Research paper

Identification of wheat *DREB* genes and functional characterization of *TaDREB3* in response to abiotic stresses

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ABSTRACT

As an important transcription factor family, DREB transcription factors play important roles in response to abiotic stresses. In this study, we identified wheat *DREB* genes at genome-level, and characterized the functions of *TaDREB* genes. Totally, there are 210 *TaDREB* genes, which can be divided into 6 subgroups. Some of these genes display tissue-specific expression patterns. Among them, the expression of three *TaDREB3* homoeologous genes is induced by abiotic stresses. Meanwhile, as alternatively spliced genes, they generate three isoforms respectively. Transcripts I and II encode DREB proteins, while transcript III does not generate DREB proteins. Transgenic Arabidopsis over-expressing *TaDREB3-AI* displayed enhanced resistance to drought, salt and heat stresses. The physical indexes and the expression of stress-related genes further verified the functions in response to abiotic stresses. Our results lay a foundation for further study of wheat *DREB* genes. Especially, our findings indicate that *TaDREB3* genes can be used for crop genetic improvement.

1. Introduction

Abiotic stresses, including heat, cold, salt and drought, have bad effects on plants growth and development. As sessile lives, plants adapt to these adverse environmental conditions at the molecular, cellular, physiological and biochemical level. At transcription level, transcription factors (TFs), such as DREB, WRKY and MYB, play key roles in these processes.

As plant specific TFs, DREB (dehydration-responsive-element-binding) proteins contain a single conserved AP2 domain and specifically bind to *cis*-element DRE (with a core sequence A/GCCGAC). So far, DREB TFs have been identified in many plants, such as *Arabidopsis thaliana* (Stockinger et al., 1997), soybean (*Glycine* max) (Mizoi et al., 2013), rice (*Oryza sativa*) (Dubouzet et al., 2003), maize (*Zea mays*) (Qin et al., 2010), barley (*Hordeum vulgare*) (Xue, 2002), and so on.

*DREB*s were divided into six subgroups (A1-A6) (Sakuma et al., 2002) and members in different subgroups participate in response to different abiotic stresses. For example, in subgroup A1, *DREB1A/CBF*s in Arabidopsis, rice and maize respond to cold stress (Dubouzet et al., 2003; Kasuga et al., 1999; Qin et al., 2004). In subgroup A2, *DREB2*

genes including *DREB2A* and *DREB2B* in Arabidopsis, rice, Mung bean (*Vigna radiate*), and tea plant (*Camellia sinensis*) participate in responses to drought and high-salt stresses (Chen et al., 2016; Dubouzet et al., 2003; Nakashima et al., 2000; Sakuma et al., 2006b; Wang et al., 2017). Additionally, *ABI4* (a member of subgroup A3) associates with ABA and sugar signals (Niu et al., 2002); *TINY* in subgroup A4 plays roles in drought, cold, ethylene, and methyl jasmonate signals (Sun et al., 2008); *RAP2.1* in subgroup A5 takes part in drought and freezing tolerance (Dong and Liu, 2010a); *RAP2.4* in subgroup A6 is engaged in drought and salt stress responses (Lin et al., 2008).

In crops, many DREB TFs have been identified to improve plants abiotic stress tolerance. For example, over-expressing *OsDREB1A*, *OsDREB1B*, *OsDREB1E*, *OsDREB1F*, *OsDREB1G*, *OsDREB2A* and *OsDREB2B* enhanced tolerance to drought and/or high salt (Chen et al., 2008; Dubouzet et al., 2003; Mallikarjuna et al., 2011; Matsukura et al., 2010; Wang et al., 2008b). Meanwhile, *OsDREB1A*, *OsDREB1B* and *OsDREB1F* regulate the freezing stress tolerance (Dubouzet et al., 2003; Ito et al., 2006; Wang et al., 2008b). Additionally, *OsDREB2B* positively regulates the tolerance to heat stresses (Matsukura et al., 2010).

Although wheat is one main world-wide cereal crop, but only a few

wheat *DREB*s have been functionally characterized presently. Over-expression of *WCBF2* and *WDREB2* improves tolerance to cold stress (Sazegari and Niazi, 2012; Takumi et al., 2008), and over-expression of *TaAIDF* enhance the tolerance to drought and osmotic stresses (Xu et al., 2008). Oppositely, *TaRAP2.1L* negatively regulate plants tolerance to cold and dehydration stresses (Amalraj et al., 2016). Among them, a *DREB2* homolog in wheat displayed alternative splicing which accumulated differently under abiotic stresses (Egawa et al., 2006). However, the function of different isoforms have not been reported yet.

To identify more wheat *DREB* genes which respond to abiotic stresses, we identified 210 wheat *DREB* genes and characterized the functions of *TaDREB3* in abiotic stress responses. This study laid a foundation for further study of *DREB*s and genetic improvement of crops.

2. Materials and methods

2.1. Sequence retrieval and bioinformatics analyses

Genome DNA, coding sequence (CDS) and protein sequences of *Triticum aestivum* (bread wheat variety *Chinese Spring*) (assembly iwgsc_refseqv1.0) were obtained from http://www.gramene.org/ and http://plants.ensembl.org/index.html. The Hidden Markov Model (HMM) of AP2 superfamily (PF00847) was acquired from Pfam database (v30.0) and set as the query in hmmsearch against wheat proteins using HMMER v3.0 (with a cut-off expected value, E-value, of 10^{-5}). SMART sequence analysis (Ivica and Peer, 2018) was conducted with a threshold of E-value $< 10^{-5}$ and proteins with only an AP2 domain was retained.

Chromosomal location was analyzed according to their annotations. Tandem duplication information was acquired from Monocots PLAZA 4.5 (https://bioinformatics.psb.ugent.be/plaza/versions/plaza v4 5 monocots/). Molecular weight (MW) and isoelectric point (pI) were predicted on http://expasy.org/tools/protparam.html. Multiple sequence alignment were conducted with MEGA6.0 (Tamura et al., 2013). An un-rooted Neighbor-Joining tree was built by MEGA6.0 (Tamura et al., 2013) with 1000 bootstrap replicates and visualized by Evolview (He et al., 2016). Gene structures were analyzed on the Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/). Conserved motifs were predicted on MEME v5.0.5 (http://meme-suite.org/tools/meme) with motif numbers set as 15 and motif width as 5–200. Gene structures and conserved motifs were visualized by Evolview (He et al., 2016).

2.2. Plant material and growth conditions

Arabidopsis (*Columbia-0*) and wheat were planted in growth chamber at 22 ˚C (light for 16 h) and 18 ˚C (dark for 8 h). Roots, stems, leaves and inflorescences of adult wheat during heading period were collected. 1-week-old wheat seedlings were treated with MS liquid medium containing 200mMNaCl, 20%PEG6000, and 25 μM ABA, respectively. Seedlings in MS liquid medium were put in 45 ˚C or 4 ˚C for 2 h to mimic heat or cold stresses. Seedlings under normal conditions were kept as control. Samples (leaves of heat, cold treatments and roots of NaCl, PEG and ABA treatments) were collected at 0 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h. Of them, 0 h seedlings serve as control. Samples were frozen in liquid nitrogen at once and stored at −80 ˚C. RNA extraction and reverse transcription were applied as previously (Niu et al., 2019).

2.3. Expression profile and alternative splicing patterns analyses

Expression levels of *DREB* genes in *Chinese Spring* were acquired from Wheat Expression Browser (http://www.wheat-expression.com/) and visualized by MeV 4.9.0. Two genes (including all copies on three chromosomes) in each subgroup was selected randomly for quantitative

PCR (q-PCR). Primer Premier 5.0 (Sing et al., 1998) was employed to design primers (primers used were listed in Table S2). Expression levels were detected by QuantStudio 7 Flex. Each experiment was repeated three times. Relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ analysis method (Livak and Schmittgen, 2001) with *TaACT* as internal reference gene (Paolacci et al., 2009).

cDNA of 2 h-treatment seedlings were used as template for *TaDREB*s expression analyses. For *TaDREB3*, cDNA of 0.5 h-, 1 h-, 4 h-, 8 h-, 12 h- and 24 h-treatment seedlings were used as template, TaA2DLPF and TaA2DLPR (both are in the 4th exon of *TaDREB3*) which generate a single product were chose for q-PCR, and TaDREB3PF (at the 5′-end of *TaDREB3* ORF) and TaDREB3OR2 (at the 5′-end of *TaDRERB3* 4th exon) which generate three products were chose for semi-quantitative PCR. The full length PCR products of *TaDREB3* using TaDREB3PF and TaDREB3OR (at the 3′-end of *TaDREB3* ORF) were ligated into cloning vector pMD18-T and sequenced. Multiple sequence alignment were applied to compare the differences between different copies and splicing variants.

