

## Some responses of dry farming wheat to osmotic stresses in hydroponics culture

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### Abstract

Osmotic stress is one of the major factors that significantly reduce yields in dry areas. Plants respond to this abiotic stress at physiological and molecular levels. Many genes are induced under stress conditions by transcription factors. Dehydration responsive element binding (DREB) protein is a subfamily of AP2/ERF transcription factors which control expression of many osmotic stress-inducible genes. In this study, 21 days old seedlings of Sardari cultivar, dry farming bread wheat transferred into hydroponics culture using Hoagland solution. Osmotic stress treatments performed with adding 100, 200 and 400 g/l poly-ethylene glycol 6000 to hydroponics culture to obtain -0.15, -0.49, and -1.76 MPa water potential, respectively. After the seedlings were withered and colorless, relative water content, dry weight, and photosynthesis measured. In addition, RT-PCR, and cDNA sequencing carried out. Molecular analysis of DREB translated protein sequence performed by DNAMAN, BLASTN, Pfam and PROSITE software. Results showed that osmotic stress decreased relative water content, root and shoot dry weight and net photosynthesis rate in comparison to control, significantly ( $P < 0.05$ ). Sequence alignment indicated 98% homology with other *Triticum aestivum* DREB protein mRNA. There was an AP2 domain in the translated protein with three  $\beta$ -sheets and one  $\alpha$ -helix and contains the Val<sub>14</sub> and Glu<sub>19</sub> amino acids. An EST Sequence deposited in NCBI GenBank database with the accession number of ES466900.

**Keywords:** AP2 domain, DREB, osmotic stress, physiological responses, wheat

### Introduction

Osmotic stress is one of the major factors that significantly reduce yields in dry areas (Trethowan et al., 2001). Wheat in some areas is usually grown on dry-agricultural fields and this often causes serious problems in wheat production (Tas and Tas, 2007). Acclimation to this stress, results from a series of integrated events occurring at physiological and molecular levels that helps in the retention and/or acquisition of water, protection of chloroplast functions and maintenance of ion homeostasis (Mohsenzadeh et al., 2006). Researchers have used various indices to categorize the symptoms of osmotic stress. These indices include changes in dry weight (Marcum et al., 1995; Huang et al., 1997), RWC (Schonfeld et al., 1988; Guan et al., 2004), net photosynthesis rate (Martin and Ruiz-Torres, 1992; Leidi et al., 1993; Flexas and Medrano, 2002) and gene expression (Shinozaki and Yamaguchi-Shinozaki, 1996; Bruce et al., 2002; Very and Sentenac, 2003).

AP2/ERF transcription factors are a large multigene family of plant-specific transcription factors with over 100 members, whose distinguishing characteristic is that they contain the so-called AP2 DNA-binding domain composed of 57-70 amino acid residues. They have been isolated from a variety of higher plants, such as *Arabidopsis thaliana*, tobacco, tomato, rice, maize, wheat and castor bean (Jofuku et al., 1994; Baker et al., 1994; Sakuma et al., 2002).

Many genes that function in stress tolerance are induced under stress conditions (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2003). Most of these stress-inducible genes are controlled by abscisic acid (ABA), but some are not, indicating the involvement of both ABA-dependent and ABA-independent regulatory systems in stress-responsive gene expression (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). Dehydration responsive element binding (DREB) protein is a subfamily of AP2/ERF transcription factors which contain one AP2 domain. It is known that the DREB genes are the key-genes conferring tolerance to water stress, in the ABA-independent pathway. The DREB transcription factor controls

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the expression of several target genes involved in the mechanism of osmotic stress tolerance (Liu et al., 2000; Latini et al., 2005; Badawi et al., 2007), by recognizing and binding to an essential cis-acting element, the dehydration responsive element (DRE) with a core motif of A/GCCGAC in the upstream of inducible genes (Yamaguchi-Shinozaki and Shinozaki, 1994; Cushman and Bohnert, 2000; Kizis et al., 2001; Shen et al., 2003; Liu et al., 2006; Wang et al., 2006).

Dry-farming is characterized by a somewhat short season and the nature of plant growth is modified by the arid conditions prevailing in dry-farming. So, the mechanisms involved in the response of plants to osmotic stress in dry-farming require further elucidation. We know that Sardari wheat is tolerant to drought because is a dry farming cultivar but the aim of this study was to get insight into some responses of dry farming wheat to osmotic stresses in hydroponics culture. It can help in understanding water stress tolerance mechanisms and defining conditions for identification of osmotic stress-inducible genes in the tolerant plants.

