 Differences in the drought stress response of DREB2 and CAT1 genes and evaluation of related physiological parameters in some bread wheat cultivars

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ABSTRACT

Abiotic stresses such as drought are among the most significant environmental stress causes in economically important crop plants including wheat (Triticum aestivum L.) and improving their yield is a major goal of plant breeding. In this study, we analysed expression of dehydration responsive element binding factor (DREB2), and an important antioxidant enzyme gene CAT1 in flag leaf of three bread wheat cultivars named Kavir, Kalheydari and Shahpasand under drought stress by RT-qPCR. In another section, physiological parameters including catalase enzyme (EC 1.11.1.6), relative water content (RWC) and chlorophyll content (a, b and total) were analysed. The cultivars were planted in a randomized complete block design with three replicates in normal and drought stress conditions on experimental field in 2013. Differential expression patterns of the genes DREB2 and CAT1 were observed in cultivars. The observed trend was the high induction in the expression of genes under drought stress. The expression of DREB2 was more than CAT1 in drought stress. The relative genes expression showed significant correlation with the catalase, RWC and chlorophyll b. Under drought stress, Kavir had higher expression of DREB2 and CAT1, activity of catalase, RWC, chlorophyll content (a, b and total) than the other cultivars. According to increasing of drought tolerance through the expression of these genes, it can be concluded that transferring of these genes may enhance drought tolerance in high-yield wheat cultivars.

Abbreviations:

DREB2: dehydration responsive element binding factor
CAT1: catalase gene
RWC: relative water content
Chl a: chlorophyll a
Chl b: chlorophyll b
Chl T: total chlorophyll
ROS: reactive oxygen species
TFs: transcription factors

Introduction

Agricultural productions have been adversely affected worldwide by environmental stresses, especially by drought. Improvement of crop plants tolerance to these stresses is practised using traditional and modern breeding methods and understanding of responses at each growth stage is essential for progress in breeding and genetic engineering. Upon exposure to abiotic stress conditions, plants undergo a variety of changes from physiological adaptation to gene expression [1]. An understanding of how plants respond to water stress at the genomic level is essential for future genetic engineering and crop breeding. Mechanisms of dehydration responses in vegetative tissues is associated with desiccation tolerance in an ABA-dependent and ABA-independent manner [2,3]. Among ABA-independent manner, drought-responsive element binding factors (DREB) have an important role [4-7]. DREB TFs play key roles in plant stress signal transduction pathway. They can specifically bind to DRE/CRT element (G/ACCGAC) and activate the expression of many stress-inducible genes [8]. Wheat DREB2 transcription factor (WDREB2) is a homologue of DREB2A in Arabidopsis [9]. The WDREB2 expression is activated by cold, drought, salt, heat shock and exogenous ABA treatment [10,11]. On the other hand, production of ROS is provoked by a variety of natural and stress stimuli and subsequently can seriously disrupt normal metabolism through oxidative damage to cellular components [12,13]. Catalase has the highest conversion efficiency among all antioxidant enzymes...
enzymes so that one molecule of catalase can remove about six million molecules of H$_2$O$_2$ per minute [14]. Under water deficit, plants can control osmotic pressure by accumulating osmolyte substances, which leads to the maintenance of turgor and higher relative water content (RWC) in leaves [15]. The content of chlorophyll can be a useful index for evaluating photosynthesis. Many reports showed that drought could lead to lower photosynthesis efficiency, damage to the photosynthetic apparatus and diminished chlorophyll content [16–19]. As an important crop in the world, wheat production is severely reduced by drought stress. Therefore, it is necessary to understand the molecular mechanism of wheat in response to drought stress to provide scientific basis for genetic improvement of stress resistance [20,21]. The aim of our work was to compare stress and non-stress environment improvement of stress resistance [20,21]. The aim of our work was to compare stress and non-stress environment differences among three wheat cultivars in some physiological traits and in the expression of protective genes DREB2 and CAT1 at the heading stage. These could be used as selection criteria in screening varieties suitable for further breeding in dry conditions and to study responses of different genotypes to the same stress conditions.

