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REVIEW

Molecular Mechanisms of Autophagy in Plants: Role of ATG8 Proteins in Formation and Functioning of Autophagosomes

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Abstract—Autophagy is an efficient way of degradation and removal of unwanted or damaged intracellular components in plant cells. It plays an important role in recycling of intracellular structures (during starvation, removal of cell components formed during plant development or damaged by various stress factors) and in programmed cell death. Morphologically, autophagy is characterized by the formation of double-membrane vesicles called autophagosomes, which are essential for the isolation and degradation of cytoplasmic components. Among autophagic (ATG) proteins, ATG8 from the ubiquitin-like protein family plays a key role in autophagosome formation. ATG8 is also involved in selective autophagy, fusion of autophagosome with the vacuole, and some other intracellular processes not associated with autophagy. In contrast to yeasts that carry a single *ATG8* gene, plants have multigene *ATG8* families. The reason for such great ATG8 diversity in plants remains unclear. It is also unknown whether all members of the ATG8 family are involved in the formation and functioning of autophagosomes. To answer these questions, the identification of the structure and the possible functions of plant proteins from ATG8 family is required. In this review, we analyze the structures of ATG8 proteins from plants and their homologs from yeast and animal cells, interactions of ATG8 proteins with functional ligands, and involvement of ATG8 proteins in different metabolic processes in eukaryotes.

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Autophagy (from Greek αὐτός – "self" and φαγεῖν – "eat") is a universal catabolic process of intracellular degradation of macromolecules and organelles. It serves as an important regulatory mechanism for removal of damaged organelles, intracellular pathogens, and some long-lived, abnormal, or aggregated proteins in specialized lytic compartments – lysosomes and vacuoles. Autophagy appears to be a highly conserved process, since it has been found in all eukaryotic organisms [1]. Earlier studies of autophagy in starving yeast suggested that autophagic degradation could be a compensatory mechanism induced by a deficit of cell energy sources [2]. Later studies broadened our understanding of the functional importance of autophagy in eukaryotes. Thus, autophagy was found to play a key role in the development of heart diseases, neurodegeneration disorders, infections, aging, and cancer [3], which significantly raised interest in the role of autophagy in human physiology. Several types of autophagy have been discovered, including selective removal of intracellular components. The discovery of autophagic (*ATG*, autophagy-related) genes opened a new page in studies of molecular mechanisms of this process [1]. It is important to note that autophagy might play a "dual" role in cells either by promoting cell survival via degradation of stress-damaged proteins and organelles or by initiating programmed cell death when the damage is too severe to be repaired [4].

Understanding of autophagy mechanisms at the cellular and molecular levels has proved to be a difficult task. Although considerable progress has been achieved in

Abbreviations: AIM, ATG8-interacting motif; ATG genes and proteins, autophagic gene and proteins; ATI1/ATI2, ATG8-interacting proteins 1 and 2; CVT pathway, cytoplasm-to-vacuole targeting pathway; ER, endoplasmic reticulum; GABARAP, γ-aminobutyric acid A receptor-associated protein; GATE-16, Golgi-associated ATPase enhancer 16 kDa; PAS, pre-autophagosomal structure; PE, phosphatidylethanolamine; PI3P, phosphatidylinositol 3-phosphate; PI3K 1, phosphatidylinositol 3-kinase complex 1; ROS, reactive oxygen species; TOR, target of rapamycin protein kinase; UB, ubiquitin; UBL, ubiquitin-like protein.

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autophagy studies in yeast and animals [5], little is still known on the autophagy mechanisms in plants. Recent data demonstrated the importance of this process in the life of plants. Plants require autophagy for both normal development during organ morphogenesis and ontogenesis and in response to detrimental environmental factors [6-9]. For example, autophagy is essential for cell survival during oxidative stress and in the presence of excessive reactive oxygen species (ROS) [10, 11].

Plants have two types of autophagy: microautophagy and macroautophagy (Fig. 1). In microautophagy, removed components are captured directly by the vacuole via tonoplast invagination and subsequent release of the cytoplasmic component-containing vesicles in the vacuole lumen. Examples of microautophagy are deposition of storage proteins in the forming wheat endosperm [12, 13] or release of resin particles into the vacuole in guayule [14]. Microautophagy is also observed in some plant species during degradation of starch granules and storage proteins [15, 16]. Considering the fact that the vacuole represents up to 90% of cell volume of the fully developed plant cell, some researchers believe that microautophagy plays an even more important role that had been considered before.

Macroautophagy (hereafter, autophagy) is characterized by the formation of autophagosomes – specialized double-membrane vacuoles that transport damaged or oxidized cell components to the vacuole [1]. Autophagosome formation is initiated in the cytoplasm with the formation of a cup-like membrane structure, called phagophore or isolation membrane, that elongates, engulfs cytoplasmic macromolecules and organelles, and then closes into a vesicle with the formation of a mature autophagosome. The outer membrane of the mature autophagosome eventually fuses with the tonoplast. This releases the autophagic body (cargo enveloped by the autophagosome inner membrane) into the vacuole lumen, where it is degraded by resident acidic hydrolases. The degradation products can be transported into the cytoplasm [17]. In yeasts, autophagosomes form a pre-autophagosomal structure (PAS) that is usually located near the site of nucleus–vacuole connection [18, 19]. PAS formation is accompanied by the transport of endoplasmic reticulum (ER) proteins into the Golgi apparatus. Multiple loci of autophagosome formation have been found in mammalian cells, e.g. ER, mitochondria, Golgi apparatus, and nuclear and plasma membranes [20]. The sites and the mechanisms of autophagosome formation in plants remain unknown. However, it has recently been found that in plants the ER serves as a platform for autophagosome formation [21].

Molecular mechanisms of autophagy are complex and involve numerous ATG proteins. The central role belongs to the ubiquitin-like protein ATG8, whose activity was found to be essential for autophagosome formation. ATG8 is located on the autophagosome membrane and is often used as an autophagosome marker in eukaryotic cells [22]. ATG8 regulates membrane elongation during autophagosome biogenesis. It is also important in selective autophagy and in the process of autophagosome fusion with the vacuole. In contrast to yeast, mammalian and plant ATG8 proteins are encoded by multigene families. Thus, in *Arabidopsis* ATG8 proteins are encoded by nine genes; in rice and maize – by five genes; in soya – by eleven genes [23]. The necessity for such large *ATG8* gene families in plants remains obscure, and its understanding requires more information on the number, structure, and expression of *ATG* genes in different species.

This review describes molecular mechanisms of autophagy in eukaryotic cells, the major stages in autophagosome formation, and proteins that control these stages. Special emphasis is placed on the structure and physicochemical properties of the key autophagic protein ATG8. The diversity of ATG8 proteins, their role, and properties in plant cells are discussed.

MOLECULAR MECHANISMS OF AUTOPHAGY

The discovery of *ATG* genes in *Saccharomyces cerevisiae* yeast was the first breakthrough in the studies of autophagy mechanisms. By now, more than thirty (~35) *ATG* genes have been identified [1]. *ATG* genes have also been found in higher eukaryotes, including plants. Thus, several *ATG* homologs were found in *Arabidopsis thaliana* [24]. *ATG* genes have also been identified in various crop plants, such as soya, tomatoes, tobacco, wheat, rice, maize, etc.

