The Endosomal System of Plants: Charting New and Familiar Territories[1]

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A BRIEF HISTORY OF ENDOCYTOSIS IN PLANTS

Endocytosis is defined as the uptake of molecules from the extracellular milieu through the formation of a vesicle at the plasma membrane (PM). This transport event circumscribes both soluble (fluid phase endocytosis) and membrane-bound cargos. The initial internalization process is followed by a series of transfer steps, mainly vesicle mediated, which carry the cargo molecules through internal (endosomal) compartments, culminating in either degradation of the cargo in the lytic compartment or in the recycling of membrane components to the PM. Considering its relevance in the medical field (e.g. clearance of cholesterol from the blood, insulin, and iron uptake) it is not surprising that endocytosis has been the subject of intense research in mammalian cell biology. However, as reflected by the paucity of PubMed entries, comparatively little work has been done on endocytosis in plants. The main reason for this has been the reluctance of the plant community to accept the operation of this fundamental cellular transport process.

Almost 30 years ago, it was suggested that turgor pressure may prevent membrane invagination at the PM of a plant cell with a wall (Cram, 1980). However, endocytosis was demonstrated in protoplasts a few years later with the help of electron dense tracers (Hillmer et al., 1986; Tanchak and Fowke, 1987). Accordingly, it was then calculated that turgor pressure varies and fluctuates enormously throughout the plant and that turgor values below 5 bar do not impede endocytosis (Saxton and Breidenbach, 1988; Gradmann and Robinson, 1989). Even in guard cells, where the highest turgor values have been recorded, there are clathrin-coated pits at the PM (Doohan and Palevitz, 1980) and the internalization of fluorescently labeled PM proteins has been measured (Meckel et al., 2004). Despite such arguments, doubts about plant cells performing endocytosis continued to be fueled by erroneous observations on fluid phase endocytosis using the sulfonated dye Lucifer Yellow (Oparka and Hawes, 1992; Baluska et al., 2004; Aniento and Robinson, 2005). Only with the introduction of amphiphilic styryl dyes, especially FM4-64, which inserts into the outer lipid bilayer of the PM and then moves in a temperature-dependent manner to the tonoplast (Bolte et al., 2004; Meckel et al., 2004), can one say that the dissenting voices on plant endocytosis have finally been silenced.

Another factor that contributed to the delayed interest in plant endocytosis was the lack of obvious cargo molecules, making a role for this process in the physiology of the plant unclear. This problem has gradually disappeared over the last 5 to 6 years as more and more receptors at the PM have been discovered and their internalization demonstrated (Russinova and de Vries, 2006; Altenbach and Robatzek, 2007). Nevertheless, from a cell biological point of view, it is curious that in some of these articles, which can certainly be regarded as classics in the rapidly developing field of plant endocytosis (e.g. Gifford et al., 2005; Robatzek et al., 2006), no reference to the clathrin-internalization machinery that is essential for receptor internalization in mammalian cells is mentioned. However, the recent demonstration of the clathrin dependency of the uptake of the PIN-FORMED (PIN) family of auxin efflux transporters (Dhonukshe et al., 2007) suggests that it is only a question of time before the ring closes and unequivocal evidence linking the clathrin machinery to receptor internalization is provided.

PLANT ENDOCYTOSIS TODAY

As evidenced by the rapid proliferation of review articles in the last 4 years, plant endocytosis research has really broken out of the dormancy period (Geldner, 2004; Murphy et al., 2005; Samaj et al., 2005, 2006b; Baluska et al., 2006; Geldner and Jurgens, 2006; Lam et al., 2007b; Muller et al., 2007). Even a book on this subject has been published (Samaj et al., 2006a). A whole battery of PM receptors, principally belonging to the Leu-rich repeat type of receptor kinases (LRKs), have been identified and are involved in a variety of mor-

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phogenetic responses, including meristem patterning, cell differentiation, and defense signaling. Both ligand-dependent (FLS2; Robatzek et al., 2006) and ligand-independent, constitutive (BRI1, ACR4; Shah et al., 2002; Gifford et al., 2005) LRK internalization have been recorded. In mammalian cells, ligand-induced, receptor-mediated endocytosis regulates signaling activity at the cell surface (receptor down-regulation), but many of these receptors continue or even start signaling after internalization. A similar situation has also recently been described for several plant PM receptors, e.g. the ACR4 and BRI1 receptors (Gifford et al., 2005; Geldner et al., 2007). As with cell surface receptors in mammalian cells, autophosphorylation of the cytosolic domain of the SERK3/BAK1 receptor is also induced by ligand binding, the subsequent dephosphorylation occurring after internalization (Shah et al., 2002). All of these receptors undergo recycling to PM after internalization (Russinova and de Vries, 2006). Thus, a whole new research area of postinternalization, endosomal-based receptor signaling is emerging, a phenomenon that seems to have developed separately from the similar situation in mammalian cells (Geldner et al., 2007).

