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Linkage of autophagy to fungal development, lipid storage and virulence in *Metarhizium robertsii*

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Abbreviations: Atg, autophagy-related; ORF, open reading frame; LD, lipid droplet; ATMT, *Agrobacterium tumefaciens*-mediated transformation; TEM, transmission electron microscopy; PLIN1, perilipin; PLIN2/ADRP, perilipin 2/adipose differentiation-related protein; PLIN3/TIP47, perilipin3/tail-interacting protein of 47 kDa; MPL1, Metarhizium perilipin-like protein 1; TUB2, tubulin beta-2; BEN, benomyl-resistance protein; RFP, red fluorescent protein; WT, wild type; PDA, potato dextrose agar; MM, minimum medium; CM, complete medium; SDB, Sabouraud dextrose broth; YPD, yeast extract peptone dextrose; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; LT_{50}, median lethal time; AP, appressorium; CO, conidium

Autophagy is a highly conserved process that maintains intracellular homeostasis by degrading proteins or organelles in all eukaryotes. The effect of autophagy on fungal biology and infection of insect pathogens is unknown. Here, we report the function of *MrATG8*, an ortholog of yeast *ATG8*, in the entomopathogenic fungus *Metarhizium robertsii*. *MrATG8* can complement an *ATG8*-defective yeast strain and deletion of *MrATG8* impaired autophagy, conidiation and fungal infection biology in *M. robertsii*. Compared with the wild-type and gene-rescued mutant, *Mratg8*Δ is not inductive to form the infection-structure appressorium and is impaired in defense response against insect immunity. In addition, accumulation of lipid droplets (LDs) is significantly reduced in the conidia of *Mratg8*Δ and the pathogenicity of the mutant is drastically impaired. We also found that the cellular level of a LD-specific perilipin-like protein is significantly lowered by deletion of *MrATG8* and that the carboxyl terminus beyond the predicted protease cleavage site is dispensable for MrAtg8 function. To corroborate the role of autophagy in fungal physiology, the homologous genes of yeast *ATG1*, *ATG4* and *ATG15*, designated as *MrATG1*, *MrATG4* and *MrATG15*, were also deleted in *M. robertsii*. In contrast to *Mratg8*Δ, these mutants could form appressoria, however, the LD accumulation and virulence were also considerably impaired in the mutant strains. Our data showed that autophagy is required in *M. robertsii* for fungal differentiation, lipid biogenesis and insect infection. The results advance our understanding of autophagic process in fungi and provide evidence to connect autophagy with lipid metabolism.

Introduction

Autophagy is a self-eating process that maintains intracellular homeostasis by degrading proteins or organelles and is conserved in all eukaryotes.[1] It plays a crucial role in establishing infections by plant pathogenic fungi such as *Magnaporthe oryzae*,[2-5] *Fusarium graminearum* or *Colletotrichum* spp,[6,7] and the mammalian pathogens like *Cryptococcus neoformans*, *Candida albicans* and *Aspergillus fumigatus*.[8] Insect pathogenic fungi like *Metarhizium robertsii*, *M. acridum* and *Beauveria bassiana* are widely used in the biological control of different insect pests and are model organisms to study the mechanisms of insect-fungus interactions.[9,10] Several virulence-related genes have been characterized in these fungi,[11] but none associated with autophagy.

A variety of proteins in the autophagy pathways have been identified and functionally characterized.[12] Thus far, 36 autophagy-related (Atg) proteins have been identified in yeast[13] and 23 in the plant pathogen *M. oryzae*.[4] Of these, Atg8 has been identified as one of the key autophagic proteins involved in multiple cellular processes including autophagy, lipid metabolism and membrane

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trafficking.[14] The *ATG8* gene encodes a highly conserved ubiquitin-like protein that is associated with the autophagosome membrane and microtubule, and is used as a marker of autophagy.[13] Gene-deletion studies have revealed the involvement of *ATG8* in differentiation and development in filamentous fungi.[12] In *M. oryzae*, MoAtg8 mediates autophagic cell death and is required for fungal infection.[2] Deletion of *AoATG8* in *Aspergillus oryzae* leads to the impairment of conidiation and aerial hyphal growth.[15] Disruption of *ATG8* in the plant pathogen *Ustilago maydis* abolishes autophagosome accumulation in the vacuoles and impairs fungal growth and virulence.[16] Besides *ATG8*, serial gene deletions of other *ATG* genes in *M. orzyae* leads to loss of virulence in 16 of 22 null mutants, due to impairment of appressorium differentiation/maturation and conidial programmed cell death.[4] It is unclear whether Atg-mediated autophagy is also required for fungal differentiation and virulence in the insect pathogens.

During autophagy, Atg8 binds to phosphatidylethanolamine on the phagophore membrane and remains there through the maturation process of the autophagosome.[13] This membrane binding is facilitated by the carboxyl-terminal glycine residue in Atg8.[17] It remains undetermined whether the C-terminal Gly residue is essential for Atg8 function in filamentous fungi. In mammalian cells, autophagy is known to regulate lipid metabolism,[18,19] although its effect on lipid breakdown or biogenesis that results in the turnover of cellular lipid droplets (LDs) is still unknown.

In this study, the ortholog of yeast *ATG8* gene, designated as *MrATG8* was deleted and complemented in the insect pathogenic fungus *M. robertsii*. We found that *MrATG8* is required for conidiation, appressorium differentiation, LD accumulation and virulence in *M. robertsii*. In addition, the orthologous genes of yeast *ATG1* (encoding a serine/threonine protein kinase), *ATG4* (encoding a cysteine protease) and *ATG15* (encoding a triglyceride lipase), labeled as *MrATG1* (MAA_03501), *MrATG4* (MAA_04475) and *MrATG15* (MAA_00506),[20] were also deleted in *M. robertsii*. These mutants showed similar defects in cellular LD storage and virulence although, in contrast to *Mratg8*Δ, appressorium formation was not affected. The results of this study improve the understanding of the effects of autophagy on cell differentiation, lipid storage and virulence in the insect pathogenic fungus *M. robertsii*.

Results

***MrATG8* gene characterization and protein localization.** The sequence of *S. cerevisiae* Atg8 protein was used as query in Blast searches against the *M. robertsii* genome database,[20] and the retrieved putative protein with 78% amino acid identity was designated as MrAtg8 (MAA_02674). To determine whether *MrATG8* gene is functionally related to the yeast *ATG8*, the complete *MrATG8* ORF was transformed into the *Atg8*Δ yeast strain. After nitrogen starvation for 18 d, the *MrATG8*-rescued yeast strain survived and grew normally similar to the wild type (WT) while the *Atg8*Δ mutant and control that was transformed only with the empty vector did not survive (**Fig. 1A**). All strains grew equally well on a nutrient-rich medium (**Fig. 1B**). This is not surprising, considering that MrAtg8 is highly conserved among fungi, plants and insects (**Fig. 1C**). Transcription of *MrATG8* was also highly activated in response to nitrogen starvation that is similar to the observations in yeast and other filamentous fungi.[12] In addition, the gene was also transcribed by the fungus when grown in a nutrient-rich medium, Sabouraud dextrose broth (SDB), and during conidiation and appressorium formation (**Fig. 1D**), which is consistent with studies in mammals that show the constitutive occurrence of autophagy.[21]

Similar to the pattern of Atg protein localization in yeast and Magnaporthe cells,[22-24] punctate localization of MrAtg8 was observed in conidia, germlings, appressoria and hyphal bodies (fungal cells harvested from the hemolymph of infected insect) by N-terminal fusion with the red fluorescent protein (RFP) (**Fig. 2A–D**). In contrast, the diffused signal is present in the conidia of a strain transformed only with the *RFP* gene (**Fig. 2E**). Relative to the conidia, stronger signals were observed in the mother cell of germling and appressorium, indicating an increased occurrence of autophagy in these cells. By fluorescent staining, we also found that MrAtg8 was not localized on lipid droplets (LDs) (**Fig. S1**).

Effects of MrAtg8 on conidiogenesis and appressorium formation. To study *MrATG8* gene function, targeted gene replacement was performed to delete *MrATG8* in *M. robertsii* via *Agrobacterium tumefaciens*-mediated transformation (ATMT) (**Fig. S2A**). Deletion of *MrATG8* in drug-resistant transformants was verified by PCR (**Fig. S2B**) and confirmed by RT-PCR analyses (**Fig. S2C**). For mutant complementation, the complete *MrATG8* ORF and its promoter region were amplified and transformed into *Mratg8*Δ and the resulting mutant was designated as Comp. When cultured on different media, we found that the WT, *Mratg8*Δ and Comp could sporulate on a complete medium (CM) (**Fig. 3**). While there was no difference between the WT and Comp on minimal medium (MM) and potato dextrose agar (PDA) that no conidiophores were produced by the null mutant on these media (**Fig. 3**; **Fig. S3**). This suggests that MrAtg8 affects fungal conidiation in a nutrient-dependent manner. To examine the effect of MrAtg8 on infection-structure differentiation, conidia of the WT, *Mratg8*Δ and Comp of *M. robertsii* were harvested from CM and used for appressorium induction either on a plastic hydrophobic surface or locust hind wings.[25] Sixteen hours postinoculation, the WT and Comp produced appressoria on both surfaces while most of the conidia of *Mratg8*Δ germinated without germ-tube tip swelling, indicating a significant failure to form appressoria either on host or nonhost surfaces (**Fig. 4A–H**).

