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COMPARATIVE ANALYSIS OF EXPRESSION PROFILES OF ANTIPORTER ENCODING GENE (*GhNHX1*) UNDER DIFFERENT CONCENTRATIONS OF NaCl IN COTTON (*Gossypium hirsutum* L.)

Shader Alizada*, Kamila Aliyeva

Department of Biology, Baku State University, Baku, Azerbaijan

Abstract. Salt stress supresses the productivity of plants. One important way to mediate the harmful effects of Na^+ in plants is the compartmentation of Na^+ into vacuoles, which is mediated by NHX-like Na^+/H^+ antiporters. In this study, the expression level of *GhNHX1* gene encoding antiporter was evaluated under 100 mM and 200 mM concentration of NaCl in 31 cotton genotypes. As a result of the ANOVA analysis, a significant differences was recorded in the expression level of the gene under 0 mM and 200 mM stress conditions. A decrease in the expression level of this gene was observed in highly sensitive varieties (Ganja-182 and Carisma) and both an increase and a decrease in tolerant varieties. Such diversity in tolerant varieties shows that the resistance of samples is controlled by separate dominant genes.

Keywords: *Cotton, salt stress, GhNHX1, tolerant.*

***Corresponding Author:** Shader Alizada, Baku State University, Baku, Azerbaijan, Tel.:+994505332224, e-mail: shader622@mail.ru*

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1. Introduction

Soil salinity is an important abiotic factor that restricts agricultural productivity and product quality in many parts of the world. Salinity causes stress on plant tissues, affecting mineral and water uptake, enzyme activities, photosynthesis and metabolism. The soil's high solute concentrations cause a water deficit, while ion-specific stressors caused by changing K^+/Na^+ ratios. In salt-affected plants, Na^+/H^+ antiporters play an important role in maintaining the cytoplasmic K^+/Na^+ ratio by pumping Na^+ out of cells or into organelles, primarily vacuoles (Tian *et al.*, 2010). Thus, increasing the vacuole's efficacy in compartmentalizing more Na^+ is a viable technique for overcoming both Na^+ toxicity and the osmotic impact produced by excessive salinity (Jabeen *et al.*, 2022).

There are several transporters and channels that assist the passive passage of Na^+ across the plasma membrane. The high affinity K^+ transporter HKT1, LCT1 and other nonselective cation channels such as cyclic nucleotide-gated channels and glutamate-activated channels are among the most essential transporters (Khan *et al.*, 2010). The compartmentalization of Na^+ into vacuoles not only helps to avoid the toxic effects of Na^+ in the cytoplasm, but it also maintains an osmotic potential for water absorption

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utilizing Na^+ as an osmoticum. Intracellular NHX antiporters are classified into two types based on their subcellular localization: tonoplast localized NHXs and endosome localized NHXs (Jia *et al.*, 2018). NHXs are powered by proton (H^+) electrochemical gradients produced by two kinds of H^+ pumps: H^+-ATPase and H^+-PPase (Cui *et al.*, 2020).

The *NHX* genes have been identified and cloned from over 60 plant species (Bassil & Blumwald, 2014; Mishra *et al.*, 2014) and overexpression of *NHX* isoforms can affect salt tolerance in several plant species (Rodríguez-Rosales *et al.*, 2009; Roy *et al.*, 2014). In tobacco *TaNHX3* ectopic overexpression increased salt stress tolerance by enhancing relevant physiological processes (Lu *et al.*, 2014). Overexpression of the *StNHX1* gene in transgenic soybean improves salt tolerance and the Na^+, malondialdehyde contents of the leaves of the transgenic lines were significantly lower as compared with the wild-type plants (Chen et al., 2014). He *et al.* determined that in cotton Arabidopsis antiporter gene increases fiber output and improves photosynthetic efficiency in salt conditions (He *et al.*, 2005).

