J. Plant Biol. (2011) 54:339–350
DOI 10.1007/s12374-011-9176-5

REVIEW

See How I Eat My Greens—Autophagy in Plant Cells

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Received: 2 July 2011 / Revised: 28 July 2011 / Accepted: 10 August 2011 / Published online: 20 August 2011

Abstract Eukaryotic cells have two conserved pathways for degrading polypeptides. One is the highly selective ubiquitin–proteasome system, and the other is autophagy, a bulk degradation pathway to a lytic compartment. Autophagy in plant cells has important roles in development and responses to abiotic and biotic stresses. Furthermore, plant autophagy has been implicated in vacuole biogenesis and Golgi-independent targeting of cytoplasmic materials to vacuoles. Here I present four questions that are frequently asked by plant scientists interested in autophagy. The first question relating to tools for plant autophagy research is relatively easy to answer, while the others are open questions about regulation of autophagy, autophagic cargoes, and potential differences of plant autophagic routes from corresponding metazoan pathways. This review will discuss recent progress that may provide the answers for the latter questions.

Keywords Autophagy-related · *Atg* genes · Selective autophagy · ATG8 · Microautophagy

Abbreviations

AIM	ATG8-interacting motif
AMPK	adenosine monophosphate-activated protein kinase
Atg	autophagy-related
GFP	green fluorescent protein
LIR	LC3-interacting region
LV	lytic vacuole
MDC	monodansylcadeverine
PB1	Phox-Bem1p
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PSV	protein storage vacuole
RCBs	Rubisco-containing bodies
SnRK	Snf1-related protein kinase
TOR	target of rapamycin
UBA	ubiquitin-associated
UPS	ubiquitin–proteasome system

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Many biologists may notice an increasing number of research articles with the term “autophagy” (Table 1) included in the titles and abstracts. Some of them may recall their undergraduate class of cell biology, possibly decades back, briefly introducing autophagy as a self-eating process. An electron micrograph showing partially degraded mitochondria in rat hepatocytes is one of the earliest snapshots of autophagy (Ashford and Porter 1962), but details of autophagic machineries were not revealed until the last decade of the twentieth century (Yang and Klionsky 2010). Molecular genetics approach in yeast identified ~34 *Autophagy-related* (*Atg*) genes. The yeast *Atg* gene products participate in different steps of macroautophagy (Fig. 1a; see Table 1), which are as follows: (a) regulation of autophagy induction, (b) phagophore (see Table 1) initiation, (c) vesicle expansion and autophagosome (see Table 1) formation, (d) docking and fusion of the autophagosome with vacuole, and (e) breakdown of cytoplasmic materials in autophagic bodies (see Table 1). A common set of Atg proteins is required for various types of autophagy and often cited as “core” or “canonical” Atg proteins which are further grouped into four groups: Atg1-Atg13 complex, the class III phosphatidylinositol

Table 1 This a set of common terms used in plant autophagic research

Terms	Definition
Autolysosome	In animal cells, it means an autophagosome fused with the lysosome. For plant cells lacking lysosomes, an autolysosome may be defined otherwise as a small (usually less than 5 μm in diameter) acidic compartment fused with an autophagosome formed by macroautophagy. Acidotropic dyes are used to visualize autolysosomes (Moriyasu and Ohsumi 1996; Moriyasu et al. 2003), but by definition, not all of the structures stained with the dyes are autolysosomes.
Autophagic body	A transient vesicle with single membrane in the vacuole, formed as a result of autophagy. In macroautophagy, the membrane of autophagic bodies corresponds to the outer membrane of autophagosomes (see Fig. 1a, c). In microautophagy, the membrane of autophagic bodies is assumed to originate from vacuolar membrane (tonoplast) (Fig. 1d, e). Inhibitors of vacuolar proteases or proton pumps are used to stabilize GFP-ATG8-labeled autophagic bodies.
Autophagic flux	The dynamic process of autophagosome synthesis, delivery of autophagic substrates to a lytic compartment, and degradation of autophagic substrates inside the lytic compartment (Mizushima et al. 2010). It is considered a more reliable indicator of autophagic activity than measurements of autophagosome numbers.
Autophagosome	A cytosolic compartment with a double membrane, containing cytoplasmic constituents (see Fig. 1a, c). It mediates macroautophagy.
Autophagy	A process by which cytoplasmic constituents are sequestered and degraded in a lytic compartment (vacuole and lysosome) in a cell. It includes both macroautophagy and microautophagy. Occasionally, autophagy refers to a Golgi-independent targeting of cytoplasmic materials to vacuole. In this case, autophagic cargoes do not have to be degraded in vacuole.
ATG8	A ubiquitin-like protein and a marker for autophagy. It is targeted to autophagic membrane by conjugation to the membrane lipid, phosphatidylethanolamine (PE). The lipid-conjugated form is called ATG8-PE. One class of mammalian ATG8 homologues is called LC3 and its conjugated form is called LC3(II), while free LC3 is LC(I).
ATG8-ATG12 conjugation systems	A group of highly conserved proteins required for conjugation of ATG8 to PE (see Fig. 1b). It consists of two ubiquitin-like protein tags, ATG8 and ATG12; their respective targets, PE and ATG5; a common E1 activating enzyme, ATG7; their respective E2 conjugating enzymes, ATG3 and ATG10; and ATG16, a protein interacting with ATG5 by non-covalent bonds. The ATG16-ATG5-ATG12 forms an oligomeric complex and is required for efficient ATG8-PE formation. Atg7 and Atg5 null animals and plants are frequently used to test whether a biological process is dependent on the canonical macroautophagy.
Macroautophagy	A type of autophagy by which cytosolic vesicles with a double- or multiple-membrane capture cytoplasmic constituents and deliver to the vacuole/lysosome (see Fig. 1a, c).
Microautophagy	A type of autophagy by which cytoplasmic constituents are directly imported into the vacuole/lysosome by protrusion or invagination of the membrane (see Fig. 1d–f).
Phagophore	Also known as isolation membrane. It is a cup-shaped membranous structure in the cytoplasm (see Fig. 1a, c), which will grow and engulf a portion of cytoplasm to form an autophagosome.
Urea-SDS-PAGE	12% SDS-PAGE containing 6 M urea in a resolving gel. It is frequently used to separate faster-migrating ATG8-PE from free ATG8, especially yeast ATG8. Urea helps better resolve proteins with a lower molecular weight. For an optimal separation of *Arabidopsis* ATG8s from ATG8s-PE, urea is absolutely needed and it is advised to use a resolving gel about 8 cm or longer.

