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Protein trafficking in plant cells: tools and markers

Dongmei Zhu[1], Mengdi Zhang[1], Caiji Gao[2] & Jinbo Shen[1*]

[1]*State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Hangzhou 311300, China;*
[2]*Guangdong Provincial Key Laboratory of Biotechnology for Plant Development, School of Life Sciences, South China Normal University (SCNU), Guangzhou 510631, China*

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Eukaryotic cells consist of numerous membrane-bound organelles, which compartmentalize cellular materials to fulfil a variety of vital functions. In the post-genomic era, it is widely recognized that identification of the subcellular organelle localization and transport mechanisms of the encoded proteins are necessary for a fundamental understanding of their biological functions and the organization of cellular activity. Multiple experimental approaches are now available to determine the subcellular localizations and dynamics of proteins. In this review, we provide an overview of the current methods and organelle markers for protein subcellular localization and trafficking studies in plants, with a focus on the organelles of the endomembrane system. We also discuss the limitations of each method in terms of protein colocalization studies.

protein subcellular localization, organelle markers, endomembrane system

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Introduction

Consistent with other eukaryotic cells, plant cells have membrane-bound organelles that perform specific functions critical for cell survival. A number of these organelles can be thought to be "connected" by vesicle-mediated movement of proteins and lipids between them, hence the termed "the endomembrane system". Membrane trafficking in the endomembrane system plays a fundamental function, supporting cell proliferation, cellular polarization, maintenance of cellular homeostasis and specific demands for higher order function in multicellular organisms (Morita and Shimada, 2014). In plant cells, the endomembrane system mainly includes the nuclear envelope, the endoplasmic reticulum (ER), the Golgi apparatus, the *trans*-Golgi network or early endosome (TGN/EE), the prevacuolar compartment/multivesicular body or late endosome (PVC/MVB/LE), vacuole, plasma membrane (PM), and different types of transport vesicle. Plant cells have a highly complex endomembrane system that is largely conserved, but with differs from that of yeast or mammalian model. Contrary to mammalian and yeast cells, plant cells contain two types of vacuoles that have distinct morphology and functions: lytic vacuoles (LVs) and protein storage vacuoles (PSVs) (Paris and Rogers, 1996). The plant LV is functionally equivalent to the animal lysosome and the yeast in terms of protein degradation and has an acid pH, while the PSV is unique to plant cells for protein storage with a neutral pH environment (Jiang et al., 2001; Jiang and Rogers, 2003). The conventional protein transport pathways in the plant endomembrane system are summarized in Figure 1.

Biosynthetic pathway to the plant vacuole

In the secretory pathway to plant LVs, soluble proteins are firstly translocated into the ER lumen. If the proteins are not

*Corresponding author (email: jshen@zafu.edu.cn)

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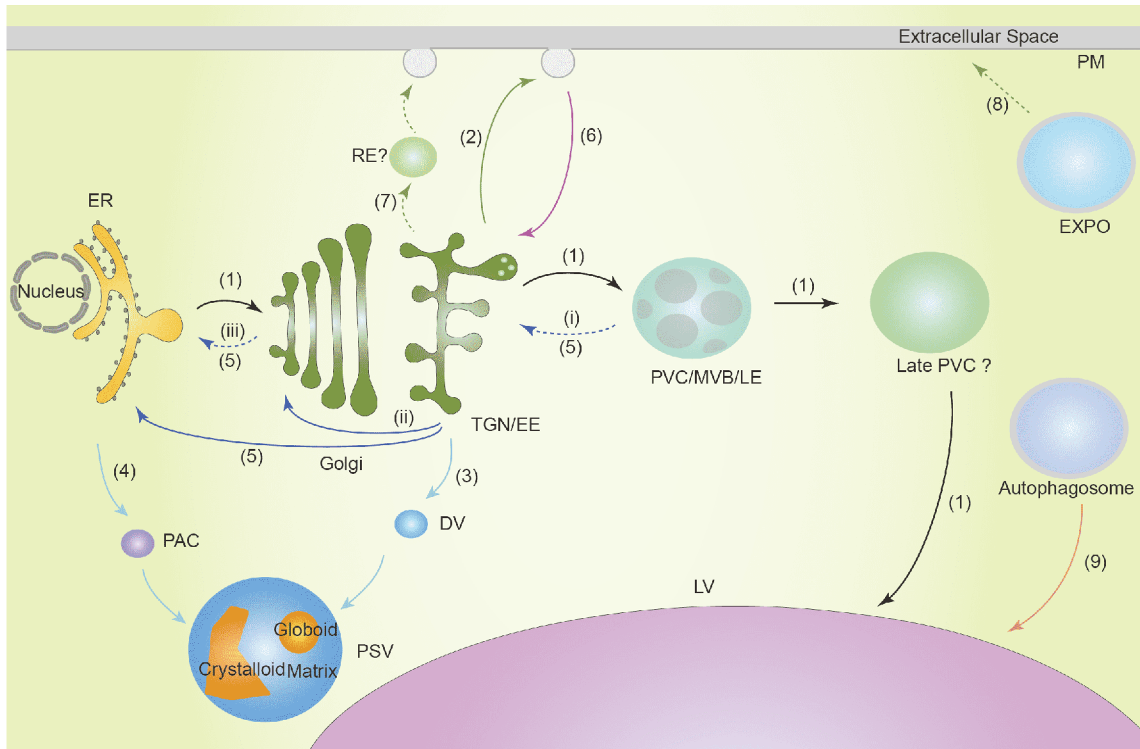

Figure 1 The plant endomembrane system and working model of conventional endosomal trafficking routs. In the plant secretory pathway, soluble proteins containing an N-terminal signal peptide entry into the ER and then transport to the Golgi apparatus. Sorting events happens at the TGN/EE: (1) proteins destined for LV are sorted from TGN/EE en route to PVC/MVB/LE, and later deposited into LV (black solid arrow); (2) proteins without vacuolar sorting signal will be secreted outside of the cell from the TGN via the "default pathway" (green solid arrow). Proteins destined for PSV are sorted (3) from TGN/EE via DVs (dense vesicles) (light blue solid arrow) or (4) via a Golgi-independent pathway directly from ER through PAC (light blue solid arrow); (5) proteins can be recycled from either (i) MVB/PVC/LE, (ii) TGN/EE, or (iii) Golgi as retrograde protein transport (dark blue dashed arrow); (6) in the endocytic pathways (purple solid arrow), proteins are internalized from the plasma membrane or extracellular space and first reach the TGN/EEs. From there they either move to PVC/MVB/LE for further transport to the LV for degradation, or (7) they are recycled back from TGN/EE to the PM by recycling endosome dependent (green dashed arrow) or independent pathway (green solid arrow); (8) protein secretion can be mediated by UPS routes, such as EXPO mediated secretion (green dashed arrow); (9) in autophagy, cytoplasmic materials are delivered via the autophagosome into the LV for degradation (red solid arrow). DV, dense vesicle; ER, endoplasmic reticulum; EXPO, exocyst-positive organelle; MVB/PVC/LE, multivesicular body/prevacuolar compartment/late endosome; LV, lytic vacuole; PAC, precursor accumulating vesicle; PM, plasma membrane; PSV, protein storage vacuole; RE, recycling endosome; TGN/EE, *trans*-Golgi network/early endosome; UPS, unconventional protein secretion.

retained in the ER, they will be transported to the Golgi apparatus for further processing. Proteins with vacuolar sorting signals, also named as cargo proteins, are recognized by vacuolar sorting receptors (VSRs) in the TGN/EE and further transported into PVC/MVBs, where receptors are recycled back to the TGN/EE by the retromer complex for another round of cargo binding, while cargo proteins are finally sorted into the LVs. This soluble vacuolar protein sorting pathway has often been used as a typical paradigm in discussions on VSR-mediated vacuolar protein transport (Robinson et al., 2008a; Rojo and Denecke, 2008; Shen et al., 2013a). Nevertheless, an alternative model for cargo protein sorting and receptor recycling has also been proposed recently with new emerging data, which suggests that such receptor-cargo sorting could initiate already in the ER or the *cis*-Golgi and the receptors dissociate with the cargo in the TGN from where the VSRs are transported back to the ER (Früholz et al., 2018; Künzl et al., 2016; Niemes et al., 2010a, 2010b; Robinson, 2014, 2018; Robinson and Neuhaus, 2016).

Multiple mechanisms are responsible for transporting storage proteins into PSVs (Robinson and Neuhaus, 2016; Shen et al., 2018a; Vitale and Hinz, 2005): (i) storage proteins may be sorted into dense vesicles (DVs) in the *cis*-Golgi possibly requiring the function of VSRs (Hillmer et al., 2001; Hinz et al., 1999; Robinson et al., 1998; Shimada et al., 2003; Shimada et al., 1997) or via receptor homology-transmembrane-RING H2 domain proteins (RMRs) as sorting receptors (Jiang et al., 2000; Park et al., 2005; Shen et al., 2011); (ii) storage proteins, such as 2S albumin and 11S globulin in pumpkin seeds (Shimada et al., 2002) or globulin in rice endosperm (Takahashi et al., 2005), can also reach PSVs directly from the ER bypassing the Golgi complex mediated by precursor-accumulating (PAC) vesicles; (iii) similarly, the ER-derived dark intrinsic protein (DIP) organelles might serve as transport vesicles for proteins targeting

to PSVs in tobacco seeds (Jiang et al., 2000); (iv) in wheat and maize, PSV transport of prolamins to the PSV can also be achieved through autophagic fusion of protein bodies (PBs), which are derived from ER-released storage protein aggregates, with PSVs (Levanony et al., 1992; Li et al., 1993; Rubin et al., 1992); Finally, (v) the sorting of 7S vicilin and prolegumin in pea seeds may also involve the formation of detergent-resistant aggregates which bind to the DV membrane (Hinz et al., 1997; Robinson et al., 1998).

