Dynamics of Autophagosome Formation

Junmarie Soto-Burgos  
*Iowa State University*

Xiaohong Zhuang  
*The Chinese University of Hong Kong*

Liwen Jiang  
*The Chinese University of Hong Kong*

Diane C. Bassham  
*Iowa State University, bassham@iastate.edu*

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Dynamics of Autophagosome Formation

Abstract
Autophagy, literally defined as “self-eating,” functions as a degradation process by recycling cytoplasmic contents under stress conditions or during development. Upon activation of autophagy, a membrane structure known as a phagophore forms and expands, finally closing to form a double-membrane vesicle called an autophagosome (Fig. 1; Lamb et al., 2013; Yin et al., 2016). The completed autophagosome, which contains the autophagic cargo, is delivered to the vacuole (plants and yeast) or lysosome (animals). The outer membrane fuses with the vacuolar/lysosomal membrane, and the inner membrane and contents are released into the vacuole/lysosome as an autophagic body and are degraded by hydrolases. The breakdown products are transported back into the cytoplasm for reuse by the cell (Yang and Bassham, 2015).

Disciplines
Cell Biology | Developmental Biology | Genetics | Plant Biology

Comments
Update on Autophagy

Dynamics of Autophagosome Formation1[OPEN]

Junmarie Soto-Burgos,a,2 Xiaohong Zhuang,b,2 Liwen Jiang,b and Diane C. Basshama,3

aDepartment of Genetics, Development and Cell Biology, Iowa State University, Ames, Iowa 50011
bCentre for Cell and Developmental Biology, State Key Laboratory of Agrobiotechnology, School of Life Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China
ORCID IDs: 0000-0002-2480-8210 (X.Z.); 0000-0001-7411-9360 (D.C.B.).

Autophagy, literally defined as “self-eating,” functions as a degradation process by recycling cytoplasmic contents under stress conditions or during development. Upon activation of autophagy, a membrane structure known as a phagophore forms and expands, finally closing to form a double-membrane vesicle called an autophagosome (Fig. 1; Lamb et al., 2013; Yin et al., 2016). The completed autophagosome, which contains the autophagic cargo, is delivered to the vacuole (plants and yeast) or lysosome (animals). The outer membrane fuses with the vacuolar/lysosomal membrane, and the inner membrane and contents are released into the vacuole/lysosome as an autophagic body and are degraded by hydrolases. The breakdown products are transported back into the cytoplasm for reuse by the cell (Yang and Bassham, 2015).

The initial identification of many autophagy-related (ATG) genes in yeast (Tsukada and Ohsumi, 1993; Thumm et al., 1994; Harding et al., 1995) was key in beginning to understand the mechanism by which autophagy occurs. The core machinery for autophagosome formation includes ATG1, which forms a complex with ATG13 for the induction of autophagy (Kamada et al., 2000); two ubiquitin-like conjugates, ATG12-ATG5 and ATG8-PE, which are recruited to the phagophore assembly site and play an important role in autophagosome formation (Yin et al., 2016); and ATG9, which may function in the recruitment of other ATG components and membrane to the forming autophagosome (Reggiori et al., 2005). In plants, autophagy has been well studied as a response to stress conditions, including nutrient deficiency (Doelling et al., 2002; Hanaoka et al., 2002), salt and drought stress (Liu et al., 2009), heat stress (Zhou et al., 2013; Yang et al., 2016), oxidative stress (Xiong et al., 2007), hypoxia (Chen et al., 2015), pathogen attack (Liu et al., 2005; Lai et al., 2011), and endoplasmic reticulum (ER) stress (Liu et al., 2012; Fig. 2). In this review, we summarize recent advances in our understanding of the dynamics of plant autophagy, focusing on regulation of autophagy and mechanisms of autophagosome formation.

REGULATION OF AUTOPHAGY

The autophagy pathway is highly conserved among all eukaryotes. In plants, it is activated during development and in response to stress, and a basal level of autophagy is important for cellular homeostasis (Wang et al., 2017). Appropriate activation of autophagy is critical in balancing growth with stress tolerance, and better understanding and
SnRK1 Activates Autophagy in Response to Abiotic Stress

