Chapter 12

Autophagy in Plant Pathogenic Fungi

Huan-Bin Shi, Shuang Liang, Lu-Yao Huang, Xiao-Hong Liu, Xue-Ming Zhu, Ya-Hui Zhao and Fu-Cheng Lin

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Abstract

Autophagy is a ubiquitous and conserved process in eukaryotic cells from yeasts to mammals. It also appears to play vital roles in plant pathogenic fungi, impacting growth, morphology, development, and pathogenicity. In this chapter, we have introduced a new concept to delineate the role of autophagy in homeostasis of plant pathogenic fungi and in their interaction with host cells, in breach of host barrier, and in the mechanisms of plant fungal infection.

Keywords: plant pathogenic fungi, autophagy, selective autophagy, process and function, molecular mechanism

1. Introduction of plant pathogenic fungi

Filamentous fungi play important roles in health care, agriculture, and bioprocessing. There are thousands of species of plant pathogenic fungi that collectively are responsible for 70% of all known plant diseases. An improved understanding of the molecular mechanisms of plant fungal pathogenesis will ultimately lead to better control of plant fungal diseases. However, until recently, the complexity of fungal pathogen-plant host interactions made for slow progress in understanding both the mechanisms of plant host resistance and fungal pathogenesis. Over the last 40 years, the development and application of productive approaches based on genetics and molecular biology has led to the cloning and analysis of many plant resistance genes and fungal pathogenesis determinants to reveal a plethora of fungus-plant molecular interactions [1–3]. Diverse plant organs present different obstacles to

infection by potential fungal pathogens, and therefore successful pathogens have evolved specific strategies, especially infection structures, that are able to break through host plant roots, stems, leaves, flowers, or other special tissues. These infection structures are usually modified hyphae specialized for the invasion of the target host plant tissue(s).

The typical life cycle of plant pathogenic fungi includes an asexual and a sexual stage. In the asexual stage, pathogens often repeatedly produce many asexual spores during the crop growing season; these play important roles in the spread of plant disease. The asexual spores germinate into filamentous hyphae, which can differentiate into a series of complex infection structures such as infection cushions, haustoria, appressoria, penetration pegs, and others [4]. This stage plays an important role in the spread of plant disease. When the vegetative growth stage reaches a certain period of time, fungi begin to enter the sexual stage to form various sexual spores, which are the initial sources of infection during the next disease cycle, in addition to giving rise to offspring and aiding pathogenic fungi in adverse environments. In favor of pathogens undergoing successful sporulation and infection, autophagy plays important roles in nutrient homeostasis. However, studies examining the role of autophagy are still not advanced in plant pathogenic fungi. With the publication of the genome sequence of *Saccharomyces cerevisiae* in 1996, the use of yeast as a model system to study the molecular mechanisms of autophagy came to the fore. The noteworthy discovery of the role of autophagy, following its induction by nutrient starvation, in the development of turgor in the appressorium represents a milestone emphasizing the importance of recognizing the role of autophagy in the formation of infection structures in plant pathogenic fungi. This achievement adds to our fundamental knowledge of both plant fungal pathogenesis and the biological roles of autophagy [5]. In the last two decades, our knowledge has advanced remarkably and autophagy has been examined in organisms from yeast to plant pathogenic fungi, such as *Magnaporthe oryzae*, *Colletortrichum* spp., *Fusarium* spp., and *Ustilago maydis*.

The rice blast fungus, *M. oryzae*, a filamentous ascomycete fungus, is the causal agent of rice blast disease, the most destructive disease of rice worldwide [6]. *M. oryzae* differentiates a special infection structure, the appressorium, to rupture the strong cuticular layer, which is the first defense barrier of the plant host, and gain entry to colonize into plant tissue [4, 7]. The appressorium is a flattened, hyphal structure that is used to enter host cells during infection. It generates colossal intracellular turgor pressure (as much as 8.0 MPa), allowing it to penetrate the leaf cuticle. The mechanical forces that generate a mature appressorium and deliver its penetration peg have been confirmed by researchers worldwide. This enormous turgor in the appressorium is a consequence of the accumulation of very large quantities of glycerol in the cell, and potential sources for glycerol biosynthesis are lipids and glycogen, as well as sugars, trehalose, and mannitol, in the conidium [8]. The sequenced genomes of *M. oryzae* have been provided online [9].

Colletotrichum is the causal agent of anthracnose and other diseases on leaves, stems, and fruits of numerous plant species, including several important crops. The damage caused by *Colletotrichum* spp. extends to important staple food crops, including bananas, cassava, and sorghum. In addition, it is particularly successful as a postharvest pathogen because latent infections, which are initiated before harvest, do not become active until the fruit has been

stored or appears on the market shelf. *Colletotrichum* is highly significant as an experimental model in studies of fungal development, infection processes, host resistance, signal transduction, and the molecular biology of plant-pathogen interactions. *Colletotrichum* can differentiate the specialized infection structure, the appressorium, as well. Dozens of laboratories are studying the biology and pathology of various species of *Colletotrichum* all over the world. The sequenced genomes of *C. graminicola* and *C. higginsianum* have been provided online [10].

