Review of Autophagy-related Approaches for Improving Nutrient Use Efficiency and Crop Yield Protection

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Abstract

Autophagy is a eukaryotic catabolic pathway essential for growth and development. In plants, it is activated in response to environmental cues or developmental stimuli. However, in contrast to other eukaryotic systems, we know comparatively little mechanistically, regarding the regulation of this important and complex pathway, or the full complement of the molecular players involved in it. In the framework of the COST (European Cooperation in Science and Technology) action TRANSAUTOPHAGY (2016-2020), we decided to review our current knowledge of autophagy responses in vascular plants, with emphasis on knowledge gaps. We also assess here the potential of translating the acquired knowledge to improve crop plant growth and development in a context of growing societal and environmental challenges for agriculture in the near future.

Introduction

During the life span of a eukaryotic cell, a catabolic pathway known as autophagy degrades dysfunctional or unnecessary cellular components as a way of recycling macromolecules' building blocks and ensuring cellular homeostasis (Klionsky *et al.*, 2016). In essence, autophagy consists in the translocation of cytoplasmic components (cargo) into the vacuole (yeast and plant) or the lysosome (animal) and their subsequent degradation (Li and Vierstra, 2012). In plants, autophagy is a central regulator of fitness, longevity and

fecundity, as well as a major housekeeping mechanism underpinning plant tolerance to various biotic and abiotic stresses (Minina *et al.*, 2018). Plant cells decrease their dependency on external sources of nutrients by recycling their contents *via* autophagy (Guiboileau *et al.*, 2013; Minina *et al.*, 2013b). Furthermore, autophagy increases viability of cells under stress conditions by a quick removal of damaged macromolecules and organelles (Bassham *et al.*, 2006; Li and Vierstra, 2012; Michaeli *et al.*, 2016), modulation of immune response and targeting of virulence factors or entire pathogens (Hafren *et al.*, 2017; Haxim *et al.*, 2017; Lenz *et al.*, 2011). Thus, autophagy defines important agricultural traits, i.e. tolerance to macro-nutrient depletion, drought, heat, oxidative and salt stress, as well as immune response to pathogen infection. Although most of the research so far has been performed in the model plant *Arabidopsis thaliana*, the involvement of autophagy in a variety of agricultural traits generates great interest in the development of tools for efficient modulation of autophagy in plants. In this manuscript, we will review the current knowledge regarding autophagy in plants, its functional mechanisms and physiological roles and highlight possible uses for autophagy manipulation as potential enhancers of plant yield and tolerance.

Types of autophagy in plants

Autophagy can be generically distinguished into microautophagy and macroautophagy (Galluzzi *et al.*, 2017). Other variants of autophagy such as chaperone-mediated autophagy (CMA) (Kaushik and Cuervo, 2012), secretory autophagy (Ponpuak *et al.*, 2015) in mammalian cells, and cytoplasm-to-vacuole transport (CVT) in yeast (Reggiori *et al.*, 2004) are cell type-specific, and have not been described so far in plant cells. Both microautophagy and macroautophagy can be selective or non-selective in plants. Microautophagy is characterized by a direct invagination of the tonoplast (vacuolar membrane) to take up the cellular components to be degraded. A well-described example in plants is the functional accumulation of anthocyanins through microautophagy-derived inclusion bodies in the plant vacuole (Chanoca *et al.*, 2015). Anthocyanins are a diverse family of flavonoid pigments synthesized in the cytoplasm, stored in the vacuole, acting as antioxidants and involved in plant tissues responses to environmental cues. These pigments

are stored in the vacuole as densely packed 3-10 μm vesicles generated through a microautophagy process (Chanoca *et al.*, 2015). The molecular mechanisms of membrane dynamics driving microautophagy are not well understood in plants, but seem not to require any of the gene products involved in macroautophagy. Macroautophagy (hereafter autophagy) is characterized by the *de novo* formation of a double membrane organelle, the autophagosome, wrapping defined cytoplasmic components for degradation. The initiation, elongation, maturation and fusion of the autophagosome with the vacuole is marshalled by a conserved set of proteins encoded by autophagy-related (*ATG*) genes (Tsukada and Ohsumi, 1993). Notably, plant-specific autophagic pathways as defined by the cargo type do exist. A well-described example is chlorophagy or the autophagic degradation of whole chloroplasts (up to 5-10 μm in size, mean volume of 20 μm^3) damaged by UV light (Izumi *et al.*, 2017). The molecular mechanism and the complement of ATG proteins involved in the formation of these uncommonly large autophagosomes may be specific to the plant kingdom.

Core autophagy complexes and their regulation in plants

Autophagy is a tightly regulated cellular response, which can be activated rapidly and transiently in eukaryotic cells. The formation of the autophagosome is a complex, dynamic, and stepwise process resulting in the engulfment of cytoplasmic material and its translocation to the vacuole. The molecular machinery that executes and regulates autophagy was first characterized in yeast (Tsukada and Ohsumi, 1993). About half of the more than 36 *ATG* genes characterized to date encode core autophagy proteins, and appear to be well conserved in most studied multicellular organisms, including plants (Galluzzi *et al.*, 2017; Klionsky *et al.*, 2016). Autophagy is a powerful catabolic process that needs to be quickly fine-tuned to fit the temporary requirements of cells under variable conditions. In animal cells, autophagy seems to oscillate with astonishingly high frequency (Nazio *et al.*, 2016). To prevent unwanted autophagic activity, most of the ATG proteins are synthesized in an inactive form and require activation by post-translational modification and recruitment into complexes (Ohsumi, 2014). Activation of autophagy is regulated by sensors of cellular nutrient state (Liu and Bassham, 2010) and stress (Minina *et al.*, 2013a; Wang *et al.*, 2015a; Yang *et al.*, 2016).The pre-autophagosomal membrane or phagophore

is initiated in response to a given internal or external cellular stimulus, then elongates and enwraps the cytoplasmic cargo. The closed phagophore matures into an autophagosome, and then fuses with the vacuole/lysosome. Each of the mentioned steps is under the control of specific autophagy complexes made of core autophagy proteins, whose assembly, subsequent subcellular localization and activity are directly or indirectly regulated by stress signaling pathways. Four main complexes are known to be required for autophagosome initiation and formation, namely the ATG1 complex, the VPS34 complex, the ATG9 complex, and the ATG8 conjugation systems (Figure 1).

The ATG1 complex is thought to be essential in transmitting stress signals to the site where the autophagosome will be formed, most likely at an organelle contact site involving the endoplasmic reticulum (ER) (Antonioli *et al.*, 2017; He and Klionsky, 2009; Nascimbeni *et al.*, 2017). In yeast and mammalian cells, the ATG1 complex is a trimeric heterocomplex made of a catalytic subunit (ATG1/ULK, a serine/threonine kinase), regulatory subunits (ATG13 and ATG101), and scaffold subunits (ATG11 or ATG17 in yeast, and FIP200/RB1CC1 RB1 inducible coiled-coil 1 in animals) (Galluzzi *et al.*, 2017). The structure, function and regulation of the ATG1 complex are not well understood in plants. The Arabidopsis genome, for example, encodes three full-length ATG1 proteins (ATG1a, locus AT3G61960; ATG1b, locus AT3G53960; ATG1c, locus AT2G37840), and a C-terminus truncated ATG1 variant called ATG1t (locus AT1G49180) (Suttangkakul *et al.*, 2011), whose function is not yet clear. The Arabidopsis genome also encodes two functional ATG13 homologues, ATG13a and ATG13b, and a single ATG101 (Suttangkakul *et al.*, 2011). Intriguingly, no functional or structural homologue of ATG17/FIP200 has been found yet in deciphered plant genomes. A potentially bifunctional protein containing structural domains related to yeast ATG11 and ATG17 is present in plants. In Arabidopsis, this protein was dubbed an ATG11 homologue since it is required for selective degradation of mitochondria *via* autophagy (Li *et al.*, 2014). However, whether the plant ATG11-related protein acts as a scaffold protein within a *bona fide* ATG1 complex remains to be clarified. If the plant ATG11-related protein functions only in selective autophagy, the plant scaffold protein required for non-selective autophagy is still to be identified.

The clustering and activation of the ATG1 complex at the phagophore initiates the recruitment of other autophagy complexes and in particular the class III VPS34 complex. The class III VPS34 complex involved in autophagy contains the catalytic subunit PI3kinase (PI3K), the regulatory subunits ATG6/Beclin-1 and ATG14, and the scaffold subunit VPS15. As compared to other multicellular organisms, plants have the peculiarity of expressing a single and essential PI3K of the class III type. The structure of the VPS34 complex involved in autophagy is not known in plants. Remarkably, ATG14 is absent in the plant lineage. Yeast ATG14 and its functional homologues in other eukaryotic systems only share resemblance at their N-terminal coil-coiled domain (~200 first amino acids), whereas the C-terminus of these proteins appearing highly divergent (Itakura *et al.*, 2008). ATG14 is known to determine the localization of the VPS34 complex, and to be required for both basal and induced autophagy in yeast and animals (Diao *et al.*, 2015; Fan *et al.*, 2011). Phosphorylation of ATG14 by the ATG1 kinase activates the catalytic activity of PI3K, which catalyzes the production of the membrane lipid PI3P (phosphatidylinositol-3-phosphate) essential for phagophore initiation and expansion (Baskaran *et al.*, 2014). Whether a functional counterpart of ATG14 exists in plants awaits experimental evidence.

