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Review

Autophagy in plants and phytopathogens

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ABSTRACT

Plants and plant-associated microorganisms including phytopathogens have to adapt to drastic changes in environmental conditions. Because of their immobility, plants must cope with various types of environmental stresses such as starvation, oxidative stress, drought stress, and invasion by phytopathogens during their differentiation, development, and aging processes. Here we briefly describe the early studies of plant autophagy, summarize recent studies on the molecular functions of *ATG* genes, and speculate on the role of autophagy in plants and phytopathogens. Autophagy regulates senescence and pathogen-induced cell death in plants, and autophagy and pexophagy play critical roles in differentiation and the invasion of host cells by phytopathogenic fungi.
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1. Introduction

Unlike animals, plants are sessile organisms that must endure or overcome variable and severe environmental conditions. For example, the growth of seedlings in nitrogen-poor soils or in the shade indicates that plants have mechanisms for coping with nitrogen- and carbon-starvation. Under nutrient-poor conditions the bulk degradation and recycling of macromolecules is integral to the plant's ability to adapt to its environment. Autophagy is the major system responsible for the degradation of organelles and cytosolic macromolecules in the vacuole, and is therefore assumed to be an extremely important function in plants. To date, two types of autophagy, called microautophagy and macroautophagy, have been reported in plants [1]. In microautophagy, cytoplasmic components are engulfed by an invaginated vacuolar membrane, causing the formation of intravacuolar vesicles, which are then digested by resident vacuolar hydrolases. During macroautophagy, bulk cytosolic constituents and organelles are sequestered into a double-membrane structure called an autophagosome. The outer membrane of the autophagosome then fuses with the vacuolar membrane and thus delivers the inner membrane structure, the autophagic body, into the vacuolar lumen for degradation. In animals the subsequent fusion of the autophagosome with the lysosome, an acidic compartment containing hydrolytic enzymes, produces the autolysosome, but whether this structure exists in all plants is not yet clear.

Early studies of autophagy, which were primarily based on morphological observations using electron microscopy, provided our first insights into the structural components and characteristics of the process in plants. More recently, genetic analyses have greatly expanded our knowledge of the mechanisms and physiological roles of autophagy. In particular, the identification in the yeast *Saccharomyces cerevisiae* of *ATG* genes, which are essential for autophagosome formation, contributed significantly to the development of methods that have allowed the molecular dissection of autophagy in higher organisms. The knowledge gained from yeast research and advances in technology have contributed to the development of autophagy-monitoring systems in plants. The isolation of autophagy-defective plants has greatly invigorated research in this field. Similar genetic approaches with plant pathogenic fungi have contributed to our understanding of the role of autophagy in plant pathogenicity. In this review we first describe the early morphological observations of plant autophagy, and then summarize recent findings relating to the identification and characterization of the plant autophagy genes. Finally, we speculate on the physiological roles of autophagy in both plants and phytopathogens.

2. Morphological observations of plant autophagy

Since the middle of the 20th century, many autophagy-related phenomena have been reported in plant cells, largely as a result

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of electron microscopic observations [2]. Several decades ago electron microscopists had already observed inclusions of cytoplasmic constituents, wrapped in membranes, in vacuoles. The entire sequence of invagination and formation of intravacuolar vesicles (microautophagy) had been documented in corn root meristem cells [3]. The presence of autophagosome-like structures, portions of cytoplasm surrounded by two continuous membranes, was reported earlier by Villiers [4]. However, the described structures often contained partially degraded cytoplasmic constituents and were already acidic, and were therefore probably autolysosomes. Such autolysosome-like structures were often found in root tip meristem cells [2].

In the late 1970s Marty proposed that autophagy involves the formation of lytic vacuoles, again using electron microscopic images [5,6]. By observing the root meristem cells of *Euphorbia*, they found that small vesicles called provacuoles became tubular structures that produced digitate extensions, which formed cage-like structures to sequester portions of the cytoplasm. Adjacent bars of the cage then fused with each other and engulfed the cytoplasmic constituents completely, making autophagosome-like structures. Their cytochemical analyses indicated that the provacuoles and autophagosomes were acidic and contained lysosomal acid hydrolases, suggesting that these structures finally turned into lytic vacuoles.

Studies in the 1980s that utilized transmission electron microscopy suggested that autophagy is involved in chloroplast degradation. It was reported that chloroplasts are taken up by large vacuoles during senescence [7,8]. In addition, it has been proposed that autophagy plays a role in the quality control of chloroplasts. A T-DNA insertion mutant of *PPI40*, which encodes the putative plastid inner membrane translocator Tic40, results in many abnormal chloroplasts in which the thylakoids have not developed. These abnormal chloroplasts were observed in the vacuolar lumen, where they were partially degraded [9]. However, the mechanism by which these abnormal chloroplasts are transported to the vacuolar lumen remains unclear.

Morphological studies have further suggested that autophagic processes execute developmental programmed cell death in plants [10–12]. This implies that embryogenesis, seed germination, xylem differentiation, root development, and petal senescence all involve autophagic cell death.

Electron microscopy was one of the earliest tools used to characterize autophagy, and is also one of the most reliable methods for monitoring the structures of autophagy. However, the results described above provide only static, one-shot images of autophagy. In order to learn more about autophagy, it has been necessary to combine electron microscopy with other techniques such as immuno-EM and tomographic analyses.

