**Pexophagy is critical for fungal development, stress response, and virulence in *Alternaria alternata***

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**Abstract**
*Alternaria alternata* can resist high levels of reactive oxygen species (ROS). The protective roles of autophagy or autophagy-mediated degradation of peroxisomes (termed pexophagy) against oxidative stress remain unclear. The present study, using transmission electron microscopy and fluorescence microscopy coupled with a GFP-AaAtg8 proteolysis assay and an mCherry tagging assay with peroxisomal targeting tripeptides, demonstrated that hydrogen peroxide (*H₂O₂*) and nitrogen depletion induced autophagy and pexophagy. Experimental evidence showed that *H₂O₂* triggered autophagy and the translocation of peroxisomes into the vacuoles. Mutational inactivation of the *AaAtg8* gene in *A. alternata* led to autophagy impairment, resulting in the accumulation of peroxisomes, increased ROS sensitivity, and decreased virulence. Compared to the wild type, Δ*AaAtg8* failed to detoxify ROS effectively, leading to ROS accumulation. Deleting *AaAtg8* down-regulated the expression of genes encoding an NADPH oxidase and a Yap1 transcription factor, both involved in ROS resistance. Deleting *AaAtg8* affected the development of conidia and appressorium-like structures. Deleting *AaAtg8* also compromised the integrity of the cell wall. Reintroduction of a functional copy of *AaAtg8* in the mutant completely restored all defective phenotypes. Although Δ*AaAtg8* produced wild-type toxin levels in axenic culture, the mutant induced a lower level of *H₂O₂* and smaller necrotic lesions on citrus leaves. In addition to *H₂O₂*, nitrogen starvation triggered peroxisome turnover. We concluded that Δ*AaAtg8* failed to degrade peroxisomes effectively, leading to the accumulation of peroxisomes and the reduction of the stress response. Autophagy-mediated peroxisome turnover could increase cell adaptability and survival under oxidative stress and starvation conditions.

**Keywords**
Atg8, autophagy, peroxisome, pexophagy, ROS detoxification, stress tolerance, virulence
1 | INTRODUCTION

*Alternaria alternata* is a necrotrophic fungal pathogen causing diseases in more than 100 plant species (Kusaba & Tsuge, 1995). The tangerine pathotype of *A. alternata* produces a host-selective toxin termed *Alternaria citri* toxin (ACT) to kill host cells (Tsuge et al., 2013). To achieve successful colonization, *A. alternata* also needs other weapons to overcome the physical obstacles of the plant cells before the invasion. Recent studies with *A. alternata* have shown that cell wall-degrading enzymes and appressoria are required for full virulence (Hatzigapapas et al., 2002; Ma et al., 2019). In addition, the ability of *A. alternata* to detoxify reactive oxygen species (ROS; e.g., $\ce{O3^-, H2O2, OH^+, 1O2}$) is required for virulence (Lin et al., 2009).

*A. alternata* infection on citrus results in the accumulation of hydrogen peroxide ($\ce{H2O2}$) and induces lipid peroxidation (Lin et al., 2011). To survive in the oxidizing environment, *A. alternata* relies on complex yet well-regulated networks to mitigate oxidative stress. *A. alternata* can resist $\ce{H2O2}$ at a concentration as high as 30mM (Lin & Chung, 2010). Studies have demonstrated that the Yap1 bZIP transcription factor plays a crucial role in ROS resistance (Lin et al., 2009). *A. alternata* can produce low levels of $\ce{H2O2}$ via an NADPH oxidase (Nox) complex containing two major oxidases, NoxA and NoxB, and one regulatory component, NoxR (Yang & Chung, 2012, 2013). Low-level $\ce{H2O2}$ produced by Nox regulates fungal growth, conidiation, and ROS resistance in *A. alternata*.

Oxidative stress and nutrient depletion can induce autophagy (Filomeni et al., 2015; Kroemer et al., 2010). Autophagy is a self-digestion pathway. Two major types of autophagy, macroautophagy and microautophagy, have been described in the budding yeast *Saccharomyces cerevisiae* (Kraft et al., 2009). Macroautophagy (hereafter referred to as autophagy) begins with an endoplasmic reticulum (ER)-derived pre-autophagosomal structure called the phagophore assembly site. A double-membrane autophagosome is formed and later fused with the vacuole to degrade the enclosed cargos (peroxisomes or other organelles) (Li et al., 2021). Microautophagy is completed by combining cargos with the vacuolar membrane, in which cargos are degraded within the vacuole. Autophagy is critical to maintaining cell homeostasis and cell adaptation to environmental stress. In *S. cerevisiae*, autophagy is regulated by more than 30 autophagy-related (Atg) proteins (Xie & Klionsky, 2007); however, only 18 or 19 Atg homologues are present in the filamentous fungi (Khan et al., 2012; Zhu et al., 2019). The Atg8 gene encodes a ubiquitin-like protein that is conjugated with phosphatidylethanolamine (PE) to form Atg8-PE for the elongation of autophagosomal membranes (Ichimura et al., 2000; Yin et al., 2020). Atg8 is highly conserved in eukaryotes and often used as a marker to monitor autophagosome formation (Klionsky et al., 2007). Atg8 is also required for the selective recruitment of organelles, including mitochondria and peroxisomes, and autophagosome–vacuole fusion (Chun & Kim, 2018; Kirkin & Rogov, 2019; Till et al., 2012).

Autophagy is required for cell survival. Under normal conditions, a cell maintains a low level of autophagy, which scavenges and degrades unwanted components and damaged organelles to supply nutrients for short-term survival (Oku & Sakai, 2010; Pollack et al., 2009; Singh & Cuervo, 2011). However, prolonged autophagy could lead to cell death. Thus, maintaining autophagy homeostasis is critical for growth, proliferation, and survival in all eukaryotic cells (Chun & Kim, 2018; Qi et al., 2021). In fungi, autophagy plays a role in hyphal growth, conidiation, oxidative stress resistance, and virulence (Hou et al., 2020; Lv et al., 2017; Nitsche et al., 2013; Wang et al., 2021; Zhu et al., 2019). Although autophagy has been thought to be required for oxidative stress resistance, the underlying mechanism remains obscure.

