



Changes in the expression pattern of two aquaporin genes in barley shoots under salinity stress

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Article Info

Article type:

Research article

Article history:

Received: July 8, 2024

Revised: September 22, 2024

Accepted: October 28, 2024

Published online:

December 31, 2024

Keywords:

Advanced breeding line,
Aquaporin,
Gene expression,
Genotype,
RT-PCR

Abstract

Objective: Barley (*Hordeum vulgare* L.) is an important crop with wide adaptation and tolerance to abiotic stresses. Salinity is one of the major abiotic stresses affecting crop growth and production. We assessed the changes in the expression pattern of HvTIP2;3 and HvTIP4;1 genes from the aquaporin family in the shoot of salt-tolerant cultivar Sahara3771, an advanced breeding line (A-Line), and the salt-sensitive cultivar Clipper.

Methods: A split-plot experiment, based on a randomized complete block design with three replications, was conducted in a hydroponic culture system under greenhouse conditions. The studied genotypes were cultured under normal and 100 and 200 mM NaCl treatments. Twenty-four hours, three days, and three weeks after salinity treatment, leaf samples were harvested for RNA extraction. Real-time PCR was conducted using gene-specific primers, and $\Delta\Delta C_t$ was used in the analysis of variance.

Results: The results revealed significant effects of genotypes and sampling times on the expression pattern of the HvTIP4;1 gene. Salinity $\times$ genotype, salinity $\times$ sampling time, genotype $\times$ sampling time, and salinity $\times$ genotype $\times$ sampling time interactions were also significant ($p < 0.01$). The expression pattern of the two studied genes was not similar in the tolerant genotypes, implying different tolerance mechanisms in these genotypes.

Conclusion: The studied barley genotypes showed different salt-tolerance mechanisms. These findings provide the foundation for future work to dissect the role of HvTIP2;3 and HvPIP1;4 genes, as well as other aquaporins containing tonoplast intrinsic protein (HvTIP) coding genes, in conferring adaptation to various stresses.

Cite this article: Pouyanmehr M, Mohammadi, SA, Toorchi M. 2024. Changes in the expression pattern of two aquaporin genes in barley shoots under salinity stress. J Plant Physiol Breed. 14(2): 223-235. <https://doi.org/10.22034/JPPB.2024.62391.1339>



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Publisher: University of Tabriz

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Introduction

Salinity following drought is one of the major stresses that cause considerable yield loss in major crops worldwide (Beltrán 1999). It was reported that about 20% of agricultural land, *i.e.*, more than 45 million hectares, is under salinity effects (Allbed and Kumar 2013). Crops grown in saline soils are unable to uptake enough water and nutrition to maintain their biological activities, thereby experiencing a significant decline in yield-related traits. Moreover, the accumulation of toxic ions in different parts of crops causes severe reduction in the growth characteristics and even crop death (Roy *et al.* 2014; Atta *et al.* 2023).

Barley (*Hordeum vulgare* L.), as one of the most important crops, was domesticated nearly 10000 years ago and has a large gene pool conferring tolerance to salinity. It has a high level of adaptability to various environmental conditions (Kim *et al.* 2016). In addition, a low number of chromosomes, diploid genome, self-pollination, short life cycle, availability of genome sequence, and the possibility of cultivation in a wide range of climates, have made barley one of the important model plants for genetic research related to salinity and other types of abiotic stresses (Walia *et al.* 2007; EL Sabagh *et al.* 2019).

One of the major breeding objectives of plant breeding programs under changing climate conditions is the development of abiotic stress-tolerant genotypes. A mutagenic nature of tolerance to abiotic stresses, such as salt stress, empowers plants to flourish under different conditions. Plants respond to salinity stress by adapting specific mechanisms and activating various salt tolerance genes to cope with various stresses, such as oxidative and osmotic stresses induced by salinity. Due to the complex genetic nature of salinity tolerance, its improvement is quite complex, and advancement has been less than expected over the past few decades. Therefore, mapping and characterization of the genes conferring tolerance to salinity are crucial for developing tolerant genotypes (Flowers 2004; Afzal *et al.* 2023). The genes whose expression changes significantly under stressful conditions are essential players in plants, and the determination of their function and expression changes in different genotypes of crops is one of the main aims of genetic studies (Ligaba *et al.* 2011).