3. Molecular machinery required for plant autophagy

Progress in understanding the mechanistic basis of autophagy has been greatly facilitated by the discovery of the *ATG* genes. Most of the *ATG* genes, which are essential for autophagosome formation in yeast, were first identified in 1993 and 1994 [13,14]. Since then, with the unification of nomenclature and the isolation of new yeast autophagy-related genes, the number of *ATG* genes has increased to 32 [15–19]. The genes essential for autophagosome formation are *ATG1-10, 12-14, 16-18, 29*, and *31*, and their products can be divided into five functional groups: the Atg1 protein kinase complex, the PI3 kinase complex specific for autophagy, the Atg9 complex, and two ubiquitination-like systems, the Atg8 lipidation system and the Atg12 protein conjugation system. The two ubiquitination-like reactions have been well investigated in many organisms. It is known that Atg8 is lipidated with the membrane phospholipid phosphatidylethanolamine (PE). In this process, the C-terminal extension of the newly synthesized Atg8 protein is cleaved by Atg4, resulting in a truncated Atg8 with an exposed Gly residue at its C-terminus. This truncated Atg8 is then activated by an E1 enzyme, Atg7, through a thioester bond between the Gly residue of Atg8 and a Cys residue of Atg7. Atg8 is then transferred to a Cys residue of the E2 enzyme Atg3, and finally, Atg8 is conjugated to the head group of PE [20]. This lipid conjugate is essential for autophagosome formation. In the Atg12 conjugation system, Atg12 is activated by Atg7 and then transferred to the Cys residue of the E2-like enzyme Atg10, before it is finally conjugated to Atg5 via an isopeptide bond between the C-terminal Gly residue of Atg12 and a Lys residue of Atg5. The Atg12–Atg5 conjugate is also essential for autophagosome formation [21]. Recently, this complex was shown to function as an E3-like enzyme during Atg8 lipidation [22,23].

The comprehensive sequencing of the *Arabidopsis* genome has allowed a genome-wide search for plant homologs of the yeast *ATG* genes. Hanaoka et al. [24] and Doelling et al. [25] identified about 30 *Arabidopsis ATG* genes *(AtATG)*. Among these are homologs of all the yeast *ATG* genes except *ATG14, 16, 17, 18, 29*, and 31. More recently, an *ATG16*-like gene lacking the WD40 repeats and eight *ATG18*-like genes have been found in the *Arabidopsis* genome (Table 1). Among the *Arabidopsis ATG* genes, *AtATG1, 4, 8, 12, 13*, and *18* are each present at multiple loci (Table 1). For example, there are nine different loci for *AtATG8*. It is unclear why so many paralogs exist in plants, but it may be that the functions of these genes have differentiated over time, as plants evolve strategies to survive under severe environmental conditions. Alternatively, the multiple loci may simply represent functional redundancy. Indeed, *AtATG4a* and *AtATG4b* are redundant [26]. Recently, *ATG* genes have also been identified in some crop species such as rice and maize [27–29].

Sequence homologies between the AtATG proteins and the corresponding yeast Atg proteins are not very high, and only several AtATG proteins can complement the corresponding yeast *atg* mutants. However, most of the essential residues in the yeast Atg proteins are conserved in the AtATG proteins, suggesting that the molecular basis of the core autophagy machinery is essentially the same in plants and yeasts. This prediction has been supported by experiments using *Arabidopsis* T-DNA insertion mutants of the *ATG* genes. In the *AtATG7* mutant *(Atatg7-1)*, the wild-type AtATG7 protein was able to complement the mutant phenotype but a mutant version of the protein, AtATG7C/S, which contains a substitution in a catalytically active Cys residue, could not. This suggested that AtATG7 functions as an E1 enzyme in ATG conjugation reactions [25]. By isolation of a double knockout mutant *(Atatg4a4b-1)* with T-DNA insertions in both the *AtATG4a* and *AtATG4b* genes, which encode the processing and deconjugation enzyme that targets AtATG8, it has also been demonstrated that the putative PE-conjugated AtATG8s and the AtATG8-AtATG3 intermediates are present in wild-type plants but not in *Atatg4a4b-1* [26]. In addition, the C-terminus of the nascent form of all nine AtATG8 homologs was cleaved in an AtATG4-dependent manner, and an Ala substitution of the C-terminal Gly residue of the proteins resulted in defects in their behavior [26]. These results indicate that in plant autophagy, a ubiquitination-like ATG8 lipidation system functions like the homologous system in yeast.

Mutant analyses have also shown that AtATG12–AtATG5 conjugates are essential for plant autophagy [30,31]. Using anti-AtATG12b antibodies, that recognize both AtATG12a and AtATG12b, and anti-AtATG5 antibodies AtATG12–AtATG5 conjugates were detected in wild-type and *AtATG10/Atatg10-1* (heterozygous) plants. In *Atatg4a4b-1* plants, AtATG12–AtATG5 conjugates were also detected. This result was expected because Atg4 is not involved in Atg12–Atg5 conjugation in yeast. By con-

Table 1
Arabidopsis Atg homologs.

