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REVIEW

Crossing paths: recent insights in the interplay between autophagy and intracellular trafficking in plants

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Autophagy fulfills a crucial role in plant cellular homeostasis by recycling diverse cellular components ranging from protein complexes to whole organelles. Autophagy cargos are shuttled to the vacuole for degradation, thereby completing the recycling process. Canonical autophagy requires the lipidation and insertion of ATG8 proteins into double-membrane structures, termed autophagosomes, which engulf the cargo to be degraded. As such, the autophagy pathway actively contributes to intracellular membrane trafficking. Yet, the autophagic process is not fully considered a *bona fide* component of the canonical membrane trafficking pathway. However, recent findings have started to pinpoint the interconnection between classical membrane trafficking pathways and autophagy. This review details the latest advances in our comprehension of the interplay between these two pathways. Understanding the overlap between autophagy and canonical membrane trafficking pathways is important to illuminate the inner workings of both pathways in plant cells.

Keywords: amphisomes; autophagy; endomembranes; lipids; non-canonical autophagy; secretory pathway; vacuolar degradation; vesicle trafficking

As eukaryotic organisms have evolved to withstand a variety of environmental challenges to their survival and reproduction capacities, a number of cellular regulatory mechanisms have been developed to cope with the everchanging requirements for the maintenance of homeostasis. Throughout evolution and the advent of increasingly complex multicellular organisms, cells have come to be specialized and therefore composed of a multitude of varying organic elements. Their abundance in each cell type needs to be regulated to guarantee organismal fitness. To maintain adequate levels of very diverse cellular components, intracellular recycling is a necessary process. In plants, the autophagy pathway ensures the recycling of faulty cellular components, from misfolded proteins to dysfunctional organelles, to maintain the correct cellular function. The ability of this pathway to target a large array of cellular components for degradation is also crucial for energy conservation under nutrient stress. This enables the cell to recycle components for harvesting energy and to limit the abundance of growth promoting constituents to participate in the reprograming and adaption to a lack of nutrients.

The autophagy pathway is initiated by a number of signaling events that spark the association of autophagy-related proteins (ATG) ATG1, ATG13, ATG11 and ATG101 into a complex [1]. The assembly of this complex can be triggered by diverse stimuli,

Abbreviations
COPII, coat protein complex II; ER, endoplasmic reticulum; ESCRT, endosomal sorting complexes required for transport; EE, early endosomes; EVs, extracellular vesicles; LE, late endosomes; MVB, multivesicular body; PAS, phagophore assembly site; PI3P, phosphatidylinositol 3-phosphate; PM, plasma membrane; PSV, protein storage vacuole; ROP, Rho family GTPases of plant; TGN, *trans*-Golgi network; TPC, TSET/TPLATE complex; TSPO, tryptophan-rich sensory protein/translocator.

with the most widely reported and used being nutrient starvation [1]. Nutrient-sensing-dependent induction of autophagy is dependent on target of rapamycin and SNF1-related protein kinase 1 kinases. The inhibition of target of rapamycin kinase by the nutrient-deficiency or by chemical components (e.g. AZD8055) induces the formation of the ATG complex and thereby autophagy [2]. During carbon starvation, SNF1-related protein kinase 1 may also participate in the induction of autophagy by regulating the ATG1 kinase's phospho status or, alternatively, by directly phosphorylating ATG6 [3].

These induction steps of the pathway lead to the initiation of the formation of the double-lipid-bilayer vesicle termed the phagophore. This stage occurs on the endoplasmic reticulum (ER) and involves: (a) the delivery of membranous material in order to complete the double-membrane vesicle by ATG9; (b) the local activity of the phosphatidylinositol-3-phosphate kinase complex to ensure vesicle identity; and (c) the functionalization of ATG8 proteins by the adjunction of a phospholipid, phosphatidylethanolamine (PE), to their C-termini, enabling their anchoring to the membrane and subsequent integration of their future cargo into the autophagosome.

After its closure, the autophagosome is targeted to the lytic vacuole via FYCO (i.e. FYVE and coiled-coil containing) proteins that recognize ATG8 and phosphatidylinositol-3-phosphate (PI3P) [1]. Trafficked along microtubules, the autophagosome can then fuse to the vacuole by way of v-SNARE proteins, thus completing its fate [1].

