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Temporal and spatial expression and function of *TaDlea3* in *Triticum aestivum* during developmental stages under drought stress

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ABSTRACT

Drought stress is a major factor limiting wheat growth and productivity. Late embryogenesis abundant (LEA) proteins are tolerant to water-related stress. To reveal the regulatory mechanisms of LEA proteins under drought stress, we cloned a novel group 3 LEA gene, namely, *TaDlea3*, from wheat (*Triticum aestivum* L.) Shaanhe 6. Subcellular localization assay showed that TaDlea3 protein accumulated in the cytoplasm. Quantitative real-time polymerase chain reaction results revealed that *TaDlea3* expression was induced by drought stress. Western blot results indicated that TaDlea3 protein expression gradually increased with drought stress during four different developmental stages. Under normal conditions, no obvious phenotype difference was observed between the transgenic and wild-type seedlings. Meanwhile, the overexpression of *TaDlea3* in *Arabidopsis* resulted in enhanced tolerance to drought stress, as determined by the assessment of antioxidant enzyme activities. Our results provide a basis for highly detailed functional analyses of LEA proteins and offer a promising approach for improving the tolerances of wheat cultivars to drought stress through genetic engineering.

1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important crops in the world. Food shortage becomes severe along with the increase in world population. The growing demand for food is paralleled by marked losses of arable land because of the increasing soil destruction by abiotic environmental conditions [1]. Drought or water-deficit stress is a major environmental factor that influences wheat yield and quality. Therefore, the response mechanism of wheat to drought stress must be determined and the drought tolerance of different breeding wheat cultivars must be improved [2].

Recently, potential stress-related genes capable of enhancing plant tolerance to abiotic stresses are identified. The expression of late embryogenesis abundant (LEA) protein is closely linked to the acquisition of tolerance against abiotic stresses [3]. Based on sequence similarity and conserved domains, LEA proteins are classified into at least five groups [4,5]. They are highly hydrophilic and rich in Glu, Asp, and Lys residues. LEA proteins are related to membrane stability by facilitating water retention and ion flow. They also protect cytoplasmic components during drought stress through their function of binding water molecules on their hydrophilic surface, thereby preventing further damage to cellular proteins [6–9]. Most LEA proteins are cytosolic proteins and are highly expressed under stress conditions, such as drought, high salt, low temperature and abscisic acid. The expression of *SmLEA* from *Salvia miltiorrhiza* Bunge enhances salt and drought tolerance in *Escherichina coli* and *S. miltiorrhiza* [10], and the expression of a group 3 LEA gene, *HVA1*, from barley (*Hordeum vulgare* L.) confers tolerance to deficits in soil water and salt stress in transgenic rice plants. Under diverse stress conditions, the overexpression of soybean group 3 LEA PM2 enhances salt tolerance in *E. coli* cells and stabilizes enzyme activity. Moreover, the overexpression of *OsLEA3-2* from rice (*Oryza sativa*) in yeast, *Arabidopsis*, and rice improves growth performance under salt- and osmotic-stress conditions compared with control conditions [11,12]. Despite various studies on LEA proteins, their precise molecular functions remain unknown. These proteins have been proposed to protect other proteins from aggregation and stabilize the membrane or other cellular

Abbreviations: LEA, late embryogenesis abundant; EST, expressed sequence tag; qRT-PCR, quantitative real-time polymerase chain reaction; FC, field capacity; ORF, open reading frame; GFP, green fluorescence of green fluorescent protein; WT, wild type; WC, water content; MDA, malondialdehyde; CAT, catalase; POD, guaiacol peroxidase; SOD, superoxidase dismutase.

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A

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1    TGAGATTTGGCCCTTCCGATCGAGTGATTTCAGTTCGTGTTTGTT
46   AGTGAGAGAGAAGACAAGCGGAGATGGCCTCCAACCAGAACCAGG
1                                M  A  S  N  Q  N  Q
91   CGAGCTACATGGCCGGCGAGACCAAGGCCCGCACCGAGGAGAAGA
8    A  S  Y  M  A  G  E  T  K  A  R  T  E  E  K
136  CCGGGCAGATGATGGACAAGGCGGGCCAGGCCACGGAGGCCACCA
23   T  G  Q  M  M  D  K  A  G  Q  A  T  E  A  T
181  AGCAGAAGGCCGGCGAGACGGCCGAGGCCACCAAGCAGAAGGCCG
38   K  Q  K  A  G  E  T  A  E  A  T  K  Q  K  A
226  GCGAGGCCAAGGACAAGACGGCCCAGACGGCTCAGGCGGCCAAGG
53   G  E  A  K  D  K  T  A  Q  T  A  Q  A  A  K
271  ACCGCGCCGCCGAGAGCAAGGACCAGACCGGCAGCTTCCTCGGCG
68   D  R  A  A  E  S  K  D  Q  T  G  S  F  L  G
316  AGAAGACTGAGGCGGCCAAGCAGAAGGCGTCGGAGACGGCGCAGT
83   E  K  T  E  A  A  K  Q  K  A  S  E  T  A  Q
361  ACGCGCAGGATAGGTCCTCCGACGCCGCGCAGTACACCAAGGAAT
98   Y  A  Q  D  R  S  S  D  A  A  Q  Y  T  K  E
406  CCGCCGTTGCCGGCAAGGACAAAACCGGCAGCGTGCTCCAGCAGG
113  S  A  V  A  G  K  D  K  T  G  S  V  L  Q  Q
451  CCGGCGAGACGGTGGTGAGCGCGGTGGTGGGCGCCAAGGACGCGG
128  A  G  E  T  V  V  S  A  V  V  G  A  K  D  A
496  TGGCGAACACGCTGGGCATGGGCGGTGACAACACCACCAAGGACA
143  V  A  N  T  L  G  M  G  G  D  N  T  T  K  D
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158  T  T  T  R  N  H  *
586  TTTCCT
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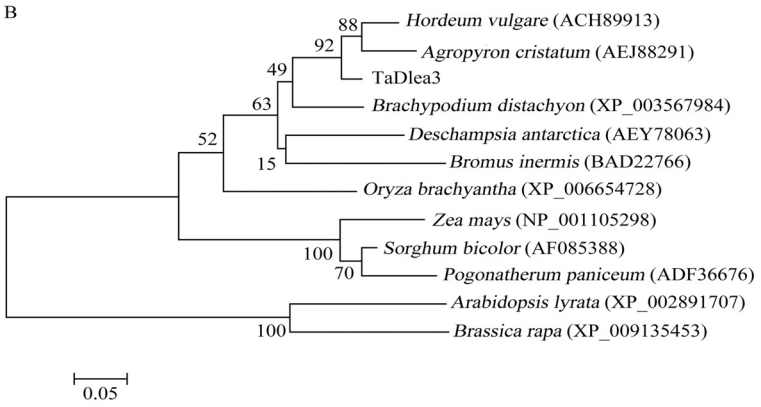
B



C

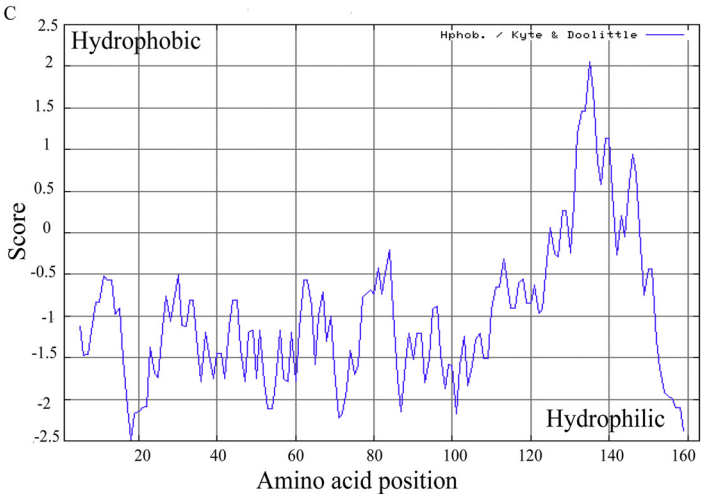


Fig. 1. Sequence analysis of *TaDlea3*. (A) The nucleotide sequence of *TaDlea3* cDNA together with its predicted amino acid sequence, underlines show eight 11-mer motifs in the TaDlea3. (B) Phylogenetic tree of TaDlea3 with other closely related LEA proteins, constructed by MEGA5.0 software. (C) Hydropathy analysis of TaDlea3 protein.

