Plant Science 275 (2018) 49–59

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Isolation and identification of wheat gene *TaDIS1* encoding a RING finger domain protein, which negatively regulates drought stress tolerance in transgenic *Arabidopsis*

Yan Liu[1], Liqun Li[1], Li Zhang, Qian Lv, Yi Zhao, Xuejun Li*

State Key Laboratory of Crop Stress Biology in Arid Areas and College of Agronomy, Northwest A&F University, Yangling, Shaanxi, 712100, People's Republic of China

ARTICLE INFO

Keywords:
TaDIS1
Overexpression
Drought stress
Negative regulation
Wheat

ABSTRACT

Drought stress is a major factor that limits the yield and quality in wheat. In this study, we identified an orthologue of the rice gene *OsDIS1* (*Oryza sativa* drought-induced SINA protein 1) in wheat (*Triticum aestivum* L.) called *TaDIS1*. *TaDIS1* encodes a putative 301 amino acid protein with a C3HC4 RING finger conserved domain at the N-terminal and a SINA domain at the C-terminal. *TaDIS1* contains three exons and two introns. qRT-PCR analysis showed that *TaDIS1* expression was induced by PEG6000, NaCl, and abscisic acid (ABA) treatment. We generated *TaDIS1*-overexpressing transgenic *Arabidopsis* lines. Under drought stress conditions, the transgenic *Arabidopsis* plants had a lower germination rate, relative water content, and proline contents, with higher water loss, chlorophyll loss, relative electrical conductivity, and malondialdehyde contents compared with the wild type. The antioxidant enzyme (superoxide dismutase, peroxidase, and catalase) activity levels were lower in the transgenic plants. The *TaDIS1*-overexpressing plants had shorter roots with greater growth inhibition in response to mannitol treatment than the wild type, with increased hypersensitivity to ABA during seed germination and early seedling growth. The expression of stress-related genes in transgenic plants under drought stress suggests that *TaDIS1* may function negatively in drought stress by regulating the stress response-related genes.

1. Introduction

Plants are subjected to a wide variety of abiotic stresses, which have many severe adverse effects on plant growth and development. Abiotic stresses are responsible for global yield reductions of over 50% in the major crop plants [1]. Thus, in order to survive and reproduce better, plants must develop effective mechanisms to adapt to stressful environments [2]. Plants can modulate their phenotypes according to changes in physiological, biochemical, molecular, and genetic information, thereby allowing them to tolerate abiotic stresses [3]. Many abiotic stress-induced genes have been identified in previous studies of various plants, and their roles in stress responses have been well documented [4–7]. Numerous studies have also shown that plants can acquire tolerance to abiotic stress by re-regulating their metabolism and gene expression [8–10]. Therefore, isolating and cloning the key stress resistance genes is important for the development of new wheat varieties via conventional breeding and genetic engineering strategies [11].

The ubiquitin/26S proteasome system is one of the main mechanisms employed by plants to control their growth and development, as well as for responding to biotic and abiotic stresses [12–15]. E3 ligase is the most important enzyme among the ubiquitin-mediated protein degradation pathways [16]. In the RING E3 ligase group, RING finger proteins are known to play important roles in responses to abiotic stresses [17]. Some progress has also been made in the cloning and functional characterization of RING domain type E3 ubiquitin ligase-related genes, where their functions in the response to drought stress have been elucidated in *Arabidopsis*. For example, C3H2C3-type RING E3 ubiquitin ligase AtAIRP1 in *Arabidopsis* is a positive regulator of the abscisic acid (ABA)-dependent response to drought stress [18]. *Arabidopsis RGLG2* encodes a RING E3 ligase, which can interact with *AtERF53* and it negatively regulates the drought stress response by mediating the transcriptional activity of *AtERF53* in *Arabidopsis* [19]. *Arabidopsis* C3HC4-RING finger E3 ubiquitin ligase AtAIRP4 may act as a positive regulator of ABA-mediated drought avoidance and as a negative regulator of salt tolerance [20]. In rice, many RING finger proteins are also involved with the responses and adaptations to abiotic stress. According to previous studies, the overexpression of *Oryza sativa* RING domain-containing protein 1 (*OsRDCP1*) and *O. sativa* chloroplast

* Corresponding author at: Northwest A&F University, 3 Taicheng Rd, Yangling, Shaanxi Province, 712100, People's Republic of China.
E-mail address: xuejun@nwsuaf.edu.cn (X. Li).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.plantsci.2018.07.017
Received 28 January 2018; Received in revised form 27 July 2018; Accepted 29 July 2018
Available online 31 July 2018
0168-9452/

Table 1
Gene-specific primers used in this study.

Gene	Upstream primer (5′-3′)	Downstream primer (5′-3′)
TaDIS1-P1	ACTGACTATGAGTTCACTGGCAATC	GACGATGGCTAAAACAGGCTAC
TaDIS1-P2	GGCCTCGGCTGCTTATACTG	AGCAGACAGGGCATTCCAAA
18SrRNA	AACACTTCACCGGACCATTCA	CGTCCCTGCCCTTTGTACAC
TaDIS1-P3	TCGGTACCCTCGAGGGATCCATGGCCTCGGCTGCTTATA	GACTGCAGGTCGACAAGCTTCTCTTCTTTCCAAATCCTCCC
TaDIS1-P4	GGACTCTTGACCATGGCCTCGGCTGCTTATACTG	TCAGATCTACCATGGCCTCTTCTTTCCAAATCCTCCC
AtActin	GGTAACATTGTGCTCAGTGGTGG	AACGACCTTAATCTTCATGCTGC
DREB2 A	CTGGAGAATGGTGCGGAAGA	CAGATAGCGAATCCTGCTGTTGT
RD29A	GTTACTGATCCCACCAAAGAAGA	GGAGACTCATCAGTCACTTCCA
RD29B	GGAGTTCAAGATTCTGGGAAC	CATCAAAGTTCACAAACAGAGGC
P5CS1	GCGCATAGTTTCTGATGCAA	TGCAACTTCGTGATCCTCTG

targeting RING E3 ligase 1 (*OsCTR1*) improve drought tolerance in transgenic plants [21,22]. The RING finger E3 ligase gene *OsDSG1* controls seed germination and stress responses in rice [23]. The *OsSDIR1* gene is a functional orthologue of *Arabidopsis SDIR1* and it encodes a functional E3 ligase. The overexpression of *OsSDIR1* greatly improves drought tolerance in transgenic rice [24].

OsDIS1 (*Oryza sativa* drought-induced SINA protein 1) encodes a C3HC4 RING finger E3 ligase. The expression of *OsDIS1* is upregulated by drought treatment. The overexpression of *OsDIS1* reduces drought tolerance in transgenic rice plants and RNA interference (RNAi) to silence *OsDIS1* enhances drought tolerance. Microarray analysis has shown that a large number of drought-responsive genes are induced or suppressed in *OsDIS1* overexpressing plants under normal and drought conditions. Alternatively, RNAi transgenic plants with reduced *OsDIS1* expression levels can be generated for rice production if transgenic rice cultivars are allowed to grow in the field [25].

Most of the previously characterized RING finger type E3 ligases were identified in *Arabidopsis* or rice, and little is known about the RING E3 ligases in wheat. In our investigations of new genes involved with wheat defense responses, we isolated the *TaDIS1* gene based on homologous cloning. *TaDIS1* encodes a C3HC4 RING finger protein. We investigated the expression patterns of *TaDIS1* and its response to stress stimuli using quantitative real-time PCR (qRT-PCR), and we studied its functions in drought tolerance by overexpressing *TaDIS1* in *Arabidopsis* plants. Phenotypic analyses indicated that the overexpression of *TaDIS1* decreased drought tolerance in transgenic *Arabidopsis*, which exhibited an ABA hypersensitive response. These findings suggest that *TaDIS1* might participate in the negative regulation of drought stress tolerance in wheat plants.

2. Materials and methods

2.1. Plant material and stress treatment

Bread wheat (*Triticum aestivum* L. cv. Chinese Spring) was used in this study. The seeds were surface sterilized with 1% sodium hypochlorite for 15 min and rinsed three times with distilled water. The sterilized seeds were then grown in Petri plates containing two layers of filter paper wetted with distilled water in a growth chamber at 22 °C. Ten-day-old wheat seedlings were subjected to various abiotic stresses. In order to mimic drought, salinity, and ABA stress treatments, seedlings were transferred into solutions containing 20% PEG6000, 200 mM NaCl, and 100 μM ABA, respectively. Leaves were collected at 0, 3, 6, 9, 12, and 24 h after various treatments for gene expression analysis. Harvested plants were dropped immediately into liquid nitrogen and stored at −80 °C until RNA extraction. Wheat seeds were sown in the field to obtain different tissues from the growing wheat plants. Conventional agricultural management was maintained during growth and development. Different tissues were collected from wheat plants at the booting stage to determine tissue-specific expression levels and the samples were stored at −80 °C until use.

2.2. Isolation of the TaDIS1 gene

The complete cDNA sequence of the rice *OsDIS1* gene [25] was obtained from GenBank by searching with the GenBank accession (AK058336). The rice sequence was used to BLAST against the wheat genomic database at Unite de Recherche Genomique Info (URGI; https://urgi.versailles.inra.fr/blast/). Many genomic sequences shared high identity with *OsDIS1* and they were assembled into integrated contig sequences. A pair of gene-specific primers was designed to cover the open reading frame (ORF) sequence based on the assembled contig sequence in order to amplify the full-length *TaDIS1* cDNA sequence. Total RNA was isolated from the young leaves of Chinese Spring plants using TRIzol reagent according to the manufacturer's instruction (TaKaRa, China). First-strand cDNA was synthesized using a PrimeScript RT Reagent Kit with gDNA Eraser (Tiangen, Beijing, China) according to the manufacturer's recommended protocol. PCR was performed using the cDNA as the template with the primer *TaDIS1*-P1 (Table 1). The PCR products were purified and cloned into the pEASY-T1 vector and confirmed by sequencing.