Secretory pathway

If the soluble proteins lack a vacuolar sorting signal, they will traffic through the TGN/EE and then be secreted outside of the cell into the extracellular space (ECS) by the "default pathway", which is defined as the classical or conventional protein secretion pathway. It is now reported that plants also show different types of unconventional protein secretion (UPS) pathways, including a Golgi-bypass pathway for signal peptide-lacking cytosolic proteins (Cheng et al., 2009; Zhang et al., 2011) and secretion pathways that are mediated by specific organelles including the central vacuole (Hatsugai et al., 2009), PVC/MVBs (Nielsen et al., 2012; Nielsen and Thordal-Christensen, 2013), and a double-membrane organelle termed EXPO (exocyst-positive organelle) (Ding et al., 2014; Wang et al., 2010).

Endocytic and recycling pathways

Endocytosis is a major route for the entry of membrane proteins, lipids, and extracellular materials into the cell via a series of endosomal compartments and thus plays an essential role in cell-to-cell communication and cellular responses to environmental stimuli (Murphy et al., 2005). Many PM localized integral membrane proteins, including nutrient transporters, ion channels, and receptors proteins have been identified as endocytic cargoes. These include leucine-rich repeat receptor-like kinases (RLKs) such as brassinosteroid (BR) insensitive 1 (BRI1) and flagellin sensing 2 (FLS2), auxin carriers PIN-FORMED 1 and 2 (PIN1 and PIN2), auxin transporter protein 1 (AUX1), a boron transporter (BOR1), an iron-regulated transporter 1 (IRT1), a plant aquaporin (PIP2;1), an ammonium transporter (AMT1;3), and members of the PHT1 family of high-affinity Pi transporters (Barberon et al., 2011; Bayle et al., 2011; Cardona-López et al., 2015; Geldner et al., 2001; Geldner et al., 2007; Kalinowska et al., 2015; Kasai et al., 2011; Kleine-Vehn et al., 2006; Kleine-Vehn and Friml, 2008; Spallek et al., 2013; Spitzer et al., 2009).

A key regulator for the endocytosis of integral membrane proteins is ubiquitination. Plant membrane proteins, such as misfolded proteins or activated receptors, are usually ubiquitinated and delivered into the TGN/EE via endocytosis. Thus, the secretory and endocytic pathways merge at the TGN/EE and their cargoes are passed on to the PVC/MVBs by different sorting machineries. In plants, two endocytic pathways: clathrin-mediated endocytosis (CME) and membrane microdomain-associated endocytosis, have been identified (Fan et al., 2015). Similar to animal cells, CME is the main route for the entry of extracellular material into plant cells. Flotillin and remorin are well-characterized PM membrane microdomain marker proteins (Li et al., 2012; Raffaele et al., 2009).

Endocytosed PM proteins are further sorted into the intralumenal vesicles (ILVs) of PVC/MVBs, which then fuse with vacuoles to deliver their contents to the lumen for degradation. The formation of ILVs occurs by invagination of the endosomal limiting membrane and the sorting of ubiquitinated membrane cargoes into PVC/MVBs is facilitated by the endosomal sorting complex required for transport (ESCRT) machinery (Gao et al., 2017; Henne et al., 2011). Endocytosed PM proteins without a ubiquitin tag or after deubiquitination can also be recycled back to the PM from the TGN or recycling endosome (RE) (Valencia et al., 2016). Endocytosis and recycling of membranes has a profound developmental importance, which contributes to the maintenance of the overall lipid and protein distribution between PM and secretory compartments. Recently, data is accumulating that demonstrate multiple localizations of ESCRT components, e.g., at the PM, TGN, and even nucleus, indicating their other potential functions beyond sorting at the PVC/MVBs (Li et al., 2019; Scheuring et al., 2011; Wang et al., 2017).

Autophagy

Macroautophagy (hereafter simply autophagy) is another conserved degradative pathway for the delivery of cytoplasmic materials into the lytic vacuole of the plant cell. Autophagy is characterized by the formation of a double-membrane structure called the autophagosome. Over the past years, the molecular components, formation as well as the membrane origin of autophagosomes in plant cells have been discussed in a number of reviews and papers (Liu and Bassham, 2012; Soto-Burgos et al., 2018; Zhuang et al., 2018; Zhuang and Jiang, 2014) and will not be covered here.

Protein trafficking in the endomembrane system is tightly regulated by multiple machineries and mechanisms in the cell. To understand the functional role(s) of a particular protein in the plant cell, it is necessary to know the specific organelle which harbors this protein and its trafficking rout (s) at particular times in different developmental stages and under particular environmental conditions. Multiple tools have been developed to identify the subcellular localization of proteins and their trafficking pathways, and a broad range of marker proteins, which predominantly locate at one spe-

cific organelle, have been identified and are widely used (Dangol et al., 2017; Li et al., 2012; Nelson et al., 2007; Shen et al., 2013b). In this review, we firstly explain the basic principles of commonly used techniques and recent advances on the protein subcellular localization and trafficking analysis. We also consider their advantages and challenges. Then we proceed to summarize previous well-identified organelle specific protein markers for the plant endomembrane compartments. Finally, we close our review with a brief commentary on issues and considerations for protein co-localization studies in plants.

Tools for protein subcellular localization and trafficking analysis

In this section, we start by focusing on immunochemical methods that require specific antibodies for assessing protein distribution. This includes organelle fractionation, immunofluorescence (IF), and immunoelectron microscopy (IEM). Such protein localization information may also contribute to our understanding of protein trafficking mechanisms, because the localization of a protein in a particular endomembrane compartment reflects its enrichment there due to the transport pathway. Then we describe the expression of fluorescence protein fusion (FPF) constructs using a variety of state-of-the art microscopy imaging techniques to visualize the FPF at higher resolution and fast speed to study the protein subcellular localization and dynamic transport in plants. These methods are also complemented by transient expression methods and pharmaceutical treatments, which can be applied both to cell cultures and intact tissue preparations. Each of the methods described is highly flexible with regard to accommodating particular experimental questions and constraints.

Organelle fractionation

Organelle fractionation is a technique to isolate specific organelles from cell/tissue homogenates. Proteins are then identified down-stream by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting or mass spectrometry (MS) to analysis to obtain protein information in specific organelle. Organelle isolation can be accomplished by the techniques of differential centrifugation and density-gradient centrifugation (Graham, 2001). Differential centrifugation is based on the principle that the particles separate according to their mass with the heaviest particles sedimenting first. Base on this concept, centrifugation at different speed allows the separation of organelles and even macromolecules into different "fractions" (Figure 2A). Usually, differential centrifugation is followed by density-gradient centrifugation, which is more sensitive and widely used (Figure 2B). In this process, the cellular components are separated by density in a gradient of dense substance, such as sucrose. The cell lysate is layered at the top of a density gradient substance. During the centrifugation, cell components move through the gradient until they reach their equilibrium density. Once the different fractions are collected, the target proteins localization can be analyzed by SDS-PAGE and immunoblotting with fractions being monitored for known organelle markers (Figure 2B).

A problem with organelle fractionation is that the collected fractions are not pure and organelle distributions overlap extensively, even with a careful selection of density gradients (Dunkley et al., 2006). Moreover, this method requires a series of specific antibodies for individual organelle in the SDS-PAGE immunoblotting analysis.

Immunofluorescence

Immunofluorescence (IF) is a technique relying on the use of antibodies to label a specific target protein (antigen). They are tagged with a fluorophore, which then allows visualization of the protein localization in the sample under the fluorescence microscope. Depending on whether the fluorophore is conjugated to the primary or the secondary antibody, IF methods are divided into direct IF, in which the antibody against the target protein is directly conjugated to a fluorophore, or indirect IF, which uses two antibodies (Kiernan, 1999). The primary antibody binds directly to the target and a fluorophore-conjugated secondary antibody binds indirectly by using the primary antibody as a bridge to the targets (Figure 3A). Generally, the indirect IF is the most common and cost-effective approach, because fluorescently labeled secondary antibodies are relatively inexpensive, flexible to change multiple colors, and compatible with any primary antibody that are home-made. Full protocols for IF in plants have been published (Paciorek et al., 2006; Sauer et al., 2006), so we will not discuss them here.

IF can be used for labeling multiple antigens in the same sample, thus in this regard, IF is especially useful for protein co-localization analysis in the cell. Traditionally, double IF has been achieved by using two different color fluorophore-conjugated secondary antibodies against primary antibodies raised from different host species to avoid cross-reactivity between primary and secondary antibodies (Figure 3B). In this way, the localization of the target protein can be identified in double IF with one known marker protein antibody.