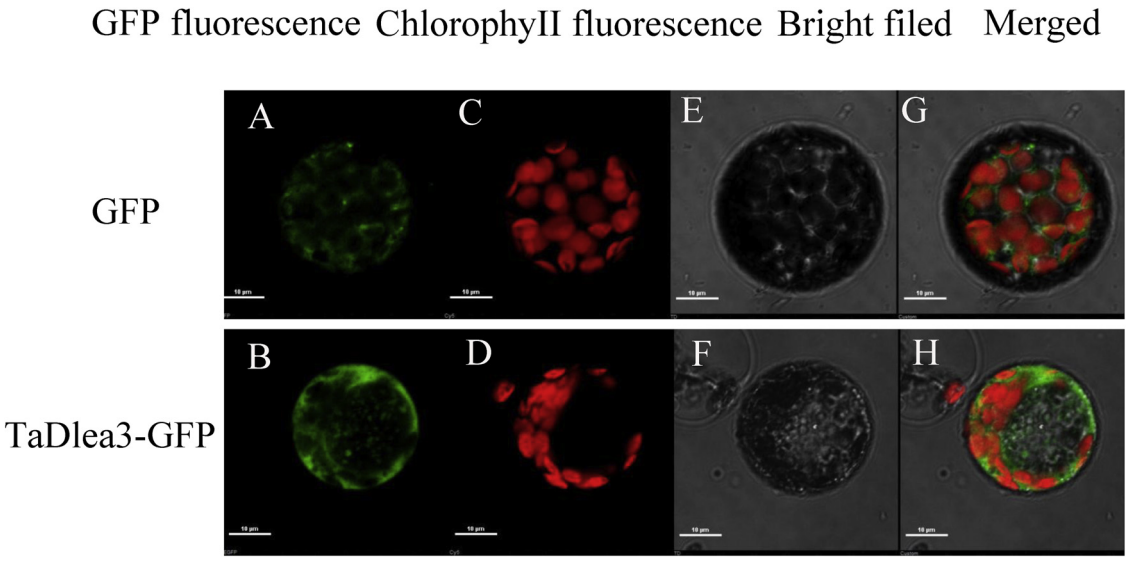


Fig. 2. Subcellular localization of TaDlea3 in tobacco protoplast. (A, B) Green fluorescence of green fluorescent protein (GFP) and TaDlea3-GFP fusion protein. (C, D) Red auto-fluorescence of chloroplasts. (E, F) Bright-field images of protoplasts. (G) and (H) Merged images. *Bar* 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

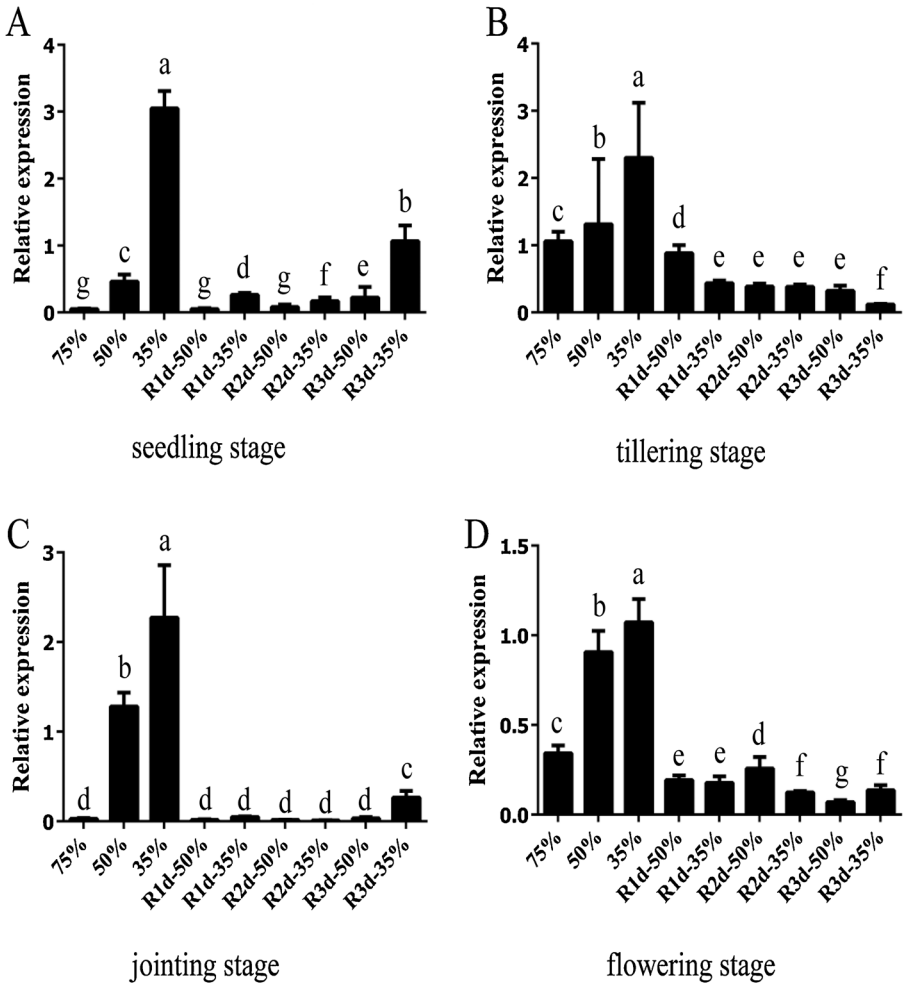


Fig. 3. Relative expression profiles of the *TaDlea3* genes in the different levels of water stress (75%, 50%, 35% FC) and 1, 2 and 3 days after recovery (R1d-50%, R1d-35%, R2d-50%, R2d-35%, R3d-50% and R3d-35%) during different stages, (A) seedling stage, (B) tillering stage, (C) jointing stage, (D) flowering stage. The transcript level of 18S was used as a reference. Mean values and standard errors (bar) were shown from three independent experiments and statistically significant differences are indicated with different letters (one-way ANOVA, followed by Duncan's test at $p < 0.05$).

components in the presence of abiotic stress by acting as molecular chaperones or molecular shields [13]. *E. coli* expressing *RcLEA* from *Rosa chinensis* shows high tolerance to low and high temperatures, high salt, and oxidative stress. Purified RcLEA protein protects lactate dehydrogenase (LDH) and citrate synthase from inactivation or aggregation and prevents the denaturation of soluble *E. coli* proteins during various stress treatments [14]. Furthermore, the WZY2 protein from wheat maintains the survival rates of cells, maintains LDH activity, and prevents protein aggregation under temperature stress [15].

Drought stress or water deficit is the most important factor constraining the yield of wheat. Group 3 LEA proteins are characterized by highly conserved 11- amino- acid repeat motif of "TAQAAKEKAGE" and have attracted attention in genetic stress-resistance breeding. Many group 3 LEA genes are resistant to drought stress in plants; however, the isolation and functions of these genes in wheat have been poorly established [16,17]. New group 3 LEA proteins associated with drought tolerance must be identified to provide useful markers for the selection of drought-tolerant genotypes by breeding [18]. In this study, we isolated a novel group 3 LEA gene, *TaDlea3*, from winter wheat Shaanhe 6 (GenBank Accession Number: KP233216). We also investigated *TaDlea3* transcript and protein expression in response to drought stress during four different developmental stages and its function in transgenic *Arabidopsis*. Our results indicated that *TaDlea3* might play important roles in resistance to water-deficit stress.