The membrane-based events that occur from the genesis to the degradation of the autophagosome, as well as the necessity of having a membrane-based system to target large membranous-compartments for degradation, tightly link the autophagy pathway with other intracellular membrane trafficking routes.

At large, intracellular membrane trafficking enables the targeting of membrane-integral and membrane-associated proteins, as well as the transport of specific lipids and other vesicle-associated metabolic compounds to intra- and extracellular compartments. In addition, vesicles may also transport in their lumen cargos that are indirectly bound to membrane-associated components [4]. This dynamic membrane-compartmentalized process needs to be tightly-regulated, and the autophagy pathway is essential in the coordination of membrane-trafficked components under various environmental stimuli. Highlighting the potential for co-regulation of membrane-trafficking and autophagy, studies focusing on the identification and/or prediction of ATG8-interacting proteins have identified a number of components involved in membrane trafficking [5,6].

In this review, we focus on the crosstalk between membrane-trafficking pathways and autophagy-associated degradation. We emphasize how autophagy is involved in all major membrane compartment involved in membrane trafficking and discuss future research avenues that may advance our understanding of this interaction.

ER-based interplay and phagophore biogenesis

ATG9 and ER-associated membrane source

The importance of supplying membranous material to the forming phagophore is at the source of the initiation of the canonical autophagy pathway. This membranous material is essential for supplying lipids for the double-membrane composition of the nascent phagophore. The trimeric transmembrane protein ATG9 [7] is considered to carry out a central role in this process [8]. In interaction with ATG2 and ATG18, it supplies membranous material from the ER to the phagophore, which drives its expansion [8,9]. The pre-autophagosomal structure (PAS) that is formed appears to be tightly-linked to the vesicle-mediated ER-export pathway involving the coat protein complex II (COPII). Indeed, the PI3P-associated protein FYVE2 was shown to interact with components of COPII, notably Sar1 isoforms and Sec24a, along with the autophagy machinery components ATG18a and ATG8a, contributing to the expansion of the phagophore [10]. The Sar1d isoform was found to regulate, along with Rab GTPase Rab-D2a, the formation of ATG8e punctae [11]. This reinforces the importance of the ER as a membrane source, as well as the ER-export machinery for PAS formation in plants, as also reported in other kingdoms [12]. Autophagy has also been proposed to be essential for direct ER-to-vacuole trafficking of vacuolar-resident proteins, notably via ATG8-interacting proteins ATI1- and/or ATI2-positive vesicles [13,14].

ER homeostasis via ER-phagy

ER-associated proteins have been reported to be involved in the generation of autophagosomes. Nck-associated protein 1 vesicles were shown to be ER-associated and appeared to regulate the formation of fluorophore-fused ATG8e punctae during mechanical stress [15]. The involvement of ER-associated proteins in the autophagic pathway is crucial for their

proteostasis via the mechanism termed ER-phagy or the degradation of ER-associated cellular components. ER-phagy is a crucial process for ER-based quality control. The autophagy-based ER quality control process is ensured by the C53 protein, which associates with ATG8 at the ER after forming a complex with E3 UFM1-protein ligase 1 and DDRGK domain containing 1. This complex is triggered by stalled ribosomes and guarantees the selective autophagic degradation of mistranslated proteins [16]. The ER-membrane localized factor inositol requiring 1–1 has also been shown to regulate ER-stress-associated autophagy by mediating the RNA-transcript degradation of negative-regulators of autophagy such as AT1G66270/BGLU21 (β-glucosidase 21), AT2G16005/ROSY1/ML (MD2-related lipid recognition protein) and AT5G01870/PR-14 (pathogenesis-related protein 1) [17]. The ER-localized reticulon family proteins RTN1 and RTN2 in maize have also been discovered to be involved in ER-phagy by associating to ATG8 via their multiple ATG8-interacting motifs. This interaction was shown to increase during ER stress (here dithiothreitol or tunicamycin treatments), suggesting the importance of reticulon proteins for the maintenance ER homeostasis [18]. The atlastin GTPase root hair deffective 3 has also been demonstrated as being an ER-localized autophagy receptor involved in ER-phagy induced by ER stress [19].

