Postprint: Rice OsZAT12 Gene Response to Abiotic Stress and Plant Hormones

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Abstract

C2H2 zinc finger proteins constitute an important class of transcription factors in eukaryotes, playing vital roles in plant growth and development as well as in abiotic stress responses. Our laboratory previously cloned the rice C2H2 zinc finger protein OsZAT12, which is specifically expressed in rice roots and localized to the nucleus; heterologous overexpression of OsZAT12 resulted in dwarf Arabidopsis plants. To further investigate the function of OsZAT12 in rice, this study analyzed the promoter elements and transcriptional activity of OsZAT12 and employed qRT-PCR to examine its expression patterns under abiotic stress and plant hormone treatments. The results demonstrated that OsZAT12 contains two typical C2H2 zinc finger domains and one EAR motif, possesses transcriptional repression activity, and its promoter harbors elements associated with abiotic stress and plant hormones. Treatments of wild-type rice with abiotic stresses and hormones revealed that low-temperature stress (4°C) and ABA treatment significantly downregulated OsZAT12 expression, whereas osmotic stress (20% PEG 6000), BR, or IAA treatment significantly upregulated its expression. These findings indicate that OsZAT12 mediates rice responses to multiple abiotic stresses and hormonal changes. Using overexpression vectors containing the 35S promoter and CRISPR/Cas9 gene editing technology, we obtained homozygous OsZAT12-overexpressing and OsZAT12-knockout plants, respectively. Phenotypic analysis of OsZAT12-overexpressing rice showed that, compared with the wild type, plant height was significantly reduced at the tillering, heading, and maturity stages. In contrast, OsZAT12-knockout plants exhibited no significant difference in plant height from the wild type, but showed significantly lower panicle number per plant and seed setting rate. These results demonstrate that OsZAT12 influences the establishment of agronomic traits including plant architecture, panicle architecture, and seed setting rate in rice. Further experiments revealed that overexpression of OsZAT12 decreased rice sensitivity to exogenous ABA, whereas knockout plants displayed the opposite

effect. In summary, we speculate that the impact of OsZAT12 on plant growth and development may be associated with its regulation in response to multiple abiotic stress and hormone signals. This study provides an experimental basis for utilizing OsZAT12 in molecular design breeding for stress-tolerant and high-yield rice.

Full Text

Preamble

OsZAT12 Gene Responses to Abiotic Stresses and Phytohormones in Rice (Oryza sativa)

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Abstract: C2H2 zinc finger proteins constitute an important class of transcription factors in eukaryotes that play crucial roles in plant growth, development, and responses to abiotic stresses. Our laboratory previously cloned the rice C2H2 zinc finger protein gene *OsZAT12*, which is specifically expressed in rice roots, localized to the nucleus, and causes dwarfism when heterologously overexpressed in Arabidopsis. To further investigate the function of *OsZAT12* in rice, we analyzed its promoter elements and transcriptional activity, and examined its expression patterns under abiotic stress and phytohormone treatments using qRT-PCR. The results show that *OsZAT12* contains two typical C2H2 zinc finger domains and one EAR motif, exhibits transcriptional repressive activity, and its promoter contains elements related to abiotic stress and phytohormones. Treatments of wild-type rice revealed that low temperature stress (4°C) and ABA treatment significantly down-regulated *OsZAT12* expression, whereas osmotic stress (20% PEG 6000) and phytohormone BR or IAA treatment significantly up-regulated its expression. These findings indicate that *OsZAT12* mediates rice responses to multiple abiotic stresses and hormonal changes. Using overexpression vectors containing the 35S promoter and CRISPR/Cas9 gene editing technology, we obtained homozygous *OsZAT12* overexpression and knockout plants. Phenotypic observation of *OsZAT12* overexpression rice showed significantly reduced plant height at tillering, heading, and maturity stages compared to wild type, while *OsZAT12* knockout plants showed no significant difference in plant height but significantly lower panicle number and seed setting rate per plant. These results demonstrate that *OsZAT12* affects the establishment of agronomic traits including plant architecture, panicle morphology, and seed setting rate. Further experiments revealed that overexpressing *OsZAT12* reduces rice sensitivity to exogenous ABA, whereas knockout plants show the opposite response. In summary, we hypothesize that

OsZAT12 influences plant growth and development through its regulation in response to multiple abiotic stress and hormonal signals. This study provides an experimental basis for molecular design breeding of stress-tolerant and high-yield rice using *OsZAT12*.

