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ORIGINAL ARTICLE

# *OsMSR15* Encoding a Rice C2H2-type Zinc Finger Protein Confers Enhanced Drought Tolerance in Transgenic Arabidopsis

**Xin Zhang[1,2,†], Bin Zhang[1,2,†], Ming Juan Li[1,2], Xu Ming Yin[1], Li Fang Huang[1], Yan Chun Cui[1], Man Ling Wang[1] and Xinjie Xia[1,*]**

[1]*Key Laboratory for Agro-ecological Process in Subtropical Region, Institute of Subtropical Agriculture, CAS, Hunan 410125, China*
[2]*Graduate School of the Chinese Academy of Sciences, Beijing 100049, China*

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**Abstract** Zinc nger proteins (ZFPs) play important roles in plant responses to biotic and abiotic stresses. Through microarray analysis, an *Oryza sativa* L. multi-stress-responsive gene, *OsMSR15*, was identied and subsequently cloned from rice Pei'ai 64S (*Oryza sativa* L.). Expression of *OsMSR15* was strongly up-regulated by cold, drought and heat stresses in different tissues at different developmental stages of rice. OsMSR15 contains two C2H2-type zinc nger motifs, a nuclear localization signal (B box), a Leu-rich domain (L-box) and a conserved EAR-motif close to its C-terminus. The OsMSR15-GFP fusion protein was localized to the nucleus. Yeast-one hybrid assay showed that OsMSR15 possesses transcriptional activation ability. Expression of *OsMSR15* in Arabidopsis conferred drought tolerance, and transgenic plants showed hypersensitivity to exogenous ABA during the seed germination and post-germination stages. Transgenic plants also showed higher levels of free proline, less electrolyte leakage and increased expressions of a number of stress-responsive genes, including *LEA3*, *RD29A*, *DREB1A* and *P5CS1* under drought stress. The obtained results indicate that *OsMSR15* is an important regulator involved in plant response to drought stress.

**Keywords:** ABA, Arabidopsis, Drought stress, Cys2/His2-type zinc nger protein, *OsMSR15*

†These authors contributed equally to this work.

*Corresponding author; Xinjie Xia
Tel : +86-0731-84619769
E-mail : jxxia@isa.ac.cn

## Introduction

Abiotic stress such as salt, drought and low temperature often adversely affect plant growth and development, as well as yield. During the adaptation to these stresses, plants perceive changes in the environment and initiate a number of cascade of transcription, and consequently leading to the production of protective proteins and metabolites (Chinnusamy et al. 2004; Kodaira et al. 2011). A series of genes are activated at the transcriptional level during these responses and adaptations. Among them, transcriptional factors play essential role in regulation of downstream genes whose products can protect cells from damage by stresses. For instance, some typical transcription factors, AP2/ERF, bZIP, NAC, MYB, WRKY and ZFP have been confirmed to be involved in stress response via transcriptional regulation modulation (Fernández-Calvo et al. 2011; Todaka et al. 2012; Tripathi et al. 2014). AtTGA4, a bZIP transcription factor, was induced by both drought and low nitrogen stresses, and overexpression of AtTGA4 simultaneously improved drought resistance and reduced nitrogen starvation in Arabidopsis (Zhong et al. 2015). Nucleus-localized transcriptional repressor *GmWRKY27* positively regulates salt and drought tolerance in soybean hairy roots, and its role in the tolerance may be attributed partly to suppressing the expression of *GmNAC29*, which functions negatively in abiotic stress (Wang et al. 2015).

The plant hormone ABA is the central regulator in the adaptive response of plants to abiotic stresses and activates a complex regulatory network enabling plants to cope with water stress conditions (Nakashima and Yamaguchi-Shinozaki 2013). ABA is produced under water deficit conditions and increased ABA levels activate signaling cascade to induce changes in multiple physiological processes such as stomatal

closure, accumulation of compatible solutes and alteration of gene expression (Finkelstein et al. 2002). Exogenous application of ABA induces the expression of a number of genes that respond to dehydration and cold stress. However, some other genes are induced by dehydration and cold stresses but do not respond to exogenous ABA treatment. Therefore, two distinct pathways (ABA dependent and ABA independent) are involved in stress-responsive gene expression. Majority of transcription factors function in either one of these pathways (Yoshida et al. 2014).

Zinc finger proteins (ZFPs) constitute a large family of eukaryotic transcription factors (Sakamoto et al. 2004). The C2H2-type zinc nger proteins are classic zinc finger proteins containing at least one C2H2 zinc nger motif with the consensus of $CX_{2–4}CX_3FX_5LX_2HX_{3–5}H$ which contains two pairs of specific Cys and His residues that bind tetrahedrally to a zinc ion. Comparisons of the reported plant C2H2-type zinc nger proteins revealed the highly conserved QALGGH motif in the zinc-nger helices, which is unique in plant C2H2-type zinc finger proteins. A genome wide annotation analysis of C2H2-type zinc nger proteins found 189 genes in rice and 176 genes in Arabidopsis (Englbrecht et al. 2004; Agarwal et al. 2007). In plants, the C2H2-type zinc nger proteins are integrally involved in various developmental processes, such as the regulation of plant growth, development, the transduction of hormone signals and responses to biotic and abiotic stresses (Payne et al. 2004; Xiao et al. 2009; Weingartner et al. 2011; Shi et al. 2014b).

A number of C2H2-type zinc finger proteins were shown to be involved in the defense response of plants to different abiotic stresses. In Arabidopsis, *ZAT7* showed enhanced expression mainly in roots during salinity stress and positively mediated salt stress tolerance via suppressing repressors of defense responses genes (Ciftci-Yilmaz et al. 2007). Soybean *SCOF-1* was confirmed to play a role in regulating the expression of cold-regulated genes and enhancing cold tolerance in transgenic plants (Kim et al. 2011). In petunia, over-expression of *ZPT2-3* showed increased tolerance to drought stress (Sugano et al. 2003). Recently, a new member of this family, *SlZF2*, was identied in *Solanum lycopersicum* and showed the ability in improving salt tolerance by maintaining photosynthesis and increasing polyamine biosynthesis (Hichri et al. 2014).

