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BRIEF COMMUNICATION

Duplicated *OsATG9* Genes Antagonise Autophagy to Balance Growth and Drought Tolerance in Rice

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Keywords: drought | gene duplication | genetic variation | grain development | OsATG | stress response

ABSTRACT

Gene duplication events frequently occur during eukaryotic genome evolution, often leading to functional redundancy for organism survival in complex environments. However, whether duplicate genes evolve diverse functions remains unclear. In this study, we explored the roles of *autophagy-related gene 9 OsATG9a* and *OsATG9b* in rice development and drought stress responses. Autophagy, an evolutionarily conserved degradation pathway, plays a critical role in multiple biological processes by recycling cellular components. We found both OsATG9a and OsATG9b involved in autophagy, with functional redundancy affecting traits like grain size, plant height, tiller number, primary branch number, and panicle length. Notably, *OsATG9b* exhibited a distinct response to drought stress. The *osatg9a* mutant displayed a lower survival rate than wild type (WT) after drought stress, similar to other *osatg* mutants, while the *osatg9b* mutant showed the opposite. Moreover, autophagy flux decreased in *osatg9a* mutant but increased in *osatg9b*, surpassing WT response. Overexpression of *OsATG9b* resulted in lower survival rates and reduced autophagy induction under drought stress. Moreover, the response of ABA related genes in *osatg9a* and in *osatg9b* were opposite compared with WT. These suggest that OsATG9a promotes autophagy during drought stress, while OsATG9b negatively impacts it, representing a newly evolved function in rice by differently regulating ABA pathway. Our findings provided insights into the functional divergence of duplicate genes during evolution.

Yiming Li, Yuantai Liu and Mengzhao Shi contributed equally to this study.