One of the most important considerations in IF is the specificity and affinity of the primary antibody for its antigen, since it is the main cause of failure. During an IF experiment, negative controls should also be included to increase confidence in the antibody specificity: (i) the use of pre-immune serum instead of primary antibodies; (ii) pre-absorption of primary antibodies with the antigen; (iii) apply

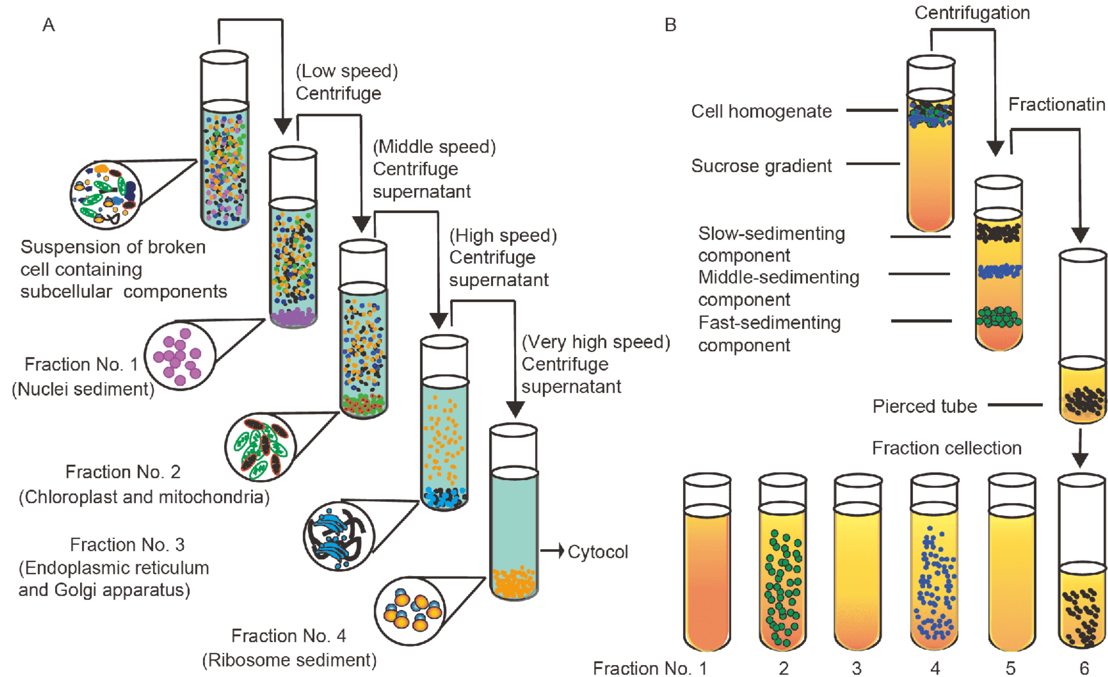

Figure 2 Fractionation of cellular components. A: Differential centrifugation. The homogenate is first subjected to low speeds of centrifugation (800*g*–1000*g*) which will sediment unbroken cells and the largest cell organelle, and nucleus. The pellet is collected while the supernatant, which contains other cellular components, is further subjected to medium-speed (10,000*g*–20,000*g*) to sediment mitochondria, chloroplasts. The supernatant is again centrifuged to high-speed (100,000*g*) resulting in fragments of ER, Golgi, and microsome in the pellet. The final centrifugation is done by further spinning at very high-speed (200,000*g*) which results in the ribosome sediments. The supernatant left is cytosol. B: Density-gradient centrifugation. The homogenate is laid on top of a density gradient medium, e.g., sucrose solution. This is prepared in a centrifuge tube by layering sucrose solutions denser towards the bottom of the tube. When centrifuged at high speed, each subcellular component will move through the density gradient until reaches a position where a density equals that of the sucrose solution. A series of distinct bands will eventually be produced, with those closest to the bottom of the tube containing the highest density components. Finally, they can be separated into "fractions", either by using a fine pipette or by piercing the bottom of the tube and collecting the fractions as the liquid drips out.

the secondary antibody directly, omitting the primary antibody; and (iv) the use of a mutant which lacks the target protein, if available.

Immunoelectron microscopy

Immunoelectron microscopy (IEM) is the highest resolution technique for identifying the subcellular localization of a protein, because it reveals the localization of the endogenous protein at the ultra-structural level under transmission electron microscopy (TEM). This technique follows the similar procedures of the indirect IF, but the secondary antibodies are conjugated with gold particles, instead of a fluorophore. The electron-dense gold particle can be observed under TEM as a black dot, which indirectly labels the protein of interest. Similar to the double IF labeling, double IEM experiments can be achieved by using two different host species of primary antibodies in combination with their respective secondary antibodies conjugated to gold particles of different sizes (e.g., 6, 10, or 15 nm in diameter). Based on the different sizes of gold particles, the localization of the two target proteins can be distinguished from each other (Lin et al., 2015; Zhuang et al., 2013).

Beside the quality of the primary antibody specificity, the success of IEM techniques also depends on the quality of preservation of protein antigen and the organelle structures. High pressure freezing/freeze substitution fixation (HPF/FS) is a relatively new fixation procedure that is highly suitable for IEM. This technique also allows routine EM post-staining of sections with uranyl acetate and lead citrate to improve observation of structural details without disturbing the immunobinding.

An inherent limitation to the IEM is that ultra-thin sections can produce misleading images as a thin slice of organelles may not give an accurate view of its three-dimensional (3D) structure. To overcome this limitation, serial sections can be cut which are then compiled into a whole-cell 3D at nanometer level resolutions using electron tomography (Austin and Staehelin, 2011). Combined with HPF/FS and immunogold labeling, electron tomographic analysis provides novel and detailed morphological information that elucidate the 3D organization of the organelles with quantitative parameters, such as surface area, volume, and density (Cui et al., 2019; Otegui et al., 2001; Seguí-Simarro et al., 2004).

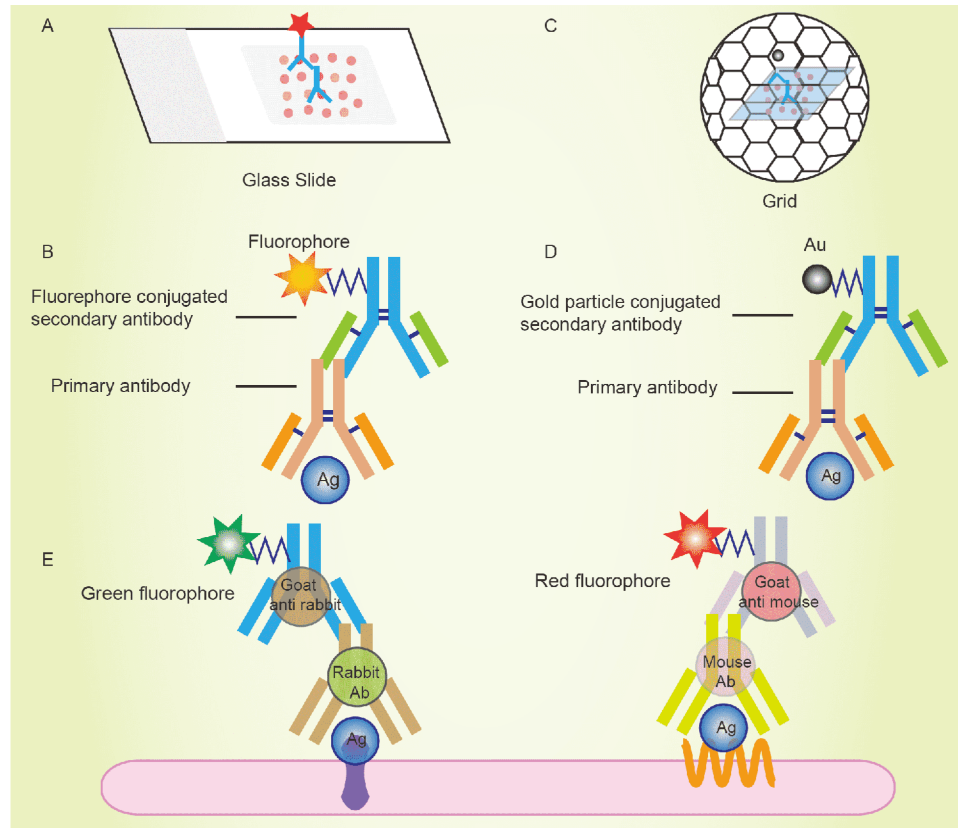

Figure 3 Membrane-bound proteins are detected using protein immunolabeling techniques. A, B: In IF, the sectioned sample is mounted in the glass slide and the fluorescence signal can be observed under CLSM microscope (A). Indirect IF uses two antibodies, the primary and the secondary. The primary antibody directed against the target antigen is unconjugated, while the secondary antibody is conjugated with the fluorophore and is directed against the primary antibody (B). C, D: In IEM, the ultra-sectioned sample is mounted in the grid for observation under TEM (C). The colloidal golds are conjugated to the secondary antibodies (D). E: Double immunolabeling. Double detection of two different target proteins in the same sample relies on the primary antibody for each target being raised in different host species to avoid crossreactivity between the secondary antibodies. confocal laser scanning microscopy (CLSM); TEM, transmission electron microscopy; IF, immunofluorescence; IEM, immunoelectron microscopy.

