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

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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 conservde 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 ATG8*s suggest that some *ATG8*s 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 KVY55 and the *scatg8* mutant KVY5 (MATa *leu2 ura3 trp1 lys2 his3 suc2-△9△atg8::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 ^{o}C in SC medium supplemented with 0.67% [w/v] YNB (yeast N base without NH_4SO_4 and amino acids), 2% (w/v) galactose, 0.5% (w/v) NH_4SO_4, and Ura DO Supplement. When the yeast grew to the logarithmic metaphase of growth (OD_{600} = 1), yeast cells were centrifugal collected, washed and incubated for another 5h in 0.67% YNB medium without amino acids, galactose and NH_4SO_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.

^{15}N-labeling and determination of ^{15}N 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 ^{15}N for 5 d by adding 10 atom% excess $Na^{15}NO_3$ to the IRRI solution. Then the plants were washed thoroughly with distilled H_2O, and transferred in the field for further growth. Two-d ^{15}N labeled plants were harvested 2 d for ^{15}N uptake measurements. The ^{15}N 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 ^{15}N and total N content using isotope ratio mass spectrometer 100. ^{15}N content of each sample was calculated as a % of total N, which was calculated as atom% or $A\%_{sample} = 100 \times (^{15}N) / (^{15}N + ^{14}N)$ [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 $DW_{grain}/ (DW_{remain}+ DW_{grain})$. N harvest index (NHI) for assess grain filling with N was calculated as $N\%_{grain} \times DW_{grain}/ (N\%_{remain} \times DW_{remain}+ N\%_{grain} \times DW_{grain})$. 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 $(A\%_{grains} \times N\%_{grains} \times DW_{grains})/ [(A\%_{remain} \times N\%_{remain} \times DW_{remain}) + (A\%_{grains} \times N\%_{grains} \times DW_{grains})]$. 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 *OsATG8*s 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 pI 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 amino-peptidase1 (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 s*GFP-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 conatin 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, 23]. 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:HI 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 [5, 23, 42]. 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 *ATG8*s [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 *ATG8*s 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 *ATG8*s 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*

