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Membrane trafficking in plants: new discoveries and approaches

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The general organization and function of the endomembrane system is highly conserved in eukaryotic cells. In addition, increasing numbers of studies demonstrate that normal plant growth and development are dependent on specialized tissue and subcellular-specific components of the plant membrane trafficking machinery. New approaches, including chemical genomics and proteomics, will likely accelerate our understanding of the diverse functions of the plant endomembrane system.

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Abbreviations

ABA	abscisic acid
ARF	ADP-ribosylation factor
BFA	brefeldin A
CPY	carboxypeptidase Y
DRP1	DYNAMIN-RELATED PROTEIN1
EMS	ethane methyl sulfonate
ER	endoplasmic reticulum
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
OSM1	OSMOTIC SENSITIVE STRESS MUTANT1
PIN	PIN-FORMED
PM	plasma membrane
ROR2	REQUIRED FOR mlo RESISTANCE2
SA	salicylic acid
SCD1	STOMATAL CYTOKINESIS DEFECTIVE1
SNAP25	SYNAPTOSOMAL-ASSOCIATED PROTEIN25
SNARE	soluble-N-ethyl-maleimide-sensitive fusion protein attachment protein receptor
SYP121	SYNTAXIN OF PLANTS121
TGN	trans-Golgi network
TIP	TONOPLAST-INTRINSIC PROTEIN
sortin	sorting inhibitor

Introduction

In eukaryotes, the establishment and maintenance of intracellular organelles, including those of the endomem-

brane system, are dependent upon the proper trafficking of macromolecules and membrane fusion. The plant endomembrane system or secretory pathway, which is critical for biosynthetic and endocytic trafficking to the plasma membrane (PM) and vacuole, is comprised of the endoplasmic reticulum (ER), Golgi apparatus and intermediate organelles such as the prevacuolar compartment [1]. Far from being static, the morphology, localization and function of these endomembrane compartments are highly dynamic throughout plant growth and development. In addition to carrying out more 'traditional' roles (e.g. vacuole protein deposition and cell wall biosynthesis), secretory membrane trafficking mechanisms have recently been shown to be involved in a variety of plant-specific processes, including abscisic acid and auxin signaling, plant development, tropic responses, and pathogen defense. In this short update, we highlight a few recent advances in our understanding of unique plant-specific aspects of endomembrane system function. We also discuss new approaches for the study of secretory membrane trafficking and biogenesis within the endomembrane system.

Role of the secretory pathway during stress responses

Environmental stresses, be they biotic or abiotic, cause significant intracellular restructuring in plants [2]. Certain molecules need to be transported to, or removed from, a given compartment. Recent studies demonstrate that proteins that are required for secretory vesicle targeting and fusion play an important role in plant defense against pathogens and stress adaptation.

Pathogen responses

Central to the process of secretory vesicle targeting and fusion is a family of membrane-associated proteins known as SNAREs [3]. In general, fusion occurs through the formation of a four-helix-containing SNARE complex (SNAREpin); three of the helices are contributed by two or three SNAREs on the target membrane (t-SNAREs) and one by a SNARE on the donor vesicle (v-SNARE). The role that SNAREs play in membrane fusion, and the wide range of predicted genes encoding SNAREs and associated regulatory factors in *Arabidopsis*, has been reviewed extensively recently [3–5].

AtSNAP33, an *Arabidopsis* homolog of the mammalian t-SNARE SNAP25, is localized at the cell plate during cytokinesis and at the PM in non-dividing cells [6]. In addition to displaying slight defects in cytokinesis, *Atsnap33* mutants are extremely dwarfed and develop necrotic lesions, which resemble those induced by various

