Genome-wide functional analysis reveals that autophagy is necessary for growth, sporulation, deoxynivalenol production and virulence in *Fusarium graminearum*

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Autophagy is a conserved cellular recycling and trafficking pathway in eukaryotic cells and has been reported to be important in the virulence of a number of microbial pathogens. Here, we report genome-wide identification and characterization of autophagy-related genes (ATGs) in the wheat pathogenic fungus *Fusarium graminearum*. We identified twenty-eight genes associated with the regulation and operation of autophagy in *F. graminearum*. Using targeted gene deletion, we generated a set of 28 isogenic mutants. Autophagy mutants were classified into two groups by differences in their growth patterns. Radial growth of 18 Group 1 ATG mutants was significantly reduced compared to the wild-type strain PH-1, while 10 Group 2 mutants grew normally. Loss of any of the ATG genes, except *FgATG17*, prevented the fungus from causing Fusarium head blight disease. Moreover, subsets of autophagy genes were necessary for asexual/sexual differentiation and deoxynivalenol (DON) production, respectively. *FgATG1* and *FgATG5* were investigated in detail and showed severe defects in autophagy. Taken together, we conclude that autophagy plays a critical role in growth, asexual/sexual sporulation, deoxynivalenol production and virulence in *F. graminearum*.

*Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schweinitz) Petch) is a homothallic filamentous ascomycete fungus and the causal agent of Fusarium head blight (FHB) or head scab disease of wheat, barley, rice and other small grain cereals worldwide1–3. Damage from head scab results in reduced yield, discolored, shriveled “tombstone” kernels, contamination with mycotoxins, and reduction in seed quality4. *F. graminearum* produces Trichothecene mycotoxins, such as nivalenol (NIV) and deoxynivalenol (DON) and an estrogenic mycotoxin, zearalenone (ZEN). Contamination of cereals and feeds with these mycotoxins sporadically causes food and feed-borne intoxication in man and farm animals5, 6. FHB is one of the most economically important diseases of grain cereals7 and is not controlled well by any current strategies. It is therefore important to understand the infection mechanisms of *F. graminearum* to guide development of more durable control strategies against FHB.

The term “autophagy” was first used by Christian de Duve in 1963 on the occasion of the Ciba Foundation Symposium on Lysosomes7. Autophagy is required for maintaining the homeostasis of eukaryotic cells and plays an important role in normal development and differentiation8. To date, 38 ATG genes (Autophagy-related Genes) have been identified in *Saccharomyces cerevisiae*, and the biological properties of most of the corresponding Atg proteins have now been characterized9, 10. Autophagy can be divided into three main types based on recognized mechanisms and functions—macroautophagy, microautophagy, and chaperone-mediated autophagy11, 12. Macroautophagy is generally referred to as simply autophagy, and is the most well characterized process of the three processes, used for sequestration and degradation of cytosolic components in a process that uses specialized cytosolic double membrane vesicles called autophagosomes, which ultimately fuse with the lysosome/vacuole, releasing the contents of the vesicle and subsequently breaking down, as proteolysis occurs8, 12–14. Chaperone-mediated autophagy degrades soluble proteins that contain a motif biochemically related to the

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pentapeptide KFERQ11,15. Although autophagy can be nonspecific, there are many selective forms of autophagy in S. cerevisiae, including the cytoplasm-to-vacuole (Cvt) targeting pathway, mitophagy, pexophagy, and other specific forms that target specific organelles. In filamentous fungi, autophagy has been shown to be involved in vegetative growth, asexual/sexual differentiation, environmental stresses, and virulence16–22. In the rice blast fungus Magnaporthe oryzae, a set of 22 isogenic mutants differing by a single component of the predicted autophagic machinery of the fungus showed that autophagy is necessary for rice blast disease15. The Mgtg8 mutant impaired in autophagy arrests conidial cell death and this renders M. grisea non-pathogenic23. Liu et al. have independently shown that the autophagy genes, MgATG1, MgATG4, MgATG5, MgATG8 and MgATG9 are required for pathogenesis in M. oryzae18,24–26. In the corn smut fungus Ustilago maydis, autophagy is also involved in pathogenicity27. Recently, Yanagisawa and colleagues have analyzed the function of Aoatg1 and detected the Cvt pathway in Aspergillus oryzae28. More recently, in the endophytic fungus Harpospora oryzae29, it has been shown that autophagy is required for vegetative growth, sporulation and virulence29. Selective autophagy may also be significant in pathogenesis, because it has been shown that Atg26-mediated pexophagy is necessary for appressorium-mediated plant infection in the hemi-biotrophic plant pathogenic fungus Colletotrichum orbiculare16. Similarly, He et al. revealed that Atg24-assisted mitophagy in foot cells is necessary for proper asexual differentiation and efficient conidiogenesis in M. oryzae30.

