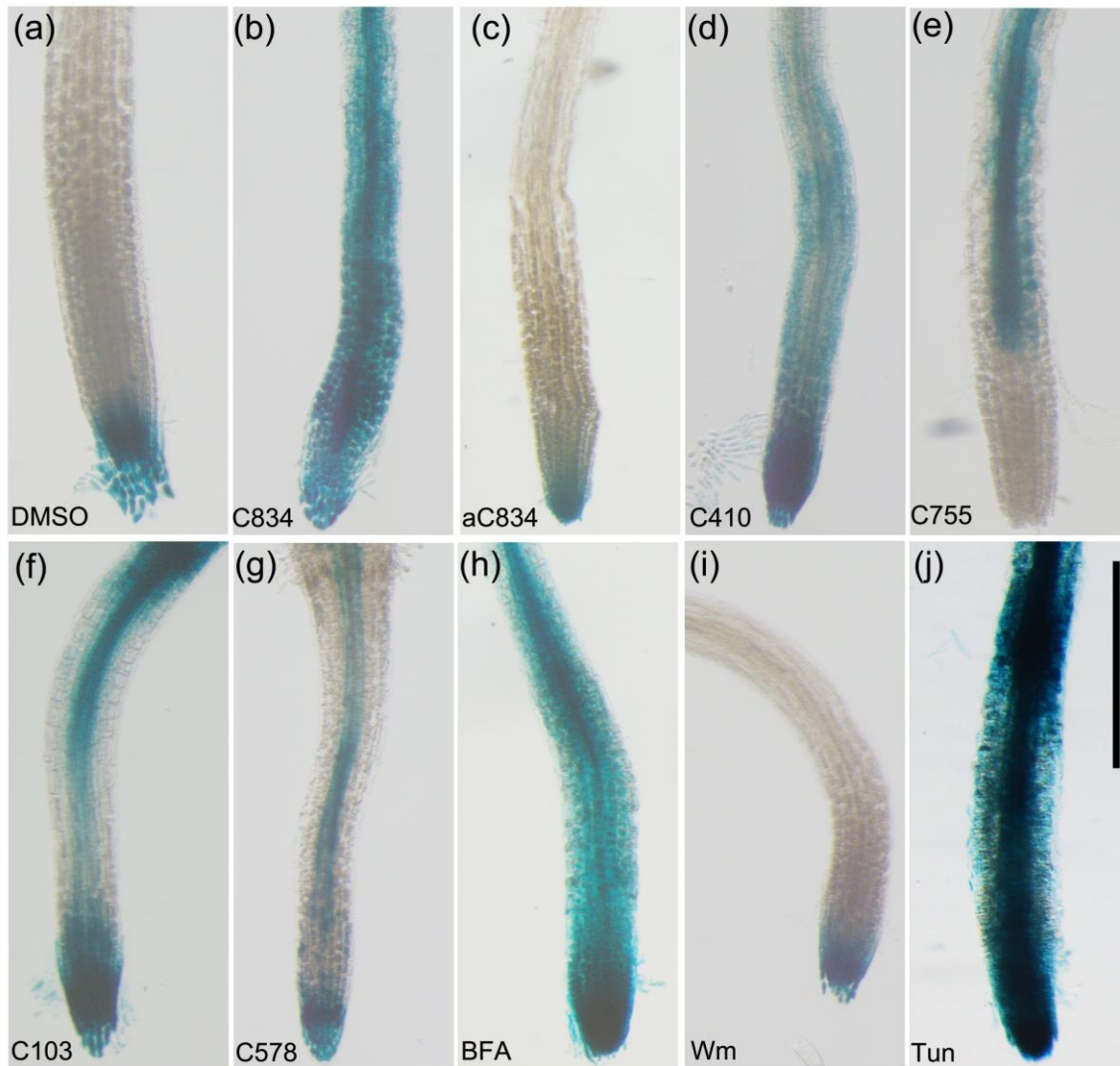


Figure 7. Expression of *ASAI::GUS* is induced by inhibitors of tonoplast trafficking.

