RESEARCH LETTER

Analysis of autophagy in *Aspergillus oryzae* by disruption of *Aoatg13*, *Aoatg4*, and *Aoatg15* genes

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Abstract

Autophagy is a degradation system in which cellular components are digested via vacuoles/lysosomes, and involved in differentiation in addition to helping cells to survive starvation. The autophagic process is composed of several steps: induction of autophagy, formation of autophagosomes, transportation to vacuoles, and degradation of autophagic bodies. To further understand autophagy in the filamentous fungus *Aspergillus oryzae*, we first constructed *A. oryzae* mutants defective for the *Aoatg13*, *Aoatg4*, and *Aoatg15* genes and examined the resulting phenotypes. The Δ*Aoatg13* mutant developed conidiophores and conidia, although the number of conidia was decreased compared with the wild-type strain, while conidiation in the Δ*Aoatg4* and Δ*Aoatg15* mutants was not detected. The Δ*Aoatg15* mutants displayed a marked reduction of development of aerial hyphae. Moreover, autophagy in these mutants was examined by observation of the behavior of enhanced green fluorescent protein (EGFP)–AoAtg8. In the Δ*Aoatg13* mutant, the slight accumulation of EGFP–AoAtg8 in vacuoles, preautophagosomal structures (PAS), and autophagosomes was observed, whereas only PAS-like structures were detected in the Δ*Aoatg4* mutant. In the Δ*Aoatg15* mutant, autophagic bodies accumulated in vacuoles, suggesting that the uptake process proceeded. We therefore propose that the level of autophagy is closely correlated with the degree of differentiation in *A. oryzae*.

Introduction

In eukaryotes, macroautophagy (autophagy) is a conserved degradation process that mediates the trafficking of cytosolic proteins and organelles into lysosomes/vacuoles for bulk degradation (Reggiori & Klionsky, 2002). Although the process appears to predominantly recycle macromolecules and aid cell survival during periods of nutritional starvation, autophagy is also involved in development and differentiation in numerous eukaryotes, including yeasts, plants, and mammals, among others (Levine & Klionsky, 2004). This involvement may have resulted from the autophagic degradation of damaged organelles and cytosol for constitutive cell clearance and cellular remodeling during development and differentiation.

The autophagic process proceeds sequentially through several steps, involving the induction of autophagy, formation of autophagosomes, fusion of autophagosomes to lysosomes/vacuoles, and degradation of autophagic bodies (Mizushima, 2007; Pollack *et al*., 2009). In *Saccharomyces cerevisiae*, the induction of autophagy results from inactivation of the target of rapamycin (Tor) kinase, allowing formation of the Atg1 kinase complex composed of Atg1, Atg13, and Atg17 (Funakoshi *et al*., 1997; Kamada *et al*., 2000; Kabeya *et al*., 2005). The association of Atg13 with Atg1, which is essential for autophagy, is prevented by phosphorylation of Atg13 in a Tor kinase-dependent manner under conditions suitable for growth. In starvation conditions, Atg13 is dephosphorylated by inhibition of Tor kinase activity, allowing it to associate with Atg1 (Kamada *et al*., 2000).

The induction of autophagy induces the formation of cup-shaped isolation membranes, which subsequently elongate and sequester cytosol and/or organelles within double-membrane vesicles termed autophagosomes. *Saccharomyces cerevisiae* Atg8 is a ubiquitin-like protein that is essential for the formation of autophagosomes and is localized in pre-autophagosomal structures (PAS) and the membranes of

autophagosomes and autophagic bodies, and has been used as a marker for these organelles (Suzuki *et al.*, 2001). A critical event for autophagy involves the conjugation of the carboxy (C)-terminal glycine of Atg8 with phosphatidylethanolamine (PE), which is mediated by a ubiquitination-like system composed of Atg4 (cysteine protease), Atg7 (E1-like protein), and Atg3 (E2-like protein) (Ichimura *et al.*, 2000; Kirisako *et al.*, 2000). Atg4 cleaves newly synthesized Atg8 to expose the C-terminal glycine for conjugation with PE, and also cleaves Atg8-conjugated PE (Atg8-PE) to recycle Atg8. *Aspergillus oryzae* AoAtg8 also has a conserved glycine residue at the C-terminus, suggesting the evolutionary conservation of this conjugation system (Kikuma *et al.*, 2006).

Following the formation of autophagosomes, the outer membranes of autophagosomes fuse to vacuolar/lysosomal membranes and deliver single-membrane vesicles, called autophagic bodies, into the lumen of the vacuoles/lysosomes. The subsequent breakdown of the vesicle membranes allows degradation of the autophagic body contents by vacuolar hydrolases. In the vacuoles of *S. cerevisiae*, the protein Atg15, which contains a putative lipase active-site motif, is predominantly responsible for the degradation of autophagic bodies (Epple *et al.*, 2001, 2003; Teter *et al.*, 2001). Although the process leading to the degradation of autophagic bodies has been well studied, it is unclear if the identical process is used by filamentous fungi, such as *A. oryzae*.