moellendorffii (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

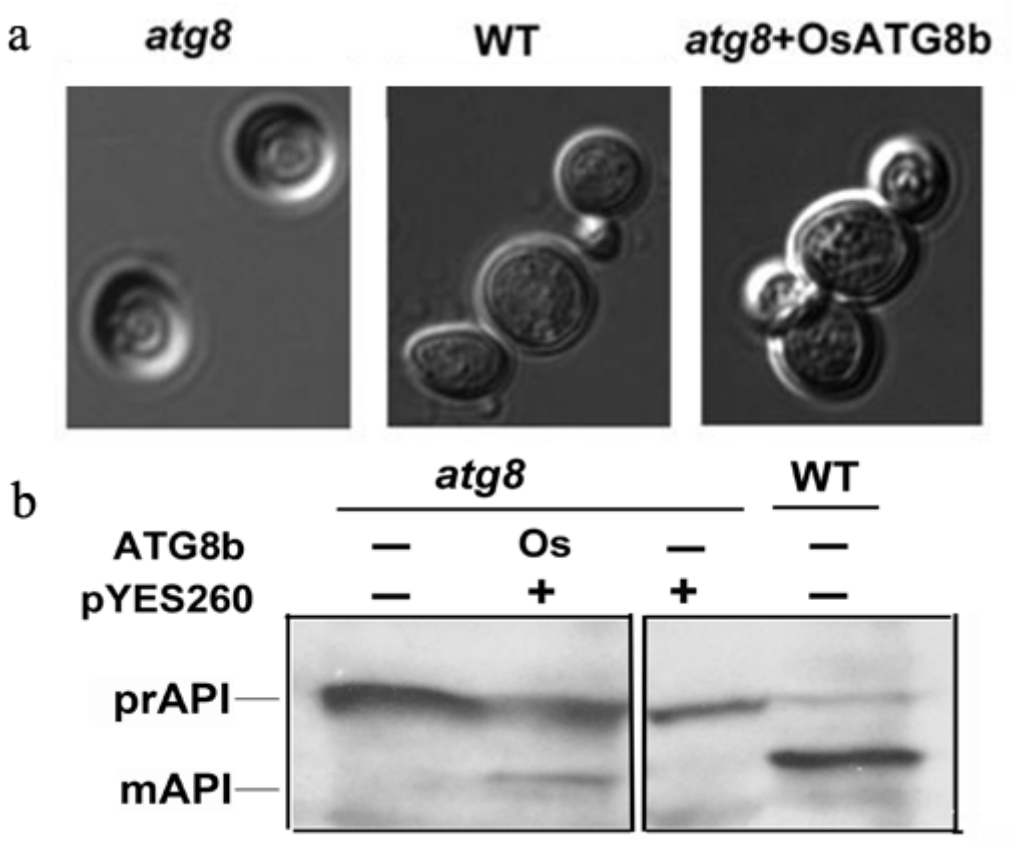

Figure 1

Functional complementation of yeast atg8 mutant by OsATG8b. OsATG8b cDNA was cloned into the plasmid pYES260 and expressed in yeast atg8 mutant KVY5 strain. a OsATG8b restores normal growth of the yeast scatg8 mutant under N starvation. Growth defect of scatg8 yeast cell (left), compared with normal growth of wild-type (WT) yeast cell (middle), and scatg8 yeast cells transformed with OsATG8b (right). b API protein identification by western blot. Yeast cells were grown to mid-log phase to be collected and washed, incubated in the presence of 2% galactose to induce OsATG8b expression, and then incubated for another 5 h in nutrient deprivation medium, and harvested for protein extraction. Proteins were then resolved by SDS-PAGE followed by immunoblotting with anti-APE1 antibody.