Zhuang *et al.* (2015) reported that efficient water transport in plants is a major process by which plants can maintain their basic physiology and tolerate dehydration conditions under salinity stress. Water transport at the cellular level is controlled by channel proteins called aquaporins (AQPs). These proteins have a central role in transporting small molecules and water across the membranes, and their regulatory function has been shown in various crop species (Johanson *et al.* 2001; Chaves *et al.* 2009; Sun *et al.* 2024). The studies on various crop plants reported altered expression levels of

aquaporin genes under salt stress (Yamada *et al.* 1995; Li *et al.* 2000; Kawasaki *et al.* 2001). A high level of isoform multiplicity in aquaporins has challenged efforts to assess the regulatory functions of these proteins in the water transport events under abiotic stresses (Kirch *et al.* 2000; Suga *et al.* 2002).

Forrest and Bhawe (2008) reported that salinity, drought, and cold stresses affect the expression profile of genes encoding aquaporins in crops. Barley's aquaporins contain tonoplast intrinsic protein (*HvTIP*), a NOD26-like intrinsic protein (*HvNIP*), plasma membrane intrinsic protein (*HvPIP*), and small basic intrinsic protein (*HvSIP*) (Ligaba *et al.* 2011). The expression of the barley aquaporin gene *HvPIP2;5* in yeast enhanced its tolerance under high salt and osmotic stress. In *Arabidopsis*, overexpressing *HvPIP2;5* under high salt and osmotic stresses improved stress tolerance at the germination stage and root growth compared to the wild type. It was reported that *HvPIP2;5* overexpression enables plants to cope with stress and recover after a 3-week drought period, while the control plants wilted and died under drought stress (Alavilli *et al.* 2016). Salami *et al.* (2017) reported differential expression of *HvTIP2;3* and *HvTIP4;1* genes in the root of salt-susceptible and salt-tolerant barley genotypes under different levels of NaCl.

The present study aimed to analyze the changes in the expression profile of two aquaporin genes in the shoots of three barley genotypes under different levels of salinity stress and exposure times.

Materials and Methods

Plant materials and experiment conditions

Three barley genotypes, including the salt-tolerant Sahara3771 cultivar and an advanced breeding line (A-Line) produced through crossing the Sahra and Kavir cultivars, and the salt-sensitive Clipper, were used in this study. Sahara3771 is a six-rowed and tall winter-type landrace from Algeria, and Clipper is a two-rowed and spring-type variety from Australia (Karakousis *et al.* 2003). Plants were grown under greenhouse conditions (16/8 hours day/night, 600-800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, and 25 ± 3 °C day and 17 ± 3 °C night temperature, and relative humidity of 45/60% day/night) using a hydroponic culture system. A split-plot experiment based on a randomized complete block design with three replicates was used. After seed germination, the 7-day-old seedlings were transferred into a hydroponic system and irrigated daily using a modified Hoagland nutrient solution. Seven days after transferring seedlings, plants were exposed to 0 (control), 100, and 200 mM NaCl in daily increments of 50 mM NaCl. In addition to NaCl, CaCl_2 was added to maintain a $\text{Na}^+/\text{Ca}^{2+}$ concentration ratio of 10:1. For RNA extraction, 24 hours, 3 days, and 3 weeks after salt treatments, leaf samples were harvested.