The ascomycete *Fusarium graminearum* (teleomorph *Gibberella zeae*) is a highly destructive pathogen of all cereal species. It causes *Fusarium* head blight, a devastating disease on wheat and barley. Infection causes shifts in the amino acid composition of wheat, resulting in shriveled kernels and contamination of the remaining grain with mycotoxins, primarily deoxynivalenol. Mycotoxins in grain can affect human and animal health when they enter the food chain. The other important *Fusarium* species, *F. oxysporum*, can induce susceptibility in more than 120 types of plants such as cotton, tomato, and banana. The infection process begins when the pathogen reaches the surface of plant roots, subsequently resulting in the penetration and colonization of this fungus. Ultimately, the pathogens will diffuse in the xylem vessels, which can result in both local and systemic defense responses in the host plant. The typical disease symptoms in the infected plant cells are slow growth, browning, wilting, and finally the death of the host. In addition to the economic importance of *Fusarium* spp., *Fusarium* species also serve as key model organisms for biological and evolutionary researches [11].

U. maydis, a member of the smut fungi, infects certain important crops such as wheat, maize, and barley. Smut fungi are obligate parasite pathogens, thus they live in their host plants to obtain necessary nutrition for their sexual life cycle. The haploid yeast-like form of *U. maydis* can be propagated on artificial media. However, this form is unable to cause disease. Two compatible haploid strains fuse and generate a dikaryotic filament to cause infection. The dikaryotic filament penetrates the host cell via invagination of the plasma membrane. Unlike the systemic infections caused by other smut fungi, *U. maydis* is unable to cause systemic disease and can only infect the above-ground portions of the host plant maize [12].

In addition, the genetic models *Aspergillus* spp., *Podospora anserina*, and *Sordaria macrospore* are discussed in this chapter. We enumerate progress in studying autophagy and describe the many differences between single cell yeasts and multiple cell fungi in the context of this process.

2. Autophagy: functional roles in eukaryotes

Autophagy is an intracellular degradation system that is highly conserved in eukaryotic cells, which degrade proteins and organelles in the vacuole/lysosome. Studies examining the functions of autophagy have increased significantly in the last decade. Autophagy has a wide variety of functions in eukaryotic cells and intensive studies have shown that autophagy is not only involved in nutrient recycling but also in other cellular processes such as cellular differentiation, growth, and pathogenicity [13–18].

- Autophagy is a general term for the degradation of cytoplasmic components within lysosomes. This process is quite distinct from general endocytosis-mediated lysosomal degradation of extracellular and plasma membrane proteins [19]. There are three types of autophagy that are classified based on the different ways in which a substrate in the cytoplasm can be transferred into the vacuole/lysosome: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). In general, we often consider the term "autophagy" to refer to macroautophagy. In recent years, given the selectivity of substrates for degradation, autophagy has been divided into two types: selective autophagy and nonselective autophagy [16–18, 20]. The unique organelle, called the autophagosome, mediates autophagy but where and how autophagosomes emerge has been a major question. In yeast, more than 38 Atg (autophagy-related) proteins have been identified, and most of them gather at a site that can be identified by fluorescence microscopy as a punctate spot very close to the vacuolar membrane. As autophagosomes are generated from this site, it is called the "preautophagosomal structure" (PAS) [21, 22]. However, the structure of the PAS has not been characterized until now.

2.1. Autophagy/autolysosomal events

In mammals, the regulation of autophagy is highly complicated. Limited numbers of studies examining autophagy in filamentous fungi have extended the knowledge gleaned from *S. cerevisiae*, the model organism for the study of autophagy [23]. Generally, autophagy consists of four sequential steps: (1) induction of autophagy; (2) recruitment of ATG proteins by phagophore assembly site proteins, the rapid formation of two-layer autophagosomal membrane structures, and isolation of the cytoplasm and organelles; (3) fusion of autophagosomes with lysosomes/vacuoles, in which the inner membrane of the autophagosome and the cytoplasm-derived materials are contained in the autophagosome; and (4) degradation of autophagic bodies in the vacuole into macromolecules that will be recycled. Autophagy facilitates the recycling of cytoplasmic components as nutrients to support cell survival. This feature is remarkably different from the ubiquitin-proteasome system, which only can specifically recognize ubiquitinated proteins for proteasomal degradation. Autophagy can selectively or nonselectively degrade proteins and fragments of organisms to maintain essential activity and viability in response to nutrient limitation.

