A rice autophagy gene OsATG8b is involved in nitrogen remobilization and grain quality

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SUBJECT AREAS
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Abstract

Background: Enhancing nitrogen (N) use efficiency is a potential way of reducing excessive nitrogen application and increasing yield. Autophagy is a conservation degradation system in the evolution of eukaryotic cells and plays an important role in plant development and stress response. Autophagic cores are two conjugation pathways that attach ATG8 to PE and ATG5 to ATG12, which then help with vesicle elongation and enclosure. Rice has six ATG8 genes, which have not been functionally confirmed so far. Results: Autophagy activity of OsATG8b was confirmed through the complementation of the yeast autophagy defective mutant scatg8 and by observation of autophagosome formation in rice. The autophagy activity is higher in OsATG8b-OE lines and lower in OsATG8b-RNAi than that in ZH11. 15N pulse-chase analysis revealed that OsATG8b-OE plants conferred higher N recycling efficiency to grains, while OsATG8b-RNAi transgenic plants exhibited lower N recycling efficiency and poorer grain quality. Conclusion: Autophagic role of OsATG8b was experimentally confirmed and OsATG8b-mediated autophagy is involved in N recycling to grains and contributes to the grain quality, indicating OsATG8b may be a potential gene for molecular breeding and cultivation of rice.

Background

Nitrogen (N) is one of the most limiting nutrients for crop yield. Increasing N utilization efficiency (NUE) is not only important for increasing yield and reducing production cost, but also for avoiding environmental pollution and keeping sustainable agriculture [1, 2]. Therefore, it is very important to find effective genes to improve NUE and yield. Plant N utilization involves complex mechanisms of
absorption, translocation, assimilation and remobilization. Of those steps, N remobilization plays an important role during seed filling [2, 3]. At the vegetative stages, most N uptake is directed to leaves, in which most proteins are synthesized. During the reproductive stage, leaf proteins degrade rapidly to amino acids and small peptide, which are transported to seeds [3]. N remobilization of cereals in senescent leaves accounted for 50-90% of the grain N content [4]. 26S proteasome/ubiquitin system and autophagy are two main pathways of protein degradation [5, 6]. Autophagy can degrade proteins, bulk organelles and cytosolic macromolecules with low selectivity and high throughput [7].

Autophagy is a conservative degradation system in the evolution of eukaryotic cells. In the process of autophagy, the cytoplasm and organelles are separated by bilayer vesicles called autophages and transported to vacuoles of yeast and plant cells or lysosomes of animal cells for degradation and recycling [8-10]. More than 30 autophagy-related genes (ATGs) have been identified in yeast, and 17 of which are necessary for autophagy formation [10, 11]. Recently, orthologs of most yeast core ATG genes have been found in Arabidopsis and rice [12-16]. ATG8 is one of the core proteins for forming autophagosome. It covalently binds to membrane lipid phosphatidylethanolamine (PE) through ubiquitin-related binding system [11]. ATG8 is a scaffold for membrane expansion and elongation during autophagosome formation [17, 18]. Yeast ATG8 also participates in the cytoplasm-to-vacuole targeting (CVT) pathway. Vacuole hydrolases, such as the precursor of aminopeptidase 1 (APE1), are selectively transported into the vacuole to produce mature APE1 [19]. Unlike yeast with a single copy of ATG8 gene, plants usually have a ATG8 family, such as nine genes in Arabidopsis [12], five in maize [20], and six in rice [16]. The different expression patterns of Arabidopsis ATG8s suggest that some
ATG8s possess functional diversity besides possible redundancy [21]. Like yeast and animals, plant autophagy plays an important role in nutrient recycling under N- and C-starvation conditions [9, 22]. Currently, research on autophagy often focus on the remobilization of N [23-26]. Most Arabidopsis ATG genes are up-regulated by N-starvation and during leaf senescence [13, 27]. Loss function of Arabidopsis autophagy (atg5, atg7, atg10 and atg13a atg13b) caused hypersensitive to N-limiting conditions in Arabidopsis, and accelerated senescence even under N-rich conditions [14, 28, 29]. Overexpression of AtATG8f and GmATG8c made Arabidopsis more tolerant to both N- and C-starvation [21, 24]. Autophagy mutants of Arabidopsis and maize (atg5 and atg7 in Arabidopsis and atg12 in maize) showed reduced seed yield, seed N content, and N remobilization efficiency (NRE) [23, 25, 26]. About 50% of remobilized N of Arabidopsis is proven to come from autophagy [23]. These researches showed that autophagy plays a central role in N remobilization.

Since the contribution of autophagy to plant physiology largely comes from the study of Arabidopsis, little is known about crop autophagy except maize. Rice is an important cereal crop for world population, especially in Asia. Currently, little is known on the contribution of autophagy to rice productivity. Only rice OsATG7 plays a role in NUE at the vegetative stage [30]. However, the male sterility of osatg7 limits research on autophagy-mediated N recycling to grains in rice.