The genomic DNA sequence was cloned to further analyze the structure of the *TaDIS1* gene. Genomic DNA was extracted from the young leaves of field-grown Chinese Spring plants using a modified CTAB method [26]. We used 1 μ of the genomic DNA as a template for PCR amplification with the primer *TaDIS1*-P1. The PCR products were purified and cloned into the pEASY-T1 vector and confirmed by sequencing.

2.3. Bioinformatics analysis

The sequence obtained was spliced using DNAMAN software to obtain the full length of the target gene. The protein encoded by the target cDNA was predicted based on the amino acid sequence and BLAST was performed via the NCBI website to analyze the sequences of the ORFs and conserved domains. Alignments were obtained with ClustalX (version 1.83) and GeneDoc software. A phylogenetic tree was constructed using ClustalX and MEGA6.0 (http://www.megasoftware.net/mega.html). The basic physical and chemical properties of the TaDIS1 protein were predicted using the Protparam tool (http://www.expasy.org/tools/protparam.html). The grand average of hydropathy (GRAVY) was predicted for the TaDIS1 protein using the Hplob./Kyte & Doolittle algorithm in ProtScale online software. The secondary structure of the *TaDIS1* protein was predicted using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). The conserved domains were analyzed using the PROSITE online database (http://expasv.org/prosite/).

2.4. qRT-PCR analysis of TaDIS1 expression patterns

Total RNA was extracted from different organs and various stress-treated materials (see above) using Trizol according to the manufacturer's instructions (Tiangen, Beijing, China), before it was reverse

transcribed into cDNA using a PrimeScript RT reagent Kit with gDNA Eraser (Tiangen, Beijing, China). After first-strand cDNA synthesis, qRT-PCR was performed using a Light Cycler® 96 detection system (Roche, Switzerland) with Faststart Essential DNA Green Master (Roche, Germany), according to the manufacturer's instructions. The qRT-PCR reactions were performed using the primer *TaDIS1*-P2 (Table 1) and bread wheat 18S rRNA was used as an internal reference (Table 1). The relative expression level was calculated using the $2^{-\Delta\Delta t}$ method [27]. Three biological replicates were analyzed for each sample and each with three technical replicates.

2.5. Prokaryotic expression analysis of TaDIS1

According to the *TaDIS1* cDNA sequence, a specific primer pair for *TaDIS1*-P3 (Table1) supplemented with the *Bam*HI and *Hind*III sites (underlined) was designed to amplify the coding region. The cDNA sequence was used as the template for PCR. After confirming by sequencing, the coding sequence of *TaDIS1* was introduced into the expression vector pCold-tf, which was also digested using the same restriction enzymes (TaKaRa Biotechnology, Japan), and the recombinant plasmid was then transformed into the *Escherichia coli* BL21(DE3) strain. The expression of the recombinant protein was performed as described previously [28]. Total protein and target protein were detected by SDS-PAGE [29].

2.6. Construction of a transgene vector and plant transformation

To obtain transgenic *Arabidopsis* plants, the *TaDIS1* ORF was amplified by RT-PCR using the primer *TaDIS1*-P4 (Table 1; *Nco*I site is underlined) and cloned into the *Nco*I restriction sites of *pCambia-1302* under the control of the CaMV35S promoter. The recombinant construct was confirmed by sequencing. The recombinant plasmid *pCambia-1302-TaDIS1* was then introduced into *Agrobacterium tumefaciens* strain GV3101. Finally, the transformation of *Arabidopsis* was performed using the floral dip method [30]. Seeds harvested from transgenic plants were screened on Murashige and Skoog (MS) medium supplemented with 50 mg/L hygromycin. The homozygous T3 progeny were confirmed by PCR analysis. Representative homozygous T3 progeny were used in further experiments.

2.7. Stress tolerance assays with TaDIS1-overexpressing plants

The wild type (WT) and *TaDIS1* overexpression lines were used to evaluate various types of stress tolerance. WT and *TaDIS1* overexpression lines were grown in plastic containers filled with humus soil and cultivated in a greenhouse with a light:dark cycle of 16:8 h at 22 °C for 25 days. In the drought tolerance assay, 25-day-old plants were not watered for 2 weeks and they were then watered for 3 days. The phenotypic analysis was then conducted. In the germination and root growth analyses, seeds of the WT *Arabidopsis* and *TaDIS1* overexpression lines were sown on 1/2 MS medium supplemented with 150 mM mannitol for the drought stress assay, with 200 mM NaCl for the salt stress assay, and with 2 μM ABA for the signal molecule response assay. After cold treatment for 3 days, the plates containing seeds were incubated in a growth chamber at 22 °C with a light:dark cycle of 16:8 h. Germination and seedling growth were examined after 8 days. The root length was measured after growth for 8 days on 1/2 MS medium. The germination rate after ABA treatment was scored each day for 8 days. The stress tolerance assays were performed as described previously [31] with slight modifications.

2.8. Physiological characteristics of the transgenic Arabidopsis plants

To further evaluate the mechanism responsible for the drought stress response in *TaDIS1* overexpression lines, the rosette leaves of 30-day-old plants grown in soil were sampled to assay their water loss rate. Water losses were determined as described by Liang et al. [32]. The detached leaves of the overexpression and control plants were placed on filter paper at a constant temperature (22 °C) and humidity (70%), and the weights of the leaves were measured at specific time intervals. The percentage weight loss was calculated based on the initial weight of the plants. The WT and overexpression lines subjected to drought for 10 days were sampled to detect the activities of antioxidant enzymes, i.e., superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), where the non-stressed plants were used as controls. In order to extract CAT, SOD, and POD, 0.1 *g* samples of leaves were mixed with 0.8 mL of phosphate-buffered saline (0.05 mol/L, pH 7.8) on ice. The crude extract was centrifuged at 10,000 × *g* for 10 min at 4 °C. The enzyme activity assays were performed using a UV–V is spectrophotometer (UV-1700, Mrcy, Japan) at 25 °C [33]. The total chlorophyll contents of the WT and transgenic *Arabidopsis* plants were measured according to a previously reported protocol [34]. The malondialdehyde (MDA) contents were analyzed using the thiobarbituric acid method [35]. The proline contents were determined as described by Ryu et al. [36]. Electrolyte leakage and the relative water content (RWC) were analyzed according to Wang et al. [37].

2.9. qRT-PCR assay of the stress-related gene

To further investigate the molecular mechanism responsible for stress tolerance, the expression levels of marker genes were detected in the WT and *TaDIS1* overexpression plants under drought stress. The leaves of *Arabidopsis* seedlings were detached after drought stress for 10 days. Total RNA was extracted and reverse transcribed into cDNA using a PrimeScript RT reagent Kit with gDNA Eraser (Tiangen, Beijing, China). qRT-PCR was performed using specific primers (Table 1) to determine the expression levels of marker genes comprising *DREB2 A, P5CS1*, *RD29A*, and *RD29B*, and *AtActin* was used as a reference gene.

2.10. Statistical analysis

All of the data were subjected to analysis of variance (ANOVA) using SPSS (SPSS Inc., Chicago, IL, USA). Significant differences were tested using Duncan's test at a $P < 0.05$ or $P < 0.01$. Figures were prepared using Sigmaplot 12.5.

3. Results

3.1. Isolation of TaDIS1 and sequence analysis

TaDIS1 was cloned from bread wheat and the full length cDNA comprising 1147 bp was obtained by PCR. The cDNA sequence of *TaDIS1* contained an ORF of 906 bp, which encoded a protein of 301 amino acid residues with a predicted molecular weight of 33.87 kDa and an isoelectric point of 7.52. The 5′ and 3′ untranslated regions (UTRs) comprised 109 bp and 132 bp (Fig. 1A), respectively. The cDNA sequence has been submitted to GenBank (accession no. MG592714). The cDNA sequence was BLASTed against the wheat genome database to establish the genomic organization. The results of BLASTn searches using the wheat genome database showed that IWGSC_4AS_v2 shared highest identity (99%) with the nucleotide sequence of *TaDIS1*, thereby suggesting that the *TaDIS1* gene is located on chromosome 4AS in wheat. The genomic organization of *TaDIS1* based on the alignment between the cDNA sequence and the corresponding genome sequence is shown in Fig. 1B. Three exons and two introns were identified in the DNA sequence.

