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Quantitative gene expression analysis of *NHX1* and *HvPIP2;1* in barley (*Hordeumvulgare*L.) under salinity stress

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Abstract

Plant sodium transporters activity and plasma membrane intrinsic proteins are the most important salt tolerance mechanisms in plants. In the present study, the expression pattern of genes encoding Na^+/H^+ exchanger (*NHX1*) and a plasma membrane intrinsic protein (*HvPIP2;1*) was investigated in three barley genotypes (Sahara3771 and an Iranian advanced line as salt tolerance and Clipper as salt susceptible) by the quantitative Real-time-PCR. The plants were exposed to 0, 100 and 200 mM NaCl at the seedling stage and root samples were harvested 24 hour, 3 days and 3 weeks after salt treatment. The results indicated that root length, fresh and dry weight were decreased by increase of salt concentration and duration. In response to 200 mM NaCl, mRNA level of *NHX1*gene showed slight increase in Sahara3771 and about 7-fold increase in advanced line, whereas there was no changes in Clipper compare with control. In all three genotype, expression of *HvPIP2;1* decreased during the 24 h of after salt treatment, but increased thereafter. In general, the mRNA levels of the studied genes in Sahara3771 and advanced line as salt tolerant genotypes were higher than Clipper (salt susceptible). Suggesting, this may be related to their greater ability to sequester Na^+ into sub-cellular compartments and/or maintain K^+ homeostasis and better water adjustment ability.

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Introduction

Barley (*Hordeumvulgare*, L.) is a highly adaptable cereal grain and ranks 5th among all crops for dry matter production in the world(FAO, 2012).Salinity is one of the most important environmental parameter that determines the success or failure of plants establishment. Nearly, 7% of the world's total land affected by salinity (Flower *et al.*, 1997). Salt tolerance of barley varies during different plant growth stage and seedling stage is the most sensitive growth stageof barley to salinity (Greenway, 1965).

High concentration of salt in the soil affect water extraction by roots (Munns and Tester, 2008).High concentration of salt affects plant at morphological, cellular, biochemical and molecularlevels by inhibition of enzymatic activity, membrane disorganization, inhibition of cell division and expansion and reduction of photosynthesis (Mahajan and Tuteja, 2005) that finally leads to reduced growth, yield and quality. Plant adaptation to salinity are of three distinct types;osmotic salt tolerance, Na^+ and Cl^- exclusion and accumulation of Na^+ and Cl^- in tissues. Plants maintain their osmotic and ion homeostasis by absorption, transportation and compartmentation of water and solutes (Ligaba and Katsuhara, 2010). The stress signalsare first perceived by the receptors and then transduced in the cell to switch on the stress responsive genes for mediating stress tolerance. Understanding the mechanism of stress tolerance is important for crop improvement.

Water transporter proteins, such as the aquaporins (AQPs) are functional in plant in response to salinity (Chaumont and Tyerman, 2014). Osmotic water transport across membranes is mediated by this protein channels which also known as major intrinsic proteins (MIPs). Aquaporins have now been found in nearly all living organisms and in plants, are members of a conserved family of MIP(Fujiyoshi*et al.*, 2002). They transport low molecular weight neutral solutes and gases (including ammonia and carbon dioxide) (Bienert *et al.*, 2008) along with water. Plants AQPs have been classified into five subfamilies according to their subcellular localization and sequence Similarities. Members of the PIP subfamily are abundant in the plasma membrane (tonoplast) (Heymann and Engel, 1999). Aquaporins provide a unique molecular entry point into the water relations of plants and establish fascinating connections between water transport, plant development and the adaptive responses of plants to their ever-changing environment (Maurel and Chrispeels, 2001). It has been indicated that expression of MIPs genes is influenced by abiotic stresses such as drought, salt, metals and etc. and their expression is differentially regulated in various plant organs (Alexanderssonet *al.*, 2005;sakurai*et al.*, 2005).

Expression of *HvPIP2;1* gene 24 hour after salt treatment decreased in both 100 and 200 mMNaCl treatment level. Katsuhara*et al.* (2003) reported that *HvPIP2;1* which is shown to transport water when expressed in Xenopus oocytes, resulted in increased shoot/root ratio and raised salt sensitivity in transgenic rice. Two aquaporin genes (*PIP* and *TIP*) preferentially expressed in the salt gland cells were rapidly induced in response to increasing salt concentration, which suggests that aquaporins are involved and contribute to the reabsorption of water during salt removal in *Avicenniaofficinalis* salt glands (Tan *et al.*, 2013). Analysis of 33 rice MIP gene expression at different growth stages and in different plant organs also showed that gene expression varied with plant organ and growth stage (sakurai*et al.*, 2005). Also the rice *OsPIP1; 3* was found to be up regulated in tolerant cultivar under water deficit (Lian*et al.*, 2004). Transgenic Arabidopsis plants expressing an antisense copy of the pip1b gene showed reduced expression of several *PIP1* homologs and provided definitive evidence for the contribution of aquaporins to plasma membrane water transport (Kaldenhoff*et al.*, 1998). Surprisingly, these antisense plants showed an increased root mass, whereas the development of the shoot was unchanged. Even though this phenotype might be related to the old observation that the root/shoot ratio of plants adjusts in response to their water

status, it directly emphasizes how membrane transport can influence the developmental plasticity of plants (Maurel and Chrispeels, 2001).