Recently, two autophagy-related genes, FgATG15 and FgATG8, have been functionally characterized in F. graminearum30–32. FgATG15 is involved in fungal growth, aerial hyphae production, conidia production and germination and important for lipid turnover and plant infection33. FgATG8 is related to linear growth rate, formation of aerial mycelium, use of storage lipid droplets, growth over an inert plastic surface, infection and formation of reproductive structures34. To further understand the biological roles of autophagy in morphogenesis and plant infection, we identified all 26 ATG genes, except the previously reported FgATG8 and FgATG15, in the genome of F. graminearum. We generated targeted deletion mutants of 28 ATG genes and demonstrated that loss of any of the ATGs, except FgATG17, prevents the fungus from causing head blight disease. Moreover, we observed that autophagy is important for vegetative growth, asexual/sexual differentiation and DON production. We conclude that autophagy plays a critical role in growth, sporulation, deoxynivalenol production and virulence in F. graminearum.

Results
Identification of ATG genes in F. graminearum. We first carried out a genome-wide search for Atg protein-encoding genes in the F. graminearum genome database using S. cerevisiae functional annotations as a guide and, in this way, we defined a set of 28 ATG genes, which are described in detail in Table S1. Non-selective macroautophagy, often referred to simply as autophagy, is a dynamic process35, but can be conceptually divided into several steps, based on studies in yeast. According to molecular analysis of a battery of autophagy-related genes36–38, the predicted genes involved in autophagy of F. graminearum could be functionally separated into those that putatively play a role in the induction of autophagy (FgATG1, FgATG13 and FgATG17), vesicle nucleation (FgATG18, FgATG20, FgATG24 and FgATG29), autophagosome expansion (FgATG3, FgATG4, FgATG5, FgATG7, FgATG8, FgATG10, FgATG12 and FgATG16), docking and fusion, and recycling (FgATG2, FgATG9, FgATG15, FgATG18 and FgATG22). By reference to Atg proteins specific for selective autophagy in yeast37,38, FgATG11, FgATG20, FgATG23, FgATG24, FgATG26, FgATG27, FgATG28, FgATG33 and FgATG37 may be required for mitophagy, pexophagy, or the Cvt pathway in F. graminearum. However, homologs of yeast ATG19, ATG21, ATG25, ATG30, ATG31, ATG32, ATG34, ATG36, ATG38, ATG39, ATG40 and ATG41 were not found in F. graminearum (Table S1).

Autophagy is required for proper vegetative growth in F. graminearum. To determine the functional putative ATG genes in F. graminearum, we generated targeted gene deletion mutants of 28 ATGs, which were confirmed by PCR and Southern blot. Confirmation of four mutants, ΔFgatg1, ΔFgatg5, ΔFgatg20 and ΔFgatg24, by Southern blot analysis is shown in Fig. S1. Based on differences in growing patterns, the 28 ATG mutants could be divided into two groups. In Group 1, colonies showed a statistically significant difference from the wild-type strain PH-1 in radial growth under nutrient-rich conditions (PDA plates). These included ΔFgatg1, ΔFgatg3, ΔFgatg4, ΔFgatg6, ΔFgatg7, ΔFgatg8, ΔFgatg9, ΔFgatg10, ΔFgatg11, ΔFgatg14, ΔFgatg15, ΔFgatg20, ΔFgatg22, ΔFgatg23, ΔFgatg24, ΔFgatg29 and ΔFgatg33 (Fig. 1A,B). For example, colony diameters of the ΔFgatg1 and ΔFgatg20 mutants were (5.83 ± 0.02) cm and (5.02 ± 0.03) cm after incubation for 3 days on PDA at 25 °C, respectively, which was significantly smaller than (6.68 ± 0.09) cm of the PH-1 strain. In Group 2 mutants, no significant difference in growth rate was observed compared to the wild-type strain. These mutants included ΔFgatg2, ΔFgatg12, ΔFgatg13, ΔFgatg16, ΔFgatg17, ΔFgatg18, ΔFgatg26, ΔFgatg27, ΔFgatg28 and ΔFgatg37 (Fig. 1C,D). On PDA plates, colonies of the wild-type strain PH-1 produced dense aerial mycelium, while colonies of most ATG mutants in the two groups (except ΔFgatg11, ΔFgatg23, ΔFgatg27, ΔFgatg28, ΔFgatg29, ΔFgatg33 and ΔFgatg37) showed significantly decreased development of aerial mycelium compared to PH-1 (Fig. 1A,C). These results indicate that autophagy is necessary for proper vegetative growth in F. graminearum.

