Independent losses and duplications of autophagy-related genes in fungal tree of life

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SIGNIFICANCE

Autophagy, an intracellular degradation process critical for cellular homeostasis, is important for the growth, development, and pathogenesis in fungi. Although autophagic process is generally considered to be conserved, our comparative genomics analysis showed only 20 of the 41 autophagy-related (*ATG*) genes are highly conserved, including most but not all the yeast core-autophagy-machinery genes. Our results showed that independent losses and duplications of *ATG* genes have occurred throughout the fungal kingdom and variations in autophagy exist among different lineages and possibly different developmental stages.

SUMMARY

Autophagy is important for growth, development, and pathogenesis in fungi. Although autophagic process is generally considered to be conserved, the conservation and evolution of *ATG* genes at kingdom-wide remains to be conducted. Here we systematically identified 41 known *ATG* genes in 331 species and analyzed their distribution across the fungal kingdom. In general, only 20 *ATG* genes are highly conserved, including most but not all the yeast core-autophagy-machinery genes. Four functional protein groups involved in autophagosome formation had conserved and non-conserved components, suggesting plasticity in autophagosome formation in fungi. All or majority of the key *ATG* genes were lost in several fungal groups with unique lifestyles and niches, such as Microsporidia, *Pneumocystis*, and *Malassezia*. Moreover, majority of *ATG* genes had A-to-I RNA editing during sexual reproduction in two ascomycetes and deletion of *FgATG11*, the *ATG* gene with the most editing sites in *Fusarium* affected ascospore releasing. Duplication and divergence also was observed to several core *ATG* genes, such as highly divergent *ATG8* paralogs in dermatophytes and multiple *ATG15* duplications in mushrooms. Taken together, independent losses and duplications of *ATG* genes have occurred throughout the fungal kingdom and variations in autophagy exist among different lineages and possibly different developmental stages.

INTRORDUCTION

Autophagy is an intracellular degradation process that is critical for cellular homeostasis (Farre and Subramani, 2016). It is a nutrient recycling mechanism for dealing with the superfluous and damaged organelles (Feng et al., 2014). The autophagy process consists of three sequential steps: cargo sequestration, degradation, and utilization of degradation products (Mizushima, 2007). For cargo sequestration, the pre-autophagosomal structure (PAS) is activated to generate the autophagosome with double membranes. PAS is very close to the vacuolar membrane (Yoshimori and Noda, 2008). In the degradation step, autophagosomes fuse with lysosomes or vacuoles and form the degrading structure known as autophagolysosomes for cargo degradation (Eskelinen and Saftig, 2009). In the last step, the nutrient monomeric units deriving from the degradation of macromolecules are exported to the cytosol for recycling (Mizushima, 2007). According to the cargoes and degradation manner, autophagy is divided into three primary types: macroautophagy, microautophagy, and chaperone-mediated autophagy (Mizushima et al., 2008). The macroautophagy is primarily a non-selective process and commonly referred to as autophagy.

The autophagic machinery consists of the autophagy-related (*ATG*) genes (Feng et al., 2014). As a model for autophagy studies, many groundbreaking and fundamental knowledge of the autophagic processes have been characterized in the budding yeast *Saccharomyces cerevisiae*, including the identification of the *ATG* genes such as *ATG1* and *ATG8* (Feng et al., 2014). *ATG1* is the first *ATG* gene that was shown to be essential for the accumulation of autophagic bodies and autophagy in yeast (Tsukada and Ohsumi, 1993). It encodes a protein kinase that is involved in the recruitment and release of other autophagy-related proteins from the PAS (Yoshimori and Noda, 2008). *ATG8* encodes an ubiquitin-like protein that is essential

for autophagosome formation and selective cargo recruitment. The GFP-Atg8 fusion is commonly used as a marker to follow autophagy (Xie et al., 2008; Shpilka et al., 2011).

Autophagy has a variety of functions in eukaryotes. In mammalian cells, autophagy is known to be involved in embryonic development, cell differentiation, immunity, and inflammation (Mizushima and Levine, 2010; Levine et al., 2011; Kanayama and Shinohara, 2016). In plants, autophagy is important for seedling establishment, plant development, senescence, stress resistance, metabolism, and reproduction (Liu et al., 2005; Bassham et al., 2006; Avila-Ospina et al., 2014; Michaeli et al., 2016). In fungi, autophagy affects growth, morphology, development, lipid turnover, and sexual reproduction (Kershaw and Talbot, 2009; Pollack et al., 2009; Nguyen et al., 2011; Josefsen et al., 2012; Liu et al., 2012; Duan et al., 2013; Voigt and Poggeler, 2013; Lv et al., 2017). In fungal pathogens, autophagy is also known to be important for plant or animal infection. For example, deletion any of the 16 *ATG* genes required for nonselective autophagy in the rice blast fungus *Magnaporthe oryzae* affected its virulence (Kershaw and Talbot, 2009). Mutants of many *ATG* genes were defective in toxin production and plant infection (Nguyen et al., 2011; Wang et al., 2011; Josefsen et al., 2012; Lv et al., 2017) in another ascomycete *Fusarium graminearum*, the main causal agent of Fusarium head blight (FHB). In human pathogen *Cryptococcus neoformans*, knockdown of *ATG8* results in attenuated virulence in a mouse model of infection (Hu et al., 2008). In the corn smut fungus *Ustilago maydis*, *ATG1* and *ATG8* are required for the full virulence and complete symptom development (Nadal and Gold, 2010).

Although many *ATG* genes are conserved from yeast to humans (Meijer et al., 2007), several of them such as *ATG25* are yeast specific (Monastyrska et al., 2005). Furthermore, during the study of kinome in *F. graminearum* (Wang et al., 2011), we found that the Atg1

kinase, an essential regulator of autophagy, is not present in some of the fungal genomes. Considering the fact that major knowledge of autophagy is based on yeast studies and the importance of autophagy in fungal development and pathogenesis, it is important to investigate the conservation and distribution of *ATG* genes across the fungal kingdom to better understand the evolution, and regulation of autophagy in fungi. In this study, we systematically identified and analyzed 41 known *ATG* genes in 331 fungal species. The distribution of each *ATG* gene was analyzed across the fungal kingdom. Massive and specific losses of *ATG* genes were observed in fungi belonging to different phyla. Duplication of several *ATG* genes, including *ATG8*, *ATG15*, *ATG18*, *ATG20*, and *ATG22*, also were observed in different fungal groups. In Arthrodermataceae, two Atg18 homologs are likely derived from an ancient duplication event because of their phylogenetic relationship. We also found that majority of the *ATG* genes had multiple A-to-I RNA editing sites during sexual reproduction in two filamentous ascomycetes. Deletion of *ATG11*, the *ATG* gene with the most RNA editing sites in *Fusarium graminearum*, had no effect on growth, infection, conidiation, and ascus development but resulted in defects in ascospore releasing. Taken together, our results showed that independent losses and duplication of *ATG* genes have occurred in fungal tree of life, and some unrelated fungi with unique growth niches and reduced genome sizes lack any or key *ATG* genes and autophagy. Furthermore, post-transcriptional modifications by RNA editing may affect stage-specific functions of *ATG* genes and autophagy during sexual reproduction in fungi.

