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Evaluating the expression of *Hv TIP2;3* and *Hv TIP4;1* in barley genotypes under different levels of salinity stress

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Abstract

Barley is the most salt tolerant cereal and is grown in a wide range of climatic conditions. To improve the plant tolerance to salinity, expression analysis of genes involved in stress tolerance could be effective in identification and development of tolerant genotypes. In this study, for evaluation of salinity effect on expression of *HvTIP2;3* and *HvTIP4;1* genes (encoding channel proteins in the membrane) in the root of barley, three genotypes *viz*., Clipper (salt susceptible), Sahara3771 (salt tolerant) and advanced breeding line (a salt tolerant line derived from a cross between Kavir and Sahara genotypes) were planted under 0, 100 and 200 mMNaCl. Analysis of variance revealed non-significant differences among genotypes, salinity levels and sampling stages for *HvTIP2;3* and *HvTIP4;1* genes expression pattern, whereas genotype x salinity interaction for *HvTIP2;3* and genotype x sampling stage interaction were significant for both of the genes studied. The expression of *HvTIP 2;3* gene in the 100 mMNaCl, was increased in salt susceptible genotype Clipper and decreased in tolerant genotypes compared with control. Mean comparison of genotype and sampling stage combination showed that the expression level of *HvTIP4;1* gene 3 weeks after salinity stress was increased in Sahara and advanced breeding line and decreased in Clipper. The study revealed that these genes are affected under salinity stress, and their effective utilization may increase salinity tolerance in plants.

Key words: Aquaporins, Expression pattern, Tonoplast intrinsic proteins, Salinity stress

Introduction

Under field conditions, commercially grown crops achieve an average of only about 50% of their potential yield due to the negative effects of abiotic environmental stresses (Hatfield and Walthall, 2015). Among abiotic stresses, salinity is one of the most severe, stresses affecting more than 800 million hectares of land throughout the world (Munnsand Tester 2008) and about 15 mha of land in Iran (FAO 2007). Development of salinity tolerant crops is now an important priority due to the rapid growth of the world population and the urgent need to maintain food security (Witzel et al. 2009). Salinity has a negative impact on root growth in many plant species, including barley which is the most salt-tolerant cereal crop (Hill et al. 2016).

Barley is the world's fourth most important cereal after wheat, rice, and corn and is cultivated in many regions of the world because of its high adaptation and tolerance to environmental conditions (FAO 2013). It is one of the most salt tolerant crops (Jiang et al. 2006). The barley malting cv. Clipper and the North African LR Sahara have contrasting root growth phenotypes in response to the early phase of salinity stress (Hill et al. 2016).

Salinity is one of the most significant environmental challenges limiting plant productivity. In the short term, salt stress is first perceived by the root system, inducing osmotic stress and causing reduced water availability. In the long term, salt stress induces ion toxicity due to nutrient imbalances in the cytosol (Acosta-Motos et al. 2017). The plant vacuole is a membrane-bound organelle that can occupy up to 90% of the total cell volume, and has multiple functions, including storage of nutrients and

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metabolites, generation of turgor, protein degradation, and plant defense (Gao et al. 2015).

Adversities such as drought, salinity or chilling affect water uptake and transport, and numerous plant MIP_S (Major intrinsic proteins), and are reported to be differentially regulated under such stresses (Forrest and Bhave 2008). Major intrinsic proteins or Aquaporins are membrane channels that facilitate the transport of water and small neutral molecules across biological membranes of almost all living organisms (Li et al. 2014; Maurel et al. 2015). MIP proteins in barley contain tonoplast intrinsic protein (Hv TIP), a NOD26-like intrinsic protein (Hv NIP), plasma membrane intrinsic protein (Hv PIP) and small basic intrinsic protein (Hv SIP) (Ligaba et al. 2011). The effectiveness of a water transporter, such as the aquaporins, is an important component of the plant response to stress. Expression *of Hv TIP* genes induce membrane proteins (Kaldenhoff et al. 2006).