Salts (mainly NaCl) present in soils can enter the cytosol of plants, where they are toxic to important physiological and biochemical processes, and inhibit plant growth and development, thereby significantly reducing yield. Growth inhibition by Na^+ and Cl^- is among the most common effect of soil salinity (Tester and Davenport, 2003). Restricting Na^+ influx into cells may improve plant growth under salinity stress. Plants cells must be able to transport Na^+ out of the cytoplasm to the external medium or sequester it to the vacuole to tolerate high levels of Na^+ ions. Sodium transport into vacuoles can be accomplished by the operation of tonoplast bound *NHX* proteins, which function as Na^+/H^+antiporters, driven by the electrochemical gradient of protons generated by the vacuolar H^+-ATPase and H^+-PPase. The compartmentalization of Na^+ into vacuoles not only averts the deleterious effects of Na^+ in the cytoplasm, but also allows the plants to use Na^+ ions as an osmoticum, helping to maintain an the osmotic potential that drives water into the cell. Thus, *NHX* antiporters have important roles in the plant's response to salt stress (Rodriguez-Rosales *et al.*, 2009). The DNA sequences encoding *NHX* proteins have been discovered in more than 60 plant species, including gymnosperms and dicotyledonous and monocotyledonous angiosperms. The expression of most of these NHX genes was induced by NaCl treatment (Pardo *et al.*, 2006).

Ligaba and Katsuhara (2010) investigated the response of salt-tolerant and salt-sensitive barley cultivars to salt stress at both physiological and molecular levels. Analysis of gene expression using quantitative RT-PCR showed that transcripts of Na^+/H^+antiporters (*HvNHX1*, *HvNHX3* and *HvNHX4*) was higher in roots of salt tolerate cultivar than I743 a salt sensitive one with prolonged exposure to salt. They suggested that better performance of tolerate cultivar during salt stress may be related to its greater ability to sequester Na^+ into sub-cellular compartments and/or maintain K^+ homeostasis. The vacuolar Na^+/H^+ exchange in *Arabidopsis thaliana* is regulated by the SOS pathway and by interaction with the calmodulin-like protein CaM15 (Qiu *et al.*, 2004). Extrusion of Na^+ through the plasma membrane by a wheat Na^+/H^+antiporter (*TaSOS1*) has been experimentally investigated (Xu *et al.*, 2008). The overexpression of the vacuolar NHX antiporter*AtNHX1* from Arabidopsis improved the salt resistance of wheat (Xue *et al.*, 2004). A putative wheat Na^+/H^+ antiporter, *TaNHX1*, conferred salt tolerance to transgenic Arabidopsis plants (Brini *et al.*, 2007).

Hordeumvulgare is one of the most salt-tolerant crops (Munns, 2001). As said before salt tolerance responses mainly is a result of salt tolerance genes activation. There is a gap about the importance degree of AQPs and Na^+/H^+ antiporter in response to salinity, and their activity based on salinity duration and level especially in root tissue. The aim of the study was to analyze the effect of salinity on the expression of *PIP*and Na^+/H^+antiporter in roots of tolerant and susceptible barley genotypes under different salinity levels and durations.

Materials and methods

Plant materials and experimental condition

Three genotypes of barley namely Clipper, Sahara3771 and an Iranian advanced line were used. Sahara3771 is a six-row salt tolerance landrace from Algeria. Clipper is an improved Australian two-row variety and susceptible to salinity. Seeds of Clipper and Sahara3771 were obtained from the University of Western Australia. Iranian advanced line is a highly salt tolerate line and produced in Plant and Seed Improvement Institute from a cross of Kavir and Sahra cultivars.

Seeds were surface-sterilized in 10% sodium hypochlorite for 15 min and then washed thoroughly with distilled water for 15 minutes. Until the appearance of first green leaf, seeds were germinated

on wet filter papers for 48-72 hours. Seedlings at similar germination stage were transferred to gravel under hydroponic system. Plants were irrigated with half-strength Hoagland solution for three day and then with full-strength. Hoagland nutrient solution composed of 6 mM KNo_3, 5mM $Ca(NO_3)_2$, 2mM $MgSo_4$, 100 nM $ZnSO_4$, 8 mM H_3BO_3, 2 mM $MnCl_2$, 2 mM $CuSO_4$, 2mM H_2MoO_4, 4 mg/l Fe-EDTA, 6 mM Kh_2Po_4.The salt stress was imposed when seedlings were in 3 leaf stage. The NaCl concentration was initially 50 mM and then increased the following day to the final concentration of 100 mMNaCl for 100mM treat and the next day to 200 mM for 200mM treat level. CaCl2 was also added along with NaCl to maintain Na^+/Ca^{2+} concentration ratio of 10: 1 on a molar basis. After salt treatment seed bed washed with water every 2 week and drenched with full-strength Hoagland again. Plants were grown in greenhouse controlled conditions at 28/18◦C day/night temperature, with a photoperiod regime of 16/8 h day/night, and 70% relative humidity. The experiment was conducted in factorial layout based on randomized complete block design with three replications.

For RNA extraction, **r**oots of 3 plants in each replication were harvested separately from control and salt-stressed plants at 24 h, 3 day and 3 week after the salt treatment, immediately frozen in liquid nitrogen and transferred to -80 °C till RNA extraction.