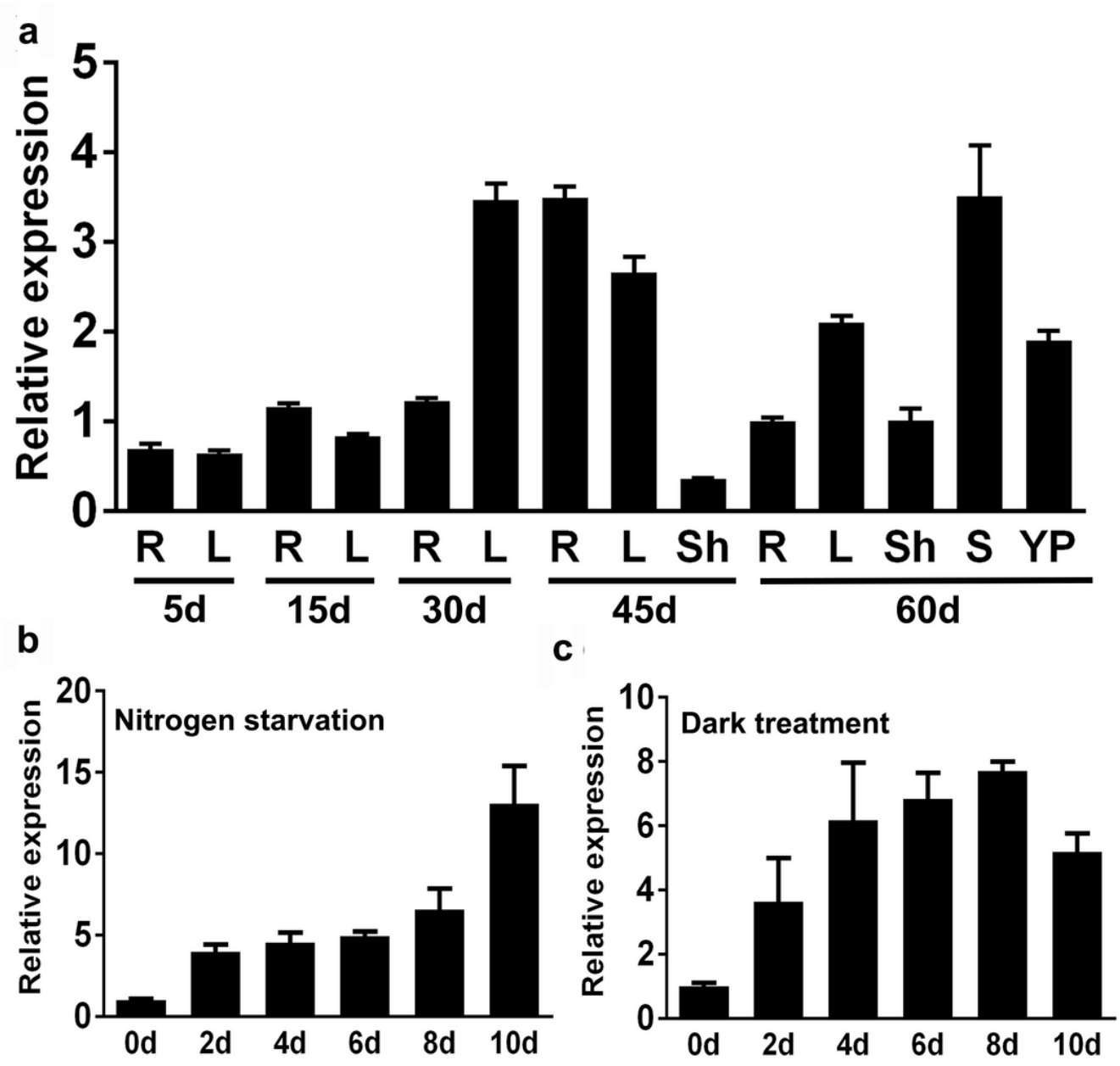

Figure 2

Expression patterns of OsATG8b in developing tissues of rice by qRT-PCR analysis. a Expression of OsATG8b in developing tissues. Total RNA was isolated from roots (R), sheaths (Sh), young panicles (YP), stems (S), and leaves (L) at different growth stages. OseEF-1a was used as an internal reference. b, c Expression profiles of OsATG8b in a N-starvation solution (b) and in darkness condition (c). Total RNA was isolated from 5-leaf seedlings treated for 2, 4, 6, 8, and 10 d with N-starvation and darkness.