In our study, we functionally analyzed OsATG8b in rice. Complementation of a yeast atg mutant and subcellular localization analysis demonstrated the role of OsATG8b on autophagy process. In addition, we characterized the OsATG8b role in N remobilization by generating transgenic plants with over-expression and knockdown of OsATG8b. Phenotypic and $^{15}$N-partitioning analysis showed that OsATG8b plays a
role in N remobilization and grain quality. This result may provide strategic
guidance for N application in rice molecular breeding and production.

Methods

**Plant materials and growth conditions**

From spring to autumn, the *japonica* rice cultivar Zhonghua11 (ZH11) and transgenic plants were grown in a controlled paddy and permitted by the South China Botanical Garden. In winter, they were grown in a greenhouse at 28 °C for a 14-h (light) and 10-h (dark) per day. Seedlings were grown in the nutrient solution of International Rice Research Institute (IRRI).

**Quantitative real-time RT-PCR (qRT-PCR)**

Rice total RNA isolation, cDNA synthesis, qRT-PCR were performed as previously description [16]. Relative gene expression was normalized to the expression level of e-EF-1a with triplicate repeat. All primers are listed in Table S1.

**Complementation of yeast scatg8 mutants**

OsATG8b cDNA was cloned downstream of promoter GAL1 of the yeast vector pYES260. Wild-type yeast K Vy55 and the scatg8 mutant K Vy5 (MATa leu2 ura3 trp1 lys2 his3 suc2-Δ9Δatg8::HIS3) were gifts from Dr. Yoshinori Ohsumi (Tokyo Institute of Technology, Japan). The vector was transformed into scatg8 according to the LiAc/SS-DNA/PEG TRAFO protocol. Yeast were cultured and shaked at 30 °C in SC medium supplemented with 0.67% [w/v] YNB (yeast N base without NH₄SO₄ and amino acids), 2% (w/v) galactose, 0.5% (w/v) NH₄SO₄, and Ura DO Supplement. When the yeast grew to the logarithmic metaphase of growth (OD₆₀₀ = 1), yeast cells were centrifugal collected, washed and incubated for another 5h in 0.67% YNB
medium without amino acids, galactose and NH$_4$SO$_4$ for nutrient deprivation to induce autophagy. The collected cells were used for immunoblotting with anti-APE1 antibody, and immunoblot analysis process were followed the description [31].

**Scanning electron microscopy (SEM)**

Seeds were sputter-coated with gold/palladium in six different 30-s pulses (Hitachi JEE–420) and analyzed by scanning electron microscope (Hitachi S-3000N).

**Subcellular localization of OsATG8b protein fused with green fluorescent protein (GFP) derivatives**

*GFP-OsATG8b* and *sGFP-OsATG8b* were constructed to analyze the subcellular localization of OsATG8b in yeast and rice respectively. For yeast subcellular localization, the fused construct was inserted downstream of promoter *GAL1* in pYES260 vector. For rice subcellular localization, the fused construct was inserted downstream of *35S* promoter in pCAMBIA1301 [32]. For root imaging, 7-d seedlings were treated with 1 μM concanamycin A for 6 h at 28°C in darkness and the 5 mm root from the root tip were cut for observation. The GFP fusion protein was analyzed by confocal laser scanning microscope (ZEISS-710 Meta). The images presented are average projections of 8-20 optical sections.

**Generation of OsATG8b-overexpression and -RNAi transgenic plants**

*OsATG8b* cDNA was cloned into downstream of *35S* promoter in pCAMBIA1301 for overexpression. For construction of the RNAi vector, a 230-bp fragment was amplified with primers *OsATG8b*-Ri-F and *OsATG8b*-Ri-R and inserted in vector pTCK303 [33]. These vectors were transformed into *A. tumefaciens* EHA105 and then introduced into ZH11.

**Antibodies**

Antibodies of OsATG8b were made with 6 x His-OsATG8b proteins as antigen, which
were purified using a Ni column (Novagen) and injected directly into rabbits by the Beijing ComWin Biotech Co., Ltd.

**Protein extraction and immunoblot analysis**

Two weeks old seedlings were used for total cell extracts, and were ground in liquid N. The powders were extracted with the lysis buffer (25mM Tris-HCl pH7.5, 1mM EDTA, 1% Triton X-100, 150mM NaCl and Complete Protease Inhibitor Cocktail from Roche). The solution was then centrifuged at 13,000g for 20 min at 4°C, and the supernatant was used as total protein. The supernatant were run by SDS-PAGE with or without 6 M urea, and then transferred to NC membranes for immunoblot analysis. The membranes were blocked and then incubated with mouse GFP antibodies (Santa cruz) at a dilution of 1:1000, while rabbit serum of OsATG8b was diluted by 1:500.

**¹⁵N-labeling and determination of ¹⁵N content**

Rice plants were grown in IRRI solution in a greenhouse with a 16h (light) and 8h (dark) cycling. At 40 days after germination (DAG), plants were labeled with ¹⁵N for 5 d by adding 10 atom% excess Na¹⁵NO₃ to the IRRI solution. Then the plants were washed thoroughly with distilled H₂O, and transferred in the field for further growth.

