

Chemical Effectors of Plant Endocytosis and Endomembrane Trafficking

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Abstract Plant endocytosis and endomembrane trafficking relies on the coordination of a highly organized and dynamic network of intracellular organelles. Membrane trafficking and associated signal transduction pathways provide critical cellular regulation of plant development and response to environmental stimuli. However, the efficiency of studies on this complex network has been hampered due to the rapid and dynamic nature of endomembrane trafficking as well as gene redundancy and embryonic lethality in mutagenesis-based strategies. Chemical genomics emerged in recent years as a complementary approach to illuminate biological functions through the integration of organic chemistry, biology, and bioinformatics to overcome gene redundancy. The approach presents significant advantages in dosage dependence and reversibility, which offers the ideal ability to study dynamic endomembrane trafficking processes in real time. In this chapter, several successful examples of chemical screening focused on the endomembrane system is presented to illuminate the efficiency and power of chemical genomics in dissecting endomembrane trafficking and its regulation of plant development and environmental responses. Perspectives are also presented to suggest directions for future development of this field.

Keywords Endocytosis • Chemical biology • Chemical genomics • Endomembrane trafficking • Bioactive compounds • High-throughput screening

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1 Endomembrane Trafficking in Plants Cells

Endocytosis is a complicated and dynamic cellular process for the uptake of extracellular molecules or internalization of plasma membrane proteins and lipids through the formation of closed vesicles (Low and Chandra 1994; Robinson et al. 2008). After internalization, vesicles are either fused with lytic compartments for degradation or recycled back to the plasma membrane. Endocytosis is facilitated by the highly organized endomembrane system which provides the functional compartments necessary for the exchange of proteins, lipids, and polysaccharides via transport intermediates (Jürgens 2004; Šamaj et al. 2005). Several endomembrane trafficking pathways converge in the continuous endomembrane system. The secretory route starts from the endoplasmic reticulum (ER), operates progressively through the *cis*, medial and *trans*-Golgi network (TGN), and finally delivers cargoes to the PM or vacuole (Bassham et al. 2008). While anterograde transport serves as the primary route for newly synthesized proteins from ER to the Golgi and endosomes, retrograde transport mainly functions to maintain the localization of ER or Golgi-resident proteins or components of the trafficking machinery (Hicks and Raikhel 2010) (Fig. 1). Endomembrane trafficking is well studied in animal systems for its importance in the medical field, such as nutrient uptake, cholesterol clearance from blood, and down regulation of many receptor-mediated signalling processes (Murphy et al. 2005; Robinson et al. 2008).

Multiple endomembrane trafficking pathways have been elucidated in animal systems of which clathrin-dependent endocytosis is most widely studied. Clathrin-independent pathways, including caveolae/lipid raft-mediated endocytosis, fluid-phase endocytosis, and phagocytosis have also been investigated in different cells, although the mechanisms are not as clear as the clathrin-dependent pathway due to the lack of specific markers (Šamaj et al. 2004; Murphy et al. 2005). Research on plant endocytosis has lagged for many years due to controversy about the possibility of endocytosis in plant cells in which membrane invagination could be hindered because of the turgor pressure from the cell wall (Cram 1980). In the past three decades, endocytosis in plant cells began to be gradually accepted with the utilization of electron dense tracers, styryl dyes, filipin-labeled plant sterols, the fluid-phase marker Lucifer Yellow, and fluorescent protein-tagged markers in different endomembrane compartments (Hillmer et al. 1986; Geldner et al. 2001; Baluška et al. 2002, 2004; Geldner et al. 2003; Grebe et al. 2003; Hurst et al. 2004). With the help of these techniques, it is now accepted that turgidity is not an obstacle for endocytosis in plant cells (Gradmann and Robison 1989). Even in highly turgid cells such as guard cells, endocytosis can be detected, as indicated by the recent investigation of the potassium channel KAT1 which undergoes constitutive endocytosis and recycling in guard cells (Sutter et al. 2007).

Consistent with animal systems, endocytosis in plant cells is mediated by clathrin-dependent and clathrin-independent pathways, although the existence of caveolae in plant cells is still not clear (Murphy et al. 2005). Multiple endomembrane compartments and the corresponding marker proteins are recognized