S. cerevisiae	*A. thaliana*
Atg1	AtATG1a, AtATG1b, AtATG1c
Atg2	AtATG2[a]
Atg3	AtATG3
Atg4	AtATG4a[a], AtATG4b[a]
Atg5	AtATG5[a]
Atg6	AtATG6[a]
Atg7	AtATG7[a]
Atg8	AtATG8a, AtATG8b, AtATG8c, AtATG8d, AtATG8e, AtATG8f, AtATG8g, AtATG8h[b], AtATG8i[b]
Atg9	AtATG9[a]
Atg10	AtATG10[a]
Atg12	AtATG12a, AtATG12b
Atg13	AtATG13a, AtATG13b
Atg14	Not identified
Atg16	AtATG16L[c]
Atg17	Not identified
Atg18	AtATG18a[a], AtATG18b, AtATG18c, AtATG18d, AtATG18e, AtATG18f, AtATG18g, AtATG18h
Atg29	Not identified
Atg31	Not identified

[a] T-DNA insertional knockout mutants of these genes have been published.
[b] These AtATG8 homologs are plant-specific molecules that lack the C-terminal extension after the Gly residue.
[c] Unpublished data.

trast, AtATG12–AtATG5 conjugates were not detected in *Atatg5-1*, *Atatg7-1*, or *Atatg10-1* plants, which are deficient in the E1-like and E2-like enzymes that target AtATG12. Instead, the levels of unconjugated AtATG12s were higher in these mutants compared with control plants. Additionally, in the T-DNA mutant of *AtATG10 (Atatg10-1)*, wild-type AtATG10 was able to restore the generation of AtATG12–AtATG5 conjugates, whereas AtATG10C/S, in which the catalytically active Cys residue was replaced with a Ser residue, could not. This provided further evidence that AtATG10 functions as an E2 enzyme in the Atg12 conjugation reaction [32]. These results have been supported by the in vitro reconstitution of both the AtATG8 and AtATG12 conjugation systems using recombinant proteins [33]. Recombinant AtATG8s were conjugated to PE in a manner dependent on AtATG7, AtATG3, and ATP. The AtATG8 conjugates were deconjugated by recombinant AtATG4a and AtATG4b. Similarly, recombinant AtATG12 was conjugated to AtATG5 in a manner dependent on AtATG7, AtATG10, and ATP. All of these results support the idea that the two ATG conjugation systems in *Arabidopsis* are very similar to those in yeasts and mammals. In yeast it is known that Atg16 makes an Atg16·Atg5–Atg12 complex via an interaction with Atg5, and that this complex is essential for autophagosome formation [34]. However, whether the plant ATG16 homologs are involved in the formation of autophagosomes remains unclear.

It is also likely that the PI3 kinase complex plays a role in plant autophagy, since silencing of the *ATG6* gene in tobacco and *Arabidopsis* plants resulted in reduced autophagy [35,36]. However, one of the constituents of the PI3 kinase complex that is specific for autophagy, Atg14, has not yet been isolated in *Arabidopsis*.

The Atg9 complex that includes Atg2 and Atg18 also seems to be involved in plant autophagy. The *Arabidopsis* T-DNA mutant *Atatg9-1* shows autophagy-defective phenotypes such as early senescence [24]. The T-DNA insertion mutant *Atatg2-1* and transgenic plants carrying an RNAi construct that targets *AtATG18a* have defects in autophagosome formation [37,38]. It is still unclear whether AtATG9 forms an oligomer that interacts with the AtATG2–AtATG18a complex, as its homolog does in yeast.

Although the Atg1 homologs AtATG1a-1c and the Atg13 homologs AtATG13a and 13b have been identified in *Arabidopsis*, it has not yet been determined whether these homologs play roles in autophagy. So far, homologs of Atg17, Atg29, and Atg31, which are constituents of the Atg1 kinase complex, have not been found in *Arabidopsis*. Recently in mammals, homologs of Atg17 with low sequence similarity were identified by biochemical analyses, suggesting that novel mammal-specific molecular mechanisms, distinct from those in yeast, exist for the induction of autophagy [39]. The isolation of proteins in plants that are functionally related to these proteins would help to elucidate the mechanisms controlling induction of plant autophagy.

4. Monitoring of plant autophagy

Until quite recently the research community lacked adequate methods for the mechanistic analysis of autophagy in plants. This changed with the identification of *AtATG* genes and concurrent advances in technology. The use of *Atatg* mutants has spurred the development of several methods for monitoring autophagy in plants, combining molecular techniques with morphological observations.

In the search for a reliable method for monitoring plant autophagy, it was anticipated that the ubiquitin-like protein Atg8 could serve as a suitable molecular marker [26,31,40]. Atg8 is modified by conjugation with the lipid PE molecule via ubiquitination-like reactions. Consequently, Atg8 behaves as an intrinsic membrane protein, and is involved in autophagosome formation in its PE-conjugated form. The PE-conjugated form of Atg8 is localized to the autophagosome and its intermediates, and eventually, some of it is transferred to the vacuole during autophagy. Therefore in yeast, Atg8 is a useful molecular marker for monitoring the autophagic process [41]. In mammalian cells, the Atg8 homolog LC3 is also used as a molecular marker for monitoring the autophagic process [42].

