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ORIGINAL PAPER

Identification and characterization of two rice autophagy associated genes, OsAtg8 and OsAtg4

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Abstract Autophagy is an intracellular process for vacuolar degradation of cytoplasmic components. The molecular machinery responsible for yeast and mammalian autophagy has begun to be elucidated at the cellular level. A genome-wide search revealed significant conservation among autophagy genes in yeast and *Arabidopsis*. Up till now, however, there is no report about rice autophagy associated genes. Here we cloned *OsAtg8* and *OsAtg4* from *Oryza sativa* and detected their expression patterns in various tissues. Immunoblotting analysis showed that carboxyl terminus of OsAtg8 can be cleaved in yeast cell. Mutation analysis revealed that the conserved Gly117 residue of OsAtg8 was essential for its characteristic C-terminal cleavage as similar to that found in mammalian and yeast Atg8. We further proved that OsAtg8 interacted with OsAtg4, and this interaction was not affected by the conserved Gly117 mutation. Our results demonstrate that Atg8 conjugation pathway is conserved in rice and may play important roles in rice autophagy.

Keywords Autophagy · Cleavage · Interaction · *OsAtg8* · *OsAtg4*

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Introduction

Autophagy is a highly regulated process for bulk degradation of proteins and organelles, and it has been shown to be essential for differentiation and development as well as for cellular maintenance [1]. With the advantages of yeast genetics, the molecular components of autophagy have been revealed and the autophagy is well characterized in yeast [2–6]. An essential protein in the yeast autophagic pathway is Atg8. After a series of post-translational modification including the characteristic C-terminal cleavage, Atg8 is covalently conjugated to phosphatidylethanolamine (PE) and localizes to autophagosome membranes, which promotes the formation of autophagosome and their delivery to the vacuole for subsequent degradation [6–11]. One of the proteins involved in the post-translational modification of yeast Atg8 is Atg4, which is a cysteine protease that cleaves off the C-terminal amino acid of Atg8 to expose a C-terminal glycine [11]. The C-terminal glycine of yeast Atg8 is strictly conserved in all mammalian and *Arabidopsis* homologues [12–15].

For plant, protein degradation is important to adapt to various severe environmental conditions, such as nutrient deprivation [16]. A recent genome-wide search revealed significant conservation among autophagy genes (Atgs) in yeast and *Arabidopsis*, indicating that the molecular basis of autophagy is well conserved in yeast and plants. In *Arabidopsis*, 25 Atg genes that are homologous to 12 of yeast Atg genes were found [15, 17–19]. Among them, AtAtg7 and AtAtg9 have been shown to be involved in *Arabidopsis* autophagy [17], AtAtg8 and AtAtg4 are essential for *Arabidopsis* autophagy [18–21]. Up till now, however,

there are no reports about rice autophagy associated genes.

In this report, we isolated *OsAtg8* and *OsAtg4*, two autophagy associated genes in rice, and detect their expression patterns in several tissues. Immunoblotting analysis showed that carboxyl terminus of OsAtg8 can be cleaved in yeast cell, but mutation of Gly-117 into Ala of OsAtg8 results in the abrogation of C-terminal cleavage. We further proved that OsAtg8 interacted with OsAtg4, and this interaction was not affected by the conserved Gly117 mutation. Our results demonstrate that Atg8 conjugation pathway is conserved in rice and may play important roles in rice autophagy.

Materials and methods

Plant materials

Rice (*Oryza sativa*, cv Zhenxian 97B) was grown under white fluorescent light (wavelength 390–500 nm, 150 μEm-2s-1, 12 h photoperiod) at 25°C and 70% relative humidity. Young leaves, young roots and leaf sheaths were prepared from 2-week-old seedlings, and mature leaves, mature roots and spikes were taken from mature plants grown in the field. All materials were frozen in liquid nitrogen and stored at –80°C before the operation.

Semi-quantitative RT-PCR analysis

Total RNAs were extracted from mature leaves, young leaves, mature roots, young roots, leaf sheaths and spikes using TRIZOL reagent (GIBCO BRL, USA). First-strand cDNA was generated using Superscript II reverse transcriptase (Invitrogen). Two pairs of primers (named OsAtg8-a/b and OsAtg4-a/b; see Table 1) were designed for OsAtg4 and OsAtg8 tissue expression pattern analysis. The templates were amplified at 95°C for 5 min, followed by 28 cycles of amplification (94°C for 30 s, 58°C for 30 s, 72°C for 30 s), then 72°C for 10 min. The rice *Actin* gene (Genebank Acc. No.: *X16280*) using specific primers Actin-a/b (Table 1) was also amplified as control. PCR products were analyzed on 1% agarose gels.

