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REVIEW

Organelle Identification and Characterization in Plant Cells: Using a Combinational Approach of Confocal Immunofluorescence and Electron Microscope

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Abstract The plant secretory and endocytic pathways consist of several functionally distinct membrane-bounded compartments. The ultra structures of the endoplasmic reticulum, the Golgi apparatus, and central vacuoles have been well characterized via traditional structural electron microscope (EM). However, the identification of plant prevacuolar compartments (PVCs) and early endosomes (EEs) had not been achieved until more recently because of the lack of specific markers for these organelles. Recent development of fluorescent reporters for PVCs and EEs expressing in transgenic tobacco BY-2 cells and *Arabidopsis* plants has allowed their dynamic characterization in living cells via confocal microscopy and drug treatment, which led to their subsequent morphological identification via structural and immunogold EM. Thus, in this review, we will use our studies on PVCs and EEs as examples to present an efficient approach for organelle identification in plant cells via primary characterization of fluorescent-marked organelles in living cells and their dynamic response to drug treatments, which then serves as the basis for subsequent immunogold and structural EM studies for organelle identification. Such strategy thus represents a powerful approach in future research for the identification of novel organelles and transport vesicles in plant cells.

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Introduction

The plant secretory and endocytic pathways consist of several functionally distinct membrane-bounded compartments in which endoplasmic reticulum (ER), Golgi apparatus, and the central vacuole have been well characterized. However, recent evidences suggested that several yet-to-be identified organelles and transport vesicles are also involved in protein trafficking in the secretory and endocytic pathways of plant cells. For example, even though their existence and function in plant cells have been proposed [2, 3], the identity of prevacuolar compartments (PVCs) and early endosomal compartment (EEs) remained elusive for many years until recently [13, 26–28, 35, 45, 54].

PVCs are membrane-bounded intermediate organelles that mediate protein traffic between the Golgi apparatus and lytic vacuoles (LVs) in the plant secretory pathway [3] (Fig. 1) with dual functions: (1) sorting proteins to LVs and (2) recycling mistargeted or receptor proteins back to trans-Golgi network (TGN; Fig. 1). Under electron microscope (EM), PVCs are membrane-bound organelles about 200 to 500 nm in diameter, with internal vesicles within their limiting membrane, thus also termed multivesicular bodies

(MVBs) [54]. In contrast, early endosomes (EEs) were identified as tubular–vesicular structures that are *trans*-Golgi localized [13, 27], also with dual functions: sorting secreted proteins to plasma membrane (PM) for secretion and receiving internalized molecules from PM via poorly characterized endocytosis in plant cells (Fig. 1).

Traditionally, EM studies have been commonly used for organelle identification because it is a direct way to define the cellular structures of interest. This technology had been developed for more than 70 years with several plant organelles being identified in the late 1940s and 1950s. However, identification of plant organelles via EM was only limited to several well-characterized organelles including ER, Golgi apparatus, chloroplast, mitochondrion, vacuole, and protein body. This situation has been changed rapidly after the discovery and application of green fluorescent protein (GFP) as well as the development and improvement of the confocal laser scanning microscope (CLSM). By making GFP fusion with the target protein to form a fluorescent reporter expressing in transgenic cells or plants, the trafficking of the GFP reporter and the dynamics of the GFP-marked organelles in living transgenic cells can be easily traced and studied in high resolution with CLSM (for a review, see also [38] and [5]). The dynamics of several plant organelles have been characterized in transgenic suspension cultured cells or transgenic plants using this approach, including the Golgi apparatus marked by Man1-GFP in transgenic BY-2 cells [36] and ST1-YFP in tobacco mesophylly cells [6]. Similarly, fluorescent markers for PVCs and EEs were also developed recently in transgenic tobacco BY2 cells [27, 54] and in *Arabidopsis* root cells [13], leading to the structural identification of PVC and EE via immunogold EM in plant cells. Therefore, the aim of this review was to summarize our research experience over the past years in characterizing and identifying plant PVCs and EEs using a combination of both CLSM and immunogold EM. Such approach will be essential and efficient in future research for the characterization and identification of novel organelles or transport vesicles in the plant secretory, endocytic, and exocytic pathways.