RNA extraction and real-time PCR analysis

Total RNA was isolated using an ice-cold RNX_PLUS extraction kit (Sina Clone Company). The RNA samples were treated with RNase-free DNaseI and incubated at 37 °C for 30 min to eliminate possible DNA contamination. A 1% agarose gel electrophoresis and PicoDrop spectrophotometer were used to assess the quality and quantity of RNA samples, respectively. The cDNA was synthesized using the RevertAidTM Reverse Transcriptase kit (Fermentase, Germany). The real-time PCR was performed using *Hv TIP2;3* and *Hv TIP4;1* genes' specific primers, cDNA as templates in an Illumina Real-Time PCR system (Illumina, SanDiego, CA, USA), and SYBR® (Invitrogen, 2006) green PCR reagent (Qiagen, Hilden, Germany). Normalization of the gene transcripts was done based on the α -tubulin as a reference gene. The primer sequences for amplification of the studied genes and α -tubulin are presented in Table 1.

Table 1. The primer sequences used for amplifying *HvTIP2;3* and *HvTIP4;1* as subject genes, and the *tubulin- α 2* gene as the reference gene.

Gene	Reverse primers	Forward primers
<i>HvTIP2;3</i>	GTGCCGAGGGATCCCTTC	CTACTGGGTTGCGCAGCTC
<i>HvTIP4;1</i>	CGGTGCTGTACGTGGTGG	CACCGACAATAAGGCCGGT
<i>α-tubulin2</i>	AGCATGAAGTGGATCCTTGG	AGTGTCTGTCCACCCACTC

Data analysis

The C_t values of target and reference genes for each genotype under different treatments and exposure times were recorded at three replications. The ΔC_t values were calculated by subtracting the gene C_t from that of α -tubulin2 in each genotype under different NaCl treatments and exposure times in each replication. The $\Delta\Delta C_t$ for each genotype was then calculated by subtracting its ΔC_t under salt treatments from that under normal conditions (Formula 1). In addition, the $\Delta\Delta C_t$ was calculated by subtracting the ΔC_t of the salt-tolerant genotypes (Sahara3771 and A-Line) from that of the salt-susceptible genotype (Clipper) (Formula 2).

$$\Delta\Delta C_t = (C_t \text{ Target} - C_t \alpha\text{-tubulin})_{\text{salinity x}} - (C_t \text{ Target} - C_t \alpha\text{-tubulin})_{\text{salinity 0}} \quad (1)$$

$$\Delta\Delta C_t = (C_t \text{ Target} - C_t \alpha\text{-tubulin})_{\text{tolerant genotype}} - (C_t \text{ Target} - C_t \alpha\text{-tubulin})_{\text{susceptible genotype}} \quad (2)$$

The $2^{-\Delta\Delta C_t}$ was used for the analysis of variance (ANOVA) using a linear model of a split-plot experiment based on a randomized block design. Duncan's multiple range test with a critical probability level of $p \leq 0.05$ was used for mean comparisons. Statistical analyses were performed using SAS 9.3 software (SAS, 2011).

Results

The melting curves of the studied genes are presented in Figure 1. The melting curve of all products showed a single peak under the testing temperature, indicating that all primers amplified specific fragments.

