Chemical Genetic Dissection of Membrane Trafficking

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Abstract

The plant endomembrane system is an extensively connected functional unit for exchanging material between compartments. Secretory and endocytic pathways allow dynamic trafficking of proteins, lipids, and other molecules, regulating a myriad of biological processes. Chemical genetics—the use of compounds to perturb biological processes in a fast, tunable, and transient manner—provides elegant tools for investigating this system. Here, we review how chemical genetics has helped to elucidate different aspects of membrane trafficking. We discuss different strategies for uncovering the modes of action of such compounds and their use in unraveling membrane trafficking regulators. We also discuss how the bioactive chemicals that are currently used as probes to interrogate endomembrane trafficking were discovered and analyze the results regarding membrane trafficking and pathway crosstalk. The integration of different expertises and the rational implementation of chemical genetic strategies will improve the identification of molecular mechanisms that drive intracellular trafficking and our understanding of how trafficking interfaces with plant physiology and development.

Contents

Cargo: a protein or lipid that is transported from one location to another in vesicles

ER: endoplasmic reticulum

TGN/EE: *trans*-Golgi network/early endosome

MVB/PVC: multivesicular body/prevacuolar compartment

PM: plasma membrane

Compartment maturation: a change in compartment identity that results from the addition of components that modify membrane composition and function

1. INTRODUCTION

Eukaryotic cells are composed of different membrane-delimited compartments that are functionally connected and constantly interact in order to exchange material. Additionally, an array of dynamic vesicles transport lipids as well as soluble, membrane-embedded, and membrane-associated proteins and other molecules. In this review, we focus on the trafficking of such cargoes within the essential compartments of plant cells, the so-called endomembrane system. This system is critical for cellular organization, which ultimately affects the structuring of tissues, organs, and the whole organism—processes that underlie many aspects of plant development and physiology.

The endomembrane system comprises a subset of compartments (**Figure 1**): the endoplasmic reticulum (ER), the Golgi apparatus, the *trans*-Golgi network (TGN), the vacuole, and the endosomal compartments. The latter include the early endosome (EE), which in plants is equivalent to the TGN (22, 108), and the late endosome or multivesicular body/prevacuolar compartment (MVB/PVC). The plasma membrane (PM) and the external domains of the cell, composed of the apoplast and the cell wall, are natural extensions of the endomembrane system, as these are the final destinations for many of the molecules that are trafficked through the endomembrane network (**Figure 1**). Although functional connections are present between the endomembrane system and peroxisomes, mitochondria, nuclei, and chloroplasts (31, 44, 51, 74, 97), these organelles are not canonically considered part of the endomembrane system.

Exchange of material between compartments is believed to proceed by at least two mechanisms that coexist and may cooperate with each other: vesicle or tubule trafficking and compartment maturation (**Figure 1**). Plant cells have different types of vesicles (reviewed in 67); morphologically, the vesicles differ in size, shape, and coating components, but more importantly, they differ in their cargoes and in the journey they take to deliver those cargoes (**Figure 1**). The existence of

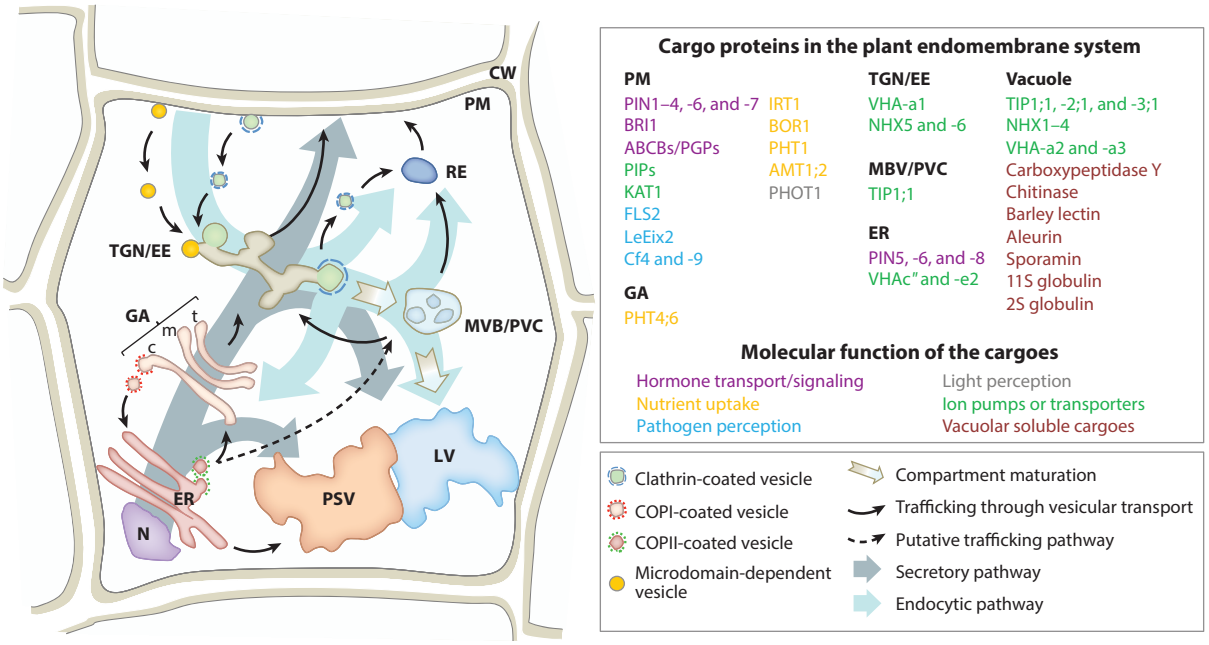

Figure 1

Schematic view of a plant cell, depicting the endomembrane system compartments and their interconnection through vesicular transport and endosomal maturation. Arrows indicating trafficking directions represent the secretory and endocytic pathways. De novo synthesized cargo proteins are targeted by different pathways and are ultimately delivered to resident compartments by the secretory pathway. The endocytic pathway internalizes PM cargoes through regulated trafficking and delivery to endosomal compartments or to the vacuole. Clathrin-coated and microdomain-dependent (clathrin-independent) vesicles mediate endocytosis from the PM. Clathrin-coated vesicles formed at the TGN/EE mediate secretory trafficking to the PM and endocytic recycling pathways. In the secretory pathway, COPII- and COPI-coated vesicles mediate anterograde trafficking from the ER to the GA and retrograde trafficking from the GA to the ER, respectively. Also shown are the principal resident compartments and molecular functions of a selected set of cargo proteins identified in plants that have been used to study the endomembrane system. Abbreviations: CW, cell wall; ER, endoplasmic reticulum; GA, Golgi apparatus [with *cis* (c), medial (m), and *trans* (t) subcompartments]; LV, lytic vacuole; MVB/PVC, multivesicular body/prevacuolar compartment; N, nucleus; PM, plasma membrane; PSV, protein storage vacuole; RE, recycling endosome; TGN/EE, *trans*-Golgi network/early endosome.

tubules has been shown in animal cells but is still controversial in plants (81). Therefore, we refer to delivery structures as vesicles for the remainder of this review.

The second process, compartment maturation, occurs as a result of the ability of compartments to change their identity. Organelles acquire new properties that are conferred by the recruitment of protein effectors and changes in their membrane composition, which converts them into another compartment class. In plants, the TGN/EE matures by gaining the proper machinery to become the MVB/PVC (17, 88). Similarly, the Golgi cisternae (i.e., the *cis*, medial, and *trans* subcompartments; **Figure 1**) mature progressively, as described for yeast and animals (19, 40).

Cargo proteins have a sort of bar code that identifies their destination. This bar code is known as the sorting signal and usually corresponds to a short peptide sequence within the cargo. Cargo proteins are packed into vesicles that bud off from a donor compartment. Such vesicles travel to find a target acceptor compartment, which is followed by fusion. Vesicle formation, trafficking, and fusion as well as compartment maturation are carried out by a set of proteins that we refer to here as trafficking machinery. This machinery includes vesicle-coating proteins; adaptor proteins and cargo receptors; and other accessory proteins, such as small GTPases, cytoskeleton

Trafficking machinery: a set of proteins that drive, execute, and regulate trafficking of vesicles and tubules within the endomembrane system

Cargo receptor: a transmembrane protein in which the luminal portion recognizes and binds a soluble cargo protein, allowing it to be packed into a vesicle

binding proteins, and phosphoinositide-metabolism enzymes (5, 63). Several detailed reviews have discussed the structure, mechanism, and cellular function of the main set of plant trafficking machinery proteins (39, 70, 104).

The proteins that constitute each endomembrane system compartment, known as resident proteins, are synthesized by ribosomes attached to the ER. In this compartment, de novo synthesized proteins acquire their energetically stable folded conformation and posttranslational modifications (10). From there, they are guided through a particular trafficking pathway to the final subcellular location where they carry out their function. This anterograde trafficking route is termed the secretory pathway (**Figure 1**). The diversity of final destinations along the secretory pathway implies the existence of at least one trafficking route for each compartment. Different proteins targeted to the same compartment may follow different trajectories involving different routes and intermediate stations (40, 42, 47, 68, 76).

After synthesis, protein cargoes follow the secretory pathway to their resident compartment (**Figure 1**). From there, they can also be trafficked to a different compartment. For instance, using a pathway known as endocytosis, PM proteins are internalized into vesicles that are further transported to other destinations, including the TGN/EE and MVB/PVC (**Figure 1**). Some proteins go from either the TGN/EE or MVB/PVC back to the PM by a recycling route. For others, the endocytic pathway ends at the vacuole, where they are degraded (4, 49, 50, 98). Depending on the nature of the protein, endocytic trafficking can be constitutive (33, 98) or induced by physiological and environmental factors (4, 77, 98). Endocytosis regulates the abundance of receptors or transporters at the PM as well as several signaling mechanisms that mediate sensing or responses to the environment, regulating nutrient uptake, hormonal transport, and/or signaling (4, 6, 48, 98). Therefore, the route taken by a particular cargo depends on its molecular function and the cellular processes involved. For a complete review of plant endocytosis, cargoes, and the corresponding trafficking machinery, we highly recommend the recent review by Paez Valencia et al. (63).

Most of the molecular functions of the trafficking machinery members are conserved throughout eukaryotes. In plants, the proteins that form part of the trafficking machinery are part of large gene families. Distinct members of the gene families often participate in tissue-specific mechanisms (86, 94, 105). Furthermore, plants display peculiarities regarding the different trafficking pathways, types of cargoes transported, and compartment identity and function, increasing the complexity of the endomembrane system (32, 43, 68, 81). This complexity implies that the trafficking machinery is selective, accurate, specific, and tightly regulated. In the following sections, we review the strategies for studying the endomembrane system and its complexity. We focus on how chemical genetics (**Figure 2**) provides unique and powerful approaches to tackle the challenges of plant cell biology.

Resident proteins: the constituents of endomembrane system compartments

Secretory pathway: the route taken by de novo synthesized proteins from the ER to the location where they carry out their function

Endocytic pathway: the route taken by PM-localized proteins to intracellular compartments (the endosomes and the vacuole)

Chemical genetics: a combination of approaches that use small compounds to perturb a particular biological pathway and identify the gene products involved

2. STRATEGIES FOR STUDYING MEMBRANE TRAFFICKING

Many modern technologies and resources were first developed using *Saccharomyces cerevisiae* as a biological model, and the study of the endomembrane system was no exception. *S. cerevisiae* is a very simple unicellular eukaryotic system and has many common pathways that are conserved in multicellular and more complex organisms (54). Schekman and colleagues (58–60) used a combination of biochemical, microscopic, and genetic approaches to isolate *sec* mutants and assign the underlying trafficking machinery genes to sequential steps in the secretory pathway. Since then, knowledge of the molecular players involved in different trafficking pathways in other organisms, including plants, has grown explosively. However, the greater complexity and larger number of trafficking machinery genes in plants compared with yeast complicate their discovery using traditional reverse genetic approaches.