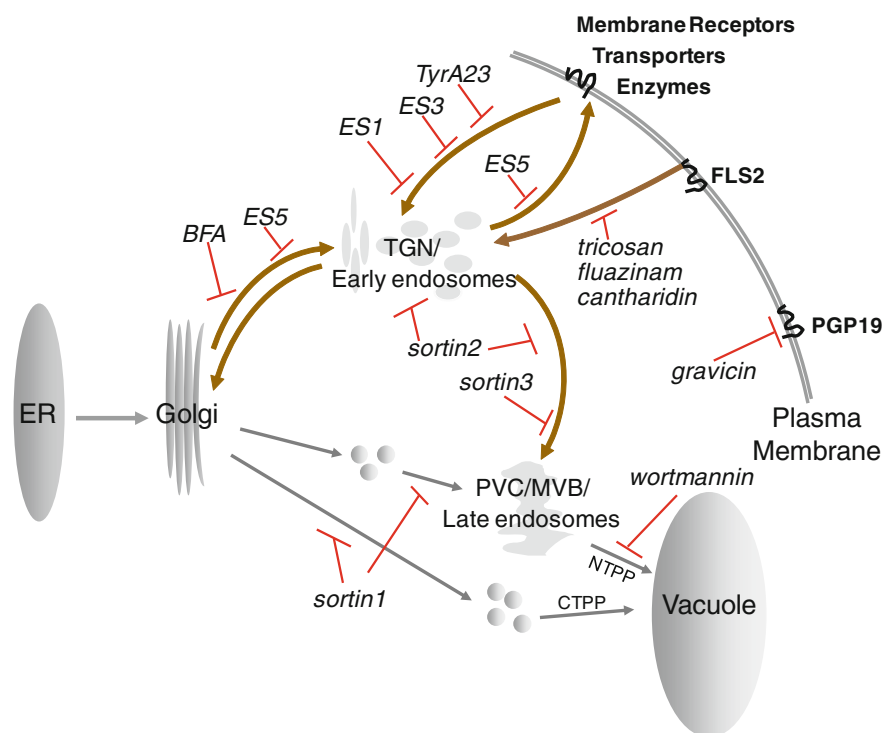


Fig. 1 A basic overview of the endomembrane system in plants. Membrane compartments (gray color) are ER, Golgi, TGN/early endosomes, PVC/multivesicular bodies (MVB)/late endosomes or other intermediate compartments, and vacuole. In plants, endosomes are contiguous with the TGN and PVC/MVB. These schematic symbols represent a continuum of intermediate compartments defined by distinct cargoes and vesicle transport components. Potential routes of vesicle transport are indicated (brown arrows) and include endocytosis, recycling to the plasma membrane, and transport toward the vacuole. Vacuole transport can utilize the N-terminal pro-peptide (NTPP), C-terminal pro-peptide (CTPP), and other routes. Also indicated are endomembrane processes perturbed by novel compounds including sortin1 (NTPP and CTPP targeting and vacuole biogenesis) (Zouhar et al. 2004; Rosado et al. 2011), sortin2 (endosome involved in trafficking to the vacuole) (Norambuena et al. 2008), sortin3 (possible late endosome compartment) (Chanda et al. 2009), gravicin (cognate target is the auxin-related multidrug P-glycoprotein 19, PGP19) (Rojas-Pierce et al. 2007), TyrA 23 (blocks cargo recruitment into CCVs from PM) (Chanda et al. 2009), endosidin1 (ES1) (perturbs an early SYP61/VHA-a1 endosome compartment) (Robert et al. 2008), ES3 (blocks protein trafficking from the PM by affecting TGN/EE compartment), ES5 (blocks recycling), ES7 (perturbation of secretory pathways) (Drakakaki et al. 2011), and several inhibitors (triclosan, fluazinam and cantharidin) of flg22-induced FLS2 endocytosis (Serrano et al. 2007). Several well-established bioactive molecules are BFA that causes agglomeration of Golgi and TGN and wortmannin (Wort) that affects late trafficking to the vacuole. Recent high-throughput screens in plants (see text) will provide a suite of compounds affecting other endomembrane trafficking processes. Figure modified from Hicks and Raikhel (2010) with permission

within endocytic routes. By the observation of sterol tracers, the first station for cargo delivery is the early endosome (EE). In animal systems, Rab5 GTPase has been established as the convenient marker gene for EE (Tonkin et al. 2006). In Arabidopsis, two Rab5 homologs-RabF1 (Ara6) and RabF2b (Ara7) were identified and co-localized with the sterol tracer FM4-64 after short time uptake (Merigout et al. 2002). In plants, the TGN is expected to be functionally equivalent to the EE, which is labeled by markers including the membrane-integral V-ATPase subunit, VHA-a1 (Dettmer et al. 2006), and the plant-specific syntaxin SYP61 (Robert et al. 2008). The TGN also functions as a sorting compartment through which endocytic vesicles are either sorted to late multivesicular bodies (MVBs) or pre-vacuolar compartment (PVC) for targeting to the lytic vacuole or recycled to the plasma membrane. In plants, the vacuolar sorting receptor BP-80 and pre-vacuolar syntaxin SYP21 (PEP12) are recognized as marker genes for PVC (Bowers and Stevens 2005; Rosado et al. 2011), whereas the ARF-GEF GNOM is widely accepted as the marker for putative recycling endosome (Marles et al. 2003).

As evidenced by proliferating research articles and reviews in the last few years, plant endocytosis impacts many critical events during the plant life cycle, including embryo patterning, lateral organ differentiation, root hair formation, hormone signal transduction, and defense responses (Morita et al. 2002; Carter et al. 2004; Voigt et al. 2005; Robatzek et al. 2006; Geldner et al. 2007; Dhonukshe et al. 2008a). Endosomes are essential compartments for protein targeting, recycling, and degradation, and they are of vital importance for processes including auxin signalling and transport (Dhonukshe et al. 2006, 2007a; Sauer and Kleine-Vehn 2011), BR signalling (Geldner et al. 2007; Robert et al. 2008), boron uptake (Takano et al. 2008), blue light responses (Christie 2007), and plant immunity (Robatzek et al. 2006; Sharfman et al. 2011) among others.

Among the well-characterized roles of endosomes, the most widely studied one is the maintenance of polar localization of auxin transporters, namely the PIN proteins. Unlike their animal counterparts, plant cells have no tight junction-like barriers to restrict lateral diffusion between the apical and basolateral membrane domains. Constitutive endocytosis functions as a powerful mechanism to counteract lateral diffusion of PIN proteins within the plasma membrane, thus the maintenance of polar distribution. The establishment of polar localization of PIN proteins consists of three steps: non-polar secretion, clathrin-dependent endocytosis, and subsequent polar recycling, similar to transcytosis in animal cells (Dhonukshe et al. 2008a, see also Chap. 8 by Nodzynski et al. in this volume). This mechanism ensures the dynamic regulation of PIN polarity in response to developmental and environmental stimulus, for example, in the rapid reorientation of PIN3 in columella cells during gravitropic response (Norambuena et al. 2009; Kleine-Vehn et al. 2010) and switch of PIN polarity during embryogenesis (Benková et al. 2003; Beutler et al. 2009). Mutations of Rab5 or clathrin subunits result in disruption of endocytosis and auxin-related developmental defects (Dhonukshe et al. 2007a, 2008a). Endocytosis also plays a key role in signal transduction processes. Although the brassinosteroid (BR) receptor BRI1

(BRASSINOSTEROID-INSENSITIVE 1) undergoes constitutive endocytosis independent of ligand binding, endosomal localization of this receptor and other endosome components are probably important in signal transduction, as evidenced by the positive effects on BRI1 signalling of fungal toxin, brefeldin A (BFA) (Geldner et al. 2007). Unlike BRI1, endocytosis of flagellin receptor FLAGELLIN-SENSITIVE 2 (FLS2) is induced by ligand (Chinchilla et al. 2007). After internalization, FLS2 is targeted for degradation by ubiquitination which subsequently terminates the signal transduction process (Gohre et al. 2008). So in this case, endocytosis works as a negative feedback of the signalling process. Thus, the dynamic trafficking machinery and balance of cargo localization in the endomembrane system needs to be well maintained for proper development and growth regulation during the plant life cycle.