2.2. The induction and inhibition of autophagy

Like yeast or mammals, fungal autophagy is typically induced by nutrition (e.g., carbon and nitrogen) starvation [24]. Conversely, autophagy is inhibited by the mammalian target of rapamycin (mTOR), a central cell growth regulator that is connected with growth factor and nutrient sensing. It has been demonstrated that the molecular mechanism is regulated by the mammalian autophagy-initiating kinase Ulk1, a homolog of yeast Atg1 [25]. Under nutrient-rich conditions, TOR kinase is activated and phosphorylates Atg13. Phosphorylated Atg13 does not possess sufficient affinity for the Atg1 kinase and cannot form a complex with Atg1. Thus, the process of autophagy is inhibited [26, 27].

Rapamycin is an inducer of autophagy as inhibition of mTOR mimics cellular starvation by blocking signals required for cell growth and proliferation. Fungi can also be induced by treatment with rapamycin, often with concentrations between 200 and 500 ng/ml in fungal culture [23, 24, 28, 29].

2.3. Autophagy in nutrient recycling

Under nutrient-limited conditions, autophagy can serve as a nutrient recycling pathway to support cell survival [30]. This role was demonstrated in the autophagy-deficient *Aspergillus fumigatus* Δ*Afatg1* mutant strain that experienced limited growth on a nutrient starvation medium (i.e., water-agarose), while the wild-type strain could maintain growth on the same medium. After reconstituting *AfATG1* back into the mutant, the complemented strain exhibited the same phenotype as the wild-type strain, suggesting that autophagy can recycle nutrients for growth [31]. For fungi, the colony margin mycelium is more active than the central mycelium and the inner hyphal network recycles nutrients to complement the growing tips [32]. When autophagy was blocked in Δ*Moatg1* mutants of *M. oryzae*, growth of the Δ*Moatg1* mutants was assessed on minimal media lacking either of the two crucial nutrients, nitrogen and carbon, and Δ*Moatg1* mutant colonies spread more slowly than those of the wild-type strain in both the cases [5]. These studies provide evidence that autophagy is required to support growth.

2.4. Autophagy in cellular degradation

Filamentous fungi have no lysosomes, like mammals, but the vacuoles play a similar role as degradative organelles. Hyphal vacuolation has been shown to increase rapidly in nutrient-starved *Aspergillus oryzae* mycelia [33]. It has been demonstrated that autophagic bodies can be visualized in vacuoles under starvation conditions; however, autophagic bodies cannot be detected in autophagy-blocked mutants, such as in the *A. fumigatus* Δ*Afatg1* mutant or in the *M. oryzae* Δ*Moatg1*, *4*, *5*, and *9* mutants [5, 31, 34–36]. Mon1 or Ypt7 are essential for vesicle fusion and vacuole morphology in yeast, and deleting the homologous genes in *M. oryzae* results in autophagy blockage in the mutants [37, 38].

2.5. Autophagy in cellular differentiation

In eukaryotic cells, autophagy is involved in cellular differentiation and development [14]. In plant pathogenic fungi, blocked autophagy impacts the phenotypes and morphologies of fungi. In the *M. oryzae* autophagy-deficient mutant Δ*Moatg8*, sucrose or glucose supplementation suppresses conidiation defects but the appressorium loses the ability to penetrate [39]. Deletion of *MoATG4* or *MoATG8* results in nuclear degeneration during appressorium formation and a drastic reduction in asexual conidiospore formation [34, 39]. Deletion of the lipase gene *ATG15* in *F. graminearum* results in reduced degradation of storage lipids [40]. Deletion of autophagy genes also inhibits the formation of sexual reproductive organs such as protoperithecia in *P. anserina* and perithecia in *M. oryzae* [5, 41]. These findings strongly suggest that autophagy is needed for proper cellular differentiation in several species of filamentous fungi. However, autophagy does not always appear to be involved in cellular differentiation.

For example, hyphal differentiation in Δ*atg9* mutant of the filamentous yeast *Candida albicans* does not affect the formation of chlamydospores [42]. Thus, there may be diverse roles for autophagy in the fungal differentiation of different species.

3. Macroautophagy-related genes studied in plant pathogenic fungi

Macroautophagy is the most widely studied type of autophagy. Many *ATG* genes involved in this process have been identified and characterized in yeast, plant, and mammals [43]. Additionally, it has been demonstrated that autophagy is a conserved catabolic pathway in plant pathogenic fungi and plays vital roles in development and differentiation among plant pathogens [44]. In contrast to yeast, many plant pathogenic fungi form special infection structures that can rupture the plant cuticle to gain entry to internal tissue, ultimately causing plant disease. The appressorium is known as a typical feature of some of the most important cereal pathogens such as the devastating rice blast disease-causing fungus, *M. oryzae*. Macroautophagy-related genes have been studied extensively in the plant pathogens *M. oryzae*, *Aspergillus* spp., *Colletotrichum* spp., *Fusarium* spp., and *U. maydis*.