The phagophore initiation and expansion requires input of specific lipids and proteins. The membrane source of these materials is still under debate, but they are more likely channeled to the site of autophagosome formation through ATG9-containing vesicles (Abada and Elazar, 2014; Karanasios *et al.*, 2016). ATG9 is the only transmembrane protein among all known ATG proteins (Reggiori *et al.*, 2004). The heterodimer complex ATG2-ATG18 regulates the polytopic ATG9 vesicle-mediated cycling and tethering to and from the growing phagophore. Although plants seem to encode single ATG9 and ATG2 homologues, a diversified multigenic family encodes the PI3P-binding ATG18-related proteins (up to 8 in Arabidopsis as compared to 1 in yeast and 4 in mammals). The resting cellular localization of ATG9 and the full complement of its interacting partners during autophagy-dependent membrane dynamics are not yet understood in plants.

The phagophore membrane expansion also requires the recruitment of lipidated ATG8-related protein. Soluble ubiquitin-like ATG8 becomes membrane-anchored through conjugation to the membrane lipid PE (phosphatidylethanolamine). This modification occurs through an ubiquitylation-like cascade regulated by the protease ATG4, the E1

activating enzyme ATG7, the E2 conjugating enzyme ATG3, and the E3 ligase complex comprising ATG5/ATG12/ATG16 (Pengo *et al.*, 2017; Sanchez-Wandelmer *et al.*, 2017). Apart from *bona fide* ATG16, whose plant orthologue has not yet been characterized, all the other components of this cascade are expressed and active in plants. ATG8-related members are relatively more diversified in plants, with some C-terminally truncated isoforms that are unique to plants (Bassham *et al.*, 2006; Li *et al.*, 2016a).

Autophagy is regulated at many steps through post-translational modification (PTM) such as ubiquitylation, phosphorylation, acetylation, glycosylation and lipidation of ATG proteins. Many ATG proteins in other organisms were shown to undergo complex and multi-layered regulation through PTM. ATG proteins can be differently modified at multiple sites whereby one type of PTM can depend on another modification. An example is the phosphorylation-dependent ubiquitylation that leads to degradation of target proteins(Lin *et al.*, 2002). By acting as a degradation signal, ubiquitylation not only regulate cargo of selective autophagy, but also the autophagy machinery itself. The abundance of mammalian ATG1/ULK1, ATG6/VPS30/beclin-1 (BECN1) and ATG12, for example, were reported to be regulated by ubiquitylation. ULK1 controls the autophagic flux together with ATG13 and is ubiquitylated by the E3 ligase NEDD4L (Nazio *et al.*, 2016) making NEDD4L a negative regulator of autophagy. BECN1, a positive regulator of autophagy induction, is a target of multiple E3 ligases (Shi and Kehrl, 2010; Xia *et al.*, 2013; Xu *et al.*, 2014). By removing the ubiquitin chains, deubiquitylating enzymes such as USP10, USP13, USP14 and USP19 can counteract the E3 ligase activity and rescue BECN1 from degradation, and thus act as positive regulators of autophagy (Jin *et al.*, 2016; Liu *et al.*, 2011; Xu *et al.*, 2016). These recent studies reveal molecular details of a tight regulation of autophagic activities through PTM. Since not all of these regulators are conserved in plants, whether and how PTM regulates plant ATG proteins awaits intensive future studies.

Maturation and fusion of the autophagosome with the lytic compartment involves vectorial movement of the matured autophagosome toward the vacuole in plants, and specific tethering of the autophagosome to the tonoplast. F-actin nucleating and branching ARP2/3 complex was shown in yeast to be associated to the autophagosome (Reggiori *et al.*, 2005). In mammalian cells, WASP homolog associated with actin, membranes and microtubules

(WHAMM), WAS protein family homolog (WASH) and junction-mediating and regulatory protein (JMY) were reported to regulate autophagy (Coutts and La Thangue, 2015; Kast *et al.*, 2015; King *et al.*, 2013; Xia *et al.*, 2013; Zavodszky *et al.*, 2014). In plants, only the WASP family verprolin (WAVE) homologous complex has been shown to be involved in autophagosome movement within the cytoplasm (Wang *et al.*, 2016a). One of the WAVE subunits, NAP1, changes its localization from the cytoplasm to ER membrane under mechanical stress (Wang *et al.*, 2016a). This localization change triggers the ARP2/3 dependent F-actin nucleation on the phagophore, which is important for its expansion and ultimately for the maturation of the autophagosome (Wang *et al.*, 2017a; Wang *et al.*, 2016a). Loss-of-function *nap1* mutant Arabidopsis seedlings (lacking a functional WAVE complex) form less autophagosomes and are more sensitive to salt and nitrogen-deficiency stresses (Wang *et al.*, 2017a; Wang *et al.*, 2016a) .

Selective autophagy in plants

Autophagy was initially considered a bulk, non-selective process. It later became evident that autophagy selectively degrades diverse cellular cargoes under various conditions (Anding and Baehrecke, 2017; Li and Vierstra, 2012; Michaeli *et al.*, 2016; Veljanovski and Batoko, 2014; Yang and Bassham, 2015). Selective autophagy typically utilizes cargo receptors that directly or indirectly bind specific cargo, and tether it to the forming autophagosome through interaction with core autophagy proteins (mainly ATG8) (Farre and Subramani, 2016; Kellner *et al.*, 2017; Zaffagnini and Martens, 2016). In mammals, multiple cargo receptors were identified, including p62/ SQSTM1 and NBR1 that were implicated in the selective autophagy of protein aggregates and organelles (Anding and Baehrecke, 2017; Zaffagnini and Martens, 2016). p62 and NBR1 bind both ubiquitin and the mammalian ATG8 homologue, LC3, thus docking ubiquitinated substrates to the autophagosome. Arabidopsis NBR1 and its tobacco homologue JOKA2 are functional hybrids of mammalian p62 and NBR1, capable of binding ATG8 and ubiquitin. Both were shown to play a role in nutrient deficiency and abiotic stress tolerance (Hafren *et al.*, 2017; Svenning *et al.*, 2011; Zhou *et al.*, 2013; Zhou *et al.*, 2014a; Zientara-Rytter *et al.*, 2011).

A fascinating cross talk between the major cellular degradation pathways, autophagy and

the proteasome, was uncovered with the discovery of both non-selective (starvation-induced) and selective autophagy mediated degradation of the 26S proteasome in Arabidopsis (Marshall *et al.*, 2015). While the proteasome subunit RPN10 was previously shown to facilitate the recognition of ubiquitinated targets, Marshall *et al.* found that it can also bind ATG8 (Marshall *et al.*, 2015). They further demonstrated that RPN10 is needed for inhibition-induced selective degradation of inactive 26S proteasome complexes (proteaphagy), suggesting a role for RPN10 as an autophagy cargo receptor. Intriguingly, in a recent study, mammalian p62 was shown to mediate selective starvation-induced autophagosomal uptake of proteasomes (Cohen-Kaplan *et al.*, 2016; Marshall *et al.*, 2016). Whether NBR1 or other plant cargo receptors have similar functions awaits further research.

Recent studies have demonstrated the selective degradation of peroxisomes by autophagy (Pexophagy). Peroxisomes are highly dynamic organelles, housing oxidative metabolic pathways, such as photorespiration and fatty acid β-oxidation, produce reactive oxygen species and contain important antioxidative components (Sandalio and Romero-Puertas, 2015). During seedling establishment, in a light-dependent manner, there is a functional transition from glyoxysomes, peroxisomes present in seeds and harboring the glyoxylate cycle and β-oxidation, to leaf type peroxisomes, containing photorespiration enzymes. Recent evidence shows that pexophagy takes place during this metabolic remodeling combined with peroxisomal protease LON2 activity (Young and Bartel, 2016). Pexophagy also mediates the turnover of peroxisomes damaged by H_2O_2 accumulation in old tissues, , under favorable and stress conditions, regulating the quality and number of peroxisomes (Shibata *et al.*, 2014; Shibata *et al.*, 2013). Pexophagy occurs at a higher rate in green tissues and appears to be more marked than other types of selective autophagy due to the highly oxidative peroxisomal metabolism (Yoshimoto *et al.*, 2014). Pexophagy is aided by autophagy receptors, although the plant autophagy receptor/adaptor protein linking ATG8 to damaged peroxisomes has not been identified. Some evidence, though controversial, suggests the involvement of NBR1 (Zhou *et al.*, 2013). Recently, 9 peroxines (PEXs, peroxisomal membrane proteins) have been identified as possible ATG8 binding proteins, two of which, AtPEX6 and AtPEX10, were shown to interact with ATG8 by bimolecular fluorescence complementation (BiFC) (Xie *et al.*, 2016). The signal involved in triggering

pexophagy has not yet been identified, although oxidized catalase has, controversially, been proposed as a possible candidate (Shibata *et al.*, 2013).