$\ce{H2O2}$ is an essential signal under normal physiological conditions. To maintain proper cellular functions, cells have to keep intracellular ROS at a low level by removing excess ROS and oxidatively damaged components. Studies with *A. alternata* have demonstrated that the number of peroxisomes decreases upon exposure to environmental $\ce{H2O2}$ (Wu et al., 2021), suggesting that $\ce{H2O2}$ may induce the turnover of peroxisomes via pexophagy, a selective autophagic pathway involved in the degradation of peroxisomes in vacuoles. Peroxisomes are membrane-enclosed microbodies engaged in a number of metabolic functions in eukaryotic cells (Okamoto et al., 2020). Peroxisome formation can be induced by external stimuli such as $\ce{H2O2}$, starvation, hypoxia, and metabolic needs (Asakura et al., 2009; Germain & Kim, 2020; Wanders & Waterham, 2006). Peroxisomes are initiated from the ER, and their numbers are controlled by de novo synthesis and degradation (Dimitrov et al., 2013; Till et al., 2012). Proteins containing conserved serine-lysine-leucine (SKL) tripeptides at the C-terminus are preferentially transferred to peroxisomes (Brocard & Hartig, 2006). In phytopathogenic fungi, peroxisomes are required for conidium germination, appressorium formation, cell wall integrity, cell viability, biotin biosynthesis, siderophore and toxin production, $\ce{H2O2}$ uptake and accumulation, and virulence (Falter & Reumann, 2022; Wu et al., 2021). Although autophagy has also been shown to play an important role in phytopathogenic fungi (Liu et al., 2007, 2008; Long et al., 2011; Nadal & Gold, 2010; Richie & Askew, 2008), little is known about the role of pexophagy in fungal pathogenesis. A study with *Colletotrichum orbiculare* has uncovered that Atg26-mediated pexophagy is required for infection (Asakura et al., 2009). Pexophagy has also been reported in other pathogenic fungi including *Magnaporthe oryzae*, *Penicillium chrysogenum*, and *Aspergillus oryzae* (Deng et al., 2013; Kurztkowski & Gebska-Kuczerowska, 2016; Todokoro et al., 2015).

ROS have dual functions, regulating cell survival and death (Mittler, 2017). Excess ROS are toxic to cells. High intracellular ROS levels could damage proteins, lipids, and nucleic acids, resulting in cell death (Schieber & Chandel, 2014). On the other hand, low levels of ROS are required for numerous biological functions as they can serve as signalling molecules, activating proper metabolism and cellular defence to environmental stress. Because $\ce{H2O2}$ treatment decreases the number of peroxisomes, probably due to the increase of peroxisome turnover (Wu et al., 2021), we hypothesize that degradation of peroxisomes via pexophagy may contribute to $\ce{H2O2}$ resistance. The objective of the present study...
was to determine whether or not autophagy or pexophagy acts as a protective mechanism under oxidative stress. We have provided genetic evidence to support the notion that pexophagy plays a critical role in oxidative stress in the tangerine pathotype of A. alternata.

2 | RESULTS

2.1 | Autophagy is induced by nitrogen starvation in A. alternata

Autophagy formation in wild-type (WT) A. alternata was examined by transmission electron microscopy (TEM) (Figure 1a). When the fungus was grown in potato dextrose broth (PDB; nutrient-rich medium), many peroxisomes appearing as dark spots and lipid bodies appearing as grey spots were observed in the cytosol. In minimal medium (MM; nutrient-poor medium), autophagosome-like structures were observed, and the number of peroxisomes was reduced considerably. In nitrogen-depleted MM (MM-N), many autophagic vacuoles containing intracellular material were observed.

To further confirm the occurrence of autophagy, a green fluorescent protein (GFP)-AaAtg8 fusion protein was expressed in WT to yield the WT/GFP-AaAtg8 strain. The A. alternata Atg8 gene (AaAtg8, 472 bp) encodes a protein of 119 amino acids similar to an autophagy-like protein. AaAtg8 contains an LC3 domain between amino acids 13 and 116 and a ubiquitin core at the C-terminus. WT/GFP-AaAtg8 was used to monitor the intracellular localization of AaAtg8 and autophagic flux under various nutrient conditions (Figure 1b). When grown in PDB, WT/GFP-AaAtg8 displayed weak green fluorescence widely distributed along the fungal hyphae. An ER-derived pre-autophagosomal structure required for autophagosome initiation was observed as a bright green fluorescent dot in the cytoplasm. When grown in MM, WT/GFP-AaAtg8 emitted bright green fluorescence widely distributed in the fungal hyphae. After staining by 7-amino-4-chloromethylcoumarin (CMAC), producing blue fluorescence, vacuoles were also visible in the hyphae. When grown in MM-N, WT/GFP-AaAtg8 displayed green fluorescence as distinct patches overlapping with blue fluorescence, indicating that GFP-AaAtg8 accumulated in the lumen of the vacuoles. When grown in MM-N, WT expressing GFP alone showed uniform green fluorescence in the cytoplasm excluding vacuoles. No autofluorescence was observed. Total proteins were extracted from WT/GFP-AaAtg8 grown in PDB, MM, or MM-N and analysed by western blot using an anti-GFP antibody (Figure 1c). The results revealed that GFP-AaAtg8 was intact in the WT/GFP-AaAtg8 strain grown in PDB. Some GFP-AaAtg8 proteins were cleaved to release free GFP in the WT/GFP-AaAtg8 strain grown in MM. Significantly less GFP-AaAtg8 protein accompanying more free GFP were detected in the WT/GFP-AaAtg8 strain grown in MM-N. Free GFP constituted 12%, 36%, and 70% in protein samples prepared from WT/GFP-AaAtg8 grown in PDB, MM, and MM-N, respectively.