3.1. The Atg1 kinase complex

In *S. cerevisiae*, Atg1, which encodes a serine/threonine kinase, forms a complex with Atg13, Atg17, Atg29, and Atg31 that are required for PAS formation. The Atg1 complex initiates the formation of the autophagosome by forming a scaffold to recruit other complexes [45]. Homologs of the yeast *ATG1* gene have been identified and characterized in many filamentous fungi including *Colletotrichum lindemuthianum* [46], *M. oryzae* [5], and *P. anserina* [41]. Random insertional mutagenesis in *C. lindemuthianum* clones produced mutations in a putative serine/threonine protein kinase named Clk1 that is associated with pathogenicity in the common bean. Clk1 is homologous to Atg1 in *S. cerevisiae*, although its role in autophagy has not been reported [46]. Disruption of the *ATG1* gene results in blockage of the autophagy process as confirmed by a defect in autophagosome formation and the absence of autophagic bodies in the vacuole in *P. anserina* [47]. In *M. oryzae*, deletion of *MgATG1*(*MoATG1*) causes reduced conidiation, delayed spore germination, and loss of pathogenicity on barley and rice due to interrupted autophagy [5]. Similarly, deletion of *BcATG1* inhibits autophagosome accumulation in the vacuoles of nitrogen-starved cells. A null mutant of *BcATG1* is defective in vegetative growth, conidiation, sclerotial formation, and appressorium formation [48]. Functional analysis of *AoATG1* in *A. oryzae* shows that conidiation and aerial hyphae are reduced significantly. Overexpression of AoAtg1 leads to decreased conidiation and excessive development of aerial hyphae [49]. These data are consistent with the role of *S. cerevisiae ATG1* in the induction of autophagy.

Upon starvation induction, Atg13 is rapidly dephosphorylated and subsequently activates Atg1 kinase activity. Atg13 binds to the C-terminus of Atg1 and this interaction occurs in a constitutive manner [50]. Data from *M. oryzae* show that *MoATG13* and *MoATG29* are not essential for turgor generation and rice blast formation [51]. Although the *AoATG13* deletion

mutant exhibits a reduction in conidiation and aerial hyphae, the mutant still exhibits autophagic activity in *A. oryzae* [52]. Thus, unlike *S. cerevisiae* Atg13, there may be another Atg13 in these plant pathogens.

3.2. Atg9-mediated vesicle transport

In yeast, Atg9 is a transmembrane protein consisting of six transmembrane domains with the amino and carboxyl termini exposed in the cytosol. Atg9 is positioned on the PAS and peripheral sites, shuttling and delivering membrane to form the phagophore. Atg1-Atg13 and Atg2-Atg18 facilitate Atg9-mediated vesicle transport from the PAS to peripheral sites [53], and the return of Atg9-mediated vesicle transport depends on Atg11, Atg23, and Atg27 [54, 55]. A new report indicates that Atg9 is a direct target of Atg1 kinase. Phosphorylated Atg9 is essential for the recruitment of Atg8 and Atg18 to the PAS and extension of the isolation membrane [56].

The functional characterization of Atg9 homologs in filamentous fungi has been documented. In *M. oryzae*, knockout of 23 autophagy-related genes confirmed that *MoATG9* is essential for rice blast formation [51]. Dong et al. also characterized the MoAtg9-mediated trafficking process in detail by observing the fluorescent localization of MoAtg9 in the wild-type strain and null mutants of *MoATG*1, 2, 13, and 18. MoAtg9 cycling depends on MoAtg1, 2, and 18 but not on MoAtg13. The null mutant of *MoATG9* exhibits similar phenotypes to the *MoATG1* deletion mutant, such as poor sporulation and appressorium formation, blockage of autophagy, and lack of pathogenicity on susceptible rice [35].

3.3. The two ubiquitin-like systems in autophagy

The process of autophagy involves two ubiquitin-like systems, the Atg12 and Atg8 conjugation systems. Both play key roles in bending and extension of the autophagosome membrane [45]. In the Atg12-Atg5-Atg16 ubiquitin-like system, Atg5 can directly bind the membrane, a process that is negatively regulated by Atg12 and positively regulated by Atg16 [57]. Identification of the proteins associated with the autophagosome has been performed in filamentous fungi. Disruption of the gene *BbATG5* resulted in abnormal conidia and reduction in growth, germination, blastospore formation, conidiation, and virulence in *Beauveria bassiana* [58]. Disruption of *MoATG5* in *M. oryzae* caused loss of pathogenicity, reduced conidiation, and perithecia formation [34]. In *Trichoderma reesei*, *TrATG5* gene knockout resulted in reduced conidiation and abnormal conidiophores [59]. Null mutants *Moatg12* and *Moatg16* also result in loss of pathogenicity in *M. oryzae* [51]. We can conclude that the Atg12 ubiquitin-like system is associated with conidiation and pathogenicity in plant pathogens.

