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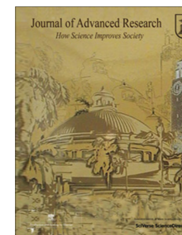

ORIGINAL ARTICLE

Roles of dehydrin genes in wheat tolerance to drought stress

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Physiological parameters and expression levels of drought related genes were analyzed in early vegetative stage of two bread wheat cultivars (Sids and Gmiza) differ in drought tolerance capacity. Both cultivars were imposed to gradual water depletion started on day 17 till day 32 after sowing. Sids, the more tolerant cultivar to drought showed higher fresh and dry weights than the drought sensitive genotype, Gmiza. Under water stress, Sids had higher membrane stability index (MSI), lower accumulated H_2O_2 and higher activity of the antioxidant enzymes; catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and superoxide dismutase (SOD) than Gmiza. On the other hand, the differential expression patterns of the genes *dhn*, *wcor* and *dreb* were observed due to water deficit intensity according to cultivar's tolerance to drought. The DNA sequence alignment of *dun* showed high similarity of about 80–92% identities with other related plants. The most striking overall observed trend was the highly induction in the expression of *dun*, *wcor* and *dreb* in leaves of the tolerant genotype, Sids under severe water stress.

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Introduction

Drought is a wide world problem constraining global crop production seriously [1]. Common wheat possesses a very complex and huge genome with size of $\sim 16 \times 10^9$ bp, which consists of about 90% repeated sequences [2]. Under drought stress, H_2O_2 generated as a result of electron leakage from the photosynthetic and respiratory electron transport chains to oxygen or during photorespiration resulting from the oxygenase activity of Rubisco. Also, drought results in changes in antioxidant enzymes activity. High antioxidant activities could be interpreted as symptoms of oxidative stress or damage, the plant upregulates antioxidant enzymes because it produces more reactive oxygen species (ROS) [3]. Conversely, high antioxidant activity could be interpreted as higher tolerance to oxidative stress.

Alteration of gene expression is always involved in preparing plants for an existence under stress. Under conditions of water deficit (dehydration), numerous processes are modified or impaired resulting in growth inhibition [4] to cope with osmotic changes in their tissues. Modification of gene expression results in a strict control of the physiological and biochemical responses to stress. Identification and isolation of these genes

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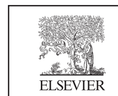

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are essential for developing tolerant crops. Expression profiling has become an important tool to investigate responses of an organism to environmental changes at the transcriptional level [5]. One of these adaptations is to produce proteins called dehydrins. Dehydration responsive element binding proteins (DREBs) constitute a large family of TFs that induce the expression of a large number of functional genes and impart stress endurance to plants [6]. The dehydration responsive element (DRE) as a cis-acting element was found in the promoter regions of many drought- and low-temperature-inducible genes [7]. All *dreb* genes feature three conserved regions, an EREBP/AP2 DNA binding domain, an N-terminal nuclear localization signal, and conserved Ser/Thr rich region adjacent to the EREBP/AP2 domain. *dreb tfs* play key roles in plant stress signaling transduction pathway, they can specifically bind to DRE/CRT element (G/ACCGAC) and activate the expression of many stress inducible genes. In several cases, the overexpression of these *dreb/cbf* genes was associated with retardation of plant growth under non stressful conditions [8]. It was suggested that the overexpression of these genes under stress-inducible promoter but not constitutive promoter may be a solution to avoid the change in plant growth [9]. Dehydrins *dhn* are associated with crucial protective functions [10]. *dhn* are part of the late embryogenesis abundant proteins (LEA) [11]. They are produced in response to abscisic acid (ABA) [10]. They are primarily distributed in cytosol, nucleus and plasma membranes [12]. Dreb controls expression of cold-regulated (*cor*) gene. Wcor410 is a dehydrin that accumulates around the plasma membrane and is present in lesser amounts in the intercellular space [13]. It is up regulated by low temperatures, dehydration, ABA, salt, wounding and polyethylene glycol. So, the present work is aimed at studying the expression of *wdhn*, *dreb* and *wcor410* in order to find their roles in drought tolerance of two wheat cultivars that differentially tolerate water stress.

Material and methods

Plant material and growing conditions

Seeds of two bread wheat (*Triticum aestivum*) cultivars, (Sids 4, and Gmiza 10) were obtained from Wheat Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt. The seeds were sown in a mixture of sand and clay culture (1:1) in plastic pots (25 cm diameter) under field conditions in greenhouse. Ten seeds of each cultivar were sown per pot. Seedlings, shortly after seedling emergence, were thinned to five per pot (11 days after sowing, 11 DAS) and watered by tap water twice in week. On the 17th DAS, the pots of each cultivar were divided into two groups: one was left as control and irrigated with tap water and the other was restricted to drought by withholding water up to the end of the experiment (33 DAS).

The experimental design was a complete block randomized design and repeated twice in triplicates so that the mean values ± SE values were of $n = 6$. The design consisted of 144 pots [2 cultivars × 2 sets (2 treatments) × 3 pots per set (3 replications per treatment each pot contained 5 plants) × 2 experimental repetitions × 6 intervals]. At each interval starting from the 17th DAS every 3 days up to the 33rd DAS, the full analysis of variance (ANOVA) was calculated at $p = 0.05$ for 24 samples [2 cultivars × 2 treatments × 6 replications]. At harvest, samples from different pots of each cultivar were collected and used for fresh and dry weight determinations while leaves from the remaining individuals were collected, frozen immediately in liquid nitrogen and stored at −80 °C for subsequent analyses.

Activity assay of antioxidant enzymes

Frozen leaf samples were ground in liquid nitrogen and homogenized in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA, 5 mM β-mercaptoethanol and 4% (w/v) polyvinylpyrrolidine-40 (PVP-40). The homogenate was centrifuged at 30,000×*g* for 30 min at 4 °C. The supernatant was used for assay of the antioxidant enzymes catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and superoxide dismutase (SOD). CAT activity was assayed according to the method of Aebi [14], GPX activity was assayed according to the method of Chance and Maehly [15], APX activity was assayed according to the method of Nakano and Asada [16] and SOD activity was measured according to Giannopolitis and Ries [17].

Membrane stability index and H_2O_2 content

The Membrane stability index (MSI) was determined indirectly by measuring the electrical conductivity according the protocol of Kocheva et al. [18]. Leaf membrane stability was estimated using the equation, $MSI = [1-(C_1/C_2)] \times 100$ [19]. Relative Injury percentage was calculated as: $RI(\%) = 100-\{[1-(T_1/T_2)]/[1-(C_1/C_2)] \times 100\}$, where C and T refer to electrical conductivity of control and drought treated samples, and the subscript 1 and 2 refer to electric conductivity readings before and after autoclaving, respectively. H_2O_2 was measured according to Alexieva et al. [20].