2.4. Plasmid construction and Arabidopsis transformation

Full length sequences of two isoforms (*TaDREB3*-AI and *TaDREB3*-AII) which encode DREB proteins were cloned, using sequenced recombined plasmids with pMD18-T as template, and TaDREB3OEPF and TaDREB3OEPR as primers (Table S2). PCR program is as follows: 94 ˚C for 5 min, 40cycles (94 ˚C for 30 s, 58 ˚C for 30 s, 72 ˚C for 80 s), 72 ˚C for 10 min. Products were ligated into over-expression vector pCAMBIA1300 using recombination reactions and transformed into *Agrobacterium tumefaciens* GV3101. Target genes were then transformed into Arabidopsis using floral dip method (Clough and Bent, 2010). T3 generation plants of two independent lines in Arabidopsis were used for further analysis.

2.5. Abiotic stress treatments of transgenic Arabidopsis

5-week-old Arabidopsis were treated with different abiotic stresses. For drought treatment, plants were deprived of water for 15 d and then re-watered for 5 d. For salt stress, plants were irrigated with PNS nutrient solution containing 200 mM NaCl for 12 d. For heat stress, plants were treated at 45 ˚C for 1 d and recovered under normal conditions for 5 d. Each experiment (containing about 36 plants) was repeated three times and the survival rates were counted.

2.6. Physical indexes and stress-related gene expression analyses

Leaves were collected after 10 d (drought stress), 7 d (salt stress), and 10 h (heat stress) for physical indexes measurements, respectively. Diaminobenzidine (DAB) and NBT (nitrobluetetrazolium) were used to stain the H_2O_2 and $O_2^{\cdot -}$ in the leaves as described previously (Jin et al., 2014). Chlorophyll content, proline content, MDA content, relative electrolytic leakage and activities of antioxidant enzymes including POD, CAT and SOD were detected as described (Chiang et al., 2015; Guo et al., 2018; Xue et al., 2010). Relative transcription level of target genes was calculated according to Livak and Schmittgen (2001) Ct means were normalized with *GAPDH* (*At1g13440*) expression (Czechowski et al., 2005). The primers used were designed using Primer Premier 5.0 (Table S2).

2.7. Statistical analysis

The significance of all data was calculated by SPSS20 using Student's *t*-test (Inc, 2011).
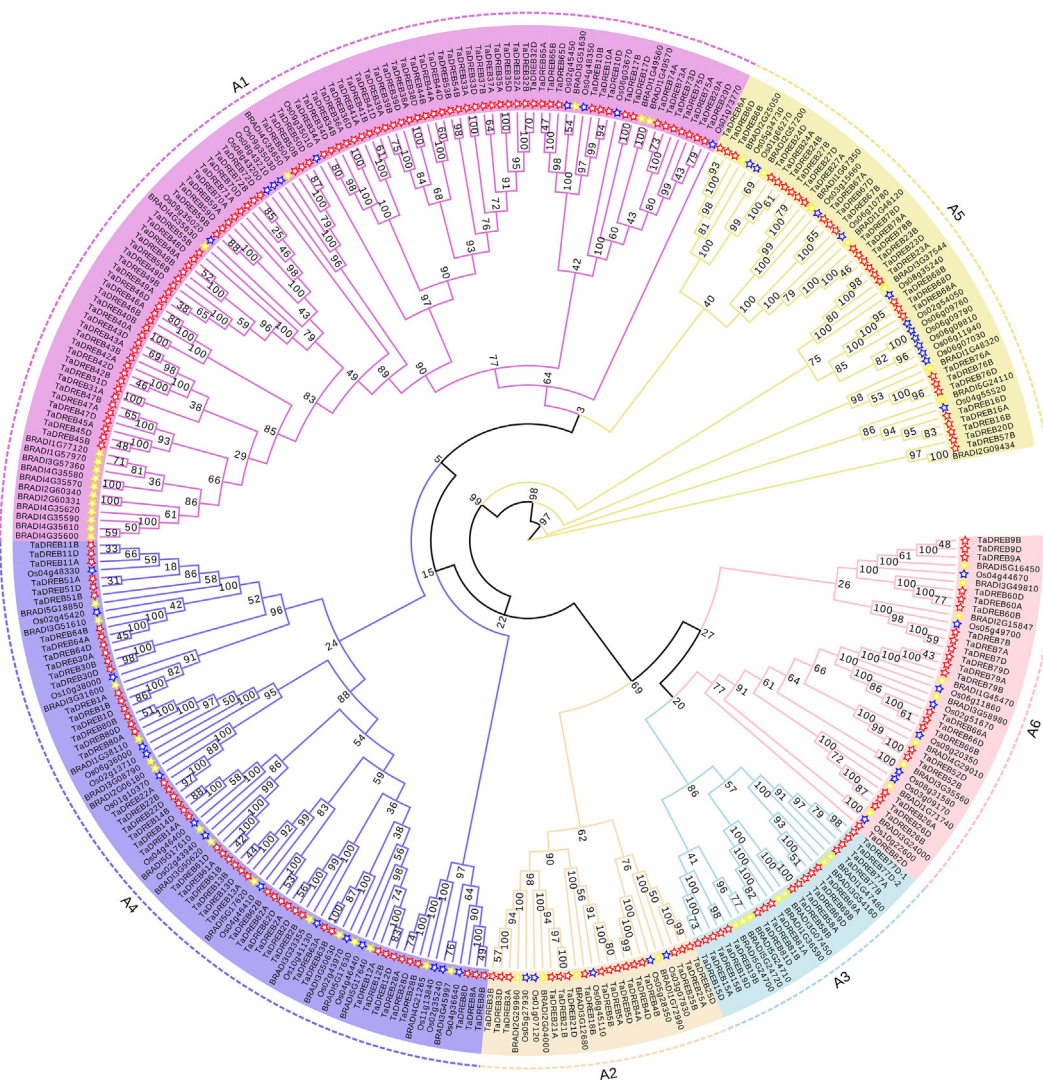
Fig. 1. Phylogenic analyses of *DREB*s in wheat, rice and *Brachypodium distachyon*. Genes in these three species were marked with stars in red, blue and yellow, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Identification of TaDREBs

A total of 210 *TaDREB* genes were identified (Table S1), and they distribute unevenly on 21 chromosomes (Fig. S1). Among them, 24 sets (108 *TaDREB*s) were tandem duplicated genes (Fig. S1). They were designated as *TaDREB1A* to *TaDREB82D* according to their chromosome location and genomic homology. Each protein contains a conserved AP2 domain (Fig. S2). The protein length is between 150 and 1422 amino acids and the molecular weight ranges from 16.5 kDa to 158.0 kDa with an average of 28.15 kDa. The GRAVY of most proteins is negative, indicating that most of them are hydrophilic. The isoelectric points varies from 4.39 to 11.90 and the mean is 6.43.

To investigate the evolutionary relationships of *TaDREB*s, a phylogenic tree of *DREB*s from wheat, rice and *Brachypodium distachyon* was constructed (Fig. 1, and Supplementary files: Tree 1). More *TaDREB*s clustered with *BdDREB*s rather than *OsDREB*s, indicating closer relationships between *TaDREB*s and *BdDREB*s, consistent with the fact that the rice diverged earlier (46 Mya) than wheat and *Brachypodium distachyon* (38 Mya) in the grasses (Gaut, 2002; Huo et al., 2009). Three conserved motifs namely YRG, WLG and RAYD and two key amino acids V14 and E19 which play essential roles in DRE specific

recognition (Sakuma et al., 2002; Zhao et al., 2012) were identified by alignment of the conserved AP2 domains (Fig. S2).

Besides, 15 conserved motifs were identified by MEME and wherein TaDREBs in the same subgroup showed similar motifs (Fig. S3). All TaDREBs have motifs 1, 2, 4, 9 and 11. On contrary, motif 3, 8, 10 and 14 were found only in members of subgroup A1, motif 6 was found only in A1 and A4, motif 13 in A3 and A6, motif 15 in A4 and A3, while motif 5 was not in subfamily A1, motif 7 not in A2 and A3, motif 12 not in A2, A3 and A5, respectively, indicating that the DREBs in the same subgroup might possess similar functions.

Gene structures showed that most *TaDREB* (1 8 3) genes encoding members in subgroups A1, A4, A5 and A6 have one exon (Fig. S3).

3.2. Expression profiles of TaDREB genes

As gene expression correlation correlates to protein functions, we analyzed the expression profiles of *TaDREB*s. TPM (Transcripts Per Million) values of TaDREBs were obtained from Wheat Expression Browser. A total of 13 tissues from Chinese Spring were studied, i.e. seedling leaves/shoots, seedling roots, vegetative leaves/shoots, vegetative roots, vegetative spike, reproductive leaves/shoots, reproductive roots, reproductive spike and reproductive grain without treatments, vegetative leaves/shoots and vegetative roots after abiotic stress

treatment, and vegetative leaves/roots and reproductive spike after disease treatment.

