Journal of **A**pplied **B**iotechnology **R**eports

Original Article

Gene Expression Patterns of some Transcription Factors (Zpt2-1, CBF4, bHLH) under Salt Stress in Alfalfa by Using qPCR

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Abstract

Researchers need continuously improve their views on mechanisms of salt tolerance in crop plants in order to overcome their limitations and improve yields under stress conditions including salt stress. One of the most important methods of controlling stresses in plants is through making adjustments during the process of gene transcription. Transcription factors adjust the degree of expression in many genes by binding to DNA and are thus very important in salt tolerance of plants. Therefore, the present research studied the role of transcription factors bHLH, CBF4, and Zpt2-1in salt tolerance by investigating their expression patterns under salt stress in the leaf and root tissues of the Yazdi (salt tolerant) and the Diabloverde (sensitive to salinity) genotypes of Alfalfa. Results showed that short-term salt stress affects on expression patterns of the CBF4, Zpt2-1, and bHLH genes in leaf and root tissues of both genotypes. Using qRT-PCR (Real-Time PCR) analysis, it was shown that the transcription factors Zpt2-1 and CBF4 in the salt tolerant genotype (Yazdi) were expressed at a higher level in root tissues. In other words, it seems that higher expression levels of the transcription factors Zpt2-1 and CBF4 were accompanied by greater salt tolerance. This finding can help plant breeders to use these transcription factors for selecting salt-tolerant genotypes in alfalfa.

Keywords: *Medicago sativa*, Real-Time PCR, Salt Tolerance

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Submission Date: 3/25/207
Accepted Date: 5/14/2017

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important forage plants, and the extent of distribution indicates that it is capable of producing high yields in a wide range of soil and climate conditions and produce good quality forage even under harsh weather conditions [1]. Plants respond differently to environmental stresses at morphological, anatomical, cellular, and molecular levels [2]. When plants are exposed to stress, their signaling system at the molecular level induces expression of specific genes against the harmful effects of environmental stresses, and the products produced by some of these genes participate in protecting plants and in maintaining their cellular structure [3]. Proteins that have well-specified roles in the process of protecting plant cells against stress damage are chaperone and osmotic adjustment proteins [4], proteins that form ion channels [5], transporter proteins [6], and antioxidant and detoxification proteins [7]. Expression of these functional proteins is strongly controlled by specific transcription factors. It has been demonstrated that many families of transcription factors play roles in the stress signaling pathways in plants, among which bZIP proteins (Abscisic Acid Response Element Binding Factors or ABREs) [8], MYC-like bHLH proteins and MYB proteins [9], and WRKY proteins [10] have the most obvious roles in responding to biotic/abiotic stresses. Some studies revealed changing of gene expression pattern on a large scale under salt stress conditions [11] and that different genotypes show variable responses under control and salinity stress conditions [12]. In the past several years, annual alfalfa (*Medicago truncatula*) has attracted the interest of researchers as a very desirable model plant for genetic studies due to its small and diploid genome, self-fertilization, and short life cycle [13]. These characters have made annual alfalfa a suitable plant for identifying genes involved intolerance against abiotic stresses and breeding for these genes [13]. The large number of molecular studies conducted on this plant has yielded a huge volume of data related to genome sequencing and gene expression analysis that is stored in databases [14]. Li *et al*., [15] have indicated that CBF4 and MYB112 play key role in tolerance to abiotic stresses in *Medicago* species. Also, Merchan *et al*., [16] showed the TFIIIA-like TF, Zpt2-2, is involved in recovery responses to salinity and cold stress in *M. truncatula*. De Lorenzo *et al*., [17] reported that, overexpression of MtZpt2-1 or MtZpt2-2 in the sensitive genotype of *M. truncatula* led to significantly increase in root growth under salt stress, as well have a major role in adaptive responses. Despite the large amount of information on the effects that salinity has on transcriptome pattern of *M. truncatula*, there are few studies regarding the mentioned area on the Iranian alfalfa cultivars. Therefore, this research was carried out using Real-Time PCR to study expression patterns of genes related to the transcription factors Zpt2-1, CBF4, and bHLH under salt stress in alfalfa.