Tonoplast intrinsic proteins regulate water movement across vacuolar membranes. *TIP* genes are associated with plant tolerance to some abiotic stresses that engender water loss, such as high salinity and drought (Wang et al. 2011). In barley, under salinity stress, expression of *Hv TIP* genes family is altered. Expression of these genes induces membrane proteins such as vacuole membrane proteins. Water and solute transport through the membrane occurs through these proteins and thus the plants maintain their osmotic condition and ions uptake under drought condition by means of uptake and transport of water as well solutes (Ligaba et al. 2011).

One of the effects of salt stress is the induction of osmotic stress in plants, which results in disruption of plant water balance. Therefore, study of the molecular mechanisms involved in osmotic regulation ability of barley as a salinity tolerant species could help to understand the involved mechanism. The aim of this work was to study the expression of *MIP* genes in barley under salt stress using real-time PCR.

Materials and methods

Plant materials

The plant material consisted of three barley genotypes; Sahara 3771, Clipper and an advanced breeding line (salt tolerance). Sahara 3771 is native to Algeria, winter type, six-rowed, tall and tolerance to salinity, and Clipper is spring type, two rowed and salt sensitive and bred in Australia (Widodo et al. 2009). Clipper and Sahara3771 seed was provided by the University of Western Australia and seed of advanced breeding line was obtained from Seed and Plant Improvement Institute, Karaj, Iran. The experiment was conducted through a factorial split plot based on randomized complete blocks with two replicates in three levels, viz., 0, 100 and 200 mMNaCl. Three plant genotypes were grown in a hydroponic system. After establishment, plants were exposed to salt stress. Sampling from the roots was done 24 hours, three days and three weeks, separately, after salinity imposition, for RNA extraction.

RNA extraction and real-time PCR analysis

RNA was extracted from root samples using ice-cold RNX_PLUS extraction kit from Sinacloncompany. To remove DNA, 2 µl DNase buffer and 2 µl Dnaseenzyme were added to samples. After a brief centrifugation, the samples were placed for 30 min. at 37°C. Then one microliter of EDTA was added to the tubes, and the samples were placed for 10 min at 65°C. The quality of extracted RNA was checked using 0.8% agarose gel electrophoresis and spectrophotometry. cDNA was synthesized with Fermentas kit (Fermentas, Hanover, MD). To amplify *Hv TIP2;3* and *Hv TIP4;1* genes, real-time PCR was performed using the synthesized cDNA and gene specific primers *Hv TIP2;3* = CTACTGGGTTGCGCAGCTC, GTGCCGA GGGATCCCTTC and *Hv TIP4;1* = CACCGACAAT AAGGCCGGT, CGGTGCTGTACG TGGTGG. Real-time PCR reaction was stained with SYBR Green.

Data analysis

Comparative analysis of the data was used to compare the expression of genes based on following formula (Livak and Schmittgen 2001).

$$2^{-\Delta\Delta CT} = (CT\ \text{Target} - CT\ \alpha\text{-}tubulin)\ \text{salinity x} - (CT\ \text{Target} - CT\ \alpha\text{-}tubulin)\ \text{salinity 0}$$

$C_{T\ Target}$ and $C_{T\alpha\text{-}tubulin}$ are data obtained from Real-Time PCR for subject and reference gene, respectively. Analysis of the data was done using SAS 9.1 software, preceded by examining normal distribution as well as variance homogeneity of the data.

Results and discussion

Expression of salinity responding genes

Examination of amplification curve of the subject and reference genes revealed that amplification had been amply successful, lacking no non-specific amplification

in the circles. Various *C*T (Threshold Cycle) for different salinity treatments indicates the differences in expression of subject genes under different levels of salinity (Fig. 1).

