Quantitative expression analysis of *P5CS* and *BADH* genes in cultivated Wheat Plants under Salt and ABA treatments

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ABSTRACT

Plants encounter various stresses such as drought and salinity which adversely affect growth, development and crop productivity. The expression of the genes Delta -1- pyrroline – 5 - carboxylate synthetase (*P5CS*) and Betaine aldehyde dehydrogenase (*BADH*) extends throughout various protective mechanisms in plants and allows them to adapt to unfavorable environmental conditions. P5CS and *BADH* genes expression patterns in the wheat cultivars Mahuti and Alamut were studied under salt and ABA treatments using a qRT-PCR technique. Results showed that gene expression patterns were significantly different in these two cultivars. Mahuti had significantly higher expressions of *P5CS* at 3h compared to other time courses, while Alamut had significantly higher expressions of *P5CS* at 72h. In addition, these result showed that the *P5CS* gene expression increased after treatment of ABA and NaCl in both Mahuti and Alamut cultivars. Similarly, Mahuti had significantly higher expressions of *BADH* at 72h compared to other time courses, while Alamut had significantly higher expressions of *BADH* at 24h. It is likely that the difference in gene expression patterns between Mahuti as a salt tolerant cultivar and Alamut as a salt sensitive cultivar is due to different signaling pathways that activate *P5CS* and *BADH* expressions. Lack of significant difference between these genes expression profile under salt and ABA treatment suggest that *P5CS* and *BADH* are not induced by the stresses used in this study. It is also possible that different concentrations of NaCl and ABA are required to cause a change in the expression of *P5CS* and *BADH* genes.

Key words: *P5CS, BADH, qRT-PCR*, Salt stress, *Triticumaestivum.*

Abbreviations:
ABA: Abscisic Acid, RT-PCR: Real Time Polymerase Chain Reaction,
P5CS: Delta -1- pyrroline – 5- carboxylate synthetase, BADH: Betaine aldehyde dehydrogenase

INTRODUTION

Unfavorable environmental conditions such as drought and salinity are among the most common abiotic factors affecting agricultural communities and limiting crop productivity in arable lands (Zhang et al. 2006; Abebe et al. 2003). Losses of agricultural productivity due to abiotic stresses can reduce average yields by 65% to 87% (Boyer 1982**;** Piero et al. 2009). Nowadays, it is well

known that abiotic stresses lead to a series of morphological, physiological, biochemical and molecular changes in plant growth and productivity (Wang et al. 2001). Conditions of drought and salinity may induce similar cellular damages such as osmotic stress, leading to a disturbance of homeostasis and can increase the production of reactive oxygen species (ROS) (Wang et al. 2003**;** Serrano et al. 1999**;** Zhu 2001). However plants have various extended protective mechanisms that allow them to adapt to unfavorable environmental conditions (Zhu 2002). Studies show that NaCl stress, resulting in the disruption of photosynthetic mechanisms via a combination of superoxide and H_2O_2 mediated oxidation (Hernandez et al. 1995). Numerous studies have been done to investigate the response of plant cells to saline environments and three basic strategies have been determined as protective mechanisms (Hasegawa et al. 2000). These strategies are as follows: Firstly, the prevention of active Na^+ influx; secondly, potentially damaging stress factor is drought, which leads to common phenomena such as wilting of leaves and stems in corps. Thirdly, ABA plays a critical role in plant response and regulation of water status and induces expression of genes that regulate tolerance to cellular dehydration (Luan 2002). Recent studies show that in many ways ABA has a pivotal regulatory function in plant response to drought and salt stresses. Studies such as Silva-Ortega et al. (2008) show that activity and expression of *P5CS* gene during salt stress in Cactus pear decreased dramatically. The core aim of this research was to assess possible changes in *BADH* and *P5CS* expression patterns in two wheat cultivars (Mahuti as the salt-tolerant and Alamut as the salt sensitive cultivars) under salt and ABA stresses.

MATERIALS AND METHODS

Plant Materials and Growth Conditions NaCl is the principal cause of soil salt stress (Hasegawa et al. 2000**;** Blumwald et al. 2000**;** Niu et al. 1995). This experiment was carried out in a greenhouse to test the effects of different salinity levels (0 mM as control, 50, 100, or 200 mM NaCl) and different periods of exposure to salinity (0, 3, 6, 10, 24, 72 h) on the expression of *BADH* and *P5CS* genes in two wheat plants. In this experiment, Mahuti (one of the most salt-tolerant Iranian wheat cultivars), (Ghavami et al. 2006) and Alamut (salt sensitive) cultivars were used.