Cytologically, the presence of clathrin-type lattices and coated pits at the PM of plant cells has been known for a long time (for review, see Robinson and Hillmer, 1990) and is generally assumed to reflect the operation of a clathrin-dependent endocytosis. Orthologs of many of the well-characterized elements of this machinery in mammalian cells (clathrin heavy and light chains, adaptins, and scaffolding proteins) have been detected in plants (Holstein, 2002; Barth and Holstein, 2004; Holstein and Oliviusson, 2005), but the crucial interaction of an AP-2-like adaptor complex with the cytoplasmic domain of an LRK remains to be demonstrated. Nevertheless, this can be deemed as being very likely based on studies involving the internalization of the human transferrin receptor (hTfR), which, when expressed in Arabidopsis (*Arabidopsis thaliana*) protoplasts, is inhibited by tyrphostin A23, a Tyr analog that prevents the interaction between the YTRF endocytosis motif in the hTfR cytosolic tail and the μ2 subunit of the AP-2 complex (Ortiz-Zapater et al., 2006). Moreover, hTfR has also been shown to directly interact with an Arabidopsis μ-adaptin, providing a tangible link between an internalized receptor (albeit a foreign one) and elements of the clathrin internalization machinery in plants (Ortiz-Zapater et al., 2006).

THE EARLY AND RECYCLING ENDOSOME(S)

With endocytosis established as an essential plant cell function and examples of endosomal-based receptor signaling now at hand, a reliable road map of the endocytic system is now urgently needed. As endosomes can be classified by either kinetic (early versus late), structural (tubular versus multivesicular), or functional (sorting versus recycling) criteria (Sachse et al., 2002; Perret et al., 2005), there is obviously some overlap between the different terms. Thus, care needs to be taken in defining plant endosomes, especially when analogies to other systems are drawn.

When we trace a primary endocytic vesicle formed at the PM, the first station to which it delivers its cargo is classically defined as the early endosome (EE). In the early days, such a kinetic definition could only be applied using nonspecific, electron-dense markers, e.g. cationic ferritin or heavy metal salts. Based on a number of studies using such tracers in protoplasts and a variety of cell types, the so-called partially coated reticulum (PCR) arose as the prime candidate for the plant EE. This is a tubular-vesicular structure with clathrin-budding profiles, which is often found in the vicinity of Golgi stacks (for review, see Robinson and Hillmer, 1990). The PCR is, however, structurally indistinguishable from the trans-Golgi network (TGN), the main sorting station of the secretory pathway, making it necessary to simultaneously visualize TGN and endosomal markers to determine if both structures are identical, overlapping, or unrelated (Geldner, 2004).

VHA-a1, one of three isoforms of a membrane-integral V-ATPase subunit, turned out to be an almost ideal tool as it is specifically localized to the TGN (Dettmer et al., 2006). Its functional GFP fusion, in combination with uptake of the styryl dye FM4-64, allows one to determine if and how fast endocytic cargo reaches the TGN. Indeed, rapid and complete colocalization of both markers indicated that the TGN, and EE, at least at the resolution of the confocal laser scanning microscope, are subdomains of one and the same compartment, and are therefore equivalent to the PCR. Analysis of rice (*Oryza sativa*) SECRETORY CARRIER MEMBRANE PROTEIN1 (SCAMP1) showed that SCAMP1-positive organelles, which become labeled prior to the multivesicular body (MVB)/prevacuolar compartment (PVC) by FM4-64 in tobacco (*Nicotiana tabacum*) BY-2 cells, are also the TGN. This provides further, independent support for a function of the TGN in the endocytic pathway (Lam et al., 2007a).

The TGN also belongs to the secretory pathway, suggesting the presence of functional subdomains such as has been described for mammalian endosomes where combinations of different RAB proteins define subdomains responsible for individual sorting steps (Gruenberg, 2001). Indeed, it has recently been shown that Rab-A2 and Rab-A3 GTPases define a trans-Golgi endosomal membrane domain that overlaps with, but is not identical with, the VHA-a1 domain in Arabidopsis root tip cells (see Fig. 1A; Chow et al., 2008). Whereas the VHA-a1 domain is the earliest destination for FM4-64, the Rab-A2/A3 domain lies on the exocytic route to the PM, potentially providing for rapid recycling to the PM (Chow et al., 2008). This would also provide an explanation for the puzzling observation that late endosomal compartments and the tonoplast are only stained by FM4-64 after >1 h whereas the TGN/EE is already labeled after a few minutes.

If we accept that the TGN and the EE are functional subdomains of one compartment, our map of the plant endocytic system is, at first glance, greatly simplified as it includes only one compartment responsible for the sorting of both newly synthesized and endocytosed material. From the TGN/EE, internalized material could be directed either into the recycling pathway to the PM or into the late endosomal pathway leading to the vacuole. On the downside, this scenario makes experimental approaches more difficult as rapid colocalization of PM proteins with FM4-64 is only indicative of recycling when assayed in the presence of the protein synthesis inhibitor cycloheximide, a drug that is likely to affect flow along the endocytic pathway. Further experiments addressing the sorting and recycling will thus require one to differentiate between newly synthesized and recycling cargo molecules in undisturbed cells, possibly through the use of photoconvertible fluorescent proteins (Dhonukshe et al., 2007).

How do these results fit in with other markers that have frequently been labeled as early endosomal? The small Rab-GTPase Rab5 is a recognized marker for the EE in mammalian cells (Zerial and McBride, 2001). Arabidopsis has three closely related proteins, AtRABF1 (ARA6), AtRAB-F2a (RHA1), and AtRAB-F2b (ARA7), which have been located on two overlapping populations of endosomes when transiently overexpressed as (X)-FP constructs in protoplasts (Ueda et al., 2001, 2004). However, it should be noted that neither the degree nor the time course of colabeling of AtRABF1 and AtRABF2b with internalized FM4-64 have been analyzed in a comparative manner. Thus, it is premature to classify the two endosome populations as early versus late (Murphy et al., 2005; Jaillais et al., 2008). Furthermore, immunolocalization using antibodies against endogenous ARA6 and ARA7 (Haas et al., 2007) as well as a GFP-ARA7 fusion protein (Reichardt et al., 2007) have shown unequivocally that both proteins reside on MVBs that are usually referred to as late endosomes (LEs; see below). In addition, the function of plant Rab5 homologs is quite different from their mammalian counterpart, being almost the same as that of the yeast (*Saccharomyces cerevisiae*) Rab5 homolog VPS21 in that, like ARA7, they are required for the sorting of vacuolar proteins (Horazdovsky et al., 1994; Kotzer et al., 2004) and are found in a prevacuolar, late endosomal compartment (Gerrard et al., 2000; Lee et al., 2004).