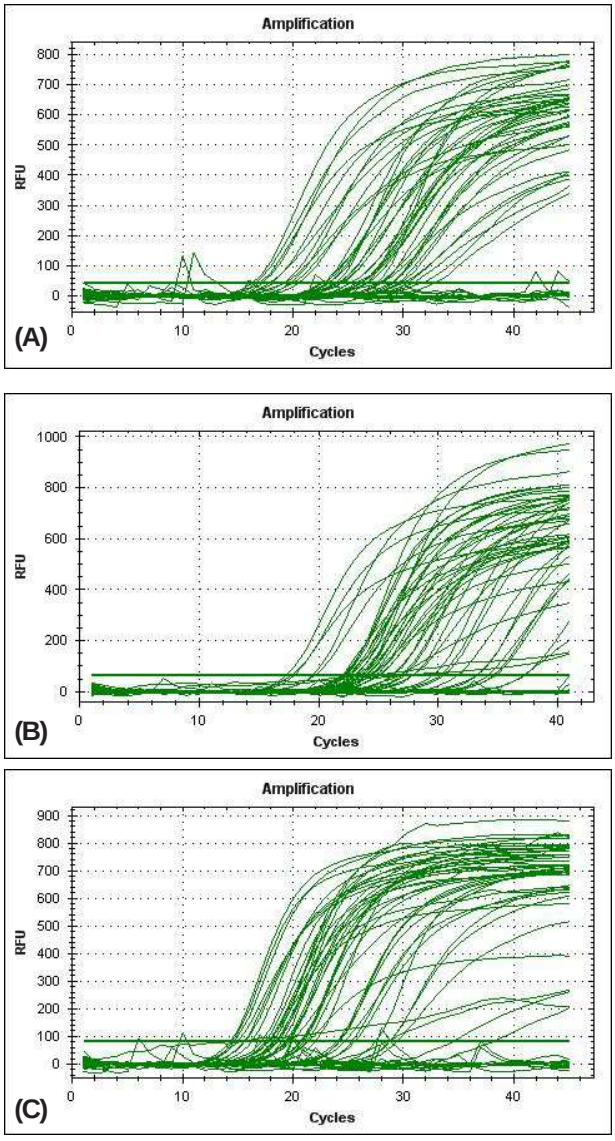

Fig. 1. The amplification curve A:*Hv TIP2;3*; B:*Hv TIP4;1*; C: α-*tubulin* genes in barley genotypes under salinity levels 0, 100 and 200 mMNaCl

Considering SYBR Green (a fluorescence dye used to mark amplified threads) has the ability of binding to double-stranded segments like paired primers and non-specific threads, analysis of the melting curve was used in order to pinpoint the performance of specific primers. In order to achieve melting curve, the temperature in polymerase chain reaction was increased gradually. At low temperatures, all amplified products rendered double-stranded, and SYBR Green bound to them and, therefore, fluorescence emission was high. As temperature rose and approached the melting point respective to amplified strands, the products became annealed and single stranded. Thus, fluorescence emission dropped sharply. In this method, the fluorescence intensity was measured and drawn against the temperature. At melting point, 50 percent of hydrogen bonds in double-stranded DNA were broken and fluorescence emission suddenly changed. In this curve, each individual peaks indicates a melting point corresponding to a specific product (QIAGEN 2005). Accordingly, a single peak on the melting curves indicates specific performance of primers on target and reference genes.

The melting curve for reference *á-tubulin* and subject genes *Hv TIP2;3* and *Hv TIP4;1* in barely genotypes Clipper, Sahara 3771 and advanced breeding line under 0, 100 and 200 mMNaCl treatments is provided in Fig. 2. As it can be seen on the melting curves, presence of a single peak above the threshold for reference α-tubulin indicates the specific amplification of in PCR. As for *Hv TIP2;3* and *Hv TIP4;1,* besides the main peak corresponding to specific amplification of the gene, there were small peaks which could not bias the estimation of the target gene's concentration.

Analysis of variance in expression of Hv TIP2;3 and Hv TIP4;1

Analysis of variance of salinity treatments and control on the expression of *Hv TIP2;3* and *Hv TIP4;1* genes for the mentioned genotypes measured in three different occasions, 24 hours, three days and three weeks after salt stress imposition, based on $2^{-\Delta\Delta CT}$ data is provided in Table 1. No significant difference was detected between replicates, genotypes, levels of salinity and occasions of sampling concerning changes in expression of the mentioned genes. Interaction of genotype x salinity for *HvTIP2;3*, and bilateral interaction of genotype x sampling stage for both genes was significant. Interaction of salinity x sampling stage interaction, and the trilateral interaction of genotype x salinity x sampling stage was also significant for the said genes.