Three-day-old seedlings expressing *ASAI::GUS* were transferred to media containing either (a) DMSO, (b) 55 μM C834, (c) 71 μM aC834, (d) 62 μM C410, (e) 88 μM C755, (f) 79 μM C103, (g) 40 μM C578, (h) 25 μM BFA, (i) 33 μM Wm and (j) 5 $\mu\text{g}/\text{mL}$ Tunicamycin for 48 h before tissue staining. Scale bar = 500 μm

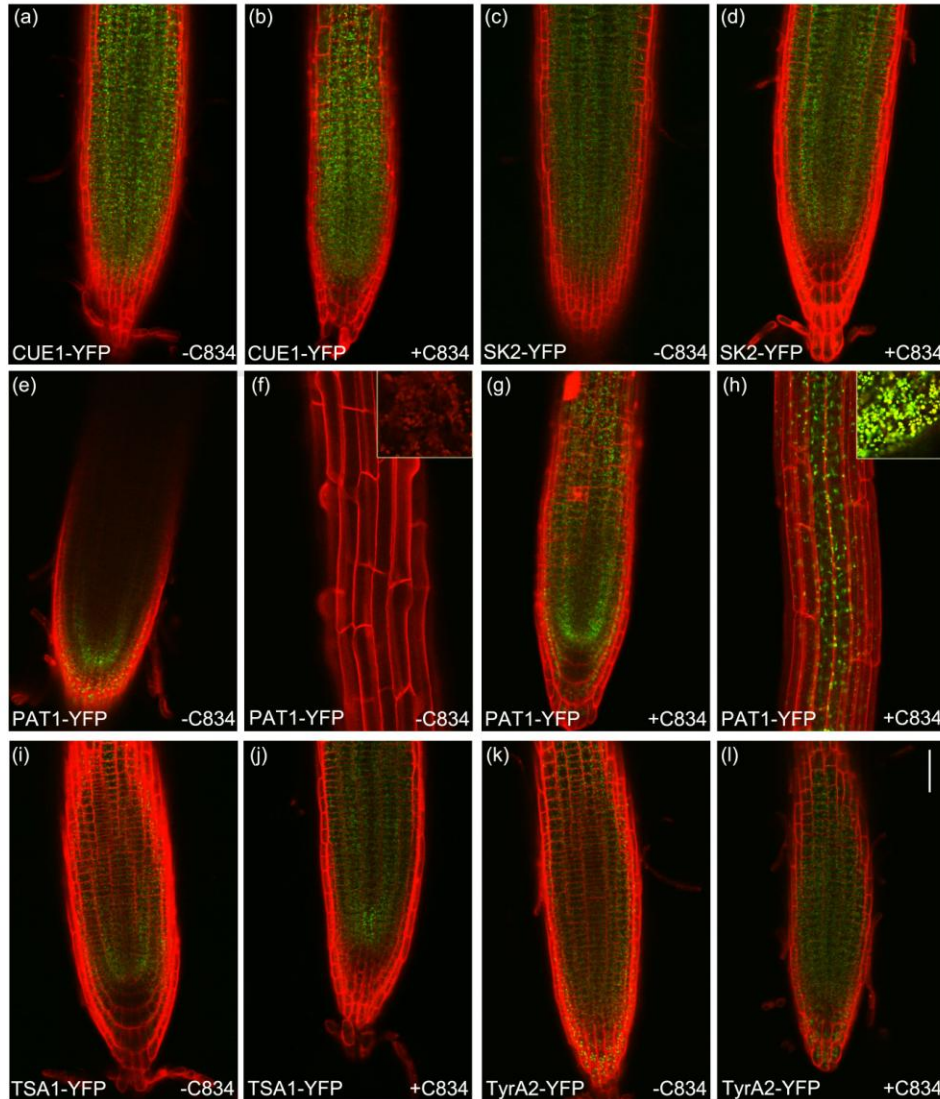


Figure 8. C834 induces the amounts of PAT1. 3-day-old seedlings expressing either CUE1-YFP (a-b), SK2-YFP (c-d), PAT1-YFP (e-h), TSA1-YFP (i-j) or TyrA2-YFP (k-l) were transferred to media containing either DMSO (-C834) or C834 for 48 h and imaged using confocal microscopy. Cell wall was stained with propidium iodide (red). Only PAT1-YFP shows a drastic increase in protein levels after C834 treatment (compare e-f with g-h). PAT1-YFP signal can be seen up to the hypocotyl after C834-treatment, where it co-localizes with the chloroplasts (h, inset). Bar = 200 μ m