RNA isolation and cDNA preparation

Total RNAs were extracted from 500 mg of shoot samples by using easy-BLUETM TRI-reagent (iNtrON Biotechnology) according to Chomczynski et al. [21] DNA was removed from RNA samples by using RNase-free DNaseI (Thermo scientific) following the instruction protocol. Purity of total RNA was assessed at 260/280 nm. Quality of the RNA was assured with ethidium-bromide stain analysis by agarose gel electrophoresis. About 0.1–1 μg DNA-free RNA in distilled water (DEPC-treated) were added into Maxime RT PreMix tubes with Oligo dT primer to a total volume of 20 μl. The tubes were left for 2 min at room temperature and then the pellet was dissolved by pipetting. The cDNA synthesis reaction was performed using Thermal counter. The mixture was incubated at 45 °C for 60 min followed by 5 min at 95 °C. The reactant was diluted by adding 20–50 μl sterile water then stored at −20 °C for RT-PCR. To check if the cDNAs were properly synthesized, 18S rRNA of Arabidopsis (amplicon length is about 800 bp) was amplified by PCR conditions. These results were used as positive controls of cDNAs.

Primer design and DNA analysis

Specific primer pairs were designed to recognize a conserved regions which were predetermined using alignment of dehydrin genes sequences from wheat that are available at the GenBank.

The alignment for these identified genes was performed by CLUSTALW. The primer pair for *dhn* and *wcor* genes was designed by PRIMER 3 program (http://fokker.wi.mit.edu/primer3/input.htm) and was checked by OligoAnalyzer 3.1, while the primer pair for *dreb* gene was designed according to the sequence of *Triticum aesitivum dreb* gene (*wdreb*) available in the NCBI database under the accession number AB193608 (Table 1) [22]. The PCR conditions were modified according to the primer properties, and the PCR products.

Semi-quantitative PCR

Semi-quantitative PCR of the three genes (*dhn*, *wcor* and *dreb*) was performed in 50 μl reaction mixtures containing 16 μl RNase–DNase-free water, 25 μl Dream Taq Green PCR Master Mix (2×) (Thermo Scientific Cat. No. #K1081) and 10 μl of each of the forward and reverse primers (10 mM each) as indicated in Table 1, 1 μl cDNA template.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as follows: initial denaturation at 95 °C for 5 min followed by (26–40) cycles of denaturation at 95 °C for 30 s, annealing at (43–58 °C) for (30–45 s) for different template cDNAs and genes specific primers were optimized, extension at 72 °C for 30 s, and final extension for 10 min at 72 °C. For semi-quantitative analysis, 10 μl of PCR products was collected after different cycles before reaching the plateau phase. The PCR cycles were adjusted to be in the linear range by viewing the RT-PCR product after each cycle on agarose gels. For ascertaining equal RNA loading in RT reaction, 18S rRNA was used as an internal control and the fold expression of the target genes was normalized accordingly. The resolved gel images were introduced into LabImage V 2.7.2 to measure the band volumes. Each experiment was repeated three times. Optical densities of bands that represent *Arabidopsis 18S* rRNA were obtained for normalization of data. Ratio of gene band intensity to 18S rRNA band intensity was calculated.

Isolation and identification of DNA sequence of dhn and wcor genes

PCR product for *dhn* was extracted from agarose gels while *wcor* was isolated from PCR mix by ISOLATE PCR and Gel Kit (Cat. No.: BIO-52029) following the instruction protocol. The extracted PCR products were sequenced in both directions by GATC Biotech. Ltd. http://www.gatc-biotech.com/en/home.html, ORF was found by Six-frame translation, http://www.bioline.com/calculator/01_13. A database analysis was done for each sequence in NCBI and BioEdit Sequence Alignment Editor Software, Version 7.04.1 using Megablast (nucleotides) search to test out the homology of the sequences. DNA alignment was done by multalin (http://bioinfo.genotoul.fr/multalin/multalin.html).

Statistical analysis

The experimental design exhibited that water regimes were in main plots, and wheat genotypes in sub plots with three random replications of two experiments. For individual experiments, standard error of means of each ratio was calculated from $n = 6$ and analysis of variance (ANOVA) was carried out at every interval at 0.05 significance level [23].

Results

Growth parameters

As Shown in Table 2, both cultivars showed gradual increase in the fresh weight with time progress. Drought resulted in decreases in fresh weight of both cultivars, the magnitude of decrease was more pronounced in Gmiza than in Sids especially at the last harvest. Shoot dry weight also showed a gradual increase in both cultivars (Table 2). At the end of the experiment there was a significant decrease in dry weight in Gmiza cultivar compared with control, otherwise drought caused insignificant decrease in dry weight in both cultivars. The results also show that there was a progressive increase in the leaf area in both cultivars under normal conditions (Table 2). Sids had larger leaf area than Gmiza during the whole experiment. Drought decreased Sids leaf area comparing control value only after 26 and 30 DAS. Conversely, drought stress caused insignificant decrease in leaf area in Gmiza comparing control values. Surprisingly, the largest increase and the greatest decrease in leaf area were observed in watered and drought stressed Sids plants respectively.

Membrane stability index (MSI) and H_2O_2 content

In Table 3, withholding water resulted in severe damage to membranes of Gmiza comparing with control while Sids did not encounter any damage in MSI along the experiment. On the other hand, after three days of withholding water, Sids and Gmiza cultivars had H_2O_2 content significantly higher than control, the magnitude of increase was most pronounced in Gmiza. Only on 30 and 33 DAS there was significant increases in the H_2O_2 content in accumulated in Gmiza due to drought comparing with control values. However, there was not any significant difference in the H_2O_2 content between watered and drought stressed Sids plants.

Antioxidant enzymes activity

Drought stress enhanced significantly CAT activity in Sids plants compared with its control all over the experiment (Table 4). Conversely, drought stress inhibited CAT activity in Gmiza plants compared with its control; this inhibition

Table 1 Primer pairs and numbers of cycles used for amplification of different genes.

Gene name	Forward primer	Reverse primer	No. of cycles
dhn	5′ATGGAGCACCAGGGGC3′	5′GCAGCTTGTCCTTGATCTTG3′	33
wcor	5′ATGGAGGATGAGAGGAG3′	5′GCTTGTCCTTGATCTTG3′	40
dreb	5′AAGAAAACAGGCGACAAGAT′3	5′ACGAAGCACAAAAAACTAGC′3	27
18S RNA	CCACCCATAGAATCAAGAAAGAG	GCAAATTACCCAATCCTGAC	25

Table 2 Changes in shoot fresh weight, dry weight and leaf area of two wheat cultivars (Sids, sd and Gmiza, gm) as a result of drought by withholding water. n = 6, Data are means ± SE, LSD was calculated at 0.05.