To date, few C2H2-type zinc finger proteins from rice have been isolated and functionally characterized. *ZFP179*, a typical C2H2-type zinc finger gene, was isolated from rice and was highly inducible by NaCl, PEG 6000 and ABA treatments (Sun et al. 2010). Constitutive expression of *ZFP179* in rice elevated the expression of several defense-responsive genes under salt stress and enhanced rice tolerance to salt stress. A novel ABA- and $H_2O_2$-responsive C2H2-type zinc finger gene, *ZFP36*, was identified to play a key role in ABA-induced antioxidant defense response and tolerance of rice to drought and oxidative stresses (Zhang et al. 2014). In addition, DST showed to be a negative regulator in the mediation of responses to $H_2O_2$ homeostasis and drought stress (Huang et al. 2009). Mutation of the DST caused the accumulation of $H_2O_2$ in guard cells and triggered stomatal closure, consequently resulting in enhanced drought tolerance in rice.

To identify and clone stress tolerance genes, we analyzed the genome expression profiles of leaf and panicle organs of rice Pei'ai 64S at different developmental stages under multiple stresses by microarray (unpublished data) and identified genes of interesting. In this study, a novel C2H2 type zinc nger gene *OsMSR15* (GenBank accession: EU717839.1) was isolated and functionally characterized. Our data showed that *OsMSR15* was inducible by cold, drought and heat treatments, and expression of *OsMSR15* in Arabidopsis showed enhanced tolerance to drought stress and ABA sensitivity compared with wild type plants. These results indicate that *OsMSR15* plays important roles in drought stress tolerance in plants.

## Results

### Expression of *OsMSR15* under Different Stress Conditions

In our microarray analysis of rice Pei'ai 64s under cold, heat and drought stresses, we noted one gene, designated *OsMSR15*, inducible by cold, heat and drought stresses in different tissues of rice at different developmental stages (Fig. 1). Under the cold treatment condition, the expression level of *OsMSR15* in leaf showed 9.3- and 10.8-fold up-regulation in the seeding and panicle forming stages, respectively. Under the heat treatment condition, the expression levels of *OsMSR15* increased 13.7-fold in the leaf of rice at the panicle-forming stage. In the panicle of rice, the expression of *OsMSR15* increased 5.3- and 9.2-fold in panicle-forming and heading stages under drought stress, respectively. The microarray

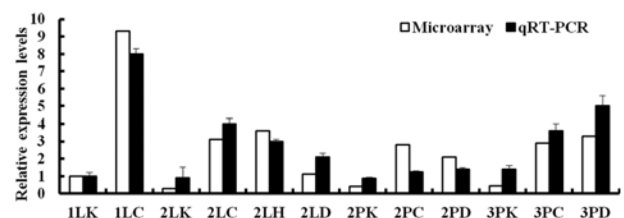


**Fig. 1.** Relative expression levels of *OsMSR15* in leaves and panicles of *indica* rice Pei'ai 64S at different developmental stages under different stress conditions. 1, Seedling stage; 2, booting stage; 3, heading stage; K, control; L, leaf; P, panicle; C, cold; H, heat; D, drought. Error bar represents SE for three independent experiments.

results were further validated by qRT-PCR, and the fold changes observed were similar to those revealed by the microarray analysis, suggesting that *OsMSR15* is a multiple stress-responsive gene in rice.

Cloning and Sequence analysis of *OsMSR15*

We cloned the full-length cDNA of *OsMSR15* from rice Pei'ai64s for further functional analysis. The 902bp cDNA sequence shows 99% of sequence identity to the predicted cDNA of LOC_Os03g41390 in the rice annotation database (http://rice.plantbiology. msu.edu/ index.shtml). The *OsMSR15* gene contains a complete ORF of 714bp, and encodes a putative protein of 238 amino acids with a calculated molecular mass of 24.59 kDa and a p*I* of 8.90. OsMSR15 contains two C2H2-type zinc fingers, with the plant specific