Plasmid construction and site-directed mutagenesis

We subcloned OsAtg8 to pGBKT7 vector (Clontech) by adding a 6 × His tag (CATCATCACCATCACCAT) to their C terminus using the primers pGBKT7-OsAtg8-a and pGBKT7-OsAtg8-b. To construct pGBKT7-OsAtg8-G117A-His, we used site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit was from Stratagene) according to the manufacturer's protocol using desired mutation primers and templates. The full-length ORF of ATG4 was amplified by PCR and subcloned into pGADT7 vector (Clontech). All names of the primers and the mutants of amino acids are given in Table 1. All plasmids were confirmed by sequencing.

Yeast transformation and western blotting

pGBKT7-OsAtg8-His and pGBKT7-OsAtg8-G117A-His were introduced to the *Saccharomyces cerevisiae* strain AH109 via the lithium acetate transformation method according to the manufacturer's instructions. The transformant was grown at 30°C in 5 ml SD media

Table 1 Nucleotide sequences of primers used for the cloning and expression analyses of OsATG8, OsATG4 and the construction of wild type and mutant OsATG8, OsATG4 cDNAs

Primers	Sequences
cDNA cloning and RT-PCR analysis primers	
OsATG8-a	5′-TCGATCGGTTGGAGATGG-3′
OsATG8-b	5′-GATGAATTACGCAGAGCCG-3′
OsATG4-a	5′-GCACACGTGCGTTGTTAG-3′
OsATG4-b	5′-GCAACGTTTTCTGTACCTC-3′
OsATG4-c	5′-GTTGCTGCTCAGCTTTGCT-3′
OsATG4-d	5′-GATGTTGCCATCCCCTGAG-3′
Actin-a	5′-CCATTGGTGCTGAGCGTT-3′
Actin-b	5′-TAGGAATGGAAGCTGCGGG-3′
Plasmid construction primers	
pGBKT7-OsATG8-a	5′-GG**CCATGG**AGATGGCCAGGACTTC-3′
pGBKT7-OsATG8-His-b	5′-GC**GTCGAC**TTA*ATGGTGATGGTGATGATG*CGCAGAGCCGAATG-3′
pGADT7-OsATG4-a	5′-GC**GAATTC**ATGACGAGCTTGCCTG-3′
pGADT7-OsATG4-b	5′-GC**CTCGAG**TCACTAAAGAATCTGCC-3′
pGBKT7-OsATG8-G117A-His-a	5′- CATGACTTACAGTGGCGAGAACACATTCGCCTCTGCG-3′
pGBKT7-OsATG8-G117A-His-b	5′- CGCAGAGGCGAATGTGTTCTCGCCACTGTAAGTCATG-3′

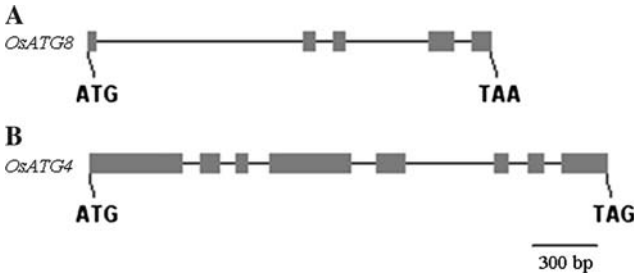

Fig. 1 Genomic structure of *OsAtg8* and *OsAtg4*. (**A**) The genomic structure of *OsAtg8*. (**B**) The genomic structure of *OsAtg4*. Exons are denoted as gray boxes, introns are shown as straight horizontal lines

lacking both tryptophan and leucin with shaking (250 rpm). Yeast whole-cell lysates were prepared by breaking the cells in 0.2 M NaOH and 1% (v/v) 2-mercaptoethanol. Then proteins were precipitated by addition of trichloroacetic acid; and after centrifugation, samples were washed with cold acetone and resuspended for immunoblotting analysis [15]. The proteins were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with phosphate-buffered saline containing 0.2% Tween 20 and 5% non-fat dry milk for 1 h, the membrane was probed with a specific mouse anti-Myc antibody (1:1000) or anti-His antibody (1:1000) for 2 h and then washed and exposed to horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (1:10000) for 1 h. The bound antibodies were visualized with a Photope-HRP Western detection kit according to the manufacturer's instructions (Cell Signaling Technology).