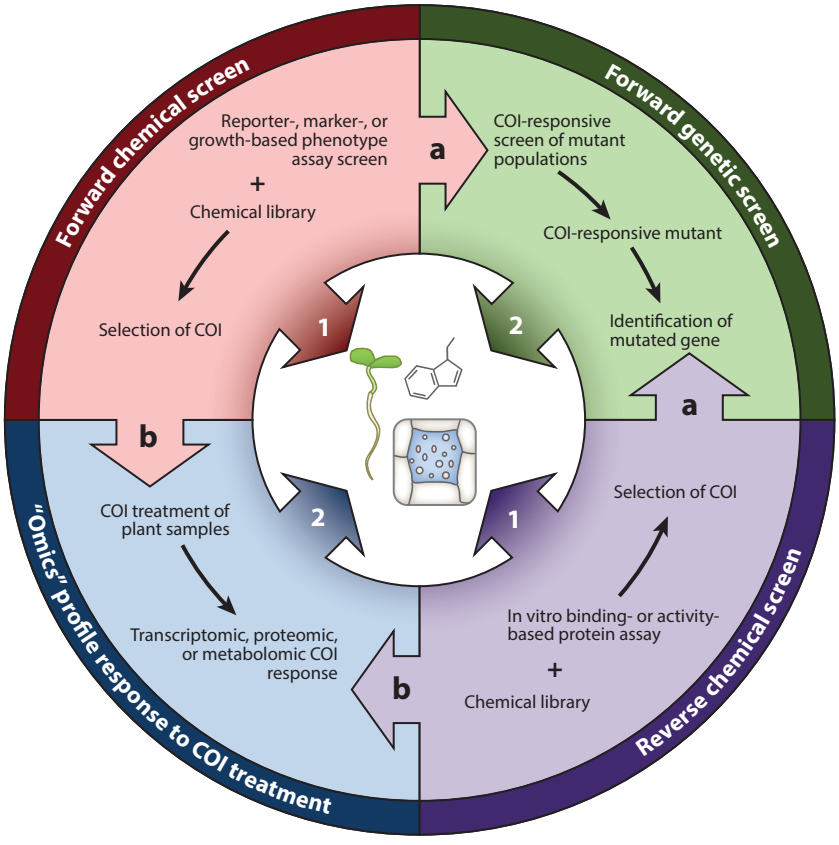

Figure 2

The interconnected strategies of chemical genetics. The use of small compounds as tools to study a biological process involves a set of interconnected strategies with complementary purposes and outcomes. A forward chemical screen (*upper left*) uses chemical libraries to identify a COI that is able to perturb a biological process, resulting in an altered phenotype at the molecular, cellular, developmental, or physiological level. Similarly, a reverse chemical screen (*lower right*) is used to obtain COIs that directly affect a particular target molecule (usually a protein) by interfering with its function. Both forward and reverse chemical screens identify COIs that can be directly used to study biological pathways (*arrows labeled 1*). As complementary experimental strategies, COIs may be used as the starting point for performing a forward genetic screen (*upper right*; *arrows labeled a*) or in a high-content profile of transcriptomics, proteomics, or metabolomics in response to COI perturbation (*lower left*; *arrows labeled b*). These last two approaches lead to the identification of COI-cognate target molecules and/or molecular players and pathways related to the COI-target pathway (e.g., through the discovery of COI-resistant mutants or the differential accumulation of certain transcripts, proteins, or metabolites) (*arrows labeled 2*). Abbreviation: COI, compound of interest.

2.1. *Arabidopsis thaliana*, the Workhorse in Plant Genetics

Since the introduction of the widely used model plant *A. thaliana*, genetics and cell biology have been used systematically to study plant physiology, growth, and development (54). The amenability of *A. thaliana* to genetic experimentation, including the plethora of mutants and transgenic fluorescent marker lines, has helped to establish the current view of the endomembrane system in plants (**Figure 1**), and the feasibility of large-scale screens has made this model organism the workhorse in plant genetics (2).

Forward and reverse genetics have been the traditional routes for assigning gene function. After the *A. thaliana* genome sequence was released, new strategies led to the widespread use of genetics to understand all aspects of plant biology (2, 8). Insertional mutagenesis (1, 89) facilitated reverse genetic approaches by selecting putative related genes based on known pathways in other organisms (e.g., yeast) for subsequent analysis of the phenotypes of individual plants. The drawbacks of such classical genetic approaches include complications related to (*a*) the lethality of mutations in essential genes and (*b*) gene redundancy, which is present in almost all gene families with homology to yeast *sec* genes. Nonetheless, many aspects of the functional importance of plant endocytosis, protein secretion, vacuolar morphology and targeting, and endosomal sorting have been studied using genetic approaches. As discussed below, *A. thaliana* genetics has been essential in establishing the current view of the plant endomembrane system.

Forward genetics: the identification of a gene responsible for a particular phenotype of a mutant

Reverse genetics: the identification of a rendered phenotype that results from mutation of a particular gene

Classical genetics: the use of mutants to understand gene function, including forward and reverse genetics

HTS: high-throughput screen

2.2. Shine On, You Crazy Cell!

Green fluorescent protein (GFP) was first identified in 1962 but was not introduced into *Arabidopsis* plants until the 1990s (34). GFP and its spectral variants made possible the illumination of organelles and proteins in the intracellular membranes, permitting the in planta visualization of protein trafficking and organelle morphology dynamically in vivo. Countless examples of the successful use of fluorescently tagged proteins have helped pave the way for the rich understanding of the plant endomembrane system (11), and many applications of this technology are rapidly emerging (92, 109). Additionally, fluorescent versions of markers for different endomembrane compartments are instrumental in understanding biological processes that are not necessarily directly related to the intrinsic function of the marker protein itself. **Figure 1** presents several cargo proteins that have been used to study the endomembrane system.

Fluorescently tagged protein marker lines have been used as phenotyping tools in high-throughput screens (HTSs). In such HTSs, ethyl methanesulfonate (EMS)–mutagenized seedling populations are screened for defects in protein trafficking, organelle morphology, and organelle dynamics, helping to unravel different aspects of endomembrane trafficking pathways and to discover new trafficking machinery components (117). Plant cell biology research has moved beyond the observation of single phenomena, allowing the screening of mutagenized plant populations in combination with chemical libraries to shed light on complex biological questions that could not be investigated previously.

2.3. The Druggable Plant Endomembrane System

Many studies have demonstrated the essential role of endomembrane trafficking in plants. However, its very importance creates an irritating difficulty for cell biologists: Mutations in genes that encode trafficking machinery proteins often lead to lethality at the embryo stage or at early stages of seedling development. When feasible, investigators therefore must design inducible or partial loss of function; however, owing to the extensive gene redundancy in plants, loss of function rarely renders detectable phenotypes, and multiple mutants are thus required for analysis. Neither of these problems is easy to overcome in a reasonable time frame.

The systematic use of chemical compounds to study biological phenomena is called chemical biology. The use of small compounds that affect endomembrane system processes at different steps has been instrumental in uncovering the regulatory mechanisms involved in intracellular transport. Several chemicals widely used in cell biology have been adopted by plant scientists based on their well-described effects on yeast and animal cells. The examples described below, which are now commonplace, give a glimpse into what can be achieved by using chemical tools to study the endomembrane system.

Brefeldin A (BFA) is a fungal metabolite that was isolated independently by several research groups from 1958 to 1970 (7). It initially captured the attention of researchers because of its interesting capacity to inhibit virus multiplication, in addition to its antifungal and antibiotic properties. After the discovery that BFA impedes protein transport from the ER to the Golgi apparatus, perturbing Golgi apparatus function and morphology, much attention was given to its intracellular effects on protein synthesis and transport along the secretory pathway in plant cells (68). BFA was shown to specifically inhibit the activity of the guanine nucleotide exchange factor (GEF) for the ADP-ribosylation factor (ARF) (80, 87). These early results proved the importance of determining the mode of action (MoA) of BFA, as understanding the molecular effects of the drug would later have a profound impact on efforts to unravel endomembrane dynamics.

MoA: mode of action

Bioactive chemical: a synthetic or natural organic compound that can interfere with a biological process

In *A. thaliana*, the broad use of BFA began when Busch et al. (12) identified an ARF-GEF protein with high homology to yeast Sec7p as the cognate target of BFA. A BFA-defective-response mutant pointed to the close relationship between endomembrane trafficking and auxin transport (12, 93), but the exact nature of this relationship came to light when Geldner et al. (33) demonstrated that auxin transport facilitator PIN-FORMED (PIN) proteins constitutively traffic from the PM to internal compartments in a BFA-dependent manner. It is now clear that *A. thaliana* uses a BFA-sensitive ARF-GEF, the GNOM protein, for protein trafficking from endosomes to the PM.

BFA became the favorite compound for studies of *A. thaliana* endosomal trafficking. BFA interferes differentially with constitutive PIN1 endocytic recycling depending on the concentration of the drug (33, 46). These differential subcellular responses were the basis for forward genetic screens to find new regulators of PIN trafficking machinery and polarity establishment (30, 99, 100). BFA is clearly an excellent example of the results that can be achieved by using a bioactive chemical to understand important aspects of endomembrane trafficking and their impact on signaling pathways.

Other successful chemical tools are broadly used to study the plant endomembrane system. Tyrphostin A23 (TyrA23) was described as an inhibitor of endocytosis of the transferrin receptor in mammalian (3, 16) and plant cells (62). A study by Crump et al. (16) showed that it inhibits the interaction between the ADAPTOR COMPLEX 2 (AP2) subunit μ2 and PM receptors in vitro. However, explaining its inhibitory effect on clathrin-mediated endocytosis has yet to unequivocally demonstrate AP2 μ2 as the TyrA23 target in vivo. Nevertheless, TyrA23 has been used for more than a decade as an endocytosis inhibitor to demonstrate the dynamic nature of PM protein trafficking (14, 25, 29). A recent report showed that TyrA23 inhibits endocytosis because of its mitochondrial uncoupling activity, increasing cytosolic pH and affecting several protein components of the clathrin-mediated endocytosis machinery (20). These results shed light on the nonspecific effects of TryA23 action on endocytosis in *A. thaliana* (20), highlighting the myriad of alterations a chemical may exert in vivo despite the clear specific effect it may exhibit in vitro. Therefore, results coming from the use of TyrA23 should be carefully interpreted.

Another compound, concanamycin A, which is an inhibitor of vacuolar ATPases (38), has been extremely informative in understanding vacuolar ATPase function in plant vacuole morphology and trafficking (22, 23). In a study using concanamycin A, Viotti et al. (108) proposed that the TGN/EE is the compartment where the secretory and the endocytic pathways merge, highlighting a dual role of this plant organelle. Other good examples of chemicals originally characterized in models other than plants include wortmannin, a well-known phosphatidylinositol kinase inhibitor that has been used to study MVB/PVC and vacuolar trafficking in plant cells (53, 103), and dynasore, a small molecule that inhibits dynamin function (52).

2.4. Chemical Genetics: Shedding Light on the Dark Side of the Cell

Forward chemical genetics: the identification of bioactive compounds that affect a biological process

Reverse chemical genetics: the identification of bioactive compounds that bind to a particular protein and affect its function

Lead compounds: small compounds that arise from a chemical genetic screen and exhibit specific biological activity

COI: compound of interest

The use of chemical biology as a high-throughput approach that adds to the knowledge of "omics" strategies has been termed chemical genomics (37, 79). Chemical genetics is the use of chemical molecules (mostly organic) to understand biological processes from the molecular point of view and to identify the function(s) of gene products (61, 102). The yield of both forward and reverse chemical genetics is a set of small compounds that cause biological perturbations manifested as reproducible phenotypes. These are the so-called lead compounds, from which usually just a few compounds of interest (COIs) are selected for further characterization (**Figures 2** and **3**).

Chemical genetic approaches have several advantages. A particular chemical compound could inhibit or enhance a biological process by working as a pathway antagonist or agonist. The underlying principle is that a particular bioactive compound physically interacts with a target molecule (likely a protein) and impairs its function. In this sense, chemical perturbation is similar to a genetic mutation. COIs are tools of great interest because they allow investigators to interfere with biological pathways in ways that classical genetics cannot accomplish. This point is especially important for studies of the endomembrane system, which require a high degree of spatial and temporal regulation (and often reversibility). Nevertheless, COIs induce cellular and/or physiological phenotypes that can be equivalent to phenotypes of genetic mutants, thus offering a broad spectrum of possibilities. A COI also has the potential to interact with multiple proteins, particularly related proteins belonging to a gene family, thereby overcoming the challenges of gene redundancy. Ideally, COIs perturb protein function rapidly in a dose-dependent and reversible manner, allowing investigators to tune the severity of the phenotype and turn their effect on or off, respectively. Consequently, phenotypes can be induced in a reasonable time frame at any stage of plant development, allowing one to dissect the potential differential effects that membrane trafficking might have at distinct developmental stages and overcoming the lethality or detrimental growth defects present in loss-of-function mutants in trafficking machinery genes. Such features are exactly what a cell biologist needs to manipulate and study endomembrane trafficking.