The analysis of the amino acid sequence indicated that the TaDIS1 protein contained a C3HC4-type RING finger domain at the N-terminal (amino acids 53–89) and a conserved SEVEN IN ABSENTIA (SINA) domain at the C-terminal (amino acids 106–166) (Fig. 2A). Further alignments with wheat, rice, and *Arabidopsis* SINA proteins showed that this protein contained conserved RING finger and zinc finger motifs,

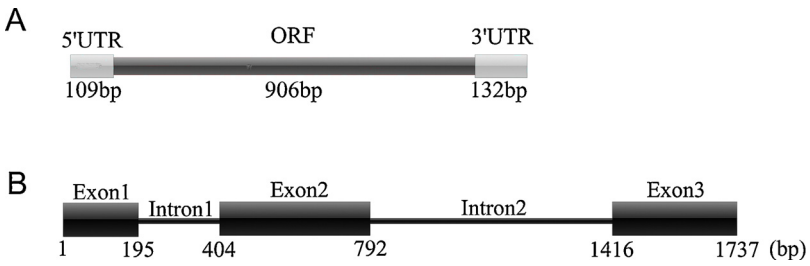

Fig. 1. Gene structure analyses for *TaDIS1*.
A, Schematic representation of the corresponding cDNA structure. The 5′ UTR and 3′ UTR, and the ORF are indicated by white and gray boxes, respectively.
B, Genomic organization of *TaDIS1*. Black boxes indicate exons encoding amino acids and black lines indicate introns. Numbers represent the lengths of each region in base pairs.

and it shared high sequence identity (92%) with SINA in rice, which was identified as a homologue of *Drosophila* SINA [38], thereby suggesting that the cloned *TaDIS1* is highly conserved (Fig. 2A). Thus, we designated the gene as *TaDIS1* in this study. Phylogenetic analyses showed that *TaDIS1* shared high homology with the E3 ubiquitin-protein ligase SINA from some monocots such as *Oryza sativa* (*OsDIS1*, BAG86666.1), *Triticum urartu* (EMS68731.1), *Brachypodium distachyon* (XP_003557906.1), *Aegilops tauschii* (XP_020178176.1), *Hordeum vulgare* (BAK05403.1), and *Setaria italica* (XP_004958578.1), but low identity with the human and *Drosophila* genes (Fig. 2B). The GRAVY value determined for TaDIS1 was −0.316, which suggests that TaDIS1 is a hydrophilic protein (Fig. S1 A). Secondary structure prediction analysis of TaDIS1 protein showed that the protein contained four main secondary structures comprising 29.24% α-helices, 37.87% random coils, 21.59% extended strand, and 11.30% β-turns (Fig. S1B).

3.2. Expression patterns of TaDIS1 in wheat

Organ-specific analysis demonstrated that the expression levels of *TaDIS1* were relatively higher in the leaf and young spike, with the highest expression levels in the latter. By contrast, *TaDIS1* was expressed at very low levels in the root and stem (Fig. 3A). We also investigated the expression patterns of *TaDIS1* under various abiotic stresses. After treatment with 20% PEG6000 to mimic drought stress, the *TaDIS1* transcript number increased gradually to peak at 12 h, after which a decrease occurred (Fig. 3B). Under treatment with ABA, the situation was similar to that with the drought treatment but the peaks occurred at different times (Fig. 3C). The abundance of *TaDIS1* transcripts changed very little until 24 h after NaCl treatment, whereas they increased dramatically by approximately three times after 24 h (Fig. 3D). However, there were no significant differences in the expression levels of *TaDIS1* in the control plants at different time points. Thus, *TaDIS1* was strongly induced by exogenous treatment with ABA and PEG but only weakly induced by salt. These results indicate that *TaDIS1* is responsive to multiple stresses, but especially drought stress. Therefore, we investigated the role of *TaDIS1* in drought tolerance in further functional analyses in this study.

3.3. Prokaryotic expression analysis of TaDIS1

In order to express and further characterize the TaDIS1 protein, the coding region of the *TaDIS1* gene was subcloned into the expression vector pCold-tf. The recombinant plasmid was expressed in the *E. coli* BL21 strain. SDS-PAGE gel analysis showed that the pCold-tf-TaDIS1 fusion protein was expressed successfully in *E. coli* BL21(DE3) when induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). The induced recombinant plasmid pCold-tf-TaDIS1 product had a relative molecular weight of about 90 kDa. The induced expression vector pCold-tf product had a relative molecular weight of about 55 kDa. The specific protein band was consistent with the theoretical molecular mass and the target protein was expressed efficiently 1 h after induction, whereas its expression increased with the induction time whereas the control did not express the fusion protein. This band was not produced by the pCold-tf and noninduced cells (Fig. 4). These results confirmed that the TaDIS1 protein was expressed successfully in the prokaryotic organism *E. coli* BL21(DE3).

3.4. Overexpression of TaDIS1 decreased the drought tolerance of transgenic Arabidopsis plants

The *TaDIS1* gene was introduced into *Arabidopsis* to study its function. Three transgenic lines (T3) were obtained by hygromycin screening, which were designated as OE-1, OE-2, and OE-3 (Fig. 5A). All three transgenic plants and the WT control were grown for 3 weeks under normal conditions, where all of the plants grew well with no obvious phenotypic changes. Watering was then stopped for 2 weeks and most of the leaves wilted and withered on the *TaDIS1* overexpression plants, whereas the WT plants continued growing normally. After rewatering for 3 days, the *TaDIS1* overexpression plants did not recover to normal growth and most of them died, whereas the WT plants resumed their normal growth (Fig. 5B). Furthermore, the *TaDIS1* overexpression plants exhibited higher electrolyte leakage and greater water loss in the detached rosette leaves, but the RWC was lower than that in the WT under drought stress, as shown in Fig. 5C–E. Thus, the overexpression of *TaDIS1* reduced the tolerance of drought stress.

Under drought conditions, the chlorophyll and proline contents of the *TaDIS1* overexpression plants were much lower than those of the WT control (Fig. 6A, C), and the *TaDIS1* overexpression plants accumulated much more MDA than the WT plants (Fig. 6B). However, under normal conditions, these indicators did not differ in the transgenic and WT plants. We also analyzed the antioxidant enzyme activity levels in plants under drought stress, where the results showed that the SOD, POD, and CAT activities were lower in the transgenic plants compared with the WT plants (Fig. 6D–F). However, there were no significant differences in the levels of these enzymes in the WT and transgenic plants under control conditions. These results suggest that the activity levels of antioxidant enzymes might play important roles in the ability to resist drought stress in *TaDIS1* overexpression plants. Therefore, *TaDIS1* may have been a negative regulator of the drought stress response in transgenic *Arabidopsis*.

3.5. Effects of TaDIS1 overexpression on seed germination and seedling growth under drought stress

We examined germination and seedling growth in WT and *TaDIS1* overexpression lines under drought stress to further understand the abiotic stress response in *TaDIS1* overexpression plants. Seeds from WT and *TaDIS1* transgenic *Arabidopsis* lines were germinated and grown in 1/2 MS medium as well as 1/2 MS medium containing mannitol or NaCl to evaluate their stress tolerance. Under normal conditions, the germination rate and seedling growth were similar in the WT and *TaDIS1* transgenic lines. However, in the presence of 150 mM mannitol, the seedlings from the transgenic line exhibited more growth inhibition than the WT seedling (Fig. 7A), while the same result was found in the presence of 200 mM NaCl (Fig. S2 A). Statistical analysis showed that the *TaDIS1* overexpression line plants grown on 1/2 MS medium supplemented with 150 mM mannitol had a lower germination rate than the control plants (Fig. 7C), while the same result was obtained in the presence of 200 mM NaCl (Fig. S2C). The root length did not differ significantly between the WT and transgenic line plants under non-

