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Trafficking routes to the Plant Vacuole: connecting alternative and classical pathways.

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running title: *Alternative pathways to the vacuole.*

Highlights: Recent discoveries have found uncharacterised trafficking pathways to the plant vacuole. Soon, these alternative routes might become classical routes. This review aims at summarising our general understanding in this field.

Abstract

Due to the numerous roles plant vacuoles play in cell homeostasis, detoxification and protein storage, the trafficking pathways to this organelle have been extensively studied. Recent evidence however suggests that our vision of transport to the vacuole is not as simple as previously imagined. Alternative routes have been identified and are being characterised. Intricate interconnections between routes seem to occur in various cases, complicating the interpretation of data. In this review, we aim to summarise the published evidence and linking the emerging data with previous findings. We give the current state of information on alternative and classical trafficking routes to the Plant vacuole.

Key words: Alternative routes, Membrane protein, Multivesicular bodies, Protein sorting, Trafficking pathways, Vacuole, VSR,

Abbreviations: VSR (Vacuolar Sorting Receptor), MVB (MultiVesicular Bodies), PVC (PreVacuolar Compartment), LPVC (Late PreVacuolar Compartment), ER (endoplasmic reticulum), AP (Adaptor Complex), PSV (Protein Storage Vacuole), DVs (Dense Vesicles), PAC (Precursor Accumulating Vesicles), PB (Protein Bodies).

Introduction:

Describing vacuolar sorting mechanisms is a difficult task especially because vacuolar organization is far from well understood. The topic is often associated with the trafficking of vacuolar sorting receptors but some vacuolar proteins, in particular membrane proteins, escape this association. This review summarizes recent advances in vacuolar sorting characterization, highlighting the knowledge gaps.

1. CLASSICAL ROUTE TO THE VACUOLE: VSR as a model

1a. Vacuolar sorting receptors.

Transport of soluble vacuolar cargo to the vacuole is commonly described using the conventional pathway involving Vacuolar Sorting Receptor (VSR) (De Marcos Lousa *et al.*, 2012). Plant VSRs are type I transmembrane proteins which appear to have evolved independently from the widespread sortilin/vps10 class of receptors and the mammalian mannose 6 phosphate receptor family (de Marcos Lousa and Denecke, 2016). VSRs are typically found in plants including green algae as well as the supergroup of Stramenopiles, Alveolates and Rhizaria (SAR). Similar to the other two classes, VSRs bind and release cargo with their large luminal domains whilst their cytosolic C-terminus controls the trafficking of the receptor to and from prevacuoles.

Although the C-terminal part of VSRs has been well studied and motifs important for trafficking of the receptor have been identified (see below), the characteristics of N-terminal luminal domain of VSRs are still unclear. The interaction between vacuolar cargo and the receptor is affected by calcium concentrations, oxidising conditions and pH *in vitro*. Experimental evidence to support their relevance *in vivo* however remains to be established. The luminal domain of VSRs has been shown to bind to a specific NPIR motif found in the N-

terminus of many soluble vacuolar cargo. In addition to this NPIR motif, different sorting signals, such as C-terminal and internal motifs, were also found in various other types of soluble cargo. Therefore, the VSR luminal domain can recognise a range of vacuolar sorting signals, all of which have yet to be identified. To understand the mechanism of cargo-receptor interaction, elucidation of the crystal structure of the luminal domain of VSR1 was attempted after overexpression in bacteria (Luo et al., 2014). It revealed a folding mechanism between two sub-domains, triggered by ligand binding. Despite identifying some important amino acids, only a portion of the luminal domain has been crystallised, unfortunately missing the real binding site of the NPIR motif. Therefore, further investigation is still needed to understand the real mechanism allowing the VSR luminal domain to bind a diversity of vacuolar cargoes. One possibility would be that various vacuolar cargo are transported by different isoforms of VSRs. Indeed, seven isoforms have been identified in *A.thaliana* which have been classified in three groups. While group 1 and 2 can complement each-other in knockout mutants, group 3 appears to be different (Zouhar et al., 2010) and could possibly represent receptors for new types of vacuolar cargo.

Efforts to understand the trafficking of vacuolar receptors, rather than the binding to the cargo, have been much more conclusive. Many studies have led to the identification of two major signals in the cytosolic tail of vacuolar sorting receptors the YxxΦ motif and the dileucine motif (Braulke and Bonifacino, 2009; de Marcos Lousa and Denecke, 2016). Surprisingly, despite the structural differences between plant, yeast and mammalian homologous lysosomal/vacuolar receptors, these two motifs are well conserved across these organisms (de Marcos Lousa et al., 2016). The YxxΦ motif is mostly based on the presence of a tyrosine residue and a hydrophobic residue in position +3 of the tyrosine, both of which are essential for anterograde trafficking of lysosomal/vacuolar receptors to the prevacuolar compartment (PVC) (daSilva *et al.*, 2006). However, in plant VSRs, the hydrophobic residue (in this case a leucine in the YMPL motif of plant receptors) has a dominant role in retrograde trafficking to rescue VSRs from degradation in the vacuole (Foresti *et al.*, 2010). Indeed, a leucine mutant of VSR2 is still able to traffic to the late compartments (anterograde trafficking), but is unable to recycle and thus reaches a more distal compartment (termed late prevacuole or LPVC) as well as the central vacuole itself (Foresti *et al.*, 2010). Although this does not exclude a minor role in anterograde trafficking, it demonstrates that anterograde and retrograde trafficking are controlled by different machineries that are connected by at least one motif YxxΦ.

Until now, the function of the N-terminal (luminal) and C-terminal (tail) domains of VSRs have been studied independently and the signal transduction between the two domains remains elusive. It is not clear how the binding of cargo to the N-terminal domain of VSRs triggers the trafficking cycle of VSRs from early to late compartments. Dimerisation of the receptors has been proposed to be a prerequisite for the trafficking of VSRs but not for binding to the cargo (Kim *et al.*, 2010). This suggests that cargo binding might lead to a dimerization of the receptor which would be the signal for VSRs to start trafficking. Although this represents an attractive hypothesis, evidence shows that fluorescent fusions devoid of a luminal VSR domain can still complete a full VSR transport cycle, indicating that the tail may exhibit an autonomous function in trafficking. Therefore, it is still unclear whether VSRs continuously traffic between early and late organelles, or if binding to vacuolar cargo is required prior to trafficking being initiated. Despite this uncertainty, studying the C-terminal tail of VSR has shed light on specific events of transport cycle that can now be mapped, as outlined below.

1b. Stage 1: early steps of transport to the vacuole

The classical route to the vacuole involves binding of the soluble cargo in early compartments and release in late compartments. Upon cargo release, the receptors are recycled to early compartments for a new round of binding while vacuolar cargo proceeds to the vacuole.

At early stages, VSRs are transported from the ER to the Golgi in a COPII-dependent route, as VSR trafficking can be inhibited by a GTP-restricted mutant of Sar1 (H71L) or overexpression of the guanine nucleotide exchange factor Sec12, both indicative of a canonical COPII-mediated ER to Golgi transport (Gershlick *et al.*, 2014).

Despite this simple model being established and accepted, the compartment where VSRs bind their cargo is still controversial. Due to the accumulation of proteins in ER protein bodies, it was first assumed that receptors start their journey by binding their cargo in the ER. In agreement with this hypothesis, a trapped VSR luminal domain fused to an ER retention signal can also retain vacuolar cargo in the ER (Watanabe *et al.*, 2004; daSilva *et al.*, 2005; Niemes *et al.*, 2010*a*). However, since ER retention signals are thought to be retrieval signals capturing proteins from the Golgi cisternae (Pelham *et al.*, 1988), VSR-ligand binding could either occur in the ER, the Golgi or in both compartments. Recent experiments suggest that sorting occurs mostly in the Golgi cisternae (Gershlick *et al.*, 2014). When a vacuolar sorting signal and an ER retention signal were placed on two different cargo molecules, the fusion proteins were

partially found in the ER and in the vacuole and the function of both signals was compromised. This can only be explained by the presence of mutually exclusive binding to either vacuolar and ER receptors (VSR or ERD2) in the same compartment. Most likely, the cis-Golgi would be a good candidate as HDEL-cargo can also be detected in this compartment in electron microscopy (Phillipson *et al.*, 2001). However the results do not rule out that low affinity ligand-VSR binding might be happening in the ER lumen (Künzl *et al.*, 2016). A recent report showing that glycosylation of the VSR luminal domain is crucial for cargo-receptor interaction (Shen *et al.*, 2014) adds to the growing list of conditions that affect ligand-binding, but it is unclear how glycans contribute to the binding pocket of the receptor.

1c. Stage 2: Trafficking from post-golgi organelles.

There is currently great uncertainty and disagreement in the field about how VSRs proceed after ligand-binding. Results obtained from VSR sorting mutants strongly suggest that ligand-release takes place at the PVC, from which receptors recycle whilst cargo moves on to the LPVC which is a mature version of PVC depleted for VSRs (Foresti *et al.*, 2010). However, the route taken to and from the PVC is subject to debate. The involvement of the TGN (*trans*-Golgi network) and the PVC in vacuolar sorting have been discussed at various levels and two models have been proposed (discussed in Kang and Hwang, 2014).

One model suggests that VSRs are selectively recruited at the TGN via AP-1 and AP-4 complexes and this would depend on the presence of the YxxΦ motif. This is supported by various evidence including *ap-1* and *ap-4* knockouts affecting vacuolar sorting as well as the VSR tyrosine mutant (in the YxxΦ motif) being unable to traffic in the TGN (Foresti *et al.*, 2010). In addition, direct interaction between VSR tails and both AP-1 and AP-4 mu subunits has been shown (Oliviusson *et al.*, 2006; Gershlick *et al.*, 2014; Nishimura *et al.*, 2016). Therefore, the first model suggests that VSR molecules are transported from the TGN to the PVC via clathrin coated vesicles using AP-1/AP-4 adaptor complexes (Fuji et al., 2016). In the PVC, VSR and cargo dissociate and VSR recycle to earlier compartments via the retromer complex. Further supporting this model, Vps29 recycling mutants accumulate VSRs in the PVC (Kang *et al.*, 2012). The recycling stages of VSRs appear to be limiting steps of the trafficking cycle, as VSRs accumulate in the PVC at steady state (daSilva *et al.*, 2005; Oliviusson *et al.*, 2006; Shen *et al.*, 2014).