Autophagosome formation proceeds in several stages: (i) autophagy induction; (ii) vesicle nucleation, autophagosome expansion and maturation; (iii) auto-

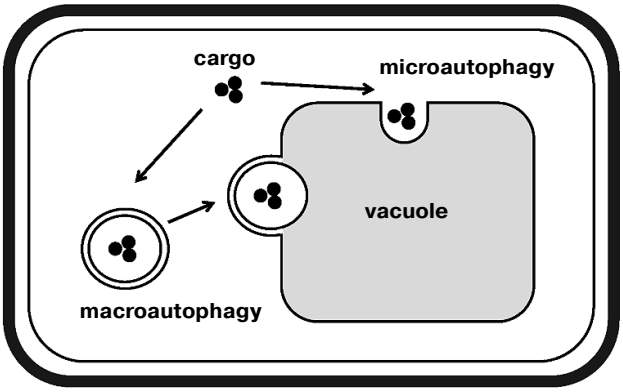

Fig. 1. Types of autophagy in plant cells. Microautophagy is a direct uptake of cell components by the vacuole via tonoplast invagination. Macroautophagy is a formation of specialized double-membrane vacuoles – autophagosomes – that engulf intracellular components and transport them to the vacuole for subsequent degradation.

phagosome docking and fusion with vacuole; (iv) autophagic body degradation. There are three groups of proteins required for autophagosome assembly: 1) ATG9 complex including ATG1, ATG2, ATG9, ATG13, ATG18, and ATG27; 2) phosphatidylinositol 3-kinase complex 1 (PI3K 1) including ATG6/VPS30/Beclin1, ATG14, VPS15, and VPS34; 3) two ubiquitin-like complexes: ATG3, ATG4, ATG5, ATG7, ATG8, ATG10, ATG12, and ATG16 [1, 25-28] (Fig. 2).

Although plant *ATG* genes are similar to those in yeasts, some plant *ATG* homologs are represented by multigene families. Thus, the *ATG1* family in *Arabidopsis* includes four genes, *ATG12*, *ATG13*, and *ATG4* families – two genes each, *ATG18* family – eight, and *ATG8* family – nine genes [24, 29]. Although no data have been obtained so far that could explain such redundancy, the diversity of *ATG* genes might provide higher specificity and multifunctionality of autophagy in plants. Plant ATG proteins have been extensively studied in *Arabidopsis*; however even in this plant the role of some of these proteins in autophagy remains unclear.

Regulation of autophagy induction. It is known that starvation can activate autophagy in organisms and cell cultures. In yeasts and mammals, autophagy induction by starvation is mediated by TOR (target of rapamycin) protein kinase, which acts as a negative autophagy regulator. Rapamycin and other TOR inhibitors are widely used for autophagy induction [30]. Under normal physiological conditions, TOR catalyzes ATG13 hyperphosphorylation. Hyperphosphorylated ATG13 has a low affinity for the ATG1 serine/threonine protein kinase and inhibits autophagy. Starvation leads to ATG13 dephosphorylation and ATG1–ATG13 complex formation and autophagy initiation [31]. A similar mechanism was observed in *Arabidopsis* cells, which suggests high evolutionary conservatism of the initiation process [32]. *Arabidopsis* has only one *TOR* gene [33]. *AtTOR* knockout mutants are not viable. Experimental data obtained for *AtTOR* knockdown and overexpressing mutants suggest that TOR can regulate the size of plant cells and organs. Until recently, no direct evidence has been obtained that proved the involvement of TOR in autophagy regulation in plants [34]. However, Liu and Bassham [33] found that *AtTOR* RNAi plants with decreased expression of *AtTOR* exhibit constitutive autophagy and autophagosome accumulation, thereby corroborating the hypothesis that TOR acts as a negative autophagy regulator in plants. The ATG1–ATG13 complex includes different additional proteins in different organisms, e.g. ATG11, ATG17, ATG29, and ATG31 in yeasts and FIP200/ATG17 and ATG101 in animals. Plant complexes contain orthologs of the animal proteins. Two *ATG13* and three *ATG1* genes were found in *Arabidopsis*. Moreover, a unique *ATG1t* gene was identified that is present in all flowering plants, but it is absent in mosses, animals, and yeasts. It was shown that in plants, ATG13 (and, probably, the entire kinase complex) controls autophagosome formation from the phagophore and delivery of autophagosome to the vacuole [35].

ATG9 complex. ATG9 is the only transmembrane ATG protein that is conserved among all eukaryotes. It has six transmembrane domains, and its N- and C-termini are exposed into the cytosol. ATG9 is required for the lipid delivery to the forming autophagosome. In yeast, ATG23 and ATG27 are transported together with ATG9 to the PAS [36, 37]. ATG9 is removed from the membrane by ATG1 kinase and two peripheral membrane proteins, ATG2 and ATG18. The lack of these proteins results in ATG9 accumulation in the membrane [38]. In yeast, ATG18 is also involved in retrograde transport from the vacuole [39]. Using *Arabidopsis atg9* mutants, ATG9 was shown not to be strictly required for autophagy, but it contributes considerably to the autophagic flux [40].

Vesicular nucleation. Vesicular induction and nucleation are regulated by PI3K complex 1 (PI3K 1) composed of the PI3 kinase catalytic subunit VPS34, membrane-anchoring activating kinase VPS15, and regulatory subunit ATG6 (also known as Beclin1 in mammals). An additional fourth subunit ATG14 was found in yeasts and mammals. It is known that VPS34 produces phosphatidylinositol 3-phosphate (PI3P). PI3P is required for autophagosome formation [41], although its exact role in this process remains unknown. The presence of PI3P in the membrane might allow to distinguish between phagophore and other endomembrane compartments. PI3P might also serve as a marker for molecular factors responsible for autophagosome fusion with the tonoplast [32]. PI3-kinase inhibitors wortmannin and 3-methyladenine suppress formation of the PI3P-containing membranes, and therefore inhibit autophagy [42].

In yeasts, ATG6 is required not only for autophagy, but also for vacuolar protein sorting (VPS). The activity of ATG6 depends on ATG14 (in autophagy) or VPS34 (in VPS). The role of Beclin1 in vesicular transport in mammals has not been proven [43]. However, recent studies identified the UVRAG protein that interacts with Beclin1 and mediates vesicular transport in endocytosis, which suggests Beclin1 involvement in the same process [44]. Moreover, three different Beclin1 complexes were found in mammals, some of these complexes (e.g. Ambra1, ATG14, UVRAG, and Rubicon) being mutually exclusive [45]. In plants, ATG6 performs additional physiological functions; in particular, it participates in hypersensitivity response [46].

In addition to PI3 kinases, PI3 phosphatases, such as myotubularin-related protein 3 (MTMR3) and Jumpy (MTMR4), also participate in autophagy [47, 48] by negatively regulating the formation and the size of autophagosomes. The balance between PI3 kinases and PI3 phosphatases controls autophagy initiation by changing local PI3P concentrations [5]. Although PI3K complex 1 proteins ATG6, PI3K, VPS15, and UVRAG have

been identified in plants, no data have been published on their interactions or their role in autophagy.

Autophagosome expansion and maturation. Two ubiquitin-like complexes are responsible for autophagosome expansion and maturation by regulating the size, expansion rate, and curvature of the growing autophagosome. It is interesting that all proteins involved in these processes are conserved and have homologs in eukaryotes, including plants.