Yeast two-hybrid interaction analysis

pGADT7-OsAtg4 and pGBKT7-OsAtg8-His or pGBKT7-OsAtg8-G120A-His were co-transformated into AH109 as above. Transformants were growth on

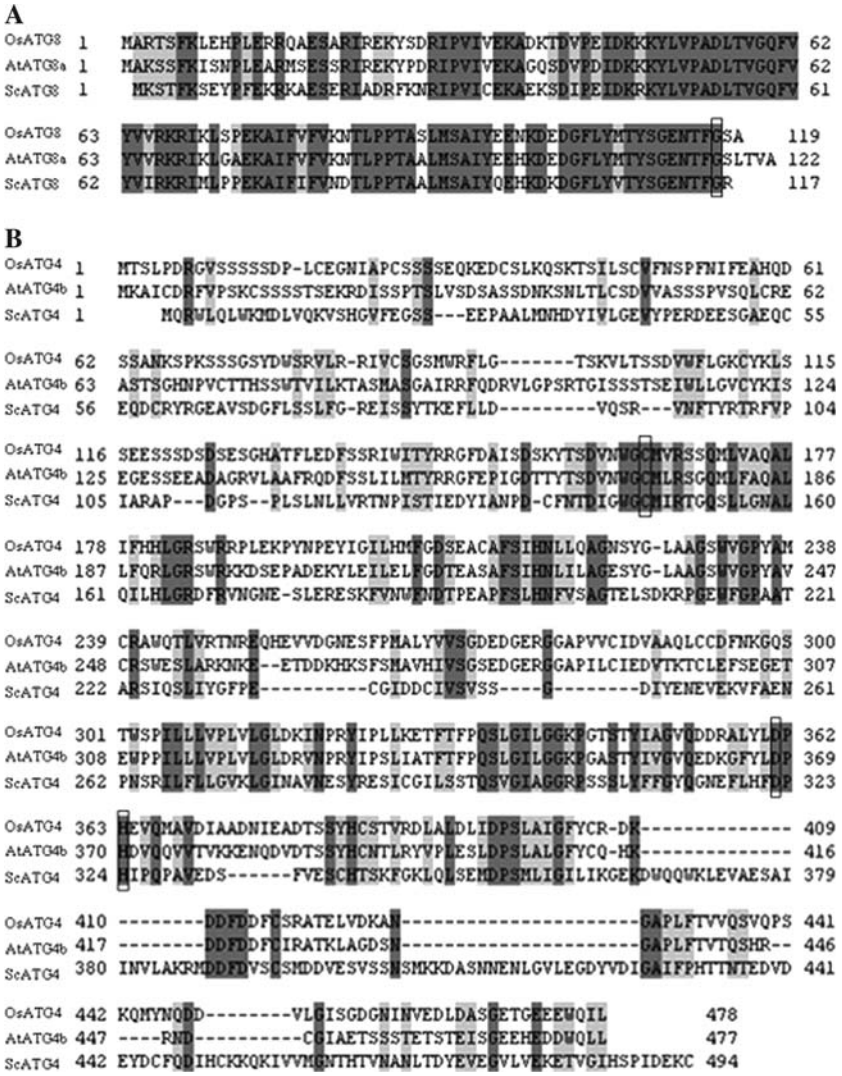

Fig. 2 Sequence comparison of OsAtgs and their homologues. (**A**) Sequence comparison of OsAtg8 (*ABB77258*) and its *Arabidopsis* (*NP_567642*, 84% identity), yeast (*AAT92889*, 73% identity) homologues; (**B**) Sequence comparison of OsAtg4 (*ABB77259*) and its *Arabidopsis* (*BAB88384*, 56% identity), yeast (*P53867*, 26% identity) homologues. Identical residues are shaded in black, similarity residues are shaded in gray. The conserved residues are boxed

SD media lacking both tryptophan and leucine at 30°C for 72 h. Interactions between BD and AD constructs were then assessed by selection upon media additionally lacking both adenine and histidine. The addition of 40 μM *x*-α-gal (Glycosynth, Warrington, UK) to the media also allowed the activity of α-galactosidase to be assessed via production of a blue precipitate. Yeast selection was performed over a period of 72 h at 30°C.