Materials and Methods

Cultivation conditions

The present research was conducted in the laboratory and greenhouse of the Safiabad Agriculture Research Center in

Dezful and in the Central Biotechnology Laboratory of Ramin Agriculture and Natural Resources University of Khuzestan Province in 2015-2016. The salt-tolerant genotype Yazdi and salt-sensitive Diabloverde genotypes were grown in an aquaculture system. The seeds were first disinfected and kept in Petri dishes in a germinator in the dark for two days to germinate. The germinated seeds were then planted on a styrofoam sheet with holes seven centimeters apart. The plot contained cocopeat, perlite and sand in the ratio of 2:3:3. There were three plants in each experimental plot [18]. During the first 30 days after planting, one-fourth strength Hoagland solution was applied to all the pots for the seedlings to be completely established. Following that, salt stress was applied on the 31st day after planting by using 180 mM salt, and samples were taken after 24 hours [15]. The Hoagland solution free of salt (NaCl) was used for the control and the Hoagland solution containing 180 mM salt for the stress treatments. Temperature was kept at 22-30˚C and relative humidity at 70 percent, and normal light intensity and a 16:8h light:dark photoperiod was used during the experiment.

Sampling of RNA extraction

Samples were taken from leaves and roots of both the salt-sensitive and salt-tolerant genotypes to be used in the molecular studies. Two bulk mixed samples (from leaves of 10 plants) and two bulk mixed samples (from roots of 10 plants) were taken for each genotype. The samples were first placed in an envelope that contained liquid nitrogen and was covered with aluminum foil, then quickly transferred to a liquid nitrogen container. It must be mentioned that root samples were immediately washed first with distilled water and then with water treated with DEPC. Following that, all samples were immersed in liquid nitrogen and then storage in -80 degrees Celsius (- 80˚C) freezer.

RNA extraction

Ribospin Plant kits (GeneAll, South Korean) were used to extract RNA by following the company protocol [19]. The RNA extracted from the samples was treated with the DNase I enzyme to minimize the genomic DNA contamination, a NanoDrop 2000C spectrophotometer (Thermo Scientific, USA) was employed to determine the quantity and purity of the extracted RNA, and electrophoresis of the samples was performed on 1% agarose gel in 1X MOPS buffer to determine the quality of the RNA samples.

Synthesis of cDNA

First strand cDNA was synthesized using 2-Step RT-PCR kits (made by the VIVANTIS Company) according to the instructions of the manufacturing company.

Designing the primers

The gene sequences for the transcription factors Zpt2-1, CBF4, and bHLH were extracted from the NCBI database, and the Primer Quest program in the IDT (Integrated DNA Technologies) site was employed to design the primers of the desired genes. Then, they were sent to the SinaClon Company (Karaj, Iran) to be synthesized. For designing the primers, their lengths (100-130 bp), GC percentage (40-60 percent), their complementary to each other, and also the probability of their ring formation were considered. The base concentration of the synthesized primers was 122 pM/µl, which was diluted to12 pM/µl by adding sterile water. It must be mentioned that the actin gene, which usually has a relatively constant expression level in leaf and root tissues under control and stress conditions, was used as the internal control gene in the qRT-PCR reaction [15]. This gene which has the same expression level under both control and stress conditions, was used to normalize expression levels of the studied genes. Table 1 lists the sequences of the forward and reverse primers for the amplification of the genes coding for the transcription factors of interest in this research.

Table 1. Sequences of used primers for CBF4, bHLH, Zpt2-1 and actin as reference gene.

Gene	primer	sequence (5'→3')	Product size
CBF4	F	GTGGGTTTGCGAAGTAAGAG	127
	R	ACAAGCAGACCTTCCTCTC	
bHLH	F	TCACAGTCTAGCAGAAAGGG	110
	R	CCAACATTACTGCCATTCCC	
Zpt2-1	F	AGCTGTTATGTCCGCAACCA	166
	R	CCCAAAGCCTGTCCAGTAGG	
Actin	F	GGATCTTGCTGGTCGTGATCT	116
	R	CTGGTGGAGCCACAACCTTA	

Real-Time PCR

Real-Time PCR was used to confirm the selected genes as candidate genes for salt tolerance in alfalfa. It was performed for each gene using the SYBR Premix Ex Taq IIsolution produced by the Takara Company containing SYBR Green and the specific primer designed for the related gene. The reaction for each sample was carried out with two biological and two technical replicates using a StepOnePlus Real-Time PCR System (from Applied Systems).