2.2 | Generation and verification of AaAtg8-impaired A. alternata strains

To investigate the biological functions of AaAtg8, two split marker fragments were generated by PCR and used to replace the entire open reading frame (472 bp) of AaAtg8 with a hygromycin resistance cassette (Hyg) (Figure S1a). After transferring the split marker fragments into WT protoplasts, two transformants (designated ΔAaAtg8-D1 and -D2) were independently recovered. Southern blot analyses of genomic DNA revealed different hybridization patterns between WT and transformants (Figure S1b). An AaAtg8 probe identified the expected 1.8-kb band in the genomic DNA of WT. A 3.9-kb, instead of 1.8-kb, band was detected in the genomic DNA of the two transformants. An additional band greater than 4.2 kb was also detected in the D2 transformant. No bands were detected in the WT DNA, and a 3.9-kb band was detected in both D1 and D2 transformants using a Hyg-specific probe. The 4.2-kb band was again detected in DNA purified from the D2 transformant, indicating that D2 contained an ectopic insertion of Hyg. The Cp17 strain was identified after mutant protoplasts were transformed with a functional copy of AaAtg8 controlled by its native promoter. Southern blot analyses using either the AaAtg8 or the Hyg probe revealed that Cp17 and WT had similar hybridization patterns.

2.3 | AaAtg8 is required for autophagy formation and maintaining cell wall integrity

The autophagy process was monitored by staining hyphae with monodansylcadaverine (MDC) and MM 4-64 (Figure 2a). MDC, commonly used to detect autophagosomes, produced blue fluorescence in the hyphae. MM 4-64, widely used for tracking endocytosis, produced red fluorescence. Hyphae incubated in MM-N and 2 mM phenylmethylsulfonyl fluoride (PMSF) for 4 h were stained with MDC and MM 4-64 and examined by fluorescence microscopy. Merging images of WT hyphae revealed that blue and red fluorescence patches were overlapping (Figure 2a), indicating the occurrence of autophagic degradation within the lysosomal vacuoles. ΔAaAtg8 hyphae after staining with MDC and MM 4-64 displayed overlapping fluorescence only in certain sections of the hyphae, indicating a role of AaAtg8 in the formation of autophagy. TEM analyses revealed that autophagic vacuoles were observed in conidia and hyphae of WT but rarely found in those of ΔAaAtg8 under nitrogen-depleted conditions (Figure 2b). Several empty vacuoles were observed in conidia and hyphae of ΔAaAtg8. Conidia and hyphae of WT had significantly more autophagic vacuoles than those of ΔAaAtg8 (Figure 2c). TEM analyses also revealed that ΔAaAtg8 had a significantly thinner cell wall than WT (n = 15), particularly conidia (Figure 2d). Sensitivity assays revealed that ΔAaAtg8 had increased sensitivity to the cell wall-disrupting agents Congo red (200 μM) and calcofluor white (75 μM) (Figure S2).
Induction of autophagy by nitrogen starvation in *Alternaria alternata*. (a) Transmission electron microscopy images of wild-type (WT) hyphae grown in potato dextrose broth (PDB), minimal medium (MM), or nitrogen-depleted MM (MM−N) for 4 h, showing lipid bodies (L), peroxisomes (P), autophagic vacuoles (AV), and autophagosomes (AP). (b) WT expressing a GFP-AaAtg8 fusion protein or GFP alone was grown in PDB, MM, or MM−N for 4 h and treated with 2 mM phenylmethylsulfonyl fluoride (PMSF). The vacuoles were stained with 7-amino-4-chloromethylcoumarin (CMAC) and examined microscopically. Phagophore assembly sites (indicated by arrows) were observed in the hyphae grown in PDB. Scale bars, 10 μm. (c) Western blot analysis of proteins prepared from WT expressing GFP-AaAtg8 with an anti-GFP antibody. The percentage of free GFP band intensity in relation to total intensity is also shown. An SDS-PAGE gel after Coomassie blue staining is shown to ensure equal loading of the samples.
AaAtg8 is required for autophagy and cell wall integrity. (a) Hyphae of the wild type (WT) and ΔAaAtg8 were cultured in nitrogen-depleted minimal medium (MM−N) for 4 h in the presence of 2 mM phenylmethylsulfonyl fluoride (PMSF), stained with monodansylcadaverine (MDC, for detecting autophagosomes) or MM 4-64 (for staining vacuoles), and examined microscopically. Scale bars, 25 μm. (b) Transmission electron microscopy images of WT and ΔAaAtg8 hyphae grown in MM−N and spores grown in potato dextrose broth, showing autophagic vacuoles (AV), vacuoles (V), and peroxisomes (indicated by arrows). (c) Quantitative analysis of autophagic vacuoles of hyphae and spores (n = 30). (d) Cell wall width (μm) of the hyphae and spores (n = 15). The significance of tests was analysed by one-way analysis of variance and Tukey’s HSD post hoc test (**p < 0.01; *p < 0.05).
2.4 | AaAtg8 is required for fungal growth and development

When grown on PDA and MM, ΔAaAtg8 reduced growth by 19.7% and 33.3%, respectively, compared to WT (Figure S3a). The Cp17 strain, recovered from a ΔΔAaAtg8 strain after reintroducing a copy of WT AaAtg8, displayed WT growth. On MM, ΔAaAtg8 produced fewer aerial hyphae than WT and Cp17 (Figure S3b). WT and Cp17 produced club-shaped conidia with dark pigmentation. However, ΔAaAtg8 produced slender, small conidia with light pigmentation (Figure 3a). ΔAaAtg8 mycelium tended to break to make hyphal fragments. Quantitative analysis of conidia formation revealed that ΔAaAtg8 had reduced conidiation by as much as 95% (Figure 3b). Conidia produced by ΔAaAtg8 were significantly smaller than those produced by WT and Cp17 (Figure 3c). Conidia produced by WT and Cp17 germinated to form germ tubes, often producing nonmelanized enlargements resembling appressorium-like structures at the end of the germ tubes. Less than 30% of ΔAaAtg8 conidia germinated to form germ tubes (Figure 3d), of which only 10% formed appressorium-like structures (Figure 3e).

