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OsADR3 increases drought stress tolerance by inducing antioxidant defense mechanisms and regulating *OsGPX1* in rice (*Oryza sativa* L.)

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ABSTRACT

The C (Cys) 2H (His) 2-type transcription factor is one of the most important transcription factors in plants and plays a regulatory role in the physiological responses of rice to abiotic stresses. A novel rice C2H2-type zinc finger protein, abscisic acid (ABA)-drought-reactive oxygen species (ROS) 3 (OsADR3), was found to confer drought stress tolerance by enhancing antioxidant defense and regulating *OsGPX1*. Overexpression of *OsADR3* in rice increased tolerance to drought stress by increasing ROS scavenging ability and ABA sensitivity. In contrast, CRISPR/Cas9-mediated knockout of *osadr3* increased the sensitivity of rice to drought and oxidative stress. An exogenous ROS-scavenging reagent restored the drought-stress tolerance of *osadr3*-CRISPR plants. Global transcriptome analysis suggested that *OsADR3* increased the expression of *OsGPX1* under drought stress. Electrophoretic mobility shift, yeast one-hybrid, and dual-luciferase reporter assays revealed that *OsADR3* modified the expression of *OsGPX1* by directly binding to its promoter. Knockdown of *OsGPX1* repressed ROS scavenging ability under drought stress in *OsADR3*-overexpression plants. These findings suggest that *OsADR3* plays a positive regulatory role in drought-stress tolerance by inducing antioxidant defense and associated with the ABA signaling pathway in rice.

1. Introduction

Plants are subject to various abiotic stresses, with drought being one of the most important, as it influences plant growth and can lead to severe crop losses [1]. Approximately 43% of the cultivated land area worldwide is subject to drought conditions, limiting the planting area [2]. Understanding the mechanisms of drought tolerance in crops will allow maintaining or improving their yield and quality in areas subject to drought stress.

Reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO), and singlet oxygen ($^1O_2$), play essential roles in signal transduction during plant drought stress [3]. However, high levels of ROS may cause irreversible damage to cells, which is one of the most harmful effects of drought stress in rice, *Oryza sativa* L. [4]. To balance ROS production and destruction, plants have evolved an antioxidant system that includes both antioxidant enzymes, such as ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX), and catalase (CAT), and antioxidant compounds, such as ascorbate (ASC) and glutathione (GSH), to scavenge ROS [5]. High ASC and GSH contents or ASC/dehydroascorbate (DHA) and GSH/oxidized glutathione (GSSG) ratios in tissues play a central sensing role in maintaining the ASC-GSH cycle under stress [6]. Identifying genes regulating the activities of antioxidant enzymes and antioxidant compounds content will be essential for increasing plant stress tolerance.

The rice glutathione peroxidase (*OsGPX*) gene family contains five members distributed in various organelles: *OsGPX1* and *OsGPX3* in the mitochondria, *OsGPX2* and *OsGPX5* in the cytosol and chloroplast, and *OsGPX4* in the chloroplast [7]. The *OsGPX* family plays crucial roles in preventing H_2O_2 accumulation and protecting cell membranes against ROS-induced damage [5].

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Previous study has investigated the role of GPX under abiotic and biotic stresses as well as in signal transduction and as redox sensor proteins [8]. For instance, *OsGPX1*, encoding mitochondrial GPX, increases rice tolerance to salt stress by regulating seed germination and plays a role in ROS scavenging as well as in root and shoot development [9,10]. Silencing of *OsGPX3* impairs normal plant development and increases H_2O_2 accumulation in young plants [9]. However, the regulatory genes of *OsGPX* and transcription factors (TFs) that bind the *OsGPX* promoter are unknown.

C2H2-type TFs are a class of proteins defined by the QALGGH motif and are known to efficiently promote the stress tolerance in crops. For example, *ZFP36*, *ZFP179*, and *ZFP245* are central regulators of ROS and abscisic acid (ABA) signaling transduction, and dramatically increase the activities of superoxide dismutase and peroxidase under drought and oxidative stresses [11–13]. *ZFP182* and *ZFP252* are transcription regulators that regulate the expression of stress-responsive genes and accumulation of osmoprotectants via the ABA pathway to improve drought- and oxidative-stress tolerance in rice [14,15].

In the present study, a novel rice C2H2-type zinc-finger protein, ABA-drought-ROS 3 (*OsADR3*, *LOC_Os03g55540*), which is preferentially expressed in the leaf tissues of lowland rice (LR) in response to different abiotic stresses [16], was identified. The previous study has demonstrated that upland rice (UR) has evolved to be more drought-tolerant than LR, although previous studies suggested that LR might harbor more drought-tolerant genes than UR [16]. To test this hypothesis, we transferred or knocked out *OsADR3* in the UR variety IRAT109 to determine whether it could promote drought tolerance. We evaluated the functions of the drought-tolerant gene *OsADR3* and rice genetic resources for breeding drought-tolerant cultivars, providing a theoretical basis for improving the molecular mechanisms of rice drought tolerance.

2. Materials and methods

2.1. Plant materials, growth conditions, and stress treatments

Rice cultivars Nipponbare and IRAT109 (*O. sativa japonica*) were used for qRT-PCR analysis under various stresses and phytohormone treatments, and IRAT109 was used for all transgenic experiments. For qRT-PCR, rice seeds were first sterilized with 10% NaClO, germinated at 32 °C for 3 days, and then grown in Hoagland nutrient solution with a 14-h light/10-h dark photoperiod, a 28 °C (light)/22 °C (dark) temperature range, 200 μmol m^{-2} s^{-2} light intensity, and 80% relative humidity. Four-week-old plants were subjected to dehydration (20% PEG6000 (w/v)), salt (200 mmol L^{-1} NaCl), cold (4 °C), H_2O_2 (2% v/v), ABA (100 μmol L^{-1}), and drought (no watering) treatments. Their leaves were harvested at 0, 1, 3, 6, 12, 24, and 48 h after the beginning of treatments. All harvested leaf samples were rapidly frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

For drought stress tolerance assays, uniformly germinated seeds of wild type (WT), *OsADR3*-OE, and *osadr3*-CR lines were sown in pots containing sterilized soil and grown in a greenhouse with 12-h light/12-h dark photoperiod, 28 °C (light)/26 °C (dark) temperature conditions, 200 μmol m^{-2} s^{-2} light intensity, and 80% relative humidity. Four-week-old plants had their watering withheld and were re-watered after 21 days of the drought stress treatment.

For exogenous ABA treatment, seeds of WT, *OsADR3*-OE, and *osadr3*-CR lines were sterilized with 10% NaClO and cultured on 1/2 MS medium with or without 10 μmol L^{-1} ABA under a 14-h light/10-h dark photoperiod and 28 °C (light)/22 °C (dark) temperature conditions for 7 days.

For osmotic stress treatment, seeds of WT, *OsADR3*-OE, and *osadr3*-CR lines were sterilized with 10% NaClO and cultured on 1/2 MS medium, with or without 500 mmol L^{-1} mannitol under a 14-h light/10-h dark photoperiod and 28 °C (light)/22 °C (dark) temperature conditions, for 7 days.

For oxidative stress treatment, the seeds of WT, *OsADR3*-OE, and *osadr3*-CR lines were germinated at 32 °C for 3 days, and then grown in Hoagland nutrient solution with or without 2% (v/v) H_2O_2 under a 14-h light/10-h dark photoperiod, 28 °C (light)/22 °C (dark) temperature conditions, 200 μmol m^{-2} s^{-2} light intensity, and 80% relative humidity for 7 days.

Treatments were applied every day, and the phenotypes and physiological indices of the WT and transgenic plants under the experimental stresses were recorded.

2.2. RNA extraction and qRT-PCR analysis

Total RNA was extracted from rice tissues by the TRIzol method (Thermo Fisher Scientific, Waltham, MA, USA) and treated with DNase I to eliminate any DNA contamination. The quality of the total RNA was assessed using a NanoDrop 2000 (Thermo Fisher Scientific, Shanghai, China). First-strand cDNA (10 μL) was synthesized according to the instructions for the PrimeScript RT Master Mix (Takara, Beijing, China). qRT-PCR primers were designed with Primer Premier 5.0 software (PREMIER, Palo Alto, CA, USA), based on the *OsADR3* transcript sequence (Table S1). qRT-PCR was performed as previously described [17].

2.3. Multiple sequence alignment and phylogenetic analysis

A multiple sequence alignment was performed for the amino acid sequences of the C2H2 conserved domain of *OsADR3* and some C2H2-type zinc-finger proteins from multiple species using MEGA 7.0 software (https://www.megasoftware.net/) [18]. Unrooted trees were constructed by the maximum-likelihood method with the following parameters: Poisson correction, pairwise deletion, 1000 bootstrap replicates.

2.4. Plasmid construction and generation of OsADR3-OE and osadr3-CR plants

To construct the *OsADR3*-OE plasmid, the open read frame of the *OsADR3* was cloned in the *Asc* I and *Pac* I sites of the pMDC32 binary vector using a specific primer (Table S2). For the construction of the *osadr3-CR* plasmid, the target sites were identified with CRISPR-P 2.0 (http://cbi.hzau.edu.cn/cgi-bin/CRISPR). The plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105. *A. tumefaciens*-mediated transformation of IRAT109 was performed as described previously [19]. Knockout lines were confirmed by PCR sequencing with primers 5′-ATGTCGAGCGCGTCGTCCAT-3′ and 5′-TTACGCGGTGAGAAGCCGG-3′.

2.5. Transcriptional activation assay in yeast

The full-length ORF of the *OsADR3* was inserted into the *Bam*H I/*Sal* I site of the pGBKT7 vector to generate the *OsADR3*-GAL4 BD vector. The transcriptional activation activity of *OsADR3* was determined as previously described [20]. The trans-activation activities of each protein were evaluated according to growth status of yeast cells and activity of β-galactosidase. The primers used for *OsADR3* trans-activation analysis are presented in Table S2.

2.6. Subcellular localization

The coding sequences of the *OsADR3* were fused to green fluorescent protein (GFP) reporter coding sequences and were inserted into the *Hind* III/*Bam*H I site of pJIT163-GFP plasmids. The plasmids were transformed into isolated rice protoplasts using polyethylene

glycol (PEG)-mediated transformation methods [21]. The nuclear-localized protein fused with mCherry was used to stain nuclei. GFP and chlorophyll signals were detected using a Leica TCS-SP2 AOBS laser scanning confocal microscope (Leica, Heidelberg, Germany).

2.7. Osmotic stress tolerance assay with E. coli

The effect of 1000 μmol L^{-1} sorbitol on the growth of *E. coli* strain DH5α containing the empty pET32a plasmid as a control vector and the pET32a-*OsADR3* recombinant plasmid was investigated by shake cultivation in liquid cultures. *E. coli* was inoculated into LB medium containing the same concentration of sorbitol, and then cultured under the same conditions to logarithmic phase (~4 h). The growth situation of the bacterial suspension was measured by flow cytometry using the Guava Easy Cyte HT system (Merck Millipore, Darmstadt, Germany).

2.8. Yeast one-hybrid assay

Y1H assays were performed to verify the physical interactions between promoters and TFs. The promoter fragment of *OsGPX1* was cloned into the pHIS2 vector. The primers used are described in Table S2. The CDS of *OsADR3* were inserted into the pGADT7 vector to generate recombinant pGAD-*OsADR3* constructs. The Y1H assay was performed according to the manufacturer's instructions (Matchmaker Gold Y1H Library Screening System; Clontech Laboratories, Mountain View, CA, USA). pGAD-Rec2-53 and Phis2-*OsGPX1*-Promoter (pHIS2.1), pGAD-*OsADR3* and pHIS2.1, and pGAD-Rec2-53 and pHIS2.1 were used as negative controls. pGAD-Rec2-53 and p53HIS2 were provided in the kit as positive controls. The plasmids were co-transformed into yeast Y187 strains which were then plated on SD-Trp/-Leu/-His medium containing either 0 mmol L^{-1} 3-AT (3-amino-1,2,4-triazole) (control) or 40 mmol L^{-1} 3-AT as described previously [22].

2.9. Electrophoretic mobility shift assay (EMSA)

Histidine (His)-*OsADR3* fusion proteins were obtained by *in vitro* prokaryotic expression. The cDNAs encoding full-length *OsADR3* were cloned into pET28a to generate His-fusion recombinant vectors, which were then expressed in *E. coli* BL21 (DE3) (TransGen Biotech Co., Ltd., Beijing, China, CD601-02). 0.2 mmol L^{-1} isopropyl b-D-1-thiogalactopyranoside was applied to induce protein expression. His-fusion proteins were purified with a His-tagged Protein Purification kit (P2229S, Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

The *OsGPX1* promoter fragment containing CACAAATAGTG motifs was synthesized by TSINGKE (Beijing, China). The EMSA Probe Biotin Labeling kit and Chemiluminescent EMSA kit (GS008 and GS009; Beyotime Biotechnology, Shanghai, China) were used for the EMSA, which were performed according to the manufacturer's instructions. Unlabeled probes were used for probe competition, and His protein was used as a negative control.