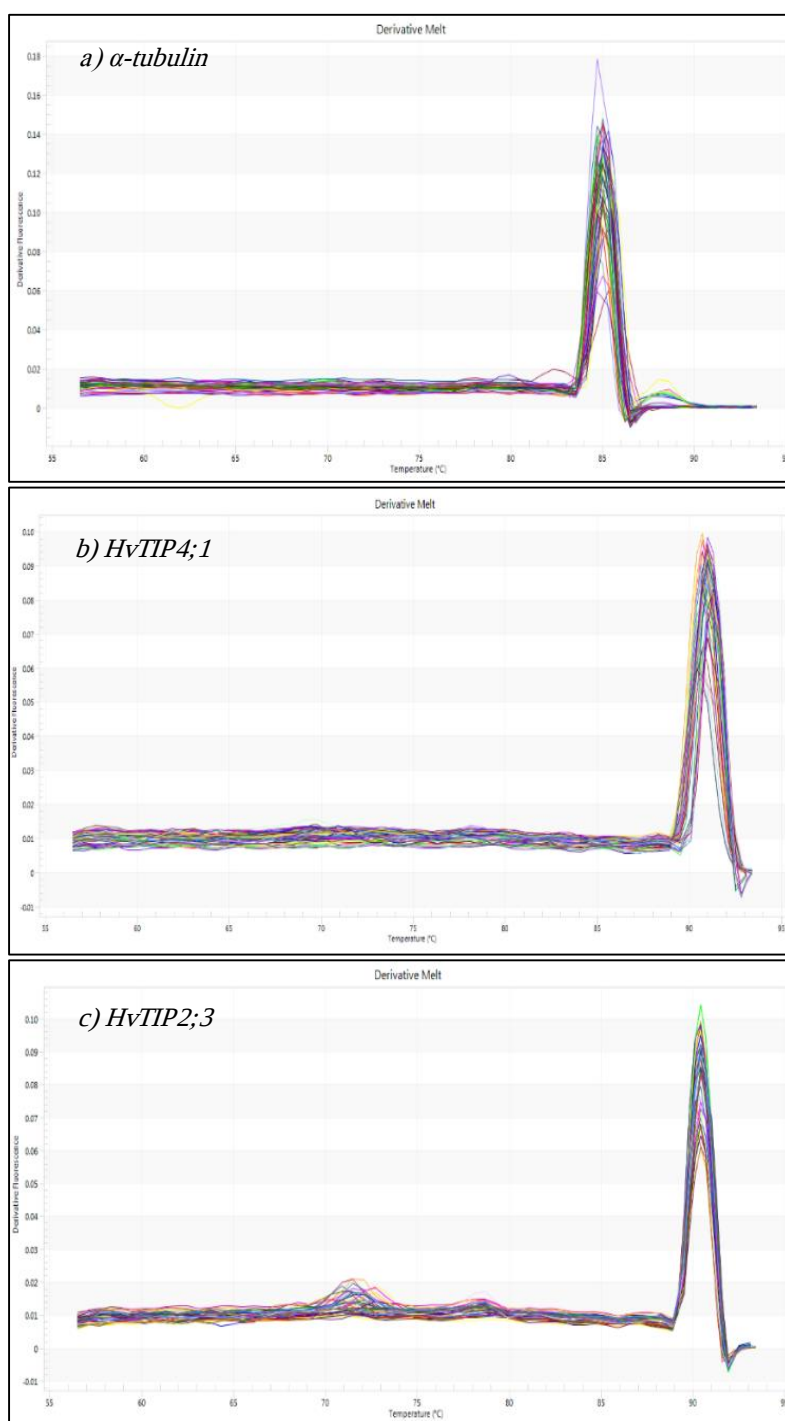


Figure 1. The melting curve of α -tubulin (a), *Hv TIP4;1*(b) *Hv TIP2;3*, and (c) genes in barley genotypes (Sahara3771, Clipper, and A-Line) under 0, 100 and 200 mM NaCl treatment.

The results of ANOVA showed significant effects of genotypes ($p \leq 0.01$), salinity $\times$ genotype ($p \leq 0.05$), and genotype $\times$ time ($p \leq 0.01$) on the expression pattern of *HvTIP2;3* gene. In the case of *HvTIP4;1* gene, the genotypes and sampling times on the expression pattern were significant ($p \leq 0.01$). The salinity $\times$ genotype, salinity $\times$ sampling time, genotype $\times$ sampling time, as well as salinity $\times$ genotype $\times$ sampling time interactions were also significant ($p \leq 0.01$) (Table 2).

Table 2. Analysis of variance for *HvTIP4;1* and *HvTIP2;3* genes in three barley genotypes under three different levels of NaCl (100-0, 200-0, and 100-200) and three sampling times (24 h, third day, and third week).