2.5 | AaAtg8 contributes to A. alternata virulence

Pathogenicity tests assayed by placing a small piece of fungal mycelium or conidial suspensions on detached calamondin leaves revealed that ΔAaAtg8 barely induced necrotic lesions 6 days postinoculation (dpi). In contrast, WT and Cp17 induced distinct necrosis. When assayed on the wounded leaves before inoculation, ΔAaAtg8 induced visible necrosis with sizes much smaller than those caused by WT and Cp17 at 5 dpi (Figure 4a,b). 3,3’-Diaminobenzidine (DAB) staining revealed that WT induced \( \text{H}_2\text{O}_2 \) accumulation around the infection sites as early as 1 dpi, whereas ΔAaAtg8 induced \( \text{H}_2\text{O}_2 \) accumulation concurrently with the development of microscopic lesions at 3 dpi (Figure 4c). Light microscopy observation revealed that the WT conidia germinated and produced microscopic lesions on the surface of the leaves at 1 dpi, which merged to form considerable necrosis. Conidia and hyphal fragments of ΔAaAtg8 germinated and produced microscopic lesions at 2 dpi (Figure 4d). Toxin assays using culture filtrates or purified toxin on detached calamondin leaves revealed that ΔAaAtg8 produced WT toxin levels (Figure S4a). High-performance liquid chromatography (HPLC) analysis of toxin purified from culture filtrates of all test strains resulted in a major peak at retention time (\( t_r \)) 7.9 min and four minor peaks at \( t_r \) 2.9, 4.1, 10.4, and 12.4 min (Figure S4b). The \( t_r \) 7.9 min peak was not detected in samples purified from Richard’s medium only or a toxin-deficient mutant (ΔΔAaACTT6). Each peak was collected and placed on calamondin leaves, revealing that only the \( t_r \) 7.9 min peak resulted in necrosis (Figure S4c).

2.6 | Autophagy is involved in resistance to ROS

Sensitivity assays on PDA revealed that ΔΔAaAtg8 had increased sensitivity to \( \text{H}_2\text{O}_2 \), tert-butyl-hydroperoxide, cumyl hydroperoxide, and diethyl malonate (Figure S5a). However, ΔAaAtg8 displayed WT sensitivity to superoxide-generating compounds (potassium superoxide [\( \text{KO}_2 \), 3 mg/ml] and menadione [0.2 mM]) and singlet oxygen-generating compounds (rose Bengal [30 \( \mu \text{M} \)] and eosin Y [150 \( \mu \text{M} \)]) (data not shown). The Cp17 strain displayed WT growth in all tests. Reverse transcription-quantitative PCR (RT-qPCR) analyses revealed that expression of the NoxA-coding gene and the redox-responsive transcription factor Yap1-coding gene was down-regulated in ΔAaAtg8 (Figure 5b).

2.7 | Autophagy is required for the detoxification of ROS

Fluorescence microscopy revealed the occurrence of visible green fluorescent spots overlapping with CMAC fluorescence in the hyphae of the WT/GFP-AaAtg8 strain after being treated with 15 mM \( \text{H}_2\text{O}_2 \) for 8 h (Figure 6a). Without \( \text{H}_2\text{O}_2 \), the WT/GFP-AaAtg8 strain displayed uniform green fluorescence in the hyphae. Western blot analyses revealed that free GFP levels increased when the WT/GFP-AaAtg8 strain was treated with \( \text{H}_2\text{O}_2 \) (Figure 6b). Fluorescence microscopy showed that the WT hyphae, after being exposed to 15 mM \( \text{H}_2\text{O}_2 \) for 8 h and being stained with 2′,7′-dichlorofluorescein diacetate (DCFHDA), emitted green fluorescence, indicating the accumulation of \( \text{H}_2\text{O}_2 \) and other ROS (Figure 6c). Compared to WT, the ΔΔAaAtg8 hyphae after DCFHDA staining emitted a much brighter green fluorescence upon exposure to \( \text{H}_2\text{O}_2 \).

2.8 | AaAtg8 is required for the turnover of peroxisomes under stress conditions

After culturing in PDB without \( \text{H}_2\text{O}_2 \), WT/mCherry-SKL displayed red fluorescence as small bright sparks, indicative of peroxisomes, in the cytoplasm (Figure 7a). When peroxisomes were localized in vacuoles, fluorescence became fainter, showing diffuse red signals. After WT/mCherry-SKL was cultured in PDB amended with 15 mM \( \text{H}_2\text{O}_2 \) for 8 h, many peroxisomes (visible as red fluorescent signals) were transferred to the vacuoles, indicating the occurrence of pexophagy. In the absence of \( \text{H}_2\text{O}_2 \), ΔAaAtg8/mCherry-SKL also displayed distinct red fluorescence in the cytoplasm. Upon exposure to 15 mM \( \text{H}_2\text{O}_2 \) for 8 h, red fluorescence was hardly localized in the vacuoles of ΔAaAtg8. Although most WT/mCherry-SKL hyphae still displayed red fluorescence as small bright sparks after incubation in PDB for 24 h, some hyphae contained diffuse red fluorescence in the vacuoles (Figure 7b). After incubation in PDB with \( \text{H}_2\text{O}_2 \) for 24 h, many WT/mCherry-SKL hyphae displayed weak or no fluorescence (data not shown). Roughly 50% of WT hyphae after \( \text{H}_2\text{O}_2 \) treatment for 24 h were collapsed, showing no red fluorescence.

Aside from \( \text{H}_2\text{O}_2 \), the effect of nutrients on the degradation of peroxisomes was examined. The conidia of ΔΔAaAtg8/mCherry-SKL, fleshly collected from PDA, showed visible red fluorescence,
**Figure 3** AaAtg8 is associated with fungal growth and development. (a) Conidia (Cn) produced by two ∆AaAtg8 mutants (D1 and D2) were much smaller and slender than those produced by the wild type (WT) and the Cp17 strain. Conidia germinated to produce a germ tube (Gt), which formed a nonmelanized enlargement resembling an appressorium-like structure at the end of the germ tube (indicated by an arrow). ∆AaAtg8 produced more hyphal fragments (Hf) than WT and Cp17. (b) Quantity of conidia produced by different strains of *Alternaria alternata*. (c) The size (μm²) of conidia. (d) Percentage of germinated conidia of different strains grown in potato dextrose broth (PDB) or liquid minimal medium (MM). (e) Percentage of the formation of appressorium-like structures. The significance of the difference between means was analysed using Tukey’s HSD post hoc test (*p* ≤ 0.01). Means (*n* = 30) indicated by the same case letters are not significantly different.
whereas no red fluorescence was observed in the conidia of WT/mCherry-SKL (Figure 8a). After incubation in MM for 24 h, the hyphae of WT/mCherry-SKL emitted red fluorescence, many of which were localized in the vacuoles (Figure 8b). In contrast to WT, ΔAaAtg8/mCherry-SKL emitted strong fluorescence as distinct sparkles in the hyphae grown in MM. A close examination revealed that the red sparkles were localized outside of the vacuoles of ΔAaAtg8. When incubated in MM-N, WT/mCherry-SKL had no visible vacuoles and showed diffuse red fluorescence, whereas ΔAaAtg8/mCherry-SKL had visible vacuoles and distinct red sparkles, which were not localized in the vacuoles (Figure 8c).