Imbibed seeds were kept in the dark for 24 h at 4^o C and germinated for 3 days at 22^o C. Seedlings were then grown hydroponically and irrigated with a modified Hoagland solution (Kerepesi and Galiba 2000) When plants had reached the 2–3 leaf stage, salinity treatments (control, 50, 100, or 200 mM NaCl) were applied. All solutions contained $CaCl_2$ to maintain a Na^+:Ca^{2+} ratio below 10:1. In order to investigate ABA treatment on the *P5CS* gene, an ABA solution (100 mM) was prepared and then sprayed onto leaf tissues. It should be noted that this experiment was conducted with three individual groups; salinity treatment, ABA treatment and control. Sampling was done after 0, 3, 6, 10, 24, and 72 h from treatments. Tests were carried out on three biological factors and two technical replicates.

RNA Extraction and cDNA Synthesis

Total RNA was extracted using RNX-Plus buffer (CINNAGEN, Iran). Briefly, about 100 mg of tissue was ground in liquid nitrogen. The ground powder was transferred to1 ml of RNX-Plus buffer in an RNase-free microtube, it was mixed thoroughly and then left at room temperature for 5 min. An aliquot of 0.2 ml chloroform was added to the slurry and mixed gently. The mixture was centrifuged at 13,200 × *g* at 4^o C for 15 min, the supernatant was then transferred to a new tube and precipitated with an equal volume of isopropanol for 15 min on ice. The RNA pellet was washed using 75% ethanol, briefly dried and resuspended in 50 µl of RNase-free water. The purified total RNA was quantified by Nano-Drop ND 1000 Spectrophotometer (Wilmington, USA). DNase treatment was carried out using Fermentas (Fermentas, Leon-Rot, Germany) DNase Kit according to the manufacturer's instructions. Five µg of DNase-treated RNA was used for the first strand cDNA synthesis, using 100 pmol oligo-dT (18 mer), 15 pmol dNTPs, 20 U RNase inhibitor, and 200 U M-Mulv reverse transcriptase (all from Fermentas) in a 20-µl final volume.

Quantitative Real-Time PCR Analysis

Primer was designed using Allele ID 7 software for internal control, *BADH* (AY050316.1) and *P5CS* (AY888045.1) genes. Wheat elongation factors α (M90077) gene was used as the internal

control (whose expression proved not to be influenced by salt and ABA stress) for data normalization (Caldana *et al*. 2007**;** Jain *et al*. 2006**;** Nicot *et al*. 2005) (Table 1). Relative real-time PCR was performed in a 20-µl volume containing 3 µl cDNA, 10 µl Syber Green buffer (Takara), and 4 pmol of each primer.

Amplification reactions were done in a lineGeneK thermal cycler (Bioer, China) with initial denaturing of 94^{o} C _for 2 min, followed by 40 cycles of 94^{o} C for 10s, annealing temperature (Ta) of each primer pair for 15s and 30s of extension at 72 ^{o}C . After 40 cycles, the specificity of each amplification was checked based on melting curves from heating the amplicons from 50 to 95 ^{o} C. All amplification reactions were repeated twice under identical conditions, in addition to a negative control and five standard samples. To ensure that the PCR products were generated from cDNA and not from the genomic DNA, proper control reactions were run without reverse transcriptase treatment.

For quantitative real-time PCR data, calculations were made for relative expression of *BADH* and *P5CS* based on the threshold cycle (C_t) method. The C_t for each sample was calculated using Line-gene K software and the method reported by Larionov *et al*. (2005). Accordingly, the fold expression of target mRNAs over the reference values were calculated by the equation $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001), where ΔC_t was determined by subtracting the corresponding internal control C_t value from the specific C_t of the targets (*BADH* or P5CS), and $\Delta\Delta C_t$ was obtained by subtracting the DCT of each sample in the experiment from that of the control sample.