2.10. Dual-luciferase reporter assay

The *OsGPX1* promoter fragment containing the CACAAATAGTG motif was inserted into pGreenII 0800-LUC vectors to generate the reporter construct. 35Spro:*OsADR3* effectors were generated by recombining *OsADR3* into the pGreenII 62-SK vector. Transformation and dual-luciferase (LUC) activity determination was performed as described previously [23]. The recombinant plasmids were introduced into *A. tumefaciens* GV3101 which were then cultured to an OD at 600 nm of 0.15. The reporter and effector were combined in equal volumes, maintained at 20 °C without shaking for 3 h, transfected into *Nicotiana benthamiana* leaves, and incubated for 60 h. The LUC and *Renilla* luciferase (REN) activity levels were determined using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Transactivation was expressed as the ratio of LUC: REN. At least 15 replicates (five biological replicates × three technical replicates) were evaluated per experiment.

2.11. siRNA synthesis and transient expression assays in rice protoplasts

The siRNA fragment of *OsGPX1* (F: 5′-CAGUUGUAUGAGAA GUACA-3′; R: 5′-UGUACUUCUCAUACAACUG-3′) was synthesized by TSINGKE. Rice plants were grown in the dark at 28 °C for 1–2 weeks. When plants were 4–8 in. tall, protoplasts from leaf and stem tissue were isolated as described previously [24]. Introduction of siRNA into protoplasts was performed essentially as described [25]. To evaluate osmotic stress in rice protoplasts, the protoplasts were cultured on the base of the cell culture plates and WI solution containing 0.5 mmol L^{-1} mannitol, 20 mmol L^{-1} KCl_2, 4 mmol L^{-1} MES, pH 5.65. The protoplasts were treated with a high concentration of mannitol; WI solution was replaced with buffer containing 1 mol L^{-1} mannitol, 20 mmol L^{-1} KCl_2, 4 mmol L^{-1} MES, pH 5.65.

2.12. H_2O_2 detection by confocal laser scanning microscopy

H_2O_2 production in protoplasts was visualized using the H_2O_2-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA; Thermo Fisher Scientific) as described previously [26]. The fluorescent signals were detected using Leica IMAGE software (Leica Microsystems, Wetzlar, Germany).

2.13. RNA sequencing (RNA-seq) analysis

Total RNA was extracted from leaves of four-week-old rice plants under drought and normal conditions at three time points. The RNA samples (3 μg) from the three biological replicates were mixed for each genotype and sent to Beijing Biomarker Technologies Co., Ltd. (Beijing, China) for sequencing. Construction of the sequencing libraries was performed according to the manufacturer instructions of the Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA). DEGs responding to drought stress were defined by a ≥2-fold expression change and false discovery rate (FDR) < 0.01, and genes which were down-regulated or up-regulated between WT and OE plants were subjected to further gene ontology (GO) enrichment analysis. Heat maps were created with HemI 1.0 software and based on the expression data [27]. The expression levels of selected DEGs were verified by qRT-PCR.

2.14. Physiological measurements

Fresh weight, shoot height, root length, germination rate, and survival rate under each treatment were measured as described previously [28]. Six plants of each line were used per replicate, with three replicates for each line. Free Pro content in leaves was measured by the sulfosalicylic acid method [29] in the leaves of four-week-old seedlings for each line. Malondialdehyde (MDA) content was measured as described previously [30]. Total protein extract from rice leaves was used for APX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), GPX, CAT, and GR activity assays. Content of total soluble protein was measured by the Bradford method [31]. For GPX activity, 0.05 g fresh leaves were ground to powder in liquid nitrogen and 1 mL potassium phosphate buffer (pH 7.5) was added. GPX and GR activities were further measured as described [9,32]. One unit of GR was

defined as the amount of enzyme that reduced 1 optical density min^{-1} at 340 nm and one unit of GPX was defined as the amount of enzyme that degraded 1 μmol of GSH min^{-1}. For analysis of APX activity, 2 mmol L^{-1} vitamin C (AsA) was added to the extraction buffer and the homogenate was centrifuged at 12,000×*g*. APX activity was determined as previously described [33]. One unit of APX activity was defined as the amount of enzyme that degraded 1 μmol of AsA min^{-1}. The enzyme activity of CAT was determined as described previously [34]. One unit of CAT was defined as the amount of enzyme that degraded 0.1 mol of H_2O_2 min^{-1}. The assay for MDHAR and DHAR activities was performed as described previously [35,36]. One unit of MDHAR activity was defined as the amount of enzyme that oxidizes 1 nmol of NADH min^{-1} at 25 °C and one unit of DHAR activity was defined as the amount of enzyme that produces 1 nmol of AsA min^{-1} at 25 °C.

To determine the content of H_2O_2, 1 g of leaf tissue was ground to powder in liquid nitrogen and 2 mL of 100 mmol L^{-1} K-phosphate buffer (pH 6.8, containing 0.1 mmol L^{-1} EDTA) was added. Total soluble protein content was measured by the Bradford method [31]. The content of H_2O_2 was measured using the Amplifu Red (Sigma-Aldrich, St. Louis, MI, USA) method [37]. ASC and DHA were measured by high-performance liquid chromatography as described previously [38]. The contents of GSH and glutathione disulfide in 3% sulfosalicylic acid extract were determined as previously described [39,40].

For quantification of endogenous ABA content, leaves (0.3 g) were ground to powder in liquid nitrogen and then homogenized in 4 mL 80% methanol containing 1 mmol L^{-1} butylated hydroxytoluene as an antioxidant. After overnight incubation at 4 °C, the mixture was centrifugated for 10 min at 5000×*g* and 4 °C. The supernatant was collected, dried under nitrogen gas, and washed with 0.2 mL 100 mmol L^{-1} disodium hydrogen phosphate solution (pH 9.2). The resulting supernatant was extracted with 0.2 mL ethyl acetate and dried under nitrogen gas. The extract was dissolved in 0.5 mL 80% methanol. Quantification of ABA was performed using enzyme linked immunosorbent assays, as described previously [41].

2.15. Statistical analyses

Differences among treatments were evaluated using analysis of variance in SPSS 19.0 software (SPSS Inc., IBM, https://www.ibm.com/cn-zh/analytics/spss-statistics-software) and Microsoft Excel 2016 (Microsoft, Redmond, WS, USA). Statistically significant differences ($P < 0.05$ or $P < 0.01$) were identified based on Student's *t*-tests.

3. Results

3.1. Expression profiles of OsADR3 in UR and LR

In a previous cDNA microarray analysis, *OsADR3* was identified as a candidate gene preferentially expressed in LR [16]. As shown in Fig. 1A, the expression levels of *OsADR3* were higher in the LR cultivar Nipponbare (Nip) than in the UR cultivar IRAT109 under the treatments, and for at least one time point in each treatment, expression was significantly higher in Nip than in IRAT109, indicating that *OsADR3* is a gene preferentially expressed in LR. For each treatment, *OsADR3* showed higher expression levels under the drought, ABA, H_2O_2, and polyethylene glycol (PEG) treatments than under the salt and cold treatments, in both UR and LR. Interestingly, the expression level of *OsADR3* differed significantly between the two cultivars at some time points under the treatments. For instance, after the drought treatment, *OsADR3* transcript levels rapidly increased in Nip, reaching a peak after 3 h, whereas in IRAT109 *OsADR3* transcript levels slowly increased and peaked after 12 h. In the H_2O_2 treatment, the *OsADR3* transcript levels peaked after 12 h in both cultivars but were significantly higher in Nip than in IRAT109. Thus, the expression of *OsADR3* was induced in response to abiotic stresses or phytohormones, preferentially in LR compared with UR.

To investigate the tissue-specific expression of *OsADR3* in the two rice cultivars, its transcript levels in multiple organs during the seedling and heading stages were measured by qRT-PCR. As shown in Fig. 1B, *OsADR3* was expressed in different tissues at different stages, mainly in leaves at the seedling stage. There were no significant differences between the two cultivars at the seedling stage. However, the expression levels of *OsADR3* were higher in Nip leaves and panicles than in IRAT109 at the heading stage. In addition, *OsADR3* expression was lower in the root than in other tissues at the seedling and the heading stages. Thus, *OsADR3* was expressed predominantly in leaf tissues at the seedling stage.

3.2. Sequence and phylogenetic analysis of OsADR3

The *OsADR3* gene contains a complete open reading frame (ORF) of 810 bp. The predicted protein of *OsADR3* contains 269 amino acids, with a calculated molecular mass of 28.3 kDa. Multiple sequence alignments among some of the reported C2H2-type zinc-finger proteins (Fig. S1) and homology searches in the GenBank database (National Center for Biotechnology Information, U. S. National Library of Medicine), revealed that the OsADR3 protein comprised two C2H2-type zinc-finger conserved domains, both with a plant-specific QALGGH motif homologous to that of many C2H2-type zinc-finger proteins (Figs. S1A, S2). Phylogenetic analysis of *OsADR3* and some known rice C2H2 TFs indicated that *OsADR3* clustered with *OsMSR15* (Fig. S1B), a regulator of rice response to water stress [15,42]. The phylogenetic relationships among 174 C2H2 proteins of rice, *Arabidopsis*, soybean, maize, wheat, and cotton are shown in Fig. S3. The phylogenetic tree displayed five clades (I to V), and *OsADR3* was clustered in clade II.

3.3. OsADR3 is a transcription factor and confers osmotic stress tolerance in protokaryon cells

To identify the transcriptional activation role of *OsADR3*, a yeast one-hybrid assay was performed, in which *OsADR3* was fused to the GAL4 DNA-binding domain and expressed in yeast cells. The yeast strain AH109 transformed with pGBKT7-*OsADR3* and the negative control pGBKT7 grew normally on the synthetic dextrose minimal medium (SD) without tryptophan (-Trp). Cells containing only the pGBKT7-*OsADR3* also grew normally on SD without tryptophan/histidine/adenine (-Trp/-His/-Ade), but negative control cells did not. Cells containing pGBKT7-*OsADR3* exhibited β-galactosidase activity (Fig. 2A). Thus, *OsADR3* showed transcriptional activation activity in yeast cells.

OsADR3 showed a B-box function as a putative nuclear localization signaling (NLS) motif, suggesting that *OsADR3* plays a role in the nucleus (Fig. S1A). To investigate the subcellular localization of the OsADR3 protein in rice cells, the transient expression of OsADR3-green fluorescent protein (GFP) in rice protoplasts was detected using confocal laser scanning microscopy. As shown in Fig. 2B, concurrent NLS-mCherry staining showed that the OsADR3-GFP fusion protein was localized specifically to the nucleus, evidence that OsADR3 is a nuclear protein.

To further investigate the role of *OsADR3* under osmotic stress, the growth of *E. coli* containing the plasmid 2× 35S::*OsADR3* was analyzed quantitatively in liquid media, by flow cytometry to evaluate the defensive properties of *OsADR3* to osmotic stress treatments. The results supported the idea that overexpression of