More than a decade ago, Natasha V. Raikhel envisioned the need for alternative strategies to overcome the challenges associated with studying the mechanisms that underlie the endomembrane systems in plants. She anticipated that chemical biology would bring invaluable tools for plant cell biologists, and her foresight was fruitful in the discovery and use of chemical tools. Successes (and mistakes) paved the route followed by her laboratory in identifying membrane trafficking modulators among thousands of small compounds [see her autobiographical article published in this volume (73)]. Indeed, the multiple success stories have motivated several leading researchers to join the challenge. In the following sections, we review the substantial contribution that chemical genetic screens have made to the membrane trafficking field.

3. HOW DO WE SEARCH FOR BIOACTIVE CHEMICALS THAT PERTURB ENDOMEMBRANE TRAFFICKING?

The chemical space of available cell-permeable organic molecules includes millions of structurally diverse compounds (75). Therefore, theoretically, the chances of finding selective and specific COIs to perturb specific processes are good. The crux of the problem is how to find the right compound.

In forward chemical genetics, the ability of thousands of chemicals (37) to perturb biological processes in *A. thaliana* is tested using an HTS (**Figure 2**). As in genetic screening, in which thousands of mutants are systematically screened for a particular defect, the chemical HTS scores phenotypes that should be carefully selected based on the biological process of interest (**Figure 3**). The screen should score easily identifiable phenotypes related to the specific process under study,

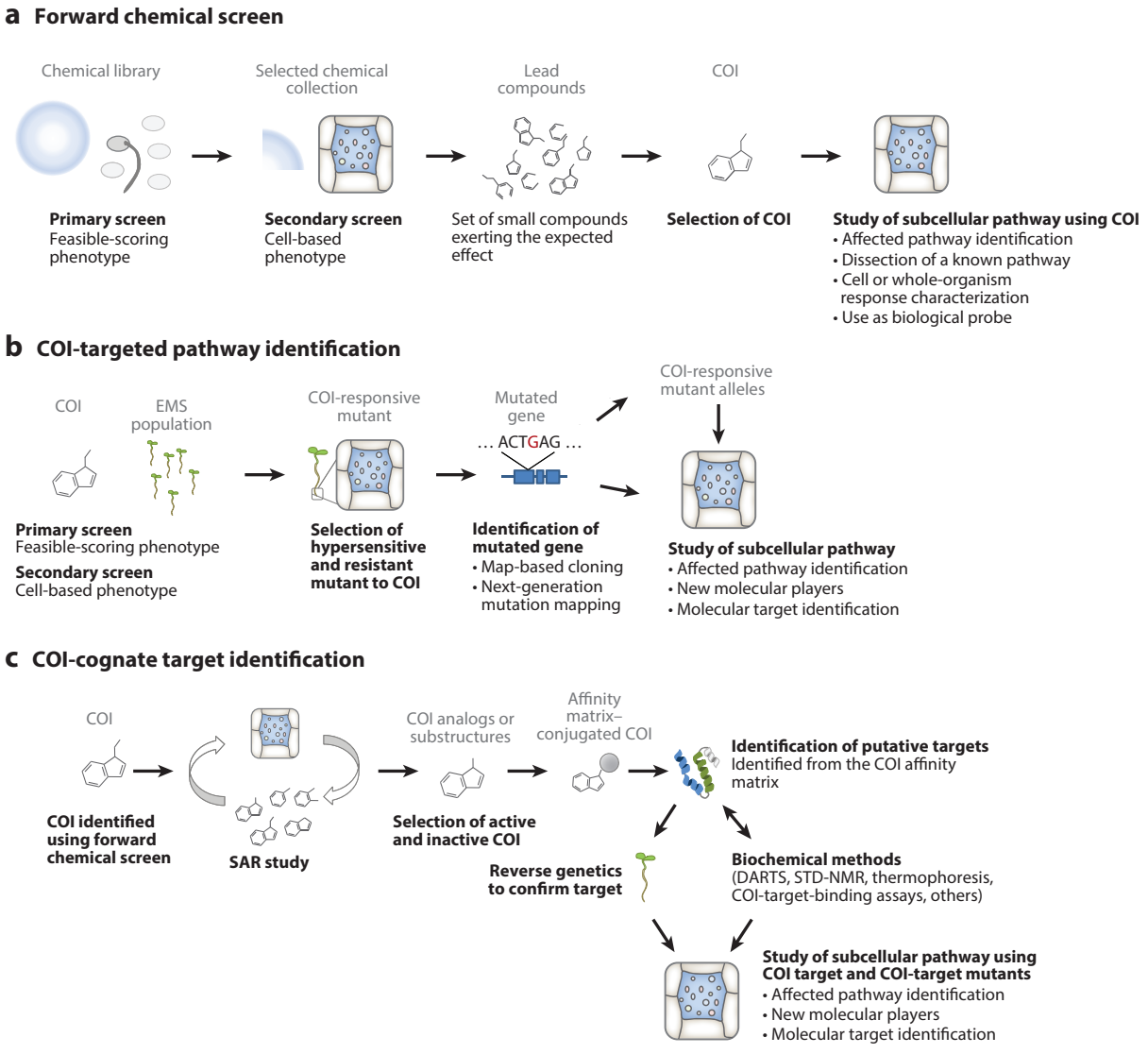

Figure 3

Chemical genetic strategies used to study the endomembrane system in plants. (*a*) A forward chemical genetic screen is the most widely used chemical genetic approach in studies of the plant endomembrane system. Two sequential screens are usually designed. The first is a primary screen based on an easy-to-score phenotype that is closely related to and informative about subcellular perturbations. Then, after candidate selection, a secondary screen is used to evaluate subcellular phenotypes to obtain hits. Hits fulfilling dose-response and reversibility effects become lead compounds. A group of lead compounds is selected for use as tools in the study of the endomembrane system. (*b*) A forward genetic screen is used to obtain information regarding the mode of action and/or cognate targets through a search for COI-responsive mutants. Hypersensitive and resistant mutants are used to identify genes that encode proteins related to and required for COI effects, respectively. (*c*) COI-target identification involves a series of steps, starting with the identification of dispensable chemical moieties within the COI structure using SAR analysis, in which COI structural analogs or substructures are tested for bioactivity. COI analogs or substructures are then used to construct affinity matrices that can identify COI-binding proteins, and candidates are confirmed using biochemical or reverse genetic approaches. Abbreviations: COI, compound of interest; DARTS, drug affinity-responsive target stability; EMS, ethyl methanesulfonate; SAR, structure-activity relationship; STD-NMR, saturation transfer difference nuclear magnetic resonance.

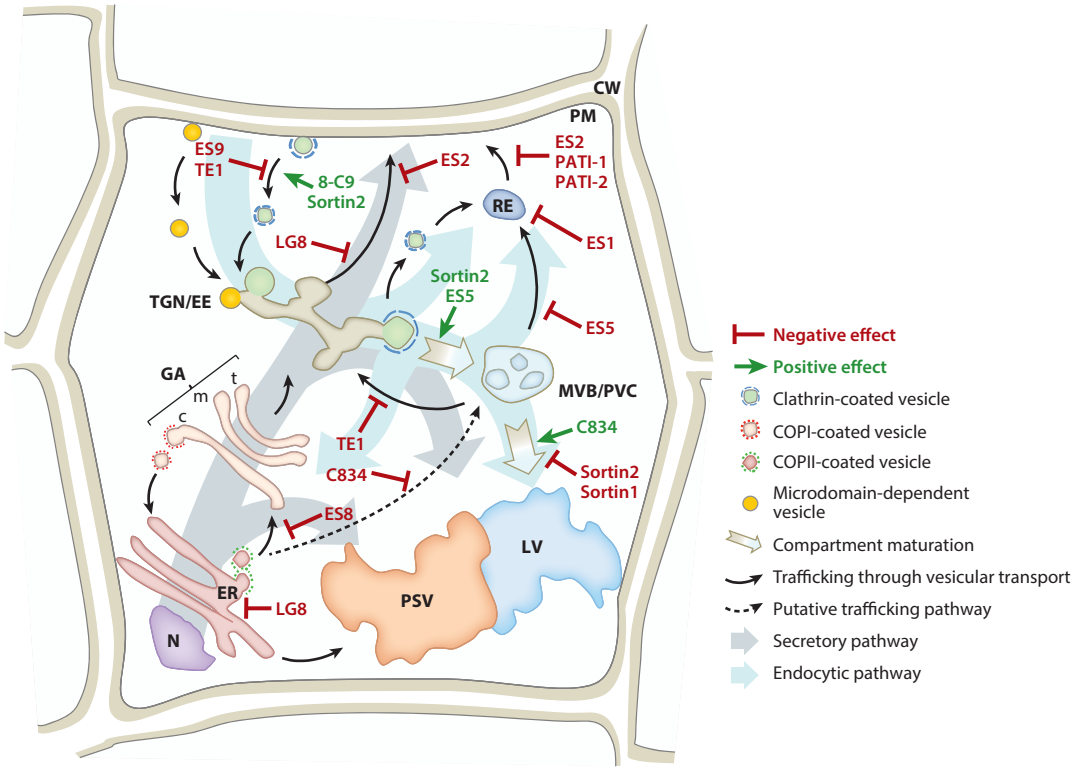

Figure 4

Chemical toolbox for studying plant endomembrane trafficking processes. Several of the lead compounds identified by means of chemical genetic approaches are depicted in the context of the plant endomembrane system (for details, see **Table 2**, below). Chemicals that have a negative effect on a particular trafficking pathway or compartment morphology are shown in red; those that have a positive effect on a particular trafficking pathway are shown in green. Abbreviations: CW, cell wall; ER, endoplasmic reticulum; ES, endosidin; GA, Golgi apparatus [with *cis* (c), medial (m), and *trans* (t) subcompartments]; LV, lytic vacuole; MVB/PVC, multivesicular body/prevacuolar compartment; PATI, polar auxin transport inhibitor; PM, plasma membrane; PSV, protein storage vacuole; RE, recycling endosome; TE, trafficking and endocytosis inhibitor; TGN/EE, *trans*-Golgi network/early endosome.

allowing the screening of thousands of compounds to select candidate hit molecules. HTS chemical hits should then be characterized in terms of the selectivity, dose dependence, and reversibility of their effect (**Figure 3**). Based on these criteria, a hit becomes a lead compound that possesses the features that will be suitable and useful for further research, and is thus deposited in what is termed the chemical toolbox (**Figure 4**). Lead compounds could be further used as baits to find new players and molecular regulators or as probes to manipulate biological processes (**Figures 2** and **3**).

Microscopy technologies allow investigators to follow the trafficking of fluorescent-cargo markers, compartment morphology, and compartment dynamics in vivo. Several markers (**Figure 1**) can be visualized simultaneously, making it possible to distinguish between different compartment populations and their interactions. However, although it is possible to use confocal microscopy to perform large screens examining such subcellular processes, doing so requires enormous amounts of effort, resources, and time. Instead, physiological responses related to endomembrane trafficking have been evaluated as the primary HTS criteria (**Table 1**). This shortcut has cleverly overcome the technical difficulty of testing in vivo cargo trafficking in *A. thaliana* seedlings in a high-throughput manner.