Autophagy plays a critical role in asexual/sexual reproduction in F. graminearum. We next examined conidiogenesis of the wild-type strain and 11 ATG mutants by growing cultures in MBL liquid medium. The PH-1 strain typically produced (28.10 ± 2.64) × 10^6 macroconidia per milliliter from such cultures. Conidiation of ΔFgatg1, ΔFgatg5, ΔFgatg8, ΔFgatg9, ΔFgatg11, ΔFgatg13, ΔFgatg14, ΔFgatg15, ΔFgatg16, ΔFgatg20 and ΔFgatg24 was significantly reduced (Fig. 2A). The ΔFgatg15, ΔFgatg20 and ΔFgatg24 mutants cultured for 4 days in the 1% MBL medium produced hardly any macroconidia. The ΔFgatg11 mutant produced (15.59 ± 3.35) × 10^6 macroconidia per milliliter, a decrease of 44.52% compared to PH-1. Conidiation of ΔFgatg1,
Defects of ATG mutants in hyphal growth. (A) Colonies of the Group 1 mutants. (B) Bar chart showing colony diameters of PH-1 and the Group 1 mutants. (C) Colonies of the Group 2 mutants. (D) Bar chart showing colony diameters of PH-1 and the Group 2 mutants. The wild-type strain PH-1 and ATG mutants were grown on PDA plates. Photographs were taken after incubation on PDA plates at 25 °C for 3 days. Linear bars in each column denote standard errors of three experiments. An asterisk indicates significant difference of colony diameter ($P < 0.05$).

ΔFgatg5, ΔFgatg8, ΔFgatg9, ΔFgatg13, ΔFgatg14 and ΔFgatg16 was only 1.25%, 1.10%, 0.68%, 7.01%, 3.17%, 0.85% and 0.53% that of the wild-type strain, respectively.

Sexual reproduction plays a critical role in FHB epidemic and disease cycle, thus we determined the sexual development of the wild-type strain and two ATG mutants on self-mating carrot agar cultures. The wild-type strain PH-1 produced abundant perithecia after two-week self-fertilization. By contrast, ΔFgatg1 and ΔFgatg5 mutants completely failed to form any perithecia under the same culture conditions (Fig. 2B). These results suggest that autophagy is important for asexual/sexual sporulation in $F. graminearum$. 

Figure 1. Defects of ATG mutants in hyphal growth. (A) Colonies of the Group 1 mutants. (B) Bar chart showing colony diameters of PH-1 and the Group 1 mutants. (C) Colonies of the Group 2 mutants. (D) Bar chart showing colony diameters of PH-1 and the Group 2 mutants. The wild-type strain PH-1 and ATG mutants were grown on PDA plates. Photographs were taken after incubation on PDA plates at 25 °C for 3 days. Linear bars in each column denote standard errors of three experiments. An asterisk indicates significant difference of colony diameter ($P < 0.05$).
Deoxynivalenol (DON) is known to be an important virulence determinant in *F. graminearum*. Therefore DON production was measured in wheat kernels infected by PH-1 and several ATG mutants. The wild-type strain PH-1 produced (1102.04 ± 198.54) milligrams DON per kilogram inoculated wheat kernels. The levels of DON production in the all tested mutants, including ∆Fgatg2, ∆Fgatg3, ∆Fgatg4, ∆Fgatg7, ∆Fgatg8, ∆Fgatg12, ∆Fgatg13, ∆Fgatg15, ∆Fgatg16, ∆Fgatg20, ∆Fgatg22, ∆Fgatg24 and ∆Fgatg26, were significantly reduced (P < 0.05) (Fig. 4). Among them, the ∆Fgatg13, ∆Fgatg22, ∆Fgatg24 and ∆Fgatg26 mutants produced only (2.12 ± 0.56), (4.90 ± 1.30), (1.89 ± 0.33) and (4.38 ± 2.47) milligrams DON per kilogram inoculated wheat kernel powder, respectively, which was significantly less than that in PH-1 infections (Fig. 4). These results suggest that these genes are involved in positive regulation of DON biosynthesis in *F. graminearum*. 