The assembly of the first complex, ATG12–ATG5, starts with conjugation of the ATG12 C-terminal glycine, first with a cysteine residue of the E1-like enzyme ATG7, and then with a cysteine residue of the E2-like enzyme ARG10 via disulfide bridge formation. Eventually, ATG12 forms an amide bond with ATG5 [49]. The ATG12–ATG5 complex interacts with the C-terminus of ATG16 (ATG16L in mammals), which oligomerizes and forms large subunits. The ATG12–ATG5–ATG16 complex binds to the phagophore and provides its expansion and bending. The ATG12–ATG5 complex does not undergo deconjugation, and it is formed constantly and independently of surrounding conditions. In mammals, ATG12–ATG5–ATG16L1 complexes are predominantly located on the isolation membrane and dissociate from it immediately after autophagosome formation [50]. The number of these complexes is low, and it is highly unlikely for them to cover the whole membrane [51].

The second complex, ATG8–PE, includes a unique ubiquitin-like ATG8 protein and phosphatidylethanolamine (PE) as a lipid. The ATG8 is synthesized as a precursor, and it requires a complex of posttranslational modifications (Fig. 2). In particular, the C-terminal tail of ATG8 is cleaved by the cysteine proteinase ATG4, as shown in rice [52]. The cleavage exposes a glycine residue that covalently binds to the amino group of PE, the major phospholipid of autophagosomes, in a reaction catalyzed by ATG7 and ATG3 (E1- and E2-like proteins, respectively) [53]. The ATG8–PE complex could be located in

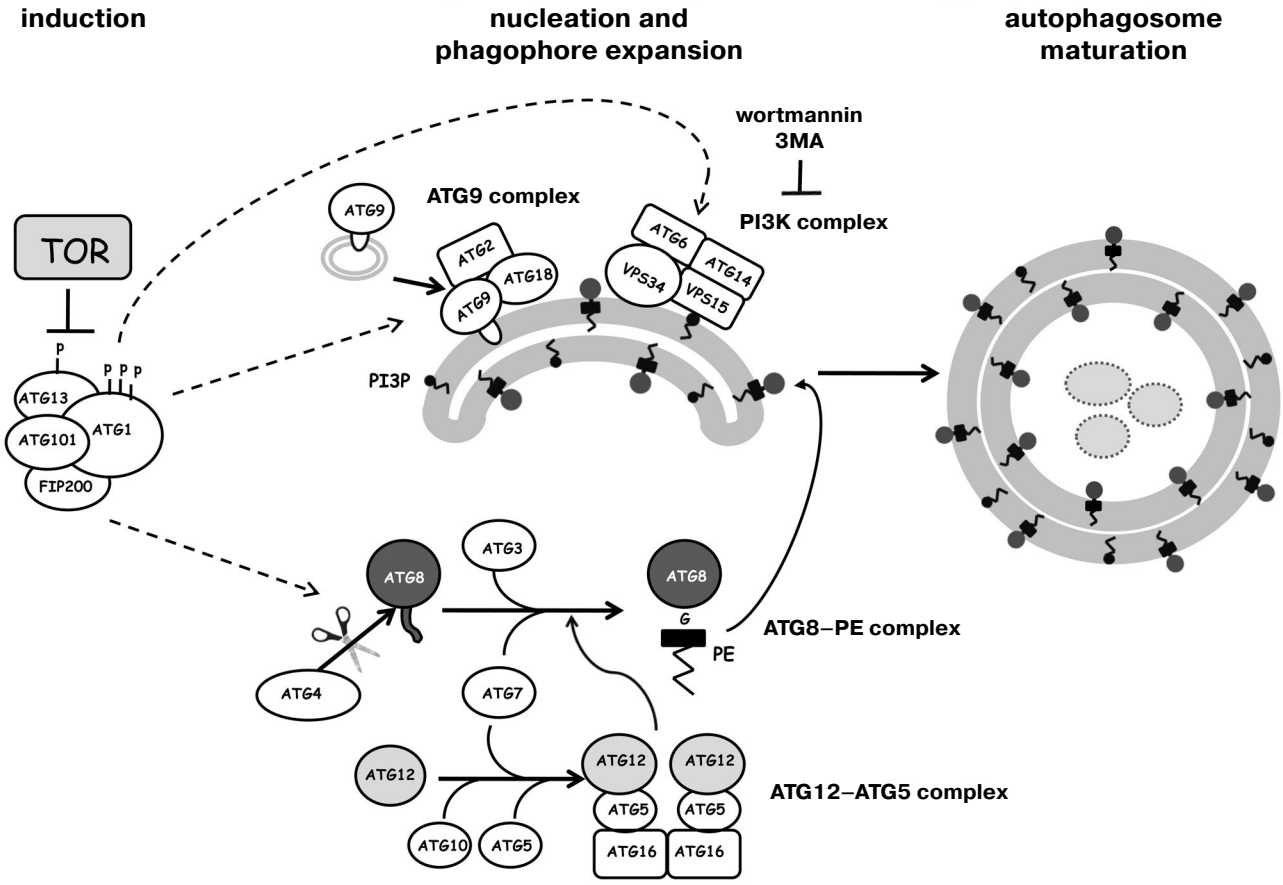

Fig. 2. Molecular mechanisms of autophagosome formation. **Induction**: inhibition of the negative autophagy regulator TOR kinase results in ATG13 dephosphorylation. ATG13 binds to ATG1 and forms a large complex including additional proteins ATG101 and FIP200, thus initiating a platform for autophagosome formation. **Nucleation and phagophore expansion**: ATG9 protein complex delivers lipids; nucleation is provided by PI3K 1 that produces PI3P. Two ubiquitin-like complexes, ATG12–ATG5 and ATG8–PE, regulate autophagosome size, curvature, and expansion rate. ATG8 interacts with PE in a ubiquitin-like reaction. **Autophagosome maturation**: after the autophagosome is formed, proteins involved in autophagosome formation dissociate from it. The mature autophagosome decorated with ATG8 is transported to the vacuole/lysosome for fusion.

either the outer or the inner autophagosomal membrane [54], which makes ATG8 a convenient molecular marker for monitoring macroautophagy [54, 55]. The ATG8–PE complex in the outer membrane is deconjugated by ATG4. The inner membrane complex is degraded in the vacuole [56]. It was found that some *atg* mutants with deficiencies in phagophore formation still had the ATG8–PE complex. This suggests that ATG8 can be lipidated in the absence of isolation membrane formation [57]. However, it remains obscure where ATG8 undergoes lipidation and how the formed complex is incorporated into the membrane. *In vitro* studies demonstrated that the ATG8–PE complex is capable of binding to the membrane and hemifusion [58].

The two conjugating systems share the E1-like ATG7 enzyme that is required for both cascades. ATG7 activates ATG12 in one of the complexes and ATG8 in another. It has been shown that the ATG12–ATG5 complex interacts with ATG3 *in vitro* and promotes ATG8 transfer from ATG3 to PE [59]. Moreover, ATG12–ATG5–ATG16(L) might be involved in determining the site of lipidation [60].

After the autophagosome is formed, most of the proteins dissociate from it. The normal course of autophagy requires the autophagosome to be formed completely before its fusion with the vacuole. The ATG5–ATG12–ATG16 complex and a portion of the ATG8–PE complexes on the outer membrane are capable of preventing premature phagophore fusion [61]. Recent studies showed that in addition to its protective function, a layer of ATG8 proteins on a phagophore surface serves for the attachment of ATG8-interacting proteins that play an important role in autophagosome formation and selection of removed components [62].