Gene expression analysis

Total RNA was extracted using the RNX-Plus Kit (Cinna Gen, Iran) following the manufacturer recommendation with some modifications. cDNA synthesis was performed in a 20µl volume reaction containing 2µl total RNA using the Revert Aid first strand cDNA synthesis kit (Thermo Scientific, America).

Gene expression was studied by the quantitative real-time PCR technique using gene specific primers (Table 1). Real-time qPCR was done for each gene in total volume of 10µl by adding 1µl of the cDNA, 0.7µl forward and reverse primers (5ng/µl), 3.5µl ddH_2O and 4µl SYBG premix Ex *Taq*™II PCR master mixture (TAKARA, Japan). The analysis was carried out with three replicates for each sample. qPCR was performed on the C1000™ Thermal Cycler system (Bio-Rad) with PCR conditions of 94°C for 5 minutes, 40 cycles of 94°C for 45 seconds, primer specific annealing temperature for 45 seconds, and 72°C for 45 seconds and final extension at 72°C for 5 minutes.

Data analysis

Experiments were repeated three times and two technical replicates were used per each RNA sample. Relative quantification of genes calculated based on ΔΔCt mathematical method (Livak and Schmittgen, 2001), from estimation of interaction of gene and salt treatment effects. All datastatistically analyzed using SAS and mean comparison was performed by Duncan′s multiple range test with critical value of $P<0.05$ using MSTAT-C software.

Results

Effect of NaCl treatment on plant growth

Analysis of variance revealed significant difference among genotypes, salt durations and their interactions for root length, fresh and dry weight. Significant differences were observed among salt levels and duration interaction for root dry weight. The advanced lines showed the highest root length under salinity treatments and its root length was significantly higher than that of Sahara3771 and Clipper. Compared with control, salt stress did not significantly affect the root length in advanced lines. There was no significant difference between Sahara3771 and Clipper for root length. Maximum root dry weight was observed three weeks after salt treatment, the differences between three sampling stages were not significant. The advanced line's fresh weight was significantly higher than that of Sahara 3771 and Clipper, but the differences for root dry weight were not significant. Compared with Clipper as salt susceptible genotype, root parameters in Sahara3771 was less affected by 100 mMNaCl

treatment and advanced line was less affected by this level of NaCl compared with Sahara3771 and Clipper. In all the genotypes, leaf symptoms were increased by 200 mMNaCl treatment.

Expression pattern of NHX1 gene under salt stress

The expression levels of *NHX1* gene in the root of three barley genotypes were evaluated under control and 100 and 200 mMNaCl treatments by quantitative RT-PCR. However, the*NHX1* was expressed at both 100 and 200 mMNaCl concentrations, but the transcripts level of *NHX1* were significantly enhanced by increased level of NaCl from 100 to 200 mM.

Table 1. Gene specific primers for the amplification of *a-tubulin*, *NHX1* and *HvPIP2:1* genes and their annealing temperature.

Gene	Primer sequence	Annealing temperature (°C)
a-Tubulin 2	5'-AGTGTCCTGTCCACCCACTC-3'	65
	5'-AGCATGAAGTGGATCCTTGG-3'	
NHX1	5'-TACGGTTTTCTGCCTCTGTCACA-3'	68
	5'-ACAA CATCTGGTCATACTGCCG-3'	
HvPIP2:1	5'-GCTAGCTTAGCAATGGCCAAGGAC-3'	65
	5'-GTCGGACTGGTGCTTGTACC-3'	

The result indicated that increase in mRNA level of *NHX1*from 100 to 200 mMNaCl was significantly higher than the difference from 0 to 100mM (Fig. 1). Under 100 mMNaCl at all sampling stages, the level of *NHX1*gene expression in salt susceptible cultivar Clipper was higher than that of Sahara3771 and advanced line. However, under 200 mMNaCl, the transcript level of this gene in Clipper did not show significant increase compared to 100 mMNaCl, but there was a slight increase in Sahara 3771 and about 7-fold increase in advanced lines. The comparison of *NHX1* transcript levels at 24 h, 3 day and 3 week revealed that under 100mM NaCl, the level of mRNA was higher at 3 week, but under 200 mM treatment, significant difference was not observed between 3 day and 3 week. In average for all the genotypes, maximum level of *NHX1* gene expression was obtained 3 day after NaCl treatment.

Table 2. Mean square of root properties of studied genotypes under salinity stress.

SV	df	Mean square			
		RL	RLSHL	RWW	RDW
Replication	2	42.921**	0.119^{ns}	0.156^{ns}	0.004 ^{ns}
Stage	2	72.852**	3.238**	2.492**	0.066**
Line	2	23.925*	1.892**	0.771**	0.021**
Treat	2	17.472 ^{ns}	0.076^{ns}	0.276*	0.007*
Stage*Line	4	22.154*	0.714**	0.559**	0.017**
Stage*Treat	4	18.694*	0.081^{ns}	0.187*	0.006*
Line*Treat	4	3.432^{ns}	0.109^{ns}	0.065^{ns}	0.003 ^{ns}
Stage*Line* Treat	8	5.854 ^{ns}	0.055 ^{ns}	0.105^{ns}	0.003^{ns}
Error	52	6.969	0.092	0.056	0.002
CV (%)		20.48	27.46	77.06	78.74

Abbreviations; RL: Root length, RLSHL: Root length to shoot length ratio, RWW: Root wet weight and RDW: Root dry weight.