For a general and comprehensive glossary, refer to Klionsky et al. 2010

(PI) 3-kinase complex, Atg8 and Atg12 conjugation systems (Fig. 1b; see Table 1), and the Atg9 recycling system (Yang and Klionsky 2010).

Based on sequence similarity to yeast Atg proteins, Atg homologues in higher eukaryotes were initially identified and biochemically characterized, which demonstrated that macroautophagic machineries are highly conserved throughout eukaryotes. Subsequently, forward and reverse genetics studies supported roles of mammalian *Atg* genes in various biological processes, such as development (Mizushima and Levine 2010), immunity and inflammation (Levine et al. 2011), protection from cancers and neurodegeneration (Mizushima et al. 2008), and stress responses (Kroemer et al. 2010). Not surprisingly, additional factors needed for metazoan autophagy have been identified using genetic screens and interactome studies (Behrends et al. 2010; Lipinski et al. 2010; Tian et al. 2010), indicating more elaborate autophagic machineries in higher eukaryotes.

Recent investigation on plant autophagy has been based largely on electron microscopic observation and/or reverse genetics of *Atg* genes. As plant *Atg* genes turned out to

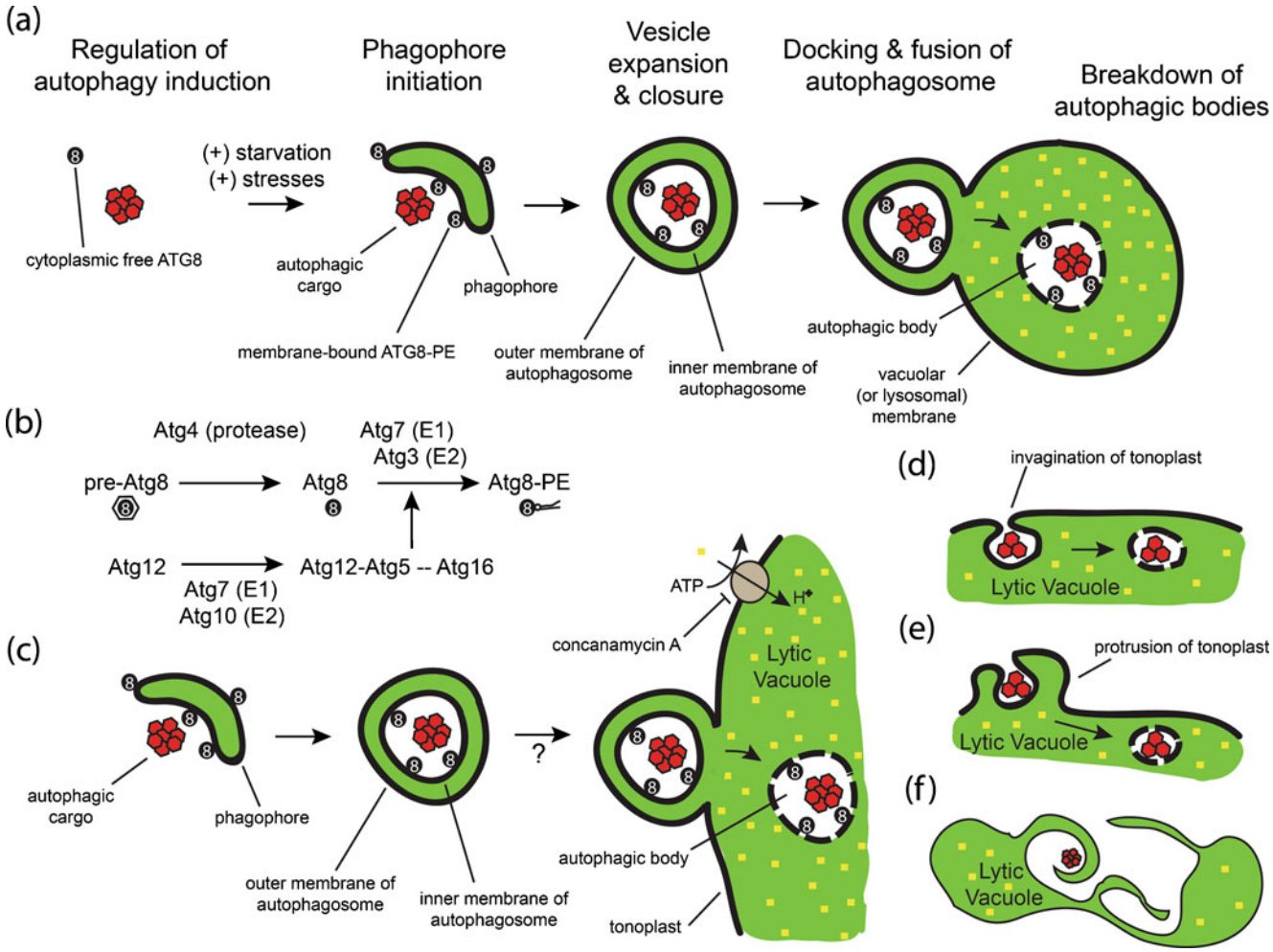

Fig. 1 Autophagic processes in eukaryotes and a proposed diversity of plant autophagy. All the organelles and molecules shown here are not to scale. **a** Steps of macroautophagy in yeast and metazoans. Of known Atg proteins, only Atg8 proteins (shown in *black circles with the number 8*) were shown to indicate the autophagic structures that can be labeled with GFP-Atg8/LC3. **b** Highly conserved Atg proteins involved in the conjugation of ubiquitin-like Atg8 and Atg12 protein tags. The Atg12–Atg5 conjugate, interacting with the Atg16(-like) protein non-covalently, is required for efficient Atg8-PE conjugation. **c** A model of the plant macroautophagic route. In this model, an autophagosome is directly fused with the lytic vacuole. Not shown here is the possibility that plant autophagosomes are fused with a prevacuolar compartment. Origin and destination of plant autolysosomes (see Table 1) are unclear and thus not depicted here. A proton pump on the tonoplast that is inhibited by concanamycin A is shown in a *gray blob*. **d**–**f** Possible modes of microautophagy by lytic vacuoles in plant cells. **d** Microautophagy by a small-scale invagination of tonoplast. **e** Microautophagy by a small-scale protrusion of tonoplast. **f** Microautophagy by arm-like projection of the lytic vacuole. See text for details

have various roles in development, metabolism, cell stress responses, and programmed cell death (Bassham et al. 2006; Thompson and Vierstra 2005), more plant scientists get interested in autophagy. This review focuses on frequently asked questions about autophagy in plant cells. Answers for some questions look obvious, while answers for others call for extensive research in future. Beyond the scope of this review, readers may refer to excellent reviews on the functional aspects of plant autophagy (Hayward et al. 2009; Herman and Schmidt 2004; Marty 1999; Muntz 2007; Reumann et al. 2010; Robinson et al. 1998; Talbot and Kershaw 2009; van Doorn et al. 2011b; Yoshimoto et al. 2010).

What Tools Are Available for Studying Plant Autophagy?