**Autophagy is required for full virulence in *F. graminearum***. Virulence assays were performed by point inoculation of flowering wheat heads with mycelial plugs from the wild-type PH-1 and ATG mutants. At 14 days post-inoculation (dpi), PH-1 caused typical scab symptom on inoculated and nearby spikelets. The rate of infected spikelets inoculated with most ATG mutants was significantly reduced compared to PH-1 (P < 0.01), while FgATG17 in Group 2 was fully virulent (Fig. 3). Atg17, as a component of the Atg1 complex, is important for induction of autophagy by starvation in *S. cerevisiae*. Therefore, we determined the role of FgATG17 in autophagy and DON production in *F. graminearum*. We found that GFP-FgAtg8 proteolysis was not blocked in the ∆Fgatg17 mutant after induction of autophagy in MM-N liquid medium with 2 mM PMSF for 4 h (Fig. S4A). To determine whether DON production in the ∆Fgatg17 mutant was impaired, an enzyme-linked immunosorbent assay (ELISA) was performed. The results showed that DON production between PH-1 and the ∆Fgatg17 mutant had no significant difference (Fig. S4B). These data suggested that FgATG17 is not essential for autophagy involved in FgAtg8 and DON production in *F. graminearum*. Furthermore, we observed that ∆Fgatg1, ∆Fgatg3, ∆Fgatg6, ∆Fgatg7, ∆Fgatg14, ∆Fgatg15, ∆Fgatg20, ∆Fgatg24 in Group 1 and ∆Fgatg2, ∆Fgatg12, ∆Fgatg13, ∆Fgatg16 in Group 2 only caused mild infection in point-inoculated spikelets but did not spread to nearby spikelets (Fig. 3A,C). Although scab symptoms developed in nearby spikelets inoculated with the other ATG mutants, the percentage of diseased spikelets was dramatically decreased by contrast to PH-1 (Fig. 3B,D). In addition, the phenotypic defects of ∆Fgatg1, ∆Fgatg7, ∆Fgatg13 and ∆Fgatg20, such as growth, conidiation and virulence, could be fully complemented by re-introduction of the corresponding ATG genes, respectively. For instance, in Fig. S3, the defects in vegetative growth of ∆Fgatg1, ∆Fgatg7, ∆Fgatg13 and ∆Fgatg20 could be complemented by re-introduction of FgATG1, FgATG7, FgATG13 and FgATG20 respectively (Fig. S3). These results indicate that autophagy is required for full virulence to wheat by *F. graminearum*.
Introduction of \( FgATG1 \) and \( FgATG5 \) into \( M. \) oryzae \( \Delta Moatg1 \) and \( \Delta Moatg5 \) complements their phenotypic defects respectively. It has been reported that \( MoATG1 \) and \( MoATG5 \) are necessary for conidiation, normal development and pathogenicity in the rice blast fungus \( M. \) oryzae\(^{18,25} \). To determine whether \( FgATG1 \) and \( FgATG5 \) can functionally complement defects in \( \Delta Moatg1 \) and \( \Delta Moatg5 \) mutants, the full length coding sequence of \( FgATG1 \) (under the control of the \( MoATG1 \) native promoter) and \( FgATG5 \) (under the control of the \( MoATG5 \) native promoter) were transformed into \( \Delta Moatg1 \) and \( \Delta Moatg5 \) mutants, respectively. The complementation transformants, \( \Delta Moatg1/FgATG1 \) and \( \Delta Moatg5/FgATG5 \), were identified. Phenotypic analysis showed that \( \Delta Moatg1 \) and \( \Delta Moatg5 \) mutants cultured on CM plates for 12 days formed sparse aerial mycelium, but \( \Delta Moatg1/FgATG1 \) and \( \Delta Moatg5/FgATG5 \) strains produced dense aerial hyphae, which were identical to the wild-type strain Guy11 (data not shown). Compared to \( (3.42 \pm 0.41) \times 10^7 \) conidia per plate produced by the wild-type strain Guy11, conidiogenesis in \( \Delta Moatg1 \) and \( \Delta Moatg5 \) mutants was significantly reduced \( (P < 0.05) \).
only produced \((7.73 \pm 1.57) \times 10^4\) and \((5.35 \pm 1.14) \times 10^5\) conidia respectively. The \(\Delta Moatg1/FgATG1\) strain produced \((2.62 \pm 0.38) \times 10^7\) conidia per plate approximately 76.6% of that of the wild-type strain (Fig. 5A).