Expression pattern of HvPIP2;1gene under salt stress

The results indicated that with increased concentration and duration of NaCl treatment, the transcript level of*HvPIP2;1*gene was increased. The maximum level of this gene was observed in the advanced line followed by Sahara3771, 3 week after 200 mMNaCl treatment. Advanced lines 3 day and 24hour samples of 200 mM treated and then Sahara3771's 3 day samples in 200 mMNaCl treatment respectively had the next positions.

Table 3. Mean square of *NHX1*and *HvPIP2:1* genes expression of studied genotypes under salinity stress.

SV	df	Mean square	
		NHX1	HvPIP2:1
Replication	1	0.005**	0.039ns
Stage	2	1.280**	9.321**
Line	2	0.581**	5.077**
Treat	2	2.963**	0.024ns
Stage*Line	4	0.717**	2.310**
Stage*Treat	4	1.619**	9.576**
Line*Treat	4	2.741**	15.814**
Stage*Line* Treat	8	0.518**	1.042**
Error	26	0.518	0.016
CV (%)		1.08	1.27

In all three genotypes, expression of *HvPIP2;1*gene decreased 24 h after 100 and 200 mMNaCl treatment, but increased 3 day and 3 week after 200 mMNaCl treatment. In the advanced line under 100 mMNaCl,*HvPIP2;1*genewas expressed earlier than *NHX*1 gene. It seems that in the advanced line water adjustment in response to salinity was the major mechanism compared to Na^+ compartmentation. In average of salt treatments, mRNA level of *HvPIP2;1*in the advanced line was significantly higher than that of Sahara3771 and Clipper, but the difference of Sahara3771and Clipper was not significant (Fig. 2).

Discussion

Although barley in known as one of the more salt tolerate crops, variation does exist among different genotypes. In this study, the root growth response of three genotypes (Sahara3771 and Iranian advanced line as salt tolerant genotypes and Clipper as salt susceptible genotype)compared 24 h, 3 day and 3 week after treatment with 100 and 200 nMNaCl.The results confirmed that the advanced line and Sahara3771's root growth compared to Clipper was less affected by salt treatments,but two salt tolerant genotypes showed differential responseto salinity stress. Widodo *et al.* (2009) compared Sahara and Clipper under hydroponic condition and reported that Sahara was less affected by the presence of 100 mMNaCl treatment, there being an absence of leaf symptoms even after 5 week exposure, but an obvious reduction in biomass was seen in Clipper. In our experiment based on root growth parameters, the advanced line showed higher tolerance to salt stress compared to Sahara3771.

Advanced line had 1.6-fold bigger root length to shoot height ratio compared to Sahara3771 and Clipper. It was suggested thathigh root to shoot ratio could be used as salinity tolerance index such as K^+/Na^+ ratio. In addition, the advanced line showed increase in *NHX1* and decrease in *HvPIP2;1*genes expression in response to salinity. Katsuhara *et al.* (2003) reported over-expression of a barley aquaporin *HvPIP2;1*increased shoot/root mass ratio resulted in salt sensitivity in transgenic rice plants.

Salinity as an abiotic stress makes Na^+ toxicity, induces osmotic stress to plants and disturbs plant water balance. In the present study, the expression of the genes involved in maintenance of ionic balance and osmotic homeostasis under salt stress in salt sensitive and tolerate genotypes and its relation with root growth was assessed. Various studies were also reported differential response of different barley cultivars to salt stress evaluating different barley cultivars based on yield and ion accumulation (Royo and Aragüe´s, 1999;Royo*et al.*, 2000;Leonova*et al.*, 2005).