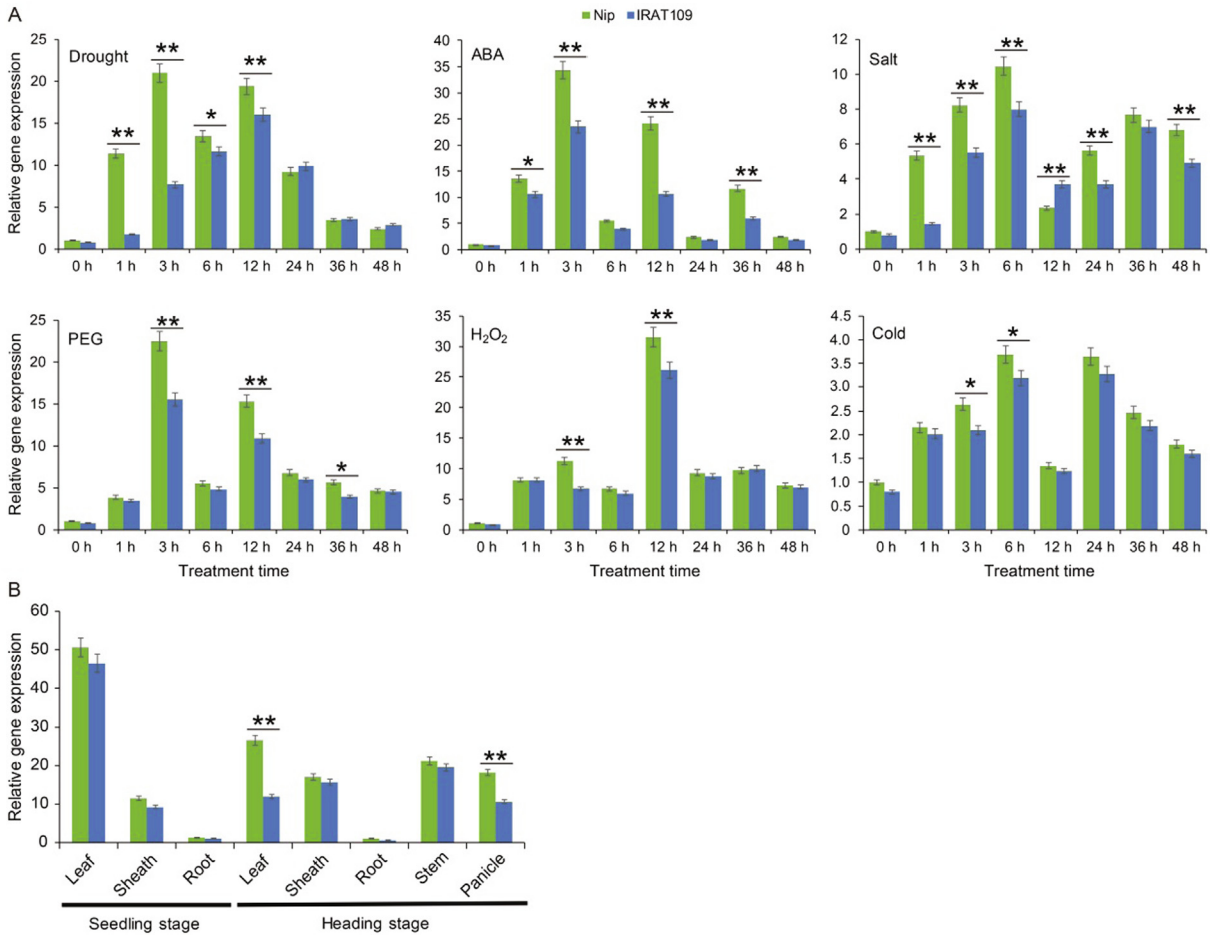

Fig. 1. Expression profiles of *OsADR3* in UR cultivar IRAT109 and LR cultivar Nip. The relative expression levels of *OsADR3* were measured by qRT-PCR. (A) Relative gene expression levels of *OsADR3* under multiple treatments. Four-week-old plants were subjected to dehydration (20% PEG6000 (w/v)), salt (200 mmol L^{-1} NaCl), cold (4 °C), H_2O_2 (2% v/v), ABA (100 μmol L^{-1}), and drought (exposure of roots to air) treatments. Leaves were harvested after treatment for 0, 1, 3, 6, 12, 24, 36, and 48 h. Three replicates were used per treatment. Expression values were normalized to the rice *β-actin* gene. Error bars indicate mean ± SD based on three replicates. Asterisks indicate corresponding genes significantly upregulated or downregulated compared with the control (*, $P < 0.05$; **, $P < 0.01$; Student's *t*-test). (B) Relative gene expression levels of *OsADR3* in different tissues at different stages. Four-week-old seedlings were used for root and leaf samples at the seedling stage. Rice at the heading stage (135 days after sown) was used for root, stem, sheath, leaf, and panicle samples. Three replicates were used per treatment. All expression values were normalized to the rice *β-actin* gene. Error bars indicate ± SD based on three replicates. Asterisks indicate genes significantly up-regulated or down-regulated compared with the control (*, $P < 0.05$; **, $P < 0.01$; Student's *t*-test).

OsADR3 in prokaryotes increases their ability to tolerate osmotic stress (Fig. 2C and D).

3.4. Overexpression of OsADR3 conferred tolerance to drought stress in rice

To evaluate the biological role of *OsADR3*, the full-length ORF of *OsADR3* (Fig. 3A) was overexpressed in the UR cultivar IRAT109. qRT-PCR analysis showed that *OsADR3* was overexpressed in the two transgenic lines (OE-7 and OE-9) (Fig. 3B), and there were no obvious morphological variations under normal growth conditions at the seedling stage (Fig. 3C). The results of the later phenotypic and physiological analyses after drought stress, and followed by a 7-day recovery period (Fig. 3C–E), revealed that *OsADR3* overexpression increased plant fresh weight and survival rates after the drought treatment. As shown in Fig. 3F–H, after drought stress, rice plants overexpressing *OsADR3* accumulated more proline and soluble sugar and less MDA.

Drought-stress treatments applied to WT and transgenic rice during the grain filling stage (rice water-sensitive period) showed that leaves and kernels of the *OsADR3* transgenic rice were not significantly affected, whereas most of the kernels of the WT rice had dried up (Fig. S4A–D). These results suggested that *OsADR3* also increased drought tolerance during the rice filling stage.

3.5. Overexpression of OsADR3 increased expression of stress-associated genes under drought stress

To further identify the mechanisms and downstream targets of *OsADR3*, RNA-seq analysis was performed using leaves from WT and *OsADR3*-OE (OE-9) rice lines under normal and drought stress conditions. In total, 5398 and 4777 up-regulated DEGs were identified in the leaves of WT and *OsADR3*-OE rice lines after exposure to drought stress for 3 and 12 h, respectively (Fig. 4A– C). As shown in Fig. 4A–C, 24 up-regulated genes were identified when the global transcriptome of *OsADR3*-OE rice was compared with that of the WT after drought stress for 3 h, and 57 up-regulated genes were identified after drought stress for 12 h. Similar to up-regulated DEGs, only six down-regulated genes were identified as present in the OE vs. OE-S, WT vs. WT-S, and OE-S vs. WT-S groups when the global transcriptome of *OsADR3*-OE rice was compared with that of the WT after drought stress for 3 h, and 79 down-regulated genes were identified in the same group comparisons after drought stress for 12 h (Fig. S5A and B).

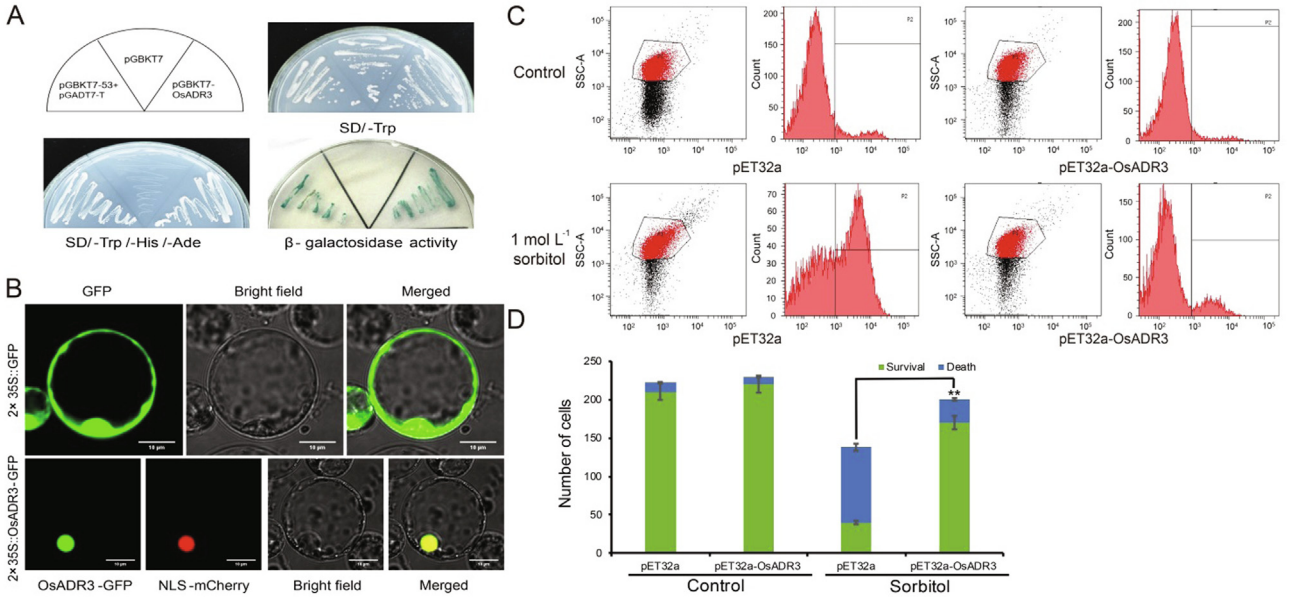

Fig. 2. Molecular characterization and osmotic stress tolerance assay in E. *coli* of *OsADR3*. (A) Transcriptional activation assay of *OsADR3* in yeast cells. Vectors pGBKT7 (negative control) and pGBKT7-53+pGADT7-T (positive control) were expressed in yeast. Plates were incubated for 3 days and subjected to β-galactosidase assay. (B) The *OsADR3-GFP* fusion gene was targeted to the nucleus in rice protoplasts (scale bar, 10 μm). A nuclear-localized protein (red) was applied to mark the nucleus. The empty GFP vector was extensively localized in both the nucleus and the cytoplasm of the rice protoplast, and used as negative control. (C) Measurement of the apoptosis of E. *coli* transformed by empty pET32b plasmid versus E. *coli* transfected with the pET32a-*OsADR3* by PI staining and a flow cytometry system under normal culture condition or 1 mol L^{-1} sorbitol stress. (D) Numbers of cells that survived or died under normal conditions, and under 1 mol L^{-1} sorbitol stress. Values are means ± SE. ** indicates significant differences at $P < 0.01$.

The transcript levels of those DEGs were further investigated. As shown in Fig. 4B and D, *LOC_Os02g44500* and *LOC_Os08g29910* were identified as DEGs after both 3- and 12-h drought stress treatments. Interestingly, *LOC_Os02g44500* is a *GPX* gene, named *OsGPX1* [43] or *OsGPX3* [9,10] in previous studies, and thus named *OsGPX1* in the present study. GO analysis after 3 and 12 h revealed that the DEGs affected by the overexpression of *OsADR3* were enriched mainly in detoxification, rhythmic processes, and antioxidant activities (Figs. S6, S7). These results indicated that *OsADR3* was involved in the detoxification and ROS scavenging mechanisms of rice under drought stress.

Some previously identified rice stress-responsive genes involved in various pathways, including *POD1*, *OsGSTU40*, *OsAMTR1*, *OsARF16*, *CYP76M5*, *SD25*, *CYP71D10*, *OsRMC*, *OsPM1*, *ATL36*, *RCE1*, and *OsRLCK318* [44–52], were also up-regulated at 3 or 12 h of stress treatment. Expression levels of these genes under normal or drought conditions was further verified by qRT-PCR in the transgenic rice seedlings. In agreement with the analysis of DEGs, the expression of these genes was up-regulated and down-regulated or unchanged in two *OsADR3*-OE lines and two *osadr3*-CR lines, respectively (Fig. 4E, F).

3.6. Loss of function of OsADR3 impairs rice tolerance to drought and osmotic stress tolerance

To further investigate the physiological functions of *OsADR3*, the CRISPR/Cas9 system was used to edit *OsADR3*. Two target sites within the CDS were designed and respectively integrated into the CRISPR/Cas9 editing vector (Fig. 5A). Two homozygous mutants (CR-2 and CR-6) were identified by sequencing (Fig. 5A). CR-2 contains a T insertion and CR-6 contains an A deletion in the CDS, which caused a frameshift mutation (Fig. 5A).

In contrast to *OsADR3*-OE rice seedlings under drought stress, rice seedlings without *OsADR3* were more sensitive to drought stress (Fig. 5B), and knockout of *OsADR3* reduced plant fresh weight and survival rates after drought treatment for 7 days (Fig. 5C and D). Further physiological analyses revealed that loss of function of *OsADR3* reduced the content of free proline and soluble sugar and increased levels of MDA in leaves (Fig. 5E–G).

Osmotic stress is one of the main factors causing damage to plants under drought stress [53]. As shown in Fig. 5H, under the 20% PEG6000 treatment, *OsADR3*-OE seedling tolerance to osmotic stress was higher than that of WT. In contrast, the growth of *osadr3*-CR seedlings was inhibited by 20% PEG6000 compared to WT. The survival rate and relative water content of *OsADR3*-OE seedlings were significantly higher than those of the WT, whereas lower survival rate and relative water content were observed in *osadr3*-CR seedlings (Fig. 5I and J). Under the 500 mmol L^{-1} mannitol treatment, the growth of *OsADR3*-OE seedlings was less inhibited (Fig. S8A), exhibiting higher germination rate and shoot length, than the WT seedlings (Fig. S8B and C). The *osadr3*-CR seedlings were more sensitive to mannitol treatment (Fig. S8A), and the germination rate and shoot length were markedly lower than those of the WT (Fig. S8B and C).