Fusion with vacuole/lysosome. Fusion of autophagosome with lysosome (in animals) or vacuole (in plants and fungi) is the terminal stage of the autophagosome life cycle. It was suggested that this fusion requires Rab7 protein [63] that provides microtubule plus-end-directed transport of autophagosomes upon its simultaneous association with FYCO and LC3 [64]. In mammals, some SNARE factors, such as VAM7, Vti1b, and VAM9, were found to be involved in fusion [65, 66]. In yeasts, these proteins are t-SNARE, VAM3, Vtip, Sec18 [67], and a small GTPase factor Ypt7 [54]. Autophagosome fusion with the lysosome is promoted by the UVRAG–VPS34–Beclin1 PI3-kinase complex 1 and inhibited by the Rubicon–UVRAG–VPS34–BECLIN1 PI3-kinase complex [68, 69]. It remains unknown, what factors and what mechanisms regulate the interaction between these complexes.

Therefore, the molecular mechanism of autophagosome formation is an extremely complex multistage process that involves numerous components, such as cell organelles, specific proteins (ATG and others), and various factors including signaling molecules and lipids. Besides, it requires an intrinsically organized platform for autophagosome formation and transport within the cell.

STRUCTURAL AND PHYSICOCHEMICAL PROPERTIES OF ATG8 FAMILY PROTEINS

ATG8 proteins are strictly conserved and have been found in all eukaryotic organisms, e.g. alga, lower plants, cultured grains, yeasts, and mammals including humans. In contrast to fungi that carry only one *ATG8* gene, plants and animals have multigene *ATG8* families [58]. In animals, the *ATG8* family can be subdivided into several subfamilies: (i) microtubule-associated protein 1 light chain 3 (MAP1 LC3); (ii) γ-aminobutyric acid A receptor-associated protein (GABARAP); (iii) Golgi-associated ATPase enhancer of 16 kDa (GATE-16) [70]. Proteins of each subfamily play different roles at different stages of autophagosome formation [71]. In *Arabidopsis*, nine ATG8 isoforms (a-i) have been identified that form three subfamilies [71]. Soya has eleven ATG8 isoforms [72], wheat – nine [73], rice and maize – five isoforms [74] (Fig. 3).

Plant *ATG8* genes consist of five exons and four introns of different length. In the wheat *Triticum aestivum*, the first *ATG8* gene subfamily includes three homeologous genes and codes for two proteins of 119 a.a. each. The second subfamily codes for one ATG8g protein of the same size. Genes of the third group code for three proteins: h, i, and j. ATG8 proteins usually contain a ubiquitin domain (pfam: PF02991), N-terminal microtubule-binding site, ATG7-binding site, and C-terminal glycine residue that is required for ATG8 lipidation. However, some plant isoforms lack additional amino acid residues after the conserved C-terminal glycine residue, which indicates that ATG8 can interact with autophagosomal membrane in the absence of ATG4. These are *At*ATG8h-i from *Arabidopsis*, *Os*ATG8d from rice, and *Ta*ATG8h, i, and j isoforms from wheat ([71, 74], Ryabovol et al., unpublished data) (Fig. 3). Phylogenetically, plant ATG8 proteins can be subdivided into two clades. Proteins of the first clade, which includes most of the ATG8 family members, are highly homologous (80-100%). It was suggested that genes coding for the ancestor and descendant proteins of this clade often underwent duplications in the course of evolution. The second clade includes one or two representatives from various plant species. The ancestors of this group and the following generations have been subjected to a rapid evolutionary process both before and after separation of monocotyledons and dicotyledons [73].

The 3D structures of some proteins from the animal ATG8 family (MAP LC3 [75], GABARAP [76], GATE-16 [77], and yeast ATG8 [78]) have been characterized by NMR and crystallography. ATG8 proteins are composed of the N-terminal helicase domain and C-terminal ubiq-