Deletion of *MrATG8* leads to impaired formation of autophagic bodies and lipid droplets. Using transmission electron microscopy (TEM), autophagic bodies were not visible in the vacuoles of *Mratg8*Δ following nitrogen starvation while they were observed in the Comp cells that was similar to the WT (**Fig. 5A–C**). Our TEM analysis also found that, the accumulation of LDs in conidia was also significantly impaired in *Mratg8*Δ which is in contrast to the WT and Comp (**Fig. 5D–F**). This was confirmed by fluorescent staining using a LD-specific dye Bodipy (**Fig. 5G–L**). Thus, consistent with the studies in mammalian

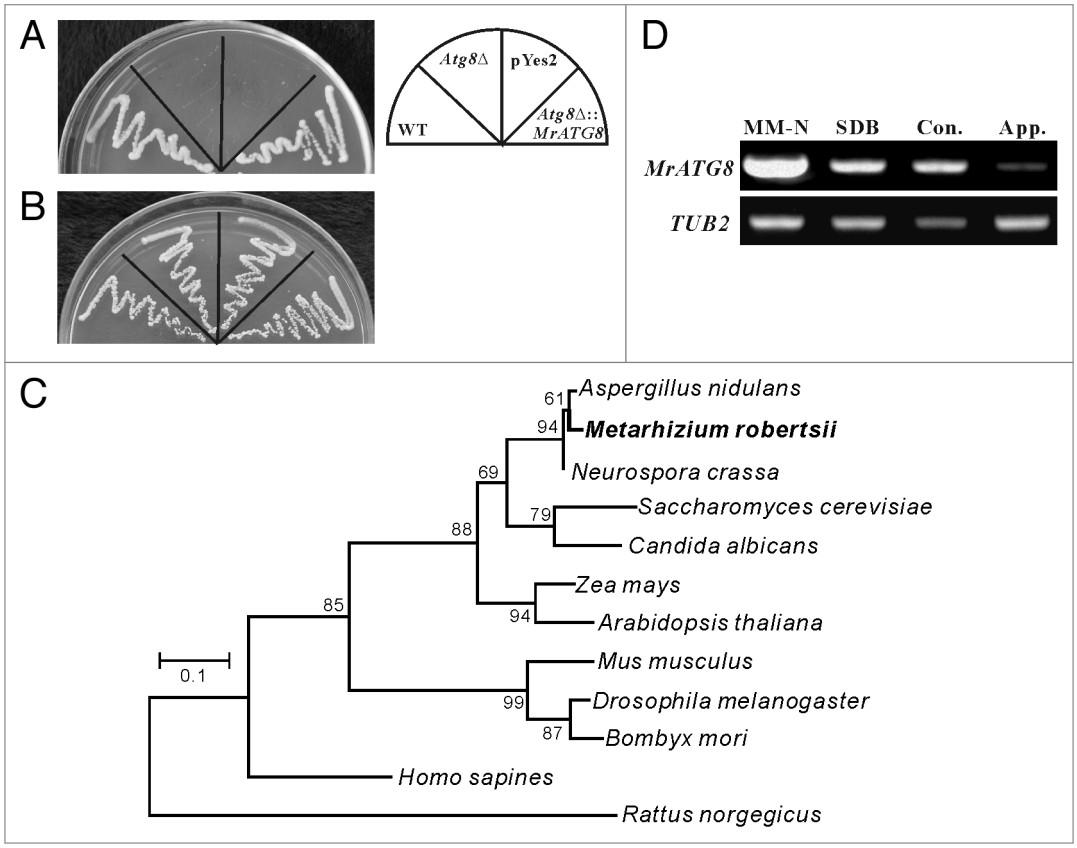

Figure 1. Characterization of *MrATG8* gene function, evolution and expression. (**A**) Complementation of yeast *ATG8* gene with *MrATG8*. In contrast to the wild-type (WT) yeast, *Atg8*Δ and the mutant transformed with the pYes2 vector did not survive after nitrogen starvation, however, the mutant transformed with the *MrATG8* gene survived like the WT. (**B**) All yeast strains grew equally well on a nutrient-rich YPD medium. (**C**) Phylogenetic analysis indicating that the *MrATG8* of *M. robertsii* is highly similar to the orthologs from various fungi, plants and insects. (**D**) RT-PCR analysis of *MrATG8* expression by the fungus under various conditions. MM-N, mycelia cultured in MM-N medium for 4 h; SDB, mycelial sample cultured in SDB for 24 h; Con., conidia harvested from CM cultures; App., appressoria induced on locust hind wings. *TUB2*, tubulin β-2 gene, was used as a reference.

cells,[18,19] the association of autophagy with lipid metabolism is also observed in *M. robertsii*.

Lipid storage is controlled by LD-specific proteins like perilipin.[26] We previously identified a mammalian perilipin-like protein Mpl1 that regulates LD storage, appressorium turgor pressure and virulence in *Metarhizium*.[27] To investigate whether the impaired LD accumulation in *Mratg8*Δ is connected to *MPL1* gene transcription and protein expression, we performed RT-PCR and western blot analyses. Interestingly, the transcription of *MPL1* in *Mratg8*Δ was not significantly different when compared with the WT and Comp (**Fig. 6A**). However, consistent with the reduced numbers of LDs in *Mratg8*Δ cells (**Fig. 5E and H**), Mpl1 protein level was also lower than in the WT and Comp cells (**Fig. 6B**). This suggests that impaired lipid storage in *Mratg8*Δ is concomitantly associated with reduced Mpl1 protein levels.

The carboxyl-terminal glycine residue in MrAtg8 is essential for its function. In yeast, the C-terminal glycine residue of Atg8, which is exposed by Atg4 protease cleavage, is essential for membrane binding, and therefore the autophagosome formation and cytoplasm-to-vacuole targeting pathway.[17] This conserved Gly residue (G116) is also present in MrAtg8 followed by Thr and Ala residues instead of an Arg residue in yeast Atg8 (**Fig. 7A**). To study the roles of MrAtg8 C-terminal residues G116, T117 and A188, five variants were generated by designing PCR primers to introduce mutations (**Table S1**) in the *MrATG8* gene. The acquired mutants were designated as M8-1 (G116 replaced with A116), M8-2 (deletion of G116), M8-3 (deletions of T117 and A118), M8-4 (deletions of G116 and T117) and M8-5 (deletions of G116, T117 and A118) (**Fig. 7A**). Growth tests on PDA medium showed that only the growth of M8-3 was similar to the WT (**Fig. 7B**). M8-3 could also form appressoria (a differentiation rate at 82.22%) on a hydrophobic surface similarly to the WT (88.4%) (**Fig. S4**) and kill insects in a similar manner like the WT (**Table 1**), indicating that G116 residue is crucial to maintain the complete function of MrAtg8. In addition, we found that fusion of the RFP protein either on the N or C terminus of MrAtg8 had no apparent alteration in conidiation of transformants in comparison to the WT (**Fig. 7B**).