pathogens [6]. Transcription of AtSNAP33 is induced by challenge from several virulent and avirulent bacterial species and is correlated with the expression of the secreted pathogenesis-related protein PR-1 [7]. Induction of AtSNAP33 in infected tissues is salicylic-acid independent, whereas systemic induction is dependent on salicylic acid [7]. Taken together, these findings imply that AtSNAP33 is involved in the defense response. Consistent with this, functionally homologous SNAREs from *Arabidopsis* and barley, SYNTAXIN OF PLANTS121 (SYP121) and REQUIRED FOR mlo RESISTANCE2 (ROR2), respectively, are required for non-host resistance (i.e. basal defense) against powdery mildew [8^{••}]. SYP121/ROR2 were identified in genetic screens for *Arabidopsis* and barley mutants that have increased susceptibility to penetration and infection by powdery mildew. ROR2 interacts with the barley homolog of AtSNAP33, HvSNAP34, suggesting that it may function in exocytosis. However, morphological evidence indicates that SYP121/ROR2 may have other pathogen-defense-related roles, such as the production of large vesicles at infection sites that stain positively for hydrogen peroxide, a compound that can function in cell wall strengthening, defense signaling and antimicrobial activity [8^{••}].

ABA signaling and abiotic stress responses

Abscisic acid (ABA) is an important signaling molecule in the regulation of stomatal movements and responses to drought, cold and salt stresses [9]. The PM t-SNARE from tobacco, NtSyr1, plays a role in ABA responses in guard cells. Cleavage of NtSyr1 by *Clostridium botulinum* neurotoxin type C, as well as the introduction of the cytosolic domain of NtSyr1 into guard cells, inhibits responses to ABA that involve potassium and chloride channels [10]. Ectopic overexpression of the cytosolic fragment of NtSyr1 in transgenic plants also blocks SNARE-mediated secretory trafficking between the Golgi apparatus and the PM [11]. Whether NtSyr1 binds to and regulates the activity of potassium and chloride channels directly and/or mediates their intracellular trafficking, as has been demonstrated for mammalian syntaxin 1A, remains to be determined [12,13]. NtSyr1 is a homolog of SYP121, suggesting a general role for this t-SNARE in abiotic and biotic stress responses.

Similarly, another t-SNARE, OSMOTIC SENSITIVE STRESS MUTANT1 (OSM1)/SYP61, which is associated with the trans-Golgi network (TGN) and the prevacuolar compartment [14], controls ABA-mediated and non-ABA responses to salt and osmotic stress [15[•]]. OSM1/SYP61 was identified in a screen for salt-tolerance in *Arabidopsis*. Mutant *osm1* plants are sensitive to drought and various salts, and the stomata of the mutants display defects in ABA-induced guard-cell closure [15[•]].

A role for the small Rab GTPase AtRabG3e (from the Rab7 family of small GTPases) in salt and osmotic stress

responses has also been demonstrated recently [16]. Rab GTPases are a large family of endomembrane-compartment-specific small GTP-binding proteins that regulate secretory membrane trafficking, including the docking and fusion of transport vesicles [17]. Transgenic plants that constitutively express AtRabG3e accumulate high levels of salt in their vacuoles and have increased resistance to salt and osmotic stress [16]. Members of the *Rab7* gene family have been shown to function in the late steps of endocytosis in other eukaryotes [17]. Consistent with this, plants that overexpress AtRabG3e exhibit greater endocytic uptake of the lipophilic membrane probe FM1-43 than do wildtype plants [16]. These results provide further evidence of the role of the endomembrane system in responses to abiotic stresses.

ER bodies

Plant cells contain a variety of distinct ER-derived structures (~0.2–10 μm in size) that are much larger than COPII-coated ER-to-Golgi transport vesicles (~50–100 nm) [18]. Recent studies have shown that *Arabidopsis* contains unique ER-derived bodies known as ER bodies or precursor protease vesicles [19,20[•]]. These structures are coated with ribosomes and are primarily found in seedling epidermal cells, although their formation can be induced in rosette leaves of *Arabidopsis* under stress conditions [19]. Formation of ER bodies, which primarily contain proteases and glycosidases [19,20[•]], is under the control of NAI1, a putative basic loop-helix-loop transcription factor [21]. It is thought that under certain stress conditions (e.g. mechanical wounding), ER bodies fuse with the lytic vacuole tonoplast, thereby delivering their cargo to the vacuole lumen [18,20[•]]. The mechanisms that mediate the biogenesis of ER-bodies and their targeting and fusion with the vacuole tonoplast are currently unknown.