There are several classes of markers for assessing autophagy in yeast and mammalian cells (Mizushima et al. 2010). Although electron microscopy is the most reliable method to detect autophagosomes with double membrane, a more popular and less laborious marker is GFP (green fluorescent protein)-ATG8 fusion protein. In mammalian cells, GFP-ATG8 (also called GFP-LC3; see Table 1) decorates phagophore, autophagosome, and autolysosome (see Table 1). However, more autophagosomes and autolysosomes do not necessarily mean a higher

autophagic activity in the cells; for instance, pharmacological or genetic inhibition of autophagosome–lysosome fusion will result in the accumulation of autophagosomes labeled with the GFP-ATG8 marker. To estimate autophagic flux (see Table 1), it is possible to quantify the GFP-ATG8 puncta with and without an inhibitor of autophagosome–lysosome fusion or lysosomal degradation (Mizushima et al. 2010). Since ATG8 itself is a known target of macroautophagy and degraded in the lysosome/vacuole, GFP-ATG8 is also used as a biochemical marker for macroautophagy, especially in yeast. GFP-ATG8 is presumably cleaved in vacuoles to generate a free GFP moiety which is more stable than GFP-ATG8 (Shintani and Klionsky 2004). An immunoblot analysis with anti-GFP antibodies is useful to determine the macroautophagic degradation of GFP-ATG8, allowing estimation of autophagic activities. More frequently, the macroautophagic activity is monitored by an endogenous level of ATG8 conjugated to phosphatidylethanolamine (ATG8-PE), which can be determined by immunoblot analysis with ATG8 antibodies. The amount of ATG8-PE usually correlates with the amount of autophagic membrane (Mizushima et al. 2010). Again, a combined use of an inhibitor of autophagosome–lysosome fusion or lysosomal degradation is highly recommended for the analysis of autophagic flux by ATG8 immunoblot. This is especially important when cells are under stresses potentially inhibiting autophagic machinery.

The GFP-ATG8 turned out to be a good marker for macroautophagy in plant cells. *Arabidopsis* transgenic plants expressing *GFP-ATG8* showed cytoplasmic puncta possibly representing autophagosomes (Fig. 1c). When incubated with concanamycin A, an inhibitor of vacuolar proton pumps (thereby inhibiting degradation in vacuole), the transgenic plants accumulated numerous GFP-ATG8 dots inside the central vacuole, presumably representing autophagic bodies. Importantly, these GFP-ATG8 dots in the vacuole were not observed in various *Arabidopsis atg* mutants (see below) such as *atg7*, *atg5* (Thompson et al. 2005), *atg10* (Phillips et al. 2008), *atg12a atg12b* (Chung et al. 2010), and *atg4a atg4b* (Yoshimoto et al. 2004) mutants. It is noteworthy that GFP-ATG8 fluorescence in these mutants was mostly seen as diffuse cytoplasmic signals, although the mutant cells occasionally accumulated cytoplasmic GFP-ATG8 dots with a bigger diameter than autophagosomes of wild-type cells (Phillips et al. 2008; Yoshimoto et al. 2004). The nature of these dots is unknown, but they may represent either GFP-ATG8 protein aggregates unrelated to autophagy (Kuma et al. 2007), immature autophagosomes that fail to be closed, or even ATG7-independent autophagic vesicles. Thus cytoplasmic GFP-ATG8 foci, especially if its transgene is ectopically expressed, should be interpreted with caution and not be used as a sole indicator of autophagic activity in plant cells.