Salt stress influences water's uptake and transfer, and distinctively affects expression of *MIP* genes including *TIP* (Forrest and Bhave 2008). It has been reported that TIP proteins, located on vacuole membrane, are linked to salt tolerance of the plant

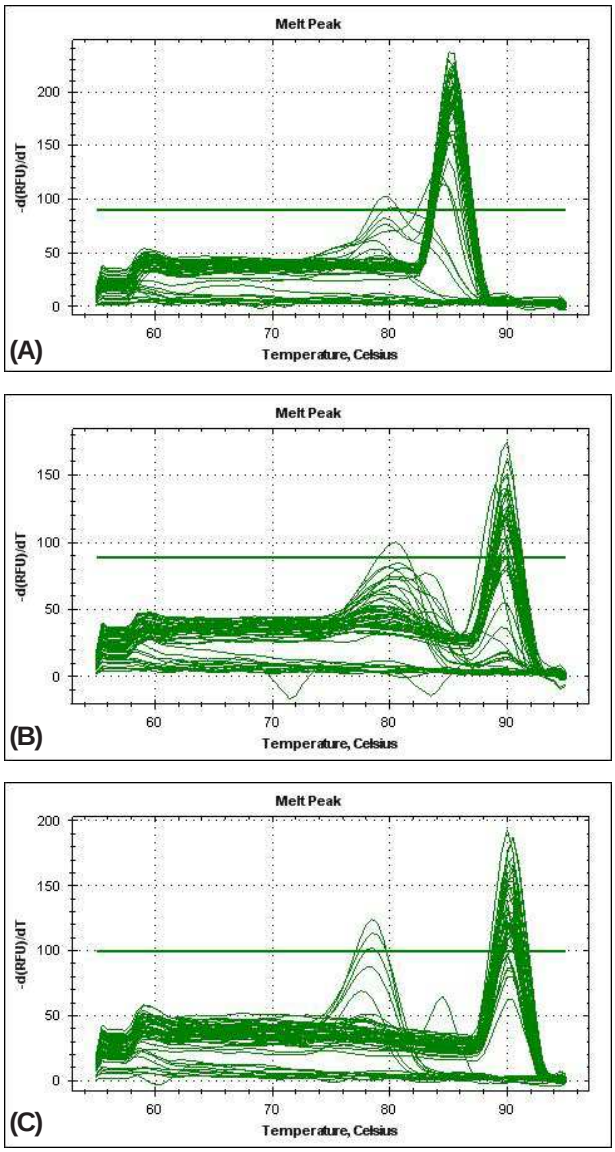

Fig. 2. The melting curve A: α-*tubulin*; B: *Hv TIP2;3*; C: *Hv TIP4;1* genes in barley genotypes under salinity levels 0, 100 and 200 mMNaCl

(Wang et al. 2011). *TIP* genes are expressed differently in different plant organelles (Tyerman et al. 2002; Zhao and Pie 2005, Ligaba et al. 2011; Gao et al. 2013; Zhang et al. 2016; Zhang et al. 2017). In studies on several salinity responding genes in barely, 92 genes experienced changes in expression (Ueda et al. 2004). Based on studies, MIP transcripts are abundant in barley's root than they are in aerial parts; *HvTIP2;3* has the most and *Hv TIP4;1* the least transcript in barley (Ligaba et al. 2011).

Change in expression of *HvTIP2;3* in the mentioned genotypes and salinity treatments in

Table 1. Analysis of variance for the expression of *Hv TIP2;3* and *Hv TIP4;1* genes in barley genotypes under salinity levels 0, 100 and 200 mMNaCl in 24h, 3 day and 3 weeks after salt treatment.

Source of variation	df	Mean squares	
		Hv TIP2;3	*Hv TIP4;1*
Replication	1	0.0003^{ns}	0.238^{ns}
Genotype	2	0.076^{ns}	1.255^{ns}
Salinity	1	0.654^{ns}	0.149^{ns}
Genotype x salinity	2	1.157*	1.006^{ns}
Error a	5	0.137	0.573
Sampling stage	2	0.163^{ns}	0.531^{ns}
Genotype x sampling stage	4	1.106**	1.348*
Salinity x sampling stage	2	0.041^{ns}	0.406^{ns}
Genotype x salinity x sampling stage	4	0.148^{ns}	0.870^{ns}
Error b	12	0.059	0.276
CV (%)		25.37	32.57

ns = Non-significant (P>0.05); * = Significant (P<0.05); ** = highly significant (P<0.01).