CLSM Studies of Plant PVCs and EEs

Fluorescent Reporters for Plant PVCs

Prevacuolar and endosomal compartments in yeast and mammalian cells had been identified and studied for more than 15 years, which was mainly due to the availability of reliable protein markers for these organelles, including the mammalian mannose-6-phosphate (M6P) receptor [25] and the yeast carboxypeptidase Y (CPY) receptor Vps10p [30] as well as the yeast syntaxin Pep12p [43]. In plant cells, several protein markers have been used to define PVCs because these proteins should reach PVCs during their trafficking in the plant secretory pathway, including the *Arabidopsis*

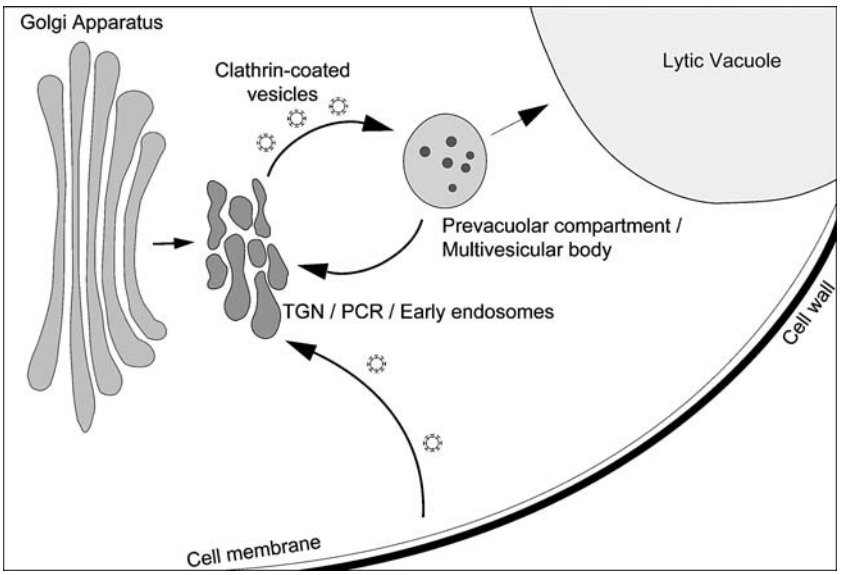

Fig. 1 Working model of the endocytic and secretory pathways in tobacco BY-2 cells. In secretory pathway, synthesized soluble proteins were exported from ER to Golgi apparatus via COP II vesicles. They were then transported to the clathrin-coated trans-Golgi network (*TGN*) before sorting into the clathrin-coated vesicles (*CCVs*) by vacuolar sorting receptor (*VSR*) proteins. Then, the soluble proteins were sorted to the prevacuolar compartments (*PVCs*)/multivesicular bodies (*MVBs*). Receptors and missorted proteins were recycled back to the TGN mediated by the retromer complex, while the soluble proteins were finally transported to the lytic vacuole (*LV*). In the endocytic pathway, internalized proteins from the plasma membrane (*PM*) first reached the TGN (an early endosome) prior to reaching the PVCs/MVBs and then LV

syntaxin AtPep12p [47], the *Arabidopsis* AtELP, and the pea BP-80 [1, 41], members of the vacuolar sorting receptor (VSR) family proteins [40]. Since VSRs were found to be concentrated on PVCs [29] and because the transmembrane domain (TMD) and cytosolic tail (CT) of BP-80 were sufficient for its correct targeting in plant cells [24], both VSRs and BP-80 reporter were subsequently used as PVC markers for PVC characterization and identification [11, 32–34, 53, 54]. Thus, transgenic tobacco BY-2 cells expressing the GFP-BP-80 reporter containing the BP-80 TMD and CT were generated which allowed studies of PVC dynamics and response to drug treatments in living cells [54].

Under confocal fluorescent microscope, the GFP-BP-80 reporters expressing in the living transgenic tobacco BY-2 cells showed typical punctate patterns (*3* in Fig. 2), and the size of each punctate signals is about 0.5 to 1 μm which might represent single PVC. Similar punctate patterns for the GFP-BP-80-marked PVCs were also found in *Arabidopsis* leaf epidermis cells [10, 16] as well as in *Arabidopsis* and tobacco protoplast [10].