As shown in Fig. S4, under normal conditions, some genes showed relatively high expression in all tissues, such as *TaDREB39B* (subfamily A1), *TaDREB81A* (A3), and *TaDREB30A* (A4), et al. During reproductive stage, a few of *TaDREBs* showed tissue-specific expression. For example, *TaDREB6B* (A5) and *TaDREB79A* (A6) were expressed specifically in leaves/shoots, *TaDREB39A* (A1), *TaDREB23D* (A5) and *TaDREB76B* (A5) were expressed only in roots, *TaDREB75D* (A1) and *TaDREB6A* (A5) were expressed exclusively in spike. Besides, some *TaDREBs* were expressed mainly in grain, including *TaDREB65B*, *Ta-DREB44B* and *TaDREB31D* in subfamily A1, *TaDREB18B* and *TaDREB5B* in subfamily A2, and *TaDREB8D* in subfamily A4, et al. Tissue-specific expression indicates that these genes might play roles in the development of these tissues. While more *TaDREB* genes showed no expression in these tissues (139 in seedling leaves/shoots, 151 in seedling roots, 99 in vegetative leaves/shoots, 101 in vegetative roots, 131 in vegetative spike, 120 in reproductive leaves, 146 in reproductive roots, 104 in reproductive spike and 112 in reproductive grain) which might functions in other biological processes.

After abiotic stress, expression of some *TaDREB* genes changed compared with none treatments (Fig. S4). In total, 34 of 80 A1 members, 8 of 16 A2 members, 3 of 17 A3 members, 24 of 47 A4 members, 13 of 29 A5 members and 8 of 21 A6 members were found changed significantly with abundance up- or down- regulated more than 1.5 folds, respectively. Expression of more *TaDREBs* in subfamily A1, A2, A4 and A5 were affected by abiotic stresses, indicating that these subfamilies might be important in abiotic stress responses.

Disease treatment led to significant expression changes (abundance changed more than 1.5 folds) in about half of *TaDREB* genes in each subfamily (Fig. S4, 38/80 in A1, 7/16 in A2, 8/17 in A3, 26/47 in A4, 12/29 in A5, and 12/21 in A6), suggesting that TaDREB family might play a key role in responses to diseases.

To further investigate the expression patterns of *TaDREBs*, twelve genes from six subfamilies were selected randomly to perform q-PCR. Their expression varies considerably and shows tissue specificity in the roots, stems, leaves and inflorescences (Fig. 2a). *TaDREB32* and *Ta-DREB44* (subfamily A1), and *TaDREB26* and *TaDREB79* (subfamily A6) are predominantly expressed in the roots and leaves; *TaDREB3* and *TaDREB25* (subfamily A2), *TaDREB14* and *TaDREB80* (subfamily A4) are mainly expressed in the leaves; *TaDREB77* and *TaDREB81* (subfamily A3) are mainly expressed in the inflorescences, while the expression of *TaDREB67* and *TaDREB78* (subfamily A5) were detected in the roots.

Furthermore, we detected the expression of these genes in seedlings under 45 °C, 4 °C, 200mMNaCl, 20% PEG and 25 μM ABA treatments. As shown in Fig. 2b, the expression of *TaDREB32* and *TaDREB44* in subfamily A1, *TaDREB25* in A2, *TaDREB77* in A3, *TaDREB80* in A4, *TaDREB67* and *TaDREB78* in A5, and *TaDREB79* in A6 were inhibited by heat, cold, salt and drought stress, *TaDREB14* in subfamily A4 was down-regulated by salt and drought stress, *TaDREB26* in subfamily A6 was suppressed by heat, cold and drought stress, while *TaDREB81* in subfamily A3 was induced by heat and cold stress. Although no regularity was found, there some meaningful results. For example, the expression of *TaDREB81A/B/D* is up-regulated by heat drastically.

3.3. Alternative splicing of TaDREB3

According to EnsemblPlants (Table S1), six sets of genes might have variant transcripts (CDs and protein sequences of their variant transcripts were listed in Supplementary files: text1). Among them, *Ta-DREB21-B/D* showed Alternative exons pattern, *TaDREB20-D*, *Ta-DREB26-A*, *TaDREB52-B/D* and *TaDREB58-A* showed Intron-retention pattern, while only *TaDREB3-A/B/D* showed Exon-skipping pattern which was found on all three chromosomes, so we selected *TaDREB3-A/B/D* for further analyses. Based on BlastN results (Table S1), *TaDREB3*

is the known *WDREB2*, with three alternative splicing forms in common wheat (Egawa et al., 2006). We amplified the cDNAs of *TaDREB3* in *Chinese Spring* by RT-PCR and cloned them into pMD-18 T respectively. Interestingly, results of DNA sequencing showed that each copy of *Ta-DREB3* is alternatively spliced gene and generates three isoforms (named I/II/III) (Fig. 3a). Consistent with previous studies of *WDREB2* (Egawa et al., 2006), two isoforms, transcripts I and II (corresponding to *Wdreb2α* and *Wdreb2γ*, respectively), encode proteins with an intact EREBP/AP2 domain (Fig. 3b showed the protein sequence of TaDREB3-AI), while transcript III (corresponding to *Wdreb2β*) showed premature translation termination and does not generate DREB proteins (Fig. 3a).

Then we analyzed the expression profiles of these isoforms by performing semi-quantitative PCR. Results showed that, after heat (Fig. 4a), cold (Fig. 4b), salt (Fig. 4c), PEG (Fig. 4d) and ABA (Fig. 4e) treatments, three alternative splicing products were changed at different levels. Heat, cold and salt stresses induce the expression of three isoforms (Fig. 4a–c). Compared to heat, cold and salt, it looks that the effect of PEG is less (Fig. 4d). In addition, ABA also induces the expression of *TaDREB3* (Fig. 4e). These results suggest that *TaDREB3* might play important roles in response to abiotic stresses.

3.4. Enhanced resistance to abiotic stresses in Arabidopsis over-expressing TaDREB3-AI

In order to analyze the function of *TaDREB3*, *TaDREB3*-AI and *TaDREB3*-AII were overexpressed in Arabidopsis, respectively. A total of 26 transgenic Arabidopsis plants over-expressing *TaDREB3*-AI were obtained. *TaDREB3*-AI-OE18 (AI-18), *TaDREB3*-AI-OE22 (AI-22) were selected for further analyses (Figure Supplementary Fig. S5).

Under drought stress, transgenic lines over-expressing *TaDREB3*-AI grew well with green leaves (Fig. 5a) while the leaves of wild type withered. After 5 days of recovery, $83.33 \pm 2.78\%$ of AI-18 and $54.63 \pm 4.24\%$ of AI-22 survived, while only $20.37 \pm 1.60\%$ of the wild type (WT) plants survived (Fig. 5a). For salt stress, the leaves of wild type wilted while most transgenic Arabidopsis over-expressing *TaDREB3*-AI were still green (Fig. 5c). Finally, over 80% of *TaDREB3*-AI-over-expressing Arabidopsis survived (Fig. 5d) while the survival rates in WT was only $40.74 \pm 4.24\%$. As to heat stress, leaves of WT were mostly desiccated while those of transgenic lines AI-18 and AI-22 were still fresh (Fig. 5e). After recovery for 5 days, over 75% of transgenic plants over-expressing *TaDREB3*-AI survived (Fig. 5f) while only about $21.30 \pm 4.24\%$ of WT continued to grow. Over-expression of *TaDREB3*-AI significantly enhanced survival rates of transgenic Arabidopsis under drought, salt and heat stresses.

Apart from survival rates, the physiological characteristics including the accumulation of reactive oxygen species (ROS), the content of chlorophyll, MDA (malondialdehyde), proline and the relative electrical conductivity, and the activities of enzymes in ROS scavenging enzymes such as POD (peroxidase), CAT (catalase) and SOD (superoxide dismutase) also reflect the stress tolerance. Then we detected these indexes in wild type and two lines of transgenic Arabidopsis over-expressing *TaDREB3*-AI (AI-18 and AI-22).