CHAPTER 4

Summary of Research Outcomes and Next Steps in Research

4.1 Summary of research outcomes

- Five novel drug-like compounds that inhibit the trafficking of the tonoplast proteins GFP-TIP2;1 at the endoplasmic reticulum (ER) were identified.
- We identified C834, a specific inhibitor of the Golgi-independent pathway for tonoplast proteins.
- We demonstrated for the first time the existence of two pathways for the targeting of members of the Tonoplast Intrinsic Protein (TIP) family of aquaporins using *in planta* assays.
- We determined that TIP3;1 and TIP2;1 are targeted to the vacuolar membrane in a Golgi-independent manner, whereas TIP1;1 is targeted via a Golgi-dependent pathway (**Figure 1**).
- We showed that Brefeldin A inhibits ER-to-Golgi traffic in Arabidopsis hypocotyl cells.
- We showed that treatment with C834 in seedlings reduced sensitivity to exogenous auxins that require transport of the hormone.
- Our studies suggest a crossroad between the Golgi-independent route for tonoplast proteins and the vacuolar accumulation of the auxin transporter PIN2 in the dark.
- We showed that treatment with chemicals that inhibit the exit of proteins from the ER induce the transcription of genes in the tryptophan biosynthetic pathway.

4.2 Next steps for future research

- It is still unclear whether vesicles are able to bypass the Golgi and merge at an intermediate compartment, such as the *trans*-Golgi network or a prevacuolar compartment.
 - One way that this can be addressed is by tracking the intracellular movement of proteins from the ER to the vacuole using live cell imaging.
- The target(s) of our chemical inhibitors are still undiscovered.
 - Characterization of mutants that are resistant to the trafficking inhibitory effects of C834 will provide information of proteins involved in the targeting of tonoplast proteins.
- It is still unclear why PIN2-GFP levels are drastically reduced in the dark after C834 treatment.
 - Characterization of *C834-resistant* mutants would confirm a link between trafficking of tonoplast proteins and targeting of PIN2 to the vacuole in the dark.
 - Experiments using light signaling mutants will determine if the apparent enhancement of PIN2-GFP vacuolar accumulation requires specific components of this pathway.
- The role of tryptophan biosynthesis in ER-stress perception is not yet understood.

- Transcript levels analyses of chaperones can be assayed to further confirm the link between these two pathways.
- We can use mutants that are defective in the perception of the unfolded protein response (UPR) machinery and test the expression of tryptophan biosynthetic genes in these mutant backgrounds.