RESULTS

The distribution of *ATG* genes varies significantly among different fungal classes: To comprehensively analyze the *ATG* genes in kingdom Fungi, we search for homologs of 36 *S.*

cerevisiae ATG genes (www.yeastgenome.org) that are categorized into core machinery, selective autophagy specific, and nonselective autophagy specific group (Lynch-Day and Klionsky, 2010), and homologs of *ATG25, ATG28, ATG30, ATG35,* and *ATG37* of *Hansenula polymorpha* or *Pichia pastoris* (Monastyrska et al., 2005; Meijer et al., 2007) (Table S1). Their orthologs in the genomes of 331 sequenced fungal species, including 227 Ascomycota, 72 Basidiomycota, and 32 lower fungi (Fig. 1A; Table S2) were identified.

Based on their conservation (Fig. 1B) and distribution (Fig. 1C) across the kingdom Fungi, these *ATG* genes were categorized into three groups. The first group contains 20 highly conserved *ATG* genes, including *ATG1-9, ATG11-13, ATG15, ATG18, ATG20-22, ATG24,* and *ATG26-27* (Fig. 1B; 1C). Their orthologs are present in over 80% of the fungal species that have been sequenced. Whereas most of them (14 out of 20) are core machinery *ATG* genes, six of them are selective autophagy-specific genes. The second group includes seven *ATG* genes, *ATG10, ATG16, ATG17, ATG28, ATG29, ATG33,* and *ATG37* that are present in Ascomycota but not in Basidiomycota or basal fungal groups (Fig. 1B; 1C). Group III consists of 14 *ATG* genes that are present only in Saccharomycetes species (Fig. 1B; 1C), including *ATG14, ATG38,* and *ATG41* that are core autophagy genes in *S. cerevisiae*.

Interestingly, examination of the 46 sequenced ascomycetous yeast species revealed that some of the *ATG* genes are not well conserved in Saccharomycetes. Among the highly conserved core machinery *ATG* genes, *ATG22* that encodes a vacuolar membrane protein is lost in the Debaryomycetaceae family (Fig. S1). The seven group II *ATG* genes, including two yeast core machinery *ATG* genes, *ATG10* and *ATG16,* were absent in 17 to 32 Saccharomycetales species (Fig. S1). Taken together, although the core machinery *ATG* genes are generally

conserved, different fungal classes differ in the conservation of selective or non-selective autophagy-specific genes.

Plasticity in the components of protein complexes involved in autophagosome formation: The formation of autophagosomes involves four complexes of Atg proteins and two ubiquitin-like (Ubl) conjugation systems (Suzuki et al., 2016). Except the Atg2-Atg8 complex, all the other functional groups consist of components that are not well conserved in fungi (Fig. 2). For the Atg1 complex, components of the Atg1-Atg13 regulatory subunit are highly conserved across the fungal kingdom. However, components of the Atg17-Atg29-Atg31 scaffolding sub-complex are only conserved in ascomycetes. For the autophagy-specific phosphatidylinositol (PI) 3-kinase (PI3K) complex, Atg14 and Atg38, are specific for Saccharomycetes (Fig. 2). Similarly, Atg41 of the Atg9 vesicle complex is present only in Saccharomycetes. For the two Ubl conjugation systems required for the formation of autophagosomes (Ohsumi, 2001; Geng and Klionsky, 2008), the E2-like enzyme Atg3 is highly conserved across the fungal kingdom in the Atg8-conjugating system. However, the E2-like enzyme Atg10 of the Atg12-conjugating system is only present in some of the Ascomycota species (Fig. 2; Table S2). Therefore, despite their conserved roles in autophagosome formation, these Atg protein complexes or systems have significant variations in compositions. Overall, among the 20 *ATG* genes involved in the autophagosome formation in *S. cerevisiae* (Mizushima, 2007; Yao et al., 2015; Suzuki et al., 2016), 12 of them are highly conserved in fungi. The other eight are either only present in Ascomycota or specific for Saccharomycetes (Fig. 2), suggesting that autophagosome formation and its regulation vary among different fungal groups.

The *ATG* genes are completely or almost completely lost in Microsporidia: Microsporidia is a phylum of spore-forming unicellular fungi that infect human and other animals (Keeling,

2009). All the Microsporidia species are obligate, intracellular pathogens. Among the 20 Microsporidia species analyzed, eleven of them have only the *ATG15* gene (Fig. 3). Six species, including *Nosema apis* and *Edhazardia aedis*, lack any *ATG* gene (Fig. 3). Interestingly, *Mitosporidium daphnia* that still possesses mitochondria (Haag et al., 2014) has six *ATG* genes (*ATG1*/*2*/*7*/*8*/*9*/*15*), which is the most among the Microsporidia species (Fig. 3). Intracellular obligate pathogens depending on host cells for growth and reproduction often have a reduced genome size (Casadevall, 2008; Corradi, 2015; Ma et al., 2016). It is likely that Microsporidia may no longer need autophagy and have lost most or all the *ATG* genes. Furthermore, differences among Microsporidia species in the number of *ATG* genes suggest that independent gene loss events likely occurred to some of them during evolution.

Most of the core machinery *ATG* genes are lost in *Pneumocystis* species: Like Microsporidia, *Pneumocystis* species are obligate, intracellular pathogens. As a genus in Taphrinomycotina, *Pneumocystis* consists of species that infect lungs of human and other mammals (Ma et al., 2016). Thirty-three out of the 41 *ATG* genes were absent in all three *Pneumocystis* species analyzed, including 12 of the 20 highly conserved group I fungal *ATG* genes (Fig. 3). Only three *ATG* genes, *ATG15*, *ATG20*, and *ATG22*, are commonly present in *P. jirovecii*, *P. murina*, and *P. carinii*. However, these three *Pneumocystis* species differ in the presence of *ATG6*, *ATG18*, *ATG21*, *ATG24*, and *ATG27* (Fig. 3). These results indicate that whereas most of the autophagy-related genes might be lost in their common ancestor, *Pneumocystis* also had species-specific gene loss events for some of the *ATG* genes.

Massive losses of *ATG* genes also occurred in *Malassezia* species: To our surprise, massive losses of *ATG* genes also were observed in all the three *Malassezia* species that have been sequenced (Fig. 3). As a genus in the Malasssezіomycetes of Ustilagomycotina, *Malassezia*

consists of free-living basidiomycete yeasts that are associated with animal skin disorders (Ashbee and Evans, 2002). *M. sympodialis*, an atopic eczema-associated pathogen, has only 7 *ATG* genes (Fig. 3). The other two *Malassezia* species have lost fewer *ATG* genes than *M. sympodialis*. *M. globosa*, a dandruff-causing fungus, and *M. pachydermatis*, a fungus frequently found on the skin and in the mucosa and ear canals of dogs, have 12 and 13 *ATG* genes, respectively (Fig. 3). It is likely that these *Malassezia* species are defective in autophagy due to the massive losses of many highly conserved *ATG* genes.