3 | DISCUSSION

Autophagy is a highly conserved and nonselective degradation process required to maintain cellular homeostasis and protect cells against stress in eukaryotes (Hale et al., 2013). This study provided...
cellular and genetic evidence to support the notion that autophagy and autophagy-mediated degradation of peroxisomes regulate cellular resistance to ROS, enhance adaptability and survival in different environments, and facilitate infectivity in the phytopathogenic fungus A. alternata. H$_2$O$_2$ triggered autophagy formation. The WT effectively reduced ROS levels in the cytosol, whereas ΔAaAtg8 failed to detoxify ROS efficiently, resulting in ROS accumulation. H$_2$O$_2$ also triggered the translocation of peroxisomes into vacuoles, indicating the degradation of peroxisomes. We concluded that autophagy is involved in ROS resistance, at least, via the degradation of peroxisomes in A. alternata. Through analysis of loss-of-function and gain-of-function mutations of the AaAtg8 gene, we also demonstrated that autophagy is involved in vegetative growth, conidial development, appressorium formation, resistance to ROS, and fungal virulence.

The Atg8 gene is highly conserved in eukaryotes and often used as a molecular marker to study the formation of autophagy.

**Figure 5** Autophagy is involved in the resistance to reactive oxygen species. (a) Deletion of AaAtg8 increased sensitivity to 20 mM hydrogen peroxide (H$_2$O$_2$), 3.75 mM tert-butyl-hydroperoxide (tBHP), 3.4 mM cumyl hydroperoxide (cumyl-H$_2$O$_2$, dissolved in ethanol), and 7 mM diethyl malonate (DEM, dissolved in methanol). Potato dextrose agar amended with ethanol (EtOH) or methanol (MeOH) was used as mock controls. The percentage of growth inhibition of ΔAaAtg8 in relation to the wild type (WT) is also shown. The significance of tests was analysed by one-way analysis of variance and Tukey’s HSD post hoc test (**p<0.01). (b) Reverse transcription-quantitative PCR analyses revealed that deletion of AaAtg8 decreased the gene expression of NoxA, encoding an NADPH oxidase, and Yap1, encoding a redox-responsive transcription factor.
**FIGURE 6**  
H₂O₂ induces the formation of autophagy, and autophagy is required for the detoxification of reactive oxygen species (ROS).  
(a) Fluorescence microscopy images of wild-type (WT) hyphae expressing a green fluorescent protein (GFP)-AaAtg8 fusion protein. Fungal hyphae cultured in potato dextrose broth (PDB) amended with or without 15 mM H₂O₂ were treated with 2 mM phenylmethylsulfonyl fluoride (PMSF) for 8 h. The vacuoles were stained with 7-amino-4-chloromethylcoumarin (CMAC) and examined microscopically. H₂O₂ treatment induced the formation of condensed green spots colocalized with vacuoles (indicated by arrows).  
(b) Western blot analysis of proteins prepared from WT expressing GFP-AaAtg8 treated with various concentrations of H₂O₂ for 8 h with an anti-GFP antibody. The free GFP band intensity in relation to total intensity is also shown. A Ponceau S stain image is shown to ensure equal loading of the samples. (c) Accumulation of ROS in WT and ΔAaAtg8 hyphae after H₂O₂ treatment. Fungal hyphae grown in PDB with or without H₂O₂ for 8 h were stained with 2′,7′-dichlorofluorescein diacetate (DCFHDA) and examined microscopically, displaying green fluorescence (indicating ROS accumulation).
Atg8 is not only essential for forming pre-autophagosomal structures but also indispensable for the expansion and maturation of autophagosomes (Nakatogawa et al., 2007). Mutation of Atg8 leads to the block of autophagy. Although autophagy has been reported to be associated with growth, conidiogenesis, cell development and differentiation, secondary metabolism, and virulence in several filamentous fungi (Liu et al., 2007, 2008; Long et al., 2011; Nadal & Gold, 2010; Richie & Askew, 2008), little is known about its role in resistance to ROS. Our results have uncovered the underlying mechanisms of autophagy-mediated ROS resistance in the tangerine pathotype of A. alternata.

Increased sensitivity to H$_2$O$_2$ in ΔAaAtg8 could be due to its inability to detoxify H$_2$O$_2$ effectively. This speculation was supported by the results that H$_2$O$_2$ triggered autophagy formation and that ΔAaAtg8 accumulated a much higher level of H$_2$O$_2$ in the hyphae than WT upon exposure to H$_2$O$_2$. Increasing H$_2$O$_2$ levels...
could increase cellular stress, leading to cell death. Moreover, we found that the expression of NoxA and Yap1 was down-regulated in ΔAaAtg8. Nox and Yap1 are required for ROS detoxification and full virulence in A. alternata (Lin et al., 2009; Yang & Chung, 2012, 2013). Nox produces low levels of H$_2$O$_2$, which in turn regulates the expression of the Yap1 gene. Yap1 regulates the glutaredoxin

**FIGURE 8** AaAtg8 is required for the turnover of peroxisomes under starvation conditions. (a) Identification of peroxisomes in ΔAaAtg8 spores expressing an mCherry fluorescent protein tagged with SKL tripeptides. No red fluorescence was observed in the wild-type (WT) spores. Spores were collected from fungal strains grown in potato dextrose broth and examined microscopically. Colocalization of peroxisomes in the vacuoles (indicated by arrows) in WT, but not ΔAaAtg8, hyphae grown in (b) minimal medium (MM) or (c) nitrogen-depleted MM (MM-N) for 24h.
and thioredoxin systems leading to \( \text{H}_2\text{O}_2 \) detoxification and cellular resistance to \( \text{H}_2\text{O}_2 \) (Ma et al., 2018; Yang et al., 2016). Feedback inhibition of Nox transcription by Yap1 enables fungal cells to maintain ROS homeostasis.