Chloroplasts represent an interesting case study for selective autophagy in plants, as they have unique turnover demands due to the photosynthetic electron chain and its oxidative byproducts. In addition, chloroplasts are the major nitrogen reservoir in mesophyll cells and thus important for nutrient recycling (Ishida *et al.*, 2014). Early studies suggested that the vacuole plays a role in chloroplast recycling (Minamikawa *et al.*, 2001; Wittenbach *et al.*, 1982) and autophagy-related and -unrelated pathways were later implicated in the degradation of chloroplast components (chlorophagy) (Ishida *et al.*, 2014; Izumi *et al.*, 2017; Martinez *et al.*, 2008; Michaeli *et al.*, 2014; Wang and Blumwald, 2014; Wang *et al.*, 2013; Xie *et al.*, 2016). Two types of vesicles, Rubisco containing bodies (RCBs) and ATG8-interacting protein1 (ATI1) bodies, were shown to participate in chlorophagy and are induced during senescence and abiotic stresses (Chiba *et al.*, 2003; Dong and Chen, 2013; Honig *et al.*, 2012; Ishida *et al.*, 2008; Michaeli *et al.*, 2014; Wada *et al.*, 2009; Yamane *et al.*, 2012). Interestingly, autophagy is also involved in the remobilization of transitory starch from chloroplasts to vacuoles via Small Starch Granules like structures (SSGLs) (Wang *et al.*, 2013). RCBs were characterized in Arabidopsis, tobacco, wheat and rice (Chiba *et al.*, 2003; Ishida *et al.*, 2008; Izumi *et al.*, 2015; Ono *et al.*, 2013; Prins *et al.*, 2008; Wada *et al.*, 2009). They were shown to deliver Rubisco and other stromal proteins to the vacuole, though their mode of cargo recognition is not known (Chiba *et al.*, 2003). ATI1 is a plant specific ATG8-binding protein localized in the ER and chloroplasts (Honig *et al.*, 2012; Michaeli *et al.*, 2014). In Arabidopsis, ATI1-labeled vesicles (ATI1-bodies) were shown to deliver plastid-targeted GFP to the vacuole. ATI1 can bind both stromal and membrane-bound chloroplast proteins, suggesting that the cargo of ATI1-bodies differ from that of RCBs (Michaeli *et al.*, 2014). Another difference is that RCBs are associated with chloroplast stromules, while ATI1 bodies initiate inside the chloroplast. In addition, the release of RCBs for the chloroplast is dependent on the ATG machinery , while ATI1 bodies bud from it in an ATG-independent manner, though their delivery to the vacuole requires active autophagy machinery (Ishida *et al.*, 2014; Michaeli *et al.*, 2014). Interestingly, two ESCRT-III subunit paralogs, were implicated in the delivery of RCBs to the vacuole, suggesting a cross talk between chlorophagy and endomembrane trafficking

304 events (Spitzer *et al.*, 2015)(Kalinowska and Isono, JXB review 2017, accepted for this
305 issue). Another chlorophagy pathway involves the vacuolar delivery of entire shrunken
306 chloroplasts (Wada *et al.*, 2009). This pathway is induced upon UV-B or high light
307 treatments (Izumi *et al.*, 2017). Information regarding selective autophagy of other types
308 of plastids is still limited, but there is evidence for RCB-like and entire plastid autophagy
309 in roots of Arabidopsis and rice (Izumi *et al.*, 2015; Nakayama *et al.*, 2012).

310 Mitophagy, the selective degradation of mitochondria by autophagy, was only recently
311 identified in plants with the characterization of an Arabidopsis ATG11-related protein.
312 Similarly to yeast, the Arabidopsis ATG11-related protein participates in the selective
313 clearance of mitochondria. Lack of ATG11-related protein in mutant Arabidopsis plant
314 resulted in mitochondria accumulation (Li *et al.*, 2014). However, a plant homolog to the
315 yeast ATG32, which recruits ATG11 to damaged mitochondria has not been identified
316 (Anding and Baehrecke, 2017; Li *et al.*, 2014). Plants also lack homologues of animal
317 mitophagy receptors such as the BCL2 interacting protein (BNIP) family members. ER-
318 phagy (reticulophagy), the selective degradation of ER by autophagy, is induced by ER
319 stress resulting from accumulation of unfolded or misfolded proteins in the ER, similarly
320 to yeast and mammals (Anding and Baehrecke, 2017; Dikic, 2017; Liu *et al.*, 2012; Yang
321 *et al.*, 2016). This process requires the ER stress sensor IRE1b, but the downstream factors
322 remain unknown (Liu *et al.*, 2012). In Arabidopsis, as in other organisms, ribophagy, the
323 autophagic degradation of rRNA, requires the nonspecific T2 endoribunuclease RNS2
324 (Bassham and MacIntosh, 2017; Floyd *et al.*, 2015; Floyd *et al.*, 2017; Hillwig *et al.*, 2011).
325 A differential role was suggested for ATG5 and ATG9 in this process, but the exact
326 mechanism of the selection of rRNA for degradation is still unknown (Floyd *et al.*, 2015)

327

328 **Methods of monitoring and manipulating autophagy in plants**

329 Monitoring autophagy in various systems has been previously described (Klionsky *et al.*,
330 2016). However, plant systems pose unique challenges requiring special modification.
331 Here we summarize some of the methods commonly used to assess and modulate
332 autophagy in plants, adding to some other excellent reviews on the topic (Bassham, 2015).

333 ***Monitoring autophagy in plants by biochemical analysis***

Assessing the formation and degradation of autophagosomes can be performed using western blot analysis. Two main approaches exist for this analysis: (i) ATG8 lipidation assay and (ii) free GFP release assay from expressed GFP-ATG8 chimera.

ATG8 is incorporated to the growing phagophore membranes through a C-terminal post-translational modification (processing followed by lipidation). Assessing the rate of ATG8 lipidation can be used as a measure of autophagosome formation. The lipidated and non-lipidated forms of ATG8 can be separated by SDS-PAGE in presence of 6 M urea followed by western blotting (Chung *et al.*, 2009; Thompson *et al.*, 2005). Expression of GFP-ATG8 can be used to visualize autophagosomes using confocal microscopy, as discussed later in this section. In addition, it is also possible to monitor the release of free GFP after proteolysis of GFP-ATG8 in the vacuole. The level of free GFP released from ATG8 indicates the relative rate of autophagosome degradation and can be used as a measure of autophagic flux (Li *et al.*, 2015; Slavikova *et al.*, 2008). GFP-ATG8 degradation in the vacuole is drastically reduced by Concanamycin A (ConcA) treatment. ConcA increases the pH of the vacuolar lumen by inhibiting the activity of the vacuolar H^+-ATPase. Therefore, ConcA treatment can result in autophagosomal bodies accumulating in the vacuole, hence reducing the proteolysis of expressed GFP-ATG8.

Imaging approaches to study plant autophagy

Live imaging of autophagosomes in plants requires both specific reporters and an adequate light microscope (LM) configuration. Multiple organic dyes such as LysoTracker (Liu *et al.*, 2005) and Monodansylcadaverin (MDC) (Contento *et al.*, 2005), have been used to label autophagosomes, based on the presumed acidity of the autophagic interior. However, their selectivity for autophagic compartments is still questionable (Klionsky *et al.*, 2016; Mizushima, 2004). Fluorescently-tagged ATG proteins are more frequently used as autophagosome markers, allowing a specific identification of autophagosomes at different stages of their maturation (Le Bars *et al.*, 2014; Suttangkakul *et al.*, 2011).

Tracking autophagosome formation and dynamics within plant cells may be complicated because : i) the lifetime of the process is very short, ii) ATG proteins are only transiently associated with the autophagosomal membranes, iii) low expression levels of potential marker proteins and their high dynamics in certain cell types. To circumvent these

limitations, one possibility is to use a light microscope equipped with highly sensitive detectors and image acquisition at a high frame rate. These conditions are met using confocal laser scanning microscopes with a resonant scanner, or a spinning disk microscope whose high-speed acquisition rate can also contribute in lowering the phototoxic effect of the imaging process (Figure 2a).

It is worth mentioning that mechanical stress could arise from tissue preparation and mounting between the microscope slide and coverslip conducive of autophagy induction (Wang *et al.*, 2016a). Having a spacer between the coverslip and the slide and performing the microscopic observations immediately following mounting can alleviate these unwanted artefacts in the experimental design. Rootchip (Grossmann *et al.*, 2011) can be a good strategy to allow long-term observation of Arabidopsis roots without affecting autophagy.