3.7. OsADR3 increased endogenous ABA levels, expression of ABA-dependent stress-responsive genes, and sensitivity to exogenous ABA in rice

As shown in Fig. S9A, under the drought conditions, the endogenous ABA level was increased in all rice seedlings and significantly increased in *OsADR3*-OE seedlings, whereas the increase in *osadr3*-CR rice seedlings was minor. As shown in Fig. S9B, genes encoding 9-*cis*-epoxy-carotenoid dioxygenase, *OsNCED4* and *OsNCED5* increased ABA biosynthesis and showed higher expression levels in the two *OsADR3*-OE rice lines than in the WT under the drought and ABA treatments. The transcript levels of genes encoding a dehydrin protein, *OsRab16C*, and an ABA-responsive factor,

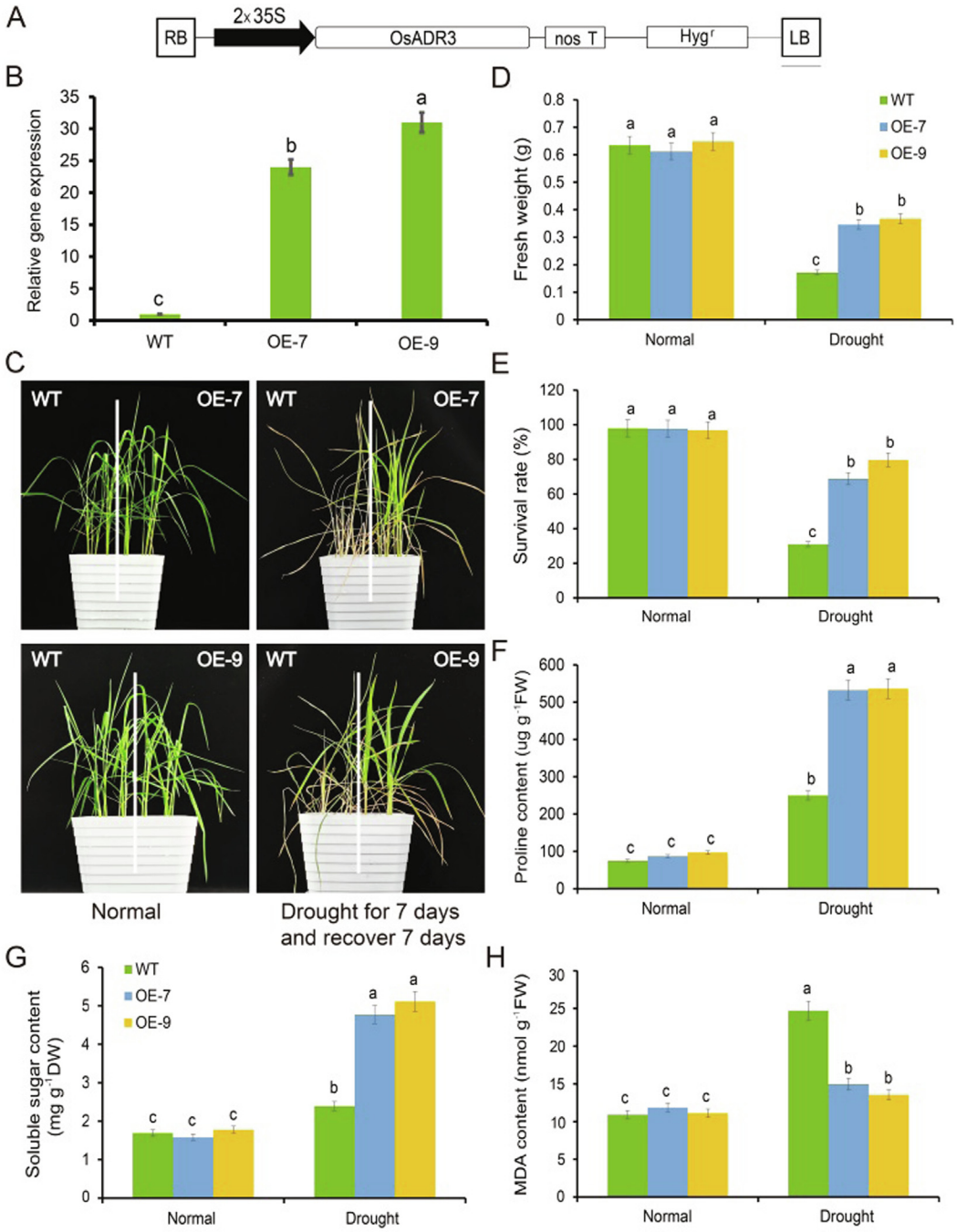

Fig. 3. Overexpression of *OsADR3* increased drought stress tolerance in rice. (A) Schematic diagrams of the overexpression constructs used for *OsADR3*-OE assay. (B) Expression analysis of *OsADR3* in WT, OE-7, and OE-9 plants. Error bars indicate ± SD (n = 10). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test. (C) Phenotype of WT, OE-7, and OE-9 rice seedlings under drought stress for 7 days and recovery 7 days. (D–H) Fresh weight (D), survival rate (E), free Pro level (F), soluble sugar level (G), and MDA content (H) of WT, OE-7, and OE-9 rice seedling leaves under drought stress. Error bars indicate ± SD (n = 10). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test.

OsRAB21, *OsLEA3*, encoding a type of late embryogenesis abundant protein, and *OsP5CS2*, encoding key enzyme responsible for proline synthesis, were all increased in the two *OsADR3*-OE rice lines compared with the WT after drought or ABA treatments (Fig. S9B). In contrast, knockout of *OsADR3* reduced the expression levels of ABA-responsive genes under drought or ABA conditions (Fig. S9B).

As shown in Fig. S9C–E, under normal conditions, the different rice seedlings showed similar growth, root length, and germination rate. However, in the 1/2 MS medium containing 10 μmol L^{-1} ABA, overexpression of *OsADR3* severely inhibited root growth at the germination stage of the rice seedlings, whereas loss of function of *OsADR3* promoted root growth and germination under ABA treatment.

3.8. OsADR3 regulated ROS accumulation under drought stress by inducing the antioxidant system

To investigate the role of *OsADR3* in antioxidant defenses under drought stress, we measured the leaf H_2O_2 content in the rice seedlings after drought stress. As shown in Fig. 6A, overexpression of

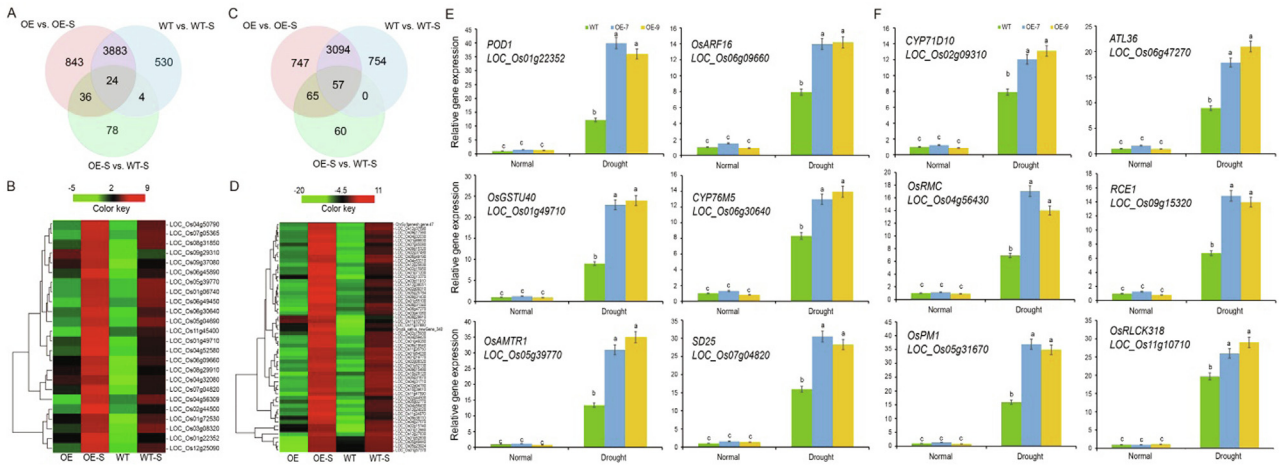

Fig. 4. Analysis of genes up-regulated by *OsADR3*. (A) and (C) show Venn diagrams of up-regulated DEGs at 3 h (A) and 12 h (C) of the OE vs. OE-S, WT vs. WT-S, and OE-S vs. WT-S comparisons. OE-S and WT-S indicate the leaves from *OsADR3*-OE and WT rice seedlings after drought stress, respectively. (B) and (D) are the transcript levels of the DEGs found in the OE vs. OE-S, WT vs. WT-S, and OE-S vs. WT-S groups after drought stress for 3 and 12 h, respectively, identified by RNA-seq analysis. Genes in blue were differentially expressed under drought stress for 3 and 12 h. (E–F) Relative expression levels of some previously known stress-related genes up-regulated by overexpression of *OsADR3* at 3 h (E) and 12 h (F) under normal or drought conditions in rice seedlings. Values are means ± SD (n = 5). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test.

OsADR3 significantly lowered the content of H_2O_2 in the rice seedlings, and knockout of *OsADR3* led to accumulation of more H_2O_2 in rice leaves under drought stress. Overexpression of *OsADR3* significantly increased the content of ASC and GSH, and maintained high ASC/DHA (2.46) and GSH/GSSG (2.29) ratios with the increasing activities of GR, APX, DHAR, and MDHAR in the *OsADR3*-OE rice seedlings under drought stress (Fig. 6B and C). In contrast, under drought stress, *osadr3*-CR showed lower ASC/DHA (1.06) and GSH/GSSG (1.11) ratios compared with WT and *OsADR3*-OE rice seedlings (Fig. 6B, C). In agreement with the activities of antioxidant enzymes participating in the ASC/GSH cycle, overexpression of *OsADR3* increased the activity of GPX, whereas *osadr3*-CR showed lower activity of GPX than WT and *OsADR3*-OE rice seedlings under drought stress. The activity of CAT and expression levels of the antioxidant genes *CatB*, *APX1*, and *APX2* were higher in leaves of *OsADR3*-OE seedlings than in those of the WT (Fig. S10). These results suggested that *OsADR3* increased drought tolerance by inducing the antioxidant system in rice.

Given that *OsADR3* reduced ROS accumulation under drought stress, we investigated whether *OsADR3* plays a role in rice oxidative stress resistance. The overexpression and loss-of-function rice lines were subjected to 2% H_2O_2 (v/v) treatment during the germination stage. Compared to the WT, the *OsADR3*-OE plants showed greater growth, germination rate (91.21%), and shoot length (3.37 cm) under the oxidative stress treatment (Fig. 6D–F). In contrast, the growth of *osadr3*-CR rice seeds was strongly inhibited by the H_2O_2 (16.85% germination rate and 0.14 cm shoot length) (Fig. 6D–F). Thus, *OsADR3* increased oxidative stress tolerance in rice.

3.9. OsADR3 regulated the expression of OsGPX1 by directly binding to its promoter

The results of RNA-seq and physiological assays in transgenic rice [43] suggested that *OsADR3* is involved in rice ROS scavenging and regulates the expression of *OsGPX1* to promote drought tolerance. To determine whether *OsGPX1* showed higher mRNA levels in *OsADR3-OE* rice seedlings than in the WT, qRT-PCR was performed for all *GPX* genes. In agreement with the results of RNA-seq, the transcript levels of *OsGPX1* were significantly higher in *OsADR3*-OE than in WT and *osadr3*-CR seedlings (Fig. 7A). However, *OsGPX4* (*LOC_Os06g08670*) and *OsGPX5* (*LOC_Os11g18170*) were repressed after drought stress in all rice lines tested. Although *OsGPX2* (*LOC_Os03g24380*) and *OsGPX3* (*LOC_Os04g46960*) were induced after drought stress, their expression levels in the WT were higher than in the *OsADR3*-OE and *osadr3*-CR rice seedlings. Thus, *OsADR3* may regulate the expression of *OsGPX1* but has no effect on the other *OsGPX*s.

To investigate how *OsADR3* regulated the expression of *OsGPX1*, we performed prediction and found that putative C2H2 TFs may bind to the *OsGPX1* promoter via the C2H2 domain [54,55]. The largest number of binding sites was found for the promoter fragment of *OsGPX1* containing the CACAAATAGTG motifs. Because *OsADR3* contains two C2H2 domains with a conserved QALGGH motif (Fig. 7B), we mutated two QALGGH motifs and performed a yeast one-hybrid assay and EMSA to determine which C2H2 domain binds the promoter of *OsGPX1* (Fig. 7C).

To determine whether OsADR3 bound to the *OsGPX1* promoter, a yeast one-hybrid (Y1H) assay was performed. The AD-OsADR3 and Phis2-*OsGPX1*-Pro vectors were constructed for the Y1H assay (Fig. 7D), and 40 mmol L^{-1} of 3-AT was added to prevent false-positive results. The results suggested that the co-transformed AD-OsADR3 and Phis2-*OsGPX1*-pro yeast strains grew on synthetic-defined (SD) medium without tryptophan, leucine, and histidine (/-Trp/-Leu/-His) containing 40 mmol L^{-1} 3-AT. In contrast, no yeast spot was detected in the negative control medium (Fig. 7E). The yeast-transformed AD-OsADR3 with two QALGGH motifs substituted with EAMRHK did not grow on SD medium (/-Trp/-Leu/-His) containing 40 mmol L^{-1} 3-AT (Fig. 7E). Thus, OsADR3 bound to the *OsGPX1* promoter via two QALGGH motifs.