Drug treatments with Brefeldin A (BFA) and wortmannin also caused the GFP-tagged PVCs to have visible changes in transgenic BY-2 cells [54]. The fungal macrocyclic lactone BFA has been widely used in studying protein

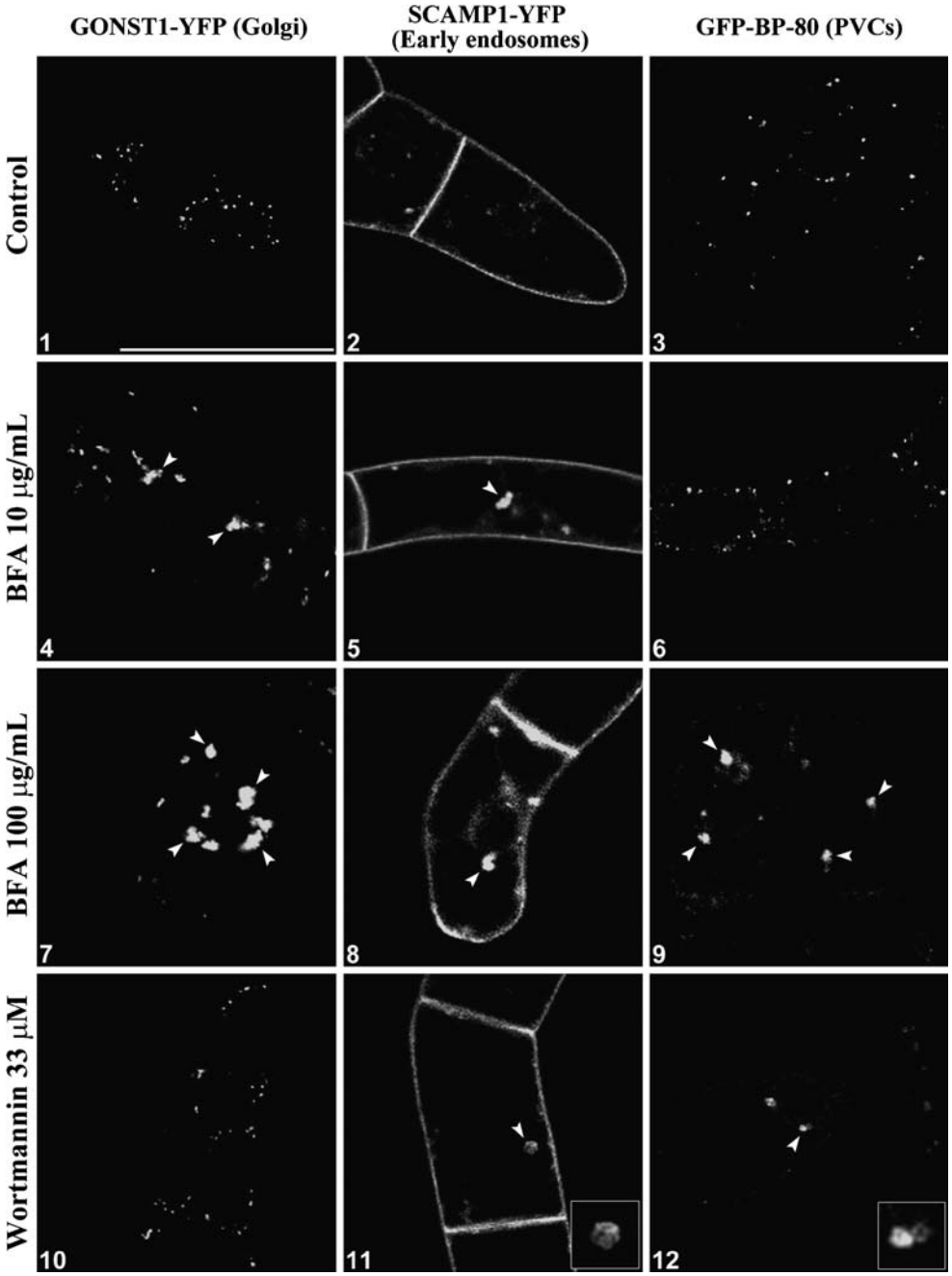

Fig. 2 Dynamics of GFP/FYP-tagged organelles. Transgenic tobacco BY-2 cells expressing various markers including Golgi marker GONST1-YFP (*1*, *4*, *7* and *10*), PM/TGN/EE marker SCAMP1-YFP (panels 2, 5, 8 and 11) and PVC marker GFP-BP-80 (*3*, *6*, *9*, and *12*) were incubated either BFA or wortmannin at indicated concentrations for 1 h before confocal imaging. Scale bar=50 μm