Expression of tagged protein to assess protein subcellular localization and trafficking

(i) Fluorescence protein fusion. The tools for subcellular localization mentioned above, i.e., co-fractionation, IF, and IEM are often technically challenging, and antibody production for immunodetection of a protein can be time-consuming and laborious. Moreover, IF and IEM entail using fixed cells, preventing observation of protein dynamic movements. At present, fluorescence proteins (FPs), such as green fluorescent protein (GFP) and its derivates, have been applied to observe the protein subcellular localization in living cells, and monitoring for changes or differences in subcellular calcium, pH, voltage, metal, glucose concentrations, or enzyme activity (Pendin et al., 2017). This has opened up the possibility of directly studying molecular turnover, transport, and molecular interactions using techniques such as Förster resonance energy transfer (FRET), fluorescence lifetime imaging, bimolecular fluorescence complementation (BiFC), fluorescence recovery after photobleaching (FRAP), and photoactivation (Fricker et al., 2006).

Nowadays, the FP fusion chimeric genes can be incorporated into stable transgenic plants or introduced into plant cells via transient expression techniques for determining subcellular localization and real-time dynamic intracellular trafficking in living cells. To observe the protein subcellular localization, the confocal laser scanning microscopy (CLSM), which takes advantage of a pinhole in front of the detector to physically block out-of-focus signals, has become a commonly used fluorescent imaging technique. Moreover, a variety of microscopy imaging techniques have been developed to observe FPF in plants at higher resolution and faster speeds. These include spinning disc microscopy (Oreopoulos et al., 2014), variable-angle epifluorescence microscopy (VAEM) (Konopka and Bednarek, 2008), super-resolution fluorescence imaging methods, such as stochastic optical reconstruction microscopy (STORM), stimulated emission depletion microscopy (STED) and structure illumination microscopy (SIM) (Komis et al., 2015), and finally light-sheet microscopy for 4D imaging (Ovečka et al., 2018; Wang, 2016).

Fluorescence correlation spectroscopy (FCS) is a minimally invasive technique used to detect the heterogeneous distribution and dynamics of fluorescently labeled proteins and lipids at the single-molecule level and on the nanosecond to second timescales (Li et al., 2016). Taken the advantages of FCS, it has now been combined with other microscopy techniques to expand the range and resolution of this technique. For example, the combination of STED and FCS has increased the *x*-*y* axis spatial resolution to 20–30 nm on live cell membranes (Clausen et al., 2015). Combining FCS with light-sheet microscopy enables the FCS detection of 4D events in cells and small organisms (Brazda et al., 2014). Moreover, FCS can be used to detect diffusion and organization not only at the PM but also in endomembranes of living cells (Kay et al., 2012; Malchus and Weiss, 2010). In addition, the dual-color variation, termed fluorescence cross-correlation spectroscopy (FCCS) can extend investigations to the examination of biochemical reactions between two molecular partners, such as reaction rates, kinetics, fractions of binding or reacting molecules, and mobilities of a complex formed. Therefore, FCS-based approaches have been become important methods in plant cell biology quantitative analysis of single proteins, protein complexes, and membrane organization, measurement of membrane protein dynamics, and quantification of biomolecular interactions during development and signal transduction in plants (Fan et al., 2013; Hao et al., 2014; Wang et al., 2015; Wang et al., 2013).

These state-of-the-art microscopy technologies will most certainly lead the FPF subcellular localization and dynamic studies into a new era. Nevertheless, the FP fusion approach also has limitations and potential experimental artifacts, which will be discussed in the last section of this review.

(ii) Correlative light and electron microscopy (CLEM). The information obtained using the traditional techniques for protein subcellular localization and the dynamic observations mentioned above comes from different samples with different sample preparation procedures. However, one hopes to obtain a complete overview of a cell at a micrometer length scale, while at the same time analyze biomolecules in this same cell at the scale of nanometers. CLEM, which combines the versatility of light microscopy with the high spatial resolution of TEM, is a perfect tool for studying the complex relation between structure and function in biology (de Boer et al., 2015).

CLEM is typically performed by correlating the images from two different microscopy modalities. In brief, ultra-thin sections or cryo-sections of tissues are mounted on finder grids, and then are incubated with fluorophore- and gold-labeled probes, then areas of interest are firstly observed under the fluorescence microscopy (FM) and further analyzed in the TEM at high resolution. In this way, fluorescence is directly correlated to subcellular structures and/or corresponding immunogold particles. Up to now, several alternative approaches using different combinations of markers are available (Sosinsky et al., 2007) and a series of correlative methods have been developed (Kopek et al., 2017). Commercial integrated microscope platforms have appeared recently, enabling inspection by FM and then EM analysis directly afterwards (Grabenbauer et al., 2005; Peddie et al., 2017). For plants, Bell et al. (2013) developed a simple method for retaining FPs after resin embedding. Using correlative light and electron microscopy, they were able to locate the same FP-labeled sieve elements in semithin and ultra-thin sections, which were also amenable to antibody labeling. Although CLEM technology is not commonly used in plant cell biology research at the present, we predict that CLEM will be one of the best tools to solve the plant cellular complexity.

(iii) Transient expression. Transient expression is a fast and simple method that requires minimum handling and allows high-throughput analyses of FPFs for subcellular localization and dynamic analysis. Unlike the stable transformation that integrates the gene into the plant cell chromosome, transient expression of a transformed gene can be achieved over a relatively short time span without passing it to the next generation. Depending on the cell type and methods of DNA presentation, several transient expression systems have been developed and used in multiple plant species. These include agrobacterium infiltration of leaf epidermal cells (Sparkes et al., 2006), biolistic bombardment of tissues or cultured cells (Ueki et al., 2009; Wang and Jiang, 2011), and polyethylene glycol (PEG)- or electroporation-mediated transformation of protoplasts (Miao and Jiang, 2007; Yoo et al., 2007).

Currently, transient co-expression in protoplasts of the FPFs (e.g., GFP fusion) with known organelle markers (e.g., red fluorescent protein (RFP) fusion) is one of the most favorable methods to determine the subcellular localization of a new protein. Beside the confocal subcellular localization analysis of the FPFs, several downstream biochemical methods have also been developed to determine the protein transport in the secretory pathway (Denecke et al., 2012). One good example is the α-amylase secretion assay (daSilva et al., 2005; Pimpl et al., 2003). When co-expressed with functional proteins in the secretory pathway, the transport of α-amylase-based reporters is quantified by the ratio of the amounts of α-amylase that was secreted to the culture medium and the α-amylase that remained in the cells. This ratio is defined as the secretion index (SI).

Pharmaceutical treatments to identify protein subcellular localization and trafficking

Pharmaceutical treatment is another powerful tool to help in understanding and confirming the subcellular localization of