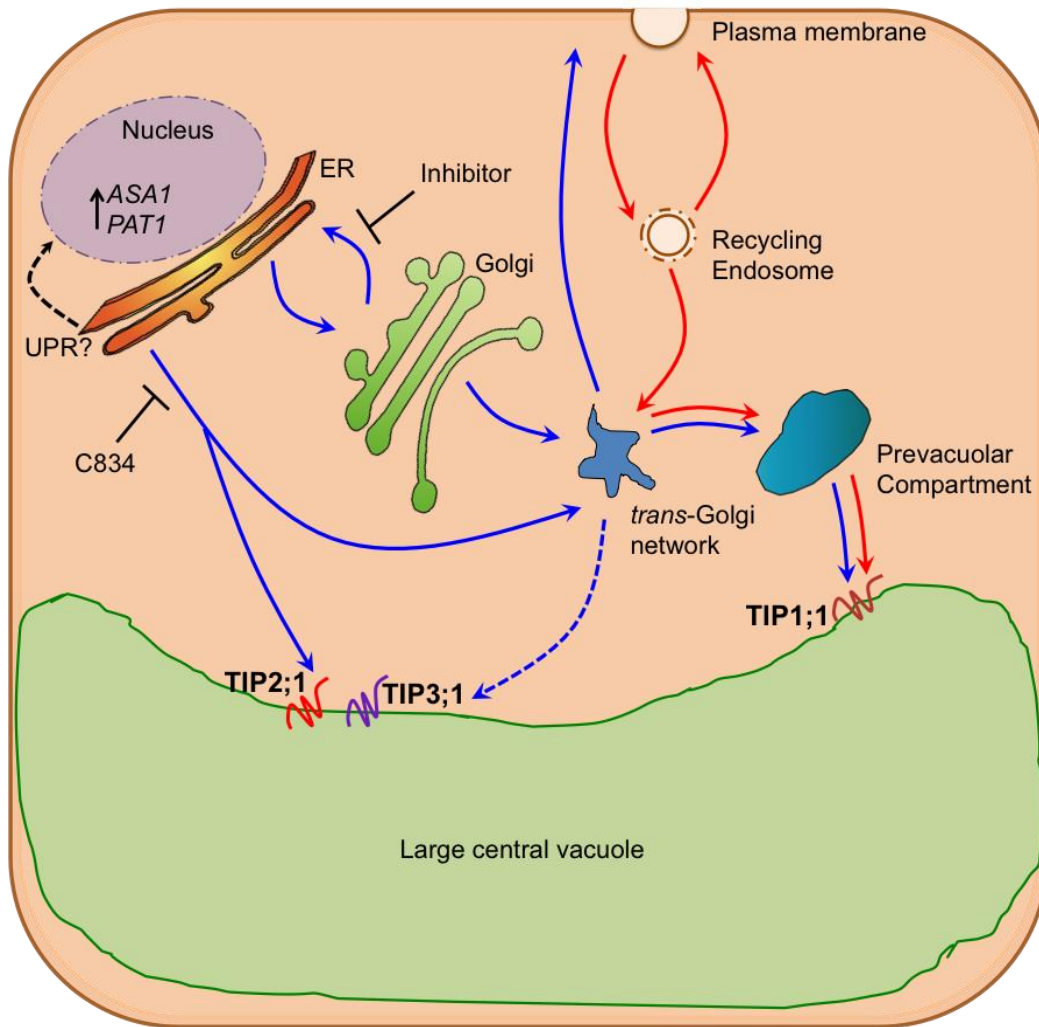
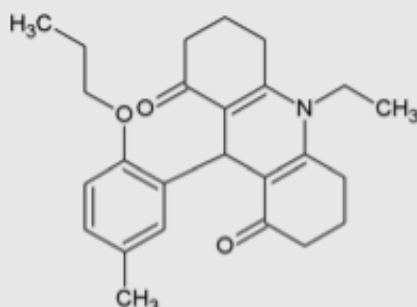
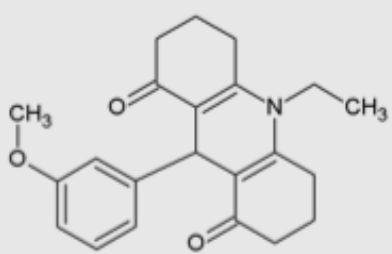


Figure 1. Updated model for the trafficking of TIPs and ER stress. TIP3;1 and TIP2;1 are targeted to the tonoplast via a Golgi-independent pathway that is C834-sensitive. In contrast, trafficking of TIP1;1 requires passage through the Golgi. Mis-localization of proteins at the ER results in induction of genes involved in tryptophan biosynthesis, including *ASA1* and *PAT1*, possibly via an unfolded protein response (UPR).

APPENDIX

TABLE 1A. Comparison of chemical structures of C834 and aC834.

C834	aC834
	
$C_{24}H_{28}BrNO_3$ 458 g/mol	$C_{22}H_{25}NO_3$ 351 g/mol
LogP = 3.60	LogP = 1.94
Rotable bonds = 2	Rotable bonds = 1