Autophagy is induced by nutrient depletion, most likely as a mechanism for nutrient recycling and generation of substrates for energy metabolism (Doelling et al., 2002; Hanaoka et al., 2002). The energy sensor Snf1-related protein kinase 1 (SnRK1) is a heterotrimeric complex that has been suggested to be a master regulator of metabolism in plants in response to nutrient and energy deficiency (Sugden et al., 1999; Baena-González et al., 2007; Crozet et al., 2014). The animal and yeast orthologs of SnRK1, AMP-activated kinase (AMPK) and Suc nonfermenting 1 (Snf1), respectively, are energy and metabolic sensors that maintain cellular homeostasis and activate autophagy under low-energy conditions (Hardie, 2011; Carroll and Dunlop, 2017). AMPK/Snf1 can regulate autophagy by inhibiting the target of rapamycin (TOR) complex (Lee et al., 2010), a negative regulator of autophagy, therefore allowing autophagy to become active, or by direct phosphorylation of ATG1, which also leads to the activation of autophagy (Wang et al., 2001; Egan et al., 2011; Kim et al., 2011). In Arabidopsis (Arabidopsis thaliana), there are two isoforms of the SnRK1 complex catalytic subunit, KIN10 and KIN11, with KIN10 being responsible for most of the SnRK1 activity (Baena-González et al., 2007; Jossier et al., 2009; Crozet et al., 2014). A kin10 kin11 double mutant is lethal, and reduced expression via virus-induced gene silencing leads to decreased activation of stress and starvation genes and to deformed leaves, flowers, and inflorescence (Baena-González et al., 2007), indicating that SnRK1 functions in development and stress responses.

Overexpression of the KIN10 gene in Arabidopsis leads to constitutive activation of autophagy (Chen et al., 2017; Soto-Burgos and Bassham, 2017), suggesting a positive role in the regulation of autophagy. KIN10 overexpression led to increased phosphorylation of ATG1 during Suc starvation, suggesting that KIN10 regulates autophagy by affecting the phosphorylation of ATG1 (Chen et al., 2017), as in mammals and yeast. Autophagy is activated during a wide range of abiotic stresses, and a kin10 knockout mutant failed to activate autophagy during most of these stresses (Soto-Burgos and Bassham, 2017). This indicates that KIN10 activates autophagy not just in response to nutrient deficiency or energy depletion as predicted, but also during other abiotic stresses, indicating a wider role for SnRK1 in regulation of autophagy than previously expected.

TOR Is a Negative Regulator of Autophagy

The TOR complex is a key regulator of the balance between growth and autophagy in all eukaryotes tested (Noda and Ohsumi, 1998; Pattingre et al., 2008; Liu and Bassham, 2010). In plants, TOR is activated when nutrients are abundant, in turn enhancing mRNA translation initiation, ribosome biogenesis, cell wall synthesis and growth and inhibiting autophagy (Deprost et al., 2007; Ren et al., 2011; Xiong and Sheen, 2015). Upon nutrient deficiency, TOR is inactivated, reducing growth and allowing the activation of autophagy (Pattingre et al., 2008).

The Arabidopsis TOR complex consists of TOR itself, a Ser/Thr kinase (Menand et al., 2002), the Regulatory Associated Protein of TOR, RAPTOR (Anderson et al., 2005; Deprost et al., 2005), which presents substrates to TOR for phosphorylation (Hara et al., 2002), and Lethal with Sec Thirteen 8, which stabilizes the complex (Moreau et al., 2012). A complete knockout of TOR is embryo-lethal (Menand et al., 2002), while knockdown by RNA interference subsequent modification of the pathway could lead to improvements in crop growth and yield. In this section, we will discuss known regulators of the autophagy pathway in plants.

SnRK1 Activates Autophagy in Response to Abiotic Stress

Figure 1. A proposed model for autophagosome formation in plant cells. Once autophagy is induced, a crescent-shaped isolation membrane named a phagophore is assembled on its membrane origin (e.g. ER). During this process, the ATG1 complex is activated, and downstream regulators (question mark) are recruited onto the initiation site. PI3P is generated on the phagophore assembly site, and ATG8 is conjugated onto the membrane to become the ATG8-PE form. Also, ATG5 and SH3P2 have been shown to localize on the phagophore structures. In addition, ATG9 vesicles are required for the efficient budding of the phagophore from the ER platform. In the subsequent steps, more ATG8 lipidation occurs, and the isolation membrane will elongate and close to form the completed double-membrane autophagosome. Finally, the autophagosome will deliver the cargos into the vacuole by fusion with the vacuole. With the help of the acidic environment and hydrolysis enzymes within the vacuole, the cargos will be degraded. Other regulators involved in the later steps after phagophore initiation are not listed.
leads to arrest of plant growth and development and constitutive autophagy (Deprost et al., 2007; Liu and Bassham, 2010). Disruption of RAPTOR in Arabidopsis similarly leads to defects in plant growth and development, although less severe than those of a TOR knockout, as well as constitutive autophagy (Anderson et al., 2005; Deprost et al., 2005; Pu et al., 2017). Overexpression of TOR blocks autophagy induced by nutrient starvation, salt, and osmotic stresses, while autophagy induced by oxidative and ER stress is not affected (Pu et al., 2017). Regulation of autophagy can therefore be TOR dependent or independent (Fig. 2) depending on the environmental stress to which the plant is subjected.