## Materials and Methods

### Plant material and growth conditions

Nine days germinated seeds of Sardari cultivar, dry farming bread wheat (*Triticum aestivum* L.), were transferred into hydroponics culture using Hoagland solution and kept for 12 days in growth chamber conditions with 16 h light, 500  $\mu\text{mol m}^{-2}\text{s}^{-1}$  fluorescent light, 22/15 °C day/night temperature and 60% humidity.

### Osmotic stress treatments

Osmotic stress treatments were performed with adding 100, 200 and 400 g/l poly-ethylene glycol 6000 (PEG 6000) to hydroponics culture to obtain -0.15, -0.49 and -0.76 MPa water potential (Michel and Kaufmann, 1973), respectively. The untreated culture was used as control. The seedlings were withered and colorless after 5 days. Seedlings from each treatment and control were harvested, quickly immersed in liquid nitrogen and stored at -20 °C for RNA extraction.

### RWC and dry weight measurements

The percentage of relative water content (RWC) was calculated as:

$$\text{RWC} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$$

Variables were the fresh weight of harvested leaves which were cut to 1cm segments (FW); the weight of leaf segments soaked in water at 4°C in

the dark for 24 h (TW); and dry weight of the segments baked at 80 °C for 24 h (DW).

### Photosynthesis measurement

Photosynthesis rate (AN) was determined by using Photosynthesis System HCM-1000 (Heinz Walz, Effeltrich, Germany). The middle part of the youngest, fully expanded leaf of all plants was placed in a gas-exchange cuvette (1010-M; Heinz Walz), while CO<sub>2</sub> concentration and flow rate were kept constant at 360 ppm. Plants were illuminated in the cuvette with 1800  $\mu\text{mol m}^{-2}\text{s}^{-1}$  PAR at 20° C during measurements. Data were automatically collected every minute after photosynthesis rate was stabilized. The rate of photosynthesis was expressed as  $\mu\text{mol CO}_2\text{ m}^{-2}\text{s}^{-1}$ .

### DNA extraction and primer design

Leaf tissue (200 mg) was ground to a fine powder in liquid nitrogen, and DNA was extracted using modified CTAB method. Primers were designed by Oligo5 software and using consensus of alignment of DREB genes for wheat from NCBI GenBank.

### RNA extraction and RT-PCR analysis

Seedling leaf samples were ground in liquid nitrogen and total RNA was extracted using Aurum™ Total RNA Mini Kit (Bio-RAD). First-strand cDNA was synthesized from extracted RNA and by cDNA Synthesis system Kit (Roche). Primer sequences for the DREB transcripts were forward primer:

5' -AAGAAGTGGGAAGGAGGAAA-3' and reverse primer: 5' -CTAAACCCATCATCACCA-3'.

Wheat actin was used as a positive control for the RT-PCR with 5' -GACCCAGACAACCTCGCAACT-3' as the forward primer and 5' -CTCGCATATGTGGCTCTTGA-3' as the reverse primer. The PCRs were carried out in 20- L solution comprising 10 ng of samples cDNA, 1 x PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.25 mM each dNTP, 2  $\mu\text{M}$  of each primer, and 1 U Taq DNA polymerase. The PCR profiles for both genes were an initial denaturation at 94 °C for 5 min followed by 35 amplification cycles (94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min) and final extension at 72 °C for 10 min. The PCR amplification products were separated in 1% (w/v) agarose gels.

### Purification of PCR product and sequencing

PCR products were purified using High Pure PCR Product Purification Kit (Roche), and then sequenced (MWG, Germany).

### Cloning

DREB sequence was cloned in pTZ57R/T plasmid and DH5 $\alpha$  strain of *E. coli* using Ins TMPCR Product T/A Cloning Kit (Fermentas). Plasmid extraction was carried out by AccuPrep<sup>®</sup> Plasmid Extraction Kit (BIONEER) to confirm the cloning via PCR and gel electrophoresis.