Low levels of \( \text{H}_2\text{O}_2 \) can trigger autophagy, which acts as an antioxidative response (Filomeni et al., 2015; Nah et al., 2017). Autophagy increases cellular adaptation to ROS stress via recycling ROS-damaged proteins and organelles (Goncalves et al., 2017). Inhibition of the autophagy process can result in the accumulation of high levels of ROS, resulting in cell death (Shi et al., 2021). Peroxisomes are essential organelles in eukaryotic cells. The number of peroxisomes is influenced by various environmental factors, including oxidative stress and nutrient availability (Oku & Sakai, 2010; Sakai et al., 2006). Under normal conditions, excess and damaged peroxisomes are degraded via a selective autophagy mechanism, termed pexophagy, to maintain functional peroxisomes in cells. Degradation of peroxisomes could also provide additional nutrients to cells and serve as a survival strategy under stress conditions. We have previously demonstrated that \( \text{H}_2\text{O}_2 \) triggers peroxisome turnover by pexophagy in \( A. \text{alternata} \) (Wu et al., 2021). Pexophagy may provide nutrients during fungal conidiation and host plant invasion (Ding et al., 2018). In the present study, we observed that peroxisomes were degraded in response to \( \text{H}_2\text{O}_2 \) treatment and starvation, indicating the occurrence of pexophagy in \( A. \text{alternata} \). TEM observation also revealed that hyphae had abundant autophagic vacuoles containing intracellular material, and the number of peroxisomes was reduced under nutrient-depleted conditions. Because deletion of \( \text{AAaAtg8} \) led to the accumulation of peroxisomes, it is likely that autophagy impairments impact peroxisome turnover. Compared to WT, the \( \Delta\text{AAaAtg8} \) hyphae became darker, indicating cell death, after \( \text{H}_2\text{O}_2 \) treatment for 24h. The results showed that autophagy-mediated peroxisome turnover could increase cell adaptability and survival under oxidative stress conditions. \( \text{AAaAtg8} \) is required to maintain the normal function of pexophagy; accumulation of excess peroxisomes could have adverse effects on fungal development and adaptation. In addition to Atg8, several Atg-coding genes have been demonstrated to be involved in pexophagy (Asakura et al., 2009; Tadokoro et al., 2015). Because impairment of the autophagy process led to the accumulation of ROS and peroxisomes, we concluded that autophagy facilitates ROS detoxification via an efficient turnover of peroxisomes.

Autophagy is required to form conidia in many fungi (Bartoszewska et al., 2011; Kikuma et al., 2007). Autophagy has been demonstrated to regulate the expression of the \( \text{brIa} \) gene, which encodes a transcription factor required for conidiation in \( A. \text{oryzae} \) (Kikuma et al., 2007). It has also been speculated that autophagy might provide essential materials and energy to support the germination of conidia and the formation of appressoria or other invasive structures because plant surfaces are nutrient-deprived environments (Deng et al., 2009; Kikuma et al., 2007). \( A. \text{alternata} \) has no known sexual stage, and the production of conidia is critical for the completion of its life cycle. Deleting \( \text{AAaAtg8} \) reduced the number and size of conidia and their ability to germinate and form appressorium-like structures, even when \( A. \text{alternata} \) was cultured in nutrient-replete conditions. The results suggest that low-level autophagy is required for conidiogenesis. Moreover, autophagy might be involved in maintaining cell wall integrity because deletion of \( \text{AAaAtg8} \) reduced cell wall thickness and increased sensitivity to cell wall-disrupting compounds (Congo red and calcofluor white).

Pathogenicity tests assayed on detached citrus leaves revealed that deletion of \( \text{AAaAtg8} \) resulted in fungal strains with a severe reduction in virulence, supporting the notion that autophagy is required for \( A. \text{alternata} \) pathogenesis to citrus. The penetration and colonization stages are defective in the \( \Delta\text{AAaAtg8} \) strains as they induced necrotic lesions more slowly than WT on citrus leaves with or without wounding before inoculation. This could be a multifaceted combination of deficiencies, including slow growth, poor germination of conidia, a reduced formation of appressoria, reduced autophagy, and poor viability, as seen in the \( \Delta\text{AAaAtg8} \) strains. Although \( \Delta\text{AAaAtg8} \) could produce ACT in axenic cultures, we could not completely rule out the possibility that \( \Delta\text{AAaAtg8} \) produces lower amounts of ACT on citrus leaves due to its slow growth. Microscopic observations indeed revealed a delayed appearance of necrotic lesions on leaf spots inoculated with \( \Delta\text{AAaAtg8} \).

The reduced virulence of \( \Delta\text{AAaAtg8} \) could also be due to poor transportation of nutrients between cells. \( A. \text{alternata} \) is a multicellular organism. The growth of new hyphae and germination of conidia might depend on nutrient supplies from old cells. Autophagy might play an essential role in recycling endogenous organelles and nutrient trafficking along hyphal filaments (Shoji et al., 2006). Defects in the autophagy pathway might prevent adequate nutrition and eventually lead to hyphal collapses (Deng et al., 2012; Fricker et al., 2008). \( A. \text{alternata} \) infection increases accumulation of \( \text{H}_2\text{O}_2 \) and cell death of host cells (Lin et al., 2011). Although the oxidative burst, characterized by the accumulation of \( \text{H}_2\text{O}_2 \), is one of the effective strategies to defend against biotrophs and hemibiotrophs (Heller & Tudzynski, 2011), a necrotrophic pathogen such as \( A. \text{alternata} \) can turn \( \text{H}_2\text{O}_2 \)-induced cell death to its benefit (Howlett, 2006; van Kan, 2006). \( A. \text{alternata} \) produces ACT to kill plant cells before colonization and obtains nutrients exclusively from dead cells (Kohmoto et al., 1993). Our previous studies have demonstrated that the ability to mitigate ROS toxicity is required for \( A. \text{alternata} \) virulence. However, the reduction of virulence in \( \Delta\text{AAaAtg8} \) is not due to high levels of \( \text{H}_2\text{O}_2 \) at the site of infection. DAB staining revealed that \( \Delta\text{AAaAtg8} \) induced a much lower level of \( \text{H}_2\text{O}_2 \) than WT. It is tempting to speculate that lower levels of \( \text{H}_2\text{O}_2 \) could be mainly due to a lower level of ACT.