Materials and methods

Plant materials and experimental design

The experiment was carried out at a research field of Shahid Bahonar University of Kerman, Iran (30°15’ N and 56°58’ E, 1753.8 m ASL). The mean annual precipitation and temperature were 154.1 mm and 25.5 °C, respectively. In this experiment, three wheat cultivars, namely Kavir, Kalheynadi and Shahpasand, were cultivated in a randomized complete block design (RCBD) with three replicates in normal and drought stress environments on experimental field in 2013. The cultivars used in this study were provided from the Seed and Plant Improvement Institute – Cereal Research Department of Iran – Karaj (Source: http://www.iranwheat.ir). Each plot containing four rows with 2 m length with 20 cm distance from each other. The distance of planting on row was considered 5 cm. Agricultural operations such as fertilization, weeding and spraying were carried out uniformly and equally to all replicates. In order to create water stress due to climate conditions of the region, irrigation was cut from the shooting stage in drought stress environment. Sampling was done from flag leaf conducted after signs of stress in drought environment. The leaf samples from the normal and drought-treated plants were harvested, flash-frozen in liquid nitrogen, and stored at −80 °C until used for RNA isolation. Variations in DREB2 and CAT1 genes expression, catalase enzyme, RWC and chlorophyll content (a,b and total) under stress and non-stress environment among three cultivars were investigated.

Gene expression analysis

Total RNA (0.1 g) was extracted from flag leaves of all cultivars in two environments using RNeasy extraction kit (Qiagen USA, Valencia, CA) according to the company’s protocol. DNA was removed from RNA samples by treatment with RNase-free DNase I (Thermo Scientific, Valencia, USA) following the instruction protocol. Purity of total RNA was assessed by measuring the ratio of 260/280 nm (A260/280 > 2.0). Quality of RNA was assessed on agarose gel (1.2%) electrophoresis. First-stranded cDNA of all RNA samples were synthesized using cDNA synthesis kit by M-MuLV reverse transcriptase and random hexamer primer (Roche Applied Science GmbH, Germany) according to the company’s recommendation. Furthermore, two negative control reactions (no reverse transcriptase and no template control) were prepared. Gene-specific primers used in real-time qPCR experiments were designed by Gene Runner software (4.0.9.6 Beta) and checked by the BLAST program in wheat sequences available in the NCBI database to ensure that the primers amplify the desired unique cDNA segment (Table 1). Quantitative PCR (RT-qPCR) with SYBR Green was performed on a Rotor Gene 3000 machine (Corbett Research, Australia). 20 μL PCR reactions contained 2 μL cDNA, 10 μL 2 × SYBR Premix Ex Taq II (TaKaRa, Dalian, China) and 100 nmol/μL primers and appropriate amounts of sterile, double-distilled water. The reactions were mixed gently and incubated at 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. A dissociation step from 72 to 94 °C with ramping increments of 0.1 °C/s was added to assess the amplification specificity for each amplicon through melting curve analysis. 18S rRNA primers were used as reference gene to normalize the total amounts of cDNA present in each reaction. All gene expression analyses were conducted in at least three independent biological and two technical replicates accompanied by negative controls. Data were exported from the Rotor Gene 3000 to Excel analysis sheet and analysed by LinReg PCR (v.2013.0) software

| Gene   | Accession number | Table 1. The primer sequence used in qPCR reaction and the accession number in NCBI. |
|--------|------------------|------------------------------------------------------------------------------------------|
| DREB2-f | AB193608.1       | 80 GGG GCC GAC TTT TCT TTC TC                                                          |
| DREB2-r |                   | TCG CAA TCT TGT CGC CGT TT                                                            |
| CAT1-f  | D86337.1          | 140 CAT CTG GCT CTC CTA CGT G                                                        |
| CAT1-r  |                   | AGA ACT TGG AGG GCC CGT A                                                               |
| 18S rRNA-f | AY049040.1       | 107 GCC TAG TAA GCC CGA GTC AT                                                         |
| 18S rRNA-r |                 | GCG ATC CGA ACA CTT CAC C                                                               |
using a window of linearity. The efficiency of amplification and the initial amount of each amplicon were determined by LinReg PCR [22].