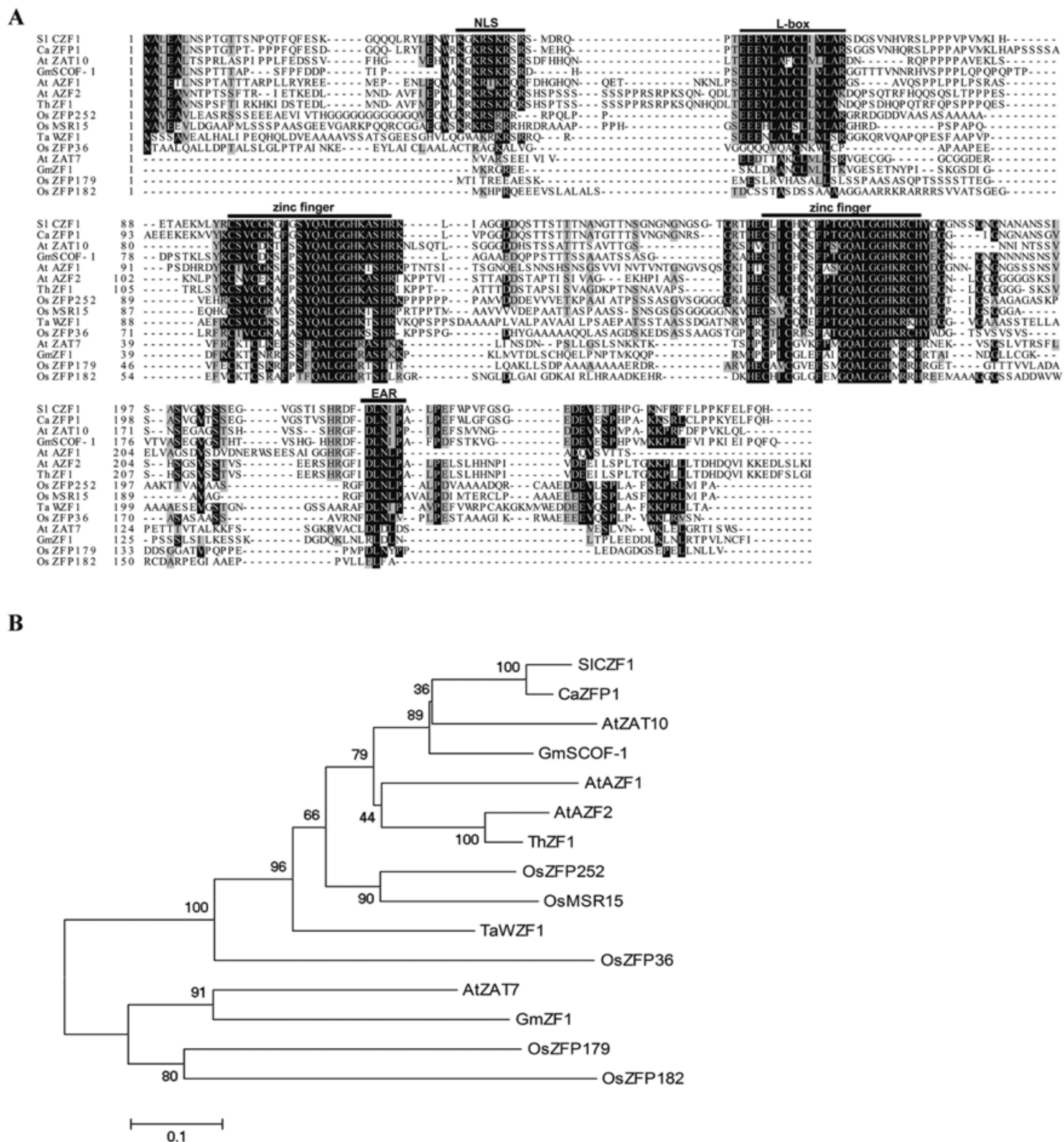


**Fig. 2.** Analysis of the deduced amino acid sequence of OsMSR15. (A) Multiple sequence alignment of OsMSR15 and other stress-responsive C2H2-type zinc nger proteins. Black boxes indicate the positions at which the residues are identical, and grey boxes highlight the residues that are similar to each other. (B) Phylogenetic analysis of OsMSR15 and its related proteins. The Neighbor-Joining tree was constructed with MEGA (version 4.1). The accession numbers of the corresponding amino acid sequences are as follows: AtAZF1 (AED98346.1), AtAZF2 (AAG10143.1), AtZAT10 (AEE30870.1), AtZAT7 (AEE78112.1), OsZFP36 (AAR89021.1), OsZFP179 (AAL76091.1), OsZFP182 (AAP42461.1), OsZFP252 (AAO46041.1), ThZF1 (*Thellu-ngiella halophile*, ABI74621.1), SlCZF1 (*Solanum lycopersicum*, ACG50000.1), TaWZF1 (*Triticum aestivum*, BAA03902.1), GmZF1 (*Glycine max*, XP_003547427.1) and CaZFP1 (*Capsicum annuum*, AAP41717.1).

QALGGH sequence in each zinc finger domain. Comparisons of the amino acid sequences between OsMSR15 and some previously reported two zinc finger proteins of other plants revealed that protein OsMSR15 has a putative nuclear localization signal (NLS), a Leu-rich region (L-box) located at the N-terminus and an EAR-motif located at the C-terminus. OsMSR15 shares a high degree of similarity to *Oryza sativa* OsZFP252 (57.3%), *Arabidopsis thaliana* AtAZF1 (44.5%) and AtAZF2 (40.7%), and *Thellungiella halophile* ThZF1 (43.3%) (Fig. 2A). To investigate the evolutionary relationship among these proteins involved in stress responses, a phylogenetic tree was constructed using Neighbor-Joining method with the full-length amino acid sequences. The result revealed that OsMSR15 was more closely related to OsZFP252, AtAZF1, AtAZF2 and ThZF1 than to other plant C2H2-type zinc finger proteins (Fig. 2B).

OsMSR15 is Localized in the Nucleus

Sequences analysis showed that protein OsMSR15 contains a nuclear localization signal (NLS) at the N-terminus, suggesting that OsMSR15 may target to the nucleus. In order to examine the subcellular localization of the OsMSR15 protein in plant cells, the full-length ORF of *OsMSR15* was fused in frame to the GFP reporter gene to generate 35S:OsMSR15-GFP, and the construct was delivered into the onion epidermal cells by particle bombardment. The results of transient expression showed that GFP signal was observed only in the nucleus of the 35S:OsMSR15-GFP transformed cell, while the onion epidermal cells transformed with the control GFP expression plasmid showed ubiquitous distribution of GFP signal (Fig. 3), suggesting that OsMSR15 is a nuclear-localized protein.

OsMSR15 Functions as a Transcriptional Activator in Yeast Cells

The transcriptional activity of OsMSR15 was examined using a yeast hybrid system. The ORF of *OsMSR15* was ligated in frame to the sequence encoding GAL4 DNA-binding domain (GAL4-DB) in the pGBKT7 vector, and the construct was delivered into yeast strain Y2H Gold. The yeast transformants were examined for their growth on selection medium (SD/-Trp or SD/-Trp/-His/-Ade/X-a-gal/AbA) based on activation of the *HIS3*, *ADE2*, *AUR1-C* and *MEL1* reporter genes in yeast strain Y2H Gold. Yeast strains with the pGBKT7-OsMSR15 vector grew well on both SD/-Trp and SD/-Trp/-His/-Ade/X-a-gal/AbA media. However, the negative control strains transformed with pGBKT7 only grew on the SD/-Trp medium and did not grow in the absence of histidine and adenine (Fig. 4). These dates indicate that OsMSR15 has the transcriptional activity in yeast cells.

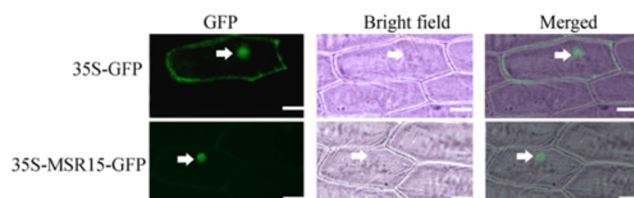


**Fig. 3.** Nuclear localization of the OsMSR15 protein. Constructs carrying 35S-GFP (upper panel) and 35S-OsMSR15-GFP (lower panel) were delivered into onion epidermal cells. Transformed cells were observed by optical (middle) and uorescence microscopy (left). Arrows indicate cell nuclei. Scale bar, 200 µm.