Like in yeast, the GFP-ATG8 appears to be cleaved in the plant vacuole, raising a possibility that free GFP is a sensitive marker for autophagic activity in plants. When anti-GFP antibodies were used to detect the GFP-ATG8 and the free GFP moiety in GFP-ATG8 transgenic *Arabidopsis*, an immunoblot band corresponding to the free GFP was detectable from a wild-type extract (Slavikova et al. 2005) but not from any *atg* mutants tested (Chung et al. 2010). This result is consistent with the lack of diffuse and punctate GFP signals in the mutant vacuole, suggesting that the free GFP in wild type is derived from the vacuole. It will be interesting to see if the free GFP level quantitatively correlates with autophagic activities in plant tissues.

Quantification of endogenous ATG8-PE in plants by the ATG8 immunoblot analysis has been difficult, since there are multiple isoforms of ATG8 (ATG8s) in plants. For example, there are at least eight immunoreactive ATG8s in *Arabidopsis* (Yoshimoto et al. 2004). Furthermore, crude extracts from *atg7* single and *atg4a atg4b* double mutants contain at least two protein species co-migrating with putative ATG8s-PE in a urea-SDS-PAGE (see Table 1) (Chung et al. 2010; Thompson et al. 2005; Yoshimoto et al. 2004), making it technically hard to determine what protein bands represent ATG8s-PE in the urea-SDS-PAGE. Recently this problem was solved using a combination of membrane fractionation with the delipidation reaction of ATG8s-PE (Chung et al. 2010). The putative ATG8s-PE were enriched in wild-type membrane fractions and cleavable by phospholipase D, whereas their co-migrating protein species in *atg* mutants were mostly found in a soluble fraction and likely unstable. Thus a change in the state level of ATG8-PE in ATG8 immunoblot analysis is not sufficient to demonstrate a change in autophagic activity in *Arabidopsis*; it is recommended to test the effect of an inhibitor of vacuolar degradation, to show the biochemical nature of faster-migrating protein bands in the urea-SDS-PAGE, and/or to use an independent method (e.g., GFP-ATG8 immunoblot analysis) in combination.

Acidotropic fluorescent dyes are also available for visualizing autophagic structures (see autolysosome in Table 1), although they are less specific than the GFP-ATG8 marker. If possible, techniques using these markers have to be complemented with others involving more specific autophagic markers. Once thought to be a specific marker for autophagosomes, monodansylcadeverine (MDC) has a higher affinity to lysosomes in mammalian cells and does not stain autophagic vesicles with neutral lumen (Mizushima 2004). In *Arabidopsis* protoplasts, MDC-labeled structures are mostly co-localized with GFP-ATG8e puncta (Contento et al. 2005), and both cytoplasmic GFP-ATG8e dots and MDC-labeled structures were absent in protoplasts prepared from an *ATG18a-RNAi*

line (Xiong et al. 2005). It is not known whether *Arabidopsis atg* knockout mutants also lack the MDC-labeled structures. Various versions of LysoTracker and neutral red are also common markers for acidic compartments in plant cells (Inoue et al. 2006; Moriyasu et al. 2003). More details on the fluorescent dyes and other tools for plant autophagy research were described in a recent review (Mitou et al. 2009).

Pharmacological inducers of yeast and animal macroautophagy include rapamycin and other inhibitors of TOR (target of rapamycin) complex. Lithium, ABT737, and SMERs (small molecular enhancer of rapamycin) appear to act independently of TOR complex (Mizushima et al. 2010). Unfortunately, rapamycin is an inefficient inhibitor for plant TOR complexes including *Arabidopsis* TOR (Mahfouz et al. 2006; Menand et al. 2002), preventing its uses as an inducer of macroautophagy. It will be interesting to see if other TOR inhibitors and known TOR-independent inducers of autophagy work in plant cells. Notably green algae *Chlamydomonas* is sensitive to rapamycin which affects the modification and accumulation of its ATG8 (Perez-Perez et al. 2010), implying a role of TOR complex in regulating autophagy (see below).

Inhibitors of plant autophagy are useful for investigating a potential role of autophagy. These are also good tools to identify a specific macroautophagic step that developmental and environmental factors and a genetic lesion can affect (Mizushima et al. 2010). Inhibitors commonly used for plant samples include concanamycin A inhibiting a vacuolar proton pump (see Fig. 1c; Yoshimoto et al. 2004), E-64 inhibiting cysteine proteases (Inoue et al. 2006; Takatsuka et al. 2004), inhibitors of PI 3-kinases (e.g., 3-methyladenine, wortmannin, LY294002) in tobacco-cultured cells (Takatsuka et al. 2004), and *Arabidopsis* roots (Inoue et al. 2006). These inhibitors, however, are not specific for autophagy (Mizushima et al. 2010). For example, most PI 3-kinase inhibitors can inhibit both class III and class I PI 3-kinases as well as other targets in cells.

Likewise, one should consider the specificity of genetic materials; *Arabidopsis atg6* null mutants are defective not only in autophagy but also in pollen germination, possibly due to abnormal vesicle trafficking (Fujiki et al. 2007). Similar phenotypes were observed in the mutants of *Arabidopsis* VPS34 PI 3-kinase (Lee et al. 2008), consistent with the view that ATG6 and VPS34 are in the same PI 3-kinase complex. Interestingly the *atg6* homozygous mutation is lethal, whereas knockout mutants defective in ATG8/ATG12 conjugation pathways are viable and do not differ in their developmental architectures from that of wild type. This implies that *atg6* and mutations corresponding to components of the PI 3-kinase complex cause phenotypes not directly related to autophagy. Thus *atg7*, *atg5* and other mutations in ATG8/ATG12 conjugation pathway appear to be genetic materials of choice when one wants to investigate specifically plant autophagy. Mutants and knockdown plants with a compromised function of the ATG9 complex appear to share common phenotypes with those of ATG8/ATG12 conjugation pathway (Hanaoka et al. 2002; Inoue et al. 2006; Xiong et al. 2005), although a direct comparison by quantitative measurement is still needed to reveal how similar these two classes of mutants are in their respective downstream targets.