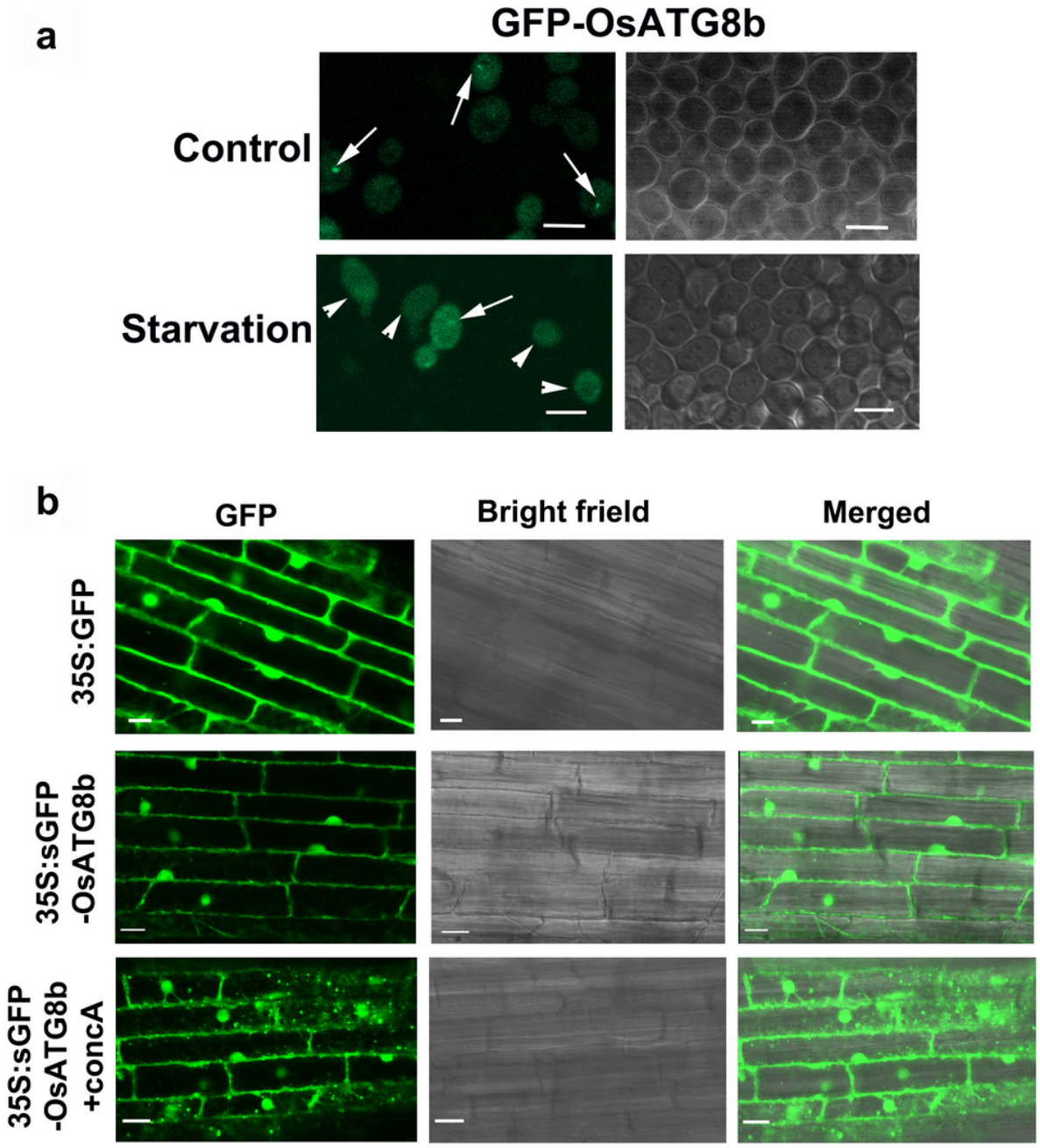

Figure 3

Subcellular localization of OsATG8b. a Subcellular localization of GFP-OsATG8b in yeast cells. Yeast cells were grown to mid-exponential phase and then incubated in the presence of 2% galactose to induce GFP-OsATG8b expression. Cells grown in both the control and starvation media were visualized in a confocal laser scanning microscope. Arrows indicate the pre-autophagosomal structure (PAS) and arrowheads indicate GFP within vacuoles. Bars = 5 μm. b Subcellular localization of sGFP-OsATG8b in roots of transgenic rice. Fresh root samples of transgenic rice plants expressing 35S: sGFP-OsATG8b were excised and observed immediately (0 h) and after 6-h treatment with 1 μM concanamycin A. The

region at approximately 5 mm from the root tip was observed in a laser scanning confocal microscope. Simultaneously obtained sGFP fluorescence images and DIC images are shown. Bars = 10 μm.