	Days after sowing (DAS)					
	17	20	23	26	30	33
Fresh weight (g per plant)						
F wt						
sd	0.262 ± 0.001	0.437 ± 0.085	0.573 ± 0.087	0.651 ± 0.094	0.683 ± 0.143	1.064 ± 0.033
sd*	0.262 ± 0.001	0.362 ± 0.039^s	0.373 ± 0.120^s	0.502 ± 0.018^s	0.558 ± 0.144^s	0.878 ± 0.070^s
gm	0.172 ± 0.018	0.247 ± 0.050	0.323 ± 0.018	0.388 ± 0.014	0.395 ± 0.010	0.651 ± 0.058
gm*	0.172 ± 0.018	0.257 ± 0.044	0.265 ± 0.010	0.283 ± 0.069^s	0.272 ± 0.075^s	0.279 ± 0.077^s
LSD	0.021	0.056	0.076	0.066	0.090	0.066
Dry weight (g per plant)						
sd	0.043 ± 0.003	0.065 ± 0.011	0.111 ± 0.015	0.118 ± 0.012	0.134 ± 0.012	0.190 ± 0.016
sd*	0.043 ± 0.003	0.077 ± 0.004^s	0.069 ± 0.022^s	0.077 ± 0.002^s	0.102 ± 0.012^s	0.153 ± 0.016^s
gm	0.039 ± 0.001	0.041 ± 0.008	0.049 ± 0.020	0.070 ± 0.006	0.076 ± 0.018	0.120 ± 0.007
gm*	0.039 ± 0.001	0.028 ± 0.006^s	0.035 ± 0.004^s	0.049 ± 0.014^s	0.070 ± 0.014	0.064 ± 0.003^s
LSD	0.004	0.008	0.013	0.011	0.015	0.012
Leaf area (cm^2)						
sd	6.84 ± 0.37	7.09 ± 0.12	8.14 ± 0.22	9.05 ± 0.43	11.21 ± 0.41	10.65 ± 0.50
sd*	6.84 ± 0.37	7.06 ± 0.35	7.99 ± 0.19	7.18 ± 0.33^s	7.69 ± 1.05^s	8.81 ± 0.14^s
gm	4.30 ± 0.11	6.57 ± 0.56	6.74 ± 0.73	7.73 ± 0.48	6.63 ± 0.21	7.19 ± 0.06
gm*	4.30 ± 0.11	5.23 ± 0.22^s	6.43 ± 0.06	6.79 ± 0.23	5.55 ± 0.14^s	5.40 ± 0.08
LSD	1.18	1.11	1.11	1.27	0.73	1.88

Values followed by an ^{s} are significantly different at 0.05 from their corresponding control.
An * represents cultivars grown under drought.

Table 3 Changes in membrane stability index and H_2O_2 content of two wheat cultivars (Sids, sd and Gmiza, gm) as a result of drought by withholding water. n = 6, Data are means ± SE, LSD was calculated at 0.05.

	Days after sowing (DAS)					
	17	20	23	26	30	33
Membrane stability index (MSI)						
MSI						
sd	94.04 ± 0.51	93.04 ± 0.45	96.74 ± 0.38	84.52 ± 0.72	89.07 ± 0.54	90.99 ± 0.76
sd*	94.04 ± 0.51	91.03 ± 0.33^s	99.33 ± 0.67^s	80.33 ± 0.60^s	86.83 ± 0.73	86.05 ± 3.44
gm	95.68 ± 0.09	95.68 ± 0.09	97.76 ± 0.38	85.33 ± 2.32	83.18 ± 0.91	91.21 ± 0.61
gm*	95.68 ± 0.09	86.46 ± 0.11^s	74.52 ± 0.72^s	57.42 ± 0.42^s	48.19 ± 1.35^s	48.19 ± 0.53^s
LSD	1.39	1.51	1.89	4.07	3.49	7.13
H_2O_2 content (μmole g^{-1} fresh weight)						
sd	1.40 ± 0.09	0.78 ± 0.11	0.10 ± 0.01	1.92 ± 0.16	1.94 ± 0.16	2.38 ± 0.33
sd*	1.40 ± 0.09	1.76 ± 0.13^s	0.44 ± 0.08^s	2.74 ± 0.05	3.51 ± 0.47^s	2.85 ± 0.44
gm	1.62 ± 0.14	0.91 ± 0.07	0.37 ± 0.04	3.35 ± 0.13	3.11 ± 0.36	2.67 ± 0.36
gm*	1.62 ± 0.14	2.17 ± 0.08^s	1.02 ± 0.19^s	4.16 ± 0.92	6.64 ± 0.70^s	4.83 ± 0.44^s
LSD	0.33	0.23	0.32	1.17	1.37	1.17

Values followed by an ^{s} are significantly different at 0.05 from their corresponding control.
An * represents cultivars grown under drought.

was significant and most pronounced starting from the 26th DAS. Similarly, drought stress enhanced GPX activity in Sids plants compared with the control values all over the experiment (Table 4). Contrarily, drought stress inhibited GPX activity in Gmiza plants compared with the control; but this inhibition was insignificant at all intervals. Generally, Sids has higher activity of SOD than Gmiza. Moreover, drought resulted in a significant increase in SOD activity comparing control until the end of the experiment (Table 4). However Gmiza plants encountered nonsignificant decrease in SOD activity during withholding water throughout the experiment. In the same pattern, Sids generally has higher activity of APX than Gmiza. Nonetheless, APX activity was inhibited in both Sids and Gmiza cultivar due to drought, however this inhibition was significant only from 30 DAS onward comparing control values. The magnitude of inhibition in APX activity was greater in Gmiza than in Sids.

Responses of dhns, wcor and dreb to drought stress

Drought stress increased the transcript level of *dhn* in both cultivars after three days stress by about 2-folds (Fig. 1A). Nevertheless, after six days of stress, the mRNA level of *dhn* declined slightly in Gmiza but increased by 3-folds in Sids. Drought

Table 4 Changes in CAT, GPX, SOD and APX activities of two wheat cultivars (Sids, sd and Gmiza, gm) as a result of drought by withholding water. $n = 6$, Data are means ± SE, LSD was calculated at 0.05.