Keywords: rice, C2H2 zinc finger protein, *OsZAT12*, abiotic stress, ABA

Introduction

Zinc finger proteins are a widely studied family of transcription factors in eukaryotes that can be classified into types such as C2H2, C2HC, C2C2, C2C2C2C2, and others based on the number and position of cysteine (C) and histidine (H) residues (Laity et al., 2001). C2H2-type zinc finger proteins are the most common class and play essential roles in various metabolic pathways, plant growth and development, and responses to abiotic stresses (Ballerini et al., 2020; Yin et al., 2020; Rodas et al., 2021; Zhang et al., 2021). To date, 189 and 176 C2H2-type zinc finger proteins have been identified in rice and Arabidopsis, respectively. These proteins feature a characteristic zinc finger domain sequence of $CX_{2\ 4}CX_3FX_5LX_2HX_{3\ 5}H$ (where C = cysteine, F = phenylalanine, H = histidine, L = leucine, and X = any amino acid) (Englbrecht et al., 2004; Agarwal et al., 2007). Based on the number, sequence, and arrangement of zinc finger domains, the 176 Arabidopsis C2H2-type zinc finger proteins can be divided into sets A, B, and C, with set C comprising those containing multiple discrete zinc finger domains (Pabo et al., 2001; Englbrecht et al., 2004; Ciftci-Yilmaz & Mittler, 2008). Set C can be further subdivided into C1, C2, and C3 based on the number of amino acids between the two histidines in the zinc finger signature sequence, with the C1 family further classified into C1-1i, C1-2i, C1-3i, C1-4i, and C1-5i subgroups (Englbrecht et al., 2004). Research on the C1 family has primarily focused on the C1-1i and C1-2i subfamilies (Englbrecht et al., 2004; Ciftci-Yilmaz & Mittler, 2008).

The C1-2i subfamily in the dicot Arabidopsis includes ZAT5–7, ZAT10–12, ZAT18, and AZF1–3. These proteins contain two zinc finger domains with three amino acids between the two histidines, and most members possess nuclear localization signals and an EAR motif (ethylene-responsive element binding factor-associated amphiphilic repression motif), primarily participating in various biotic and abiotic stress responses (Lippuner et al., 1996; Meissner et al., 1997; Englbrecht et al., 2004; Sakamoto et al., 2004; Mittler et al., 2006; Liu et al., 2013; Shi et al., 2014; Yin et al., 2017). In Arabidopsis, *AtAZF2* shows strong responses to both salt and drought stress treatments, whereas *AtAZF1* and *AtAZF3* exhibit weaker responses to abiotic stresses (salt, drought, and cold), and only *AtAZF2* can be induced by ABA, possibly due to the presence of ABA-responsive elements in its promoter (Sakamoto et al., 2004). Overexpressing *AtZAT18* enhances drought tolerance in Arabidopsis, while mutating *AtZAT18* reduces plant tolerance to drought stress (Yin et al., 2017). Constitu-

tive overexpression of *AtZAT10* in transgenic Arabidopsis inhibits growth but enhances tolerance to drought, salt, heat, and osmotic stress, and also increases transcription of ROS homeostasis-related genes such as *AtAPX1* and *AtAPX2* (Sakamoto et al., 2004; Mittler et al., 2006). Interestingly, both *AtZAT10* knockout and RNAi interference plants also show increased tolerance to salt and osmotic stress, though the regulatory mechanism remains unclear (Mittler et al., 2006). Beyond Arabidopsis, C2H2 transcription factors have been reported in other dicots. The pea *St* (*stipules reduced*) gene regulates stipule size by affecting cell division and expansion (Moreau et al., 2018). The tomato *H* (*hair*) gene encodes a C2H2-type zinc finger protein, and overexpressing this gene significantly increases trichome number on leaves (Chang et al., 2018). Alfalfa *MtSUP* (*SUPERMAN*) is also a C2H2 zinc finger protein expressed mainly in meristems, stamens, and carpel margins, and mutation of this gene in alfalfa affects floral organ number, morphology, and fruit development (Rodas et al., 2021).