Higher resolution autophagic structures can be visualized with transmission electron microscopy (TEM) (Figure 2b-c). Correlative light and electron microscopy (CLEM) protocols, allowing LM and TEM observations of the same sample can be used to combine the localization of ATG proteins with light microscopy and the identification of the labelled membranes with TEM (Marion *et al.*, 2017).

Indirect (using anti-GFP antibodies) (Figure 2b) or direct (using specific antibodies against plant ATG8) (Chung *et al.*, 2010) TEM immunogold labelling of ATG8 can provide ultrastructural details of ATG8 membrane-bound structures including autophagosomes. A convenient and feasible processing method for ATG8 immunogold labeling is freeze-substitution (FS) followed by cryoembedding in an acrylic resin (Figure 2c). Several protocols of FS have been developed, providing excellent ultrastructure and high sensitivity immunogold labelling of various antigens, including membrane-bound molecules (Andreu *et al.*, 2007; Bernal *et al.*, 2007; Derrien *et al.*, 2012; Segui-Simarro *et al.*, 2011; Segui-Simarro *et al.*, 2003). This strategy has revealed the localization of ATG8 in autophagosomes and autolysosomes in various plant cells and tissues such as maize aleurone (Reyes *et al.*, 2011), Arabidopsis root (Zhuang *et al.*, 2013), or *Brassica napus* tapetum (Figure 2c), a tissue with high autophagy activity during late pollen development (Hanamata *et al.*, 2014; Papini *et al.*, 2014). The development of antibodies against plant

ATG proteins, with high specificity and sensitivity, will help to identify the components and ultrastructural organization of autophagic structures in diverse plant cells and systems.

Approaches for manipulating plant autophagy

Since autophagy is a very dynamic process, it needs to be and is in fact tightly regulated at multiple levels: transcriptional, post-transcriptional, translational and post-translational (Feng *et al.*, 2015; Kraft *et al.*, 2008)

Targeting transcriptional regulation of plant *ATG* genes

Some ATG proteins are either actively incorporated into autophagosomes as their integral part or are engulfed together with the cargo destined for degradation (Nakatogawa, 2013; Nakatogawa *et al.*, 2012). Autophagy is constitutively active at the basal level in most types of plant cells, playing a housekeeping role. Hence, *ATG* genes are constitutively transcribed, albeit at lower levels (Pu *et al.*, 2017). Interestingly, expression of multiple plant *ATG* genes goes up under stress, e.g. under starvation conditions, coinciding with upregulation of autophagic activity (Chung *et al.*, 2010; Minina *et al.*, 2013b; Rose *et al.*, 2006). Thus, identification of master regulators influencing the expression of *ATG* genes is an important step towards the development of autophagy-modulating tools. Multiple transcription factors regulating *ATG* gene expression in animal cells have already been identified (Feng *et al.*, 2015). Although there is no doubt that such transcription factors also exist in plants, information about them is still very scarce. For example, it has been demonstrated that induced expression of Arabidopsis' *ATG* genes upon *Botrytis cinerea* infection is directly mediated by the transcriptional activator AtWRKY33 (Lai *et al.*, 2011). In addition, the tomato transcription factor HsFA1a binds the promoters of *ATG10* and *ATG18f* to activate their transcription upon drought stress (Wang *et al.*, 2015a).

To date, phenotypic studies of the role of autophagy in plants have been based on comparing the performance of either *ATG*-knockout or knockdown lines to wild–type plants under various unfavorable conditions (Kim *et al.*, 2012). All these studies collectively indicate a potential benefit of upregulated autophagy for stress tolerance and plant fitness. While ectopic overexpression of *ATG* genes in yeast did not seem to have an effect on autophagic activity (Ma *et al.*, 2007), a growing body of evidence indicates that

overexpression of *ATG* genes might be successfully used for upregulation of autophagy in other model organisms, including plants (Minina *et al.*, 2018; Pyo *et al.*, 2013; Scott *et al.*, 2007; Wang P *et al.*, 2016; Wang *et al.*, 2017b; Xia *et al.*, 2012). These results indicate that the level of the core ATG proteins are a limiting factor of autophagic activity in plant and animal cells, but not in yeast. A possible explanation of this phenomenon is the difference in the number of phagophore assembly sites (PAS), where the core ATG proteins are active. While yeast has a single PAS, animal and plant cells do not seem to have a limit in the number of PASes. Thus, availability of a higher amount of the core ATG proteins might stimulate the formation of a higher number of PASes, leading to increase formation of autophagosomes (Minina *et al.*, 2018). The predicted benefit of enhanced autophagy for plant fitness, fecundity, biomass and stress tolerance has been described in the recent studies (Minina *et al.*, 2018; Wang *et al.*, 2016b). Further development of this approach is required as there might be penalties for constitutive upregulation of plant autophagy in most of the tissues as well as benefits of tissue/organ-specific stimulation of autophagic activity.

Targeting post-transcriptional regulation of plant *ATG* genes

Although multiple examples of miRNA regulating autophagy are known for animal models (Feng *et al.*, 2015), almost nothing is known about post-transcriptional regulation of autophagy in plants. Indirect evidence of possible regulation of autophagy by miRNA *via* the stress sensor SnRK1 was demonstrated in the study by Confraria *et al* (Confraria *et al.*, 2013). So far, post-transcriptional silencing of plant *ATG* genes has only been implemented by using artificial *ATG*-specific RNAi constructs (Kim *et al.*, 2012).

Targeting translational regulation of plant ATG genes

Under stress conditions, autophagy degrades cytoplasmic content together with ribosomes, thus downregulating the translation of most mRNAs, including ATG mRNAs (Bassham and MacIntosh, 2017; Kraft *et al.*, 2008). Importantly, selective degradation of ribosomes, ribophagy, under normal conditions positively affects the efficacy of the translational machinery by controlling ribosome quality (Mathis *et al.*, 2017). Artificial modulation of autophagy at the translational level has not yet been attempted due to numerous challenges regarding the specificity of this approach.

453 Pharmacological modulation of plant autophagy

454 As compared to animals, pharmacological manipulation of autophagy in plant has not been
455 comprehensively tested in part due to poor cellular accessibility of many of the described
456 chemical modulators. Paradoxically, some of the natural chemicals tested for their
457 modulation of animal cell autophagy, are plant-derived and we know nothing about their
458 potential effect on plant autophagy (Fleming *et al.*, 2011; Vakifahmetoglu-Norberg *et al.*,
459 2015; Wang *et al.*, 2017c). There are several compounds that have been demonstrated to
460 either inhibit or stimulate plant autophagy ((Klionsky *et al.*, 2016), Table 1). Drug
461 treatment can be a quick and a relatively easy method to modulate autophagy activity in
462 plants. The important disadvantages of pharmacological modulation of plant autophagy are
463 potential off-target effects of the drugs currently available (Table 1), issues with drug
464 stability and tissue/cell-permeability. Nevertheless, this approach has a very important
465 practical benefit, as it may be applicable for agricultural purposes in the countries that do
466 not allow cultivation of genetically modified organisms.

467

468 **Autophagy responses to abiotic stress in plant**

469 Plant stress has been defined by Lichtenthaler (1996) as "any unfavorable condition or
470 substrate that affects or blocks a plant metabolism, growth or development". A common
471 feature of abiotic stresses such as high salinity, drought and osmotic stress is their ability
472 to induce, at the cellular level, a transient or permanent physiological water deficit,
473 conducive of energy limitation in plants. Low energy level in plant tissues is sensed by a
474 subfamily of serine/threonine kinases known as SnRK1 (SNF1-related kinase),
475 homologous to the yeast SNF1 (Sucrose Non-Fermenting-1) and the animal AMPK
476 (Adenosine MonoPhosphate-activated protein Kinase). Plant SnRK1 act as metabolite
477 sensors to constantly adapt metabolism to the supply of, and demand for, energy, and are
478 central integrators of a transcriptional network for stress and energy signaling (Bakshi *et*
479 *al.*, 2017; Emanuelle *et al.*, 2015; Jossier *et al.*, 2009; Nukarinen *et al.*, 2016). SnRK1-
480 dependent restoration of energy homeostasis and promotion of tolerance to adverse
481 conditions is partly achieved through an induction of catabolic processes and a general
482 repression of anabolism (Emanuelle *et al.*, 2015; Soto-Burgos and Bassham, 2017).