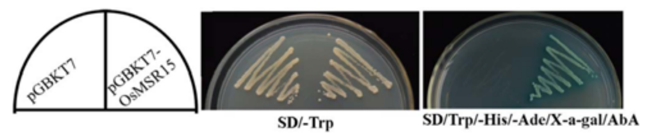


**Fig. 4.** Transactivation assay of OsMSR15 in yeast. The vector pGBKT7 or pGBKT7-OsMSR15 was delivered into yeast strain Y2H Gold and examined on SD/-Trp and SD/-Trp/-His/-Ade/X-a-gal plates containing 125 ng/ml AbA.

Enhanced Drought Tolerance of Transgenic Arabidopsis

To assess the in vivo function of *OsMSR15*, transgenic Arabidopsis plants expressing *OsMSR15* were generated. qRT-PCR analysis showed that *OsMSR15* was expressed in different lines analyzed (Fig. 5), and two independent transgenic lines, L-2 and L-3, were chosen for further study. A root growth assay was conducted to test the effect of *OsMSR15* on drought tolerance of plants at the early seedling stage. In the presence of increased concentration of mannitol, root growth of transgenic plants was less inhibited compared with that of the wild type plants after treatment for ten days (Fig. 6A, B). Only 12% of the wild type plants survived after the drought treatment, while 38% (L-2) and 42% (L-3) transgenic plants survived and could resume

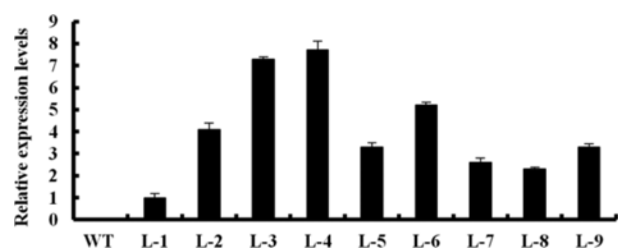


**Fig. 5.** Expression level of *OsMSR15* in transgenic and wild type Arabidopsis. Total RNAs were extracted from the homozygous transgenic lines of T3 generation and wild type Arabidopsis for qRT-PCR analysis. The Arabidopsis gene *AtACTIN2* was used as an internal control. Error bars represent SE for three independent experiments.

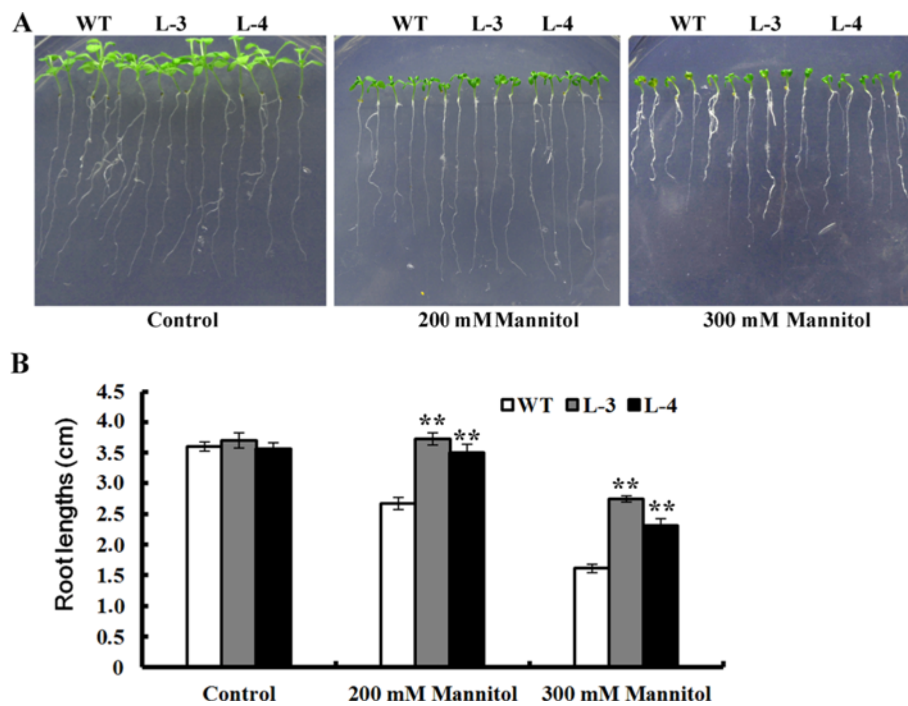


**Fig. 6.** Phenotypes of wild type and transgenic Arabidopsis seedlings grown on 1/2 MS medium supplemented with mannitol. (A) 3-day-old wild type and transgenic seedlings were transferred to new solid medium supplemented with 200 or 300 mM mannitol. Photographs were taken after 10 days treatment. (B) Measurements of root lengths for plants shown in A. All values are means ±SD from three independent experiments (n=5). ** represent signicant differences from the WT at values of P<0.01, as determined by Student's t test.