Statistical analysis

Bootstrap tests were employed for analyzing Real-Time PCR results to study significant differences in gene expression levels of each genotype (both under salt-stress and stress-free conditions) as follows. The threshold cycles for the various samples were first normalized using the reference gene (actin in the present research), and then relative differences between expression levels of the target genes were determined using $2-\Delta\Delta Ct$ estimates (as explained by Schmittgen and Livak) [20] and through employing the REST software produced by Pfaffl [21]. In order to evaluate efficiencies of primers, Real-Time PCR reaction was done using various cDNA concentrations for different primers. Primers efficiencies were calculated around 1.95-2.05. Finally, differences in threshold cycles were used as the criterion for comparing the genotypes:

$\Delta\Delta Ct = A - B$

A = (Ct of the gene of interest under stress conditions minus Ct of the reference gene)

B = (Ct of the gene of interest under control conditions minus Ct of the housekeeping gene under control conditions)

Results and Discussion

Effects of salt stress on expression pattern of the gene for the transcription factor Zpt2-1

Salt stress significantly increased Zpt2-1 in leaf and root tissues of both the Yazdi and the Diabloverde genotypes (Fig. 1A). Expression patterns of these transcription factors in root tissues were higher compared to those in leaf tissues. Studies by researchers, which led to the identification of the mentioned gene as an effective transcription factor effective in increasing tolerance to salt in alfalfa, were conducted on root tip and root node tissues [16].

Zpt2-1 expression significantly increased in leaf tissues in response to salt stress in both the Yazdi (salt tolerant) and the Diabloverde (salt sensitive) genotypes. However, the increases in gene expression under salt stress were not identical in two alfalfa genotypes. That is, the increases in expression of the Zpt2-1 gene in leaf tissues of the Yazdi genotype under salt stress was 5.37 fold higher than that of corresponding plants under control conditions, while the increases for the leaf tissues in the Diabloverde genotype were 3.48 fold. As in the case of leaf tissues, results obtained from qRT-PCR in root tissues of two alfalfa genotypes indicated that there were significant differences between Zpt2-1 gene expression levels under salt stress compared to the control group. However, as in the leaf tissues, the increases in gene expression under salt stress were not identical in the two alfalfa genotypes. Increases in expression of the Zpt2-1 gene in root tissues of the Yazdi genotype under salt stress were 12.42 fold higher compared to the corresponding plants under control plants, while increases in the gene expression for root tissues of the Diabloverde genotype were 2.71 fold greater compared to the corresponding plants under stress free conditions. Results indicate the importance of the mentioned gene in increasing salt tolerance especially in root tissues. Therefore, this gene could be a suitable candidate in complementary studies for improving salt tolerance in alfalfa. These results are in agreement with the observations from other studies [16, 17, 22, 23]. Gene expression analysis using microarray technology in *M. truncatula* has resulted in the identification of various genes such as homologous genes of COLD-REGULATEDA1 (CorA1; MtCorA1), which are considered a part of the target gene for the transcription factor MtZpt2, as genes that are involved in salt tolerance [17]. Based on the findings of researchers, the transcription factors MtZpt2-1 and MtZpt2-2 (of the zinc-finger type of transcription factors)significantly increased root growth under salt stress conditions and, hence, played an important part in the adaptation of *M. truncatula* to the salt stress [17].

So far, 384 genes have been reported with different expression levels under control and salt stress conditions by comparing transcription patterns of root tissues in *M. truncatula* under these two different conditions [22]. These genes include a homologous gene of COLD-REGULATEDA1 whose role in resistance to cold had been confirmed previously and the genes for the two transcription factors MtZpt2-1 and MtZpt2-2 that have key roles in resistance to salt.

Other researchers also reported that the MtZpt2-1 gene from the C2H2 zinc-finger family increased nodule formation and root regeneration in the genus *Medicago* after application of salt stress [17].

Merchan *et al*., [16] studied the level and content of transcriptome in *M. truncatula* under salt stress; and using antisense transgenic plants showed that Mszpt2-1 formed a part of a signal transfer pathway for improving plants following salt stress.

Wang *et al*., [23] studied salt stress in the salt tolerant JEMALONG A17 and the salt sensitive R108 genotypes of *M. truncatula*, and noticed that the expression level of the MtZpt2-1 gene (a transcription factor belonging to the TFIIIA family of transcription factors) in the JEMALONG A17 salt tolerant genotype was greater than sensitive genotype. They reported that the reason for tolerance to the salt stress in the JEMALONG A17 genotype was the greater abundance of stress response elements in the promoter sequence of the tolerant genotype.

Analysis of the MtZpt2-1 promoter by these researchers suggested that there were several types of cis-elements associated with response to abiotic stresses that increased expression level of the MtZpt2-1 gene under salt stress conditions. These elements included two types of MYB-core elements and ABA responsive elements (ABREs).