**Catalase enzyme extraction and assay**

Catalase activity (EC 1.11.1.6) was determined according to Dhindsa et al. [23], as the rate of destruction and drop in the absorption of hydrogen peroxide at 240 nm (ε = 39.4 mmol/L/cm). One unit of catalase activity was defined to be equivalent to the amount of enzyme required to degrade 0.1 μmol H₂O₂ /min/mg protein. Concurrently, the protein content of the extracts was also determined according to the standard procedure of Bradford (1976) [24], using BSA (Sigma, USA) as a standard.

**Determination of relative water content (RWC)**

The fresh weight (FW) of wheat flag leaves was measured immediately after cutting from the plants. The flag leaves were then soaked in distilled water in dishes for 24 h at room temperature under low-light conditions. Then the leaves were quickly and carefully dried with filter paper to determine turgid weight (TW). Dry weight (DW) was obtained after oven-drying the leaf samples for 72 h at 70 °C. RWC was calculated from the equation of Tambussi et al. [25]: RWC (%) = [(FW − DW)/(TW − DW)] × 100.

**Photosynthetic pigments**

Chlorophylls (Chl a and Chl b) were extracted and measured according to Lichtenthaler and Buschmann [26]. To achieve highly purified and less contaminated sample, flag leaves chlorophyll were extracted with 80% acetone and then estimated spectrophotometrically, by reading absorbance at 646.8 and 663.2 nm in WAP spectrophotometer model S2000 UV/vis. The chlorophyll content was calculated according to the following equations:

\[
\text{Chl a (mg / mL)} = (12.25A_{663.2} - 2.79A_{646.8})
\]

\[
\text{Chl b (mg / mL)} = (21.21A_{646.8} - 5.1A_{663.2})
\]

\[
\text{Chl T (mg / mL)} = \text{Chl a} + \text{Chl b}
\]

**Statistical analysis**

Two experiments were conducted out in an RCBD with three replicates in the normal and drought stress conditions. All experimental data were analysed as mean ± standard error of at least three experimental and two technical replicates. Each value was, therefore, a mean of six estimations (n = 6). Factorial analysis of variance (ANOVA) was applied; F values were calculated whereby two main effects and interactions were considered. Differences among treatment means were assessed by Duncan’s multiple range test at P ≤ 0.05, thus a correlation coefficient between DREB2 gene, CAT1 gene, catalase enzyme, RWC and chlorophyll content was also calculated [27].

**Results and discussion**

Among abiotic stresses, drought is a major factor responsible for yield loss in agriculture. Drought tolerance is a polygenic complex of traits, including a range of morpho-physiological and biochemical adaptations. Previous research works indicated that many genes are involved in drought response [28,29]. DREBs are important transcription factors that regulate the expression of many stress-inducible genes [30]. Data analysis of DREB2 expression (Table 2) revealed significant difference in the stress, cultivar effects (P ≤ 0.01) and interaction effect (P ≤ 0.05). Significance of interaction effect indicates different response of the Kavir, Kalheydar and Shahpasand cultivars to drought stress conditions. The mean comparison analyses of expression of DREB2 in three cultivars, Kavir, Kalheydar and Shahpasand, showed significant differences in the whole condition of normal and stress (P ≤ 0.05). According to the results of comparisons of mean based on Multiple Rang Duncan’s test (Figure 1), the relative expression of DREB2 for cultivar × stress effect in the Kavir and Kalheydari to Shahpasand was approximately 1.5-fold in stress environment, while in non-stress or normal environment, expression between these cultivars was roughly in one statistical level. Our results from RT-qPCR showed that transcript level of DREB2 was upregulated significantly under drought stress (Table 1; Figure 1). This result is in agreement with those in [7,10,31–33]. The level of mRNA of

| S.O.V     | df  | DREB2       | CAT1       | RWC         | Catalase   | Chl a       | Chl b       | Chl T       |
|-----------|-----|-------------|------------|-------------|------------|-------------|-------------|-------------|
| Stress    | 1   | 10.59**    | 8.56**     | 9546.4**    | 0.056**    | 215.5**     | 146.27**    | 643.4**     |
| Cultivar  | 2   | 0.52**     | 0.39**     | 1067**      | 0.023**    | 91.85**     | 25.92**     | 242.1**     |
| Cultivar × Stress | 2 | 0.35**     | 0.23**     | 53.2        | 0.008      | 4.16        | 3.07        | 10.04       |
| Error     | 10  | 0.05       | 0.06       | 68.2        | 0.002      | 12.92       | 4.45        | 17.02       |

**Table 2.** Variance analysis of the expression DREB2, CAT1 genes and some physiological traits in drought stress for leaf in three cultivars of wheat.