Is Plant Autophagy Mechanistically Similar to Yeast and Mammalian Autophagy?

Unfortunately we do not have a clear answer for this question. It is true that plants have a set of genes with sequence similarities to yeast and mammalian *Atg* genes, and that many of the genes were shown to have biochemical functions related to autophagy in plant cells. Nevertheless, plant cells are different from yeast and mammalian cells, and this may result in a potential difference in plant autophagic pathway. Plant cells have multiple types of vacuoles serving various functions (Marty 1999; Zouhar and Rojo 2009). Plant lytic vacuoles (LVs; see Fig. 1c) are a functional equivalent of metazoan lysosomes, but typical plant LVs are bigger than animal lysosomes. These structural and functional differences in lytic compartments may lead to differences in the plant autophagic pathway from metazoan counterpart.

We do not understand what constitutes the major route for autophagic degradation in plant cells. For example, the origin of autophagic membrane in mammalian cells has been debated for decades, and recent studies indicated that it can be derived from membranes of the endoplasmic reticulum (ER), mitochondria, or even plasma membrane (Hailey et al. 2010; Hayashi-Nishino et al. 2009; Ravikumar et al. 2010). It is unknown where and how the phagophore forms in plant cells. Furthermore, it is unclear how endocytic compartments such as trans-Golgi network (functions as an early endosome in plant cells) and prevacuolar compartment (functions as a late endosome in plant cells) interacts with autophagic vesicles. To understand the dynamics of plant autophagic membranes, potentially informative is a time-lapse analysis of autophagic markers like GFP-ATG8 with a reference to other organellar markers (Toyooka et al. 2006).

A hallmark of autophagic vesicles is cytoplasmic and intravacuolar ultrastrucutres containing cytoplasmic materials (e.g., ribosomes) within membrane, which have been described from a variety of non-transgenic plant species (Aubert et al. 1996; Levanony et al. 1992; Otegui et al. 2005; Reyes et al. 2011; Rose et al. 2006; Toyooka et al. 2001; Van der Wilden et al. 1980; Zheng and Staehelin

2011). Although a few of these structures can be considered cytoplasmic autophagosomes with double membrane (Otegui et al. 2005; Reyes et al. 2011; Rose et al. 2006; Toyooka et al. 2001), we do not have compelling evidence supporting that macroautophagy is a prevalent type of autophagy in plants. In almost all eukaryotes, much less is known about microautophagy (see Table 1) than macroautophagy, due to the lack of a specific marker for microautophagy (Mijaljica et al. 2011).

Microautophagy (Fig. 1d–f) may play a bigger role in plant cells than previously thought, given that typical plant cells have the central vacuole occupying a large volume. There are several reports of plant ultrastructures reminiscent of microautophagy (Bassham et al. 2006; Levanony et al. 1992; Saito et al. 2002). Recently, transformation of protein storage vacuole (PSV) into LV was investigated in germinating tobacco roots using high-pressure freezing and freeze-substitution techniques (Zheng and Staehelin 2011). PSV were initially fused and they formed arm-like projections as their contents are degraded and volumes decrease, like a flat ball (see Fig. 1f). Interestingly, multi-lamellar type autophagic vacuoles were identified and appeared to be derived from concentric sheets of preexisting PSV membrane. These autophagic vacuoles were later engulfed by the pre-LVs, giving rise to mature LVs where cytoplasm sequestered in the autophagic vacuoles is degraded. Such types of vacuolar/lysosomal extensions or "arms" leading to microautophagy have been observed in both yeast and mammalian cells (Mijaljica et al. 2011).

Similar phenomenon was described for the plastids of senescing *Dendrobium* petal mesophylls (van Doorn et al. 2011a). The authors observed plastids with variable diameters (0.5 to 5.7 μm) often having a large invagination which forms an intraplastidal space as they designated. The space can take up to half the volume of its plastid and appears continuous with cytoplasm through a narrow opening (less than 0.2 μm in diameter). van Doorn et al. proposed a (micro)autophagy-like process in the invaginated plastids, based on observation that an acid phosphatase activity, a marker for an organelle showing autophagic activity, was detected in some of the intraplastidic spaces. However, the activity in the space does not seem to exceed that in the cytoplasm, suggesting that the intraplastidal space is not specialized for a lytic compartment. Additionally it has to be seen whether the last step in a microautophagy-like process takes place—the complete sequestration of the intrasplastidal space from cytoplasm.

Another big gap in our knowledge of plant autophagic routes relates to the requirement of known *Atg* genes for various types of autophagy. Although GFP-ATG8 and ATG8 lipidation are reliable markers for ATG7-dependent macro-autophagy in yeast, animals, and plants (see above), evidence supports the presence of ATG7-independent autophagy in mammalian cells (Nishida et al. 2009). Nishida et al. detected autophagosomes in *Atg7*- and *Atg5*-deficient mouse cells treated with etoposide. It is not known whether plant *atg* mutants defective in ATG8/ATG12 conjugation system contain any kinds—functional or non-functional, and ATG7-dependent or ATG7-independent—of autophagosomes when observed under electron microscopy. However, Ohsumi and colleagues showed that, unlike wild-type vacuole, concanamycin A-treated *atg4a atg4b* double mutants do not accumulate autophagic bodies in their root vacuoles, consistent with the data obtained using GFP-ATG8 and concanamycin A (Yoshimoto et al. 2004). Combined with the presence of GFP-ATG8 dots in *atg7* and other mutants (see above), this observation supports the claim that the accumulation of autophagic bodies by concanamycin A treatment is a more reliable indicator of functional macroautophagy in plant cells than the accumulation of autophagosomes in cytoplasm.