Table 1 Chemical collections and screens that have been used to find bioactive chemicals that perturb the endomembrane system in plants

Chemical collection	Number of compounds	Phenotype-based screens	Screen hits[a]	Lead compounds	Reference(s)
Screens designed to directly identify new modulators of the endomembrane system					
ChemBridge DIVERSet	4,800	Primary screen: secretion of vacuole-targeted carboxypeptidase Y in *Saccharomyces cerevisiae* Secondary screen: GFP:δ-TIP tonoplast fluorescent marker distribution and vacuole morphology in *Arabidopsis thaliana*	14	Sortin1 and Sortin2	115
ChemBridge DIVERSet	10,000	Primary and secondary screens: gravitropic response in *Arabidopsis thaliana* Tertiary screen: GFP:δ-TIP tonoplast fluorescent marker distribution and vacuole morphology in hypocotyl epidermal cells in *Arabidopsis thaliana*	4	Gravacin and pyocyanin	95
MicroSource Spectrum chemicals with known biological activity	2,106	Primary screen: pollen germination and pollen tube polarized growth in *Nicotiana tabacum* Secondary screen: mislocalization of the pollen polar-located GFP:RIC1 marker in *Nicotiana tabacum*	16	ES1/prieurianin, isothiocyanate erysolin, and cantharidin	78
Compounds derived from EXO2	No information	Primary screen: GA and TGN/EE morphology visualized by ST-YFP and VHA-a1-mRFP, respectively, in *Arabidopsis thaliana*	1	LG8	91
ChemBridge DIVERSet	20,000	Primary screen: pollen germination and pollen tube polarized growth in *Nicotiana tabacum* Secondary screen: localization and abundance of PM protein markers in *Arabidopsis thaliana* (PIN2::PIN1:GFP, PIN2::PIN2:GFP, and BRI1::BRI1:GFP)	123	PMRA and endosidins	28
Sigma-TimTec Myria	10,000				
ChemBridge NOVACore	10,000				
LATCA	3,650				
CLICKables library	2,768				
Pollen growth inhibitors (28)	360	Primary screen: tonoplast protein trafficking and localization in hypocotyl epidermal cells in *Arabidopsis thaliana*	5	C834, C410, C755, C103, and C578	76
PMRA (28)	123	Primary screen: PIN1:GFP accumulation in BFA bodies in root stele cells in *Arabidopsis thaliana*	1	ES8	27
PMRA (28)	123	Primary screen: uptake of endocytic tracer FM4-64	1	ES9	20
Screens that have indirectly identified modulators of the endomembrane system (trafficking and/or compartment morphology)					
Korea Chemical Bank small organic compounds	6,500	Primary screen: seedling growth phenotype and development of pin-formed inflorescences in *Arabidopsis thaliana*	1	BUM	45

(*Continued*)

Table 1 (*Continued*)

Chemical collection	Number of compounds	Phenotype-based screens	Screen hits[a]	Lead compounds	Reference(s)
HitFinder small-molecule library	10,000	Parallel primary screens: coleoptile curvature and auxin transport from coleoptile apical tips in *Zea mays*	18	Groups A and B	55, 96
MicroSource Discovery System NatProd collection	720	Primary screen: circadian clock rhythm of the GIGANTEA::LUC transcriptional reporter measured in constant darkness in *Arabidopsis thaliana* seedlings	2	ES1/prieurianin and prieurianin acetate	101
LATCA	3,600	Primary screen: vein pattern of the first rosette leaf in *Arabidopsis thaliana*	9	PATI-1–4, OVP1 and -2, and HYVP1–3	13
Natural product molecules	800				
Other commercially available compounds	1,120				

Abbreviations: BFA, brefeldin A; ES, endosidin; GA, Golgi apparatus; LATCA, Library of Active Compounds on *Arabidopsis* (collected by Sean Cutler, University of California, Riverside); PATI, polar auxin transport inhibitor; PM, plasma membrane; PMRA, plasma membrane recycling compound set A; TGN, *trans*-Golgi network.

[a]The number of compounds that alter or impair the screen-based phenotype.

Inspired by the important role of trafficking to the vacuole in the response to gravistimulation, Raikhel and colleagues (95) performed the first *A. thaliana* chemical HTS with the aim of finding endomembrane system modulators. Reinforcing the link between the two processes evidenced earlier by trafficking machinery mutants (reviewed in 94), the screen successfully identified four compounds that affect membrane trafficking to the vacuole and vacuole morphology (95).

Pollen is another convenient model to study polar secretion as well as an excellent model to study tip growth (112). A proof-of-concept chemical HTS demonstrated that pollen germination and pollen tube growth phenotypes are robust criteria for successfully finding modulators of endomembrane trafficking (78). Indeed, years later, an HTS that included 45,000 diverse chemicals identified a subset of 360 compounds as pollen growth inhibitors (28). Further deep characterization of these compounds in *A. thaliana* seedlings led to the identification of 123 PM recycling modulators (28) (**Table 1**). Both subcollections have been instrumental for subsequent screens focused on secretory targeting of vacuolar proteins (76), PM polarity establishment (27), and endocytic trafficking (20), demonstrating their potential for plant cell biology research. Interestingly, other HTSs that were set up for different purposes but also used growth or physiological phenotypes as screening parameters have also identified endomembrane regulators (**Table 1**). These findings strengthened the idea of using sequential screens to narrow down the chemical library and obtain specific modulators. Certainly, physiological primary screens help to reduce the load on time-consuming endomembrane-specific secondary screens. Nonetheless, the ideal phenotype has to be closely related to the cellular pathway under study.

One should also consider that lead compounds act at a cellular level yet manifest phenotypes in the whole organism. Clearly, paying attention to such phenotypes provides clues regarding the affected molecular or physiological pathways. In essence, the outcome of a chemical genetic screen is

equivalent to that of a classical genetic screen: Both screens trigger perturbations—by chemical interactions and gene mutations, respectively—that result in altered phenotypes caused by impaired function of one or more cognate targets. Bioactive compounds are able to mimic loss-of-function mutants. For instance, endosidin 1 (ES1) is able to phenocopy the mutant *brassinosteroid-insensitive 1-1* (*bri1-1*), restricting the pool of the brassinosteroid receptor BRI1 by inhibiting its recycling to the PM (78) (**Tables 1** and **2**). In the same sense, the effect of gravacin on the gravitropic response mimics the loss of function of the auxin transporter proteins, which turned out to be gravacin's cognate molecular target (84) (**Table 2**).

However, there are risks associated with focusing on such pleiotropic phenotypes, and unrelated effects may be stumbled upon. For instance, subcellular defects induced by endosidins in *A. thaliana* root cells make perfect sense with inhibition of pollen tube growth (28). In the case of ES2, the molecular MoA is closely related to the secretory pathway, exactly as the design of the HTS strategy predicted. However, experience has revealed that this is not always the case. Thus, investigating the MoAs of prioritized COIs becomes a vital task (see Section 4). For instance, ES7 was discovered as an inducer of gaps in the cell plate (28). The cell plate is synthesized during cytokinesis, where abundant material is secreted to the new cell border. Therefore, it was initially believed that ES7 could be an inhibitor of the secretory pathway (28). However, further characterization revealed that ES7 actually inhibits the biosynthesis of callose, a structural component of the cell plate and the main constituent of the pollen tube cell wall (65). Luckily, it turned out that the effect of ES7 is quite specific for callose deposition during cytokinesis in epidermal root cells (65), and ES7 therefore became an unprecedented tool to interrogate the cell plate formation process in the absence of callose deposition.

We believe that the lesson from these experiences is to be attentive and aware of the phenotypes induced by COIs. Molecular pathways unrelated to each other could bring about similar phenotypes. Designing HTSs and extrapolating cause-and-effect phenotypes is risky, raising the need to test the MoA of the bioactive chemical in question before drawing any conclusions. Searching for known mutants that mimic the effect of the chemical is a useful complementary approach. Alternatively, it is beneficial to track all possible phenotypes, as that should lead to both the pathway targeted by the COI and the likely cognate targets.

4. UNDERSTANDING SMALL-COMPOUND MODES OF ACTION IN THE CONTEXT OF ENDOMEMBRANE CHEMICAL GENETICS

After COIs have been identified, one of the challenges is discovering how they exert their effects, and we expect that knowledge of the MoAs of small molecules will increase exponentially because of the number of molecules already available and the increased awareness, acceptance, and popularity of chemical genetic HTSs (**Tables 1** and **2**). Characterization of the effects of COIs in the context of subcellular phenotypes is often informative enough to dissect particular or interconnected pathways, as discussed below (see Section 5). Once the pathway affected by a COI has been identified, the COI can be used as bait to find other molecular players. Moreover, characterizing the cognate target of a COI increases our understanding of the biological significance of the plant's response to that COI. Several methods have been developed to understand the MoAs of COIs, but in plants, chemical genetics is still in its infancy. Multiple approaches, including genetic and biochemical methods, have already provided hints regarding the proteins directly affected by COIs, leading to successes in precisely pinpointing the molecular targets of some COIs.

Forward genetic screens have been instrumental in helping to identify COI targets. The rationale is that mutants exhibiting hypersensitivity or resistance to the COI may yield information about the MoA (**Figure 2**). For instance, a resistant mutant could arise from a mutation in the

Table 2 Bioactive compounds affecting plant endomembrane trafficking processes that were identified by chemical genetic screens

Compound	Subcellular effects	Tissue or whole-organism phenotypes[a]	Molecular target	Reference(s)
Sortin1	Induces the secretion of CPY in *Arabidopsis* suspension cells and seedlings; alters vacuole morphology in *Arabidopsis* hypocotyl cells without altering GA or ER morphology	Reduces root length (although this effect is reversible)	NI	85, 115
Sortin2	Alters vacuole morphology in *Arabidopsis* hypocotyl cells; induces the secretion of CPY in hypocotyl and root *Arabidopsis* seedlings; induces endocytosis from the PM; induces a wortmannin-sensitive protein trafficking pathway to the vacuole	Reduces root length (although this effect is reversible); reduces viability in tobacco and *Arabidopsis* suspension cells; induces lateral root formation in an $SCF^{TIR1/ABFs}$-independent manner	NI	57, 69, 107, 115
ES1/prieurianin	Stabilizes the actin cytoskeleton, thereby affecting endosomal trafficking processes; induces the formation of SYP61- and VHA-a1-positive intracellular agglomerations of PM endocytosed cargoes (PIN2, AUX1, and BRI1); changes the morphology of RABA1c TGN/EE endosomes	Reduces polar growth of trichomes, hypocotyl epidermal cells, and pavement cells; inhibits seedling growth; induces BRI1 loss-of-function phenotypes; shortens circadian period	NI	72, 78, 101
ES2	Reduces PIN2 recycling; inhibits PIN2 exocytosis to the PM by interfering with EXO70/exocyst function; enhances protein accumulation at ARA7-positive MVB/PVCs and targeting to the vacuole; interferes with EXO70A1 polar localization	Inhibits seedling growth, pollen germination, and pollen tube and root hair elongation; reduces root gravitropic response	EXO70	113
ES3	Induces PIN2 intracellular accumulation; disrupts ROP6-GFP PM localization	Alters ROP-mediated cotyledon pavement cell polarity	NI	28
ES5	Enhances PM trafficking of cargoes (PIN1 and -2, BRI1, BRL3, and PIP2) to the vacuole without affecting trafficking of soluble vacuole cargoes; modifies localization of TGN/EE, GA, and MVB/PVC markers; interferes with PIN2 recycling to the PM	Impairs response to gravitropic stimulation in a dose-dependent manner	NI	28
ES7	Induces defects in cell plate formation by inhibiting callose deposition; perturbs YFP-RABA2a and YFP-RABA2e localization in late developmental stages of cell plate formation	Inhibits seedling growth; causes the collapse of the root apical meristem patterning and disorganized cell division planes in root and cotyledon epidermal cells; reduces stomatal density; arrests cell cytokinesis; inhibits callose synthase activity	NI	18, 28, 65

(*Continued*)

Table 2 (*Continued*)

