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Expression Profile of Some Dehydration Responsive Element-binding Factor under Drought Stress Induced by PEG in Wheat Seedlings

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Abstract: Plants respond to drought stress with producing signals that cause to expression of genes with stress tolerance. Molecular and genomic studies show that several genes with various functions are induced by drought stress and various transcription factors are involved in the regulation of stress-inducible genes. For this reason the main goals of this research are study on expression patterns of genes which their products act as transcription factor for expression of drought tolerant genes by quantitative method (Real-time PCR). Therefore 10 days-seedling of wheat were sampled in various treatments of drought stress (induced by PEG) from tolerant variety (Zagros) and susceptible line (N8019).The expression of three genes consisted *CRTBF2*, *DREB6* and *DBF* was studied. Results showed that expression of *CRTBF2* and *DBF* genes was significantly higher in susceptible line compared to tolerant variety that indicating *CRTBF2* functions as a repressor of some drought-induced genes in seedling of N8019. The expression of *DREB6* gene in tolerant variety was increased which indicate the role of the product of this gene as a transcription factors to induce some drought-responsive genes.

Key words: *CRTBF2* · *DREB* · *DBF* · Transcript · Wheat

INTRODUCTION

Plants are sessile organisms; they are directly exposed to environmental stresses such as drought, high salinity and low temperature. Many genes respond to drought stress at the transcriptional level and the products of these genes function in the stress response and tolerance [1, 2]. Genes induced during dehydration stress conditions are to function not only in protecting cells from dehydration by the production of important metabolic proteins (functional proteins) but also in the regulation of genes for signal transduction in the dehydration stress response (regulatory proteins). The functional proteins contain water channel proteins, chaperons, proteases and enzymes for the synthesis of osmoprotectants (compatible solutes, sugars, proline, etc.) [3]. The regulatory proteins contain transcription factors, protein kinases and enzymes for the synthesis of the plant hormone abscisic acid (ABA). So far, various kinds of functional proteins such as enzymes for the synthesis of osmoprotectants were overexpressed in plants to improve the stress tolerance [4, 5]. However, it seems that the engineering of one enzyme is not enough as many kinds of stress responses are necessary for plants to survive in severe stress conditions [6].

Sometimes a transcription unit is called a "regulon". Regulon biotechnology, by controlling the expression of the regulon system, is expected to improve the tolerance against stresses in plants [3]. More than half of the drought-inducible genes are also induced by high salinity and/or ABA treatments, indicating the existence of significant crosstalk among the drought, high-salinity and ABA responses [7].

Many transcription factor genes were found among the stress-inducible genes, suggesting that various transcriptional regulatory mechanisms function in the drought, cold or high-salinity stress signal transduction pathways [8, 4, 9]. These transcription factors could regulate various stress inducible genes cooperatively or separately and may constitute gene networks. Functional analysis of these stress-inducible transcription factors should provide more information on the complex regulatory gene networks that are involved in responses to drought, cold and high salinity stresses [10].

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Potential *cis*-acting DNA elements have been analyzed by comparing their expression profiles with those of the promoter sequences of stress-inducible genes [7, 8, 4]. The promoter of some drought, high-salinity and cold inducible genes, Responsive to Dehydration 29A (*RD29A*), Cold-Regulated 78 (*COR78*) and Low-Temperature- Induced 78 (*LTI78*), has been found to contain two major cis-acting elements, the ABA-responsive element (ABRE) and the dehydration-responsive element (DRE)/C-repeat (CRT), that are involved in stress-inducible gene expression [11].

Transcription factors belonging to the AP2/ERF (APETALA2 / ethylene-responsive element binding factor) family that bind to DRE/CRT have been isolated and termed DREB1/CBF and DREB2 [8, 12, 13]. The conserved DNA-binding motif of DREB1/CBF and DREB2 is A/GCCGAC [14]. The *DREB1/CBF* genes are quickly and transiently induced by cold stress and their products activate the expression of target stress-inducible genes. The *DREB2* genes are induced by dehydration, leading to the expression of various genes that are involved in drought stress tolerance [12].