What do we know about candidates for a recycling endosome (RE) in plants? The adenosine ribosylation factor (ARF)-guanine nucleotide exchange factor (GEF) GNOM acts to mediate the recycling of the auxin efflux regulator PIN1 to the PM (Geldner et al., 2003). Unfortunately, the exact localization of this important player is still unclear, but GNOM neither colocalizes with EE nor with LE/MVB markers (Chow et al., 2008). It could therefore mark an additional compartment likely to serve as an equivalent of the mammalian RE. However, GNOM, being brefeldin A (BFA) sensitive and wortmannin insensitive (Geldner et al., 2003; Jaillais et al., 2008), behaves much more like the early endosomal markers described above than the LE markers described in the following section.

LEs

Several characteristic features of LEs in nonplant cells are also recognizable in an organelle in plant cells, which is distinct from the EE just described. In mammalian and yeast cells, LEs are enriched in phosphatidylinositol-3-P (PI-3P; Gillooly et al., 2000). The ring finger FYVE domain is known to specifically bind to PI-3P, and (X)-FP-tagged versions of FYVE have become sensors for the presence of PI-3P (Kutateladze et al., 1999). This is also the case in plants, whereby a most useful variant is a YFP-2xFYVE dimer (Park et al., 2003; Vermeer et al., 2006). In BY-2 cells, this colocalizes together with RABF2b to FM4-64-positive, punctate structures. This strongly suggests that PI-3P is also enriched in the plant LE.

Characteristic of the LE in yeast is also the SNARE Pep12p (Pelham, 2000). Its ortholog in plants PEP12/AtSYP21 has also been shown to reside on post-Golgi dense and small (<100 nm) structures but missed from endoplasmic reticulum and Golgi (Conceicao et al., 1997). Subsequent work in which numerous GFP-tagged SNAREs were transiently expressed in Arabidopsis protoplasts has established a total of 12 SNAREs (e.g. AtSYP21, AtSYP22, AtVAM712, and AtVTI11) as being resident on a structure that is distinct from the EE and the Golgi apparatus in Arabidopsis (Uemura et al., 2004).

Sorting receptors for acid hydrolases (Vps10p) in yeast and mammalian cells (mannosyl 6-P receptor) are recycled to the TGN from a LE compartment that also lies on the biosynthetic route to the lytic compartment of the cell (see also below). This occurs through interaction with a cytosolic protein complex named retromer (Seaman, 2004, 2005). In yeast, retromer consists of a large subunit with three proteins, Vps35p, Vps29p, and Vps26p, and a small subunit with Vps5p and Vps17p (Reddy and Seaman, 2001). The cytosolic tail of Vps10p interacts with Vps35p, and the Phox (PX) domains in Vps5p and Vps17p bind to PI-3P (Yu and Lemmon, 2001). Homologs to these proteins are present in mammals, whereby the roles of the small subunit proteins are assumed by the sorting nexins SNX1 (=Vps5p) and SNX2 (=Vps17p). Retromer polypeptides have been detected in plants (Oliviusson et al., 2006; Shimada et al., 2006; Jaillais et al., 2007), and evidence has been presented for an interaction with vacuolar sorting receptor (VSR)-At1 (Oliviusson et al., 2006). The large subunit proteins of retromer are clearly on the surface of a MVB (Oliviusson et al., 2006), and this also appears to be the location for the plant homolog of Vps5 (=AtSNX1; Jaillais et al., 2006). However, unlike Jaillais et al. (2007), we see no a priori reason to name such structures as SNX1 endosomes; they could equally well be termed retromer endosomes. AtSNX1 also colocalizes with RABF2b (ARA7) to a wortmannin-

sensitive compartment in both Arabidopsis roots and BY-2 cells (Jaillais et al., 2008; see also Fig. 2C). In confirmation of the prevacuolar nature of this putative LE is the observation that it also received PM proteins (PIN2, BRI1) apparently destined for degradation as the tonoplast also showed weak labeling with (X)-FP-tagged versions of these proteins (Jaillais et al., 2008; see also Fig. 1D).