Another cue for the necessity of a functional macroautophagy pathway for ER homeostasis is the observed perturbations exhibited in the *atg5* mutant [20] The *atg5* mutant is impaired in the ATG8-lipidation pathway, thereby *bona fide* hindering the association of ATG8 to the phagophore [21]. Havé *et al.* [20] combined transcriptomic, proteomic and lipidomic data to dissect the many levels of alterations to nitrogen- and carbon-stress responses in *atg5*. With regard to ER-based alterations, they observed an increase in thioredoxins and ER-associated chaperones in the *atg5*, pointing to higher levels of ER-stress when ATG8-lipidation is not possible. In maize, a multi-omics analysis of an *atg12* mutant, putatively impaired in the lipidation and subsequent functionalization of ATG8 proteins, revealed many disruptions protein accumulation and metabolic pathways [22,23]. These studies demonstrated a strong accumulation of ER- and Golgi-resident proteins along with peroxisome, proteasome components, under both carbon starvation [22] and nitrogen starvation [23].

Because the autophagy pathway and the ER appear to be intimately inter-dependent in ensuring their mutual homeostasis, this relationship appears to hold true for other sections of the secretory pathway. Indeed, the strong involvement of the autophagy pathway in ER-based processes along with the interdependency of the different membrane trafficking compartments that defines the cycling function of the secretory pathway strongly suggests the potential participation of the autophagy pathway in other compartments.

Golgi- and post-Golgi-associated autophagic turnover

In plants, the autophagy pathway has rarely been reported to associate with *cis*-Golgi apparatus components, although this has been widely reported in other eukaryotic model organisms [24]. A notable report of a plant Golgi-apparatus component involved in autophagic regulation is the tryptophan-rich sensory protein/translocator (TSPO), which has been shown to regulate the amounts of the aquaporin PIP2;7. TSPO was shown to interact and mediate the autophagic degradation of PIP2;7 in the endomembrane system [25]. TSPO had also been reported to undergo itself autophagic degradation [26]. It should be noted that, considering the cycling of the COPII components between the ER and the Golgi apparatus and their involvement in the formation of the PAS [10,11], it is difficult to exclude them for Golgi-based autophagy pathway events.

With regard to the *trans*-Golgi network (TGN)/ early-endosomes (EE), a number of components have been found to participate in autophagic degradation. It is important to note that this compartment is very dynamic spatially, as well as in its nature, in the sense that it is at the crossroads of exocytosis, coming from the Golgi apparatus, and endocytosis, coming from the plasma membrane (PM). Moreover, a maturation process may occur for some TGN/EE vesicles, which will become late endosomes (LE)/multivesicular bodies (MVB). LE/MVB and endocytosis-derived clathrin-coated vesicles may be shuttled to the vacuole independently of the autophagy pathway [4]. This implies that it may be challenging to decipher the exact degradation path taken by vesicles from these highly-dynamic compartments.

Nevertheless, recent advances in understanding the interoperations of the autophagic pathway and these different post-Golgi compartments in plants have been made [27]. Notable contributions to this understanding were recently achieved by identifying the involvement of the small GTPase Rab8a under oomycete infection conditions in *Nicotiana benthamiana* [28]. Rab GTPases comprise a large family of proteins involved in membrane trafficking between many different compartments [29,30]. Rab8 belongs to the Rab-E1 subfamily [31] and has been shown to be involved in the

resistance to *Pseudomonas syringae*^{DC3000} in *Arabidopsis thaliana* [32]. RabE1 localizes to the Golgi apparatus and regulates the trafficking of cargo from Golgi to post-Golgi compartments [33]. The oomycete *Phytophthora infestans* secretes into the host the effector protein PexRD54, which is able to hijack the host's autophagy pathway to apparently redirect the cargo to the pathogen interface. PexRD54 appeared to use Rab8a to mediate its autophagosome-forming capacity and potentially shuttle lipid-droplets to the pathogen interface [28]. These observations performed under plant-pathogen interaction conditions reveal the strong connection between the endomembrane trafficking and autophagy pathways because they are readily manipulable to the advantage of parasitic pathogens.