Results and discussion

Identification and isolation of OsAtg8 and OsAtg4

In this report, we use the amino acid sequence of AtAtg8a (GenBank acc. No.: *NM118319*) and AtAtg4b (GenBank acc. No.: *AB073171*) to search the rice nr database (http://www.ncbi.nlm.nih.gov), and two cDNA sequences were obtained, respectively. Two pairs of primers (named OsAtg8/4-A and B; see Table 1) were designed based on two cDNA sequences and used for PCR amplification, cDNAs reverse transcribed from rice total RNAs used as templates. The PCR products were cloned into the pGEM-T vector (Promega) and sequenced using the BigDye terminator sequencing kit and ABI377 sequencer (PerkinElmer Life Sciences) according to the manufacturer's instructions. According the *Arabidopsis* homologues, the two novel cDNAs were named *OsAtg8* and submitted to GenBank (GenBank acc. No.: *DQ269983*) and *OsAtg4* (GenBank acc. No.: *DQ269984*), respectively. *OsAtg8* localizes to chromosome 7 and consists of five exons and four introns (Fig. 1A). Sequence comparison indicates that OsAtg8 shows 84% and 73% amino acid identities with the *Arabidopsis* and yeast homologues, respectively, and all members contain the conserved C-terminal Gly residue (Fig. 2A). *OsAtg4* localizes to chromosome 4 and consists of eight exons and seven introns (Fig. 1B). OsAtg4 shows low similarity to yeast and *Arabidopsis* Atg4, but the Cys-164, Asp-361 and His-361 of OsAtg4 are strictly conserved in all homologues (Fig. 2B), which constitute the conserved geometry observed in the canonical catalytic triad of cysteine protease [22]. These results demonstrated the autophagy associated genes are conserved in rice.

Expression analysis

Multi-tissue RT-PCR was performed to examine the tissue distribution of *OsAtg8* and *OsAtg4*. As showed in Fig. 3, *OsAtg8* and *OsAtg4* could be detected from mature leaves, young leaves, mature roots, young roots, leaf sheaths, and spikes which indicated they are constitutive expressed gene and maybe have important role in rice.

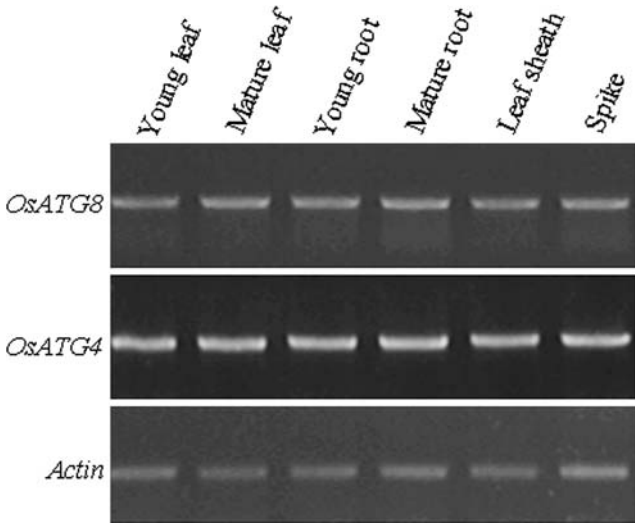

Fig. 3 Expression pattern analyses of *OsAtg8* and *OsAtg4* in various tissues. Multi-tissue RT-PCR was performed to examine the tissue distribution of *OsAtg8* and *OsAtg4*. The tissues are indicated above the panels and rice *actin* gene was used as an internal control to show the consistent amount of beginning RNA for RT-PCR

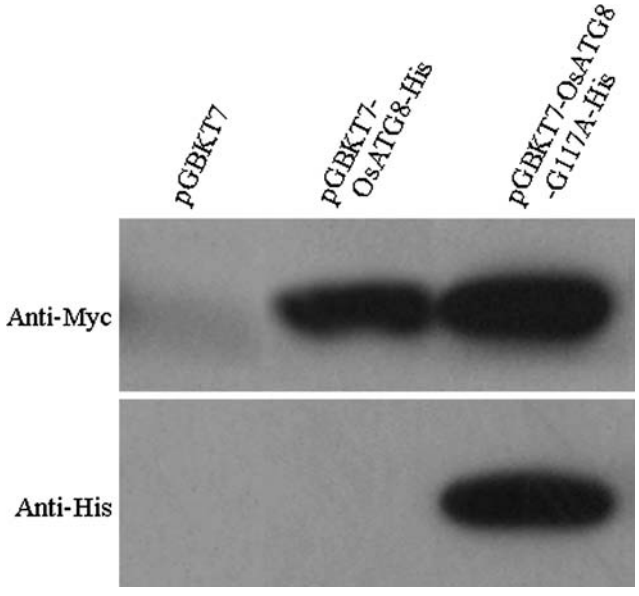

Fig. 4 Expression of OsAtg8 and its mutant in yeast. Yeast AH109 was transformed with expression vectors pGBKT7 (lane 1), pGBKT7-OsAtg8-His (lane 2) or pGBKT7-OsAtg8-G117A-His (lane 3), respectively. Cell lysates were analyzed by Western blotting with anti-Myc antibodies or anti-His antibodies