RAPTOR interacts with ribosomal p70 S6 kinase (S6K) in response to osmotic stress signals, suggesting a role for S6K in the TOR signaling pathway and plant stress responses (Mahfouz et al., 2006). Several other proteins have also been shown to interact with RAPTOR or TOR in vitro, including Arabidopsis Me2-like1 (Anderson et al., 2005) and transcription factor E2Fa. Tap42/α4 is an effector of TOR in yeast and mammals, and its Arabidopsis homolog, Tap46, has been identified as a downstream target of TOR. Tap46 is phosphorylated by TOR and interacts with protein phosphatase type 2A, a regulator of autophagy in yeast (Ahn et al., 2011). Overexpression or reduction of expression of Tap46 correlates with TOR activity (Ahn et al., 2015), suggesting that Tap46 is a positive regulator of the TOR pathway. Silencing of the TAP46 gene using virus-induced gene silencing in tobacco (Nicotiana tabacum) led to induction of autophagy, as in the TOR RNAi plants (Ahn et al., 2011), indicating that it can negatively regulate autophagy. This suggests that Tap46 acts as a positive effector in the TOR signaling pathway, leading to the regulation of autophagy.

Recently, a connection between the TOR signaling pathway and SnRK1 complex has been demonstrated. KIN10 interacts with RAPTOR in vivo and can phosphorylate RAPTOR in vitro, like its mammalian orthologs (Nukarinen et al., 2016). Blocking TOR activity in a kin10 mutant led to activation of autophagy, while inhibition of SnRK1 activity in a raptor1b mutant failed to block the constitutive autophagy observed in this mutant (Soto-Burgos and Bassham, 2017). SnRK1 therefore acts upstream of the TOR signaling pathway in the regulation of autophagy (Soto-Burgos and Bassham, 2017), at least under conditions in which activation of autophagy is TOR dependent.

The ATG1 Kinase Complex

A major regulator of autophagy and a downstream substrate of TOR in yeast and animals is the ATG1/ATG13 kinase complex. ATG1 is the catalytic subunit of the
complex and activates autophagy in response to nutrient depletion (Diaz-Troya et al., 2008; Mizushima 2010). In mammals, AMPK promotes autophagy by phosphorylating Ulk1 (ATG1 homolog) upon Glc starvation (Kim et al., 2011). In Arabidopsis, KIN10 overexpression results in an increase in ATG1 phosphorylation, suggesting that this mode of regulation is conserved (Chen et al., 2017). In yeast, TOR phosphorylates ATG13 in nutrient-rich conditions, causing a decrease in its affinity for ATG1, preventing their association and therefore repressing autophagy. Under starvation conditions, TOR becomes inactive, leading to the dephosphorylation of ATG13, allowing ATG1 to associate with ATG13 and activate autophagy (Nakatogawa et al., 2009; Kamada et al., 2010). In mammals, unlike in yeast, ATG1 associates with ATG13 under all conditions, indicating that the regulatory mechanism of ATG1/ATG13 differs between mammals and yeast (Lee et al., 2007).

In Arabidopsis, ATG1 and ATG13 are present in multiple copies, which are most likely functionally redundant. During nutrient starvation, ATG1a is hyper-phosphorylated, potentially by SnRK1 (Chen et al., 2017), while ATG13a is hypo-phosphorylated (Suttangkakul et al., 2011), suggesting that the ATG1 complex functions in a manner similar to yeast in terms of autophagy regulation. Disruption of ATG13 leads to phenotypes similar to those of mutants in other core autophagy genes, with hypersensitivity to nutrient starvation and accelerated senescence. atg13 mutants have defects in the formation of autophagic bodies, suggesting that the complex acts upstream of autophagosome formation (Suttangkakul et al., 2011). ATG1a associates with autophagic bodies and is delivered to the vacuole for degradation, indicating that the ATG1 complex is a substrate of autophagy. A negative feedback mechanism is therefore proposed to exist to reduce activated ATG1 complex levels after induction of autophagy by nutrient deficiency (Suttangkakul et al., 2011). This turnover might be an attempt to reset autophagic induction by requiring the incorporation of freshly activated ATG1 kinase during each round of phagophore assembly (Suttangkakul et al., 2011). Based on this evidence, and by comparison with ATG1 complex functions in animals and yeast, we hypothesize that the ATG1 complex may regulate autophagy in Arabidopsis via its phosphorylation by TOR and/or SnRK1 (Fig. 2).