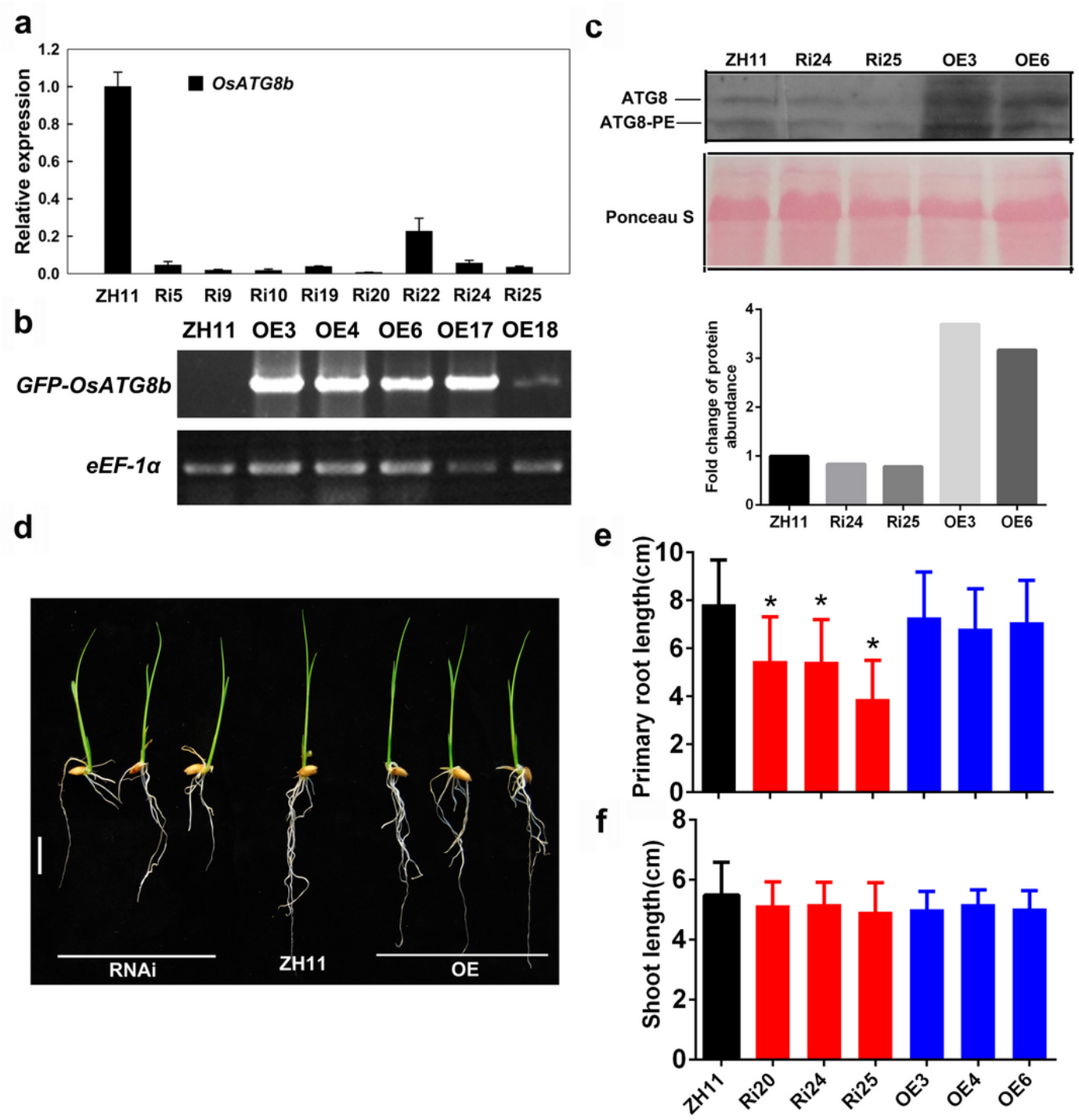

Figure 4

OsATG8b affects root growth at seed germination stage. a, b Expression levels of OsATG8b in OsATG8b-RNAi (Ri) (a), and OsATG8b-overexpression (OE) lines (b) measured by qRT-PCR and semi-qRT-PCR analyses respectively. OseEF-1a was used as an internal reference. c Immunoblot detection of the ATG8

and ATG8 lipidation level in ZH11, OsATG8b-RNAi and OsATG8b-OE lines. Total protein extracts were subjected to SDS-PAGE in the presence of 6M urea and immunoblotted with OsATG8b antibody. The fold change of ATG8-PE was quantified using Image J software v1.45. d-f Seedlings (d) and statistical analysis of root (e) and shoot length (f) of OsATG8b-RNAi, OsATG8b-OE lines and ZH11 at 7 d after germination in water. Bar = 1 cm. Results are the means ± SD from eighty plants. *, $p<0.05$ (t-test): significantly different from ZH11.