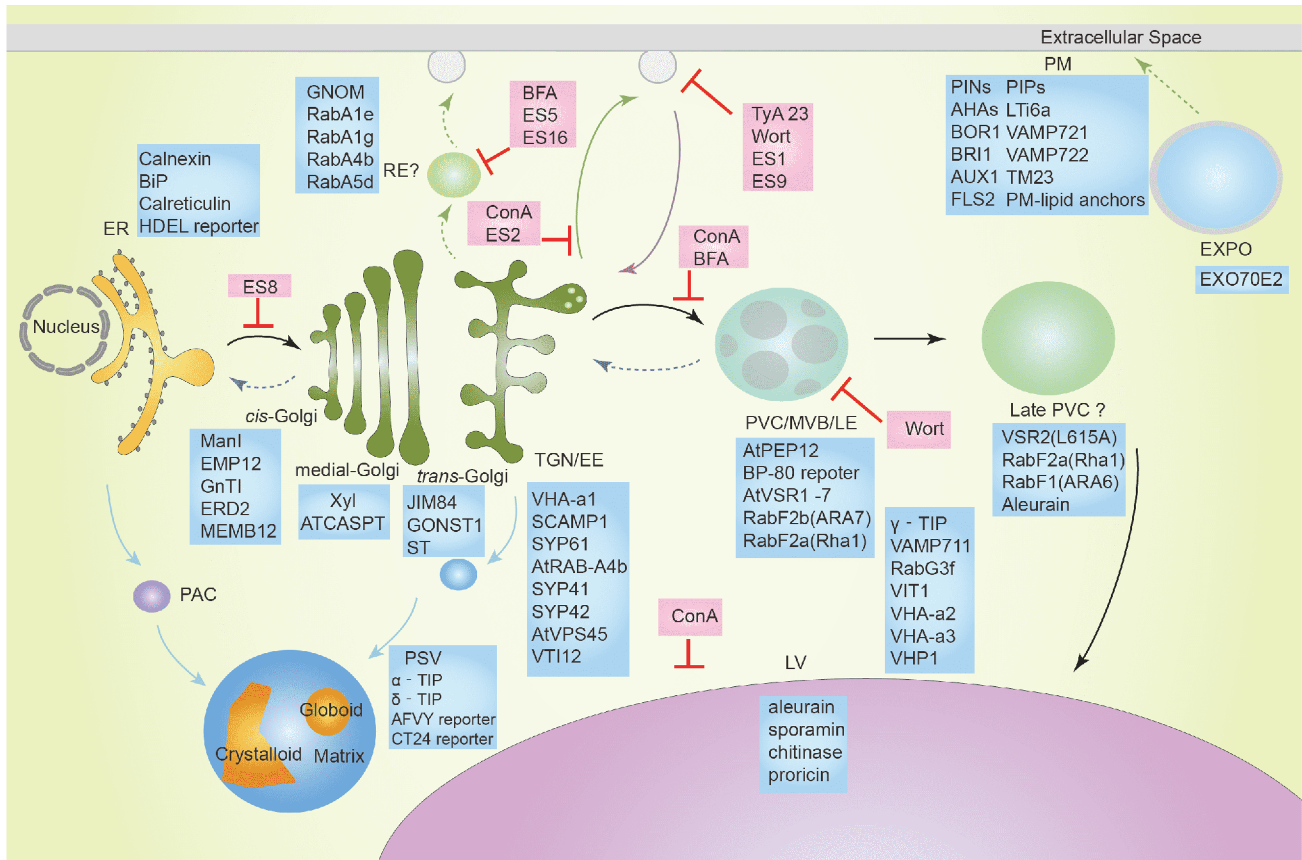

Figure 4 A working model including compartments, markers, and inhibitors in the endomembrane system. The markers for each compartment are indicated in blue boxes and the inhibitors are noted in purple box adjacent to the compartment or route they affect. BFA blocks trafficking from endosomes to plasma membrane, causes formation of endosomal aggregates (BFA compartment), and causes redistribution of Golgi proteins to ER; wortmannin inhibits PI3K, and causes enlarged PVC/MVB/LE; concanamycin A inhibits vacuolar ATPase preventing vacuolar degradation, and blocks transport out of TGN/EE; TyA23 inhibits endocytosis; endosidins (ESs) are small molecules disrupting specific trafficking pathways: early ER–Golgi secretion (ES8), endocytosis (ES1 and ES9), exocytosis (ES2), and recycling between TGN-PM (ES2, ES5, and ES16).

proteins by perturbing protein trafficking pathways. A variety of drug inhibitors have been developed for defining protein localization on endosomal compartments and have been summarized in Table 1, and the trafficking pathways for each drug have been indicated in Figure 4.

(i) Brefeldin A (BFA). The BFA is a lactone antiviral agent produced by the fungus *Penicillium brefeldianum*, which inhibits a subset of Sec7 domain-containing ADP-ribosylation factor (ARF) guanine nucleotide exchange factors (ARF-GEF) (Jackson and Casanova, 2000). BFA has been described affecting secretory pathway (Nebenführ et al., 2002) and causing redistribution the protein from the Golgi apparatus to the ER (Geldner, 2004). BFA treatment results in the formation of large intracellular endosomal and *trans*-Golgi compartment aggregates, called BFA bodies or BFA compartments (Robinson et al., 2008b). The BFA effect is reversible after washing out the drug and the "BFA bodies" gradually disappear. Proteins that accumulate in the BFA bodies include TGN proteins (e.g., VHA-a1 and SCAMP1) (Lam et al., 2007), and the post-Golgi ARF GNOM (Geldner et al., 2003), whereas PVC/MVBs are excluded as observed by the markers ARA7 or BP-80 (Tse et al., 2006).

Given that the TGN/EE is found in the core of BFA compartments, many PM proteins that recycle between PM and TGN/EE have been reported to be sensitive upon the BFA treatment. The appearance of fluorescently tagged PM proteins in BFA bodies in the presence of the protein synthesis inhibitor cycloheximide (CHX) has been utilized to monitor endocytosis and recycling in plants, and to assess how endocytosis is regulated in response to different environmental cues (Geldner et al., 2001; Naramoto et al., 2014).

(ii) Concanamycin A (ConA) and Bafilomycin A (BafA). ConA and BafA are membrane-permeable macrolide antibiotics that bind to the V-ATPase subunits C (Huss et al., 2002) and A (Wang et al., 2005), respectively, and thereby inhibit proton transport activity at the compartments membrane. Although V-ATPases have been found throughout the endomembrane system, including the ER, Golgi, and PVC/MVB in plants , the most prominent role of the V-ATPase that are well defined is to maintain acidic environment of the vacuole and TGN/EE (Kluge et al., 2003; Sze et al., 1999).

Table 1 Examples of chemical treatment to study endosomal trafficking in plants

Inhibitor	Function/target on plant cells	Ref.
Brefeldin A (BFA)	Inhibitor of a subset of Sec7 domain-containing ARF-GEF; blocks trafficking from endosomes to plasma membrane; causes redistribution of Golgi proteins to ER; results in formation of intracellular endosomal aggregates (BFA body or BFA compartment)	(Geldner et al., 2003; Robinson et al., 2008b; Sato et al., 1995; Tse et al., 2006)
Concanamycin A (ConA) /Bafilomycin A (BafA)	Inhibits vacuolar ATPase; blocks transport out of TGN/EE; produces alkaline condition in vacuole and TGN/EE; prevents vacuolar degradation	(Dettmer et al., 2006; Zhuang et al., 2013; Zhuang et al., 2017)
Tyrphostin A23	Inhibits clathrin-mediated endocytosis; binds with the *Arabidopsis* clathrin heavy chain; causing cytosolic acidification	(Dhonukshe et al., 2007; Dejonghe et al., 2016)
Wortmannin	Inhibits PI3K; causes enlarged PVC/MVB; blocks autophagy pathway	(Corvera et al., 1999; Le Bars et al., 2014; Miao et al., 2006; Tse et al., 2004; Vermeer et al., 2006; Zhuang et al., 2017)
Endosidin1 (ES1)	Causes formation of endosomal aggregates; blocks endocytic trafficking	(Robert et al., 2008)
ES2	Binds to the exocyst complex subunit EXO70; inhibition of exocytosis and endosomal recycling; bnhancement of plant vacuolar trafficking	(Zhang et al., 2016)
ES3, ES5, and ES7	Inhibits protein trafficking from the PM, vacuolar targeting and recycling; affect cell polarity and callose deposition during cell plate maturation	(Drakakaki et al., 2011)
ES8	Affects secretory pathways exclusively toward the basal plasma membrane of the cell	(Doyle et al., 2015)
ES9	A mitochondrial uncoupler; induces cytoplasmic acidification; interference with clathrin mediated endocytosis;	(Dejonghe et al., 2016)
ES16	Affects the small GTPase RabA proteins; perturbs apically localized plasma membrane proteins trafficking as well as biosynthetic secretion	(Li et al., 2017)

Thus, ConA treatment produces alkaline condition mainly in the vacuole and TGN/EE lumen. A morphological study by TEM after ConcA or BafA treatment showed massive vacuolation of the Golgi apparatus and aggregations of vesicles in BY-2 cells or *Arabidopsis* root cells (Robinson et al., 2004). Moreover, ConA treatment interfered with the trafficking of endocytic and secretory cargos (Dettmer et al., 2006), which is in agreement with the phenotype observed in the V-ATPase mutant (Luo et al., 2015). Because ConA treatment prevents acidification of the vacuolar lumen and thereby allows for the detection of autophagic bodies in the LVs, it also been used to monitor autophagic processes in plant cells (Lin et al., 2015; Zhuang et al., 2013; Zhuang et al., 2017).

(iii) Tyrphostins A23 (TyrA23). TyrA23 was originally identified as a Tyr kinase inhibitor of the epidermal growth factor receptor, and was later described as a specific inhibitor of the interaction between the receptor and the clathrin machinery in mammalian cells (Banbury et al., 2003). TyrA23 is a well-described inhibitor of clathrin-mediated endocytosis affecting the endocytosis of PIN proteins (but not FM4-64) (Dhonukshe et al., 2007). However, the endocytosis-inhibiting activity of TyrA23 has recently been found to be due to its protonophoric characteristics, causing cytosolic acidification and endocytic block (Dejonghe et al., 2016), indicating that TyrA23 may not be a specific inhibitor of clathrin-mediated endocytosis in plant cells. Elevating the concentration of TyrA23 above that typically used to inhibit endocytosis (30–50 μmol L^{-1}) has induced the dissociation of the TPLATE complex from the PM (75 μmol L^{-1}; (Van Damme et al., 2011) and the inhibition of flg22-elicted reactive oxygen species formation (100 μmol L^{-1}; (Smith et al., 2014a, 2014b).

(iv) Wortmannin. Wortmannin is an inhibitor of phosphatidylinositol-3 kinase (PI-3 kinase, Vps34p in yeast) (Corvera et al., 1999). Distinct from mammals and yeast where phosphatidylinositol-3-phosphate (PI3P) distributes at early endosomes, the PI3P in plant is mainly found in the late endosomal PVC/MVB membrane (Vermeer et al., 2006). Thus, disruption of the PI-3 kinase activity should affect the

PVC/MVB morphology or function in plants. Indeed, upon wortmannin treatment, the chimeric PI3P sensor YFP-2xFYVE is rapidly released from PVC/MVBs and PVCs/MVBs become vacuolated as ring-like structures when observed under confocal microscopy labeled by VSR or ARA7 (Oliviusson et al., 2006; Tse et al., 2004). The ring-like structure is formed by the homotypic fusions of PVCs/MVBs as well as heterotypic fusions between the TGN and PVC/MVB as observed under TEM (Wang et al., 2007, Wang et al., 2009). In plants, wortmannin treatment inhibits protein trafficking to the plant vacuole (daSilva et al., 2005) and induces DVs to fuse with the PM in the developing seed of mung bean (Wang et al., 2012).