	Days after sowing (DAS)					
	17	20	23	26	30	33
CAT activity (μmole min^{-1} g^{-1} fresh weight)						
CAT						
sd	0.303 ± 0.008	0.873 ± 0.037	2.155 ± 0.223	1.881 ± 0.011	3.003 ± 0.082	2.229 ± 0.551
sd*	0.303 ± 0.008	2.812 ± 0.100^s	6.477 ± 0.740^s	5.413 ± 0.117^s	5.126 ± 0.058^s	4.955 ± 0.073^s
gm	0.242 ± 0.011	1.961 ± 0.084	1.537 ± 0.264	2.942 ± 0.139	3.457 ± 0.087	3.124 ± 0.398
gm*	0.242 ± 0.011	1.433 ± 0.233	1.333 ± 0.203	0.964 ± 0.122^s	1.671 ± 0.091^s	1.865 ± 0.034^s
LSD	0.280	0.581	1.501	0.471	0.547	1.034
GPX activity (nmole min^{-1} g^{-1} fresh weight)						
sd	13.92 ± 2.28	13.87 ± 0.91	47.58 ± 4.13	57.41 ± 0.49	94.62 ± 2.51	50.64 ± 1.46
sd*	13.92 ± 2.28	78.58 ± 1.42^s	124.73 ± 7.20^s	152.97 ± 2.62^s	141.23 ± 4.00^s	97.58 ± 10.18^s
gm	8.85 ± 0.32	16.70 ± 1.51	55.56 ± 0.63	42.49 ± 1.76	82.45 ± 2.38	67.34 ± 1.77
gm*	8.85 ± 0.32	28.79 ± 0.67^s	56.58 ± 3.03	32.81 ± 1.47^s	70.20 ± 1.25^s	67.44 ± 0.91
LSD	5.43	4.38	13.44	5.50	10.44	41.18
SOD activity (Unit g^{-1} fresh weight)						
sd	65.4 ± 0.6	127.8 ± 1.4	251.5 ± 4.2	282.2 ± 3.4	253.1 ± 4.0	269.1 ± 10.2
sd*	65.4 ± 0.6	352.9 ± 16.0^s	390.5 ± 4.5^s	371.9 ± 11.0^s	338.5 ± 4.0^s	396.9 ± 3.7^s
gm	42.5 ± 1.7	91.6 ± 1.2	244.4 ± 37.6	251.3 ± 6.9	188.5 ± 9.4	210.8 ± 4.1
gm*	42.5 ± 1.5	84.3 ± 1.6	204.9 ± 3.8	169.7 ± 2.3^s	170.0 ± 7.4	210.2 ± 5.0
LSD	5.4	26.8	60.7	32.1	25.8	22.5
APX activity (nmole min^{-1} g^{-1} fresh weight)						
sd	32.57 ± 0.67	39.30 ± 3.59	32.43 ± 1.20	26.66 ± 1.04	22.63 ± 1.19	24.69 ± 1.33
sd*	32.57 ± 0.67	33.62 ± 5.44	22.77 ± 4.76^s	25.28 ± 1.51	10.54 ± 1.99^s	10.54 ± 0.67^s
gm	14.20 ± 0.33	30.69 ± 6.90	13.11 ± 3.81	15.89 ± 1.60	14.81 ± 3.60	19.20 ± 1.18
gm*	14.20 ± 0.33	22.43 ± 6.30	5.32 ± 1.68	6.01 ± 1.18^s	4.99 ± 0.33^s	2.39 ± 0.67^s
LSD	10.03	11.01	9.07	7.64	5.51	3.56

Values followed by an ^{s} are significantly different at 0.05 from their corresponding control.
An * represents cultivars grown under drought.

stress for 10 days resulted in an increase in the transcript level of *dhn* gene by 2-folds again in Gmiza however it reached the highest value in Sids as it went up by about more than 4-folds. On the other hand, after 2 days of rehydration, the transcript level of *dhn* declined to the minimum level in both cultivars. It is worth mentioning that the early expression of *dhn* gene after 26 cycles in the drought stressed Sids plants was after 6 days drought.

The transcript level of *wcor* gene was over regulated following drought stress for 3 days by 2-folds in Gmiza (Fig. 1B). However, there was an increase in about 30% in Sids as compared with control transcript levels. On the other hand, after 6 days drought the increase in the *wcor* transcript level was restricted to Sids cultivar as there was a decline in the mRNA level of *wcor* in Gmiza plants by about 40% as compared with control. Afterward, there was up-regulation in the mRNA level of *wcor* gene in both cultivars as a result of drought stress for 10 days in both cultivars but the increase in the expression level was highly significant in Sids plants. On other hand, rewatering both plants resulted in sharp reduction in the transcript level to reach minimum level during treatment period. It is noticed that there was a dense early expression for *wcor* gene after 37 cycles in the droughted Gmiza after 6 days drought.

Drought stress differentially regulated the expression level of *dreb* gene in both cultivars (Fig. 1C). After 3 days stress, the mRNA level in Gmiza went up 4.5 times of the control level but decreased slightly in Sids plants. However 6 days stress led to a sharp decrease in the *dreb* mRNA level in Gmiza and significant increase in case of Sids plants. The increase in the *dreb* transcript level continued up to the maximum value after 10 days stress while the transcript level of the droughted Gmiza plants was slightly higher than the transcript level of control plants. Afterward, the transcript level of *dreb* went down to lowest values in both cultivars after 2 days rehydration.

Characterization of tadhn and wcor

The sequence analysis results showed that the fragment size of *tadhn* was 307 bp; (G + C) content was 58.63% (Fig. 2). The predicted molecular weight for this fragment was 93.888 kDa for single strand and 187.111 kDa for double strand. This fragment encoded a polypeptide of 102 amino acids (20.4% Gly). A comparison of the deduced protein sequence of *tadhn* with known *dhn*s sequences from wheat, *Hordeum vulgare* and *Prunus persica* showed the conserved Ser-repeat in the N-terminus, Y-segment and has one Lys-rich region at the C-terminus of the protein (K-segments). The predicted protein was found to have similarity with other dehydrin proteins by about 35–79%. The similarity was 35% with *P. persica* dehydrin, AAC49658; 79% with *T. aestivum* dehydrin, BAF30987; 68% with *T. aestivum* LEA D-11 dehydrin, BAC01112 and *H. vulgare* subsp. spontaneum dehydrin5, AAQ55342.

Results of BLAST search using Megablast (nucleotide) indicated that the *Tadhn* DNA sequence shared high nucleotide sequence homology with wheat and other plants at about 91–96% identity. The similarity percentages were 96% with