Although filamentous fungal autophagy has been studied in *Podospora anserine*, *Magnaporthe grisea*, *M. oryzae*, *A. oryzae*, and *Aspergillus fumigatus* (Pinan-Lucarré *et al.*, 2003, 2005; Dementhon *et al.*, 2004; Veneault-Fourrey *et al.*, 2006; Liu *et al.*, 2007, 2010; Richie *et al.*, 2007; Dong *et al.*, 2009; Kershaw & Talbot, 2009; Lu *et al.*, 2009), the autophagic process in filamentous fungi is poorly understood. In the present study, we identified the *A. oryzae atg* gene homologues *Aoatg13*, *Aoatg4*, and *Aoatg15*, which were proposed to be involved in the induction of autophagy, formation of autophagosomes, and degradation of autophagic bodies, respectively. Subsequently, we generated deletion mutants of these genes and analyzed the resulting phenotypes of these *A. oryzae* mutants. Additionally, autophagy in these mutants was visualized by expressing enhanced green fluorescent protein (EGFP)–AoAtg8 in *Aoatg13*-, *Aoatg4*-, and *Aoatg15*-deletion backgrounds in an attempt to further understand the autophagic process in filamentous fungi.

Materials and methods

Strains and growth media

The *A. oryzae* strains used in this study are listed in Table 1. The *A. oryzae* wild-type strain RIB40 was used as a DNA donor, while strain NSRku70-1-1 (*niaD*$^-$, *sC*$^-$, *adeA*$^-$, and *ku70*$^-$) (Takahashi *et al.*, 2006) was used to disrupt the *Aoatg4*, *Aoatg13*, and *Aoatg15* genes. Strain NSRku70-1-1 transformed with *adeA* (NSRku70-1-1A) (Higuchi *et al.*, 2009) was used as a control for the phenotypic assay. M medium [0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, and 2% glucose (pH 5.5)] supplemented with 0.15% methionine (M+m) was used as a selective medium for disrupting the *Aoatg4*, *Aoatg13*, and *Aoatg15* genes. Czapek–Dox (CD) medium [0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, and 2% glucose (pH 5.5)] supplemented with 0.0015% methionine (CD+m) was used as a selective medium for identifying positive clones of the Δ*Aoatg4*, Δ*Aoatg13*, and Δ*Aoatg15* mutants expressing EGFP–AoAtg8. CD and CD+m media lacking sodium nitrate (CD − N and CD+m − N, respectively) were used for inducing autophagy.

Table 1. Strains of *Aspergillus oryzae* used in this study

Strains	Genotypes	References
RIB40	Wild type	
GEGA8	*niaD*$^-$ (P*Aoatg8–egfp–Aoatg8 niaD*)	Kikuma *et al.* (2006)
NSRku70-1-1	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*argB* Δ*ku70*::*argB*	Takahashi *et al.* (2006)
NSRku70-1-1A	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ *adeA* Δ*argB* Δ*ku70*::*argB*	Higuchi *et al.* (2009)
Δ*Aoatg13*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*Aoatg13*::*adeA* Δ*argB* Δ*ku70*::*argB*	This study
DA13EA8	*niaD*$^-$ (P*Aoatg8–egfp–Aoatg8 niaD*) *sC*$^-$ *adeA*$^-$ Δ*Aoatg13*::*adeA* Δ*argB* Δ*ku70*::*argB*	This study
Δ*Aoatg4*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*Aoatg4*::*adeA* Δ*argB* Δ*ku70*::*argB*	This study
DA4EA8	*niaD*$^-$ (P*Aoatg8–egfp–Aoatg8 niaD*) *sC*$^-$ *adeA*$^-$ Δ*Aoatg4*::*adeA* Δ*argB* Δ*ku70*::*argB*	This study
Δ*Aoatg15*	*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*Aoatg15*::*adeA* Δ*argB* Δ*ku70*::*argB*	This study
DA15EA8	*niaD*$^-$ (P*Aoatg8–egfp–Aoatg8 niaD*) *sC*$^-$ *adeA*$^-$ Δ*Aoatg15*::*adeA* Δ*argB* Δ*ku70*::*argB*	This study

Construction of disruption mutants

The plasmid pgΔAoatg4 was constructed to disrupt the *Aoatg4* gene using the Multisite Gateway cloning system. The upstream and downstream 1.5-kb regions of the *Aoatg4* gene were amplified by PCR using the primer pairs *att*B4-upAoatg4-F (5′-GGGGACAACTTTGTATAGAAAAGTTG TTTAGGGGGTTACGGCATGG-3′) and *att*B1-upAoatg4-R (5′-GGGGACTGCTTTTTTGTACAAACTTGTTTTGGGTG TAGTCGGTGTG-3′), and *att*B2-downAoatg4-F (5′-GGGG ACAGCTTTCTTGTACAAAGTGGGAACTAAACACCCGAT