2 Chemicals Known to Perturb Endomembrane Trafficking

Our knowledge of the mechanisms regulating plant endomembrane trafficking has been enriched with the help of technological advancement. Due to high quality confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM), together with the development of histological dyes, contrast-enhancing techniques, and innovative imaging techniques, the ability to image endomembrane structure has been greatly enhanced (Serrano et al. 2007, see also Chap. 1 by Šamajová et al. in this volume). In recent years, the introduction of genetically encoded fluorophores, such as GFP and its mutated isoforms (Chalfie et al. 1994), have further facilitated the observation of subcellular membrane structures. GFP labeling of marker proteins has provided details of the real-time intracellular dynamics within live cells. In the model plant *Arabidopsis*, the large number of available T-DNA inactivation mutants or ethyl methane sulfonate (EMS)-induced point mutation stocks provide valuable genetic resources to screen for specific genes involved in endomembrane trafficking process. However, only a limited number of marker lines are available, almost all based on the published literature, with which is possible to describe the continuous and complicated endomembrane system. Also, fusions with fluorescent protein could impair normal subcellular localization due to structural alteration. Moreover, T-DNA insertions in many plant endomembrane system genes are lethal since many genes involved in endocytosis are of vital importance during embryo patterning or meristem differentiation (Xu and Scheres 2005; Dhonukshe et al. 2008a), although a few point mutants are viable and exert clear phenotypes (Jaillais et al. 2007). In many cases, no phenotypes are observed in loss-of-function mutants due to redundancies in gene functions. In the case of proteins which may have several functions in a cell, point mutants may exhibit phenotypes only in a particular gene function, which further limits the characterization of gene properties.

Compared to the classic technologies mentioned above, chemical biology which has emerged in the past 5–10 years serves as a complementary approach to

illuminate biological functions through the integration of organic chemistry, biology, and bioinformatics, and displays high capacity to overcome important limitations inherent to mutation-based genetic screening (Sharma and Swarup 2003; Aikawa et al. 2006; Robert et al. 2009). Several terms of chemical biology have been defined according to application scope. While chemical genetics makes use of small molecules to identify protein targets through the induction and modulation of phenotypes, the term chemical genomics explores a wider field with the combination of many modern genomics tools, such as high-throughput screening approaches, sequence and expression databases, and next-generation sequencing for the identification of cognate targets (Hicks and Raikhel 2012). Chemical genomics displays advantages over mutagenesis screening through the easy handling of compounds and rapid screening methods. Dosage dependence and reversibility provide the means to circumvent problems of lethality and redundancy (Drakakaki et al. 2009; Hicks and Raikhel 2009; Norambuena et al. 2009). Chemical genomics also offers the ideal ability to study dynamic endomembrane trafficking processes in real time which often is within minutes (Dhonukshe et al. 2007a). This can be achieved by the observing interference of GFP-tagged protein trafficking through endomembrane compartments via confocal microscopy (Drakakaki et al. 2011; Hicks and Raikhel 2012). High-throughput chemical primary screening can also expand the capacity of chemical genomics by developing automated microscopy. In this manner, endomembrane phenotypes of large EMS populations could be scored instead of using time-consuming manual confocal microscopy. These developments may further facilitate the systematic understanding of the endomembrane trafficking machinery (Hicks and Raikhel 2012).

Even before the advent of chemical screening approaches in plants, a variety of inhibitors including BFA and wortmannin were used intensively to define endosomal compartments and illustrated corresponding biological functions. The fungal toxin BFA inhibits ADP ribosylation factor (ARF) GTPases by interacting with their associated guanine nucleotide exchange factors (GEFs) (Dambournet et al. 2011). In *Arabidopsis*, the cognate *in vivo* target of BFA includes the BFA-sensitive ARF-GEF, GNOM, which is a key component involved in cargo recycling (Geldner et al. 2003). BFA has been variously described as an inhibitor of secretion (Nebenfuhr 2002) and endocytosis (Baluška et al. 2002; Geldner 2004) and has been used to dissect trafficking pathways between different endomembrane compartments (Geldner et al. 2003; Grebe et al. 2003; Kleine-Vehn et al. 2008). Another inhibitor Tyrphostin A23, but not Tyrphostin A51, interferes with the recognition of the Yxx Φ internalization motif in the cytosolic domain of plasma membrane receptors by the μ -adaptin of the AP-2 adaptor complex (Titapiwatanakun et al. 2009; Dambournet et al. 2011) and is widely used as a competitive inhibitor of receptor Tyr kinases in mammalian cells due to its structural similarity to Tyr (Aniento and Robinson 2005). In plants, tyrphostin A23 has been reported to inhibit endocytosis in protoplasts (Ortiz-Zapater et al. 2006), prevent the accumulation of PIN2 in BFA compartments in *Arabidopsis* roots (Dhonukshe et al. 2007a), and interfere with cytokinesis and cell plate formation

(Reichardt et al. 2007), indicating that both endocytosis and Golgi-based secretion are targets of the inhibitor. Other inhibitors, such as the phosphatidylinositol-3/4 kinase (PI-3/4 kinase) inhibitor wortmannin, interfere with protein trafficking to the plant vacuole (Dhonukshe et al. 2008b; Takáč et al. 2012) and with endocytosis (Irani and Russinova 2009). Concanamycin A (Conc A) and Bafilomycin A (BafA) bind to V-ATPase subunits c and a (Huss et al. 2002; Wang et al. 2005) leading to massive alteration of Golgi morphology, aggregation of vesicles and a block of transport from the TGN/EE to the vacuole (Strompen et al. 2005; Dettmer et al. 2006). Other compounds affecting endomembrane trafficking are listed in Table 1. Thus, small molecules play important roles in understanding endomembrane compartmentalization and machinery of endomembrane trafficking.

However, most of the inhibitors mentioned above interfere with multiple membrane trafficking processes that have a broad range of functions. In the case of BFA, which is extensively used as endocytosis inhibitor, the compound targets a wide range of endomembrane trafficking processes including ER-to-Golgi, endosome-to-MVB/PVC, and recycling back to the plasma membrane. BFA compartments are actually composed of remnant Golgi stacks surrounding TGN vesicles, and partially of MVB fragments (Satiat-Jeunemaitre et al. 1996; Merigout et al. 2002). To better elucidate the highly specific trafficking routes supported by different endomembrane compartments, including responses to either environmental stimuli or developmental signals, it is important to develop chemicals showing more selectivity for specific targets or particular functions. A step forward is to generate extensive small molecule collections that cover a wide range of protein targets within networks of interests. This would present substantial and diverse phenotypes for systematic organization and provide plentiful information for further studies. Here, we will introduce several successful examples of chemical screens which target the endomembrane system resulting in new inhibitors of endocytosis and endomembrane trafficking.