The orthologous genes of *DREB1/CBF* have been found in many crop plants such as canola, broccoli, tomato, alfalfa, wheat, barley, corn and rice [5]. These indicate that the DREB1/CBF regulon system is ubiquitous in the plant kingdom and the "DREB technology" with controlling the expression of the DREB1/CBF regulon system is expected to improve the tolerance against stresses in crop plants. So far the *DREB1/CBF* genes of *Arabidopsis* have been successfully used to engineer abiotic stress tolerance in a number of different species [3].

Sakuma *et al.* [15] studied DNA binding specificity of the *ERF/ AP2* domain of *Arabidopsis DREBs*, transcription factors involved in dehydration and cold inducible gene expression. They showed that DRE/CRT is a *cis*-acting element that is involved in gene expression responsive to drought and low-temperature stress in higher plants. *DREB1A/CBF3* and *DREB2A* are transcription factors that specifically bind to *DRE/CRT* in *Arabidopsis*. They precisely analyzed the DNA-binding specificity of *DREBs* and showed that both *DREBs* are specifically bound to six nucleotides (A/GCCGAC) of *DRE*. Overexpression of *DREB1A* (*CBF3*) or *CBF1* (*DREB1B*) in transgenic *Arabidopsis* induced strong expression of target stress inducible genes and resulted in improved tolerance to drought, high salt and freezing [16-18]. The DREB2 protein is expressed under normal growth conditions and is activated in the early stage of the osmotic stress response through post-translational modification. The dehydration inducible *CBF/DREB1* may function in next stage of the osmotic stress response [10].

The determination of expression pattern of transcription factor proteins in response to drought may leads to understanding more about mechanisms involved in such stresses tolerance. In this study, we have investigated the changes of expression pattern of three transcription factor genes in the growing wheat seedlings under drought stress in susceptible and tolerant plants.

MATERIALS AND METHODS

Plant Material and Stress Treatments: The seeds of two genotypes of wheat (*Triticum aestivum* L.) consisting the drought tolerance cv. Zagros and the drought sensitive line N8019 Supplied by Gorgan Research Institute. Seeds were sterilized in 5% (v/v) sodium hypo chloride for 10 min and thoroughly washed with sterile water. Three replicates of 20 grains were placed on germination paper, separately at different osmotic potential (0, -0.25, -0.5, -0.75, -1, -1.25 and -1.5 MPa). Poly ethylene glycol (PEG 6000) was used to induce different osmotic potential using Michel and Kaufmann [19] method. Grains were grown for a 10 days period in growth chamber with a 16h/8h photoperiod and an average temperature 25 ?. After 10 days, sampling from seedlings were carried out and samples were quickly freezed in liquid nitrogen and stored in -80°C.

RNA Extraction: Total RNA was extracted from 0.3 g of seedling tissue by using lithium chloride ($LiCl_3$) method [20]. The seedlings were ground in liquid nitrogen using sterile pestle and mortar prior to RNA extraction. Concentration of each RNA sample was measured using spectrophotomer. Only the RNA samples with 260/280 ratio (an indication of protein contamination) between 1.8 and 2 were used for analysis.To confirm the RNA quality, the RNA was electrophoresed on 1.5% agaros gel containing ethydium bromide.

RT-PCR Analysis: After DNase treatment, the first strand cDNA was prepared according to the Fermentase reverse transcriptase enzyme instruction. First strand cDNA synthesis was performed using the oligo(dT_{18}) primer. The reaction began by incubating 2 μg of total RNA, 0.5 μg oligo (dT_{18}) primer and adding of DEPC treated water to reach 11 μl of volume following by incubating for 5 min at 70°C. The tubes was placed on ice before adding the cDNA synthesis mix which consisted of 4 μl cDNA