The internal vesicles in MVB/LE are a consequence of the need for a selective degradation of membrane proteins that may be related to the necessity to reduce signaling at the cell surface (receptor down-regulation). Such proteins become ubiquitinated in their cytosolic domains, providing a signal for their entry into invaginating vesicles in the endosomes (Gruenberg and Stenmark, 2004). Responsible for recognizing these proteins and for delivering them into the invaginations are four protein complexes known as endosomal sorting complexes required for transport (ESCRTs) 0 to 4 (Hurley and Emr, 2006; Williams and Urbe, 2007). These complexes are attached to the membrane surface via interaction with either PI-3P (via Vps27p in ESCRT 0; via Vps36p in ESCRT 2) or PI-3,5P (via VPs24p in ESCRT 3). Ubiquinated cargo molecules are initially recognized by Vps27p of the ESCRT 0 complex. Morphologically, the presence of ESCRT can be seen through the presence of a double-layered, adaptor-free clathrin plaque that also interacts with Vps27p of the ESCRT 0 complex (Sachse et al., 2002; Raiborg et al., 2003). Homologs to ESCRT proteins have been identified in plants (Spitzer et al., 2006; Winter and Hauser, 2006). Among these, both VPS23 the *ELCH* gene product and VPS4/SKD1, an AAA-ATPase associated with the ESCRT 4 complex, have been shown to associate with the surface of a MVB/LE and colocalize with RABF1 (ARA6) and RABF2b (ARA7; Spitzer et al., 2006; Haas et al., 2007). A thick plaque has also been seen on the surface of the multivesicular plant PVC (Tse et al., 2004), but its identity as clathrin remains to be demonstrated. Curiously, in mammalian cells, ESCRT 0/clathrin plaque is more typical of early/sorting endosomes than of LEs that tend to have more of the other ESCRT complexes (Williams and Urbe, 2007).

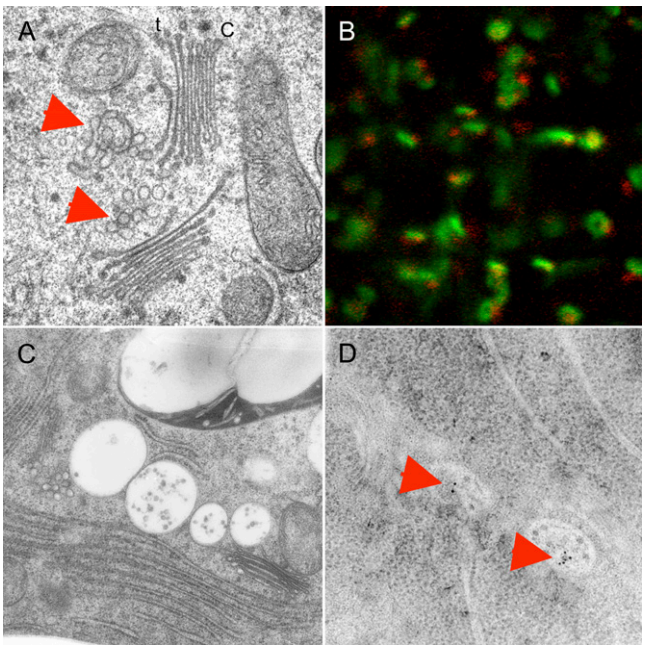

Figure 1. Endosomal compartments and the Golgi apparatus in Arabidopsis. A, Two Golgi stacks with typical cis (c)-to-trans (t) polarity. The TGN/EE is a tubular-vesicular cluster (indicated by arrowheads) at the trans-face of the stacks. B, Differential fluorescent labeling of the Golgi apparatus. The green signal (GFP) is from the trans-Golgi stack marker sialyl transferase. This is visible in face view as a doughnut structure or in side view as a sausage-like structure. The attached red signal (mRFP) is the TGN marker VHA-a1. C, A group of three mulitivesicular bodies (=LE or PVC). D, Positive BRI1 immunogold labeling of the internal vesicles in LE (see arrowheads; micrograph courtesy of Corrado Viotti). A, B, and D are from root cells; C is from the integument. A, C, and D are from high-pressure-frozen/freeze-substituted samples. Magnification bars are 200 nm in A, C, and D, and 1 μM in B.

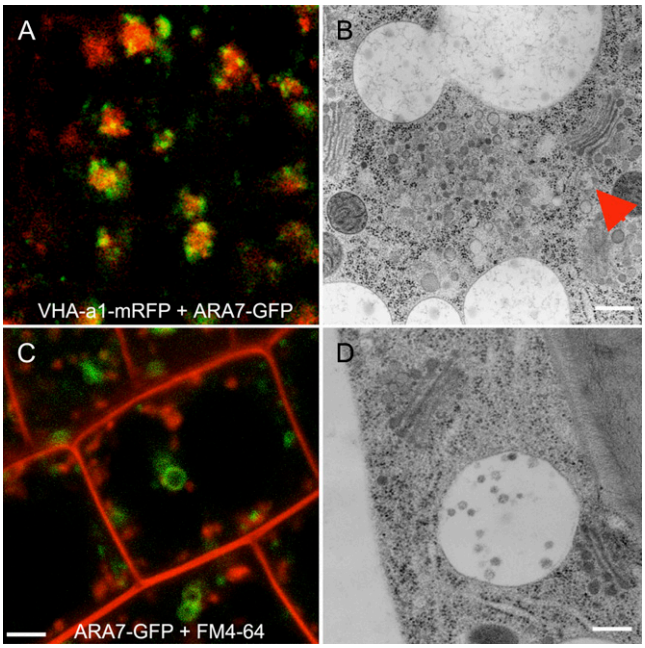

Figure 2. The effects of inhibitors on endosomal compartments in Arabidopsis. A and B, BFA. The core of the BFA compartment contains elements of the TGN as indicated by the centrally located aggregates of VHA-a1-mRFP. Outside of the core lie both Golgi stacks (clearly visible in the EM: B) as well as LE structures (monitored with ARA7-GFP in A). The arrowhead in B indicates an MVB/LE. Seedlings were incubated in 25 (B) or 50 (A) μg mL^{-1} BFA for 30 min. C and D, Wortmannin. This drug leads to a characteristic dilation of LE compartments, and can be monitored with VSR or with the Rab GTPase ARA7-GFP (see C), or visualized in the EM (compare the sizes of the MVB in relation to Golgi stacks in B and D). Wortmannin also blocks endocytic uptake of FM4-64; seedlings were therefore stained with FM4-64 for 5 min prior to incubation in 20 μM wortmannin for 60 min. All pictures are from root cells. B and D, From high-pressure-frozen/freeze-substituted samples (courtesy of York-Dieter Stierhof). Magnification bars are 10 μM for A and C and 200 nm in B and D.