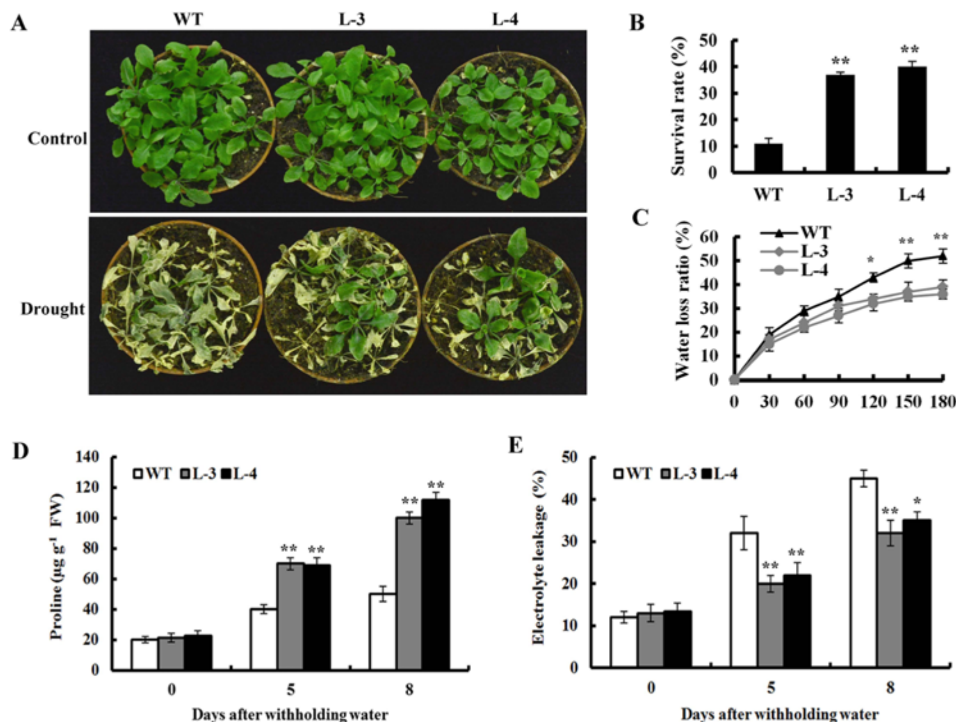


**Fig. 7.** Phenotypic and physiological changes in wild type and transgenic Arabidopsis under the drought stress condition. (A) Three-week-old wild type and transgenic Arabidopsis plants withheld water for 12 d and then allowed to recover for 4 d. (B) Survival rates of wild type and transgenic Arabidopsis plants after recovery for 4 d following drought treatment. (C) Water loss from detached leaves between wild type and transgenic plants. Leaves weights were measured at the indicated time points in triplicate, and three measurements were averaged at each time point. (D, E) Proline concentrations and relative electrolyte leakage in wild type and transgenic Arabidopsis plants after drought treatment. All values are means ± SD from three independent experiments. * and ** indicate signicant differences between WT and transgenic plants at P<0.05 and P<0.01, respectively.

growth after re-watering (Fig. 7A, B).

The increased drought tolerance of the transgenic plants was further confirmed by measuring changes in water loss ratio, electrolyte leakage and proline contents. The water loss ratios were estimated, and leaves of the transgenic plants showed a slower rate of water loss than wild type plants during dehydration process (Fig. 7C). Electrolyte leakage was used to evaluate cell membrane integrity. As shown in Fig. 7E, electrolyte leakage in transgenic plants was signicantly lower than that in wild type plants after the drought stress. Proline contents were significantly increased after the drought treatment in both transgenic and wild type plants; however, compared to wild type plants, higher levels of proline accumulation were detected in transgenic plants under the drought stress condition (Fig. 7D). The above results obtained showed that expression of *OsMSR15* confers the drought tolerance of transgenic Arabidopsis.

Increased ABA Sensitivity of Transgenic Arabidopsis

To determine whether or not the *OsMSR15* is involved in regulating the response of transgenic Arabidopsis to ABA, we examined the ABA sensitivity of transgenic plants relative to wild type plants during the germination and seedling stages. Without treatment with ABA, the seed germination rates of transgenic plants were similar to that of wild type plants. In the presence of ABA, the germination of both wild type and transgenic seeds was inhibited significantly (Fig. 8A). However, the ABA inhibition of transgenic seed germination was more severe than that of the wild type. At the fourth day after sown on MS agar medium containing 0.6 μM ABA, only 24% (L-3) and 21% (L-4) seeds of the transgenic plants germinated, and however 51% of the wild type seeds geminated. On the other hand, under the control condition, the leaf opening and greening rates of transgenic plants showed no obvious differences when compared to wild type plants. However, less opening and greening leaves were observed in the transgenic plants than those in the wild-type plants when exposed to 0.6 μM ABA for 7 days (Fig. 8B).

Altered Expression of Stress-responsive Genes in Transgenic Arabidopsis

To elucidate the possible molecular mechanisms of *OsMSR15* in stress response, the expressions of known stress-responsive genes were assessed in wild type and transgenic plants following the drought treatment and ABA exposure. The transcription levels of *LEA3*, *RD29A* and *P5CS1* showed no significant difference between the wild type and transgenic plants under control condition, while the analyzed genes all showed higher expression levels in the transgenic plants under the drought and ABA conditions (Fig. 9A, B,

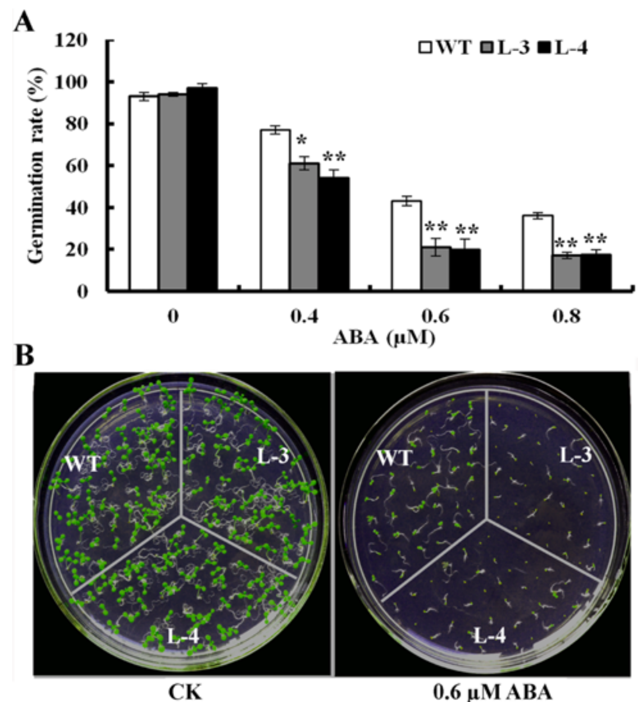


**Fig. 8.** Transgenic Arabidopsis showed increased sensitivity to ABA. (A) Seed germination in response to ABA in wild type and transgenic Arabidopsis. Seeds from wild type and transgenic Arabidopsis were germinated on 1/2 MS agar medium without or with different concentrations of ABA for 4 days. Each value represents the mean ± SE of three replicates (n = 4). * and ** represent signicant differences from the WT at values of $P < 0.05$ and $< 0.01$, respectively, as determined by Student's t test. (B) Growth vigor of wild type and transgenic seedlings under the normal condition (left) and after exposure to 0.6 μM ABA for 7 days (right).