***ATG1* and 8 other highly conserved *ATG* genes are absent in a nematode trapping fungus:** *ATG1* encodes a protein kinase that is essential for the initiation of autophageosomes and formation of cytoplasm-to-vacuole targeting vesicles (Cvt, a selective autophagy). Interestingly, *Drechslerella stenobrocha* (Orbiliomycetes) appears to lack a distinct *ATG1* ortholog (Table S2). Bidirectional blastp and tblastn searches also failed to identify *ATG1* gene in its genome. *D. stenobrocha* also lacks 8 other highly conserved *ATG* genes, including *ATG4*, *ATG5*, *ATG9*, *ATG11*, *ATG15*, *ATG18*, *ATG20*, and *ATG21*. Although it still has 16 *ATG* genes, the loss of *ATG1* and 8 other highly conserved *ATG* genes likely eliminated autophagy in this fungus. *D. stenobrocha* is a nematode trapping fungus that forms constricting rings (Liu et al., 2014). It favors environments rich in organic compounds and its genome is 11 Mb smaller than that of *Arthrobotrys oligospora*, a close-related nematode trapping fungus (Liu et al., 2014). Loss of *ATG* genes in *D. stenobrocha* may be related to its adaption to the organic compound-rich environments and reduction in the genome size.

Many of the *ATG* genes are duplicated in Mucoromycotina fungi: Mucoromycotina contains several species that have been used for the production of fermented soy food in Asia and Africa (Dolatabadi et al., 2016). Some Mucoromycotina species are emerging life-threatening human

pathogens (Roden et al., 2005). Seven highly conserved group I fungal *ATG* genes (*ATG8*, *ATG13*, *ATG15*, *ATG18*, *ATG20*, *ATG21*, and *ATG24*) were duplicated in Mucoromycotina species (Fig. 3), which may be related to the ancient genome duplication events in Mucoromycotina (Schwartze et al., 2014). Interestingly, some of these duplicated *ATG* genes have more than two copies. For example, *ATG24* have three copies in almost all the Mucoromycotina species analyzed, indicating that duplications of *ATG24* likely occurred twice before the divergence of these species. Therefore, both genome-wide and gene-specific duplications events may have contributed to the expansion of these *ATG* genes in Mucoromycotina.

Ancient duplication and divergence of *ATG8* in dermatophytes: Atg8 is a ubiquitin-like protein that is essential for the generation of double-membrane autophagosomes (Shpilka et al., 2011). The *ATG8* orthologs are present in all the fungal genomes analyzed but Microsporodia and *Pneumocystis* species. Notably, several dermatophyte fungi in Arthrodermataceae, including *Trichophyton rubrum,* one of the causal agent of athlete's foot, have two *ATG8* genes (Fig. 4A). Sequence alignment and phylogenetic analysis showed that Atg8a is highly similar to other fungal Atg8 proteins. In contrast, Atg8b has a high sequence divergence with typical fungal Atg8 proteins and appears to be unique to these dermatophytes (Fig. 4A; 4B). Although their amino acid sequences are not highly similar, both Atg8a and Atg8b have an ubiquitin-like protein folding (Fig. 4C). Furthermore, both *ATG8a* and *ATG8b* were expressed in *T. rubrum* based on the published RNA-seq data (Xu et al., 2015) although the later had a much lower expression level (Fig. 4D). It is possible that both *ATG8* alleles encode ubiquitin-like proteins and have different or overlapping functions in Arthrodermataceae species.

***ATG20* is duplicated in the fission and budding yeasts but not in filamentous fungi:** In *S. cerevisiae*, *ATG20* (*SNX42*) and *SNX41* are two members of the sorting nexin family. Homologs of these two genes are also present in most of the Saccharomycetes species except the members of the Dipodascaceae family, such as *Geotrichum candidum* and *Yarrowia lipolytica* (Fig. S1; Table S2). Phylogenetic analysis revealed that *ATG20* and *SNX41* are two paralogs derived from a duplication occurred in the common ancestor of Saccharomycetaceae after its divergence from Dipodascaceae (Fig. 5A).

Most filamentous ascomycetes and basidiomycetes had only one *ATG20*. However, the Schizosaccharomycetes species also have two *ATG20* homologs (Fig. S1; Table S2). Phylogenetic analysis showed that the two *ATG20* paralogs in *S. cerevisiae* and *S. pombe* were derived from independent gene duplication events (Fig. 5A). The two paralogs in Schizosaccharomycetes species are likely derived from a duplication event occurred in their common ancestor after its divergence from other Taphrinomycota lineages. In *S. cerevisiae*, whereas Atg20 is required for both pexophagy and pexophagy-independent endosomal retrieval trafficking, Snx41 is only involved in the latter (Hettema et al., 2003; Deng et al., 2012). The two *ATG20* paralogs also differ in their functions in *S. pombe* (Zhao et al., 2016). *MoATG20*, the only *ATG20* ortholog in *M. oryzae*, was shown to play roles in both protein sorting (Snx41 function) and pexophagy (Atg20 function) (Deng et al., 2013). The dual functions of Atg20 proteins are likely conserved in other filamentous fungi with a single *ATG20* homolog.

***ATG18* is also duplicated in the ascomycetous yeasts but not in filamentous fungi:** Atg18 and Atg21 are two paralogous proteins with WD-40 repeats in *S. cerevisiae* (Krick et al., 2008; Nair et al., 2010). Whereas Atg18 is involved in both autophagy and Cvt pathways, Atg21 is only required for the latter (Barth et al., 2001; Stromhaug et al., 2004). Paralogous Atg18 and

Atg21 also are present in most Saccharomycetes species except *Y. lipolytica* (Fig. S1; Table S2). Phylogenetic analysis showed that *ATG18* and *ATG21* also were from a duplication in the last common ancestor of Saccharomycetaceae (Fig. 5B). Most filamentous fungi have a single *ATG18* ortholog. Interestingly, *S. pombe* and other Schizosaccharomycetes have three genes that are homologous to *ATG18* or *ATG21* (Table S2). Phylogenetic analysis showed that these three *ATG18*/*21* homologs likely are derived from two separate duplication events occurred in the common ancestor of Schizosaccharomycetes (Fig. 5C). The duplications of these *ATG* genes in unicellular yeasts but not in multicellular filamentous fungi may be related to their differences in life styles or vegetative growth and development.

Lineage-specific gene duplications of two *ATG* genes required for autophagic body breakdown: Two *ATG* genes, *ATG15* and *ATG22*, are involved in the breakdown and release of autophagic bodies (Lynch-Day and Klionsky, 2010). Our analysis showed that some Chytridiomycota species have one but others have two *ATG15*. Whereas majority of Ascomycetes have only one, most Agaricomycetes have three *ATG15* homologs, which are likely derived from two separate, sequential gene duplication events occurred in the common ancestor of Agaricomycetes fungi (Fig. 6A). In addition to these three ancient paralogs, some Agaricomycetes species also have recent duplications of *ATG15* (Fig. 6A). For example, *Pisolithus microcarpus* (Pm), an ectomycorrhizal fungus, has five *ATG15* genes, including three ancient alleles and two alleles derived from recent gene duplication events.