Furthermore, wortmannin is also useful for autophagy studies. As the activation of PI3Ks is responsible for autophagosome biogenesis (Blommaart et al., 1997), inhibition of PI3K with wortmannin has also been used to block autophagy, in which the formation of autophagosome-related structures labeled by ATG5, ATG8, or SH3 domain-containing protein (SH3P2) are suppressed upon wortmannin treatment in plant (Le Bars et al., 2014; Zhuang et al., 2017; Zhuang et al., 2013).

(v) Endosidins (ES). In addition to the traditional trafficking inhibitors mentioned above, hundreds of small molecules affecting different aspects of endomembrane trafficking in plants have been recently discovered through recent chemical genetic screens (Dejonghe and Russinova, 2017). Specifically, we are interested here in a subset of compounds, known as endosidins that affect endocytosis and endosomal function. Endosidin 1 (ES1) was reported to be an EE compartment inhibitor, interfering selectively with receptor-mediated endocytosis (Robert et al., 2008). Most recently, ES2 has been revealed to bind to the EXO70 (exocyst component of 70 kD) subunit of the exocyst complex, resulting in inhibition of exocytosis and endosomal recycling in both plant and human cells and enhancement of plant vacuolar trafficking (Zhang et al., 2016). The molecules ES3, ES5, and ES7 affect cell polarity by inhibiting protein trafficking from the PM, vacuolar targeting and recycling, and callose deposition during cell plate maturation, respectively (Drakakaki et al., 2011). ES8 affects secretory pathways, exclusively toward the basal plasma membrane of the cell, thereby affecting PIN1 trafficking and auxin distribution (Doyle et al., 2015), whereas ES16 specifically perturbs apically localized PM proteins through regulation of the small GTPase RabA proteins (Li et al., 2017). ES9 is a protonophore that interferes with clathrin mediated endocytosis through cytoplasmic acidification and its binding with the *Arabidopsis* clathrin heavy chain (Dejonghe et al., 2016). Taken together, after the full characterization and target identification, these small molecules should become valuable and to be widely used research tools in the future.

Organelle markers for the plant endomembrane compartments

At present, a set of fluorescent organelle markers for plants have been constructed by fusing well-studied short targeting sequences to fluorescent proteins. The nuclear localization signal (NLS) from SV40 enables the attached FP to locate in the nucleus (Grebenok et al., 1997). The C-terminal three amino acid residues, SRL, are necessary and sufficient for targeting FP to the peroxisome (Mano, 1999). The mitochondrial transit peptide consists of the first 66 amino acids of the β-subunit of the F1-ATP synthase of *Nicotiana plumbaginifolia* together with the N-terminus of the FP fusion protein for the mitochondrial matrix targeting (Duby et al., 2001). The N-terminal transit peptide of Rubisco activase RecA is fused to the N-terminus of the FP for targeting to the plastid stroma (Köhler et al., 1997).

Although a variety of proteins have been identified in specific organelles and are used as organelle markers in the endomembrane system (Geldner et al., 2009; Shen et al., 2013a), some of the proposed marker proteins are not very specific and need extra evidence of proof, especially when the targeting mechanism is unclear. In the next section, we specifically focus on the well-identified organelle markers in the plant endomembrane system as summarized in Figure 4 that can be used to validate the location of new proteins.

Endoplasmic reticulum

The endoplasmic reticulum (ER) lumen is a specialized organelle compartment dedicated to import, folding and assembly of proteins in the secretory pathway of eukaryotic cells. The presence of native ER chaperones, including BiP (Lee et al., 2002), calreticulin (Denecke et al., 1995), and calnexin (Irons et al., 2003), have been demonstrated in plants by IF or IEM. It is currently known that the C-terminal tetrapeptide (K/HDEL) retrieval signal is responsible for soluble proteins, such as BiP, remaining in the ER lumen (Denecke et al., 1992). Proteins escaping the ER and carrying the K/HDEL signal are retrieved by a receptor termed ER retention defective 2 (ERD2) or K/HDEL receptor that recognizes the signal (Lee et al., 1993; Semenza et al., 1990). When the K/HDEL tetrapeptide was fused to the C-terminus of sp-GFP (GFP fusion with N-terminus signal peptide), GFP was retained in the ER (Boevink et al., 1996). Thus sp-XFP-HEDL has now been widely used as a fluorescent ER marker. Residence of the calnexin in the ER is due to its transmembrane domain (TMD) and cytosolic tail (CT). The GFP-calnexin-TMD/CT fusion (GFP-CNX) is an established ER membrane marker for confocal imaging analysis (daSilva et al., 2005). FPFs of the domains of membrane protein calreticulin (Brandizzi et al., 2003) and calnexin (daSilva et al., 2005), and soluble protein BiP (Kim et al., 2001) are now

widely used as ER markers.

As observed under confocal microscopy with the ER marker sp-GFP-HDEL in *Arabidopsis* and tobacco leaf cells, the ER appears as a relatively immobile polygonal tubular network with variously shaped cisternae at the cell cortex, and with other more mobile tubules streaming through the cytoplasm (Matsushima et al., 2002; Matsushima et al., 2003). However, in *Arabidopsis* seedlings and roots, novel ER derived mobile "ER bodies" as well as the tubular network have also been observed (Hawes et al., 2001; Matsushima et al., 2003). The formation of ER bodies and their potential functions have been summarized recently (Nakano et al., 2014).

Golgi apparatus

The Golgi apparatus is composed of several stacked cisternae near the outer edges of the ER and organized into three biochemically distinct sub-compartments: the *cis*-Golgi, medial-Golgi, and *trans*-Golgi. The *cis*-Golgi is the face closest to the ER. In IF, a monoclonal JIM84 antibody, which was raised against a carrot coated vesicle fraction, can specifically label the *trans*-Golgi face in the pea root-tip cells (Satiat-Jeunemaitre and Hawes, 1992). The Golgi apparatus in living plant cells was first visualized with two different fluorescent constructs: a GFP fusion to the putative *Arabidopsis* K/HDEL receptor ERD2, and to the TMD of a rat sialyl-transferase, a mammalian Golgi glycosylation enzyme (ST-GFP) (Boevink et al., 1998). A few reports based on EM suggests that the ERD2 is the *cis*-Golgi marker and ST is the *trans*-Golgi marker (Brandizzi et al., 2002a).

Subsequently, glycosidases and glycosyltransferases for the processing of *N*-linked oligosaccharides in the plant secretory pathway have been shown to localize at the Golgi. A soybean α-1,2 mannosidase I (ManI), the first enzyme involved in the *N*-linked oligosaccharide pathway, and the plant *N*-acetylglucosaminyltransferase I (GnTI) have also been fused to GFP and found to be targeted to the *cis*-Golgi in tobacco plants (Saint-Jore-Dupas et al., 2006). An *N*-glycan GFP-tagged β-1,2 xylosyltransferase (XylT) is associated with Golgi stacks in IEM, and is preferentially located in the medial cisternae of tobacco BY-2 cells (Ito et al., 2018), with the TMD and CT regions being sufficient to sustain the Golgi retention (Dirnberger et al., 2002). Moreover, the expression of a Golgi nucleotide sugar transporter 1 (GONST1-YFP) in BY-2 suspension cells has also been used as a *trans*-Golgi marker (Baldwin et al., 2001).

Golgi-resident proteins with properties different from the aforementioned Golgi markers have also been found. ATCASP is a putative Golgi matrix protein. In BY-2 cells, ATCASP localizes to intermediate cisternae between *cis*-Golgi SYP31 and *trans*-Golgi ST markers with partial overlap, indicating the enrichment of ATCASP in the medial-Golgi cisternae (Renna et al., 2005). RER1B is a homologue of yeast Rer1p, which is responsible for retrieving a subset of ER membrane proteins from the Golgi to the ER, and also localizes to the *cis*-Golgi (Sato et al., 1995). Recently, a new Golgi localized *Arabidopsis* endomembrane protein 12 (EMP12) has been identified. Both endogenous EMP12 and GFP-EMP12 fusion localized to the *cis*-Golgi face as identified by IF and IEM, respectively (Gao et al., 2012). Finally, a systematic analysis of (soluble *N*-ethyl-maleimide sensitive factor attachment protein receptors) SNARE molecules in *Arabidopsis* has identified 9 Golgi localized proteins, with SYP31, SYP32, or MEMB12 fused to XFP have now been widely used as Golgi specific markers (Geldner et al., 2009; Uemura et al., 2004). However, to which side of the Golgi stacks the proteins are localized needs to be determined. Most recently, Parsons et al., (2019) have determined the sequential localization of resident proteins across the Golgi cisternae by mass spectrometry, and suggested that sequence-based characteristics of transmembrane regions, rather than discrete rules, guide proteins to sub-compartments location within the Golgi stack.