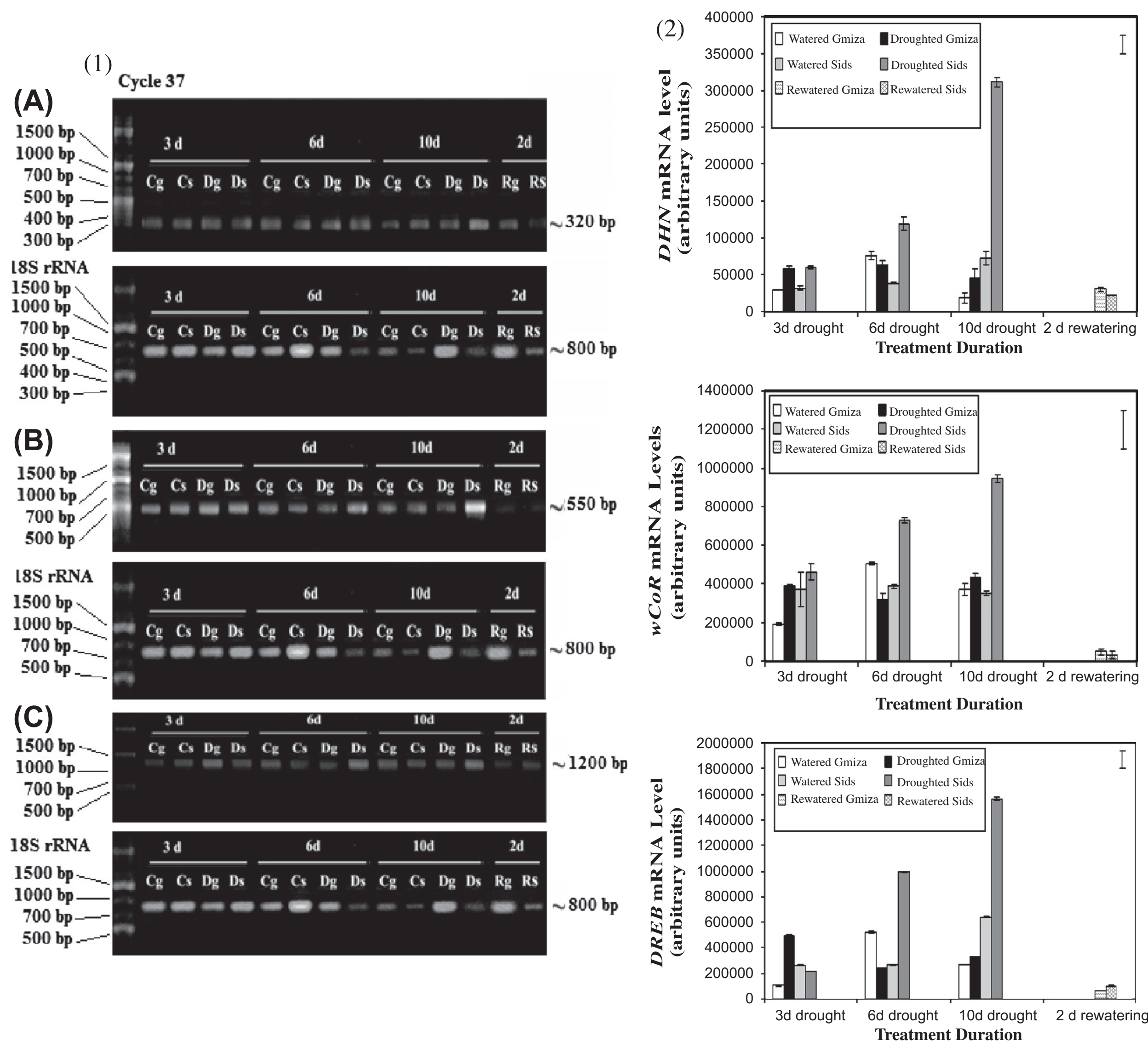

Fig. 1 Semi-quantitative RT-PCR of *dhn* (A), *wcor* (B) and *dreb* (C) of two wheat cultivars (Sids and Gmiza) as a result of drought by withholding water. (1) Quantification of expression in terms of band volumes. (2) The relative expression in the bands of each treatment after normalization with the internal control *18S rRNA* compared to the control treatment. C, control; D, drought; g, Gmiza; s, Sids; R, rehydrating.

T. aestivum wcor726, U73213; 95% with *T. aestivum wdhn* gene, FN393741; DHN14, AB272228; 93% with *Lophopyrum elongatum* dehydrin AF031250; 91% *T. aestivum* rab15B, X62476 and 91% with *T. turgidum* subsp. Durum *dhn5*, AM180933.

Similarly, the fragment size of *wcor* was 429 bp; (G + C) content was 55.94% (Fig. 3). The predicted molecular weight for this fragment was 130.722 kDa for single strand and 261.259 kDa for double strand. This fragment encoded a polypeptide of 144 amino acids (with 17.12% Glu and 15% Ser) and the predicted protein with molecular weight of 16.26 kDa. The deduced amino acid sequences corresponding to the partial genomic fragment which was similar to previously reported DHNs. The amino acid sequence of *wcor* contains Ser-repeat and has two Lys-rich regions (K-segments). The predicted deduced protein was found to 45 to 86% similarity with other dehydrins. 78% similarity with *H. vulgare* dehydrin8, AAD02259; 81% with *T. aesitivum wcor410*, AEJ88292; 62% with *Oryza sativa* SK3-type dehydrin, ABS44866 and 45% with DHN2-like protein in *Zea mays*.

Results from BLAST search using Megablast (nucleotide) indicated that the DNA sequence was highly identical to *dhn* sequence from wheat and other plants at about 80–92% identity. The similarity percentages were 89% with *T. aesitivum wcor410c*, U73211; 89% with *T. turgidum* subsp. Durum *dhn11*, AJ890140; 86% with *T. aesitivum wcor410b*, U73210; 85% with *T. aesitivum WZY1-2*, EU124658; 82% with *H. vulgare* dehydrin8, AF181458 and 77% with *Oryza sativa* SK3-type dehydrin, *EF444534*.

Discussion

The present results revealed that the drought stress significantly declined the fresh weight in both cultivars but only Sids could tolerate the stress and retained its fresh weight at the end

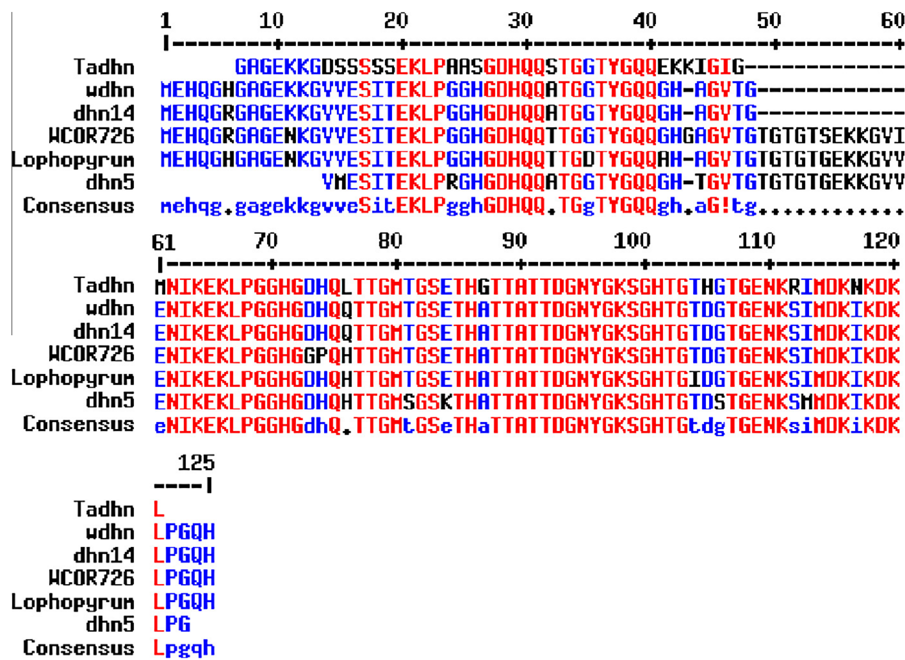

Fig. 2 Alignment of the deduced amino acids of *Tadhn* with the most closely related DHNs from wheat (*T. aesitivum*, *wcor*726, U73213), (*T. aestivum wdhn*, FN393741), (*dhn*14, AB272228), (*Lophopyrum elongatum* dehydrin, AF031250), (*T. aestivum rab*15B, X62476) and (*T. turgidum* subsp. Durum *dhn*5, AM180933).