Thus, all evidence points to the LE in plants as being multivesiculate in morphology and enriched in PI-3 phosphates, bearing the SNARE PEP12/SYP21, having polypeptides of the retromer and ESCRT complexes, as well as Rab5-type GTPases (ARA6, ARA7, RHA1) at its surface. However, whether it is correct to assign the term sorting endosome to the plant LE as Jaillais et al. (2008) have done is a moot point. In mammalian cells, the term sorting endosome is commonly used as synonymous with EE where sorting to either the PM, the RE, or the LE occurs (Maxfield and McGraw, 2004).

While it seems that the plant LE possesses the machinery for recognizing and internalizing membrane proteins destined for degradation, and through the presence of retromer there is clearly a capacity for recycling receptors to the TGN, there is no evidence for a recycling of proteins to the PM. The presence of PM proteins on the internal vesicles (see Fig. 1D) argues strongly against a recycling function and in favor of them being transported to the vacuole for degradation. In fact, vacuolar accumulation has been observed for BOR1-GFP (Takano et al., 2005), BRI1-YFP (Geldner et al., 2007), and PIN2-GFP (Laxmi et al., 2008). It would thus appear that, in plants, sorting and internalization are spatially separated events: the former event occurring in the EE, where as a consequence recycling to the PM occurs, and the latter restricted to the LE.

CONVERGENCE OF ENDOCYTIC AND BIOSYNTHETIC PATHWAYS TO VACUOLES

The connection of post-Golgi protein trafficking to endocytosis has been well studied in mammalian cells and yeast (Le Roy and Wrana, 2005; Murphy et al., 2005; Bonifacino and Rojas, 2006). However, the possible convergence of endocytic and biosynthetic pathways leading to the vacuole has been slow to establish in the plant literature, because traditionally these two pathways were always studied independently of each other (Lam et al., 2007b). Evidence for a merging of secretory and endocytic protein trafficking came from an investigation of the localization of VSRs in relation to FM4-64 uptake in BY-2 cells (Tse et al., 2004).

VSRs are type I integral membrane proteins that mediate protein transport from Golgi apparatus to vacuole in plant cells (Jiang and Rogers, 2003). The first studied VSR protein, pea (*Pisum sativum*) BP-80, was shown to localize to both Golgi and a morphologically undefined PVC in pea root tip cell (Paris et al., 1997). Its homolog, AtELP, had a similar distribution in Arabidopsis root tip cells (Ahmed et al., 1997). A later study on the relative distribution of VSR proteins between Golgi and PVC demonstrated that VSR were predominantly concentrated in PVC in various plant cells (Li et al., 2002). Since a construct containing the transmembrane domain and cytoplasmic tail (termed BP-80 reporter) colocalized with endogenous VSR (Jiang and Rogers, 1998), both VSR and the BP-80 reporter were thus used as markers, resulting in the identification of MVB as the PVC that became vacuolated in the presence of wortmannin (Tse et al., 2004; see also Fig. 2). The MVB nature of PVC was subsequently confirmed in both developing Arabidopsis seeds (Otegui et al., 2006) and germinating mung bean (*Vigna radiata*) cotyledons (Wang et al., 2007). Similarly, GFP fusions with individual transmembrane domains and cytoplasmic tails of the seven Arabidopsis VSRs were also found to localize to PVCs in transgenic BY-2 cells (Miao et al., 2006). Using either VSR or VSR reporters as markers for PVC, several plant proteins were shown to localize to PVCs. These include the retromer complex (see above), AtMTP11, an integral membrane protein that functions in manganese transport and tolerance in Arabidopsis (Delhaize et al., 2007), and the SNARE PEP12/AtSYP21 (Conceicao et al., 1997).

With the TGN now being recognized as assuming the functions of the EE, it remains to be determined whether biosynthetic traffic from the Golgi apparatus to the PVC/LE goes via the EE. It is generally thought that VSRs mediate the incorporation of acid hydrolases into clathrin-coated vesicles (CCVs) via the μ-adaptins of an AP-1-like adaptor complex (Happel et al., 2004; Robinson et al., 2005). Recruitment of CCV coat proteins at the TGN requires the activity of ARF1, and expression of an ARF1 mutant fixed in the GTP form inhibits the export of vacuolar cargo out of the Golgi apparatus (Pimpl et al., 2003). Thus, it would seem that the TGN has two entry portals: one for endocytic traffic and the other for biosynthetic cargo. However, whether the CCV seen budding at the TGN are exclusively involved in transport to the PVC/LE, or whether some represent recycling vehicles to the PM, has not yet been resolved. Indeed, based on separation of trans Golgi RabA2/A3- and VHA-a1-marked compartments, especially at cytokinesis, it might be prudent not to consider the TGN as a single EE (Chow et al., 2008).

INHIBITORS OF THE ENDOCYTIC PATHWAY

A variety of inhibitors including BFA and wortmannin have been instrumental in defining endosomal compartments. The results obtained have to be interpreted with great care as localization of their molecular targets can vary between cell types and their specificity might only hold true within a certain range of concentrations.