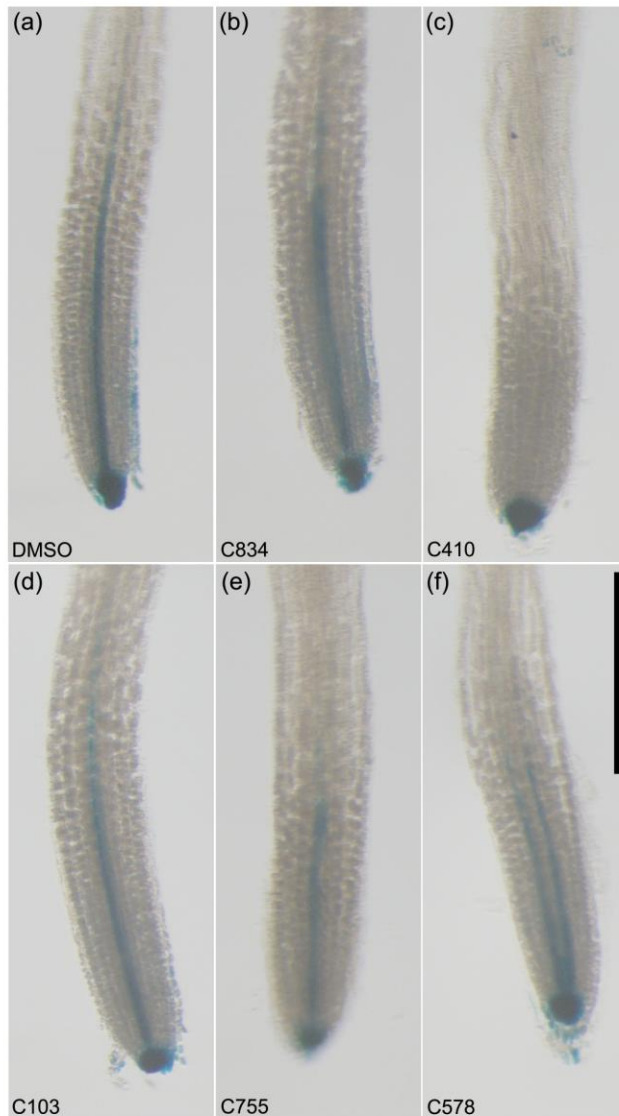


Figure 1A. Effect of chemical inhibitors on *DR5::GUS* expression. Three-day-old seedlings expressing *DR5::GUS* were transferred to media containing either (a) DMSO, (b) 55 μ M C834, (c) 62 μ M C410, (d) 79 μ M C103, (e) 88 μ M C755 and (f) 80 μ M C578 for 48 h. Only C410 reduces the auxin response at the root tip (compare c with a). Scale bar = 500 μ m

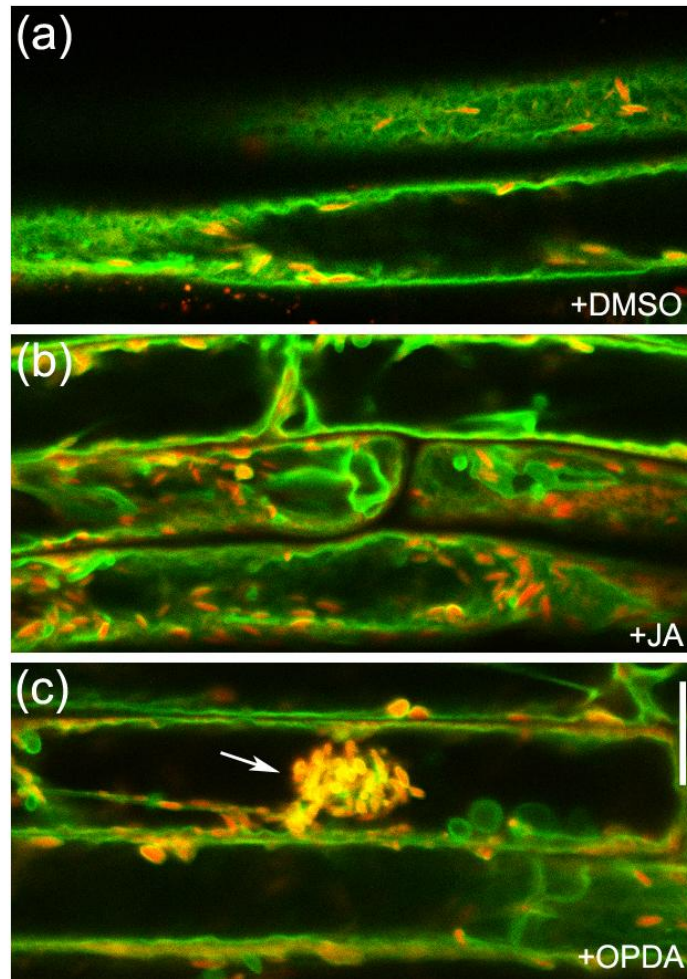


Figure 2A. Jasmonic acid induces the amounts of ER bodies. Three-day-old seedlings expressing both the tonoplast marker GFP-TIP2;1 (green) and the ER marker mCherry-HDEL (red) were transferred to media containing either (a) DMSO, (b) 50 μ M jasmonic acid (JA) or (c) 10 μ M OPDA for 48 h. Seedlings treated with JA or its precursor OPDA showed an increase in the number of ER bodies (compare b with c). Furthermore, the ER bodies seem to aggregate after OPDA treatment (c, arrows). No drastic phenotype was seen for the tonoplast marker. Scale bar = 10 μ m