Compound	Subcellular effects	Tissue or whole-organism phenotypes[a]	Molecular target	Reference(s)
ES8	Induces PIN1 intracellular agglomeration, interfering with PIN1 basal polarity without altering the polarity of other apical-localized proteins; interferes with secretion of PIN1 (but not that of PIN2) to the PM	Disrupts auxin response distribution pattern in roots and impairs seedling development; *gnom-like 1* mutants display ES8 resistance	NI	27
ES9	Uncouples the mitochondrial electrochemical proton gradient; inhibits clathrin-mediated endocytosis by acting as a protonophore, leading to cytoplasm acidification; reduces PM recruitment of clathrin and associated adaptors; reduces accumulation of phosphatidylinositol 4,5-bisphosphate at the PM	No information available	NI	20
Gravacin	Induces GFP-TIP2;1 accumulation in ER-like structures specifically in root cells; increases MDR1/PGP19-GFP abundance at the PM and induces its accumulation in unidentified FM4-64-negative vesicular structures	Reduces root and hypocotyl gravitropic curvature; inhibits polar auxin transport	PGP19	84
TENin1	Inhibits endocytosis of PIN2, causing protein accumulation in the MVB/PVC	Inhibits root gravitropic bending	NI	66
CESTRIN	Reduces CESA mobility at the PM and induces its agglomeration inside the cell in SYP61-positive endosomes	Alters anisotropic growth of hypocotyls in *Arabidopsis*; reduces cellulose content	NI	111
BUM	Enhances PIN1 expression and polar localization in root cortex and epidermal cells	Reduces root and hypocotyl growth; interferes with auxin response and polar transport; induces the formation of pin-like inflorescences	ABCB1	45
PATI-1 and -2	Affect PIN2 endosomal cycling in *Arabidopsis* root epidermal cells	Disrupt normal vein pattern formation in leaves; eliminate hook formation and auxin response patterning in etiolated *Arabidopsis* seedlings	NI	13
C834	Disturbs the trafficking of GFP-TIP2;1 and TIP3;1-YFP to the tonoplast, inducing their accumulation at the ER; enhances PIN2-GFP trafficking to the vacuole in the dark	Reduces root growth and suppresses root hair formation	NI	76
C410	Disturbs the trafficking of GFP-TIP2;1, TIP3;1-YFP, and TIP1;1-YFP to the tonoplast; inhibits PM targeting of the marker PIP2A-GFP, inducing its agglomeration at the ER	No information available	NI	76
C755	Disturbs the trafficking of GFP-TIP2;1, TIP3;1-YFP, and TIP1;1-YFP to the tonoplast, inducing their agglomeration at the ER and in other cytoplasmic structures	No information available	NI	76

(*Continued*)

Table 2 (*Continued*)

Compound	Subcellular effects	Tissue or whole-organism phenotypes[a]	Molecular target	Reference(s)
C103	Disturbs the trafficking of TIP2;1-YFP, TIP3;1-YFP, and TIP1;1-YFP to the tonoplast; inhibits PM targeting of the marker PIP2A-GFP, inducing its agglomeration at the ER; affects ER structure	No information available	NI	76
C578	Disturbs the trafficking of the protein storage vacuole marker TIP2;1-YFP to the tonoplast, inducing its agglomeration at the ER; induces the mislocalization of GA and TGN/EE markers to an unidentified cytoplasmic structure; affects ER structure	No information available	NI	76
LG8	Changes TGN/EE and ER morphology; prevents BRI-GFP secretion, reducing its localization to the PM and inducing its accumulation to TGN/EE-like compartments	Reduces primary root growth; increases lateral root density	NI	91
8-C9	Induces internalization of PIN1 and -2 into small vesicles; increases the number of PIN2 agglomerations in cotreatment with BFA	Inhibits seedling growth; disrupts auxin response and cell division at the root tip, changing root apical meristem patterning	NI	56
Group B chemicals[b]	Partially inhibit formation of PIN2 BFA bodies in *Arabidopsis* roots	Disrupt auxin response at the root tip; inhibit seedling growth; one compound induces agravitropic growth	NI	56

Abbreviations: BFA, brefeldin A; ER, endoplasmic reticulum; ES, endosidin; GA, Golgi apparatus; MVB/PVC, multivesicular body/prevacuolar compartment; NI, not identified; PATI, polar auxin transport inhibitor; PM, plasma membrane; TGN/EE, *trans*-Golgi network/early endosome.
[a]Phenotypes other than those observed in the original screening indicated in **Table 1**.
[b]Includes three non-structurally-related chemicals.

COI-binding pocket of the putative target protein, which would create a plant that no longer responds to the COI as the wild type does. There have been a few examples of the use of genetic approaches to identify a molecular cognate target (see below). On the other hand, a hypersensitive mutant could help to identify proteins in the COI-affected pathway or in any pathway related to it. This approach does not guarantee the successful identification of the COI-target molecule; however, it does provide useful information regarding the COI-perturbed pathway. The examples presented below highlight the way forward in chemical genetics: combining parallel experimental approaches to maximize the information obtained when using bioactive compounds.

4.1. Using Structure-Activity Relationships to Determine the Mode of Action of a Compound of Interest

SAR: structure-activity relationship

The number of available cell-permeable molecules and the development of organic synthesis methods have enabled researchers to test the bioactivity of structures that are chemically related to a COI. These analogs are either precursors or substructures of a COI or are COI-related structures with different substitutions. Structure-activity relationship (SAR) studies provide information on

the chemical structural features that determine COI bioactivity (**Figure 2**). SAR studies attempt to establish a relationship between the structural features of a chemical and its biological activity, which then enables investigators to find crucial substructures that explain the biological activity of the COI or to find chemical moieties that are dispensable for COI biological activity.

The SAR approach was instrumental for understanding the MoA of Sortin2 in *S. cerevisiae*. Norambuena et al. (57) determined key structural features of Sortin2 by employing analogs and substructures of this compound. The use of such molecules, along with the identification of yeast mutants with differential sensitivity to Sortin2, defined its effect on the crosstalk of secretory and endocytic cellular pathways (57, 107). A combination of SAR analysis and mutants has also been useful for obtaining new insights regarding the MoAs of other small molecules in *A. thaliana* (84, 85, 113).

Sortin1 was identified in a chemical HTS aimed at obtaining compounds that interfere with vacuolar sorting of cargo proteins, particularly N-terminal-propeptide-mediated transport to the lytic vacuole (115) (**Tables 1** and **2**). Rosado et al. (85) set up a forward genetic approach to find hypersensitive Sortin1-responsive mutants. The *sortin1 hypersensitive* (*s1h*) mutants displayed both root elongation and vacuolar morphology defects at Sortin1 concentrations where the wild type was only partially sensitive in terms of root growth inhibition and was insensitive to vacuole morphology changes. Additional Sortin1-induced phenotypes in *s1h* mutants were similar to defects displayed by mutants related to flavonoid synthesis and vacuolar transport (71). *s1h* mutants had particular phenotypes in seed coat color and reduced accumulation of anthocyanins and flavonoids in the vacuole. These observations helped to guide the search for the MoA of Sortin1; most likely, the Sortin1 phenotype is triggered by defects in flavonoid synthesis or transport (85). The Sortin1 SAR analysis suggested that Sortin1 binds to two different protein pockets, either in the same protein or in two interacting proteins. Additionally, Sortin1 substructures differentially modulate vacuolar morphology and anthocyanin accumulation in the vacuole, uncoupling these two phenotypes. Even though it was not possible to clearly demonstrate the exact subcellular molecular target(s) of Sortin1 in vacuolar trafficking, the study of how Sortin1 exerts its effects contributed to our understanding of the close relationship between vacuole morphology and anthocyanin accumulation. The take-home message is that smart genetics and SAR analysis can complement each other in sophisticated ways to narrow down the MoAs of bioactive compounds (**Figure 3**).

Plant tropisms, including the responses to light and gravity, all depend on the hormone auxin. Auxin is transported from cell to cell in a polar fashion by the concerted action of auxin carriers, among them the PIN proteins (48) and the ATP-binding cassette (ABC) transporters (9) that participate in auxin efflux from the cell. Both types of carriers are polarly localized at the PM as a result of endocytic trafficking. Polar auxin transport is involved in plant development and environment responses, including the gravitropic response. Surpin et al. (95) identified gravacin as a compound that interferes with the response to gravistimulation (**Tables 1** and **2**). Rojas-Pierce et al. (84) subsequently carried out a forward genetic screen to isolate gravacin-resistant mutants (*grav-r*) and determined that a mutation in the ABCB19/P-GLYCOPROTEIN 19 (PGP19) protein was responsible for the gravacin-resistant phenotype. Additionally, the advantages of *A. thaliana* genetics enabled the analysis of further *pgp19* mutant alleles, confirming that PGP19 is involved in the gravacin response. Binding assays of this compound and a SAR-identified inactive structural analog demonstrated that PGP19 is a target of gravacin (84). This experience clearly exemplifies how classical genetics can powerfully influence the final outcome of chemical genetics. Overall, it is clear that target identification should be achieved by combining organic chemistry (e.g., the synthesis of structural analogs followed by SAR analysis) and biochemical methods to reinforce the conclusions arising from classical forward genetics.

4.2. Fishing the Cognate Target with a Chemical Bait

Alternatives to genetic approaches to clarify the MoA of a specific bioactive compound include identifying COI-interacting proteins. The most commonly used method involves affinity purification of COI-binding proteins. SAR analysis needs to be applied to find chemical moieties dispensable for COI activity. The goal is to find COI analogs that maintain biological activity, allowing the insertion of a cross-linking moiety within the COI-analog structure to generate an affinity matrix. Moreover, having inactive COI analogs is essential for subtracting nonspecific proteins in pull-down assays. Only a few studies have used this strategy to identify molecular targets in plants (21). Zhang et al. (113) recently provided a beautiful example of this direct approach, using it to study the MoA of ES2 in the endomembrane system by identifying its molecular target.

ES2 appeared as one of the lead compounds of a chemical genetic screen aimed at identifying molecules that alter the localization and dynamics of endosomal cargoes (28) (**Tables 1** and **2**). SAR analysis was key for designing ES2 modifications without altering its biological activity and for finding an ES2 inactive analog. Affinity matrices were generated using these two compounds. Comparing the proteins eluted from pull-down assays enabled the authors to fish out specific ES2-interacting partners. Proteomic analysis detected several peptides with homology to the EXOCYST 70G2 (EXO70G2) protein, one of the components of the exocyst complex (15). The exocyst complex is conserved among eukaryotes and plays important roles in vesicle trafficking in the secretory pathway. In *A. thaliana*, it belongs to a large family with 23 members (15). Another member, EXO70A1, also bound to the ES2 affinity matrix. Consistently, *exo70a1* mutant alleles were found to be resistant to the inhibitory effect of ES2 on root growth, further confirming EXO70A1 as one of the cognate targets (113). Several alternative chemical approaches have elegantly confirmed that ES2 binds to EXO70A1.

ES2 represents the first specific compound that binds to the EXO70 subunits of the exocyst complex. Inhibiting this conserved eukaryotic protein complex expands the repertoire of drugs, paving the way for studies of exocyst-dependent processes in a broad range of eukaryotes and potentially overcoming challenges posed by the highly redundant EXO70 protein family in plants. ES2 is a novel tool that can be used to affect a pathway that delivers cargoes from intracellular compartments to the PM and beyond (**Figure 4**). This route has not been extensively studied in plants, even though it is the route taken by components of the cell wall, the feature of plant cells that provides the structural matrix to tissues and establishes contact with neighboring cells.

5. CHEMICAL SCREENS HAVE BROUGHT POWERFUL HARDWARE FOR DISSECTING MEMBRANE TRAFFICKING

Several lead compounds that perturb endomembrane trafficking pathways have been described from chemical HTSs, thus filling up the endomembrane system chemical toolbox (**Figure 4**). Besides COI target identification, the bioactive compound could be used to interrogate biological processes. The use of COIs as probes has undoubtedly enriched our understanding of plant membrane trafficking. In the following, we detail the biological insights that have been gained by using COIs identified in chemical HTSs.

5.1. Dissecting Endomembrane Trafficking Pathways

The TGN/EE and MVB/PVC endosomal compartments have been morphologically identified by electron microscopy. Under a regular confocal microscope, they appear as moving organelles of different shapes that can be identified by marker proteins (63). Perturbing trafficking pathways

often results in aggregations of compartments that are commonly called bodies. For instance, the well-described BFA bodies are distinct from ES1 bodies in number, shape, and type (78). Although both bodies accumulate protein cargoes originating from the PM by clathrin-mediated endocytosis, the composition of the accumulated membranes in the aggregates seems to be different (78). The high-content screening performed by Drakakaki et al. (28) described five distinctive membrane aggregates induced by chemical treatments. The diverse intracellular body-phenotypes collection manifests the underlying complexity of membrane trafficking, in which different perturbations culminate in subtle differences. Interestingly, the effects of short and permanent treatments on membrane trafficking pathways differ (28), suggesting that endomembrane trafficking can regulate and respond to external perturbations (70).