BFA

The fungal macrocyclic lactone BFA inhibits the function of ARF GTPases by interacting with their associated GEFs (Jackson and Casanova, 2000). BFA has been variously been described as an inhibitor of secretion (Nebenfuhr et al., 2002) or as an inhibitor of endocytosis (Geldner, 2004), depending upon where the BFA-sensitive ARF-GEFs are located: early Golgi or post-Golgi (Richter et al., 2007; Teh and Moore, 2007).

The term BFA compartment was originally introduced by Lippincott-Schwartz et al. (1991) in studies on mammalian cells to describe a structure formed from trans-Golgi and endosomal elements that remained behind after the rest of the Golgi had been resorbed into the endoplasmic reticulum. Working with maize (*Zea mays*) root cells, Satiat-Jeunemaitre and Hawes used the same term to describe a somewhat different structure: a large cluster of vesicles surrounded by more or less intact Golgi stacks (see Satiatjeunemaitre and Hawes, 1994 for a description of the early literature). Exactly this is the kind of structure that is induced by BFA in Arabidopsis roots (Geldner et al., 2003; Grebe et al., 2003; Paciorek et al., 2005; see also Figure 2, A and B).

Because Arabidopsis roots are in frequent use as an experimental system, it is appropriate to ask the following: What is in the BFA compartment and what is not? As seen in Figure 2B, the TGN marker VHA-a1 is present in the core of the structure, whereas the rest of the Golgi stacks, as monitored for with the marker sialyl transferase, lie at the surface (see also Grebe et al., 2003). Indeed, the proton-pumping activity of the V-ATPase in the TGN is required for the formation of BFA compartments and for transport of FM4-64 to LEs and, eventually, to the tonoplast (Dettmer et al., 2006). The post-Golgi ARF GNOM also accumulates in the core (Geldner et al., 2003), whereas MVB/PVC are excluded as judged by the localization of the appropriate markers (ARA7; BP-80; see Fig. 2A), and is confirmed by thin sections in the electron microscope (EM) where MVBs are not seen in the core (see Fig. 2B). In contrast, Jaillais et al. (2006, 2008) have claimed that MVBs do enter the BFA compartment based on the formation of VSR (BP-80) and AtSNX1-containing aggregates. However, since it has been shown that the PVC in root tip cells from a number of organisms form aggregates in response to high concentrations (50–100 $\mu g\ mL^{-1}$) of BFA (Tse et al., 2006), we would maintain that the BP-80/AtSNX1 aggregates do not represent the BFA compartment, in which PM proteins that constitutively recycle between the PM and the EE accumulate along with the endocytic tracer FM4-64 (see above). These PM proteins include members of the PIN and AUX families of auxin transporters (Geldner et al., 2001, 2003; Grebe et al., 2002, 2003; Dhonukshe et al., 2007), a protein involved in gravity sensing (ARG1; Boonsirichai et al., 2003), a PM H^+-ATPase (Geldner et al., 2001), the boron transporter BOR1 (Takano et al., 2005), and the brassinosteroid receptor BRI1 (Geldner et al., 2007).

PM-derived sterols also accumulate in the BFA compartment (Grebe et al., 2003), confirming that endocytosis is still in operation during the formation of the BFA compartment. Since, more or less intact Golgi stacks surround the BFA compartment, and these bear a BFA-resistant ARF-GEF (Richter et al., 2007), it can be assumed that membrane trafficking in the early secretory pathway is also intact during the formation of the BFA compartment. Indeed, fluorescence recovery after photobleaching experiments showed that transport of newly synthesized proteins to the PM was not significantly inhibited by BFA (Grebe et al., 2003). Given that the TGN/EE is found in the core of BFA compartments, it will be important to determine how and where newly synthesized and recycling PM proteins can be discriminated.

Indeed, the presence of cell wall polysaccharides in the BFA compartment (Satiatjeunemaitre and Hawes, 1992) may reflect the accumulation of secretory vesicles, rather than being interpreted as endocytic in origin (Baluska et al., 2002). Nevertheless, the huge numbers of vesicles constituting the BFA compartment in Arabidopsis roots can only be the result of a continual vesicle input from both secretory and endocytic pathways.

Concanamycin A and Bafilomycin A

Concanamycin A (ConcA) and Bafilomycin A (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, thus inhibiting proton transport. Numerous pharmacological studies in mammalian cells have established that acidification is crucial for many functions of the secretory and endocytic pathways including the dissociation of receptor-ligand complexes (Forgac, 1999), recruitment of proteins involved in vesicle formation (Aniento et al., 1996; Maranda et al., 2001), and transport between different endosomal compartments (Clague et al., 1994; Tawfeek and Abou-Samra, 2004). In tobacco cells, inhibition of the V-ATPase interfered with secretion and leads to missorting of vacuolar proteins, suggesting that the functionality of a nonvacuolar compartment depends on V-ATPase function (Matsuoka et al., 1997). Interestingly, V-ATPase activity in the Golgi was found to be more sensitive to ConcA than to BafA1, whereas the tonoplast activity was equally sensitive to both inhibitors (Matsuoka et al., 1997). Electron microscopy of chemically fixed BY-2 cells showed that both ConcA and BafA lead to massive vacuolation of the Golgi apparatus (Robinson et al., 2004). Similarly, high-pressure-frozen Arabidopsis root cells treated with ConcA displayed changes in Golgi morphology and aggregations of vesicles (Dettmer et al., 2006) resembling the situation observed in *vha* mutant cells (Dettmer et al., 2005). In agreement with the proposed dual function of the TGN, ConcA blocks both the trafficking of newly synthesized proteins to the PM and the transport of FM4-64 from the TGN/EE to the vacuole (Dettmer et al., 2006). Moreover, the formation of the typical large BFA compartments does not occur in cells pretreated with ConcA (Dettmer et al., 2006). The effects of treatments with 1 to 2 μM ConcA for 2 to 4 h are largely reversible but care needs to be taken as ConcA effects are easily observed in root epidermal and cortex cells but are much weaker in deeper cell layers (Y. Stierhof and K. Schumacher, unpublished data). In addition, ConcA is fairly unstable and appropriate controls for activity have to be included if the absence of effects is interpreted.