Regulation of Autophagy by IRE1 during ER Stress

Autophagy is induced by ER stress, in which accumulation of unfolded and misfolded proteins within the ER activates the unfolded protein response (UPR; Liu et al., 2012; Liu and Howell, 2016). Although repression of TOR activity leads to activation of autophagy during some abiotic stresses, autophagy induced by ER stress seems to be independent of TOR (Pu et al., 2017), as autophagosomes are formed normally during ER stress in TOR overexpression lines. Instead, ER stress-induced autophagy depends on inositol-requiring enzyme-1 (IRE1), an ER stress sensor that activates the UPR (Cox and Walker, 1996; Mori et al., 1996; Chen and Brandizzi, 2013). During ER stress, IRE1 is activated by oligomerization and autophosphorylation (Korennykh et al., 2009). After activation, IRE1 splices an mRNA encoding a membrane-associated basic Leu zipper transcription factor (bZIP60; Nagashima et al., 2011). The spliced bZIP60 mRNA is translated, producing an active protein that is translocated into the nucleus and upregulates UPR genes such as BIP (Iwata and Koizumi, 2005; Deng et al., 2011).

Two IRE1 genes have been identified in Arabidopsis, IRE1a and IRE1b (Koizumi et al., 2001; Deng et al., 2011; Moreno et al., 2012), and a mutant defective in IRE1b is unable to form autophagosomes after inducing ER stress using dithiothreitol (DTT) or tunicamycin, indicating that IRE1b is required for the induction of autophagy by ER stress (Liu et al., 2012). Mutations in either IRE1a or bZIP60 have no effect on autophagy during ER stress, suggesting that only IRE1b is involved in the regulation of autophagy and that its bZIP60 splicing activity is not required (Liu et al., 2012). The addition of chemical chaperones or overexpression of molecular chaperones inhibited activation of autophagy by DTT or tunicamycin, and expression of a misfolded protein mimic in the ER was sufficient to induce autophagy via IRE1b activity. The accumulation of unfolded proteins in the ER, presumably recognized by IRE1b, is therefore a key event in activating autophagy during ER stress (Yang et al., 2016).

Excessive heat is a major factor that causes ER stress, as indicated by the splicing of bZIP60 mRNA by IRE1b (Deng et al., 2011) and by up-regulation of BIP (Leborgne-Castel et al., 1999). The induction of autophagy by heat stress is also mainly due to the accumulation of misfolded proteins (Yang et al., 2016). Autophagy activation is significantly reduced in an ire1b mutant during heat stress, compared to the wild type, indicating that the autophagy response during heat stress is dependent on IRE1b (Yang et al., 2016) and is most likely primarily acting as an ER stress response.

Other Possible Regulators

Most of the identified regulators of plant autophagy act posttranslationally, and relatively little is known about transcriptional regulation of autophagy-related genes. In tomato (Solanum lycopersicum), the transcription factor HsfA1a has been shown to induce drought tolerance by the activation of ATG genes and the induction of autophagy (Wang et al., 2015a). The Arabidopsis WRKY33 transcription factor has also been suggested to regulate autophagy. WRKY33 is important for plant resistance to necrotrophic pathogens (Zheng et al., 2006), and a yeast two-hybrid screen showed that WRKY33 interacts with ATG18a, a core autophagy component (Lai et al., 2011). Furthermore, a wrky33 mutant was defective in up-regulation of ATG18a and induction of autophagy upon infection with Botrytis cinerea (Lai et al., 2011). Silencing of WRKY33 in tomato led to reduced ATG gene expression and autophagosome accumulation during heat stress (Zhou et al., 2014), suggesting that it also functions in abiotic stress responses.
Several new pathways for regulation of autophagy in plants have been identified recently. First, the glyceraldehyde-3-phosphate dehydrogenases (GAPDH) have been shown to negatively regulate autophagy (Han et al., 2015; Henry et al., 2015). In Arabidopsis, there are multiple isoforms of GAPDH, including chloroplastic photosynthetic (GAPA1, GAPA2, and GABP) and cytosolic glycolytic (GAPC1 and GAPC2) enzymes (Zaffagnini et al., 2013). Mutants in the GAPDH isoforms GAPA1 and GAPA1 have constitutive autophagy, suggesting that GAPDH can negatively regulate autophagy (Henry et al., 2015). In tobacco, silencing of GAPCs activated autophagy, whereas overexpression of GAPCs inhibited oxidative-stress-induced autophagy (Han et al., 2015). Furthermore, GAPCs interact with ATG3 in vivo and in vitro, but upon oxidative stress this interaction weakens (Han et al., 2015). Disruption of GAPDHs led to enhanced disease resistance (Han et al., 2015; Henry et al., 2015), although whether this is related to autophagy is unclear. Together, these data suggest that GAPDH negatively regulates autophagy through interaction with ATG3.