Atg8 is another ubiquitin-like protein associated with autophagosome formation throughout the autophagy process and is well conserved in most model organisms and higher eukaryotes. It is associated with the autophagosome membrane and has been used as a marker for autophagy [60]. The amount of Atg8 can regulate the volume of autophagosomes by controlling phagophore expansion [61, 62]. The Atg12-Atg5-Atg16 complex acts like an E3 ligase, catalyzing Atg8 to phosphatidylethanolamine (PE) on the membrane [57].

Deletion of the *ATG8* gene in fungi detrimentally inhibits autophagy and affects cellular growth and differentiation [63]. Deletion of *FgATG8* in the necrotrophic plant pathogen *F. graminearum* results in loss of perithecia, reduced conidiation, and collapse of the aerial hyphae [64]. Lipid utilization is dependent on autophagy in *F. graminearum* for providing nutrients for the nonassimilating portion of the fungi. Although the ability to infect plants is not affected in the null mutant *Fgatg8*, plant colonization cannot be seen from spikelet to spikelet as with the wild-type fungus [64]. In *F. oxysporum*, the autophagy-related gene *FoATG8* is involved in nuclear degradation after hyphal fusion and the control of nuclear distribution [65]. The null mutant *Smatg8* in *S. macrospore* results in fruiting body loss, impaired ascospore germination, and the ability to undergo hyphal fusion [66]. In *M. oryzae*, loss of *MoATG8* blocks autophagic conidial cell death leading to impaired appressorium formation, loss of pathogenicity, and reduced conidiation [67]. Consistent with its function in *M. oryzae*, *CoATG8* is also involved in normal conidiation, appressorium formation, and pathogenicity in *C. orbiculare* [68]. Disruption of the *AoATG8* gene causes severe defects in the formation of aerial hyphae and conidia by affecting the autophagy process in *A. oryzae* [69].

The Atg12 and Atg8, two ubiquitin-like conjugating systems, share the same E1-like activating enzyme, Atg7, but have different E2-like conjugating enzymes: Atg10 and Atg3, respectively [45]. In *M. oryzae*, *MoATG7*, *MoATG10*, and *MoATG3* are all associated with pathogenicity. Deletion of any of these causes a loss of pathogenicity in *M. oryzae* [51]. Atg4, a cysteine protease, is responsible for the first cleavage of Atg8 to expose its C-terminal glycine and the second cleavage from PE to recycle Atg8 [47]. In *M. oryzae*, the cleavage event mediated by MoAtg4 is also conserved as observed in *in vitro* assays. Disruption of MoAtg4 blocks autophagy and causes defects like other null mutants of autophagy-related genes in *M. oryzae* [36]. In *Sordaria macrospora*, SmAtg4 is also capable of cleaving the SmAtg8 precursor [66]. Deletion of *AoATG4* results in the loss of aerial hyphae and reduced conidiation, resulting from the destruction of autophagy in *A. oryzae* [52].

3.4. The PI3K complex

Another protein complex required for autophagy in yeast is the phosphatidylinositol 3-kinase (PI3K) complex that involves Vps15, Vps34, Atg6, and Atg14 [45]. All of these proteins, with the exception of Atg14, are conserved in filamentous fungi. In *S. cerevisiae*, this complex is not specific to autophagy and is also involved in the cytoplasm-to-vacuole targeting pathway (CVT) pathway and a fairly diverse array of signaling and membrane transport events including Golgi to vacuole transport [61]. Thus, it is likely that autophagy may be impacted by other cellular signaling events. There have been few studies examining this complex in plant pathogens except in *M. oryzae*. A null mutant of *MoATG6* resulted in the loss of pathogenicity [50].

3.5. Membrane fusion

The membrane fusion of the autophagosome and vacuole requires many proteins. The autophagosome first docks with the surface of a vacuole, and then the outer membrane fuses with the vacuolar membrane. The proteins involved in this step are mostly those

proteins which are implicated in membrane fusion, such as the SNARE family proteins and the homotypic fusion and vacuolar protein sorting (HOPS) tethering complex [70, 71]. Once inside the vacuolar lumen, the single-membrane vesicle turns into the autophagic body.