Mratg8 is essential for full virulence of *M. robertsii*. We performed both injection and immersion assays to examine the effects of *MrATG8* deletion on fungal virulence against the silkworm larvae. The values of median lethal time (LT_{50}) were estimated and compared among the WT, *Mratg8*Δ and gene rescued

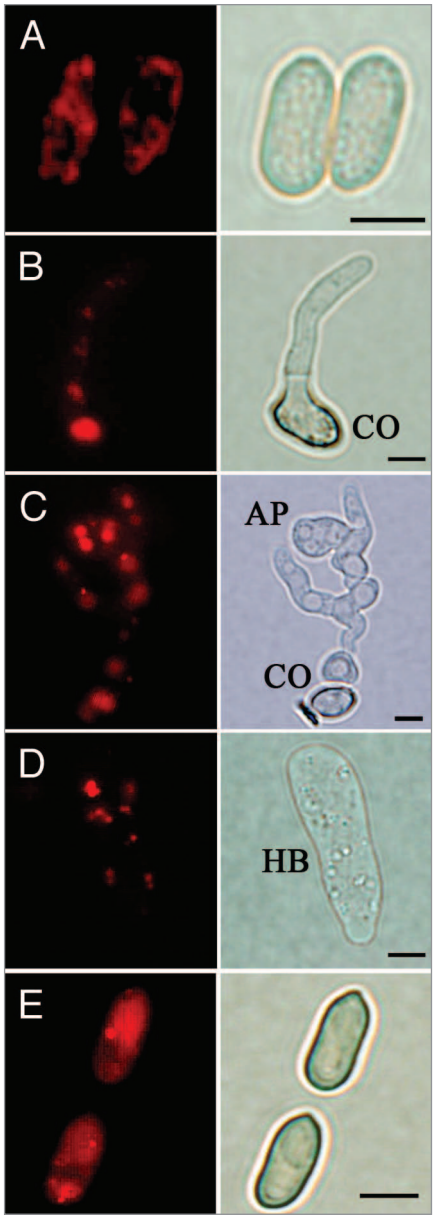

Figure 2. Cellular localization of MrAtg8 in various cell types of *M. robertsii*. An RFP-MrAtg8 fused construct was introduced into the wild-type strain to examine the localization of MrAtg8 in fungal cells under various developmental stages. Punctate RFP signals were detected in conidia (**A**), germlings (**B**), appressorium (**C**) and hyphal bodies (**D**). However, the strain only transformed with an *RFP* gene demonstrated a diffused RFP signal in conidia (**E**). CO, conidium; AP, appressorium. Hyphal bodies (HB) were harvested from the hemolymph of fungal infected silkworm larvae. Scale bar: 5 μm.

mutant Comp. Injection of the WT and Comp spores killed the insects in five days but *Mratg8*Δ only caused 41% mortality (**Fig. 8A**). Thus, the difference between the WT (LT_{50} = 3.0 ± 0.30) and *Mratg8*Δ (LT_{50} = 5.0 ± 0.35) (χ^2 = 47.42, p = 0), and between *Mratg8*Δ and Comp (LT_{50} = 3.5 ± 0.27) (χ^2 = 42.42, p = 0) was significant although the difference between the WT and Comp was not significant (χ^2 = 0.22, p = 0.64). After topical infection, WT and Comp killed insects in six days but *Mratg8*Δ failed to cause even 50% mortality (**Fig. 8B**). The difference was thus significant between the WT and *Mratg8*Δ (χ^2 = 63.1, p = 0) and between the mutant and Comp (χ^2 = 54.5, p = 0) (**Table 1**). We found that deletion of *MrATG8* delayed fungal development and mycosis on insect cadaver, although conidia were produced on insect cuticles (**Fig. 8C**). Microscopic observations indicated that the conidia of *Mratg8*Δ were encapsulated and heavily melanized by insect hemocytes 16 h postinjection, while both the WT and Comp could escape from the hemocyte nodules up to 24 h postinjection and produce branched mycelia (**Fig. 9**). These observations indicate that deletion of *MrATG8* impaired fungal defense-response against host immunity thereby lowering virulence.

Characterization of various autophagy-related genes. Similar to the plant pathogen *M. orzyae* (**Table S2**), 23 yeast-like *ATG* genes were identified in *M. robertsii* by whole-genome survey.[20] To determine whether other autophagy-related genes are required for fungal development and pathogenicity, the orthologous genes of yeast *ATG1* (MAA_03501, designated as *MrATG1*, 46% identity), *ATG4* (MAA_04475, *MrATG4*, 40% identity) and *ATG15* (MAA_00506, *MrATG15*, 38% identity) were deleted in *M. robertsii*, respectively. TEM analysis showed that, similar to *Mratg8*Δ, all three null mutants had defects in LD accumulation in conidia (**Fig. 10A**). While all three mutants could similarly produce conidia on CM medium only the WT and *Mratg15*Δ could sporulate on PDA medium and *Mratg1*Δ and *Mratg4*Δ lost their abilities to sporulate. For gene-complemented mutants, their sporulation abilities on PDA were restored (**Fig. S5**). Most significantly, we found that the deletion of these three genes did not impair appressoria formation by the mutants when compared with the WT (**Fig. 10A**; **Fig. S6**). Consistent with the observation in *Mratg8*Δ, our western blot analysis revealed that the LD surface protein Mpl1 was downregulated in these three mutants when compared with the WT, particularly in *Mratg4*Δ (**Fig. 10B**). In contrast to *Mratg8*Δ, topical infection of *Mratg1*Δ, *Mratg4*Δ and *Mratg15*Δ caused 58%, 82% and 91% insect mortality, respectively. A significant difference ($p < 0.05$) was observed between each mutant and the WT in the time taken to kill insect hosts (**Table 1**).

Discussion

The most primordial function of autophagy is to recycle proteins and organelles within cells as an adaptation to nutrient deprivation.[1,13] Current understanding of autophagy has extended its function to cell differentiation, polarity, lipid metabolism, immunization and aging.[28,29] Autophagy has been studied in a few species of filamentous fungi, especially the plant pathogen *M. oryzae*.[12] Deletion of various autophagy-related genes revealed that autophagy is required for fungal conidiation, infection structure formation and virulence.[4,12] In this report, we conducted a comprehensive study of autophagy-related genes in an insect pathogenic fungus *M. robertsii* by deleting *MrATG1*, *MrATG4*, *MrATG8* and *MrATG15* genes. Disruption of any of these four genes reduced conidial LD accumulation and impaired fungal virulence against silkworm larvae but only *Mratg8*Δ lost the

ability to form appressoria. Most significantly, we found that the LD-specific perilipin-like protein, Mpl1, was downregulated in the mutants specifically in the conidia of *Mratg4*Δ and *Mratg8*Δ consistent with impaired LD accumulation. We also found that the localization of MrAtg8 was punctate in the various cell types.

In yeast cells, Atg8 is cleaved by the cysteine protease Atg4 to remove its C-terminal residues and expose Gly116 for Atg8 conjugation with phosphatidylethanolamine (PE) which results in targeting to autophagosomal membranes.[30] Although Atg8 is highly conserved in eukaryotes, the C-terminal residues after Gly116 differ in their composition and length in different organisms (**Fig. 7A**). Our experiments with protein mutations revealed that the amino acids following Gly116 are dispensable for MrAtg8 function. Interestingly, we found that the mutation of MrAtg8 that deleted all residues beyond Gly116 still rescued *Mratg8*Δ phenotype, i.e., mutant M8-3 (**Fig. 7**; **Fig. S4**). This mutation did not require the function of protease MrAtg4. Thus, it is surprising that the Atg8 orthologs have not evolved with Gly as the last residue. Consistent with the successful cleavage of human microtubule-associated protein 1 light chain 3 (LC3, an ortholog of yeast Atg8) C terminus tagged with FLAG or His (6),[31] MrAtg8-RFP could rescue *Mratg8*Δ phenotype (**Fig. 7B**), suggesting that the residue length of MrAtg8 C terminus does not prevent the protease cleavage. Mutagenesis of LC3 with Ala substituted for G120 (G120A) does not result in cleavage of the mutant protein,[31] emphasizing the importance of the C-terminal Gly residue. Similar results were obtained with the mutated MrAtg8 (G116A) strain failing to rescue the *Mratg8*Δ phenotype (mutant M8-1, **Fig. 7**). The nonsporulation phenotypes of M8-2, M8-4 and M8-5 mutants confirmed that the C-terminal Gly is crucial for MrAtg8 function, including conjugation with PE.

Genes affecting the virulence of *Metarhizium* have been functionally identified from those mediating conidial adhesion, controlling cuticle penetration and invading host immunities.[9,32] Similar to plant pathogenic fungi,[12,33] which require autophagy for infections, deletions of autophagy genes in *M. robertsii* also led to reduced pathogenicity. However, while the *Moatg8* null-mutant of *M. oryzae* formed appressoria,[2,4] *Mratg8*Δ lost the ability to produce an infection structure. Taken together with the reduced level of LD in conidia, the significantly decreased virulence of *Mratg8*Δ might be due to the impaired ability of the fungus to penetrate insect cuticle during topical infection. To some extent, it is similar to the reduced virulence of *M. robertsii* with deletion of the perilipin-like protein gene, *MPL1,* that leads to decreased turgor pressure within the appressoria.[27] However, in our injection assays that bypass insect cuticle *Mratg8*Δ showed a significantly reduced virulence while *Mpl1*Δ killed insects like the WT strain.[27] This could be due to the inability of *Mratg8*Δ to counteract insect defense responses (**Fig. 9**).