Two-d ¹⁵N labeled plants were harvested 2 d for ¹⁵N uptake measurements. The ¹⁵N labeled plants were further grown in the field to maturity, and grains and remains were separated for N recycling assess. A dry-weight (DW) of each sample was assayed for ¹⁵N and total N content using isotope ratio mass spectrometer 100. ¹⁵N content of each sample was calculated as a % of total N, which was calculated as atom% or A%_{sample} = 100 \times (¹⁵N) / (¹⁵N + ¹⁴N) [26].

**NUE and N recycling efficiency (NRE) calculations**
Factors of calculation for NUE and NRE were followed description [23, 26]. The HI (harvest index) for evaluation yield was defined as the $DW_{\text{grain}} / (DW_{\text{remain}} + DW_{\text{grain}})$. N harvest index (NHI) for assess grain filling with N was calculated as

$$N\%_{\text{grain}} \times DW_{\text{grain}} / (N\%_{\text{remain}} \times DW_{\text{remain}} + N\%_{\text{grain}} \times DW_{\text{grain}}).$$

NUE was then calculated as the NHI/HI ratio, and NUE of different genotypes was compared. The efficiency of N recycling to grains was showed by $^{15}$NHI ($^{15}$N harvest index), which was caculated by

$$(A\%_{\text{grains}} \times N\%_{\text{grains}} \times DW_{\text{grains}}) / [(A\%_{\text{remain}} \times N\%_{\text{remain}} \times DW_{\text{remain}}) + (A\%_{\text{grains}} \times N\%_{\text{grains}} \times DW_{\text{grains}})].$$

The $^{15}$NHI:HI ratio was used to compare NRE of different transgenic plants.

**Quantification of soluble proteins**

Total protein concentration and starch content were determined as description [34,35].

**Results**

**OsATG8b restores autophagy activity in yeast scatg8 mutant**

Six OsATG8s have been identified in the rice genome [16]. The ATG8 phylogenetic tree generated from amino acid sequences showed that plant ATG8s are clustered into two main subgroups. Subgroup I cover the most of the plant $ATG8$ family members, comprising OsATG8a, b and c. Subgroup II covers 1–3 plant $ATG8$ family members from each species, containing OsATG8d, e and f (Fig. S1). The existence of two subgroups may imply specific functions to each, besides possible redundancy. OsATG8b is encoded by a single gene (Os04g0642400) in rice. It is a soluble protein of 119 amino acids, with a predicted pl of 8.78. OsATG8b shares 81.8% amino acid identity with yeast ScATG8, 71.4% identity with human HsGABARAP, and 86.9%
identity with *Arabidopsis* AtATG8a (Fig. S2a). Like other ATG8 proteins, OsATG8b has a conserved gly residue at the C-terminus for PE-conjugation (Fig. S2a). A 3D model prediction revealed that corresponds to the functional domains of yeast ScATG8, OsATG8b also contains a C-terminal ubiquitin-like domain and an N-terminal helical domain and two characteristic hydrophobic pockets named the W-site and the L-site (Fig. S2a and b) like yeast ScATG8 [36]. This implies that OsATG8b may have the autophagic function, similar to yeast ScATG8.

To verify the autophagic function of *OsATG8b*, we investigated whether *OsATG8b* rescues defects of *ATG8*-deficient (*scatg8*) yeast KVY5 [37]. *OsATG8b* cDNA containing the entire ORF was driven by the yeast GAL1 promoter in a plasmid (pYES260) and expressed in *scatg8* yeast. *OsATG8b* can rescue the growth of the *scatg8* yeast cells under N starvation (Fig. 1a). In yeast, the precursor aminopeptidase1 (prAPE1) was delivered to the vacuole for processing into mature APE1 (mAPE1) through the Cvt/autophagy pathway [19]. Thus, we monitored the protein levels of both prAPE1 and mAPE1 after 5 h of starvation. Both wild-type yeast and *scatg8* cells complemented with *OsATG8b* accumulated mAPE1. In contrast, mAPE1 was detected in neither *scatg8* cells nor the *scatg8* cells transformed with the empty vector (Fig. 1b). This suggests that prAPE1 was delivered to the vacuole and processed to mAPE1. These results confirmed the autophagy role of *OsATG8b* and showed that *OsATG8b* is a functional homologue of yeast ScATG8.

**OsATG8b expression is induced by N- and C-starvation**

To determine the spatial and temporal expression pattern of *OsATG8b*, we employed qRT-PCR and the GUS reporter assay to examine *OsATG8b* expression. qRT-PCR analysis showed that *OsATG8b* transcripts accumulated in all studied organs, including roots, stems, leaves, leaf sheaths and panicles at different growth stages.
(Fig. 2a). Consistent with these results, in the OsATG8b promoter-GUS analysis, GUS activity was predominantly detected in all of the above-mentioned rice organs, and section analysis showed that OsATG8b is expressed in all tested cells (Fig. S3), thus suggesting that OsATG8b is expressed ubiquitously. Notably, OsATG8b transcript levels were higher in roots and leaves of plants at 45 days after germination (DAG) than in those of plants at other growth stages. At 60 DAG, OsATG8b transcript was relatively abundant in stems, leaf and panicle (Fig. 2a). The expression level of OsATG8b was also examined under N deficiency and darkness treatment for C starvation, respectively (Fig. 2b, c). OsATG8b transcript level increased in response to both N deficiency and darkness treatments. When rice seedlings were subjected to the N-free treatment, the expression level of OsATG8b gradually increased, peaking at 10 d after treatment application. Similarly, darkness treatment rapidly induced a rough three-fold increase in OsATG8b expression within 2 d after treatment. Taken together, these results suggest that OsATG8b may play a crucial role in regulating multiple developmental processes and in response to nutrient stresses.