Multitudes of unrelated cellular pathways converge on the autophagy machinery to signal a diversity of stimuli. Indeed, activated SnRK1 induces the catabolic pathway autophagy by inhibiting its negative regulator TOR (Target Of Rapamycin) complex in plants (Chen *et al.*, 2017; Soto-Burgos and Bassham, 2017). A crucial feature of autophagy is that it is a highly regulated and dynamic process, able to sense intracellular stress within minutes and rapidly initiate an appropriate response to cope with the damage (Antonioli *et al.*, 2017). High salinity and osmotic stress induce autophagy in plant tissues within a couple of hours of incubation into stress-induction medium (Liu *et al.*, 2009; Vanhee *et al.*, 2011b). Accordingly, many core *ATG* genes are transcriptionally upregulated by various abiotic stresses (Bassham *et al.*, 2006; Wang *et al.*, 2015a; Zhou *et al.*, 2014a). Conversely, autophagy-deficient plants are more sensitive to abiotic stresses (Liu *et al.*, 2009). Recent evidence also suggest that ectopic overexpression of defined plant core *ATG* genes can confer tolerance to various types of stresses and improve growth performance under nutrient starvation conditions (Minina *et al.*, 2018; Wang *et al.*, 2017b)

The plant adaptation responses to abiotic stresses involve phytohormones-dependent signaling cascades, including that of the stress hormone abscisic acid (a growth negative regulator) and that of brassinosteroid (a growth promoting regulator) to reprogram its metabolism (Mair *et al.*, 2015). When subjected to an abiotic stress, plants have to balance between maintaining growth and competitiveness on the one hand, and ensuring survival on the other hand (Claeys and Inze, 2013). This delicate and vital process involves hormone-regulated master regulators, some of which have been characterized recently.

ABI1 (ABA insensitive 1) and PP2CA (protein phosphatase 2C-A) are negative regulators of ABA-dependent signaling, and the two phosphatases were shown to dephosphorylate and inactivate SnRK1. The repressive action of protein phosphatases, established negative regulators of the ABA signaling pathway, is blocked by their ABA-dependent interaction with ABA receptors (Emanuelle *et al.*, 2015). ABA-dependent signaling results in the expression of effector proteins regulating different aspects of plant physiology. The polytopic transmembrane protein TSPO is a multi-stress regulator, transiently induced by water-related stress and ABA treatment in plants (Guillaumot *et al.*, 2009). The induced Arabidopsis TSPO protein is also rapidly (within 48 hours) degraded, suggesting a time-limited role for it during stress. Plant TSPO may act as an autophagy cargo receptor for a

514 diverse set of cargo such as cytoplasmic free porphyrins and defined water channels
515 (Veljanovski and Batoko, 2014). AtTSPO interacts with a highly expressed plasma
516 membrane water channel, aquaporin PIP2;7, during osmotic stress. The aquaporin-TSPO
517 complex is targeted by autophagy for degradation in the vacuole, thus preventing PIP2;7
518 from reaching the plasma membrane and possibly protecting the cell from water loss
519 (Hachez *et al.*, 2014). However, constitutive expression of TSPO can be detrimental to
520 plant growth and development (Guillaumot *et al.*, 2009; Vanhee *et al.*, 2011a). This is
521 probably due to its intrinsic free heme binding capacity, and the consequence of this
522 cytoplasmic heme titration on ROS scavenger enzymes activity (Batoko *et al.*, 2015;
523 Vanhee *et al.*, 2011b). Enhanced ROS accumulation could generate ER-stress and chronic
524 UPR (Unfolded Protein Response) followed by cell death (Petrov *et al.*, 2015). The free
525 heme/porphyrin detoxification function of TSPO may be required only transiently, when
526 the plant cell needs to manage stress-induced ROS, and probably ROS-dependent signaling
527 events (Batoko *et al.*, 2015).

528 Plant TSPO is also upregulated by the growth-promoting hormone brassinosteroid (Nolan
529 *et al.*, 2017). Brassinosteroid (BR) plasma membrane receptor BRI1 (Brassinosteroid
530 Insensitive 1) and the downstream signaling components regulate the activity of the
531 transcription factor BES1 (BRI1-EMS Suppressor 1) (Li and Nam, 2002). BR inhibits the
532 activity of the kinase BIN2 that negatively regulates BES1 by phosphorylation. BES1
533 master transcriptional activity promotes plant growth, and its deregulation was shown
534 recently to enhance plant survival instead of growth during abiotic stress (Nolan *et al.*,
535 2017). During osmotic stress for example, BES1 is ubiquitylated and interacts with the
536 ubiquitin-binding receptor protein DSK2 (Dominant Suppressor of Kar2), a known
537 autophagy cargo receptor in higher eukaryotes (Lee *et al.*, 2013). BES1 is therefore
538 targeted for autophagy-mediated degradation as a response to abiotic stress. DSK2's
539 autophagy receptor activity is regulated by phosphorylation, the latter being catalyzed by
540 the BIN2 kinase. Loss-of-function *dsk2* mutant plants accumulate BES1 proteins, have
541 altered global gene expression profiles and compromised survival during abiotic stresses
542 (Nolan *et al.*, 2017). Consistently, constitutively active BR signaling mutant plants are
543 more sensitive to abiotic stress, suggesting that reducing growth during abiotic stress is a
544 vital mechanism for plant to survive during abiotic stresses. Although BES1 abundance

545 can be regulated by the ubiquitin proteasome system (Lin *et al.*, 2011), autophagy appears
546 to be a key pathway in achieving this tricky physiological and metabolic balance between
547 growth and survival.

548

549 **The role of autophagy in plant-pathogen interactions**

550 Autophagy is a central regulator of plant innate immunity. It can either act as survival or
551 cell death pathway in response to invading microbes with different pathogenic (i.e.
552 biotrophic or necrotrophic) lifestyles. Because of the co-evolutionary battle with their
553 hosts, several pathogens have developed various countermeasures to suppress, evade or
554 subvert autophagy processes to the benefit of infection. In addition, some eukaryotic
555 microbes require their own autophagy machinery for successful pathogenesis (Hofius *et*
556 *al.*, 2017). Most studies demonstrating the role of autophagy in plant-microbe interactions
557 have considered autophagy as a largely unspecific ("bulk") process. However, recent
558 reports indicate that plants are able to explore selective autophagic mechanisms to
559 effectively fend off microbial intruders, whereas some pathogens overcome plant
560 immunity by hijacking autophagy pathways for selective removal of host components
561 (Clavel *et al.*, 2017; Dagdas *et al.*, 2016; Hafren *et al.*, 2017; Haxim *et al.*, 2017). In this
562 section, we will briefly discuss the role of autophagy in different immunity- and disease-
563 related contexts, including the hypersensitive response (HR) to avirulent pathogens as well
564 as infections with virulent fungal, viral, oomycete and bacterial species. More
565 comprehensive reviews on the topic are available from (Hofius *et al.*, 2017; Minina *et al.*,
566 2014; Zhou *et al.*, 2014b) and in this Special Issue on Plant Autophagy from Leary et al.
567 (Leary *et al.*, 2018).

568 Pathogen recognition by the plant immune system often results in HR, a localized form of
569 programmed cell death (PCD) activated by intracellular immune receptors [known as
570 resistance (*R*) genes] (Coll *et al.*, 2011). HR levels were reduced in autophagy-deficient
571 mutants infected with avirulent bacteria and oomycetes, or enhanced in autophagy-
572 stimulated transgenic plants upon virus challenge (Coll *et al.*, 2011; Hackenberg *et al.*,
573 2013; Han *et al.*, 2015; Munch *et al.*, 2014; Munch *et al.*, 2015). Hence, autophagy acts
574 locally as a positive regulator of HR. Autophagy mutants were also shown to display

575 unrestricted cell death upon HR induction (Liu *et al.*, 2005; Yoshimoto *et al.*, 2009)
576 suggesting that autophagy can contribute to the confinement of HR, thus minimizing
577 damage to healthy, non-infected tissue (Hofius *et al.*, 2011). This pro-survival effect of
578 autophagy might be linked to its homeostatic role in eliminating potentially noxious by-
579 products of systemic responses triggered during infection (Coll *et al.*, 2014; Hofius *et al.*,
580 2011; Munch *et al.*, 2014; Yoshimoto *et al.*, 2009).

581 An additional pro-survival role of autophagy in immunity has been revealed in the context
582 of plant defense against necrotrophs, which deliberately activate cell death to retrieve
583 nutrients from the host. Autophagy-deficient mutants displayed enhanced disease-
584 associated cell death and pathogen growth upon infection with different necrotrophic fungi
585 (Katsiarimpa *et al.*, 2013; Lai *et al.*, 2011; Lenz *et al.*, 2011; Li *et al.*, 2016b), whereas
586 plants with elevated level of autophagy showed increased resistance (Minina et al. 2018).
587 Besides restricting disease-associated necrotic cell death, autophagy may also contribute
588 to basal defense against necrotrophs by modulating hormone levels or eliminating toxic
589 cellular constituents induced as part of the disease response (Lai *et al.*, 2011). Some
590 necrotrophic fungi have therefore evolved mechanisms to overcome autophagy-mediated
591 defenses in plants. For example, secretion of the phytotoxin oxalic acid by *Sclerotinia*
592 *sclerotiorum* results in unrestricted host cell death via autophagy inhibition (Kabbage *et*
593 *al.*, 2013).