The alternative model proposes that VSRs steady state is found at the TGN instead of the PVC (Niemes *et al.*, 2010*b*). In this model, only the cargo proceeds to post TGN organelles whilst the receptor is recycled at the TGN (Niemes *et al.*, 2010*b*; Künzl *et al.*, 2016). This cargo transport is mediated by maturation of the TGN into the PVC and therefore excludes active transport of vacuolar proteins (both cargo and receptor) from the TGN. To support this model, retromer components such as sorting nexins were found to be located at the TGN instead of the PVC in tobacco protoplasts (Niemes et al., 2010; Scheuring et al., 2011; Stierhof et al., 2013). Additionally, using FRET-FLIM experiments with nanobody epitope interaction, VSRs were found to bind the cargo only in the ER and Golgi, but not in post-Golgi organelles (Künzl et al., 2016). Despite its attractiveness, the model cannot explain the segregation of secretory bulk flow of soluble proteins from that of soluble vacuolar proteins (Dorel *et al.*, 1989; Denecke *et al.*, 1990), and neither does it give a role for AP complexes in vacuolar trafficking. Finally, the effect of selective VSR tail mutants in the YxxΦ motif can also not be explained using this model.

Discrepancy between these two models could reflect the use of various models (knockout in *A.thaliana* vs overexpression in Tobacco protoplast…) but nevertheless requires further investigation to establish the so-called vacuolar trafficking pathway.

1d. Stage 3: fusion with the vacuole : LPVC and vacuolinos and other small vacuoles.

Whether VSRs reach the PVC or not (model I or II respectively), vacuolar cargo do reach the prevacuolar compartment and are delivered to the vacuole by fusion with this compartment. The mechanism of fusion involves a cascade of Rab5 and Rab7 GTPases (Cui *et al.*, 2014; Singh *et al.*, 2014). Interestingly, growing evidence shows that fluorescent vacuolar cargo accumulates in discrete punctate structures prior to fusion with the vacuole. Their visualisation can be enhanced using a Rab7NI mutant, demonstrating that fusion of these structures to the vacuole is dependent on an active Rab7GTPase (Bottanelli *et al.*, 2012). In normal conditions, comparable structures have been seen upon expression of Aleurain-GFP marker in *A.thaliana* roots (Fluckiger *et al.*, 2003; Jaillais and Gaude, 2007; Gendre *et al.*, 2011)*, Arabidospis* protoplasts (Miao *et al.*, 2008) as well as tobacco protoplasts (Scheuring *et al.*, 2012). Initially, these structures were described as colocalising with markers of the PVC/MVBs such as VSR2 and SNX1 and sensitive to wortmanin and overexpression of Ara7QL (Jaillais *et al.*, 2007; Miao *et al.*, 2008; Jia *et al.*, 2013). However, by refining the analysis using weak expression of similar markers, these structures were found to label the

LPVC (Foresti *et al.*, 2010). In contrast to the classical PVC that is enriched in VSRs (schematic 1), the LPVC is depleted in VSRs but enriched in Rab5 small GTPases. Weak expression of organelle markers are essential to visualise the LPVC as their overexpression lead to organelle fusion events similar to those observed upon treatment with wortmannin or expression of constitutively active mutant Ara7QL (Bottanelli *et al.*, 2012; Jia *et al.*, 2013). Most studies however use strong promoters to express and visualise these organelle markers, which results in mislabelling organelles. Therefore, establishing lines that express PVC and LPVC markers at low levels will allow distinction between these two organelles (PVC and LPVCs) in current models, leading to meaningful conclusions on vacuolar sorting pathways.

The existence of this new organelle is not trivial as it sheds light on the late steps of vacuolar delivery. The LPVC is formed by selective retrieval of VSRs from the PVC and accumulation of cargo molecules. Consistent with this hypothesis, a VSR recycling defective mutant in the YxxΦ motif of VSRs (L615A) is now found to accumulate in the LPVC instead of the PVC (Foresti *et al.*, 2010). Interestingly, the presence of PVC and LPVC organelles is comparable to early endosome (EE) and late endosomes (LE) described for the mammalian lysosomal field and would be consistent with a cascade of Rab5-Rab7 progressing from the PVC to the LPVC before fusing to the vacuole. This model is not without challenges as it is unclear how membranes would be recycled from the central vacuole and how new PVCs would be replenished after they mature into LPVCs. The mechanisms of vacuolar fusion seem to be more complex than expected and the role of the LPVC as an intermediate organelle between the PVC and the vacuole needs to be further evaluated.

Other types of punctate structures accumulating vacuolar cargo have been described in petunia petal epidermis. These structures accumulating Aleu-GFP are completely separated from the anthocyanins-rich central vacuole and do not share markers with the tonoplast (Verweij *et al.*, 2008; Faraco *et al.*, 2017). Due to their resemblance with small vacuoles, the name “vacuolinos” has been proposed (Faraco *et al.*, 2017). Further investigation has shown that the trafficking of tonoplast localised PH1-PH5 pumps transit through the vacuolinos in a SNARE dependent pathway (Faraco *et al.*, 2014; 2017). In contrast, before reaching the vacuole, a-, d- and g- TIPs from Arabidopsis or Petunia accumulate in similar structures which are distinct from vacuolinos and CV. These observations, together with additional data using a collection of mutants affecting biogenesis and fusion of vacuolinos, have led the authors to propose the existence of multiple vacuoles in a single cell (Faraco et al., 2017). This hypothesis

is supported by the identification of individual markers for each type of vacuole (vacuolinos, CV and PSV). Although vacuolinos are confined to Petunia petal epidermis, the co-existence of different types of vacuoles in a single cell has been speculated by many and represents a future path worth investigating.

2. GOLGI to VACUOLE TRAFFICKING PATHWAY (Golgi-DEPENDENT ROUTE).

The classical view described above, with VSR as a model, is still considered as the only or major route for delivering vacuolar proteins. But this has been somewhat challenged by a growing number of reports describing alternative routes to the vacuole. In the following paragraphs, we will give an overview of various alternative pathways that are starting to emerge for vacuolar transport routes.

2a. The case of AP-3

AP-3 is an adaptor complex similar to AP-1 and AP-4. In yeast, the AP-3 dependent pathway to the vacuole was first described 20 years ago. It is known as the ALP (alkaline phosphatase pathway) pathway, as opposed to the classical CPY (carboxypeptidase) pathway (Valls *et al.*, 1990; van Voorst *et al.*, 1996; Cowles *et al.*, 1997; Jørgensen *et al.*, 1999). Despite using similar proteins, the two pathways have been shown to be independent as the inhibition of the ALP pathway affects ALP transport without affecting the transport of CPY (Cowles *et al.*, 1997). In mammalian cells, the AP-3 pathway has also been very well described and is involved in transport to the lysosome or related organelles, such as the melanosome and platelet dense granules or lytic granules. Mutation in the AP-3 complex leads to relocation of lysosomal proteins and genetic disorder (Dell'Angelica *et al.*, 1997; Nakatsu *et al.*, 2004; Assoum *et al.*, 2016).

In both yeast and mammals, AP-3 has an established role in transport of specific vacuolar/lysosomal proteins directly from the Golgi, therefore bypassing the post-golgi organelles (Rous *et al.*, 2002; Reusch *et al.*, 2002; Bowers and Stevens, 2005; Feraru *et al.*, 2010). Yet, in plants, a role for AP-3 in vacuolar sorting is only starting to emerge.

In Arabidopsis, the localisation of AP-3 is still unclear. AP-3β subunit was found to be mostly cytosolic with discrete punctate structures, rarely localising with endomembranes (Feraru *et al.*, 2010). Although these structures have not been identified, Lee et al (2007) have

demonstrated that AP-3 interacts with two TGN proteins, VTI12 and EPSIN2. Therefore it would appear that AP-3 could be localising partially at the TGN, similarly to yeast and mammals where AP-3 and AP-1 colocalise on the same membranes but on distinct regions of *trans*-Golgi network or recycling endosomes (Cowles *et al.*, 1997; Odorizzi *et al.*, 1998; Peden *et al.*, 2004). Likewise, we would expect AP-3 to localise and act at the level of the TGN in plants, similarly to AP-1 and AP-4. Yet, this hypothesis is questioned by the finding that in *AP-3β* mutants, SUC4 (a sucrose transporter) was arrested in the Golgi rather than in the TGN (Wolfenstetter *et al.*, 2012), suggesting that in plants, AP-3 could also be involved in a direct route from the Golgi to the Vacuole.

Despite this uncertainty on AP-3 localisation in plants, the role of AP-3 in vacuolar transport is established as an alternative pathway to the classical AP-1/AP-4 pathway. Various evidence suggests that transported substrates are different for these two routes.

While AP-3 does not seem to interact with VSR2, as shown with a yeast two hybrid experiment (Gershlick *et al.*, 2014), *AP-3β* knock-out mutant mistarget membrane proteins such as vacuolar invertase, PIN1, PIN2, BRI1, plasma membrane aquaporin and ATPases or membrane proteins essential for lytic vacuole biogenesis (Feraru *et al.*, 2010; Zwiewka *et al.*, 2011; Pertl-Obermeyer *et al.*, 2016). While storage protein delivery is not affected in this mutant' seeds, the transition from PSV to lytic vacuole was compromised with enlargement of RabF2b/Ara7 positive compartments (Feraru *et al.*, 2010). Therefore, it appears that AP-3 could act in parallel to the classical pathway with a double function: the rapid delivery of essential proteins for the biogenesis of the vacuole (mostly membrane proteins) and the recycling of plasma membrane proteins such as PINs. An interplay between the AP-1/AP-4 route and the AP-3 route cannot be excluded, as it has already been shown in mammalian cells (Hirst *et al.*, 2012). In agreement with this, signals such as YxxΦ motif and dileucine motifs involved in the classical pathway, seem to also be recognised by the AP-3 pathway in mammals. Although this has not yet been tested in plants yet, similar evidence could explain an overlap between the two pathways in plants. Nevertheless, other unidentified signals should be present on cargo proteins to selectively recruit membrane protein cargos to AP-3 subdomains, separate from the AP-1/AP-4 subdomains. Finally, a putative receptor for unconventional soluble vacuolar cargo using the AP-3 pathway still awaits identification.