**GFP-OsATG8b is localized to autophagosomes**

To determine whether OsATG8b is an autophagy marker for rice, GFP was fused to its N-terminus and transformed into scatg8 yeast cells. Under control conditions, GFP-OsATG8b was mainly localized to the cytosol with punctate distribution, whereas after starvation it accumulated within the vacuole of yeast (Fig. 3a). These data suggest that OsATG8b may be localized to the autophagosomes of cytosol in the control conditions and translocate from the cytosol to the vacuole in an autophagy-dependent manner after starvation in yeast. To further verify the above result in rice, sGFP-OsATG8b was also transiently expressed in rice protoplasts, but
the data showed that the sGFP-OsATG8b fusion protein was localized to the membrane, cytoplasm, and nucleus (Fig. S4), similar to the free sGFP control. To further confirm sub-cellular localization, transgenic rice expressing sGFP-OsATG8b were generated under control of 35S promoter (Fig. 3b). The 5mm roots from tip were cut and immediately observed by LSCM. In sGFP-OsATG8b, GFP fluorescence was detected in the cytoplasm and nucleus; however, after 6h of incubation in the darkness with concanamycin A (an inhibitor of vacuolar H+-ATPase to help observation of autophagic bodies through increasing vacuolar pH [38, 39]), many vesicles with strong GFP signal and the spread of faint GFP signal were observed (Fig. 3b). These results indicate that the sGFP-OsATG8b-labeled puncta located in autophagosomes and the sGFP-OsATG8b can be used to visualize the progression of autophagy in rice, and overexpression of OsATG8b could increase the autophagic activity. Immunoblot analysis using proteins isolated from either ZH11 or transgenic sGFP or sGFP-OsATG8b rice plants showed that the OsATG8b antibodies recognized the endogenous as well as the GFP fusion proteins (Fig. S5). Meanwhile, we performed a sGFP-ATG8 processing assay by the levels of free GFP moiety in anti-GFP immunoblots. The results showed the ATG8 has already conjugated onto the autophagosome membrane and is able to be completed or delivered into the vacuole (Fig. S5).

**OsATG8b affects root growth at grain germination**

To further investigate the function of OsATG8b, OsATG8b over-expression (OsATG8b-OE) and RNA-interference (OsATG8b-RNAi) transgenic lines were generated. RT-PCR analysis showed that OsATG8b expression increased in OsATG8b-OE lines and reduced in OsATG8b-RNAi lines (Fig. 4a, b). The OsATG8b-RNAi construct was targeted specifically to the non-conserved 5’ end of OsATG8b outside the ubiquitin
domain to avoid interference with other OsATG8 proteins. Three of the OsATG8b-RNAi lines (Ri20, Ri24 and Ri25) and three of the OsATG8-OE lines (OE3, OE4 and OE6) were selected for subsequent analysis. In order to observe the effect of altered OsATG8b expression to OsATG8a and OsATG8c, we detected the expression of OsATG8a and OsATG8c in the shoots and roots of the transgenic rice seedling at four leaf stage. The results showed there is no significant difference of OsATG8a or OsATG8c transcript level among ZH11, the OsATG8-OE lines or OsATG8b-RNAi lines (Fig. S6). To confirm whether autophagic activities is altered in the OsATG8b-RNAi and OsATG8b-OE lines, we examined the ATG8 autophagic activities in OsATG8b-RNAi, OsATG8b-OE and ZH11 lines using OsATG8b antibodies (Fig. 4c). The immunoblot analysis showed the expression levels of ATG8-PE (representing the forming or completed autophagosomes) and cytosolic ATG8 form were remarkably increased in OsATG8b-OE lines compared with ZH11 lines, and the quantified results showed there is slight decrease (about 17-20%) of them in OsATG8b-RNAi comparing with that in ZH11. These results indicated the autophagic activity is higher in OsATG8b-OE lines and a little lower in OsATG8b-RNAi than that in ZH11. In addition, we observed that the roots of OsATG8b-RNAi line were much shorter than those of ZH11 and OsATG8b-OE lines (Fig. 4d, e) at 7 DAG when germinated in water. To reveal how N affects autophagy in rice, growth of OsATG8b-RNAi and OsATG8b-OE lines were measured under low (0.2 mM N) and high (5 mM N) N levels. Under low and high N levels, the OsATG8b-RNAi and OsATG8b-OE lines appeared relatively normal phenotype and exhibited similar growth rate when compared with ZH11 at 30 or 60 DAG (Fig. S7). Neither root nor shoot length showed any significant difference among these lines (Fig. S7).