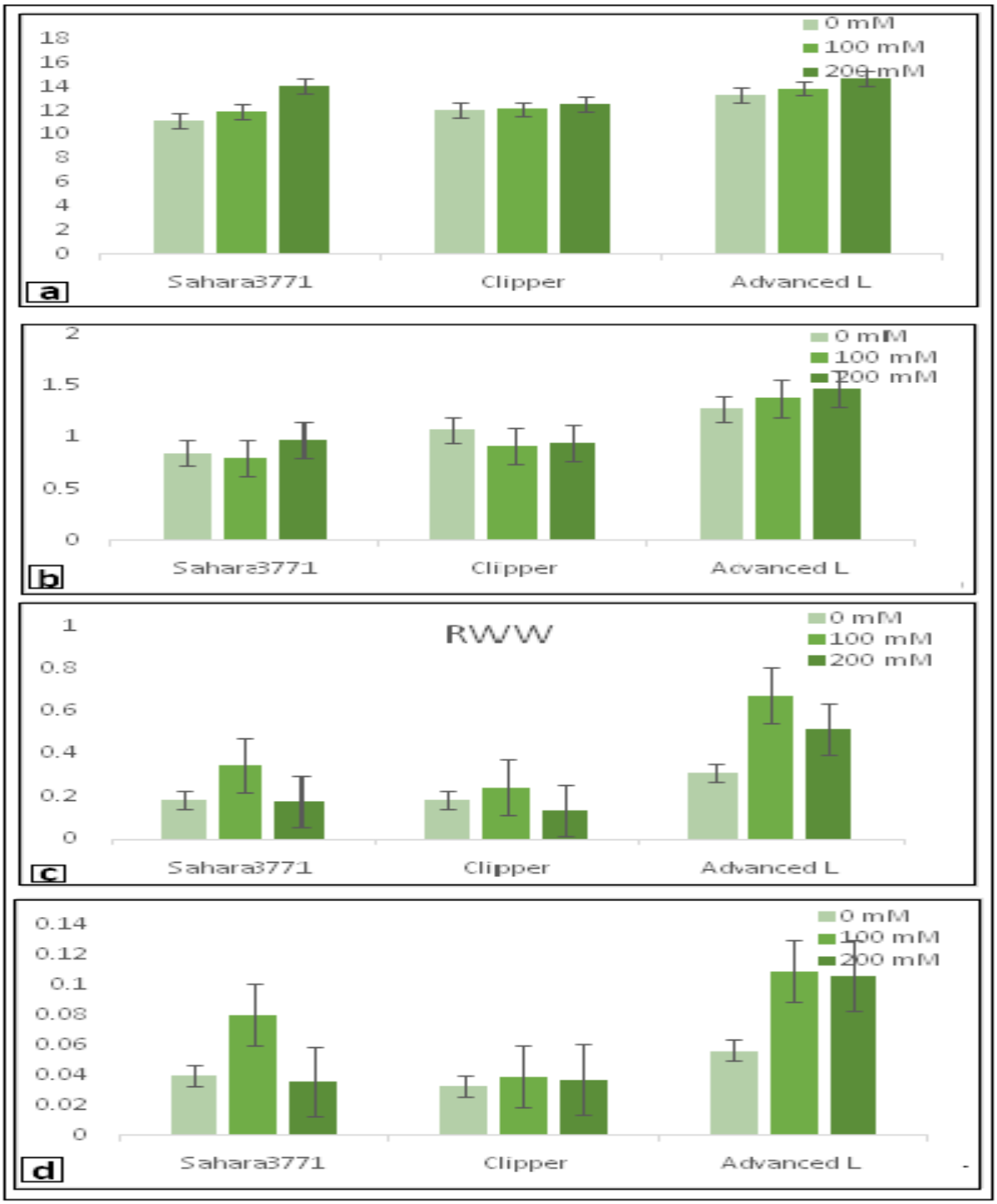

Fig. 1. Effect of NaCl concentrations on root length (a), root length to shoot height (b), fresh (c) and dry weight (d).

Under saline environment, plants will accumulate Na^+ ions to some extent, due to the strong driving force for its entry. Metabolite toxicity of Na^+ occurs through inhibition of various enzymes that require K^+ as cofactor (Tester and davenport, 2003). Such enzymes are sensitive to high Na^+/K^+ ratio. The ability of plants to maintain a high cytosolic K^+/Na^+ ratio is likely to be one of the key determinants of plant salt tolerance (Maathuis and Amtmann, 1999). The extrusion of Na^+ into the vacuole across the tonoplast membrane of Na^+ inside vacuoles is a strategy used by many plants such barley to survive salt stress (Garbarino and Dupont, 1989; Munns and tester, 2008). At the cellular level, Na^+ accumulation in vacuoles will lower the amount of toxic Na^+ ions in the cytoplasm, and lower osmotic potential in the vacuole to maintain turgor pressure and cell expansion in saline conditions. There is some identified functions of *NHX*1Antiporters include salt tolerance by Na^+compartmentation, K^+ homeostasis and cellular pH regulation (Rodríguez-Rosales *et al.*, 2009).Results showedsignificant up regulation of *NHX1* gene under salt stress especially under severe stress in tolerant genotypes;Sahara3771 and advanced line. Ligaba and Katsuhara (2010) also reported increased number of *NHX1*transcripts in root of barley under 100 mMNaClafter 24 h.