2b. Transport from Golgi to the vacuole: Dense Vesicles

Several pathways connecting the ER or even the Golgi directly to the vacuole seem to usually share a common trait: they all use electron dense vesicles. This characteristic has given its name to Dense Vesicles (DVs) as seen by electron microscopy in pea cotyledons (Hohl *et al.*, 1996), and this was later confirmed with density gradients (Hinz *et al.*, 1999*a*). Dense vesicles are slightly bigger than clathrin coated vesicles (average of 130 nm compared to around 60 nm (Dhonukshe *et al.*, 2007) and appear to mature from the side of the cis-Golgi to the trans-Golgi where they bud off. They accumulate specific storage proteins, such as prolamins and globulins (vicilin and legumin), and sucrose binding protein (SBP) (Craig *et al.*, 1979; Hohl *et al.*, 1996; Wenzel *et al.*, 2005; Robinson *et al.*, 2005). In addition, they seem to be devoid of BP80/VSRs but contain RMRs, putative vacuolar receptors (Hinz *et al.*, 1999*a*, 2007; Hillmer *et al.*, 2001). The mechanism by which DVs fuse to the PSV (protein storage vacuole) remains elusive (Vitale and Raikhel, 1999). It was suggested that DVs fuse directly with the PSV (Herman and Larkins, 1999*a*; Liu *et al.*, 2013). However DVs in rice endosperm were found to fuse to different types of prevacuolar compartments before fusing with the PSVs (Shen *et al.*, 2011). These prevacuolar compartments contain RMRs due to the fusion with DVs, but are not labelled with VSRs. Moreover, as in the classical pathway, fusion of DVs with PSVs depend on Rab5 proteins (Fukuda *et al.*, 2013; Liu *et al.*, 2013). In agreement with this, Wang and collaborators have reported that, in late stages of bean cotyledon development, globulin 8s is found in DVs and also in novel forms of partitioned MVBs with one side packed with storage proteins and the other packed with internal vesicles (Wang *et al.*, 2012). Again, these partitioned MVBs were labelled with Rha1, but the authors have not investigated the role of this protein.

In the light of this evidence, DVs represent an alternative pathway for proteins destined for the vacuole. Although the fate of DVs still requires further investigation, the similarities between DVs and LPVCs are intriguing. Indeed, the LPVC was defined in tobacco transgenics as enriched in Soluble vacuolar proteins, depleted in vacuolar receptors and labelled by Rab5 GTPases, characteristics similar to DVs (Foresti *et al.*, 2010). Growing evidence also show an increasing interconnection between MVBs and DVs in rice (Shen *et al.*, 2011; Liu *et al.*, 2013). Hence, the link between LPVC and DVs could represent a new point of convergence leading to the merge of the lytic and storage protein trafficking pathways before fusion to the vacuole (Figure 1). Various reports give indication in favour of this statement. Indeed, while DVs have been reported to pack storage proteins at level of the Cis-Golgi, they were reported as partially coated with clathrin after progressing to the trans-Golgi network (von Lüpke *et al.*, 2008).

Additionally, Aleurain, which predominantly binds to VSR and traffics through the classical pathway, could also be detected in DVs (Hinz *et al.*, 2007). All this evidence suggests that pathways to the vacuole are flexible and interconnected and views on a strict segregation between lytic and storage vacuolar trafficking pathways might have to be reconsidered (Jiang *et al.,* 2002).

3. ER - VACUOLE TRAFFICKING PATHWAY (Golgi-independent route)

Both AP-3 and dense vesicles routes are described as Golgi-dependent pathways to the vacuole. In parallel, more evidence is pointing to the existence of Golgi-independent routes for unconventional vacuolar cargo.

3a. Direct ER to Vacuole trafficking

Several direct trafficking events between the ER and the vacuoles have been studied in detail but they are generally not considered as part of a unique molecular mechanism.

One of the mechanisms that is involved in the direct transport of storage protein precursors from the ER to PSV, i.e. by-passing the Golgi, involves precursor-accumulating (PAC) vesicles. PAC vesicles have been described during the maturation of *C. maxima* seeds but were also described in other plants such as *O. sativa* (Hara-Nishimura *et al.*, 1998). In this organism, PAC vesicles were reported to contain storage proteins, such as glutelin and α-globulin, as well as an ER-resident protein such as BIP (Takahashi et al., 2005; Pelham, 1990; Vitale and Denecke, 1999). For this reason, and their larger size compared to Golgi-derived dense vesicles, PAC vesicles were suggested to derive directly from the ER and transport proteins directly to the vacuole directly. The presence of VSR molecules and other hyperglycosylated proteins in PAC vesicles, however, has questioned this hypothesis, indicating that they might not by-pass the Golgi where glycosylations occur (Shimada *et al.*, 2002). In addition, the Golgi-mediated vacuolar transport of a BiP deletion mutant lacking the HDEL motif suggests that the presence of BiP in PAC vesicles is not sufficient evidence to prove a direct trafficking from ER membranes (Pimpl et al., 2006). The origin of PAC vesicles and the involvement of the Golgi in this pathway is therefore still unclear.

Nevertheless, other types of ER to vacuole transport have been reported. One of them involves protein bodies (PBs). PBs differ in shape and size from the electron-dense core PAC vesicles. Yet, their content is very similar as PBs have been reported to accumulate storage proteins and ER proteins. Two types of PBs have been described. In *O. sativa,* glutelin is stored

in PB type II (PB-II) and transported to the vacuole, while prolamin is deposited in PB type I (PB-I), a sub-domain of the ER. Calreticulin, a protein with an ER-retention signal at the C-terminus, (Pelham, 1990; Vitale and Denecke, 1999) has been found in both PB types with a small portion also reported in PSVs in rice callus and mesophyll cells (Torres *et al.*, 2001). This finding has prompted the authors to suggest an alternative pathway from the ER directly to the vacuole via PBs. However in tobacco leaf protoplasts, calreticulin has been shown to follow a classical COPI and COPII route (Phillipson *et al.*, 2001; Pimpl *et al.*, 2006). These apparent discrepancies could be explained in terms of cell maturation: although in early stages (seed development) calreticulin could be accumulating in PB and transported to the vacuole in a Golgi-independent pathway, such protein would follow a more classical route in established and mature cells. It was recently hypothesized that in leaves, PBs do not detach from the ER but rather dynamically interact with the ER to exchange proteins (Saberianfar *et al.*, 2016). Another factor could be cell type: while glutelin could be sorted to PSV by-passing Golgi cisternae in rice (Torres *et al.*, 2001), a Golgi-dependent sorting was described in castor beans (Jolliffe *et al.*, 2004) and pea (Hinz *et al.*, 1999*b*). Such differences may reflect the high flexibility of the alternative trafficking pathways from the ER to the vacuole, adjusting the routes with the needs of cell types.

3b. Autophagic related processes

Autophagy is the main process for organelle degradation in most eukaryotes and hence plays a major role in cell homeostasis (Michaeli *et al.*, 2016/2; Liu and Bassham, 2012). In addition, autophagy related mechanisms appear to also be involved in a number of trafficking events, such as the direct ER-vacuole trafficking (Robinson *et al.*, 1998; Herman and Larkins, 1999*a*; Michaeli *et al.*, 2014). Indeed, PBs have been reported to become surrounded by autophagic membrane in the cytosol after their release from the ER (Herman and Larkins 1999). This autophagosome then fuses with the tonoplast, releasing PBs in the vacuole. Early evidence of the presence of PBs engulfed in vacuoles comes from electron microscopy observations of storage protein delivery by the ERvt pathway (Levanony *et al.*, 1992; Coleman *et al.*, 1996). Interestingly, prolamins can be delivered to vacuole via the Golgi-dependent pathway in early stages of development, and then switch to autophagy mediated delivery in later stages when accumulation in PBs is increased (Levanony *et al.*, 1992). This again shows the flexibility and interconnection between pathways depending on the cell status.

Autophagy has also been proposed for the delivery of other types of cargos and vesicles originating from the ER such as rubber and anthocyanins (Pourcel *et al.*, 2010/1; Herman and Schmidt, 2004; Chanoca *et al.*, 2015). Autophagy of ER-derived compartments can also be induced by stress or overexpression of proteins (Bassham *et al.*, 2006). In stress induced events, Atg8, the main protein involved in classical autophagy processes, has been shown to be recruited to ER membrane and to the vacuole (Liu *et al.*, 2012). Despite this observation, the role of the main autophagy regulators such as Atg8 in the process of ER-vacuole trafficking is questionable as knock out mutants of Atg proteins appear not to disturb seed formation in Arabidopsis (Liu and Bassham, 2012). In addition, Atg8 is not present with prolamin-containing PBs engulfed in maize seeds vacuoles (Reyes *et al.*, 2011). Therefore, it seems that despite the existence of autophagy like processes involved in vacuolar trafficking, the main autophagy regulators do not seem to play a role. New regulators of this pathway hence await identification.