To confirm whether OsADR3 binds to the *OsGPX1* promoter, EMSA was performed. The 33-bp *OsGPX1* promoter fragments and mutant fragments were labeled with biotin as WT probe and mutant probe (Fig. 7F), and *OsGPX1* promoter fragments without biotin label were used as competitors. The purified His-OsADR3 fusion proteins were used in the experiment. The EMSA displayed dark stripes of the DNA–protein complexes after the OsADR3 proteins and labeled WT probes were co-incubated (Fig. 7G). In the presence of competitor probes with the same sequence, these complexes formed at a very low rate (Fig. 7G). The dark stripe completely disappeared when the *OsGPX1* promoter fragments or QALGGH motifs of OsADR3 were mutated (Fig. 7G). The Y1H and

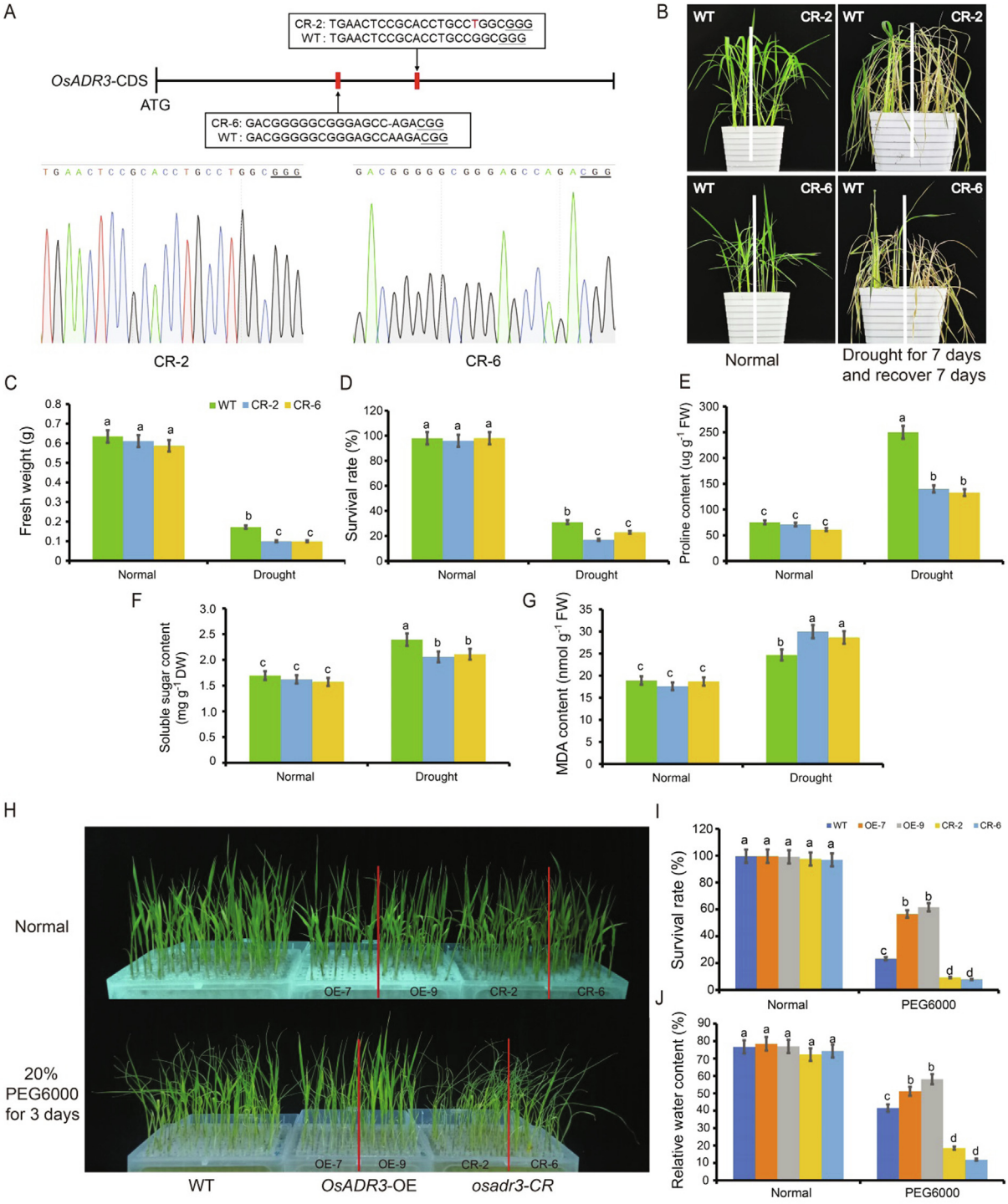

Fig. 5. Knockout of *osadr3* increased drought sensitivity in rice. (A) CRISPR/Cas9-edited *osadr3* mutants. Three underlined letters indicate the protospacer adjacent motif (PAM) region. The red letter in CR-2 indicates a 1-bp insertion and the single line in CR-6 indicates a 1-bp deletion. (B) Phenotypes of WT, CR-2, and CR-6 rice seedlings under drought-stress for 7 days and recovery 7 days. (C–G) Fresh weight (C), survival rate (D), free proline level (E), soluble sugar level (F), and MDA content (G) of WT, CR-2, and CR-6 rice seedling leaves under drought stress. Error bars indicate ± SD (n = 10). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test. (H) Phenotypes of WT, OE-7, OE-9, CR-2, and CR-6 rice seedlings under 20% PEG6000- mediated osmotic stress for 3 days. (I, J) Survival rate (I) and relative water content (J) of WT, OE-7, OE-9, CR-2, and CR-6 rice seedlings under osmotic stress. Error bars indicate ± SD (n = 10). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test.

EMSA assays support the inference that OsADR3 directly binds to the *OsGPX1* promoters via two QALGGH motifs.

To evaluate whether OsADR3 activates the *OsGPX1* promoter *in vivo*, a transient expression assay was constructed in *N. benthamiana* leaves. The *OsGPX1* promoter fragment containing the CACAAATAGTG motif was introduced in the pGreenII 0800-LUC vector to generate a reporter plasmid. The *OsADR3* coding sequence (CDS) was fused to a pGreenII 62-SK vector to generate an effector

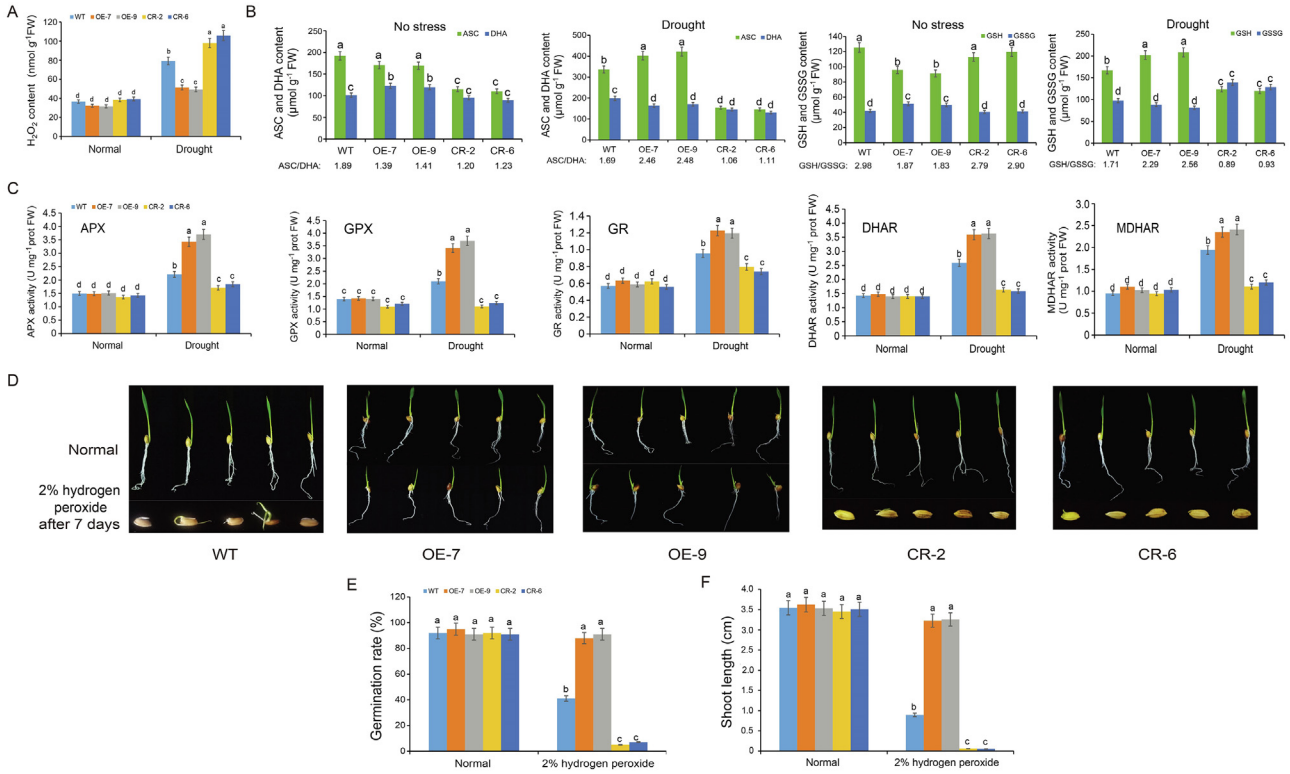

Fig. 6. Analysis of ROS homeostasis in WT, *OsADR3*-OE, and *osadr3*-CR rice seedlings. (A) H_2O_2 content in leaves of WT, *OsADR3*-OE, and *osadr3*-CR four-week-old rice seedlings under normal conditions or exposure to drought stress for 12 h. Values are means ± SD (n = 5). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test. (B) Levels of GSH, GSSG, ASC, and DHA, and ratios of GSH/GSSG and ASC/DHA in the leaves of WT, *OsADR3*-OE, and *osadr3*-CR four-week-old rice seedlings under normal condition or exposure to drought stress for 12 h. Values are means ± SD (n = 5). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test. (C) Activities of GPX, APX, GR, DHAR, and MDHAR in leaves of WT, *OsADR3*-OE, and *osadr3*-CR rice seedlings under normal conditions or exposure to drought stress for 12 h. Values are means ± SD (n = 5). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test. (D) Phenotypes of WT, *OsADR3*-OE, and *osadr3*-CR four-week-old rice seedlings under normal conditions or exposure to oxidative stress (2% (v/v) H_2O_2 for 7 days). (E) Germination rates of WT, *OsADR3*-OE, and *osadr3*-CR seeds under normal conditions and oxidative stress for 7 days. Values are means ± SD (n = 5). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test. (F) Shoot lengths of WT, *OsADR3*-OE, and *osadr3*-CR rice seedlings under normal conditions or oxidative stress for 7 days. Values are means ± SD (n = 5). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test.

construct (Fig. 7H). Dual-luciferase assay revealed that OsADR3 induced *OsGPX1* expression under normal and drought conditions and that drought stress increased this induction (Fig. 7I). Thus, OsADR3 activated *OsGPX1* expression by directly binding to its promoter.

3.10. Silenced OsGPX1 represses the ROS scavenging ability of OsADR3-OE plants

To investigate whether *OsADR3* confers drought stress tolerance in rice by regulating *OsGPX1* expression, we knocked down *OsGPX1* in *OsADR3*-OE plants by transforming siRNA-*OsGPX1* into rice protoplasts. The H_2O_2 contents in the rice protoplasts were loaded with H2DCF-DA and visualized by confocal laser scanning microscopy, which showed that silencing *OsGPX1* in the *OsADR3*-OE plants accumulated more H_2O_2 in the rice protoplasts (Fig. 8A and B). The negative siRNA supplied by the manufacturer was used as a negative control. qRT-PCR and RT-PCR analyses revealed that RNAi-mediated silencing in rice protoplasts substantially reduced the expression of *OsGPX1* (Fig. 8C and D), demonstrating the effectiveness of the experiments. These results suggest that knockdown of *OsGPX1* partly reduced the ROS scavenging ability of *OsADR3*-OE plants under drought stress and that *OsADR3* conferred drought-stress tolerance in rice by regulating *OsGPX1* expression.

3.11. Exogenous GSH restored the drought tolerance of osadr3-CR plants.

To confirm that the deficiency of drought tolerance in *osadr3*-CR plants was caused by an impaired antioxidant system, we used exogenous glutathione to treat the leaves of WT and *osadr3*-CR plants exposed to PEG6000-simulated drought stress, to investigate whether increased ROS scavenging ability could restore the drought tolerance of *osadr3*-CR plants. Under drought stress for 3 days, the leaves of *osadr3*-CR plants were significantly more damaged than those of the WT (Fig. 8E). However, when 5 mmol L^{-1} GSH was added to the PEG6000 solution, the leaves of WT plants showed better drought tolerance and the phenotype of the leaves of *osadr3*-CR plants returned to the same level as those of the WT (Fig. 8F). Exogenous glutathione significantly lowered the content of H_2O_2 and increased the activity of GPX in the leaves of WT and *osadr3*-CR plants (Fig. 8G and H). These results suggested that exogenous glutathione restored the antioxidant system and drought stress tolerance of *osadr3*-CR plants.