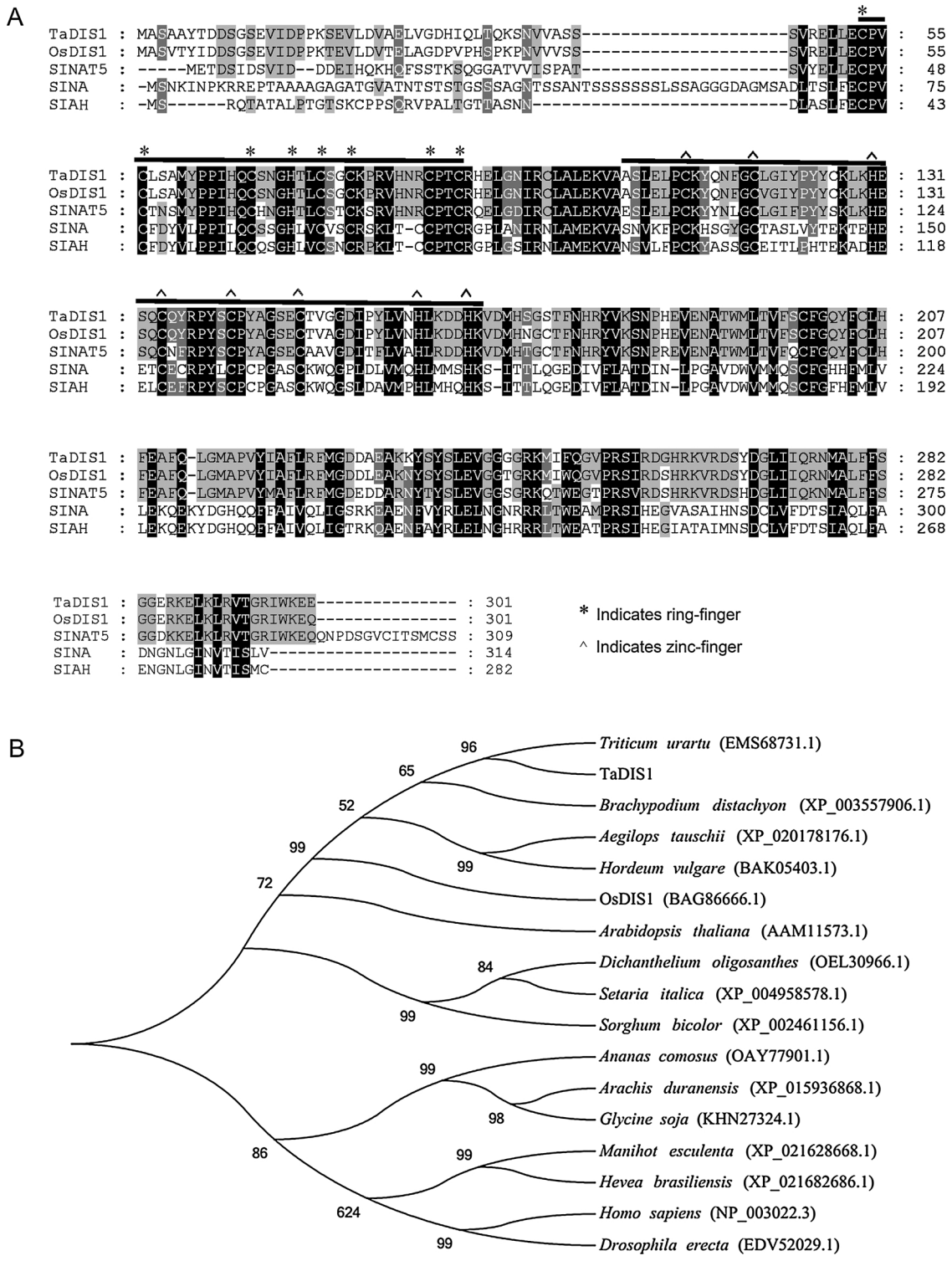

Fig. 2. Protein sequence and phylogenetic analyses of TaDIS1 protein.
A, Multiple alignment of TaDIS1 and other SINA E3 ligase proteins comprising *Arabidopsis* SINAT5, rice OsDIS1, *Drosophila* SINA, and human SIAH. The highly conserved amino acid residues among the proteins examined are shaded. The conserved RING-finger and zinc-finger motifs are indicated by asterisks and carets, respectively. The solid black line shows the RING domain (53–89) and the SINA domain (106–166).
B, Phylogenetic tree constructed using MEGA6.0 for TaDIS1 and other closely related SINA E3 ligase proteins.

stress conditions (Fig. 7B, D). However, the *TaDIS1* overexpression line plants had shorter primary roots compared with the control plants under drought stress (Fig. 7B, D), while the same result was obtained in the presence of 200 mM NaCl (Fig. S2B, S2D). These results suggest that the *TaDIS1* overexpression plants exhibited hypersensitivity to drought stress.

3.6. TaDIS1overexpression plants exhibited hypersensitivity to ABA

ABA signaling is mainly regulated by abiotic stresses in plants [39]. The increased expression of *TaDIS1* in response to ABA treatment suggests that *TaDIS1* might play a role in the ABA signaling pathway. Thus, in order to determine whether ABA participated in the response

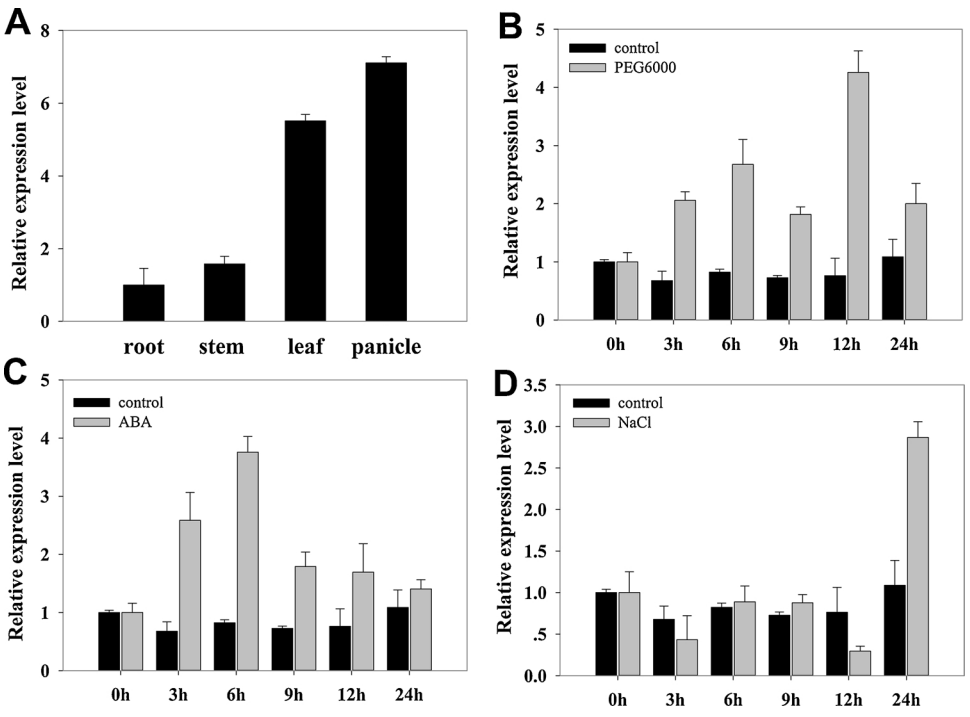

Fig. 3. Expression patterns of *TaDIS1* in wheat. **A**, Tissue-specific expression of *TaDIS1* in root, stem, leaf, and panicle tissues in wheat. **B–D**, Expression patterns of *TaDIS1* under different abiotic stresses: **B**, 20% PEG6000; **C,** 100 μM ABA; and **D**, 200 mM NaCl. *AtActin* was used as an internal control. Data represent mean ± SD (n = 3).

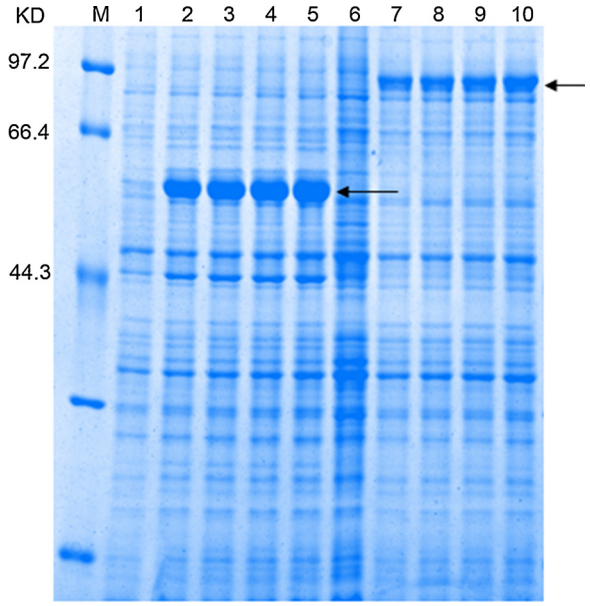

Fig. 4. Prokaryotic expression results. SDS-PAGE of the recombinant proteins expressed in *E. coli* BL21(DE3): lanes 1–5, total protein from *E. coli* BL21(DE3) containing pCold-tf after induction with 0.3 mM IPTG at 37 °C for 0, 1, 2, 4, and 6 h, respectively; Lanes 6–10, total protein from *E. coli* BL21(DE3) containing pCold-tf-TaDIS1 after induction with 0.3 mM IPTG at 37 °C for 0, 1, 2, 4, and 6 h, respectively; Lane M, protein molecular mass standards. The corresponding fusion proteins are indicated by arrows.

to abiotic stresses in transgenic plants, we examined the response of *TaDIS1* overexpression plants to treatment with exogenous ABA during the germination and post-germination growth periods. When grown on 1/2 MS medium without ABA, there was no significant difference in seedling emergence between the WT and transgenic seeds, but when grown on 1/2 MS medium containing 2 μM ABA, seedling emergence was inhibited more severely in the transgenic plants than the control plants (Fig. 8A). In addition, the transgenic line seeds grown on ABA-containing medium had a lower germination rate than the WT seeds (Fig. 8B). Therefore, the *TaDIS1* overexpression transgenic plant seeds were more sensitive to ABA in the germination stage than the WT. These observations indicate that the *TaDIS1* transgenic line exhibited ABA hypersensitivity during post-germination growth and that *TaDIS1* might be regulated partly by ABA signaling during abiotic stress responses in plants.

3.7. Overexpression of TaDIS1 affects the expression of stress-responsive genes

The transcript levels of four stress-related genes were compared in WT and transgenic *Arabidopsis* plants by qRT-PCR with gene-specific primers to elucidate the molecular mechanism related to *TaDIS1* in drought stress tolerance (Table 1). Under normal conditions, the transcript levels of *RD29A, RD29B, P5CS1* and *DREB2A* did not differ significantly between the control and overexpression lines. However, after drought stress, the transcript levels of all four genes were induced at much higher levels, but the mRNA levels of these genes were significantly lower in the overexpression seedlings than the controls (Fig. 9). These results agreed with the stress-sensitive phenotypes of *TaDIS1* overexpression plants grown in soil. Therefore, *TaDIS1* may function negatively in drought stress by regulating the stress response-related genes.