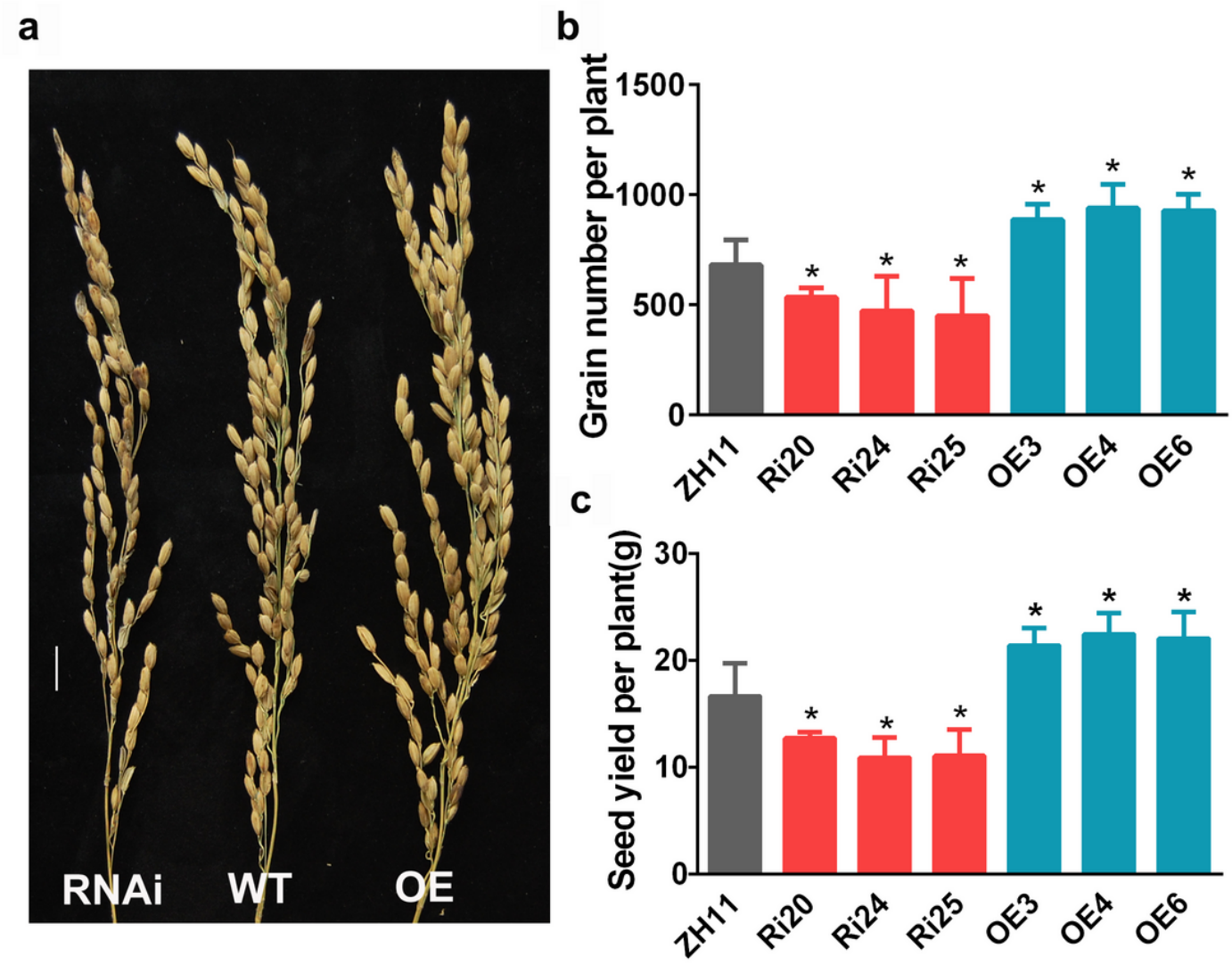

Figure 5

OsATG8b affects rice grain yield. OsATG8b-RNAi (Ri) and OsATG8b-OE (OE) lines and ZH11 were grown in the paddy field with normal fertilizer. a–c Panicle architecture (a), grain number (b), and grain yield (c) per plant of OsATG8b-RNAi and OsATG8b-OE lines and ZH11. Bar = 1 cm. Results are the means ± SD from eighty plants. *, $p<0.05$ (t-test): significantly different from ZH11.