ES1 has revealed the existence of distinct endosomal populations (78). Indeed, it defined a particular endocytic trafficking pathway taken by the PM cargoes AUXIN RESISTANT 1 (AUX1), PIN2, and BRI1, which differs from the pathway taken by PIN1 and PIN7 (78). A later study showed that ES1 indeed impairs actin filament dynamics, explaining its effect on cell polarity and seedling growth (101). The effect on the cytoskeleton could also explain its influence on vesicular trafficking (26). However, the inhibitory effect of ES1 relies on the endocytic cargoes instead of on a generalized effect on trafficking (78). This specificity is consistent with this compound causing the aggregation of particular endosomal pools (78). Therefore, it would be expected that particular pools of actin may be associated with specific membrane trafficking pathways. Novel actin modifications could affect specific motor or associated proteins, thus distinguishing between different actin organizations.

In plants, two distinctive vacuoles—the lytic vacuole and the protein storage vacuole—may coexist in the same cell; in other types of cells, however, they fuse, generating the central vacuole that occupies the majority of the volume of the cell (114) (**Figure 1**). Different target pathways for soluble vacuolar proteins correlate with cargo sorting signals and cargo receptors for each compartment, and therefore different trafficking machinery is involved (reviewed in 68, 116). Because the two vacuoles have different functions, their repertoires of resident proteins also differ.

The tonoplast intrinsic proteins (TIPs) were instrumental in defining the two different vacuoles. The presence of TIP1;1 (also called γTIP) is specific to the lytic vacuole, whereas TIP3;1 (also called αTIP) defines the protein storage vacuole (41). The presence of these membrane proteins correlates with particular populations of vacuolar soluble proteins (64). Consequently, after synthesis at the ER, vacuolar resident proteins follow different trafficking pathways, as shown in **Figure 1** (42).

New evidence came to light with the search for and use of modulators of trafficking to the vacuole from a chemical genetic screen. These chemicals were elegantly selected for their effect of inducing the retention of vacuolar membrane proteins in the ER (76) (**Tables 1** and **2**). The selected bioactive compounds show a variety of effects on protein targeting (**Table 2**). Interestingly, one of these compounds, C834, selectively inhibits the targeting of proteins directed to the vacuole in a Golgi apparatus–independent pathway without affecting either the Golgi apparatus–dependent or other trafficking pathways. The use of C834 thus confirmed the existence of two vacuolar targeting pathways in *A. thaliana*, suggesting a link between the secretory and endocytic pathways that proteins use to traffic to the vacuole. As depicted in **Figure 1**, these two routes intersect and share compartments and flux of vesicles, and thus they also share cargoes. Therefore, it is challenging to decipher how vesicle flux is regulated and how the molecular components become recruited, including the mechanisms that recognize the protein sorting signals. The use of C834 could contribute to the dissection of these processes, because most likely it is targeting specific members of the trafficking machinery for the delivery of vacuolar membrane proteins.

5.2. The Root of Crosstalk Pathways

Lead compounds discovered in chemical HTSs have been extremely useful in uncovering the crosstalk between different signaling pathways. As discussed above, studies using genetic mutants or chemical tools have shown that endomembrane system trafficking affects plant development. Chemical screens are usually designed to find chemical inhibitors because the phenotypic screens are typically related to the impairment of functions, such as reduced growth or diminished responses to stimuli. Nonetheless, a few examples of chemicals that are able to enhance specific phenotypes have been described (**Figure 4**, **Table 2**).

Sortin2 is a small compound that induces particular pathways. Initially described because of its ability to inhibit the trafficking of a vacuolar protein (115), Sortin2 was further characterized as an inductor of clathrin-mediated endocytosis from the PM toward the vacuole (69). Combinations of Sortin2 with well-known drugs, such as BFA and wortmannin, showed that it is also able to accelerate endocytic trafficking from the TGN/EE to the MVB/PVC. Overall, analysis of the effect of Sortin2 on protein trafficking indicated that it most likely modulates the crosstalk between the endocytic and secretory pathways (69). This particular COI also shed light on a previously unknown role of endocytic trafficking in plant development. The Sortin2 induction of endocytic trafficking results in stimulation of lateral root initiation. Signaling mediated by the phytohormone auxin and its receptor complex $SCF^{TIR/AFBs}$ has been described as the central hub for lateral root formation during normal plant development (24, 106). However, Sortin2-induced endocytosis enhances lateral root formation via an $SCF^{TIR/AFBs}$-independent pathway, which depends on trafficking toward the vacuole and involves an unknown mechanism (69). Sortin2 induces lateral root initiation and has different requirements than auxin-dependent lateral root formation does. Sortin2-induced endocytosis is at the heart of auxin-independent lateral root initiation. Finding the mechanism targeted by Sortin2 and its cognate target will open new avenues of research and clarify the nature of this novel lateral root formation pathway.

Chemical tools allowed the recent discovery that endomembrane trafficking could be regulated by cytoplasmic pH. Dejonghe et al. (20) set up a small screen using FM4-64 uptake as a proxy for endocytosis (**Table 1**) and found that ES9 is a highly specific inhibitor (20): It consistently perturbed several endocytosis components, including the AP2 and TPLATE subunits, and reduced the levels of phosphoinositides in the PM (**Table 2**). It turned out that ES9 changes cytoplasmic pH, strongly affecting the dynamics of endocytosis. The fact that the broadly used compound TyrA23 was also affecting cytoplasmic pH revealed that perturbation at this level could affect protein trafficking. The two compounds behave as mitochondrial uncouplers, although their MoAs are independent of mitochondrial function (20). Therefore, the MoAs of such inhibitors represent newly discovered effects that cytoplasmic acidification has on clathrin-mediated endocytosis.

5.3. What Do We Have in Our Hands Today?

The identification of endomembrane trafficking bioactive chemicals was a major contribution to the broader scientific community because this collection includes potentially interesting chemicals that could affect other processes that require protein trafficking, such as tip growth, cell wall deposition, and developmental processes (**Table 1**). The bioactivity of identified chemicals is also applicable to animal cells and yeast (20, 28, 101, 107, 113, 115), most likely because of the conservation of endomembrane system mechanisms as the headquarters of eukaryotic cells. Indeed the conservation of these mechanisms was pivotal for finding Sortin1 and Sortin2, originally identified in an HTS based on the phenotype of mis-sorting of a soluble vacuole cargo in *S. cerevisiae* (115) (**Table 1**). The effects of Sortin1 and Sortin2 on endocytosis and trafficking to the vacuole, respectively, are conserved in yeast and *A. thaliana* (57, 69, 107, 115) (**Table 2**). The

common effects exerted by bioactive chemicals on different organisms constitute an advantage. Indeed, a chemical HTS in *A. thaliana* found new growth regulators, the effect of which could be efficiently translatable to monocots and dicots (83). Lead compounds could therefore be useful in opening up research on endomembrane trafficking in other plant species, which could lead to the discovery of novel and distinctive pathways of plants with body structures and physiological strategies different from those of *A. thaliana*.

6. FUTURE ROADS AND PERSPECTIVES

Although the compartments of the endomembrane system appear to be fairly stable, vesicles are constantly budding off from different compartments and traveling around to reach diverse stations. The number of pathways feeding this dynamically active equilibrium needs to be tightly regulated. Perturbing these pathways clearly affects the normal movement of cargo proteins (**Figure 4**, **Table 2**). Indeed, bioactive chemicals could induce multiple subcellular phenotypes within just a few hours. In this scenario, chemical genetics is a versatile tool that can be used in different ways depending on the needs of the study and the other available tools. If a particular study requires using a different method to perturb processes, an HTS could be performed. If a bioactive chemical has already been characterized, a new screen could be performed around a diverse, structurally oriented chemical library based on SAR analysis to find the most specific and selective chemical.

The advantages of conditional phenotypes are evident when adding or removing the COI and should be exploited as part of the validation process. Having several probes that affect different pathways or different levels of interconnected pathways can be useful to identify cargo trafficking pathways and their impact on plant physiology. Therefore, a chemical toolbox that includes selective and specific lead compounds covering a range of bioactivities is under construction. Combining hierarchized chemical effects builds a picture of upstream and downstream chemical-targeted pathways (82). Adding new members to this toolbox is challenging, but researchers of intricate biological processes greatly appreciate having a large number of tools. There is a general consensus that these bioactive compounds are highly useful, especially when studying subcellular phenotypes such as endomembrane morphology, cell polarity, and intracellular trafficking. Despite the importance of such molecules, the plant endomembrane field needs more COIs, with higher specificity and undiscovered MoAs, and such tools should help with investigations into unexplored areas.

Reverse chemical genetic approaches involve using a known protein in the screening strategy to find small compounds (**Figure 2**). The obvious advantage is the specificity of the screen for a particular target pathway, thus bypassing the target identification step. Mammalian research has provided some beautiful examples of this method. Pitstops are bioactive molecules found in a reverse chemical genetic screen aimed at obtaining molecules that alter the interaction between the clathrin terminal domain and many of the associated proteins involved in the clathrin-coated pit, inhibiting clathrin cage formation and endocytosis (109). The screen involved using an enzyme-linked immunosorbent assay to find compounds that interfere with the association with amphisin, an accessory protein containing a clathrin box motif (90). The outcome of this approach was two small compounds, Pitstop1 and Pitstop2, that selectively inhibit receptor-mediated endocytosis (109). A further example of a compound found in a reverse chemical genetic approach being applied to the study of endosomal systems is dynasore, which inhibits the small GTPase dynamin and thereby perturbs endocytosis (52). This screen used a straightforward in vitro colorimetric assay to detect dynamin GTPase activity.

These two examples give a glimpse into how simple approaches can be applied to any protein-protein interaction or to any small GTPase. Membrane trafficking implies the interplay

of cargo receptors, RAB proteins, and soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins, conferring specificity and directionality to the vesicle journey through a given trafficking pathway. SNARE proteins present in the vesicles and target membrane interact, creating the driving force for membrane fusion. RAB proteins are small GTPases whose active isoform is recruited to uncoated vesicles, conferring vesicle identity. Therefore, finding lead compounds that specifically perturb the function of a particular RAB or the interactions of SNARE partners will likely result in precise and subtle modifications. Having a toolbox containing those specific molecules would provide unparalleled opportunities to increase our understanding of vesicle trafficking.

RAB proteins: small GTPases with an active isoform that is recruited to uncoated vesicles, conferring vesicle identity

Soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins: mediators of vesicle fusion through protein-specific association of a v-SNARE (vesicle) with two or three t-SNAREs (acceptor compartment)

The effect of a particular compound is based on its interaction with a cognate target that is part of a biochemical or cellular process. The strength of interaction depends on the complementarity of their structures, as determined by their physicochemical properties. The specificity and/or selectivity of the drug is determined by the number of putative cognate targets that display some degree of complementarity. Information on three-dimensional protein structures is still limited; however, in silico modeling based on crystal structures has become a powerful tool for structural biologists. Hayashi et al. (36) used this type of approach to systematically design chemical compounds that could affect auxin signaling. The output was a specific auxin antagonist able to abolish auxin-induced responses by interacting with all members of the TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/AUXIN SIGNALING F-BOX PROTEIN (AFB) auxin receptor family (35). Intelligently designed chemicals that are able to impair, agonize, or antagonize a given protein may be selective enough to alter a membrane trafficking pathway. Using these chemicals will enable us to go further toward answering the biological question of how this pathway affects cellular function and organism physiology. The key to the design is to ensure binding specificity. Therefore, we should also aim for structural studies of members of the trafficking machinery.