AGAAACGA-3′) and *att*B3-downAoatg4-R (5′-GGGGACAACTTTGTATAATAAAGTTGAACGATTCCGACGCCTGC-3′), respectively. The underlined sequences are the Multisite Gateway *att*B recombination sites. The amplified *att*B-flanked upstream and downstream fragments were introduced into pDNOR^{TM}P4-P1R and pDNOR^{TM}P2R-P3, respectively, using the Gateway BP Clonase Reaction Mix (Invitrogen, Japan), generating the Entry Clone plasmids pg5′upAoatg4 and pg3′downAoatg4, respectively. The plasmids pg5′upAoatg4, pg3′downAoatg4, the Entry Clone plasmid containing the *A. oryzae adeA* gene as a selective marker (constructed in our laboratory), and the Destination vector pDEST^{TM}R4-R3 (Invitrogen) were then subjected to the Gateway LR reaction using the Gateway LR clonase reaction mix (Invitrogen) to generate pgΔAoatg4. Using plasmid pgΔAoatg4 as a template, the sequence containing the deletion cassette, which consisted of the upstream region of *Aoatg4* (1.5 kb), the *adeA* gene (2.0 kb), and the downstream region of *Aoatg4* (1.5 kb), was amplified by PCR with the primers *att*B4-upAoatg4-F and *att*B1-upAoatg4-R, and then transformed into *A. oryzae* NSRku70-1-1. The disruption of the *Aoatg4* gene was confirmed by Southern blotting using a 1.5-kb fragment of the region of upstream as a probe, which was generated by PCR with the primers *att*B4-upAoatg4-F and *att*B1-upAoatg4-R (see Supporting Information, Fig. S4).

The plasmids pgΔAoatg13 and pgΔAoatg15 for disruption of the *Aoatg13* and *Aoatg15* genes, respectively, were constructed by the identical method used for the disruption of *Aoatg4*. The upstream and downstream 1.5-kb regions of the *Aoatg13* gene were amplified by PCR using the primer pairs *att*B4-upAoatg13-F (5′-GGGGACAACTTTGTATAGAAAAGTTG GGTATCCACCTGACTGTTTTC-3′) and *att*B1-upAoatg13-R (5′-GGGGACTGCTTTTTTGTACAAACTTG GATCCTCCTGCGACATACAA-3′), and *att*B2-downAoatg13-F (5′-GGGGACAGCTTTCTTGTACAAAGTGGTTGCATA ACTGAAGCCCGTAG-3′) and *att*B3-downAoatg13-R (5′-GGGGACAACTTTGTATAATAAAGTTGAATTGCGCACTCTGA ACTTGG-3′), respectively. The upstream and downstream 1.5-kb regions of the *Aoatg15* gene were amplified by PCR using the primer pairs *att*B4-upAoatg15-F (5′-GGGGACAACTTTGTATAGAAAAGTTGAGACCATGAACAACGAGGA-3′) and *att*B1-upAoatg15-R (5′-GGGGACTGCTTTTTTGTACAAACTTGAGCACAACGACGCGTACATA-3′), and *att*B2-downAoatg15-F (5′-GGGGACAGCTTTCTTGTACAAAGTGGGAG AGGTACCTTATACTTCAC-3′) and *att*B3-downAoatg15-R (5′-GGGGACAACTTTGTATAATAAAGTTGGACATCAACCC CAAGGTCAT-3′), respectively. All primers were based on the *A. oryzae* genome database. The PCR reactions were performed using the genomic DNA of *A. oryzae* RIB40 as a template. Transformation of *A. oryzae* was carried out using a standard method, as described previously (Jin *et al.*, 2004).

For the phenotypic analysis of all disruptants, hyphae or conidia were point inoculated on M+m, dextrin–polypeptone–yeast extract (DPY), and potato dextrose (PD) (Nissui, Japan) agar media, and plates were then incubated for 4 days at 30 °C. NSRku70-1-1A was used as a control.

Visualization of autophagy in the disruption mutants

To visualize autophagy, the pgEGA8 plasmid containing the *A. oryzae niaD* gene as a selection marker and the *egfp* gene-linked *Aoatg8* gene (Kikuma *et al.*, 2006) were introduced into the disruption mutants. Conidia or hyphae from the disruption mutants were cultured in a glass-based dish (Asahi Techno Glass Co., Japan) using 100 μL CD+m medium for 24 h at 30 °C. The medium was then replaced with either fresh CD+m medium (control) or CD+m − N (for the induction of autophagy), and the cells were further incubated for 4 h at 30 °C. The strains were then observed with an IX71 confocal laser scanning microscope (Olympus Co., Japan).

Results

Disruption of *Aoatg13* decreases autophagy levels

To investigate the effects of defects in signal transduction in autophagy, we first identified the *ATG13* homologue in *A. oryzae*, *Aoatg13*, from the *A. oryzae* genome database (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao) using the BLAST algorithm. *Aoatg13* (DDBJ accession number AB586123) contained two introns and three exons, and encoded a predicted polypeptide of 974 amino acids with a calculated molecular mass of 104 kDa. AoAtg13 displayed 24% identity to Atg13 of *S. cerevisiae*, and an Atg13 family domain was identified in the Pfam database (http://pfam.sanger.ac.uk/) (Fig. S1).

To determine the function of *Aoatg13*, we disrupted *Aoatg13* by replacement with the selective marker *adeA*, which was confirmed by Southern blot analysis (Fig. S4). When the Δ*Aoatg13* mutant was grown on PD and DPY agar media, the colonies appeared slightly green in color (Fig. 1a) and generated conidia, unlike the Δ*Aoatg8* mutant (Kikuma *et al.*, 2006). This result suggested that autophagy occurs in the Δ*Aoatg13* mutants. To confirm this speculation, we generated an Δ*Aoatg13* strain expressing EGFP–AoAtg8 (DA13EA8). *Saccharomyces cerevisiae* Atg8 and its orthologues, which are anchored in the membranes of autophagosomes and autophagic bodies, have been used as markers for visualization of autophagy in various organisms (Kabeya *et al.*, 2000; Pinan-Lucarré *et al.*, 2003; Yoshimoto *et al.*, 2004; Monastyrska *et al.*, 2005; Kikuma *et al.*, 2006). In a previous study, we showed that the *A. oryzae* Atg8 orthologue, AoAtg8, was a useful marker for detecting autophagy in *A. oryzae* (Kikuma *et al.*, 2006). When strain