Under normal conditions, leaves of both WT and transgenic Arabidopsis showed no difference. After drought, salt and heat stresses, all leaves were stained with blue of $O_2^{\cdot-}$ by NBT (Fig. 6a) or brown of H_2O_2 by DAB (Fig. 6b) wherein WT was deeper than transgenic lines, indicating WT accumulated more ROS than 35S-*TaDREB3*-AI. The chlorophyll contents declined in all leaves, but those in transgenic Arabidopsis were higher than in WT (Fig. 7a), suggesting a better stay green trait (Xue et al., 2010); proline accumulated in all leaves while transgenic lines showed higher levels than WT (Fig. 7b), suggesting more stable cellular content of transgenic plants (Shao et al., 2006); MDA concentration (Fig. 7c) and relative electrical conductivity (Fig. 7d) increased and the heightened levels in transgenic Arabidopsis were lower than WT, suggesting a deeper oxidation degree of plasma membranes in non-transgenic lines than transgenic lines(Guo et al.,
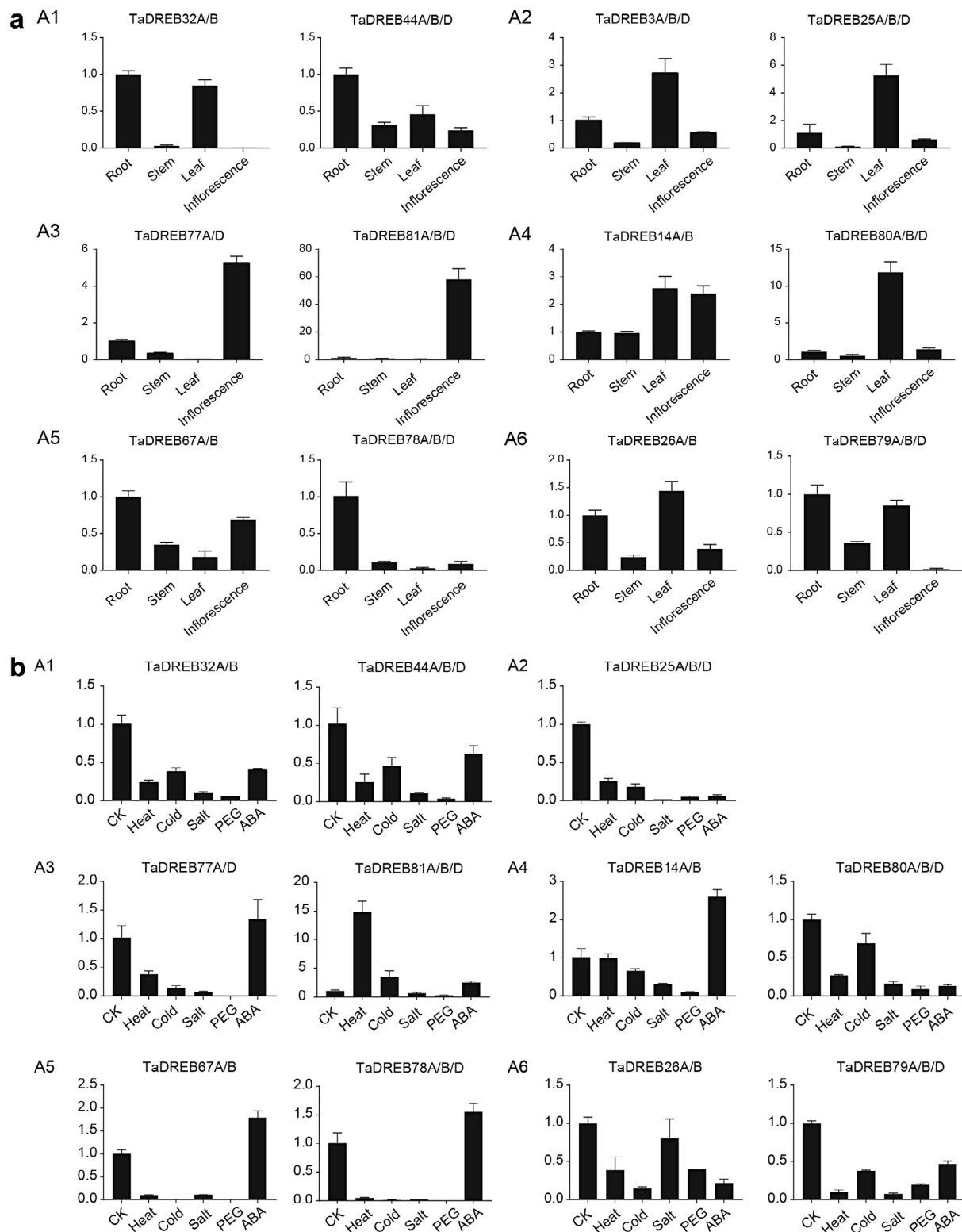
Fig. 2. Expression pattern of *TaDREBs* in different tissues (a) and under different treatments (b). Each experiment was repeated three times and the error bars represent the standard deviations (**, P < 0.01 versus control).

2018; Sathiyaraj et al., 2011); activities of POD (Fig. 7e), CAT (Fig. 7f) and SOD (Fig. 7g) rose in all lines and higher in transgenic lines than WT, implying better capability of removing active oxide in cells which was in accordance with histochemical staining assays(Wu et al., 2018). These results further suggested that over-expression of *TaDREB3*-AI improved the tolerance of transgenic Arabidopsis to drought, salt and heat stresses.

The transcription levels of stress-responsive genes were also detected (Sakuma et al., 2006b). qRT-PCR results showed that under normal conditions, the expression of *RD29A*, *RD19*, *HsfA3*, *LEA*, *RAS*1 and *HSP70* in wild type and transgenic Arabidopsis over-expressing *TaDREB3*-AI showed no significant differences (Fig. 8). But after
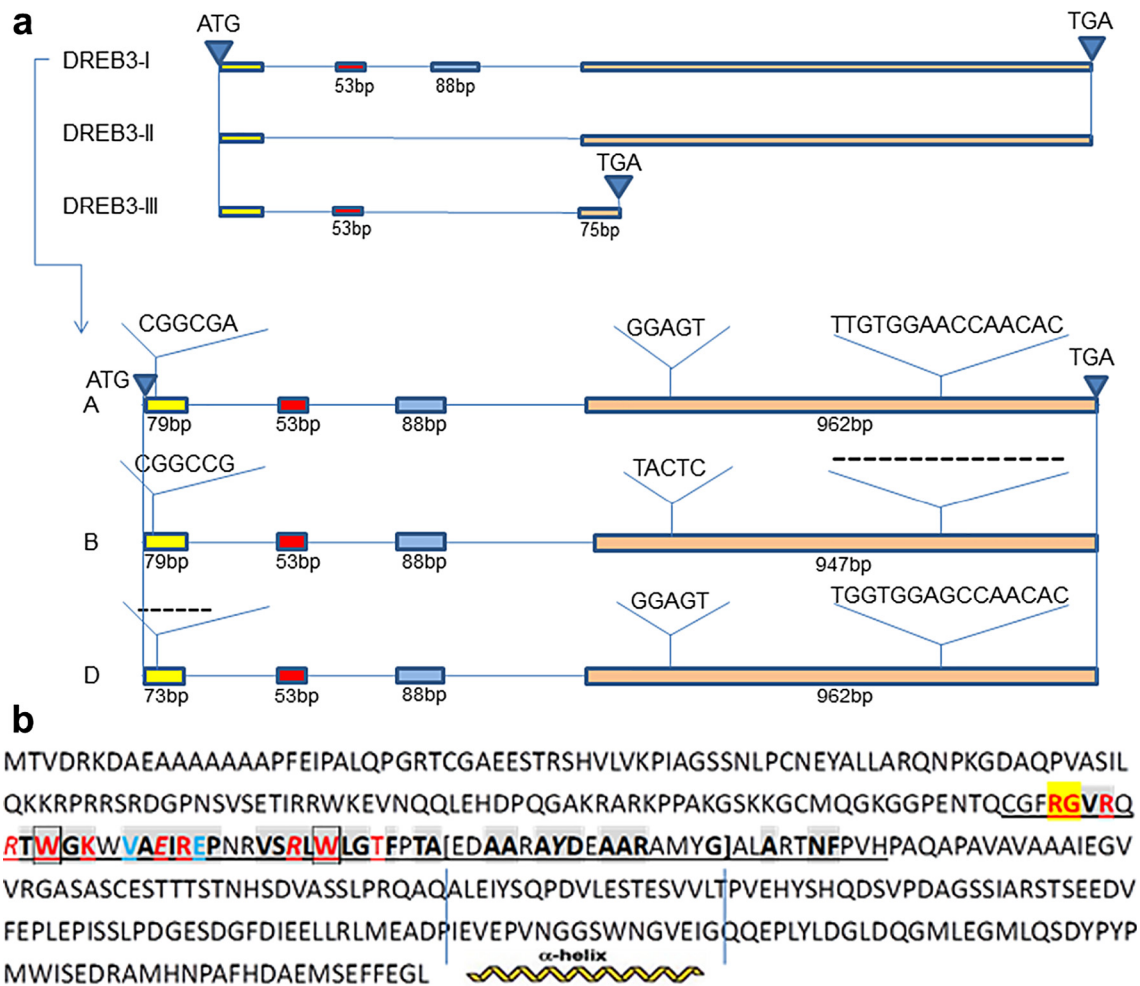
Fig. 3. Alternative splicing patterns and three copies of *TaDREB3* (a) and amino acid sequence of TaDREB3-AI (b). The EREBP/AP2 conserved domain is in grey with underline. The highly conserved amino acid residues are in bold. The GCC-box specific recognition sites are in boxes. V14 and E19 are blue. Italic residues help to maintain geometrical configurations of DREB. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

drought, salt and heat stresses, their expression was up-regulated differently in both plants with a higher degree in transgenic Arabidopsis (Fig. 8). These results indicate that over-expression of *TaDREB3*-AI might improve the stress tolerance of transgenic plants via enhancing the expression of stress-responsive genes downstream.