**Notes:** **Significant differences at P ≤ 0.05 or P ≤ 0.01, respectively, as based on F test**
DREB2 was sharply increased in the tolerant cultivar, Kavir, but decreased in the more sensitive one, Shahpasand. The relatively higher level of DREB2 expression could point to the presence of other protective mechanisms that manifest under field conditions. Under the stress conditions, production of ROS is greatly increased and the oxidative burst occurs. The leading role in protecting the plants from ROS belongs to antioxidant enzymes. Catalase is one of the major systems in the plant for the enzymatic removal of hydrogen peroxide in peroxisomes [34]. In our study, we also investigated the effects of drought stress on the expression of CAT1 encoding antioxidant enzymes at the mRNA level. The results of ANOVA for CAT1 are provided in Table 2. As it is evident, the main effects of stress and cultivar were, respectively, significant at 1% and 5% statistical levels, meanwhile the interaction effect was not significant. Based on the comparison mean analyses of the two stress and non-stress conditions, CAT1 expression in the Kavir and Kalheydari was greater than Shahpasand cultivar, while classified under two different statistical levels (a, b). The CAT1 expression of Kavir, Kalheydari and Shahpasand was 1.53, 1.44 and 1.05, respectively (Figure 2(A)). The CAT1 relative expression of Kavir and Kalheydari was approximately 1.37-fold to Shahpasand. According to all cultivars, the relative gene expression of CAT1 was significant at 1% statistical level in stress and non-stress conditions as the value of gene expression was threefold in stress to non-stress condition (Figure 2(B)). The results of ANOVA on catalase antioxidant enzyme revealed that the main effects of stress and cultivar were significant at 1% statistical level (Table 2). However, the interaction effect was not significant which was in agreement with CAT1 results. According to Duncan’s mean analyses (Figure 3(A)), Kavir cultivar has significantly the greatest level of catalase enzyme activity among all cultivars. Drought stress increased the rate of catalase enzyme threefold in comparison to normal condition (Figure 3(B)). We detected a significant increase in expression during drought stress in three cultivars (Table 1; Figures 2(A) and 3(A)). The increase of catalase enzyme activity in plants under abiotic stresses was also reported in other studies [35–43]. The results suggested that antioxidant capacity was higher in drought-resistant cultivar (Kavir) than in drought-sensitive cultivar (Shahpasand) under drought stress. Thus, upregulated expression of CAT1 was in accordance with upper catalase activity in drought-resistant cultivar throughout the drought stress period. These finding is in accordance with the results of Wang et al. [44] that the upregulated expression of CAT was associated with higher catalase activity in the pre-acclimation followed by post-anthesis heat stress in wheat. Since the CAT1 gene is upregulated under drought stress conditions, it can be used as a candidate gene for the improvement of drought tolerance in wheat. RWC is known as an indicator of damage by drought stress and a positive correlation has also been
reported between RWC index with wheat yield under drought stress [45,46]. Therefore, this trait can be used as one of the best indicators of equilibrium water in plant breeding strategies under stress [47]. Analysis of variance of RWC revealed that the main effects of stress and cultivar were statistically significant at 1% statistical level but the interaction effect was not significant (Table 2). The mean analyses according to Duncan’s test for cultivar effect revealed that the Kavir cultivar has a significant difference with Kalheydari and Shahpasand at 5% statistical level. The RWC for Kavir, Kalheydari and Shahpasand was 66.63, 46.32 and 41.5, respectively. According to Duncan’s test (Figure 4(A)), drought stress reduced significantly the RWC of leaf, so that a 2.6-fold reduction in leaf RWC was seen in comparison to normal condition in all three cultivars. According to Kamoshita et al. [48], genotypes that can keep a higher RWC are more resistant to drought. The greater decrease of RWC due to drought-induced stress is connected mainly with the capacity of more tolerant genotypes to better absorb soil water and to prevent water loss through stomata [49]. Also, the results of RWC on three wheat cultivars revealed that the tolerant cultivar (kavir) keeps a larger amount of water than the sensitive (Shahpasand) and RWC decreased significantly in drought stress condition. These results are in agreement to the results about decrease in RWC in stress condition [47,50–52]. The results of ANOVA for Chl a revealed that the main effects of stress and cultivar were significant at 1% statistical level only, and the interaction was not significant (Table 2); therefore, mean analyses based on Duncan’s test were done. Overall, the results of Chl a in the sum of all three cultivars showed that the amount Chl a reduced 1.58-fold at 1% statistical level in comparison to non-stress condition (Figure 5(B)). The contents of Chl a in the Kavir, Kalheydari and Shahpasand were 19.61, 15.15 and 11.81, respectively, in all conditions. Multiple Rang Duncan’s test showed a significant difference between the Kavir and other cultivars at 5% statistical level (Figure 5(A)). Based on ANOVA on Chl b (Table 2), the main effects of cultivar and drought stress were significant at 5% and 1% levels, respectively. However, the interaction effect of cultivar and stress was not significant and only the single effects can be interpreted. According to Duncan’s test (Figure 5(A)), a significant difference was seen between the Kavir and Shahpas, and at
Stress on chlorophyll (a, b and total) contents in all cultivars and response to drought stress in Kavir had the highest chlorophyll (a, b and total) content under drought stress which is in agreement with previous results in abiotic stresses [53–56]. According to Figure 5, we found that Kavir cultivar had higher genes expression, catalase enzyme activity, RWC, and Chlorophyll content than the other cultivars under drought stress at the heading stage. This suggests that drought-resistant wheat cultivars resisted the damage caused by drought stress more effectively. This finding could be applied in predicting drought-resistant wheat cultivars. A clear correlation was also demonstrated between the loss of RWC, chlorophyll contents, increase of catalase and the expression of DREB2 and CAT1 genes in wheat cultivars. These results will greatly contribute to the improvement of plant resistance through genetic breeding and genetic manipulation by plant breeders.