trafficking in the plant secretory and endocytic pathways. The Golgi apparatus is likely the initial site in response to BFA [37, 49] in which BFA (at 5–10 μg/mL) induced the Golgi apparatus to form ER–Golgi hybrid structures [44] and BFA compartments (*4* and *7* in Fig. 2) [48, 53, 54], . BFA at higher concentrations (50–100 μg/mL) also caused PVCs to form aggregates. BFA at 5 to 10 μg/mL, which normally induced the Golgi to form aggregates, did not cause visible change of the GFP-tagged PVCs (*6* in Fig. 2), but BFA at 50–100 μg/mL induced the punctate PVCs to form aggregates in transgenic BY-2 cells (*9* in Fig. 2) [53]. Similar BFA-induced PVC-derived aggregates were also formed in various plant cell types in response to BFA treatments at 50–100 μg/mL, including root tip cells of *Arabidopsis* mung bean and pea (Fig. 3). Thus, this is likely a general response in plant cells.

In contrast, PVCs and Golgi show distinct responses to wortmannin treatment. Wortmannin inhibits phosphatidylinositol 3-kinase (PI3 kinase) activity [52] and causes swelling of endocytic compartments in mammalian cells [7]. Homologs of PI3 kinase were also identified in plants including *Arabidopsis*, *Brassica napus*, *Medicago truncatula*, and *Glycine max* [12, 18, 19, 57]. Similarly, in mammalian system, wortmannin inhibits phosphatidylinositol 4-kinase activity [31] and causes the GFP-tagged PVCs to form ring structures in living transgenic BY-2 cells (*12* in Fig. 2) [34, 54] and in *Arabidopsis* cells [33]. Similar wortmannin-induced PVC-ring structures in various plant cell types were also detected, including the late ensodomes/PVCs labeled by AtSNX1-mRFP in *Arabidopsis* root tips [22, 23], AtVSR-labeled PVCs in suspension cultured cells of rice and *Arabidopsis*, root tips of tobacco, mungbean, and pea [33, 34].

Take together, these results indicated that the GFP-tagged PVCs can be relatively easy identified in transgenic living cells based on their distinct response to treatments of BFA and wortmannin under CLSM.

Fluorescent Reporters of EEs

Endocytosis in plant cells has been demonstrated by the internalization of membrane-impermeable molecules in the past. Cationic ferritin [51], heavy metal [21], biotinylated molecules [20], and the more reliable lipophilic styryl dyes