In yeast, Atg8 is processed by the cysteine protease Atg4 to expose the C-terminal glycine residue for membrane targeting.[34] MoAtg4 of *M. orzyae* interacts with MoAtg8 and deletion of *MoATG4* leads to a significant reduction in conidiation, delay in appressorium formation and a lower turgor pressure.[24] Similarly, *Mratg4*Δ lost the ability to sporulate on PDA medium and also

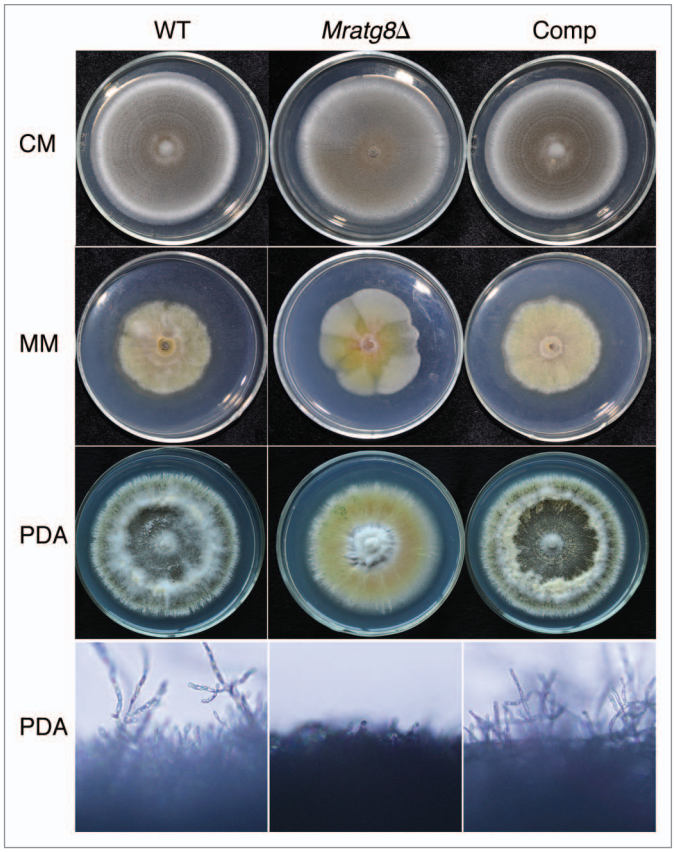

Figure 3. Phenotypic characterization on different media. The wild-type (WT), *Mratg8*Δ and complemented mutant (Comp, *Mratg8*Δ complemented with the *MrATG8* gene) strains sporulated uniformly on a complete medium (CM) while in contrast to the WT and Comp, *Mratg8*Δ failed to produce conidia either on a nutrient-poor minimum medium (MM) or a nutrient-rich potato dextrose agar (PDA), indicating the loss of ability to form conidiophores.

showed a significantly reduced virulence when compared with the WT strain. Consistent with reduced virulence in *M. orzyae* with *MoATG1* deletion[3,4] and *F. graminearum* with *FgATG15* (FGSG_02519) deletion,[6] *Mratg1*Δ and *Mratg15*Δ of *M. robertsii* were also slower in killing insects when compared with the WT strain. This could be, in part, due to the reduced turgor pressure of appressoria caused by impaired LD accumulation in conidia. It is interesting to note that Atg15 extends yeast life span by mediating intravacuolar membrane disintegration.[35] The role of MrAtg15 in *M. robertsii* remains to be determined.

Although autophagy is highly conserved in eukaryotic cells, differences have been observed between yeasts and mammalian cells.[36] When compared with yeasts,[13] our genome survey indicated that 11 of 34 yeast *ATG* orthologs (*ATG10*, *ATG14*, *ATG19*, *ATG23*, *ATG25*, *ATG28*, *ATG30*, *ATG31*, *ATG32*, *ATG33* and *ATG34*) are not present in the genomes of *M. robertsii* and *M. oryzae* (**Table S2**), implying that different mechanisms are employed by the yeasts and filamentous fungi for degradation of proteins or organelles. In particular, *ATG32* and *ATG33* essential for the specific autophagic elimination of mitochondria (mitophagy),[37] are not present in *M. robertsii* or *M. oryzae*. Interestingly,

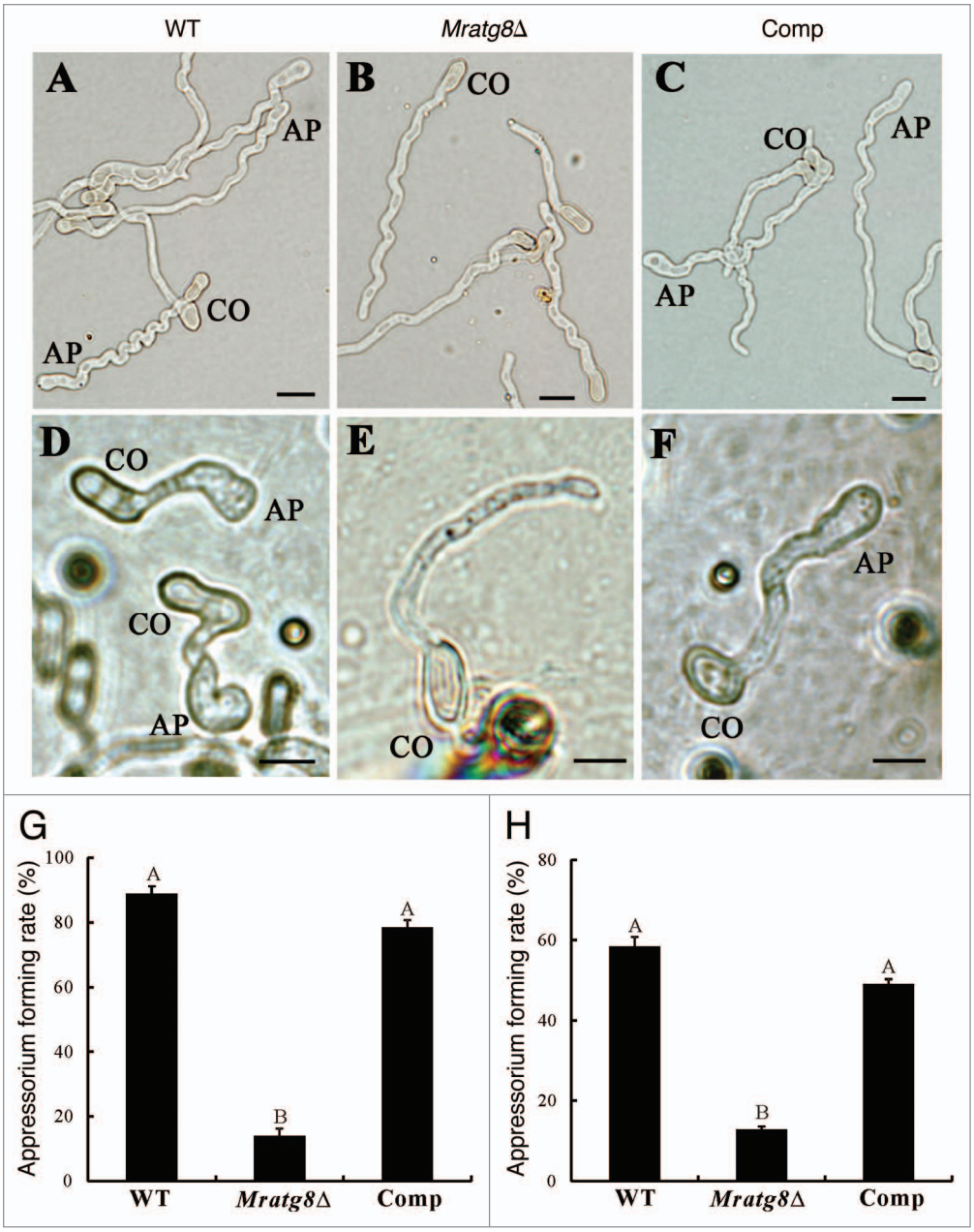

Figure 4. Appressorium induction. Conidia of the wild-type (WT), *Mratg8*Δ and Comp mutant harvested after 20 d on CM plates were subjected to appressorial induction for 16 h either on a hydrophobic surface (**A–C**; sterile plastic Petri dishes in six cm diameter containing 3 ml of 0.0125% yeast extract) or on locust hind wings (**D–F**). In contrast to the WT (**A and D**) and Comp (**C and F**), appressorium production by *Mratg8*Δ (**B and E**) were significantly impaired on the surfaces of either plastic Petri dishes (**G**) or locust hind wings (**H**). The same letter labeled within (**G or H**) means that no statistical difference was detected between the samples at a level of $p < 0.01$. AP, appressorium. CO, conidium. Scale bar: 5 μm.

the ortholog of yeast *ATG11*, a mitophagy receptor,[38] is present in the genomes of *M. robertsii* (MAA_04312, 20% identity) and *M. oryzae* (MGG_04486, 22% identity). Three of five *ATG*s, *ATG25*, *ATG28* and *ATG30*, are also absent in *Metarhizium* or *Magnaporthe* (**Table S2**). The protein products of these genes are required for vacuolar degradation of peroxisomes, i.e., the pexophagy.[39] Yeast *ATG14* encodes the autophagy-specific subunit of phosphatidylinositol-3-kinase complex.[40] The ortholog is also absent in filamentous fungi (**Table S2**), suggesting an alternative strategy in filamentous fungi to form the proper kinase complex to phosphorylate phosphatidylinositol, an essential process in autophagy.