Under the same culture conditions, the \(\Delta Moatg5/FgATG5\) strain produced \((2.24 \pm 0.16) \times 10^7\) conidia per plate, respectively. The \(\Delta Moatg1/FgATG1\) strain produced \((2.62 \pm 0.38) \times 10^7\) conidia per plate approximately 76.6% of that of the wild-type strain (Fig. 5A).

Figure 4. The ATG mutants were involved in DON production in *F. graminearum*. Levels of DON in PH-1 and 13 ATG mutants were detected in infected wheat kernels at 25 days post-inoculation (dpi). All the tested mutants produced significantly less DON than PH-1. Linear bars in each column represent standard errors of four repeats. Different capital letters indicate a significant different DON level \((P < 0.01)\).

Figure 5. *FgATG1* and *FgATG5* complement the phenotypic defects of *M. oryzae \(\Delta Moatg1\) and \(\Delta Moatg5\) mutants respectively. (A) Bar chart showing the conidial production of the strains. Error bars in each column represent standard errors of three independent experiments. Different letters in each column indicate the significant difference of conidiation \((P < 0.05)\). (B) Infection assays on the barley leaves. Leaves of 10-day-old barley seedlings were inoculated with mycelial plugs (0.5 cm) and examined at 7 days post inoculation (dpi). The complemented strains (\(\Delta Moatg1/FgATG1\) and \(\Delta Moatg5/FgATG5\)) were fully pathogenic to barley leaves.
In this study, we identified 28 putative autophagy-related genes in *F. graminearum* ATG protein-encoding genes as a guide. Using targeted gene deletions, the predicted physiological functions of *S. cerevisiae* ∆Fgatg1 and ∆Fgatg5 mutants were functionally characterized at a global scale. We found that autophagy is required for vegetative growth, asexual/sexual sporulation, DON production and pathogenicity in *F. graminearum*. In addition, we investigated the biological function of *FgATG1* and *FgATG5* and found that the autophagic process was blocked in ∆Fgatg1 and ∆Fgatg5 mutants. Taken together, we conclude that autophagy plays a critical role in many important physiological functions of *F. graminearum*.

**Discussion.**

In this study, we identified 28 putative autophagy-related genes in *F. graminearum* using the known genome-wide set of *S. cerevisiae* Atg protein-encoding genes as a guide. Using targeted gene deletions, the predicted *F. graminearum* ATG genes were functionally characterized at a global scale. We found that autophagy is required for vegetative growth, asexual/sexual sporulation, DON production and pathogenicity in *F. graminearum*. In addition, we investigated the biological function of *FgATG1* and *FgATG5* and found that the autophagic process was blocked in ∆Fgatg1 and ∆Fgatg5 mutants. Taken together, we conclude that autophagy plays a critical role in many important physiological functions of *F. graminearum*.

Autophagy is a conserved process from budding yeasts to mammalian cells, and a major cellular pathway for degradation of long-lived proteins and cytoplasmic organelles, important for maintaining homeostasis of cells. Autophagy is triggered by starvation stress, and leads to rearrangement of subcellular membranes to