In contrast to *ATG15*, most Agaricomycotina and Pucciniomycotina fungi have only one but many Ascomycetes have two *ATG22* genes. It appears that *ATG22* was duplicated in the ancestor of filamentous ascomycetes, resulting in two ancestral paralogs, *ATG22a* and *ATG22b* (Fig. 6B). Whereas *ATG22a* is well-conserved, *ATG22b* was lost in most branches of

Pezizomycotina, including all the Sordariomycetes species (Fig. 6B). Interestingly, two copies of *ATG22* are present in *Puccinia* rust fungi (Fig. 6B) but none of the smut fungi in Ustilaginomycotina has *ATG22* (Table S2). These data suggest that filamentous or higher fungi may have different approaches in the releasing of recycled nutrients by lineage-specific duplication of *ATG15* or *ATG22*.

Most *ATG* genes are subjected to RNA editing during sexual reproduction in *Fusarium graminearum* and *Neurospora crassa*: Autophagy is known to be important for sexual reproduction in fungi (Wang et al., 2011; Voigt and Poggeler, 2013; Lv et al., 2017). Recently, it has been reported that A-to-I RNA editing occurs specifically during sexual reproduction in *F. graminearum*, *N. crassa*, and other filamentous ascomycetes (Liu et al., 2016; Liu et al., 2017). The genes involved in autophagy were significantly enriched among the genes with nonsynonymous editing events in these two fungi, suggesting that A-to-I RNA editing likely plays a stage-specific role in autophagy during sexual reproduction. When the published strand-specific RNA-seq data were analyzed, transcripts of most *ATG* genes had A-to-I RNA editing events in both *F. graminearum* and *N. crassa* (Fig. 7A). In *F. graminearum*, among the 24 *ATG* genes with A-to-I RNA editing, 22 of them had nonsynonymous editing resulting in amino acid changes. In *N. crassa*, all the 23 *ATG* genes with A-to-I RNA editing had nonsynonymous editing events (Fig. 7A). Many of these *ATG* genes had multiple editing events that specifically occurred during sexual reproduction, such as 27 editing sites in *FgATG11* and 31 editing sites in *NcATG2*. Interestingly, three *ATG* genes in *F. graminearum* (*FgATG7, FgATG15*, and *FgATG28*) and six *ATG* genes in *N. crassa* (including 5 selective autophagy genes) had stop-codon-loss editing events, which will cause read through and possibly produce longer proteins than normal ones specifically during sexual reproduction (Fig. 7B).

In comparison between *F. graminearum* and *N. crassa*, a total of 10 editing events identified in 9 *ATG* genes were found to be conserved, including a stop-loss editing site in *ATG28* (Fig. 7). Two of these conserved editing sites in *ATG26* and *ATG13* resulted in the recoding of serine to glycine, which may affect the phosphorylation or structure of these Atg proteins. Interestingly, a conserved editing event resulting in the isoleucine to valine (I32V) recoding was identified in *ATG8*, leading a similar consequence to the difference between Atg8a and Atg8b in dermatophytic fungi. Taken together, these data suggest that RNA editing may affect autophagy during sexual reproduction in both *F. graminearum*, *N. crassa*, and other filamentous ascomycetes.

Mutations affecting the I32V and I41V editing events in *FgATG8* had no obvious effects on its function during sexual reproduction: To determine the importance of two editing events at A94 and A121 (resulting in the I32V and I41V nonsynonymous changes), we first generated the non-editable alleles of *FgATG8* (FGSG_10740) by introducing the T93 to G and T120 to C mutations. Because of the preference of U at the -1 position for A-to-I editing (Liu et al., 2016), the T93G and T120C mutations likely will eliminate the editing of A94 and A121 in *FgATG8* transcripts. The resulting *FgATG8*^{G93} and *FgATG8*^{C120} alleles were transformed into the *Fgatg8* mutant generated by the split-marker approach (Catlett et al., 2002; Josefsen et al., 2012). When assayed for sexual reproduction, whereas the *Fgatg8* mutant was sterile, both the *Fgatg8*/*FgATG8*^{G93} and *Fgatg8*/*FgATG8*^{C120} transformants were similar to the wild type in perithecium formation and ascospore development (Fig. S2). We then introduced the A94 to G and A121 to G mutations to *FgATG8* to generate the edited *FgATG8*^{G94} and *FgATG8*^{G121} alleles and transformed them into the *Fgatg8* mutant. The resulting *Fgatg8*/*FgATG8*^{G94} and *Fgatg8*/*FgATG8*^{G121} transformants also were normal in sexual reproduction (Fig. S2). These

results indicated that mutations affecting the two A-to-I editing sites in *FgATG8* individually had no obvious effects on its functions during sexual reproduction. Because the editing levels at A94 (10.3%) and A121 (13.0%) in *FgATG8* were relatively low, it is not surprising that mutations affecting these two editing sites had no obvious effects under laboratory conditions. However, nonsynonymous editing events with low editing levels tend to confer to protein heterozygosity advantage in fungi (Liu et al., 2017). Therefore, it remains possible that these two editing events are advantageous for FgAtg8 functions during sexual reproduction under field conditions.

Deletion of the *FgATG11* results in sexual-specific defects in *F. graminearum*: Transcripts of *FgATG11* (FGSG_15734) had 27 A-to-I RNA editing sites, the most among all the edited *ATG* genes in *F. graminearum*. Two of these editing sites are conserved in *NcATG11* of *N. crassa* (Fig. 7). These data suggested that editing of this selective autophagy specific gene may be important for its function during sexual reproduction. To test this hypothesis, we generated the mutants deleted of *FgATG11* by the split marker approach (Catlett et al., 2002). Four *Fgatg11* deletion mutants were identified after screening over a dozen hygromycin-resistant transformants. All of them had the same phenotypes described below although only data for one *Fgatg11* mutant were presented here. The *Fgatg11* mutants had no obvious defects in growth rate, plant infection, DON production, conidiation, and conidia germination (Fig. 8A-F). Interestingly, although they were also normal in the formation of perithecia, asci, ascospores, and ascospore germination (Fig. 8G-I), the *Fgatg11* mutants were defective in ascospore releasing (Fig. 8J). Whereas massive ascospores were forcibly discharged from mature perithecia in the wild type, only a small portion of the ascospores were ejected from *Fgatg11* perithecia (Fig. 8J). These results showed that *FgATG11* and selective autophagy play a stage-specific role during sexual reproduction in *F. graminearum*. Based on published RNA-seq data, *FgATG11* is

constitutively expressed. Bioinformatics analysis also showed that *FgATG11* lacked stage-specific alternative splicing or transcription start/stop sites during sexual reproduction. Therefore, it is likely that stage-specific RNA editing may be related to or responsible for its functions during ascosporogenesis.

