Functional Analysis of the ATG8 Homologue Aoatg8 and Role of Autophagy in Differentiation and Germination in Aspergillus oryzae

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Autophagy is a well-known degradation system, induced by nutrient starvation, in which cytoplasmic components and organelles are digested via vacuoles/lysosomes. Recently, it was reported that autophagy is involved in the turnover of cellular components, development, differentiation, immune responses, protection against pathogens, and cell death. In this study, we isolated the ATG8 gene homologue Aoatg8 from the filamentous fungus Aspergillus oryzae and visualized autophagy by the expression of DsRed2-AoAtg8 and enhanced green fluorescent protein-AoAtg8 fusion proteins in this fungus. While the fusion proteins were localized in dot structures which are preautophagosomal structure-like structures under normal growth conditions, starvation or rapamycin treatment caused their accumulation in vacuoles. DsRed2 expressed in the cytoplasm was also taken up into vacuoles under starvation conditions or during the differentiation of conidiophores and conidial germination. Deletion mutants of Aoatg8 did not form aerial hyphae and conidia, and DsRed2 was not localized in vacuoles under starvation conditions, indicating that Aoatg8 is essential for autophagy. Furthermore, Aoatg8 conditional mutants showed delayed conidial germination in the absence of nitrogen sources. These results suggest that autophagy functions in both the differentiation of aerial hyphae and in conidial germination in A. oryzae.

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transformed with _adeA_ (6), was used as a control for the phenotypic assay. Czapke Dox (CD) medium (0.5% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, and 2% glucose (pH 5.5)) was used as a selective medium and for microscopic observations. Methionine (0.0015%) was added to select for positive clones when the Δ_atg8-1-1 strain was transformed with plasmids harboring the _niaD_ gene as a selection marker. M medium [0.2% NH₄Cl, 0.1% (NH₄)₂SO₄, 0.05% KCl, 0.05% NaCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, and 2% glucose (pH 5.5)] supplemented with 0.15% methionine was used as a selective medium for disrupting the _Ao_atg8_ gene. CD medium lacking sodium nitrate (CD- N) or CD medium containing 200 ng/ml rapamycin (CD + R) was used for inducing autophagy. The transformation of _A. oryzae_ was carried out using the standard method (7).

**Construction of _A. oryzae_ strains for visualizing autophagy.** To clone the promoter, gene, and terminator regions of _AoAtg8_ with the Multisite Gateway cloning system (Invitrogen Co., Tokyo, Japan), four primers were designed: with the Multisite Gateway promoter, gene, and terminator regions of _AoAtg8_. The resulting fragment was blunt ended and inserted into the SmaI site of pUNA, consisting of the _PamyB_ gene as a selection marker. M medium [0.2% NaNO₃, 0.02% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, and 2% glucose (pH 5.5)] supplemented with 0.15% methionine was used as a selective medium for disrupting the _Ao_atg8_ gene using the Multisite Gateway cloning system. The genome databases using the BLAST algorithm. The _niaD_ gene homologue, we searched the genome databases using the BLAST algorithm. The _niaD_ gene homologue, we searched the

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**RESULTS**

Isolation of the _A. oryzae_ _Atg8_ gene. _S. cerevisiae_ _Atg8_ is an essential protein in the autophagic pathway, and orthologues of _Atg8_ in other organisms have been used as specific markers of autophagy (8, 19, 27, 35). In order to isolate the _A. oryzae_ _ATG8_ gene homologue, we searched the _A. oryzae_ EST and genome databases using the BLAST algorithm. The _ATG8_ gene was confirmed on October 26, 2017 by guest http://ec.asm.org/ Downloaded from

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**TABLE 1. Strains of _A. oryzae_**

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**Autophagy in _Aspergillus oryzae_**

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homologue in *A. oryzae*, Aoatg8, contains two introns and three exons and encodes a polypeptide of 118 amino acids with a calculated molecular mass of 14 kDa. Aoatg8 (DDBJ accession number AB246664) displays similarity to Atg8 of *S. cerevisiae* (79% identity) (14), AtAtg8a of *Arabidopsis thaliana* (NCBI accession number AAM70188) (73%), and LC3 of *Homo sapiens* (NCBI accession number NP_115093) (29%) and shows even higher similarity to its orthologues from the ascomycete fungi *Aspergillus nidulans* (NCBI accession number EAA62312) (98%) and *P. anserina* (98%) (27) and the basid-
iomycete fungus *Ustilago maydis* (NCBI accession number EAL64271) (86%).