3 Chemical Genomic Screens to Dissect Vacuole Transport and Biogenesis

The earliest screen for plant bioactive molecules affecting endomembrane trafficking was focused on vacuole transport and biogenesis. Taking advantage of the evolutionary conservation between plants and the budding yeast *Saccharomyces cerevisiae*, a library of 4,800 diverse compounds was screened for those that induced aberrant secretion of carboxypeptidase Y (CPY) in yeast through a high-throughput 96-well assay. This screen identified 14 compounds, termed sorting inhibitors (sortins), which affected the trafficking of proteins to the vacuole in yeast and would phenocopy the vacuole protein sorting (*vps*) mutant phenotypes (Bowers and Stevens 2005) (Table 1). Application of two of these compounds (sortin 1 and sortin 2) in *Arabidopsis* seedlings resulted in similar effects on CPY secretion and

Table 1 Summary of compounds affecting endomembrane trafficking

Compounds	Organisms	Target	Mode of action	Source
BFA	Plant/animal	ARF-GEFs	Block exocytosis/endomembrane recycling	Dinter and Berger (1998), Robinson et al. (2008)
Wortmannin	Plant/animal	PI3Ks and PI4Ks	Block vacuolar trafficking, MVBs and endocytosis	Robinson et al. (2008), Takáč et al. (2012)
Typhostin A23	Plant/animal	Cargo recognition (YXXΦ) by μ subunit of AP complex	Blocks cargo recruitment into CCVs from PM, TGN	Ortiz-Zaoater et al. (2006), Robinson et al. (2008)
Concanamycin A	Plant/animal	V ATPase	Block trafficking at TGN, endosome acidification	Robinson et al. (2008)
Dynasore	Animal	GTPase activity of dynamin	Blocks endocytosis	Macia et al. (2006)
Sortin 1	Yeast/plant	Unknown	Affects vacuole biogenesis	Zouhar et al. (2004)
Sortin 2	Yeast/plant	Unknown	Interference with ESCRT complex components	Zouhar et al. (2004), Norambuena et al. (2008)
Sortin 3	Yeast	Unknown	Possibly target late endosomal compartments	Chanda et al. (2009)
Endosidin 1	Plant	Unknown (stabilizes actin)	Blocks trafficking at the TGN	Robert et al. (2008), Toth et al. (2012)
Endosidin 3	Plant	Unknown	Blocks protein trafficking from the PM by affecting TGN/EE compartment	Drakakaki et al. (2011)
Endosidin 5	Plant/animal	Unknown	Blocks recycling	Drakakaki et al. (2011)
Endosidin 7	Plant	Unknown	Disturbs cell plate formation, cell wall maturation and expansion through the perturbation of secretory pathways	Drakakaki et al. (2011)
Gravicin	Plant	PGP19 and other unknown target	Inhibition of auxin transport and trafficking to the vacuole	Surpin et al. (2005), Rojass-Pierce et al. (2007)
LY294002	Plant/animal	PI3Ks	Blocks MVBs, endocytosis	Lee et al. (2008)

(continued)

Table 1 (continued)

Compounds	Organisms	Target	Mode of action	Source
Bafilomycin A	Plant/animal	V ATPase	Blocks trafficking at the TGN, causes endosome acidification	Robinson et al. (2008)
Sulfonamide 16D10	Animal	V ATPase	Blocks endocytosis and exocytosis, causes endosome acidification	Nieland et al. (2004)
Monensin	Plant/animal	Ionophore	Blocks exocytosis, disrupts trafficking at Golgi	Diner et al. (1998)
Cantharidin	Plant/animal	PP2A-specific inhibitor	Blocks FLS2 endocytic trafficking	Serrano et al. (2007)
Triclosan	Plant	Enoyl-acyl carrier protein (acp) reductases (ENRs)	Blocks FLS2 endocytic trafficking, flg22-induced oxidative burst; inhibitor of fatty-acid synthesis	Serrano et al. (2007)
Fluazinam	Plant	Unknown	Blocks FLS2 endocytic trafficking, flg22-induced oxidative burst	Serrano et al. (2007)
Exo1 and Exo2	Animal	Unknown	Block exocytosis, ER to Golgi transport	Nieland et al. (2004)
Cobtorin	Plant	Unknown	Affects cortical microtubule alignment	Yoneda et al. (2007)
Triclosan, Fluazinam and Cantharidin	Plant	PP2A (cantharidin)	Affect flg22-mediated FLS2 endocytosis	Serrano et al. (2007)
Morlin	Plant	Unknown	Affects cytoskeletal organization/ interaction with cellulose synthase	DeBolt et al. (2007)

vacuole biogenesis, and additional defects of root development. This suggests that it is possible to take advantage of simpler single cell eukaryotes such as yeast to dissect endomembrane trafficking pathways in multi-cellular organisms. However, the fact that only two of the 14 sortins were active in a plant system may reflect the difference in chemical uptake, drug targets, and the evolutionary differentiation between plants and yeast. Further studies utilizing electron microscopy of sortin 1 effects showed that unlike other drugs such as BFA, this compound only showed specific interference with vacuole morphology but not broad disruption of other endomembrane organelles such as ER and Golgi (Zouhar et al. 2004). This indeed sets a good example of a chemical screen to identify specific inhibitors. More recent studies using GFP fusion markers support the preliminary screening conclusion that sortin 1 primarily affects vacuole biogenesis (Zouhar et al. 2004).

The follow-up genetic screen for hypersensitive lines in EMS-mutagenized GFP: δ -TIP Arabidopsis collections identified mutants defective in both vacuole biogenesis and accumulation of anthocyanin (Rosado et al. 2011). The defects of anthocyanin accumulation in vacuoles can be mimicked by the known glutathione inhibitor buthioninesulfoximine, and this demonstrates that sortin 1 or its metabolite may serve as a xenobiotic leading to the depletion of glutathione pools by oxidative stress. Based on the previous evidence that anthocyanins are transported to the vacuole as glutathione conjugates via ATP-binding cassette (ABC)-type transporters (Zhao and Dixon 2009) and defective anthocyanin accumulation results in tonoplast vesiculation caused by autophagy (Rosado et al. 2011), this suggests a working model that anthocyanin transport to the vacuole is essential for normal vacuole biogenesis through the combination of oxidative stress and autophagic processes. In this case, chemical genomics provides a tool to discover new pathways linking vacuole trafficking and vacuole biogenesis to secondary metabolism and oxidative response (Rosado et al. 2011; Hicks and Raikhel 2012).

The genetic characterization of sortin 2 was carried out by screening for sortin 2 hypersensitivity in a yeast haploid deletion library. The resulting hypersensitive mutants are mainly defective in the endomembrane system, such as some of the ESCRT complex components. Further bioinformatics analysis and subcellular localization showed higher expression of genes in the most hypersensitive mutants that are annotated as TGN and endosome related. This reflects the essential functions of these compartments for vacuole targeting or secretion (Norambuena et al. 2008). Thus, this approach verified the role of above compartments in the regulation of vesicular trafficking toward vacuole.