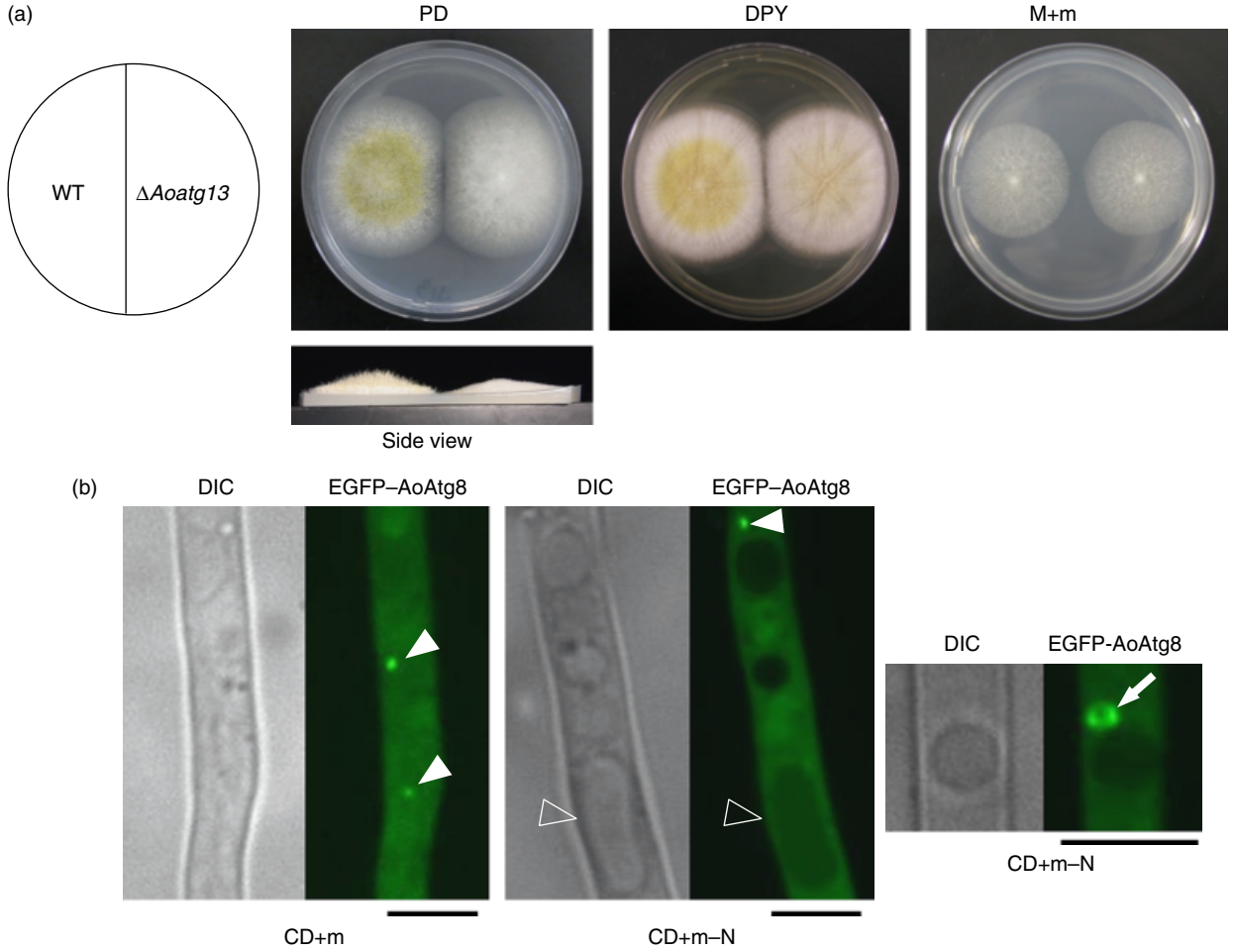

Fig. 1. Phenotype of the Δ*Aoatg13* mutant. (a) Images of the NSRku70-1-1A [wild type (WT)] and Δ*Aoatg13* strains after growth on PD, DPY, and M+m agar plates for 4 days at 30 °C. (b) The DA13EA8 strain expressing EGFP–AoAtg8 was grown on CD+m medium for 24 h and then shifted to CD+m − N medium. After incubation for 2 h, hyphae were visualized by DIC and confocal laser scanning microscopy. The arrowheads and arrow indicate PAS-like structures and an autophagsome, respectively. The black arrowhead indicates a vacuole slightly accumulated EGFP–AoAtg8. Scale bars = 5 μm.

DA13EA8 was cultured in CD+m medium, EGFP–AoAtg8 was localized in PAS-like structures, but was also diffused in cytoplasm. After growth for 24 h at 30 °C in CD+m medium, the mutant was shifted to nitrogen-deprived medium (CD+m − N) to induce autophagy. Following the induction of autophagy under starvation conditions, the fluorescence of EGFP–AoAtg8 was predominantly observed in PAS-like structures, but could also be seen to a lesser extent in vacuoles (Fig. 1b, CD+m − N). Furthermore, ring-like structures, which appeared to be autophagosomes, were observed in the vicinity of vacuoles. These observations indicated that the autophagic process proceeded to completion in the Δ*Aoatg13* mutant, although the induction of autophagy was limited compared with the wild-type strain (Kikuma *et al.*, 2006).