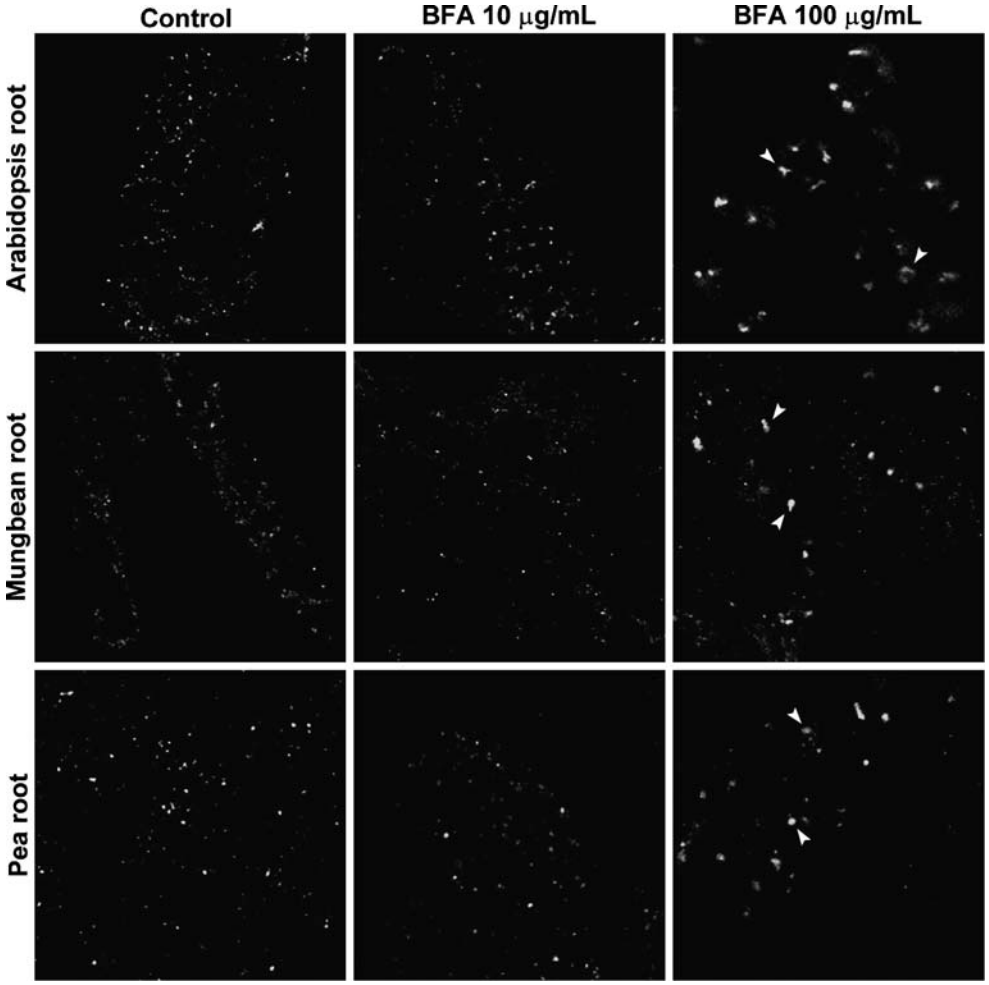

Fig. 3 BFA-induced VSR-positive aggregates in plant cells. Root tip cells of *Arabidopsis*, mungbean, and pea were treated with BFA at indicated concentrations for 2 h before fixation and used for immunofluorescent detection using the PVC marker VSR antibodies. *Arrowheads* indicate examples of BFA-induced VSR-positve aggregates. Scale bar=50 μm

FM4-64 and FM1-43 [15, 54–56] have been used to trace the process of endocytosis in plant cells. However, all these endocytic organelles connecting PM and vacuoles/lysosomes were labeled by these molecules and dyes on their path from PM to vacuole. For example, FM4-64 labeled both the Golgi apparatus and PVCs during the uptake process [4]. Therefore, these molecules and dyes are not the reliable markers for defining a particular organelle in the endocytic pathway.

Secretory carrier membrane proteins (SCAMPs) are the post-Golgi, integral membrane proteins mediating endocytosis in mammalian cells [27]. The rice SCAMP1 was recently cloned and used as probe to study the endocytosis in plant cells. Both YFP-SCAMP1 and SCAMP1-YFP fusions expressing in transgenic tobacco BY-2 cells were localized to PM and punctate cytosolic organelles (*2* in Fig. 2) which were identified as EEs because the internalized endosomal marker FM4-64 dye reached the SCAMP1-positive organelles prior to reaching the VSR-positive PVCs in BY-2 cells [27]. The pattern and size of the cytosolic SCAMP1-YFP signals are similar to the GFP-BP-80 signals in transgenic tobacco BY-2 cells. However, these two reporters, as well as Golgi reporter, show high degree of separation in confocal immunofluorescent studies [27]. In BFA and wortmannin treatment studies, the responses of the SCAMP1-YFP-labeled organelles were different from the Golgi and PVCs. The SCAMP1-YFP signals became enlarged in cells treated with either low (5–10 μg/mL) or high (50–100 μg/mL) concentrations of BFA, whereas the punctate signals of SCAMP1-YFP were transformed into spherical in shape upon wortmannin treatment. Therefore, EEs marked by the SCAMP1-YFP reporter can be easily distinguished from Golgi and PVC upon various drug treatments in tobacco BY-2 cells [27]. Similarly, other fluorescent fusion proteins labeling endosomes were also used for the study of the plant endocytosis, including the human transferring receptor (hTfR) expressing in *Arabidopsis* protoplasts [39], the V-ATPase-GFP fusion in *Arabidopsis* root cells [13], and the PIN2–GFP fusion in the root epidermis cells of *Arabidopsis* [14]. Interestingly, EE markers SCAMPs and Rab GTPases also highlighted the cell plate during cytokinesis in tobacco BY-2 cells and in *Arabidopsis* root cells [8, 26].