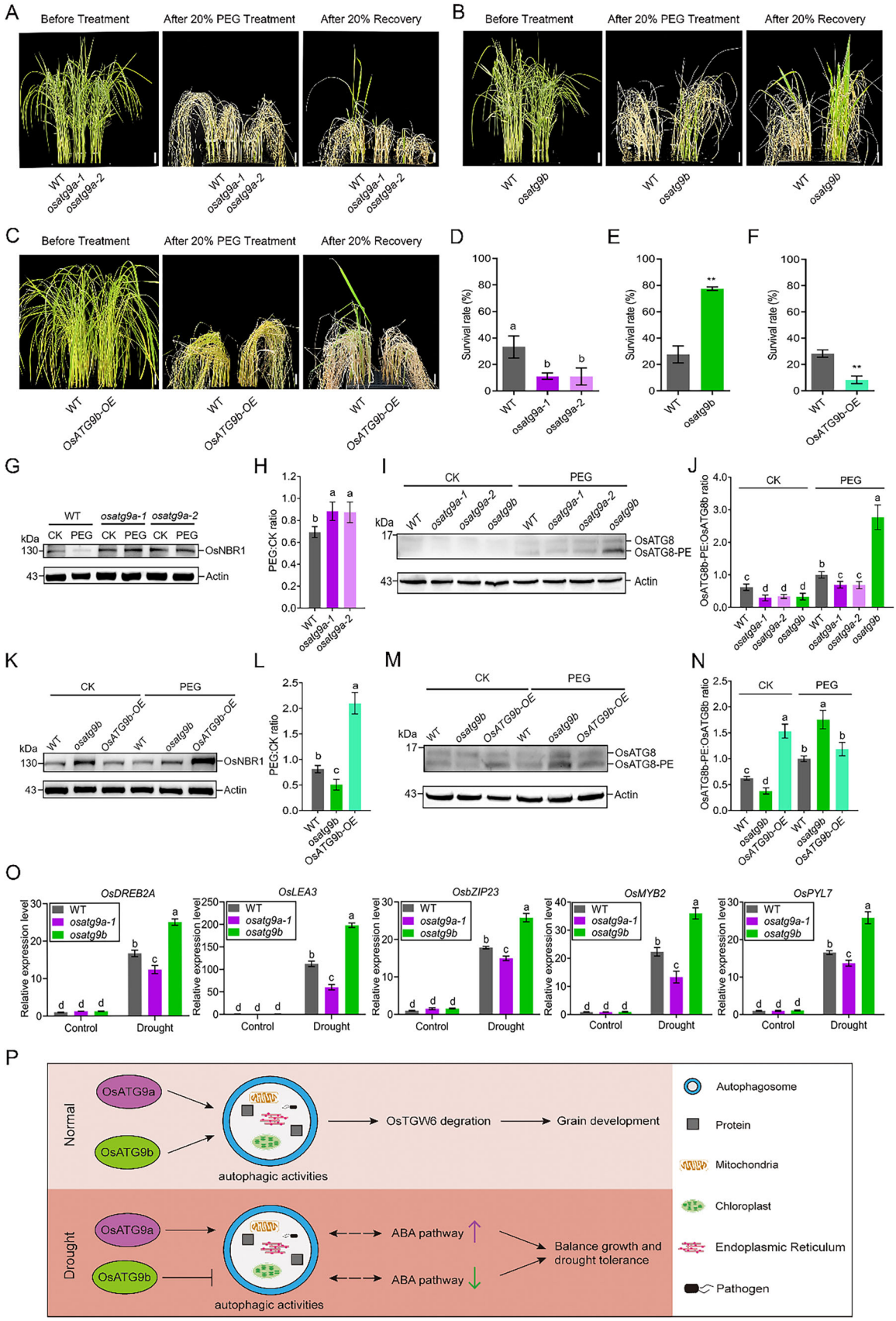

FIGURE 1 | Legend on next page.

Gene duplication drives functional innovation in evolution, enabling organisms to develop new mechanisms to cope with environmental challenges. One such mechanism is autophagy – a conserved intracellular degradation pathway that not only supports normal development but is also strongly induced by abiotic stresses. Indeed, when plants face drought, autophagy is activated to recycle cellular components (Yagyu and Yoshimoto 2024). Autophagy initiation involves conserved ATG genes (AuTophaGy related genes), with ATG9 facilitating autophagosome formation (Zhuang et al. 2017). While most plants possess a single *ATG9* gene, rice experienced a segmental duplication that yielded two paralogs, OsATG9a and OsATG9b, with positive selection (Ka/Ks > 1) (Xia et al. 2011). This raises the question of whether these duplicates have evolved distinct functions, particularly in the context of drought-induced autophagy, which remains poorly understood in rice. In our previous study, we observed that OsATG9b plays a role in determining grain size and quality in rice. Mutations of *OsATG9b* result in smaller grains and increased chalkiness, whereas its overexpression enhances grain size and quality (Liu et al. 2023). Considering *OsATG9b* is the duplicated gene from *OsATG9a* and they experienced positive selection, we hypothesized potential divergent functions of these genes during rice development and in response to environmental stressors.

Firstly, we constructed the evolution analysis of ATG9s from 21 species, which revealed that OsATG9a and OsATG9b belong to different clusters (Supporting Information S1: Figure S1). The superfamily domain of ATG9 is highly conserved across different species (Supporting Information S1: Figure S2). Using fluorescent-tagged constructs, we showed that both OsATG9a and OsATG9b localise to the Golgi apparatus and colocalize with each other, as well as with the core autophagy marker OsATG8b (Supporting Information S1: Figures S3 and S4). Furthermore, OsATG9a share the similar expression pattern with OsATG9b (Supporting Information S1: Figure S5). To assess their functional roles, we then analyzed two new *osatg9a* single mutants, two *osatg9a osatg9b* double mutants, and previous published *osatg9b* line to confirm that, OsATG9a also positively regulates autophagy and affects rice grain development by degrading TGW6 and other agronomic traits (Supporting Information S1: Figures S6–S9), mirroring OsATG9b's roles in rice development (Liu et al. 2023).

Given that OsATG9b originated from a duplication of OsATG9a with a Ka/Ks ratio > 1 (Xia et al. 2011), it is plausible that OsATG9b has evolved distinct functions compared to OsATG9a under abiotic stress. To explore this hypothesis, we subjected WT, *osatg9a-1*, *osatg9a-2*, *osatg9b*, *osatg9a osatg9b-1* and *osatg9a osatg9b-2* plants to 20% PEG (simulating drought) and soil-drying conditions. Surprisingly, *osatg9a-1* and *osatg9a-2* exhibited significantly lower survival rates than WT, whereas *osatg9b*, *osatg9a osatg9b-1* and *osatg9a osatg9b-2* mutants displayed higher survival rates than WT (Figure 1A,B,D,E, Supporting Information S1: Figure S10). These results suggested that OsATG9a positively regulates drought stress response, while OsATG9b negatively regulated it, potentially epistatic to OsATG9a. We then assessed the stomatal movement, water-loss rates, drought-related physiological parameters, and transcript levels of drought-resistance marker genes in the leaves after 20% PEG treatment (Supporting Information S1: Figures S10–S13). Consistent with survival rate, *osatg9a* mutants were more drought-sensitive, whereas *osatg9b* and *osatg9a osatg9b* mutants were more drought-resistant than WT.