DISCUSSIONS

Autophagy is a dynamic and highly inducible degradation system that responds to environmental and physiological changes (Mizushima and Levine, 2010; Farre and Subramani, 2016) and involves various autophagy-related genes (Feng et al., 2014). Investigation of the conservation and evolution of *ATG* genes in fungi is important for better understanding of the formation, evolution, and regulation of autophagy. In this study, we systematically identified and analyzed the *ATG* genes in fungi with sequenced genomes. Our large-scale analysis revealed that only 20 *ATG* genes are highly conserved across the fungal kingdom, which is approximately half of those *ATG* genes characterized in the budding yeast, a model for studying autophagy. Interestingly, 14 *ATG* genes based on yeast homologs are unique to Saccharomycetes species and the other 7 are only present in Ascomyctoa. We also showed that important functional groups involved in the autophagosome formation contains conserved and non-conserved Atg components. In fact, many of the fungal species lacks the non-conserved components still possess autophagy process. These non-conserved Atg proteins may be dispensable or replaceable in particular species, such as the absence of *ATG17* in the human pathogen *Cryptococcus neoformans*, which requires autophagy during infection (Hu et al., 2008). Mammalian cells lack Atg17 ortholog but have a functional counterpart (Hara and Mizushima, 2009).

The *ATG* genes were completely or almost completely lost in Microsporidia and *Pneumocystis* that belong to different fungal lineages (Fig. 9). The massive losses of *ATG* genes are likely associated with their nutrient acquisition styles. As obligate intracellular pathogens, Microsporidia and *Pneumocystis* directly acquire nutrients from the host cell. The autophagy recycling system may be no longer required for these fungi to grow and reproduce within their hosts. These fungi have relative small genomes and lack many genes involved in primary metabolism and cellular signaling (Corradi, 2015; Ma et al., 2016). Most likely, the *ATG* genes were lost independently in these fungi as parts of their genome reduction during the evolution. Differences among *Pneumocystis* species in the presence of a few specific *ATG* genes indicate that besides massive gene loss events occurred in their common ancestor, some of the *ATG* genes may be lost after their divergence. Similarly, some Microsporidia species differed from the majority of them in the distribution of *ATG* genes. Therefore, independent gene loss events likely occurred not only among different fungal lineages but also in a species-specific manner for at least some of these *ATG* genes in these obligate pathogens.

It is notable that massive losses of *ATG* genes are not a general feature of obligate or intracellular pathogens. The rust (basidiomycetes) and powdery mildew (ascomycetes) fungi are obligate plant pathogens that still have the core *ATG* genes but they are not intracellular pathogens. Some intracellular chytrid pathogens of animals such as the frog pathogen *Batrachochytrium dermatitidis* still have the core *ATG* genes but they are not obligate pathogens. Some chytrids, such as *Synchytridium endobioticum*, are obligate, intracellular plant pathogens but none of them has been sequenced. However, *Rozella allomycis* beloning to Cryptomycota is an obligate intracellular pathogen of other chytrids that still has most of the core *ATG* genes. The massive losses of *ATG* genes is also not a common feature related to infection of human or

animal cells, because facultative pathogens such as *C. neoformans*, *Histoplasma capsulatum*, *Sporothrix schenckii*, and *Blastomyces dermatitidis* still have most of the *ATG* genes (Casadevall, 2008). In comparison with other *ATG* genes subjected to massive losses, the retaining of *ATG15* in *Pneumocystis* and some Microsporidia species is also an interesting phenomenon. It is possible that the Atg15 lipase may have functions in other biological processes that are not directly related to autophagy. Interestingly, duplication of *ATG15* was observed in most Agaricomycetes species that tend to live in environments with limited lipid sources.

The third group of fungi with massive losses of *ATG* genes consists of three *Malassezia* species. Being smaller than 9-Mb, their genomes are among the smallest genomes in the free-living basidiomycetes and filamentous ascomycetes (Xu et al., 2007; Coelho et al., 2013). Hence, the loss of *ATG* genes in *Malassezia* may be also associated with its reduction in genome size. However, unlike Microsporidia or *Pneumocystis*, *Malassezia* species are not obligate fungal pathogens although they have slow growth rate and lack sexual reproduction. *Malassezia* fungi can use lipids as the sole carbon source but fail to ferment sugars, and have reduced numbers of glycosyl hydrolase and fatty acid synthase genes (Coelho et al., 2013). With adaptation to animal skin environments with continuous lipid supplies, autophagy may be no longer essential for their growth and survivals in this niche. Strikingly, the nematode trapping fungus *D. stenobrocha* lacks *ATG1* and 8 other *ATG* genes, likely being defective in autophagy. However, autophagy is essential for nematode trapping in another Orbiliomycetes species, *A. oligospora* (Chen et al., 2013), suggesting that losses of *ATG* genes likely occurred recently in *D. stenobrocha*. Nevertheless, unlike *D. stenobrocha*, *A. oligospora* forms extensive 3-D adhesive networks to trap nematodes instead of constricting rings. In general, nematode-trapping fungi

that form adhesive nets are more abundant in relatively poor soil and constricting ring-forming fungi favor environments rich in organic matters. Loss of *ATG* genes in *D. stenobrocha* may be related to its adaption to the organic compound-rich environments and its genome is also 11-Mb smaller than that of *A. oligospora* (Liu et al., 2014). Taken together, all the massive loss of *ATG* genes observed in specific fungal lineages or groups are likely associated with their reduction in the genome size and adaptation to specific modes of nutrient acquirements.

Unlike massive losses of *ATG* genes that are observed in specific fungal groups as described above, our analysis showed that losses of specific or individual *ATG* genes have occurred independently throughout the fungal kingdom. Some of these species- or lineage-specific *ATG* gene loss events appeared to occur relatively recently because of their differences in distribution among closely related species. Even in Saccharomycetes, seven group II *ATG* genes were lost in 17 to 32 species. In higher fungi (filamentous ascomycetes and basidiomycetes), species- or lineage-specific (independent) losses were observed for several core and selected autophagy genes, including *ATG10*, *ATG16*, *ATG22*, and *ATG33*. Some of these *ATG* gene loss events may be also related to their adaption to life or infection cycles, such as the loss of *ATG22* in Ustilagomycotina species. Many smut fungi have the free-living yeast form but obligate biotrophic hyphal form.

Besides gene losses, fungi may also gain specific *ATG* genes during evolution (Fig. 9). The basal fungal groups, such as Chytridiomycota and Mucoromycotina, have most of the group I highly conserved *ATG* genes. However, they lack all the *ATG* genes that are specific for Saccharomycetes and likely are evolved after their divergence from the other ascomycetes. *ATG22*, a highly conserved core machinery *ATG* gene, is present in most of the Ascomycota and Basidiomycota species but absent in the basal fungal groups. Chytridiomycota and

Mucoromycotina also lack *ATG10* and *ATG16*, two other core machinery *ATG* genes that are only conserved in Ascomycota. However, unlike *ATG22*, *ATG10* and *ATG16* are absent in Basidiomycetes. Lineage-specific gain of *ATG* genes also was observed for some selective autophagy specific genes, such as *ATG19* and *ATG34* in *Saccharomyces*. It is possible that the different conservation of these *ATG* gene are due to different *ATG* gene gained/evolved in the different stages of fungal evolution.