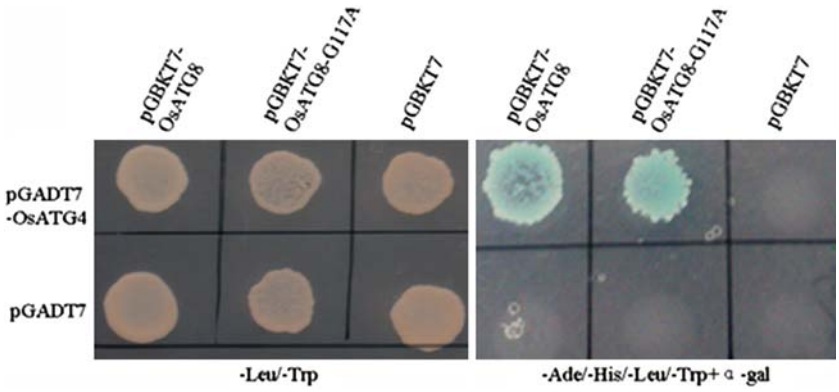

Fig. 5 OsAtg8 and its mutant interact with OsAtg4 in a yeast two-hybrid analysis. Yeast strain AH109 co-transformed with the indicated pGBKT7 and pGADT7 plasmids. Yeast was grown on the -Leu-Trp media (**A**) or -Leu/-Trp/-His/-Ade addition of 40 μM *x*-α-gal media (**B**)

Expression of OsAtg8 and its mutant in yeast

Carboxyl terminus of mammalian LC3, which is the homologues of yeast Atg8, can be cleaved by a cysteine protease—ATG4, and the cleavage leads to the exposition of Gly120 [22–25]. To investigate whether OsAtg8 also undergoes the C-terminal cleavage in yeast cells, we constructed pGBKT7-OsAtg8-His, which produced fusion proteins containing an N-terminal c-Myc epitope tag and a C-terminal 6×His tag. When pGBKT7-OsAtg8-His protein was expressed in yeast, a band was recognized by immunoblotting with anti-Myc antibody, but not with anti-His antibody (Fig. 4, lane 2). These results demonstrated that the C-terminal His6 tag was removed from fusion proteins upon expression and the C-terminal cleavage occurred in OsAtg8, consistent with the observation in mammalian LC3 [12]. Yeast Atg8 and OsAtg8 may have similar structure and the C-terminal of OsAtg8 was hydrolyzed by yeast Atg4 because AH109 is not an Atg4 deficient strain.

The only known residue for post-translational modifications of yeast Atg8 and rat LC3 is the conserved Gly-120, which is conserved in OsAtg8 (Fig. 2A). It has been shown that mutation of Gly-120 into Ala of LC3 resulted in the abrogation of a serial of post-translational modifications [11, 12]. To clarify in a straightforward manner whether cleavage of the carboxyl terminus of OsAtg8 in yeast requires Gly117, we constructed pGBKT7-OsAtg8-G117A -His to determine the effect of this mutation on their processing. Expression of mutant pGBKT7-OsAtg8-G117A-His, in which Gly117 of OsAtg8 were changed to Ala, resulted in a single band that was recognized by both anti-Myc and anti-His antibodies (Fig. 4, lane 3), which is in agreement with the obligatory role of conserved Gly-117 residue for the C-terminal cleavage of OsAtg8. These results demonstrated that the Atg8 post-translational modification pathway is conserved in rice.

OsAtg8 and its mutant interact with OsAtg4 in a yeast two-hybrid analysis

Atg4 interacts with Atg8 and hydrolyzes its C-terminal in yeast, a direct interaction between their rice homologues would strengthen the hypothesis that both rice genes are in the autophagy pathway. Mutation of Gly-117 into Ala of OsAtg8 resulted in the abrogation of its C-terminal cleavage, but we did not know whether this mutation affected the interaction between OsAtg4 and OsAtg8. To identify any interaction between OsAtg4 and OsAtg8 or its mutants, we performed a two-hybrid assay. As showed in Fig. 5, Atg4 interacts with Atg8 and its mutants, indicating the conserved Gly117 mutation does not affect this interaction. This cleavage reaction may include two-step mechanism involving the recognition of the OsAtg8 core and the subsequent recognition of the OsAtg8 tail by OsAtg4. The tail region of OsAtg8 is crucial for hydrolysis, but it is not indispensable to the interaction between the core region of OsAtg8 and OsAtg4.

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