![Image](https://example.com/image.png)
sequester cargo for delivery to the lysosome or vacuole, where sequestered material is then degraded and recycled\textsuperscript{45, 48}. In addition to its homeostatic functions, autophagy is necessary for many aspects of development in multicellular organisms\textsuperscript{49}. Previously, it has been observed that ATG mutants are impaired in mycelial growth and pathogenicity in several fungal species\textsuperscript{17, 18, 50, 51}. In *F. graminearum*, deletion of *FgATG8* results in impairment of mycelial growth, usage of storage lipid droplets, formation of asexual/sexual spores and infection\textsuperscript{20}. Similarly, *FgATG15* is involved in aerial hyphal growth, conidiogenesis, lipid droplet degradation and virulence\textsuperscript{31}. In this study, the mutants of the 28 autophagy-related genes in *F. graminearum* were classified into two groups according to the rate of vegetative growth on PDA medium. Deletion of any of the genes in Group 1 resulted in a statistically significant difference in linear growth on the PDA plates compared to the wild-type strain PH-1 (Fig. 1A,B). However, the mutants of the ATGs in Group 2 had similar growth rates compared to an isogenic wild-type strain (Fig. 1C,D). This suggests that genes associated with Group 1 play wider roles in cellular viability and hyphal growth than those in Group 2. However, since multiple gene deletion mutants of Group 2 have not been generated and analyzed in the study, it is unknown whether genes of group 2 are redundant or functionally overlap. Therefore, we reasoned that further clarification of gene function in Group 2 proteins, for example in cellular viability and hyphal growth than those in Group 2. However, since multiple gene deletion mutants of Group 2 have not been generated and analyzed in the study, it is unknown whether genes of group 2 are redundant or functionally overlap. 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Figure 7. *FgATG1* and *FgATG5* were involved in autophagy in *F. graminearum*. (A–C) GFP-FgAtg8 localization in the PH-1, ΔFgatg1 and ΔFgatg5 mutants. PH-1 (A), ΔFgatg1 (B) and ΔFgatg5 (C) expressing GFP-FgAtg8 were grown in liquid CM medium at 25 °C for 24 h, and then shifted to liquid MM-N medium with 2 mM PMSF for 4 h. The vacuoles of hyphal cells of different strains were stained by CMAC (7-amino-4-chloromethylcoumarin) and examined by fluorescence microscopy. Scale bars = 5 μm. (D) GFP-FgAtg8 proteolysis assays of PH-1, ΔFgatg1 and ΔFgatg5. Mycelia were harvested from liquid CM cultures after incubation in a 180 rpm-shaker at 25 °C for 24 h. Autophagy was induced after nitrogen starvation for 4 h in MM-N liquid medium with 2 mM PMSF. Mycelia were collected at the indicated times and total proteins were extracted for the analysis of Western blotting by anti-GFP. Anti-GAPDH was shown as a control. Full-length blots were presented in Supplementary Figure S2.

conidial production of 11 tested ATG mutants was analyzed statistically and showed that these mutants produced significantly less or even no conidia (Fig. 2A). Also, we found that perithecium development of ΔFgatg1 and ΔFgatg5 was completely blocked (Fig. 2B). Sporulation is associated closely with the energy metabolism of organisms\textsuperscript{52}. Since autophagy provides nutrients by recycling cytoplasmic materials, a deficiency in this process probably reduces the production of conidia in consequence of the lack of energy and nutrients. Based on our
pathogenicity assays, we found that most ATG mutants, but not FgATG17, exhibited a decrease in infection of wheat spikelets (Fig. 3). This affected not only symptom expression but also the ability of the fungus to spread to new spikelets. This suggests that the ability to colonize plant tissue is impaired, as well as the ability to produce spores to infect new hosts. Given these defects, it seems likely that autophagy is absolutely necessary for success of F. graminearum in the field and that the fungus would be unable to survive and cause disease without the operation of autophagy. Consistent with this loss of ability to colonize wheat tissue, we found that DON production of 13 tested ATG mutants was significantly reduced in comparison with PH-1 (Fig. 4), indicating that autophagy is necessary for fueling secondary metabolism in F. graminearum. However, the analysis of dynamic DON production for each of the ATG mutants was not carried out in the study. We cannot preclude at this stage that the lower levels of DON are associated with a delay in biosynthesis, rather than a reduction in the ability to synthesize the secondary metabolite. To date, at least 15 TRI genes in F. graminearum encoding trichothecene biosynthetic enzymes and regulators have been identified. We found that the expression levels of TRI5 (trichodiene synthase), TRI6 (transcription regulator) and TRI10 (transcription regulator) genes in mycelium cultured for 24 h were significantly decreased in ΔFgatg20 and ΔFgatg24 mutants (data not shown). The data suggest that reduction of DON biosynthesis of ATG mutants is associated with the low expression of the TRI genes in these mutants. Since deoxynivalenol (DON) was an important virulence determinant in F. graminearum, and DON is necessary to suppress plant defense enabling the pathogen to break through the rachis node, the reduction of DON production in these mutants may be a cause of their loss of virulence or may be associated with the relative lack of infection ability of the mutants.