Second, hydrogen sulfide has been linked to the regulation of autophagy. Hydrogen sulfide is an important signaling molecule in mammalian systems, and emerging data suggest that this is also true in plants. It has been identified as a component of the ABA signaling pathway (García-Mata and Lamattina, 2010) and has roles in regulation of photosynthesis (Chen et al., 2011) and tolerance to copper (Zhang et al., 2008), aluminum (Zhang et al., 2010), and boron (Wang et al., 2010) stress. DES1 is an 1-Cys desulfhydrase that is involved in the production of hydrogen sulfide and the degradation of Cys (Alvarez et al., 2010). A mutation in the DES1 gene impedes sulfide generation in the cytosol and promotes the accumulation of ATG8 and ATG8-PE, indicating activation of autophagy. Furthermore, addition of exogenous sulfide to a des1 mutant or genetic complementation of DES1 gene prevented the accumulation and lipidation of ATG8 proteins (Alvarez et al., 2012). Recently, it was demonstrated that the negative regulation of autophagy by sulfide is independent of reactive oxygen species, and sulfide therefore probably regulates autophagy by an alternative pathway (Laureano-Marín et al., 2016).

Third, the plant Bax inhibitor-1 (BI-1) has recently been shown to interact with ATG6 in vivo and in vitro and to positively regulate autophagy (Xu et al., 2017). Silencing of tobacco BI-1 reduced the autophagy activity induced by virus infection or oxidative stress, while overexpression of BI-1 increased autophagy activity and caused autophagy-dependent cell death (Xu et al., 2017). BI-1 therefore has both autophagy-dependent prosurvival and prodeath effects, depending on the conditions.

As research progresses, more information becomes available about how autophagy is regulated in plants. Although new discoveries have been made, further research is needed to fully understand how the autophagy pathway is controlled under different conditions and how the regulatory components are coordinated to determine the degree of autophagy activation (see Outstanding Questions).

**Dynamics of Autophagosome Formation**

After autophagy is activated, a conserved autophagosome formation process has been observed in plant cells, which involves several steps: initiation, expansion, maturation, and degradation (Fig. 1; Liu and Bassham, 2012). Autophagosomes may fuse with endosomes for further maturation prior to reaching the final destination, the vacuole, to acquire degradative enzymes, including proteases and lipases, for cargo degradation (Cui et al., 2016). Each step requires a dynamic membrane deformation process to give rise to the newly formed membrane, elongate the membrane for cargo sequestration, and fuse with other endomembrane compartments like endosomes and finally the vacuole/lysosome. In the following, we will provide an update on membrane dynamics during autophagosome formation, and we apologize to authors whose primary work cannot be cited here due to the space limitation.

**Phagophore Initiation**

Recent studies on yeast and mammalian cells have greatly advanced our understanding of the underlying mechanisms of autophagosome formation, and multiple membrane sources have been reported (Lamb et al., 2013). A typical preautophagosome structure, which is characterized by an open cup-like double membrane with highly curved edges, is called a phagophore or isolation membrane. It has been shown that the phagophore arises from an omega-shaped structure on an ER subdomain, called the omegasome (Axe et al., 2008). The highly curved shape of the phagophore membrane can be achieved by lipid composition and/or asymmetric distribution or by scaffolding through membrane curvature sensing proteins. So far, a general model for phagophore initiation can be described by the following sequence of events: ATG1 and PI3K complexes are initially recruited to the omegasome, leading to phosphatidylinositol 3-phosphate (PI3P) production, as well as recruitment of the ATG12-ATG5-ATG16 complex and other downstream regulators, which further facilitates the conjugation of ATG8 to phosphatidyethanolamine on the nascent phagophore membrane and its detachment from the ER platform (Lamb et al., 2013).