Table 1. Sequences of primers used for real-time PCR amplification and the resulting product size.

primer	sequence	Amplicon length (bp)	Ta
BADH-F	CATCACTGACATCAACACATC	180	54.2
BADH-R	TCCTCGGCTAATCTCTGG		
EF-α-1F	TTCACTCTTGGAGTGAAGCAGAT	107	55.2
EF-α -1R	GACCTCCTTGACAATTTCTTCATAA		
P5CS-F	ACAGATGATAAAGTAGCAGAGAC	167	53.9
P5CS-R	AGACCTTCAACACCCACAG		

Statistical Analysis

Statistical analysis was done using MINITAB 14 (Minitab, Inc., Pennsylvania, USA) and SAS6.12 software (SAS institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was used to investigate significant differences in *BADH* and *P5CS* genes expressions under different NaCl concentrations, ABA treatments and different time courses. Duncan's multiple range test procedure (SAS 6.12) was used to group NaCl concentrations and ABA treatments or time courses based on the average expression levels of the genes of interest in each condition. The Pearson correlation (PC) coefficient was used to measure the relationship between gene expressions under the various treatments (including NaCl concentration and ABA treatment) and different times. A Paired t test was calculated to determine significant differences in gene expression profiles between the wheat cultivars.

RESULTS

The quantitative expression patterns for the BADH and P5CS genes under treatments (different concentrations of NaCl and ABA treatment) and different time courses are shown in Figures. 1 and 2, respectively. Analysis of variance showed significant differences between P5CS gene expressions in the two wheat cultivars Mahuti and Alamut under different time courses. Analysis of variance (ANOVA) was used to investigate differences in the BADH and P5CS genes expression profile under different times,

NaCl concentrations and ABA treatments. There was a significant difference between Mahuti (Mean: 2.4962) and Alamut (Mean: 1.7822) (P≤ 0.01).
Mahuti had significantly a higher expression of *P5CS* at 3h compared to other time courses, while Alamut had significantly a higher expression of P5CS at 72h. In addition, these results showed that the P5CS gene expression increased after treatment of ABA and NaCl in both Mahuti and Alamut cultivars. Similarly, Mahuti had significantly higher expressions of BADH at 72h compared to other time courses, while Alamut had significantly a higher expression of BADH at 24h.

However, analysis of variance for the investigation of differences in the P5CS gene expression in different wheat cultivars under different NaCl concentrations and ABA treatments showed no significant differences (P≤0/05) in the P5CS transcription levels in the two cultivars at 50, 100, 200 mM NaCl and 100 mM ABA treatments. Evaluation of relationships between the GSTF1 expression levels in different NaCl concentrations and ABA treatments using the PC coefficient showed no significant correlation between gene expression profiles at 50, 100 and 200 mM NaCl concentrations 100 mM ABA treatment in cultivars.

Discussion

It is now clear that *BADH* and *P5CS* genes family encode a diverse group of multi-functional proteins that catalyze a variety of reactions (Chen J. *et al.* 2010**;** Dixon and Edwards 2010) Accuracy of the mentioned phrases is supported by the results of various studies (Chen J. *et al*., 2002; Proceill et al., 2005; Silva Ortega *et al*., 2008 and Xiang *et al*., 2008). *P5CS* gene expression was the lowest at 50 mM NaCl, 72 hours after the treatment. At 200 mM NaCl the highest and lowest *P5CS* gene expression were observed 3 h and 6 h after treatment, respectively. In the salt resistant cultivar, *BADH* expression was the highest at 50 mM NaCl, and 72 h after the stress. The lowest BADH gene expression was observed with 200 mM NaCl and 6 h after salt stress. The highest and lowest BADH gene expressions were observed with 100 mM NaCl 24 h and 3 h after the treatment, respectively. Analysis of variance showed that BADH gene expression was not significant in different salt concentrations and time of treatment. In the salt-sensitive cultivar, BADH gene expression was the highest at 100 mM NaCl and 24 hours after the salt stress.

In 200 mM NaCl treatment, the highest and lowest levels of gene expression occurred 3 and 10 h after the application of salt stress. Similar results have been reported (Yushiba et al., 1997 Paranova et al., 1997 and Silva-Ortega et al., 2008). However, in other studies it was shown that the expression of these genes under drought stress did not increase significantly (Brey 1993 and 1997; Chandler et al., 1994; Skriver et al., 1990 'Braha'm et al., 2003).

Research has shown that gene expression under water stress and ABA can be influenced by the growth stage (Ingram et al., 1996; Shinozaki and Yamaguchi-Shinozaki, 1996). According to the results, similarity of gene expression pattern of BADH under salinity and ABA treatment suggested that common stress-signaling pathways may exist. On the other hand, it seems that differences in gene expression pattern in Mahuti as a salt tolerant cultivar and Alamut as a salt sensitive cultivar is due to different signaling pathways that activate P5CS expression.