In monocots, the *ZjZFN1* gene from zoysiagrass encodes a C2H2-type zinc finger protein whose expression is induced by salt stress, cold, and ABA. Heterologous overexpression of *ZjZFN1* in Arabidopsis revealed that this gene enhances salt stress resistance by affecting reactive oxygen species accumulation and transcription of salt stress-responsive genes (Teng et al., 2018). Drought stress induces expression of wheat *TaZFP1B*, and wheat overexpressing *TaZFP1B* shows significantly increased resistance to drought stress (Cheuk et al., 2020). Several rice C2H2 zinc finger proteins have also been documented, including ZFP182, ZFP36, ZFP179, ZFP245, and ZFP252. Overexpressing *ZFP182* enhances plant tolerance to salt stress (Zhang et al., 2012). Overexpressing *ZFP36* increases antioxidant enzyme activity and enhances rice tolerance to drought and oxidative stress, whereas *ZFP36* RNAi plants show lower antioxidant enzyme activity and increased sensitivity to drought and oxidative stress (Zhang et al., 2014). Overexpressing *ZFP179* improves salt tolerance in rice, and transgenic seedlings show increased sensitivity to exogenous ABA (Sun et al., 2010). Overexpressing *ZFP252* increases tolerance to salt and drought stress, and under these treatments, transgenic plants show higher expression of abiotic stress-related genes such as *OsDREB1A*, *OsP5CS*, and *OsProT* than wild type and *ZFP252* silenced lines, suggesting these genes may be downstream targets of ZFP252 (Xu et al., 2008).

OsZAT12 belongs to the C1-2i subfamily of C2H2 zinc finger proteins, which play crucial roles in various metabolic pathways and in plant growth, development, and abiotic stress responses (Ballerini et al., 2020; Yin et al., 2020; Zhang et al., 2021; Rodas et al., 2021). Our laboratory previously cloned the *OsZAT12* gene (Chen et al., 2019), which is specifically expressed in rice roots and localized to the nucleus; heterologous overexpression of *OsZAT12* in Arabidopsis results in dwarf plants with inhibited root growth. As a staple food crop, rice development and morphogenesis are affected by biotic/abiotic stresses and phytohormones, ultimately influencing yield. Given that *OsZAT12* may play important roles in plant growth, development, and abiotic stress responses, its functions

in rice development and stress responses remain unclear. Therefore, this study first analyzed the promoter elements and transcriptional activity of *OsZAT12* and examined its expression patterns under abiotic stress and phytohormone treatments using qRT-PCR. We then obtained homozygous *OsZAT12* overexpression and knockout plants using 35S promoter vectors and CRISPR/Cas9 gene editing technology. Our results demonstrate that *OsZAT12* participates in rice responses to multiple abiotic stress and hormonal signals, affecting agronomic traits including plant architecture, panicle morphology, and seed setting rate. These findings lay the foundation for further investigation of the molecular mechanisms by which *OsZAT12* participates in different abiotic stress responses and ABA signal transduction pathways.

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1 Materials and Methods

1.1 Experimental Materials

Japonica rice "Zhonghua 11" (WT) was stored in our laboratory. The binary vector pCAMBIA1301 was obtained from our laboratory. CRISPR editing-related vectors pYLCRISPR/Cas9Pubi-H, pYLgRNA-OsU6a/LacZ, pYLgRNA-OsU6a, pYLgRNA-OsU3/LacZ, and pYLsgRNA-OsU3 were kindly provided by Dr. Yaoguang Liu' s laboratory at South China Agricultural University. *Escherichia coli* DH5α competent cells and *Agrobacterium tumefaciens* EHA105 competent cells were maintained in our laboratory.

1.2 Conserved Domain Analysis of OsZAT12 and Its Homologous Genes

C1-2i subfamily members from Arabidopsis and rice were identified in the NCBI and TAIR databases, and their amino acid sequences were exported. Multiple sequence alignment was performed using Clustal X 1.83 software, and images were generated using DNAMAN 6.0 software. The genes used for multiple sequence alignment in this study were: *AtAZF1* (At5g67450), *AtAZF2* (At3g19580), *AtAZF3* (At5g43170), *AtZAT5* (At2g28200), *AtZAT6* (At5g04340), *AtZAT7* (At3g46090), *AtZAT10* (At1g27730), *AtZAT11* (At2g37430), *AtZAT12* (At5g59820), *AtZAT18* (At3g53600), *OsZFP252* (AAO46041.1), *OsZFP245* (AAQ95583), *OsZFP182* (NP001051718.1), *OsZFP179* (AAL76091.1), and *OsZFP36* (AAP51130.1).

1.3 Transcriptional Activity Analysis of OsZAT12

The dual-luciferase reporter system combines firefly luciferase (LUC) and renilla luciferase (REN) detection systems and is commonly used to analyze transcription factor activity. This study used an Arabidopsis protoplast transient expression system for dual-luciferase assays. Arabidopsis protoplast extraction followed the method of Wu et al. (2009), and dual-luciferase activity was de-

tected according to the Promega Dual-Luciferase Reporter Assay System kit (Cat. No. E1910) instructions. The LUC/REN ratio was calculated to analyze the transcriptional activation/repression activity of OsZAT12.