Polarized membrane trafficking

Cytokinesis

Cytokinesis and cell expansion are highly dependent upon polarized membrane trafficking. Plants utilize several distinct cytokinetic mechanisms. During 'conventional' somatic cytokinesis, new cell walls are formed after nuclear division. By contrast, in some other cell types, such as *Arabidopsis* syntical-type endosperm and pollen, new cell wall formation is independent of karyokinesis. Cytokinesis in these cells is 'unconventional', with multiple cell walls being assembled simultaneously between sister and non-sister nuclei. The formation of new cell walls during both somatic and syntical-endosperm cytokinesis involves cell plate assembly and maturation. Although the assembly of both types of cell plate is highly dependent upon polarized membrane trafficking and fusion, the formation of the two types of cell plates appear to involve morphologically distinct stages [22,23]. Two recent studies in which *Arabidopsis* meristematic cells and pollen were analyzed at

high-resolution by electron tomography of high-pressure frozen and freeze-substituted material have provided additional insight into the dynamic process of somatic cell plate formation and microsporocyte cellularization [24^{••},25^{••}]. It has been suggested that post-meiotic cellularization of microsporocytes, in contrast to cytokinesis in syntical endosperm, is mediated by the invagination of the PM (i.e. formation of a cleavage furrow) rather than by cell plate assembly. Electron tomographic analysis of *Arabidopsis* pollen development has revealed, however, that pollen cellularization is indeed mediated by the formation of a cell plate, albeit one that develops centripetally as opposed to in the centrifugal direction of cell plate maturation as observed in other cell types [25^{••}].

Cell plate intermediates

The formation of dumbbell-shaped cell plate membrane intermediates accompanies the fusion of individual somatic cell plate vesicles [23]. Formation of these structures is hypothesized to be mediated by the constrictive force of dynamins, which are mechanochemical GTPases that form multimeric rings around the necks of budding clathrin-coated vesicles and other membrane tubules [26]. Members of the plant-specific dynamin-related protein family, DRP1 (formally called *Arabidopsis* DYNAMIN-LIKE PROTEIN1 [ADL1]/phragmoplastin [27]), are necessary for the formation of somatic and syntical cell plates [28]. These proteins have been shown to form rings and spirals that constrict wide tubules of syntical-type cell plates during *Arabidopsis* endosperm cellularization [22]. Similar electron-dense rings and spirals have been observed in the neck region of the dumbbell-shaped somatic cell plate membrane precursors, but attempts to confirm by immunocytochemistry whether these rings or spirals are composed of DRP1A produced ambiguous results [24^{••}]. Constriction of fusing cell plate vesicles into dumbbell-shaped structures leads to a 50% reduction in the starting volume of these vesicles, and has been postulated to be critical for the assembly of stable cell plate domains [24^{••}]. Further analysis of cell plate formation in *drp1* mutants, which have broken and irregular cell walls [28], may reveal the biochemical mechanism for the formation of dumbbell intermediates and their role in cell plate maturation.

Cell expansion

Following cytokinesis, the expansion of the two daughter cells occurs through either diffuse polar or tip growth [29]. Diffuse polar growth, which occurs in the majority of plant cells, is characterized by the uniform incorporation of new PM and cell wall material across the expanding cell surface. By contrast, tip growth is mediated by the fusion of exocytic vesicles with the expanding apex of the cell, resulting in the unidirectional and highly asymmetric growth of cell types such as root hairs and pollen tubes. Like the process of cell plate assembly, diffuse and tip