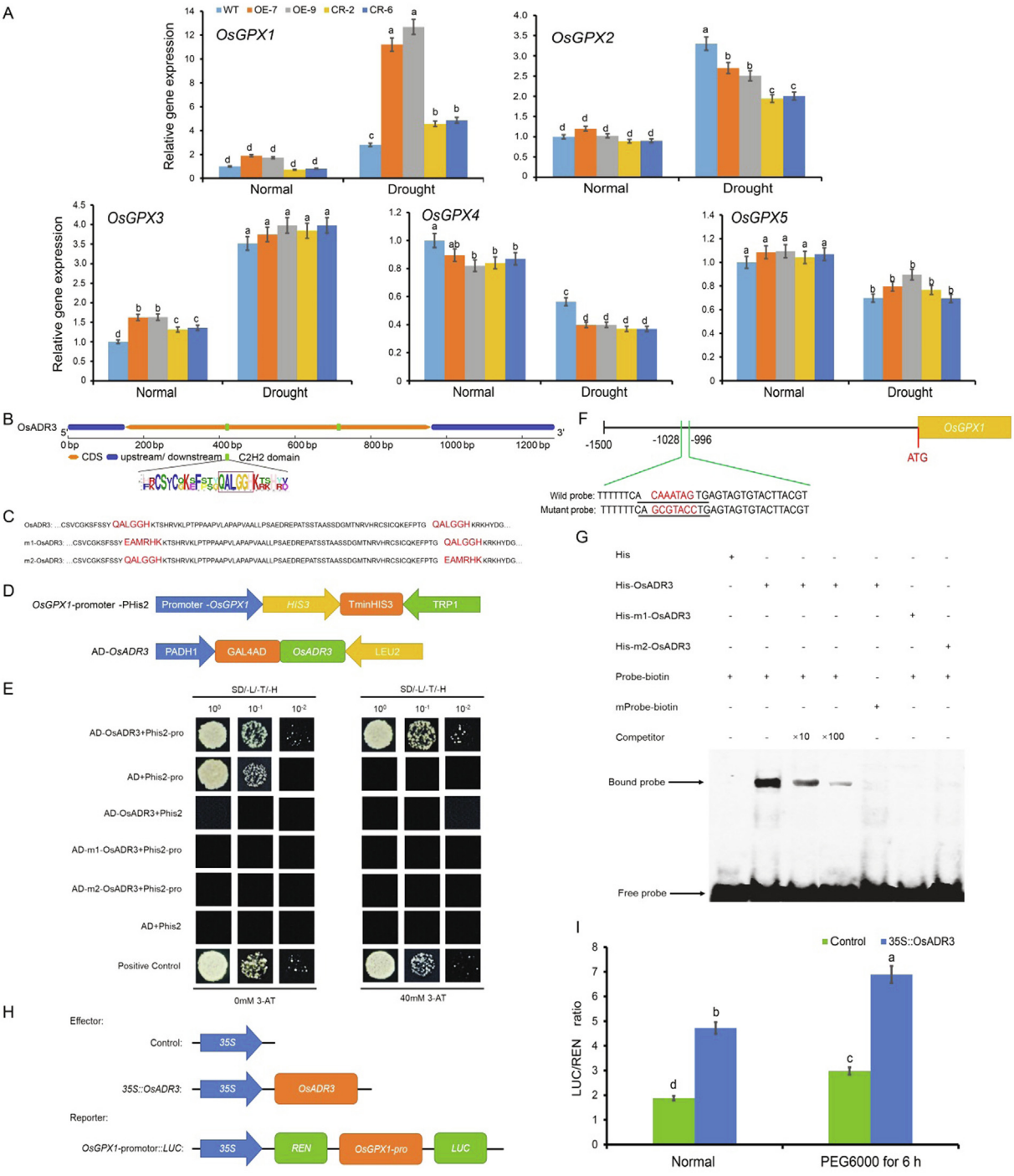

Fig. 7. OsADR3 directly binds to the promoter of *OsGPX1* and regulates *OsGPX1* expression. (A) Expression analysis of all *OsGPX* genes in WT, *OsADR3*-OE, and *osadr3*-CR plants cultivated under normal conditions or drought stress. Measurements were performed after 12 h of drought. Values are means ± SD (n = 5). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test. (B) Gene structure of *OsADR3*. Exons, untranslated regions, and C2H2 domains are marked by orange double-sided wedge, blue rounded-corner rectangles, and green rounded-corner rectangles, respectively. The scale bar at the bottom indicates the lengths of the exon, intron, and untranslated regions. (C) Amino acid sequence of the C2H2 domain used in Y1H and EMSA assays. The putative motifs (QALGGH) for DNA binding are in red. m1-*OsADR3* and m2-*OsADR3* represent the mutant OsADR3 proteins. Two putative motifs QALGGH were replaced with EAMRHK, respectively. (D) AD-OsADR3 and *OsGPX1*-promoter-PHis2 vectors used for yeast one-hybrid assay. Phis2, AD, AD-OsADR3, and *OsGPX1*-promoter-PHis2 represent the p53HIS2 vector and pGADT7-Rec2 vector, *OsADR3* gene fused to the pGAD vector, and promoter of *OsGPX1* fused to the Phis2 vector, respectively. (E) Yeast one-hybrid assay displayed *OsADR3* directly binds to the promoter of *OsGPX1* via its two QALGGH motifs. The AD and the Phis2-pro, AD-OsADR3 and Phis2, AD-m1-OsADR3 and Phis2-pro, AD-m2-OsADR3 and Phis2-pro, and AD and Phis2 to represent negative control, respectively. Yeast were grown on a SD/-Leu/-Trp/-His plate containing 0 mmol L^{-1} 3-AT (3-amino-1,2,4-triazole) (as a control) or 40 mmol L^{-1} 3-AT. (F) Diagram of the wild-type and mutated probes used for electrophoretic mobility shift assay (EMSA). The wild probe is a putative OsADR3 transcription factor binding site (CACAAATAGTG) on the *OsGPX1* promoter. In the mutant probe, the putative binding site sequence CACAAATAGTG was replaced with CAGCGTACCTG. (G) EMSA assays displayed an interaction between His-OsADR3 protein and the *OsGPX1* promoter via its two QALGGH motifs. (H) Schematic diagrams of the effector and reporter constructs used for a dual-luciferase assay. (I) Dual-luciferase assay. Transient expression assay of the promoter activities co-transformed with effector and reporter constructs in *Nicotiana benthamiana* leaves. Values are means ± SD (n = 6). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test.

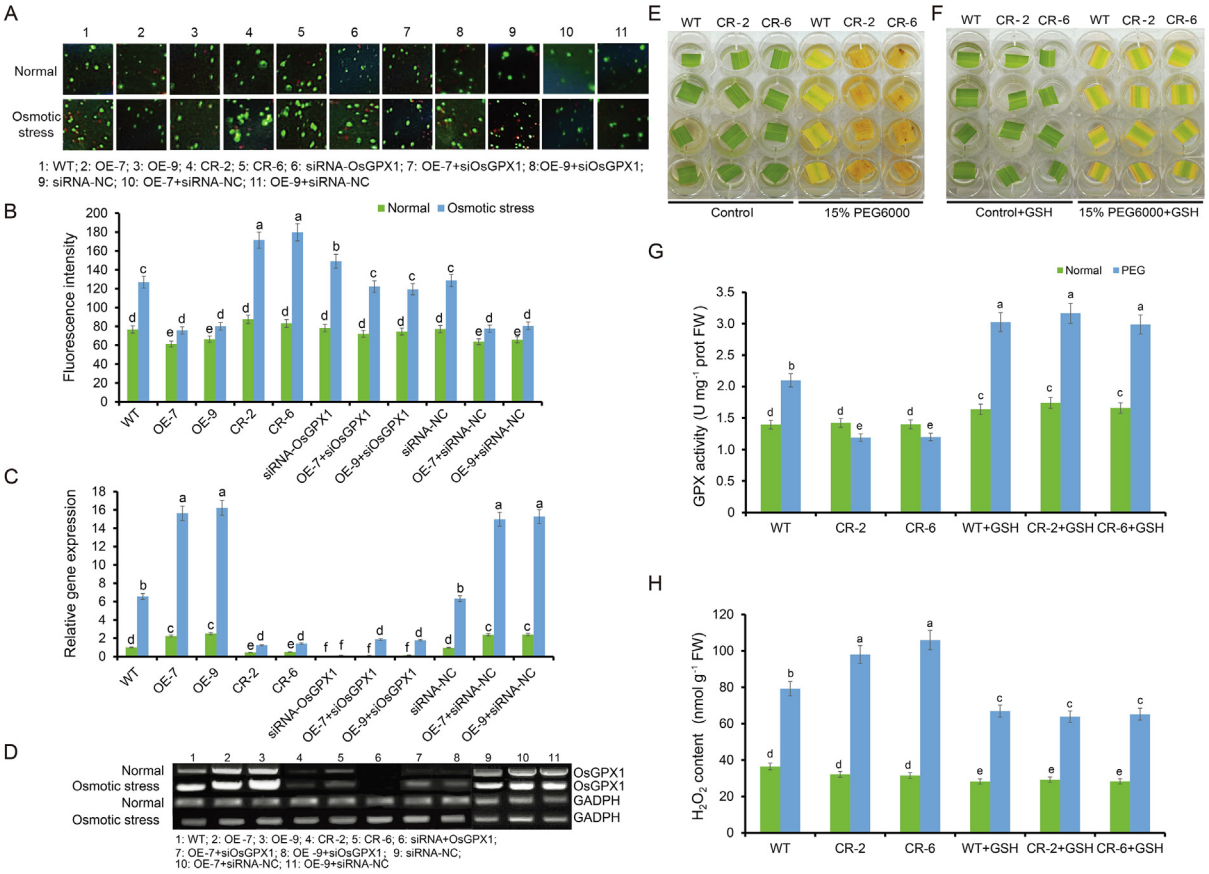

Fig. 8. Silenced *OsGPX1* repressed the ROS scavenger ability of *OsADR3*-OE plants under drought and exogenous GSH restored the drought tolerance of *osadr3*-CR plants. (A, B) Production of H_2O_2 in protoplasts from WT plants, *OsADR3*-OE plants, *osadr3*-CR plants, *OsGPX1*-RNAi plants, and *OsADR3*-OE+*OsGPX1*-RNAi plants. Protoplasts were treated with osmotic stress medium (+mannitol) or normal incubation medium for 2 h and then loaded with H_2DCF-DA (2′, 7′-dichlorodihydrofluoresceindiacetate) for 10 min. Images were presented as mean pixel intensities. Fifty protoplasts per treatment were observed for three independent replicates· H_2O_2 was visualized by confocal microscopy (A), and fluorescence intensity was measured with Leica IMAGE software (B). Values are means ± SD (n = 5). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test. (C) Expression analysis of *OsGPX1* in rice protoplasts of WT plants, *OsADR3*-OE plants, *osadr3*-CR plants, *OsGPX1*-RNAi plants, and *OsADR3*-OE+*OsGPX1*-RNAi plants cultivated under normal conditions or osmotic stress. Values are means ± SD (n = 5). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test. (D) RT–PCR analysis of *OsGPX1* expression in rice protoplasts of WT plants, *OsADR3*-OE plants, *osadr3*-CR plants, *OsGPX1*-RNAi plants, and *OsADR3*-OE+*OsGPX1*-RNAi plants cultivated under normal conditions or osmotic stress. *GADPH* was analyzed as a control. (E, F) Detached leaves from 4–week–old WT and *osadr3*-CR plants exposed to 15% PEG6000 with or without exogenous GSH (5 mmol L^{-1}) for 3 days to indicate the stress tolerance. (G, H) Activities of GPX (G) and H_2O_2 content (H) in the leaves of WT and *osadr3*-CR rice seedlings under normal conditions or exposure to 15% PEG6000 with or without exogenous GSH for 3 days. Values are means ± SD (n = 5). Lowercase letters indicate significant differences at $P < 0.05$. Statistical significance was determined by Student's t-test.

4. Discussion

Multiple sequence alignments and phylogenetic analysis indicated that *OsADR3* belongs to the C2H2 zinc-finger family. Some zinc-finger proteins are transcriptional activators [13,56]. Other zinc-finger proteins, also harboring a DLN-box/EAR-motif, have shown transcription-repressive activities and can repress the expression of osmotic stress-related and ABA-repressive genes in plants, such as *ZPT2-3* in petunia [57] and *STZ/Zat10*, *AZF1*, and *AZF2* in *Arabidopsis* [58]. In the present study, molecular characterization revealed that *OsADR3* is a TF located in the nucleus and is a transcriptional activator (Fig. 2). Thus, *OsADR3* is controlled by multiple signaling pathways and regulates unknown genes under plant drought stress to exercise various molecular functions in response to environmental stresses.

As the expression background of *OsADR3* in the UR was lower than that in the LR (Fig. 1), the results of transgenic experiments, particularly the analysis of differentially expressed genes, were precise and convincing. The present study overexpressed or knocked out *OsADR3* in WT plants, and the phenotype and physiological experiment indicated that *OsADR3* increased drought and osmotic stress tolerance (Figs. 3, 5). The finding that overexpressed or knocked-out *OsADR3* did not affect plant growth under normal conditions suggested that *OsADR3* offers practical benefits for molecular breeding to increase rice drought tolerance.

The synthesis of osmotic protectants is controlled by the corresponding functional genes, such as free Pro and *LEA* biosynthesis genes [59]. In our study, the content of osmotic protectants and the expression of the functional genes examined in the *OsADR3*-OE lines were higher than those of the WT line under drought stress. Moreover, the expression of many stress-related genes was up-regulated in *OsADR3*-OE plants (Fig. 4). Stressed (S) and non-stressed seedlings of transgenic and WT lines displayed many identical DEGs after the drought stress treatment for 3 or 12 h (3883 and 3094, respectively), indicating that most of the DEGs were produced by the stress conditions and might not be associated with *OsADR3* overexpression. For this reason, in the present study, only DEGs present in the OE vs. OE-S, WT vs. WT-S, and OE-S vs. WT-S groups were further investigated. These DEGs have been shown to respond to drought or osmotic stress. For instance, *OsPM1 is* an ABA influx carrier and plays an important role in drought response [44]. Overexpression of *OsGSTU40* increased

drought tolerance, as reflected in germination, root growth assay, and whole plant growth [60]. These results suggest that *OsADR3* regulates downstream genes or is an upstream modulator that control the content of osmo-protectants, thereby increasing rice drought stress tolerance.