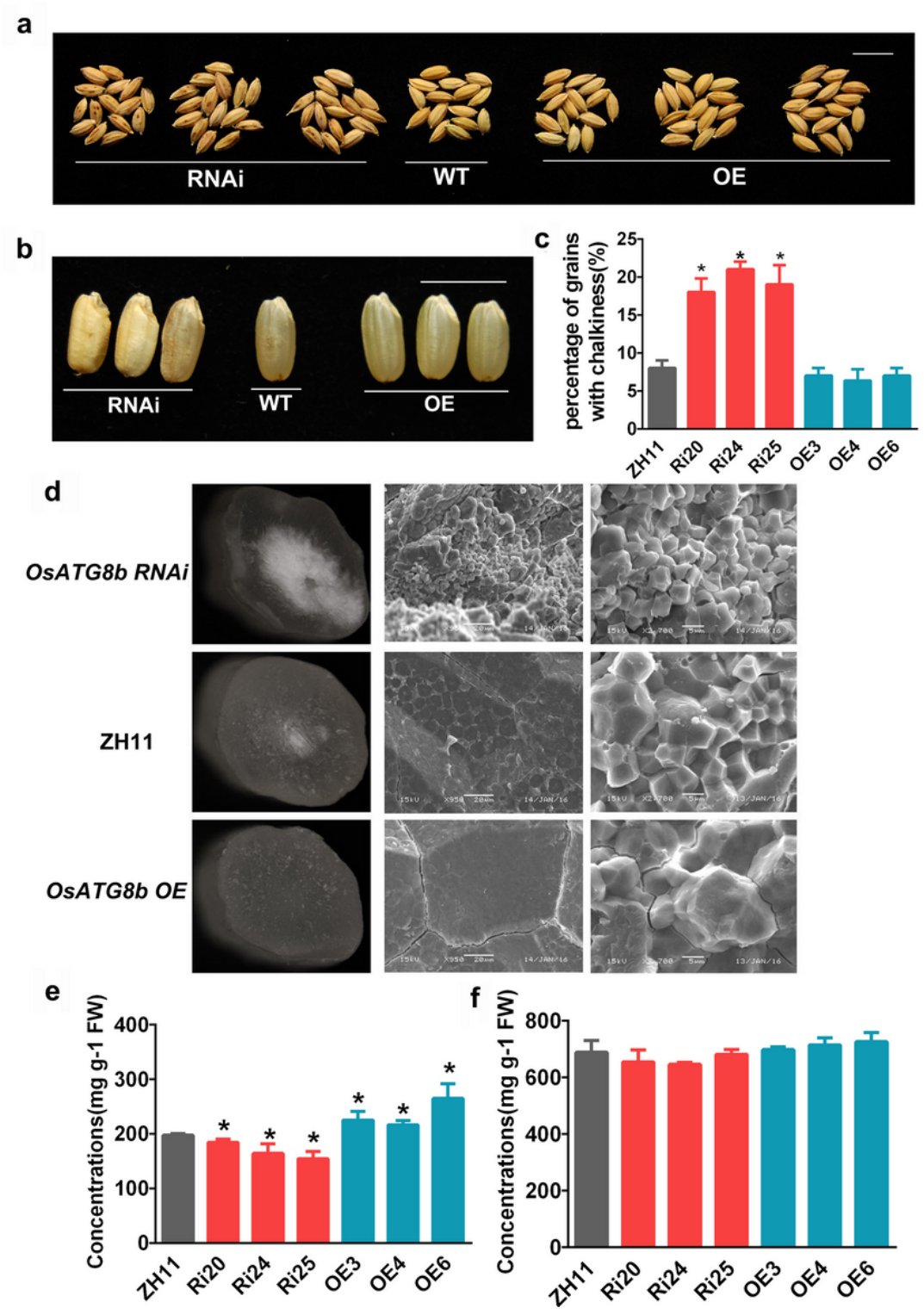

Figure 6

OsATG8b-mediated autophagy affects grain quality in rice. OsATG8b-RNAi (Ri) and OsATG8b-OE (OE) lines and ZH11 were grown in a paddy field under normal growth conditions. a Seed grains. OsATG8b-RNAi lines produced grains with brown spotted hulls. Bar = 1 cm. b Hulled OsATG8b-RNAi rice lines showed a chalky endosperm phenotype. Bar = 1 cm. c Percentage of hulled rice with chalkiness. d Scanning electron micrographs of cracked mature caryopses of rice grains under different

magnifications. Endosperms of OsATG8b-RNAi lines had small, loosely packed starch granules, which differed markedly from the large, tightly packed starch granules of ZH11 and OsATG8b-OE lines. e Soluble protein concentration in grains. f Starch concentration in grains. Results are the means ± SD from five plants. *, p<0.05 (t-test): significantly different from ZH11 (c, e, f).