Regarding the fact that the LE/MVB compartment is readily able to shuttle cargo to the vacuole [4], an underlying consideration is how autophagy may interplay with this pathway. Recently, it was shown in plants that autophagosome may fuse to MVBs to form 'amphisomes' [34]. This process has already been described in mammalian and yeast cells [35–37] but had not thoroughly been characterized in plants. In *A. thaliana*, it was now shown that cell-death-related endosomal FYVE/SYLF protein 1 (CFS1, also called FYVE2) acts as an adaptor between ATG8a and the endosomal sorting complex required for transport (ESCRT)-I complex present at MVBs [10,34,38]. ESCRT complexes have been shown to be involved in autophagic turnover in other model organisms and there is now some evidence of the involvement of ESCRTs in plant autophagy, although much has yet to be discovered [27,39–41]. The BAR-domain containing protein SH3P2 has been proposed to behave as ubiquitin-binding protein enabling the endocytosis of ubiquitinated PM-associated proteins, in effect working as a substitute for the missing ESCRT-0 in plants, in addition to interacting with VPS23, a component of ESCRT-I [42]. SH3P2 has also been shown to interact with ATG8 along with PI3P and was proposed to participate in phagophore maturation [43,44]. In good agreement with Zhao et al. [34], who identified SH3P2 on the outer-surface of autophagosomes and showed an interaction between SH3P2 and CFS1, SH3P2 was also found to associate to amphisome-like structures [43]. SH3P2 was also shown to be a target of the bacterial effector protein XopL, which actively induced the degradation of SH3P2 via its E3-ligase activity and thereby dampens autophagic degradation [45]. This is consistent with previous studies reporting that FREE1/FYVE1/CFS1 was a plant-specific component of the ESCRT machinery associated with SH3P2 and was essential for MVB formation, as well as autophagosome degradation [46,47]. This exhibits the pivotal role of SH3P2 and the ESCRT machinery as a whole in both autophagosome formation/maturation. Other ESCRT paralogs have been previously shown to take part in the autophagic turnover of cellular components, notably the ESCRT-III paralogs charged multivesicular body protein1 (CHMP1A and CHMP1B), which were proven to be essential for the autophagic degradation of chloroplasts (i.e. chlorophagy) [48].

Bearing in mind that these post-Golgi compartments may potentially be directed-to or coming-from the PM, the involvement of endo- and exocytosis pathways is intimately linked to these compartments, as well as their potential regulation and crosstalk with the autophagy pathway. In terms of components canonically considered to be involved in exocytosis, recent contributions to the literature have shed light on the interplay between the EXOCYST complex and autophagy. It has been shown that EXO70 isoforms are involved in autophagic turnover, notably EXO70B1 and EXO70B2, which participate in shuttling autophagosomes to the vacuole by way of their interaction with ATG8 [49,50]. For EXO70E2, it has been shown that, under non-stressed conditions, it does not associate to autophagosomes, although, once stress is induced, by way of starvation or the salicylic acid analog BTH, EXO70E2 and ATG8 appear to colocalize in the vacuole [51]. Moreover, the selective autophagy receptor Next to BRCA1 gene 1 protein was revealed to mediate the trafficking of EXO70E2 to the vacuole by specifically recruiting it to ATG8 [52]. EXO70D isoforms have been demonstrated to be involved in the targeting for degradation of type-A ARR proteins *via* ATG8 [53].

These observations highlight the crosstalk between EXOCYST-positive vesicles and autophagosomes, suggesting a virtual switchboard of sorts that, according to the current stress signaling events taking place, would direct different cargo either to the vacuole or the apoplast. This idea is also supported by the presence of large number of EXO70 isoforms in plants, suggesting a high-level of specialization for each individual isoform [54]. Rho family GTPases of plants (ROPs) based signaling events have recently been shown to mediate autophagosome formation via the EXOCYST subunit Sec5. The ROP8 isoform along with Sec5 are proposed to interact with the autophagy machinery proteins ATG1, ATG13, ATG6 and ATG8 to regulate autophagosome formation [55].