Sources of variation	df	Mean squares	
		<i>HvTIP2;3</i>	<i>HvTIP4;1</i>
Replication	1	2.97 ^{ns}	0.01 ^{ns}
Salt	2	1.14 ^{ns}	2.49 ^{ns}
Error 1	2	0.34	0.32
Genotype	2	44.17 ^{**}	3.40 ^{**}
Salt $\times$ Genotype	4	5.17 [*]	1.41 ^{**}
Sampling time	2	0.37 ^{ns}	1.62 ^{**}
Salt $\times$ Sampling time	4	0.14 ^{ns}	0.90 ^{**}
Genotype $\times$ Sampling time	4	11.55 ^{**}	1.75 ^{**}
Salt $\times$ Genotype $\times$ Sampling time	8	1.62 ^{ns}	3.83 ^{**}
Error 2	24	1.72	0.20
CV%		16.28	0.76

ns: Non-significant; *: Significant at probability level of 0.05; **: Significant at probability level of 0.01.

HvTIP2;3 gene expression

The amplification of the *HvTIP2;3* gene was significantly higher on the A-line compared to the salt-tolerant Sahara3771 and salt-susceptible Clipper cultivars (Figure 2). Non-significant differences were observed among genotypes 24 hours after exposure to 100 mM NaCl compared to the control (0 mM NaCl). The 200 mM NaCl compared to the control downregulated the expression of *HvTIP2;3* gene in Sahara3771 and Clipper cultivars, and the amount of reduction in Sahara3771 was significantly higher than in Clipper. The level of *HvTIP2;3* gene transcripts on the salt-tolerant A-Line was upregulated at 200 and 100 compared to 0 mM NaCl, and 100 compared to 0 mM NaCl, and it was significantly higher than that of the Sahara3771 and Clipper cultivars at 200 compared to 100 and 0 mM NaCl (Figure 3A). With prolonged sampling time, the expression of *HvTIP2;3* was significantly increased in A-Line, whereas Sahara3771 showed the largest decrease at three weeks after salt treatment (Figure 3B). In general, the transcript level of the *HvTIP2;3* gene in Sahara3771 was significantly reduced under prolonged salt treatment compared to the control. In case of the Clipper, 24 hours and three days of exposure to salinity reduced the transcript level of the *HvTIP2;3* compared to control, but the transcript level increased under long salt exposure (three weeks).

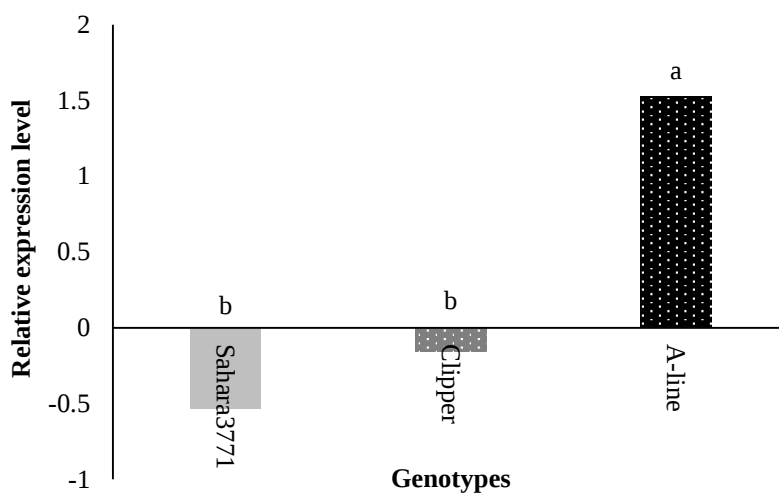


Figure 2. The expression level of the *HvTIP2;3* gene in three barley genotypes.