2. Materials and methods

2.1. Plant material and growth

Seeds of winter wheat Shaanhe 6, were obtained from the College of Life Science of Northwest A&F University. The seeds were grown in Petri dishes at 25 °C/18 °C (day/night) with a 16-h photoperiod. For dehydration treatment, seedlings grown under hydroponic conditions for 10 days were placed on dry filter paper under 70% humidity and dim light. The leaves were harvested at 48 h.

2.2. Isolation of the TaDlea3 gene

The total RNA from 100 mg of leaves collected from dehydration stress-treated seedlings was isolated using the Trizol Reagent (TaKaRa, China) according to the instructions of the manufacturer. First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa, China) according to the recommended protocol of the manufacturer. PCR was performed using the cDNA as the template with the forward primer (5′-TGAGATTTGGCCCTTCCGAT-3′) and the reverse primer (5′-AGGAAAGCAAATTAAGCGCGAAC-3′).

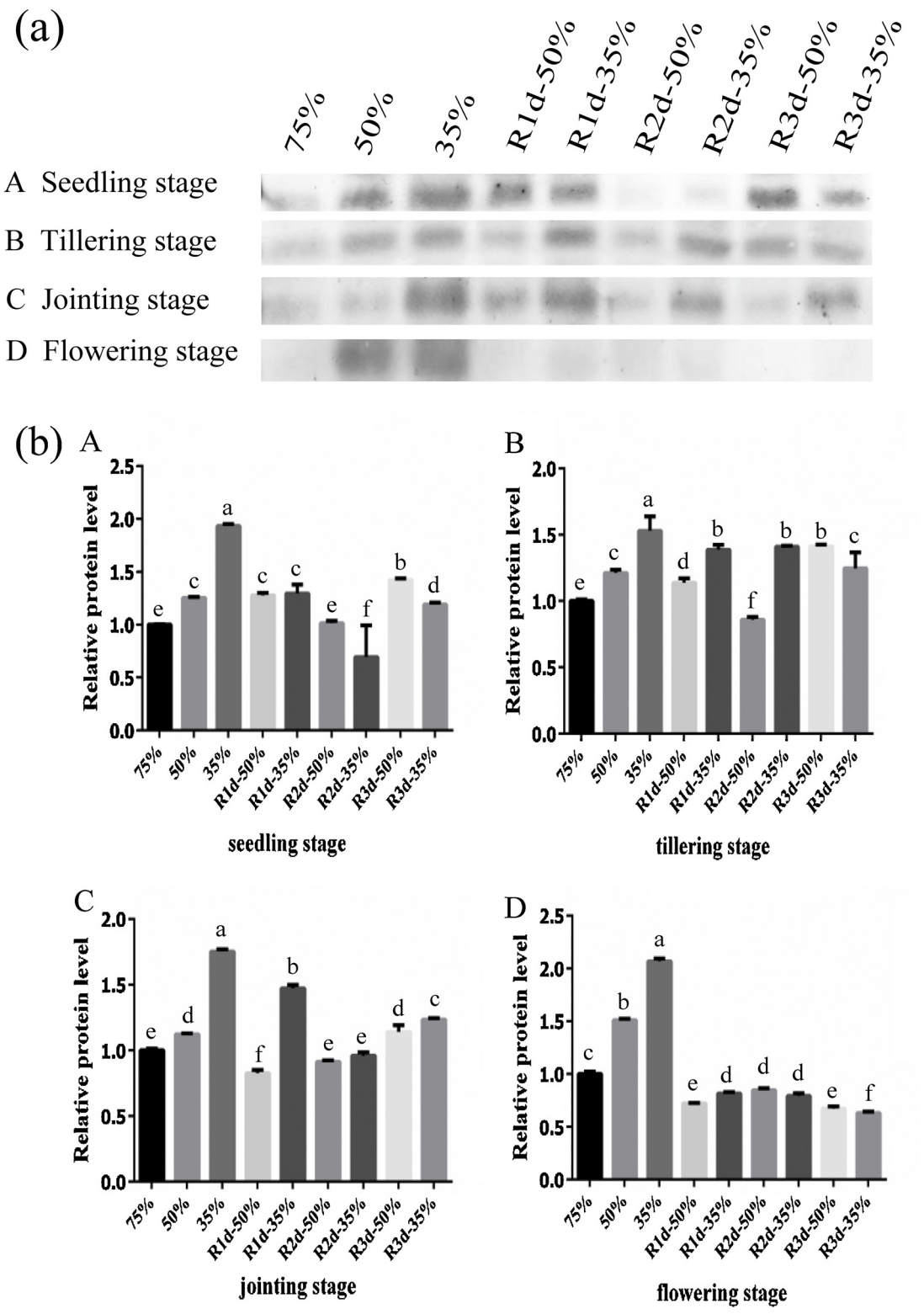


Fig. 4. The accumulation of TaDlea3 protein in the leaves of Shaanhe 6 of the different levels of water stress (75%, 50%, 35% FC) and 1, 2 and 3 days after recovery (R1d-50%, R1d-35%, R2d-50%, R2d-35%, R3d-50% and R3d-35%) in different stages. (a) Western blot analyses of (A) seedling stage, (B) tillering stage, (C) jointing stage, (D) flowering stage. (b) The intensity of the bands on the blot was quantified using densitometry. For comparison of TaDlea3 protein relative accumulation, the accumulation of TaDlea3 protein at 75% FC was an internal standard equal 1. Statistically significant differences are indicated with different letters (one-way ANOVA, followed by Duncan's test at $p < 0.05$).

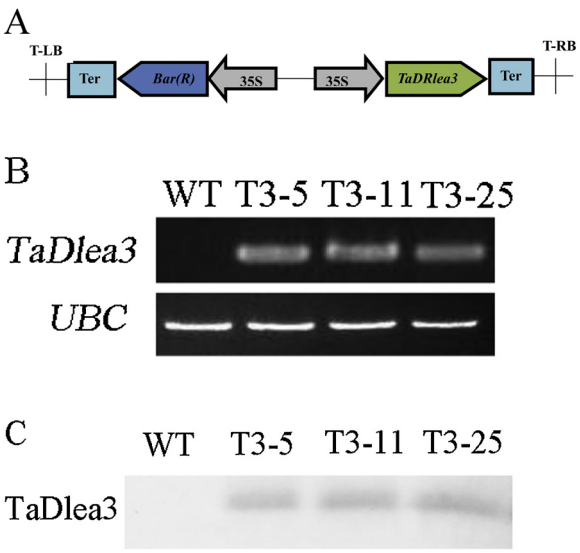


Fig. 5. Characterization of *TaDlea3* overexpressing plants. (A) Diagram of the T-DNA region of the binary vector pCAMBIA 3301:TaDlea3; (B) The transcript level of *TaDlea3* overexpressing in transgenic *Arabidopsis* and wild type plants assayed by semi-quantitative RT-PCR. The expression of UBC (NM_122477) was used as an internal control. (C) Western-blot analysis of the three independent transgenic lines and wild-type line.

The PCR products were purified and cloned into the pMD18-T vector for sequencing.