If ATG8 is involved in autophagosome formation in plant cells, the protein should be detected on ultrastructure representing autophagic membrane. Such data were unavailable until a recent report by Otegui and colleagues that an autophagosome-like structure, decorated by anti-ATG8 antibodies, was observed in developing starchy endosperm cells of maize (Reyes et al. 2011). The major discovery of the report was, however, the presence of atypical PSVs in maize aleurone. In the starchy endosperm cells, zeins, the major storage proteins in maize endosperm, are synthesized on the ER and accumulate in the protein bodies which are extension of ER membrane and contain protein aggregates consisting of various types of zeins. By contrast, in the aleurone cells surrounding the starchy endosperm, protein bodies are not formed and zein-containing inclusions are found in atypical PSVs with multilayered intravacuolar membranes. Golgi-processed glycoproteins were found in the matrix of the PSVs, implying that Golgi-mediated trafficking contributes to the biogenesis of the PSVs. Strikingly, the PSVs also contain cytoplasmic materials (e.g., ribosomes) and ER-targeted proteins together with zein inclusions, indicating that the PSV biogenesis also requires Golgi-independent transport of cytoplasmic constituents, i.e., "broad-sense" autophagy (see Table 1). Neither immunoelectron microscopy nor YFP-ATG8 co-labeling experiment supported the localization of ATG8 on the PSVs, however, suggesting that ATG8 is not involved in the autophagic delivery of zein and other cytoplasmic materials into the atypical PSVs. Further investigation using maize *atg* mutants is needed to confirm the ATG8-independent autophagy in maize aleurone cells.

To summarize, it is likely that autophagic pathways in plants somewhat differ from those in animals, possibly due to the difference in their respective lytic compartments.

Details of the difference are unknown, although micro-autophagy may play an important role. Functional redundancy (e.g., *Atg7*-independent autophagic routes) and interaction with the endocytic pathway are possible, all of which can make it hard to define the roles of canonical and new *Atg* genes in the plant autophagy, thus demanding an efficient method for screening more *Atg* genes in plants.

How Is Plant Autophagy Regulated?

Regulation of autophagy is a research area that is filled with question marks but has great potential in drug discovery and other applications. Frequently cited proteins regulating autophagy induction include Atg1, TOR, and adenosine monophosphate-activated protein kinase (AMPK) complexes. These kinase complexes form a current model of autophagy induction by nutrient starvation in mammalian cells (Egan et al. 2011; Neufeld 2010).

The Atg1 complex is a master regulator in the model. Atg1 is the catalytic subunit of the complex which also contains Atg13 and FIP200/Atg17. Although very few proteins are known as substrates of the Atg1 kinase activity except for components of its complex (Dorsey et al. 2009; Mizushima 2010), the Atg1 complex is thought to be an upstream regulator of other Atg proteins involved in multiple autophagic steps. In yeast, *ATG1*, *ATG13*, and *ATG17* are at the top of genetic hierarchy of *Atg* genes for organizing pre-autophagosomal structure (Suzuki et al. 2007). Furthermore, Atg1 and FIP200 appear to be also important for the Atg7-independent autophagy in mammalian cells (Nishida et al. 2009), and FIP200 is required for TOR-independent autophagy induced by lithium (Hara et al. 2008).

Plant genomes have putative homologues for all of the three components. In *Arabidopsis*, there are three genes encoding full-length ATG1-like proteins, one for a truncated ATG1-like, two ATG13-like, and one FIP200-like protein. Mutants lacking two ATG13-like proteins showed phenotypes similar to those of mutations defective in the conjugation of ATG8 and ATG12 (unpublished manuscript by Suttangkakul, Li, Chung, and Vierstra). The *atg13a atg13b* double mutant did not have autophagic bodies labeled with GFP-ATG8 but showed a normal pattern of ATG8 lipidation, indicating that ATG8-PE conjugation is necessary but not sufficient for autophagy.

The TOR kinase complex negatively regulates autophagy induction. When yeast cells are supplied with nutrients, the TOR complex phosphorylates Atg13. This phosphorylation suppresses the Atg1 kinase activity and interaction between Atg1 and Atg13, resulting in a minimal level of autophagy. In response to starvation, yeast TOR kinase activity is inhibited, Atg13 is rapidly dephosphorylated, Atg1 interacts with Atg13, Atg1 kinase activity increases, and autophagy is induced. In mammalian cells, the regulation of Atg1 complex by TOR complex is more complicated and perhaps calls for further investigation (Mizushima 2010). Under nutrient-rich conditions, a rapamycin-sensitive mTOR (mammalian TOR) complex is active and phosphorylates Atg13 and ULK1 (one of mammalian Atg1 homologues). mTOR interacts with Atg1 complex where ULK1 is still associated with Atg13 unlike in yeast. Under starvation, the mTOR complex does not interact, and the ULK1 kinase activity increases and phosphorylates itself, ATG13, and FIP200. The changes in ULK1 complex somehow lead to phagophore initiation and autophagy induction.

A possible regulatory role of plant TOR complex in autophagy induction was recently investigated (Liu and Bassham 2010). *Arabidopsis* transgenic plants with reduced *TOR* message showed more MDC-stained bodies and GFP-ATG8e-labeled puncta. The GFP-ATG8e puncta in the *TOR* RNAi plants likely result from a higher rate of autophagosome formation rather than from inhibition of downstream events, since autophagic bodies can be seen by concanamycin A treatment and the expression of some *ATG* genes increases in the RNAi plants. The data suggest that *Arabidopsis* TOR is a negative regulator of autophagy. The RNAi plants are unique in that they are genetically manipulated to show a higher autophagic activity in contrast with conventional *atg* knockouts. However, the RNAi line did not show obvious phenotypes except for reduced seedling root growth which was reported previously. Notably more serious developmental phenotypes were associated with independent RNAi lines for *Arabidopsis TOR* gene (Deprost et al. 2007). Thus more investigation is needed to determine whether a higher autophagic activity in the RNAi plants has any physiological consequences.