Aoatg4 is essential for autophagosome formation

To evaluate the process of autophagosome formation in *A. oryzae*, we next identified the *ATG4* gene homologue, *Aoatg4*, from the *A. oryzae* genome database using the BLAST algorithm. *Aoatg4* (DDBJ accession number AB586122) contained four introns and five exons, and encoded a predicted polypeptide of 356 amino acids with a calculated molecular mass of 14 kDa. AoAtg4 displayed 41% identity to Atg4 of *S. cerevisiae* and, as determined from the Pfam database, had a peptidase family C54 motif (Fig. S2).

To examine the function of *Aoatg4* in *A. oryzae*, we constructed a strain with a disrupted *Aoatg4* gene using the identical strategy to that for the *Aoatg13* gene (Fig. S4). Hyphae of the Δ*Aoatg4* mutant were then grown on PD,

DPY, and M+m agar media for 4 days at 30 °C. The Δ*Aoatg4* mutant generated white colonies on all media, indicating that the mutants did not form normal aerial hyphae or conidia (Fig. 2a), which is the identical phenotype to the *Aoatg8*-deletion mutant (Kikuma *et al.*, 2006).

Next, we tested whether *Aoatg4* was essential for autophagy in *A. oryzae*. To visualize autophagy in the Δ*Aoatg4* mutants, we constructed strain DA4EA8 expressing EGFP–AoAtg8 in the Δ*Aoatg4* background, which displayed a similar phenotype as the Δ*Aoatg4* strain. While EGFP–AoAtg8 was transported to vacuoles in the wild-type strain (Fig. 2b, WT) (Kikuma *et al.*, 2006), EGFP–AoAtg8 in the DA4EA8 strain localized to PAS-like structures, but not to vacuoles, even under starvation conditions (Fig. 2b, Δ*Aoatg4*). Interestingly, dot structures with large diameters compared with normal PAS-like structures were observed (Fig. 2b, arrow). Taken together, these observations suggest that the Δ*Aoatg4* mutant is defective in autophagy, and AoAtg4 is essential for autophagosome formation in *A. oryzae*.

Aoatg15 is required for digestion of autophagic bodies

Autophagic bodies are single-membrane vesicles formed in the lumen of vacuoles as a result of the fusion of autophagosomes with vacuolar membranes. *Saccharomyces cerevisiae* Atg15 is a putative lipase essential for the lysis of autophagic bodies. We identified the *ATG15* gene homologue in *A. oryzae* using the BLAST algorithm, and found that *Aoatg15* (DDBJ accession number AB586124) contained one intron and two exons, and encoded a predicted polypeptide of 591 amino acids with a calculated molecular mass of 64 kDa. AoAtg15 showed 35% identity to Atg15 of *S. cerevisiae*