C). The expression of *DREB1A* was higher compared to wild type plants under the normal condition, and *DREB1A* was more strongly induced in transgenic plants under the drought condition. Under ABA treatment, transgenic plants did not promote a higher expression of *DREB1A* compared to wild type plants (Fig. 9D).

## Discussion

Although the roles of some C2H2-type zinc finger proteins have been identified to be related to stress and developmental processes, the functions of C2H2-type ZFPs from rice involved in stress response are largely unknown (Sun et al. 2010; Huang et al. 2012). In this study, as a novel C2H2-type zinc finger protein gene, *OsMSR15* was cloned from rice and then functionally characterized. *OsMSR15* was inducible by cold, drought and heat stresses, suggesting that *OsMSR15* may be involved in stress tolerance. Homology comparison revealed that the OsMSR15 had high identity with other C2H2-type ZFPs, and shared two zinc finger motifs which

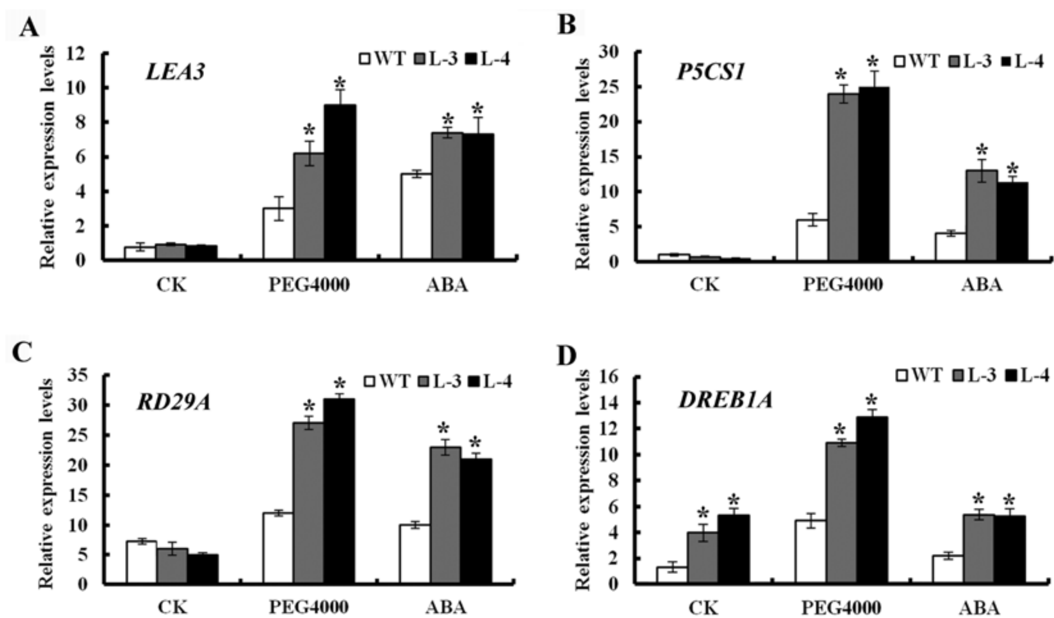


**Fig. 9.** Relative expression levels of stress-responsive genes in wild type and transgenic Arabidopsis under the drought and ABA treatments. Three-week-old wild type and transgenic seedlings were treated with water (CK), 10% PEG4000 and 100μM ABA for 2 h, respectively. Transcript levels of *RD29A*, *LEA3*, *P5CS1* and *DREB1A* were measured by qRT-PCR. *AtACTIN2* was used as an internal control. Error bars represent SD for three independent experiments and asterisks indicate the signicant difference of $P < 0.05$ compared with the wild type.

were proved to be critical for DNA-binding activity (Fig. 2). Yeast hybrid assays indicated that OsMSR15 was an activator of transcriptional activity (Fig. 4). Subcellular localization analysis also revealed that OsMSR15 localized at nuclei (Fig. 3). Based on these observations, OsMSR15 is likely to function as C2H2-type transcription factor in plant cells and may play an important role in signaling pathway in rice under abiotic stresses.

Previous studies have demonstrated that constitutive expression of C2H2-type ZFPs in plants is associated with enhanced tolerance to abiotic stresses (Kim et al. 2011; Shi et al. 2014a). For example, transgenic Arabidopsis plants expressing *GsZFP1* are more tolerant to cold and drought stresses (Luo et al. 2012). Strong induction of *OsMSR15* expression by heat, cold and drought stresses suggests that this gene might be involved in stress tolerance. As expected, transgenic Arabidopsis plants exhibited an enhancement in tolerance to drought stress. In addition, transgenic Arabidopsis plants also showed significantly improved sensitivity to exogenous ABA (Fig. 8). These results suggest that *OsMSR15* is likely to function as a positive regulator in mediating an ABA-dependent signaling pathway for improving tolerance to drought stress. Similar results were reported for *ZmSNAC1*, *OsZFP179*, *OsMYB48-1* as well as others, which suggests that overexpression of these genes conferring increased sensitivity to ABA can increase stress tolerance (Sun et al. 2010; Lu et al. 2012; Xiong et al. 2014).

Plants have developed sophisticated mechanisms to adapt to various stresses. Cell membranes are among the rst targets of adverse stresses, and the maintenance of membrane integrity under abiotic stress conditions is a major component of environmental stress tolerance in plants (Bhaskaran and Panneerselvam 2013). In our work, the electrolyte leakage of transgenic plants was lower than that of wild type plants, indicating that introduction of the *OsMSR15* gene decreased membrane damage under the drought stress condition. Osmotic adjustment is a fundamental cell tolerance response to osmotic stress and can be realized by the accumulation of osmolytes. The increased content of proline in transgenic Arabidopsis could help to adjust the intracellular osmotic potential, thus making the plants have higher water retention capacity (Merewitz et al. 2012; Filippou et al. 2014). Consistently, the leaves of transgenic Arabidopsis had a lower water loss ratio than those of wild type plants, suggesting that expression of *OsMSR15* in transgenic plants resulted in an increased ability to retain water under the drought stress condition. Microscopic check showed that no remarkable difference was observed in the stomatal density and size between the wild type and transgenic plants (data not shown). Since proline can act as an antioxidant to regulate cell membrane stability (Niu et al. 2012), the greater content of proline in transgenic plants may contribute to the lower electrolyte leakage of transgenic plants under stress conditions. Thus, the enhanced drought tolerance of transgenic Arabidopsis maybe partly contributed by the enhanced proline accumulation although the underlying mechanism is yet to be fully

understood.