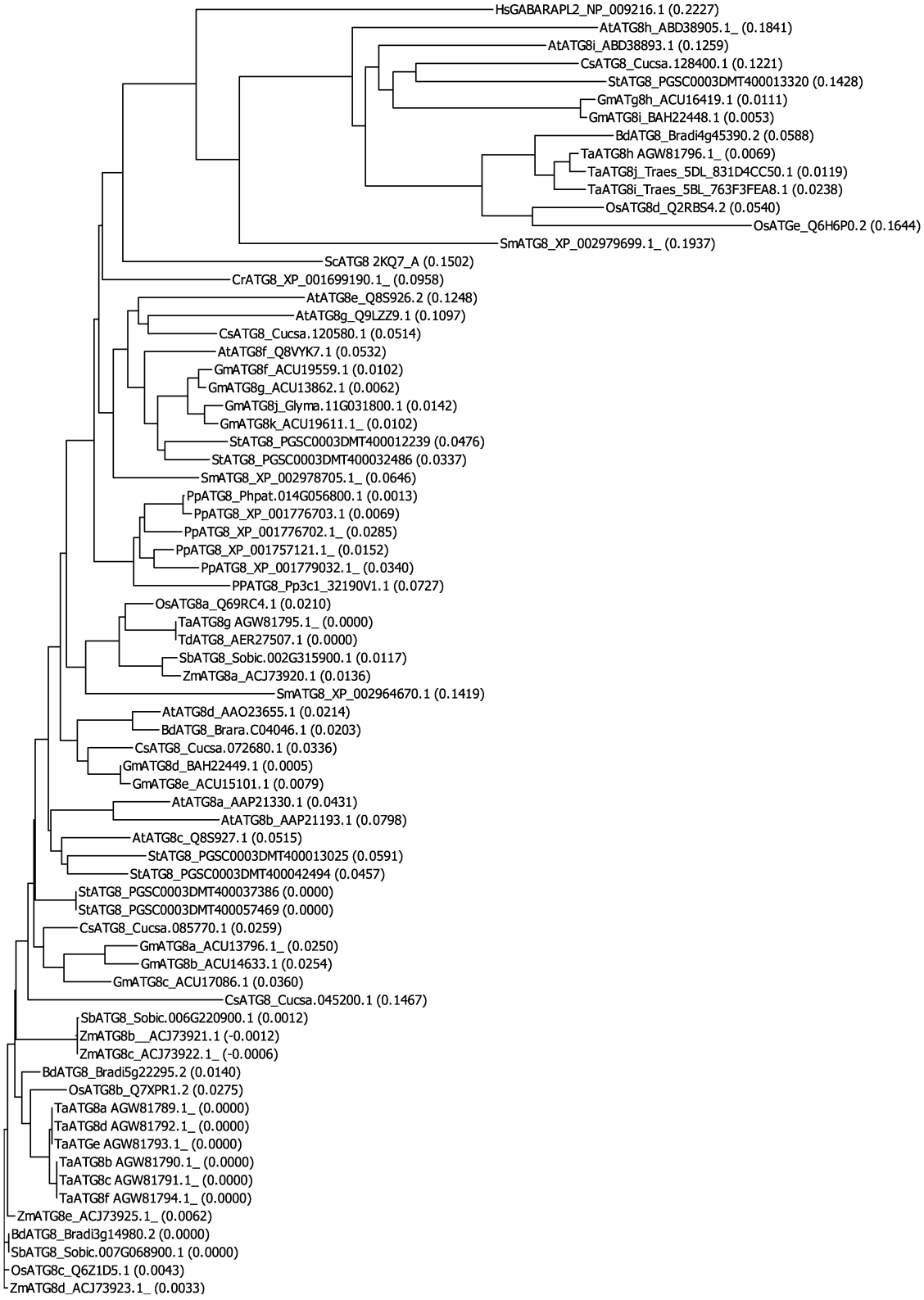

Fig. 3. Phylogenetic tree of the ATG8 protein family (created using the Vector NTI program). The tree includes ATG8 families from plants, yeasts, and humans: nine isoforms from *A. thaliana*, four – from *Brachypodium distachyon*, one – from *Chlamydomonas reinhardtii*, four – from *Cucumis sativus*, eleven – from *Glycine max*, five – from *Oryza sativa*, six – from *Physcomitrella patens*, three – from *Selaginella moellendorffii*, seven – from *Solanum tuberosum*, three – from *Sorghum bicolor*, one – from *Triticum dicoccoides*, ten – from *Triticum aestivum*, five – from *Zea mays*, ATG8 from *Saccharomyces cerevisiae*, and GABARAPL2 from *Homo sapiens*. The data were taken from the GenBank, SwissProt, and Phytozome databases (protein ID numbers are shown next to protein names).

uitin domain. The ubiquitin core consists of a four-stranded central β-sheet (β1-β4). Between these strands, there are two α-helices (α3 and α4). The folding of this domain resembles the folding of ubiquitin (hence the name "ubiquitin domain") [79] (Fig. 4). ATG8 belongs to a superfamilily of ubiquitin-like protein. Ubiquitin (UB) and ubiquitin-like (UBL) proteins form a large superfamily that includes about 20 protein families. Although ubiquitin proteins fullfil various functions in cells by performing posttranslational modifocations, all UB and UBL proteins are characterized by the presence of the "β-grasp" folding (Fig. 4). Such folding allows UB proteins to assemble rapidly, stabilizes their spatial structures, and provides resistance to environmental factors, e.g. high temperature [80]. Interestingly, despite the fact that the 3D structures of ATG8 and ubiquitin are very similar, their amino acid sequences differ greatly. Indeed, the identity between amino acid sequences of GABARAP and ubiquitin is only 7% [81]. The ubiquitin domain in ATG8 proteins is mostly conserved among the members of the family and might play an important role in protein–ligand interactions [79].

The N-terminus of ATG8 consists of two α-helices (α1 and α2) (Fig. 4). Unlike the ubiquitin domain, the N-terminal region is not conserved and varies among ATG8 proteins from different organisms, being the major unique feature for the ATG8 family [79]. It is responsible for tubulin binding and protein oligomerization during autophagosome formation [82].

It has been shown that in animals, GABARAP is involved in the intracellular traffic of the $GABA_A$ receptor and could exist in two conformations – open and closed. In its closed conformation, the structure of the GABARAP protein is silmilar to that of GATE-16. The N-terminal domain is packed against the C-terminal ubiquitin-like domain forming a compact fold. In the open conformation, the ten N-terminal amino acid residues that form the α1 helix in the closed conformation acquire extended configuration and distance themselves from the ubiquitin-like domain. The open conformation supports intermolecular interactions of β-sheet type, where residues 2-4 interact with β2 of the adjacent GABARAP molecule. Residues 1-5 form polar and nonpolar bonds with another protein molecule. The open conformation is stabilized by high salt concentrations, and presumably, other proteins (e.g. tubulin) or membranes [82].

The structure of LC3, a mammalian ortholog of ATG8, is similar to the stuctures of GATE-16 and GABARAP in closed conformation. Amino acids of the α1 helix in LC3 are basic, whereas in GATE-16 and GABARAP, most amino acids in the α1 helix are acidic. The α2 helices in LC3, GATE-16, and GABARAP contain mostly acidic, neutral, and basic amino acids, respectively. The differerence in the electrostatic surface potential of these three homologs might explain the variety of functions performed by the proteins in mammalian cells [79].

Despite the presence of numerous ATG8 isoforms [71] that might function at various stages of autophagosome formation in plants, none of the plant ATG8 3D structures has been completely solved so far. Two brief communications have been published on the structure of APG8A (LC3 homolog from *Arabidopsis*) [83, 84]. However, in *At*APG8A, only 85% of signals have been assigned. It was not possible to determine positions of 16 out of 110 amide protons, 11 of these protons belonging to the N-terminal domain. The lack of information on the ATG8 structure could result from protein instability and changes in its physical and chemical properties. For example, recombinant *At*APG8A obtained by Chae et al. [83] did not exhibit stable 3D structure. Recently, we purified recombinant ATG8g from *T. aestivum*. The protein was unstable in buffer solutions and aggregated dur-

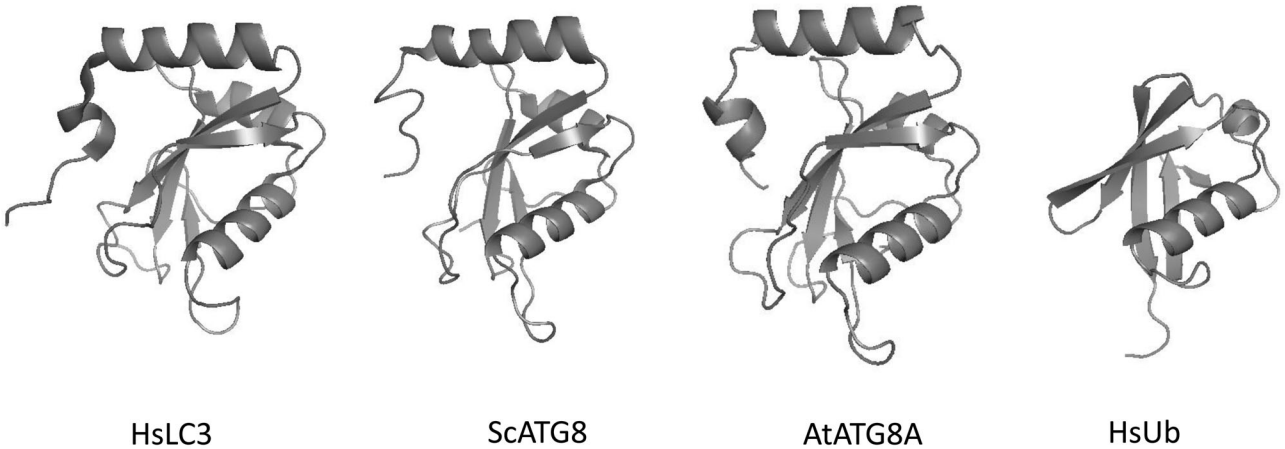

Fig. 4. 3D structures of the ubiquitin proteins: human *Hs*LC3 (PDB: 1V49), yeast *Sc*ATG8 (PDB: 2KWC), *Arabidopsis At*ATG8A (predicted with the I-Tasser server; C-score, 0.56), human ubiquitin *Hs*Ub (PDB: 1UBI). All models were built with the PyMOL program.

ing purification (Ryabovol et al., unpublished data). The same properties were observed earlier for yeast ATG8 [78]. It was also found that ATG8 displays higher stability after forming a complex with a ligand, which can be explained by restricted mobility of the α2 helix involved in intermolecular interactions. Unlike yeast and plant ATG8 proteins, animal ATG8s are stable in solutions even at high concentrations [78]. Analysis of spatial structures and physicochemical properties of ATG8 proteins reveals their common domain organization in all eukaryotes. However, the observed mobility of structural elements indicates that formation of intermolecular interactions by plant ATG8 might be specific for this family of proteins, but this suggestion needs further corroboration.