**Visualization of autophagy in A. oryzae.** To visualize autophagy in *A. oryzae*, we constructed the CEDA8 strain coexpressing vacuolar carboxypeptidase Y (CPY)-EGFP and DsRed2-AoAtg8 fusion proteins. After growing for 24 h at 30°C in the CD medium, CEDA8 was cultured in the nitrogen-deprived medium (CD–N) or CD medium containing rapamycin (CD+R) to induce autophagy. During the growth in CD, DsRed2-AoAtg8 was localized to the PAS-like structures found in the vicinity of vacuoles (Fig. 1A). DsRed2-AoAtg8 was not localized in vacuoles when autophagy was not induced, although CPY-EGFP was observed in the vacuoles (Fig. 1A, lower left panels). When autophagy was induced, DsRed2-AoAtg8 was translocated to vacuoles (Fig. 1A, lower right panels). EGFP-AoAtg8 expressed in the GEGA8 strain showed behavior similar to that of DsRed2-AoAtg8 in CEDA8 (data not shown).

In order to find autophagic isolation membranes and autophagosomes in *A. oryzae*, EGFP-AoAtg8 was observed using a confocal laser scanning microscope. GEGA8 was cultured for 24 h at 30°C in CD medium on glass base dishes. When cells grown in CD were shifted to CD–N and cultured for an additional 2 h, ring-like and cup-shaped structures reminiscent of autophagosomes and isolation membranes, respectively, were observed (Fig. 1B). Moreover, some spherical structures were seen in the center of the ring-like and cup-shaped structures in differential interference contrast (DIC) images. In *P. anserina*, lipid bodies are accumulated during cell death by incompatibility (26). The spherical structures accumulated under starvation conditions or during treatment with rapamycin in CEDA8 (Fig. 1A, lower right panels) were also stained by Nile red (data not shown), indicating that the structures are most likely lipid droplets.

**Induction of autophagy during the formation of aerial hyphae, conidiation, and conidial germination.** Recently, it has been suggested that autophagy is involved in development and differentiation in various eukaryotes (15). To observe autophagy in the development and differentiation of *A. oryzae*, conidia of GEGA8 expressing EGFP-AoAtg8 were cultured under noninduced conditions of autophagy (the presence of nitrogen sources and the absence of rapamycin). Interestingly, in swollen conidia, germlings, and germ tubes, EGFP-AoAtg8 was localized in vacuoles, even under nutrient-rich growth conditions (Fig. 2).

When autophagy is induced, the isolation membranes sequester cytoplasm and organelles nonselectively (12, 13). Therefore, we predicted that autophagy could be observed by detecting fluorescent proteins inside vacuoles, which had been incorporated from the cytoplasm. We obtained the CEDR1 strain, which constitutively expresses CPY-EGFP in vacuoles and DsRed2 protein in the cytoplasm. DsRed2 in CEDR1 was observed in vacuoles 12 h after the shift to the starvation condition (see Fig. 4D, left panels). In *A. oryzae*, conidiation occurs through the characteristic morphogenesis of differentiated cells. Aerial hyphae are formed from foot cells, and some differentiate into conidiophores, which are composed of vesicles, phialides, and conidia (Fig. 3A). To investigate whether autophagy is induced in differentiated cells of the aerial hyphae, CEDR1 was grown on minimal agar medium (CD) on glass slides for 3 days at 30°C and observed with a fluorescence microscope. In conidiophores growing on CD medium, DsRed2 was localized to vacuoles in stalk, conidiophore vesicles, and phialides, whereas it was...
localized to cytoplasm in aerial hyphae that have not developed into conidiophores (Fig. 3B). These results suggest that autophagy is involved in conidiation and the formation of conidiophores.