Tyrphostins

As described by Aniento and Robinson (2005) tyrphostins are structural analogs of Tyr that have been successfully used as competitive inhibitors for receptor Tyr kinases in mammalian cells. It has been reported that they can inhibit both endocytosis and vesiculation at the TGN (Holen et al., 1995; Austin and Shields, 1996). In particular tyrphostin A23 (but not A51, which therefore acts as a negative control) seems to interfere with the recognition of the classical YxxΦ internalization motif in the cytosolic domain of PM receptors by the μ-adaptin of the AP-2 adaptor complex (Banbury et al., 2003). Two articles have suggested that tyrphostin A23 could be a useful inhibitor of endocytosis in plants. First, as already mentioned, the internalization of the hTfR when expressed in Arabidopsis protoplasts is inhibited by tyrphostin A23 (Ortiz-Zapater et al., 2006). Second, tyrphostin A23 has been reported to inhibit the internalization of PIN2 (but not FM4-64) in Arabidopsis roots, in particular preventing its accumulation into the BFA compartment (Dhonukshe et al., 2007). Since the appearance (in the confocal laser scanning microscope) of various TGN markers remained unchanged after tyrphostin A23 treatment, Dhonukshe et al. (2007) claimed that tyrphostin A23 ''did not show any apparent effects on other subcellular trafficking processes'' (p. 526).

Unfortunately, this positive impression is not upheld by recent data coming from different laboratories. First, Arabidopsis roots pretreated with tyrphostin A23 are unable to form a BFA compartment. Second, tyrphostin A23 severely impaired cell plate development (I. Reichardt and G. Juergens, unpublished data). This indicates that tyrphostin A23 is also acting at the level of the Golgi apparatus, since Golgi-based secretion is required for both the formation of the BFA compartment (see above) as well as for cytokinesis (Reichardt et al., 2007). Tyrphostin concentrations are also very important: As judged by vitality stains, tobacco protoplasts die within 30 min after exposure to 100 μM tyrphostin A23 (Dogbevia and Pimpl, unpublished data); Arabidopsis roots die when treated with 35 μM tyrphostin for longer than 2 h (Reichardt and Jürgens, unpublished data). Therefore, great caution should be taken when these drugs are used without the appropriate controls.

Wortmannin

This is an inhibitor of phosphatidylinositol-3 kinase (PI-3 kinase; Vps34p in yeast; Corvera et al., 1999). PI-3 kinase is essential for the formation of internal vesicles (Fernandez-Borja et al., 1999) and for the attachment of the small subunit of retromer via the PX domains in Vps5p and Vps17p and the sorting nexins (see above). Treatment with wortmannin, leads to a release of the sorting nexins, and therefore to a perturbation of Vps10p and mannosyl 6-P receptor-based receptor recycling in yeast and mammals (Burda et al., 2002; Arighi et al., 2004). As a consequence, LEs lose their clathrin plaques, have fewer internal vesicles, and swell (Bright et al., 2001; Sachse et al., 2002).

Vps34p homologs have been described in plants (Welters et al., 1994; Das et al., 2005), and wortmannin is an efficient inhibitor of protein trafficking to the plant vacuole (daSilva et al., 2005, and refs. therein). It also targets the PVC/LE that then dilates as can be moni-