growth are both dependent upon polarized membrane trafficking. Indeed, several membrane trafficking factors, including the Sec1-like protein KEULE [30], the putative Rab guanine nucleotide exchange factor (GEF) STOMATAL CYTOKINESIS DEFECTIVE1 (SCD1) [31[•]], and members of the DRP1 protein family [28,32], are common molecular components of the membrane trafficking machinery utilized during both polarized cell expansion and cytokinesis. In addition to inhibited cell plate assembly, *drp1* mutants display cell-specific defects in diffuse and tip-type polarized cell expansion; the cells of *drp1* mutants have abnormal cell walls and show a marked accumulation of PM, perhaps because of inhibited membrane recycling. One DRP1 isoform, DRP1C, accumulates in the tips of expanding root-hair cells [32]. Likewise, distribution of the *Arabidopsis* Rab GTPase RabA4b, which is associated with a novel secretory compartment that is distinct from the TGN, is highly polarized at the tips of growing root hairs [33]. The localization of DRP1C and RabA4b within the apex of the expanding root hairs is correlated with tip growth.

Asymmetric localization of proteins and sterols

Polarized membrane trafficking is also required to maintain the asymmetric localization of proteins within distinct domains of the PM. Vesicle trafficking between the PM and endosomes is critical for the proper localization and function of PIN-FORMED (PIN) and AUXIN-RESISTANT1 (AUX1) proteins, which are required for polarized auxin transport [34]. Recycling of these and other proteins from the endosomes back to the PM is actin-dependent and sensitive to the fungal metabolite brefeldin A (BFA), an inhibitor of ADP-ribosylation factor (ARF)-GEFs. ARF-type GTPases are critical regulators of the formation of secretory transport vesicles. The *Arabidopsis* protein GNOM is a BFA-sensitive ARF-GEF that is required for the proper localization of PIN1. Importantly, it has been shown that expression of a BFA-resistant form of GNOM, GN^{M696L}, in transgenic plants renders PIN1 trafficking and localization insensitive to BFA [35^{••}]. However, the trafficking of other PM proteins, including other members of the PIN protein family, remained sensitive to the drug [35^{••}]. These studies indicate that there are multiple and perhaps overlapping PM protein recycling pathways in plant cells that are regulated by distinct BFA-sensitive and BFA-resistant ARF-GEFs.

Membrane sterols are required for proper polarized secretory trafficking in animal cells [36,37]. Very little is known about sterol trafficking in plants, but it has been shown recently that sterol endocytosis in plants is dependent upon endosomes that contain ARA6 (a small Rab-type GTPase) and is mediated through cytoskeletal networks [38[•]]. BFA treatment of plants that expressed green fluorescent protein (GFP)-labeled ARA6 resulted in the co-localization of sterols, ARA6::GFP and PIN2

[38[•]]. In another study, a specific mutation in *STEROL METHYLTRANSFERASE1* (*SMT1*) of *Arabidopsis* caused several notable cell polarity defects [39[•]]. These defects included altered organellar position and mislocalization of the PIN1 and PIN3 proteins [39[•]]. However, polarized trafficking and localization of AUX1 are not affected in the *smt1* mutant, again highlighting the complexity of polarized membrane trafficking in plant cells.

New approaches to the study of trafficking in the endomembrane system

When compared to other eukaryotes that have completely sequenced genomes, *Arabidopsis* contains a relatively large number of genes that are predicted to be involved in membrane trafficking processes [40]. To fully understand the workings of the plant endomembrane system, we need to examine the function(s) of each of these genes. Knockouts for many of these genes are lethal and thus inherently difficult to study [32,41,42]. Hence, new approaches have been developed to study the functions of genes that are associated with membrane trafficking processes, several of which are highlighted in Figure 1.