The generation of post-Golgi vesicles may also occur through the process of endocytosis, where the PM will invaginate and bud off into to the cytosol in order to form endosomes. Endocytosis may be performed via clathrin-mediated endocytosis, which can involve the TSET/TPLATE complex (TPC) directly from the PM or by way of ER–PM contact sites [56]. The TPC has

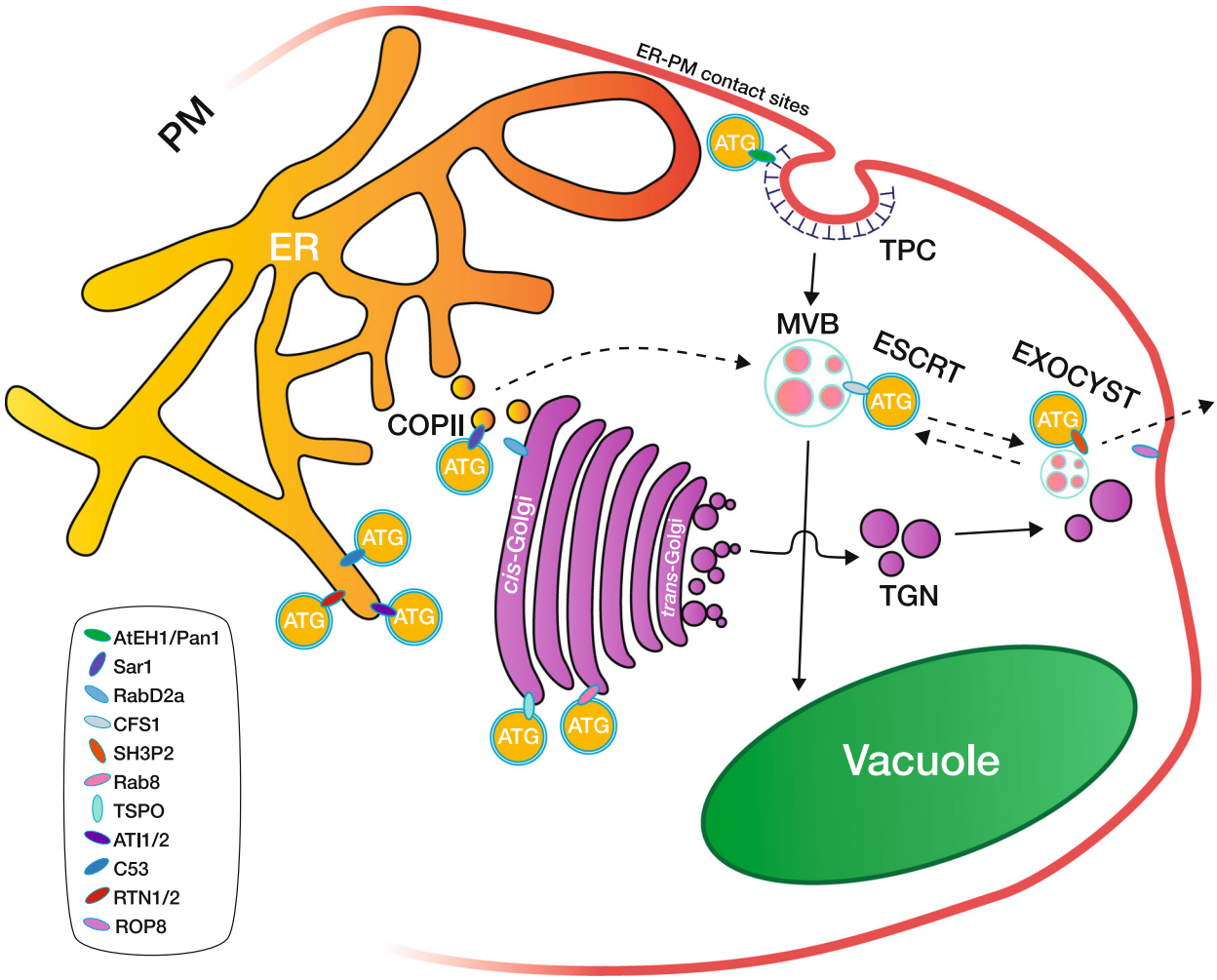

Fig. 1. Schematic of the interplay between autophagy pathway and membrane trafficking in plants. Simplified graphical representation of membrane-based components involved in plant autophagy. PM, plasma membrane; ER, endoplasmic reticulum; MVB, multivesicular body; TPC, TSET/TPLATE complex; ESCRT, endosomal sorting complex required for transport; TGN, *trans*-Golgi network; COPII, coat protein complex II; ATG, autophagosome. Box decodes protein icons.

been shown to be implicated in the regulation of autophagosome formation as well as endocytic trafficking [57,58]. The TPC member AtEH1/PAN1 was shown to interact at ER–PM contact sites along with VAP27-1 and the actin cytoskeleton and drives autophagosome formation at ER–PM contact sites [58].