594 Intracellular pathogens in animals are often subject to direct targeting and elimination by
595 the autophagy machinery in a process referred to as xenophagy (Levine *et al.*, 2011;
596 Mostowy, 2013; Paul and Munz, 2016). In plants, viruses are the only pathogens with
597 intracellular replication, but the anti- and pro-viral functions of autophagy in host immunity
598 and viral pathogenesis have only recently begun to emerge (Clavel *et al.*, 2017). Most
599 strikingly, selective autophagy mechanisms were discovered as integral part of the innate
600 immune response against different DNA viruses. The cargo receptor NBR1 mediates
601 autophagic degradation of non-assembled capsid proteins and viral particles of *Cauliflower*
602 *mosaic virus* (CaMV), providing a first example of xenophagy in plants (Hafren *et al.*,
603 2017). Likewise, the virulence factor βC1 of *Cotton leaf curl Multan virus* (CLCuMuV) is
604 selectively targeted during infection (Haxim *et al.*, 2017). However, recruitment of this

viral suppressor of RNA silencing (VSR) to autophagosomes seems to involve direct interaction with ATG8 rather than distinct cargo receptors. The potyviral HCpro and cucumoviral 2b proteins, representing VSRs of RNA viruses, were also shown to undergo autophagic clearance but the link between their binding to the host protein rgsCaM and the autophagy machinery is unclear (Nakahara *et al.*, 2012). In contrast to the examples from DNA viruses, the biological relevance of the autophagic processes for antiviral immunity against RNA viruses remains to be shown.

As part of their counter defense, some viruses trigger the autophagic degradation of host antiviral RNA silencing pathway components (Cheng and Wang, 2017; Derrien *et al.*, 2012). In addition, virus-induced activation of bulk autophagy seems to benefit virus survival and particle production via suppression of disease-associated cell death and promotion of plant fitness (Hafren *et al.*, 2017). Hence, viral measures to interfere with xenophagic targeting may influence the pro-viral effects of bulk autophagy, implying a potential trade-off between suppression of antiviral autophagy and host survival.

The hemibiotrophic oomycete pathogen *Phytophthora infestans* was also shown to be targeted by NBR1-dependent autophagy processes as part of the host defense (Dagdas *et al.*, 2016). In turn, the *P. infestans* effector protein PexRD54 can outcompete the interaction of the NBR1 tobacco homolog Joka 2 with an ATG8 protein, which led to the speculation that *P. infestans* hijacks the autophagy pathway to selectively remove defense components or to recycle and deviate nutrients to the intracellular infection structures (Dagdas *et al.*, 2016). The role of autophagy during infection with the strictly biotrophic downy mildew oomycete and powdery mildew fungal species still remains unclear, probably because of the use of different autophagy-deficient mutant backgrounds and pathogen species or plant age-dependent alterations in cellular homeostasis and hormone signaling (Hofius *et al.*, 2009; Lenz *et al.*, 2011).

Similarly, the functions of autophagy during virulent bacterial infection are not well understood. There is the prevailing view that autophagy promotes plant susceptibility to infection with *Pseudomonas syringae* (Hofius *et al.*, 2017; Kwon *et al.*, 2013; Lenz *et al.*, 2011). The recent identification of the *Ralstonia solanacearum* AWR5 effector, which

inhibits the negative autophagy regulator TOR, further suggests that bacteria can exploit autophagy activation to enhance virulence (Popa *et al.*, 2016)

Autophagy as a facilitator of nutrient recycling and remobilization in plants

It is generally accepted that autophagy is involved in nutrient recycling and that it is induced under nutrient starvation. This role has been suspected since the early stages of autophagy research, when de Duve observed autophagosome structures in the livers of rats submitted to nutrient starvation (Deter *et al.*, 1967). Further, the possibility to induce autophagy for nutrient recycling in yeast using starvation was used by Oshumi and colleagues to set up a mutant screening strategy that permitted the discovery of the *ATG* genes (Takeshige *et al.*, 1992). In mice, the importance of autophagy in nutrient recycling was demonstrated by the strong impact of autophagic activity on newborn survival (Kuma *et al.*, 2004). In plants, hypersensitivity to carbon and nitrogen starvation has been established as a basic phenotype of *atg* mutants, characterized originally in Arabidopsis (Doelling *et al.*, 2002; Ishizaki *et al.*, 2005; Phillips *et al.*, 2008; Thompson *et al.*, 2005), but also shown in maize (Li *et al.*, 2015). However, our knowledge of the underlying molecular details of such interplay is limited.

Both carbon and nitrogen starvation are known to induce autophagy (Avila-Ospina *et al.*, 2016; Rose *et al.*, 2006). Expression of *ATG* genes was shown to increase upon carbon and nitrogen starvation in many plant species, including Arabidopsis, maize, tobacco, wheat and the model algae *Chlamydomonas reinhardtii*, as well as increased lipidation of ATG8 (Caldana *et al.*, 2011; Li *et al.*, 2015; Pei *et al.*, 2014; Perez-Perez *et al.*, 2010; Thompson *et al.*, 2005; Zientara-Rytter *et al.*, 2011). In addition, crossing *atg* mutants with starch deficient mutants was shown to exacerbate their starvation phenotype, demonstrating the tight link between autophagy and carbon supply under starvation (Izumi *et al.*, 2013). Links between autophagy and other nutrient deficiencies are less documented. Induction of some autophagy-related genes (*ATG8* and *Joka2*) in roots of tobacco plants grown in sulfur deficient conditions (Zientara-Rytter *et al.*, 2011) suggested that sulfur starvation induces autophagy activity. Indeed, it was recently shown in Arabidopsis that limited sulfur supply decreases soluble sugars, downregulates TOR activity, as demonstrated by downregulation

of its downstream target S6K, and increases level of the lipidated ATG8a (Dong *et al.*, 2017). Induction of autophagy under phosphorus starvation has also been suggested in the model algae *Chlamydomonas reinhardtii* and marine algae *Emiliania huxleyi* (Couso *et al.*, 2017; Shemi *et al.*, 2016). In Arabidopsis, it has been proposed that in the absence of phosphate, selective autophagy (with PUB9 as E3 ligase) is involved in degradation of auxin accumulation repressor, leading to auxin accumulation and lateral roots growth (Deb *et al.*, 2014).

We do not know what triggers the induction of autophagy-related genes during limitation of certain nutrients, however the signal, at least for nitrogen, carbon and sulfur starvation, is probably TOR-dependent (Dong *et al.*, 2017; Pu *et al.*, 2017; Rexin *et al.*, 2015). Interestingly, the level of hydrogen sulfide, a recently identified negative regulator of autophagy, drops during sulfur limitation, and, at least, in such conditions might be one of the triggers (Gotor *et al.*, 2013; Laureano-Marin *et al.*, 2016).

It is considered that starvation induces non-specific autophagy (i.e. bulk degradation) of cytoplasmic components for nutrient remobilization. In mammals, however, the selective degradation of lipid bodies under starvation was demonstrated in hepathocytes (Singh *et al.*, 2009). In addition, selective degradation of ald6p under nitrogen starvation has been demonstrated in yeast (Onodera and Ohsumi, 2004). The diversity of the cytoplasmic components dedicated for degradation by autophagy, including protein aggregates, membranes, organelles, suggests that in addition to C and N molecules, many other mineral nutrients could be released from the process. These compounds can then be used for the cell's own metabolism, to sustain respiration for example (Barros *et al.*, 2017) or dedicated for the whole organism after remobilization. Whether phosphate, iron, zinc, sulphur or potassium can be recycled though autophagy is not documented. Also it is unknown whether some selectivity exists in the cargos degraded by autophagy under starvation conditions. It is likely, for example, that under dark conditions, chloroplasts could be preferentially targeted and that autophagy could participate in starch degradation (Wang and Liu, 2013; Wang *et al.*, 2015b). Under low nitrate availability, autophagy would mainly participate in protein degradation but not starch degradation as proteins accumulated in *atg* mutants while starch was depleted (Guiboileau *et al.*, 2013). Under

carbon starvation, the situation is singularly the opposite, with increased usage of free amino acids, presumably as an alternative carbon source for respiration (Avin-Wittenberg *et al.*, 2015).

At the whole plant level, autophagy is an essential process for nitrogen remobilization from leaf to seeds as shown by the ^{15}N pulse-chase experiments performed in Arabidopsis (Guiboileau *et al.*, 2012). Based on this Arabidopsis study, pulse-chase labelling strategy was used on maize *atg12* mutants that showed accordingly lower N mobilization to the seeds (Li *et al.*, 2015). Both studies thus confirmed that autophagy manages nutrient resources in source leaves and that its role for seed formation and seed filling is fundamental. In Arabidopsis, the composition of *atg* mutant seeds is strongly modified as their nitrogen content mainly relies on the post anthesis nitrate uptake rather than N remobilization from leaves (Guiboileau *et al.*, 2012). Because of their poor N remobilization capacity, *atg* mutant display lower yield and lower harvest index. Whether increasing autophagy activity in the source leaves during senescence could conversely increase plant performance in seed production and seed quality is then a major issue to be investigated.