Many studies have demonstrated that overexpression of transcription factors in plants activate the expression of stress/ABA-responsive genes, which in turn enhances tolerance to various stresses (Ren et al. 2010; Shi et al. 2014a). The expression of *DREB1A*, *RD29A*, *LEA3* and *P5CS1* genes is induced by external stimuli and plays important roles in plant response to abiotic stresses. The DREB/CBF transcription factors play critical roles in cold, salt and drought stress responses via binding to the C-repeat/dehydration-responsive *cis*-acting element of several stress-responsive genes. Ectopic expression of DREB/CBF genes leads to enhanced expression of downstream stress-responsive genes and increased tolerance to some kinds of abiotic stresses. We noted that OsMSR15 could activate expression of *DREB1A* in Arabidopsis under the normal condition as well as under the drought stress condition, indicating the positive regulation of *DREB1A* contributed to *OsMSR15*-mediated drought stress resistance. *P5CS1* is the rate-limiting enzyme of proline synthesis in plants. *RD29A* and LEA3 are known for its involvement in responses to drought and salt stresses (Nakashima et al. 2009). *P5CS1*, *RD29A* and LEA3 also had a higher expression levels in transgenic plants in drought stress condition, compared to wild type plants (Fig. 9). The differences in drought tolerance between the wild-type and transgenic plants might be due to the reinforced expression of the above-mentioned genes and possibly other stress-responsive genes in the transgenic plants. The results suggest that the drought tolerance exhibited by transgenic plants might be conferred by the coordinated work of the proteins encoded by these genes. We speculate that *OsMSR15* may positively regulate the expression levels of some stress-responsive genes under drought stress condition. However, expression of *OsMSR15* could not enhance the expression of stress-responsive genes, including *P5CS1*, *LEA3* and *RD29A* in transgenic plants under the normal growth condition. One possible explanation is that *OsMSR15* may mediate the activation of such stress-responsive genes accompanied by other stress-responsive regulators.

ABA plays diverse roles in plant development and the adaption to environmental stresses such as drought, high salinity and low temperature. Under abiotic stress conditions, this hormone is rapidly accumulated and then functions as a secondary messenger in abiotic stress signaling (Peleg and Blumwald 2011; Lee and Luan 2012; Liu et al. 2015). Our data showed that Arabidopsis plants expressing *OsMSR15* had signicantly increased sensitivity to exogenous ABA, indicating that OsMSR15 might play a role in the ABA signal transduction pathway during the stress responses. Moreover, it was shown that the *P5CS1*, *RD29A* and *LEA3* were all more highly expressed in transgenic plants compared with wild type plants under either drought stress or ABA treatment, suggesting that the regulation of these stress responsive genes by *OsMSR15* might be ABA dependent under drought stress. We also note that the transcript levels of *DREB1A* were not altered in either transgenic or wild type plants under ABA treatment. Because it is well known that *DREB* genes are mainly involved in ABA independent signal transduction pathway, the *DREB1A*-regulated expression of some stress responsive genes may be controlled by *OsMSR15* in an ABA-independent manner. Altogether, it is suggested that *OsMSR15* may play important roles in response to drought stress both in ABA-dependent and -independent pathways.

Overall, OsMSR15 is characterized as a C2H2-type transcription factor, which is localized in the nucleus. Expression of *OsMSR15* resulted in an enhancement in drought tolerance of the transgenic Arabidopsis by activating transcription of some stress-responsive genes. Our data suggest that OsMSR15 probably functions as a positive transcription factor in the complex regulatory systems for drought stress response.

## Materials and Methods

### Plant Material and Growth Conditions

Seeds of rice Pei'ai 64S (*Oryza sativa* L.) were sterilized with 0.1% $HgCl_2$. After washing three times with sterile water, they were kept in water for 3 days at 25°C (with daily water changes), and then germinated in an incubator at 37°C in darkness for 48-72 h. Germinated seeds were sown on plastic pots filled with soil in the greenhouse at 28°C/22°C (day/night) with a 16-h photoperiod. Rice stress treatments and microarray analysis were performed as described previously (Xu et al. 2011). Seeds of *Arabidopsis thaliana* (Columbia 0) were surface-sterilized with 10% (v/v) bleach and 0.1% (v/v) Triton X-100 for 25 min and then washed three times with sterile water. After stratication at 4°C for 3 d in darkness, Arabidopsis seeds were sown on 1/2-strength Murashige and Skoog (MS) medium supplemented with 1% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8). When seedlings achieved the four-leaf stage, they were transplanted into soil and placed in a growth room at 26°C /22°C (day/night) with 65% relative humidity under a 16-h photoperiod.

### Subcellular Localization of the OsMSR15 Protein in Onion Epidermal Cells

The coding sequences of *OsMSR15* were rst amplied using primer pairs, 5'-AAGCTTTCTTTGCCCATTACTCTACTCC-3' (forward, *Hind*III site underlined) and 5'-CCATGGAAGCAGGGATCA-TTAGC-3' (reverse, *Noc*I site underlined). After verifying by sequencing, the PCR fragment was digested with *Hind*III and *Noc*I and ligated into the pJIT163-GFP vector to obtain a transcriptional fusion of OsMSR15 and GFP under the control of the CaMV 35S promoter. The fusion (CaMV35S:OsMSR15-GFP) and control (CaMV35S:GFP) plasmids were delivered into onion epidermal cells by particle-bombardment. After bombardment, the bombarded tissues were incubated on 1/2 MS agar medium in darkness for 24-36 h. The GFP signal was observed with a Leica MZ16FA uorescent

stereomicroscope.

