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Functional analysis of AoAtg11 in selective autophagy in the filamentous fungus *Aspergillus oryzae*

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ABSTRACT

Autophagy is a highly conserved cellular degradation process in eukaryotes and consists of both non-selective and selective types. Selective autophagic processes include pexophagy, mitophagy, and the cytoplasm-to-vacuole targeting (Cvt) pathway of yeast, in which particular vacuolar proteins, such as aminopeptidase I (Ape1), are selectively transported to vacuoles. Although selective autophagy has been mainly studied in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*, there is evidence for selective autophagy in filamentous fungi; however, the details are poorly understood. In *S. cerevisiae*, Atg11 is a selective autophagy-specific protein that recognizes and transports substrates to the pre-autophagosomal structure (PAS). Here, we first identified an *ATG11* homologue in the filamentous fungus *Aspergillus oryzae* and analyzed the localization of the corresponding protein, designated AoAtg11, fused to enhanced green fluorescent protein (EGFP). Imaging analysis revealed that AoAtg11–EGFP was localized to PAS-like structures. We next constructed an *Aoatg11* disruptant of *A. oryzae* and showed that AoAtg11 is involved in pexophagy and mitophagy. In addition, AoAtg11 was found to be dispensable for non-selective autophagy and for transporting AoApe1 to vacuoles. Taken together, these results suggest that AoAtg11 is a selective autophagy-specific protein in *A. oryzae*, and has distinct molecular functions from that of *S. cerevisiae* Atg11.

Introduction

Macroautophagy, or autophagy, is a highly conserved process that utilizes a membrane transport system to degrade unnecessary cellular proteins and organelles in vacuoles and lysosomes. In general, autophagy functions to recycle nutrients under starvation conditions to facilitate cell survival. However, autophagy also plays physiological roles in cell differentiation, cell cycle control, and suppression of neurodegeneration and cancer (Levine & Klionsky 2004; Shintani & Klionsky 2004; Huang & Klionsky 2007; Mizushima *et al.* 2008; Lee *et al.* 2012).

Among the different types of autophagy that have been characterized to date, non-selective (or simple) autophagy is most well studied. In this autophagic process, membrane elongation is initiated in the pre-autophagosomal structure (PAS), which is comprised of numerous autophagy-related proteins (Suzuki *et al.* 2001). The extended double membrane

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enwraps cellular components and forms vesicles called autophagosomes, which then fuse with the vacuolar membrane and release autophagic bodies into the vacuole, leading to their degradation.

Recently, several selective autophagic processes, such as pexophagy, mitophagy, and the cytoplasm-to-vacuole targeting (Cvt) pathway, which transport specific substrates to the vacuole, have been identified in mammals and yeasts. Selective autophagy is thought to play important roles in quality control and maintenance of the cellular environment by finely regulating the amount of proteins and organelles.

Pexophagy (macropexophagy) was first observed in *Pichia pastoris* as a process that selectively degrades peroxisomes in the vacuole. The receptor protein involved in pexophagy is PpAtg30 in *P. pastoris* and Atg36 *Saccharomyces cerevisiae* (Nazarko *et al.* 2009; Motley *et al.* 2012). In addition, the adaptor protein Atg11, which is a component of PAS and is found in both organisms, is required for transportation of peroxisomes to the PAS.

Mitophagy is a selective autophagic process for the degradation of mitochondria and functions by a similar mechanism to macroautophagy, in which mitochondria are engulfed by the autophagosome membrane and transported to the vacuole. As mitochondria produce not only adenosine triphosphate, but also reactive oxygen species (ROS), malfunctioning mitochondria must be effectively eliminated to avoid cellular damage. In *S. cerevisiae*, such mitochondria are recognized by a receptor protein, mitochondrial outer membrane protein Atg32, which binds to Atg11 (Kanki *et al.* 2009).