Besides, 19 plants over-expressing *TaDREB3*-AII were acquired and *TaDREB3*-AII-OE1 (AII-1), *TaDREB3*-AII-OE6 (AII-6) were selected for further analyses (Supplementary Fig. S5). Under normal condition and after drought salt and heat stresses, transgenic lines over-expressing *TaDREB3*-AII showed no difference with WT (Supplementary Fig. S6), suggesting that *TaDREB3*- AII might have no actual work in plant tolerance to heat, salt and drought stresses.

4. Discussion

Adverse environmental conditions could lead to crop reduction of both yield and quality. Plants have to evolve a serious of reaction to respond to stresses for their immovable. At molecular level, DREBs, one plant specific TF family, play key roles in stress resistance.

4.1. DREB genes play key roles in abiotic stresses

So far, many DREB TFs in different plants have been proved to improve tolerance to abiotic stresses. Most of them belong to subgroup A1 and A2. In Arabidopsis, *DREB1A/CBF3*, is induced by cold and over-

expression of it improved tolerance to dehydration, high-salt and low-temperature stresses in transgenic Arabidopsis (Kasuga et al., 1999; Liu et al., 1998), rice (Latha et al., 2018; Oh et al., 2005; Ravikumar et al., 2014), tobacco (Kasuga et al., 2004), and potato (Behnam et al., 2007; Watanabe et al., 2011), et al. *DREB1B/CBF1* enhanced drought and freezing tolerance in transgenic Arabidopsis (Jaglo-Ottosen et al., 1998), potato (Movahedi et al., 2012), and tomato (Hsieh et al., 2002; Lee et al., 2010). *DREB1C* negatively regulates the transcription of *DREB1A* and *DREB1B*, and plays a key role in freezing tolerance in Arabidopsis (Novillo et al., 2004). Transgenic plants over-expressing *OsDREB1A*, *OsDREB1B* and *OsDREB1*F displayed enhanced tolerance to drought, high-salt and freezing stresses (Dubouzet et al., 2003; Ito et al., 2006; Wang et al., 2008b). Over-expression of *OsDREB1G* remarkably improved transgenic plants tolerance to water deficit stress while *OsDREB1E* slightly improved (Chen et al., 2008). *DREB1* genes in other plants are also related with stress resistance. For instance, heterologous expression of *ZjDREB1.4*, a *DREB1* gene from zoysiagrass (*Zoysia japonica Steud.*), enhances transgenic Arabidopsis tolerance to low and high temperature stresses (Feng et al., 2019). Transgenic Arabidopsis over-expressing potato *CBF1* showed higher tolerance to freezing and drought stresses than wild type (Li et al., 2018).

Over-expression of Arabidopsis *DREB2A* in subgroup A2, whose transcripts are accumulated by drought and salt stresses, significantly enhanced drought, heat tolerance by many regulating water stress-inducible and heat-shock-related genes (Sakuma et al., 2006a; Sakuma
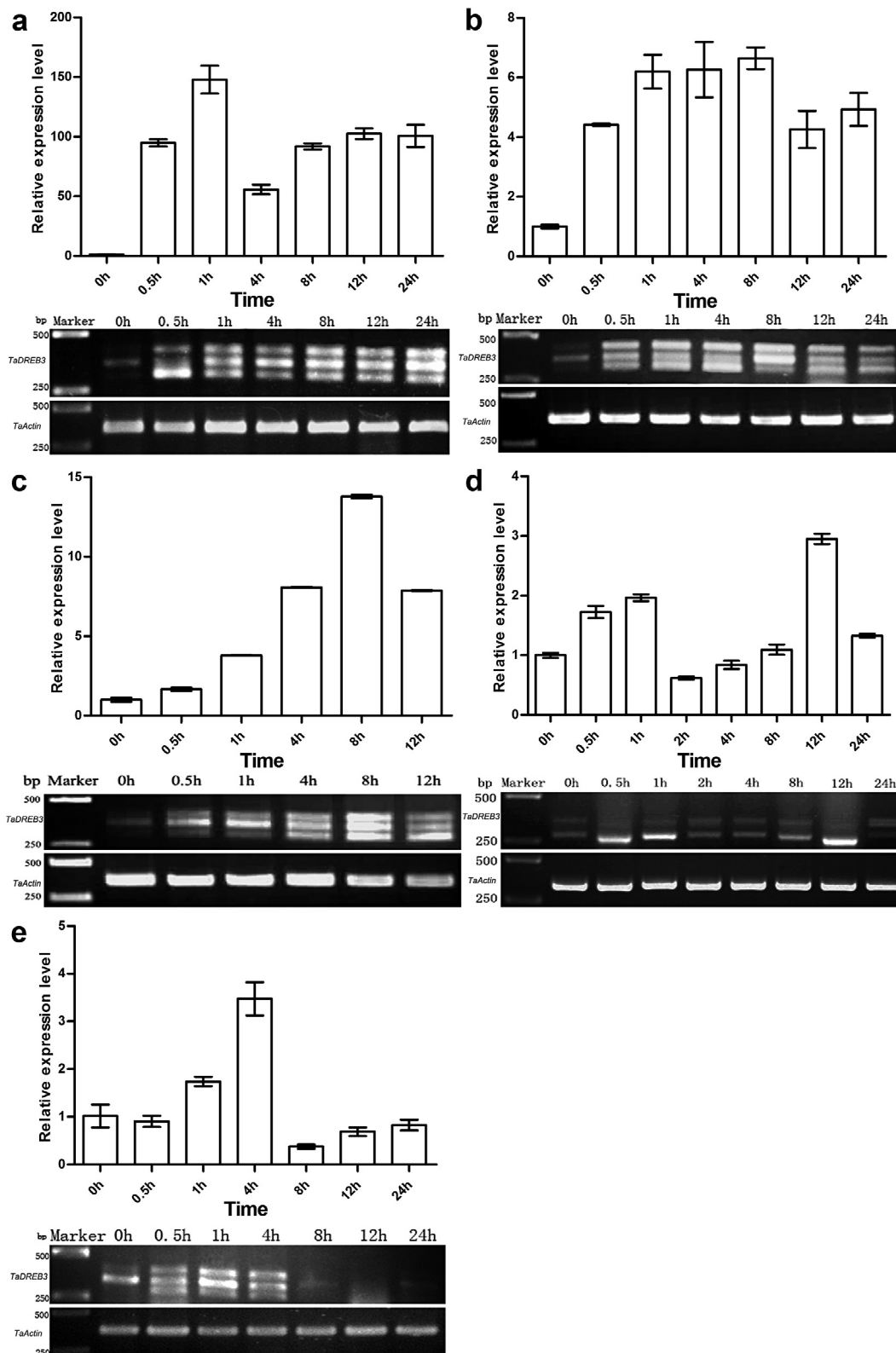
Fig. 4. Expression pattern of *TaDREB3* under different treatments. (a) 45 °C; (b) 4 °C; (c) 200 mM NaCl; (d) 20% PEG6000; (e) 25 μM ABA.

et al., 2006b). Besides, its homologous genes are also involved in stress responses. In rice, *OsDREB2A* is markedly induced by drought, high-salt and ABA treatments and transgenic plants over-expressing *OsDREB2A* showed higher survival rates than wild type plants under severe drought and salt stresses (Cui et al., 2011; Dubouzet et al., 2003; Mallikarjuna et al., 2011). Transgenic rice over-expressing *OsDREB2B*