To address the need for an autophagy-monitoring system in plants, a construct encoding the GFP protein fused to the N-terminus of the AtATG8 protein (GFP-AtATG8) was generated and expressed in transgenic *Arabidopsis* plants. The plants were observed using a confocal laser scanning microscope. In wild-type roots expressing GFP-AtATG8 under nutrient-rich conditions, GFP fluorescence was observed in many ring-shaped and punctate structures, which corresponded to autophagosomes and their intermediates, respectively. By contrast, these structures were not observed in the roots of *Atatg4a4b-1* plants expressing GFP-AtATG8, although some small dot-like structures, possibly corresponding to protein aggregates, were observed. Under conditions of nitrogen-starvation, GFP-AtATG8 was delivered to the vacuolar lumen in wild-type roots. On the other hand, in *Atatg4a4b-1* plants, none of the GFP-AtATG8 proteins, including GFP-AtATG8i, which has no C-terminal extension after the Gly residue, were delivered to the vacuoles. This suggested that the AtATG4s are essential for autophagosome formation not only as processing enzymes, but also as enzymes that deconjugate the putative PE-conjugated AtATG8s. These results suggest that AtATG8s are suitable for monitoring the autophagic process in plants [26].

In mammalian cells it is known that the Atg12–Atg5 conjugate dissociates from the autophagosome when its formation has been completed, which makes it a good marker for monitoring autophagosome formation [43]. As in mammalian cells, it is expected that AtATG5 could provide a useful marker for monitoring the process of plant autophagosome formation.

Approaches that utilize chemical inhibitors of processes essential to autophagy have also been developed in plants. When *Arabidopsis* roots are treated with the V-ATPase inhibitor concanamycin A, many spherical bodies can be observed in the vacuolar lumens of wild-type root cells by conventional light microscopy, but not

in *Atatg4a4b-1* root cells [26]. Electron microscopy revealed that these spherical bodies contain cytoplasmic structures such as mitochondria, endoplasmic reticulum, and Golgi bodies, suggesting that these structures are most likely plant autophagic bodies. Concanamycin A is known to raise the pH in vacuolar lumens when exogenously added to plant cells. Under such high pH conditions vacuolar hydrolases cannot act, resulting in the accumulation of autophagic bodies in the vacuoles. Treatment with concanamycin A is therefore a very easy way to detect whether autophagy occurs in plants. Using these monitoring systems, it is now possible to show that AtATG proteins are responsible for autophagy.

A similar approach utilizes the cysteine protease inhibitor E-64d to detect the autophagy process in plant cells. E-64d is a membrane-permeable inhibitor of cysteine proteases of the papain family. When *Arabidopsis* roots were treated with E-64d, many aggregated (rather than spherical) structures accumulated in the vacuolar lumens of wild-type cells, but not in those of *Atatg* cells [38,44]. This result indicated that these aggregated structures were autophagic bodies that were probably partially degraded. It seems that E-64d does not fully prevent degradation of the membranes of the autophagic bodies in vacuolar lumens. An interesting point is that there is a difference between *Arabidopsis* and tobacco cells in their responses to E-64d treatment. In cultured tobacco cells under conditions of sucrose starvation, E-64d treatment caused the accumulation of membrane vesicles around the nucleus, not in the vacuolar lumens as occurred in *Arabidopsis*. These vesicles were acidic and contained partially degraded cytoplasmic constituents, cysteine protease activity, and acid phosphatase, which is a marker enzyme of lysosomes [45]. Thus, the structures observed in tobacco cells after E-64d treatment seemed to be autolysosomes. The reason for this difference still remains to be elucidated.

As in mammals, LysoTracker and monodansylcadaverine (MDC) staining methods have been tested for monitoring autophagy in various plants including *Arabidopsis*, tobacco, and barley [35,36,46]. However, LysoTracker is a fluorescent acidotropic probe that functions as a label of acidic organelles in living cells, and it is therefore not specific for autophagy. Indeed, senescence-associated vacuoles (SAVs) are labeled by LysoTracker, even though these SAVs cannot be autophagic compartments because they are formed in the *Atatg7* mutant [47]. Thus, caution is necessary when interpreting LysoTracker staining, to ensure that autophagic activity is clearly distinguished from other events that increase lysosomal or vacuolar activity. MDC was thought to label autophagosomes and autolysosomes in mammalian cells, however, recent studies indicate that it was not suitable as an autophagy-specific marker. MDC-positive structures apparently do not always co-localize with autophagosomes labeled with the GFP-ATG8 homolog [48]. It seems that MDC labels acidic compartments such as lysosomes and late endosomes rather than autophagosomes. Therefore, careful observations are required, despite a previous report that MDC-positive structures co-localized with autophagosome-related structures labeled with GFP-AtATG8e in *Arabidopsis* suspension cells [40].

5. Physiological roles of autophagy in whole plants

Arabidopsis atg mutants such as *Atatg7-1* [25], *Atatg9-1* [24], *Atatg4a4b-1* [26], *Atatg5-1* [31], *Atatg10-1* [32], and *Atatg2-1* [38,44], and transgenic plants expressing silencing constructs such as *AtATG6*-RNA interference (RNAi) [36] and *AtATG18a*-RNAi [37] are all defective in autophagy, but are able to complete normal embryogenesis, germination, cotyledon development, shoot and root elongation, flowering, and seed production under normal nutrient-rich conditions. The exception is the *Atatg6* mutant, in which pollen does not germinate [49–51]. It is known that yeast Atg6 is one of the constituents of the PI3 kinase complex and has multiple functions in addition to its role in autophagy, such as vacuolar protein sorting [52]. Therefore, defects in pollen germination in the *Atatg6* mutant cannot necessarily be ascribed to defects in autophagy. The data suggest that AtATG6 plays a role in phosphoinositide signaling during pollen germination in addition to its role in autophagy.