Conclusions and perspectives

Many parts of the membrane trafficking system appear to be crucial for autophagosome formation and autophagic turnover, from the generation of the phagophore, which may be located in the ER, in ER–PM contact sites, or in association with endosomes, to the maturation of the phagophore into a fully formed autophagosome and its fusion to MVBs to form amphisome-like structures before being shuttled to the vacuole. The function of autophagy pathway is highly dependent on membrane trafficking components and, reciprocally, autophagy appears to regulate many of these components to maintain homeostasis in a highly dynamic membrane trafficking/exchange system (Fig. 1 and Table 1).

The complex interplay between endomembranes and the autophagy pathway in plants still has many facets to be revealed. In mice, it has been shown that the autophagy pathway is crucial for the formation of exosomes or extracellular vesicles (EVs), in a process termed 'ecretory autophagy' [59,60]. The autophagy-pathway-dependent EVs were shown to act as decoy vesicles carrying a receptor recognized by the pore-forming α-toxin from *Staphylococcus aureus* [60]. Moreover, it was also observed that

Table 1. Summary of recently identified endomembrane-associated components involved in the autophagy pathway.

	References
ER-associated components	
C53	[16]
ATI1/2	[13,14]
RTN1/2	[18]
Sar1d/Sec24	[10,11]
Cis-Golgi components	
TSPO	[25,26]
Rab8	[28]
Rab-D2a	[11]
Post-Golgi components	
EXOCYST	[49,50,52,53,55]
SH3P2	[42–45]
AtEH1/Pan1	[57,58]
FREE1/FYVE1	[46,47]
FYVE2/CFS1	[10,34,38]
ROP8	[55]

the production of these EVs blocked lysosomal degradation, indicating that the autophagy machinery contributes to the generation of EVs and that autophagosomes may be redirected to the extracellular environment when the cell cannot degrade autophagic cargos in the lysosome, such as upon bafilomycin A treatment [60]. These observations have echoes in *A. thaliana*, where blocking vacuolar targeting of prevacuolar compartment cargos to the protein storage vacuole (PSV) in a *mon1* mutant line resulted in apoplastic mislocalization of PSV components such as 2S-albumin, 12S-globulin and the artificial seed protein GFP-CT24 [61]. Mon1-Ccz1 complex in yeast was furthermore shown to target pre-autophagosomal structures [62]. Other mutants of components of vacuolar trafficking such as AtNHX5 and AtNHX6 were found to induce secretion into the apoplast of PSV-targeted components [63]. This prompts further research about the role of the autophagy pathway in other vacuolar-targeting