Table 1: sequence and some characteristics of specific primers

Name	Sequence	Tm	GC%	Product size (bp)	Accession no.
CRT/BF2	5'-GACAACCGATGACGAGAAGG-3'	60.6	55	128	AY572831
	5'-ACAGGCCCTCCGAGTAGAAC-3'	60.6	60		
DBF	5'-CGGAGATGCAGCTTCTTGATT-3'	61.7	47.6	139	DQ021908
	5'-TCACTTTGGACGAGCTGTGG-3'	62.4	55		
DREB6	5'-AGGCACCAGACACAAGCAC-3'	59.8	57.8	137	AY781361
	5'-ACATGGGCCTTTGGACCT-3'	60.3	55.5		
GADPH	5'-TCACCACCGACTACATGACC-3'	60	50	121	EF592180
	5'-ACAGCAACCTCCTTCTCACC-3'	60	55		

reaction buffer, 2 µl dNTP (10 mM 4 dNTP mix), 20 U Rnase inhibitor and DEPC-treated water to final volume of 19 µl. Then tubes were incubated for 5 min at 37°C. 200 U Revert Aid enzyme (MMLV-RT) were added and reactions were incubated for 60 min at 42°C. Finally for stopping of reaction, the tubes placed in incubator for 10 min at 70°C.

Primer Designing and Real Time PCR Analysis: Primers were designed using primer 3 (www.embnet.sk/cgi-bin/primer3_www.cgi) to obtain 18-21 bp length, 59 and 61°C melting temperature and GC content between 55% and 65% avoiding hairpins and complementarity between primers. The Primers, wherever possible, were designed based on 3' untranslated region (3'-UTR) of for transcription factor genes. The primer names, sequences and the accession no which used to primer designing are given in Table 1. GAPDH (d-glyceraldehyde-3-phosphate dehydrogenase) was used as reference gene to normalize data.

The PCR mixture contained 5µl of diluted cDNA, 10µl of 2X SYBR Bio Pars (GUASNR) PCR Master Mix and 1 µl of each gene-specific primer (10 pmol) in a final volume 20µl with double distilled water. All the PCRs were performed under following conditions: 2 min at 94°C and 35 cycles of 10s at 95°C and 10s at 60°C. The specificity of amplicons was verified by melting curve analysis (55 to 95°C) after 35 cycles. Three technical and two biological replicates for each sample were used for real-time PCR analysis. Relative expression was computed using following formula which presented by Pfaffl and Hegeleit, [21], based on its real-time PCR efficiency (E) and the crossing point (CP) difference (Δ) of an treated sample (under stress) versus control ($\Delta CP_{control-sample}$) for both target and reference genes.

$$Ratio = \left(E_{target}\right)^{\Delta CP_{target(control-sample)}} \Big/ \left(E_{ref}\right)^{\Delta CP_{ref(control-sample)}}$$

The REST[1] software [22] used this formula to calculate ratio between the amount of target molecule and reference molecule *GADPH* within the same sample. In this model the target gene expression was normalized by *GADPH* expression which is a non-regulated reference gene. The normalized value was then used to compare differential gene expression in different samples.

RESULT

Analysis of the expression pattern of *CRTBF2* showed it has been up regulated gradually in -0.25 to -1.25 Mpa in drought tolerant variety in (Zagros) and then there was sharp increase *in its* transcript in Zagros by increasing of osmotic potential to -1.5 Mpa (Fig. 1). There was a sharp increase in *CRTBF2* transcripts in drought susceptible variety (N8019) by increasing of osmotic potential to -0.25 Mpa followed by a high steady state to -1 Mpa. Its transcripts declined sharply in N8019 in -1.25 to -1.5 Mpa (Fig. 2).

Analysis of *DREB6* transcript accumulation in wheat seedling showed that there was a sharp increase in *DREB6* transcripts in drought tolerant variety (Zagros) by increasing of osmotic potential to -0.25 Mpa followed by high steady state to -0.75 Mpa of PEG osmotic potential (Fig. 3). Its transcripts gradually declined to -1 Mpa and increased in -1.25 Mpa and decrease in -1.5 Mpa. It was down-regulated in susceptible drought variety at -0.25 to -1 Mpa, followed by a sudden increase at -1.25 Mpa and had steady state of transcript to -1.5 Mpa. Its transcripts level vas almost higher in tolerant cultivar than in susceptible one.