1.4 OsZAT12 Promoter Analysis

The 2000 bp sequence upstream of the *OsZAT12* gene start codon (ATG) was analyzed using online promoter analysis tools PLACE (http://www.dna.affrc.go.jp/PLACE/) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to predict and analyze regulatory elements in the *OsZAT12* promoter sequence.

1.5.1 Rice Seed Sterilization and Hydroponic Seedling Culture

Rice seeds were dehusked and placed in 50 mL centrifuge tubes, surface-sterilized with 75% ethanol for 1 min, then with 2.5% sodium hypochlorite for 50 min, and rinsed five times with sterile water. Seeds were subsequently cultured on 1/2 MS solid medium. Five-day-old sterile seedlings were transferred to 96-well PCR plastic plates (with bottoms removed) for hydroponic culture (16 h light/8 h dark, 28°C day/24°C night). Rice nutrient solution was prepared according to the "International Rice Research Institute rice nutrient solution" formula and changed every 5–6 days.

1.5.2 Abiotic Stress Treatments

Fourteen-day-old rice seedlings were used for abiotic stress treatments. Low temperature stress: seedlings were placed in a 4°C illuminated incubator (16 h light/8 h dark). Osmotic stress: seedlings were placed in rice nutrient solution containing 20% PEG (polyethylene glycol) 6000. Oxidative stress: seedlings were placed in rice nutrient solution containing 20 mol L^{-1} methyl viologen (MV). Salt stress: seedlings were placed in rice nutrient solution containing 100 mmol L^{-1} NaCl. Whole seedlings were collected at 0, 0.5, 1, 3, 6, 12, 24, and 48 h after treatment. Untreated materials were sampled simultaneously as controls. All samples were snap-frozen in liquid nitrogen and stored at -80°C. Except for low temperature treatment, all other stress treatments were conducted in a plant growth chamber (16 h light/8 h dark, 28°C day/24°C night).

1.5.3 Phytohormone Treatments

Fourteen-day-old rice seedlings were used for phytohormone treatments. The culture solution was supplemented with 100 mol L^{-1} ABA (abscisic acid), 1 mol L^{-1} 24-eBL (2,4-epibrassinolide), or 1 mol L^{-1} IAA (indole-3-acetic acid). Whole seedlings were collected at 0, 1, 24, and 48 h after treatment. Untreated materials were sampled simultaneously as controls. All samples were snap-frozen in liquid nitrogen and stored at -80°C. Hormone-treated materials were maintained in a plant growth chamber (16 h light/8 h dark, 28°C day/24°C night).

1.5.4 RNA Extraction and First-Strand cDNA Synthesis

RNA extraction was performed according to the Huayueyang Ultra-fast Plant RNA Extraction Kit (Cat. No. 0416-50) instructions. First-strand cDNA synthesis was performed according to the Toyobo Reverse Transcription Kit (Cat. No. FSQ-301) instructions.

1.5.5 qRT-PCR Detection of OsZAT12 Gene

qRT-PCR in this study used the SYBR dye method (GenStar 2× RealStar Green Fast Mixture, Cat. No. A301-10). The rice *eEF-1a* gene was used as an internal reference (qPCR-eEF-1a-F: 5'-GCACGCTCTTCTTGCTTTC-3'; qPCR-eEF-1a-R: 5' -AGGGAATCTTGTCAGGGTTG-3'). Relative expression of *OsZAT12* was calculated using the $2^{-}\Delta\Delta$CT method (Livak & Schmittgen, 2001) (qPCR-OsZAT12-F: 5' -GACCTGAACCATCCACCCTG-3' ; qPCR-OsZAT12-R: 5' -CGGTATCCAAGAACTGGTGGAA-3').

1.6 Construction of OsZAT12 Overexpression and CRISPR/Cas9 Vectors

Since *OsZAT12* is specifically expressed in rice roots (Chen et al., 2019), wild-type rice root cDNA was used as template. The *OsZAT12* gene was amplified using primers OsZAT12-F: 5'-CGGGATCCATGAAGAGGTTTGCA-3'(BamHI site) and OsZAT12-R: 5' -AACTGCAGCTAGTAGCCGACGCA-3' (PstI site) and cloned into the pCAMBIA1301 vector to generate the overexpression vector pCAMBIA1301::35S::OsZAT12.

Knockout targets for *OsZAT12* were designed using the CRISPR-GE website (http://skl.scau.edu.cn/) developed by Dr. Yaoguang Liu' s laboratory at South China Agricultural University. To improve knockout efficiency, a dual-target strategy was employed. Two targets were designed: target 5' -CATGAGGCGCCACCGCGCCA-3' with U6 promoter and target 5' -TGCGACGACATGAGCATCAG-3' with U3 promoter. Vector construction followed the method of Ma et al. (2015).