4 Chemical Investigation of Development and Defense Responses

Several studies have clearly demonstrated the crucial role of endosomes in several plant processes including cell fate specification, abscisic acid and auxin signalling, tropic responses, and pathogen defense (Carter et al. 2004; Surpin and Raikhel 2004).

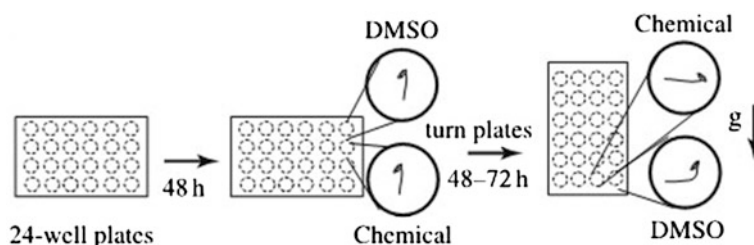


Fig. 2 Screen for chemicals that affect gravitropism. The chemical library was screened in a 24-well format, and seedlings were scored for gravitropic response of both roots and hypocotyls after reorientation. Chemicals dissolved in 20 % DMSO were added to wells. Control wells contained an equivalent concentration of the solvent. The gravity vector (g) is indicated by an arrow (on the right)

Classical mutant screens have shown that the endomembrane system is intimately involved in auxin-dependent responses such as gravitropism. Mutants in genes encoding SNARE (Soluble *N*-ethylmaleimide-sensitive adaptor protein receptor) proteins are both agravitropic and defective in vacuolar morphology (Morita et al. 2002; Yano et al. 2003). Furthermore, polar auxin transport which is crucial for tissue specification and organ formation throughout plant development is highly dependent on endomembrane systems. Recent studies have revealed the constitutive endocytosis and recycling of PIN1 by a clathrin-dependent mechanism (Dhonukshe et al. 2007b; Robert et al. 2010; Kitakura et al. 2011). Disruption of the dynamic cycling between the plasma membrane and endosomal compartments via either pharmacological treatment or interference with gene function results in loss of proper PIN polarity and disruption of normal developmental processes (Jaillais et al. 2007; Kleine-Vehn et al. 2010).

A screen based upon the gravitropic response of *Arabidopsis thaliana* roots and hypocotyls was performed to identify chemical effectors. A chemical library (ChemBridgeDIVERSet) was screened in a 24-well format, and seedlings were scored for both root and hypocotyl responses after reorientation (Fig. 2). The secondary screen was performed under the same conditions as the primary screen, but at variable compound concentrations. The impact on the morphology and targeting to endomembrane compartments was tested using GFP fusion markers, so the effect of these compounds could be used to examine the link between gravitropism and endomembrane system morphology (Norambuena et al. 2009; Robert et al. 2009). The semi-high-throughput primary screen with 10,000 diverse compounds led to the identification of four compounds affecting vacuolar targeting and morphology and shoot gravitropism (Surpin et al. 2005). One of the screens resulted in the identification of gravicin as a strong inhibitor of gravitropism via disruption of auxin signalling as well as inhibition of δ -TIP delivery to the tonoplast (Table 1). Subsequent screens for resistant and hypersensitive mutants to gravicin resulted in the identification of the multi-drug transporter PGP19 as the

cognate target for the inhibition of gravitropism but, interestingly, not for tonoplast mistargeting (Rojas-Pierce et al. 2007).

PGP 19 is an ATP-binding cassette protein that functions as an auxin transporter and participates in the regulation of gravitropic response through interaction with PIN1 (Noh et al. 2001; Titapiwatanakun et al. 2009). However, a second unknown target is responsible for the mistargeting of δ -TIP from the tonoplast to an ER-like compartment. The identification of PGP19 as the gravicin target showed the clear advantage of chemical genomics to dissect links between different pathways. However, the observation that gravicin displays distinct gravitropic and tonoplast targeting defects indicates the potential for multiple targets when dealing with small molecules, possibly due to in vivo metabolism of these compounds resulting in multiple active products and leading to the challenge of defining these active forms (Rojas-Pierce et al. 2007).

The induction of pathogen immunity in plants involves several pathways, including pathogen-associated molecular patterns (PAMP) pathways and the resistance (R-mediated) pathway (Chisholm et al. 2006; Jones and Dangl 2006; Bernoux et al. 2011). In Arabidopsis, the LRR-RLK receptor FLAGELLIN-SENSITIVE 2 (FLS2) is an established receptor for bacterial flagellin (flg22). Using GFP-tagged FLS2, it has been shown that plasma membrane localized FLS2 undergoes endocytosis upon ligand induction. A potentially phosphorylated threonine residue in the juxta membrane region of the receptor is required for both signalling and endocytosis. After internalization, GFP-FLS2 accumulates in intracellular vesicles and is later targeted for degradation, which subsequently attenuates the signalling process (Robatzek et al. 2006). Recently, a chemical screen using a library composed of 120 small molecules with known biological activities was performed to identify candidate compounds which could modify elicitor-responsive gene expression. Seven-day-old Arabidopsis seedlings were used in the screen by growing them in submerged culture in 96-well microtiter plates. In this way it was easy to monitor the transcriptional activation of GUS fused to early PAMP-responsive genes (Serrano et al. 2007). Four compounds were identified that inhibit GUS reporter activity. Among them, three compounds clearly affected flg22-mediated FLS2 endocytosis (Table 1), and two of the three chemicals, triclosan and fluazinam, also strongly inhibited the production of flg22-stimulated reactive oxygen species (Serrano et al. 2007). Thus, the identified chemicals may decrease elicitor-responsive gene induction by interfering with endocytic processes of PAMP receptors at the cell periphery. The chemical cantharidin was identified in the screen, and it induced sustained rather than transient accumulation of reactive oxygen species upon flg22 treatment and inhibition of FLS2 endocytosis (Serrano et al. 2007). These compounds can be used for further investigation on the role of FLS2 endocytosis in downstream signalling and elicitor-mediated immune response.