To survive in harsh environments with oxidative stress, \( A. \text{alternata} \) has protective mechanisms to ensure invasive growth and successful colonization. The direct contribution of autophagy to detoxifying or repressing the ROS-mediated plant defence barriers could be important in \( A. \text{alternata} \). Yet, the overall impacts of autophagy on growth, conidiogenesis, differentiation, and nutrient utilization contribute to the reduced virulence of \( \Delta\text{AAaAtg8} \). In conclusion, the current results confirm further that the ability to detoxify ROS via autophagy plays a vital role in the oxidative stress response and \( A. \text{alternata} \) pathogenesis in citrus.
4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal strains and culture conditions

The WT strain of *A. alternata* used in this study has been previously characterized (Lin et al., 2009). WT expressing an mCherry fluorescent protein tagged with serine-lysine-leucine tripeptides (designated WT/mCherry-SKL) was used to monitor the location of peroxisomes as previously described (Wu et al., 2021). Fungal strains were inoculated as a toothpick point on PDA (Difco) or MM (Chung et al., 2020) agar and incubated at 28°C. Conidia were harvested from the fungal strains after culturing on PDA with constant fluorescent light for 3 to 5 days. Conidium germination and appressorium formation were assayed by culturing conidia in PDB (Difco) or liquid MM in 96-well microtitre plates. To study autophagy formation, spores were inoculated in PDB for 16h. Mycelium was harvested, washed three times with Milli-Q water, and incubated in PDB, MM, or MM-N in the presence of 2mM PMSF for 4 h. PMSF blocks protease activity in vacuoles to facilitate the observation of autophagic bodies (Takeshige et al., 1992). For ROS stress response assays, mycelium, after being cultured in PDB for 16h, was harvested, shifted to PDB or PDB containing H₂O₂, and incubated for 8 or 24 h.

4.2 | Sensitivity assays

Fungal sensitivity was assessed on PDA containing a test compound on a 90mm ×15mm Petri dish and incubated under constant fluorescent light at 28°C. Unless otherwise indicated, all compounds were dissolved in water. Test compounds included calcofluor white (200μM dissolved in dimethyl sulphoxide), Congo red (75μM dissolved in ethanol), H₂O₂ (20mM), tert-butyl-hydroperoxide (3.75mM), cumyl hydroperoxide (3.4mM dissolved in ethanol), and diethyl malonate (7mM dissolved in methanol). All treatments had at least three replicates, and experiments were repeated at least three times.

4.3 | Molecular procedures and fungal transformation

Fungal genomic DNA was isolated using a Genomic DNA Mini Purification kit (BioKit). All primers used in this study are listed in Table S1. The *AaAtg8* gene (accession number OK617334) was identified from the complete genome sequence of *A. alternata*. Functional motifs were identified via motif scan available at https://myhits.isb-sib.ch/cgi-bin/motif_scan. The entire coding region of WT *AaAtg8* (472bp) was replaced by a hygromycin phosphotransferase gene cassette (Hyg), which conferred resistance to hygromycin, using a split marker approach (You et al., 2009). Split marker fragments were generated by two-step PCR with gene-specific primers. PCR fragments were mixed and transformed into protoplasts prepared from WT by the CaCl₂ and polyethylene glycol (PEG)-mediated method as previously described (You et al., 2009). Fungal transformants appearing on regeneration medium (RMM) amended with 200μg/ml hygromycin (InvivoGen) were picked and evaluated by Southern blot analyses using an *AaAtg8*- or a Hyg-specific gene probe. The probes used for Southern blot analyses were produced by PCR to incorporate digoxigenin (DIG)-11-dUTP (Roche Applied Science) into the probes with gene-specific primers: Hyg3 and Hyg4 (for the *Hyg* probe) and Atg8 southern F and Atg8 southern R (for the *AaAtg8* probe). Immunological assays identified the probes following the manufacturer’s instructions (Roche Applied Science). A 1598-bp fragment including full-length *AaAtg8* and its endogenous promoter region was amplified from the WT genome with primers BamHI-Atg8 comp-F2 and Atg8 comp-R2-EcoRI. After cleavage with BamHI and EcoRI endonucleases, the DNA fragment was cloned into pCB1532 containing a sulfonylurea resistance gene cassette to result in a complementation construct. The resulting plasmid was transformed into protoplasts prepared from one *AaAtg8*-deficient mutant (designated Δ*AaAtg8*). Transformants were selected on RMM supplemented with 5 μg/ml of sulfonylurea (chlorimuron ethyl; Chem Service). Protoplasts prepared from Δ*AaAtg8* were transformed with a pCB1532/mCherry-SKL plasmid (Wu et al., 2021) to generate Δ*AaAtg8*/mCherry-SKL. A synthetic GFP (sGFP) coding gene was translationally fused in-frame at the 5’ end of the *AaAtg8*-coding sequence by fusion PCR with primers Atg8Pro_F_BamHI, sGFP_F, sGFP_R_NEW, sGFP_Atg8_F, and Atg8_R_XhoI and cloned into the BamHI/Xhol sites of pCB1532 to generate a construct containing an *AaAtg8* promoter-GFP-*AaAtg8* fragment. The resulting plasmid was transformed into WT protoplasts. A fungal strain expressing a GFP-*AaAtg8* protein (WT/GFP-*AaAtg8*) was identified from RMM amended with sulfonylurea, examined by PCR, and further confirmed microscopically for green fluorescence.