Many SNARE proteins have been characterized in plant pathogens especially in *M. oryzae* and *F. graminearum*. In *M. oryzae*, MoVam7 is essential for vacuolar membrane fusion and vacuolar maturation [72]. MoSec22, an R-SNARE protein, is involved in endocytosis. Both MoVam7 and MoSec22 are required for growth, conidiation, and pathogenicity in *M. oryzae* [73]. Recently, another syntaxin protein, MoSyn8, has been identified for its role in regulating intracellular trafficking in *M. oryzae* [74]. In *F. graminearum*, FgVam7, a homolog of Vam7, plays an important role in regulating cellular differentiation and virulence [75]. Roles for these SNARE proteins in fungal development have been explored in depth but their functions in the autophagy process must be further evaluated. It has been reported that the HOPS subunit MoVps39 is crucial for pathogenicity in *M. oryzae* due to its role in anchoring G protein signaling. Rab GTPases also function in vesicle-vacuolar fusion [76]. In *M. oryzae*, MoYpt7, a homolog of Ypt7 in yeast, has been confirmed to be required for autophagy by affecting membrane fusion and assembly of the mature vacuole [37]. MoMon1, an ortholog of *S. cerevisiae* Mon1, is also required for membrane fusion in mature vacuolar formation [38]. Consistent with the results in *M. oryzae*, it has also been demonstrated that the *F. graminearum* homologs of Ypt7 and Mon1 are conserved in vacuole fusion and autophagy [77, 78].

3.6. Degradation of the autophagic body

The last step during autophagy is the degradation of the autophagic body in the vacuoles and recycling of cellular macromolecules to supply nutrient. Two autophagy-related proteins, Atg15 and Atg22, have been identified in yeast and are involved in this process [45]. Atg15 is a putative lipase involved in the lysis of CVT bodies, autophagic bodies, and incorporated peroxisomes in the vacuole [79]. Atg15 is well conserved in yeast and filamentous fungi, but it contains a distinct repeat motif at its C-terminus in front of the Ser/Thr-rich region [80]. In *A. oryzae*, AoAtg15 is required for autophagosome formation and the lysis of autophagic bodies. A null mutant of *Aoatg15* exhibits differentiation defects for the aerial hyphae and conidia [52]. Disruption of *FgATG15* causes a delay in lipid body degradation and utilization and abnormal development of the conidia and aerial hyphae formation [81]. In *M. oryzae*, *MoATG15* is also involved in pathogenicity [53]. On the other hand, Atg22 is an integral vacuolar transmembrane protein with structural similarity to permeates. It may function as a transporter in the export of recycled amino acids from the vacuole to the cytosol [82]. In *A. nidulans* and many other filamentous fungi, multiple paralogs of Atg22-like proteins have been identified [80]. In *P. anserina*, the *pspA/idi-6* gene encoding serine protease A is the functional ortholog of the *S. cerevisiae* vacuolar protease B (Prb1). Both of these proteases are involved in autophagy [47].

4. Selective autophagy in plant pathogenic fungi

With the exception of bulk autophagy, which is nonselective, eukaryotic cells possess several types of selective autophagy to maintain homeostasis during stress. These processes include the CVT pathway (which is only found in *S. cerevisiae*), pexophagy (which targets peroxisomes), mitophagy (the specific elimination of mitochondria), nucleophagy (which targets the nucleus), reticulophagy (which mediates the turnover of the endoplasmic reticulum), and ribophagy (the specific elimination of ribosomes) [83]. Here, we provide a brief introduction to the mechanisms that have been identified and the development of selective autophagy in plant pathogenic fungi.

4.1. Pexophagy

Peroxisomes share enzymes involved in the β-oxidation of fatty acids and the production and degradation of H_2O_2 or other reactive oxygen species (ROS); thus, homeostasis in peroxisomes plays an important role in survival and development. Researchers have found that peroxisomes exhibit high variability under changing circumstances because their numbers can be rapidly increased when their functions are required but then can be quickly recycled when they are not essential to avoid wasting energy [84]. There are two biological processes that explain this phenomenon: the process of peroxisome growth and division and the specific degradation of peroxisomes by autophagy, known as pexophagy. Studies of methylotrophic yeasts have shown that two distinct, selective modes are exploited for pexophagy: macropexophagy and micropexophagy. It appears that high levels of ATP activate micropexophagy, while lower levels induce macropexophagy. Macropexophagy is initiated at a specific PAS (different from the CVT pathway); newly synthesized membrane wraps around and sequesters the peroxisomes one by one and then forms a double membrane pexophagosome, which is ultimately delivered to the vacuole. In contrast to macropexophagy, a cluster of peroxisomes is swallowed by vacuolar sequestering membranes (VSMs) in micropexophagy. Meanwhile, the micropexophagy-specific membrane apparatus (MIPA), which mediates fusion between the tips of the invagination vacuoles, extends from the PAS. Finally, membrane scission occurs on the inner side of the vacuolar membrane and the peroxisomes are cracked [85].