SUMMARY POINTS

1. Endomembrane system trafficking is tightly regulated and selective, depending on the cargo proteins, trajectory, and destination.
2. Endomembrane trafficking pathways are diverse and complex because of the diversity of cargo proteins, the variety of destinations, and the types and dynamics of endomembrane compartments.
3. The dynamics, efficiency, and selectivity of plant membrane trafficking affect homeostasis, development, physiology, and responses to environmental challenges.
4. Genetics (both forward and reverse) and broadly used bioactive chemical tools have been instrumental in gaining a mechanistic understanding of the endomembrane system and identifying the plant trafficking machinery.
5. The use of chemical genetic approaches has uncovered trafficking machinery members, demonstrating the power of this strategy.
6. Plant chemical genetics has been implemented successfully, allowing the dissection of trafficking pathways and providing novel insights into their impact on the whole organism.
7. Chemical genetics has enormous potential, pulling together areas of knowledge such as cell biology, chemistry, structural biology, genetics, and physiology.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED

1. Alonso JM. 2003. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301:653–57
2. Alonso JM, Ecker JR. 2006. Moving forward in reverse: genetic technologies to enable genome-wide phenomic screens in *Arabidopsis*. *Nat. Rev. Genet.* 7:524–36
3. Banbury DN, Oakley JD, Sessions RB, Banting G. 2003. Tyrphostin A23 inhibits internalization of the transferrin receptor by perturbing the interaction between tyrosine motifs and the medium chain subunit of the AP-2 adaptor complex. *J. Biol. Chem.* 278:12022–28
4. Barberon M, Zelazny E, Robert S, Conéjéro G, Curie C, et al. 2011. Monoubiquitin-dependent endocytosis of the IRON-REGULATED TRANSPORTER 1 (IRT1) transporter controls iron uptake in plants. *PNAS* 108:E450–58
5. Bassham DC, Brandizzi F, Otegui MS, Sanderfoot AA, et al. 2008. The secretory system of Arabidopsis. *Arabidopsis Book* 6:e0116
6. Ben Khaled S, Postma J, Robatzek S. 2015. A moving view: subcellular trafficking processes in pattern recognition receptor-triggered plant immunity. *Annu. Rev. Phytopathol.* 53:379–402
7. Betina V. 1992. Biological effects of the antibiotic brefeldin A (decumbin, cyanein, ascotoxin, synergisidin): a retrospective. *Folia Microbiol.* 37:3–11
8. Bolle C, Schneider A, Leister D. 2011. Perspectives on systematic analyses of gene function in *Arabidopsis thaliana*: new tools, topics and trends. *Curr. Genom.* 12:1–14
9. Borghi L, Kang J, Ko D, Lee Y, Martinoia E. 2015. The role of ABCG-type ABC transporters in phytohormone transport. *Biochem. Soc. Trans.* 43:924–30
10. Braakman I, Hebert DN. 2013. Protein folding in the endoplasmic reticulum. *Cold Spring Harb. Perspect. Biol.* 5:a013201
11. Brandizzi F, Irons SL, Johansen J, Kotzer A, Neumann U. 2004. GFP is the way to glow: bioimaging of the plant endomembrane system. *J. Microsc.* 214:138–58
12. Busch M, Mayer U, Jürgens G. 1996. Molecular analysis of the *Arabidopsis* pattern formation gene *GNOM*: gene structure and intragenic complementation. *Mol. Gen. Genet.* 250:681–91
13. Carland F, Defries A, Cutler S, Nelson T. 2015. Novel vein patterns in Arabidopsis induced by small molecules. *Plant Physiol.* 170:338–53
14. Chen X, Irani NG, Friml J. 2011. Clathrin-mediated endocytosis: the gateway into plant cells. *Curr. Opin. Plant Biol.* 14:674–82
15. Chong YT, Gidda SK, Sanford C, Parkinson J, Mullen RT, Goring DR. 2009. Characterization of the *Arabidopsis thaliana* exocyst complex gene families by phylogenetic, expression profiling, and subcellular localization studies. *New Phytol.* 185:401–19

16. Crump CM, Williams JL, Stephens DJ, Banting G. 1998. Inhibition of the interaction between tyrosine-based motifs and the medium chain subunit of the AP-2 adaptor complex by specific tyrphostins. *J. Biol. Chem.* 273:28073–77
17. Cui Y, Shen J, Gao C, Zhuang X, Wang J, Jiang L. 2016. Biogenesis of plant prevacuolar multivesicular bodies. *Mol. Plant* 9:774–86
18. Davis DJ, McDowell SC, Park E, Hicks G, Wilkop TE, Drakakaki G. 2015. The RAB GTPase RABA1e localizes to the cell plate and shows distinct subcellular behavior from RABA2a under Endosidin 7 treatment. *Plant Signal. Behav.* 11:e984520
19. Day KJ, Staehelin LA, Glick BS. 2013. A three-stage model of Golgi structure and function. *Histochem. Cell Biol.* 140:239–49
20. Dejonghe W, Kuenen S, Mylle E, Vasileva M, Keech O, et al. 2016. Mitochondrial uncouplers inhibit clathrin-mediated endocytosis largely through cytoplasmic acidification. *Nat. Commun.* 7:11710
21. Dejonghe W, Russinova E. 2014. Target identification strategies in plant chemical biology. *Front. Plant Sci.* 5:352
22. Dettmer J, Hong-Hermesdorf A, Stierhof Y-D, Schumacher K. 2006. Vacuolar H^+-ATPase activity is required for endocytic and secretory trafficking in *Arabidopsis*. *Plant Cell* 18:715–30
23. Dettmer J, Schubert D, Calvo-Weimar O, Stierhof Y-D, Schmidt R, Schumacher K. 2005. Essential role of the V-ATPase in male gametophyte development. *Plant J.* 41:117–24
24. Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, et al. 2005. Plant development is regulated by a family of auxin receptor F box proteins. *Dev. Cell* 9:109–19
25. Dhonukshe P, Aniento F, Hwang I, Robinson DG, Mravec J, et al. 2007. Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in *Arabidopsis*. *Curr. Biol.* 17:520–27
26. Dhonukshe P, Grigoriev I, Fischer R, Tominaga M, Robinson DG, et al. 2008. Auxin transport inhibitors impair vesicle motility and actin cytoskeleton dynamics in diverse eukaryotes. *PNAS* 105:4489–94
27. Doyle SM, Haeger A, Vain T, Rigal A, Viotti C, et al. 2015. An early secretory pathway mediated by GNOM-LIKE 1 and GNOM is essential for basal polarity establishment in *Arabidopsis thaliana*. *PNAS* 112:E806–15
28. Drakakaki G, Robert S, Szatmari A-M, Brown MQ, Nagawa S, et al. 2011. Clusters of bioactive compounds target dynamic endomembrane networks in vivo. *PNAS* 108:17850–55
29. Fan L, Li R, Pan J, Ding Z, Lin J. 2015. Endocytosis and its regulation in plants. *Trends Plant Sci.* 20:388–97
30. Feraru E, Feraru MI, Asaoka R, Paciorek T, De Rycke R, et al. 2012. BEX5/RabA1b regulates *trans*-Golgi network-to-plasma membrane protein trafficking in *Arabidopsis*. *Plant Cell* 24:3074–86
31. Flis VV, Daum G. 2013. Lipid transport between the endoplasmic reticulum and mitochondria. *Cold Spring Harb. Perspect. Biol.* 5:a013235
32. Geldner N. 2009. Cell polarity in plants: a PARspective on PINs. *Curr. Opin. Plant Biol.* 12:42–48
33. Geldner N, Friml J, Stierhof YD, Jürgens G, Palme K. 2001. Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* 413:425–28
34. Haseloff J, Siemering KR, Prasher DC, Hodge S. 1997. Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *PNAS* 94:2122–27
35. Hayashi K-I, Neve J, Hirose M, Kuboki A, Shimada Y, et al. 2012. Rational design of an auxin antagonist of the SCF^{TIR1} auxin receptor complex. *ACS Chem. Biol.* 7:590–98
36. Hayashi K-I, Tan X, Zheng N, Hatate T, Kimura Y, et al. 2008. Small-molecule agonists and antagonists of F-box protein-substrate interactions in auxin perception and signaling. *PNAS* 105:5632–37
37. Hicks GR, Raikhel NV. 2012. Small molecules present large opportunities in plant biology. *Annu. Rev. Plant Biol.* 63:261–82
38. Huss M, Ingenhorst G, König S, Gaßel M, Dröse S, et al. 2002. Concanamycin A, the specific inhibitor of V-ATPases, binds to the V_o subunit c. *J. Biol. Chem.* 277:40544–48
39. Hwang I, Robinson DG. 2009. Transport vesicle formation in plant cells. *Curr. Opin. Plant Biol.* 12:660–69
40. Ito Y, Uemura T, Nakano A. 2014. Formation and maintenance of the Golgi apparatus in plant cells. *Int. Rev. Cell Mol. Biol.* 310:221–87

41. Jauh G, Phillips T, Rogers J. 1999. Tonoplast intrinsic protein isoforms as markers for vacuolar functions. *Plant Cell* 11:1867–82
42. Jiang L, Rogers JC. 1998. Integral membrane protein sorting to vacuoles in plant cells: evidence for two pathways. *J. Cell Biol.* 143:1183–99
43. Kania U, Fendrych M, Friml J. 2014. Polar delivery in plants; commonalities and differences to animal epithelial cells. *Open Biol*. 4:140017
44. Kaur N, Reumann S, Hu J. 2009. Peroxisome biogenesis and function. *Arabidopsis Book* 7:e0123
45. Kim J-Y, Henrichs S, Bailly A, Vincenzetti V, Sovero V, et al. 2010. Identification of an ABCB/P-glycoprotein-specific inhibitor of auxin transport by chemical genomics. *J. Biol. Chem.* 285:23309–17
46. Kleine-Vehn J, Dhonukshe P, Sauer M, Brewer PB, Wisniewska J, et al. 2008. ARF GEF-dependent transcytosis and polar delivery of PIN auxin carriers in *Arabidopsis. Curr. Biol.* 18:526–31
47. Kleine-Vehn J, Dhonukshe P, Swarup R, Bennett M, Friml J. 2006. Subcellular trafficking of the *Arabidopsis* auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. *Plant Cell* 18:3171–81
48. Kleine-Vehn J, Friml J. 2008. Polar targeting and endocytic recycling in auxin-dependent plant development. *Annu. Rev. Cell Dev. Biol.* 24:447–73
49. Kleine-Vehn J, Leitner J, Zwiewka M, Sauer M, Abas L, et al. 2008. Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. *PNAS* 105:17812–17
50. Laxmi A, Pan J, Morsy M, Chen R. 2008. Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in *Arabidopsis thaliana. PLOS ONE* 3:e1510
51. Li HM, Chiu CC. 2010. Protein transport into chloroplasts. *Annu. Rev. Plant Biol.* 61:157–80
52. Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T. 2006. Dynasore, a cell-permeable inhibitor of dynamin. *Dev. Cell* 10:839–50
53. Matsuoka K, Bassham DC, Raikhel NV, Nakamura K. 1995. Different sensitivity to wortmannin of two vacuolar sorting signals indicates the presence of distinct sorting machineries in tobacco cells. *J. Cell Biol.* 130:1307–18
54. Müller B, Grossniklaus U. 2010. Model organisms—a historical perspective. *J. Proteom.* 73:2054–63
55. Nishimura T, Hayashi K-I, Suzuki H, Gyohda A, Takaoka C, et al. 2014. Yucasin is a potent inhibitor of YUCCA, a key enzyme in auxin biosynthesis. *Plant J.* 77:352–66
56. Nishimura T, Matano N, Morishima T, Kakinuma C, Hayashi K-I, et al. 2012. Identification of IAA transport inhibitors including compounds affecting cellular PIN trafficking by two chemical screening approaches using maize coleoptile systems. *Plant Cell Physiol*. 53:1671–82
57. Norambuena L, Zouhar J, Hicks GR, Raikhel NV. 2008. Identification of cellular pathways affected by Sortin2, a synthetic compound that affects protein targeting to the vacuole in *Saccharomyces cerevisiae. BMC Chem. Biol.* 8:1
58. Novick P, Ferro S, Schekman R. 1981. Order of events in the yeast secretory pathway. *Cell* 25:461–69
59. Novick P, Field C, Schekman R. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21:205–15
60. Novick P, Schekman R. 1979. Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *PNAS* 76:1858–62
61. O'Connor CJ, Laraia L, Spring DR. 2011. Chemical genetics. *Chem. Soc. Rev.* 40:4332–15
62. Ortiz-Zapater E, Soriano-Ortega E, Marcote MJ, Ortiz-Masiá D, Aniento F. 2006. Trafficking of the human transferrin receptor in plant cells: effects of tyrphostin A23 and brefeldin A. *Plant J.* 48:757–70
63. Paez Valencia J, Goodman K, Otegui MS. 2016. Endocytosis and endosomal trafficking in plants. *Annu. Rev. Plant Biol.* 67:309–35
64. Paris N, Stanley CM, Jones RL, Rogers JC. 1996. Plant cells contain two functionally distinct vacuolar compartments. *Cell* 85:563–72
65. Park E, Díaz-Moreno SM, Davis DJ, Wilkop TE, Bulone V, Drakakaki G. 2014. Endosidin 7 specifically arrests late cytokinesis and inhibits callose biosynthesis, revealing distinct trafficking events during cell plate maturation. *Plant Physiol*. 165:1019–34
66. Paudyal R, Jamaluddin A, Warren JP, Doyle SM, Robert S, et al. 2014. Trafficking modulator TENin1 inhibits endocytosis, causes endomembrane protein accumulation at the pre-vacuolar compartment and impairs gravitropic response in *Arabidopsis thaliana. Biochem. J.* 460:177–85