2.3. Bioinformatics analysis

Sequence identities were determined using BLAST on the NCBI web server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed by ClustalX and MEGA5.0 program (http://www.megasoftware.net/mega.html). The grand average of hydropathy (GRAVY) of the TaDlea3 protein was predicted using ExPASy programs (http://www.expasy.org/tools)

2.4. Expression and purification of TaDlea3

The open reading frame (ORF) of TaDlea3 was amplified using the forward primer (5′-GCCCATGGCCTCCAACCAGAACCAGGCGA-3′, NcoI site underlined) and the reverse primer (5′-GGCTCGAGGTGATTCCTGGTGGTGGTGTCCT-3′, XhoI site underlined), which correspond to the 5′ and 3′ ends. The PCR products were digested with *Nco*I and *Xho*I and ligated into the expression vector pET28a (Novagen, USA), which was digested with the same enzymes. The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3) cell according to Novagen's protocol. The recombinant constructs were confirmed by sequencing. The *E. coli* BL21 (DE3) transformants, including the PET28a/His-TaDlea3 expression vector, were incubated in Luria–Bertani (LB) medium with kanamycin (50 μg/mL) and expression of the recombinant protein was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 8 h at 37 °C. The bacterial cells were harvested by centrifugation at 5000 × *g* for 15 min at 4 °C, and the pelleted cells were re-suspended in phosphate-buffered saline (PBS, pH 7.0). The *E. coli* cells suspended in PBS were lysed by sonication and then boiled for 15 min. The supernatant was transferred to fresh tubes after centrifugation at 12,000 × *g* for 15 min at 4 °C. Soluble protein was passed over a Ni-NTA Superflow column (QIAGEN) for purification. After washing with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, and pH 8.0), the recombinant proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, and pH 8.0). The products were assessed by SDS-PAGE and dialyzed with PBS buffer more than twice. Antibodies were raised in rabbits against TaDlea3 protein by GenScript Biotechnology (NJ, USA).

2.5. Real-time PCR analysis of TaDlea3 expression during different developmental stages

Shaanhe 6 seeds were germinated in plastic pots (30 cm in diameter and 20 cm in depth) containing soil, peat and sand at a ratio of 1:1:1, and grown under greenhouse conditions. A total of 36 pots were assigned to the control and drought treatments during the seedling, tillering, jointing, and flowering stages. For the drought treatment, the control, moderate drought, and severe drought seedlings were maintained under optimal moisture levels at 75%, 50%, and 35% field capacity (FC). After the seedlings were sampled at a FC of 50% and 35%, the plants were irrigated to 75% FC and allowed to recover for 3 days. The leaves of the wheat were collected from plants exposed to different levels of water stress (75%, 50% and 35% FC) that were allowed to recover for 1, 2, and 3 days (R1d-50%, R1d-35%, R2d-50%, R2d-35%, R3d-50% and R3d-35%). The leaves from different developmental stages were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis. After performing first-strand cDNA synthesis, quantitative real-time PCR (qRT-PCR) was performed on a Bio-Rad CFX96TM real-time system using SYBR Premix Ex Taq™ II (TaKaRa, China), following the instructions of the manufacturer. The qRT-PCR reactions were performed using the forward primer (5′-GTGACAACACCACCAAGGAC-3′) and the reverse primer (5′-TGGAAACATAGTAGAAGGCTCGT-3′); and *T. aestivum* 18S rRNA (AJ272181) was used as an internal reference with the forward primer (5′-CGCGCAAATTACCCAATCCTG-3′) and the reverse primer (5′-GCCCAAGGTCCAACTACGAG-3′). The relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method [19]. Three independent experiments were performed and three technical replicates were analyzed for each sample.

2.6. Western blot analysis

The total soluble proteins from 0.5 g of leaves collected as described above for real-time PCR analysis were extracted according to a previous study [20]. The protein content was determined through the Bradford assay [21] using bovine serum albumin (BSA, 1 mg/mL) as the standard, and proteins (40 μg) were visualized by 12% (m/v) SDS-PAGE. The total soluble proteins were detected by Coomassie brilliant blue (CBB) staining and Western blot according to Omar's protocol [22]. The anti-TaDlea3 polyclonal antibody was prepared by GenScript Biotechnology, and alkaline phosphate-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA) was used as the secondary antibody. Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyphosphate (BCIP) were used for detection according to the instructions of the manufacturer. The TaDlea3 protein antibody and the secondary antibody were used at a dilution of 1:1000. Densitometric analyses of the amount of accumulated TaDlea3 protein on the immunoblots were performed using *Quantity One V 4.6.2* software (*Bio-Rad*).

2.7. Subcellular localization analysis

The TaDlea3 ORF sequence was amplified using the forward primer (5′-GCCTCGAGATGGCCTCCAACCAGAACCAG3′, Xho I site underlined), and the reverse primer (5′-CAACTAGTGAACCTCCGCCTCCGCCGTGATTCCTGGTGGTGGTG3′, Spe I site underlined). The PCR products from a double restriction-enzyme digestion were inserted into the PA7 vector containing a green-fluorescent protein (GFP) fusion at the C-terminus under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