While several studies have performed metabolic profiling of *atg* mutants (Avin-Wittenberg *et al.*, 2015; Barros *et al.*, 2017; Masclaux-Daubresse *et al.*, 2014), analysis of the changes in the metabolic fluxes are considerably less common. Metabolic flux analysis relies on determining the redistribution of label over time in order to estimate the atomic flux between pools of different metabolic species. Two approaches are commonly used: (i) radiolabeled isotopes, namely ^{14}C and ^{35}S and ^{3}H, and (ii) stable isotopes, such as ^{13}C and ^{15}N (Batista Silva *et al.*, 2016). Few works investigate primary metabolic fluxes in connection to autophagy and in plants. In the first study, etiolated wild type and *atg* mutant Arabidopsis seedlings were incubated in the presence of either uniformly labelled ^{14}C-glucose, positionally labelled ^{14}C glucoses or ^{13}C lysine in order to characterize the respiratory metabolism of these mutants (Avin-Wittenberg *et al.*, 2015). These revealed various effects, including lower protein synthesis and an accumulation of label in specific amino acids and TCA cycle intermediates. As mentioned above, the change in amino acid levels were different from that reported during nitrogen deficiency (Masclaux-Daubresse

et al., 2014). It would therefore be interesting to examine the impact of autophagy deficiency on metabolic fluxes in a range of conditions/tissues other than the etiolated seedling. These examples highlight the power of incorporating flux analyses into studies on plant autophagy, suggesting that their greater adoption will yield further insights into molecular and energetic mechanisms regulating and being modulated by autophagy.

Targeting autophagy in plant oil production

Plant oils play pivotal role in human nutrition and the potential for plant oils to replace fossil oil in chemical industry is likewise immense. To realize the full potential of using plant oils, it is crucial to optimize quantity and quality of the oil *in planta* using genetic and metabolic engineering. As in all eukaryotes, plants store their lipid reserves in specialized organelles, lipid droplets (LDs), which are especially abundant in seeds of oilseed crops. Recent research using animal and yeast systems has established that autophagy plays pivotal role in both breakdown and biogenesis of LDs (Singh *et al.*, 2009; Zhang *et al.*, 2009) and that LDs in return can regulate autophagy (Shpilka *et al.*, 2015). The process of autophagic degradation of LDs in the lysosome or lytic vacuole has been named "lipophagy" and shown to crosstalk in a number of ways with cytosolic lipolysis (Zechner *et al.*, 2017).

It has been shown that Arabidopsis mutants in beta-oxidation of fatty acids have greatly reduced seed oil content, demonstrating that turnover of lipids is an essential component for efficient seed oil accumulation (Lin *et al.*, 2004). Therefore, can manipulation of autophagy be used as a tool to improve oil crops? To-date, the evidence for the role of autophagy in biogenesis or degradation of LDs in plants is rather scarce and fragmented, encompassing only a few species. Thus, autophagy is required for the formation of LDs in tapetal cells and phospholipid editing in rice pollen (Kurusu *et al.*, 2014). Two cytological studies using electron microscopy have revealed micro- and macroautophagy-mediated engulfment of LDs in the algae *Auxenochlorella protothecoides* (Zhao *et al.*, 2014) and *Micrasterias denticulata* (Schwarz *et al.*, 2017) respectively. Finally, although autophagy does not seem to be critically required for Arabidopsis seed development, efficient mobilization of lipids upon seed germination under carbon-deprived conditions is at least partly dependent on autophagy (Avin-Wittenberg *et al.*, 2015). Clearly, more research is

754 needed to establish a solid platform for biotechnological application of autophagy in
755 regulating plant oil reserves (Elander *et al.*, 2018).

756

757 **Future perspectives**

758 The study of autophagy in plants has boomed in the last few years, and our understanding
759 of the function and regulation of this complex mechanism is steadily expanding. However,
760 much work is still needed in order to understand the many facets of autophagy and utilize
761 it for agricultural use. Primarily, it is very importance to continue deciphering the
762 mechanisms regulating autophagy in plants, as these are still only partially understood.
763 Better understanding of the regulation of autophagy will assist in the modulation of
764 autophagy on the field. In the field of selective autophagy, for example, information on the
765 role of selective autophagy in plant development is lagging behind. In addition, the cargo
766 receptors or other specificity factors involved in selective autophagy need to be further
767 identified and characterized and the role of ubiquitin-tagging in organelle degradation
768 elucidated. As a complementary approach, understanding the functional differences
769 between the different plant ATG8 isoforms would be very useful. Most of our
770 understanding of selective autophagy pathways is currently based on studies in model
771 plants, mainly Arabidopsis. More emphasis should be given to expanding the research to
772 crops and to possible specific differences in autophagy pathways and responses.

773 Translating the knowledge gained from model systems to crop plants is also a challenge
774 for understanding the interplay between autophagy and plant pathogens, which cause
775 devastating economical losses to farmers and threaten global food security. Future work
776 will help gaining additional insight into the molecular mechanisms that pathogens use to
777 exploit plant autophagy for their own benefit and deepen our understanding of the
778 autophagic components and pathways contributing to plant innate immune responses.

779 Development of artificial tools for modulating plant autophagy will allow us to control
780 crop fitness, stress-tolerance and productivity, eliminating the need in laborious and time-
781 consuming breeding process. Advances in CRISP/CAS9-based genetic editing tools and
782 high-throughput drug screens should facilitate manipulation of autophagy in crops. All this

783 may result in the production of crops with increased nutrient remobilization, able to cope
784 better with nutrient starvation and increase efficacy of agriculture and its adjustability to
785 the changing climate conditions, as well as stability under high pathogen pressure in the
786 field.

787

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Table 1. Tools for plant autophagy modulation

			Confirmation of the expected effect on autophagy		
Type of regulation	**Effect on autophagy**	**Suggest mechanism of action/target**	**Algae**	**Mosses**	**Seed plants**
Genetic regulation					
Knockout of *ATG* gene(s)	Inhibition		Possibly (Zhang *et al.*, 2014)	Yes (Mukae *et al.*, 2015), (Vera *et al.*, 2017)	Yes (Kim *et al.*, 2012)
Knockdown of *ATG* gene(s)	Inhibition		-	-	Yes (Kim *et al.*, 2012)
Overexpression of *ATG* gene(s)	Stimulation		-	-	Possibly (Xia *et al.*, 2012), (Wang *et al.*, 2017; Wang *et al.*, 2016)
Pharmacological regulation					
Rapamycin	Stimulation	An inhibitor of TOR kinase	Yes (Crespo *et al.*, 2005)	Reference to personal communication in (Menand *et al.*, 2002)	In Arabidopsis it requires the presence of FKBP12 (Ren *et al.*, 2012), (Zhang *et al.*, 2013)
AZD8055	Stimulation	TOR kinase active site inhibitor	Yes (Imamura *et al.*, 2016)	-	Yes (Dong *et al.*, 2015)
Torin1	Stimulation	TOR active site inhibitor	Yes (Imamura *et al.*, 2016)	-	Yes (Montane and Menand, 2013)
KU63794	Stimulation	TOR active site inhibitor	-	-	Yes, especially in combination with rapamycin (Deng *et al.*, 2017)