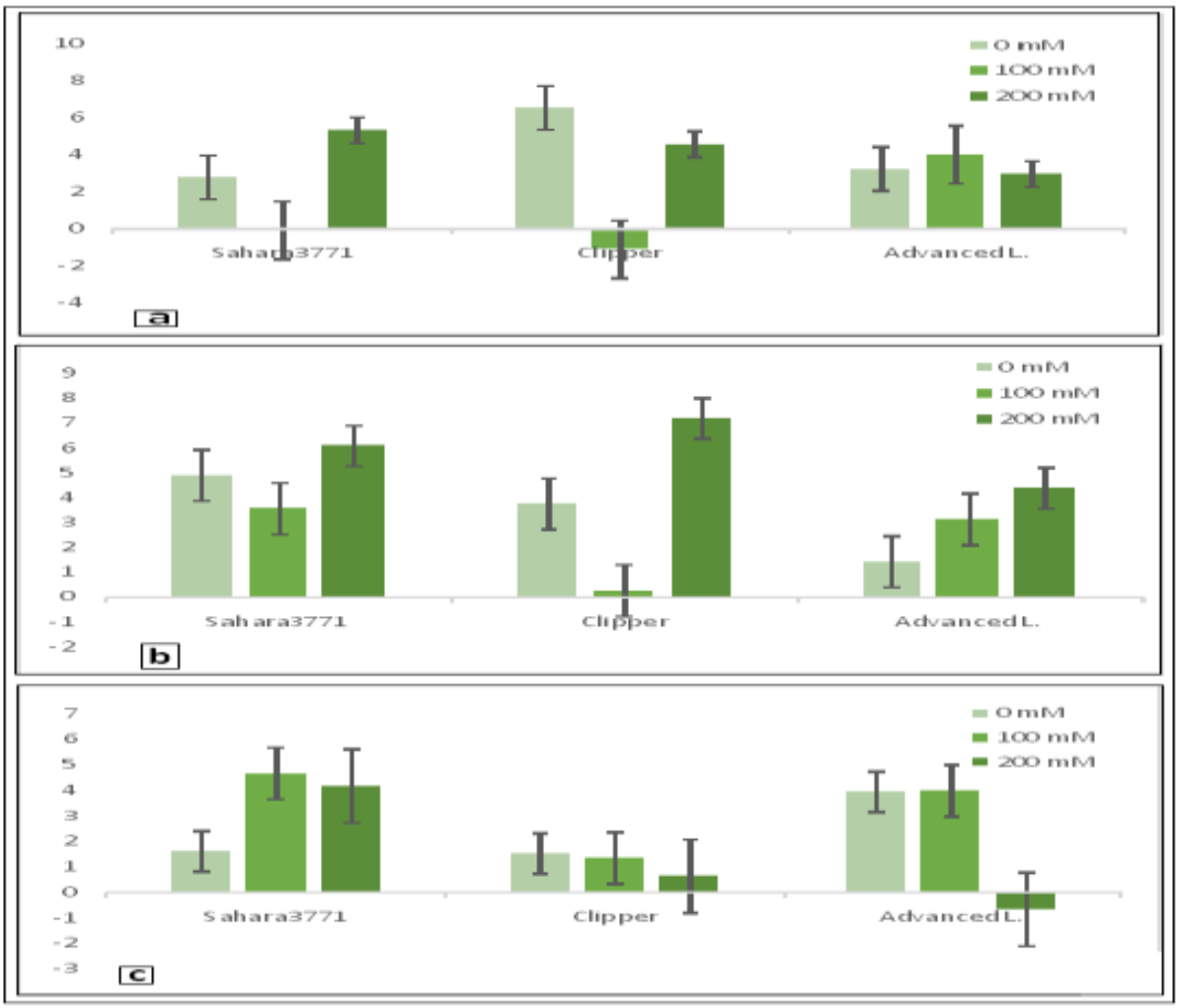

Fig. 2. Expression of NHX1 in the root of barley under different concentrations of NaCl. Semi-quantitative RT-PCR analysis of NHX1 mRNA in plants treated with 0, 100 and 200 mMNaCl for (a) 24 hour, (b) 3 day and (C) 3 weeks. α-Tubulin was used as an internal control. The final value was the average of at least three independent experiments. Values are means ± SD and bars indicate SD.

It was reported that in some plants, Na^+ accumulation in shoots inhibited by reduced transport Na^+ from root to shoot and recirculation of Na^+ out of the shoots and its storage in root or stem cell vacuoles in the shoot and these are suggested to be key factors for sustained growth during salt stress (Munns and tester, 2008).Transcript abundance of *NHX1* gene in response to salinity can trigger high activities of tonoplast Na^+/H^+antiporter (Blumwald *et al.*, 2000). Increased Na^+/H^+antiporter activity upon Na^+ addition has been reported in roots of barley (Garbarino and DuPont, 1989), especially in salt tolerant cultivars. Consistent with increase in Na^+/H^+antiporter activity, increased expression of *AtNHX1*, *AtNHX2* and *AtNHX5* genes by NaCl treatment has been reported (Yokoi *et al.*, 2002). Accumulation of sodium ion and expression level of *NHX* in root tissues might be correlated with each other.