5 Identifying New Inhibitors of Endosomes Involved in Polar Cell Growth

Polarized tip growth of pollen tubes and root hairs involves targeted transport and vesicle fusion which is necessary to form and maintain a tubular structure. This involves processes including calcium gradient-dependent cytoskeleton organization, vesicle targeting, and recycling at the apex (Drakakaki et al. 2009; Robert et al. 2009). Pollen is an ideal system for chemical screens based on the fact that endomembrane components in large tobacco pollen tubes are relatively easy to view by confocal microscopy, and the organization of endocytic and exocytic vesicles are clearly visible within the growing tip by membrane-staining styryl dyes such as FM4-64 and FM1-43 (Zonia and Munnik 2008; Robert et al. 2009). Chemical genomics is especially valuable for pollen biology because chemical phenotypes are dose dependent, reversible, and can be applied in time course experiments, whereas genetic approaches using mutants defective in endomembrane trafficking in *Arabidopsis* pollen typically result in gametophyte lethality. A high-throughput pollen chemical screen was recently developed using a 384-well microtiter plate format (Robert et al. 2008). More than 2,000 compounds from a collection of known bioactive molecules were scored for inhibition of tobacco pollen germination in the primary screen. The identified germination inhibitors in the first screen represented less than 1 % of the compounds, and could possibly affect exocytosis, endocytosis, cytoskeleton, calcium signalling, or protein targeting, among other factors. These chemicals were subsequently scored in a secondary screen for the perturbation of a plasma membrane-associated tip-focused marker GFP-ROP1-interacting partner 1 (RIP1 or ICR1 (Lavy et al. 2007)) for polarity loss phenotypes such as mislocalization and isodiametric growth. From the initial screen of 2016 chemicals, four resulted in both pollen germination inhibition and mislocalization of GFP-RIP1.

One of these bioactive compounds termed endosidin1 (ES1) was further investigated in detail in *Arabidopsis* roots with available markers which are known to recycle between the plasma membrane and endomembrane compartments (Robert et al. 2008). Two-hour treatment with ES1 resulted in specific effects on the auxin transporters PIN2-GFP (Xu and Scheres 2005) and AUX1-YFP (Swarup and Bennett 2003), and the brassinosteroid (BR) receptor BRI1-GFP (Geldner et al. 2007). In each case, intracellular aggregates termed “endosidin bodies” were formed. However, other plasma membrane markers such as PIN1-GFP and PIN7-GFP were unaffected by ES1 treatment, indicating target specificity and selectivity for endocytic pathways of diverse plasma membrane proteins (Robert et al. 2008, 2009). Differential sensitivity of plasma membrane proteins to ES1 may reflect different endocytic/recycling routes during cargo transport which is consistent with a previous study using BFA (Kleine-Vehn et al. 2008) and suggesting that AUX1, PIN2 and PIN1, PIN7 are trafficking via different pathways (Robert et al. 2008). ES1 antagonizes BR signalling as evidenced by the inhibitory effect on a BR-specific transcriptional factor when examined by quantitative PCR. Seedlings

grown in the dark in the presence of ES1 display a light-grown phenotype, similar to the BR receptor mutant *bri1-1* (Li and Chory 1997). However, in the presence of BFA, BR signalling was increased due to the increased pool of BRI1 in endosomes and other compartments, such as Golgi. Thus, ES1 appears to display action that is more specific than BFA for endomembrane compartments.

Further analysis via the examination of different endomembrane markers revealed that ES1 specifically incorporated TGN/EE compartments into endomembrane bodies defined by markers for the syntaxin SYP61 and the ATPase subunit VHA-a1, but had no effect on other markers for endosomal compartments. This indicates that the major intracellular action of ES1 was achieved through the impact on early endosomes. Although PIN2 formed agglomerates during treatments, ES1 had no effect on PIN2 polarity in the epidermis and cortex, suggesting a different mechanism of asymmetric localization between root cells organized in cell files and pollen tube growth polarity. Consistent with this phenomenon, ES1 did not induce an auxin-specific reporter nor inhibit gravitropism, which depends on PIN asymmetry. This demonstrates that endocytosis and plasma membrane polar localization mechanisms may be uncoupled by ES1. Given that PIN1, PIN2, and BRI1 are sorted through a SNX1 endosomal compartment after internalization and ES1 had no effect on GNOM endosomal compartment essential for PIN1 recycling, this revealed the specification of early endocytic compartments for different plasma membrane proteins prior to trafficking through the shared common steps in late endosomes (Robert et al. 2008; Hicks and Raikhel 2012). Thus, the identification of SYP61/VHA-a1 as an EE compartment highlights the power of bioactive chemicals to dissect the dynamic endomembrane trafficking system and allow us to investigate endocytosis, recycling, and degradation pathways in more detail. It further provides a novel inhibitor of endocytosis through the SYP61/VHA1 defined endosomal compartment.

Although the target of ES1 has not been reported, recent evidence indicates that ES1 halts the movement of FLS2-GFP endosomes after flg22 induction. This coincides with the increased bundling of actin filaments (M. Beck and S. Robatzek, unpublished data), suggesting that ES1 may target the cytoskeleton (Hicks and Raikhel 2012). A recent chemical screen for circadian clock effectors in *Arabidopsis thaliana* identified ES1 as the stimulator of shortened rhythm periodicity. Although there is no direct evidence to link ES1 action with rhythm regulation, Toth and coworkers show that ES1 primarily affects actin filament dynamics. This may later result in reduced severing and depolymerization of actin filaments in both *Arabidopsis* seedlings and mammalian cells. This stabilization of the actin cytoskeleton is the likely cause of changes in vesicular trafficking (Toth et al. 2012). Interestingly, ES1 does not act in the same manner as known actin drugs binding G-actin (e.g. latrunculin B), suggesting that it may rather target an actin-binding protein (ABP). Such an ABP could link cytoskeleton to specific vesicle transport. ES1 presents a clear pharmacological advantage linking endomembrane trafficking and cytoskeleton dynamics with developmental regulation and response to environmental stimulus. In this respect, a recent proteomic screen using BFA already identified the ABP profilin 2 as a crucial cytoskeletal protein regulating vesicular trafficking in *Arabidopsis* (Takáč et al. 2011).

6 Systematic Approach to Understand the Endomembrane Network

To fully understand the dynamic endomembrane trafficking network and obtain novel effectors of endocytosis, chemical screens need to be combined with real-time microscopy which is essential to observe the effects of compounds on markers rapidly and directly. A step forward is to generate extensive small molecule collections that cover a wide range of protein targets, so the scaled-up chemical genomics would provide systematic information through the evaluation of subcellular phenotypes based on multiple endomembrane-specific markers (Hicks and Raikhel 2010, 2012).