TGN/EE

The TGN is a specialized organelle on the trans side of Golgi stack. Electron tomography of *Arabidopsis* cells indicates that the TGN is clearly separated from Golgi apparatus and differentiated into early and late sub-compartments (Kang et al., 2011; Otegui et al., 2006). Super-resolution live imaging of the TGN-localized SNARE protein SYP43 has revealed two types of TGN in *Arabidopsis* root cells: the GA-TGNs (Golgi-associated TGNs), located on the *trans*-side of the Golgi apparatus, and the GI-TGNs (Golgi-released independent TGNs), located away from the Golgi apparatus and behaving independently (Uemura et al., 2014). It has been proposed that GA-TGN matures into the GI-TGN and then into secretory vesicles by increasing the abundance of VAMP721-dependent secretory pathway components (Uemura et al., 2019).

Time-lapse confocal imaging and IEM shown that the rice (*Oryza sativa*) homolog of animal secretory carrier membrane proteins (SCAMPs) localize to the plasma membrane and mobile tubular-vesicular structures-the TGN. Drug treatments and confocal IF demonstrated that SCAMP1-labeled organelles may represent an EE because the internalized endocytic markers FM4-64 and AM4-64 reached these organelles before PVCs (Lam et al., 2007). Thus, unlike its role in animal and yeast cells, the TGN functions as an early endosomal compartment in plant cells.

The SYP4 group (SYP41, SYP42, and SYP43) represents the plant orthologs of the Tlg2/syntaxin16 Qa-SNARE. SYP41 and SYP42 each interact with the SYP61 and VTI12 in addition to the SM (Sec1/Munc18) protein VPS45, a po-

tential regulator of vesicle fusion (Zouhar et al., 2009). The TGN localization of SYP41, SYP61, and VPS45 have been confirmed by IEM in plants (Bassham et al., 2000; Sanderfoot et al., 2001), and transgenic plants expressing these fluorescent fusion proteins have now been used as TGN markers in living cells (Li et al., 2012). Moreover, confocal colocalization experiments as well as immunogold labeling have shown that the V-ATPase subunit VHA-a1 is preferentially found in the TGN (Dettmer et al., 2006). Thus, *Arabidopsis* seedlings expressing multicolor VHA-a1 (VHA-a1-XFP) have been generated as TGN/EE marker lines (Geldner et al., 2009).

Recycling endosome (RE)?

From the TGN/EE, internalized materials can be directed either into the vacuole or into the recycling pathway to the PM. In the recycling pathway, internalized components, such as AUX and PIN proteins, are returned to the plasma membrane through a specialized RE compartment. The adenosine ribosylation factor (ARF)-guanine nucleotide exchange factor (GEF) GNOM, the most prominent regulator of recycling of PIN auxin transporters and other proteins to the PM (Geldner et al., 2003), has been proposed to act and localize to the so far elusive REs. However, super-resolution confocal live imaging microscopy with pharmacological treatments and ultra-structure analysis has identified GNOM and its closest homolog GNOM-like 1 predominantly to the Golgi apparatus (Naramoto et al., 2014). Thus, GNOM as a RE marker has been challenged. In animals, members of the RAB11/RABA GTPases have been found to regulate distinct transport routes between the recycling endosome and the Golgi or PM (Ullrich et al., 1996), while the homologous subclass in yeast, YPT31/32 has been implicated in export of secretory and endocytic cargo from the *trans*-Golgi cisterna (Jedd et al., 1997). Similarly, several plant RAB11/RABA GTPases, including the Rab A1e, Rab A1g, Rab A4b, and Rab A5d, have now been found, either as fluorescent fusion proteins or by cell fractionation techniques, to localize to REs that partially overlap with *trans*-Golgi elements (Geldner et al., 2009). Furthermore, another protein that localizes to RE is EHD1, which co-localizes to RabA and RabD positive vesicles, and functions in endocytic recycling in plant cells (Bar et al., 2013). Although we know something about marker candidates for the RE in plants, we still, unfortunately do not have an EM identification of the RE.

PVC/MVB/LE

Because VSRs are believed to function between the TGN and the LVs in sorting acid hydrolases to the vacuole, VSRs were first used as a marker to define the PVC (intermediate organelle between the TGN and the lytic compartment in animal) structure in plants (Ahmed et al., 1997; Paris et al., 1997; Sanderfoot et al., 1998). A subsequent confocal IF study with VSR antibodies demonstrated that VSRs were predominantly concentrated on PVCs, and thus the VSRs were markers for defining PVCs in multiple experiments and various plant cells including *Arabidopsis*, tobacco, and pea (Li et al., 2002). VSRs are type I integral membrane proteins that contain an N-terminal luminal region (NT), a single transmembrane domain (TMD), and a C-terminal cytoplasmic tail (CT) (Kirsch et al., 1994). A VSR reporter containing the TMD and CT regions of BP-80 was sufficient and specific to target the reporter to PVCs in plant cells (Jiang and Rogers, 1998). Therefore, both VSR antibodies and the BP-80 reporter have been used as markers for the PVCs in plants. Similarly, *Arabidopsis* GFP-AtVSR1-7 reporter fusions, containing proaleurain signal peptide (sp) linked to GFP and the TMC/CT sequences of individual *Arabidopsis* VSRs, also showed typical punctate patterns that were largely colocalized with anti-VSR labeled PVC/MVB (Miao et al., 2006). Thus, proteins containing the TMD and CT sequences of AtVSRs are widely used as PVC/MVB markers in *Arabidopsis* plant.

In addition to either VSR antibodies or VSR reporters as markers for PVC/MVB, the *Arabidopsis* Rab5 homologs RHA1 and ARA7 are also used as PVC/MVB markers (Lee et al., 2004; Sohn et al., 2003), since both ARA7 and RHA1 colocalize with the PVC/MVB marker VSR1. Overexpression of its active form, GFP-ARA7 (Q69L), leads to the formation of enlarged PVCs/MVBs shown as ring-like structure under confocal, which were likely to have arisen through their homotypic fusion (Jia et al., 2013). Thus, overexpression of ARA7 (Q69L) can also be used as a tool to identify the protein PVC/MVB localization.

An extension of the PVC/MVB-vacuole transport route is implicated by the discovery of the so-called late prevacuolar compartment (LPVC), which is the last endocytic compartment to fuse with the vacuole. This compartment lacks VSRs, but a soluble vacuolar marker accumulates in these structures which contains the Rab5 family of small GTPases Rha1/RabF2a and ARA6/RabF1 (Bottanelli et al., 2012; Foresti et al., 2010). It is proposed that the PVC/MVB matures into the LPVC by recycling of VSRs back to earlier compartments before fusion of the LPVC with the vacuole. However, this model need to be confirmed, because these authors did not provide EM images of their LPVC, and the exact site of recycling of VSRs is still controversial, with different localization of the retromer and the sorting nexins reported by different laboratories (Robinson, 2018; Robinson et al., 2012).

Vacuole

Unlike yeast vacuoles or mammalian lysosomes, plants have

two different functional vacuolar compartments, LVs and PSVs (Paris et al., 1997). The presence of different types of aquaporins (tonoplast intrinsic proteins, TIPs) can be used as marker proteins to distinguish the two kinds of vacuoles in plant cells (Jauh et al., 1999). Using antibodies labeling, α-TIP and δ-TIP specifically reside on the tonoplasts of PSVs while γ-TIP is only found on the tonoplast of LVs (Jauh et al., 1999; Paris et al., 1996; Reisen et al., 2003). However, by performing detailed developmental time courses, it is also shown that δ-TIP which is normally regarded as being confined to vegetative tissues, is also expressed in seed maternal tissues, and the embryo specific α-TIP appear to localize to both the plasma membrane and the PSV tonoplast during seed development and germination (Gattolin et al., 2011). Moreover, α-TIP also traffics from the ER to PSV-like organelles via a Golgi-independent manner in protoplasts derived from leaf tissues of *Arabidopsis* and tobacco (Park et al., 2004).

Several SNARE proteins have also been demonstrated by IEM to locate to the vacuolar membrane, including the VAMP711 and AtVam3/SYP22 (Sato et al., 1997). Plants expressing GFP-VAMP711 and GFP-SYP22 mark the LV tonoplast, and have been used to study vacuolar dynamics in *Arabidopsis* (Uemura et al., 2002). Moreover, proton pumps VHA-a2-GFP, VHA-a3-GFP, and VHP1-GFP display fluorescence preferentially at the LV tonoplast (Dettmer et al., 2006). Finally, the *Arabidopsis* vacuolar ion transporter1 (VIT1) has also been reported to localize to the LV tonoplast, with the dileucine motif mediating its tonoplast targeting (Wang et al., 2014).