exhibited significantly elevated survival rates under drought and heat-shock stresses (Matsukura et al., 2010). In soybean, *DREB2A* is highly induced by dehydration, low temperature and heat (Mizoi et al., 2013). Heterologous expression of *GmDREB2A* in Arabidopsis improved transgenic plants tolerance to heat and drought stresses (Mizoi et al., 2013). In maize (*Zea may*), the functional form of *ZmDREB2A* is induced
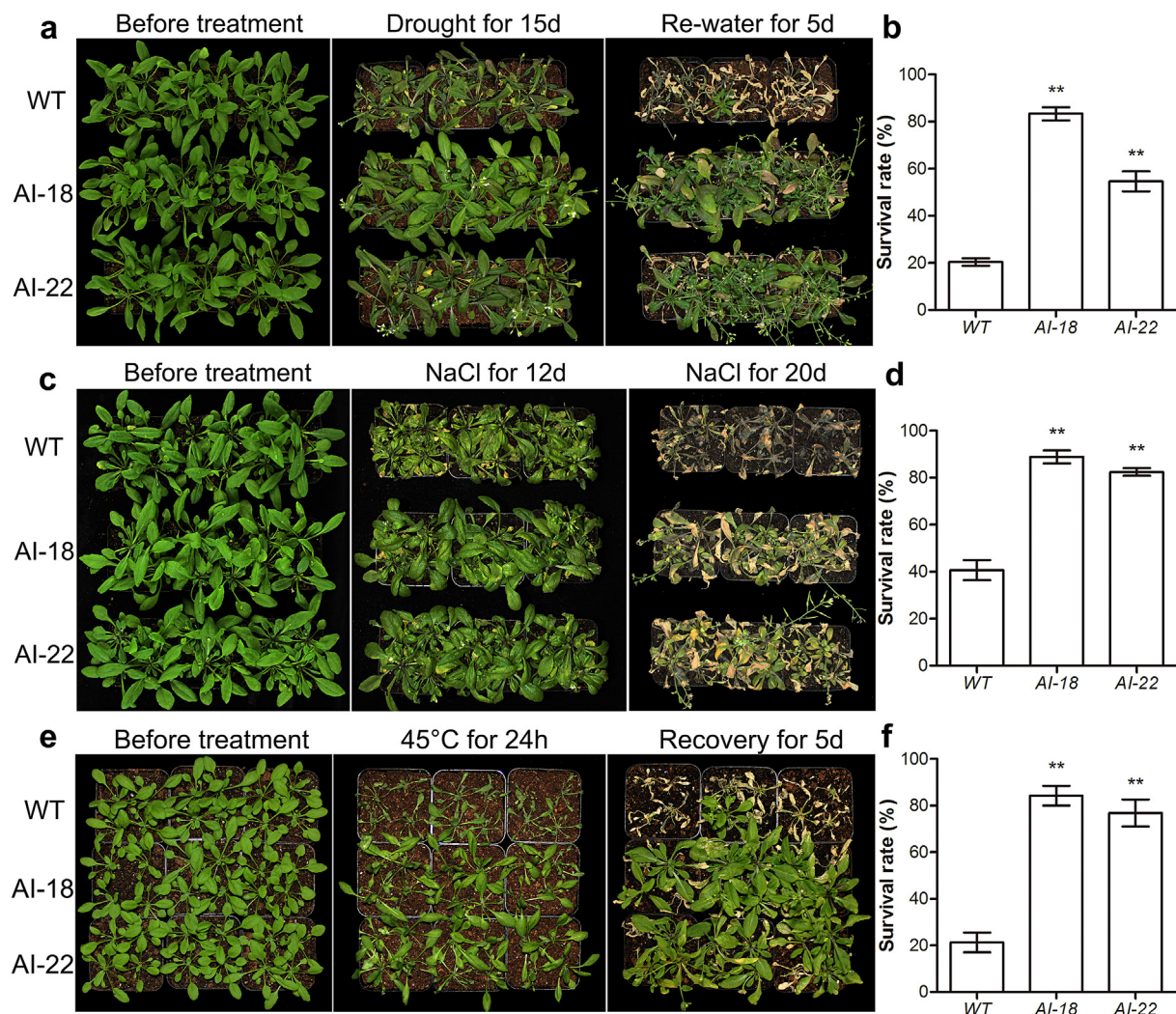
Fig. 5. Phenotypes of transgenic Arabidopsis over-expressing *TaDREB3*-AI under drought (a, b), salt (c, d) and heat (e, f) stresses. Each experiment was repeated three times with 36 plants and the error bars represent the standard deviations (**, P < 0.01 versus wild-type).

by dehydration, salt, cold and heat stresses and over-expressing it in Arabidopsis also enhanced tolerance to drought and heat stresses (Qin et al., 2010). In lettuce (*Lactuca sativa* L.), *LsDREB2A* is dramatically induced by salt and hyperosmotic stresses and over-expressing it in transgenic plants improved tolerance to high salinity (Kudo et al., 2014). Another DREB2-type transcription factor gene, *EsDREB2B* in *Eremosparton songoricum* is differentially induced by drought, salt, cold, heat, heavy metal, mechanical wounding, oxidative stress and exogenous ABA treatment. Over-expressing *EsDREB2B* in tobacco improved transgenic plants tolerance to salt, cold, heat and osmotic stresses (Li et al., 2014).

Although few members in other four subgroups have been identified, many of them are involved in responses to abiotic stresses. For example, A3-type *DREB* gene *GmSGR* in soybean, which is also involved in ABA and glucose pathways like its orthologous gene *ABI4* in Arabidopsis (Niu et al., 2002), might play a negative role in responses to salt stress (Wang et al., 2008a). Apart from *TINY* (Sun et al., 2008) in Arabidopsis, over-expression of A4 members such as *ZmDBF3* from maize (salt, drought, and freezing stresses) (Zhou et al., 2016), *StDREB1* from potato (drought, salt, and oxidative stresses) (Bouaziz et al., 2015; Bouaziz et al., 2013), and *MnDREB4A* from mulberry (*Morus alba* L.) (heat, cold, drought and salt stresses) (Liu et al., 2015), et al, also enhanced transgenic plants tolerance to abiotic stresses. A5 members *ScDREB8* (in *Syntrichia caninervis*) (Liang et al., 2017) and *StDREB2* (Bouaziz et al., 2012) improved transgenic plants salt tolerance.

Besides, over-expression of *StDREB2* also enhanced cotton tolerance to drought stress (El-Esawi and Alayafi, 2019). A-6 members are also responsive to different abiotic stresses. Over-expression of *CmDREB6* from chrysanthemum (*Chrysanthemum morifolium*) improved transgenic plants tolerance to heat stress (Du et al., 2018) while *CmERF053* improved drought stress tolerance (Nie et al., 2018). Heterologous expression of *SsDREB* from *Suaeda salsa* enhanced transgenic tobacco tolerance to salt and drought stresses (Zhang et al., 2015). *JcDREB* play a key role in salt and freezing tolerance (Tang et al., 2011).

So far, only a few *DREB* genes have been functionally characterized in wheat (Table S1). For instance, *Dreb1* gene was related to drought tolerance (Huseynova et al., 2013). Over-expressing *TaAIDFa*, an drought-, high-salt- and cold- responsive copy of Dreb1, improved transgenic plants tolerance to drought and osmotic stresses (Xu et al., 2008). *WCBF2* increased transgenic plants tolerance to freezing stress through up-regulating target genes downstream (Takumi et al., 2008). An α isoform of *WDREB2* also improved transgenic plants tolerance to cold stress (Sazegari and Niazi, 2012). Over-expressing two *TaDREB* genes (Acc. DQ353852 and Acc. DQ353853) improved the tolerance of transgenic plants to drought and frost stresses (Morran et al., 2011). *TaRAP2.1L* functions as a stress-responsive transcriptional repressor in plants tolerance to cold and dehydration (Amalraj et al., 2016), similar to its ortholog in Arabidopsis (Dong and Liu, 2010b).

In this study, we identified 210 wheat *DREB* genes at genome-level and divided them into 6 known subfamilies (A1 to A6) with 80, 16, 17,
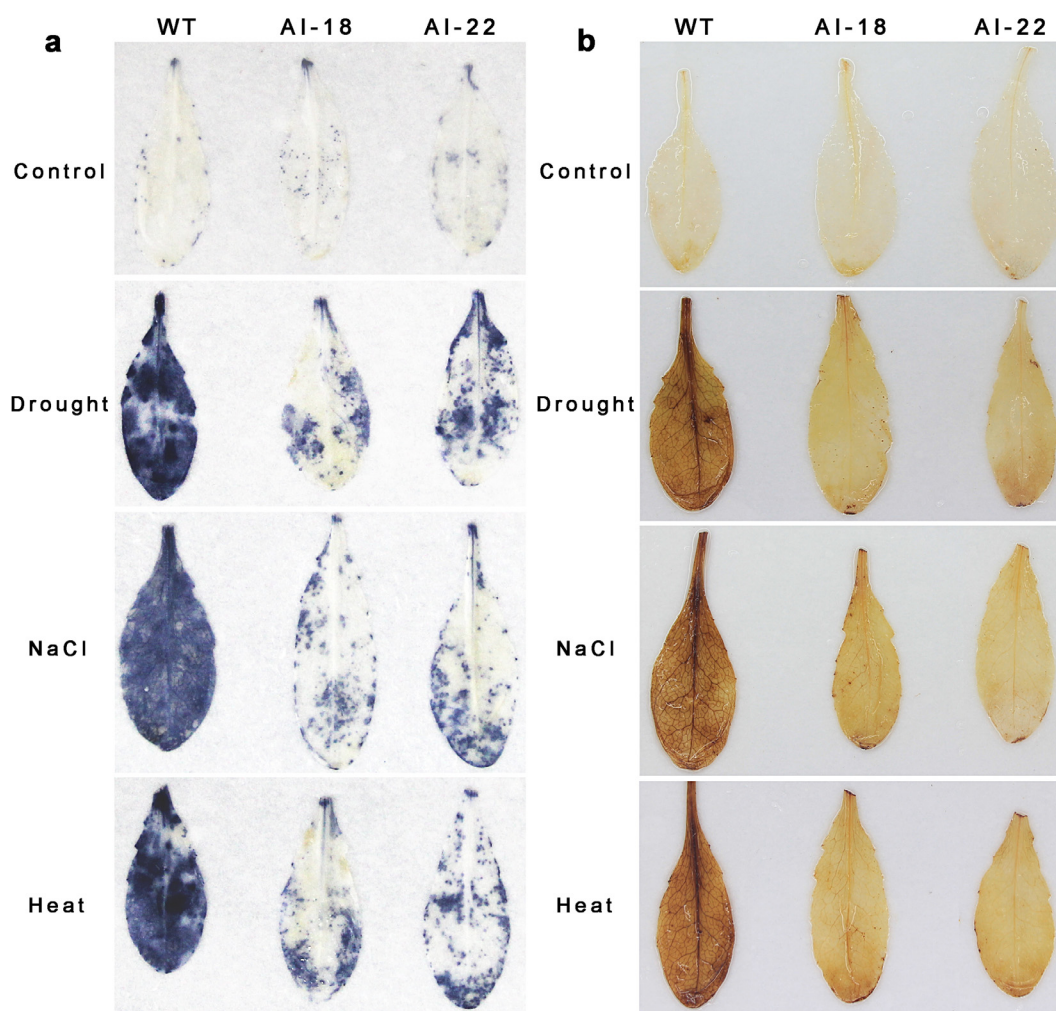
Fig. 6. Histochemical assay of $O_2^{\cdot -}$ (a) and H_2O_2 (b) under drought, salt and heat stresses.