**Phenotypes of Aoatg8 gene disruption mutants in A. oryzae.** In order to examine the function of autophagy in *A. oryzae*, we constructed strains disrupted for the Aoatg8 gene by replacement with the selective marker adeA (Fig. 4A). Disruption was confirmed by PCR and Southern blot analyses (Fig. 4A). Hyphae of the disruptant, ΔAoatg8-1-1, were grown on PD, DPY, and M plus 0.15% methionine agar media for 4 days at 30°C. ΔAoatg8-1-1 formed no aerial hyphae and conidia and showed slower growth on synthetic medium than the wild-type strain did (Fig. 4B). Conidiation is usually enhanced when *A. oryzae*...
is grown on PD medium; therefore, differences in the formation of conidia and aerial hyphae were clearly identified between ΔAoatg8-1-1 and the control strain (Fig. 4B, lower panel). These phenotypes were complemented by the introduction of a plasmid for the conditional expression of AoAtg8 under the control of the thiA promoter into ΔAoatg8-1-1. The thiA promoter is controlled by the addition of thiamine: in the absence of thiamine, the expression of a gene controlled by the thiA promoter is induced, whereas in the presence of 100 nM thiamine, it is repressed (32). This strain, CSC1-2-1, was grown on a PD medium plate with or without thiamine. In the absence of thiamine, the formation of aerial hyphae and conidiation occurred; however, in the presence of thiamine, CSC1-2-1 displayed a phenotype similar to that of ΔAoatg8-1-1 (Fig. 4C). These results suggest that Aoatg8 is involved in the formation of aerial hyphae. Moreover, conidia of the wild-type strain germinated on CD–N medium and then formed a mycelial colony. Similarly, CSC1-2-1 elongated the hyphae on CD–N medium containing thiamine but the differentiation of aerial hyphae and conidiation was inhibited and its mycelial colony was larger than that of the wild-type strain (data not shown).

Next, we tested whether ΔAoatg8-1-1 displays defects in autophagy. To visualize autophagy in ΔAoatg8-1-1, we constructed ΔAoatg8-1-1DR expressing DsRed2 in the cytoplasm. ΔAoatg8-1-1DR showed a phenotype similar to that of ΔAoatg8-1-1. During autophagy, DsRed2 expressed in the cytoplasm in CEDR1 was incorporated into autophagosomes and transported to vacuoles (Fig. 4D). By contrast, in ΔAoatg8-1-1DR, DsRed2 was homogeneously distributed in the cytoplasm under starvation conditions. This observation suggests that ΔAoatg8-1-1DR is defective in autophagy and AoAtg8 is essential for autophagy in A. oryzae.
Conidial germination of the Aoatg8 conditional mutant. Analysis of the phenotype of ΔAoaotg8-1-1 suggested that autophagy is involved in the formation of conidia and aerial hyphae. Moreover, the localization of EGFP-AoAtg8 during germination implied that autophagy is involved in conidial germination (Fig. 2 and 3). To investigate the role of autophagy in conidial germination, conidia were harvested from the CSC1-2-1 strain grown on a CD medium plate with 50 nM thiamine because the expression of AoAtg8 was decreased as much as possible. Conidia of CSC1-2-1 were cultured in liquid CD-N medium with or without thiamine, and the number of germ tubes formed was counted under a microscope (Fig. 5). The conidia started to germinate 4 h after inoculation. In the absence of thiamine, 65% of the conidia formed germ tubes by 8 h (Fig. 5, lower left panel). By contrast, when conidia were inoculated in CD-N containing thiamine, conidial germination was delayed: 40% of the conidia formed germ tubes by 8 h (Fig. 5, lower right panel). However, more than 90% of the conidia formed germ tubes 16 h after inoculation. Thus, conidial viability was not affected by the defect in autophagy. These data suggest that autophagy functions partially during the early stage of conidial germination.

DISCUSSION

Autophagy is a degradation process using vacuolar enzymes and primarily functions as a survival strategy during nutrient starvation and for the turnover of nonselective cytoplasmic contents. We isolated the A. oryzae ATG8 homologue Aoatg8 as a molecular marker and visualized autophagy in A. oryzae using fluorescent proteins. AoAtg8 is composed of a polypeptide of 118 amino acids, and its sequence is highly conserved among other eukaryotes, including humans and plants. In S. cerevisiae, the carboxy (C)-terminal glycine of Atg8 conjugates with phosphatidylethanolamine mediated by a ubiquitination-like system (5). This lipidation is essential for autophagy (10). In A. oryzae, AoAtg8 also has a conserved glycine residue at the C terminus, suggesting the evolutionary conservation of this conjugation system.

We visualized autophagy by expressing EGFP-AoAtg8 and DsRed2-AoAtg8 fusion proteins. Furthermore, we constructed complemented strains expressing EGFP-AoAtg8 in the ΔAoaotg8 mutants. In these strains, the phenotypes of the ΔAoaotg8 mutants were restored, and EGFP-AoAtg8 was localized in vacuoles under starvation conditions and during conidial germination (data not shown). Therefore, EGFP-AoAtg8
was functional and behaved in a manner similar to that of the native AoAtg8 protein. Our observations suggest that a similar system of autophagy takes place in the deuteromycete A. oryzae. In addition, we found that DsRed2 and EGFP-AoAtg8 were taken up into vacuoles in conidiophores, swollen conidia, and germlings under growth conditions. Thus, we propose that autophagy occurs during asexual differentiation and conidial germination in A. oryzae.