3c. Anthocyanins trafficking and other metabolites

Other interesting observations derived from staining of neutral red-stained bodies (NRSBs) include large bodies found inside plant vacuoles that are stained by the supravital dye neutral red. In anthocyanin accumulating cells, NRSBs appear much bigger than in other cells, suggesting a relationship with AVIs (anthocyanin vesicular inclusions), other anthocyanin accumulating bodies (Pourcel *et al.*, 2010). Anthocyanins have been proposed to be synthesized on the cytosolic side of the ER and further transported to the vacuole, which then confers typical petal colors (Saslowsky and Winkel-Shirley, 2001; Winkel-Shirley, 2002). However, the transport of anthocyanins to the vacuole is not yet understood. TT19, an Arabidopsis glutathione S-transferase ligand transporter, seem to play a specific role in the transport of anthocyanins to vacuoles. Indeed, the inhibition of TT19 lowers the amount of total anthocyanins (Poustka *et al.*, 2007; Sun *et al*., 2012). Therefore it has been postulated that TT19 induces the solubility of cytoplasmic anthocyanins, which otherwise aggregate and are engulfed by microautophagy in the vacuole (Chanoca *et al.*, 2015). This mechanism appears to be Atg-dependent as *Atg* mutants are defective in the accumulation of anthocyanins, with fewer numbers NRSBs and AVIs reported (Pourcel *et al.*, 2010). NRSBs and AVIs disappear in *Exo70B1-2* double mutants demonstrating that Exo70B1-2 is also implicated in the transport of anthocyanins from the ER to the vacuole (Kulich *et al.*, 2013) (Kulich and Žárský, 2014). NRSBs and AVIs are nevertheless not the only mechanism of anthocyanin transport to the vacuole. In *Vitis vinifera* and *Zea mays,* anthocyanins accumulate initially in small tubular or

vesicular bodies that become larger through vesicular fusion or vacuolar autophagy (Irani and Grotewold, 2005; Zhang *et al.*, 2006; Conn *et al.*, 2010; Gomez *et al.*, 2011). These bodies and tubes are composed of ER membranes or ER-derived vesicles and are localized to tonoplast invaginations deep inside the vacuole (Poustka *et al.*, 2007; Gomez *et al.*, 2011). This suggests that they may be the plant version of autophagic tubes previously described in yeast (Müller *et al.*, 2000).

Apart from anthocyanins, other secondary metabolites and hormones are also reported to be directly sorted to the vacuole from the ER. This is the case for compounds such as phenylpropanoid/flavonoids and cyanogenic glucosides (Ralston *et al.*, 2005), alkaloids (sanguinarine) and indole alkaloids (vinblastine) (Alcantara *et al.*, 2005), phytohormones, like salicylic acid (Yoshimoto *et al.*, 2009), abscisic acid glucosyl ester (ABA-GE) and its activator (AtBG1, a β-glucosidase (Lee *et al.*, 2006; Burla *et al.*, 2013), and auxin (Kulich and Žárský, 2014). However, these pathways are difficult to observe as they may involve cooperation of multiple transport mechanisms as suggested for flavonoids (Zhao, 2015). Membrane transporters, glutathione S-transferase conjugation and vesicle trafficking may be cooperating for the vacuolar sequestration of flavonoids. In fact, the only known protein involved in the membrane fusion of flavonoid-containing vesicles with vacuoles is the Golgi-localized membrane protein GFS9 (Ichino *et al.*, 2014; Zhao, 2015). Therefore, more work is required to understand how these secondary metabolites are transported to the vacuole (Kulich and Žárský, 2014).

3d. Other uncharacterized vacuolar pathways.

In addition to the above described unconventional trafficking to the vacuole, many more routes have been reported but still require clarification or remain controversial. In the following paragraph, we give a summary of the findings to date.

The Chitinase A case

In contrast to Aleurain which contains a typical N-terminal NPIR motif, Chitinase is an enzyme that carries a C-terminal vacuolar sorting determinant (CtVSD). This determinant does not

have a consensus sequence, but relies on 4-7 amino acids with hydrophobic characteristics. Chimeric proteins fused with this C-terminal signal are transported to the vacuole showing that it acts as a VSD. Other proteins such as barley lectin and phaseolin also contain a CtVSD. It has been shown that VSR affinity for this type of protein is very low, and instead another putative vacuolar receptor (RMR: Receptor Membrane RING-H2) has been proposed (Ahmed *et al.*, 2000; Park *et al.*, 2007). A fusion protein GFPChi is in part retained in the ER and also found in uncharacterised punctate structures different from those labelled by Aleu-RFP in tobacco (Stigliano *et al.*, 2013) and tomato (Di Sansebastiano *et al.*, 2014) protoplasts.

Altogether these observations suggest that Chitinase and Aleurain might use different routes to the vacuole (Stigliano *et al.*, 2013). Various reports have shown differences in Chitinase and Aleurain sorting: engineered glycosylated GFPChi appears to be sensitive to EndoH treatment whereas the glycosylated AleuGFPgl133 is not (Stigliano *et al.*, 2013). SNAREs appear to control vacuolar sorting and modulate targeting of these markers differentially (Uemura end Ueda 2014). Indeed, VTI12 and SYP51 has been reported to affect more specifically GFPChi trafficking, while VTI11 and SYP52 seem to be involved in AleuGFP transport (Sanmartin et al., 2007; De Benedictis et al., 2013). Finally, Sar1HL, an inhibitor of COPII trafficking, seem to increase the fluorescence of Chitinase in the vacuole while Aleurain trafficking is prevented. Taken together this evidence suggests that Aleurain and Chitinase might traffic through different routes. Nevertheless, other reports have shown that RFPChi transport is still dependent from components of the classical route such as Rab11, Rha1, Ara6 and Rab7 (Bottanelli *et al.*, 2011). RFPChi was found to strongly label the ER, with only weak labelling of the central vacuole (Bottanelli et al, 2011). Although these observations might only be the result of differential fluorescent fusion (GFP vs RFP), a plausible explanation is that Chitinase could be trafficking through various routes depending on the cell status and vacuole identity, as suggested above (Fluckiger *et al.*, 2003). Indeed, Chitinase can also be found in dense vesicles, budding off from the Golgi, or in ER bodies in seeds and developing cotyledons respectively (G. Hinz unpublished data). Both Aleurain and Chitinase can also be found colocalising in BFA bodies or in prevacuolar compartments upon treatment with auxin and/or acetylcholine (ACh) (Stigilano et al., 2013; Di Sansebastiano *et al.*, 2014). Auxin and ACh treatments do not alter sorting pathways like BFA treatments (Stigliano *et al.*, 2013) but simply change the sorting specificity, emphasizing compartments characterized by PIN1 and PIN7 (Kleine-Vehn *et al.*, 2006; Geldner, 2009). These observations again suggest a close connection between pathways.

493
494
495
496 ***Cardosines***
497 Even if the trafficking of Chitinase may appear exceptional, an increasing number of
498 proteins may soon be reported as trafficking through alternative pathways. Cardosin A, a
499 vacuolar aspartic proteinase, is characterized by two domains: a plant specific insert (PSI)
500 domain and a C-terminal region. Both domains act as vacuolar sorting determinants (VSDs)
501 but each of them is involved in distinct routes to the vacuole (Tormakangas et al., 2001). A
502 working model for Cardosin A trafficking suggests that the C-terminus mediates a COPII-
503 dependent ER-to-Golgi pathway to the vacuole while the PSI domain mediates either a COPII-
504 dependent or COPII-independent vacuolar trafficking pathway in a non-glycosylated or
505 glycosylated form, respectively. Again, the relevance of the PSI-mediated pathway depends on
506 the type of tissue and the metabolic activity of the organs (Pereira *et al.*, 2008, 2013).
507
508 ***Membrane proteins examples***
509 Although most studies have concentrated on the sorting of storage and lytic proteins to
510 the vacuole, membrane proteins have also been studied and found to use various routes. As
511 already described above, VSRs and RMR proteins traffic via different pathways (classical or
512 DVs).
513 AtRMR1 and -2. AtRMR2 homodimers and AtRMR2/AtRMR1 heterodimers have
514 been recently shown to assemble in the ER and bind different vacuolar cargos (Occhialini *et*
515 *al.*, 2016). Two different pathways sort AtRMRs to the TGN, either a Golgi-dependent or
516 Golgi-independent pathway (Occhialini *et al.*, 2016). Comparative studies emphasizing
517 common characteristics between lysosomes and vacuoles also point out that the percentage of
518 N-glycoproteome is much higher in lysosomal/PM membrane than in the tonoplast (Pedrazzini
519 *et al.*, 2016; Pompa *et al.*, 2017). Based on this observation, the authors propose that the major
520 trafficking pathway to the tonoplast might be bypassing the Golgi apparatus.
521 Other reports on more membrane proteins again support the presence of multiple pathways to
522 the tonoplast. α-TIP, the SNARE VAMP3 and CBL6 were all found to be COPII independent
523 in tobacco epidermis, suggesting a direct ER-vacuole transport (Bottanelli *et al.*, 2011).
524 However, while α-TIP trafficking is sensitive to Rab mutants (Rha1, ara6 and Rab7), Vam3 is
525 only sensitive to Rab7 mutant and CBL6 is not affected by any of these mutants. Moreover,

additional evidence comes from the fact that BFA affects the sorting of TIP1;1, but not of TIP3;1 (α-TIP) and TIP2;1 in *A. thaliana* hypocotyls (Rivera-Serrano et al. 2012).

PIN proteins also traffic through different pathways: PIN2 (as well as AUX1) recycling appears to be BFA insensitive whereas PIN1 and 3 appear to be BFA sensitive and dependent on the GNOM pathway (Geldner *et al.*, 2003; Kleine-Vehn *et al.*, 2006; Ding *et al.*, 2011). The inhibition caused by this molecule also defines a link between the BFA-insensitive pathway and PIN2 recycling (Rivera-Serrano *et al.*, 2012). However, the BFA treatment disrupts most of the pathways exhibiting these markers (Kleine-Vehn *et al.*, 2006; Drakakaki *et al.*, 2009; Ding *et al.*, 2011).

Golgi-mediated trafficking may play a role in controlling and modifying another kind of compartment formed directly from the ER membranes, the dark-induced protein (DIP) vesicles. These compartments are characterized by the presence of DIP aquaporin (specifically α-TIP; (Neuhaus and Rogers, 1998) and RMR-like proteins, and are formed with the direct contribution of the ER and the Golgi (Jiang *et al.*, 2000, 2001). DIP vesicles are the main system for transporting crystalloid elements to PSVs (Vitale and Hinz, 2005).

A small amount of DIP aquaporin is present in the PSV tonoplast. In contrast, α- and γ-aquaporin are typically targeted to CVs and are absent from DIP vesicles (Jiang *et al.*, 2000). DIP vesicles are surrounded by a double membrane that fuses with PSVs, delivering the inner membrane that forms an independent compartment inside PSVs. DIP vesicles probably transport specific membrane proteins and hydrolytic enzymes (Isayenkov, 2014).