4. Discussion

Drought is one of the major abiotic stresses that directly affect plant growth and development, and ultimately the yield [40]. Plants have evolved various mechanisms for responding to drought stress in order to maintain their normal activities, particularly molecular responses [41]. The ubiquitin/26S proteasome system is a versatile post-translational modification system, which has been implicated in most aspects of growth and development, as well as in the responses to biotic and abiotic stress in plants [13,42]. In plants, the first identified SINA member was *Arabidopsis* SINAT5, which interacts with the NAC1 transcription factor and promotes its degradation to attenuate auxin signals [43]. In previous studies, several SINA E3 ligase genes were identified in various species act as key regulators of the responses to abiotic stresses [25,44]. In the current study, using a homology-based strategy,

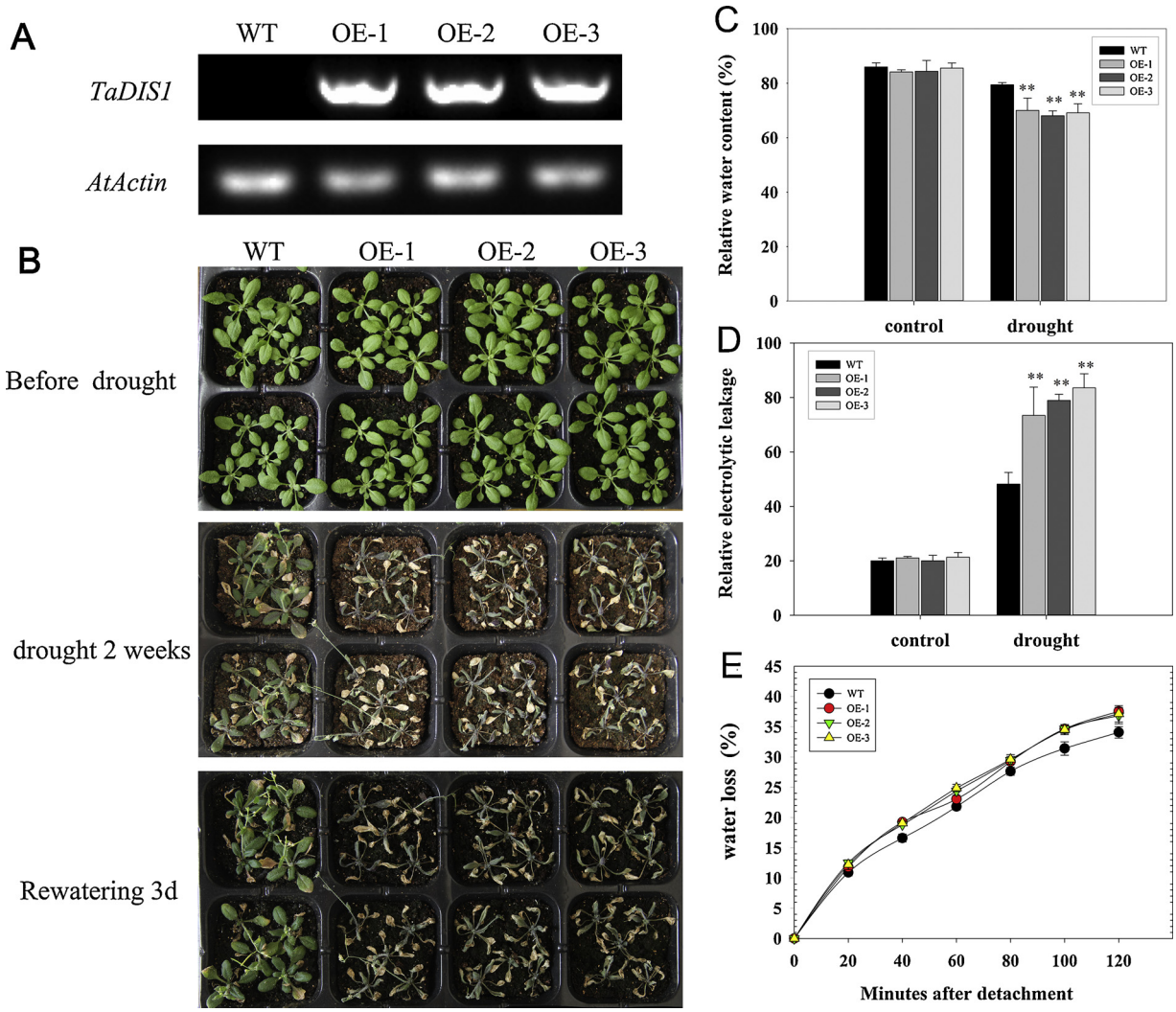

Fig. 5. Drought stress tolerance by wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsis* plants.
A, RT-PCR identification of transgenic *TaDIS1* overexpression *Arabidopsis* plants; **B**, phenotypic changes in transgenic *Arabidopsis* plants before/after drought stress; **C**, RWC of wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsi* plants under normal conditions and drought stress; **D**, relative electrolyte leakage from wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsis* plants under normal conditions and drought stress; **E**, water loss by wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsis* plants in response to dehydration. Data represent mean ± SD (n = 3), ** $P \leq 0.01$.

we cloned a new SINA E3 ligase gene from wheat called *TaDIS1*. In addition, we studied the characteristics of *TaDIS1* and its involvement with drought stress tolerance, thereby demonstrating that TaDIS1 protein is homologous to *OsDIS1* in rice, and thus we suggest that *TaDIS1* may have similar functions.

In a previous study, the expression of stress-induced genes was shown to be associated with stress tolerance [45]. Thus, *OsDIS1 is highly induced 24 h after drought treatment in a similar manner to OsDREB2 A,* and it was used as a positive control for the drought *treatment* [46] *where the results suggested that OsDIS1 is involved in the drought response in rice* [25]. In our study, we demonstrated that the expression of *TaDIS1* in wheat was significantly induced by PEG, ABA, and NaCl stresses (Fig. 3), and thus *TaDIS1* might be involved in different stress signaling pathways.

Moreover, drought stress can induce several different types of physiological stress. For example, various morphological, physiological, and biochemical parameters related to drought tolerance have been evaluated in physiological studies, such as the MDA and proline contents, RWC, and electrolyte leakage [47,48]. The accumulation of MDA is an indicator of cell membrane lipid peroxidation and it has also been used to measure the level of damage to the plasma membrane in response to abiotic stress [49]. Proline is a good indicator of stress-induced cell damage [50]. In the present study, the MDA content was higher in the transgenic plants than the wide-type plants under drought stress (Fig. 6B), thereby suggesting that damage to the cell membrane was increased in transgenic plants. The proline contents were lower in the transgenic lines than the WT plants under drought conditions (Fig. 6C), which indicates that the TaDIS1 protein did not effectively protect the cell membrane under drought stress. RWC is one of the most reliable phenotypic and physiological indicators used for evaluating the plant water status under drought tolerance [51]. Water stress causes plant water losses and reduces the RWC [52]. Membrane integrity and stability are essential for plant survival and stress resistance, and drought may damage the structure and function of the plasma membrane, thereby increasing the permeability of the cell membrane and intracellular material may leak outside the cell [53]. Assessing electrolyte leakage from cells is a common method used to assess plasma membrane damage or malfunction [54]. A lower level of electrolyte leakage indicates higher tolerance (less damage) of drought stress. We found that the *TaDIS1* overexpression plants had lower RWC levels and higher relative electrolyte leakage (Fig. 5), which suggests that the *TaDIS1* overexpression transgenic *Arabidopsis* plants had lower capacities for water retention and osmotic adjustment, and thus reduced tolerance of drought stress. Drought stress also causes plants to express

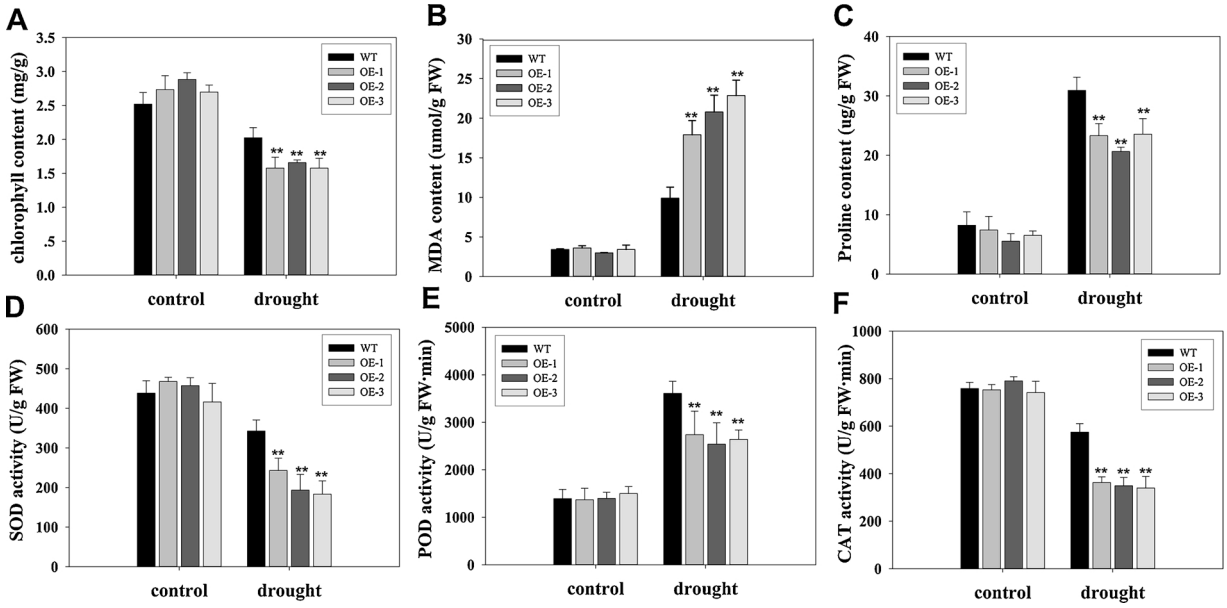

Fig. 6. Total physiological changes and activities of antioxidant enzymes in wild type *Arabidopsis* and transgenic *Arabidopsis* plants under normal conditions and drought stress treatment. **A**, Chlorophyll content; **B**, MDA content; **C**, free proline content; **D**, superoxidase dismutase, SOD; **E**, guaiacol peroxidase, POD; **F**, catalase, CAT; Each column represents the mean ± standard error based on three biological repeats. Significant differences between wild type *Arabidopsis* and transgenic *Arabidopsis* lines were determined by one-way ANOVA with Duncan's test (**$P \leq 0.01$).

numerous scavenging enzymes such as SOD, POD, and CAT, which protect plant cells from oxidative damage. We found that the activities of these antioxidant enzymes were lower in the transgenic *Arabidopsis* plants than the WT plants, which suggests that antioxidant enzymes were involved with drought tolerance. Our results showed that the overexpression of *TaDIS1* decreased the tolerance of drought in transgenic *Arabidopsis* plants.