Careful phenotypic analyses have revealed the differences between wild-type and *Atatg* mutant plants. When *Atatg* mutants are grown under nitrogen depleted or limited conditions, they predictably exhibit a drastic acceleration in starvation-induced chlorosis. Additionally, when the leaves are artificially starved by detachment and dark-treatment, the transcript levels of senescence marker genes such as *SEN1* and *SAG12* show larger increases in the mutant leaves than in the wild-type, resulting in an acceleration of artificially induced senescence [24,25,31,32]. *Arabidopsis* plants that have a T-DNA insertion in the gene encoding VTI12, whose yeast homolog is involved in docking and fusion of the autophagosome, exhibit similar accelerations of artificially induced senescence in detached leaves, suggesting that VTI12 functions in autophagy [53]. Due to early senescence, the *Atatg* mutants also develop fewer inflorescence branches, resulting in a lower seed yields [24,25]. Under nutrient depleted conditions, the root elongation of *Atatg* mutants is inhibited relative to wild-type [26]. These observations suggest that when nutrient supply from the environment is limited, autophagy is required for nutrient mobilization in plant cells.

The degradation of chloroplast proteins is accelerated in the *Atatg* mutants compared with wild-type plants [25]. Therefore, autophagy was initially not thought to be necessary for the degradation of chloroplast proteins. However, recent studies have revealed that autophagy can be involved in chloroplast degradation [54,55]. When leaves of wild-type plants expressing chloroplast stroma-targeted GFP were incubated with the V-ATPase inhibitor concanamycin A in the dark, many small vesicles exhibiting Brownian motion were observed in the vacuolar lumens. These vesicles were not co-localized with the chlorophyll. Immuno-EM analysis indicated that these vesicles contained both GFP and large subunits of Rubisco, which is one of the main endogenous proteins found in chloroplast stroma. Consequently these vesicles were named Rubisco-Containing Bodies (RCBs). The transport of RCBs to the vacuolar lumen was only seen under starvation conditions. When leaves were incubated under nutrient-rich conditions, RCBs were not detected in the vacuole, even in the presence of concanamycin A. Interestingly, the accumulation of RCBs in the vacuolar lumen is disrupted in the *Atatg5-1* mutant, indicating that RCBs are transported to the vacuole by autophagy [54]. Furthermore, in individually-darkened leaves, whole chloroplasts seem to be transported to vacuolar lumens by autophagy [55]. Putting these results together, it is likely that the degradation of chloroplast proteins occurs by autophagy in addition to chloroplast-specific or other systems of degradation. However, the degree to which autophagy contributes to the degradation of chloroplast proteins under normal conditions has not been determined.

A role for autophagy in the oxidative stress response has been suggested by experiments involving *AtATG18a* knockdown transgenic plants [37] and a rice *(Oryza sativa)* mutant disrupted in the *ATG10* gene [28]. These plants seem to be hypersensitive to oxidative stress and accumulate more oxidized proteins compared with wild-type plants. This suggests that autophagy can transport oxidized and damaged cellular components produced by oxidative stress to the vacuole, to eliminate toxic materials from the cytosol. However, whether this transport is selective is still unclear.

Some studies have suggested that autophagy is involved in the execution of cell death in cultured mammalian cells [56]. Does plant autophagy execute cell death as well? As mentioned above,

early morphological studies have long suggested that autophagic processes execute developmental programmed cell death (PCD) in plants. Until recently there was no genetic evidence for the requirement of autophagy in the execution of plant PCD. Various *Atatg* mutants that completely lack autophagy are able to accomplish normal developmental processes that were previously assumed to involve autophagic cell death [57–60]. Recently however, Hofius et al. [61] reported that autophagy has a pro-death function in *Arabidopsis* during the hypersensitive response (HR) induced by the bacterium *Pseudomonas syringae* pv. *tomato* strain DC3000, which expresses the avirulence gene *avrRps4 (Pst-avrRps4)*. HR-PCD is one of the defense responses plants employ to survive pathogen attack. When plants are infected with pathogens, the plant cells at the infection site rapidly die to prevent the spread of the pathogen. Hofius et al. detected partially reduced HR cell death rates, measured by an ion leakage assay, in autophagy-defective mutants. This contrasted with the results of Liu et al. [35], who reported that silencing of *ATG6* in *Nicotiana benthamiana* resulted in reduced autophagy, and the spread of chlorotic cell death around the infection site, after inoculation with tobacco mosaic virus. This phenotype was not due to viral movement because transient expression of the viral elicitor protein (TMV-p50) alone was sufficient to induce the spread of chlorotic cell death in leaves of the *ATG6*-silenced plants. A similar phenotype was also observed in *ATG6*- silenced *Arabidopsis* plants infected with *P. syringae* expressing *avrRpm1* [36]. However, involvement of SA in autophagy regulation may explain this discrepancy [44]. Early senescence and the pathogen-induced spread of cell death in *Atatg* mutants could be suppressed by producing double mutants that are defective in salicylic acid (SA) biosynthesis (using the *sid2* mutant) or SA signaling (using the *npr1* mutant) (Fig. 1). These results indicate that excessive SA signaling is the major cause of the early cell death phenotype. Additionally, early cell death in the *Atatg* mutants was only present in the older leaves of older plants, and not in younger plants or younger leaves of older plants. This age-dependency is likely due to developmentally controlled increases in SA levels in the *Atatg* mutants. Hofius et al. used relatively young plants that were approximately 3–4 weeks old. At this younger stage, the *Atatg* mutants show only slight increases in SA levels [44]. It is therefore possible that proper SA levels are critical for the full execution of cell death. Consistent with this possibility, the *Arabidopsis* protein EDS1, which is a modulator of the SA amplification signal, is important for the *Pst-avrRps4*-dependent induction of both SA [62] and autophagy [61]. Based on these results we now propose that autophagy is induced by SA signaling via the NPR1 protein to operate a negative feedback loop modulating SA signaling, which in turn negatively regulates senescence and pathogen-induced cell death.