47, 29 and 21 members in each subfamily in order. Of them, 73, 6, 26 and 3 *TaDREBs* in subfamily A1, A3, A4 and A5 were tandem duplicated genes, suggesting that tandem duplication events play an important role in the expansion of these subfamilies and TaDREB TFs.

TPM values (Fig. S4) and q-PCR results (Fig. 2) identified some TaDREBs with high expression levels in specific tissues, suggesting that these genes might take part in wheat growth and development. Besides, TPM analyses displayed that more members in subfamily A1, A2, A4 and A5 were affected by abiotic stresses (Fig. S4), indicating an important role of these subfamilies in responses to abiotic stresses. Further study of 12 randomly selected *TaDREBs* by q-PCR (Fig. 2) showed that their expression levels were affected by heat, cold, salt and drought stresses, although the trend and degree were different. These results also suggest that *DREBs* in wheat might participate in responses to abiotic stresses just like their orthologs in other plants. Among them, A2-type gene *TaDREB3* is induced by abiotic stresses, indicating a potential role in stress tolerance.

4.2. Over-expression of TaDREB3 in Arabidopsis improved tolerance to heat, salt and drought stresses

In *Poaceae*, alternative splicing patterns have been reported in barley (*HvDRF1*) (Xue and Loveridge, 2004), maize (*ZmDREB2A*) (Qin et al., 2010), rice (*OsDREB2B*) (Matsukura et al., 2010), wheat (*Wdreb2*) (Egawa et al., 2006), et al. Among them, *HvDRF1* and *Wdreb2* generates three isoforms (Egawa et al., 2006; Xue and Loveridge, 2004). Two of them possess entire AP2 domains and encode DREB proteins wherein

HvDRF1.1 and HvDRF1.3 showed similar transactivation (Xue and Loveridge, 2004). Multiple sequences alignment of several DREB proteins in *Poaceae* showed that *HvDRF1.1* and *TaDREB3*-I have additional 30 amino acids in their N-terminal (Supplementary Fig. S7), in consistent with *BdDREB3* (*BRADI2G29960*) which generates two isoforms and only one encodes protein with a typical AP2 domain. However, the functional difference in abiotic stresses between these two isoforms have not been reported yet.

In our study, *TaDREB3* corresponds to *Wdreb2* (Table S1). In consistent with previous study (Egawa et al., 2006), *TaDREB3* contains three introns and generates three isoforms (Fig. 3) which were all found on chromosome 1A, 1B and 1D (Fig. 1). Among them, both longer *DREB3-I* and shorter *DREB3-II* encode intact amino acid sequences while *DREB3-III* showed frameshift and abolished translation. Expression of *DREB3-I* and *DREB3-II* were differently induced by heat, cold, salt, drought and ABA treatment, suggesting different roles during stress responses (Fig. 4).

Then we studied the function of *TaDREB3*-AI and *TaDREB3*-AII through ectopically expressing them in Arabidopsis (Clough and Bent, 2010). Under normal conditions, transgenic plants over-expressing *TaDREB3*-AI and *TaDREB3*-AII grow well and showed no significant difference with wild type plants (Fig. 5 and Fig. S5).

Stresses including drought, salt and heat stresses could stunt plant growth and have a negative impact on survival. We found that transgenic plants over-expressing *TaDREB3*-AI showed better growth status and higher survival rates after dehydration, high-salt and high temperature stresses compared with wild type (Fig. 5). These results
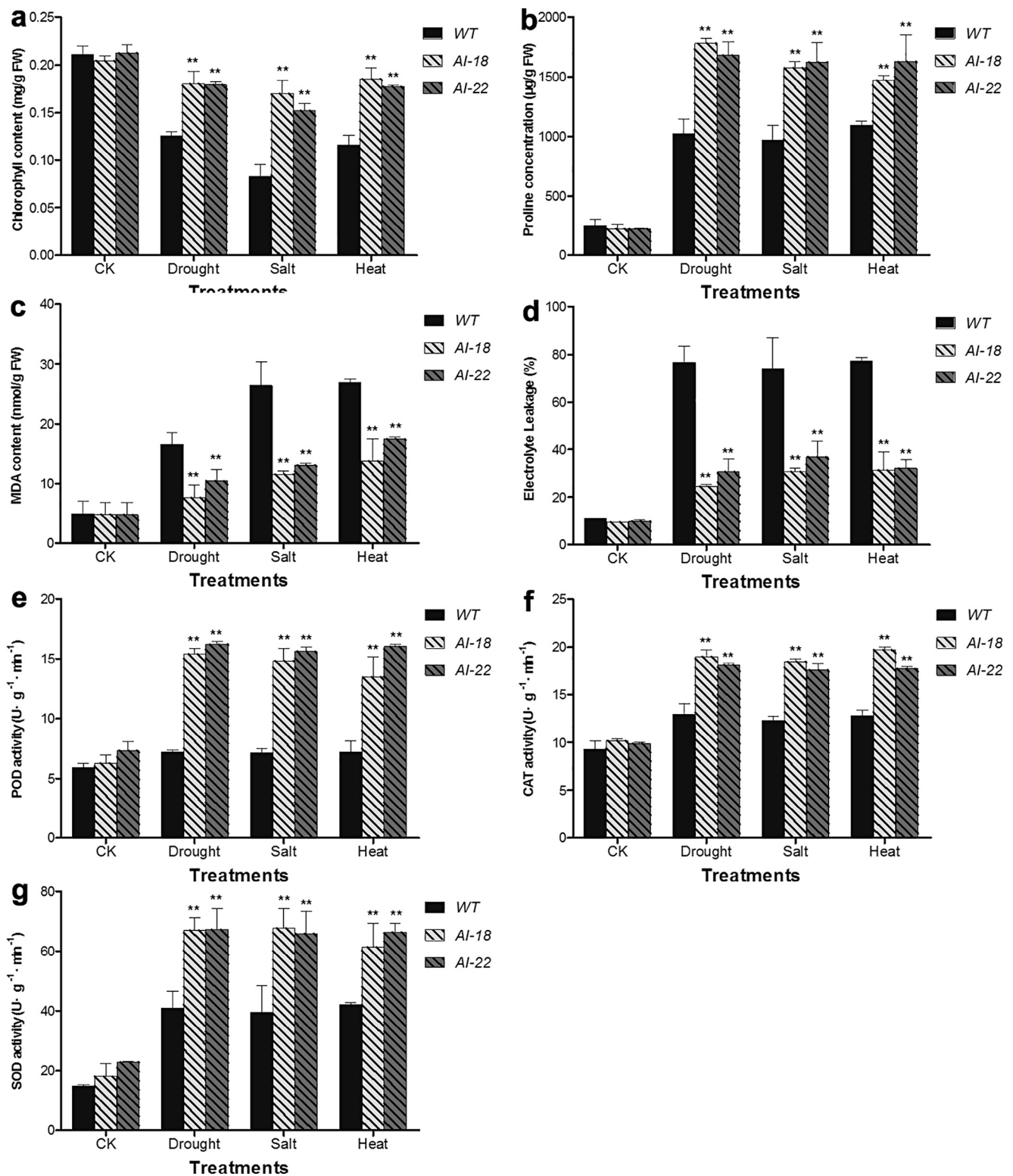
Fig. 7. Physiological analyses of Arabidopsis under drought, salt and heat stresses. Chlorophyll content, MDA content, electrolyte leakage, proline concentration, and activities of POD, CAT and SOD in WT, AI-18 and AI-22 plants under normal conditions (CK) and drought (10d), salt (7d) and heat (10 h) stresses were analyzed using leaves collected after treatment, respectively. Each experiment was repeated three times and the error bars represent the standard deviations (**, P < 0.01 versus wild-type).

suggest that *TaDREB3*-AI participate in regulating responses to drought, salt and heat stresses and might be a viable candidate for improving yield through molecular breeding.