In this regard, Drakakaki et al. (2011) developed a modified high-throughput confocal-based screen focused on the rapid cycling of plasma membrane markers to discover molecules that target endomembrane trafficking *in vivo* in a complex eukaryote. Compared with the previous semi-high-throughput screen performed by the same group, the modified screening expanded the scale of chemicals to 46,418 compounds in the primary screen. Based on the germination and growth of tobacco pollen, 360 (0.77 %) inhibitors of pollen germination were identified in the first screen by using a 384-well format and automated microscopy (Drakakaki et al. 2011). By using CLSM, the molecules were then used to challenge model cargoes represented by polar plasma membrane markers PIN2::PIN1:GFP, PIN2::PIN2:GFP, and non-polar receptor BRI1::BRI1:GFP expressed in *Arabidopsis* root tips. The secondary screen resulted in about 7,500 CLSM images representing the marker behaviors of 18,570 seedlings. About 123 small molecules (34 % out of 360) were identified in the secondary screen and these were further analyzed against plasma membrane, endosome/TGN, and other endomembrane markers. To evaluate and characterize the intracellular effects of these molecules, a scoring system of chemically induced subcellular phenotypes was developed by using hierarchical clustering that treats data points with single and multiple phenotypes. This clustering grouped structurally diverse compounds into 18 clusters, of which 15 classes represented distinct subcellular phenotypes induced by chemical treatment. This included endomembrane bodies of various qualitative size categories, morphology, aberrant localization of plasma membrane or endosomal markers, changes in the polarity of PIN proteins, altered vacuole morphology, and cell plate defects, among others. A color-coded map incorporating multiple subcellular markers treated at different time points was also developed in a non-biased manner by computational methods (Fig. 3). The resulting clustering of PIN2::PIN1:GFP and PIN2::PIN2:GFP in different groups at 24 h of treatment supported previous hypotheses that apical, basal, and basolateral targeting of plasma membrane proteins are maintained through distinct endomembrane pathways.

Further characterization of the molecules identified additional endosidins (Table 1), other than ES1. Endosidins are defined as bioactive molecules affecting trafficking or the morphology of endomembrane compartments. Among them, ES3 is a potential inhibitor of protein trafficking from the plasma membrane by

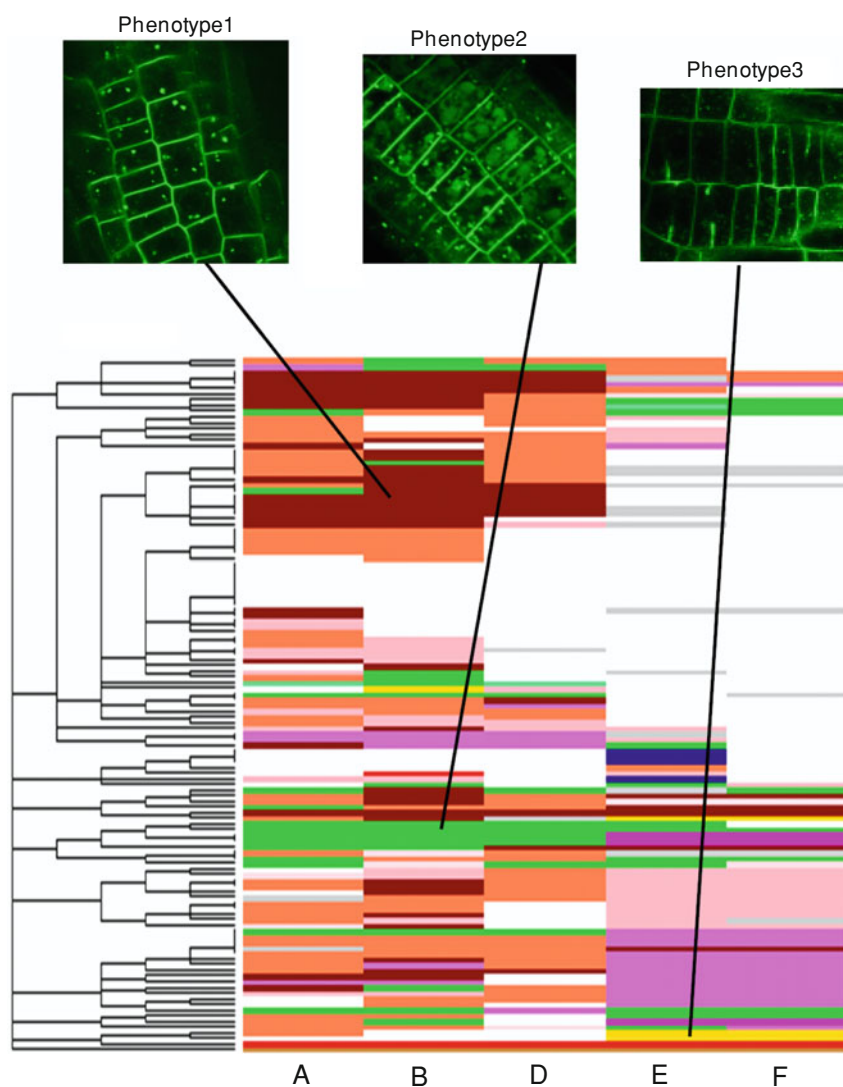


Fig. 3 An illustration of phenotypes and output from a high-content intracellular small molecule screen. The *colored map* presents the results from screening different molecules (*dendrogram at left*) on a collection of Arabidopsis lines expressing different fluorescent protein markers in roots (A–F). *Color coding* represents distinct phenotypes observed upon chemical treatment. In this display, a single color-coded phenotype can be produced within a single marker line or across multiple marker lines, which leads to clustering of bioactive compounds based on phenotype. *Dashed lines* point to clusters represented by phenotypes 1, 2, or 3. Within each cluster, more in-depth structural analysis defines molecules of similar or dissimilar structure. Thus, this approach is also potentially predictive of the activity of uncharacterized molecules. Marker lines in this example can also be screened after different time of treatment or following other treatments, such as stress or pathogen challenge. Figure modified from Hicks and Raikhel (2012) with permission

affecting TGN/EE compartments without effecting Golgi or late endosome/prevacuolar compartments. One interesting thing is that ES3 can also distinguish between two antagonist ROP-signalling pathways necessary to establish polar growth via the auxin-responsive remodeling of cytoskeleton, possibly through the affection of lipid modification. ES5 induces a strong accumulation of plasma membrane cargoes in the vacuole and also affects gravitropism in a dose-dependent manner. The further investigation of ES5 function in Hela cells demonstrated that ES5 targeted recycling in both plant and animal cells, providing insights into recycling mechanisms across kingdoms. ES7 displays a capacity to disturb cell plate formation, cell wall maturation, and expansion through the perturbation of secretory pathways. This demonstrated the ability of small molecules to dissect specificity of endomembrane system in late cytokinesis (Drakakaki et al. 2011).