INTERACTION OF ATG8 WITH LIGANDS

ATG8 interacts with various proteins in the course of autophagy. Most protein–protein interactions occur via complex formation between amino acids of the W- and L-sites of ATG8 and amino acids of the so-called AIM (ATG8 family-interacting motif), or LIR motif (LC3-interacting motif) of the receptor protein. Amino acids of the W- and L-sites are conformationally close to each other and form two hydrophobic pockets on the protein surface [62]. The AIM in ligand proteins is universal and consists of only several amino acids: X_{-3} X_{-2} X_{-1} W X_1 X_2 L. The major interactions take place between the ligand tryptophan and the W-site of ATG8 and between the ligand leucine residue and the L-site of ATG8. The AIM is highly conserved, although in some proteins, tryptophan could be substituted by tyrosine or phenylalanine, and leucine could be substituted by isoleucine or valine. Five other amino acids (X_{-3}, X_{-2}, X_{-1}, X_1, X_2) are usually acidic. To bind to the W- and L-sites, the AIM should be in an extended β-conformation with exposed hydrophobic side chains. This requirement should be taken into consideration when searching for AIMs in proteins: if potential AIM residues are buried within the molecule, they might be incapable of interacting with ATG8. AIM identification will be greatly helped by data on the protein 3D structures. In cases when the 3D structure is unavailable, prediction methods can be valuable instruments for AIM identification [62].

Humans have a few tens of proteins that can potentially interact with ATG8 [85]. Among them, autophagic proteins ATG1, ATG3, ATG4, and ATG16 can directly modify ATG8 during its lipidation with PE (see "Molecular Mechanisms of Autophagy" above). The second group includes the Bin/Amphiphysin/Rvs (BAR)-Src homology (SH)-3 domain-containing family that is involved in changing the curvature of the growing phagophore and its closure upon autophagosome maturation. The third group of AIM proteins includes FYVE and coiled-coil domain-containing adaptor proteins (FYCO1). Another large group of AIM proteins includes molecules (NBR, p62, Nix/Binp3L, etc.) that participate in selective removal of cell components [85]. AIM proteins have been identified in various organisms and now are extensively studied. Some of these proteins are highly conserved, and others are specific for each eukaryotic kingdom. In this review, we describe in details some of the plant AIM proteins.

ATG4. Cysteine proteinase ATG4 plays an important role in autophagosome formation [86]. This protein is mostly involved in the processing and delipidation of ubiquitin-like ATG8 proteins. The characteristic property of cysteine proteinases is the presence of conserved cysteine residues that can undergo fast and reversible posttranslational modification. Due to such modification, the activity of these enzymes can be regulated by the cell redox state. The redox regulation of ATG4 in HeLa cells depends on a specific cysteine residue located close to the enzyme active site. Mutation of this residue prevents ATG8 lipidation and, as a result, autophagosome formation [4].

Yeasts carry a single *ATG4* gene that codes for only one isoform of the protein (similarly to ATG8) [58]. Mammalian cells have several Atg4 protein homologs: hAtg4A/*Hs*Atg4A/*Hs*Apg4A/autophagin-2, hAtg4B/*Hs*Atg4B/hApg4B/autophagin-1, hAtg4C/HsAut11/autophagin-3, and hAtg4D/autophagin-4. Each protein modifies different ATG8 isoforms with different efficiency [87]. Satoo et al. [88] deciphered the molecular mechanisms of hydrophilic and hydrophobic interactions between LC3 and human *Hs*ATG4B. Thus, the N-terminal fragment of *Hs*Atg4B acquires an extended β-configuration and forms intermolecular β-structure with the β2-sheet of LC3. The side chains of Tyr8 and Leu11 of *Hs*Atg4B interact with the hydrophobic pockets on LC3 formed by Ile23, Pro32, Lys51, Phe52, Leu53, Val54, Leu63, Ile67, and Phe108 residues, i.e. *Hs*Atg4B interaction with LC3 involves the binding of the N-terminal AIM motif to the W- and L-sites of LC3 [88].

Wheat has two ATG4 homologs – *Ta*ATG4a and *Ta*ATG4b [73]. Two ATG4 homologs, *At*ATG4a and *At*ATG4b, have been identified in *Arabidopsis*. Recent studies showed that these enzymes have different activities and exhibit different substrate affinity [89, 90]. Thus, *At*ATG4a predominantly cleaves *At*ATG8a, *At*ATG8c, *At*ATG8d, and *At*ATG8i, whereas other isoforms are processed by *At*ATG4a-b with similar efficiencies [89]. Kinetic analysis showed that *At*ATG4a activity is twice as high as the activity of *At*ATG4b, but *At*ATG4a is more sensitive to high H_2O_2 concentration. These data suggest that *At*ATG4a has wider substrate specificity and acts as a major ATG8-activating enzyme in plants under normal conditions. When subjected to prolonged oxidative stress, plants use *At*ATG4b for autophagosome formation, i.e. similarly to animals and unlike yeast, combinations of

different ATG4–ATG8 isoforms in plants provide precise autophagy regulation in varying microenvironments [90].

ATG1–ATG13. Lee and Vierstra [91] demonstrated that ATG1 directly binds ATG8 incorporated into the phagophore membrane, presumably through the AIM in the C-terminal domain (EREYVLV in ATG1a). This might provide ATG1–ATG13 complex turnover in a course of autophagy. Interestingly, plant-specific ATG1t lacks AIM and is incapable of ATG8 binding.

FYCO1. FYCO1 proteins contain a sequence of four amino acid residues (FYVE) and a coiled-coil domain. This "module" structure allows them to interact simultaneously with ATG8 and some of PI3P molecules decorating the autophagosome surface, as well as with RAB7 Ras-bound GTPases that promote direct microtubule plus-end-directed transport. It was suggested that FYCO1 serves for autophagosome binding to the microtubule network and autophagosome delivery to the vacuole [64].

AIM PROTEINS INVOLVED IN SELECTIVE AUTOPHAGY

NBR and p62/SQSMT1. It is now commonly believed that to be removed by autophagy, cytoplasmic components should be ubiquitinated first. In mammalian cells, ubiquitinated proteins are recognized by the autophagosomal receptors NBR and p62/SQSMT1 that noncovalently interact with ubiquitin via their ubiquitin-associated (UBA) domains. Both NBR and p62/SQSMT1 have short LIR sequences for interaction with the LC3 domain and PB1 domains for self-aggregation and association with other adaptor proteins (e.g. for interaction with each other).