When encounter abiotic stresses, reactive oxygen series particularly H_2O_2 and $O_2^{\cdot-}$ generate and accumulate in plant cells (SUZUKI et al.,

2012). Excess ROS cause lipid peroxidation and produce MDA(Chen et al., 2015). Thus, the stabilities of membrane lipid were decreased which further lead to the increased relative leakage of cells and declined chlorophyll content (Chen et al., 2015). The active oxygen eliminating enzyme system including SOD, CAT and POD could
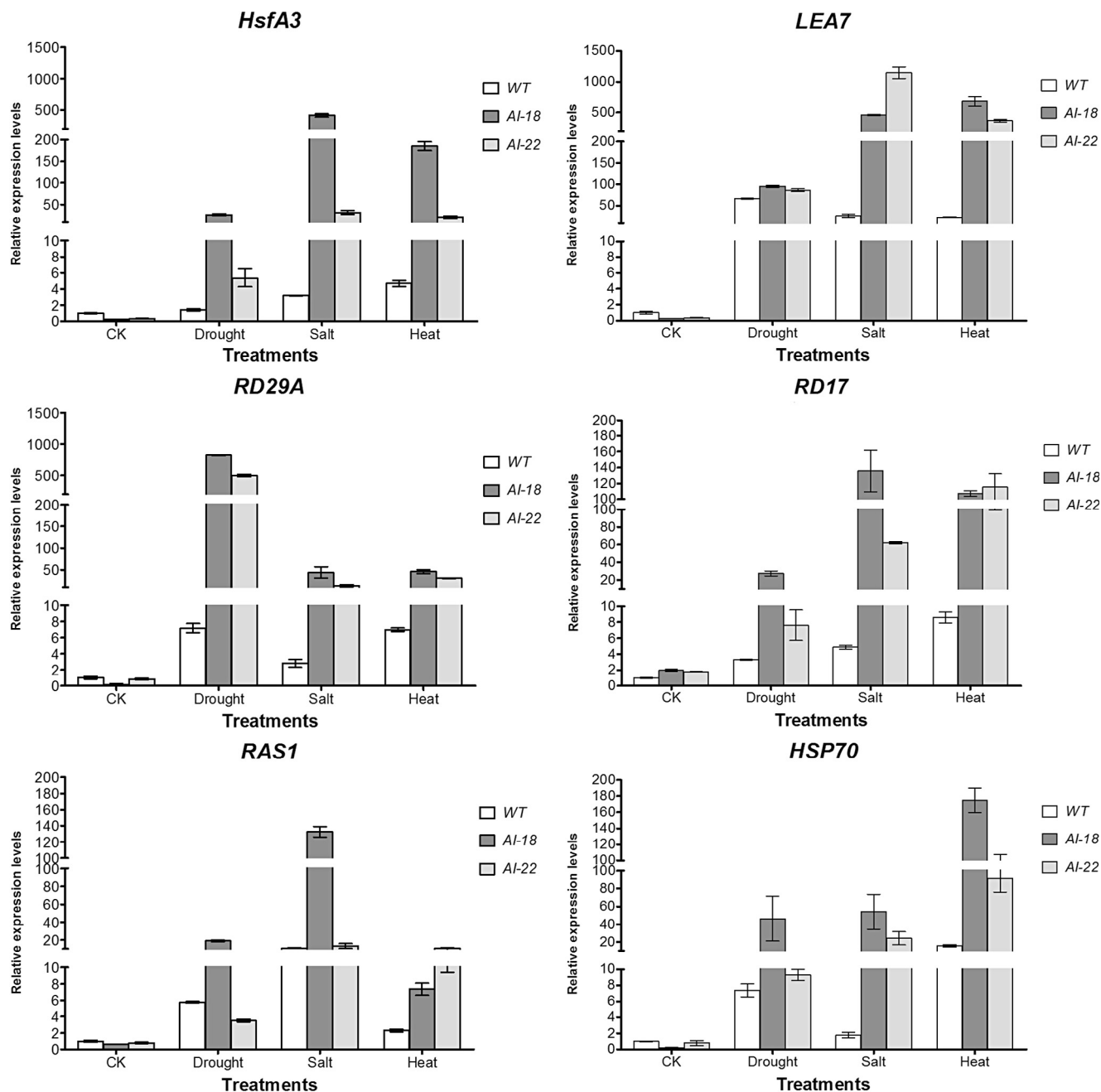
Fig. 8. The expression levels of stress-related genes in Arabidopsis. The error bars represent the standard error of triplicate experiments (**, P < 0.01 versus wild-type).

catalyze the metabolism of ROS into H_2O and O_2 to alleviate the toxicity of redundant ROS (D'Autréaux and Toledano, 2007). On the other hand, ROS also function as signal to promote proline synthesis (Skopelitis et al., 2006). As a signaling molecule in cellular homeostasis, proline plays a key role in plant recovery from stress (Szabados and Savouré, 2010).

In our study, all these physical indexes showed no difference between transgenic and wild type Arabidopsis. After stresses, the accumulation of H_2O_2 and $O_2 \cdot^-$, MDA content and electrolyte leakage in transgenic Arabidopsis were all lower than those in wild type while the chlorophyll content, proline concentration, and the activity of POD, CAT and SOD in transgenic Arabidopsis were all higher than those in wild type (Figs. 6 and 7).

The relative expression levels of some genes downstream of *DREB* were also detected. The expression of *RD29A* (*At5g52310*) and *RD17*

(*At1g20440*) is under the regulation of DREB TFs due to the DRE elements in the promoter regions, so they are extensively used in abiotic stresses as marker genes (Yamaguchi-Shinozaki and Shinozaki, 1993). The expression of *HsfA3* (*At5g03720*), one target genes of *DREB2A*, is induced by heat stress (Sato et al., 2014) and regulates the transcription of Hsp-encoding genes in turn (Schramm et al., 2008). *HSP70* (*At3g12580*) encodes a chaperone which helps to prevent incorrect folding of proteins and accelerate the degradation of unstable proteins (Wang et al., 2004). *LEA7* (*At1g52690*) encodes a LEA protein downstream of DREB and plays a key role in dehydration and cold stresses (Popova et al., 2011; Popova et al., 2015). *RAS1* (*At1g09950*) was reported to respond to salt stress (Ren et al., 2010). When plants were under stresses, the expression of these stress-responsive genes were up regulated. These stress-related protein then interact with their targets downstream to slow down the accumulation of reactive oxygen species

and maintain the cellular homeostasis, finally enhance plants tolerance to adverse environment (Agarwal et al., 2006). In this study, the expression levels of these genes in transgenic Arabidopsis over-expressing *TaDREB3*-AI were all higher than in wild type plants under heat, drought and salt stresses (Fig. 8), suggesting a better tolerance to stresses. Under normal conditions, over-expression of *TaDREB3*-AI had no impact on DREB downstream genes, implying it also require post-translational modification to be activated by stress signals just like *DREB2A* in Arabidopsis and rice (Dubouzet et al., 2003; Liu et al., 1998).

Although the expression of three isoforms of *TaDREB3* was up regulated after various abiotic stresses, only *TaDREB3*-IA showed important functions in responses to stresses like *OsDREB2B2* in rice (Matsukura et al., 2010), suggesting *TaDREB3-II* and *TaDREB3-III* might help keep constitutive activation of the transcription of *TaDREB3* without affecting plants growth and they might undergo different regulation mechanisms after transcription and translation from same *TaDREB3* pre-mRNA (Matsukura et al., 2010). Our results indicate that over-expression of *TaDREB3*-AI improved transgenic plants tolerance to heat, high salinity and drought stresses while *TaDREB3*-AII did not. The difference between these two isoform might due to the existence of additional 30 amino acids in the N-terminal which needs further verification.

5. Conclusion

210 *DREB* genes in wheat were identified and divided into 6 subgroups based on phylogenic analyses. Among them, some showed tissue-specific expression in which *TaDREB3* is induced by abiotic stresses. Belonging to subgroup A2, *TaDREB3* has four exons and generates three alternative splicing transcripts which were found in all A/B/D genomes. *DREB3-III* with frameshift showed premature termination of translation while both longer *DREB3-I* and shorter *DREB3-II* encode normal peptide chain. *TaDREB3*-AI might have a hand in improving tolerance to drought, salt and heat stresses by regulating the relative expression of some stress-responsive genes.

CRediT authorship contribution statement

Xin Niu: Investigation, Data curation, Visualization, Writing - original draft. **Tengli Luo:** Formal analysis, Validation. **Hongyan Zhao:** Validation, Data curation. **Yali Su:** Writing - review & editing. **Wanquan Ji:** Conceptualization, Methodology, Writing - review & editing. **Haifeng Li:** Conceptualization, Methodology, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Author contributions

Haifeng Li designed the project; XinNiu conducted most experiments. Haifeng Li and Xin Niu analyzed data and wrote the manuscript. All authors read and approved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2020.144514.

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