The Cvt pathway selectively delivers the vacuolar enzymes aminopeptidase Ape1 and α-mannosidase Ams1 from the cytoplasm to the vacuole independently of the secretory pathway (Yoshihisa & Anraku 1990; Klionsky *et al.* 1992). Genetic analysis has revealed that many genes required for the Cvt pathway overlap with autophagy-related genes (Klionsky *et al.* 2003), indicating that the Cvt pathway and autophagy utilize common molecular mechanisms. This speculation is supported by the observation that Ape1 is also sequestered by autophagosome-like structures and is fused to the vacuole. In the Cvt pathway, Ape1 is recognized by the receptor protein Atg19, and the resulting complex is transported to PAS by Atg11. In contrast to autophagy, which is induced by starvation, the Cvt pathway is constitutively active, even under nutrient-rich conditions (Lynch-Day & Klionsky 2010).

In filamentous fungi, such as *Aspergillus oryzae*, numerous genes involved in non-selective autophagy have been identified and analyzed (Voigt & Pöggeler 2013; Shoji *et al.* 2014). In contrast, although selective autophagy is also speculated to occur in filamentous fungi (Shoji *et al.* 2010; Yanagisawa *et al.* 2013), only a few specific genes related to this process have been identified (Asakura *et al.* 2009; He *et al.* 2013), and details of the mechanism remain unknown. In the present study, we identified a homologue of the yeast adaptor gene *ATG11* in *A. oryzae* and showed that the corresponding protein, AoAtg11, is involved in both pexophagy and mitophagy.

Materials and methods

Strains and growth media

The *Aspergillus oryzae* strains used in this study are listed in Table 1. The *A. oryzae* wild-type strain RIB40 was used as a DNA donor, and strain NSRku70-1-1 was used to disrupt the *Aoatg11* gene. Strain NSRku70-1-1 transformed with *adeA* (NSRku70-1-1A) was used as a control for phenotypic analysis. 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 *Aoatg11* gene. 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 Δ*Aoatg11* disruptants expressing EGFP-AoAtg8, EGFP-PTS1, AoCit1-EGFP, and AoApe1-EGFP. CD + m medium lacking sodium nitrate (CD + m−N) was used for inducing non-selective autophagy. CD(O) [0.3 % $NaNO_3$, 0.2 % KCl, 0.1 % KH_2PO_4, 0.05 % $MgSO_4{\cdot}7H_2O$, 0.002 %

Table 1 – *Aspergillus oryzae* strains used in this study.

Strain name	Genotype	Reference
RIB40	Wild type	Machida *et al.* 2005
NSRKu70-1-1	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB*	Escaño *et al.* 2009
NSRKu70-1-1A	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB adeA*	Escaño *et al.* 2009
PaAtg11G	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB adeA PamyB*::*Aoatg11-egfp*::*niaD*	This study
PaAtg11G-PaRAtg8	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB adeA PamyB*::*Aoatg11-egfp*::*niaD* *PamyB*::*mdsred-Aoatg8*::*AosC*	This study
ΔAtg11-3	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB* Δ*Aoatg11*::*adeA*	This study
ΔAtg11-5	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB* Δ*Aoatg11*::*adeA*	This study
PA8GAtg8	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB adeA PAoatg8*::*egfp-Aoatg8*::*niaD*	This study
ΔAtg11-PA8GAtg8	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB* Δ*Aoatg11*::*adeA PAoatg8*::*egfp-Aoatg8*::*niaD*	This study
PC1Cit1G	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB adeA PAocit1*::*Aocit1-egfp*::*niaD*	This study
ΔAtg11-PaCit1G	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB* Δ*Aoatg11*::*adeA PamyB*::*Aocit1-egfp*::*niaD*	This study
PfGPTS1	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB adeA PAofox2*::*egfp-pts1*::*niaD*	This study
ΔAtg11-PfGPTS1	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB* Δ*Aoatg11*::*adeA PAofox2*::*egfp-pts1*::*niaD*	This study
PaApe1G	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB adeA PamyB*::*Aoape1-egfp*::*niaD*	This study
ΔAtg11-PaApe1G	*niaD*⁻ *sC*⁻ *adeA*⁻ *argB*⁻ Δ*ku70*::*argB* Δ*Aoatg11*::*adeA PamyB*::*Aoape1-egfp*::*niaD*	This study