1.7 Rice Genetic Transformation and Transgenic Plant Identification

The recombinant vectors (overexpression and CRISPR/Cas9 vectors) constructed in section 1.6 were transformed into *Agrobacterium tumefaciens* EHA105 competent cells. The recombinant vectors were introduced into rice callus via *Agrobacterium*-mediated transformation following the methods of Li & Li (2003) and Wang et al. (2011).

Transgenic plants were identified by PCR. Approximately 2 cm leaf segments from T_1 generation seedlings were used for DNA extraction with TPS solution (100 mmol · L^{-1} Tris-HCl, pH 8.0; 10 mmol · L^{-1} EDTA-Na_2, pH 8.0; 1 mol · L^{-1} KCl). *OsZAT12* overexpression transgenic plants were detected by PCR

amplification using the selectable marker gene *HptII* (hygromycin phosphotransferase II) primers Hyg-F: 5' -GATGTTGGCGACCTCGTATT-3' and Hyg-R: 5' -TCGTTATGTTTATCGGCACTTT-3' . CRISPR plants were amplified using primers CRISPR-F: 5' -TCAGACAACAGAGAGGTTGGT-3' and CRISPR-R: 5' -TAGTAGCCGACGCAGTCAAC-3' to amplify the *OsZAT12* fragment containing target sites, and mutation sites were detected by sequencing.

1.8 Sensitivity Analysis of Transgenic Rice Seedlings to Exogenous ABA

Seeds of wild-type, *OsZAT12* overexpression, and *OsZAT12* knockout plants were surface-sterilized and sown on 1/2 MS medium containing different ABA concentrations (0, 1, 5, 10 mol L^{-1}). Thirty seeds of each line were sown on each ABA concentration medium. After 10 days of culture in a plant growth chamber (16 h light/8 h dark, 28°C day/24°C night), plant height and root length were photographed and measured.

1.9 Data Analysis

All experiments were performed with three biological replicates, with three technical replicates for each sample in each replicate. Data are presented as means ± standard deviation. Significance analysis was performed using one-way ANOVA in SPSS Statistics software ($P < 0.05$; **P* < 0.01).

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2 Results and Analysis

2.1 Analysis of Conserved Domains in Transcription Factor OsZAT12

The *OsZAT12* (*Os05g0114400*) coding sequence is 597 bp in full length, contains no introns, and encodes 198 amino acids (Chen et al., 2019). To further investigate the conservation and sequence characteristics of the OsZAT12 protein domains, multiple sequence alignment was performed on selected members of the C1-2i subfamily from Arabidopsis and rice. As shown in Figure 1 [Figure 1: see original paper], the domain structure of OsZAT12 is consistent with homologous proteins in Arabidopsis and rice, containing two zinc finger domains with the QALGGH conserved sequence and one EAR motif (ethylene-responsive element binding factor-associated amphiphilic repression motif). The EAR motif at the C-terminus of the protein is generally considered to confer repressive activity (Meissner & Michael, 1997; Englbrecht et al., 2004; Ciftci-Yilmaz & Mittler, 2008). These results indicate that OsZAT12 is a typical C2H2-type zinc finger protein belonging to the C1-2i subfamily and likely possesses transcriptional repressive activity.

2.2 Transcriptional Activity Analysis of Transcription Factor OsZAT12

This study used the dual-luciferase reporter system to detect the transcriptional activity of OsZAT12. *OsZAT12* was fused to an effector vector containing the GAL4 DNA binding domain (GALBD) (Figure 2 [Figure 2: see original paper]:A) and co-transformed with a reporter vector carrying the luciferase gene into Arabidopsis protoplasts. Firefly luciferase (LUC) and renilla luciferase (REN) activities were detected in control and experimental groups, and LUC/REN values were calculated. The results showed that LUC/REN values in the experimental group were significantly lower than in the control group (Figure 2:B), indicating that transcription factor OsZAT12 possesses transcriptional repressive activity.

2.3 Sequence Analysis of OsZAT12 Promoter

The 2000 bp sequence upstream of the rice *OsZAT12* gene start codon (ATG) was analyzed using online promoter analysis tools to predict regulatory elements in the *OsZAT12* promoter. The results showed that the *OsZAT12* promoter is rich in regulatory elements. In addition to basic core promoter elements (TATA box and CAAT box), the sequence contains cis-acting elements related to abiotic stress and hormones. Abiotic stress-related elements include two MYB transcription factor binding elements. Hormone-related elements include three GA-responsive elements, three ABA-responsive elements, two auxin-responsive elements, and one JA-responsive element (Figure 3 [Figure 3: see original paper]). These findings suggest that *OsZAT12* expression may be regulated by abiotic stress factors and multiple hormones.