In cotton (*Gossypium hirsutum*) 22 NHX family members were identified. A phylogenetic study classified the *GhNHX* genes into two groups: vacuolar (18) and endosomal (4). The chromosomal location of the NHX genes demonstrated that genome-wide duplication during polyploidization had a considerable effect on the amount of *GhNHX* genes. Analysis of gene structures and motifs revealed that *GhNHX* genes in the same evolutionary cluster are conserved. Furthermore, salt-induced expression patterns demonstrated that salinity affects the expression levels of the majority of *GhNHX* genes (Ma *et al.*, 2020). Fu et al. (2020) discovered 23, 24, 12 and 12 NHX genes from *G. hirsutum*, *G. barbadense*, *G. arboreum* and *G. raimondii*, respectively. Phylogenetic analysis showed that these genes were mainly divided into three class with significant subcellular localization, namely, endosome, plasma membrane and vacuole class.

Expression of genes responsive to salt stress could be species-dependent and it starts at substantially lower salt concentrations, such as 50-100 mM NaCl, which is sufficient for the majority of plant species. However, depending on the level of salt sensitivity, lower concentrations could be considered for more salt-sensitive plants (Shavrukov, 2013; Jabeen *et al.*, 2022). Intracellular NHXs normally play a role in salt stress tolerance by regulating cellular ion homeostasis (Reguera *et al.*, 2014), however, the regulating mechanism of most NHXs remains uncertain (Jia *et al.*, 2018).

The cotton samples stored in the National Genbank of Azerbaijan were mainly characterized based on morphometric descriptors and the study of salt resistance of these genotypes based on molecular-genetic markers is important for future selection programs.

2. Material and methods

The research was carried out at the Institute of Genetic Resources of the Ministry of Science and Education of Azerbaijan. 13 local (Aghdas-3, AP-317, Ganja-110, Bayragdar, Ganja-114, Barakat, Ganja-160, Ganja-182, Ganja-195, Kharabag-11, Kharabag-12, Ganja-200, Zafar) and 18 introduced (Kyrgyzstan-174 (Kyrgyzstan), Tashkent-1 (Uzbekistan), Tashkent-2 (Uzbekistan), Tashkent-3 (Uzbekistan), Navai-9 (Uzbekistan), Edessa (Turkey), Sezener-76 (Turkey), May-344 (Turkey), Beyazaltun-440 (Turkey), CSN-12 (Turkey), PG (Turkey), Flash (Turkey), Lima (Turkey), Carisma

(Turkey), Assos (Greece), Prime (Greece), Select (Greece), Cristina (Greece)) cotton varieties were used as the material of the study. Planting scheme and stress treatment were performed based on the method proposed by Basal (2010). 72 h after stress, leaves were collected from 3 different plants per cultivar and mixed to reduce transcriptome heterogeneity. Samples were immediately frozen in liquid nitrogen and stored at -70°C until RNA extraction. RNA extraction was performed using RNX-Plus Solution (Cat:EX6101) and cDNA synthesis using SinaClon (Cat:RT5201) according to the manufacturer's protocol. The sequences of *GhNHX1* gene and genes encoding beta tubulin as an endogenous stabilizing factor (Table 1) were selected from the NCBI (https://ncbi.nlm.nih.gov/) database and primer design was carried out using the internet resource (https://primer3.ut.ee/).

Table 1. Characteristics of primers used in molecular assays

Primer	Sequence	Tm	GC %
GhTUB1	F: 5′ATGGATCTGGAACCCGGTAC3′	59.35	55
	R: 5′AATCGCAATTCTCGGCTTCC3′	57.30	50
GhNHX1	F: 5′TGACAAGTGGGGGTAAAAGC3′	58	50
	R: 5′AGCCAATGTCCATTTCCTTG3′	56	45

qRT-PCR analysis was performed on Rotor Gene Q 5plex during 35 cycles (initial denaturation 95°C, 5'; 35 cycles of elongation 95°C, 15"; 58°C, 0.5'; 72°C, 1' and melting curve 72°C, 1'). The relative expression level was calculated according to the $2^{-\Delta\Delta CT}$ (Pfaffl, 2001) method. Statistical analysis was performed using SPSS v.25 software.

3. Results and discussion

Least significant difference (LSD) results of a one-way ANOVA revealed a significant difference in the expression level of *GhNHX1* gene between control and 200 mM salt stress conditions (Table 2).