Although a number of images collected by electron microscopy provide a detailed morphological description of autophagosomal structures in plant cells, studies focusing on the mechanism of phagophore initiation are relatively rare (van Doorn and Papini, 2013). Similar to the complex in yeast and animal cells, the ATG1 complex functions at an early step for phagophore formation in plants, as an atg13 mutant fails to form autophagosomal structures (Suttangkakul et al., 2011). PI3P is also crucial for autophagosome initiation, as autophagosome formation is completely blocked after PI3K inhibitor treatment (Zhuang et al., 2013; Le Bar et al., 2014), while the autophagic defect in a yeast mutant in the PI3K component atg6/Atg630 can be restored by expressing its Arabidopsis homolog ATG6 (Fujiki et al., 2007). However, the connection between the ATG1 complex and the PI3K complex as well as other...
downstream regulators remains unknown, hindering our further understanding of the current model for phagophore initiation in plants.

A recent study focusing on ATG5 dynamics provides a new model for phagophore formation in Arabidopsis (Le Bars et al., 2014). In this paper, early autophagosomal structures were labeled by ATG5-GFP fluorescent proteins and exhibit a tight connection with the ER network. Interestingly, ATG5 fusion proteins decorate the high curvature domain of the phagophore at all stages of its differentiation and finally detach from the phagophore once it is sealed. By detailed real-time and 3D imaging analysis of the growing phagophore, it was shown that ATG5-GFP is located on the growing phagophore with a toroidal disposition, raising the possibility that ATG5 and/or its related complex (ATG12-ATG16) may sense or promote the membrane deformation. This observation is consistent with that in animal cells, in which when ATG16 is artificially targeted to the plasma membrane, LC3 (animal ATG8 homolog) lipidation also occurs on the plasma membrane (Fujita et al., 2008). However, a counterpart for ATG16 in Arabidopsis has not been identified.

In another study, by tracking SH3P2, it was also shown that omegasome-like structures may be employed for phagophore formation (Zhuang et al., 2013). It was clearly observed that SH3P2 was predominantly distributed on a highly curved domain in omegasome-like structures as well as on the nascent phagophore, which is closely associated with the ER membranes (Zhuang et al., 2013). In addition, the fusion process has also been visualized through labeling with SH3P2, while SH3P2 still displays an asymmetrical distribution on the membrane. It is noteworthy that SH3P2 contains a BAR domain, a well-established membrane curvature sensor, and directly interacts with ATG8; thereby, it is also speculated that SH3P2 may function as a membrane sensor during the membrane remodeling process. It will be interesting to investigate how the interaction between ATG8 and SH3P2 regulates autophagosome membrane remodeling.

**ATG9 Vesicles**

In contrast to most other ATG proteins, ATG9 is the only transmembrane protein and moves rapidly as numerous distinct compartments throughout the cytoplasm (Yamamoto et al., 2012; Karanasios et al., 2016; Rao et al., 2016). Therefore, ATG9 vesicles have been postulated as a key contributor to deliver the membrane source or other regulators to the phagophore membrane. In yeast, autophagy is completely blocked in the atg9 mutant (Yamamoto et al., 2012). In mice, deficiency in ATG9 only leads to fewer autophagosome structures upon induction and decreased autophagic activity, suggesting that mammalian ATG9 is not crucial in basal conditions (Orsi et al., 2012). Furthermore, interaction data also show that ATG9 is associated with ATG and non-ATG regulators for phagophore initiation (Karanasios et al., 2016; Rao et al., 2016).