### Statistical analysis and computations

All physiological experiment set-ups were randomized complete block designed with three replicates. Raw data were imported to Microsoft Excel program for calculation and graphical representation. SPSS version 11.5 program was used for analysis of variance and comparison of means by Duncan's method at  $P < 0.05$ .

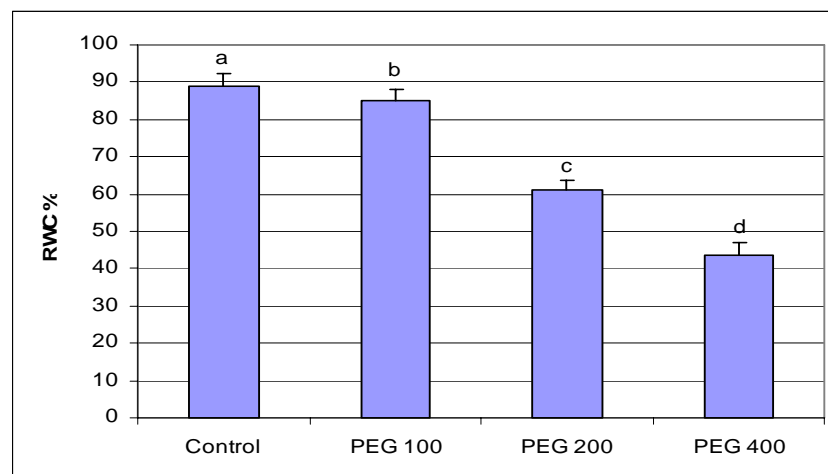
Transeq software was used for translating the nucleotide sequence to amino acid sequence. The alignment of the deduced amino-acid sequence of AP2 domain with other AP2 domain-containing carried out by protein blast search databases. Sequence similarity and several structural features

were predicted by online databases and related software including BLAST, Pfam and PROSITE.

## Results

### Plant growth and osmotic stress treatments effects on RWC and dry weight

Seeds of Sardari cultivar wheat were germinated at the rate of 88.5% in sufficient moisture and were grown under growth chamber conditions. RWC averages were 88.5% for control seedlings as the reference point for well watered plants and 84.9, 61.1, and 43.4 percent for  $-0.15$ ,  $-0.49$ , and  $-1.76$  MPa water potential, respectively. Results showed that osmotic stress decreased relative water content, significantly ( $P < 0.05$ ) (Figure 1). In addition, osmotic stress decreased both root and shoot dry weight significantly ( $P < 0.05$ ) from 0.015 and 0.033 g root and shoot dry weight, respectively in control to 0.009 and 0.019 g in plants which treated with 400 g/l polyethylene glycol 6000.



**Figure 1.** RWC in control and drought- treated plants. PEG 100, 200 and 400 g/l are equal to  $-0.15$ ,  $-0.49$ , and  $-1.76$  MPa water potential, respectively. Data are means  $\pm$ S.E. of three replicates. Treatments with the same lowercase letters were not significantly different based on mean comparison by Duncan's method at  $P < 0.05$ .

### Effects on photosynthesis rate

Figure 2 shows a significant decrease in the net photosynthesis rate as drought became severe. The most declines were seen between 100 and 200 g/l PEG treatments. In 200 g/l PEG treatment with  $-0.49$  MPa water potential the net photosynthesis rate was zero but in 400 g/l PEG with  $-1.76$  MPa water potential, the net photosynthesis rate was negative because respiration is higher.

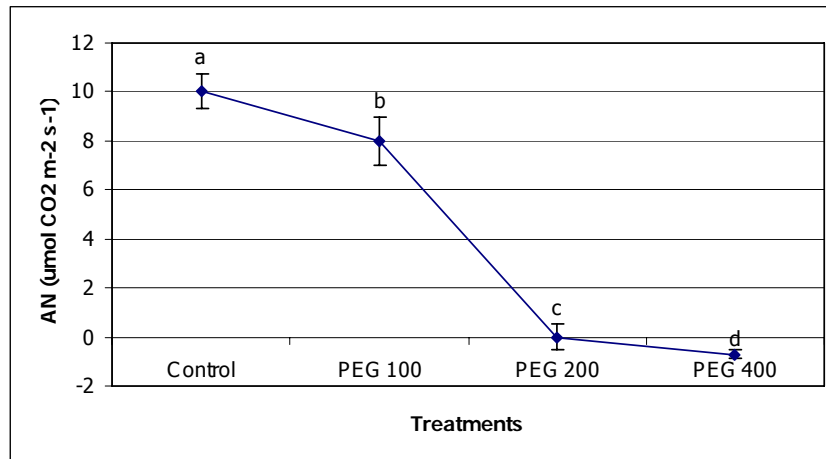
### Gel electrophoresis display

Agarose gel electrophoresis of amplified cDNA

by specific primers showed 700-bp bands for DREB gene and a 500-bp band for positive control (wheat actin) (Figure 3).