Table 2. One-way ANOVA analysis of relative expressions of *GhNHX1*

Dependent Variable: *GhNHX1*					
(I) Treatment	(J) Treatment	Mean difference (I-J)	Std. Error	95% confidence interval	
Control (0 mM)	100 mm	-5.52	6.63	-18.69	7.65
	200 mM	-16.60*	6.63	-29.77	-3.43
100 mM	0 mM	5.52	6.63	-7.65	18.69
	200 mM	-11.09	6.63	-24.26	2.09
200 mM	0 mM	16.60*	6.63	3.43	29.77
	100 mm	11.09	6.63	-2.09	24.26
*. The mean difference is significant at the 0.05 level.					

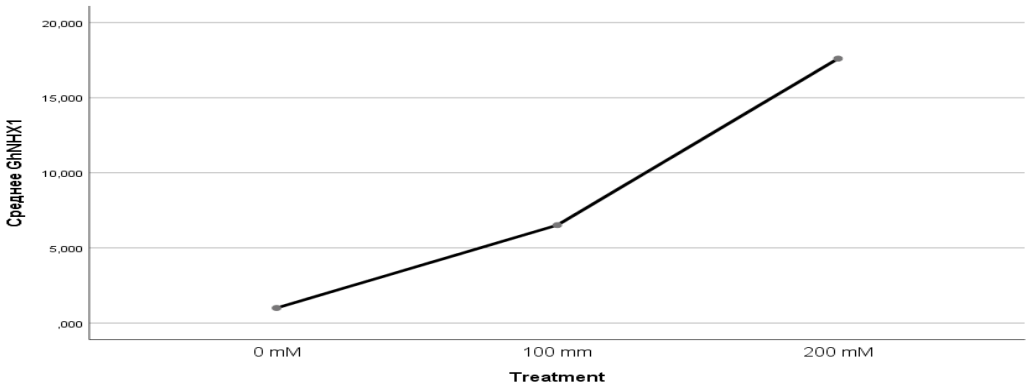
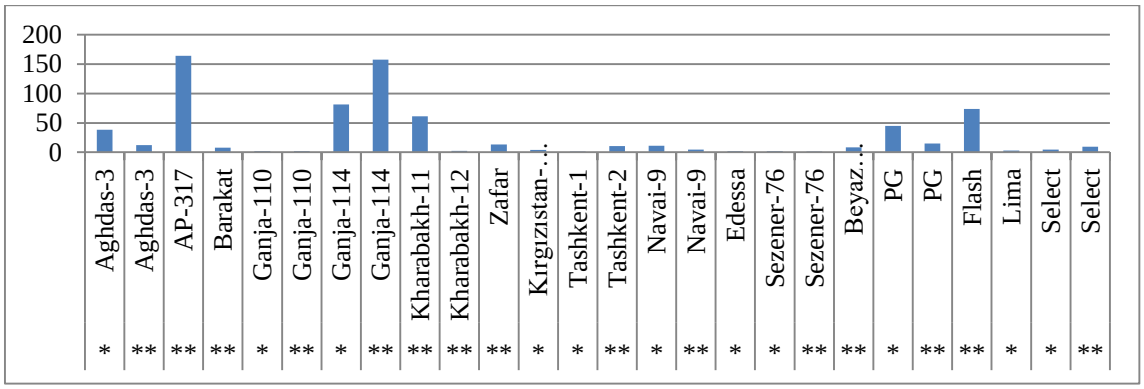


Figure 1. Changes in *GhNHX1* gene expression level with increasing salt concentration

The analysis of the mean expression value of the *GhNHX1* gene in the samples showed an increasing trend with increasing salt concentration (Figure 1).