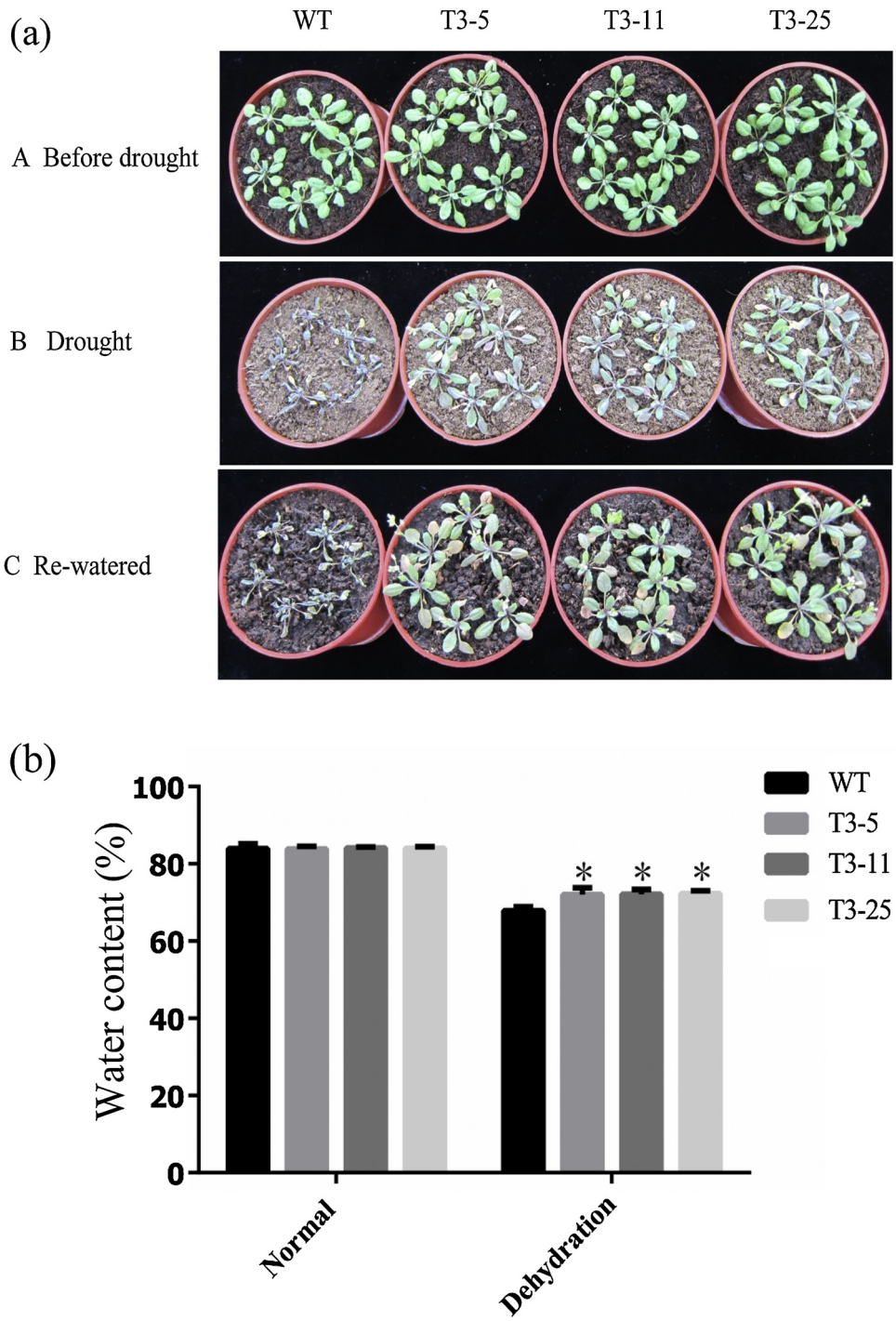


Fig. 6. (a) Drought-treatment assay of wild type and transgenic *Arabidopsis* plants. (A) One month old seedlings of the wild type and transgenic plants under normal condition. (B) were treated with drought stress (without irrigation) for 10 days. (C) then irrigated with water. 1, wild type; 2, transgenic line T3-5; 3, transgenic line T3-11; 4, transgenic line T3-25. (b) Changes in water content (WC) in the wild type and transgenic plants under normal condition and drought stress treatment. Each column represents the mean ± standard error of three biological repeats. Statistical significance of differences between the wild type and transgenic lines were determined by one way ANOVA with Duncan's test (*$p < 0.05$).

The recombinant construct was confirmed by sequencing. The recombinant plasmid was subjected to restriction digestion using *Xho* I and *Xba* I, ligated into the PA70390 expression vector, and transformed into *Agrobacterium tumefaciens* strain GV3101. The bacterial cells were harvested by centrifugation at 5500 × *g* for 10 min at 4 °C, and re-suspended in a similar volume of infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6, and 150 μM acetosyringone). The strains were incubated at 28 °C for 3 h in a dark room and diluted to obtain an OD_{600} of 1.00. The strains were then transformed into *Nicotiana benthamiana* leaf epidermal cells and cultivated for 3 days in the dark. Protoplasts were prepared after infiltration by cutting leaf discs into small pieces and incubating for 10 h in an enzyme solution (0.2 M MES, 0.8 M mannitol, 2 M

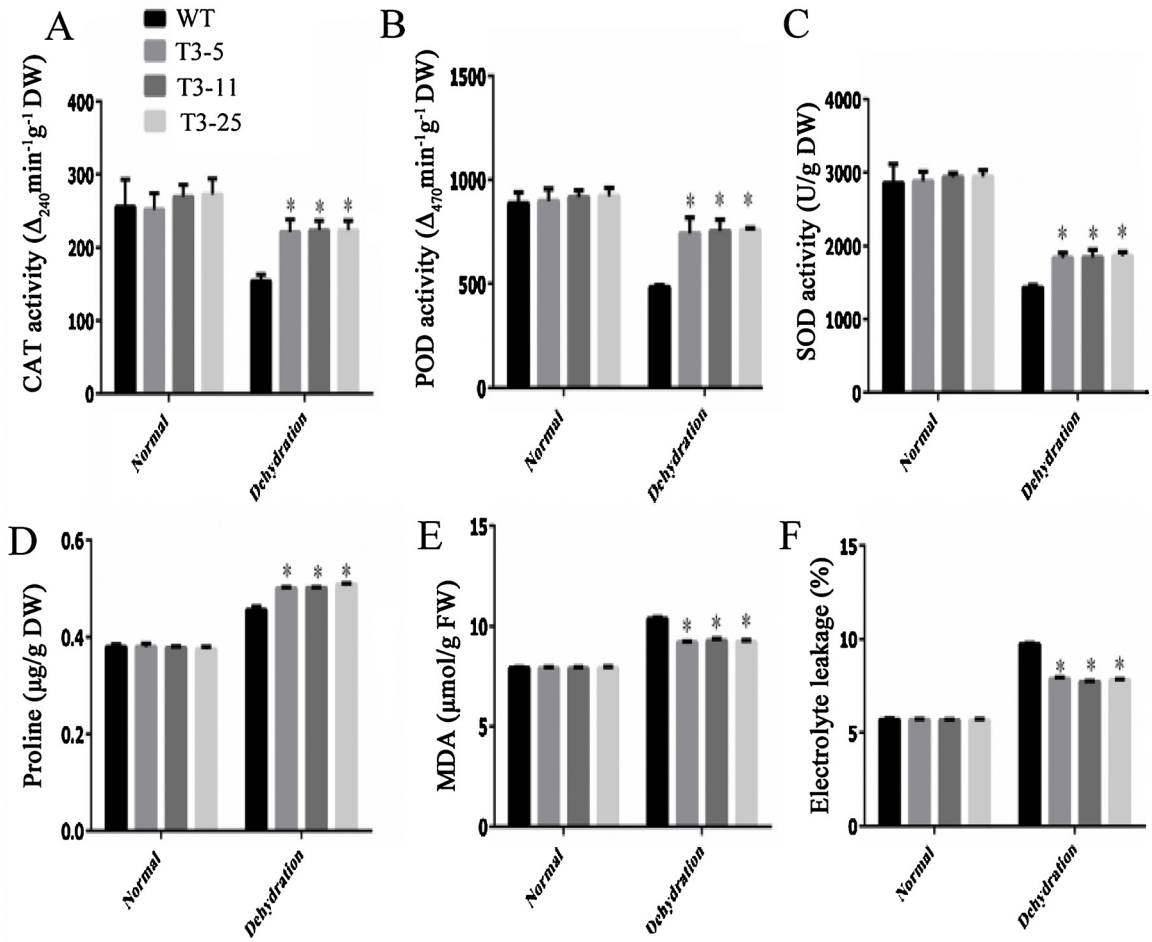


Fig. 7. The total activities of the antioxidant enzymes and physiological changes in the wild type and transgenic plants under normal condition and drought stress treatment. (A) catalase, CAT; (B) guaiacol peroxidase, POD; (C) superoxidase dismutase, SOD; (D) Free proline content; (E) Malondialdehyde content MDA; (F) Relative electrolyte leakage. Each column represents the mean ± standard error of three biological repeats. Statistical significance of differences between the wild type and transgenic lines were determined by one way ANOVA with Duncan's test (*$p < 0.05$).

KCl, 1 M $CaCl_2$, 10% BSA, and 1.5% (wt/vol) cellulase R10, and 0.4% (wt/vol) macerozyme R10, (pH 5.7)) [23,24].

2.8. Construction of transgene vectors

The TaDlea3 ORF was amplified using the TF-NcoI primer: (5′-CATGCCATGGCCTCCAACCAGAACCAGG-3′) and the TR-BstEII primer (5′-GCGGTCACCCTAGTGATTCCTGGTGGTGG-3′) and cloned into the NcoI/BstEII sites of pCAMBIA-3301 under the control of the CaMV 35S promoter. The recombinant construct was confirmed by sequencing. The recombinant plasmid was then introduced into *A. tumefaciens* strain GV3101. The transformation of *Arabidopsis* (Col-0) was performed using the floral dip method [25]. Three independent T_3 transgenic *Arabidopsis* lines were used in subsequent experiments.

2.9. Drought stress treatment of transgenic Arabidopsis plants

Control and transgenic *Arabidopsis* seeds were grown in a commercial soil and routinely maintained for 3 days in the dark at 4 °C to break dormancy. These seeds were then transferred to a growth chamber under day/night (16 h/8 h) cycles at 22 °C, with an irradiance of 100 μmol/m^2/s and 65% relative humidity. One-month-old transgenic and control lines treated with drought stress were collected to measure the activities of antioxidant enzymes, including superoxide dismutase (SOD) (EC1.15.1.1), peroxidase (POD) (EC 1.11.1.7), and catalase (CAT) (EC 1.11.1.6) as described previously [26,27]. Enzyme activity assays were performed using a UV–vis spectrophotometer (UV-2550, Shimadzu, Japan) at 25 °C. Water content (WC) was measured using the following formula: WC = (fresh weight − dry weight)/fresh weight [28]. Relative electrolyte leakage (EL) and malondialdehyde (MDA) were determined as previously described [29], and the proline content was determined following the method suggested by Zhang et al. [30].