Transmission Electron Microscopy Studies of the Plant PVCs and Early Endosomal Compartments

Ultrastructural Analysis in Normal Cells

ER, the Golgi apparatus, mitochondria, and chloroplasts are the organelles that can be easily identified under transmission electron microscope (TEM) because of their distinct morphology, abundance, and large size. However, PVCs are less abundance and smaller in size, thus difficult to be identified under TEM without proper markers. In this connection, the GFP-tagged PVCs in transgenic tobacco BY-2 cells as well as their dynamic response to drug treatments would allow relatively easier morphological identification of PVCs via both structural and immunogold EM studies using either GFP or VSR antibodies [54]. Therefore, the GFP and/or VSR antibody-labeled structures, the MVBs, were defined as the PVCs in transgenic BY-2 cells. However, the structure of the detected MVBs by VSR antibodies by immunogold EM studies was not clear because the sample preparation was mainly targeted for good preservation of antigens for immunolabeling. Thus, structural EM study was next performed to elucidate the clear structure of MVBs/PVCs in BY-2 cells: PVCs in BY-2 cells are MVBs about 200 to 500 nm in diameter with relative uniform internal vesicles of 20–50 nm inside their limiting membrane (Fig. 4c) [54], whereas the SCAMP-positive EEs are vesicular-like and clathrin-coated TGN (Fig. 4b) [27] that are morphologically distinct from the Golgi apparatus (Fig. 4a).

The nature of EES as vesicular-like clathrin-coated TGN in plant cells is consistent with previous TEM studies on a specific structure term partially coated reticulum (PCR) which was suggested as plant endosome long time ago [42], and these PCR structures, usually found near the Golgi apparatus, have been detected in various plant cell types including the leaf glands of *Phaseolus*, the root cortical cells of *Zea*, and other tissues from *Allium cepa*, *Nicotiana tabacum*, and *Beta vulgaris*. Similarly, MVB-like structures have been documented in many different cell types, including *Arabidopsis* meristem cells and root cells, pea, and pumpkin [9, 17, 41, 46, 50] (for a review, see also [35]. Therefore, the plant EEs and PVCs are vesicular and multivesicular in nature, respectively.

Ultrastructural Analysis in Drug-Treated Cells

Brefeldin A

In CLSM level, the sizes of GFP-tagged PVCs and EEs were gradually increased upon BFA treatments. Since the resolution of CLSM is limited to 200 nm, structural TEM studies were next used to elucidate the nature of these BFA-induced PVC-derived or EE-derived aggregates in plant cells. In normal conditions, the Golgi apparatus contain about five to seven linear Golgi stacks of 400 to 700 nm in size (Fig. 4a). However, the shapes of the Golgi apparatus were changed vigorously after BFA treatments. In most cases, house-shoe-shaped Golgi apparatus were found and/or a big circular membrane structure was presented adjacent to the Golgi apparatus albeit the average size of the Golgi apparatus remaining similar (Fig. 4d–g). Moreover, several