comparison to the control is provided in Fig. 3. For 100 mMNaCl, the gene is significantly less expressed in tolerant Sahara3771 and advanced breeding line than it is in sensitive Clipper. Under 200 mMNaCl, *HvTIP2;3* displayed significant down-expression in sensitive clipper, while in tolerant Sahara3771 and advanced breeding line the gene showed significant up-expression, the highest expression being of advanced breeding line. Under 100 mMNaCl treatment, the expression of *HvTIP2;3* was same in all tolerant genotypes, indicating a similar response compared to the sensitive one, Clipper. Tolerant genotypes

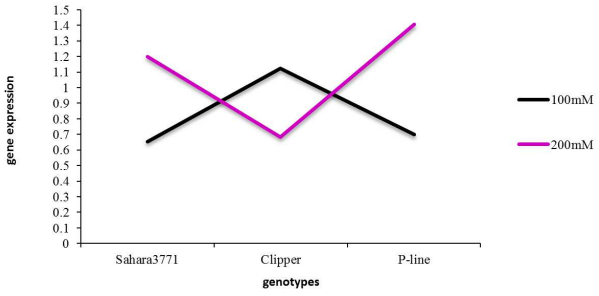

Fig. 3. Changes in gene expression *Hv TIP2;3* in the stress levels of 100 and 200 mMNaCl compared to control in barley genotypes Clipper, Sahara3771 and advanced breeding line (P-line) at average samplings stage

underwent down-expression compared to the control. For 200 mMNaCl, however, the response was the opposite. The conclusion being that reduction in *HvTIP2;3* expression in tolerant genotypes might act as a mechanism against mild salinity. Studies have suggested that *HvTIP2;3* is expressed more in normal condition than stress condition; significant reduction in expression in roots of barley Haruna-Nijo under 100 mMNaCl has been detected 24 hours after salt stress imposition (Besse et al. 2011; Ligaba et al. 2011). It has been also reported that severe salt stress (200 mMNaCl) significantly reduce hydraulic conductivity of barley roots, contrary to mild stress (100 mMNaCl) where hydraulic conductivity was barely reduced. Nonetheless, no significant difference was detected for *HvTIP* gene at mRNA level.

Comparison of the means for genotype and sampling occasion for changes in expression of *Hv TIP2;3* showed that 24 hours after salinity imposition, expression significantly increased in tolerant genotypes compared to the sensitive Clipper (Fig. 4),

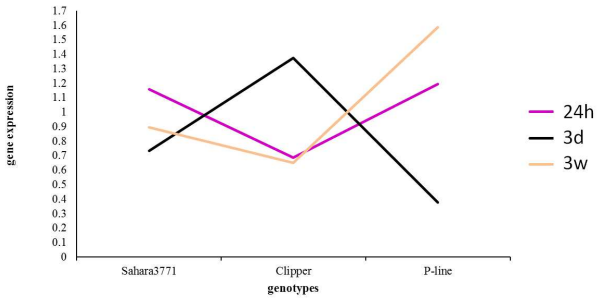

Fig. 4. Changes in gene expression *Hv TIP2;3* barley genotypes Clipper, Sahara3771 and advanced breeding line (P-line) at 24 hours, three days and three weeks after applying the average salinity stress levels

indicating that, primary response to salinity is different in tolerant genotype from sensitive ones. Three days after salinity imposition, *Hv TIP2;3* was down-expressed in Sahara3771 and advanced breeding line, a bigger reduction was observed in the later. However, a significant increase was found in Clipper. After three weeks, expression of *Hv TIP2;3* in Sahara 3771 and advanced breeding line was increased. Based on the observation it can be said that *Hv TIP2;3* is likely to be involved in early response to salinity in tolerant genotypes.