Trans-activation analysis of OsMSR15

Gene-specic primers 5'-CATATGATGGCGGTGGAGGAGGTTC-3' (forward, *NdeI* site underlined) and 5'-GAATTCAGCAGG-GATCATTAGCCTTGG-3' (reverse, *EcoRI* site underlined) were used to clone the whole open reading frame of *OsMSR15*. DNA fragments containing the whole ORF of *OsMSR15* were inserted into the *NdeI/EcoRI* site of the pGBKT7 vector to create the pGBKT7-*OsMSR15* construct. According to the protocol provided by manufacturer, pGBKT7-*OsMSR15* and the negative control pGBKT7 plasmids were used to transform yeast strain Y2H Gold containing *AUR1-C*, *ADE2*, *HIS3* and *MEL1* reporter genes. The transformed strains were streaked onto SD/-Trp or SD/-Trp/-His/-Ade/X-a-gal/AbA plates for 4 d, and the trans-activation activity of each protein was evaluated according to their growth status and the activity of a-galactosidase.

Construction of the Expression Vector and Arabidopsis Transformation

Gene-specic primers 5'-AAGCTTTCTTTGCCCATTACTCTACTCC-3' (forward, *Hind*III site underlined) and 5'-GGATCCCCA-GCTCGCCTGAATCTAC-3' (reverse, *Bam*HI site underlined) were used to clone the whole open reading frame of *OsMSR15*. The products were inserted into the PMD18-T vector (Takara), sequenced and then subcloned into the modified vector pC163 (derived from pCAMBIA1300) using the restriction enzymes *Hind*III and *Bam*HI, in which the expression of the recombinant gene is under the control of CaMV 35S promoter. Construct pC163-*OsMSR15* was introduced into *Agrobacterium tumefaciens* EHA105 cells, and Arabidopsis transformation was performed by the oral dipping method (Clough and Bent 1998). Transformed seeds were selected on 1/2 MS medium supplemented with 25 mg/L hygromycin. Transgenic lines displaying a segregation ratio of 3:1 (resistant: sensitive) were obtained. The expression levels of *OsMSR15* in the different transgenic lines were determined by qRT-PCR. T3 seeds that exhibited 100% resistance to hygromycin were used for further experiments.

## Stress Tolerance Assays for Arabidopsis

For measuring root growth under mannitol treatment, stratied seeds were germinated on 1/2 MS agar plates for 3 days. Seedlings were then transferred to 1/2 MS agar plates supplemented with different concentrations of mannitol (0, 200 and 300 mM), and the plates were maintained vertically in a growth chamber. Root length was scored at the tenth day. For drought assays, each pot was lled with equal amount of soil and growth substrate which were homogeneously and thoroughly mixed with distilled water. 3-week-old plants grown in soil were withheld water for 12 days. Photographs were taken 4 days after watering was resumed. Seedlings which did not grow were considered as dead, and survival rates were then determined. During the drought stress experiment, soil water content differed by < 5% among all pots (data not shown).

For the germination assays, seeds from wild type and transgenic plants were sown in triplicate on 1/2 MS medium in the same plates with different concentrations of 0-0.6 μM ABA. Seeds were incubated for 2 days at 4°C in darkness to break dormancy before transferring to the growth chamber. Seeds were considered germinated when radicles completely penetrated the seed coat. The germinated seeds were scored at the fourth day.

For qRT-PCR analysis of stress-responsive genes, three-week-old Arabidopsis seedlings were immersed in 1/2-strength MS solutions containing 10% polyethylene glycol (PEG), or 100 μM ABA for abiotic stresses. Samples were collected after 2 h of exposure.

**Table 1.** The primers' sequences of qRT-PCR analysis described in this article

| Gene name | Primer sequence |
|---|---|
| AtACTIN2 | F: 5'-AGGTATCGCTGACCGTATGAG-3'<br>R:5'- CATCTGCTGGAATGTGCTGA-3' |
| RD29A | F: 5'-ACGTCGAGACCCCGATAA C-3'<br>R: 5'-CAATCTCCGGTACTCCTCCA-3' |
| P5CS1 | F: 5'-AAGGCTTGTGATACGGATATG G-3'<br>R: 5'-ATGCACAAGAAGGGTTTCCA-3' |
| DREB1A | F: 5'-AACTTGCGCTAAGGACATCC-3'<br>R: 5'- CTCGGCATCTCAAACATCG-3' |
| LEA3 | F: 5'-GATTGACCCGGCTGAGCTACGA-3'<br>R: 5'-AGATGGGATTCACCACAAAAGA-3' |
| OsMSR15 | F: 5'-CTTTGCCCATTACTCTACTCCACTG-3'<br>R: 5'- CCACCTCCTCGCCGCTC-3' |

Quantitative Real-time RT-PCR (qRT-PCR) analysis

RNA isolation and quantitative real-time PCR was performed as described by Xu et al. (2011). *AtACTIN2* was used as internal controls in Arabidopsis. The total RNAs were used as templates in qRT-PCR reactions with primers for the *OsMSR15*, *LEA3*, *RD29A*, *P5CS1* and *DREB1A*. The primer pairs are listed in Table 1. The relative amounts of mRNA were calculated using the comparative threshold cycle method. All reactions were repeated for three times.

Measurement of Leaf Water Loss, Electrolyte Leakage and Proline Content

For water loss analysis, rosette leaves from wild type and transgenic Arabidopsis plants were detached from 4-week-old plants and placed on weighing dishes at a constant temperature (22°C) and humidity (50%) for the indicated periods. Weights of the samples were recorded at regular intervals. Determination of electrolyte leakage was performed as described by Qin et al. (2015). Free proline concentrations in leaf extracts from drought-stressed wild type and transgenic Arabidopsis were determined as described by Bates et al. (1973). For each sample, the measurement was repeated three times.

Statistical Analyses

All data were examined by ANOVA, using the SAS statistics program. Statistically signicant differences ($P < 0.05$ or $P < 0.01$) were computed based on the Student's t-tests. Data are the means±SD of three independent replicates.

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## Author's Contributions

ZX constructed the vector, generated the transgenic plants, performed abiotic stress treatment and drafted the manuscript; ZB, YXM and LMJ participated in function analysis of the transgenic plants; WML, HLF and CYC analyzed the data; XX designed the experiment, supervised the work and revised the manuscript. All the authors

agreed on the contents of the paper and post no conflicting interest.

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