One of the downstream effectors of TOR is yeast TAP42, which negatively regulates autophagy induction (Yorimitsu et al. 2009). Its plant homologues designated TAP46 were tested for their involvement in autophagy and other TOR-related processes (Ahn et al. 2011). Dr. Pai's group identified the tobacco *TAP46* homologue and used virus-induced gene silencing (VIGS) to downregulate the gene and also generated RNAi lines to suppress the *Arabidopsis TAP46* homologue. Protoplasts isolated from these VIGS and RNAi lines exhibited accumulation of autolysosome-like structures and GFP-ATG8e puncta. It is not clear whether autophagosome formation increases or a downstream process is blocked. Nevertheless, together with the work with TOR RNAi lines (see above), these data support the hypothesis that TOR complexes negatively regulate autophagy in plant cells.

AMPK is activated by glucose starvation and triggers autophagy. The mechanisms for this regulation were recently revealed. AMPK directly phosphorylates ULK1 (Egan et al. 2011) and Raptor (Gwinn et al. 2008), a component of the TOR complex. The consequence of these phosphorylations is opposite; TOR is inhibited while ULK1 is activated. SNF1, the yeast orthologue of mammalian AMPK, was also shown to be a positive regulator of autophagy (Wang et al. 2001). Among SnRK (Snf1-related protein kinase) homologues in *Arabidopsis*, AKIN10 and AKIN11 belong to the SnRK1 family and are *Arabidopsis* SNF1/AMPK orthologues (Baena-Gonzalez et al. 2007). Genes activated by AKIN10 include several *ATG* genes, consistent with hypothesis that the plant AMPK-like proteins are also a positive regulator of autophagy.

Although not mentioned above, there are many known autophagy regulators functioning at different levels (Liang 2010; Neufeld 2010). Some of them have putative plant homologues, but their roles in plant autophagy have not been established in most cases. Are there unknown autophagy regulators? The answer is "yes, probably," considering that autophagy is a highly regulated process which responds to a variety of environmental and developmental factors. Markers for plant autophagy show differential responses to developmental ages, organ identity, nutrient availability, and pathogens (Chung et al. 2009; Liu et al. 2005; Slavikova et al. 2005; Yoshimoto et al. 2004).

What Kinds of Cargoes Are Delivered to Vacuole by Autophagy?

Vacuolar degradation by autophagy contrasts with the ubiquitin–proteasome system (UPS) in several points. Both are pathways for degradation, but their targets, selectivity, and functions are quite different. While the UPS can process only polypeptides, autophagic cargoes include not only the individual polypeptides but also non-protein materials, protein complexes, and organelles in the cytoplasm. It is reasonable to assume that organelles bigger than the size of autophagosomes cannot be processed by macroautophagy. The diameter of typical autophagosomes ranges 0.5 to 0.9 μm in yeast and 0.5 to 1.5 μm in mammalian cells (Mizushima et al. 2002). Although we do not know the size limit of plant autophagosomes, fluorescent microscopic observation indicates that *Arabidopsis* GFP-ATG8a puncta in the cytoplasm and the central vacuole are less than 2 μm in diameter, similar to the size of mammalian autophagosomes. Therefore an intact chloroplast would not be engulfed by an ATG8-decorated autophagosome, and it was thought that a whole chloroplast cannot be delivered to the vacuole for degradation (Hortensteiner and Feller 2002). From attached *Arabidopsis* leaves undergoing dark-induced senescence, Wada et al. (2009) recently identified an intravacuolar structure that is as big as 3 to 4 μm in diameter and exhibits chlorophyll autofluorescence, implying that a whole chloroplast may be imported and degraded in the central vacuole. *atg4a atg4b* double homozygous mutants (Yoshimoto et al. 2004) that are defective in the ATG8 lipidation (Chung et al. 2010) showed a delayed loss of chloroplasts during the leaf senescence and did not have the intravacuolar structures (Wada et al. 2009), suggesting that the canonical ATG8 conjugation system is required for the hypothetical targeting of whole chloroplasts into the central vacuole. It is not known whether macroautophagy, microautophagy, or something else is responsible for the targeting. It will be interesting to see if the structure is co-localized with GFP-ATG8. In addition, the ~4-μm intravacuolar bodies may represent a special, dark-induced type of chloroplasts since they did not emit the fluorescent signal of stroma-targeted DsRed (CT-DsRed). The biochemical and ultrastructural nature of the intravacuolar bodies may reveal the origin of the structures.

In spite of the new finding, the vacuolar degradation of entire chloroplasts is not the major route for chloroplast breakdown during senescence. The same research group also showed data supporting that the canonical autophagy involving ATG8 is, at least partially, responsible for chloroplast degradation (Ishida et al. 2008). Rubisco-containing bodies (RCBs) with a diameter of 0.4 to 1.2 μm were previously detected in the cytoplasm (and occasionally in the vacuole) of naturally senescent leaves of wheat, suggesting that RCBs are an intermediate of chloroplast breakdown (Chiba et al. 2003). RCBs were also detected in detached *Arabidopsis* leaves, and treatment with concanamycin A was used to stabilize CT-DsRed-labeled RCBs in the vacuolar lumen. The vacuolar RCBs were not detected in the *atg5* mutant background and most of the vacuolar RCBs were decorated by GFP-ATG8 (Ishida et al. 2008).