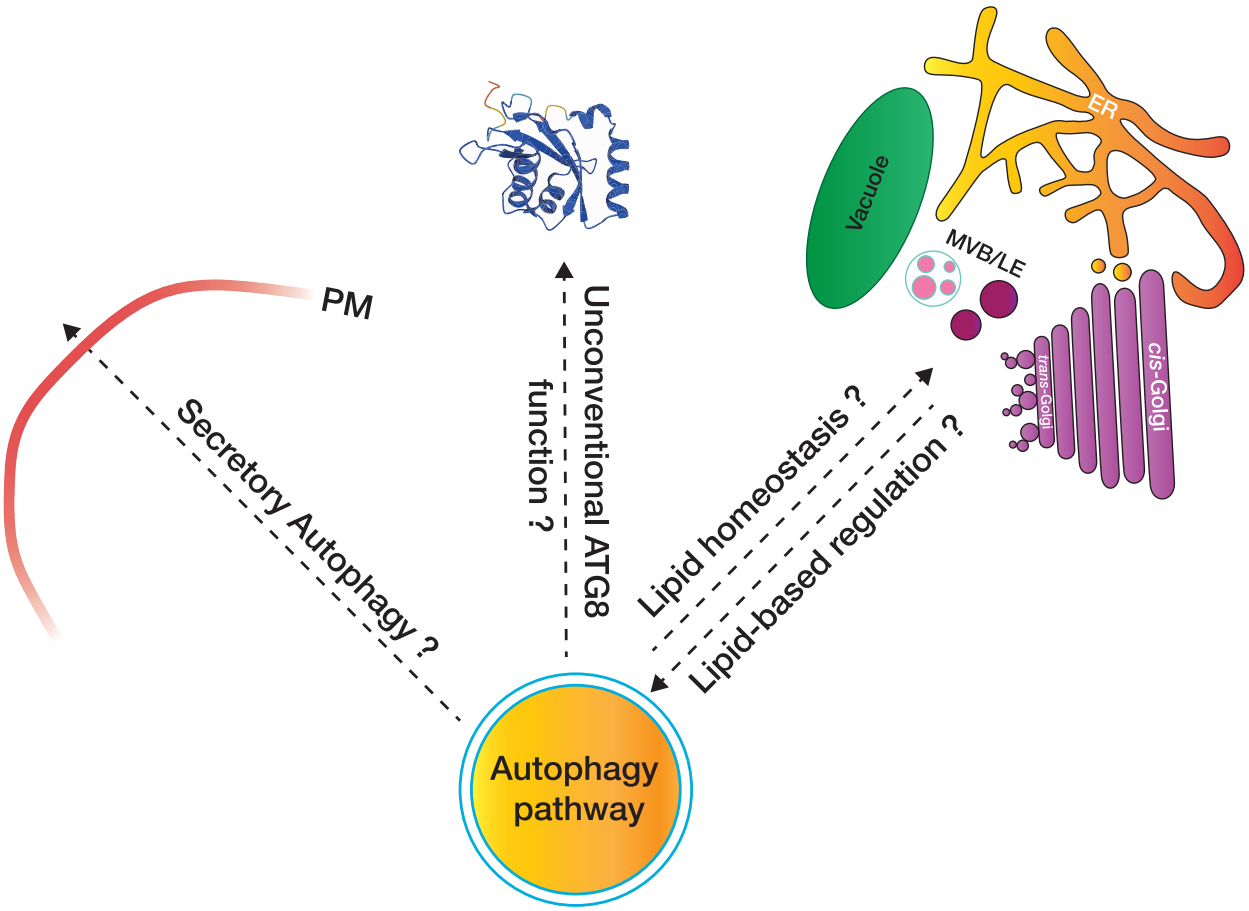

Fig. 2. Future research avenues in the study of the interplay between autophagy and membrane trafficking in plants. Arrows represent potential research angles to follow to better understand the role of the autophagy pathway in participating in membrane trafficking. MVB/LE, multi-vesicular bodies/late endosomes; ER, endoplasmic reticulum; PM, plasma membrane. Structural model of ATG8 was produced using ALPHAFOLD, ID #AF-Q8S926-F1 (https://alphafold.ebi.ac.uk).

pathways that shuttle cargos to the apoplast under various stress conditions.

An important point deserving further study is deciphering the possible existence of 'non-canonical' autophagy in plants, as it has been described in mammalian cells [64]. Interestingly, the other known functions of ATG8-like proteins are mainly related to cargo secretion into the extracellular compartment [64]. Non-canonical autophagy in mammalian cells appears to take on many different roles that are characteristic of specific cell-types, an important issue to be tackled in the plant autophagy field [65].

Notwithstanding, understanding the ins and outs of the autophagic pathway and its interaction with other membrane trafficking routes must crucially incorporate lipids, the major components of all of these pathways. The identity of intracellular vesicles is defined by both the proteins that decorate them and by the lipids that compose them [66]. Lipid identity has been shown to be consequential in many steps of the autophagy pathway [67]. A noteworthy example of the lipid-associated implications and crosstalk that may occur between the autophagy pathway and vesicle trafficking is the role of the length of very-long chain fatty acids in mediating PI3P homeostasis, a crucial lipid for both autophagosome biogenesis and LE/MVB identity [68] (Fig. 2).

Based on these perspectives, we consider two major points of future inquiry into the dynamic nature of these pathways: (a) understanding the diversity of components at the interface between the autophagy pathway and general membrane trafficking and (b) how the interplay between them may serve novel and/or redundant functions involved in cellular homeostasis and stress adaptation.

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