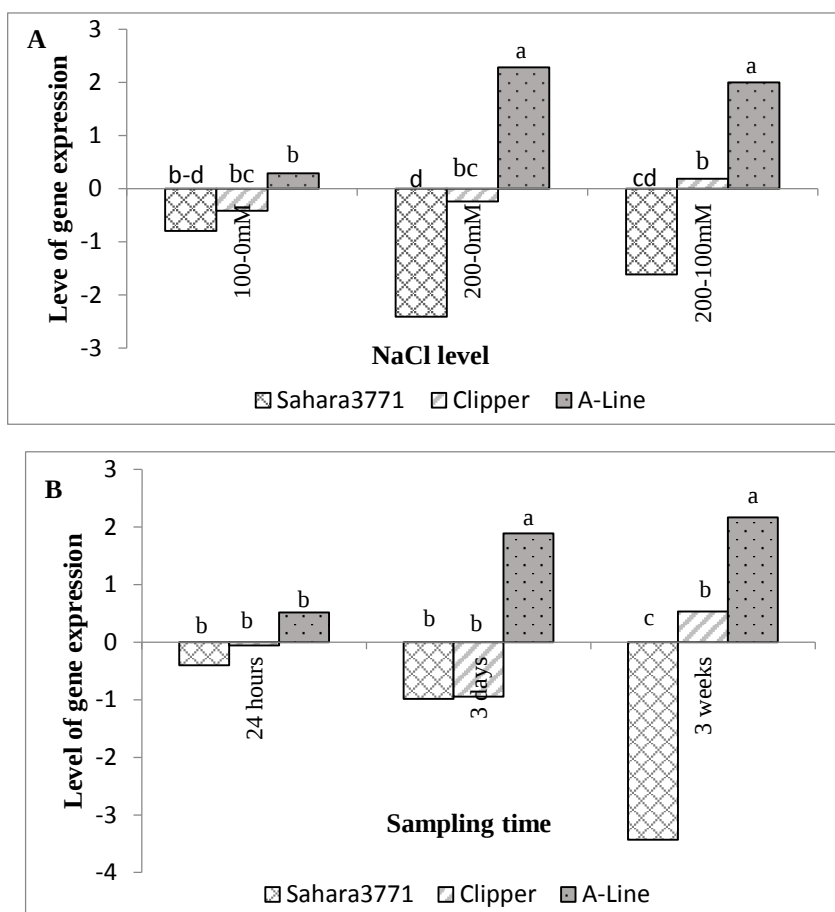


Figure 3. The expression level of *Hv TIP2;3* gene in three barley genotypes under different NaCl treatments (A), and various sampling times (B).

HvTIP4;1 gene expression

As shown in Figure 4, 24 hours after salt treatment, *HvTIP4;1* gene was significantly overexpressed in Sahara3771 under 100 mM NaCl compared to the control (0 mM NaCl), whereas the gene was upregulated in Clipper and A-line. Comparison of the expression level of the *HvTIP4;1* gene 24 hours after 200 mM NaCl treatment compared to the control showed a significant reduction in the transcript level of the gene in Sahara3771 than 100 mM NaCl treatment, whereas the expression level of the gene was significantly increased in Clipper and A-Line under 200 mM NaCl compared to 100 mM NaCl treatment.

Reduction in the transcript level of *HvTIP4;1* gene 24 hours after 100 compared to 200 mM NaCl treatment was highly significant in Sahara3771, but significant overexpression was observed in Clipper and A-Line.