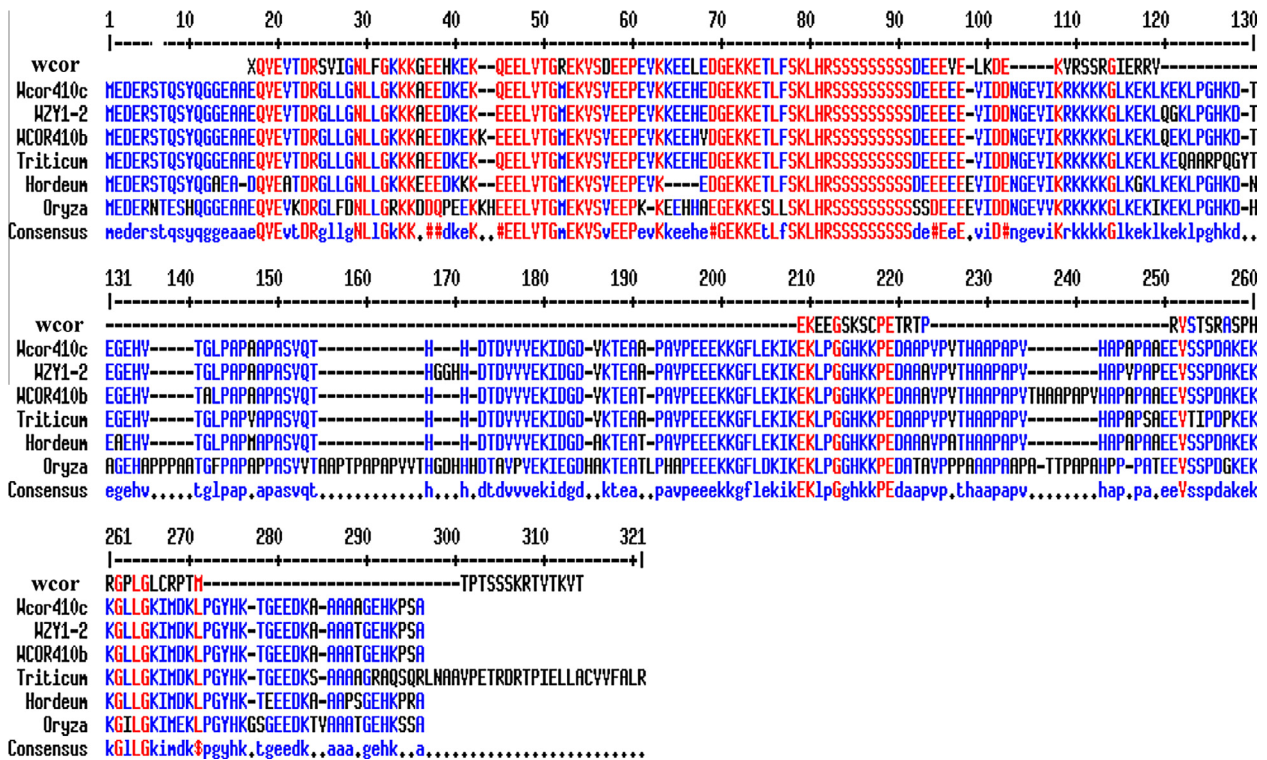

Fig. 3 Alignment of the deduced amino acids of *wcor* with the most closely related DHNs from wheat (*T. aesitivum*, *wcor410c*, U73211), (*T. turgidum* subsp. Durum *dhn11*, AJ890140), (*T. aesitivum wcor410b*, U73210), (*T. aesitivum wzy1-2,* EU124658), (*Hordeum vulgare* dehydrin, AF181458) and (*Oryza sativa* SK3-type dehydrin, EF444534).

of the experiment. Sayar et al. [24] reported that there was a decrease in the seedling fresh weight of both drought tolerant and sensitive durum wheat cultivars as osmotic potentials increased. Moreover, the dry weight of Sids was not significantly affected by drought by the end of the experiment although drought significantly decreased dry weight of Gmiza. Drought

may limit the growth of the plant organs thereby reducing meristems and finally decreasing the capacity of vegetation [25]. More increase in dry weight of Sids was detected after 16 days drought stress than the other cultivar. This means that Sids was the superior cultivar in its growth and this probably due to the vigor and strength of its genotype.

Specific leaf area is a marker for the regulation of plant leaf following abiotic stress factors including drought [26]. The specific leaf area was larger in Sids, the more tolerant cultivar, than in Gmiza the less tolerant one. According to our results, drought did not change Sids leaf area at early stress but led to a significant decrease during the period from 26 to 30 DAS. Conversely, drought stress caused insignificant decrease in leaf area in Gmiza comparing control values. Sayar et al. [24] found decrease in leaf area in both tolerant and sensitive cultivars of durum wheat linearly as osmotic potentials in PEG increased. Reduction of leaf area in the more tolerant cultivar, Sids may be a try to decrease the transpiring area as an adaptive response to water deficit for avoiding water loss.

Cell membranes are among the first targets of adverse stresses and the maintenance of membrane integrity and stability under abiotic stress conditions is a major component of environmental stress tolerance in plants [27]. Our results showed a reduction in membrane stability index in both genotypes due to mild drought stress followed by severe reduction in sensitive genotype. The decrease in MSI progressively augmented with increasing drought severity. These results are in agreement with those of Filippou et al. [28].

In addition, the present results revealed that mild drought induced H_2O_2 generation in Sids and Gmiza. H_2O_2 content sharply dropped in Sids at 23 DAS but its accumulation continued in Gmiza. These findings are in accordance with the results of Wang et al. [29] who found that exposure of plants to moderate stress can induce tolerance to severe stress. Such treatment can improve tolerance to other stress as so-called cross-acclimation to stress usually functions to rescue plants from oxidative stress and lipid peroxidation [30]. Severe drought stimulates H_2O_2 production in Gmiza plants suggesting a lack of an enhanced capacity for protection from oxidative damage caused by drought stress in this sensitive cultivar. Actually, H_2O_2 produced by photorespiration can act on the redox states of leaf antioxidant pools, implicating the possibility of photorespiratory H_2O_2 as a signal role under drought [31].