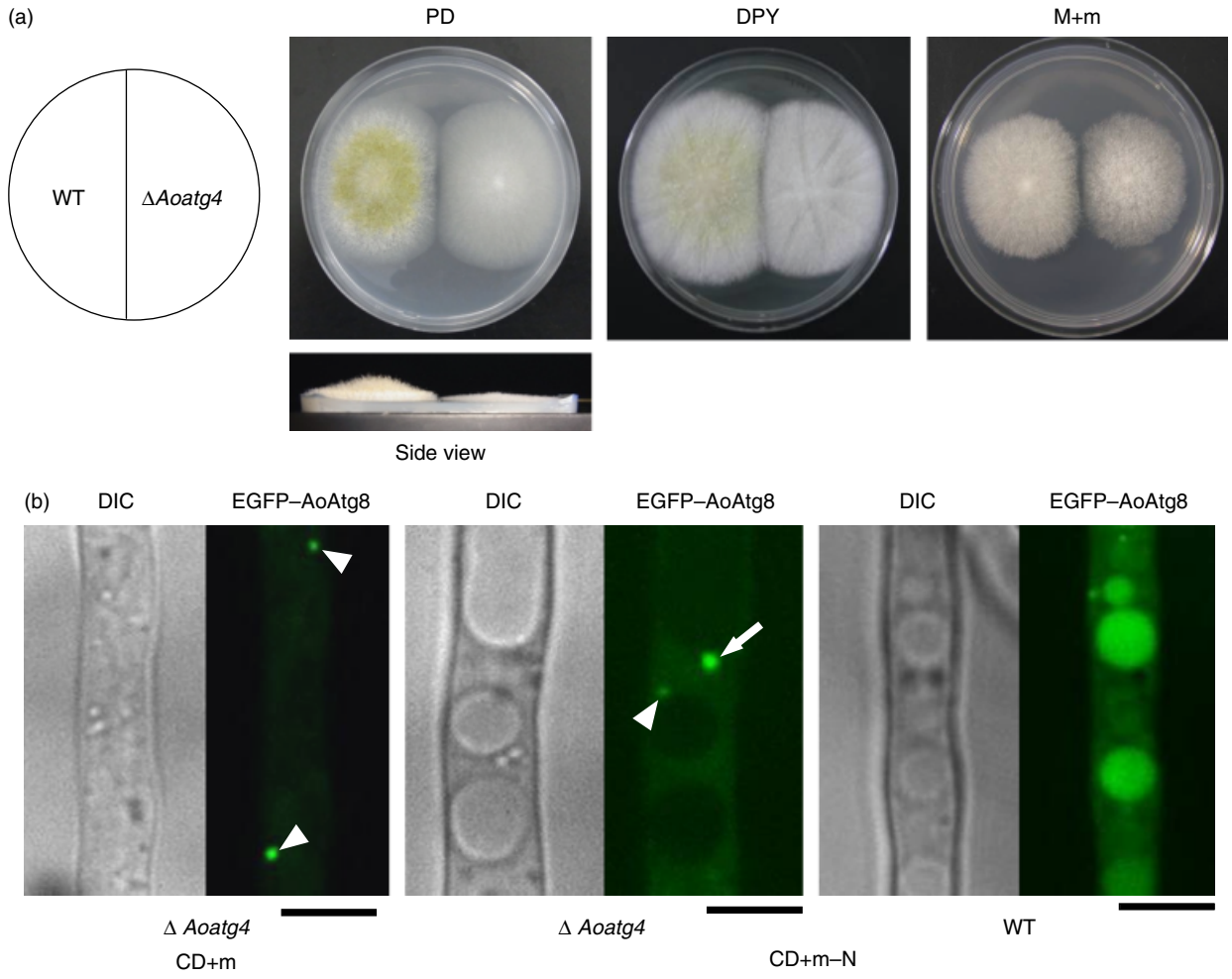

Fig. 2. Phenotype of the Δ*Aoatg4* mutant. (a) Images of the NSRku70-1-1A [wild type (WT)] and Δ*Aoatg4* strains after growth on PD, DPY, and M+m agar plates for 4 days at 30 °C. (b) The DA4EA8 and GEGA8 (Kikuma *et al.*, 2006) strains expressing EGFP–AoAtg8 were grown on CD+m medium for 24 h at 30 °C and then shifted to CD+m − N medium. After further incubation for 2 h, hyphae were visualized by DIC and confocal laser scanning microscopy. The arrowheads and arrow point to PAS-like structures and a large PAS-like structure, respectively. Scale bars = 5 μm.

and had a putative lipase domain (from the Pfam database) (Fig. S3).

The function of *Aoatg15* in *A. oryzae* was examined by constructing a strain disrupted for the *Aoatg15* gene by replacement with the selective marker *adeA* (Fig. S4). When the hyphae of the Δ*Aoatg15* mutant were grown on PD, DPY, and M+m agar media for 4 days at 30 °C, no aerial hyphae or conidia were observed (Fig. 3a), and the colony was flatter than the Δ*Aoatg13* and Δ*Aoatg4* mutants (Fig. 4). Moreover, the accumulation of vesicles in vacuoles was observed under starvation conditions (Fig. 3b).

Finally, we constructed a Δ*Aoatg15* mutant strain expressing EGFP–AoAtg8 (DA15EA8), which was then cultured for 24 h at 30 °C in CD+m medium on a glass-based dish and observed by confocal laser scanning microscopy. During the growth in CD+m, EGFP–AoAtg8 localized to the PAS-like structures found in the vicinity of vacuoles (Fig. 3c, CD+m). However, when DA15EA8 was grown under starvation conditions (CD+m − N medium), EGFP–AoAtg8 localized to autophagosomes and cup-shaped sequestering membranes (isolation membranes), while autophagic bodies accumulated in the lumen of vacuoles (Fig. 3c). These observations indicated that AoAtg15 was a vacuolar lipase for the lysis of autophagic bodies, similar to the function of *S. cerevisiae* Atg15, and normal uptake of cytosolic material into vacuoles with isolation membranes and autophagosomes occurred in the Δ*Aoatg15* mutants.