Among 38 *ATG* genes that have been identified in yeasts, 16 Atg proteins constitute the core machinery mediating the formation of the autophagosome for almost all types of autophagy. While the specialization of any selective autophagy pathway is determined by the selective cargo recognized and engulfed by the autophagosomes, this requires the help of other selective autophagy-specific *ATG* genes. In *Pichia pastoris*, phosphorylated Atg30 physically interacts directly with Atg11 and Atg17. These two proteins act as scaffolds at the PAS to recruit other proteins. In *Hansenula polymorpha*, Atg25 is essential to connect Atg11 and Atg17. In contrast, Atg30 is required for macropexophagy in *S. cerevisiae*. During the final stages of pexophagy in *P. pastoris* and *S. cerevisiae*, Atg24 is involved in the regulation of membrane fusion at the vacuolar surface. Atg26 is a sterol glucosyltransferase that synthesizes sterol glucoside and is necessary for both modes of pexophagy but not bulk autophagy. In *P. pastoris*, the protein is associated with the MIPA during micropexophagy [86].

In the cucumber anthracnose fungus *C. orbiculare*, pexophagy is required for phytopathogenicity. To penetrate host epidermal cells and cause infection, *C. orbiculare* forms a specific structure, the appressorium. Researchers showed that while appressoria could still form in the *Coatg26* deletion mutant, the infection process was severely delayed. Furthermore, deletion of *Coatg8* impaired formation of the appressoria, suggesting the important role of autophagy during infection. A GFP fusion protein was peroxisomally expressed in *C. orbiculare* to monitor peroxisome conditions. Massive fluorescent dots could be observed in the wild-type strain and *Coatg26* mutant showing that the Atg26 null mutant still possessed a normal ability to perform peroxisome biosynthesis. The authors also observed that CoAtg8-tagged phagophores could swallow the peroxisomes through diffusion of GFP inside the vacuoles in the wild-type strain. However, bright peroxisomal dots were present in the appressoria of the *Coatg26* mutant. This indicated that appressoria pexophagy of the *Coatg26* mutant was significantly impaired compared with the wild-type strain. Taken together, nonselective general autophagy is essential for early stage pathogen development and Atg26-dependent selective pexophagy is essential for later stages of infection [68].

4.2. Mitophagy

Mitochondria are the sites of oxidative metabolism in eukaryotic organisms and are the places where the energy is released by the final oxidation of carbohydrates, fats, and amino acids. Reactive oxygen species are a side-product of the mitochondria. ROS release damages mitochondrial DNA and proteins and other cellular compartments, e.g., nuclear DNA. Thus, mitochondrial homeostasis is critical for organisms. Mitochondria degradation is mediated through a selective type of autophagy, called mitophagy. In *S. cerevisiae*, Atg32 (a mitochondrial outer membrane protein) functions as a receptor protein that interacts with Atg11 and Atg8 [87]. Recent studies show that a portion of the molecular mechanism involved in mitochondrial fission participates in mitophagy [88].

In *M. oryzae*, Atg24-assisted mitophagy in the foot cells is necessary for proper asexual differentiation and functions in redox homeostasis and nutrient modulation. MoAtg24, a sorting nexin related to yeast Snx4, is only required for mitophagy. The Δ*Moatg24* strain exhibited a decreased rate of conidiophore formation and reduced aerial hyphal growth. Subcellular localization of MoAtg24-GFP under ROS stress and starvation conditions found that MoAtg24-GFP was localized to the mitochondria and studies employing Mito-GFP clearly indicated signals in the foot cells. The Bin-Amphiphysin-Rvs (BAR) and Phox homology (PX) domains of MoAtg24 are essential for its mitochondrial localization [89].

4.3. Reticulophagy and ribophagy

The ribosome is a cellular ribonucleoprotein particle that is primarily composed of RNA (rRNA) and proteins, and its only function is to catalyze amino acids for proteins according to the instructions imparted by the mRNA. Ribophagy involves degradation of the 60S ribosomal subunit and is regulated by both ubiquitination and deubiquitination. Some proteins are synthesized in the endoplasmic reticulum (ER) shortly after synthesis begins. The ER also modulates the modification and processing of protein folding and assembly and transport of

nascent peptides. Like other selective autophagy pathways, reticulophagy and nucleophagy should be highly controlled. ERphagy is differentially induced, depending on the intensity and type of the ER stress [90]. This shows that the Ypt/Rab GTPase module, formed by the Trs85 containing the Ypt1, the Atg11 effector and the TRAPPIII GEF, functions in reticulophagy. With the exception of Ypt/Rab GTPases [91], macroreticulophagy also depends on Atgs and their cargos. In conclusion, Atg9-dependent ERphagy involved autophagy of the endoplasmic reticulum, Ypt1- and core Atg-dependent phagy mediate the organization of PAS, and Ypt51-dependent phagy mediates the delivery of autophagosomes to the vacuole [92].