Clearly, more evidence is still needed to decipher interconnections between vacuolar pathways. Nevertheless, the obvious existence of alternative transport routes for these membrane proteins suggests that some classes of soluble vacuolar cargo molecules could also be using such alternative pathways. This hypothesis however does not exclude the existence of a merging point with the classical routes at various stages (Bottanelli *et al.*, 2011).

Non-vesicular ER-Vacuole transport

A Golgi-independent tonoplast biogenesis model has been proposed in which the smooth ER is involved. This ER sub-domain has a distinctive lipid composition that accumulates proteins and lipids destined for the tonoplast (Viotti *et al.*, 2013). After reaching a certain size, the smooth ER curves (Knorr *et al.*, 2012) and eventually fuses with the pre-

existing vacuolar network. When post-Golgi trafficking is blocked by BFA, provacuoles appear multi-lamellar, suggesting that a component delivered by the TGN is necessary for the separation of the provacuole from the ER or for fusion with the vacuolar network (Viotti *et al.*, 2013). The analogy with the GERL model (Golgi-associated ER from which lysosome apparently form) (Marty, 1999) first described in the late 70s (Marty, 1978) is evident. GERL models proposes that despite the formation of provacuoles in the vicinity of Golgi *trans* faces, the Golgi apparatus itself appears to be bypassed in the transport. The novelty of more recent research arises from evidence that trafficking can now be shown to fully bypass the Golgi, and merge with endocytosis and phagocytosis later.

Whilst models need to be adjusted and fine-tuned, it is now obvious that direct transport from the ER to vacuoles represent a large portion of the transport to the vacuole. The Golgi contribution in this process is still unclear, but if it occurs, it certainly differs from the classic Golgi-dependent model in ways that are slowly emerging.

Conclusions

Golgi-independent vacuolar trafficking is not exceptional but a fundamental process, which is still poorly understood, and affects the very interpretation of cell compartmentalization starting from vacuoles characterization. It is evident that their compartmental diversity is not due to a maturation process similar to that observed in Golgi cisternae. Vacuoles receive cargo molecules and membranes from multiple sources and acquire their functional specificity depending on the contribution of different donors. The ER is the most important of these donors but the plasma membrane and Golgi apparatus are also involved.

ER export has a central role in controlling the biogenesis of intermediate compartments, including endosomes. In the absence of specific pathways activated by growth, stress, starvation and/or other specific processes, post-Golgi organelles such as the TGN and the LPVC may represent hubs where trafficking events could merge. Clearly, the trafficking pathways are more complex and interconnected than previously thought. In addition, direct routes involving post-translational modifications mediating transport from the cytosol to membranes of the secretory pathway such as the tonoplast need to be explored in more depth (Batistic et al., 2012). Future studies will probably contribute to the idea that “unconventional trafficking” routes will soon become conventional.

Figure 1:

Possible routes from the Endoplasmic Reticulum (bottom) to the Vacuole (top). Depicted are multiples routes that can be adopted by various storage or lytic proteins showing classical and unconventional sorting to the vacuole.

References

Ahmed SU, Rojo E, Kovaleva V, Venkataraman S, Dombrowski JE, Matsuoka K, Raikhel NV. 2000. The plant vacuolar sorting receptor AtELP is involved in transport of NH(2)-terminal propeptide-containing vacuolar proteins in Arabidopsis thaliana. The Journal of cell biology **149**, 1335–1344.

Alcantara J, Bird DA, Franceschi VR, Facchini PJ. 2005. Sanguinarine biosynthesis is associated with the endoplasmic reticulum in cultured opium poppy cells after elicitor treatment. Plant physiology **138**, 173–183.

Assoum M, Philippe C, Isidor B, *et al.* 2016. Autosomal-Recessive Mutations in AP-3B2, Adaptor-Related Protein Complex 3 Beta 2 Subunit, Cause an Early-Onset Epileptic Encephalopathy with Optic Atrophy. American journal of human genetics **99**, 1368–1376.

Bassham DC, Laporte M, Marty F, Moriyasu Y, Ohsumi Y, Olsen LJ, Yoshimoto K. 2006. Autophagy in development and stress responses of plants. Autophagy **2**, 2–11.

Batistič O, Rehers M, Akerman A, Schlücking K, Steinhorst L, Yalovsky S, Kudla J. 2012. S-acylation-dependent association of the calcium sensor CBL2 with the vacuolar membrane is essential for proper abscisic acid responses. Cell research **22**, 1155–1168.

Bottanelli F, Foresti O, Hanton S, Denecke J. 2011. Vacuolar Transport in Tobacco Leaf Epidermis Cells Involves a Single Route for Soluble Cargo and Multiple Routes for Membrane Cargo. The Plant cell **23**, 3007–3025.

Bottanelli F, Gershlick DC, Denecke J. 2012. Evidence for sequential action of Rab5 and Rab7 GTPases in prevacuolar organelle partitioning. Traffic **13**, 338–354.

Bowers K, Stevens TH. 2005. Protein transport from the late Golgi to the vacuole in the yeast Saccharomyces cerevisiae. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1744**, 438–454.

Braulke T, Bonifacino JS. 2009. Sorting of lysosomal proteins. Biochimica et biophysica acta **1793**, 605–614.

Burla B, Pfrunder S, Nagy R, Francisco RM, Lee Y, Martinoia E. 2013. Vacuolar transport of abscisic acid glucosyl ester is mediated by ATP-binding cassette and proton-antiport mechanisms in Arabidopsis. Plant physiology **163**, 1446–1458.

Chanoca A, Kovinich N, Burkel B, Stecha S, Bohorquez-Restrepo A, Ueda T, Eliceiri KW, Grotewold E, Otegui MS. 2015. Anthocyanin Vacuolar Inclusions Form by a Microautophagy Mechanism. The Plant cell **27**, 2545–2559.

Coleman CE, Herman EM, Takasaki K, Larkins BA. 1996. The maize gamma-zein sequesters alpha-zein and stabilizes its accumulation in protein bodies of transgenic tobacco endosperm. The Plant cell **8**, 2335–2345.

Conn S, Franco C, Zhang W. 2010. Characterization of anthocyanic vacuolar inclusions in Vitis vinifera L. cell suspension cultures. Planta **231**, 1343–1360.

Cowles CR, Odorizzi G, Payne GS, Emr SD. 1997. The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. Cell **91**, 109–118.

Craig S, Goodchild DJ, Hardham AR. 1979. Structural Aspects of Protein Accumulation in Developing Pea Cotyledons. I. Qualitative and Quantitative Changes in Parenchyma Cell Vacuoles. Functional plant biology: FPB **6**, 81–98.

Cui Y, Zhao Q, Gao C, Ding Y, Zeng Y, Ueda T, Nakano A, Jiang L. 2014. Activation of the Rab7 GTPase by the MON1-CCZ1 Complex Is Essential for PVC-to-Vacuole Trafficking and Plant Growth in Arabidopsis. The Plant cell **26**, 2080–2097.

daSilva LLP, Taylor JP, Hadlington JL, Hanton SL, Snowden CJ, Fox SJ, Foresti O, Brandizzi F, Denecke J. 2005. Receptor salvage from the prevacuolar compartment is essential for efficient vacuolar protein targeting. The Plant cell **17**, 132–148.

daSilva LLP, Foresti O, Denecke J. 2006. Targeting of the plant vacuolar sorting receptor BP80 is dependent on multiple sorting signals in the cytosolic tail. The Plant cell **18**, 1477–1497.

De Benedictis M, Bleve G, Faraco M, Stigliano E, Grieco F, Piro G, Dalessandro G, Di Sansebastiano GP. 2013. AtSYP51/52 functions diverge in the post-Golgi traffic and differently affect vacuolar sorting. Mol. Plant **6**, 916–930.

Dell'Angelica EC, Ooi CE, Bonifacino JS. 1997. β3A-adaptin, a Subunit of the Adaptor-like Complex AP-3. The Journal of biological chemistry **272**, 15078–15084.

De Marcos Lousa C, Gershlick DC, Denecke J. 2012. Mechanisms and concepts paving the way towards a complete transport cycle of plant vacuolar sorting receptors. The Plant cell **24**, 1714–1732.

de Marcos Lousa C, Denecke J. 2016. Lysosomal and vacuolar sorting: not so different after all! Biochemical Society transactions **44**, 891–897.

Denecke J, Botterman J, Deblaere R. 1990. Protein secretion in plant cells can occur via a default pathway. The Plant cell **2**, 51–59.

Dhonukshe P, Aniento F, Hwang I, Robinson DG, Mravec J, Stierhof Y-D, Friml J. 2007. Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in Arabidopsis. Current

biology: CB **17**, 520–527.

Ding Z, Galván-Ampudia CS, Demarsy E, *et al.* 2011. Light-mediated polarization of the PIN3 auxin transporter for the phototropic response in Arabidopsis. Nature cell biology **13**, 447–452.

Di Sansebastiano G-P, Fornaciari S, Barozzi F, Piro G, Arru L. 2014. New insights on plant cell elongation: a role for acetylcholine. International journal of molecular sciences **15**, 4565–4582.

Dorel C, Voelker TA, Herman EM, Chrispeels MJ. 1989. Transport of proteins to the plant vacuole is not by bulk flow through the secretory system, and requires positive sorting information. The Journal of cell biology **108**, 327–337.

Drakakaki G, Robert S, Raikhel NV, Hicks GR. 2009. Chemical dissection of endosomal pathways. Plant signaling & behavior **4**, 57–62.

Faraco M, Spelt C, Bliek M, *et al.* 2014. Hyperacidification of vacuoles by the combined action of two different P-ATPases in the tonoplast determines flower color. Cell reports **6**, 32–43.

Faraco M, Li Y, Li S, Spelt C, Di Sansebastiano GP, Reale L, Ferranti F, Verweij W, Koes R, Quattrocchio FM. 2017. A Tonoplast P3B-ATPase Mediates Fusion of Two Types of Vacuoles in Petal Cells. Cell reports **19**, 2413–2422.

Feraru E, Paciorek T, Feraru MI, Zwiewka M, De Groodt R, De Rycke R, Kleine-Vehn J, Friml J. 2010. The AP-3 β adaptin mediates the biogenesis and function of lytic vacuoles in Arabidopsis. The Plant cell **22**, 2812–2824.