Taken together, the high-content intracellular screening paralleled with evaluation and elegant clustering of complex intracellular phenotypes visualized by one or multiple endomembrane-specific molecular markers permits a comprehensive view of the endomembrane network. Also, the database of phenotypic clusters presents a good resource for future investigations. Furthermore, all three ES compounds mentioned above linked intracellular trafficking to certain developmental or physiological phenotypes, which would be a challenge for forward genetic screens. Phenotypic data combined with structural databases can be used for the design of new molecules. The common effect of certain chemicals between plants and other organisms also provides the potential to use plant-based systems for drug discovery. Critically, through the assembly of clustered phenotypic data from intracellular dynamics to whole seedlings, it is possible to investigate the network of endomembrane trafficking, developmental regulation and response to environmental stimulus (Drakakaki et al. 2011; Hicks and Raikhel 2012).

7 Finding New Effectors and Pathways

Plant researchers in traditional genetics tend to prefer the one-to-one principle that chemicals should display strong activity against a single cognate target. Although this will be helpful in subsequent genetic screens to identify resistance genes, it does not necessarily block the value of compounds with well-characterized cellular effects. In the case of gravicin, although the identified resistant gene PGP19 is responsible for one of the compound effects, inhibition of gravitropism, whereas the other target for vacuole trafficking defects is unknown (Rojas-Pierce et al. 2007), it still provides a good platform for the investigation of intracellular trafficking and physiological response. Another example is the well-characterized fungal toxin BFA (Satiat-Jeunemaitre et al. 1996). The best known target of this chemical in plants is the SEC7 family member ARF/GEF (ADP-ribosylation factor/GDP/GTP exchange factor) protein GNOM (Geldner et al. 2003). Treatment with BFA results in the agglomeration of TGN/EE and part of the Golgi stacks by the disruption of Golgi-based secretion and indirect interference with endocytosis

and exocytosis (Nebenfuhr et al. 2002; Ritzenthaler et al. 2002). However, BFA is still one of the most widely used chemical inhibitors and has contributed greatly to the research literature, for example in elucidating differential sensitivities of endosomal vesicles and distinguishing between different endomembrane trafficking routes. Other cases such as ES1, ES3, ES5, and ES7, as mentioned in the above sections, have already drawn wide interest for their specific effects on particular intracellular trafficking pathways even before characterization of the cognate targets. By their direct application, these newly identified compounds provide excellent tools by being rapid, reversible, and conditional for the study of highly dynamic processes in endomembrane trafficking (Drakakaki et al. 2011).

The other challenge of chemicals in plant cell biology is target identification since targets are essential to fully understand functionality. Forward genetic screens for resistant or hypersensitive mutants are the most widely used methods for target identification after primary or secondary chemical screening. Structure–activity relationship (SAR) studies are often helpful in selecting related molecules which might be a better choice in subsequent genetic screens (Peddibhotla et al. 2007; Rojas-Pierce et al. 2007). Identification of core structures of chemicals by SAR studies also facilitates the design of biotin-tagged derivatives in subsequent affinity purification (Khersonsky et al. 2003; Wang et al. 2004). Affinity chromatography is a primary approach in the animal field and has gained some success in plant biology as well, such as the identification of the terfestatin A target (Hayashi et al. 2008). Other strategies have been developed, although most of them are only applied in mammalian systems, such as the improved affinity chromatography-tagged library, yeast-three-hybrid (Y3H), phage display, protein microarray, and transcriptome analysis (Toth and van der Hoorn 2010). Although EMS-based mutagenesis is still the most prevalent strategy in plant chemical genomics, these established methods will eventually enable plant scientists to develop more advanced technologies which are suitable for application in plants. The combination of chemical screening, proteomics analysis, next-generation sequencing, and mapping will also facilitate target identification and expand the range of chemical screening to wider application scope (Hicks and Raikhel 2009; Schneeberger et al. 2009).

In the future, to fully capitalize on the potential of chemical biology to uncover new pathways and networks in endomembrane trafficking, the development of more advanced techniques which serve automated plate preparation, seed plating, and imaging to effectively screen large, diverse chemical library will be required. Hardware and software for image-based intracellular phenotypes will simultaneously collect large sets of image and video data. It will be necessary to develop automatic image analysis tools to correlate intracellular bodies, vesicle movement, and signal intensity with different chemical treatments. An important goal is to shift from a qualitative description to quantification of vesicles and organelles upon automatic real-time tracking rather than tedious manual measurements of images (Agee and Carter 2009). An example of this approach is the recent work in zebrafish, in which neuroactive small molecules were screened by automatic record of 14 behavioral responses (Kokel et al. 2010). However, most of the

commercial tools are designed for mammalian drug screening in which the cells are relatively two dimensional (Kvilekval et al. 2010), whereas plant tissues with multiple cell layers and cell types present a much greater challenge for instrumentation and computational analysis. However, recent work from the Robatzek laboratory sets a good example of automated image capture and quantification in leaf pavement cells, inspiring scientists to devote more effort on designing such tools in plant field (Salomon et al. 2010). The Raikhel group at UC Riverside is developing image processing methods to facilitate the automatic measurement and quantification of vesicle movement during pollen germination (Ung et al. 2012).

With a large array of data collected from screening, it is necessary to design websites to assist with the structure-based hierarchical clustering of molecules. The well-established web-based systems are ChemMine (Girke et al. 2005; Backman et al. 2011) (<http://chemmine.ucr.edu/>) and ChemMineR (Cao et al. 2008) ([http://manuals.bioinformatics.ucr.edu/home/chemminer](http://manuals.bioinformatics.ucr.edu/home/chemminer;); <http://cobra20.fhcrc.org/packages/release/bioc/html/ChennineR.html>). The ChemMine portal allows users to query compound information by chemical property, structure, and activity and also allows the release of efficiently managed phenotype database from different screening projects. The most important benefit of the website is that it is an open database that allows scientists to upload their collected data and share their results with the community. As with the example of ChemMine, housing and sharing common databases is of vital importance in chemical genomics. This will permit non-expert laboratories to bypass some of the most laborious initial screening necessary for the discovery of chemicals with bioactive functions. ChemMine is housed at UC Riverside's Center for Plant Cell Biology (<http://cepeceb.ucr.edu>). Some other sites located in Europe include the Department of Plant Systems Biology at the University of Ghent (<http://www.psb.ugent.be/roots-projects/157-chemical-genetics>). This worldwide contribution and arrangement will facilitate the better utilization of chemical biology by the plant community and provide broad dissemination of information and tools such as endomembrane trafficking inhibitors.

Overall, with the development of sophisticated microscope technology, automatic software analysis, systematic data assembly, and community-based databases, chemical genomics will stand in a more powerful position in the research of endomembrane trafficking in plant cell biology.

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