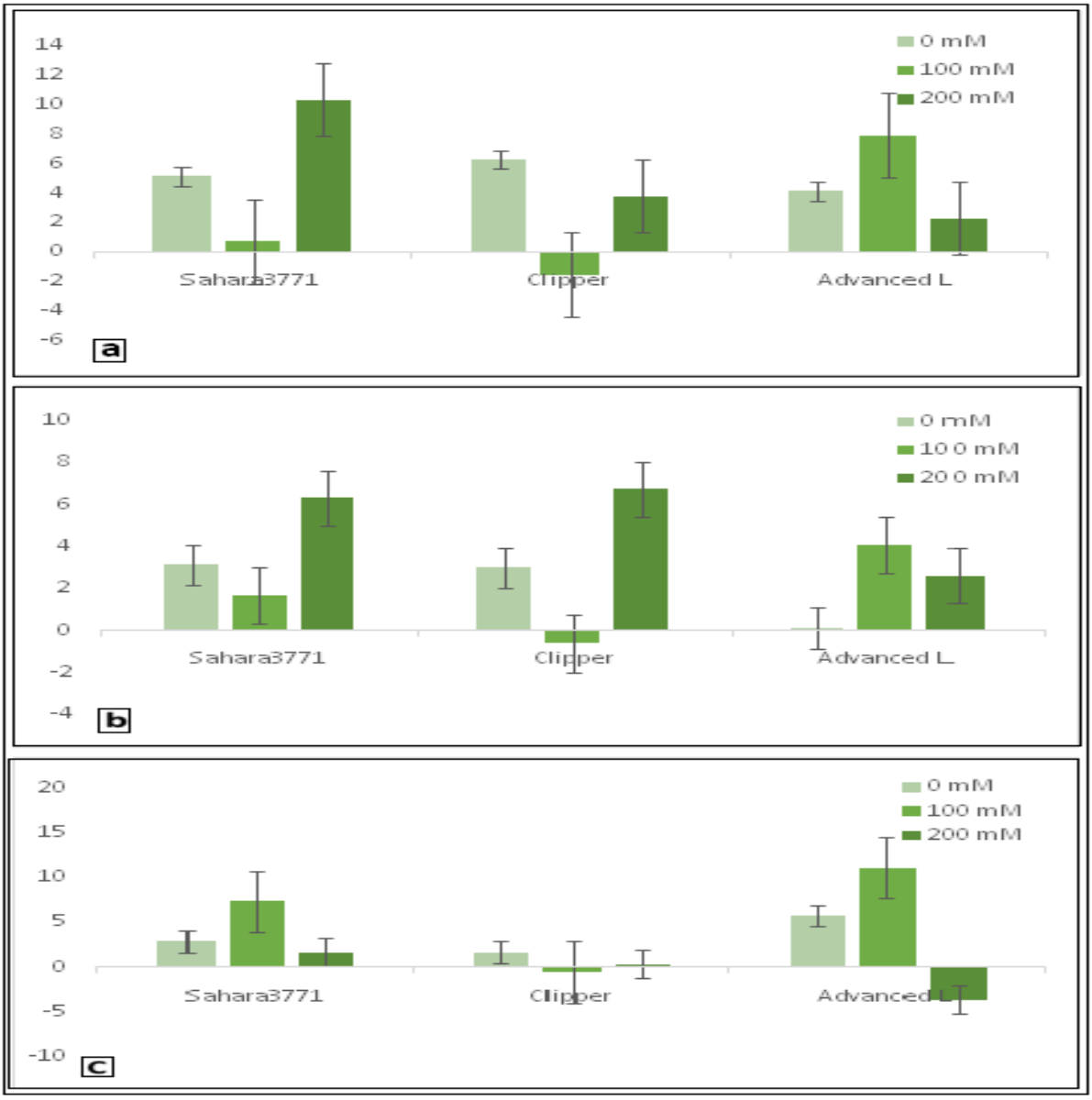

Fig. 3. Expression of HvPIP2;1 in the root of barley under different concentrations of NaCl. Semi-quantitative RT-PCR analysis of HvPIP2;1 mRNA in plants treated with 0, 100 and 200 mMNaCl for (a) 24 hour, (b) 3 days and (C) 3 weeks. α-Tubulin was used as an internal control. The final value was the average of at least three independent experiments. Values are means ± SD and bars indicate SD.

The up regulation of *NHX* gene expression might diminish Na^+ translocation from root to shoot via Na^+ accumulation in the root vacuoles (Zhang *et al.*, 2008). The advanced line showed the maximum root length and higher expression of *NHX1* gene under 200 mMNaCl3 day after treament in hydroponic environment and its expression level was was 3.3- and 7-fold higher than that of Sahara3771 and Clipper, respectively. This may suggest the association between root length and expression level of *NHX1* in root tissue. Zhang *et al.* (2008) suggested that up-regulation of *NHX1* gene expression might diminish Na^+ translocation from root to shoot via Na^+ accumulation in the root vacuoles. it was observed that *HvNHX1* was mostly induced in roots of the relatively salt tolerant monocot barley, whilst in rice, *OsNHX1* induction is observed in shoots, suggesting that the high salt tolerance in barley is related to accumulation of Na^+ in root cell vacuoles in order to limit transport to the shoot (Fukuda *et al.*, 2004). Most of the studies, except Yang *et al.* (2009) reported that over expression of *NHX* isoforms in a variety of plant species shows substantially increased in salt tolerance (Wu *et al.*, 2004; Zhang *et al.*, 2008).In the study, the maximum changes in *NHX1* gene expression was observed 3 day after NaCl treatment and 24 h after salt treatment changes in gene expression was negligible. Ligaba and Katsuhara (2010) also reported that the expression *HvNHX1*, *HvNHX3* and *HvNHX4* genes was not markedly affected by salt treatment in both salt sensitive and tolerate barley cultivars during the initial hours of salt treatment. They also reported that transcripts of Na^+/H^+antiporters (*HvNHX1*, *HvNHX3* and *HvNHX4*) was higher in roots of salt tolerate compared to salt sensitive cultivar with prolonged exposure to salt. They suggested that better performance of tolerate cultivar during salt stress may be related to its greater ability to sequester Na^+ into sub-cellular compartments and/or maintain K^+ homeostasis.