Several studies have demonstrated that stress tolerance in plants is

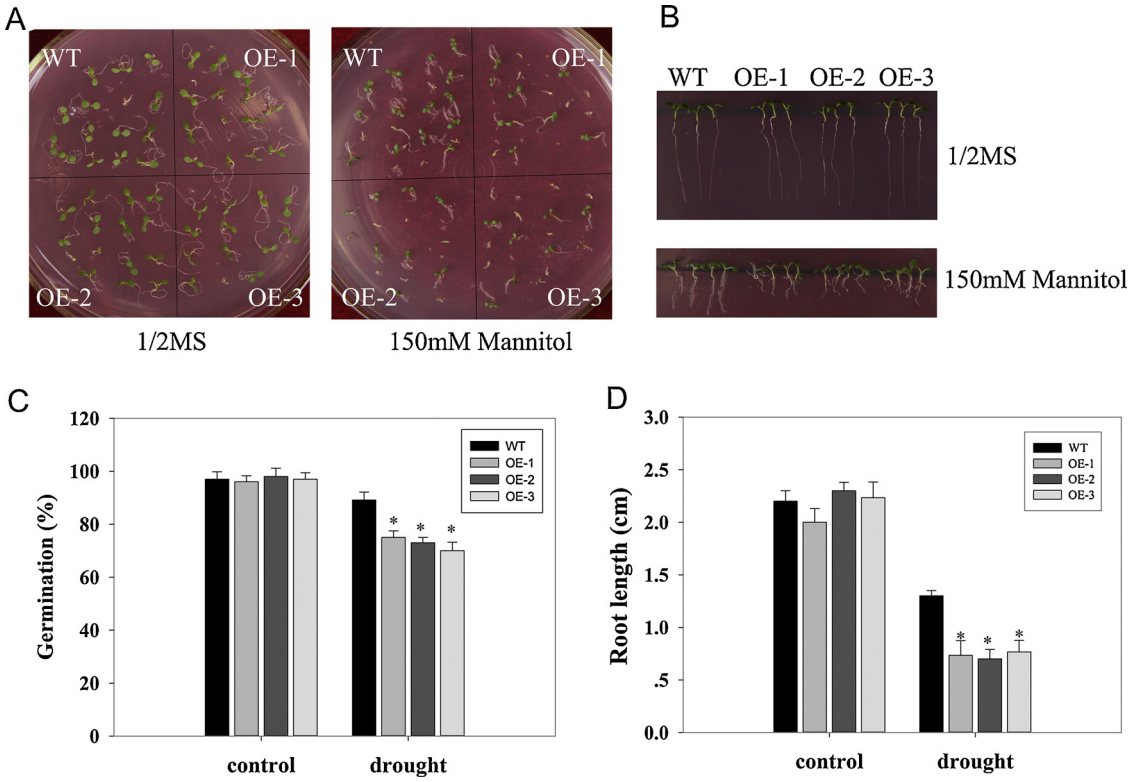

Fig. 7. Drought stress responses by wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsis* plants. **A**, Seedling growth in wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsis* plants grown on 1/2 MS medium and 1/2 MS medium containing 150 mM mannitol for 8 days; **B**, root length in wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsis* plants grown on 1/2 MS medium and 1/2 MS medium containing 150 mM mannitol, where the images were obtained after 8 days. Percentage germination and root length for the wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsis* plants in (**C**, **D**); means ± SD (n = 3). The asterisks indicate the significant differences between wild type *Arabidopsis* and transgenic *Arabidopsis* lines according to one-way ANOVA with Duncan's test (*$P \leq 0.05$).

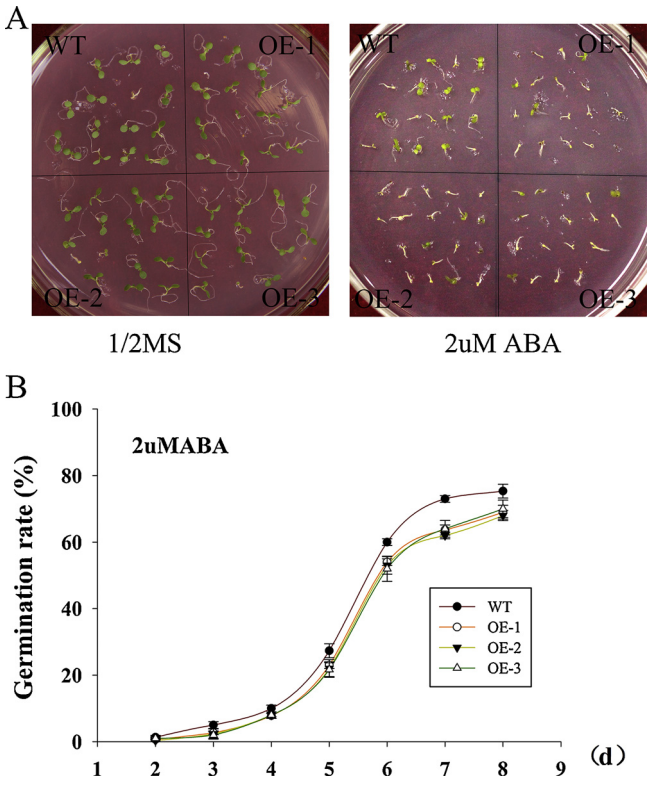

Fig. 8. Response of *TaDIS1* overexpression plants to exogenous ABA. **A,** Seedlings of wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsis* lines at 8 days after germination on 1/2 MS medium supplemented with 0 or 2 μM ABA. **B,** Percentage germination rates by seeds of wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsis* plants. Data represent means ± SD (n = 3). Three independent experiments were performed where each evaluated 80–100 seeds.

associated with germination and root growth by seedlings [55,56]. We found that the *TaDIS1* overexpression plants exhibited more growth inhibition with shorter roots, and thus the *TaDIS1* overexpression plants exhibited hypersensitivity to drought stress. Indeed, ABA is often considered to be a second messenger that modulates a wide spectrum of growth and developmental processes (such as seed maturation, dormancy, and germination) in abiotic stress conditions [57–61]. In the present study, the *TaDIS1* overexpression transgenic *Arabidopsis* seeds exhibited increased sensitivity to ABA and drought stress in the seed germination stage, which suggests that *TaDIS1* might be involved with the responses to drought and stress in an ABA-dependent manner. Further studies are required to understand how the *TaDIS1* gene is induced in response to drought stress and the role of ABA.

Four genes involved with the stress response, i.e., *RD29A*, *RD29B*, *P5CS1*, and *DREB2 A*, were analyzed by qRT-PCR (Fig. 9) to determine whether *TaDIS1* affected the expression of stress-related genes. *DREB2 A* (the gene encoding dehydration-responsive element binding protein 2 A) is thought to be one of the major transcription factors under drought stress conditions [9,62]. The abundance of *DREB2 A* is regulated by two RING-type E3 ligases comprising *DRIP1* and *DRIP2*, which negatively regulate plant drought responses by degrading the positive regulator *DREB2 A* [63]. In previous studies, it was shown that *RD29A* and *RD29B* are induced by different stresses (drought, salt, and ABA) via an ABA-dependent pathway [64,65]. *P5CS1* is involved in the rate-limiting step in proline biosynthesis and it is induced by drought in *Arabidopsis*. *AtP5CS1* is responsible mainly for proline accumulation under salt and drought stress [66]. The expression levels of these genes in the *TaDIS1* overexpression and WT plants after drought stress suggest that *TaDIS1* may regulate these stress-related genes to tolerate stress, and it could be involved with cross-talk between the complex networks of stress-responsive genes, thereby explaining the responses of *TaDIS1* overexpression plants under drought stress.

In addition, the expression of *OsDIS1* is upregulated by drought treatment. Silencing this gene in transgenic rice leads to enhanced drought tolerance whereas overexpression of the gene leads to increased drought sensitivity, which suggests that *OsDIS1* plays a negative role in drought stress tolerance by regulating the transcriptional of various stress-related genes and possibly via the posttranslational regulation of *OsNek6* [25]. Our results also indicated that *TaDIS1* is a negative regulator of the stress response in wheat and it has a similar function to *OsDIS1*, and thus silencing the *TaDIS1* gene may be lead to enhanced drought tolerance. Further studies are necessary to elucidate