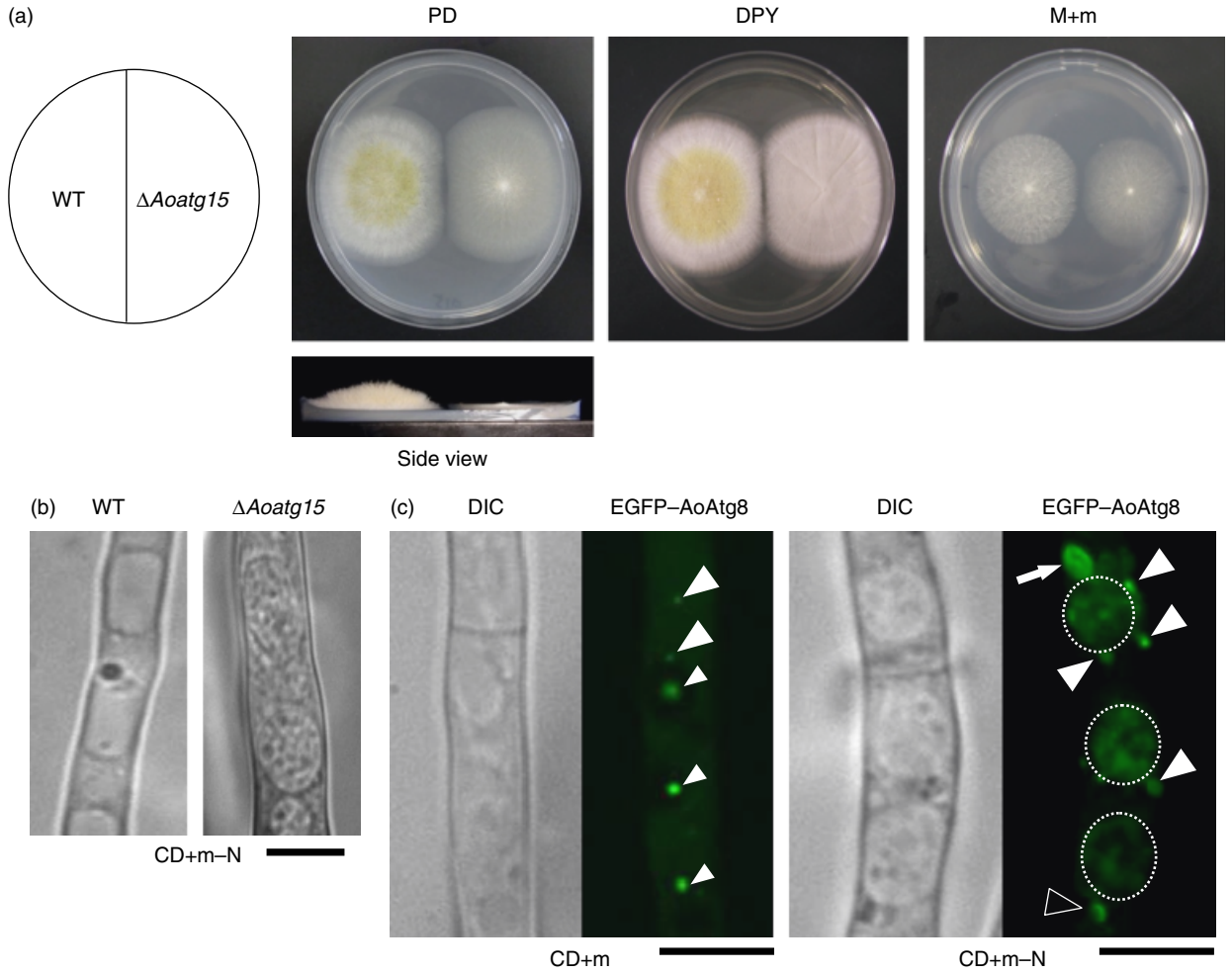

Fig. 3. Phenotype of the Δ*Aoatg15* mutant. (a) Images of the NSRku70-1-1A [wild type (WT)] and Δ*Aoatg15* strains after growth on PD, DPY, and M+m agar plates for 4 days at 30 °C. (b) The NSRku70-1-1A and Δ*Aoatg15* strains were grown on CD+m medium for 24 h at 30 °C and then shifted to CD+m − N medium. After further incubation for 2 h, hyphae were visualized by DIC microscopy. (c) The DA15EA8 strain expressing EGFP–AoAtg8 was grown on CD+m medium for 24 h at 30 °C and then shifted to CD+m − N medium. After further incubation for 2 h, hyphae were visualized by DIC and confocal laser scanning microscopy. The large and small arrowheads point to PAS-like structures and autophagic bodies, respectively. The black arrowhead and white arrow indicate an isolation membrane and an autophagosome, respectively. Autophagic body-accumulated vacuoles are encircled with dotted lines. Scale bars = 5 μm.

Discussion

In eukaryotes, autophagy is regulated by many Atg proteins which function at each step in the autophagic process. To investigate the effects of impairment of the induction step of autophagy, we first constructed an *Aoatg13*-deletion mutant, Δ*Aoatg13*. Unlike the Δ*Aoatg8* mutant, conidiation occurred in the Δ*Aoatg13* mutant, although the number of conidia produced after 4 days of culture was smaller than that of the wild-type control strain, suggesting that autophagy proceeds in the absence of *Aoatg13*. Indeed, the subtle accumulation of EGFP–AoAtg8 fluorescence in vacuoles was observed, and PAS-like and autophagosome-like ring structures were visualized in the DA13EA8 strain under starvation conditions, presumably due to the constitutive basal levels of autophagy. Intriguingly, colonies of the DA13EA8 strain appeared greener than those of the Δ*Aoatg13* mutant, and the DA13EA8 strain produced an increased number of conidia compared with the Δ*Aoatg13* mutants, but not the DA4EA8 or DA15EA8 strains. In *A. fumigatus*, the disruption of Af*atg1*, which is an orthologue of *S. cerevisiae ATG1*, causes a defect in autophagy (Richie *et al.*, 2007). Moreover, conidiation in the Af*atg1*-deletion mutant is reduced, but can be rescued by addition of nitrogen sources, such as ammonium salts or nitrates, to the culture medium. The EGFP–AoAtg8 expression plasmid contains the *A. oryzae niaD* gene encoding a nitrate reductase as a selection marker, suggesting that the nitrogen sources produced by the reduction of nitrates in the DPY and PD media may have been available to the DA13EA8 cells. In *S. cerevisiae*, Atg1 and Atg13 interact with each other, and *ATG13* disruptants are defective in autophagy; however, the defect is suppressed by the overexpression of *ATG1* (Funakoshi *et al.*, 1997; Kamada *et al.*, 2000). *Drosophila* Atg13 is also essential for autophagy, and Atg13 overexpression inhibits autophagosome expansion (Change & Neufeld, 2009). These phenotypes differ from those observed in *A. oryzae*, as autophagy was slightly induced under starvation conditions in the Δ*Aoatg13* mutant, suggesting that AoAtg13 functions as an amplifier or regulator of the signal from the *A. oryzae* Atg1 orthologue, resulting in a higher level of autophagy induction. Further studies are necessary to determine the first step of autophagy in *A. oryzae*; for example, by disrupting or overexpressing the *A. oryzae ATG1* homologue.