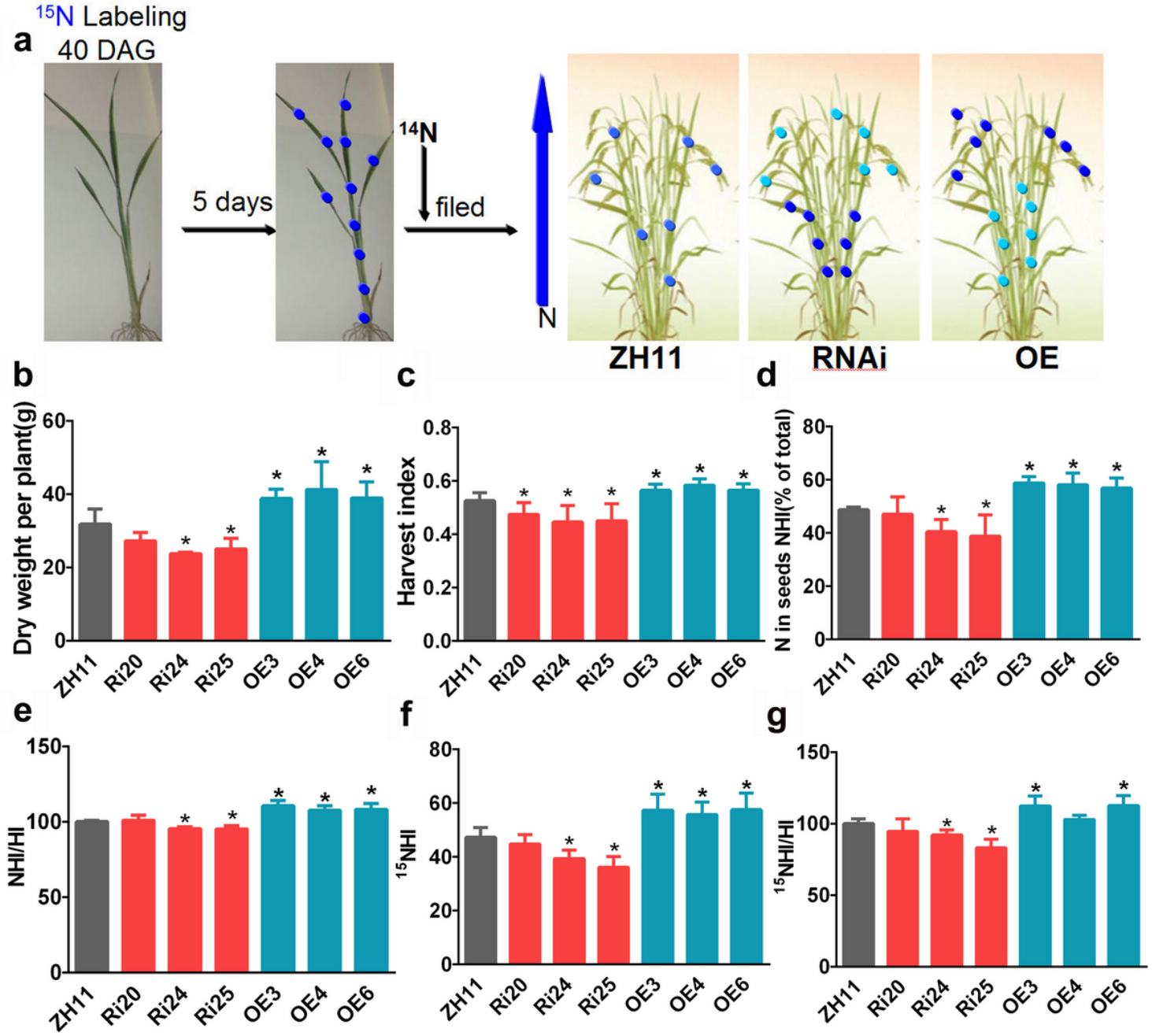

Figure 7

OsATG8b-mediated autophagy significantly affects N recycling efficiency (NRE). OsATG8b-RNAi (Ri), OsATG8b-OE (OE) lines and ZH11 were grown in IRRI solution with 14NO3- supplementation until 40 d after germination and then labeled by a 5-d pulse of 15NO3- and subsequently grown in normal conditions to mature stage. After grain filling, 15N and the 14N/15N ratio were analyzed by isotopic ratio mass spectrometry. a Overview of 15N labeling and subsequent N partitioning. The deeper the color of

blue dots, the higher the N content. b Biomass accumulation as measured by dry weight (DW) per plant. c Harvest index (HI) as measured by ratio of DW of grains to DW of the aboveground plant parts. d Nitrogen harvest index (NHI) as measured by the partitioning of total plant N in grains. e NHI:HI ratio as an estimate of N use efficiency. f Partitioning of total 15N in grains. g 15NHI:HI ratio as an indicator of N recycling efficiency. Results are the means ± SD from five plants. *, $p<0.05$ (t-test): significantly different from ZH11.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS8.jpg
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- FigureS6.jpg
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