The selectivity of targets to be degraded is another contrasting property of autophagy to the UPS. The UPS is highly selective, while autophagy was once thought to be a non-selective process. Now it is generally accepted that there are selective types of autophagy in yeast and mammalian cells (Komatsu and Ichimura 2010; Kraft et al. 2010). Known selective autophagic cargoes include protein aggregates, mitochondria, peroxisomes, ribosomes, bacteria, etc.

Two types of candidate cargoes have been investigated for selective autophagy in plant cells, namely that of chloroplasts (Reumann et al. 2010; also see above) and of protein aggregates. An oligomeric version of RFP fused with a cytochrome b5 protein, which would be localized in

the ER if it were not fused, resulted in the formation of RFP puncta in the tobacco cells (Toyooka et al. 2006). The Cyt b5-RFP puncta were not overlapped with structures labeled with any known plant organelle markers. However, when the transformed cells were starved, a diffuse RFP signal was detected from the central vacuole, and a band corresponding to the size of a free RFP moiety dominates over the full-length Cyt b5-RFP protein band in the GFP immunoblot analysis. This indicates the disposal of the fusion protein in the vacuole, possibly by autophagy. Indeed the vacuolar degradation was inhibited by treatments with a cysteine protease inhibitor E-64 and with PI 3-kinase inhibitor 3-methyladenine. The involvement of autophagy was further confirmed by colocalization with GFP-ATG8 and by immunoelectron microscopy locating anti-Cyt b5 antibodies in an autophagosome-like structure. It remains to be elucidated whether canonical *atg* mutations affects the vacuolar targeting of the protein aggregates.

Several research groups are testing hypothetical mechanisms for the selection of autophagic cargoes (Johansen and Lamark 2011; Komatsu and Ichimura 2010; Kraft et al. 2010). One hypothesis is that ubiquitinylation is important for the recognition of cargoes by autophagic machinery. In the hypothesis, p62/sequestosome1 (SQSTM1) and NBR1 act as putative receptors for selective autophagic cargoes in mammalian cells (Kirkin et al. 2009; Komatsu et al. 2007; Pankiv et al. 2007). These proteins share a very similar combination of domains and have three kinds of interacting partners: interactions with ubiquitinylated proteins through their ubiquitin-associated (UBA) domains, with LC3/ATG8 through the LC3-interacting region (LIR, also known as AIM, or ATG8-interacting motif, for a more general term), and with other p62 and/or NBR1 proteins through the Phox-Bem1p (PB1) domain. Since autophagic cargoes are often ubiquitinylated, p62 and NBR1 may mediate the molecular interaction between ubiquitinylated cargo proteins and ATG8-PE on the autophagic membrane.

Arabidopsis and tobacco p62-like proteins were identified by two independent groups (Svenning et al. 2011; Zientara-Rytter et al. 2011). Johansen and colleagues classified p62 and NBR homologues in various taxa, based on the presence of the FW domain unique to NBR1-like proteins (Svenning et al. 2011). Phylogenic tree analysis indicated that NBR1-like proteins, not p62-like proteins, are the only homologues in most non-metazoan species. Metazoans possess either NBR1-like and p62-like proteins or p62-like proteins alone. Several lines of evidence indicate that the *Arabidopsis* NBR1-like protein, AtNBR1, is more similar to mammalian p62 than NBR1, despite its name. Like mammalian p62, AtNBR1 interacts with itself via its p62-type PB1 domain, with ubiquitin via C-terminal UBA2 domain, and with six of the eight tested *Arabidopsis* ATG8 isoforms via AIM. Furthermore, AtNBR1 is delivered to the central vacuole for degradation, which requires functional AIM and PB1 domain of the AtNBR1 and *ATG7* gene. Finally mCherry-AtNBR1 puncta co-localized with some of GFP-AtATG8a puncta, and treatment with concanamycin A stabilized autophagic bodies decorated by both mCherry-AtNBR1 and GFP-AtATG8a (Svenning et al. 2011). Although the functional conservation strongly suggests a role of AtNBR1 in selective autophagy, further investigation is needed to reveal the function of *AtNBR1* gene *in planta*. The tobacco *NBR1*/*Joka2* transcript accumulates in response to nitrogen and sulfur limitation. The tobacco gene was identified from a yeast two-hybrid screen for proteins interacting with the tobacco UP9C protein which may be involved in the plant responses to sulfur deficiency (Zientara-Rytter et al. 2011). Combined with the notion that autophagy is important in the adaptation to nutrient stresses, these data indicate that plant Joka2/NBR1 homologues play a similar role in nutrient recycling. Alternatively, like animal p62/NBR1 homologues (Komatsu et al. 2007), their plant counterparts may respond to oxidative stresses which are also associated with nutritional stresses in plants (Shin and Schachtman 2004).

Concluding Remarks

The past decade has witnessed the development of cell biological and molecular genetics tools for the investigation of plant autophagy. The core machinery for ATG7-dependent autophagy is very similar to yeast and mammalian counterparts in terms of biochemical properties. Relatively well characterized *atg* mutants (e.g., *atg5*, *atg7*) are being used to reveal hitherto unknown functions of plant autophagy. One example is the roles of ATG5 and a Rab GTPase RabG3b in the tracheary element differentiation, which were recently elucidated by the thorough analysis of *atg5* mutants (Kwon et al. 2010).

Research on plant autophagy will focus on less defined steps such as regulation of autophagy induction, phagophore initiation, and fusion of autophagosomes and other compartments in plant cells. Evidence suggesting microautophagy and *ATG7*-independent autophagy in plant cells is interesting, but their mechanistic characterization may require a technological breakthrough like specific markers and a large-scale screen. Selective autophagy in plants is likely and the combination of autophagic mutants with established reporters for protein trafficking may identify selective autophagic cargoes in plant cells.

Acknowledgments This work was supported for 2 years by Pusan National University Research Grant.

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