67. Paul MJ, Frigerio L. 2007. Coated vesicles in plant cells. *Semin. Cell Dev. Biol.* 18:471–78
68. Pedrazzini E, Komarova NY, Rentsch D, Vitale A. 2013. Traffic routes and signals for the tonoplast. *Traffic* 14:622–28
69. Pérez-Henríquez P, Raikhel NV, Norambuena L. 2012. Endocytic trafficking towards the vacuole plays a key role in the auxin receptor SCF(TIR)-independent mechanism of lateral root formation in *A. thaliana*. *Mol. Plant* 5:1195–1209
70. Pizarro L, Norambuena L. 2014. Regulation of protein trafficking: posttranslational mechanisms and the unexplored transcriptional control. *Plant Sci*. 225:24–33
71. Poustka F, Irani NG, Feller A, Lu Y, Pourcel L, et al. 2007. A trafficking pathway for anthocyanins overlaps with the endoplasmic reticulum-to-vacuole protein-sorting route in Arabidopsis and contributes to the formation of vacuolar inclusions. *Plant Physiol*. 145:1323–35
72. Qi X, Zheng H. 2013. Rab-A1c GTPase defines a population of the *trans*-Golgi network that is sensitive to endosidin1 during cytokinesis in *Arabidopsis*. *Mol. Plant* 6:847–59
73. Raikhel NV. 2017. Firmly planted, always moving. *Annu. Rev. Plant Biol.* 68:1–27
74. Raturi A, Simmen T. 2013. Where the endoplasmic reticulum and the mitochondrion tie the knot: the mitochondria-associated membrane (MAM). *Biochim. Biophys. Acta* 1833:213–24
75. Reymond J-L, Awale M. 2012. Exploring chemical space for drug discovery using the chemical universe database. *ACS Chem. Neurosci.* 3:649–57
76. Rivera-Serrano EE, Rodriguez-Welsh MF, Hicks GR, Rojas-Pierce M. 2012. A small molecule inhibitor partitions two distinct pathways for trafficking of tonoplast intrinsic proteins in Arabidopsis. *PLOS ONE* 7:e44735
77. Robatzek S, Chinchilla D, Boller T. 2006. Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis*. *Genes Dev.* 20:537–42
78. Robert S, Chary SN, Drakakaki G, Li S, Yang Z, et al. 2008. Endosidin1 defines a compartment involved in endocytosis of the brassinosteroid receptor BRI1 and the auxin transporters PIN2 and AUX1. *PNAS* 105:8464–69
79. Robert S, Raikhel NV, Hicks GR. 2009. Powerful partners: Arabidopsis and chemical genomics. *Arabidopsis Book* 7:e0109
80. Robineau S, Chabre M, Antonny B. 2000. Binding site of brefeldin A at the interface between the small G protein ADP-ribosylation factor 1 (ARF1) and the nucleotide-exchange factor Sec7 domain. *PNAS* 97:9913–18
81. Robinson DG, Brandizzi F, Hawes C, Nakano A. 2015. Vesicles versus tubes: Is endoplasmic reticulum-Golgi transport in plants fundamentally different from other eukaryotes? *Plant Physiol*. 168:393–406
82. Rodriguez-Furlán C, Hicks GR, Norambuena L. 2014. Chemical genomics: characterizing target pathways for bioactive compounds using the endomembrane trafficking network. *Methods Mol. Biol.* 1174:317–28
83. Rodriguez-Furlán C, Miranda G, Reggiardo M, Hicks GR, Norambuena L. 2016. High throughput selection of novel plant growth regulators: assessing the translatability of small bioactive molecules from *Arabidopsis* to crops. *Plant Sci*. 245:50–60
84. Rojas-Pierce M, Titapiwatanakun B, Sohn EJ, Fang F, Larive CK, et al. 2007. *Arabidopsis* P-glycoprotein19 participates in the inhibition of gravitropism by gravacin. *Chem. Biol.* 14:1366–76
85. Rosado A, Hicks GR, Norambuena L, Rogachev I, Meir S, et al. 2011. Sortin1-hypersensitive mutants link vacuolar-trafficking defects and flavonoid metabolism in *Arabidopsis* vegetative tissues. *Chem. Biol.* 18:187–97
86. Sanderfoot AA, Assaad FF, Raikhel NV. 2000. The Arabidopsis genome. An abundance of soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptors. *Plant Physiol*. 124:1558–69
87. Sata M, Donaldson JG, Moss J, Vaughan M. 1998. Brefeldin A-inhibited guanine nucleotide-exchange activity of Sec7 domain from yeast Sec7 with yeast and mammalian ADP ribosylation factors. *PNAS* 95:4204–8
88. Scheuring D, Viotti C, Krüger F, Künzl F, Sturm S, et al. 2011. Multivesicular bodies mature from the *trans*-Golgi network/early endosome in *Arabidopsis*. *Plant Cell* 23:3463–81
89. Sessions A, Burke E, Presting G, Aux G, McElver J, et al. 2002. A high-throughput Arabidopsis reverse genetics system. *Plant Cell* 14:2985–94

90. Slepnev VI, Ochoa GC, Butler MH, De Camilli P. 2000. Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1 and disruption of clathrin coat function by amphiphysin fragments comprising these sites. *J. Biol. Chem.* 275:17583–89
91. Sorieul M, Langhans M, Guetzoyan L, Hillmer S, Clarkson G, et al. 2011. An Exo2 derivative affects ER and Golgi morphology and vacuolar sorting in a tissue-specific manner in *Arabidopsis*. *Traffic* 12:1552–62
92. Sparkes I, Brandizzi F. 2012. Fluorescent protein-based technologies: shedding new light on the plant endomembrane system. *Plant J.* 70:96–107
93. Steinmann T, Geldner N, Grebe M, Mangold S, Jackson CL, et al. 1999. Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* 286:316–18
94. Surpin M, Raikhel NV. 2004. Traffic jams affect plant development and signal transduction. *Nat. Rev. Mol. Cell Biol.* 5:100–109
95. Surpin M, Rojas-Pierce M, Carter C, Hicks GR, Vasquez J, Raikhel NV. 2005. The power of chemical genomics to study the link between endomembrane system components and the gravitropic response. *PNAS* 102:4902–7
96. Suzuki H, Matano N, Nishimura T, Koshiba T. 2014. A 2,4-dichlorophenoxyacetic acid analog screened using a maize coleoptile system potentially inhibits indole-3-acetic acid influx in *Arabidopsis thaliana*. *Plant Signal. Behav.* 9:e29077
97. Tabak HF, Braakman I, Zand AVD. 2013. Peroxisome formation and maintenance are dependent on the endoplasmic reticulum. *Annu. Rev. Biochem.* 82:723–44
98. Takano J, Miwa K, Yuan L, von Wirén N, Fujiwara T. 2005. Endocytosis and degradation of BOR1, a boron transporter of *Arabidopsis thaliana*, regulated by boron availability. *PNAS* 102:12276–81
99. Tanaka H, Kitakura S, De Rycke R, De Groodt R, Friml J. 2009. Fluorescence imaging-based screen identifies ARF GEF component of early endosomal trafficking. *Curr. Biol.* 19:391–97
100. Tanaka H, Kitakura S, Rakusová H, Uemura T, Feraru MI, et al. 2013. Cell polarity and patterning by PIN trafficking through early endosomal compartments in *Arabidopsis thaliana*. *PLOS Genet.* 9:e1003540
101. Tóth R, Gerding-Reimers C, Deeks MJ, Menninger S, Gallegos RM, et al. 2012. Prieurianin/endosidin 1 is an actin-stabilizing small molecule identified from a chemical genetic screen for circadian clock effectors in *Arabidopsis thaliana*. *Plant J.* 71:338–52
102. Tóth R, van der Hoorn RAL. 2010. Emerging principles in plant chemical genetics. *Trends Plant Sci.* 15:81–88
103. Tse YC, Mo B, Hillmer S, Zhao M, Lo SW, et al. 2004. Identification of multivesicular bodies as prevacuolar compartments in *Nicotiana tabacum* BY-2 cells. *Plant Cell* 16:672–93
104. Uemura T, Ueda T. 2014. Plant vacuolar trafficking driven by RAB and SNARE proteins. *Curr. Opin. Plant Biol.* 22:116–21
105. Uemura T, Ueda T, Ohniwa RL, Nakano A, Takeyasu K, Sato MH. 2004. Systematic analysis of SNARE molecules in *Arabidopsis*: dissection of the post-Golgi network in plant cells. *Cell Struct. Funct.* 29:49–65
106. Van Norman JM, Xuan W, Beeckman T, Benfey PN. 2013. To branch or not to branch: the role of pre-patterning in lateral root formation. *Development* 140:4301–10
107. Vásquez-Soto B, Manríquez N, Cruz-Amaya M, Zouhar J, Raikhel NV, Norambuena L. 2015. Sortin2 enhances endocytic trafficking towards the vacuole in *Saccharomyces cerevisiae*. *Biol. Res.* 48:100–11
108. Viotti C, Bubeck J, Stierhof Y-D, Krebs M, Langhans M, et al. 2010. Endocytic and secretory traffic in *Arabidopsis* merge in the *trans*-Golgi network/early endosome, an independent and highly dynamic organelle. *Plant Cell* 22:1344–57
109. von Kleist L, Stahlschmidt W, Bulut H, Gromova K, Puchkov D, et al. 2011. Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. *Cell* 146:471–84
110. Voss U, Larrieu A, Wells DM. 2013. From jellyfish to biosensors: the use of fluorescent proteins in plants. *Int. J. Dev. Biol.* 57:525–33
111. Worden N, Wilkop TE, Esteve VE, Jeannotte R, Lathe R, et al. 2015. CESA TRAFFICKING INHIBITOR inhibits cellulose deposition and interferes with the trafficking of cellulose synthase complexes and their associated proteins KORRIGAN1 and POM2/CELLULOSE SYNTHASE INTERACTIVE PROTEIN1. *Plant Physiol.* 167:381–93

112. Yang Z. 2008. Cell polarity signaling in *Arabidopsis*. *Annu. Rev. Cell Dev. Biol.* 24:551–75
113. Zhang C, Brown MQ, van de Ven W, Zhang Z-M, Wu B, et al. 2016. Endosidin2 targets conserved exocyst complex subunit EXO70 to inhibit exocytosis. *PNAS* 113:E41–50
114. Zheng H, Staehelin LA. 2011. Protein storage vacuoles are transformed into lytic vacuoles in root meristematic cells of germinating seedlings by multiple, cell type-specific mechanisms. *Plant Physiol.* 155:2023–35
115. Zouhar J, Hicks GR, Raikhel NV. 2004. Sorting inhibitors (Sortins): chemical compounds to study vacuolar sorting in *Arabidopsis*. *PNAS* 101:9497–9501
116. Zouhar J, Rojo E. 2009. Plant vacuoles: Where did they come from and where are they heading? *Curr. Opin. Plant Biol.* 12:677–84
117. Zwiewka M, Friml J. 2012. Fluorescence imaging-based forward genetic screens to identify trafficking regulators in plants. *Front. Plant Sci.* 3:97

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Contents

Errata

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