Fluorescently tagged marker proteins

Fluorescently tagged proteins that label specific subcellular compartments can be used to study endomembrane trafficking and biogenesis. Avila *et al.* [43[•]] successfully demonstrated the use of a fluorescently tagged marker protein to study vacuole biogenesis in plants. Specifically, an *Arabidopsis* line that constitutively expresses GFP-labeled tonoplast-intrinsic protein (δ -TIP) was subjected to ethane methyl sulfonate (EMS) treatment. Normally, GFP:: δ -TIP specifically labels the vacuolar membrane. Using screens that were based on confocal microscopy, however, it was possible to identify a large number of mutants that have altered vacuolar morphology [43[•]]. One of the major biological observations from this work is that roots and aerial tissues apparently have tissue-specific mechanisms for membrane trafficking to the vacuole and/or for biogenesis of the vacuole. Studies are underway to identify the genes responsible for the observed phenotypes, some of which will undoubtedly identify components that are necessary for vacuolar trafficking and biogenesis.

The functions of about 35% of the genes in the *Arabidopsis* genome are unknown [44^{••}]. To determine their localization, a large-scale effort to tag each unknown protein with fluorescent markers (GFP, yellow fluorescent protein [YFP], and so on) has been undertaken [44^{••}]. Future studies, including the use of fluorescently tagged organelle-specific marker-protein lines [44^{••}], will provide more detailed analyses of the subcellular distribution of the tagged unknown proteins using standard colocalization and fluorescence resonance energy transfer (FRET) approaches.

Chemical genomics

Chemical genomics relies on the use of small organic chemicals to alter or inhibit biological processes [45]. Similar to the traditional use of conditional mutants, chemicals can be used to specifically regulate the onset of mutant phenotypes. The use of chemical genomics in plant systems is an emerging field that provides alternative approaches for the study of macromolecular trafficking. Raikhel and colleagues [46[•]] have recently used a large-scale yeast screen to identify fourteen compounds (known as sorting inhibitors [sortins]) that cause a vacuolar protein, carboxypeptidase Y (CPY), to be secreted. Two of these compounds also cause the *Arabidopsis* vacuolar homolog of CPY to be secreted and result in altered vacuolar phenotypes [46[•]]. In addition to CPY, several other proteins are secreted in the presence of Sortin1. Multiple pathways exist for sorting to the plant vacuole, and Sortin1 appears to affect only one of these pathways. EMS mutagenesis is now being used to isolate mutants that are resistant or hypersensitive to sortins. This approach is likely to identify genes that are involved in vesicle trafficking to the vacuole. Genetic approaches have been used successfully to identify the targets of bioactive chemicals in *Arabidopsis* [47]. The sortins could also be used as ligands to affinity-purify the potential targets of these drugs.