The expression pattern of *DBF* in drought tolerant variety (Zagros) showed that its transcripts gradually declined in -0.25 Mpa to -1.25 Mpa and suddenly reduction in its transcripts level at -1.5 Mpa (Fig. 4). It was up-regulated in N8019 by increasing osmotic potential to -0.5 Mpa and kept a steady-state level of transcript to -1.5 Mpa.

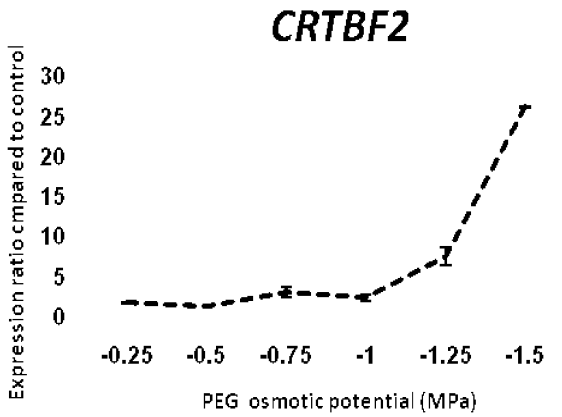

Fig. 1: Quantitative expression patterns of *DREB6* gene in seedling of Zagros in different PEG osmotic potential

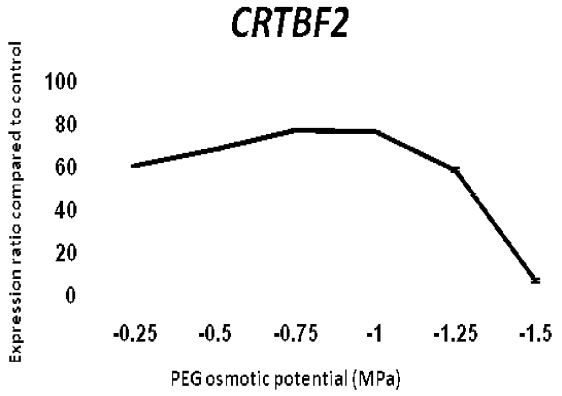

Fig. 2: Quantitative expression patterns of *DREB6* gene in seedling of N8019 in different PEG osmotic potential

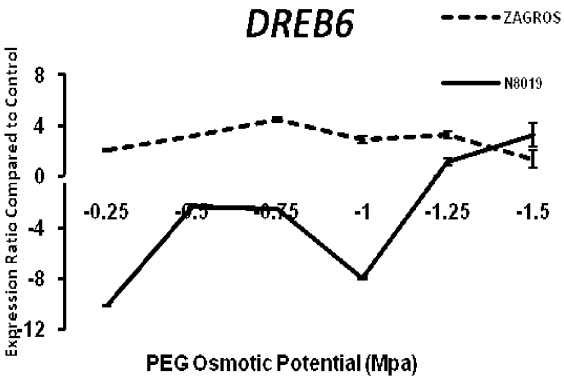

Fig. 3: Quantitative expression patterns of *DREB6* gene in seedling of Zagros and N8019 in different PEG osmotic potential

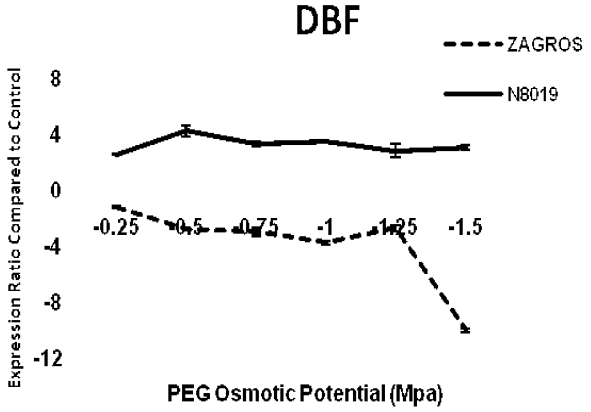

Fig. 4: Quantitative expression patterns of *DBF* gene in seedling of Zagros and N8019 in different PEG osmotic potential