We also found a variety of phenotypic differences in different organisms with disruptions of *ATG* gene orthologs. For example, *Mratg8*Δ of *M. robertsii* could not produce appressoria but *Moatg8*Δ of *M. oryzae* could form appressoria on hydrophobic surface.[2,4] Interestingly, both *Mratg8*Δ and *Moatg8*Δ showed a significant reduction in the ability to infect insect and rice hosts, respectively, while the ability of *F. graminearum Fgatg8*Δ to infect barley and wheat remained like the WT.[41] Similar to *Aoatg4*Δ and *Aoatg15*Δ of *A. orzyae*,[42] *Mratg4*Δ and *Mratg15*Δ lost the ability to form conidia. In contrast, *Moatg4*Δ and *Moatg15* of *M. oryzae* can produce conidia but exhibit significantly reduced abilities of conidgiogenesis.[4,24] There are also differences between fungal and mammalian autophagy gene-deletion mutants. For example, in contrast to the increased lipid contents in mammalian cells followed by deletions of autophagy genes,[18,21] reduced LD accumulation was evident in the four null mutants of *M. robertsii*. Compared with the WT cells, fewer LD were also observed in the conidia of *Moatg1*Δ of *M. oryzae*[3] and *Fgatg15*Δ

of *F. graminearum*.[6] Studies in different organisms indicate that autophagy has evolved multiple times. For example, autophagy in mammals is much more ancient than previously anticipated and in yeast the autophagic pathway appears to be specialized.[36] The evolution of autophagy in filamentous fungi remains to be determined. Nevertheless, our study indicated that a comprehensive understanding of the mechanisms and effects of autophagy by using various organisms as research models is resourceful.

Given that autophagy regulates lipid metabolism it is reasonable to assume a connection between lipolysis and autophagy,[18,19,21] however, the exact mechanism(s) of this connection is still unknown. In mammalian cells two distinct functions have been suggested for autophagy in LD homeostasis; one is the regulation of LD breakdown in hepatocytes and the second is the control of LD formation in adipocytes associated with adipose differentiation.[21] Because of this, inhibition or genetic knockdown of autophagy markedly increases lipid content in hepatocytes and LDs destined for degradation could be visualized within autophagic vacuoles.[18] Deletion of autophagy gene in the protozoan parasite *Leishmania major* markedly increases cellular content of phospholipids PE and phosphatidylcholine.[43] In contrast, knockdown of autophagy genes in adipocytes block LD formation due to lack of LD biogenesis.[19] Before this study, fewer LDs have been observed in the conidia of *Moatg1*Δ and *FgAtg15*Δ of the plant pathogens *M. oryzae* and *F. graminearum*, respectively.[3,6] Thus, to a certain extent fungal spores are similar to animal adipocytes where autophagy controls LD biogenesis rather than breakdown. According to the current theory, LD biogenesis involves neutral lipid accumulation within the membrane bilayer of endoplasmic reticulum followed by budding off and enwrapping by a phospholipid monolayer containing specific proteins including perilipin.[44] Except for the perilipin-like protein Mpl1, mammalian LD associated proteins like the adipose differentiation-related protein (PLIN2/ADRP, also called perilipin-2) and tail-interacting protein of 47 kDa (PLIN3/TIP47, also called perilipin-3) are not present in the genomes of *Metarhizium*[20] and other filamentous fungi (our own blast searches). Thus, perilipin-like protein is largely responsible for the regulation of lipid storage and metabolism in filamentous fungi. In mammalian cells, perilipin (PLIN1), PLIN2 or PLIN3 protein, if not bound to LDs, will be covalently ubiquitinated and targeted for proteasomal degradation.[26] In this respect, since the transcription of *MPL1* is not affected in *Mratg8*Δ (**Fig. 6A**), the reduction of Mpl1 protein in mutant conidia could be presumably due to proteasomal degradation when there is not enough LDs for Mpl1 to bind. MrAtg8 is not localized on LDs (**Fig. S1**), implying the absence of protein-protein interaction between MrAtg8 and Mpl1. It remains to be established whether the deletion of autophagy gene affects neutral lipid accumulation or the formation phospholipid monolayer. On the other hand, we cannot preclude that autophagy is involved in LD degradation in fungi. Indeed, it has been observed in *F. graminearum* that deletions of autophagy genes block LD degradation in mycelia upon nutrient deprivation.[6,41]

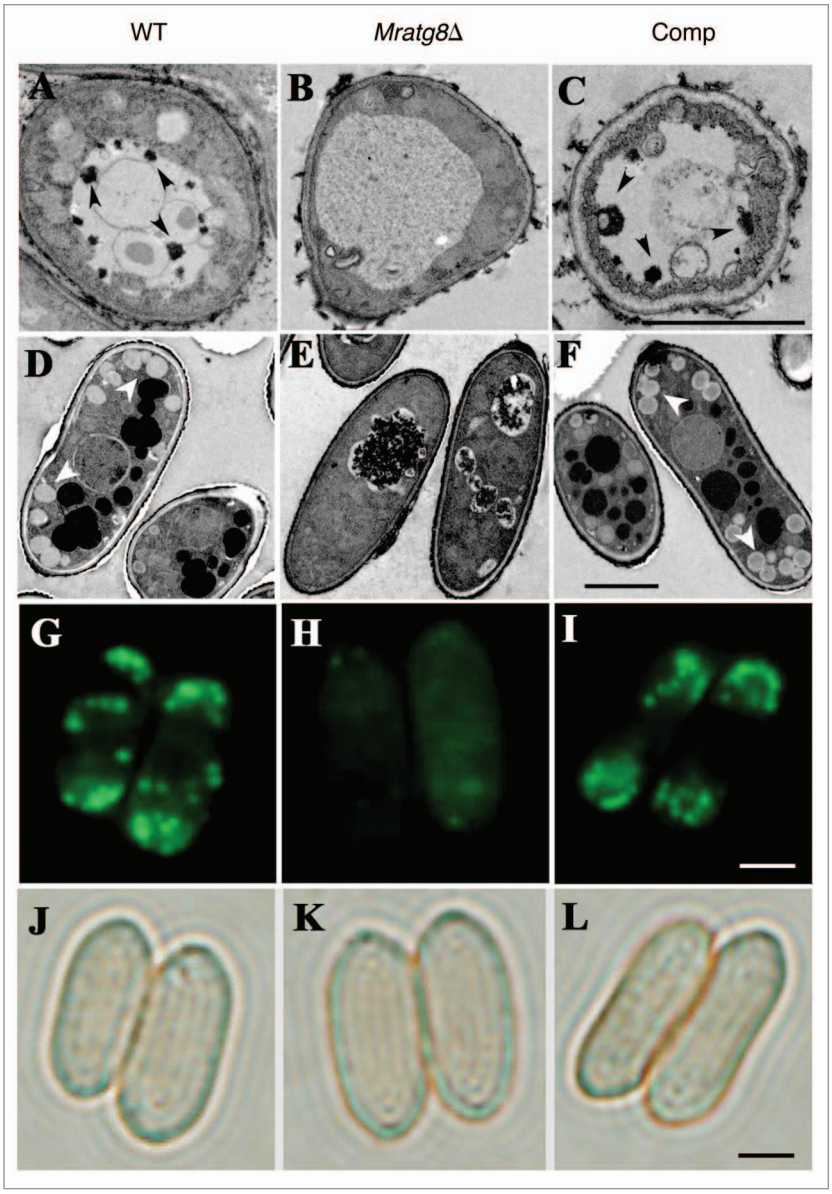

Figure 5. *MrATG8* effects on the accumulation of autophagosome and lipid droplets (LDs). After growth in an MM-N medium for 4 h, vacuolar autophagic bodies (arrows) were evident in the WT (**A**) and Comp (**C**) cells but not in the gene-deletion mutant (**B**). Conidia in the wild type (WT), *Mratg8*Δ and Comp mutant harvested after 20 d on CM plates were used for TEM analysis. In contrast to the WT (**D**) and Comp (**F**), accumulation of LDs (arrows) was significantly reduced in *Mratg8*Δ (**E**). This was confirmed by Bodipy fluorescent dye staining that shows the lack or presence of a few LDs in *Mratg8*Δ conidia (comparing **H to G and I**). (**J–L**) show the corresponding bright-field images of (**G–I**), respectively. Scale bar: 2 μm.