6. Importance of autophagy and the core Atg molecules in phytopathogenic fungi

It has recently been shown that autophagy plays critical roles in the pathogenicity of two phytopathogenic fungi, the rice blast fungus *Magnaporthe oryzae* and the cucumber anthracnose fungus *Colletotrichum orbiculare* [63,64]. Both *M. oryzae* and *C. orbiculare* form dome-shaped infection structures called appressoria that are darkly pigmented with melanin. The melanized appressorium generates enormous turgor and produces a narrow penetration hypha to breach the plant epidermal cell wall [65,66]. Melanin is a secondary metabolite that is required for the function of the appressorium, and inhibitors of melanin biosynthesis, such as tricyclazole and carpropamid, are widely used as effective compounds to protect plants from fungal diseases [67].

During appressorium formation in both *M. oryzae* and *C. orbiculare*, a nucleus in the conidium (asexual spore) divides, and one daughter nucleus moves to the appressorium while the other remains in the conidium [63,68]. Pharmacological and genetic analyses indicate that mitosis and the subsequent movement of the nucleus are required for appressorium development in *M. oryzae*. Also, the completion of mitosis always results in collapse and PCD of the conidium cell [63]. Notably, the knockout analysis of *MoATG8*, a functional homolog of yeast *ATG8*, demonstrated that this cellular event requires autophagy in *M. oryzae* [63]. The *atg8* mutant did not undergo PCD of the conidium cells, and the appressoria formed by the *atg8* mutant failed to infect rice plants, indicating that autophagy is required for the infection process of *M. oryzae*. The *atg8* mutant of *M. oryzae* exhibits a significant reduction in the production of conidia, which is a common phenotype of yeast mutants defective in autophagy. It has also been suggested

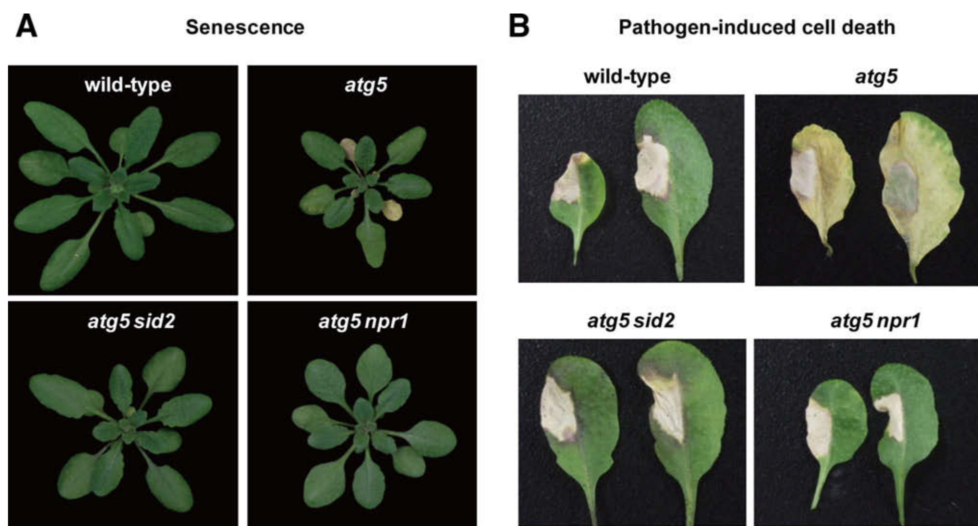

Fig. 1. Early cell death phenotypes of mutants defective in autophagy are suppressed by inactivation of the SA signaling pathway. (A) The early senescence phenotype of the *Atatg5* mutant was suppressed by reducing SA biosynthesis in the *sid2* mutant background, or by blocking SA signaling in the *nprl* mutant background. Shown are photographs of five-week-old plants grown on rockwool supplied with a rich nutrient solution under long-day conditions. (B) The spread of pathogen-induced cell death in the *Atatg5* mutant was suppressed in the *sid2* and *nprl* mutant backgrounds. The 5–8th leaves of each plant grown under short-day conditions for 8 weeks were infected with *Pst-avrRpml* (2×10^7 cfu/ml). Photographs were taken 9 days after infection.

that Atg8 functions in glycogen catabolism during conidial development of *M. oryzae* [69].

A differential screen for genes highly expressed at the appressorium formation stage resulted in the identification of an *ATG1* homolog in *M. oryzae*, and the *atg1* mutant, generated by targeted gene disruption, showed a defect in pathogenicity in common with the *atg8* mutant [70]. The *atg5* mutant of *M. oryzae* showed phenotypes similar to those of the other *atg* mutants [71]. Dong et al. [72] also showed that the integral membrane protein Atg9 showed partial co-localization with Atg8 in conidial cells of *M. oryzae*, and that this co-localization depended on Atg1, Atg2, Atg18, but not Atg13. Recently, studies on the *SPM1* gene, which is highly expressed at the appressorium formation stage, revealed that *SPM1* encodes a subtilisin-like protease required for autophagy in *M. oryzae* [73]. Spm1 shows high homology to yeast Prb1 which functions in autophagy. The expression analysis of an Spm1-GFP fusion protein suggested that Spm1 predominantly localizes inside vacuoles, and the *spm1* mutant, generated by targeted disruption, shows a defect in conidiation and pathogenicity.