Fluckiger R, De Caroli M, Piro G, Dalessandro G, Neuhaus J-M, Di Sansebastiano G-P. 2003. Vacuolar system distribution in Arabidopsis tissues, visualized using GFP fusion proteins. Journal of experimental botany **54**, 1577–1584.

Foresti O, Gershlick DC, Bottanelli F, Hummel E, Hawes C, Denecke J. 2010. A recycling-defective vacuolar sorting receptor reveals an intermediate compartment situated between prevacuoles and vacuoles in tobacco. The Plant cell **22**, 3992–4008.

Fuji K, Shirakawa M, Shimono Y, Kunieda T, Fukao Y, Koumoto Y, Takahashi H, Hara-Nishimura I, Shimada T. 2016. The Adaptor Complex AP-4 Regulates Vacuolar Protein Sorting at the trans-Golgi Network by Interacting with VACUOLAR SORTING RECEPTOR1. Plant physiology **170**, 211–219.

Fukuda M, Wen L, Satoh-Cruz M, *et al.* 2013. A guanine nucleotide exchange factor for Rab5 proteins is essential for intracellular transport of the proglutelin from the Golgi apparatus to the protein storage vacuole in rice endosperm. Plant physiology **162**, 663–674.

Geldner N. 2009. Cell polarity in plants: a PARspective on PINs. Current opinion in plant biology **12**, 42–48.

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jürgens G. 2003. The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell **112**, 219–230.

Gendre D, Oh J, Boutté Y, *et al.* 2011. Conserved Arabidopsis ECHIDNA protein mediates trans–Golgi-network trafficking and cell elongation. Proceedings of the National Academy of Sciences **108**, 8048–8053.

Gershlick DC, de Marcos Lousa C, Foresti O, Lee AJ, Pereira EA, daSilva LLP, Bottanelli F, Denecke J. 2014. Golgi-dependent transport of vacuolar sorting receptors is regulated by COPII, AP-1, and AP-4 protein complexes in tobacco. The Plant cell **26**, 1308–1329.

Gomez C, Conejero G, Torregrosa L, Cheynier V, Terrier N, Ageorges A. 2011. In vivo grapevine anthocyanin transport involves vesicle-mediated trafficking and the contribution of anthoMATE transporters and GST. The Plant journal: for cell and molecular biology **67**, 960–970.

Hara-Nishimura I, Shimada T, Hatano K, Takeuchi Y, Nishimura M. 1998. Transport of storage proteins to protein storage vacuoles is mediated by large precursor-accumulating vesicles. The Plant cell **10**, 825–836.

Herman EM, Larkins BA. 1999*a*. Protein storage bodies and vacuoles. The Plant cell **11**, 601–614.

Herman E, Schmidt M. 2004. Endoplasmic reticulum to vacuole trafficking of endoplasmic reticulum bodies provides an alternate pathway for protein transfer to the vacuole. Plant physiology **136**, 3440–3446.

Hillmer S, Movafeghi A, Robinson DG, Hinz G. 2001. Vacuolar storage proteins are sorted in the cis-cisternae of the pea cotyledon Golgi apparatus. The Journal of cell biology **152**, 41–50.

Hinz G, Colanesi S, Hillmer S, Rogers JC, Robinson DG. 2007. Localization of vacuolar transport receptors and cargo proteins in the Golgi apparatus of developing Arabidopsis embryos. Traffic **8**, 1452–1464.

Hinz G, Hillmer S, Baumer M, Hohl I I. 1999*a*. Vacuolar storage proteins and the putative vacuolar sorting receptor BP-80 exit the golgi apparatus of developing pea cotyledons in different transport vesicles. The Plant cell **11**, 1509–1524.

Hinz G, Hillmer S, Bäumer M, Hohl I. 1999*b*. Vacuolar Storage Proteins and the Putative Vacuolar Sorting Receptor BP-80 Exit the Golgi Apparatus of Developing Pea Cotyledons in Different Transport Vesicles. The Plant cell **11**, 1509–1524.

Hirst J, Borner GHH, Antrobus R, Peden AA, Hodson NA, Sahlender DA, Robinson MS. 2012. Distinct and overlapping roles for AP-1 and GGAs revealed by the 'knocksideways' system. Current biology: CB **22**, 1711–1716.

Hohl I, Robinson DG, Chrispeels MJ, Hinz G. 1996. Transport of storage proteins to the vacuole is mediated by vesicles without a clathrin coat. Journal of cell science **109**, 2539–2550.

Ichino T, Fuji K, Ueda H, *et al.* 2014. GFS9/TT9 contributes to intracellular membrane trafficking and flavonoid accumulation in Arabidopsis thaliana. The Plant journal: for cell and molecular biology **80**, 410–423.

Irani NG, Grotewold E. 2005. Light-induced morphological alteration in anthocyanin-accumulating vacuoles of maize cells. BMC plant biology **5**, 7.

Isayenkov SV. 2014. Plant vacuoles: Physiological roles and mechanisms of vacuolar sorting and vesicular trafficking. Cytology and genetics **48**, 127–137.

Jaillais Y, Gaude T. 2007. Sorting out the sorting functions of endosomes in Arabidopsis. Plant signaling & behavior **2**, 556–558.

Jaillais Y, Santambrogio M, Rozier F, Fobis-Loisy I, Miège C, Gaude T. 2007. The retromer protein VPS29 links cell polarity and organ initiation in plants. Cell **130**, 1057–1070.

Jia T, Gao C, Cui Y, Wang J, Ding Y, Cai Y, Ueda T, Nakano A, Jiang L. 2013. ARA7(Q69L) expression in transgenic Arabidopsis cells induces the formation of enlarged multivesicular bodies. Journal of experimental botany **64**, 2817–2829.

Jiang L, Erickson A, Rogers J. 2002. Multivesicular bodies: a mechanism to package lytic and storage functions in one organelle? Trends in cell biology **12**, 362–367.

Jiang L, Phillips TE, Hamm CA, Drozdowicz YM, Rea PA, Maeshima M, Rogers SW, Rogers JC. 2001. The protein storage vacuole: a unique compound organelle. The Journal of cell biology **155**, 991–1002.

Jiang L, Phillips TE, Rogers SW, Rogers JC. 2000. Biogenesis of the protein storage vacuole crystalloid. The Journal of cell biology **150**, 755–770.

Jolliffe NA, Brown JC, Neumann U, Vicré M, Bachi A, Hawes C, Ceriotti A, Roberts LM,

Frigerio L. 2004. Transport of ricin and 2S albumin precursors to the storage vacuoles of Ricinus communis endosperm involves the Golgi and VSR-like receptors. The Plant journal: for cell and molecular biology **39**, 821–833.

Jørgensen MU, Emr SD, Winther JR. 1999. Ligand recognition and domain structure of Vps10p, a vacuolar protein sorting receptor in Saccharomyces cerevisiae. European journal of biochemistry / FEBS **260**, 461–469.

Kang H, Kim SY, Song K, Sohn EJ, Lee Y, Lee DW, Hara-Nishimura I, Hwang I. 2012. Trafficking of vacuolar proteins: the crucial role of Arabidopsis vacuolar protein sorting 29 in recycling vacuolar sorting receptor. The Plant cell **24**, 5058–5073.

Kang H, Hwang I. 2014. Vacuolar Sorting Receptor-Mediated Trafficking of Soluble Vacuolar Proteins in Plant Cells. Plants **3**, 392–408.

Kim DH, Eu YJ, Yoo CM, Kim YW, Pih KT, Jin JB, Kim SJ, Stenmark H, Hwang I. 2001. Trafficking of phosphatidylinositol 3-phosphate from the trans-Golgi network to the lumen of the central vacuole in plant cells. The Plant cell **13**, 287–301.

Kim H, Kang H, Jang M, Chang JH, Miao Y, Jiang L, Hwang I. 2010. Homomeric Interaction of AtVSR1 Is Essential for Its Function as a Vacuolar Sorting Receptor. Plant physiology **154**, 134–148.

Kleine-Vehn J, Dhonukshe P, Swarup R, Bennett M, Friml J. 2006. Subcellular trafficking of the Arabidopsis auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. The Plant cell **18**, 3171–3181.

Knorr RL, Dimova R, Lipowsky R. 2012. Curvature of double-membrane organelles generated by changes in membrane size and composition. PloS one **7**, e32753.

Kulich I, Pečenková T, Sekereš J, Smetana O, Fendrych M, Foissner I, Höftberger M, Zárský V. 2013. Arabidopsis exocyst subcomplex containing subunit EXO70B1 is involved in autophagy-related transport to the vacuole. Traffic **14**, 1155–1165.

Kulich I, Žárský V. 2014. Autophagy-related direct membrane import from ER/cytoplasm into the vacuole or apoplast: a hidden gateway also for secondary metabolites and phytohormones? International journal of molecular sciences **15**, 7462–7474.

Künzl F, Früholz S, Fäßler F, Li B, Pimpl P. 2016. Receptor-mediated sorting of soluble vacuolar proteins ends at the trans-Golgi network/early endosome. Nature plants **2**, 16017.

Lee KH, Piao HL, Kim H-Y, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee I-J, Hwang I. 2006. Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. Cell **126**, 1109–1120.

Levanony H, Rubin R, Altschuler Y, Galili G. 1992. Evidence for a novel route of wheat storage proteins to vacuoles. The Journal of cell biology **119**, 1117–1128.

Liu Y, Bassham DC. 2012. Autophagy: pathways for self-eating in plant cells. Annual review of plant biology **63**, 215–237.

Liu Y, Burgos JS, Deng Y, Srivastava R, Howell SH, Bassham DC. 2012. Degradation of the endoplasmic reticulum by autophagy during endoplasmic reticulum stress in Arabidopsis. The Plant cell **24**, 4635–4651.

Liu F, Ren Y, Wang Y, *et al.* 2013. OsVPS9A functions cooperatively with OsRAB5A to regulate post-Golgi dense vesicle-mediated storage protein trafficking to the protein storage vacuole in rice endosperm cells. Molecular plant **6**, 1918–1932.