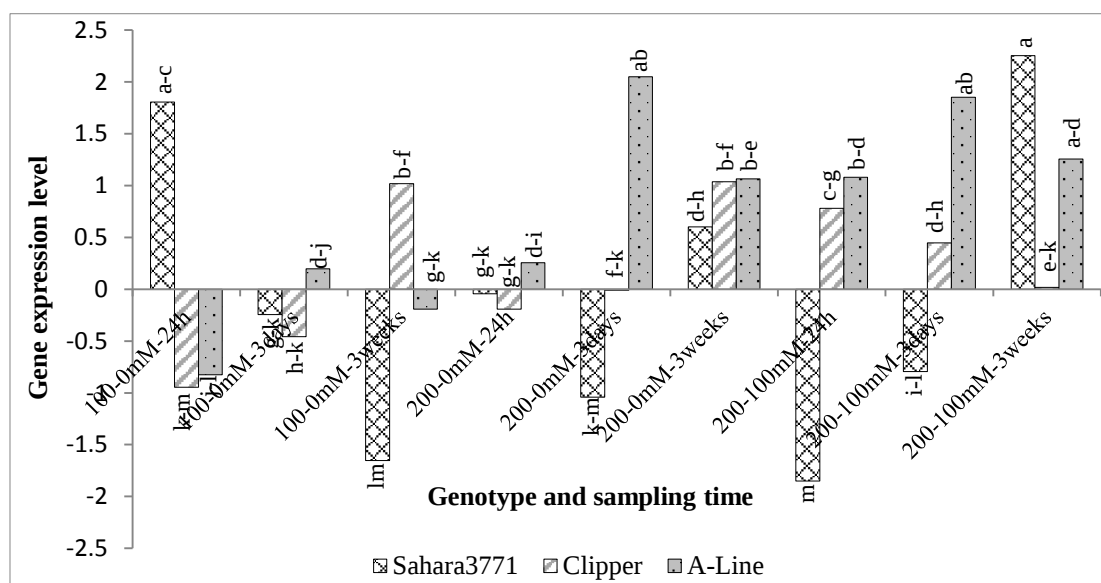


Figure 4. The expression Level of the *HvTIP4;1* gene in three barley genotypes under 0-100 mM, 0-200 mM, and 100-200 mM NaCl treatment at various sampling times. Abbreviations: 24h, 24 hours after salinity treatment; 3D, three days after salinity treatment; 3W, three weeks after salinity treatment.

Three days after salt treatment, the expression of *HvTIP4;1* gene under 100 mM NaCl compared to the control declined in all genotypes, but the reduction in Sahara3771 and Clipper cultivars was significantly higher than in A-Line. Comparison of 200 mM NaCl and the control revealed the overexpression of the gene in A-line and upregulation in Sahara3771 and Clipper cultivars, and Sahara3771 showed a higher reduction in the transcript level of *HvTIP4;1*. Assessment of gene expression in the genotypes under 100 mM compared to 200 mM NaCl, three days after salt treatment, showed a similar pattern in genotype profiles like 200-0 mM NaCl, but there was an increase in the gene transcript level in Clipper.

The analysis of *HvTIP4;1* expression three weeks after 0, 100, and 200 mM NaCl treatments revealed under-regulation of the gene in Sahara3771 and A-line, with higher reduction in the transcript level in Sahara3771 and overexpression in Clipper under 100 mM NaCl compared with the control. Expression of *HvTIP4;1* gene was significantly increased in Sahara3771 and A-Line under 200 mM NaCl compared to the control, but there was no change in the gene expression in Clipper in comparison with 100-0 mM NaCl. The expression of *HvTIP4;1* gene under 100 mM NaCl compared to 200 mM three weeks after salt treatment, showed a significant increase and decrease in Sahara3771 and Clipper, respectively, but there was no significant change in A-Line as compared to 200-0 mM NaCl. The results showed differential responses of the genotypes to salt treatments and sampling times.

Discussion

The results obtained indicate significant differences among the accessions for all the morphological traits evaluated. This finding aligns with the study conducted by Atta *et al.* (2011), who reported substantial variability among ecotypes for the majority of the yield-related characteristics in *H. sabdariffa* L. Our results showed a positive correlation among CD, NB, and DF. The CD also had a positive correlation with PH. Selection for such traits may optimize yield through their simultaneous response to salt stress by which changes in water uptake and transfer patterns induce osmotic stress in plants and disturb plant water balance (Shi *et al.* 2022). We assessed the expression of aquaporin protein-coding genes *HvTIP2;3* and *HvTIP4;1* in barley to elucidate the osmotic homeostasis in maintaining molecular mechanisms. Aquaporins are tonoplast intrinsic proteins (TIPs) that play critical roles in dehydration stress responses, plant-water relations, and crop productivity by mediating the bidirectional flux of water and other substrates across cell membranes (Hove *et al.* 2015). The TIPs located at the membrane of the plant vacuole mediate the flow of water through the tonoplast (Wang *et al.* 2011), and the number of *TIP* genes in plants ranges from 6 to 35 (Kurowska *et al.* 2019).