DISCUSSION

Plants respond to environmental stress and the trans-induced signals cause expression of numerous genes associated with stress tolerance. A number of genes have been described that respond to drought [23]. In plants, one transcription factor can control the expression of many target genes through the specific binding of the transcription factor to the *cis*-acting element in the promoters of the target genes [24].

The aim of this research is study of Expression patterns of some genes which their product is transcription factor for expression of drought tolerant genes in seedling of wheat by quantitative method (Real-time PCR). Molecular studies have shown that several genes with various functions are induced by drought stress and that various transcription factors are involved in the regulation of stress-inducible genes. The products of stress-inducible genes function not only in stress tolerance but also in stress response [10].

The present work focuses on gene expression upon PEG osmotic potential in wheat seedling. Result showed a differential expression for transcription factor genes. Some transcription factors that induce or repress the expression of several genes related to stress have been discovered.

In different study, it was shown that transcription factors have a variety of roles in the plant life cycle, in plant development [25-29] and in response of plants to abiotic or biotic stresses [16, 30-34]. Three AP2/EREBP factors, the CBF1, DREB1 and DREB2 from Arabidopsis, were demonstrated to bind to the C-repeat/DRE sequence

and to function as transcriptional activators [16, 33]. Sequence comparison and phylogenetic studies have shown that DBF proteins are not homologous to DREB-CBF1.

A stimulus-specific induction or activation of the transcription factors may regulate the relative amounts of the factor present in the cell and could be an alternative way of determining the binding of the different factors to the cis-element. DBF1 and DBF2 do not share a common stimulus of induction, with DBF1 being strongly induced by dehydration, salinity stress and ABA and DBF2 having low-level mRNA accumulation [35].

In *Arabidopsis* and some crop species (e.g., rice, wheat, barley, *Brassica*), DREB genes are rapidly induced in response to dehydration, cold and high salt [12, 36]. In contrast, the DREB2-type transcription factor *TaDREB1* from wheat responded poorly to drought, salinity and ABA [37]. The expression of *DREB2A* and its homolog *DREB2B* were induced by dehydration and high salt stress, but not by cold stress [38].

In our study, expression pattern of *CRTBF2* gene in susceptible variety (N8019) has been showed up-regulated in whole of drought stress treatments while in tolerant variety (Zagros) a little increase in transcript level has been seen only at the end of stress treatment. Totally the increase of *CRTBF2* transcripts in seedling of N8019 was higher than Zagros, therefore it will be significance studying of function of this gene on susceptible and tolerant seedling of wheat. Our result indicates that *CRTBF2* functions as a repressor of some drought-induced genes in seedling of N8019. Kiziz and pages [39] showed that overexpression of some transcription factors may resulted in a decrease in some drought-responsive promoter activity. In their study overexpression of ö DBF2 resulted in a decrease in rab17 promoter activity in maize.

The transcripts of *DBF* in N8019 were accumulated by a steady state by increasing osmotic potential. That showed adaptation response to all of drought treatment in susceptible variety (N8019), while in tolerant variety (Zagros) the transcript level was declined in whole of PEG treatments. This gene can act as a repressor of some drought-induced genes in wheat seedling. Something was found for CRTBF2 gene, too. One another transcription factors is *DREB* that binds to drought responsive Cis-acting element [40]. Expression pattern of *DREB6* in tolerant drought variety (Zagros) was showed under drought stress significantly up- regulated in all of PEG osmotic potential, while was down-regulated in susceptible drought variety (N8019) in most treatment of PEG. Therefore it seems that accumulation of *DREB6* transcripts necessarily correlated with drought tolerance in seedling of wheat.

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