2.10. Statistical analysis

All the data were processed by the analysis of variance (ANOVA) using SPSS (SPSS Inc., Chicago, IL USA). Significant differences were tested by the Duncan test at a 0.05 probability level and marked with different letters and asterisks in the figures.

3. Results

3.1. Isolation and sequence analysis of TaDlea3

We used the gene sequence of *Agropyron mongolicum lea*3 (GenBank Accession Number: FJ025815.1) as a probe for searching the EST library of wheat in the NCBI database. The obtained 50 wheat ESTs with high similarity were analyzed and assembled into a full-length ORF sequence. The predicted gene sequence was used to design PCR primers for the amplification of *TaDlea3* from wheat

cultivar Shaanhe 6 (GenBank Accession Number: KP233216). The *TaDlea3* ORF is 492 bp and encodes a 163- amino- acid protein with a predicted molecular mass of 16.9 kDa and a pI of 6.62. Motif analysis using BLASTP (NCBI) revealed that the TaDlea3 protein contained eight 11-mer amino acid motif of group 3 LEA proteins (hydrophilic and glycine-rich proteins) (Fig. 1A), and the TaDlea3 protein was classified as group 3 A (D-7 family) according to Battaglia's classification [31]. BLASTP search indicated that the putative TaDlea3 protein cluster showed significant similarity to LEA proteins in other plants and was most closely related to those from *H. vulgare* and *Agropyron cristatum* (Fig. 1B). The GRAVY value of TaDlea3 was −1.026, suggesting that the TaDlea3 protein is a hydrophilic LEA protein (Fig. 1C). Therefore, our analysis indicated that *TaDlea3* is a novel gene belonging to the group 3 LEA family of wheat proteins.

3.2. Subcellular localization of TaDlea3

To determine the subcellular localization of TaDlea3, the 35S:GFP and 35S:TaDlea3-GFP fusion proteins were expressed in *N. benthamiana* leaf epidermal cells by infiltration with *A. tumefaciens* strain GV3101 harboring the recombinant constructs. Furthermore, we delivered the chimeric constructs into *N. benthamiana* mesophyll protoplasts [23]. The expressed fusion proteins were examined with a Nikon confocal laser-scanning microscope. As shown in Fig. 2, the TaDlea3-GFP fusion protein specifically accumulated in the cytoplasm. This result was consistent with a previous report that LEA proteins are primarily localized to the cytosol and nucleus [32].

3.3. Accumulation of TaDlea3 transcript during four different developmental stages

Previous studies have shown that LEA gene expression is induced by drought stress [33]. To determine gene expression, we performed quantitative RT-PCR to assess the *TaDlea3*-expression patterns under drought-stress conditions. Measurements were obtained from the leaves of wheat seedlings exposed to different levels of water stress (75%, 50%, and 35% FC) that allowed to recover for 1, 2 and 3 days (R1d-50%, R1d-35%, R2d-50%, R2d-35%, R3d-50% and R3d-35%) during four different developmental stages (seedling, tillering, jointing, and flowering) (Fig. 3). In the seedling stage, the *TaDlea3* transcript levels were up-regulated at 50% and 35% FC, as compared with those obtained in 75% FC (control); at 35% FC, the transcript expression reached a peak. After 3 days of recovery, the *TaDlea3* transcript levels declined at 50% and 35% FC but were slightly higher in 75% FC. The *TaDlea3* transcript levels were consistent in all the four developmental stages. *TaDlea3* transcript levels were increased at 50% and 35% FC but were declined after 3 days of recovery. The transcript expression of *TaDlea3* in the flowering stage cannot reach the same level as in the three other stages. Our findings indicated that *TaDlea3* expression was highly induced by drought treatment at all four developmental stages.

3.4. Accumulation of TaDlea3 protein following drought treatment

To investigate how the TaDlea3 protein is expressed in response to drought stress, we analyzed protein synthesis during four different developmental stages using Western blotting analysis (Fig. 4A) and densitometry (Fig. 4B). In the four stages, substantial TaDlea3 was immunodetected in 50% and 35% FC samples. The strongest signals were observed at 35% FC and the weakest signals were detected at 75% FC and after 3 days of recovery. Drought stress increased the band intensity, and the signal considerably diminished after recovery. Such trend was consistent throughout the four developmental stages; however, during the flowering stage, TaDlea3 expression after 3 days of recovery was relatively weaker than that in the three other stages. This pattern was in agreement with the mRNA expression profile. These results indicated that TaDlea3 protein and *TaDlea3* transcript responded similarly among different developmental stages to drought stress.

3.5. Overexpression of TaDlea3 enhances drought tolerance in transgenic Arabidopsis thaliana plants

To analyze the roles of *TaDlea3* in plants, *TaDlea3* was inserted into a pCAMBIA 3301 vector (Fig. 5A) and was overexpressed in *Arabidopsis* under the control of a constitutive CaMV 35S promoter. Transformants were selected according to phosphinothricin-resistance, and *TaDlea3* expression in transgenic *Arabidopsis* was examined by semi-quantitative RT-PCR. A single specific band was observed in each transgenic line, while no band was observed in the wild type control (Fig. 5B). Furthermore, the transgenic *Arabidopsis* at the protein level was confirmed by Western blot analysis (Fig. 5C). The specific band was obtained from transgenic lines, whereas no signal was detected in the wild-type plants. To demonstrate whether *TaDlea3* is involved in tolerance to drought stress, one-month-old transgenic *Arabidopsis* and wild-type plants were treated with drought stress. No clear phenotype difference was observed between the transgenic and wild-type plants under normal conditions. However, the wild-type plants showed extreme wilting 10 days after water drought stress treatment, whereas the transgenic lines remained turgid (Fig. 6A). Under the control conditions, the WCs of the wild-type and transgenic plants were 83.89% and 84.09%, indicating no significant difference. Drought stress caused decreasing WCs in all plants, while transgenic plants displayed significantly higher WCs than the wild-type. Specifically, wild-type and transgenic plants leaves were 67.9% and 72.06%–72.29% with 10 days after water withholding (Fig. 6B). Therefore, *TaDlea3* overexpression improved the tolerance capability of *Arabidopsis* to drought stress.

3.6. Overexpression of TaDlea3 increased antioxidant enzyme activity during drought stress

To explore the mechanism through which *TaDlea3* overexpression improves the capability to survive in drought stress, we measured the activity of several antioxidant enzymes and drought-resistance indicators (including MDA, relative EL, and proline levels) in wild-type and transgenic plants under drought stress conditions at the seedling stage. Under normal conditions, MDA levels and relative EL in transgenic plants showed no difference with those in wild-type plants. By contrast, under drought-stress conditions, MDA levels and EL were significantly higher in wild-type plants than in transgenic plants (Fig. 7E, F). With or without drought stress treatment, the contents of proline in *TaDlea3*-overexpressing plants showed no difference or increased levels compared with the control wide-type plants (Fig. 7D).

We also detected how antioxidant enzyme activity changes in plants under drought stress. The results showed that, under drought treatment, SOD, POD, and CAT activities were increased in transgenic plants compared with those in wild-type plants (Fig. 7A–C); however, no significant difference was observed between the wild-type and transgenic plants under control conditions. These results suggested that increased antioxidant enzyme activity might play an important role in the resistance capability of *TaDlea3* overexpressing plants against drought stress.