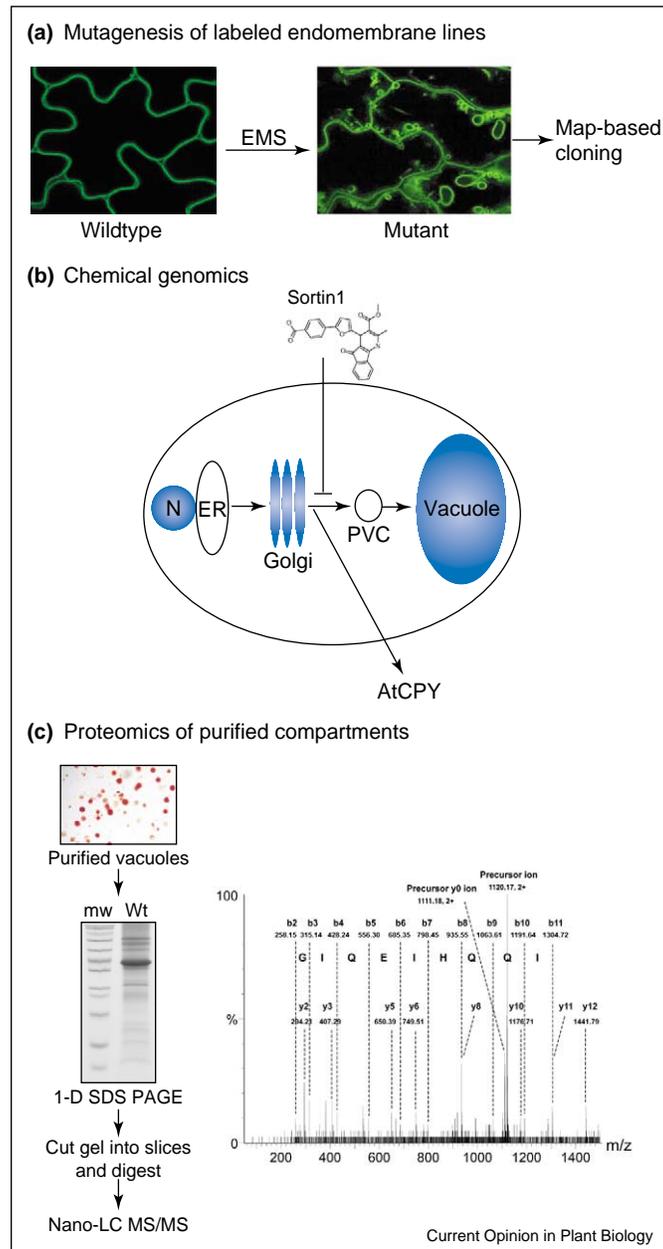
Proteomics

Proteomics approaches are required to gain a more global overview of the proteins that are required for endomembrane trafficking. As a first step to address vacuolar protein trafficking, vacuoles were purified from the rosette leaves of *Arabidopsis* and their soluble and membrane protein content was analyzed by nano-liquid chromatography/tandem mass spectrometry (nano-LC/MS/MS). A total of 402 proteins were identified from these analyses [48], including members of a putative tonoplast-localized SNAREpin complex, potential cargo proteins of various vacuolar sorting pathways (e.g. carboxy- and amino-terminal propeptide pathways [reviewed in [49]]) and many proteins of unknown function. Similar studies on other purified endomembrane compartments are likely to follow.

Conclusions and future prospects

Recent data suggest a myriad of roles for membrane trafficking components in plant development and environmental responses. The specific endomembrane components that are required in stress responses and polarized sorting, as well as the apparent tissue specificity of various membrane trafficking events, are among the interesting new findings summarized in this review. Although new approaches to study the endomembrane system will continue to provide useful insights, the use of systems biology approaches will be required to provide a full understanding and appreciation of the complexity of networks that regulate secretory membrane trafficking.

Figure 1



New approaches in studies of the plant endomembrane system. **(a)** GFP:: δ -TIP labeling of the vacuolar membrane (tonoplast) displays the *in-vivo* morphology of the vacuole in the epidermal cells of *Arabidopsis* cotyledons. EMS mutagenesis of the parental line, followed by high-throughput confocal microscopy screening, has resulted in the identification of a large number of lines that have altered vacuolar phenotypes [43*]. Map-base cloning is likely to identify genes that are required for vacuolar biogenesis. **(b)** Using a high-throughput chemical genetics screen, a large number of sorting inhibitors (sortins) have been identified that cause CPY (normally a vacuolar protein) to be secreted in yeast [46*]. In *Arabidopsis*, Sortin1 is found to block the transport of CPY from the Golgi to later compartments, resulting in its secretion. Mutant screens are being performed to identify lines that are resistant or hypersensitive to sortins. **(c)** Vacuoles were isolated from *Arabidopsis* rosette leaves and vacuolar protein was subjected to 1D-SDS PAGE (left panel) followed by nano-LC/MS/MS analyses. The right panel represents the fragmentation (MS/MS) spectrum of a peptide derived from a low-abundance tonoplast SNARE protein, SYP22 (**At5g46860**). The precursor ion was doubly charged with an m/z of 1120.17. The spectrum was matched by MASCOT database searching to a peptide, EQGIQEIHQIGEVNEIFK (amino acids 182–200), of SYP22. All matched y - and b -series ions are labeled. Amino-acid residues are assigned on the basis of the mass ladders generated by the b -series ions. From these studies, a total of 402 proteins were identified in the *Arabidopsis* vegetative vacuole [48]. The purification of specific vesicles from *Arabidopsis* for similar analyses is in progress.

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