Recently, researchers identified two Atg8-binding proteins in *S. cerevisiae*, Atg39 and Atg40, both of which are receptors for reticulophagy. ER consists of 3 subdomains in *S. cerevisiae*, specifically, the cytoplasmic ER (cytoER), the cortical ER (cER), and the perinuclear ER (pnER), i.e., the nuclear envelope (NE). Atg39 specifically localizes to the pnER/NE and induces autophagic sequestration of double-membrane vesicles as well as some intranuclear components. Thus, the Atg39-dependent pathway should also be called nucleophagy. Atg40 is primarily responsible for cER/cytoERphagy. Atg39 mutant cells die earlier than wild-type cells under prolonged nitrogen starvation, while Atg40 mutant cells do not, suggesting the physiological significance of the pnER-autophagy pathway. It is still unknown whether the generation of nuclear envelope-derived vesicles and ER fragments are coupled with their sequestration into autophagosomes [93, 94]. The processes of reticulophagy and ribophagy still need to be studied in plant pathogens.

4.4. Nucleophagy

The removal of damaged/nonessential/entire (in some circumstances) nuclei under stress is crucial for cell survival. Nucleophagy is the selective degradation of nuclear material by autophagy. It has two modes: macronucleophagy and micronucleophagy. Two processes (piecemeal microautophagy of the nucleus, PMN, and late nucleophagy) exist in *S. cerevisiae* and are considered microautophagy. In the PMN, two proteins, Vac8 (located in the vacuolar membrane) and Nvj1 (in the nuclear envelope), recognize each other at nucleus-vacuole (NV) junctions. Then, nuclear membranes that are associated with NV junctions invaginate into the vacuolar lumen. Sequestration of the nuclear cargo occurs via the fission of nuclear membranes and the vacuolar membrane, and ultimately a triple-membrane PMN vesicle is released into the vacuolar lumen and is degraded. The cargo that is sequestered by PMN includes nonessential nuclear components; PMN requires the core *ATG* genes, and specific *ATG* genes are necessary at the step in which the vacuolar membrane fuses. Conversely, late nucleophagy can occur in the absence of Nvj1, Vac8, and Atg11 [95].

Micronucleophagy has been studied in *A. oryzae*. EGFP-tagged Atg8 has been utilized to track autophagosomal structures. Large autophagosomes (1–2 mm in diameter) are formed around the nuclei and then sequester whole targets. Subsequently, nuclear material is dispersed throughout neighboring vacuoles, suggesting that autophagosomes forming close to targeted nuclei in turn sequester whole nuclei [96]. Micronucleophagy has been studied in *M. oryzae*. A dome-shaped structure, called the appressorium, penetrates host tissues. To form appressoria, spores undergo a series of autophagy steps including nuclear degeneration. In contrast

to piecemeal microautophagy of the nucleus, nuclear degradation in *M. oryzae* is dependent on core autophagy genes, and fungi, such as *A. oryzae*, possess large autophagosomes, while *M. oryzae*'s autophagosomes are smaller and more punctate [97].

In *M. oryzae*, core *ATG* genes such as *MoATG1*, *MoATG5*, and *MoATG8* were studied in the context of pathogenicity but given that these genes are essential for both selective and bulk autophagy, it is still unknown if selective autophagy has a critical function in the fungal pathogenicity of *M. oryzae* [98]. Kershaw et al. conducted a mass gene knockout in *M. oryzae* to analyze 22 *ATG* genes [51]. The results showed that *M. oryzae* becomes nonpathogenic on rice when it loses any one of the core *ATG* genes but is still pathogenic in the absence of *ATG* genes for selective autophagy (*ATG11*, *24*, *26–29*).

5. Conclusions

In this report, we have introduced a new concept to delineate the role of autophagy in homeostasis of plant pathogenic fungi and in their interaction with host cells, breach of host barrier, and in the mechanisms of plant fungal infection. More knowledge of the diverse modes of autophagy is likely to help us understand the mechanisms of fungal pathogen-plant host interactions. In addition, pathogenic fungi are multicellular organisms that undergo constant polar growth that is completely different from yeast. Due to the differences between yeast and multicellular organisms, greater divergence has been revealed in terms of the CVT pathway, SNARE proteins, and selective autophagy, as mentioned in this chapter. It is necessary to create a new system to analyze autophagy in filamentous fungi. Furthermore, with the development of metabolomics and proteomics, studies of autophagy in plant pathogenic fungi can be combined with new technologies based on the molecular mechanisms of autophagy.

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Author details

Huan-Bin Shi, Shuang Liang, Lu-Yao Huang, Xiao-Hong Liu, Xue-Ming Zhu, Ya-Hui Zhao and Fu-Cheng Lin*

*Address all correspondence to: fuchenglin@zju.edu.cn

State Key Laboratory for Rice Biology, Biotechnology Institute, Zhejiang University, Hangzhou, China

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