4. Discussion

Drought stress is one of the most important factors influencing plant growth, development and productivity [34]. In this study, we focused on LEA proteins, which are known to play a protective role in the dehydration process induced during either different developmental stages and by different environmental factors, including drought, cold and salinity [18]. LEA proteins are characterized by high hydrophilicity, high glycine content, and a lack of secondary structure; these proteins are involved in the membrane maintenance or served as molecular chaperones to protect cells against cellular dehydration damage [35]. The transcriptional regulation of LEA genes in response to abiotic stress has been reported in different plant species, including *T. aestivum* L. [36], *Vitis vinifera* L. [37], *Solanum lycopersicum* L. [38], and *O. sativa* [39]. In this study, we cloned a new LEA3 protein from winter wheat Shaanhe 6 that is located in the cytosol, which was consistent with the previously described LEA protein localization [32]. During the four developmental stages, TaDlea3 protein expression levels were similar to the *TaDlea3* transcript levels. We observed similar trends involving strong *TaDlea3* expression and increased TaDlea3 accumulation in 50% and 35% FC, reaching a peak in 35% FC. Meanwhile, the expression level decreased after rehydration. This finding was in agreement with the previous studies [40,41]. The concentration of dehydrin proteins in two varieties of winter wheat gradually increased, reaching a maximum accumulation at 20% FC. This increase was accompanied by water loss; however, after rehydration, the concentration of dehydrin proteins decreased [42].

Drought stress treatment of wheat seedlings in the seedling and tillering stages did not affect their productivity; drought at the jointing stage led to small wheat spikes and drought at the flowering stage caused wheat seeds to be shriveled. These observations agreed with the observations that drought stress has influence on grain yield, particularly during the wheat-generative phases. We observed lower levels of *TaDlea3*-gene expression in the flowering period along with weaker protein signals after rehydration than that observed at the three other growth periods. This observation indicated that nutrition was transported to the grain and leaves over time. The trend of TaDlea3 protein accumulation was also consistent with the *TaDlea3* transcription levels during all the developmental stages in the presence of drought stress treatment. Thus, the *TaDlea3* transcripts and proteins could serve as diagnostic markers of the drought response and potentially aid the marker-assisted selection of drought tolerance in wheat.

Several members of group 3 LEA proteins play significant roles in improving resistance to abiotic stresses. Overexpression of barley *HVA1* gene in transgenic mulberry plant improves abiotic stress tolerance [43], and wheat *TaLEA3* enhances drought tolerance in transgenic *Leymus chinensis* plants [44]. In the present study, we generated *Arabidopsis* transgenic plants overexpressing the *TaDlea3* gene to investigate the function of *TaDlea3* associated with drought stress tolerance. The transgenic lines exhibited the same phenotypes as wild-type variants for the majority of the morphological traits. MDA is the product of lipid peroxidation, and MDA levels are commonly used as indicators of lipid peroxidation [45]. EL through cell membranes is an indicator of membrane damage [46]. In the present study, MDA content and relative EL were lower in transgenic plants than in wide-type plants under drought stress, suggesting that the damage to the cell membrane was decreased in transgenic plants. Proline is a good indicator of stress-induced cell damage [47]. In the present study, the proline content in transgenic lines was high in wild-type plants under drought conditions, indicating that the TaDlea3 protein effectively protected the cell membrane under drought stress. Drought stress causes plants to express numerous scavenging enzymes, such as SOD, POD, and CAT, for the purpose of protecting plant cells from oxidative damage. The activities of these antioxidant enzymes in the present study were higher in transgenic *Arabidopsis* plants than in wild-type plants, suggesting that enhanced antioxidant enzyme activity was involved in the promotion increased drought tolerance. Our analysis showed that TaDlea3 plays an important role in the response to drought stress. Therefore, TaDlea3 protein could maintain cellular WC under environmental stress and might also function as a molecule protector under water limitation.

In conclusion, a novel wheat group 3 LEA gene, *TaDlea3*, was identified and characterized. The results demonstrated that *TaDlea3* expression was induced by drought stress, and TaDlea3 protein accumulation also increased under drought stress in all developmental stages. Transgenic plants overexpressing *TaDlea3* gene enhanced the resistance to drought stress. However, further studies are necessary to elucidate the precise mechanism associated with *TaDlea3*-mediated protection against drought stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2016.08.010.

References

[1] D. Golldack, I. Luking, O. Yang, Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network, Plant Cell Rep. 30 (2011) 1383–1391.
[2] W. Rong, L. Qi, A. Wang, X. Ye, L. Du, H. Liang, Z. Xin, Z. Zhang, The ERF transcription factor TaERF3 promotes tolerance to salt and drought stresses in wheat, Plant Biotechnol. J. 12 (2014) 468–479.
[3] N. Bies-Etheve, P. Gaubier-Comella, A. Debures, E. Lasserre, E. Jobet, M. Raynal, R. Cooke, M. Delseny, Inventory, evolution and expression profiling diversity of the LEA (late embryogenesis abundant) protein gene family in *Arabidopsis thaliana*, Plant Mol. Biol. 67 (2008) 107–124.
[4] M. Dalal, G.S. Kumar, K. Mayandi, Identification and expression analysis of group 3 LEA family genes in sorghum [*Sorghum bicolor* (L.) Moench], Acta Physiol. Plant. 35 (2013) 979–984.
[5] Y. Liu, Y.Z. Zheng, PM2, a group 3 LEA protein from soybean, and its 22-mer repeating region confer salt tolerance in *Escherichia coli*, Biochem. Biophys. Res. Commun. 331 (2005) 325–332.
[6] S.C. Hand, M.A. Menze, M. Toner, L. Boswell, D. Moore, LEA proteins during water stress: not just for plants anymore, Annu. Rev. Physiol. 73 (2011) 115–134.
[7] D.K. Hincha, A. Thalhammer, LEA proteins: IDPs with versatile functions in cellular dehydration tolerance, Biochem. Soc. Trans. 40 (2012) 1000–1003.
[8] T. Furuki, M. Sakurai, group 3 LEA protein model peptides protect liposomes during desiccation, Biochim. Biophys. Acta 1838 (2014) 2757–2766.
[9] E.L. Arumingtyas, E.S. Savitri, R.D. Purwoningrahayu, Protein profiles and dehydrin accumulation in some soybean varieties (*Glycine max* L. merr) in drought stress conditions, Am. J. Plant Sci. 04 (2013) 134–141.
[10] Y. Wu, C. Liu, J. Kuang, Q. Ge, Y. Zhang, Z. Wang, Overexpression of *SmLEA* enhances salt and drought tolerance in *Escherichia coli* and *Salvia miltiorrhiza*, Protoplasma 251 (2014) 1191–1199.
[11] Y. Liu, Y.Z. Zheng, Y.Q. Zhang, W.M. Wang, R.H. Li, Soybean PM2 protein (LEA3) confers the tolerance of *Escherichia coli* and stabilization of enzyme activity under diverse stresses, Curr. Microbiol. 60 (2010) 373–378.
[12] J.L. Duan, W.M. Cai, *OsLEA3-2*, an abiotic stress induced gene of rice plays a key role in salt and drought tolerance, PLoS One 7 (2012).
[13] S. Ming-Der, H. Tzung-Yang, L. Tsai-Piao, Y.I.C. Hsing, F.A. Hoekstra, Characterization of two soybean (*Glycine max* L.) LEA IV proteins by circular dichroism and Fourier transform infrared spectrometry, Plant Cell Physiol. 51 (2010) 395–407.
[14] X. Zhang, S. Lu, C. Jiang, Y. Wang, B. Lv, J. Shen, F. Ming, *RcLEA*, a late embryogenesis abundant protein gene isolated from *Rosa chinensis*, confers