Studies on P5CS show that plants exposed to biotic and abiotic stresses strongly express the gene (Chen *et al*. 1996; Jones et al. 2004; Uquillas *et al*. 2004). Nevertheless findings about BADH and P5CS function are limited (Dixon and Edwards 2010). On the other hand, Ramezani *et al.* (2012) reported that the amount of Na+ in the leaves of Alamut is more than Mahuti, so it can affect the gene expression profile.

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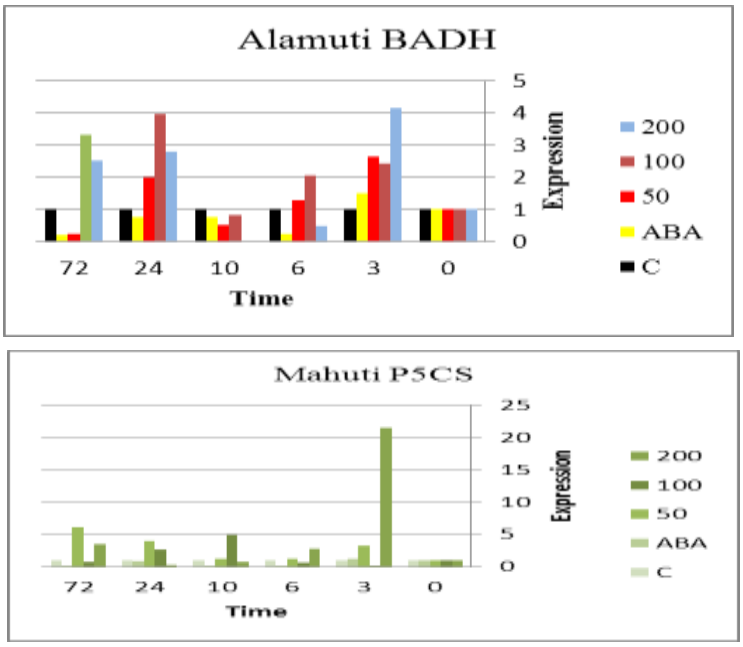

Figure 2. P5CS gene expression pattern under different NaCl concentrations (control, 50, 100 or 200 mM NaCl), ABA treatment and different times after NaCl treatment (0, 3, 6, 10, 24, 72 h).

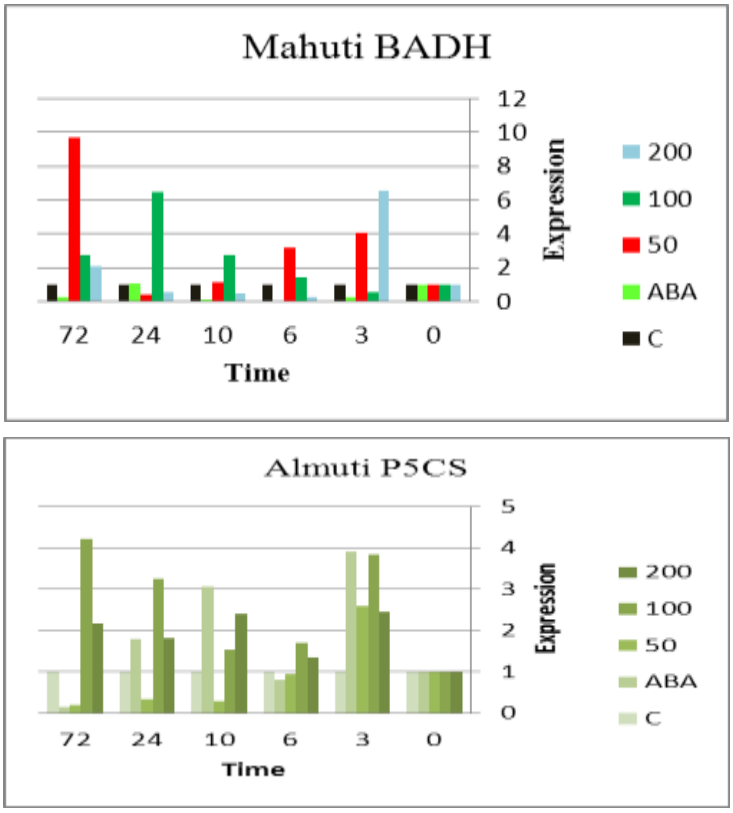

Figure 1. *BADH* gene expression pattern under different NaCl concentrations (control, 50, 100 or 200 mM NaCl), ABA treatment and different times after NaCl treatment (0, 3, 6, 10, 24, 72 h).

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