Effects of salt stress on expression patterns of transcription factor CBF4 (C-repeat binding factor)

Results obtained from studying the expression level of the CBF4 gene using qRT-PCR showed that (Fig. 1B) the expression levels of this gene in leaf and root tissues significantly increased in both the Yazdi and the Diabloverde genotypes under the influence of salt stress.

The increase in gene expression level for this transcription factor under salt stress was greater than those of all other genes studied in the present research. The increase in gene expression in root tissues of the Yazdi genotype was greater than the Diabloverde genotype, so that the increase in expression level of the CBF4 gene in root tissues of the Yazdi genotype under salt stress was 48.74 fold greater compared to stress-free conditions, while the corresponding increase in the root tissues of the Diabloverde genotype was 15.74 fold greater.

Li *et al*., [15] studied the effect of salinity on root tip tissues and reported a substantial increase in the expression levels of the mentioned gene in the genotypes under stress compared to the control.

In leaf tissues, the trend of changes in expression levels of CBF4 gene in the two studied genotypes was not similar to that in the root tissues. In other words, CBF4 expression level increased significantly in response to salt stress in both the Yazdi (salt tolerant) and Diabloverde (salt sensitive) genotypes, but the increases in gene expression under salt stress were not the same in the two alfalfa genotypes.

The estimated increase in expression level of this gene in leaf tissues of the Yazdi genotype under salt stress was 15.76 fold compared to corresponding plants under stress-free conditions, while the corresponding increase in leaf tissues of the Diabloverde genotype was 132.73 fold.

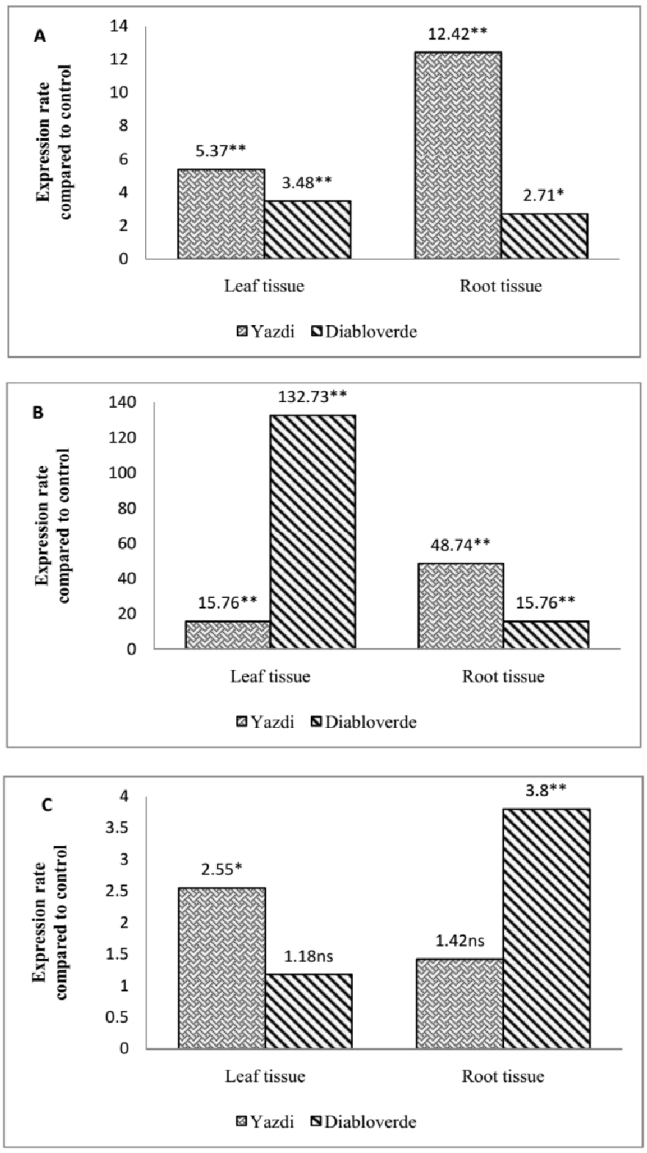

Figure 1. The relative genes expression of Zpt2-1(A), CBF4 (B), and bHLH (C) under salt stress condition (180 mM NaCl). (Yazdi, tolerate and Diabloverde, sensitive).** and * indicate significant differences respectively at P≤0.05 and P≤0.01 between treat and itself control.