In *C. orbiculare*, disruption of the *ATG8* gene also resulted in the loss of phytopathogenicity [64]. The *atg8* mutant of *C. orbiculare* shows a defect in conidiation in common with the *M. oryzae atg8* mutant. However, the *C. orbiculare atg8* mutant shows a defect in germination and appressorium development, whereas the *M. oryzae atg8* mutant retains the ability to develop appressoria. This suggests a diversity in the physiological function of autophagy between these two fungal pathogens, which both develop melanized appressoria.

7. Roles of non-selective and selective autophagy in phytopathogens

Autophagy pathways are divided into two types, selective and non-selective autophagy, depending on the manner of cargo selection. During the non-selective process, the bulk cytosol and other cytoplasmic components are randomly sequestered into autophagosomes. In contrast, during selective autophagy a specific cargo is exclusively targeted. In *M. oryzae, ATG1, ATG5*, and *ATG8* genes were first studied as mentioned. But these genes are core genes essential for both selective and non-selective autophagy. Thus, it remained unclear whether non-selective or selective autophagy has a critical function in fungal pathogenicity in *M. oryzae*. To specify the autophagy pathway required for fungal infection, a systematic gene knockout analysis of 22 *ATG* orthologs was recently performed in *M. oryzae* [74]. It was shown that *M. oryzae* becomes non-pathogenic on rice when it loses any one of the *ATG* orthologs that are involved in non-selective autophagy in yeast *(ATG1-8, 10, 13, 15-18)*. In contrast, *M. oryzae* is still pathogenic on rice in the absence of any one of the *ATG* orthologs specific for selective autophagy (*ATG11, 24, 26-29*). The data indicate that the non-selective autophagy pathway in *M. oryzae* plays a major role in rice blast disease.

A unique autophagy-related pathway, the cytoplasm-to-vacuole targeting (Cvt) pathway, exists in yeasts for the transport of aminopeptidase I to vacuoles. However, there are no clear homologs of the genes involved in this pathway *(ATG19-21, 23)* in the genome of *M. oryzae*. Thus it seems unlikely that *M. oryzae* has a functional Cvt pathway [74].

In contrast with the studies of *M. oryzae*, it has been shown that plant infection by *C. orbiculare* requires the selective autophagy of peroxisomes in the process called pexophagy [75,64]. The number of peroxisomes in a cell depends on both their synthesis and degradation. The physiological significance of peroxisome assembly is well documented due to the presence of human peroxisome biogenesis disorders. In *C. orbiculare* and *M. oryzae*, peroxisome assembly was shown to be necessary for infection of the host plant [76–78]. However, the physiological role of pexophagy has not been explored in either organism.

In the methylotrophic yeast *Pichia pastoris*, growth on methanol as the sole carbon source induces peroxisome proliferation, but changing the carbon source to glucose or ethanol promotes pexophagy. The product of the *P. pastoris ATG26* homolog *(PpATG26)* was identified as a factor that enhances pexophagy in this yeast [79]. *PpATG26* encodes a sterol glucosyltransferase with a phosphoinositide-binding domain. Via this domain, PpAtg26 is localized to a protein–lipid nucleation complex, where it promotes the elongation and maturation of an autophagosome-like structure [80]. The *Ppatg26* knockout mutant shows no obvious phenotypic defects other than in pexophagy.

The molecular analysis of a non-pathogenic *C. orbiculare* mutant, produced by plasmid insertional mutagenesis, resulted in the identification of the *ATG26* homolog *(CoATG26)* as the tagged gene [64]. The *Coatg26* mutant exhibits severe reductions in pathogenicity on the host plant cucumber, indicating a requirement of Atg26 for infection. The *Coatg26* mutant develops appressoria that are darkly pigmented with melanin, suggesting that the mutant has a defect in the host invasion step via the appressoria. Consistent with this, a host invasion assay revealed that the mutant appressoria are defective in the formation of invasive hyphae, indicating that *ATG26* of *C. orbiculare* is specifically required for effective host invasion (Fig. 2).

A GFP fusion protein was made with the peroxisomal targeting signal 1, and expressed in *C. orbiculare* cells to monitor peroxisome status. The conidia of both the wild-type and the *Coatg26* mutant showed abundant fluorescent dots representing peroxisomes, indicating normal peroxisome biogenesis in the absence of Atg26. In the mature appressoria of wild-type cells, the CoAtg8-tagged phagophores engulfed the peroxisomes, and diffuse GFP fluorescence was observed inside the vacuoles, indicating pexophagy in the infection structure. In contrast, bright peroxisomal dots were maintained in the appressoria of the *Coatg26* mutant. This indicated that pexophagy in the appressoria of the *Coatg26* mutant was significantly impaired compared with that in the wild-type. Furthermore, a domain analysis and a GFP-based localization assay of CoAtg26 showed a strong correlation between functional pexophagy and fungal pathogenicity. These data indicate that Atg26-mediated pexophagy is required for the host invasion step by *C. orbiculare*.