In M. oryzae autophagy genes can be classified into those predicted to be required for nonselective autophagy and those necessary for pexophagy, mitophagy, or the Cvt pathway. Loss of any of the 16 genes necessary for nonselective macroautophagy leads to M. oryzae being unable to cause rice blast disease, but the 6 genes necessary only for selective autophagy are dispensable for appressorium-mediated plant infection. In the present study, deletion of any of the genes required presumptively for pexophagy, mitophagy, or the Cvt pathway (FgATG11, FgATG20, FgATG23, FgATG24, FgATG26, FgATG27, FgATG28, FgATG33 and FgATG37) and genes necessary for the nonselective macroautophagy (FgATG1, FgATG2, FgATG3, FgATG4, FgATG5, FgATG6, FgATG7, FgATG8, FgATG9, FgATG10, FgATG12, FgATG13, FgATG14, FgATG15, FgATG16, FgATG18, FgATG22 and FgATG29) in F. graminearum leads to the significant reduction in virulence (Fig. 2). This suggests that both processes may be necessary for infection by F. graminearum. The functional difference of the ATGs in the two groups in plant infection between M. oryzae and F. graminearum may be due to DON production of these mutants, which is significantly reduced and which plays such a critical role in plant infection. Interestingly, Δfagatg17 mutants were fully pathogenic, suggesting that this has a non-essential function in autophagy in F. graminearum. In S. cerevisiae, Δatg17 mutants were almost completely defective in autophagy and produced few small autophagosomes that were less than half the normal size upon starvation. However, we found that FgATG17 is not essential for GFP-FgAtg8 proteolysis and DON production in F. graminearum (Fig. S4).

In S. cerevisiae, Atg1 as a serine/threonine kinase involved in regulation of autophagy by protein phosphorylation. Atg1 forms complexes with Atg13 and Atg17, which is required for the induction of autophagy. Atg5 with Atg12 and Atg16 forms the Atg12-Atg5-Atg16 conjugation complex system and plays an essential role in the formation of autophagosomes. In filamentous fungi, such as Podospora anserina, Aspergillus fumigatus and M. oryzae, homologs of ATG1 and ATG5 genes have been identified and characterized. We found that re-introduction of FgATG1 and FgATG5 into M. oryzae Moatg1 and Moatg5 mutants functionally complemented the phenotypes of the mutants, respectively, suggesting that Atg1 and Atg5 proteins in filamentous fungi probably play a conserved role in regulation of conidiogenesis and pathogenicity. We found that autophagic bodies in the ΔFgatg1 and ΔFgatg5 mutants were absent or obviously decreased under starvation conditions (Fig. 6). Moreover, disruption of FgATG1 and FgATG5 prevented movement of GFP-FgAtg8 to the vacuolar lumen when autophagy was induced (Fig. 7B, C). Consistent with this, impairment of autophagy in the ΔFgatg1 and ΔFgatg5 mutants was confirmed by GFP-FgAtg8 proteolysis assays (Fig. 7D). These results suggest that the autophagic pathway was blocked in ΔFgatg1 and ΔFgatg5 mutants. However, the detailed mechanism of how FgATG1 and FgATG5 are involved in autophagy in F. graminearum requires further study. In M. oryzae, deletion of MgATG1 gene influences the number of lipid bodies, and lipid storage in conidia in a ΔMgatg5 mutant is reduced. In Aspergillus oryzae, AoAtg1 is involved in the Cvt pathway. Hence, further studies will be necessary to reveal the relationship between autophagy and the lipid metabolism in F. graminearum.

In summary, we conclude that autophagy-related genes (except FgATG17) are involved in regulating vegetative growth, aerial mycelium formation, asexual/sexual sporulation, DON production and virulence in F. graminearum.

Materials and Methods

**Fungal strains and culture conditions.** The wild-type F. graminearum strain PH-1 and all derivative mutants in this study were cultured on PDA (potato dextrose agar, 200 g potato, 20 g dextrose, 20 g agar per 1 L water) plates at 25 °C to assess mycelial growth and colony characteristics. Conidiation assays of all strains were performed after growing 4 days in 1% mung bean liquid (MBL) medium (10 g mung beans boiled in 1 L water for 20 min). Cultures in PDB (PDA without agar) were used for genomic DNA isolation. Complete medium (CM) and minimal medium without the nitrogen source (MM-N) were used for autophagy assays.

**Generation of ATG deletion mutants.** The DNA cassettes used for the gene deletions were constructed as described previously. All PCR primers used in this study were listed in Table S2. The putative gene deletion mutants were identified and confirmed by PCR amplification and Southern blotting assays. Southern blot analysis was performed by the digoxigenin (DIG) high prime DNA labeling and detection starter Kit I (Roche, Mannheim, Germany).
Phenotypic analysis. For the vegetative growth of each colony, 5-mm plugs cut from the edge of a 3-day-old colony of each strain were placed on PDA plates and incubated at 25°C. After 3 days, the diameter of each strain was measured and recorded. For the conidiation assay, five 5-mm plugs of each strain from the edge of 3-day-old colony were inoculated in 20 mL 1% MBL. After 4 days cultivation in a 180 rpm-shaker, conidia of PH-1 were harvested by filtering through cheesecloth and directly counted using a haemocytometer. The conidial number of mutants was harvested using the same method and then centrifuged at 8000 rpm for 10 min. The harvested conidia of mutants were re-suspended in 1 mL sterile distilled water and subsequently counted with the haemocytometer. Each experiment with three replicates was independently repeated three times. For self-crossing assays, perithecial formation was assayed on carrot agar (CA) medium as previously described64.