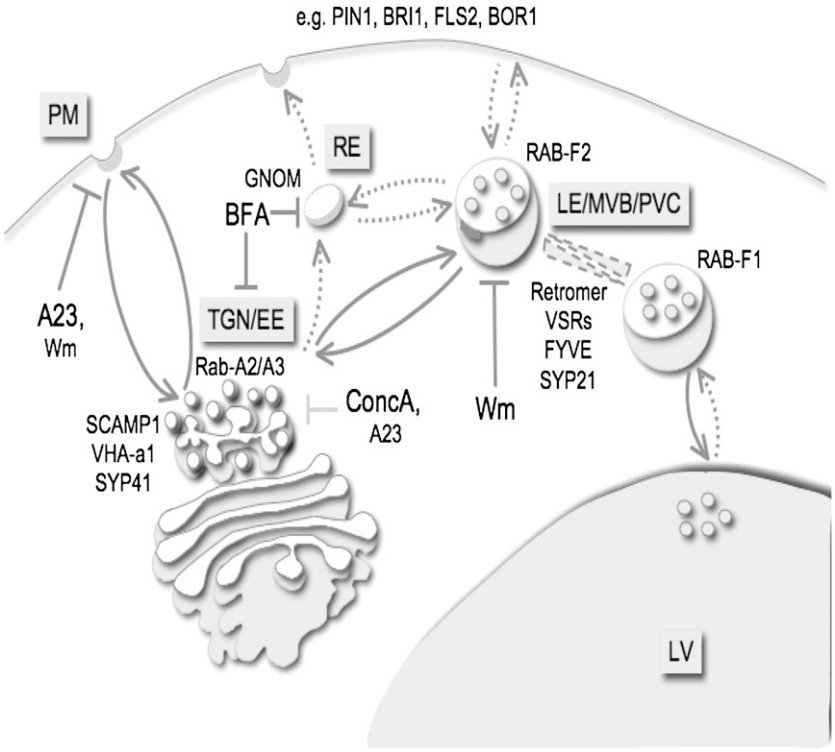

Figure 3. The endocytic pathway in plants: a working model including compartments, markers, and inhibitors. The compartments are indicated in boxes; the markers are noted adjacent to these. The targets for the four inhibitors BFA, ConcA, tyrphostin A23 (A23), and wortmannin (Wm) are indicated. Full arrows represent trafficking pathways that have been proven; dashed arrows are speculative trafficking pathways.

tored by GFP-tagged VSR or ARA7 (Tse et al., 2004; Jaillais et al., 2006, 2008; Oliviusson et al., 2006; Silady et al., 2008; see also Fig. 2, C and D). Interestingly, although the large subunit of retromer (containing VPS35, VPS29, and VPS26) is not released from the PVC (Oliviusson et al., 2006; Jaillais et al., 2008) through wortmannin treatment, it separates physically from the VSR (Oliviusson et al., 2006). In contrast to the situation in mammalian cells where wortmannin treatment causes a reduction in endosomal-bound sorting nexins (Zhong et al., 2002; Arighi et al., 2004), this does not appear to be the case for the plant SNX1 (Jaillais et al., 2008). On the other hand, Vermeer et al. (2006) have shown that the chimeric PI-3P sensor YFP-2xFYVE is rapidly released from BY-2 endosomes upon wortmannin addition.

There are still some unsolved problems with wortmannin. First, where does the extra membrane come from to allow the PVC to swell, and second, how specific are the effects of wortmannin in the plant cell? The data of Emans et al. (2002) and more recently Reichardt et al. (2007), on the uptake of the styryl dyes FM1-43 and FM4-64 suggest that, in addition to its inhibitory effect on retromer-dependent VSR recycling, wortmannin also perturbs the endocytic pathway upstream of the LE. In addition, as shown by Lam et al. (2007a) rice SCAMP1 localizes normally to a TGN-like structure when expressed in BY-2 cells, but upon treatment with wortmannin OsSCAMP1 was then detected in the dilated PVC. This might provide a tangible explanation for the mechanism of dilation, but has not found support in the article of Jaillais et al. (2008) who failed to record an effect of wortmannin on the distribution of the two TGN markers TLG2a-GFP and VHA-a1-GFP in Arabidopsis root cells.

CONCLUSION AND OUTLOOK

The map of the plant endomembrane system that has emerged during the past years is clearly not yet of high resolution and still has question marks and blind spots attached to it (see Fig. 3). However, when comparing it to maps for yeast or mammalian cells, it is becoming clear that the plant and the yeast endosomal systems seem to be organized similarly and in a way that differs from the mammalian model. Whereas Rab5 labels the mammalian EE (Zerial and McBride, 2001), its yeast homolog Vps21 as well as Arabidopsis Rab-F2s are found on LE, and both are involved in trafficking of vacuolar proteins (Horazdovsky et al., 1994; Haas et al., 2007). Similarly, PI-3P and SNX1 are important molecules for mammalian early endosomal function but seem to be characteristic of LE in both yeast and plants (Gillooly et al., 2000; Zhong et al., 2005; Vermeer et al., 2006). Moreover, a specialized isoform of the V-ATPase subunit-a is found in the TGN/EE of plants (Dettmer et al., 2006) and the late Golgi/early endosomal compartment of yeast (Manolson et al., 1994; Kawasaki-Nishi et al., 2001). In mammalian cells, receptor-ligand complexes are formed at the PM at near neutral pH and slight acidification of the EE allows the dissociation of certain ligands (Presley et al., 1997; Johnson et al., 2007), an essential prerequisite for recycling. In contrast, ligand binding in yeast and plants occurs in an acidic extracellular space and pH-dependent dissociation would therefore require much greater acidification of the early/sorting endosome. Although it remains to be determined if pH-dependent dissociation even occurs, the high density of V-ATPase complexes found in the Arabidopsis TGN/EE might reflect this demand, but this needs to be determined experimentally.

Despite these striking similarities with yeast there are also clear differences: Clathrin is dispensable in yeast (Payne and Schekman, 1985) and yeast lacks a specialized RE, whereas GNOM clearly demonstrates that such a compartment, although yet to be identified at the structural level, exists in plant cells (Geldner et al., 2003). Moreover, endocytosis of PIN1 and FM4-64 are clathrin dependent (Dhonukshe et al., 2007), suggesting that clathrin-dependent endocytosis accounts for most, if not all, of the internalization at the plant PM.

With the stations along the endocytic route becoming more clearly defined, the next important steps will be to work out their connections and to monitor the flow of different cargo molecules through the system. Keeping in mind that we are trying to describe a highly dynamic system, it will be of great importance to determine to which extend the different endosomes are connected via vesicle trafficking or if they are derived from each other by maturation. 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. We will also need highly fluorescent cargo molecules including recycling and down-regulated receptors and transporters that will allow one to trace their fates using high-resolution live cell imaging and electron microscopy. With such improved tools on the horizon, plant endocytosis is finally ready to leave its difficult childhood behind.

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