The expression of *OsADR3* was strongly induced by exogenous ABA, indicating that *OsADR3* is involved in the rice ABA signaling pathway. The phytohormone ABA plays a central role in plant responses to environmental stresses. Some TFs of rice, such as *OsABI5* [56] involved in ABA-dependent pathways increase rice seedling sensitivity to exogenous ABA. Overexpressing *OsADR3* increased rice seedling sensitivity to exogenous ABA and increased the endogenous ABA content in leaves (Fig. S9A–C), suggesting that *OsADR3* participates in the ABA-dependent signaling pathway. *OsLEA3* [61]; *OsRab16C* [62]; *OsRAB21* [63], *OsP5CS2* [64], *OsNCED4*, and *OsNCED5* [65] showed higher expression levels in two *OsADR3*-OE lines and lower expression levels in two *osadr3*-CR lines than in WT rice seedlings under drought stress or exogenous ABA treatment (Fig. S9B), indicating that *OsADR3* increased the synthesis of anti-permeable substances by regulating the expression of stress-resistance genes to increase rice tolerance under drought stress conditions. These results suggest that *OsADR3* plays a crucial role in protecting rice cells from drought stress via the ABA pathway.

Reactive oxygen species are key signal transduction molecules in plants, but their excessive accumulation can cause irreversible damage to cells [66]. In previous studies, C2H2 TFs were reported to act in the antioxidant defense system by increasing the activity of antioxidant enzymes, which are the most effective mechanisms against oxidative stress [67,68]. C2H2 zinc-finger proteins were shown to be essential for the expression and synthesis of SOD [13,69], POD [12], APX [70], CAT [71], and NADPH in rice under drought, low temperature, and high-salt-stress conditions, and conferred high tolerance to oxidative stress in rice seedlings. In the present study, overexpression of *OsADR3* increased the activity of the ROS scavenging enzymes GR, GPX, DHAR, MDHAR, CAT, and APX in rice under drought stress and H_2O_2 treatments (Figs. 6, S10). Previous studies [72–75] indicated that increased APX, GR, MDHAR, and DHAR activities maintain the ASC-GSH cycle, thereby improving environmental-stress tolerance. Generally, GSH and ASC pools showed significant alterations in response to environmental stresses, and high ratios of ASC/DHA and GSH/GSSG are a general feature of increased oxidation processes, and contents of ASC and GSH are the sensor of the ASC-GSH cycle, the main mechanism preventing ROS accumulation in plants [5,76]. Consistently, overexpression of *OsADR3* increased the contents of ASC and GSH and maintained high ASC/DHA and GSH/GSSG ratios under drought stress (Fig. 6B), suggesting that *OsADR3* can maintain the ASC-GSH cycle under drought stress. Furthermore, exogenous ROS reagent recovered the *osadr3*-CR phenotype and activity of antioxidant enzymes under drought stress (Fig. 8E–H). These results indicate that *OsADR3* can reduce ROS accumulation under drought and osmotic stress.

In plants, several TFs have been shown to bind to the promoter regions of antioxidant enzyme genes such as *APX*s and *CAT*s to activate or suppress their expression and enzyme activity involved in seed germination, disease resistance, and abiotic stresses [22,70,77]. However, the TFs involved in regulating *GPX*s-mediated ROS scavenging are unknown. In previous studies [9,10], *OsGPX1* was found to be involved in H_2O_2 homeostasis of rice and play an important role in osmotic and oxidative stress tolerance in rice. In the present study, the RNA-seq assay revealed that under both normal and drought conditions, *OsADR3* increased the expression of *OsGPX1* and overexpression of *OsADR3* increased the activity of GPX (Figs. 4, 6, 7A). *OsADR3* maintained a high level of constitutive expression in all OE lines, suggesting that the genes expressed in the OE vs. OE-S, WT vs. WT-S, and OE-S vs. WT-S groups at both 3 and 12 h after treatment might be regulatory genes for *OsADR3*. The EMSA, Y1H, and dual-luciferase reporter assay indicated that OsADR3 bound to the promoter of *OsGPX1* and regulate *OsGPX1* expression under normal and drought stress (Fig. 7). *OsADR3* as a C2H2-type TF contains two plant-specific conserved amino acid sequences, QALGGH, in the DNA-recognition motif, which is important in phytohormone responses and tolerance to abiotic stresses [70]. EMSA and Y1H assays showed that OsADR3 bound to the promoter of *OsGPX1* only if both C2H2 domains were present (Fig. 7). In tobacco, *NtERF172* confers drought resistance by regulating *NtCAT*, and knockdown of *NtCAT* in *NtERF172*-OE plants reduced drought stress tolerance and ROS scavenging ability by more than in *NtERF172*-OE lines [22]. Consistently, in the present study, transiently silenced *OsGPX1* repressed ROS scavenging ability in *OsADR3*-OE plants under osmotic stress (Fig. 8A and B). Notably, the H_2O_2 contents in the rice protoplasts of knocked-down *OsGPX1* alone were lower than those in *osadr3*-CR plants and higher than in siRNA-*OsGPX1*+*OsADR3*-OE, perhaps because *OsADR3* regulates other genes with redundant functions of *OsGPX1*. Alternatively, *OsADR3* activity may not rely solely on *OsGPX1*. These results show strong evidence that *OsADR3* increases drought and oxidative stress tolerance by modifying the expression of *OsGPX1*. Thus, *OsADR3* increases drought stress tolerance by inducing antioxidant defense mechanisms and regulating *OsGPX1* in rice, indicating its potential utility for breeding rice cultivars.

5. Conclusions

We identified a novel C2H2-type zinc finger protein, *OsADR3*, containing an NLS motif, L-box, DLN-box/EAR-motif, which was preferentially expressed in LR as a prominent regulator of the response to drought stress while causing rice to be sensitive to exogenous ABA. Overexpression of *OsADR3* increased the activities of many antioxidant defense enzymes including CAT, GPX, and APX and maintained the ASC-GSH cycle by regulating ASC/DHA and GSH/GSSG levels to reduce damage caused by drought stress and accumulation of osmo-protectants under drought stress. *OsADR3* modifies the expression of *OsGPX1* by directly binding to its promoter. *OsADR3* increases drought stress tolerance by inducing antioxidant defense and associated with the ABA signaling pathway in rice. In summary, *OsADR3* increases antioxidant defense mechanisms by regulating the expression of *OsGPX1* and maintains the ASC-GSH cycle by regulating ASC/DHA and GSH/GSSG levels, thereby increasing the drought stress tolerance of rice.

Availability of data and materials

The RNA-seq data reported here have been submitted to the NCBI database (https://www.ncbi.nlm.nih.gov/) under the accession numbers PRJNA635251.

CRediT authorship contribution statement

Jiaming Li, Minghui Zhang, Jinjie Li, Zichao Li, and Detang Zou: designed the experiments. **Jiaming Li, Luomiao Yang, Lei Lei, Xinrui Mao, Lu Li, Jingguo Wang, Hualong Liu, and Hongliang Zheng:** performed the experiments. **Jiaming Li, Jian Sun, Hongwei Zhao, and Xianwei Li:** prepared the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2020.12.005.

References

[1] J.K. Zhu, Salt and drought stress signal transduction in plants, Annu. Rev. Plant Biol. 53 (2002) 247–273.
[2] X. Liu, C.L. Burras, Y.S. Kravchenko, A. Duran, T. Huffman, H. Morras, G. Studdert, X. Zhang, R.M. Cruse, X. Yuan, Overview of Mollisols in the world: distribution, land use and management, Can. J. Soil Sci. 92 (2012) 383–402.
[3] G. Miller, N. Suzuki, S. Ciftci-Yilmaz, R. Mittler, Reactive oxygen species homeostasis and signalling during drought and salinity stresses, Plant Cell Environ. 33 (2010) 453–467.
[4] G. Miller, V. Shulaev, R. Mittler, Reactive oxygen signaling and abiotic stress, Physiol. Plant. 133 (3) (2008) 481–489.
[5] C.H. Foyer, G. Noctor, Ascorbate and glutathione: the heart of the redox hub, Plant Physiol. 155 (2011) 2–18.
[6] G. Potters, N. Horemans, M.A.K. Jansen, The cellular redox state in plant stress biology-a charging concept, Plant Physiol. Biochem. 48 (5) (2010) 292–300.
[7] R. Margis, C. Dunand, F.K. Teixeira, M. Margispinheiro, Glutathione peroxidase family-an evolutionary overview, FEBS J. 275 (2008) 3959–3970.
[8] G. Passaia, M. Margis-Pinheiro, Glutathione peroxidases as redox sensor proteins in plant cells, Plant Sci. 234 (2015) 22–26.
[9] G. Passaia, L. Spagnolo Fonini, A. Caverzan, D. Jardim-Messeder, A.P. Christoff, M.L. Gaeta, J.E. de Araujo Mariath, R. Margis, M. Margis-Pinheiro, The mitochondrial glutathione peroxidase *GPX3* is essential for H_2O_2 homeostasis and root and shoot development in rice, Plant Sci. 208 (2013) 93–101.
[10] A.L.S. Paiva, G. Passaia, A.K.M. Lobo, D. Jardim-Messeder, J.A.G. Silveira, M. Margis-Pinheiro, Mitochondrial glutathione peroxidase (*OsGPX3*) has a crucial role in rice protection against salt stress, Environ. Exp. Bot. 158 (2019) 12–21.
[11] H. Zhang, Y.P. Liu, F. Wen, D.M. Yao, L. Wang, J. Guo, L. Ni, A. Zhang, M.P. Tan, M.Y. Jiang, A novel rice C2H2-type zinc finger protein, ZFP36, is a key player involved in abscisic acid-induced antioxidant defence and oxidative stress tolerance in rice, J. Exp. Bot. 65 (2014) 5795–5809.
[12] S.J. Sun, S.Q. Guo, X. Yang, Y.M. Bao, H.J. Tang, H. Sun, J. Huang, H.S. Zhang, Functional analysis of a novel Cys2/His2-type zinc finger protein involved in salt tolerance in rice, J. Exp. Bot. 61 (2010) 2807–2818.
[13] J.i. Huang, S.J. Sun, D.Q. Xu, X. Yang, Y.M. Bao, Z.F. Wang, H.J. Tang, H. Zhang, Increased tolerance of rice to cold, drought and oxidative stresses mediated by the overexpression of a gene that encodes the zinc finger protein *ZFP245*, Biochem. Bioph. Res. Commun. 389 (2009) 556–561.
[14] J.i. Huang, S. Sun, D. Xu, H. Lan, H. Sun, Z. Wang, Y. Bao, J. Wang, H. Tang, H. Zhang, A TFIIIA-type zinc finger protein confers multiple abiotic stress tolerances in transgenic rice (*Oryza sativa* L.), Plant Mol. Biol. 80 (2012) 337–350.
[15] D.Q. Xu, J. Huang, S.Q. Guo, X. Yang, Y.M. Bao, H.J. Tang, H.S. Zhang, Overexpression of a TFIIIA-type zinc finger protein gene *ZFP252* enhances drought and salt tolerance in rice (*Oryza sativa* L.), FEBS Lett. 582 (2008) 1037–1043.
[16] H. Wang, H. Zhang, F. Gao, J. Li, Z. Li, Comparison of gene expression between upland and lowland rice cultivars under water stress using cDNA microarray, Theor. Appl. Genet. 115 (2007) 1109–1126.
[17] Z.Y. Zhang, J.J. Li, Z.S. Tang, X.M. Sun, H.L. Zhang, J.P. Yu, G.X. Yao, G.L. Li, H.F. Guo, J.L. Li, H.M. Wu, H.G. Huang, Y.W. Xu, Z.G. Yin, Y.H. Qi, R.F. Huang, W.C. Yang, Z.C. Li, Gnp4/LAX2, a RAWUL protein, interferes with the OsIAA3-OsARF25 interaction to regulate grain length via the auxin signaling pathway in rice, J. Exp. Bot. 69 (2018) 4723–4737.
[18] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874.
[19] L.I. Mei, A simple and highly efficient agrobacterium -mediated rice transformation system, Acta Biol. Exp. Sin. 36 (2003) 289.
[20] N. Zhu, S. Cheng, X. Liu, H. Du, M. Dai, D.X. Zhou, W. Yang, Y. Zhao, The R2R3-type MYB gene *OsMYB91* has a function in coordinating plant growth and salt stress tolerance in rice, Plant Sci. 236 (2015) 146–156.
[21] S.D. Yoo, Y.H. Cho, J. Sheen, *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis, Nat. Protoc. 2 (2007) 1565–1572.
[22] Q. Zhao, R.H. Hu, D. Liu, X. Liu, Y.A. Li, The AP2 transcription factor *NtERF172* confers drought resistance by modifying *NtCAT*, Plant Biotechnol. J. (2020) 2444–2455.
[23] X. Zhang, Y. Ji, C. Xue, H. Ma, Y. Xi, P. Huang, H. Wang, F. An, B. Li, Y. Wang, H. Guo, Integrated regulation of apical hook development by transcriptional coupling of EIN3/EIL1 and PIFs in *Arabidopsis*, Plant Cell 30 (2018) 1971–1988.
[24] Y. Qi, H. Wang, Y. Zou, C. Liu, Y. Liu, Y. Wang, W. Zhang, Over-expression of mitochondrial heat shock protein 70 suppresses programmed cell death in rice, FEBS Lett. 585 (2011) 231–239.
[25] J.G. Dubouzet, T. Morishige, N. Fujii, A.N. Chungil, E. Fukusaki, K. Ifuku, F. Sato, Transient RNA silencing of scoulerine 9-O-methyltransferase expression by double stranded RNA in *Coptis japonica* protoplasts, Biosci. Biotechnol. Biochem. 69 (2005) 63–70.
[26] J. Bright, R. Desikan, J.T. Hancock, I.S. Weir, S.J. Neill, ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis, Plant J. 45 (2010) 113–122.
[27] W. Deng, Y. Wang, Z. Liu, H. Cheng, Y. Xue, Z. Zhang, HemI: a toolkit for illustrating heatmaps, PLoS ONE 9 (2014) e111988.
[28] H. Xiong, J. Li, P. Liu, J. Duan, Y. Zhao, X. Guo, Y. Li, H. Zhang, J. Ali, Z. Li, H. Yang, Overexpression of *OsMYB48-1*, a novel MYB-related transcription factor, enhances drought and salinity tolerance in rice, PLoS ONE 9 (2014) e92913.
[29] W. Troll, J. Lindsley, A photometric method for the determination of proline, J. Biol. Chem. 215 (1955) 655–660.
[30] D.M. Hodges, J.M. DeLong, C.F. Forney, R.K. Prange, Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds, Planta 207 (1999) 604–611.
[31] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[32] J.G. Foster, J.L. Hess, Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere enriched in oxygen, Plant Physiol. 66 (1980) 482–487.
[33] M. Chikahiro, A. Kozi, Inactivation mechanism of ascorbate peroxidase at low concentrations of ascorbate; hydrogen peroxide decomposes compound i of ascorbate peroxidase, Plant Cell Physiol. 4 (1996) 423–430.
[34] J. Duan, M. Zhang, H. Zhang, H. Xiong, P. Liu, J. Ali, J. Li, Z. Li, *OsMIOX*, a myo-inositol oxygenase gene, improves drought tolerance through scavenging of reactive oxygen species in rice (*Oryza sativa* L.), Plant Sci. 196 (2012) 143.
[35] D.P. Drew, C. Lunde, J. Lahnstein, G.B. Fincher, Heterologous expression of cDNAs encoding monodehydroascorbate reductases from the moss, physcomitrella patens and characterization of the expressed enzymes, Planta 225 (2007) 945–954.
[36] Y. Nakano, K. Asada, Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts, Plant Cell Physiol. 22 (1981) 867–880.
[37] A.M.O. Smith, R.G. Ratcliffe, L.J. Sweetlove, Activation and function of mitochondrial uncoupling protein in plants, J. Biol. Chem. 279 (2004) 51944–51952.
[38] A.E. Eltayeb, N. Kawano, G.H. Badawi, H. Kaminaka, T. Sanekata, I. Morishima, T. Shibahara, S. Inanaga, K. Tanaka, Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol, Physiol. Plant. 127 (2006) 57–65.
[39] M.Y. Law, S.A. Charles, B. Halliwell, Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts: the effect of hydrogen peroxide and of Paraquat, Biochem. J. 210 (1983) 899–903.
[40] O.W. Griffith, Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine, Anal. Biochem. 106 (1980) 207–212.
[41] J. Yang, J. Zhang, Z. Wang, Q. Zhu, W. Wang, Hormonal changes in the grains of rice subjected to water stress during grain filling, Plant Physiol. 127 (1) (2001) 315–323.
[42] X. Zhang, B. Zhang, M.J. Li, X.M. Yin, L.F. Huang, Y.C. Cui, M.L. Wang, X. Xia, *OsMSR15* encoding a rice C2H2-type zinc finger protein confers enhanced drought tolerance in transgenic *Arabidopsis*, J. Plant Biol. 59 (2016) 271–281.
[43] T. Islam, M. Manna, T. Kaul, S. Pandey, C.S. Reddy, M.K. Reddy, Genome-wide dissection of *Arabidopsis* and rice for the identification and expression analysis of glutathione peroxidases reveals their stress-specific and overlapping response patterns, Plant Mol. Biol. Rep. 33 (2015) 1413–1427.
[44] L. Yao, X. Cheng, Z. Gu, W. Huang, S. Li, L. Wang, Y.F. Wang, P. Xu, H. Ma, X. Ge, The AWPM-19 family protein ospm1 mediates abscisic acid influx and drought response in rice, Plant Cell 30 (6) (2018) 1258–1276.
[45] C.J. Shen, S.K. Wang, S.N. Zhang, Y.X. Xu, Q. Qian, Y.H. Qi, D.A. Jiang, *OsARF16*, a transcription factor, is required for auxin and phosphate starvation response in rice (*Oryza sativa* L.), Plant Cell Environ. 36 (2013) 607–620.
[46] D.R. Nelson, M.A. Schuler, S.M. Paquette, D. Werck-Reichhart, S. Bak, Comparative genomics of rice and *Arabidopsis*. Analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and a dicot, Plant Physiol. 135 (2004) 756–772.
[47] Z.G. E, Y.P. Zhang, T.T. Li, L. Wang, H.M. Zhao, Characterization of the ubiquitin-conjugating enzyme gene family in rice and evaluation of expression profiles under abiotic stresses and hormone treatments, PLoS ONE 10 (2015) e0122621.
[48] K.S. Kothari, P.K. Dansana, J. Giri, A.K. Tyagi, Rice stress associated protein 1 (OsSAP1) interacts with aminotransferase (*OsAMTR1*) and pathogenesis-related 1a protein (OsSCP) and regulates abiotic stress responses, Front. Plant Sci. 7 (2016) 1057.
[49] S. Kumar, P.K. Trivedi, Glutathione S-Transferases: role in combating abiotic stresses including arsenic detoxification in plants, Front. Plant Sci. 9 (2018) 751.