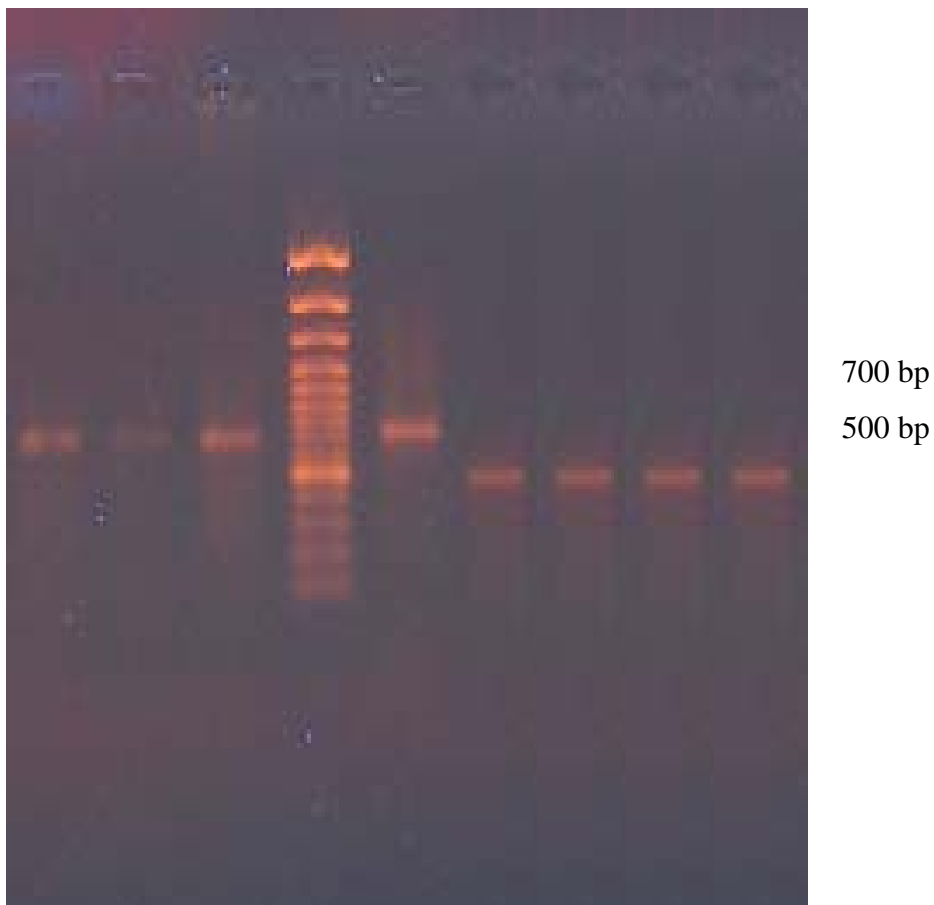
### Sequencing and bioinformatics analysis

Sequencing reported a 645 nucleotide sequence which was deposited in NCBI GenBank database with the accession number ES466900. Alignment of TaDREB sequence using blast search, indicated that it has 98% homology with DREB1 genome A and B and DREB1 mRNA of wheat.



**Figure 2.** Rate of net CO<sub>2</sub> assimilation in control and drought- treated plants. PEG 100, 200 and 400 g/l are equal to – 0.15, –0.49, and –1.76 MPa water potential, respectively. Data are means ±S.E. of three replicates. Treatments with the same lowercase letters were not significantly different based on mean comparison by Duncan’s method at  $P < 0.05$ .

0 100 200 M 400 A1 A2 A3 A4



**Figure 3.** Agarose gel electrophoresis of amplified cDNA by specific primers. cDNA constructed via RT-PCR from seedling mRNA under conditions with adding 0, 100, 200, and 400 g/l poly-ethylene glycol 6000 to hydroponics culture. M column is 100-bp ladder and A1 to A4 are expression of the actin as housekeeping gene in the four treated tissue.