Arabidopsis has the *At*NBR1 protein that is homologous to both NBR1 and p62 and has hybrid properties of these two adaptor proteins. *At*NBR1 contains two UBA domains, of which only the C-terminal one is capable of ubiquitin binding. *At*NBR1 interacts with various ATG8 isoforms via the AIM; this interaction does not depend on *At*NBR1 homopolymerization and is more efficient [92]. A new structural and functional homolog of p62 and NBR1 has been recently found in tobacco. The identified protein, Joka2, belongs to the UP9/LSU family. Similarly to p62 and NBR1, Joka2 exhibits both nuclear and cytoplasmic localization. It also has conserved domains typical of p62 and NBR1, forms homodimers, and interacts with ATG8. These structural and functional properties suggest that Joka2 might have an LIR domain as well [93]. Both NBR1 from *Arabidopsis* and Joka2 from tobacco are involved in the removal of polyubiquitinated proteins that accumulate during abiotic stress [93, 94].

TSPOs (tryptophan-rich sensory protein/peripheral-type benzodiazepine receptor domain-anchored proteins). Another group of molecules with potential AIMs is TSPO. TPSOs are membrane proteins that participate in transmembrane signaling and maintain the concentration of free porphyrins in plant cells. The level of porphyrins, which can protect cells from ROS, increases as a result of stress response when exogenous abscisic acid is added to plant cells. However, redox processes that involve free porphyrins (including hemes) can be cytotoxic because of free radical formation, especially when plants are exposed to light. The TSPO proteins have a high affinity for the heme and can neutralize free hemes and other highly reactive porphyrins if they accumulate in high concentrations. *At*TSPO was found to colocalize with ATG8e and to be transported to the vacuole during autophagic degradation [9].

AIM proteins involved in selective removal of organelles. Autophagosomes are also capable of removal of whole organelles, such as mitochondria, chloroplasts, peroxisomes, ribosomes, and ER. Recently, the role and the mechanisms of ATG8 involvement in selective autophagy of organelles became an extensively studied subject. Mitochondria are often found in autophagosomes. Some of them are removed via a nonselective process, especially during starvation. However, a strong body of evidence confirms that autophagosomes can recognize and selectively remove mitochondria in a process of so-called mitophagy. It was found earlier that Uth1 and Aup1 proteins are involved in the degradation of mitochondria in the vacuole [95, 96]. Recent studies demonstrated that in yeasts, proteins ATG11, ATG20, and ATG24 participate in mitophagy in addition to major ATG proteins [97]. Moreover, two other mitophagy-related proteins were identified in yeasts: mitochondrial receptor ATG32 located on the outer mitochondrial membrane and capable of ATG8 binding [98, 99] and mitophagy-mediating factor ATG33 [100]. Although ATG32 is not conserved in higher eukaryotes, selective removal of damaged and aged mitochondrial by mitophagy have been observed in mammals [101, 102]. The two proteins that were found to be involved in selective mitochondria removal in humans are Parkin and Nix [103, 104]. Parkin, which is an E3 ubiquitin ligase, selectively attaches to damaged mitochondria, while PINK1 (PTEN-induced kinase 1, a protein related to the development of Parkinsonism and neurodegenerative disorders) activates Parkin and mediates its binding to damaged mitochondria [4]. Nix serves as an adaptor between mitochondria and LC3 on the autophagosome membrane [104].

The existence of selective mitochondria removal and degradation in plants is still under discussion. Some authors believe that mitochondria undergo internal degradation accompanied by swelling of cristae, granule formation between the membranes, and formation of vacuole-like structures from mitochondria [105]. At the same time, researchers are looking for specific proteins and factors involved in mitophagy in plants. Recently, a new ATG11-related protein was found in *Arabidopsis*

[106]. This protein is involved in mitochondria removal by senescence-induced mitophagy. It is structurally similar to RB1CC1 (RB1-inducible coiled-coil protein 1), also known as FIP200 (FAK family interacting protein of 200 kDa). The latter is composed of the functional domain of ATG17 protein (N-terminus) and the functional domain of ATG11 protein (C-terminus) [107]. The ATG17 domain of the ATG11-related protein has the ATG8-binding motif Y–X–X–X–L/V/I–X–E–V/I–X–RR–R/K. Mutants by the *atg11* gene exhibit lower contents of autophagic vesicles with encapsulated mitochondrial proteins (COX2 and VDAC) or whole mitochondria. However, the function of ATG11 remains unknown [106]. It was suggested that some proteins from the ATG18 and ATG8 multigene families could also be involved in mitophagy in plants [108].

It has been shown [109] that in aging plants, the major photosynthesizing protein complex Rubisco can be selectively eliminated using specific structures called Rubisco-containing bodies (RCBs). RCBs (~1 μm in size) colocalize with GFP-labeled ATG8, which indicates that RCBs are degraded by autophagy. RCB autophagy could be induced by carbon starvation, but not by osmotic stress or nitrogen deficit [110]. It is suggested that autophagy plays a role in decreasing the size and content of chloroplasts in senescent cells via RCB-like mechanisms different from canonical chloroplast degradation [111]. However, no direct involvement of ATG8 in selective removal of chloroplasts and their components, as well as peroxisomes, has been demonstrated so far. In yeasts, the molecular mechanism of pexophagy is well established: the ATG36 protein acts as a mediator that can simultaneously bind peroxisome protein Pex3 and autophagic protein ATG11 (and, probably, ATG8) [112]. In tobacco cell culture, peroxisomes are degraded by the vacuolar mechanism sensitive to the PI3K complex inhibitor 3-methyladenine [113]. Additionally, abnormal peroxisomes were localized close to the phagophore-like structure that could be labeled with anti-ATG8 antibodies. This suggests that peroxisomes in plants can be selectively degraded in autophagosomes [114].

The role of ATG8 in the selective degradation of cell organelles and components in plant autophagosomes still has to be elucidated.

ATI1/ATI2. Recently, ATG8-interacting proteins ATI1/ATI2 have been identified in *Arabidopsis*. These plant-specific proteins can bind different ATG8 isoforms and associate with newly discovered cytoplasmic compartments named ATI bodies [115]. ATI bodies differ from classic autophagosomes and represent a new type of stress-induced autophagy-related compartments that presumably deliver ER components to the plant vacuole [116]. It was suggested that stress-induced ATI1 structures might be involved in either vesicular transport of functional vacuolar cargo from the cytoplasm into the vacuole (CVT pathway) or in autophagy by transporting aberrant ER proteins [32]. The ATI pathway may be an alternative autophagy mechanism, although the precise function of ATI bodies has still not been revealed. ATI proteins have two AIMs for ATG8 binding. Under normal conditions, ATI1 and ATI2 associate with the ER membrane. When cells undergo carbon starvation, ATI1 and ATI2 provide *de novo* formation of vesicles that move along the ER and finally arrive to the vacuole. Overexpression or downregulation of ATI stimulated or suppressed, respectively, seed germination in medium containing abscisic acid, a hormone that is known to inhibit germination [115].

Therefore, identification of ATG8-binding receptors will help in elucidating the mechanisms of several intracellular processes, such as selective removal of cell components during "housecleaning", maintenance of carbon and nitrogen turnovers essential for plant growth and development, and induction of immune response, in particular, by isolation and degradation of pathogens upon their entry into cells. Besides, discovery of new AIM proteins will reveal the connections between autophagy and other metabolic processes, as happened in the case of ATI bodies.