Lineage-specific duplications of certain *ATG* genes also were observed in different fungal groups. Interestingly, all the filamentous ascomycetes have a single copy of *ATG18* and *ATG20* but both of them are duplicated independently in the budding yeasts and fission yeasts. Whereas hyphae of filamentous ascomycetes have simple septa with septal pores that allow the free flow of mitochondria and other organelles between different compartments, the budding or fission yeasts are individual cells with rigid cell wall. Considering the functions of Atg18 and Atg20, their duplications may contribute to the intracellular recycling of nutrients and organelles by differentially controlling the vesicle formation in autophagy and Cvt pathways in the budding and fission yeasts. Another interesting observation is that whereas duplication of *ATG15* occurred in Agariomycetes, *ATG22* was specifically duplicated in Ascomycetes. Although Atg15 and Atg22 differ in biological functions, both of them are involved in the final stages of autophagy. The differences in these gene duplication events suggest that different fungi may differ in nutrient recycling during autophagy.

Interestingly, unlike the other fungi, the dermatophytes in Arthrodermataceae possess two *ATG8* genes. To our knowledge, this is the first observation of a group of fungi with two *ATG8* paralogs of significant sequence divergence. The *ATG8* genes have evolved to three subfamilies in animals (Shpilka et al., 2011) and also have been dramatically expanded in plants (Kellner et

al., 2016; Seo et al., 2016). The three subfamilies of animal Atg8s differ in their subcellular localization, expression profiles, and functions in the autophagy process (Shpilka et al., 2011). *Dictyostelium discoideum* also has two *ATG8* genes that have different expression profiles and functions (Matthias et al., 2016). In *T. rubrum*, the two *ATG8* genes also differed in the expression profiles, suggesting that they may also have distinct roles in the autophagy process. Because the natural habitats of the Arthrodermataceae dermatophytes are nutrient-limited, the two copies of *ATG8* may contribute to their adaptation the harsh environments. It is also possible that duplication of *ATG8* is related to the differentiation of specific ornaments on their primitive fruiting bodies or the production of arthrospores on vegetative hyphae in Arthrodermataceae fungi.

Mucoromycotina species are the only other group of fungi that have duplication of *ATG8*. However, unlike Arthrodermataceae fungi, Mucoromycetes with two *ATG8s* also have many other *ATG* genes duplicated in the genome. Ancient whole-genome duplication may be responsible for the duplication of many genes in Mucoromycetes, including the genes involved in ergasterol synthesis (Ma et al., 2009). The two *ATG8* paralogs in Mucoromycetes are highly similar to each other in amino acid sequences, which is different from Atg8a and Atg8b in Arthrodermataceae that have significant sequence divergence. It is likely that the two *ATG8* genes in Mucoromycetes have not underwent sequence and functional divergence yet due to their recent occurrence. Recent duplication of *ATG8* also was observed in three Agaricomycetes, *Pleurotus ostreatus, Sphaerobolus stellatus,* and *Dacryopinax* species DJM-731 SS1 that have two *ATG8* genes encoding proteins of highly similar amino acid sequences. It will be interesting to determine the functions of *ATG8* duplication in these fungi. Because the expression level of *ATG8* is known to be related to the size of autophagosomes in other organisms (Xie et al., 2008),

it is will be interesting to test whether two copies of *ATG8* will increase its overall expression and autophagosome size in these fungi.

Interestingly, approximately 90% of the *ATG* genes in *F. graminearum* and *N. crassa* are targeted by RNA editing during sexual reproduction. Since the A-to-I editing in fungi is generally adaptive (Wang et al., 2016; Liu et al., 2017), the editing of these *ATG* genes may be important for the autophagy function during the sexual reproduction. It is notable that although fungi have a single *ATG8* gene in general, different isoforms of Atg8 proteins can be generated by RNA editing during sexual reproduction. For example, both *F. graminearum* and *N. crassa* have two nonsynonymous editing sites in *ATG8* transcripts, which may lead to the synthesis of 4 isoforms of Atg8 proteins that may be slightly different in functions. These editing events in *ATG8* may be necessary for the fine-tuning of autophagy during sexual reproduction or provide protein heterozygosity beneficial for adaptation in the nature. *ATG13* is important for the initialization of both non-selective and selective autophagy. The Atg13 protein is targeted by the TORC1 kinase for phosphorylation. Inhibition of TORC1 leads the hypophosphorylation of Atg13 and affects its interaction with Atg1 and Atg17 for the initiation of autophagy (Farre and Subramani, 2016). In *F. graminearum*, *N. crassa*, and *N. tetrasperma*, the conserved editing event in *ATG13* results in the recoding of serine 536 to glycine, which may mimic the hypophosphorylation status of Atg13 proteins and affect autophagy during sexual reproduction. In *F. graminearum*, deletion of *FgATG11*, the *ATG* gene with the most editing sites in *F. graminearum*, had no obvious effect on growth, conidiation, plant infection, and formation of perithecia or ascospores but specifically affected forcibly discharge of ascospores, suggesting a relationship between stage-specific function and RNA editing of *FgATG11* during sexual reproduction. Deletion of *PUK1* and *AMD1*, two other genes with sexual stage-specific RNA

editing, also affected ascospore release in *F. graminearum* (Liu et al., 2016; Cao et al., 2017). Overall, our results indicated that RNA editing may affect the expression or sequence of Atg proteins and play a role in fine turning the autophagy process during ascospore formation and release. Therefore, it will be important to functionally characterize the functions of *ATG* genes and their editing events during sexual reproduction in *F. graminearum* and *N. crassa*.

METHODS

Reference *ATG* genes from yeasts and filamentous fungi: The sequences of 36 *ATG* genes of *S. cerevisiae* (Table S1) were retrieved from SGD (yeastgenome.org). The sequences of Atg25 from the methylotrophic yeast *Hansenula polymorpha* and Atg28, Atg30, Atg35, and Atg37 from the methylotrophic yeast *Pichia pastoris* (Table S1) were based on earlier publications (Monastyrska et al., 2005; Meijer et al., 2007). The *M. oryzae* and *F. oxysporum ATG* genes (Kershaw and Talbot, 2009; Corral-Ramos et al., 2015) were manually verified by both bidirectional blast and OrthoMCL (Li et al., 2003) methods. The *ATG8* and *ATG15* genes of *F. graminearum* were adapted from earlier publications (Nguyen et al., 2011; Josefsen et al., 2012). All the *ATG* genes identified in *F. graminearum* were manually annotated.