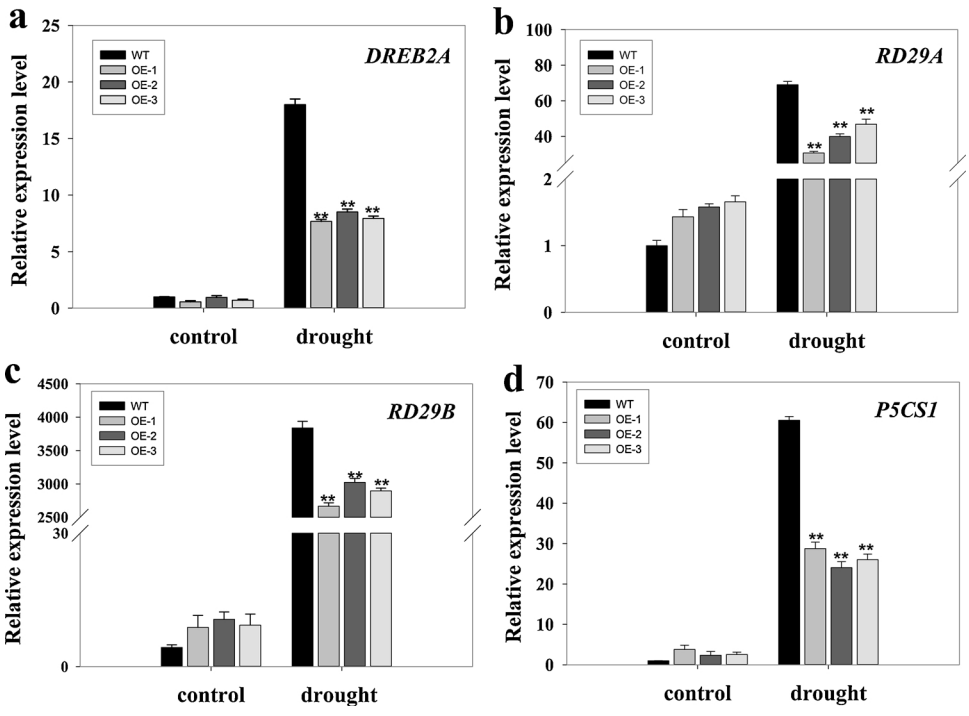

Fig. 9. Expression of stress-responsive genes in wild type *Arabidopsis* and *TaDIS1* overexpression transgenic *Arabidopsis* plants in response to drought. The 25-day-old plants grown in soil were not watered for 10 days and the expression levels of *DREB2 A*, *RD29A*, *RD29B*, and *P5CS1* were analyzed by qRT-PCR. *AtActin* was used as an internal control. Data represent means ± SD (n = 3). The asterisks indicate significant differences between wild type *Arabidopsis* control and transgenic *Arabidopsis* lines according to one-way ANOVA with Duncan's test (∗$P \leq$ 0.05; ∗∗$P \leq$ 0.01).

the precise function of *TaDIS1* by characterizing *TaDIS1*-suppressed transgenic plants or loss-of-function mutants.

In conclusion, we cloned and characterized the *TaDIS1* gene from wheat in this study. Our results demonstrated that TaDIS1 is a RING-containing protein in wheat and *TaDIS1* expression could be induced by drought stress and ABA. Furthermore, we investigated the function of *TaDIS1* under drought stress, where the results demonstrated that overexpression of *TaDIS1* increased the sensitivity to drought stress and ABA. The results obtained using transgenic plants showed that overexpression of the *TaDIS1* gene reduced the resistance to drought stress, thereby demonstrating that *TaDIS1* is a negative regulator of the drought stress response, where it may negatively function in drought stress by regulating stress-related genes. The exact functional role of *TaDIS1* in the response to abiotic stresses is not fully understood but our results provide valuable information, which may facilitate for molecular breeding and genetic engineering to improve abiotic stress tolerance in crops.

Author contributions

XL designed the project and amended the manuscript. YL and LL performed the experiments, analyzed the data, and drafted the manuscript. QL and LZ collected the data. YZ helped with image analysis. All of the authors read and approved the manuscript.

Acknowledgements

This study was supported by the National Key Research and Development Plan (No. 2017YFD0100704), the National Natural Special program for Transgenetic Wheat Breeding (No. 2016ZX08002-003) and the Collaborative Innovative Production, Teaching Research, and Application Program for Wheat Breeding and Germplasm Innovation (2016C XY-01).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2018.07.017.

References

[1] P. Rodziewicz, B. Swarcewicz, K. Chmielewska, A. Wojakowska, M. Stobiecki, Influence of abiotic stresses on plant proteome and metabolome changes, Acta Physiol. Plant. 36 (2014) 1–19.
[2] I. Ahuja, R.C.H.D. Vos, A.M. Bones, R.D. Hall, Plant molecular stress responses face climate change, Trends Plant Sci. 15 (2010) 664–674.
[3] T.K. Mohanta, T. Bashir, A. Hashem, E.F.A. Allah, Systems biology approach in plant abiotic stresses, Plant Physiol. Biochem. 121 (2017) 58–73.
[4] F. Qin, K. Shinozaki, K. Yamaguchi-Shinozaki, Achievements and challenges in understanding plant abiotic stress responses and tolerance, Plant Cell Physiol. 52 (2011) 1569–1582.
[5] H.M. Li, H.L. Jiang, Q.Y. Bu, Q.Z. Zhao, J.Q. Sun, Q. Xie, C.Y. Li, The Arabidopsis RING finger E3 ligase RHA2b acts additively with RHA2a in regulating abscisic acid signaling and drought response, Plant Physiol. 156 (2011) 550–563.
[6] X. Li, S.S. Zhang, J.X. Ma, G.Y. Guo, X.Y. Zhang, X. Liu, C.L. Bi, TaUBA, a UBA domain-containing protein in wheat (Triticum aestivum L.), is a negative regulator of salt and drought stress response in transgenic arabidopsis, Plant Cell Rep. 34 (2015) 755–766.
[7] Z.X. Zhao, G.Q. Zhang, S.M. Zhou, Y.Q. Ren, W. Wei, The improvement of salt tolerance in transgenic tobacco by overexpression of wheat F-box gene TaFBA1, Plant Sci. 259 (2017) 71–85.
[8] M. Agarwal, Y. Hao, A. Kapoor, C.H. Dong, H. Fujii, X. Zheng, J.K. Zhu, A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance, J. Biol. Chem. 281 (2006) 37636–37645.
[9] Y. Sakuma, Y. Maruyama, Y. Osakabe, F. Qin, M. Seki, K. Shinozaki, K. Yamaquchi-Shinozaki, Functional analysis of an Arabidopsis transcription factor, DREB2A, involved in drought-responsive gene expression, Plant Cell 18 (2006) 1292–1309.
[10] S. Ray, P.K. Dansana, J. Giri, P. Deveshwar, R. Arora, P. Agarwal, J.P. Khurana, S. Kapoor, A.K. Tyagi, Modulation of transcription factor and metabolic pathway genes in response to water-deficit stress in rice, Funct. Integr. Genomic 11 (2011) 157–178.
[11] T. Hirayama, K. Shinozaki, Research on plant abiotic stress responses in the post-genome era: past, present and future, Plant J. 61 (2010) 1041–1052.
[12] A. Santner, M. Estelle, The ubiquitin-proteasome system regulates plant hormone signaling, Plant J. 61 (2010) 1029–1040.
[13] W.J. Lyzenga, S.L. Stone, Abiotic stress tolerance mediated by protein ubiquitination, J. Exp. Bot. 63 (2012) 599–616.
[14] L. Perales, L. Penarrubia, M.J. Cornejo, Induction of a polyubiquitin gene promoter by dehydration stresses in transformed rice cells, J. Plant Physiol. 165 (2008) 159–171.
[15] A. Sadanandom, M. Bailey, R. Ewan, J. Lee, S. Nelis, The ubiquitin-proteasome system: central modifier of plant signaling, New Phytol. 196 (2012) 13–28.
[16] A. Dill, S.G. Thomas, J. Hu, C.M. Steber, T.P. Sun, The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation, Plant Cell 16 (2004) 1392–1405.
[17] L. Guo, C.D. Nezames, L. Sheng, X. Deng, N. Wei, Cullin-RING ubiquitin ligase family in plant abiotic stress pathways, J. Integr. Plant Biol. 55 (2013) 21–30.
[18] M.Y. Ryu, S.K. Cho, W.T. Kim, The Arabidopsis C3H2C3-type RING E3 ubiquitin ligase AtAIRP1 is a positive regulator of an abscisic acid-dependent response to drought stress, Plant Physiol. 154 (2010) 1983–1997.
[19] M.C. Cheng, E.J. Hsieh, J.H. Chen, H.Y. Chen, T.P. Lin, Arabidopsis RGLG2, functioning as a RING E3 ligase, interacts with AtERF53 and negatively regulates the plant drought stress response, Plant Physiol. 158 (2012) 363–375.
[20] L. Yang, Q.H. Liu, Z.B. Liu, H. Yang, J.M. Wang, X.F. Li, Y. Yang, Arabidopsis C3HC4-RING finger E3 ubiquitin ligase AtAIRP4 positively regulates stress-responsive abscisic acid signaling, J. Integr. Plant Biol. 58 (2016) 67–80.
[21] H. Bae, S.K. Kim, S.K. Cho, B.G. Kang, W.T. Kim, Overexpression of OsRDCP1, a rice RING domain-containing E3 ubiquitin ligase, increased tolerance to drought stress in rice (Oryza sativa L.), Plant Sci. 180 (2011) 775–782.
[22] S.D. Lim, C. Lee, C.S. Jang, The rice RING E3 ligase, OsCTR1, inhibits trafficking to the chloroplasts of OsCP12 and OsRP1, and its overexpression confers drought tolerance in Arabidopsis, Plant Cell Environ. 37 (2014) 1097–1113.
[23] G.G. Park, J.J. Park, J. Yoon, S.N. Yu, G. An, A RING finger E3 ligase gene, Oryza sativa delayed seed germination 1 (OsDSG1), controls seed germination and stress responses in rice, Plant Mol. Biol. 74 (2010) 467–478.
[24] T. Gao, Y.R. Wu, Y.Y. Zhang, L.J. Liu, Y.S. Ning, D.J. Wang, H.N. Tong, S.Y. Chen, C.C. Chu, Q. Xie, OsSDIR1 overexpression greatly improves drought tolerance in transgenic rice, Plant Mol. Biol. 76 (2011) 145–156.
[25] Y.S. Ning, C. Jantasuriyarat, Q.Z. Zhao, H.W. Zhang, S.B. Chen, J.L. Liu, L.J. Liu, S.Y. Tang, C.H. Park, X.J. Wang, X.L. Liu, L.Y. Dai, Q. Xie, G.L. Wang, The SINA E3 ligase OsDIS1 negatively regulates drought response in rice, Plant Physiol. 157 (2011) 242–255.
[26] D.H. Chen, P.C. Ronald, A rapid DNA minipreparation method suitable for AFLP and other PCR applications, Plant Mol. Biol. Rep. 17 (1999) 53–57.
[27] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method, Methods 25 (2001) 402–408.
[28] Z.B. Yang, Z.Y. Bai, X.L. Li, P. Wang, Q.X. Wu, L. Yang, L.Q. Li, X.J. Li, SNP identification and allelic-specific PCR markers development for TaGW2, a gene linked to wheat kernel weight, Theor. Appl. Genet. 125 (2012) 1057–1068.
[29] S. Yla-Herttuala, W. Palinski, M.E. Rosenfeld, S. Parthasarathy, T.E. Carew, S. Butler, J.L. Witztum, D. Steinberg, Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man, J. Clin. Invest. 84 (1989) 1086–1095.
[30] X. Zhang, R. Henriques, S.S. Lin, Q.W. Niu, N.H. Chua, Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method, Nat. Protoc. 1 (2006) 641–646.
[31] S. Zhou, W. Hu, X. Deng, Z. Ma, L. Chen, C. Huang, C. Wang, J. Wang, Y. He, G. Yang, G. He, Overexpression of the wheat aquaporin gene, *TaAQP7*, enhances drought tolerance in transgenic tobacco, PLoS ONE 7 (2012) e52439.
[32] Y.K. Liang, X. Xie, S.E. Lindsay, Y.B. Wang, J. Masle, L. Williamson, O. Leyser, A.M. Hetherington, Cell wall composition contributes to the control of transpiration efficiency in *Arabidopsis* thaliana, Plant J. 64 (2010) 679–686.
[33] Z. Hui, F.X. Tian, G.K. Wang, G.P. Wang, W. Wang, The antioxidative defense system is involved in the delayed senescence in a wheat mutant tasg1, Plant Cell Rep. 31 (2012) 1073–1084.
[34] D. Arnon, Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris, Plant Physiol. 24 (1949) 1–15.
[35] R.L. Heath, L. Packer, Photoperoxidation in isolated chloroplasts : I. Kinetics and stoichiometry of fatty acid peroxidation, Arch. Biochem. Biophys. 125 (1968) 189.
[36] J.Y. Ryu, S.Y. Hong, S.H. Jo, J.C. Woo, S. Lee, C.M. Park, Molecular and functional characterization of cold-responsive C-repeat binding factors from Brachypodium distachyon, BMC Plant Biol. 14 (2014) 15.
[37] J. Wang, B. Ding, Y. Guo, M. Li, S. Chen, G. Huang, X. Xie, Overexpression of a wheat phospholipase D gene, *TaPLDα*, enhances tolerance to drought and osmotic stress in Arabidopsis thaliana, Planta 240 (2014) 103.
[38] R.W. Carthew, G.M. Rubin, Seven in absentia, a gene required for specification of R7 cell fate in the Drosophila eye, Cell 63 (1990) 561–577.
[39] T. Yoshida, J. Mogami, K. Yamaguchi-Shinozaki, ABA-dependent and ABA-independent signaling in response to osmotic stress in plants, Curr. Opin. Plant Biol. 21 (2014) 133–139.
[40] S.M. Zargar, P. Nagar, R. Deshmukh, M. Nazir, A.A. Wani, K.Z. Masoodi, G.K. Agrawal, R. Rakwai, Aquaporins as potential drought tolerance inducing proteins: towards instigating stress tolerance, J. Proteomics 169 (2017) 233–238.
[41] M. Ashraf, Inducing drought tolerance in plants: recent advances, Biotechnol. Adv. 28 (2010) 169–183.
[42] S.L. Stone, The role of ubiquitin and the 26S proteasome in plant abiotic stress signaling, Front. Plant Sci. 5 (2014) 135.
[43] Q. Xie, H.S. Guo, G. Dallman, S. Fang, A.M. Weissman, N.H. Chua, SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals, Nature 419 (2002) 167–170.
[44] Z.Q. Fan, J.Y. Chen, J.F. Kuang, W.J. Lu, W. Shan, The banana fruit SINA ubiquitin ligase MaSINA1 regulates the stability of MaICE1 to be negatively involved in cold