ACTIVITY OF ATG8 IN CELL METABOLISM AND PLANT FUNCTIONING

ATG8 is a multifunctional protein. As discussed above, ATG8 is involved in autophagosome biogenesis: it regulates elongation of the isolating membrane and determines autophagosome size by phagophore lipidation in an ubiquitination-like process resulting in the ATG8–PE complex formation. Because of this, ATG8 is often used as an autophagosome marker in eukaryotic cells [54, 55]. In animals, ATG8 homologs are involved in different stages of autophagosome formation: the LC3 family proteins are responsible for phagophore membrane elongation, while GABARAP and GATE-16 participate in autophagosome maturation [70]. However, ATG8 is required not only for the formation, elongation, and maturation of the autophagosome isolation membrane, it also performs numerous other functions. Thus, both plant and animal ATG8 proteins can bind microtubules, which suggests interconnection between autophagosome processing and cytoskeleton activity [117, 118]. Animal homologs of ATG8 participate in intracellular transport that is not related to autophagy [117, 119, 120]. In yeasts, ATG8 is involved in the yeast-specific CVT pathway that provides the delivery of precursor proteins (e.g. aminopeptidase 1 and α-mannosidase) into the vacuole. These precursor proteins form complexes of large size that prevents their entry into the vacuole through the tonoplast. Instead, the complexes become surrounded by autophagosome-resembling vesicles. ATG8 was found to be located on the surface of CVT vesicles [121].

ATG8 also controls the docking of autophagosome and its fusion with the tonoplast, as demonstrated using liposomes with the incorporated target protein. ATG8 oligomerization in the liposomes caused liposome aggregation due to so-called tethering and membrane hemifusion, i.e. fusion of the outer layers of two different membranes with the inner layers remaining intact [58].

ATG8 also acts as a "connecting link" in the process of cargo recognition during selective autophagy (see above): it recognizes and binds universal AIMs in various ligand proteins [62]. Autophagosomes can remove insoluble protein aggregates, organelles, 26S proteasome itself, and even pathogens. Interestingly, the cargo might be ubiquitinated first and then captured by the autophagosome [122, 123]. Recent studies confirmed that when bound to the cargo through selective autophagy receptors, ATG8/LC3/GABARAP activate various regulatory and core autophagic proteins and determine the site of autophagosome formation [124-127]. Selective autophagy receptors can bind both free ATG8/LC3/GABARAP proteins and their conjugates in the content of forming autophagosomes [128]. Therefore, ATG8 serves as a universal link that provides direct recognition and binding of adaptors/receptors/target proteins to the autophagosome during the selective removal of intracellular components.

It has been proven that *ATG8* genes are expressed constitutively and universally at all stages of plant development, including seed development and germination, flowering, senescence, and death. In rice plants, *ATG8* transcripts were found in spikes, young and mature roots, and leaves [52]. In *Arabidopsis*, transcripts of nine ATG8 isoforms were identified in roots, leaves, stems, flowers, pods, and seeds [129, 130]. We demonstrated that genes of three *ATG8* subfamilies are active in wheat leaves and seedling roots. In particular, *TaATG8g* exhibited high activity in roots under oxidative stress conditions [131]. Using *Arabidopsis* transgenic GUS constructs, it was shown that under favorable conditions expression of genes from different *AtAtg8* subfamilies is organ- and tissue-specific. GUS staining was more prominent in seedling roots compared to shoots. *AtAtg8* genes were predominantly active in root zones that had finished their growth and elongation, such as vascular tissue and root cap, i.e. in areas of intense protein degradation and cell death [132]. Activity of some *Atg8* genes, e.g. *AtAtg8c*, was found in regions with active biosynthesis, such as meristem and elongation zone. *Arabidopsis* flower tissues (sepals, petals, stamens) displayed high levels of *ATG8* gene transcription [130]. Pollination activated transcription of *ATG8* homologs in senescent petunia flowers [133]. It is known that seeds synthesize and store high amounts of storage compounds that are later degraded during seed germination. At late stages of seed development, storage proteins are transported from ER into storage vacuoles by an intracellular mechanism that resembles autophagy [134, 135]. This process is accompanied by an increase in the levels of *ATG* gene expression (in particular, *ATG8*). Interestingly, high levels of *ATG* gene expression are observed even in dry and desiccated seeds [136]. All these data support the idea that ATG8 is essential for maintaining cell metabolism during plant growth and development [131].

ATG8 protein is also involved in the stress response in plants. Genes of the *ATG8* family are differentially expressed under different stresses [22]. Thus, starvation causes an increase in the levels of various *ATG* gene transcripts, including *AtATG8a-AtATG8i*, in *Arabidopsis* cell culture [137]. *AtATG8a*, *AtATG8c*, and *AtATG8g-i* genes were most upregulated during the first 12 h of cell culturing in a depleted medium, the levels of transcription being decreased after 18-20 h of starvation [137]. Changes in the expression levels of *ATG8* family genes upon sucrose and nitrogen deficit were observed in maize, soya, and wheat plants, along with an increase in the amount of processed protein [72-74, 132]. ATG8 is involved in cell response to salt and osmotic stresses [6, 138]. As demonstrated by Western blotting, treatment with osmotically active PEG-20 increased the amounts of ATG8 and ATG8–PE adducts in *Triticum dicoccoides* leaves. Mutants with the "silent" *ATG8* gene were less resistant to drought compared to the control plants [138]. It was shown recently that expression of *ATG8* genes in *T. aestivum* is affected by various factors such as salinization, drought, pathogen attack, and phytohormones [73]. Among abiotic factors, drought caused the most pronounced activation of the *ATG8a*, *g*, and *h* genes. Wheat infection with powdery mildew resulted in two peaks of transcript accumulation for these genes. Phytohormones such as ethylene and salicylic acid downregulated expression of the *ATG8* genes in mildew-resistant plants, but increased the levels of *ATG8* transcripts in mildew-sensitive plants. Methyl jasmonate inhibited *ATG8* expression in both mildew-sensitive and mildew-resistant wheat lines [73].

Some authors suggest that existing diversity of ATG8 proteins provides higher specificity and multifunctionality of autophagy in plants [108, 129, 132]; however, this hypothesis still requires experimental verification.

In plants, autophagy serves as an important catabolic mechanism that controls growth, development, aging, programmed cell death, and stress response to detrimental factors. The key step in understanding of molecular mechanisms of autophagy is solving the structure of the marker autophagic protein ATG8 and deciphering the mechanisms of its functioning in cells. The presence of various ligand-binding sites in the structure of ATG8 indicates the importance of this protein in the formation and processing of autophagosomes.

An intriguing question is the presence of multiple ATG8 isoforms in plants in contrast to a single ATG8 protein in yeasts. The reasons for such diversity and the role

of ATG8 proteins in the formation of autophagosomes and other biological processes are still widely discussed. Some of the explanations might be the following. First, different ATG8 isoforms can perform different functions in autophagosome formation, as observed in mammals. Second, these proteins can interact differently with different ligands. Thus, ATG4 was found to exhibit specificity toward certain ATG8 isoforms. Moreover, it remains unknown what isoforms interact with specific target proteins/adaptors during selective autophagy. Third, ATG8 functions might depend on the intracellular localization of these proteins. Fourth, expression of *ATG8* genes can be tissue- and organ-specific. It can also depend on the development stage or be affected by stress, especially considering the fact that regulatory regions of autophagic genes contain multiple stress-responsive elements. Therefore, the existence of many ATG8 isoforms and the presence of various ligand-interacting sites in their structure not only indicate importance of ATG8 for selective and nonselective removal of intracellular components, but also provide a molecular basis for connection of autophagy with other metabolic processes.

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