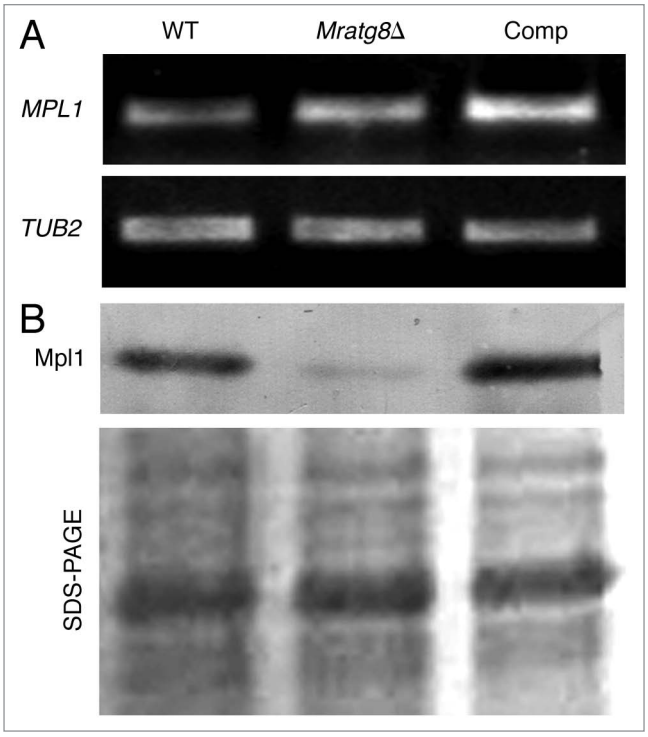

Figure 6. Transcription and protein expression of lipid droplet specific gene. RT-PCR analysis of the *Metarhizium* LD specific gene, *MPL1*. No obvious difference in gene transcription was observed among the WT, *Mratg8*Δ and Comp strains (**A**). Western blot analysis indicated that, in contrast to the WT and Comp, cellular accumulation of Mpl1 protein was significantly reduced in *Mratg8*Δ (**B**).

Collectively, our data indicated that autophagy plays an important role in the differentiation and virulence of an insect pathogenic fungus, *M. robertsii* and the fungus represents a genetically tractable new system to study the mechanisms of autophagy and how that relates to other physiological processes, including lipid metabolism.

Materials and Methods

Fungal strains and growth conditions. The wild-type strain and mutants of *M. robertsii* ARSEF 2575 (previously named as *M. anisopliae*) were routinely cultured on potato dextrose agar (PDA, Difco, 213200) at 25°C. For characterization of growth, fungi were additionally cultured on a minimum medium (MM)[27] and a complete medium (CM).[24] For liquid incubation, fungi were grown in Sabouraud dextrose broth (SDB, Difco, 238220) and MM-N (MM without the nitrogen source and agar). Yeast strains SEY6210 (WT, *MAT*α, *ura3 leu2 his3 trp1 lys2 suc2*) and the *ATG8* deletion mutant (KVY5, *apg8*Δ*-HIS3*)[22] were included in this study for gene complementation assay. These were cultured in YPD (1% yeast extract, 2% peptone and 2% glucose) and SD-N (2% glucose and 0.17% yeast nitrogen base with neither amino acids nor ammonium sulfate).

Yeast complementation. For functional complementation of yeast *Atg8*Δ gene, the full-length cDNA of *MrATG8* was amplified from a cDNA library with primers A8XbU and A8XbD (**Table S1**) and cloned into the *Xba*I site of the yeast expression vector pYES2 (Invitrogen, V825-20) under the control of the *GAL1* promoter. The resultant vector pYES-*MrATG8* was then transformed into the yeast KVY5 strain using a small-scale yeast transformation protocol.[27] The uracil prototrophic transformants were selected and verified by PCR. The complementation effect of *Metarhizium MrATG8* gene was tested in a starvation challenge

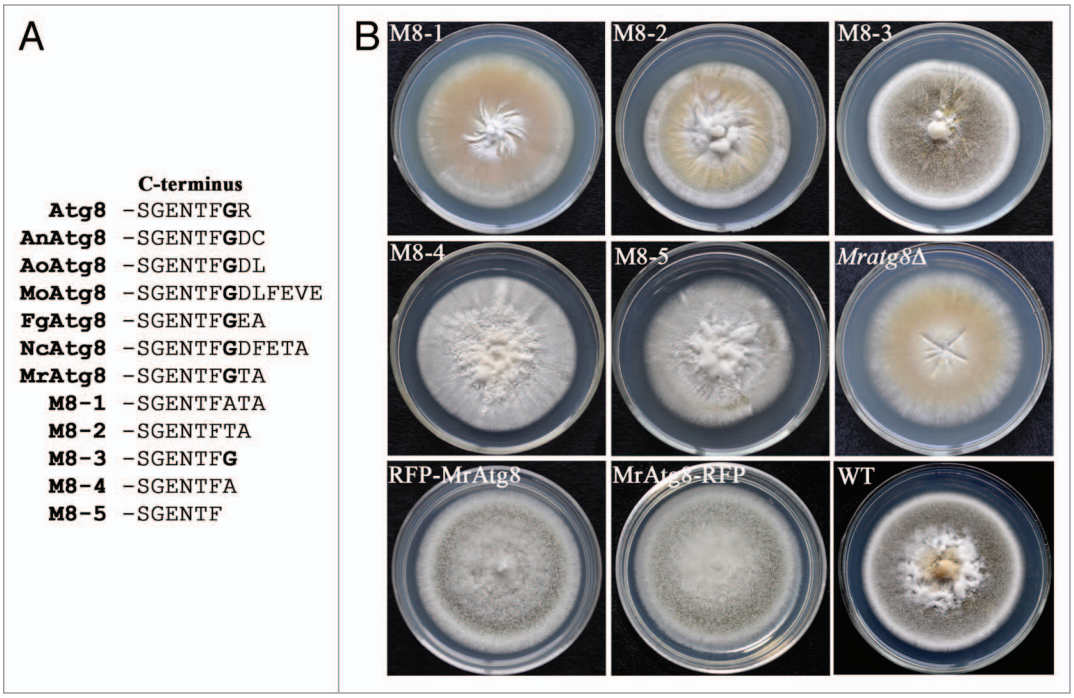

Figure 7. Mutagenesis of MrAtg8 C-terminal residues. (**A**) Alignment of MrAtg8 with the orthologs of yeast (Atg8, NP_009475), *A. nidulans* (AnAtg8, AN5131), *A. oryzae* (AoAtg8, Q2UBH5), *M. oryzae* (MoAtg8, MGG_01062), *F. graminearum* (FgAtg8, FGSG_10740) and *Neurospora crassa* (NcAtg8, NCU01545) showing the conservation of C-terminal residues and the mutation strategies of MrAtg8 to generate M8-1 to M8-5. (**B**) Phenotyping of the corresponding transformants on PDA medium. RFP-Atg8 and Atg8-RFP cultures are mutants transformed with the MrAtg8 N- and C-terminal fused RFP, respectively.

as described previously.[3] Briefly, the yeast cells (SEY6210, KVY5 and *MrATG8* transformants) were streaked on YPD plates and cultured for two days at 30°C and then moved to SD-N medium for 18 d at 30°C.

Gene deletions. Targeted gene deletion of *MrATG8* gene was performed by homologous recombination as we described before.[45] Briefly, the 5' and 3' flanking sequences of *MrATG8* gene were amplified using genomic DNA as a template with primer pairs *ATG8*UF and *ATG8*UR, *ATG8*DF and *ATG8*DR (**Table S1**), respectively. The products were digested with *Bam*HI and *Spe*I, respectively, and then inserted into the corresponding sites of the binary vector pDHt-bar (conferring resistance against ammonium glufosinate) to produce the binary vector pBar*ATG8* for *Agrobacterium*-mediated fungal transformation (ATMT). For mutant complementation, the full-length ORF plus the promoter region of *MrATG8* was amplified with the primers A8XbU and A8XbD (**Table S1**) and the purified product cloned into the

Table 1. Comparison of virulence between the wild type and mutants assayed on silkworm larvae

Strains	LT_{50} (days)	Significance*
WT	4.0 ± 0.39	-
*Mratg1*Δ	5.0 ± 0.62	$\chi2 = 12.77$; $p = 0$
*Mratg4*Δ	4.6 ± 0.34	$\chi2 = 4.86$; $p = 0.028$
*Mratg8*Δ	NC	$\chi2 = 63.12$; $p = 0$
*Mratg15*Δ	4.3 ± 0.16	$\chi2 = 8.51$; $p = 0.004$
Comp	4.5 ± 0.21	$\chi2 = 0.38$; $p = 0.54$
M8-3	4.0 ± 0.15	$\chi2 = 0.04$; $p = 0.85$

*Each mutant was compared with the wild type (WT), respectively and a Log-Rank test was conducted to examine the significance of difference between treatments. Comp, *Mratg8*Δ gene-rescued strain; M8-3, *Mratg8*Δ mutant complemented with mutagenized *MrATG8* gene by deletions of protein C-terminal T117 and A118 residues. NC, not calculated due to mortality < 50%.