OsATG8b affects grain yield and grain quality in rice
The phenotypes of OsATG8b-RNAi and OsATG8b-OE rice at the reproductive stage were investigated in the paddy field under normal N conditions. Previous studies have shown that the autophagy-defective rice mutant osatg7 displayed complete sporophytic male sterility. However, OsATG8b-RNAi and OsATG8b-OE plants produced healthy pollen grains and could be fertilized normally. The statistical results showed that grain number and grain yield per plant increased in OsATG8b-OE plants but decreased in OsATG8b-RNAi ones, compared with ZH11 plants (Fig. 5). These data indicate that OsATG8b may be involved in grain development and yield. The grains of OsATG8b-RNAi are brown-spotted hull and contain chalky endosperm (Fig. 6a, b). This showed that it produced poor quality seeds. The percentage of hulled rice with chalkiness was higher in OsATG8b-RNAi lines compared to ZH11 (Fig. 6c). SEM revealed that there are many loosely packed and small starch granules in endosperm of OsATG8b-RNAi, which differed from the large and tightly packed starch granules in ZH11 (Fig. 6d). Conversely, endosperm starch granules of OsATG8b-OE and ZH11 grains seemed larger and tighter (Fig. 6d). Compared with ZH11, soluble protein content in OsATG8b-RNAi lines was lower while that in OsATG8b-OE lines was higher (Fig. 6e). However, starch content showed no significant difference among those lines (Fig. 6f).

**OsATG8b affects N recycling to grains**

To investigate whether OsATG8b plays a role in N recycling to grains in rice, we performed a pulse-chase assay with $^{15}$NO$_3^-$, as previously conducted with *Arabidopsis* [23, 40]. $^{15}$N and the $^{14}$N/$^{15}$N ratio were measured (Fig. 7a). Plant DW was higher in OsATG8b-OE lines and lower in OsATG8b-RNAi lines than in ZH11 (Fig. 7b). This is similar to what was observed in *Arabidopsis* mutants (atg5, atg7) [13,
HI, an important productivity indicator [41], was lower in OsATG8b-RNAi lines, but higher in OsATG8b-OE lines than in ZH11 (Fig. 7c), which shows that autophagy plays an important role at the grain filling stage.

NHI is a main index of the efficiency of N distribution to grains and N grain filling [23]. The NHI of OsATG8b-RNAi was lower than that of ZH11, while that in OsATG8b-OE as higher (Fig. 7d). As the NHI/HI ratio is considered a good indicator of NUE in plants [40], we then measured the NHI/HI ratio of OsATG8b-RNAi, OsATG8b-OE and ZH11. The results showed that the NHI/HI ratio increased dramatically in OsATG8b-OE lines, but decreased in OsATG8b-RNAi lines when compared to ZH11 (Fig. 7e). These data indicate that OsATG8b-mediated autophagy plays a role in grain NUE.

On the 7th d after $^{15}$NO$_3$-labeling, $^{15}$N contents of OsATG8b-RNAi, OsATG8b-OE and ZH11 showed no significant differences. This is consistent with the normal growth of OsATG8b-RNAi and OsATG8b-OE lines under N-rich conditions (Fig. S8). The abundance of $^{15}$N in grains and remains were determined using isotopic ratio mass spectrometry, enabled us to calculate the partitioning of $^{15}$N in grains ($^{15}$NHI) by combining these values with DW and N% data. $^{15}$NHI and the $^{15}$NHI:Hl ratio, an indicator for NRE, were lower in OsATG8b-RNAi lines and higher in OsATG8b-OE lines than in ZH11 (Fig. 7f, g). Taken together, these $^{15}$N partitioning results show that OsATG8b-mediated autophagy significantly affects NRE during the grain filling stage.

Discussion

Plant autophagy plays important roles in growth and development, grain filling, response to pathogen infection and to abiotic and biotic stresses, and N recycling
All these functions have major agricultural relevance, and most ATG orthologs in crop has been identified in maize and rice [16, 20]. Here, we report that rice OsATG8b involves in N recycling to affect rice yield and quality.

**OsATG8b is a functional homologue of yeast ScATG8 and a useful autophagosome marker for rice**

Evolutionarily, autophagy is a highly conserved intracellular mechanism of degradation of cellular components in eukaryotic cells [43]. At the elongation and final enclosure stages of the autophagosome, the linkage of ATG8 to PE anchors the former to both inner and outer membranes of the phagophore [44]. Therefore, the ATG8 protein is a useful molecular marker of autophagosomes, allowing for their distinction from other cellular vesicles and intracellular membranes [12, 44]. Unlike yeast with a single ATG8, higher eukaryotes usually have an ATG8 family. Rice has six ATG8s [16], and five of their proteins have the conservative glycine in C-terminal for PE-conjugation except OsATG8f. OsATG8a, b and c belong to subgroup I of the plant ATG8 phylogenetic tree (Fig. S1), as all three proteins have extra amino acids behind the conserved Gly residue and need cleavage by ATG4 to expose the Gly residue (Fig. S2). On the other hand, OsATG8d and e belong to subgroup II (Fig. S1), as both have an innate C-terminal-exposed Gly residue, which makes OsATG8 quickly proceed conjugation with PE without ATG4 processing (Fig. S2). Expression of nine AtATG8 genes showed different patterns [21], which indicates that different ATG8 members may have multiple non-redundant functions and individual ATG8s may have specific functions.