4.4 | GFP-*AaAtg8* proteolysis assay

Crude proteins were extracted from the WT/GFP-*AaAtg8* strain, separated by SDS-PAGE, and analysed by western blot using an anti-GFP antibody. Briefly, mycelium was harvested, ground to a fine powder in liquid nitrogen, and suspended in protein lysis buffer (500μl/0.1 g dried mycelium; 50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 0.1% Triton X-100, and 1% protease inhibitor cocktail [Biokit]). The lysates were homogenized in a centrifugation tube by placing in an ice tray and incubating on a shaker for 30 min. After centrifugation at 12,000 x g for 20 min at 4°C, supernatants were mixed with 5x protein loading buffer (Biokit) and boiled for 5 min. Samples were separated by 10% SDS-PAGE and subsequently transferred to polyvinylidene fluoride (PVDF) membranes. Protein bands were visualized by staining gels with Coomassie blue or staining the PVDF membranes with Ponceau S solution (5% acetic acid, 0.1% Ponceau S). Membranes were preincubated with TSW buffer (10mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.25% gelatin, 0.1% Triton X-100, and 0.2% SDS) for 1 h, followed by incubation with a rabbit polyclonal anti-enhanced GFP (eGFP) antibody (1:5000, kindly provided by Dr. F.J. Jan) at room temperature (approximately 25°C) for
1 h and incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; H+L, Jackson ImmunoResearch) for an additional 1 h. Protein bands were visualized by incubating PVDF membranes with Western Lightning ECL Pro chemiluminescence substrate (PerkinElmer) following the manufacturer’s recommendations. Band intensities were quantified by ImageJ software (https://imagej.nih.gov/ij/).

4.5 | Extraction and analysis of fungal toxin

Fungal strains were grown in Richard’s medium (Kohmoto et al., 1993) for 22 days to evaluate the ability to produce ACT toxins. An ACT-deficient strain of A. alternata (ΔAaACTT6) (Ma et al., 2019) was included as a negative control. Richard’s medium containing agar plugs without fungal mycelium was used as a blank control. Bioassays and HPLC of the toxin were performed as previously described (Wu et al., 2020). After separation in an Ascentis C18 HPLC column (5 μm particle size silica, 4.6 mm internal diameter × 250 mm; Sigma-Aldrich), each fraction was collected, dried, and resuspended in 50 μl methanol. Toxin bioassays were performed by placing 10 μl of each sample on the surface of prewounded calamondin (Citrus mitis) leaves. Each fraction was evaluated on at least five different leaves.

4.6 | RT-qPCR

RT-qPCR was conducted to analyse gene expression in fungal strains. Fungal RNA was purified using TRizol reagent (Sigma-Aldrich), treated with RQ1 RNase-free DNase (Promega), and used for the synthesis of complementary DNA with a One-Step iScript cDNA synthesis kit (Bio-Rad). Reactions for qPCR were prepared using iQ SYBR Green Supermix (Bio-Rad) and carried out in a CFX Connect Real-Time PCR Detection System (Bio-Rad). All primers are listed in Table S1. The β-tubulin-coding gene was used as an internal control. The 2−ΔΔCT method was used to determine relative expression levels of genes. All treatments were performed with three technical replicates, and significant differences were determined by statistical analysis. Experiments were repeated at least two times.

4.7 | Virulence tests

Fungal virulence was evaluated on detached calamondin leaves by placing a small piece (1 mm) of fungal mycelium or 10 μl of conidial suspension (10^5 conidia/ml) on each spot. Leaves were either unwounded or prewounded before inoculations with a sharp needle to facilitate fungal penetration. Leaf spots treated with sterile water were used as mock controls. For lesion development, all treated leaves were kept in a plastic box for 5 to 6 days. Each strain was evaluated on more than five different leaves, and experiments were replicated at least three times. H2O2 in citrus leaves was detected using DAB (Sigma-Aldrich). Briefly, leaves were inoculated with 10 μl of conidial suspension (10^5 conidia/ml), kept in a plastic box for 1 to 3 days, placed in DAB solution (100 mg DAB and 50 μl of 37% HCl in 100 ml Milli-Q water) in the dark for 8 h, and incubated in ethanol-acetic acid–Milli-Q water (94:4:2, vol/vol/vol) for 7 days for discolouration and fixation. H2O2 reacted with DAB to form a brown stain around the foci of infection.

4.8 | Microscopy

Leaves were inoculated, hyphae and conidia were stained with lactophenol cotton blue (Sigma-Aldrich), and samples were examined with an Optiphot 2 microscope (Nikon). Conidia were photographed, and sizes were measured using ImageJ. TEM was carried out as previously described (Wu et al., 2020) to examine autophagy and organelles in hyphae and conidia. Hyphae were stained with 1 μg/ml MM 4-64 (Enzo) to track endocytosis (Fischer-Parton et al., 2000) or with 100 μM MDC (Sigma-Aldrich) to detect autophagic vacuoles such as autophagosomes, amphisomes, and autolysosomes (Biedermann et al., 1995). Hyphae were stained for 30 min and observed by a ZOE fluorescent cell imager (Bio-Rad) microscope with excitation at 588 nm and emission at 734 nm for MM 4-64 and with excitation at 335 nm and emission at 518 nm for MDC. Red fluorescence from mCherry was visualized with excitation at 543 nm and emission at 560–615 nm. Hyphae were examined after staining with 40 μM DCFHDA (Sigma-Aldrich) using excitation at 504 nm and emission at 529 nm. GFP fluorescence was visualized with excitation at 450–490 nm and emission at 505–550 nm. Vacuoles were stained with CMAC (final concentration, 100 μM; Thermo Fisher Scientific) and visualized with excitation at 353 nm and emission at 466 nm.

4.9 | Statistical analysis

All experiments with at least three replicates were conducted three times unless indicated otherwise. The significance of tests was analysed by one-way analysis of variance and Tukey’s honestly significant difference (HSD) post hoc test (**p < 0.01; *p < 0.05).

AUTHOR CONTRIBUTIONS

P.C.W., Y.K.C., J.I.Y., and K.R.C. designed the experiments. P.C.W., C.Y.L.C., H.Y.L., and X.Y.W. performed the experiments. P.C.W. performed data analysis. P.C.W. and K.-R.C. wrote the manuscript.

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CONFLICT OF INTEREST
The authors declare no competing financial or nonfinancial interests.

DATA AVAILABILITY STATEMENT
All data supporting the findings of this study are available within the paper and its supplementary data published online.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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