2.4 Expression Analysis of OsZAT12 Under Abiotic Stress Treatments

Arabidopsis *ZAT12* plays an important role in reactive oxygen and abiotic stress signaling (Davletova et al., 2005). The rice *OsZAT12* promoter contains abiotic stress-related elements (Figure 3), suggesting it may respond to abiotic stress. We therefore examined *OsZAT12* expression in rice seedlings under low temperature (4°C), osmotic stress (20% PEG 6000), oxidative stress (20 mol L^{-1} MV), and salt stress (100 mmol L^{-1} NaCl). As shown in Figure 4 [Figure 4: see original paper]:A, *OsZAT12* expression was down-regulated within 48 h of low temperature treatment, reaching its lowest level at 12 h before slightly increasing. Under osmotic stress, *OsZAT12* expression showed a trend of initial decrease followed by increase, rising from 3 h to 48 h and reaching approximately twice the untreated level (Figure 4:B). However, under 20 mol L^{-1} MV and 100 mmol L^{-1} NaCl treatments, *OsZAT12* expression showed no significant up- or down-regulation trends over time (Figure 4:C,D). These results indicate that *OsZAT12* expression responds to multiple abiotic stresses, showing different expression patterns with prolonged stress duration.

2.5 Expression Analysis of OsZAT12 Under Different Phytohormone Treatments

Phytohormones act as endogenous signaling molecules that play important roles in plant growth and development. The *OsZAT12* promoter contains multiple hormone-related elements (Figure 3), suggesting it may respond to hormonal level changes. Phytohormone treatment results showed that exogenous ABA significantly down-regulated *OsZAT12* expression. Expression decreased rapidly at 1 h, reached its lowest level at 24 h (approximately 0.1-fold of control), and slightly increased at 48 h (Figure 5 [Figure 5: see original paper]:A). Conversely, exogenous 1 mol L^{-1} 24-eBL up-regulated *OsZAT12* expression beginning at 1 h, which continued to 48 h and peaked at 12-fold of control (Figure 5:B). IAA treatment also up-regulated *OsZAT12* expression, with expression peaking at 24 h and persisting to 48 h (Figure 5:C). These results demonstrate that *OsZAT12* responds differently to various phytohormones and may participate in the regulation of rice growth and development by different hormonal signaling pathways.

2.6.1 Screening of OsZAT12 Knockout Plants

Genomic DNA of *OsZAT12* knockout plants was amplified by PCR using specific detection primers (CRISPR-F and CRISPR-R), and the resulting single PCR products were sequenced. Three homozygous *OsZAT12* knockout lines were obtained, with *oszat12-12-3* and *oszat12-8-15* showing single base insertions and *oszat12-10-10* showing a sequence deletion (Figure 6 [Figure 6: see original paper]).

2.6.2 Expression Detection in OsZAT12 Overexpression Plants

Four homozygous *OsZAT12* overexpression lines were obtained and designated OE8, OE3, OE9, and OE5. qRT-PCR results (Figure 7 [Figure 7: see original paper]) showed that compared with wild type, expression levels in overexpression lines OE8, OE3, and OE5 were significantly increased, while OE9 showed increased expression but not significantly different from wild type. Therefore, OE8 and OE3 were selected for subsequent studies.

2.7 Sensitivity Analysis of OsZAT12 Transgenic Rice to ABA

ABA, as a stress hormone, is an important regulator of plant responses to biotic/abiotic stresses (Chen et al., 2020; Bharath et al., 2021). The *OsZAT12* promoter sequence contains three ABREs (ABA responsive elements) (Figure 3), and ABA treatment suppresses *OsZAT12* expression (Figure 5:A). After obtaining *OsZAT12* overexpression and knockout plants, we further examined their sensitivity to ABA. The results showed that ABA inhibited growth of both wild-type and *OsZAT12* overexpression seedlings, with greater inhibition at higher ABA concentrations. However, plant height and root length of *OsZAT12* overexpression lines were significantly higher than wild type (Figure 8 [Figure 8:

see original paper]). *OsZAT12* knockout plant height was significantly lower than wild type under 5 mol L^{-1} ABA treatment; root length was significantly higher than wild type under low concentration ABA (1 mol L^{-1}) but showed no significant difference under higher concentration ABA (10 mol L^{-1}) (Figure 8). These results indicate that overexpressing *OsZAT12* reduces rice sensitivity to ABA, while knocking out *OsZAT12* enhances ABA sensitivity at appropriate concentrations.