The functions of transcription factors in cellular adjustments were reported. For example, it was reported that the dehydration responsive element-binding (DREB) transcription factors played an important role in the regulation of the cell cycles in response to stress [24]. Phylogenic analyses have indicated that MtCBF1 belongs to the EREBP-AP2 family of transcription factors and its expression is induced by abiotic stresses including salt, cold, drought, and abscisic acid. This gene is very similar to GmCBF2 and GmCBF1, and there is relatively great similarity (57 percent) between the amino acid sequence of its protein and that of the Arabidopsis protein AtDREB1D/CBG4 [15]. Some researchers have shown that DREB2B/CBF4 expression, which is increased by abscisic acid (ABA), plays an important role in salt, drought, and cold tolerance [25, 26]. Expression of some transcription factors is induced by one specific stress, while expression of others is induced under the influence of numerous stresses. It has been reported that AtCBF4 expression increases by drought and salinity stresses and also by increases in ABA content [25]. Other studies also used qRT-PCR to prove increased expression of AtCBF4 under cold stress [26]. Presence of the MtCBF1 and MtCBF2, MtDREB1C/CBF3, and MtDREB2A genes in annual alfalfa (*Medicago truncatula*) has been proved. Moreover, it has been shown that expression levels of MtCBF2 and MtCBF3 increases in annual alfalfa at 6 and 8˚C [27]. The MtCBF2 and MtCBF3/DREB1 genes in annual alfalfa play an important role in inducing the expression of CAS-COLD-ACCLIMATION-SPECIFIC genes that increase cold tolerance. Furthermore, expression level of the MtDREB2A gene significantly increases when roots are exposed to drought and salinity stresses [24]. Another research also used results obtained from microarray technology and showed that the MtCBF4 transcription factor played a very important role in the response to salt stress and also to other abiotic stresses in annual alfalfa [15].

Salt stress effects on expression pattern of the gene for the bHLH transcription factor

Figure 3, graph C shows bHLH expression pattern in leaf and root tissues of the Yazdi (salt tolerant) and Diabloverde (salt sensitive) genotypes. As shown in this figure, salt stress significantly increased expression of this gene in leaf tissues of the Yazdi genotype (2.55 fold) and in root tissues of the salt sensitive Diabloverde genotype (3.80 fold) compared to the same plants under stress-free conditions.

However, in the leaf tissues of the Diabloverde genotype, and also in the root tissues of the salt tolerant Yazdi genotype, no significant differences were observed under salt stress conditions compared to stress-free conditions. This result suggested that the type of the studied tissue played an important role in the expression of the mentioned gene. The bHLH transcription factors play an important role in root growth and development, and several genes in this family are involved in the response to salt stress [28]. In non-forage crops, increases in bHLH expression levels enhanced tolerance against salt and osmotic stresses [29]. It has been demonstrated that the bHLH transcription factors are involved in root growth and nodule formation in forage plants [30].

Postnikova *et al*., [31] studied the effects of salt stress on the AZGERM SALT-II (salt tolerant) and AZ-88NDC (salt sensitive) genotypes of alfalfa and identified four types of bHLH transcription factors in the salt tolerant genotype that caused differences in gene expression levels under salt stress conditions compared to the control. In three of these four types, gene expression increased and in one it reduced.

Zahaf *et al*., [32] also reported that there was a relationship between the bHLH transcription factors and adaptation of annual alfalfa to saline soils. Using microarray technology, they noticed that the expression of the regulator gene MtbHLH-658 was associated with one of the PM-ATPase genes. Pm-ATPase genes are involved in intracellular pH regulation and in maintenance of turgor and electrochemi-

cal gradient in cells, and they are expressed in response to salt stress.

Conclusion

In general, the short-term effects of salinity on expression patterns of the CBF4, Zpt2-1, and bHLH genes in the leaf and root tissues of the Yazdi and Diabloverde genotypes showed different pattern. Real-Time PCR (qRT-PCR) analysis showed that expression levels of the transcription factors Zpt2-1 and CBF4 were higher in root tissues of the salt tolerant genotype. Increased expression levels of the gene for the Zpt2-1 transcription factor in the leaf tissues of the Yazdi genotype also indicated the effective role of this transcription factor in response to salinity. In other words, it seems that the higher expression levels of the Zpt2-1 and CBF4 transcription factors were accompanied by higher tolerance in response to salt. This finding can help plant breeders to carry out complementary studies on these transcription factors to select salt tolerant genotypes in alfalfa.

Acknowledgements

This work was supported by Safiabad Agricultural and Natural Resource Research Center, AREEO, Dezful, Iran.

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