In this research, the expression pattern of two TIP genes, *HvTIP2;3* and *HvTIP4;1*, in Sahara3771 and Clipper cultivars and an advanced breeding line under 0, 100, and 200 mM NaCl after 24 hours, three days, and three weeks was studied. The transcript level of both genes was significantly affected by genotype, salt $\times$ genotype, genotype $\times$ sampling time interactions. The effect of sampling time, salt $\times$ sampling time, and salt $\times$ genotype $\times$ sampling time interaction were also significant for the *HvTIP4;1* gene. The salt treatments and sampling times differentially affected the expression of both genes in three genotypes. In general, the salt-tolerant advanced breeding line showed higher

expression of the *HvTIP2;3* gene compared to the salt-tolerant Sahara3771 and the salt-susceptible Clipper cultivars. Ligaba *et al.* (2011) analyzed tonoplast intrinsic protein isoform genes *HvTIP1;2*, *HvTIP2;1*, *HvTIP2;2*, *HvTIP2;3*, and *HvTIP4;1* at five-day-old barley seedlings under 100 mM NaCl for 24 h and reported differential regulation of gene expression in roots and shoots under 100 mM NaCl treatment. The expression of *HvTIP2;1* and *HvNIP2;1* was enhanced in the shoots under salt stress, but the transcript level of other genes was not significantly affected by salt stress. Salami *et al.* (2017) assessed the expression level of *HvTIP2;3* and *HvTIP4;1* genes in the root of Sahara3771, Clipper, and A-line genotypes when exposed to 100 and 200 mM NaCl for 24 hours, three days, and three weeks. They reported a significant effect of genotype on the expression of *HvTIP2;3*. Whereas the expression of *HvTIP4;1* was significantly affected by the genotype $\times$ salinity and genotype $\times$ sampling time interactions.

Hove *et al.* (2015) reported down-regulation of *HvPIP1;4* in barley shoots under salt treatment, and the level of transcripts in the control plants nearly doubled compared to the plants exposed to salt stress (fold change = +1.93). We also observed the down-regulation of this gene in the Clipper and A-Line under 100 mM NaCl compared to the control 24 hours after salt treatment, but a significant increase occurred in the Sahara3771 cultivar. Three days after 100 mM NaCl treatment, the level of the *HvPIP1;4* gene in all three genotypes was low, but with the prolonged salt exposure for three weeks, Sahara3771 showed a higher reduction in expression of the gene, whereas the expression of *HvPIP1;4* in Clipper was increased under 100 mM NaCl compared to the control. All three genotypes showed down-regulation of the *HvPIP1;4* gene after 24 hours of exposure to 200 mM NaCl compared to the control, but three days after salt treatment, two salt-tolerant genotypes, Sahara3771 and A-Line, showed a differential response. The level of *HvPIP1;4* gene expression significantly decreased in Sahara3771 and increased in A-Line, but a change in Clipper was negligible. Under three weeks of salt treatment, the level of *HvPIP1;4* transcripts was significantly increased in the Sahara3771 and Clipper cultivars and decreased in A-Line compared to three days.

Conclusion

Out of 11 members of the *HvTIP* gene subfamily reported in barley, we assessed the changes in transcript level of the *HvTIP2;3* and *HvPIP1;4* genes in the shoots of three barley genotypes under different levels of NaCl at various salt exposure times. Our results indicated that the expression level of each gene was differentially regulated by various levels of NaCl and different exposure times to salinity stress. This suggests that these genes with distinct mechanisms contribute to salt tolerance. Our findings provide the basis for future work aimed at dissecting the role of the *HvTIP2;3* and

HvPIP1;4 genes reported here, and other aquaporins containing tonoplast intrinsic protein (*HvTIP*) coding genes, in conferring the adaptation to various stresses such as drought, cold, salinity, etc.

Conflict of Interest

The authors declare that they have no conflict of interest concerning this article.

Acknowledgment

The authors acknowledge the financial support from the Center of Excellence in Cereal Molecular Breeding, University of Tabriz, Tabriz, Iran.

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