In plants, analysis of Arabidopsis atg9 mutants shows that autophagy is less severely blocked when compared with other atg mutants like atg5 and atg7 (Hanaoka et al., 2002). Recently, advanced-imaging analysis has provided novel insights into the roles of ATG9 in the formation of early autophagosomal structures (Zhuang et al., 2017). In Arabidopsis, ATG9 vesicles are observed as distinct mobile compartments and show transient interactions with the autophagosomal membrane after autophagic induction. Interestingly, upon benzothiazole and DTT treatments, highly dynamic extending tubules labeled by YFP-ATG8e are captured in the atg9 mutants. Moreover, 3D electronic tomography as well as dynamic confocal microscopy analysis demonstrated a direct contact between these abnormal autophagosomal structures and the ER membrane, providing clear evidence for the ER origin of autophagosomes in Arabidopsis. In addition, the PI3P effector ATG18, an ATG9-interacting protein, is also trapped on extending tubules in atg9 mutants, suggesting that in plants, ATG9 acts as a carrier to recycle regulators from the newly formed phagophore to control the elongation of autophagosomal membrane. ATG9 may also interact with other membrane remodeling proteins for the fission of the phagophore from the membrane of origin, which has also been indicated in other eukaryotic cells, including Trs85, a specific subunit of the transport protein particle III complex (Kakuta et al., 2012). It should be pointed out that such a defect has not been observed in other model organisms or other Arabidopsis atg mutants like atg5 and atg7 under the same conditions, thus revealing a unique role of ATG9 for autophagosome development on the ER membrane. However, it remains to be seen whether this is true in all types of ER-dependent autophagy or whether it is specific to benzothiazole- and DTT-induced autophagy, both drugs being able to induce ER stress. More importantly, future efforts in identifying and characterizing ATG9-interacting proteins would certainly facilitate our understanding of how ATG9 is involved in this process.

**Autophagosome Expansion, Maturation, and Degradation**

Once the phagophore is formed, it will undergo a series of steps for expansion, maturation, and finally degradation in the vacuole by fusion with endosomes and vacuole, which requires additional driving forces for membrane deformation and fusion. Recent exciting studies have uncovered several non-ATG regulators in this process, particularly the endocytic components that function in endomembrane trafficking (Zhuang et al., 2015).

The cytoskeleton may drive the membrane shaping during autophagosome formation in both yeast and mammalian cells (Kast and Dominguez, 2017). Studies demonstrating the colocalization of the autophagy markers ATG8 and JOKA2 with cytoskeletal components (Ketelaar et al., 2004; Zientara-Rytter and Sirko, 2014) have provided evidence of links between the cytoskeleton and plant autophagy. Another study also reported a role for a
subunit of the exocyst complex in autophagic membrane transport to the vacuole, as an exo70B1 mutant showed decreased amounts of intravacuolar autophagic bodies (Kulich et al., 2013), while the exocyst complex has been implicated to function in coordination of vesicle trafficking with the cytoskeleton (Synek, et al., 2014). In addition, disruption of the microtubule cytoskeleton compromised autophagosome formation upon autophagic induction (Wang et al., 2015b). What is more, when a component of the SCAR/WAVE complex named NAP1 is defective, autophagosome formation is reduced (Wang et al., 2016). NAP1 is initially ER associated and coaligns with the cytoskeleton, but when treated with constant pressure, NAP1-labeled punctae are induced and colocalize with an autophagosome marker. It is proposed that ER-associated NAP1 may activate actin polymerization to promote membrane deformation for phagophore formation and expansion. However, future work should clarify how cytoskeleton activities are coordinated for autophagosome formation.

In contrast, abnormal autophagosomal structures have been shown to accumulate in several mutants defective in endosome or vacuole trafficking, particularly the ESCRT complex, which is essential for MVB and vacuole biogenesis (Surpin et al., 2003; Kaspiarmpa et al., 2013; Kwon et al., 2013; Gao et al., 2015; Zhuang et al., 2015). It is possible that a failure in fusion with endosomes or the vacuole leads to these defects. The importance of autophagosomal fusion with endosomes was supported by other studies. A plant-specific ESCRT component, FYVE domain protein required for endosomal sorting 1 (FREE1), has been reported to associate with the ESCRT components and participate in regulating vacuolar protein transport (Gao et al., 2014; Zhao et al., 2015; Belda-Palazon et al., 2016). On the other hand, FREE1 interacts with SH3P2, and an Arabidopsis free1 mutant accumulates abnormal autophagosome-like structures, which display a higher association with the late endosome and failure in delivery of autophagosomes to the vacuole (Gao et al., 2015). Since SH3P2 binds to the autophagosome membrane and ATG8 (Zhuang et al., 2013), it is hypothesized that FREE1-SH3P2 serves as a bridge for autophagosome fusion with the endosome/vacuole. Several studies have shown that autophagosomes share the membrane tethering machineries with the endomembrane system in yeast and animal cells (Tooze et al., 2014). Therefore, identification of the components in the fusion process will be an important first step to fully understand the membrane fusion mechanism.