Why is pexophagy necessary for host invasion in *C. orbiculare?* CoAtg26-mediated pexophagy functions to recycle cellular components, and this might provide materials for the protein synthesis required for host invasion. Alternatively, the undegraded peroxisomes might have negative impacts on metabolic or structural aspects of the host invasion machinery. A cytorrhysis assay suggested that CoAtg26 contributes to the cellular integrity of the appressoria. It is likely that the integrity of the cell wall is critical for fungal pathogens to resist plant defense responses, such as defenses involving antimicrobial compounds. CoAtg26-mediated pexophagy might contribute to cell wall integrity through organelle recycling. Further experiments will be needed to elucidate the relationship between Atg26-mediated pexophagy and appressorium function at the molecular level.

As described above, melanin is essential for appressorium function [67]. Acetyl-CoA, derived from fatty acid β-oxidation in peroxisomes, is directly utilized for melanin biosynthesis in the appressoria of *C. orbiculare* and *M. oryzae*. In a mutant defective in a peroxin, Pex6, peroxisomal metabolic deficiencies lead to impaired appressorial melanization, resulting in the loss of phytopathogenicity in both *C. orbiculare* and *M. oryzae* [76,78]. If peroxisomes are excessively degraded during the early stage of infection, appressorium maturation, including melanization, might be inhibited. Therefore, peroxisome homeostasis must be strictly

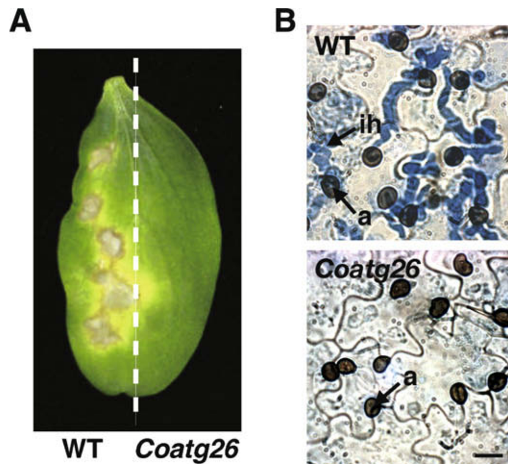

Fig. 2. (A) Inoculation assay of the *Coatg26* knockout mutant on the host plant. The left half of the cucumber cotyledon was inoculated with the wild-type strain (WT) of *C. orbiculare* as a positive control. The right half of the cotyledon was inoculated with the *Coatg26* mutant. The cotyledon was photographed 7 days after inoculation. (B) Appressoria of the *Coatg26* mutant failed to effectively develop invasive hyphae. Conidial suspensions of each strain were used to inoculate the lower surfaces of cucumber cotyledons, which were then incubated for 4 days. The invasive hyphae were stained with Trypan blue. a, appressorium; ih, invasive hypha. Bar = 10 μm.

controlled during this infection stage. It will be interesting to investigate how the Atg26-mediated pexophagy is appropriately regulated during the plant infection process.

Also, as mentioned above, the *atg8* mutant of *C. orbiculare* shows a defect in germination and appressorium formation. This phenotype is not detected in the *atg26* mutant of *C. orbiculare*. Because *ATG8* is required for both selective and non-selective autophagy pathways, the data indicate that plant infection by *C. orbiculare* involves other autophagy pathway(s) in addition to the selective pathway mediated by Atg26. The finding that non-selective autophagy is critical for the pathogenicity of *M. oryzae* suggests that *C. orbiculare* might also need non-selective autophagy for plant infection.

Future studies should focus on the regulatory aspects of autophagy during host infection by *M. oryzae* and *C. orbiculare*. It will be especially interesting to investigate how multiple autophagy pathways (pexophagy and more) are appropriately regulated during the infection process of *C. oribulare*. It will also be important to reveal how autophagy contributes to fungal pathogenicity at the molecular level. This challenge will involve the use of proteomic and metabolomic approaches to studying these fungal pathogens. We also would like to emphasize that both *M. oryzae* and *C. orbiculare* are commonly regarded as hemibiotrophic pathogens: they form melanized appressoria, establish a temporary biotrophic phase after host invasion, and subsequently switch to a necrotophic phase. It is still unknown whether other types of fungal pathogens also require autophagy systems for the establishment of plant infection. Therefore, it is important to study the roles of autophagy in other fungal pathogens, such as biotrophic and necrotrophic pathogens, that show infection strategies that differ from those of *M. oryzae* and *C. orbiculare*.

8. Concluding remarks

With the recent availability of systems for monitoring autophagy and of organisms that are defective in autophagy, major advances are being made in our understanding of autophagy in plants and phytopathogens. Autophagy-monitoring systems allow us to know when and where autophagy occurs, and under which conditions autophagy is induced. These systems will contribute to a further understanding of the physiological roles of autophagy in these organisms. However, we still cannot quantify autophagic activity. Quantification methods, such as the alkaline phosphatase assay in yeast [81], will be needed for a better understanding of the biological phenomena involving autophagy in plants and plant pathogens. Although recently accumulated data suggest that autophagy has a variety of biological functions other than nutrient recycling in plants and phytopathogens, there are still many questions that have to be addressed. Of particular interest is the question of how plants and phytopathogens have evolved autophagic processes in strategies for both infection and defense against one another. Further studies on plants and phytopathogens will involve a combination of morphological, biological, genetic, and comparative approaches, and should result in new insights into autophagy.

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