Plant ATG8s can functionally complement yeast atg8 mutant, such as those in *Arabidopsis* [21], soybean [24], and wheat [45]. In our study, OsATG8b expression restored autophagy defects in the corresponding yeast atg8 mutant (Fig. 1). This
indicated that OsATG8b has an autophagic function similar to yeast ATG8. At present, observation of GFP-ATG8 puncta has been shown to be the best and most convenient detection method for autophagic activity [46]. However, it is showed that GFP-ATG8 signal foci in cytoplasm might not be the true autophagosomes in the cytoplasm of atg4a-1atg4b-1 double mutants [12] and atg7-2 mutants [29], since the foci may be GFP-ATG8 aggregates [47, 48]. However, in the presence of concanamycin A, the mutants (atg7-2, atg5, atg10, atg4a-1atg4b-1 in Arabidopsis and atg7 in rice) always lack GFP-ATG8 labeled autophagic foci in the vacuole [12, 28, 39, 49]. This indicates vacuolar GFP-ATG8 spots should be utilized as autophagy indicator instead of GFP-ATG8 dots. [50]. The sGFP-OsATG8b puncta in vacuoles of rice root cells in the presence of concanamycin A was observed (Fig. 3b); therefore, sGFP-OsATG8b is considered to be a marker for measuring the autophagic activity of rice cells. We also detected autophages in vacuoles of sGFP-ATG8b transgenic rice (Fig. 3b). Free GFP released from fused sGFP-ATG8b also supports this transfer and accumulates in vacuoles (Fig. S5). Therefore, the sGFP-ATG8b test is a biochemical way to monitor the autophagic flux of rice cells.

**OsATG8b affects grain number and grain quality**

Arabidopsis and maize atg mutants are sensitive to nutrient-limiting condition [14, 21, 26]. However, the OsATG8b-RNAi and OsATG8b-OE lines showed relatively normal phenotype. In rice, there are six ATG8s, of which OsATG8a, OsATG8b and OsATG8c have high homology. Data from RiceXpro (Fig. S9) showed that these three genes have similar expression patterns at vegetative stage and different pattern during grain development (Fig. S9). These data indicate that OsATG8 function redundantly in response to nutrient stress at vegetative stages, but individual ATG8s may have specific functions in grain development. Indeed, in our study,
OsATG8b-RNAi lines showed a chalky endosperm phenotype and carried small, loosely packed starch granules (Fig. 6b, d), while in OsATG8b-OE lines endosperm, starch granules seemed larger and tighter (Fig. 6d). Many genes and environmental factors control the grain endosperm chalkiness of rice [51]. Starch is the main storage material in rice grains, accounting for nearly 90% of the total dry weight, while protein accounts for about 8% of the endosperm weight of rice, filling the area between starch grains [52]. Previous studies have shown that incomplete accumulation of starch and inadequate accumulation of proteins cannot fully fill the gap between starch granules, which may lead to the formation of chalk [52, 53]. Starch and protein of rice grain are products of C and N, which are transported from source organs to produce starch and protein in precise quantities and proportions [54]. C and N statuses are affected in Arabidopsis atg (atg5 and atg7) mutants [25, 55]. We showed that soluble protein content decreased in OsATG8b-RNAi lines and increased in OsATG8b-OE lines, while starch content showed no difference between these lines (Fig. 6e, f). Additionally, we showed that reduced grain quality may cause root shortening in OsATG8b-RNAi lines at the grain germination stage (Fig. 4d, e). In OsATG8b-RNAi lines, autophagic activity was slightly inhibited, grain yield and quality were reduced. The reduced grain quality may cause decreased degradation of stored proteins in the germinating grains, and then attenuate the growth rate of roots at the grain germination stage. These results indicate that OsATG8b-RNAi lines produced chalky endosperm mainly by breaking the balance between C and N in rice grains.

In the early reproductive stage, spike primordia and spikelets differentiated and developed in stem apex meristem, and apical four leaves and internodes developed in turn on mature dwarf stems and leaves. In which, all these events are maintained
mainly by the N storage in the epiphylls of dwarf stem and supplied by new soil N [56]. Therefore, spikelet number is determined by the N obtained from both recycling from leaves and root uptake. Our data showed that grain number per plant in OsATG8b-OE lines increased while that in OsATG8b-RNAi lines decreased compared with that in ZH11 in the field, indicating that OsATG8b-mediated autophagy affects grain number mainly by influencing N recycling from the dwarf stem-attached leaves to spikelet development.