We further investigate whether the divergent drought-resistance functions between OsATG9a and OsATG9b are linked to autophagy. Hence, we firstly checked the autophagy function in response to drought stress. Under 20% PEG treatment, the drought marker gene *OsERD1* (*Early-Responsive to Dehydration stress*) peaked early and then declined by Day 7 under 20% PEG (Supporting Information S1: Figure S14A,B). *OsATG9a* expression similarly rose and then fell, whereas *OsATG9b* peaked early but remained unchanged after 24 h (Supporting Information S1: Figure S14C,D). NBR1 (Next to BRCA1 gene 1 protein) is a selective autophagy substrate and it also acts as cargo receptors for degradation of other substrates, thus NBR1 can be used as the autophagy marker (Marshall and Vierstra 2018). In WT plants, OsNBR1 protein levels dropped in 24 h under PEG and soared above baseline after 3 days (Supporting Information S1: Figure S14E–H). All *OsATG* genes were upregulated at 6 h post-PEG (Supporting Information S1: Figure S15), suggesting an initial induction of autophagy followed by suppression after 1 day. By employing the same approaches using previously characterised autophagy mutants and overexpression lines (*osatg5*, *OsATG5-OE*, *osatg7*, and *OsATG8b-OE*) (Gou et al. 2019), we confirmed that autophagy contributes positively to drought tolerance (Supporting Information S1: Figures S16–S18).

FIGURE 1 | Duplicated *OsATG9* genes antagonise autophagy to balance growth and drought tolerance in rice. (A–C) Representative images of WT, *osatg9a*, *osatg9b* and *OsATG9b-OE* plants from hydroponic experiments are shown before treatment, after 7 days of exposure to 20% (w/v) PEG6000, and after 5 days of recovery in water. (D–F) Quantification of the survival rates of WT, *osatg9a*, *osatg9b* and *OsATG9b-OE* plants after recovery shown in (A–C). (G, K) Relative OsNBR1 levels in leaves of WT, *osatg9a*, *osatg9b* and *OsATG9b-OE* after 1 day of 20% PEG6000 treatment. OsNBR1 protein abundance was determined by immunoblotting, with Actin serving as a loading control. (H, L) Quantification of OsNBR1 levels based on immunoblot density analysis using Image J. (I, M) The ratio of OsATG8a-PE:OsATG8a in rice leaves after 1 day of 20% PEG6000 treatment. The proteins were detected with anti-OsATG8a antibody. Actin was used as the loading control. (J, N) Statistical analysis of the ratio of OsATG8a-PE:OsATG8a shown in (I, M). (O) Relative expression levels of ABA pathway related genes of WT, *osatg9a-1* and *osatg9b*. Each column represents the mean ± SD ($n = 3$), with different letters indicating statistically significant differences at the $p < 0.05$ level, as determined by one-way ANOVA followed by post hoc multiple comparisons. (P) Proposed model for the functions of duplicated genes *OsATG9a* and *OsATG9b*. Under normal condition, both OsATG9a and OsATG9b activate autophagic activity and promote the degradation of OsTGW6 protein to support grain development. However, under drought stress, OsATG9a activates autophagy and stimulates the ABA pathway to mitigate drought effects, while OsATG9b suppresses these processes, thereby negatively affecting drought response. The reciprocal regulation between autophagy and the ABA pathway remains unclear. Together, the opposing actions of OsATG9a and OsATG9b may help balance plant growth and drought tolerance. [Color figure can be viewed at wileyonlinelibrary.com]