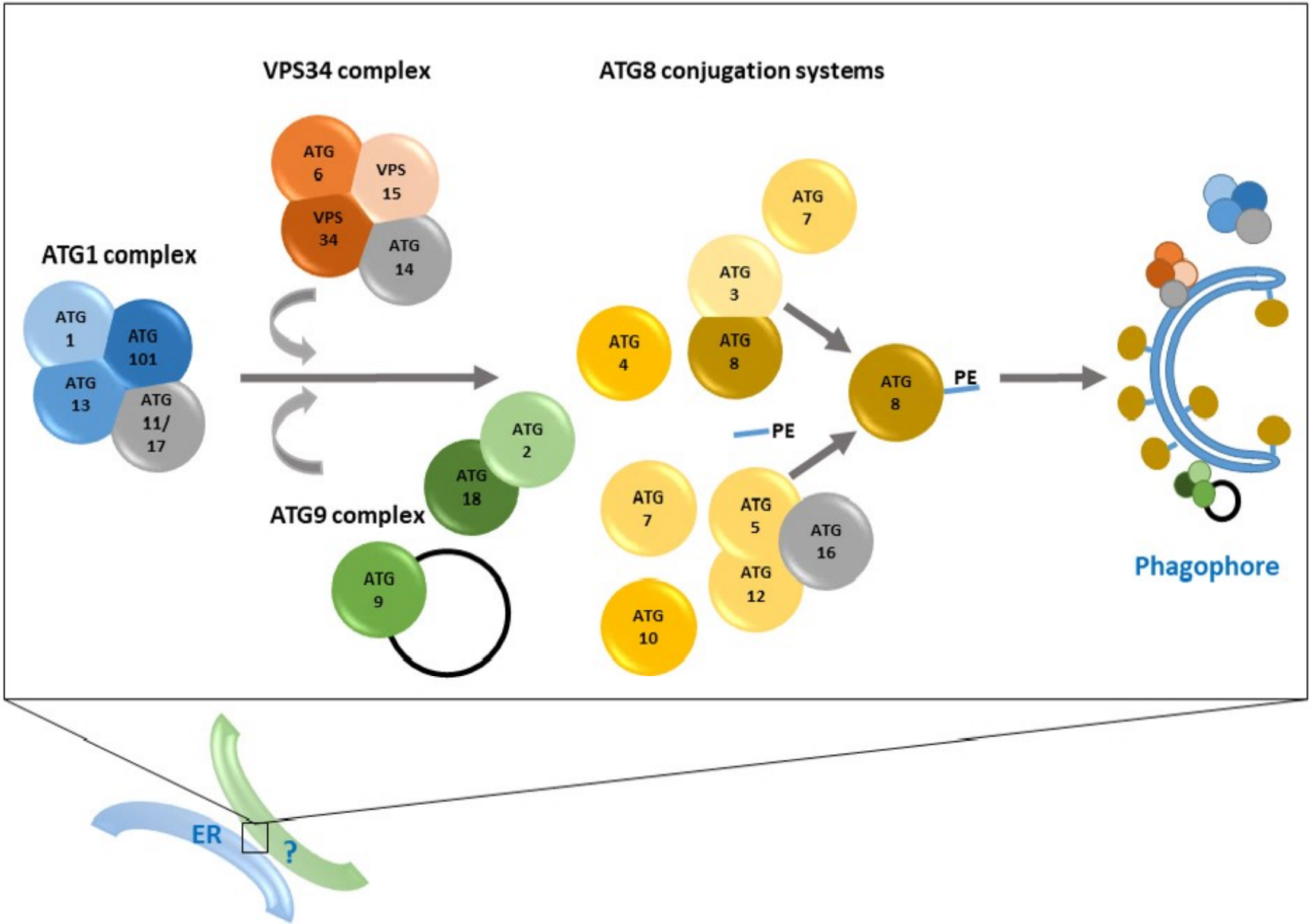
3-MA (3-methyladenine)	Inhibition, but also might lead to enhancement	A pan Phosphatidylinosytol-3 kinase (PI3K) inhibitor. Can persistently inhibit class III PI3K and transiently inhibit class I PI3K.	Yes (Jiang *et al.*, 2012)	-	Yes (Takatsuka *et al.*, 2004), (Wang *et al.*, 2013)
Wortmannin	Inhibition	A pan Phosphatidylinosytol-3 kinase (PI3K) inhibitor. Inhibit class I and III PI3K with the same efficacy.	-	-	Yes (Takatsuka *et al.*, 2004)
LY294002	Inhibition, but also might lead to enhancement	A pan Phosphatidylinosytol-3 kinase (PI3K) inhibitor. Inhibits activity of class I and class III PI3Ks and additionally influences Ca^{2+} homeostasis.	-	-	Yes (Takatsuka *et al.*, 2004)
Bafilomycin A1	Inhibition	A specific inhibitor of vacuolar H^+-ATPase	-	-	Yes. In BY-2 it gives a weaker effect than Concanamycin A (Matsuoka *et al.*, 1997)
Concanamycin A	Inhibition	A specific inhibitor of vacuolar H^+-ATPase	-	Yes (Mukae *et al.*, 2015)	Yes (Matsuoka *et al.*, 1997; Yano *et al.*, 2015)
E-64c/d	Inhibition	A cysteine-protease inhibitor, blocks degradation of autophagic cargo and ATG4 activity	Possibly (Moriyasu, 1995)	Yes (Mukae *et al.*, 2015)	Yes (Oh-ye *et al.*, 2011; Yano *et al.*, 2015)
BTH (benzothiadiazole)	Stimulation	Acts as analog of salicylic acid	-	-	Yes (Yoshimoto *et al.*, 2009)
Fumonisin B1	Stimulation	An inhibitor of sphingosine N-acyltransferase	-	-	Possibly (Qin *et al.*, 2017)

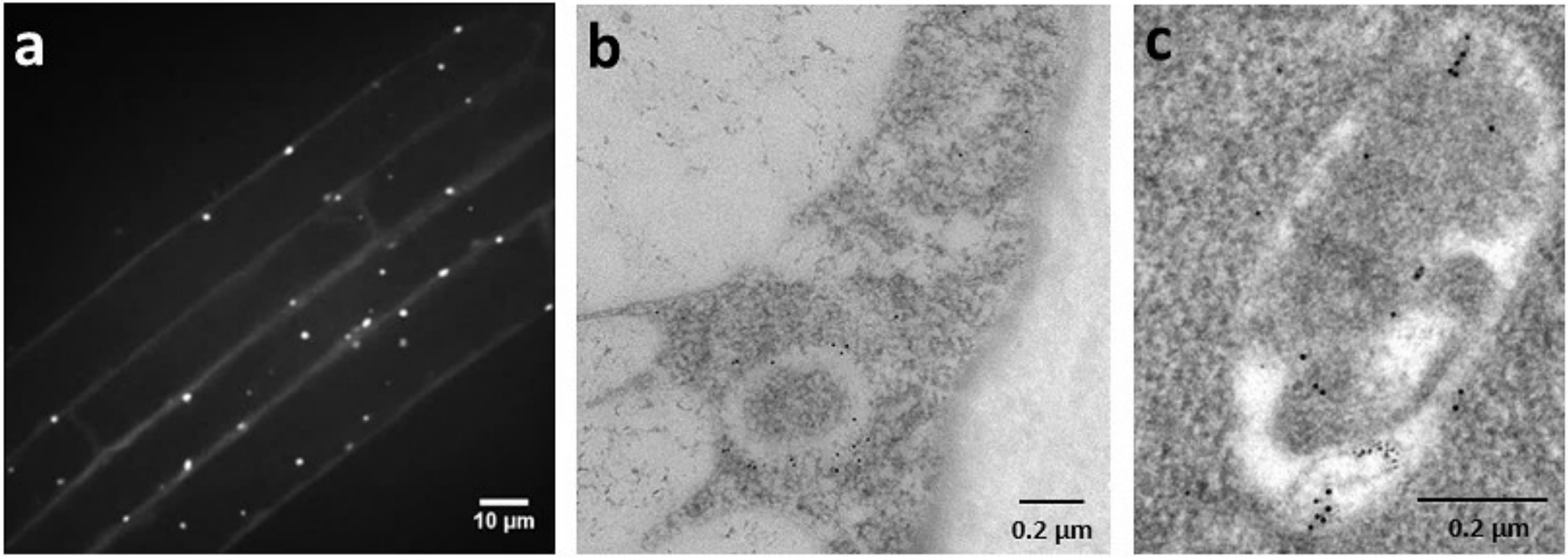
Tunicamycin	Stimulation	Induces ER stress	Possibly (Diaz-Troya *et al.*, 2011)	-	Yes (Yang *et al.*, 2016)
Polyamines	Stimulation	Spermidine was suggested to influence expression of *ATG* genes by changing chromatin structure	-	-	Possibly (Sequera-Mutiozabal *et al.*, 2016)

"-", no published data is yet available

Figure 1: Autophagic response initiation in plants: complexes in complexity. In response to a stimulus, the ATG1 complex is formed and targeted to an organelle contact site involving the endoplasmic reticulum (ER) and an unknown organelle (?). The ATG1 complex activates and recruits the VPS34 complex resulting in local PI3P (phosphatidylinositol-3-Phosphate) synthesis and enrichment within the organelles contact site. ATG9-containing vesicles (black circle) are docked to the contact site by ATG9 interaction with ATG2-ATG18 dimers, site-localized through ATG18 binding to PI3P, the input of membrane lipids and defined proteins contributing in the formation of the phagophore. The phagophore membranes are decorated with enzymatically processed and lipidated (conjugation to PE, phosphatidylethanolamine) ATG8. This process is facilitated by components of the ATG8 conjugation systems. Putative subunits of the various complexes not yet characterized in plant are illustrated in grey.

Figure 2. Imaging of plant autophagic structures and subcellular localization of ATG8 by different microscopy approaches. (a) Live imaging of ATG8-GFP reporter proteins in Arabidopsis roots observed by spinning disk confocal microscope. (b) TEM micrograph of the same plant tissues immunolabelled for ATG8 -GFP detection using anti-GFP antibodies. Note the gold particles on the autophagosome membrane. (c) TEM immunogold labelling with anti-ATG8 antibodies of a tapetal cell of *Brassica napus*.


VPS34 complex
ATG8 conjugation systems
ATG1 complex
ATG 6
VPS 15
VPS 34
ATG 14
ATG 1
ATG 101
ATG 13
ATG 11/ 17
ATG 2
ATG 18
ATG9 complex
ATG 9
ATG 7
ATG 3
ATG 4
ATG 8
PE
ATG 8
PE
ATG 7
ATG 5
ATG 16
ATG 12
ATG 10
Phagophore
ER
?


a
10 µm
b
0.2 µm
c
0.2 µm