Pathogenicity assays. Since the F. graminearum ATG mutants produced few conidia, mycelial plugs taken from the PDA plates were applied for pathogenicity assays. Agar plugs with mycelium from 3-day-old PDA plates of PH-1 or mutants were scraped off with dentiscalprum, and the middle spikelet of flowering wheat heads of the susceptible cultivar Jimaⅲ33 was inoculated. Symptomatic spikelets and quantification of infected spikelets among the whole wheat heads were determined after incubation for 14 days.

DON production assays. After immersion in water for 12 h, 50 g wet wheat kernels were sterilized for 3 times and inoculated with ten mycelial plugs (5 mm in diameter) of each strain from the edge of 3-day-old colony and incubation at 25°C for 25 days. The inoculated wheat kernels were dried at 37°C for 24 h, and then broken into powder with juicer. The powder was delivered to the company named Pribolab biological engineering co., LTD in Qingdao which performed the DON production assays.

Western blot analysis. A piece of agar blocks with mycelia of each tested strain was introduced into 20 mL of liquid CM medium. The suspension was shaken at 25°C, 180 rpm for 24 h, and then transferred into the MM-N medium in the presence of 2 mM PMSF (phenylmethylsulfonyl fluoride) for 4 h, 8 h and 12 h. Hyphae of each sample were harvested, washed with sterile distilled water, ground into powder in liquid nitrogen, and then suspended in the protein lysis buffer (50 mM Tris-Cl (pH = 7.4), 0.15 M NaCl, 1 mM EDTA, 1% Triton × 100) added 1% 100 × Protease Inhibitor Cocktail for Fungal/ Yeast Cell (Sangon, Shanghai, China). The lysate was centrifuged at 13200 rpm for 20 min at 4°C after lysis for 30 min. Afterwards, 50 μL supernatant was mixed with isovolumetric 2× protein-loading buffer and boiled for 5 min and then cooled on the ice immediately. 10–15 μL of each sample was taken for loading on 10% SDS-PAGE gels. GFP-Tag (7G9) Mouse mAb (Abmart, Shanghai, China) as primary antibody was used at a 1:5000 dilution. HiSec™ HRP-conjugated Goat Anti-Mouse IgG (H + L) (Vazyme, Nanjing, China) was applied to immunoblot analysis at a 1:10000–1:50000 dilution. FD™ FDBio-Femto Ecl chemiluminescent substrate (Fdbio science, Hangzhou, China) was used for antigen antibody detections.

Complementation analysis of Moatg1 and Moatg5 by introducing FgATG1 and FgATG5 respectively. The pCB1532-FgATG1 and pCB1532-FgATG5 vectors for the complementation of Moatg1 and Moatg5 mutants respectively were constructed as described previously63.

Confocal microscopy and transmission electron microscopy assays. Hyphae expressing the GFP-Atg8 fusion protein were cultured in CM medium at 25°C, 180 rpm for 24 h, and then transferred into the MM-N medium in the presence of 2 mM PMSF for 4 h at 25°C in a 180 rpm shaker. CMAC chemiluminescent substrate (Fdbio science, Hangzhou, China) was used for antigen antibody detections. The PDBi (7G9) Mouse mAb (Abmart, Shanghai, China) as primary antibody was used at a 1:5000 dilution. HiSec™ HRP-conjugated Goat Anti-Mouse IgG (H + L) (Vazyme, Nanjing, China) was applied to immunoblot analysis at a 1:10000–1:50000 dilution. FD™ FDBio-Femto Ecl chemiluminescent substrate (Fdbio science, Hangzhou, China) was used for antigen antibody detections.

Data Availability. All data generated or analysed during this study are including in this published article (and its Supplementary Information files).

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**Author Contributions**

W.L. and Z.W. conceived and designed the experiments, W.L., C.W. and N.Y., conducted the experiments, W.L., Y.Q. and Z.W. analysed the data. W.L., Z.Y. and N.T. wrote the paper. All authors reviewed the manuscript.

**Additional Information**

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