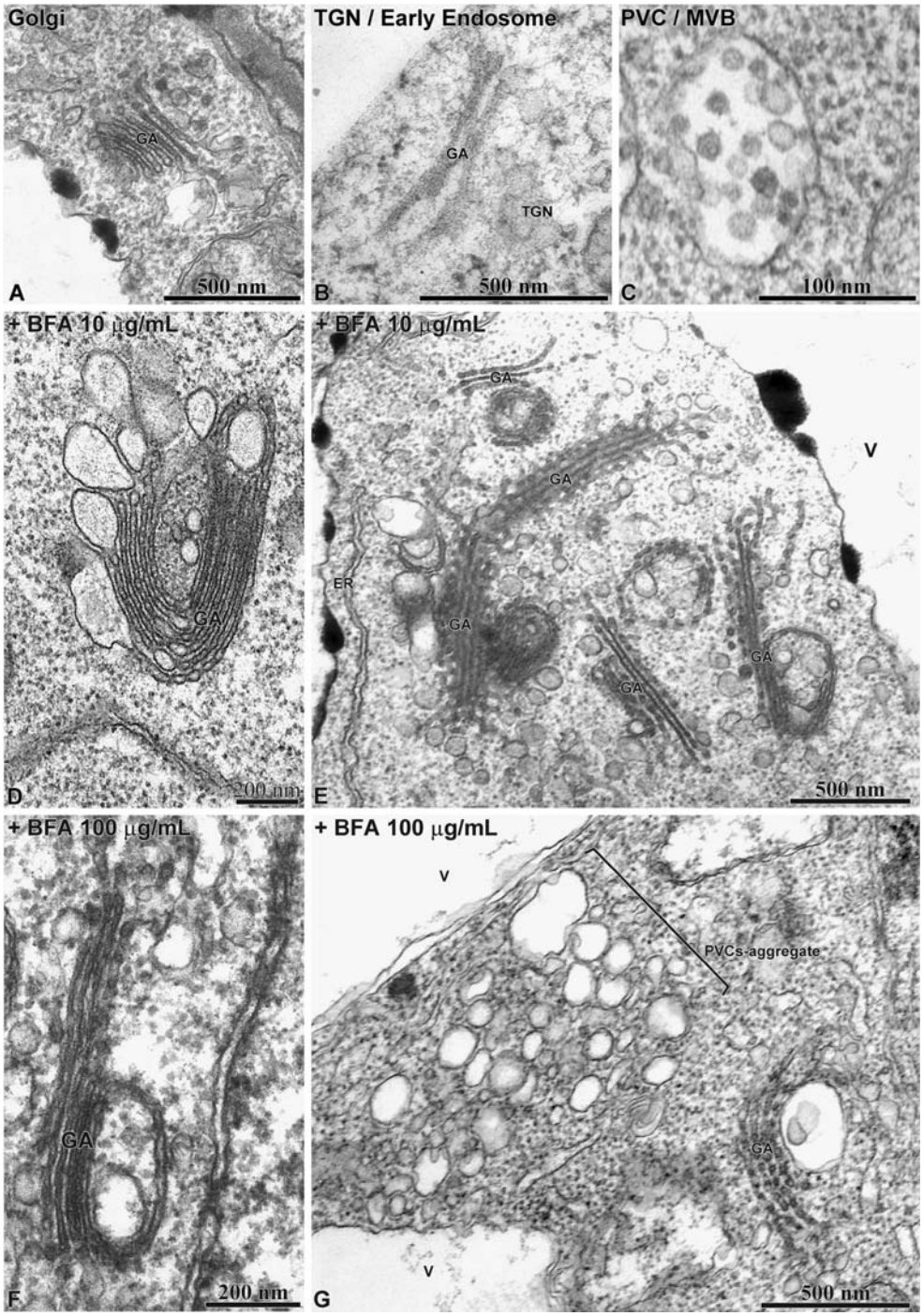

Fig. 4 Structural EM study. Untreated tobacco BY-2 cells (**a**–**c**) and BFA-treated (either at 10 or 100 μg/mL as indicated for 2 h) (**d**–**g**) were fixed and embedded in Spurr. Ultra-thin sections were prepared for TEM observation of various organelles. *GA* Golgi apparatus, *TGN* *trans*-Golgi network, *V* vacuole. Scale bars are included in respective panels of the figures

morphologically changed Golgi apparatus were found to pack closely together without any visible membrane fusion (Fig. 4e, g). This closely packed rearrangement of the Golgi apparatus may well reflect what was observed from the CLSM studies (Figs. 2 and 3) because the enlarged/aggregated GFP-tagged organelle signals in response to BFA treatment were due to the Golgi aggregation.

CLSM studies on GFP-tagged PVCs in transgenic BY-2 cells demonstrated that BFA at high concentrations caused PVCs to form aggregates where the sizes of the fluorescent signals from the GFP-tagged PVCs were at least doubled upon BFA treatment. A recent structural TEM study further demonstrated that in the presence of high concentrations of BFA, PVCs tended to form aggregates together with the closely packed Golgi apparatus (Fig. 4g) [53], forming the so-called BFA compartments that has been described in *Arabidopsis* root cells for years [45] in the presence of high concentrations of BFA (Fig. 4g) [53]. In addition, in BFA-treated cells, the sizes of the PVCs remained unchanged, but most of their internal vesicles disappeared [53] (Fig. 5).