Considerable effort has also been made to characterize the transport of soluble vacuolar proteins to the LVs and PSVs in plants. In the secretory pathway, soluble vacuolar proteins are thought to have certain vacuolar sorting determinants (VSDs), including the ssVSD (sequence-specific VSD), ctVSD (C-terminal VSD), and physical structure VSD (Neuhaus and Rogers, 1998; Vitale and Hinz, 2005). Thus, proteins destined for LVs are thought to carry ssVSD that interact with VSRs, while proteins destined to PSVs are instead thought to bear hydrophobic ctVSD or physical structure of VSD that may interact with a different receptor *Arabidopsis* RMRs or pumpkin PV72 (Ahmed et al., 2000; Jiang and Rogers, 1998; Park et al., 2005). Up to now, using the targeting sequence of the vacuole proteins, several GFP fusion reporters have been used in the protoplast transient expression, including aleurain-GFP (containing aleurain NtVSD) (Flückiger et al., 2003) , sporamin-GFP (fusion with sporamin NtVSD (Kim et al., 2001)), GFP-chitinase (fusion with chitinase ctVSD) (Flückiger et al., 2003), and GFP-AFVY (the C-terminal tetrapeptide of phaseolin (Frigerio et al., 2001) that used as markers delivered to LVs or PSV, respectively. It is noted that because of the rapid degradation of GFP in the LVs, it is difficult to explore the GFP fluorescence when expressed in the transgenic plants. Thus, mRFP fused to the ssVSS of proricin, with a linker peptide (spL-RFP) has been used as a lytic vacuole marker in transgenic plants (Frigerio et al., 2001). Similarly, GFP-CT24, which consists of a signal peptide and GFP followed by the C-terminal 24 amino acids of the α′ subunit of β-conglycinin under the control of a seed-specific promoter that is sufficient for sorting to PSVs in *Arabidopsis* seeds, has been used as a PSV marker (Fuji et al., 2007).

PM and the apoplast

Several artificial markers have been used to label the PM in protoplast transient expression, including the BP22-GFP, designed by adding three hydrophobic residues (LAL) to the TMD of the pea BP80 (Brandizzi et al., 2002b; Paris et al., 1997), and TM23-GFP, a fusion of the TMD of a human lysosomal protein (LAMP1) to GFP (Brandizzi et al., 2002b). Similarly, several PM-anchoring proteins have been recently generated, including a myristoylated and palmitoylated GFP (MAP-GFP), a prenylated GFP (GFP-PAP), a glycosylphosphatidylinositol-anchored GFP (GFP-GPI), and a phosphatidylinositol-4-phosphate-binding protein YFP (PI-YFP) to study PM lateral diffusion (Martinière et al., 2012). These minimal PM proteins consist only of a PM-anchoring domain and would have no ability to interact with other cellular constituents. R-SNAREs VAMP721 and VAMP722 which are known to be responsible for protein secretion and for extracellular defense, both have PM localization besides TGN under confocal observation of the transgenic plants in root meristematic cells (Zhang L. et al., 2011). Moreover, many endogenous proteins including the polar localized PINs , AUX1, BOR1, BRI1, FLS2, aquaporins PIP1-2, prone pump AHAs, and hydrophobic protein LTI6A (low temperature-induced protein 6A) (Cutler et al., 2000; Robinson et al., 2008a) have all been observed at the PM by FP fusion, IF and IEM studies.

The ECS or apoplast is the plant cell compartment outside the plasma membrane. In the secretion pathway, the proteins contain a signal peptide but without the vacuolar targeting sequence can be secreted from the endomembrane system by the "default secretion pathway" into the ECS. Thus, a GFP construct with the signal peptide but lacking the retrieval signal (sp-GFP or secGFP) has been proven to be a useful marker for following secretion in transient expression assay *in vivo* (Boevink et al., 1999; daSilva et al., 2005). The secretion also occurs in a stable *Arabidopsis* transformant of sp-GFP, which generated the GFP fluorescence signal in the apoplast (Zheng et al., 2004). Moreover, the secreted pathogenesis-related protein1 (PR1) have also been observed to accumulate at the apoplast under confocal analysis in *Arabidopsis* seedlings (Chung et al., 2018). Therefore, the fluorescence fusion protein PR1-RFP could be another po-

tential marker in *Arabidopsis* plants. New endogenous marker proteins for the ECS could be emerging after the proteomic analysis of proteins revealed from the apoplastic fluids of *Arabidopsis* leaves.

Issues and considerations for protein colocalization studies

The fluorescence protein fusion position

Currently, observation of FPFs in transient expression or transgenic plants is perhaps the most commonly used tool for protein localization analysis. When making the FPF constructs, FPs can link to the C-terminal, N-terminal, or insert the middle of a protein. The improper fusion of the FP might thus lead to mis-targeting of the fusion proteins. For example, the *Arabidopsis* EMP12 is a *cis*-Golgi colocalized multiple transmembrane protein. When fused to GFP GFP at its N-terminus (GFP-EMP12), the protein colocalized with the endogenous EMP12. However, GFP fusion at the C-terminus of EMP12 caused EMP12-GFP to reach the vacuole for degradation, due to the GFP blocking of the sorting signals at the EMP12 C-terminus (Gao et al., 2012).

To determine which fluorescence fusion localization is correct, the common way is to verify the function of fluorescence fusion proteins by complementation of deletion mutants. However, it could be argued that it is neither strictly necessary nor sufficient, because complementation simply indicates a sufficient portion of the fluorescence fusions is functional at the appropriate site(s) and it is not equivalent to the major steady state location of the endogenous protein. When a specific antibody is available, the distribution of an FPF signal can be compared with immunolocalization signals obtained from the endogenous protein in specific cell types.

Protein overexpression

The subcellular localization of the target protein is frequently determined by protein overexpression in most of the localization analysis techniques. However, a mis-localization can occur when the protein is overexpressed. The principal disadvantage is that the overexpression may alter the location or interactions of the native proteins and may even alter certain trafficking routes through dominant effects. For example, accumulation partially or exclusively in the ER might indicate overexpression and might saturate ER export. Furthermore, accumulation at the plasma membrane or the tonoplast could also happen because of either saturation of a retrieval trafficking pathway or a failure to recycle to the cytosol. To solve this dilemma, the fusion should be driven by the native expression signals including the upstream and downstream intergenic regions plus introns, rather than just the native or heterologous promoter regions (Colinas et al., 2008). More strictly, it is better to express the FPF at native levels in a null mutant background. Usually, the expression level of FPF can also be compared with native protein levels by SDS-PAGE immunoblotting with specific antibodies (Gao et al., 2014; Shen et al., 2018b).

This is also the same in the transient expression, in which the expression level should be strictly controlled. Time-course experiments are particularly necessary because expression proteins can be detected as early as 4 h after gene transfer, followed by a linear increase in the first 24 h (Phillipson et al., 2001). Thus, careful timing of the experiment allows the analysis of protein levels from near to the detection limit right up to the full steady state level (Künzl et al., 2016).

Recruitment by protein-protein interaction

Care must also be taken in co-localization studies, because one protein can be recruited by another via protein-protein interactions thus changing the target protein location from the original location to the marker protein location. One example for the recruitment phenomenon is the protein Exo70E2, an exocyst subunit that labels EXPO in plant cells, can positively recruit other exocyst proteins to the EXPO from the cytoplasm (Ding et al., 2014). Specific attention should be paid to autophagy-related processes that may result in protein co-localization over time, because autophagy can either non-selectively or selectively engulf cytosolic components and organelles for degradation. For example, EXPO and autophagosome have been shown to be distinct organelles under normal conditions, but EXPO fuses with autophagosomes for degradation upon autophagic induction (Lin et al., 2015). To determine whether the co-localization of two proteins is due to this recruitment phenomenon, the authors suggests the following steps: (i) single expression of the target protein or organelle marker respectively to identify their "native" localization pattern; (ii) co-expression to determine whether the two proteins are co-localization or at different positions; (iii) comparing the patterns between single expression and co-expression for consistency to avoid mis-localization caused by protein recruitment; (iv) if the recruitment occurs, it is necessary to perform an interaction assay (e.g., FRET) to understand the nature of the protein-protein interaction *in vivo* (Wang et al., 2016).

Conclusion and outlook

In this review, we have described multiple tools and the organelle markers that can be used to determine protein localization in the plant endomembrane system. We have discussed their limitations and concerns, and argue that diverse

methods and biological tests should be taken to address the questions of protein localization and trafficking routes, and all approaches can reveal useful information which finally in turn allows greater confidence to be assigned to a suggested location.

With the endomembrane compartments in the plant cells becoming more clearly defined, the next important steps will be to determine to which extent the different endosomes are connected via vesicle trafficking or if they are derived from each other by maturation (Robinson, 2018; Robinson and Neuhaus, 2016). To approach these questions, we will need to know much more about the molecular repertoire of different types of endosomes, in particular their lipid composition (Simon et al., 2014). We also need highly fluorescent cargo molecules that will allow us to trace their fates using high-resolution live cell imaging and electron microscopy. As new tools are becoming available for the analysis of endosomal structure, function and trafficking, our understanding of these important organelles will greatly expand. It is anticipated that super-resolution fluorescence microscopy with 3D structures in living cells as well as the EM tomography will become widely used tools for cell imaging to provide previously unobserved details of biological structures and processes at the nanometer scale (Komis et al., 2018; Otegui and Pennington, 2019; Schubert, 2017; Wang et al., 2019).

The multiple fluorescent proteins or antibodies of organelle markers summarized here enable straightforward compartment mapping and co-localization with genes of interest studies in live cell imaging as well as immuno-EM. In addition, the *Arabidopsis* organelle marker proteins can also be employed as reference to find their relative homologues in other species, thus providing a fast and reliable way for generating of entire sets of endomembrane marker proteins, and further promote our understanding of the conserved and specific features of subcellular organization between different plant species. Such strategy has been used to develop several fluorescent organelle markers in the monocot model rice most recently (Dangol et al., 2017; Wu et al., 2016).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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