Under starvation conditions, DsRed2 expressed in the cytoplasm was taken up into vacuoles, indicating the induction of autophagy. Therefore, autophagy can be detected by using this visualization method much more conveniently than by using an electron microscope. AoAtg8 was localized in the vacuoles within 4 h, whereas DsRed2 fluorescence in the vacuoles was observed 12 h after the shift to starvation conditions. The reason for this time difference might be the increased time needed for the fluorescence intensity inside the vacuoles to exceed that in the cytoplasm in CEDR1 during autophagy.

In P. anserina, Δidi-7ΔPaATG8 mutants show defects in the differentiation of aerial hyphae and the protopherytbe, which is a female reproductive organ (27). By contrast, A. oryzae has no sexual reproduction, and conidia are the sole reproductive organs. Here, although the differentiation of aerial hyphae and conidiation in the ΔAoAtg8 mutants were defective, the mutants could grow on minimal medium without any nitrogen source and the mycelial colony was large compared with that of the control strain. This might have been due to a defect in the differentiation of aerial hyphae; vegetative hyphae searched for nutrients around the colony because the mutants could not develop aerial hyphae or conidiophores, which were induced by nutrient starvation. Therefore, unlike P. anserina, the defect in conidiation in A. oryzae ΔAoAtg8-1-1 prohibited it from producing progeny. Thus, the effect of the deletion of Aoatg8 was more severe in A. oryzae in extreme environments (such as limited nutrients) than in P. anserina.

In A. nidulans, brlA mutants develop abnormal conidiophore stalks that are 20 to 30 times longer than those of the wild type and give the colony a “bristle” phenotype (1). Moreover, chitin synthase chsA disruptants in A. nidulans form normal aerial hyphae but cannot form conidiophores (3). In the current paper, we show that a strain defective in autophagy (ΔAoatg8-1-1) failed to form aerial hyphae and that DsRed2 expressed in the cytoplasm in CEDR1 was taken up into the vacuole during conidiophore formation. Therefore, the defect in conidiation might have been due to defects in aerial hyphae or conidiophore stalk formation, rather than in conidiophore-specific cells, such as vesicles, phialides and metulae, suggesting that autophagy is involved in the development of both aerial hyphae and conidiophores.

Thus, the formation of aerial hyphae in filamentous fungi is regulated by various factors. Although the involvement of autophagy during the differentiation of aerial hyphae and conidiophores is not yet clearly understood, autophagy might take part in the reconstitution of intracellular components during aerial hypha and conidiophore formation. Furthermore, it is thought that cells that are not in contact with the medium might acquire nutrients through the recycling of intracellular components by autophagy.

In Neurospora crassa, conidial germination requires a carbon source and a salt (30). In A. nidulans, conidial germination occurs in the presence of a carbon source alone (21, 24). We examined germination in A. oryzae and observed that conidial germination of the wild type was induced on CD medium lacking a nitrogen source (CD–N). This suggests that the nitrogen source might be stored in the conidia for germination. Furthermore, we showed that on CD-N, conidial germination of CSC1-2-1 was delayed, suggesting that autophagy was at least partly involved in supplying a nitrogen source at an early stage of conidial germination. This result agrees with the localization of AoAtg8 and DsRed2 during conidial germination. In A. nidulans and Aspergillus fumigatus, the Ras signaling pathway, which plays an important role in the control of cell growth and response to nutrients, is involved in germination and autophagy (2, 4, 24). Furthermore, in S. cerevisiae, a relationship has been reported between the Ras signaling pathway and the Tor kinase (central negative regulator of autophagy) (29), which supports our data. Thus, the involvement of autophagy in conidial germination has been demonstrated for the first time in filamentous fungi.

How autophagy plays a role in differentiation and development is of interest and remains an important question. Because yeast is unicellular, an analysis of the relationship between autophagy and differentiation is difficult. Thus, we predict that investigations of the multicellular organism A. oryzae will be more useful than those involving yeast in elucidating an overview of autophagy.

ACKNOWLEDGMENTS

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