Table 3. Correlation coefficients for the relationship between relative expression (RE) of DREB2 and CAT1 and physiological traits of Kavir cultivar.

|          | DREB2 | CAT1 | RWC | Catalase | Chl a | Chl b | Chl T |
|----------|-------|------|-----|----------|-------|-------|-------|
| Chl T    | –0.45 | –0.46| 0.86**| –0.09   | 0.96**| 0.88**|       |
| Chl b    | –0.52*| –0.56*| 0.89**| –0.15   | 0.76**| 1      |       |
| Chl a    | –0.41 | –0.41| –0.77**| –0.08   | 1      |       |       |
| Catalase | 0.76**| 0.63**| –0.25 | 1        |       |       |       |
| RWC      | –0.65**| –0.71**| 1    | 1        |       |       |       |
| CAT1     | 0.94**| 1    |     |          |       |       |       |
| DREB2    | 1     |      |     |          |       |       |       |

**,** Significant differences at $P \leq 0.05$ or $P \leq 0.01$, respectively.

Conclusion

The emphasis of our experiment was on the expression of two important genes, DREB2 and CAT1, which are involved in drought stress. Collectively, the studied genes showed significant response to drought stress, so it can be concluded that these genes are useful candidates for drought tolerance. Moreover, the level of DREB2 expression was greater than CAT1 and indicated the importance of DREB2 transcription factor. Our results showed that wheat cultivars differently responded to drought stress. In conclusion, our results indicated that Kavir cultivar had higher genes expression, catalase enzyme activity, RWC and Chlorophyll content than the other cultivars under drought stress at the heading stage. This suggests that drought-resistant wheat cultivars resisted the damage caused by drought stress more effectively. This finding could be applied in predicting drought-resistant wheat cultivars. A clear correlation was also demonstrated between the loss of RWC, chlorophyll contents, increase of catalase and the expression of DREB2 and CAT1 genes in wheat cultivars. These results will greatly contribute to the improvement of plant resistance through genetic breeding and genetic manipulation by plant breeders.

Disclosure statement

No potential conflict of interest was reported by the authors.
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