Genome sequences and RNA-seq data of different fungi: Most of the genome sequences and annotations used in this study were retrieved from the Ensembl Fungi genome database (fungi.ensembl.org/index.html) release 34. The genome sequences of *Phycomyces blakesleeanus* (Corrochano et al., 2016), *Taphrina deformans* (Cisse et al., 2013), *Gonapodya prolifera* (Chang et al., 2015), and *M. globosa* (Xu et al., 2007) were downloaded from the DOE Joint Genome Institute. The *P. jirovecii*, *P. carinii* (Ma et al., 2016), *N. antheraeae* (Pan et al., 2013), and *Hamiltosporidium tvaerminnensis* (Corradi et al., 2009) genomes. The genomes of *Mortierella*

verticillata, *Punctularia strigosozonata*, *Aspergillus ustus*, *Talaromyces cellulolyticus*, *Fomitiporia mediterranea*, *Trametes versicolor*, *Stereum hirsutum*, *Plicaturopsis crispa*, *Coniophora puteana*, *Tremella mesenterica*, and *Rhodotorula glutinis* were not well assembled or annotated. For *Colletotrichum higginsianum*, *Verticillium longisporum*, and *Allomyces macrogynus*, their genome sequences appeared to be derived from heterokaryotic or diploid hyphae. These fungal species were excluded from this analysis to avoid false negative or false positive results with *ATG* gene annotations.

The raw reads of *T. rubrum* RNA-seq data (Xu et al., 2015) were mapped to its genome by hisat2 (Kim et al., 2015). The bam file was generated by SAMtools (Li et al., 2009) and sashimi plots were generated with IGV (Thorvaldsdottir et al., 2013). The 3D structures of TrAtg8s were predicted with the I-TASSER server (Yang et al., 2015) and visualized with PyMol (pymol.org).

Identification of fungal *ATG* genes: To systematically identify fungal *ATG* genes, the in-house Perl scripts (available at github.com/wangqinhu/fungi_atg) were developed and used to retrieve and parse homology information from release 34 of Ensembl Fungi. To ensure that all the orthologs were correctly identified, four previously reported and manually verified seed sequences were applied for most *ATG* genes. For the genomes that are not included in the genome database of Ensembl Fungi, a bidirectional blastp search was performed to define the orthologs. For species with most of the *ATG* gene but lacking an *ATG8* ortholog, a tblastn search was performed against the genome sequence to identify potential mis-annotation. For *ATG1*, *ATG10*, and *ATG26*, manual annotation also was applied to correct errors introduced by automated annotation. For *Dichomitus squalens*, *Glarea lozoyensis*, *Gymnopus luxurians*, *Hydnomerulius pinastri*, *Laccaria bicolor*, *Paxillus involutus*, *Postia placenta*, *Pseudozyma*

antarctica, *Pseudozyma aphidis*, and *Sphaerobolus stellatus*, the annotation of *ATG* genes were corrected with the data available from the DOE Joint Genome Institute by blsatp or tblastn searches.

Phylogenetic analysis: The sequence alignments were generated with M-Coffee (Wallace et al., 2006) and adjusted with trimAl (Capella-Gutierrez et al., 2009). The phylogenetic relationships were inferred with the maximum likelihood method as developed in PhyML 3.1 (Guindon and Gascuel, 2003). The trees were visualized by FigTree (tree.bio.ed.ac.uk/software/figtree).

Editing sites in *ATG* genes of *F. graminearum* and *N. crassa*: The A-to-I editing data of *F. graminearum* and *N. crassa* were generated in previous studies (Liu et al., 2016; Liu et al., 2017). The editing sites in *ATG* genes were plotted with costumed *R* script. Conserved A-to-I editing sites in *F. graminearum*, *N. crassa*, and *N. tetrasperma* were also obtained from previous report (Liu et al., 2016).

Generation and characterization of the *ATG* genes mutant in *F. graminearum*: To functionally characterize the two editing sites in *FgATG8*, we generated mutant alleles of *FgATG8* carrying the T93G, A94G, T120G, or A121G mutation by overlapping PCR. The resulting $FgATG8^{G93}$, $FgATG8^{G94}$, $FgATG8^{G120}$, and $FgATG8^{G121}$ constructs were verified by sequencing analysis and transformed into the *Fgatg8* mutant generated by the split-marker approach (Catlett et al., 2002). Transformants of *Fgatg8* expressing these mutant alleles were confirmed by PCR analysis and assayed for defects in sexual reproduction as described (Josefsen et al., 2012).

To generate the *FgATG11* gene replacement mutant, its upstream and downstream flanking sequences were amplified and fused with the N-terminal portion and C-terminal portion of the hygromycin phosphotransferase (*hph*) cassette (Catlett et al., 2002), respectively, by

overlapping PCR. The resulting PCR products were transformed into the wild-type strain PH-1 as described (Zhou et al., 2011). The resulting hygromycin-resistant transformants were screened by PCR with primers (Fig. S3). Growth rate, infection assays with flowering wheat heads, disease index, conidiation, and conidium germination were assayed as previously described (Wang et al., 2011; Cao et al., 2016; Wang et al., 2018). Perithecium formation, ascus development, ascospore germination, and ascospore releasing were assayed on carrot agar plates as described (Cavinder et al., 2012; Luo et al., 2014; Yin et al., 2018).

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FIGURE LEGEND

Figure 1. Autophagy-related genes in different classes of fungi.

(**A**) The number of fungi belonging to different fungal phyla surveyed in this study. (**B**) The number of fungal species containing 0 (gray), 1 (blue), or at least 2 copies (pink) of individual *ATG* genes (*ATG1 - ATG41* labeled as 1 - 41 at the bottom). The numbers of highly conserved, conserved, and Saccharomycetes-specific *ATG* genes were shaded in red, green, and yellow, respectively. These *ATG* genes also were labelled on the top with C for core machinery, S for selective autophagy specific, and N for nonselective autophagy specific genes based on the classification of their orthologs in the budding yeast. (**C**) The conservation of *ATG* genes in different lineages across the kingdom Fungi. The dendrogram on the left shows the phylogenetic relationship of the 331 fungi belonging to labeled fungal classes or phyla. The numbers of species in each class or phylum are indicated in the bracket. Individual pie charts in the matrix show the ratio of fungal species in each fungal groups with (blue) vs. without (gray) individual *ATG* genes.

Figure 2. Conservation analysis of important complex or process involved in autophagosome formation.

For each protein complex involved in different processes of autophageosome formation as diagrammed underneath, the Atg proteins highlighted in red, green, or yellow represent the *ATG* genes that are highly conserved, conserved, or Saccharomycetes-specific, respectively. For the PI3K complex I, the components shaded in gray (Vps15 and Vps34) were not covered in this analysis.

Figure 3. **Massive losses and duplications of *ATG* genes in specific fungal groups.**

The branches of Microsporidia, *Pneumocystis*, *Malassezia*, and Mucoromycotina are in red, green, purple, and blue, respectively, in the dendrogram showing their phylogenetic relationship on the left. The copy numbers of individual *ATG* genes (*ATG1-ATG41*, labeled on the top) in each species vary from 0 to 4. *Fusarium graminearum, Magnaporthe oryzae, Neurospora crassa, Komagataela pastroris, Ogataea parapolymorpha*, and *Saccharomyces cerevisiae* are included for comparison.