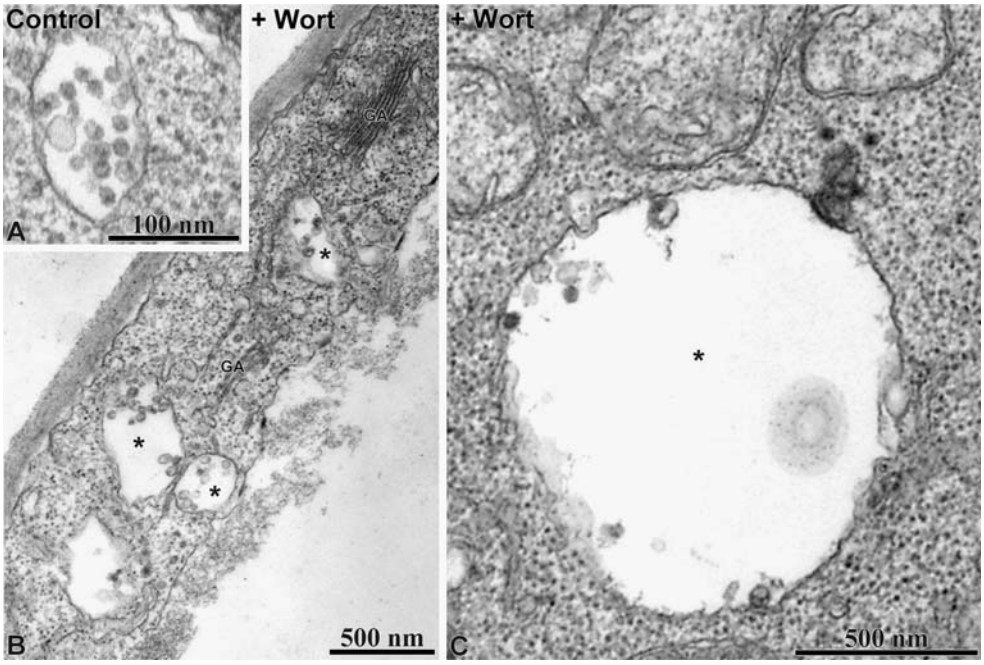

Fig. 5 Wortmannin-induced vacuolation of PVCs. Untreated (**a**) and wortmannin-treated (at 33 μM for 2 h) tobacco BY-2 cells (**b**, **c**) were chemical-fixed and embedded in Spurr. Ultra-thin sections were prepared for structural observation of PVCs. *Asterisk* indicated examples of the enlarged PVCs

Wortmannin

Under CLSM, wortmannin induces the GFP-tagged PVCs to form ring structures. The nature of such wortmannin-induced PVC enlargement was further illustrated by structural TEM studies in which enlarged PVCs with reduced number of internal vesicles and larger sizes (about five to ten times larger than the normal PVCs/MVBs) were detected in wortmannin-treated cells (Fig. 6b, c) [34, 54], indicating two possible causes of PVC enlargement: a possibility of wortmannin-induced fusion between internal vesicles and the limiting membrane of PVCs as well as wortmannin-blocked retrograde pathway.

Conclusive Remarks

The presence of prevacuolar and endosomal compartments in plant cells has been suggested for many years. However, the nature and identity of these organelles had not been solved until recent years when reliable fluorescent and antibody markers for these organelles have been available to be used for both immunofluorescent studies in transgenic living cells and structural or immunogold EM studies. Therefore, such combinational study using CLSM and structural or immunogold EM has proven to be a powerful tool for organelle characterization and identification in plant cells. Figure 6 summarizes the criteria that can be used to

			Golgi	Early endosomes	Prevacuolar Compartments
	Marker		GONST1-YFP	SCAMP1-YFP	GFP/YFP-BP-80
CLSM Level	Signal Pattern		Punctate	Punctate	Punctate
	BFA	10 μg/mL	Enlarged	Enlarged	No effect
		100 μg/mL	Enlarged	Enlarged	Enlarged
	Wortmannin	16.5 - 33 μM	No effect	Enlarged and spherical shape	Vacuolated
EM Level	Structure		Linear Golgi stacks	Tubular-vesicular structures	Multivesicular bodies
	BFA	10 μg/mL	Cup-shaped and closely packed	Aggregated	No effect
		100 μg/mL	Cup-shaped and closely packed	Aggregated	Aggregated and lost internal vesicles
	Wortmannin	16.5 - 33 μM	No effect	No effect	Enlarged and lost internal vesicles

Fig. 6 Criteria for defining distinct organelles in plant cells. Shown are summarized results that can be used to define and distinguish Golgi apparatus, early endosome, and prevacuolar compartment using CLSM and EM in plant cells. *CLSM* confocal laser scanning microscopy, *EM* electron microscopy

define various compartments based on their GFP-tagged signals and dynamic response to drugs (BFA and wortmannin) in plant cells [28]. We expect that more organelles or transport vesicles for the plant secretory, endocytic, and exocytic pathways will be characterized as more markers are developed in future studies.

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