Membrane Dynamics in Selective Autophagy

Selective autophagy involves the engulfment of specific proteins or organelles into autophagosomes, which requires receptors or adaptor proteins that bind the cargo and also interact with the ATG component(s) for the recruitment of the cargo into autophagosomes (Floyd et al., 2012; Zhou et al., 2013; Hafren et al., 2017). In this process, ATG8 is a central player for selective autophagy that decorates autophagosomes and binds to various cargo receptors (Kellner et al., 2017). Autophagosomes may develop into multiple sizes to sequester different cargos, including protein aggregates and organelles, to avoid excess damage to the cell. As such, autophagosomes will undergo drastic membrane expansion to engulf specific cargos efficiently and selectively. Here we

**Figure 3.** Summary of the characterized autophagy-mediated pathways for chloroplast material degradation. Three types of structures, RCB (A), SSGL body (B), and ATI1-PS body (C), bud off from chloroplasts with different cargos and are sequestered into ATG8-coated membranes (blue color). The receptors for engulfment of RCB and SSGL into autophagosomal membranes are presently unidentified. In addition, when cells are exposed to light-induced damage, whole damaged, dysfunctional chloroplasts can be targeted by ATG8-decorated autophagosome structures (D) to be delivered into the vacuole, but the underlying mechanism remains unknown. ATG8 and ATI1 are labeled with green and red dots, respectively.
OUTSTANDING QUESTIONS

- How is transcription of autophagy-related genes regulated?
- What upstream signals affect SnRK1 and TOR activity to regulate autophagy in response to stress?
- What is the molecular mechanism of membrane remodeling of the phagophore from its membrane origin in plant cells?
- Does ATG9 play a specific role in ER stress-induced autophagy or ER-derived autophagosome biogenesis? Are there any other membrane sources for plant autophagosomes?
- How does autophagy recognize its cargos in the plant cell and how is the ATG machinery coordinated?

will focus on one plant-specific type of selective autophagy, chlorophagy (Fig. 3). New data show that autophagy is involved in chloroplast degradation in different manners for specific chloroplast contents or the entire chloroplast, including: Rubisco-containing bodies (RCBs), small starch granule-like structure (SSGL) bodies, and ATG8-interacting Protein 1 (ATI1-PS) bodies (Michaeli and Galili, 2014).

Stromal proteins are imported into the small double membrane structures termed RCBs and eventually transported to the vacuole for degradation (Ishida et al., 2008). It has been observed that RCBs labeled by a chloroplast-targeted DsRed fluorescent protein colocalized with the GFP-ATG8 autophagosome marker. A recent study demonstrates that the ESCRT components CHMP1A and B play a direct role in the delivery of RCB cargos into the vacuole, as the chmp1 mutant accumulates plastid clusters with plastid proteins (Spitzer et al., 2015). Notably, a defect in plastid morphology is also observed in atg5 and atg7 mutants, with the accumulation of long plastid bridges and extensions. In this study, vacuolar turnover of free GFP cleaved from GFP-ATG8 is also increased in the chmp1 mutant, implying a possible role of CHMP1 in promoting the efficient sequestration of cargo from plastids into autophagosomes. Similar to RCB bodies, SSGL bodies are another type of plastid-derived small spherical structures, which are responsible for the delivery of small starch granules from chloroplasts to vacuoles in an autophagy-dependent mechanism (Wang et al., 2013). ATI1-PS bodies require a membrane-spanning protein, ATI1, which can interact with chloroplast proteins and ATG8, for the targeting of plastid proteins into the vacuole (Michaeli et al., 2014). The ATI1-PS bodies are detected in the periphery and inside of plastids, which will finally bud off from plastids into the cytoplasm independent of the core ATG machinery such as ATG5. Apart from these different pathways for chloroplast degradation, it is also of note that entire chloroplasts can be engulfed by autophagosomal structures when cells are exposed to UV light-induced damage (Izumi et al., 2017). However, how the ATG proteins sense the targeted chloroplast cargos to initiate the formation of various types of structures remains unclear.

CONCLUSIONS

Accumulating studies have begun to address the essential roles of autophagy in plant development and growth. It is apparent that plants may exhibit specific types of autophagy and autophagosomal structures. Exciting findings such as the identification of novel regulators to sense and shape the unique double membrane structures in yeast and animal cells have provided great advances in our understanding of autophagy regulation and autophagosome formation. It is very likely that unique mechanisms for autophagy regulation will be uncovered in the near future and more plant-specific cellular functions will be unraveled (see Outstanding Questions).

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