tolerance to *Escherichia coli* and *Arabidopsis thaliana* and stabilizes enzyme activity under diverse stresses, Plant Mol. Biol. 85 (2014) 333–347.
[15] W. Yang, L. Zhang, H. Lv, H. Li, Y. Zhang, Y. Xu, J. Yu, The K-segments of wheat dehydrin WZY2 are essential for its protective functions under temperature stress, Front. Plant Sci. 6 (2015) 406.
[16] D.H. Min, X.H. Zhang, Z.S. Xu, Y. Zhao, Y. Chen, L.C. Li, M. Chen, Y.Z. Ma, Induction kinetics of a novel stress-related *LEA* gene in wheat, Plant Mol. Biol. Rep. 30 (2012) 1313–1321.
[17] I.I. Vaseva, B.S. Grigorova, L.P. Simova Stoilova, K.N. Demirevska, U. Feller, Abscisic acid and late embryogenesis abundant protein profile changes in winter wheat under progressive drought stress, Plant Biol. 12 (2010) 698–707.
[18] A. Chiappetta, A. Muto, L. Bruno, M. Woloszynska, M. Van Lijsebettens, M.B. Bitonti, A dehydrin gene isolated from feral olive enhances drought tolerance in *Arabidopsis* transgenic plants, Front. Plant Sci. 6 (2015) 392.
[19] 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.
[20] M. Zhang, D. Lv, P. Ge, Y. Bian, G. Chen, G. Zhu, X. Li, Y. Yan, Phosphoproteome analysis reveals new drought response and defense mechanisms of seedling leaves in bread wheat (*Triticum aestivum* L.), J. Proteomics 109 (2014) 290–308.
[21] 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.
[22] S.A. Omar, N.I. Elsheery, H.M. Kalaji, M.K. Ebrahim, S. Pietkiewicz, C.H. Lee, S.I. Allakhverdiev, Z.F. Xu, Identification and differential expression of two dehydrin cDNAs during maturation of *Jatropha curcas* seeds, Biochemistry 78 (2013) 485–495.
[23] 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.
[24] I.E. Hernandez-Sanchez, I. Maruri-Lopez, A. Ferrando, J. Carbonell, S.P. Graether, J.F. Jimenez-Bremont, Nuclear localization of the dehydrin OpsDHN1 is determined by histidine-rich motif, Front. Plant Sci. 6 (2015) 702.
[25] X.R. 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.
[26] 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.
[27] Y.H. Cheong, G.K. Pandey, J.J. Grant, O. Batistic, L. Li, B.G. Kim, S.C. Lee, J. Kudla, S. Luan, Two calcineurin B-like calcium sensors interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis, Plant J. 52 (2007) 223–239.
[28] S.C. Park, Y.H. Kim, J.C. Jeong, C.Y. Kim, H.S. Lee, J.W. Bang, S.S. Kwak, Sweetpotato late embryogenesis abundant 14 (*IbLEA14*) gene influences lignification and increases osmotic- and salt stress-tolerance of transgenic calli, Planta 233 (2011) 621–634.
[29] Z. Li, Y. Peng, X. Ma, Different response on drought tolerance and post-drought recovery between the small-leafed and the large-leafed white clover (*Trifolium repens* L.) associated with antioxidative enzyme protection and lignin metabolism, Acta Physiol. Plant. 35 (2012) 213–222.
[30] G. Zhang, M. Chen, L. Li, Z. Xu, X. Chen, J. Guo, Y. Ma, Overexpression of the soybean *GmERF3* gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco, J. Exp. Bot. 60 (2009) 3781–3796.
[31] M. Battaglia, Y. Olvera-Carrillo, A. Garciarrubio, F. Campos, A.A. Covarrubias, The enigmatic LEA proteins and other hydrophilins, Plant Physiol. 148 (2008) 6–24.
[32] A. Candat, G. Paszkiewicz, M. Neveu, R. Gautier, D.C. Logan, M.H. Avelange-Macherel, D. Macherel, The ubiquitous distribution of late embryogenesis abundant proteins across cell compartments in *Arabidopsis* offers tailored protection against abiotic stress, Plant Cell 26 (2014) 3148–3166.
[33] Z.J. Feng, X.Y. Cui, M. Chen, G.X. Yang, Y.Z. Ma, G.Y. He, Z.S. Xu, The soybean GmDi19-5 interacts with GmLEA3.1 and increases sensitivity of transgenic plants to abiotic stresses, Front. Plant Sci. 6 (2015) 179.
[34] S.J. Oh, Y.S. Kim, C.W. Kwon, H.K. Park, J.S. Jeong, J.K. Kim, Overexpression of the transcription factor *AP37* in rice improves grain yield under drought conditions, Plant Physiol. 150 (2009) 1368–1379.
[35] K. Goyal, L.J. Walton, A. Tunnacliffe, LEA proteins prevent protein aggregation due to water stress, Biochem. J 388 (2005) 151–157.
[36] W. Zhu, L. Zhang, H. Lv, H. Zhang, D. Zhang, X. Wang, J. Chen, The dehydrin *wzy2* promoter from wheat defines its contribution to stress tolerance, Funct. Integr. Genomic. 14 (2014) 111–125.
[37] Y.Z. Yang, M.Y. He, Z.G. Zhu, S.X. Li, Y. Xu, C.H. Zhang, S.D. Singer, Y.J. Wang, Identification of the dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress, BMC Plant Biol. 12 (2012).
[38] J. Weiss, M. Egea-Cortines, Transcriptomic analysis of cold response in tomato fruits identifies dehydrin as a marker of cold stress, J. Appl. Genet. 50 (2009) 311–319.
[39] S.C. Lee, M.Y. Lee, S.J. Kim, S.H. Jun, G. An, S.R. Kim, Characterization of an abiotic stress-inducible dehydrin gene, *OsDhn*1, in rice (*Oryza sativa* L.), Mol. Cells 19 (2005) 212–218.
[40] B.E. Layton, M.B. Boyd, M.S. Tripepi, B.M. Bitonti, M.N.R. Dollahon, R.A. Balsamo, Dehydration-Induced expression of a 31-kDa dehydrin in *Polypodium polypodioides* (Polypodiaceae) may enable large, reversible deformation of cell walls, Am. J. Bot. 97 (2010) 535–544.
[41] J. Dobra, R. Vankova, M. Havlova, A.J. Burman, J. Libus, H. Storchova, Tobacco leaves and roots differ in the expression of proline metabolism-related genes in the course of drought stress and subsequent recovery, J. Plant Physiol. 168 (2011) 1588–1597.
[42] H.M. Zhang, L.S. Zhang, L. Liu, W.N. Zhu, W.B. Yang, Changes of dehydrin profiles induced by drought in winter wheat at different developmental stages, Biol. Plantarum 57 (2013) 797–800.
[43] V.G. Checker, A.K. Chhibbar, P. Khurana, Stress-inducible expression of barley *Hva1* gene in transgenic mulberry displays enhanced tolerance against drought, salinity and cold stress, Transgenic Res. 21 (2012) 939–957.
[44] L.J. Wang, X.F. Li, S.Y. Chen, G.S. Liu, Enhanced drought tolerance in transgenic *Leymus chinensis* plants with constitutively expressed wheat $TaLEA_3$, Biotechnol. Lett. 31 (2009) 313–319.
[45] S. Liping, L. Yang, K. Xiangpei, Z. Dan, P. Jiaowen, Z. Yan, W. Li, L. Dequan, Y. Xinghong, *ZmHSP16.9*, a cytosolic class I small heat shock protein in maize (*Zea mays*), confers heat tolerance in transgenic tobacco, Plant Cell Rep. 31 (2012) 1473–1484.
[46] Y. Liu, L. Wang, S. Jiang, J. Pan, G. Cai, D. Li, Group 5 LEA protein, *ZmLEA5C*, enhance tolerance to osmotic and low temperature stresses in transgenic tobacco and yeast, Plant Physiol. Biochem. 84 (2014) 22–31.
[47] P. Zhang, P. Yang, Z. Zhang, B. Han, W. Wang, Y. Wang, Y. Cao, T. Hu, Isolation and characterization of a buffalograss (*Buchloe dactyloides*) dehydration responsive element binding transcription factor, *BdDREB2*, Gene 536 (2014) 123–128.