We further assessed NBR1 protein levels and ATG8-PE levels (phosphatidylethanolamine), which is another stringent criterion for assessing autophagic flux (Marshall and Vierstra 2018) in *osatg9* mutants. The NBR1 PEG:CK ratios of *osatg9a-1* and *osatg9a-2* were higher than in WT (Figure 1G,H), consistent with the observations in *osatg5* and *osatg7* (Supporting Information S1: Figure S16E–H), indicating a reduction in autophagy activity in *osatg9a* in response to drought stress. However, the PEG:CK ratios in *osatg9b*, *osatg9a osatg9b-1* and *osatg9a osatg9b-2* were significantly lower than in WT (Figure 1K,L, Supporting Information S1: Figure S19), suggesting a much higher induction of autophagy flux in *osatg9b* and *osatg9a osatg9b* compared to WT. Using the ATG8 antibody, our immunoblotting analyses revealed that in *osatg9* mutants, reduced ATG8-PE/ATG8 ratios under non-stress conditions indicated compromised basal autophagic flux (Figure 1I,J,M,N). Paradoxically, *osatg9b* mutants exhibited significantly stronger induction of ATG8-PE conjugation after PEG treatment (Figure 1I,J,M,N), demonstrating enhanced autophagic activation.

This functional antagonism was further validated through overexpression studies. The survival rate of *OsATG9b-OE* was significantly lower than WT under PEG treatment (Figure 1C,F), and soil-drying conditions (Supporting Information S1: Figure S20A,B). Under both normal and PEG conditions, the percentages of completely open stomata and partially open stomata in *OsATG9b-OE* were higher than in WT, opposite to the trend observed in *osatg9b* (Supporting Information S1: Figure 20C), which led to higher water loss in *OsATG9b-OE* compared to WT (Supporting Information S1: Figure 20D). Concordantly, *OsATG9b* overexpression suppressed drought-induced autophagic flux, as evidenced by attenuated NBR1 degradation (Figure 1K,L). The OsATG8-PE: OsATG8 ratio revealed that under normal conditions, the ratio was higher in *OsATG9b-OE* line compared to WT (Figure 1M,N), indicating increased autophagic flux in the *OsATG9b-OE* line under normal condition. However, after PEG treatment, the ratio in the *OsATG9b-OE* line did not significantly differ from that in WT, but was lower than under normal condition (Figure 1M,N), suggesting a suppression of autophagic flux in the *OsATG9b-OE* line following drought stress. The reciprocal regulation patterns establish that OsATG9a promotes drought resilience through autophagy potentiation like canonical ATG5/7/8b components, while OsATG9b acts as a novel negative regulator by constraining autophagic flux during stress adaptation.

Gene duplicates typically undergo three evolutionary trajectories: non-functionalization, neofunctionalization/subfunctionalization (retained for functional innovation) (Lynch and Conery 2000). Evolutionarily significant duplicates often develop specialised roles, as exemplified by legume CHI1B's (*chalcone isomerase 1B*) exclusive nodulation control versus nonfunctional CHI1A (Liu et al. 2024b). Such divergence enables biological systems to expand adaptive capacity while maintaining regulatory equilibrium.

Abscisic acid (ABA) is a key phytohormone that regulates plant drought resistance and response (Haverroth et al. 2023; Liu et al. 2024a). Hence, the ABA-mediated drought responses were investigated to determine functional links between OsATG9a/9b and ABA signalling. The expression levels of ABA pathway components, including *OsDREB2A* (*AP2/EREBP transcription factor*), *OsLEA3* (*late embryogenesis abundant 3*), *OsbZIP23* (*bZIP transcription factor*), *MYB2* (*MYB Domain Protein 2*) and *OsPYL7* (*Pyrabactin Resistance-like 7, Abscisic Acid Receptor*) showed PEG-induced upregulation in WT, *osatg9a*, and *osatg9b* mutants. Strikingly, *osatg9a* exhibited attenuated induction of these genes, while *osatg9b* displayed amplified responses (Figure 1O), demonstrating divergent ABA pathway regulation. In plants, ABA and autophagy form a feedback loop via ATG8-interacting proteins, transcriptional regulation, and TOR-mediated phosphorylation to balance stress responses and growth (Gou et al. 2019). OsATG9b may affect the ABA pathway under drought conditions; it is possible that a key factor in this pathway, in turn, regulates autophagic activity or that OsATG9b's suppression of autophagy leads to the accumulation of undegraded proteins, thereby disrupting the ABA pathway and compromising drought resistance. We further proposed that OsATG9b has evolved as a critical regulator of autophagy that balances growth and drought tolerance in concert with OsATG9a (Figure 1P).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.