Luo F, Fong YH, Zeng Y, Shen J, Jiang L, Wong K-B. 2014. How vacuolar sorting receptor proteins interact with their cargo proteins: crystal structures of apo and cargo-bound forms of the protease-associated domain from an Arabidopsis vacuolar sorting receptor. The Plant cell **26**, 3693–3708.

Marty F. 1978. Cytochemical studies on GERL, provacuoles, and vacuoles in root meristematic cells of Euphorbia. Proceedings of the National Academy of Sciences of the United States of America **75**, 852–856.

Marty F. 1999. Plant Vacuoles. The Plant cell **11**, 587–599.

Miao Y, Li KY, Li H-Y, Yao X, Jiang L. 2008. The vacuolar transport of aleurain-GFP and 2S albumin-GFP fusions is mediated by the same pre-vacuolar compartments in tobacco BY-2 and Arabidopsis suspension cultured cells. The Plant journal: for cell and molecular biology **56**, 824–839.

Michaeli S, Avin-Wittenberg T, Galili G. 2014. Involvement of autophagy in the direct ER to vacuole protein trafficking route in plants. Frontiers in plant science **5**, 134.

Michaeli S, Galili G, Genschik P, Fernie AR, Avin-Wittenberg T. 2016/2. Autophagy in Plants – What's New on the Menu? Trends in plant science **21**, 134–144.

Müller O, Sattler T, Flötenmeyer M, Schwarz H, Plattner H, Mayer A. 2000. Autophagic tubes: vacuolar invaginations involved in lateral membrane sorting and inverse vesicle budding. The Journal of cell biology **151**, 519–528.

Nakatsu F, Okada M, Mori F, *et al.* 2004. Defective function of GABA-containing synaptic vesicles in mice lacking the AP-3B clathrin adaptor. The Journal of cell biology **167**, 293–302.

Neuhaus JM, Rogers JC. 1998. Sorting of proteins to vacuoles in plant cells. Plant molecular biology **38**, 127–144.

Niemes S, Labs M, Scheuring D, Krueger F, Langhans M, Jesenofsky B, Robinson DG, Pimpl P. 2010*a*. Sorting of plant vacuolar proteins is initiated in the ER. The Plant journal: for cell and molecular biology **62**, 601–614.

Niemes S, Langhans M, Viotti C, Scheuring D, San Wan Yan M, Jiang L, Hillmer S, Robinson DG, Pimpl P. 2010*b*. Retromer recycles vacuolar sorting receptors from the trans-Golgi network. The Plant journal: for cell and molecular biology **61**, 107–121.

Nishimura K, Matsunami E, Yoshida S, Kohata S, Yamauchi J, Jisaka M, Nagaya T, Yokota K, Nakagawa T. 2016. The tyrosine-sorting motif of the vacuolar sorting receptor VSR4 from Arabidopsis thaliana, which is involved in the interaction between VSR4 and AP-1M2, μ1-adaptin type 2 of clathrin adaptor complex 1 subunits, participates in the post-Golgi sorting of VSR4. Bioscience, biotechnology, and biochemistry **80**, 694–705.

Occhialini A, Gouzerh G, Di Sansebastiano G-P, Neuhaus J-M. 2016. Dimerization of the Vacuolar Receptors AtRMR1 and -2 from Arabidopsis thaliana Contributes to Their Localization in the trans-Golgi Network. International journal of molecular sciences **17**.

Odorizzi G, Cowles CR, Emr SD. 1998. The AP-3 complex: a coat of many colours. Trends in cell biology **8**, 282–288.

Oliviusson P, Heinzerling O, Hillmer S, Hinz G, Tse YC, Jiang L, Robinson DG. 2006. Plant retromer, localized to the prevacuolar compartment and microvesicles in Arabidopsis, may interact with vacuolar sorting receptors. The Plant cell **18**, 1239–1252.

Park JH, Oufattole M, Rogers JC. 2007. Golgi-mediated vacuolar sorting in plant cells: RMR proteins are sorting receptors for the protein aggregation/membrane internalization pathway. Plant science: an international journal of experimental plant biology **172**.

Peden AA, Oorschot V, Hesser BA, Austin CD, Scheller RH, Klumperman J. 2004. Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. The Journal of cell biology **164**, 1065–1076.

Pedrazzini E, Caprera A, Fojadelli I, Stella A, Rocchetti A, Bassin B, Martinoia E, Vitale A. 2016. The Arabidopsis tonoplast is almost devoid of glycoproteins with complex N-glycans, unlike the rat lysosomal membrane. Journal of experimental botany **67**, 1769–1781.

Pelham HR, Hardwick KG, Lewis MJ. 1988. Sorting of soluble ER proteins in yeast. The EMBO journal **7**, 1757–1762.

Pelham HR. 1990. The retention signal for soluble proteins of the endoplasmic reticulum. Trends in biochemical sciences **15**, 483–486.

Pereira CS, da Costa DS, Pereira S, Nogueira F de M, Albuquerque PM, Teixeira J, Faro

C, Pissarra J. 2008. Cardosins in postembryonic development of cardoon: towards an elucidation of the biological function of plant aspartic proteinases. Protoplasma **232**, 203–213.

Pereira C, Pereira S, Satiat-Jeunemaitre B, Pissarra J. 2013. Cardosin A contains two vacuolar sorting signals using different vacuolar routes in tobacco epidermal cells. The Plant journal: for cell and molecular biology **76**, 87–100.

Pertl-Obermeyer H, Wu XN, Schrodt J, Müdsam C, Obermeyer G, Schulze WX. 2016. Identification of Cargo for Adaptor Protein (AP) Complexes 3 and 4 by Sucrose Gradient Profiling. Molecular & cellular proteomics: MCP **15**, 2877–2889.

Phillipson BA, Pimpl P, daSilva LL, Crofts AJ, Taylor JP, Movafeghi A, Robinson DG, Denecke J. 2001. Secretory bulk flow of soluble proteins is efficient and COPII dependent. The Plant cell **13**, 2005–2020.

Pimpl P, Taylor JP, Snowden C, Hillmer S, Robinson DG, Denecke J. 2006. Golgi-mediated vacuolar sorting of the endoplasmic reticulum chaperone BiP may play an active role in quality control within the secretory pathway. The Plant cell **18**, 198–211.

Pompa A, De Marchis F, Pallotta MT, Benitez-Alfonso Y, Jones A, Schipper K, Moreau K, Žárský V, Di Sansebastiano GP, Bellucci M. 2017. Unconventional Transport Routes of Soluble and Membrane Proteins and Their Role in Developmental Biology. International journal of molecular sciences **18**.

Pourcel L, Irani NG, Lu Y, Riedl K, Schwartz S, Grotewold E. 2010. The formation of Anthocyanic Vacuolar Inclusions in Arabidopsis thaliana and implications for the sequestration of anthocyanin pigments. Molecular plant **3**, 78–90.

Poustka F, Irani NG, Feller A, Lu Y, Pourcel L, Frame K, Grotewold E. 2007. A trafficking pathway for anthocyanins overlaps with the endoplasmic reticulum-to-vacuole protein-sorting route in Arabidopsis and contributes to the formation of vacuolar inclusions. Plant physiology **145**, 1323–1335.

Ralston L, Subramanian S, Matsuno M, Yu O. 2005. Partial reconstruction of flavonoid and isoflavonoid biosynthesis in yeast using soybean type I and type II chalcone isomerases. Plant physiology **137**, 1375–1388.

Reusch U, Bernhard O, Koszinowski U, Schu P. 2002. AP-1A and AP-3A lysosomal sorting functions. Traffic **3**, 752–761.

Reyes FC, Chung T, Holding D, Jung R, Vierstra R, Otegui MS. 2011. Delivery of prolamins to the protein storage vacuole in maize aleurone cells. The Plant cell **23**, 769–784.

Rivera-Serrano EE, Rodriguez-Welsh MF, Hicks GR, Rojas-Pierce M. 2012. A small

molecule inhibitor partitions two distinct pathways for trafficking of tonoplast intrinsic proteins in Arabidopsis. PloS one **7**, e44735.

Robinson DG, Galili G, Herman E, Hillmer S. 1998. Topical aspects of vacuolar protein transport: autophagy and prevacuolar compartments. Journal of experimental botany **49**, 1263–1270.

Robinson DG, Oliviusson P, Hinz G. 2005. Protein sorting to the storage vacuoles of plants: a critical appraisal. Traffic **6**, 615–625.

Rous BA, Reaves BJ, Ihrke G, Briggs JAG, Gray SR, Stephens DJ, Banting G, Luzio JP. 2002. Role of adaptor complex AP-3 in targeting wild-type and mutated CD63 to lysosomes. Molecular biology of the cell **13**, 1071–1082.

Saberianfar R, Sattarzadeh A, Joensuu JJ, Kohalmi SE, Menassa R. 2016. Protein Bodies in Leaves Exchange Contents through the Endoplasmic Reticulum. Frontiers in plant science **7**, 693.

Sanmartin M, Ordonez A, Sohn EJ, Robert S, Sanchez-Serrano JJ, Surpin MA, Raikhel NV, Rojo E. 2007. Divergent functions of VTI12 and VTI11 in trafficking to storage and lytic vacuoles in Arabidopsis. Proc. Natl Acad. Sci. U S A. **104**, 3645–3650.

Saslowsky D, Winkel-Shirley B. 2001. Localization of flavonoid enzymes in Arabidopsis roots. The Plant journal: for cell and molecular biology **27**, 37–48.

Scheuring D, Viotti C, Krüger F, *et al.* 2011. Multivesicular bodies mature from the trans-Golgi network/early endosome in Arabidopsis. The Plant cell **23**, 3463–3481.

Scheuring D, Künzl F, Viotti C, Yan MSW, Jiang L, Schellmann S, Robinson DG, Pimpl P. 2012. Ubiquitin initiates sorting of Golgi and plasma membrane proteins into the vacuolar degradation pathway. BMC plant biology **12**, 164.

Shen J, Ding Y, Gao C, Rojo E, Jiang L. 2014. N-linked glycosylation of AtVSR1 is important for vacuolar protein sorting in Arabidopsis. The Plant journal: for cell and molecular biology **80**, 977–992.

Shen Y, Wang J, Ding Y, Lo SW, Gouzerh G, Neuhaus J-M, Jiang L. 2011. The rice RMR1 associates with a distinct prevacuolar compartment for the protein storage vacuole pathway. Molecular plant **4**, 854–868.