The results of antioxidant enzymes showed variation and differentiation between tolerant and sensitive genotypes. CAT enzyme showed high significant activity in Sids genotype at all intervals and reached its maximum activity at moderate drought stress. Contrarily, there was no significant response to drought stress in the sensitive cultivar Gmiza except on 30 DAS. Accordingly, Rivero et al. [32] found that CAT remained more active for a greater duration of drought stress. APX and POD might be responsible for the fine modulation of reactive oxygen species (ROS) for signaling, whereas CAT might be responsible for or the removal of excess ROS during stress [33]. Moreover, GPX activity showed significant increase in Sids genotype nearly during all the experimental period. However, there was no significant response in Gmiza to drought. Various stressful conditions of the environment have been shown to induce the activity of GPX [34]. POD, APX and glutathione reductase are involved in fine regulation of ROS and loss of their activities resulted in build up of ROS to high levels that resulted in CAT induction. It appears that there was an association between the higher antioxidant capacity and higher tolerance to drought stress in our tolerant wheat cultivar. The association between the levels/activities of antioxidant enzymes and plant drought tolerance has been previously observed [35]. This strong antioxidant system under stress conditions plays a major role in stress tolerance of both leaves and roots of many species.

It has been reported that dehydrin accumulation is correlated with dehydration when dehydrin accumulation was compared in dried cereal seedlings [36]. *dhn* data of the present results were in accordance with that observed by Lopez et al. [37] in drought-tolerant wheat compared with drought-sensitive plants. Dehydrins were thought to be associated with crucial protective functions [10]. Recently, antioxidative activity has been proposed to be an important function of dehydrins [38], since *CuCOR19* was proposed to have antioxidative activity [39] and *PvSR3* dehydrin was suggested to protect cells against oxidative damage caused by ROS and metal ions [40]. In accordance, the present results exhibited a general relationship between enzymatic antioxidants and *dhn*. Indeed, the more tolerant cultivar, Sids showed greater stimulation of antioxidant enzymes and also a 3-fold increase in the mRNA of *dhn*.

Regarding the transcript level of *wco*r gene, drought stress resulted in over regulation in Sids cultivar more than Gmiza. On the other hand, rewatering both cultivars resulted in sharp reduction in the transcript level of *wcor* gene to reach its minimum level during treatment period. Moreover, there was a dense early expression for *wcor* gene after 37 cycles in the droughted Gmiza after 6 days drought. Similarly, findings of Kurahashi et al. [41] on wild wheat *Aegilops tauschii* showed that KU-2829A, with high ABA sensitivity and drought tolerance, showed more rapid response in cold-responsive / late embryogenesis abundant (*cor*/*lea*) gene expression than the ABA-insensitive and drought-sensitive KU-2811. As indicated for *dhn*, there was a relationship between *wcor* and the efficiency of the antioxidant enzymes. The transcript level of *wcor* was increased more in the tolerant cultivar, Sids than in the sensitive one particularly under severe drought stress. The same pattern was also detected regarding the antioxidant enzymes supporting their relationship.

dreb2 homologs contribute to increase multiple stress tolerance in several plant species [42–45] expression is induced by low temperature, drought, NaCl exposure, and exogenous ABA treatment [46]. Drought stress differentially regulated the expression level of *dreb* gene in both cultivars. The increased *dreb* expression by drought was reported by Chen et al. [47]. In this context, the level of mRNA of *dreb* was sharply decreased after 6 days of dehydration in the sensitive cultivar, Gmiza but increased in the more tolerant one, Sids. Such increase continued to become maximum value after 10 days of water stress. A similar trend was also detected in enzymatic antioxidants pointing out to the presence of a general relationship between antioxidants and *dreb*. Altering the expression level of a single *dreb*/*cbf* can increase or decrease levels of expression of other transcription factors, which in turn, can lead to activation of a larger number of target genes, some of which may be directly activated not by the initially overexpressed *dreb*/*cbf* but by downstream *drebs*/*cbfs*. Several research groups have shown that raising levels of *dreb*/*cbf* expression by plant transformation enhance expression of

downstream target genes encoding late embryogenesis abundant (LEA) proteins, also known as dehydrins (DHNs), and cold-responsive (COR) proteins [48].

Conclusions

The present work is an attempt to clarify the direct relationship between *wdreb* and the two *cor* and *dhn* genes in development of drought stress tolerance in wheat using two cultivars with different drought tolerance. According to the present results, *dreb* seemed to activate the transcription process of *Tadhn* and *wcor*. Almost all genes examined in Sids and Gmiza appeared to be regulated by drought stress and subsequent rewatering in a variable manner. The most striking overall trend observed was the acute induction in expression of *dhn*, *wcor* and *dreb* in leaves tissue during severe water stress conditions in tolerant genotype Sids. Moreover, there was a general relationship of the enzymatic antioxidants with the genes *dhn*, *wcor* and *dreb*. A further study is needed to elucidate the transcription factors in relation to these antioxidant enzymes with the dreb genes. Some transcription factors that regulate the expression of several genes related to stress have been discovered [6]. Transcriptome analysis has revealed that dozens of transcription factors are involved in the plant response to various stresses [49,50]. Transcription factors control the rates of transcription to regulate the amounts of gene products (RNA and protein) available to the cell. DREBs are important plant transcription factors that regulate the expression of many stress-inducible genes [51]. So, the data presented here indicate a cross-talk between physiological and molecular tolerance mechanisms in response to drought stress.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