In *S. cerevisiae*, the delivery of Atg8 to PAS does not occur in Δ*atg4* cells (Suzuki *et al.*, 2001), which indicates that the localization of Atg8 to PAS requires the prior lipidation of Atg8, allowing the PE conjugated form (Atg8-PE) to associate with PAS. The phenotype of the Δ*Aoatg4* mutant appeared similar to that of the *Aoatg8*-deletion mutant, indicating a defect in autophagy. In the DA4EA8 strain, EGFP–AoAtg8 predominantly localized to dot-like structures, which seemed to be the PAS, although larger dot-like structures were also observed. These results suggest that the localization of AoAtg8 might be independent of PE, and may be mediated by interaction with AoAtg proteins other than AoAtg4. We speculate that the lipidation of AoAtg8 is required for the elongation of isolation membranes and formation of autophagosomes, and the larger dot-like structures was a result of the aggregation of EGFP–AoAtg8 in the Δ*Aoatg4* mutant.

In the DA15EA8 strain, PAS-like structures, autophagosomes and autophagic bodies were observed, in addition to the accumulation of autophagic bodies in the lumen of vacuoles. These observations indicate that AoAtg15 is required for degradation of autophagic bodies, but not for the stages of autophagy involving dynamic membrane rearrangements for the uptake of intracellular components into vacuoles. Notably, the Δ*Aoatg15* strain displayed a more severe developmentally impaired phenotype. Colonies of the strain were significantly flatter than the other gene-deletion mutants (Fig. 4). This phenotype might be due to defects in the lysis of lipid vesicles in vacuoles, including not only autophagic bodies, but also other lipid vesicles, such as those arising from the cytoplasm-to-vacuole (Cvt) pathway (Cvt bodies) (Klionsky & Ohsumi, 1999) and multivesicular body (MVB) pathway (MVB vesicles) (Epple *et al.*, 2003), which have been described in *S. cerevisiae*. The Cvt pathway is morphologically similar to autophagy, and numerous components of this pathway overlap with Atg proteins (Harding *et al.*, 1996; Scott *et al.*, 1996; Wang & Klionsky, 2003). The MVB pathway also serves to transport Atg15 to vacuoles, and the breakdown of intravacuolar MVB vesicles is impaired in Δ*atg15* cells (Epple *et al.*, 2003). However, as these pathways have not been identified in filamentous fungi, the Δ*Aoatg15* strain is expected to be useful for the

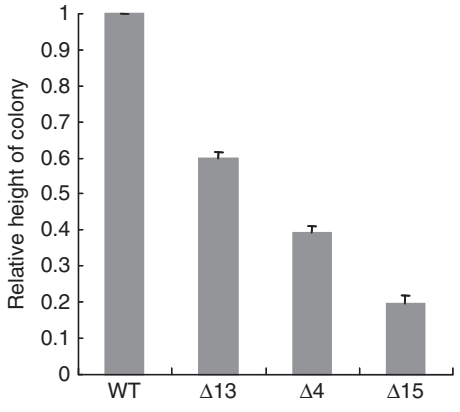

Fig. 4. Length of aerial hyphae in the colonies of deletion mutants. NSRku70-1-1A [wild type (WT)] and each deletion mutant (Δ*Aoatg13*: Δ13, Δ*Aoatg4*: Δ4, Δ*Aoatg15*: Δ15) were grown on PD agar plates for 4 days at 30 °C. The relative height of the mutant colonies to NSRku70-1-1A is shown. Error bars represent the SD for three independent experiments.

determination of the substrates for autophagy, as well as the study of vacuolar protein sorting pathways in filamentous fungi.

In the present study, we investigated the process of autophagy by disrupting the key genes in each step of autophagy in *A. oryzae*. Our results demonstrated that the formation of aerial hyphae is dependent on the level of degradation of intravacuolar lipid vesicles in autophagy, indicating that autophagy plays a key role in differentiation in *A. oryzae*. However, many details of autophagy in filamentous fungi remain poorly understood; for example, the correlation of autophagy with differentiation, the mechanism of PAS formation, and the relationship between autophagy and the transport of other vesicles to vacuoles, such as the Cvt and MVB pathways. Therefore, the establishment of methods for biochemical analysis and quantitative evaluation in *A. oryzae* are needed to determine how autophagy is precisely controlled in this organism. In addition, studies of vacuolar transport pathways are necessary to determine the effects of autophagy on morphology and physiology in filamentous fungi.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alignment of AoAtg13 and Atg13.
Fig. S2. Alignment of AoAtg4 and Atg4.
Fig. S3. Alignment of AoAtg15 and Atg15.
Fig. S4. Schema for the integration of the *adeA* gene, and Southern blotting for the *Aoatg13*, *Aoatg4*, and *Aoatg15* genes in the deletion mutants.

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