Shimada T, Watanabe E, Tamura K, Hayashi Y, Nishimura M, Hara-Nishimura I. 2002. A vacuolar sorting receptor PV72 on the membrane of vesicles that accumulate precursors of seed storage proteins (PAC vesicles). Plant & cell physiology **43**, 1086–1095.

Singh MK, Krüger F, Beckmann H, *et al.* 2014. Protein delivery to vacuole requires SAND

protein-dependent Rab GTPase conversion for MVB-vacuole fusion. Current biology: CB **24**, 1383–1389.

Stigliano E, Faraco M, Neuhaus J-M, Montefusco A, Dalessandro G, Piro G, Di Sansebastiano G-P. 2013. Two glycosylated vacuolar GFPs are new markers for ER-to-vacuole sorting. Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale **73**, 337–343.

Stierhof Y-D, Viotti C, Scheuring D, Sturm S, Robinson DG. 2013. Sorting nexins 1 and 2a locate mainly to the TGN. Protoplasma **250,** 235-240

Sun Y, Li H, Huang J-R. 2012. Arabidopsis TT19 functions as a carrier to transport anthocyanin from the cytosol to tonoplasts. Molecular plant **5**, 387–400.

Takahashi H, Saito Y, Kitagawa T, Morita S, Masumura T, Tanaka K. 2005. A novel vesicle derived directly from endoplasmic reticulum is involved in the transport of vacuolar storage proteins in rice endosperm. Plant & cell physiology **46**, 245–249.

Törmäkangas K, Hadlington JL, Pimpl P, Hillmer S, Brandizzi F, Teeri TH, Denecke J. 2001. A vacuolar sorting domain may also influence the way in which proteins leave the endoplasmic reticulum. The Plant cell **13**, 2021–2032.

Torres E, Gonzalez-Melendi P, Stöger E, Shaw P, Twyman RM, Nicholson L, Vaquero C, Fischer R, Christou P, Perrin Y. 2001. Native and artificial reticuloplasmins co-accumulate in distinct domains of the endoplasmic reticulum and in post-endoplasmic reticulum compartments. Plant physiology **127**, 1212–1223.

Uemura T, Ueda T. 2014. Plant vacuolar trafficking driven by RAB and SNARE proteins. Curr Opin Plant Biol **22**, 116-21.

Valls LA, Winther JR, Stevens TH. 1990. Yeast carboxypeptidase Y vacuolar targeting signal is defined by four propeptide amino acids. The Journal of cell biology **111**, 361–368.

Verweij W, Di Sansebastiano G-P, Quattrocchio F, Dalessandro G. 2008. Agrobacterium-mediated transient expression of vacuolar GFPs in Petunia leaves and petals. Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology **142**, 343–347.

Viotti C, Krüger F, Krebs M, *et al.* 2013. The endoplasmic reticulum is the main membrane source for biogenesis of the lytic vacuole in Arabidopsis. The Plant cell **25**, 3434–3449.

Vitale A, Denecke J. 1999. The endoplasmic reticulum-gateway of the secretory pathway. The Plant cell **11**, 615–628.

Vitale A, Hinz G. 2005. Sorting of proteins to storage vacuoles: how many mechanisms? Trends in plant science **10**, 316–323.

Vitale A, Raikhel NV. 1999. What do proteins need to reach different vacuoles? Trends in plant science **4**, 149–155.

van Voorst F, Kielland-Brandt MC, Winther JR. 1996. Mutational Analysis of the Vacuolar Sorting Signal of Procarboxypeptidase Y in Yeast Shows a Low Requirement for Sequence Conservation. The Journal of biological chemistry **271**, 841–846.

von Lüpke A, Schauermann G, Feussner I, Hinz G. 2008. Peripheral membrane proteins mediate binding of vacuolar storage proteins to membranes of the secretory pathway of developing pea cotyledons. Journal of experimental botany **59**, 1327–1340.

Wang J, Tse YC, Hinz G, Robinson DG, Jiang L. 2012. Storage globulins pass through the Golgi apparatus and multivesicular bodies in the absence of dense vesicle formation during early stages of cotyledon development in mung bean. Journal of experimental botany **63**, 1367–1380.

Watanabe E, Shimada T, Tamura K, Matsushima R, Koumoto Y, Nishimura M, Hara-Nishimura I. 2004. An ER-localized form of PV72, a seed-specific vacuolar sorting receptor, interferes the transport of an NPIR-containing proteinase in Arabidopsis leaves. Plant & cell physiology **45**, 9–17.

Wenzel D, Schauermann G, von Lüpke A, Hinz G. 2005. The cargo in vacuolar storage protein transport vesicles is stratified. Traffic **6**, 45–55.

Winkel-Shirley B. 2002. Biosynthesis of flavonoids and effects of stress. Current opinion in plant biology **5**, 218–223.

Wolfenstetter S, Wirsching P, Dotzauer D, Schneider S, Sauer N. 2012. Routes to the tonoplast: the sorting of tonoplast transporters in Arabidopsis mesophyll protoplasts. The Plant cell **24**, 215–232.

~~**Yano K, Suzuki T, Moriyasu Y**. 2007. Constitutive Autophagy in Plant Root Cells. Autophagy **3**, 360–362.~~

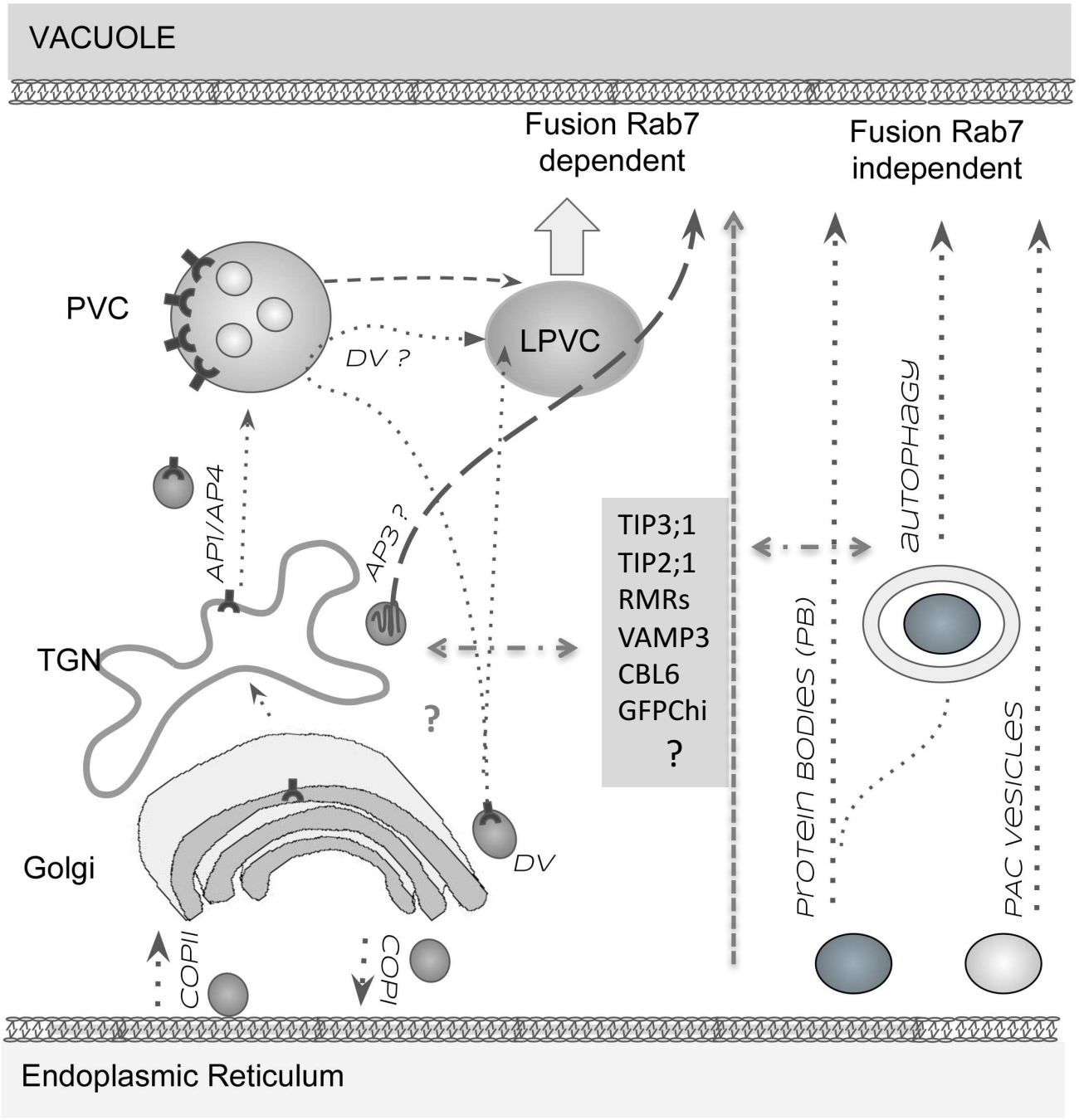
Yoshimoto K, Jikumaru Y, Kamiya Y, Kusano M, Consonni C, Panstruga R, Ohsumi Y, Shirasu K. 2009. Autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in Arabidopsis. The Plant cell **21**, 2914–2927.

Zhang H, Wang L, Deroles S, Bennett R, Davies K. 2006. New insight into the structures and formation of anthocyanic vacuolar inclusions in flower petals. BMC plant biology **6**, 29.

Zhao J. 2015. Flavonoid transport mechanisms: how to go, and with whom. Trends in plant science **20**, 576–585.

Zouhar J, Sauer M. 2014. Helping hands for budding prospects: ENTH/ANTH/VHS accessory proteins in endocytosis, vacuolar transport, and secretion. The Plant cell **26**, 4232–4244.

Zwiewka M, Feraru E, Möller B, Hwang I, Feraru IM, Kleine-Vehn J, Weijers D, Friml J. 2011. The AP-3 adaptor complex is required for vacuolar function in Arabidopsis. Cell research **21**, 1711–1722.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Endoplasmic Reticulum