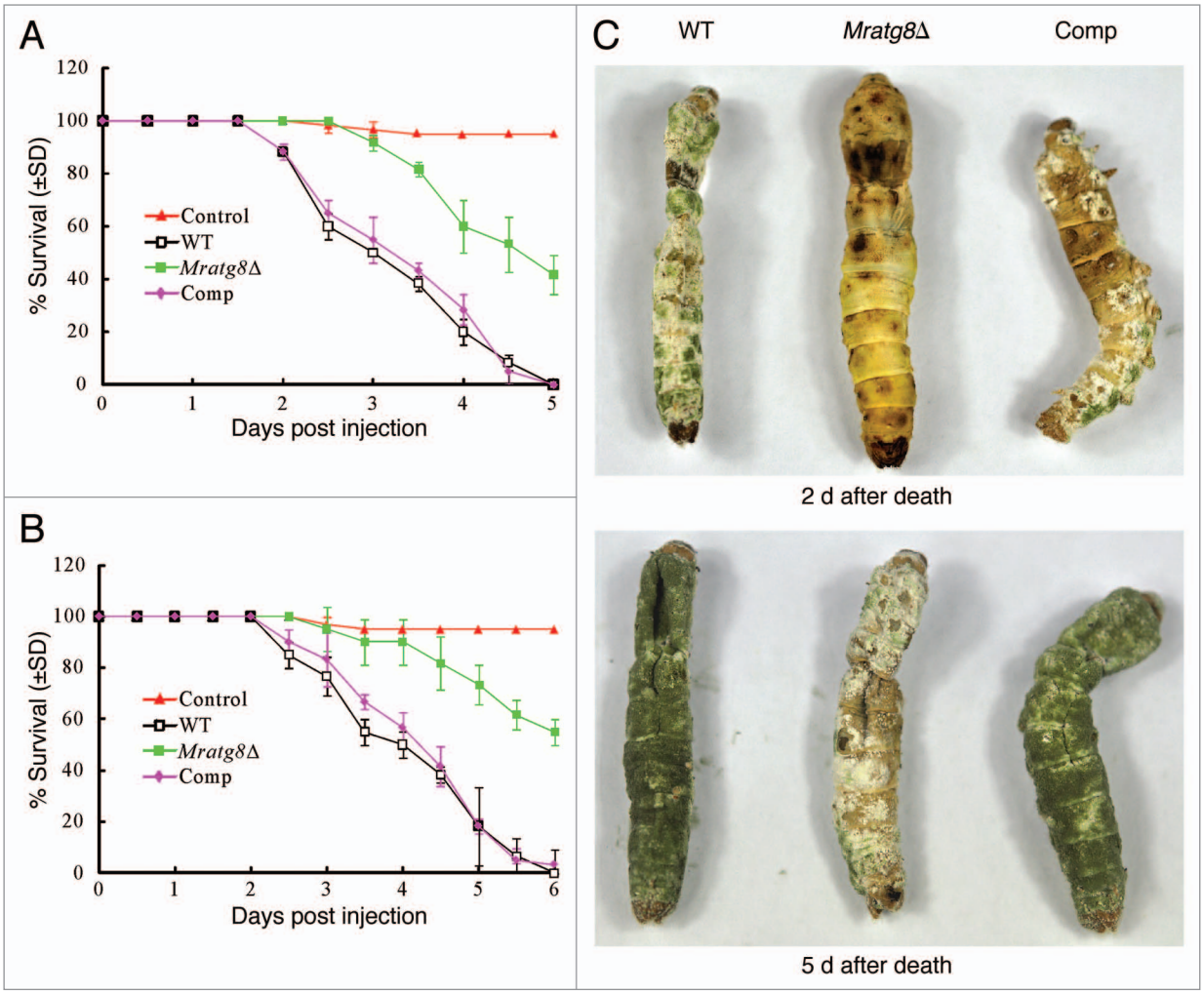

Figure 8. Insect bioassays. (**A**) Survival of silkworm larvae injected with 10 μl of 5×10^6 conidia/ml suspensions (control insects were injected with 10 μl sterilized water). Median lethal time (LT_{50}) was estimated as 3.0 ± 0.30 d for the wild type (WT), 5.0 ± 0.35 d for *Mratg8*Δ and 3.5 ± 0.27 d for Comp, respectively. (**B**) Survival of silkworm larvae following immersion in 2×10^7 conidia/ml suspensions for 30 sec (control insects were treated with sterilized water). LT_{50} values were estimated as 4.0 ± 0.39 d for the WT and 4.5 ± 0.21 d for Comp, respectively. *Mratg8*Δ caused only 45% mortality. (**C**) Development and conidiation differences among the WT, *Mratg8*Δ and Comp on insect cadavers. Insects newly killed by the fungi were kept moisturized for different times for mycosis, which showed that, in contrast to the WT and Comp, *Mratg8*Δ demonstrated defective sporulation on insect cadavers.

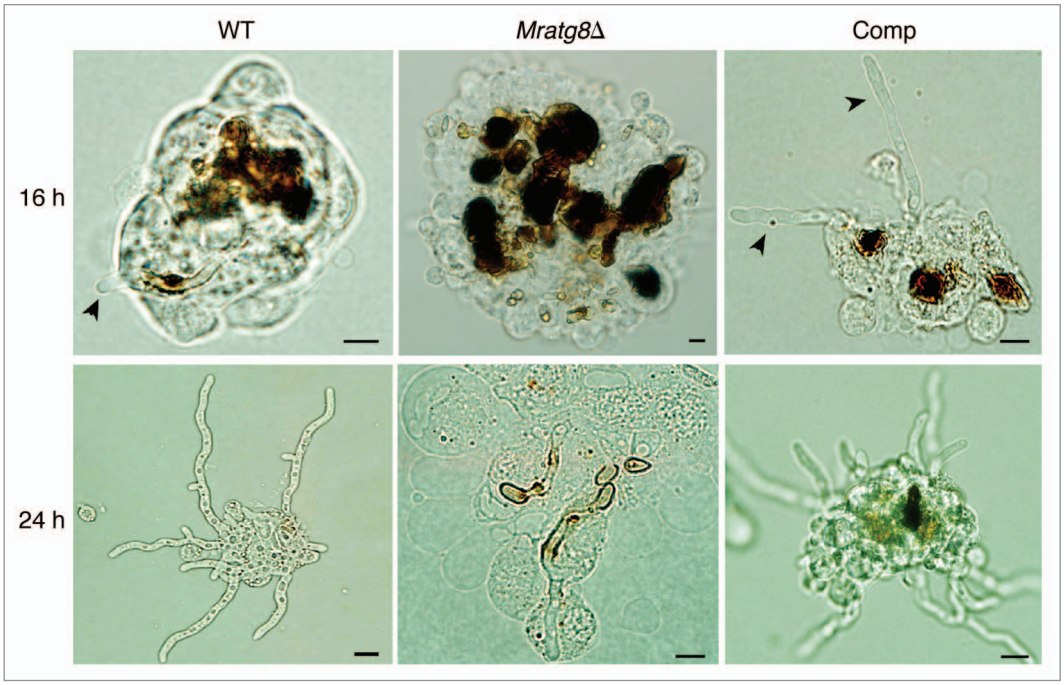

Figure 9. Fungal development in insect hemocoel. Relative to the WT and Comp, *Mratg8*Δ conidia were heavily encapsulated and melanized by insect hemocytes 16 h after injection. Arrows indicate the escaped hyphal tubes. Twenty-four hours postinjection, mycelia of the WT and Comp branched and escaped from the hemocyte nodules but not in the gene-deletion mutant. Scale bar: 10 μm.

binary vector pDHt-ben-gpdA (conferring resistance against benomyl) to produce pBen*ATG8*. To investigate the effects of other autophagy genes in *Metarhizium*, the orthologs of yeast *ATG1* (designated as *MrATG1*, MAA_03501), *ATG4* (*MrATG4*, MAA_04475) and *ATG15* (*MrATG15*, MAA_00506) were additionally deleted in *M. robertsii* for functional characterizations. Gene complementation was conducted for these mutants by amplification of individual ORF and transformation of the mutant using the benomyl selection marker gene *BEN* as described above.