2.8 Observation and Statistical Analysis of Agronomic Traits in OsZAT12 Transgenic Plants

Statistical analysis of agronomic traits showed that during tillering, heading, and maturity stages, *OsZAT12* overexpression rice exhibited significantly reduced plant height compared to wild type, while *OsZAT12* knockout plants showed no significant difference in plant height (Figure 9 [Figure 9: see original paper]:A,B,D,F). Both root length and tiller number showed no significant differences between *OsZAT12* overexpression or knockout plants and wild type (Figure 9:C,E,G). *OsZAT12* knockout plants showed significantly lower panicle number per plant and seed setting rate than wild type, while *OsZAT12* overexpression lines showed no significant difference from wild type (Figure 9:H,I). These results demonstrate that *OsZAT12* affects rice plant height, panicle number per plant, and seed setting rate.

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3 Discussion and Conclusion

Zinc finger proteins are an important family of transcriptional regulators in eukaryotes, with C2H2-type zinc finger proteins being the most common class (Takatsuji, 1999). C2H2-type zinc finger proteins typically contain 1–6 zinc finger domains and feature a QALGGH conserved sequence in the α-helix of the zinc finger structure (Sakamoto et al., 2000; Englbrecht et al., 2004; Ciftci-Yilmaz & Mittler, 2008; Wang et al., 2019). This study found that rice OsZAT12 possesses two typical C2H2 zinc finger domains and one EAR motif, showing high homology with Arabidopsis ZAT12, indicating that OsZAT12 belongs to the rice C2H2 zinc finger protein family. Most zinc finger proteins containing an EAR motif exhibit transcriptional repressive activity (Ohta et al., 2001). For example, transient expression analysis of petunia ZPT2-3 indicated it functions as a repressor (Sugano et al., 2003), while Arabidopsis ZAT12, containing an EAR motif-like sequence, may act as a repressor of AtCBF transcription factors during cold stress responses (Vogel et al., 2005). Our results also demonstrate that rice OsZAT12 protein possesses transcriptional repressive activity, confirming it is a functional transcriptional repressor.

Plant C2H2-type zinc finger proteins, as an important class of transcription factors, have been extensively studied. These transcription factors play crucial regulatory roles in plant growth, development, and responses to abiotic stresses

(Sakamoto et al., 2004; Davletova et al., 2005; Mittler et al., 2006; Rossel et al., 2007; Xie et al., 2012; Shi et al., 2014; Chen et al., 2016; Yin et al., 2017; Ballerini et al., 2020; Yin et al., 2020; Zhang et al., 2021; Rodas et al., 2021). *BnLATE* (Late flowering) in rapeseed reduces silique shattering by limiting lignin polymerization in silique walls (Tao et al., 2017). Rice *NSG1* encodes a C2H2-type zinc finger protein that, similar to Arabidopsis SUP (SUPERMAN), JAG (JAGGED), and NUB (NUBBIN) and rice SL1 (STAMENLESS1), participates in regulating rice flower development (Dinneny et al., 2004; Ohno et al., 2004; Xiao et al., 2009; Zhuang et al., 2020). Constitutive overexpression of *AtZAT10* in transgenic Arabidopsis causes growth inhibition (Sakamoto et al., 2004; Mittler et al., 2006). Our previous study (Chen et al., 2019) also found that heterologous overexpression of *OsZAT12* in Arabidopsis resulted in dwarf plants with inhibited root growth. Similar to the phenotype of heterologous *OsZAT12* expression in Arabidopsis, this study found that *OsZAT12* overexpression rice plants showed significantly reduced plant height at tillering, heading, and maturity stages. These phenotypes resemble those of Arabidopsis or rice plants under stress conditions or overexpressing stress-related transcription factors such as DREB1 (Kasuga et al., 1999), suggesting that *OsZAT12* may be a stress-related gene.