References

[1] Pennisi E. Plant genetics: the blue revolution, drop by drop, gene by gene. Science 2008;320:171–3.
[2] Li W, Zhang P, Fellers JP, Friebe B, Gill BS. Sequence composition, organization, and evolution of the core Triticeae genome. Plant J 2004;40:500–11.
[3] Nemat Alla MM, Hassan NM. Changes of antioxidants and GSH-associated enzymes in isoproturon-treated maize. Acta Physiol Plant 2007;29:247–58.
[4] Lawlor DW, Cornic G. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ 2002;25:275–94.
[5] Molina C, Rotter B, Horres R, Udupa SM, Besser B, Bellarmino L, et al. SuperSAGE: the drought stress-responsive transcriptome of chickpea roots. BMC Genomics 2008;9:553.
[6] Agarwal PK, Agarwal P, Reddy MK, Sopory SK. Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Reports 2006;25:1263–74.
[7] Shinozaki K, Yamaguchi-Shinozaki K. Gene networks involved in drought stress response and tolerance. J Exp Bot 2007;58:221–7.
[8] Lee SJ, Kang JY, Park HJ, Kim MD, Bae MS, Choi HI, et al. DREB2C interacts with ABF2 a bZIP protein regulating abscisic acid responsive gene expression and its overexpression affects abscisic acid sensitivity. Plant Physiol 2010;153:716–27.
[9] Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K. Improving plant drought salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol 1999;17:287–91.
[10] Allagulova CR, Gimalov FR, Shakirova FM, Vakhitov VA. The plant dehydrins: structure and putative functions. Biochemistry 2003;68:945–51.
[11] Wahid A, Close T. Expression of dehydrins under heat stress and their relationship with water relations of sugarcane leaves. Biol. Plant 2007;51:104–9.
[12] Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamoun N, et al. Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. Plant Cell 1998;10:623–38.
[13] Rorat T. Plant dehydrins-tissue location structure and function. Cell Mol Biol Lett 2006;11:536–56.
[14] Aebi H. Catalase *in vitro*. Method Enzymol 1984;105:121–6.
[15] Chance B, Maehly AC. Assay of catalase and peroxidase. Method Enzymol 1955;2:764–75.
[16] Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidise in spinach chloroplasts. Plant Cell Physiol. 1981;22:867–80.
[17] Giannopolitis CN, Ries SK. Superoxide dismutase I. Occurrence in higher plants. Plant Physiol 1977;59:309–14.
[18] Kocheva KV, Georgiev GI, Kochev VK. A diffusion approach to the electrolyte leakage from plant tissues. Physiol Plant 2005;125:1–9.
[19] Blum A, Ebercon A. Cell membrane stability as a measure of drought and heat tolerance in wheat. Crop Sci 1981;21:43–7.
[20] Alexieva V, Sergiev I, Mapelli S, Karanov E. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 2001;24:1337–44.
[21] Chomczynski P, Mackey K, Drews R, Wilfinger W. DNAzol: a reagent for the rapid isolation of zgenomic DNA. Biotechniques 1997;22:550–3.
[22] Fuminori K, Takumi S, Egawa C, Ishibashi M, Nakamura C. Expression pattern of the low temperature responsive genes in a dominant ABA-less-sensitive mutant of common wheat. Physiol Plant 2006;127:612–23.
[23] Snedecor W, Cochran G. Statistical methods. 7th ed. Ames, IA: The Lowa State University Press; 1980.
[24] Sayar R, Bchini H, Mosbahi M, Khemira H. Response of durum wheat (*Triticum durum* Desf.) growth to salt and drought stresses. Czech J Genet. Plant Breed 2010;46:54–63.
[25] Sankar B, Jaleel CA, Manivannan P, Kishorekumar A, Somasundaram R, Panneerselvam R. Drought induced biochemical modifications and proline metabolism in *Abelmoschus esculentus* (L). Moench Acta Bot Croat 2007;66:43–56.
[26] Monclus R, Dreyer E, Villa M, Delmotte FM, Delay D, Petit J-M, et al. Impact of drought on productivity and water use efficiency in 29 genotypes of Populus deltoids3Populus nigra. New Phytologist 2006;169:765–77.
[27] Levitt J. Response of plants to environmental stresses: chilling, freezing and high temperature stresses. New York: Academic Press; 1980.
[28] Filippou P, Chrystalla A, Vasileios F. Effect of drought and rewatering on the cellular status and antioxidant response of *Medicago truncatula* plants. Plant Sign Behav 2011;6:270–7.

[29] Wang WX, Barak T, Vinocur B, Shoseyov O, Altman A. Abiotic resistance and chaperones: possible physiological role of SP1, a stable and stabilizing protein from Populus. In: Vasil IK, editor. Plant biotechnology. Kluwer: Dordrecht; 2000. p. 439–43.
[30] Bowler C, Fluhr R. The role of calcium and activated oxygens as signals for controlling cross-tolerance. Trends Plant Sci 2000;5:241–6.
[31] Noctor GS, Veljovic-Jovanovic S, Driscoll L, Novitskaya V, Foyer CH. Drought and oxidative load in the leaves of C3 plants: a predominant role for photorespiration. Ann Bot 2002;89:841–50.
[32] Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R, Gepstein S, et al. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proc Nat Acad Sci USA 2007;104:19631–6.
[33] Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 2002;7:405–10.
[34] Kovalchuk I. Multiple roles of radicles in plants. In: Dutta Gupta SEd. Reactive oxygen species and antioxidants in higher plants. CRC Press; 2010. p. 31–44.
[35] Benesova M, Hola D, Fischer L, Jedelsky PL, Hnilicka F. The physiology and proteomics of drought tolerance in maize: early stomatal closure as a cause of lower tolerance to short-term dehydration? Plos One 2012;7:e38017.
[36] Close T, Fenton R, Moonan F. A view of plant dehydrates using antibodies specific to carboxyl terminal peptide. Plant Mol Biol 1993;23:279–86.
[37] Lopez CG, Banowetz GM, Peterson CJ, Kronstad WE. Differential accumulation of a 24-kd dehydrin protein in wheat seedlings correlates with drought stress tolerance at grain filling. Hereditas 2001;135:175–81.
[38] Hara M, Shinoda Y, Tanaka Y, Kuboi T. DNA binding of citrus dehydrin promoted by zinc ion. Plant Cell Environ 2009;32:532–41.
[39] Hara M, Terashima S, Fukaya T, Kuboi T. Enhancement of cold tolerance and inhibition of lipid peroxidation by citrus dehydrin in transgenic tobacco. Planta 2003;217:290–8.
[40] Zhang JH, Jia WS, Yang JC, Ismail AM. Role of ABA in integrating plant responses to drought and salt stresses. Field Crops Res 2006;97:111–9.
[41] Kurahashi Y, Terashima A, Takumi S. Variation in dehydration tolerance, ABA sensitivity and related gene expression patterns in D-genome progenitor and synthetic hexaploid wheat lines. Int J Mol Sci 2009;10:2733–51.
[42] Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, et al. Functional analysis of an arabidopsis transcription factor, DREB2A, involved in drought- responsive gene expression. Plant Cell 2006;18:1292–309.
[43] Qin F, Kakimoto M, Sakuma Y, Maruyama K, Osakabe Y, Phan Tran LS, et al. Regulation and functional analysis of ZmDREB2A in response to drought and heat stresses in Zea mays L. Plant J 2007;50:54–69.
[44] Kobayashi F, Ishibashi M, Takumi S. Transcriptional activation of *Cor/Lea* genes and increase in abiotic stress tolerance through expression of a wheat DREB2 homolog in transgenic tobacco. Transgenic Res 2008;17:755–67.
[45] Morran S, Eini O, Pyvovarenko T, Parent B, Singh R, Ismagul A, et al. Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotechnol J 2011;9:230–49.
[46] Egawa C, Kobayashi F, Ishibashi M, Nakamura T, Nakamura C, Takumi S. Differential regulation of transcript accumulation and alternative splicing of a DREB2 homolog under abiotic stress conditions in common wheat. Gene Genet Syst 2006;81:77–91.
[47] Chen Y, Chen P, de los Reyes B. Differential responses of the cultivated and wild species of soybean to dehydration stress. Crop Sci 2006;46:2041–6.
[48] Battaglia M, Olvera-Carrillo Garciarrubio A, Campos F, Covarrubias AA. The enigmatic LEA proteins and other hydrophilins. Plant Physiol 2008;148:6–24.
[49] Mochida K, Yoshida T, Sakurai T, Yamaguchi-Shinozaki K, Shinozaki K, Tran LSP. In silico analysis of transcription factor repertoire and prediction of stress responsive transcription factors in soybean. DNA Res 2009;16:353–69.
[50] Vinocur B, Altman A. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opin Biotechnol 2005;16:123–32.
[51] Lata C, Prasad M. Role of DREBs in regulation of abiotic stress responses in plants. J Exp Bot 2011;62:4731–48.