The expression analysis indicated that *GmTIP2;3* gene in soybean was constitutively expressed in all detected tissues, with higher levels in the root, stem and pod, and the accumulation of *GmTIP2;3* transcript showed a significant response to osmotic stresses, including 20% PEG6000 (polyethylene glycol) and 100μM ABA (abscisic acid) treatments. Yeast heterologous expression revealed that *GmTIP2;3* could improve tolerance to osmotic stress in yeast cells. Integrating these results, *GmTIP2;3* might play an important role in response to osmotic stress in plants (Zhang et al. 2016). Boursiac et al. (2005) studied the effects of salinity on hydraulic conductivity (Lp(r)) of *Arabidopsis* roots in less than two hours and 6-24 hours after salinity imposition. Results indicated that inhibition of water transference due to salinity is associated with plant's immediate response to stress, which is accompanied by changes in aquaporin's production; 100 mMNaCl caused 70% reduction in Lp(r). Later, TIP's rate of expression was studied in Arabidopsis in hydroponic condition, using RT-PCR. Results showed that *TIP2;2*, *TIP1;2* and *TIP1;1* are highly expressed in roots. Except for *PIP2;3* and *TIP2;3*, the expression rate for TIP and PIP groups of genes was static within the two hours after stress induction. The amount of aquaporin transcripts was significantly reduced in 2 to 4 hours after salinity imposition. More reduction happened 6 hours after salt stress, and the expression continued to drop for 24 hours for most of these genes. Generally, all aquaporin transcripts dropped by 25 to 60% two hours after stress induction.

Comparison of the means for genotypes and sampling occasion of *HvTIP4;1* indicated that it's expression was significantly boosted in Sahara3771, 24 hours after salinity induction, while clipper and advanced breeding line had relatively the same increase for that time period. Three days after salinity stress induction, three genotypes showed a static amount and slope of increase. The expression was lower compared to the 24-hour. Three weeks after salinity induction, rate of expression dropped significantly in sensitive Clipper, while the tolerant genotypes showed increase (Fig. 5).

In an experiment, expression of several genes including *Os TIP4;1* was investigated in roots, leaf and anther of rice, 26 and 56 days after emergence. The gene seemed to be expressed in all three organelles (Sakurai et al. 2005). Hove et al. (2015) studied MIP family expression in barley. Analysis of leaf mRNA-seq data identified notable differential expression of *HvPIP1;2* and *HvTIP4;1* under salt stress. Analyses of other gene expression resources also confirmed

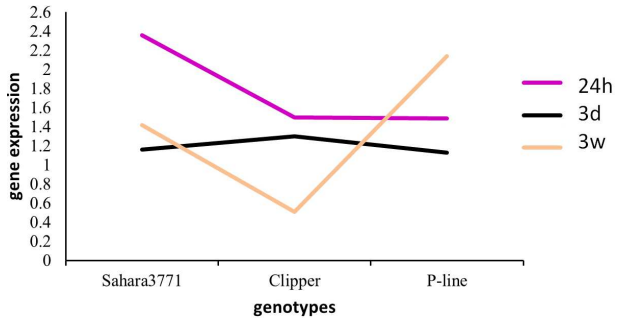

Fig. 5. Changes in gene expression *Hv TIP4;1* in barley genotypes Clipper, Sahara3771 and advanced breeding line (P-line) at 24 hours, three days and three weeks after applying the average salinity stress levels

isoform-specific responses in different tissues and/or in response to salinity, as well as some potentially inter-cultivar differences. In case of expression of some *Hv TIP* genes, it was reported that under 100 mMNaCl salinity condition for 24 hours, *HvTIP4;1* was expressed significantly highly compared with the control (Ligabaet al*.* 2011). It is validated that there is a link between gene expression and responses to abiotic stresses like salinity, as investigation on gene expression, researchers observed that after 3, 8 and 27 hours after salinity stress imposition, majority of the genes were over expressed 27 hours after stress induction. On the other hand, some genes were less expressed after 8 hours (Waliaet al*.* 2006).

Investigation of MIP expression in different organelles of *Arabidopsis* under salinity stress using RT-PCR revealed that mostly several aquaporins are expressed in the same organelle, and many of them were either over- or less-expressed dependent on different stresses (Alexandersson et al. 2005).

Authors' contribution

Conceptualization of research (RS, SAM, MM); Designing of the experiments (RS, SAM, MM); Contribution of experimental materials (SAM, SG, MM); Execution of field/lab experiments and data collection (RS, SAM); Analysis of data and interpretation (RS, SAM, SG); Preparation of manuscript (RS, SAM, SG, MM).

Declaration

The authors declare no conflict of interest.

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