Plant tolerance to abiotic stresses primarily depends on activation of stress-related molecular regulatory networks, including signal perception, hormone signal transduction pathways, and induction of signaling pathway gene expression (Dansana et al., 2014; Lima et al., 2015). Cold stress can cause damage and even death to plants (Wang et al., 2017). C2H2 zinc finger proteins can enhance plant cold tolerance by directly regulating downstream cold stress-related genes (Han et al., 2020). Tomato *SlCZFP1* enhances cold tolerance in transgenic Arabidopsis and rice by inducing constitutive expression of COR (cold-regulated) or cold-responsive genes (Zhang et al., 2011). Soybean *GmZF1* regulates cold stress resistance in transgenic Arabidopsis by binding to the *COR6.6* promoter region and up-regulating its expression (Yu et al., 2014). In banana, overexpression of *MaC2H2-2* and *MaC2H2-3* significantly suppresses transcription of *MaICE1* (inducer of CBF expression, a key component of cold signal transduction); thus, MaC2H2s may enhance banana cold resistance by suppressing *MaICE1* transcription (Han & Fu, 2019). Low temperature treatment up-regulates Arabidopsis *ZAT12* expression (Davletova et al., 2005), while overexpressing this gene down-regulates CBF (CRT/DRE binding factor) gene expression, indicating that ZAT12 negatively regulates Arabidopsis adaptation to cold stress (Vogel et al., 2005). This study shows that 4°C treatment down-regulates *OsZAT12* expression, demonstrating that ZAT12 responds differently to low temperature stress in Arabidopsis and rice, suggesting functional differences between the two. Many abiotic stresses (such as salt, cold, and drought) can induce osmotic stress in plants (Han et al., 2020). Osmotic stress causes physiological drought, ion imbalance, oxidative damage, and growth inhibition (Yamaguchi-Shinozaki & Shinozaki, 2006). Arabidopsis *ZAT10* expression is significantly up-regulated after osmotic stress treatment, especially in leaves, and both *ZAT10* overexpres-

sion Arabidopsis and *zat10* mutants show enhanced osmotic stress tolerance (Mittler et al., 2006). In Arabidopsis, ZAT10 is considered a positive regulator of osmotic stress and is regulated by MAP kinases (Nguyen et al., 2016). Sixteen C1-2i subfamily members have been identified in poplar, six of which are involved in osmotic stress responses (Gourcilleau et al., 2011). Heterologous overexpression of soybean *GmZAT4* in Arabidopsis improves tolerance to 20% PEG 6000 through the ABA pathway (Sun et al., 2019). In rice, expression of *RZF71* is significantly up-regulated after 20% PEG 6000 treatment, indicating its important role in osmotic stress responses (Guo et al., 2007). Unlike the expression patterns of Arabidopsis *ZAT10* and rice *RZF71*, this study found that *OsZAT12* expression initially decreased then gradually increased during 20% PEG 6000 osmotic stress treatment. Collectively, these results indicate that *OsZAT12* expression is regulated by abiotic stresses (such as low temperature or osmotic stress), suggesting that *OsZAT12* participates in rice responses to abiotic stress.

Promoter analysis of rice *OsZAT12* revealed the presence of hormone-related elements. Interestingly, *OsZAT12* expression was up-regulated after 24-eBL and IAA treatments, suggesting that *OsZAT12* also participates in different hormone signaling pathways. ABA is an important regulatory factor in plant abiotic stress responses and is known as the stress hormone due to its crucial roles in drought, cold, heat, salt, and waterlogging stresses (Chen et al., 2020; Bharath et al., 2021). Rice C1-2i subfamily member *ZFP179* shows up-regulated expression after 3 h of ABA treatment, then down-regulates, peaking at 24 h (Sun et al., 2010). In contrast, this study found that ABA significantly down-regulates *OsZAT12* expression, suggesting functional differences between the two genes in ABA signal transduction. Overexpressing *ZFP179* increases ABA sensitivity in rice seedlings (Sun et al., 2010), whereas this study found that overexpressing *OsZAT12* reduces ABA sensitivity. The difference in ABA sensitivity between *OsZAT12* overexpression and *ZFP179* overexpression rice seedlings may be related to their different response patterns to ABA. Interestingly, *OsZAT12* knockout plant height was significantly lower than wild type only under 5 mol L^{-1} ABA treatment; under normal conditions and low ABA concentration (1 mol L^{-1}), root length was significantly higher than wild type, while under higher ABA concentration (10 mol L^{-1}), root length showed no significant difference from wild type. We hypothesize that knocking out *OsZAT12* may reduce endogenous ABA content, and only when appropriate concentrations of exogenous ABA are applied do knockout plants exhibit enhanced ABA sensitivity. This response pattern is similar to that of the *osbglu33* rice mutant and rice plants overexpressing *ZmWRKY114* to exogenous ABA (Ren et al., 2019; Bo et al., 2020). Combined with the response patterns of *OsZAT12* to abiotic stresses (low temperature and osmotic stress) and the stress hormone ABA, we propose that *OsZAT12* participates in regulating abiotic stress and hormone signaling pathways, thereby affecting rice plant architecture development and playing an important regulatory role in panicle morphology and seed setting. Further investigation of the molecular mechanisms by which *OsZAT12* partic-

ipates in different abiotic stress responses and ABA signal transduction will provide an experimental basis for molecular design breeding of stress-tolerant and high-yield rice using *OsZAT12*.

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