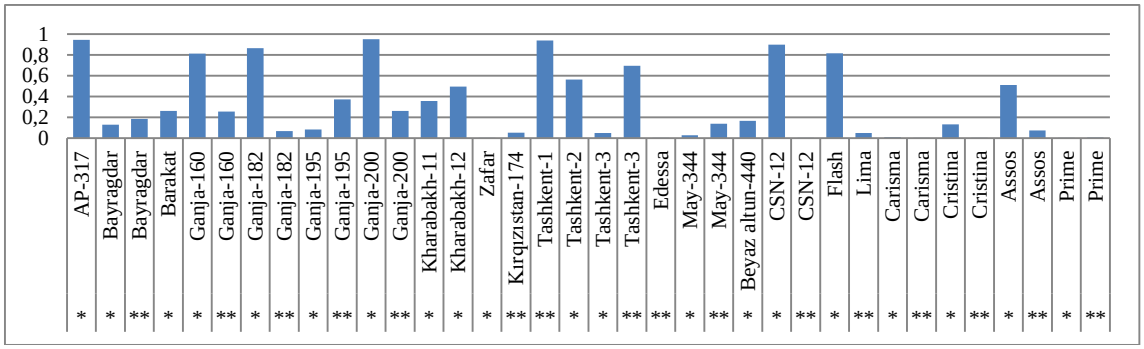
In general, the analysis of the expression level of *GhNHX1* gene under 100 mM concentration of NaCl showed an increase compared to the control in 11 cultivars and a decrease in 20 cultivars (Figure 2 and Figure 3). Under the stress condition of 200 mM concentration of NaCl salt, the expression level of this gene were increased in 15 cultivars compared to the control and decreased in 16 cultivars. In both salt concentrations, the expression level of *GhNHX1* gene increased in 7 cultivars and lines compared to the control and only decreased in 12 varieties.



* Change in expression level at 100 mM concentration of NaCl
** Change in expression level at 200 mM concentration of NaCl

Figure 2. Up-regulation of *GhNHX1* (in times)

At 100 mM concentration of NaCl the highest increase of *GhNHX1* gene expression was observed in Ganja-114 among local and in PG among introduced varieties. At 200 mM concentration of NaCl, the maximum decrease in the expression level among the local cultivars was determined in Ganja-200 and in CSN-12 from Turkey, among the introduced cultivars, compared to other samples.



* Change in expression level at 100 mM concentration of NaCl
** Change in expression level at 200 mM concentration of NaCl

Figure 3. Down-regulation of *GhNHX1* (in times)

In our previous studies, salt tolerance of these genotypes was evaluated based on seed germination parameters (Alizade & Mammadova, 2023a), total chlorophyll index (Alizade, 2023b) and morphometric parameters (Alizade *et al.,* 2023c) and tolerant (AP-317, Kyrgyzstan-174, Tashkent-2, Tashkent-3, Navai-9, Beyazaltun-440) and sensitive (Ganja-182, Carisma) varieties were identified. Although only a decrease in the expression level of the *GhNHX1* gene was observed in the susceptible cultivars, both an increase and a decrease in the expression level were observed in the tolerant cultivars. A wide diversity was also observed between tolerant and susceptible varieties in the studies conducted on the evaluation of the *GhMAPK* gene expression level on these varieties (Alizadeh, 2023d).

The expression level of *GhSOS1* in cotton roots was significantly upregulated in the presence of high concentrations of NaCl (200 mM) in salt-tolerant cultivar, while its transcript abundance was increased when exposed to low temperature and drought stresses (Chen *et al.,* 2017). In cotton transcriptomic data analysis and results of qRT-PCR showed that *GhNHXs* exhibited different expression patterns in each tissue and under different salinities (Fu *et al.,* 2020). The results of a comparative analysis of the expression level of *GhNHX1* in salt-tolerant and sensitive cotton cultivars Taghizadeh (Taghizadeh *et al.,* 2018) and her colleagues found that after salinity stress, the relative expression was significantly increased in tolerant cultivar than the sensitive cultivar.

The results of the research can be useful in the research conducted the direction of salt resistance in cotton.

4. Conclusion

The study of *GhNHX1* gene expression in 31 geographically distant varieties of cotton *G. hirsutum* under 2 different concentrations of NaCl salt revealed that it was significantly induced by salt in different varieties. The expression of *GhNHX1* is both up- and down-regulated in tolerant cultivars, while it was only down-regulated in sensitive cultivars. Such diversity in tolerant cultivars indicates that resistance has an individual potential level.

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