Figure 4. The divergence of *ATG8* genes in dermatophytes.

(**A**) Sequence divergence and phylogenetic relationship between the two Atg8 proteins, Atg8a and Atg8b, in dermatophytes. The phylogenetic tree of fungal Atg8 proteins was inferred by PhyML 3.1 and the SH-like support values of branches, from lower and higher, were designated with circles from green to red. The abbreviations for the species used in the phylogenetic analysis are listed in Table S3. Atg8b proteins are specific for Arthrodermataceae and form a distinct clade. (**B**) Sequence alignment of Atg8a and Atg8b from six dermatophytes and Atg8 orthologs from *F. graminearum* (Fg), *M. oryzae* (Mo), and *N. crassa* (Nc). (**C**) The predicted 3D structures of *T. rubrum* Atg8a and Atg8b. (**D**) IGV sashimi plots showing the coverage of RNA-seq reads for *ATG8a* and *ATG8b* in *T. rubrum*. The density of RNA-seq reads are shown in red, the gene models are shown in blue, and the junction reads are shown as arcs with the read number.

Figure 5. Independent duplication of *ATG20* and *ATG18* in Saccharomycetes and Schizosaccharomycetes.

(**A**) Phylogenetic tree of yeast Atg20 and Snx41 (two paralogs) and their orthologs from other ascomycetes and basidiomycetes. Except *Geotrichum candidum* (Gc) and *Yarrowia lipolytica* (Yl) that has a single copy, the other Saccharomycetes species have both Atg20 and Snx41 orthologs. *S. pombe* and three other Schizosaccharomycetes species also have the Atg20 and Snx41 paralogs. However, the paralogous Atg20 and Snx41 in Saccharomycetes and Schizosaccharomycetes are in different clades, suggesting their independent origins. Furthermore, both Atg20 and Snx41 clades are not clustered or aligned with the single copy of Atg20/Snx41 in their closely related species such as *Y. lipolytica* or *T. deformans*, suggesting the occurrence of sequence divergence to both Atg20 and Snx41 in these yeast species. (**B**) Phylogeny of *S. cerevisiae* Atg18 and Atg21 and their orthologs from Saccharomycetes species. (**C**) Phylogeny of *S. pombe* Atg18 proteins and their orthologs from other Schizosaccharomycetes species. The Atg18/21 orthologs from *Saitoella complicata* (Sco) and *Taphrina deformans* (Td) are included for comparison with other Taphrinomycotina species. The phylogenetic tree was inferred by PhyML 3.1, and the SH-like support values of branches, from lower and higher, were designated with circles from green to red. The abbreviations for the species used in the phylogenetic analysis are listed in Table S3.

Figure 6. Phylogenetic analysis of Atg15 and Atg22 proteins involved in autophagic body breakdown in filamentous fungi.

(**A**) Phylogeny of Atg15 homologs from different ascomycetes and basidiomycetes. Many Agaricomycetes have three Atg15 paralogs. (**B**) Phylogeny of Atg22 homologs for

representative ascomycetes and basidiomycetes to show the duplication of Atg22 in Pezizomycotina and *Puccinia* species. The phylogenetic tree was inferred by PhyML 3.1, and the SH-like support values of branches, from lower and higher, were designated with circles from green to red. The abbreviations for the species used in the phylogenetic analysis are listed in Table S3.

Figure 7. **The A-to-I RNA editing sites in *ATG* genes in *F. graminearum* and *N. crassa*.** The RNA editing sites identified in transcripts of marked *ATG* genes during sexual reproduction in *F. graminearum* and *N. crassa*. The number of RNA editing sites in each *ATG* gene is marked in the bracket. The red and blue vertical lines represent the nonsynonymous and synonymous editing sites, respectively. Black arrows mark the editing sites conserved between *F. graminearum* and *N. crassa*.

Figure 8. Phenotypes of the *Fgatg11* mutant in *F. graminearum*.

The wild-type strain PH-1 and *Fgatg11* deletion mutant of *F. graminearum* were assayed for colony growth (**A**, three-day-old cultures), virulence on wheat heads (**B,** sampled 14-days post-inoculation), disease index (**C**, diseased spikelets per wheat heads), DON production (**D**), conidiation (**E**), the germination rate of conidia (**F**), perithecium development on carrot agar plates (**G**), asci and ascospores (**H,** bar = 20 μM), the germination rate of ascospores (**I**), and ascospore discharge from perithecia at 10 days post-fertilization (**J**). The defect in ascospore discharge was detected in all the four *Fgatg11* mutant strains repeatedly. In **C, D**, E, F, and I, error bars represent the standard deviations from at least 3 replicates. Two-sided *t*-tests were

used for accessing the phenotype difference between PH-1 and *Fgatg11* mutant. "ns" means not significant.

Figure 9. Illustration of the loss and duplication of *ATG* genes in kingdom Fungi. The tree shows the evolutionary relationship of major groups of fungi. During the evolution of fungi, many *ATG* genes were gained but some were lost at different branching time. Independent losses and duplications of *ATG* genes in the fungal tree of life were labeled by the red and green patterns as marked, respectively.

SUPPORTING INFORMATION

Figure S1. The conservation of *ATG* genes in Saccharomycetes.

The dendrogram on the left shows the phylogenetic relationship of the marked Saccharomycetes species. The copy numbers of individual *ATG* genes (highly conserved, red; conserved, green; specific, yellow) in each species vary from 0 to 3. The *ATG* genes from *Fusarium graminearum, Magnaporthe oryzae,* and *Neurospora crassa* are included as the outliers for comparison.

Figure S2. Mutations affecting the editing events at A94 and A121 in *FgATG8*.

(**A**) Mating cultures of the wild type strain PH-1, *Fgatg8* deletion mutant, and transformants of the *Fgatg8* mutant expressing the non-editable $FgATG8^{G93}$ and $FgATG8^{C120}$ alleles or edited $FgATG8^{G94}$ and $FgATG8^{G121}$ alleles were assayed for perithecium formation at 8 days post-fertilization. (**B**) Asci and ascospores of PH-1 and transformants of the *Fgatg8* mutant expressing $FgATG8^{G93}$, $FgATG8^{C120}$, $FgATG8^{G94}$, and $FgATG8^{G121}$ alleles. Bar = 20 μM.

Figure S3. Diagram of the *FgATG11* gene and its gene replacement construct.

Primer pairs F1/R2 and F3/R4 were used to amplify the upstream and downstream flanking sequences of *FgATG11*, respectively. The N- and C-portions of the hygromycin phosphotransferase (hph) cassette were amplified with primer pairs HYG-F/HY-R and YG-F/HYG-R. The overlapping PCR products amplified by primer pairs F1/HY-R and YG-F/R4 were co-transformed into the wild-type strain PH-1. Hygromycin-resistant mutants were screened with primer pairs H852/H850, F5/R6, F7/H855-R, and H856-F/R8.