stress response, Front. Plant Sci. 8 (2017).
[45] Y. Hong, W. Zhang, X. Wang, Phospholipase D and phosphatidic acid signalling in plant response to drought and salinity, Plant Cell Environ. 33 (2010) 627–635.
[46] G. Mallikarjuna, K. Mallikarjuna, M.K. Reddy, T. Kaul, Expression of OsDREB2A transcription factor confers enhanced dehydration and salt stress tolerance in rice (Oryza sativa L.), Biotechnol. Lett. 33 (2011) 1689–1697.
[47] C. Dong, Y. Ma, M. Wisniewski, Z.M. Cheng, Meta-analysis of the effect of over-expression of CBF/DREB family genes on drought stress response, Environ. Exp. Bot. 142 (2017) 1–14.
[48] H.W. Zou, X.H. Wang, C.L. Huang, J.S. Chen, X.H. Zhang, C. Luo, R. Yu, Z. Wu, Stress-inducible expression of a gene encoding C-repeat binding factor 4 (CBF4) from Arabidopsis improved performance of transgenic maize under drought condition, Plant Omics 7 (2014) 94–101.
[49] M. Mirzaee, A. Moieni, F. Ghanati, Effects of drought stress on the lipid peroxidation and antioxidant enzyme activities in two canola (Brassica napus L.) cultivars, J. Agri. Sci. Technol. 15 (2013) 593–602.
[50] P. Zhang, P.Z. Yang, Z.Q. Zhang, B. Han, W.D. Wang, Y.F. Wang, Y.M. Cao, T.M. Hu, Isolation and characterization of a buffalograss (Buchloe dactyloides) dehydration responsive element binding transcription factor, BdDREB2, Gene 536 (2014) 123–128.
[51] C. Patanè, D. Scordia, G. Testa, S.L. Cosentino, Physiological screening for drought tolerance in Mediterranean long-storage tomato, Plant Sci. 249 (2016) 25–34.
[52] P. Rampino, S. Pataleo, C. Gerardi, G. Mita, C. Perrotta, Drought stress response in wheat: physiological and molecular analysis of resistant and sensitive genotypes, Plant Cell Environ. 29 (2010) 2143–2152.
[53] M. Bajji, J.M. Kinet, S. Lutts, The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat, Plant Growth Regul. 36 (2002) 61–70.
[54] M.B. Murray, J.N. Cape, D. Fowler, Quantification of frost damage in plant tissues by rates of electrolyte leakage, New Phytol. 113 (2006) 307–311.
[55] Z. Ren, Z. Zheng, V. Chinnusamy, J. Zhu, X. Cui, K. Iida, J.K. Zhu, RAS1, a quantitative trait locus for salt tolerance and ABA sensitivity in Arabidopsis, Proc Natl. Acad. Sci. 107 (2010) 5669–5674.
[56] J. Zhu, B.H. Lee, M. Dellinger, X. Cui, C. Zhang, S. Wu, E.A. Nothnagel, J.K. Zhu, A cellulose synthase-like protein is required for osmotic stress tolerance in Arabidopsis, Plant J. 63 (2010) 128–140.
[57] R.R. Finkelstein, S.S. Gampala, C.D. Rock, Abscisic acid signaling in seeds and seedlings, Plant Cell 14 (2002) S15–S45.
[58] A. Himmelbach, Y. Yang, E. Grill, Relay and control of abscisic acid signaling, Curr. Opin. Plant Biol. 6 (2003) 470–479.
[59] Z. Xie, P. Ruas, Q.J. Shen, Regulatory networks of the phytohormone abscisic acid, Vitam. Horm. 72 (2005) 235–269.
[60] Q. Huang, Y. Wang, Overexpression of TaNAC2D displays opposite responses to abiotic stresses between seedling and mature stage of transgenic Arabidopsis, Front. Plant Sci. 7 (2016) 163.
[61] Q. Huang, Y. Wang, B. Li, J. Chang, M. Chen, K. Li, G. Yang, G. He, TaNAC29, a NAC transcription factor from wheat, enhances salt and drought tolerance in transgenic Arabidopsis, BMC Plant Biol. 15 (2015) 268.
[62] K. Nakashima, K. Yamaguchi-Shinozaki, Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses, Plant Physiol. 149 (2009) 88–95.
[63] F. Qin, Y. Sakuma, L.S.P. Tran, K. Maruyama, S. Kidokoro, Y. Fujita, M. Fujita, T. Umezawa, Y. Sawano, K.I. Miyazono, M. Tanokura, K. Shinozaki, K. Yamaguchi-Shinozaki, Arabidopsis DREB2A-interacting proteins function as RING E3 ligases and negatively regulate plant drought stress–responsive gene expression, Plant Cell 20 (2008) 1693–1707.
[64] J.K. Zhu, Salt and drought stress signal transduction in plants, Annu. Rev. Plant Biol. 53 (2002) 247–273.
[65] M. Kasuga, Q. Liu, S. Miura, K. Yamaguchi-Shinozaki, K. Shionzaki, Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor, Nat. Biotechnol. 17 (1999) 287–291.
[66] L. Szabados, A. Savoure, Proline: a multifunctional amino acid, Trends Plant Sci. 15 (2010) 89–97.