RT–PCR and western blot analyses. Total RNA was extracted using a TRIzol Reagent (Invitrogen, 10296-028) from mycelia grown in MM-N medium and SDB, conidia from CM plates and appressoria differentiated on locust hind wings after 24 h of culture. RT-PCR was performed using the primers *ATG8*F and *ATG8*R as described previously.[46] The tubulin β-2 gene, *TUB2*, (MAA_02081) was amplified using primers *TUB*F and *TUB*R (**Table S1**) and served as an internal positive control. To detect the expression of the mammalian perilipin-like Mpl1,[27] which is a lipid droplet specific protein, the predicted antigenic peptide RAD SLG DKT LDR IDE RFP IVK KPT SC was synthesized, conjugated with keyhole limpet hemycyanin and used to raise polyclonal antibodies in New Zealand white rabbits. The conidia of the WT and the various mutants were harvested from the CM plates, washed three times in PBS buffer and homogenized in PBS with silica beads (1 mm in diameter, BioSpec Products Inc., 11079110z) using FastPrep (MP Biomedicals, 116004500). Equal amounts of proteins (10 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gel), transferred to polyvinylidene fluoride (PVDF) membranes and probed with the antibodies as previously described.[45]

***MrATG8* gene point-mutations.** C-terminal Gly residue is essential for Atg8-mediated autophagy.[17] To verify the importance of Gly116 and other terminal amino acid(s) in MrAtg8, five variants of MrAtg8 were created by PCR using different primers (**Table S1**). Based on the sequence of *MrATG8* gene, primer pairs A8XbU/A8XbD1, A8XbU/A8XbD2, A8XbU/A8XbD3, A8XbU/A8XbD4 and A8XbU/A8XbD5 were designed, to introduce the following mutations in *MrATG8* respectively; substitution of Gly116 to Ala116, deletion of Gly116, deletions of Thr117 and Ala118, deletions of Gly116 and Thr117, and deletions of Gly116, Thr117 and Ala118 (**Fig. 7A**). Amplicons were inserted individually into the *Xba*I site of plasmid pDHt-ben-gpdA to generate pBenAtg81, pBenAtg82, pBenAtg83, pBenAtg84 and pBenAtg85, for transformation of *Mratg8*Δ by ATMT. The resulting transformed strains were designated as M8-1, M8-2, M8-3, M8-4 and M8-5, respectively.

***MrATG8* gene fusions.** Primers RfpXbF and RfpXbR (**Table S1**) were used to amplify the ORF of the red fluorescent protein (*RFP*) gene using plasmid pMT-m*RFP1* (Fungal Genetic Stock Center) as the template. The amplified *RFP* fragment was then ligated into the *Xba*I site of the vector pDHt-ben-gpdA to create pBen*RFP* for direct transformation of the WT strain. In addition, to generate the *RFP-MrATG8* and *MrATG8-RFP* fusion constructs, fusion PCRs were performed with corresponding primers. In the first PCR, primer pairs RfpXbF-RfA81 and RfA82-A8XbD were used to amplify the *RFP* gene without stop codon from the plasmid pBen*RFP* and *MrATG8* from a cDNA library, respectively. The second PCR was performed with the primers RfpXbF and A8XbD using the PCR products from above as templates and the resulting fusion product was cloned into the *Xba*I restriction site of vector pDHt-ben-gpdA to produce p*RFP-ATG8*. The same strategy was employed to generate p*ATG8-RFP* by using the primer pairs A8XbU-A8Rf1 and A8Rf2-RfpXbR in the first PCR that amplified *MrATG8* without the stop codon and the primers A8XbU and RfpXbR in the second PCR amplification. The resulting fusion product was cloned into the *Xba*I site of pDHt-ben-gpdA to generate p*ATG8-RFP*. The orientation of each construct was verified by additional PCRs and sequencing.

All these constructs were transformed into the *Mratg8*Δ mutant strain by ATMT. To localize the distribution of MrAtg8 protein, p*RFP-ATG8* was also introduced into the wild-type strain of *M. robertsii*.

Fluorescent staining and microscopy. Fluorescence microscopy was performed on an Olympus BX51 microscope (Olympus, BX51-33P). For lipid-droplet staining, conidia harvested from CM plates were stained with the Bodipy dye (Invitrogen, D3922) as described previously.[27] Images of cells were captured with an Olympus DP71 CCD camera using the Image-Pro® Express software.

Transmission electron microscopy (TEM) analysis. For TEM observations, fungal conidia collected from 14-d-old CM plates were cultured in SDB liquid medium at 25°C and 200 rpm for 24 h. The mycelia were harvested, washed with sterilized distilled water for three times, and transferred into a MM-N liquid medium containing 4 mM phenylmethylsulfonyl fluoride.[3] After incubation at 25°C and 200 rpm for 4 h, fungal cultures were collected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C overnight. The samples were then rinsed three times with phosphate buffer and fixed overnight in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.0) at 4°C. After rinsing three times with phosphate buffer, the samples were dehydrated in a gradient ethanol series, infiltrated with a graded series of epoxy resin in epoxy propane, and then embedded in Epon 812 resin. The ultrathin sections were stained in 2% uranium acetate followed by lead citrate and visualized under a transmission electron microscope (Hitachi, H-7650) operating at 80 kV.

Appressorium induction and insect bioassays. Appressoria of the WT and mutant strains of *Metarhizium* was induced either on locust hind wings or on sterile plastic Petri dishes (6 cm in diameters, Fisher Scientific, 14-793-07) containing 3 ml of 0.0125% yeast extract.[25] The induction rates of appressorium differentiation were quantified by examining different microscopic fields of at least 300 spores for each strain. The differences between different strains were subject to a t-test analysis. To investigate the effect of *MrATG8* mutation on fungal virulence, insect bioassays were conducted against the newly emerged fifth instar silkworm, *Bombyx mori*.[45] Conidia of the WT, *Mratg8*Δ and gene complementary mutants harvested from CM plates were applied topically by immersing the larvae for 30 sec in an aqueous suspension containing 1×10^7 conidia/ml. Each treatment had three replicates with 20 insects each and the experiments were repeated twice. Mortality was recorded every 12 h. Median lethal time (LT_{50}) was calculated by Kaplan-Meier analysis with the program SPSS (Ver. 13.0). Additional insects were injected and bled at different times to observe fungal development within insect hemocoel.[32]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

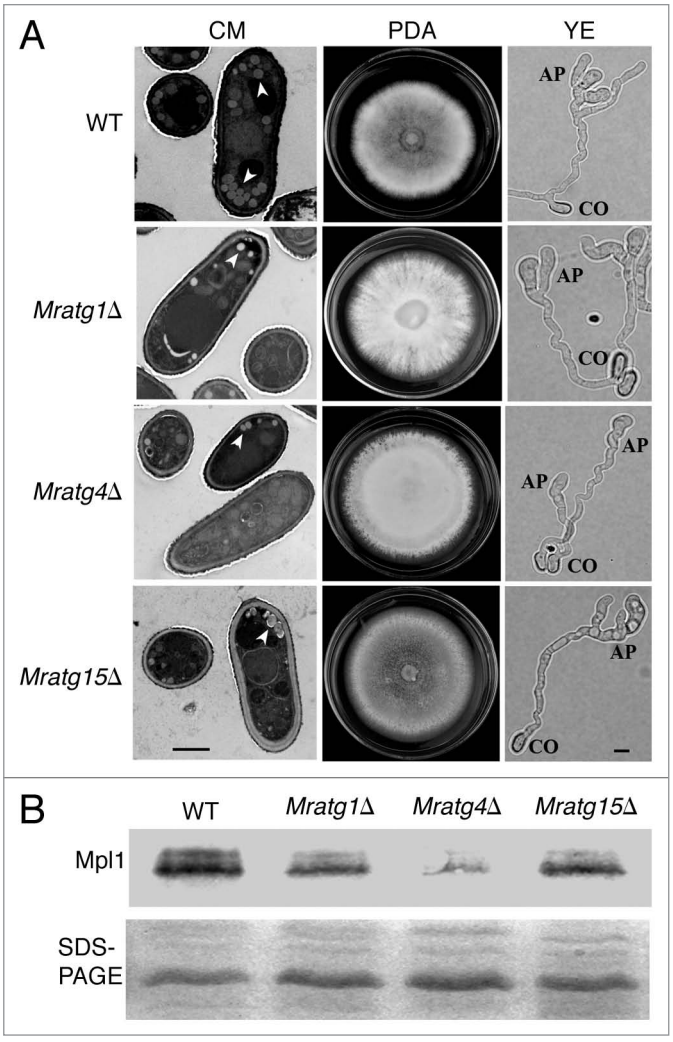

Figure 10. Phenotypic characterization of the various autophagy gene deletion mutants and western blot analysis. (**A**) Compared with the wild type (WT), accumulation of lipid droplets (arrows) was reduced in the conidia of *Mratg1*Δ, *Mratg4*Δ and *Mratg15*Δ. *Mratg1*Δ and *Mratg4*Δ lost abilities to sporulate on the potato dextrose agar (PDA). Like the WT, all three mutants formed appressoria on the hydrophobic plastic surface in yeast extract (YE) medium. CO, conidium; AP, appressoirum; scale bar: 2 μm. (**B**) Western blot analysis showing the reduced level of Mpl1 protein in the conidia of *Mratg1*Δ, *Mratg4*Δ and *Mratg15*Δ in comparison to the WT.

Acknowledgments

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/autophagy/article/23575

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