**OsATG8b-mediated autophagy is involved in N recycling to grains**

Grain yield is affected by both soil N and remobilized N during reproductive stage [4]. To increase the NUE and crop yield, traditional methods focus on the operation of basic genes for N uptake and assimilation, such as *NRT, NR*, etc [57]. In the grain filling process, leaf organic N supply is more important because it contributes to plant N economy and limits the demand for exogenous N after flowering [14]. That is to say, the available N of grain was obtained from existing organic storage through recycling rather than soil sources. Recently, studies on *Arabidopsis* and maize have showed that autophagy is the main factor affecting N recycling from senescent leaves to seeds [23, 26]. N recycling in senescent leaves was suppressed in osatg7 at the vegetative stage, but male-sterility of osatg7 limited evaluation of autophagy on both N economy and grain yield [58]. Thus, we analyzed N recycling contributed by autophagy from the plant remains to grains in rice by over-expression and RNA interference of OsATG8b. Immunoblotting analysis results showed autophagy activity is higher in OsATG8b-OE lines and a little lower in OsATG8b-RNAi than that in ZH11. Previous studies showed that OsATG8b antibody can also recognize OsATG8a and OsATG8c (Fig. S10). In OsATG8b RNAi lines, the band recognized by OsATG8b antibody represents the total OsATG8s, including
OsATG8a, OsATG8b and OsATG8c, so it is difficult to observe obvious difference of OsATG8b protein level with this method. Therefore, in our study, OsATG8b-RNAi lines showed slightly inhibited autophagic activity, which leads to reduced NRE from vegetative tissues to developing grains and finally results in reduced grain yield and quality. Meanwhile, reduced grain quality may cause decreased degradation of stored proteins in the germinating grains, and then slow down the root growth at the grain germination stage. Conversely, OsATG8b-OE plants have higher yield and increased NRE (Fig. 6, 7), and higher autophagic activity (Fig. 4c). So higher autophagic activity causes increased NRE, which leads to better grain yield. These results confirm autophagy play a crucial role in the N recycling process in rice. Therefore, improving N recycling by operating autophagy may be a useful strategy to increase rice yield.

Conclusion

We identified the rice gene OsATG8b and characterized its role in N recycling by generating its over-expression and knockdown transgenic plants. Our study showed that OsATG8b can complement the function defect of yeast scatg8 mutant and that OsATG8b is localized to autophagosomes in yeast and rice. The autophagy activity is higher in OsATG8b-OE lines and a little lower in OsATG8b-RNAi than that in ZH11. 15N pulse-chase analysis revealed that OsATG8b over-expression transgenic rice plants conferred higher N recycling efficiency to grains, while OsATG8b knockdown transgenic plants has lower N recycling efficiency and poorer grain quality. OsATG8b-mediated autophagy is involved in N recycling to grains in rice. This result may provide strategic guidance for N application in rice molecular breeding and cultivation.
Abbreviations

APE1: aminopeptidase 1; CVT: cytoplasm-to-vacuole targeting; DAG: days after germination; DW: dry weight; HI: harvest index; IRRI: International Rice Research Institute; LSCM: laser-scanning confocal microscopy; mAPE1: mature APE1; N: nitrogen; NHI: nitrogen harvest index; NUE: nitrogen use efficiency; NRE: nitrogen recycling efficiency; OE: over-expression; ORF: open reading frame; OsATG8b: Oryza sativa autophagic related gene 8b; PE: phosphatidylethanolamine; qRT-PCR: quantitative real-time PCR; RNAi: RNA-interference.

Declarations

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Data of this study are included in this article and its additional files. The material that support the findings of this study are available from the corresponding author on request.

**Competing interests**

The authors declare no competing interests.

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Author’s contributions

MZ and TF designed the research. TF, WY, XZ, XX, YX and ML performed experiments. TF, MZ, XF, KX and CT analyzed data. TF and MZ wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary Files

Supplementary material 1: **Fig. S1** Phylogenetic tree of ATG8s by amino sequence alignment of different species. *Glycine max* (Gm), *Arabidopsis thaliana* (At), *Saccharomyces cerevisiae* (Sc), *Selaginella moellendorfii* (Sm), *Oryza sativa* (Os), *Homo sapiens* (Hs), *Solanum lycopersicum* (Sly), *Triticum aestivum* (Ta), and *Brachypodium distachyon* (Bd). Deduced amino acid sequences were aligned by
CLUSTALX; the phylogenetic tree was generated by the neighbor-joining method and constructed using MEGA4.

Supplementary material 2: Fig. S2 Alignment of ATG8 amino acid sequence and 3D model of OsATG8b. a Alignment of ATG8 amino acid sequences from rice, Arabidopsis, human, and yeast. Arrows indicate the C-terminal glycine residue, which is processed by ATG4 cysteine protease. Residues constituting W- and L-sites are colored red and green, respectively. Sc, S. cerevisiae; Hs, Homo sapiens; At, Arabidopsis thaliana; Os, Oryza sativa. b 3D models of OsATG8b. Two hydrophobic pockets responsible for the recognition of Trp and Leu are labeled W-site and L-site, respectively, and circled.