The Pfam program shows that the deduced amino-acid sequence of AP2 domain including three  $\beta$ -sheets and one  $\alpha$ -helix with the valine<sub>14</sub> and glutamic acid<sub>19</sub> amino acids (Figure 4). According to protein blast search databases, our deduced amino-acid sequence of AP2 domain has

98 to 100 % identities with other wheat, 92 to 93 % identities with *Avena sativa*, *Festuca arundinacea* and *Poa pratensis*, 87 to 89 % identities with *Sorghum bicolor*, *Cynodon dactylon* and *Zea mays* and 80 to 83 % identities with *Populus euphratica* and *Glycine max*.

*A Y R G V R Q R T W G K W V A E I R E P N R G N R L W L G S*  
*F P T A V E A A R A Y D D A A R A M Y G A K A R V N F S E Q*  
S P D A N S

**Figure 4.** The deduced amino-acid sequence of AP2 domain analysed by Pfam program. AP2 domain has three  $\beta$ -sheets and one  $\alpha$ -helix, respectively showed by italic. The valine<sub>14</sub> and glutamic acid<sub>19</sub> amino acids of AP2 domain are typed with bigger font.

## Discussion

Wheat crop responds to osmotic stress in the form of changes in various physiological and biochemical processes. As mentioned in results, osmotic stress decreased relative water content; both root and shoot dry weight and net photosynthesis rate, significantly. These results are confirmed by other researches (Mohsenzadeh et al., 2006; Gill et al., 2002; Flexas et al., 2002). Based on these data, it is possible to define three levels of drought stress: mild, moderate and severe. If RWC is reduced to below 5-7% of the control ones, plants looked healthy and decrease in growth rate was not significant. Moderate reduction of RWC for 10–25% affected biomass and the stress is severe when RWC decreased by more than 25%. Lawlor (2002) suggests that decreased ATP concentration at low RWC impairs protein synthesis, though it may increase the synthesis of certain types of proteins (Flexas, 2002). The moisture content of dry-farm wheat, the chief dry-farm crop, is more important. In this study, RWC as an indicator of the level of response to osmotic stress showed the mild, moderate and severe drought stress for 100, 200, 400 g/l PEG treatments conditions, respectively. Maintenance of high plant water status and the net photosynthesis rate in 100 g/l PEG treatment is an indication of osmotic stress resistance and it is necessary for Sardari cultivar as dry farming wheat and it may be due to gene expression induced by transcription factors like DREB protein. The recognition of the superior quality of wheat grown under osmotic stress stimulates faith in the great profitableness of dry-farming which is growth without irrigation under a limited rainfall.

As figure 3 shows, TaDREB gene in Sardari wheat is expressed both under osmotic stress and control conditions and it is suggested that this gene

is also responsible for the non-stress physiology or growth and development of the plant. This observation is similar to that of Latini and co-workers (Latini et al., 2005). In addition, we know that the DREB genes are controlled by the ABA-independent pathway and the responsiveness of plants to osmotic stress is critically mediated by the increase in ABA levels.

The primary and secondary protein databases shows that our Sardari DREB deduced amino-acid sequence of AP2 domain is very similar to other wheat plant and slightly different with other plants. This similarity of the protein motif suggests similar functions. In particular, understanding regulatory gene networks in stress response cascades depends on successful functional analyses of cis-acting elements (Yamaguchi-Shinozaki and Shinozaki, 2005). According to Shen and co-workers research, in different wheat cultivars, the TaDREB1 gene is induced by low temperature, salinity and drought; and the expression of some genes that contains DRE motifs in its promoter is closely related to the expression of TaDREB1. The results suggest that TaDREB1 functions as a DRE-binding transcription factor in wheat (Shen et al., 2003). Four genes encoding putative ethylene-responsive element binding factor (ERF)/AP2 domains were cloned from *Brassica napus*, and these genes could be induced by low temperature, ethylene, drought, high salinity, abscisic acid and jasmonate treatments (Liu et al., 2006).

The obtained 645 nucleotide sequence from cDNA was also amplified from genomic DNA with the same primers and it means that this segment of sequence has no intron. This study is the first research of DREB gene on an Iranian plant.

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