[50] T.S. Serra, D.D. Figueiredo, A.M. Cordeiro, D.M. Almeida, T. Lourenço, I.A. Abreu, A. Sebastián, L. Fernandes, B. Contreras-Moreira, M.M. Oliveira, N.J. M. Saibo, *OsRMC*, a negative regulator of salt stress response in rice, is regulated by two AP2/ERF transcription factors, Plant Mol. Biol. 82 (2013) 439–455.
[51] S. Vij, J. Giri, P.K. Dansana, S. Kapoor, A.K. Tyagi, The receptor-like cytoplasmic kinase (*OsRLCK*) gene family in rice: organization, phylogenetic relationship, and expression during development and stress, Mol. Plant 1 (2008) 732–750.
[52] P. Guzmán, The prolific ATL family of RING-H2 ubiquitin ligases, Plant Signal. Behav. 7 (2012) 1014–1021.
[53] M. Farooq, A. Wahid, N. Kobayashi, D. Fujita, S.M.A. Basra, Plant drought stress: effects, mechanisms and management, Agron. Sustain. Dev. 29 (1) (2009) 185–212.
[54] C.N. Chow, T. Lee, Y.C. Hung, G.Z. Li, K.C. Tseng, Y. Liu, P.L. Kuo, H.Q. Zheng, W.C. Chang, PlantPAN3.0: a new and updated resource for reconstructing transcriptional regulatory networks from ChIP-seq experiments in plants, Nucleic Acids Res. 47 (2019) D1155–D1163.
[55] R.C. Omalley, S.C. Huang, L. Song, M.G. Lewsey, A. Bartlett, J.R. Nery, M. Galli, A. Gallavotti, J.R. Ecker, Cistrome and epicistrome features shape the regulatory DNA landscape, Cell 165 (2016) 1280–1292.
[56] F. Wang, W. Tong, H. Zhu, W. Kong, R. Peng, Q. Liu, Q. Yao, A novel Cys2/His2 zinc finger protein gene from sweetpotato, *IbZFP1*, is involved in salt and drought tolerance in transgenic *Arabidopsis*, Planta 243 (2016) 783–797.
[57] S. Sugano, H. Kaminaka, Z. Rybka, R. Catala, J. Salinas, K. Matsui, M. Ohme-Takagi, H. Takatsuji, Stress-responsive zinc finger gene *ZPT2-3* plays a role in drought tolerance in petunia, Plant J. 36 (2003) 830–841.
[58] H. Sakamoto, K. Maruyama, Y. Sakuma, T. Meshi, M. Iwabuchi, K. Shinozaki, K. Yamaguchi-Shinozaki, *Arabidopsis* Cys2/His2-type zinc-finger proteins function as transcription repressors under drought, cold, and high-salinity stress conditions, Plant Physiol. 136 (2004) 2734–2746.
[59] J.-K. Zhu, Abiotic stress signaling and responses in plants, Cell 167 (2016) 313–324.
[60] D. Srivastava, G. Verma, A.S. Chauhan, V. Pande, D. Chakrabarty, Rice (*Oryza sativa* L.) tau class glutathione S-transferase (*OsGSTU30*) overexpression in *Arabidopsis thaliana* modulates a regulatory network leading to heavy metal and drought stress tolerance, Metallomics Integrated Biometal Sci. 11 (2019) 375–389.
[61] T.Z. Hu, *OsLEA3*, a late embryogenesis abundant protein gene from rice, confers tolerance to water deficit and salt stress to transgenic rice, Russ. J. Plant Physiol. 55 (2008) 530–537.
[62] C.D. Buchanan, P.E. Klein, J.E. Mullet, Phylogenetic analysis of 5′-noncoding regions from the ABA-responsive rab16/17 gene family of sorghum, maize and rice provides insight into the composition, organization and function of *cis*-regulatory modules, Genetics 168 (2004) 1639–1654.
[63] M. Ganguly, K. Datta, A. Roychoudhury, D. Gayen, D.N. Sengupta, S.K. Datta, Overexpression of *Rab16A* gene in indica rice variety for generating enhanced salt tolerance, Plant Signal. Behav. 7 (2012) 502–509.
[64] J. Hur, K.H. Jung, C.H. Lee, G. An, Stress-inducible *OsP5CS2* gene is essential for salt and cold tolerance in rice, Plant Sci. 167 (2004) 417–426.
[65] G. Zhu, N. Ye, J. Zhang, Glucose-induced delay of seed germination in rice is mediated by the suppression of ABA catabolism rather than an enhancement of ABA biosynthesis, Plant Cell Physiol. 50 (2009) 644–651.
[66] N. Suzuki, S. Koussevitzky, R. Mittler, G. Miller, ROS and redox signalling in the response of plants to abiotic stress, Plant Cell Environ. 35 (2012) 259–270.
[67] J.G. de la Garma, N. Fernandez-Garcia, E. Bardisi, B. Pallol, J.S. Rubio-Asensio, R. Bru, E. Olmos, New insights into plant salt acclimation: the roles of vesicle trafficking and reactive oxygen species signalling in mitochondria and the endomembrane system, New Phytol. 205 (2015) 216–239.
[68] C. Xing, Y. Liu, L. Zhao, S. Zhang, X. Huang, A novel MYB transcription factor regulates ascorbic acid synthesis and affects cold tolerance, Plant Cell Environ. 42 (2019) 832–845.
[69] R. Mittler, Y. Kim, L. Song, J. Coutu, A. Coutu, S. Ciftci-Yilmaz, H. Lee, B. Stevenson, J.K. Zhu, Gain-and loss-of-function mutations in *Zat10* enhance the tolerance of plants to abiotic stress, FEBS Lett. 580 (2006) 6537–6542.
[70] F. He, H.G. Li, J.J. Wang, Y. Su, H.L. Wang, C.H. Feng, Y. Yang, M.X. Niu, C. Liu, W. Yin, *PeSTZ1*, a C2H2-type zinc finger transcription factor from *Populus euphratica*, enhances freezing tolerance through modulation of ROS scavenging by directly regulating *PeAPX2*, Plant Biotechnol. J. (2019) 2169–2183.
[71] Z. Zhang, H. Liu, C.e. Sun, Q. Ma, H. Bu, K. Chong, Y. Xu, A C2H2 zinc-finger protein OsZFP213 interacts with OsMAPK3 to enhance salt tolerance in rice, J. Plant Physiol. 229 (2018) 100–110.
[72] T. Wu, W.R. Lin, C.H. Kao, C. Hong, Gene knockout of glutathione reductase 3 results in increased sensitivity to salt stress in rice, Plant Mol. Biol. 87 (2015) 555–564.
[73] Y. Kim, I. Kim, S.Y. Shin, T.H. Park, H.M. Park, Y.H. Kim, G.S. Lee, H. Kang, S.H. Lee, H. Yoon, Overexpression of dehydroascorbate reductase confers enhanced tolerance to salt stress in rice plants (*Oryza sativa* L. *japonica*), J. Agron. Crop Sci. 200 (2014) 444–456.
[74] T. Chou, Y. Chao, C.H. Kao, Involvement of hydrogen peroxide in heat shock- and cadmium-induced expression of ascorbate peroxidase and glutathione reductase in leaves of rice seedlings, J. Plant Physiol. 169 (2012) 478–486.
[75] H. Yeh, T. Lin, C. Chen, T. Cheng, H. Chang, T. Lee, Monodehydroascorbate reductase plays a role in the tolerance of Chlamydomonas Reinhardtii to photooxidative stress, Plant Cell Physiol. 60 (2019) 2167–2179.
[76] K. Asada, The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 601–639.
[77] M.K. Giri, N. Singh, Z.Z. Banday, V. Singh, H. Ram, D. Singh, S. Chattopadhyay, A. K. Nandi, *GBF1* differentially regulates *CAT2* and *PAD4* transcription to promote pathogen defense in *Arabidopsis thaliana*, Plant J. 91 (2017) 802–815.