Supplementary material 3: Fig. S3 OsATG8b expression pattern in pOsATG8b::GUS transgenic rice. a OsATG8b promoter-GUS staining in tissues of various rice organs, including leaf, sheath, stem, ligule, and spikelet. Bars = 1 mm. b GUS-staining analysis on leaf, sheath, culm, and anther sections. Bars = 50 μm.

Supplementary material 4: Fig. S4 Subcellular localization of sGFP-OsATG8b in rice protoplasts. Bars = 1 μm.

Supplementary material 5: Fig. S5 Immunoblot detection of the vacuolar delivery of GFP in GFP-OsATG8b lines and immunoblot analysis with OsATG8b antibodies. a Total proteins extracted from 14 DAG seedlings of GFP-OsATG8b, GFP and ZH11 lines were subjected to immunoblot analysis with GFP antibody. b OsATG8b antibodies recognize the endogenous proteins as well as the GFP fusion proteins in wild-type (ZH11) and GFP-OsATG8b transgenic lines.

Supplementary material 6: Fig. S6 The expression of OsATG8a and OsATG8c in ZH11, OsATG8b-OE and OsATG8b-RNAi lines. The seedlings of ZH11, OsATG8b-OE and OsATG8b-RNAi at four leaf stage were divided into the shoots and roots. OseEF-
1a was used as an internal reference.

Supplementary material 7: **Fig. S7** *OsATG8b*-RNAi (Ri) and *OsATG8b*-OE (OE) lines appeared relatively normal phenotype and exhibited similar growth rate when compared with ZH11 at 30 and 60 d after germination (DAG). *a*, *b* Phenotype of *OsATG8b*-RNAi and *OsATG8b*-OE plants grown under low (LN, 0.2 mM N) (*a*) and high N contents (HN, 5 mM N) (*b*) at 30 DAG. *c*, *d* Root (*c*) and shoot (*d*) length of *OsATG8b*-RNAi and *OsATG8b*-OE plants grown under both low (LN, 0.2 mM N) and high N contents (HN, 5 mM N) at 30 DAG. *e*, *f* Phenotype of *OsATG8b*-RNAi and *OsATG8b*-OE plants grown under low (LN, 0.2 mM N) (*e*) and high N contents (HN, 5 mM N) (*f*) at 60 DAG. *g*, *h* Root (*g*) and shoot (*h*) length of *OsATG8b*-RNAi and *OsATG8b*-OE plants grown under both low (LN, 0.2 mM N) and high N contents (HN, 5 mM N) at 60 DAG.

Supplementary material 8: **Fig. S8** $^{15}$N-labeling efficiency of ZH11, *OsATG8b*-RNAi (Ri), and *OsATG8b*-OE (OE) lines. Plants at 40 d after germination were labeled for 5 d with $^{15}$NO$_3^-$, harvested 7 d later, and then assayed for $^{15}$N content in seedlings. Results are the means ± SD from three plants.

Supplementary material 9: **Fig. S9** Spatio-temporal expression of *OsATG8a*, *OsATG8b* and *OsATG8c* in various tissues/organs throughout the entire plant growth in the field. Data was obtained from RiceXpro (http://ricexpro.dna.affrc.go.jp/).

Supplementary material 10: **Fig. S10** OsATG8b antibody cannot distinguish OsATG8a, OsATG8b and OsATG8c. The proteins of OsATG8a, OsATG8b and OsATG8c were expressed in *E.coli*, and immunoblotting with anti-OsATG8b antibody.

Supplementary material 11: **Table. S1** All primes used in this study.
Figures

**Figure 1**

Functional complementation of yeast atg8 mutant by OsATG8b. OsATG8b cDNA w;
Expression patterns of OsATG8b in developing tissues of rice by qRT-PCR analysis
Figure 3

Subcellular localization of OsATG8b. Subcellular localization of GFP-OsATG8b in
OsATG8b affects root growth at seed germination stage. a, b Expression levels of OsATG8b in OsATG8b-RNAi (ZH11, RI5, RI9, RI10, RI19, RI20, RI22, RI24, RI25) and wild-type plants (ZH11, OE3, OE4, OE6, OE17, OE18). c, d Western blot analysis of ATG8 and ATG8-PE proteins in ZH11, RI24, RI25, OE3, and OE6 lines. e, f Root and shoot lengths of different lines (ZH11, RI20, RI24, RI25, OE3, OE4, OE6). Results are the means ± SD from eighty plants. *, p<0.05 (t-test): significantly different from ZH11.
OsATG8b affects rice grain yield. OsATG8b-RNAi (Ri) and OsATG8b-OE (OE) lines
OsATG8b-mediated autophagy affects grain quality in rice. OsATG8b-RNAi (Ri) and OsATG8b-OE (OE) lines and ZH11 were used. The figures show the percentage of grains with chalkiness. The error bars represent the means ± SD from five plants. *, p < 0.05 (t-test): significantly different from ZH11 (c, e, f).
OsATG8b-mediated autophagy significantly affects N recycling efficiency (NRE). C

Supplementary Files

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