Expression of *HvPIP2;1*gene 24 hour after salt treatment decreased in both 100 and 200 mMNaCl treatment level. Katsuhara *et al.*(2003) reported that over expression of*HvPIP2;1* which is shown to transport water when expressed in Xenopus oocytes, resulted in increased shoot/root mass ratio and raised salt sensitivity in transgenic rice. Similarly, over expression of Arabidopsis plasma membrane *PIP1b* aquaporin in tobacco resulted in a negative effect during drought stress, causing faster wilting (Aharon *et al.*, 2003). These findings suggest that AQPs may mediate water loss from plant cells. Horie*et al.*(2011) reported that under hypertonic conditions induced by NaCl treatment, *HvPIP2* facilitate water efflux. High concentration of NaCl (200mM) suppressed expression of *PIP* isoforms in barley (Katsuhara*et al.*, 2002). The decrease in gene expression of the *PIPs* observed at 200mM NaCl treatment could be an adaptation mechanism by which plants minimize water loss through efflux to survive under severe salt stress conditions. Such a high salt concentration might have also induced serious injuries to plant cells. *HvPIP2; 1* have water transport activity. Similar results have been reported by researchers in barley (Ligaba and Katsuhara, 2010) and *Aeluropuslittoralis* (Rezaeimashaei *et al.*, 2014). In the study, an obvious enhance in *HvPIP2;1*transcripts copy number was observed 3 days and 3 weeks after salt treatment. Enhanced accumulation *HvPIP2; 1* transcripts may suggest a role in salt stress tolerance. Multiple lines of evidence indicated that several MIP isoforms have been shown to enhance salt and draught stress tolerance in transgenic plants (Ligaba and Katsuhara, 2010).In total it may be concluded that by long term stress plant changes its stress tolerance strategy and tries to adapt to severe stress by adjusting water relation. Tan *et al.*(2013) studiedthe expression pattern of two aquaporin genes (*PIP* and *TIP*) preferentially expressed in the salt gland cells and induced in response to increasing salt concentration. Their results suggested that aquaporins are involved and contribute to the reabsorption of water during salt removal in *Avicenniaofficinalis* salt glands and maybe the same pathway initiating in barley roots by long term salinity.

Conclusion

In conclusion, higher expression of antiporter genes involved in Na^+/H^+ exchange under salt stress condition in Sahara3771 and advanced line suggest that the high salt tolerance on these genotypes could be attributed to the intracellular compartmentation of Na^+ into the vacuole. Severe *HvPIP2;1* gene expression induction by long term 200 mMNaCl treatment may be caused primarily by the accumulation of Na^+ in root hence by Na toxicity.

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Reference

Aharon R, Shahak Y, Rozalina S, Wininger B, Kapulnik Y, Galili G. 2003. Over expression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress, Plant Cell **15,** 439–447.

Alexandersson E, Fraysse L, Sjo¨vall-Larsen S, Gustavsson S, Fellert M, Karlsson M, Johanson U, Kjellbom P. 2005. Whole gene family expression and drought stress regulation of aquaporins. Plant Molecular Biology **59**, 469–484.

Bienert GP, Thorsen M, Schussler MD, Nilsson HR, Wagner A, Tamas MJ, Jahn TP. 2008. A subgroup of plant aquaporins facilitate the bi-directional diffusion of As $(OH)_3$ and $Sb(OH)_3$ across membranes, BMC Biology **6,** 1-15.

Blumwald E, Aharon GS, Apse MP. 2000. Sodium transport in plant cells. Biochimicaet Biophysica Acta **1465,** 140-151.

Brini F, Hanin M, Mezghani I, Berkowitz G, Masmoudi K. 2007. Overexpression of wheat Na^+/H^+antiporter TNHX1 and H^+-pyrophosphatase TVP1 improve salt- and drought-stress tolerance in *Arabidopsis thaliana* plants. Journal of Experimental Botany **58,** 301–308.

Chaumont F, Tyerman SD. 2014. Aquaporins: Highly Regulated Channels Controlling Plant Water Relations. Plant Physiology **164,** 1600–1618.

FAO. 2012. Faostat. FAO, Rome. www.faostat.fao.org.

Flowers TJ, Garcia A, Koyama M, Yeo AR. 1997. Breeding for salt tolerance in crop plants, the role of molecular biology. Acta Physiologiae Plantarum **19,** 427-433.

Fujiyoshi Y, Mitsuoka K, de Groot BL, Philippsen A, Grubmuller H, Agre P, Engel A. 2002. Structure and function of water channels. Current Opinionin Structural Biology **12,** 509–515.

Fukuda A, Chiba K, Maeda M, Nakamura A, Maeshima M, Tanaka Y. 2004. Effect of salt and osmotic stresses on the expression of genes for the vacuolar H+pyrophosphatase, H^+-ATPase subunit A, and Na^+/H^+antiporter from barley. Journal of Experimental Botany **55,** 585-94.

Garbarino J, Dupont FM. 1989. Rapid induction of Na^+/H^+ exchange activity in barley root tonoplast. Plant Physiology **89,** 1-4.

Greenway H. 1965. Plant response to saline substrates. Growth and ion uptake throughout plant development in two varieties of *Hordeumvulgare*. Australian Journal of Biological Science**18,** 763–779.

Heymann JB, Engel A. 1999. Aquaporins: phylogeny, structure, and physiology of water channels. News in PhysiologicalSciences**14,** 187–193.

Horie T, Kaneko T, Sugimoto G, Sasano S, Panda SK, Shibasaka M, Katsuhara M. 2011. Mechanisms of water transport mediated by PIP aquaporins and their regulation via phosphorylation

events under salinity stress in barley roots. Plant and Cell Physiology **52,** 663–675.

Kaldenhoff R, Grote K, Zhu JJ, Zimmermann U. 1998. Significance of plasmalemmaaquaporins for water-transport in *Arabidopsis thaliana*. Plant Journal **14,** 121–128.

Katsuhara M, Akiyama Y, Koshio K, Shibasaka M, Kasamo K. 2002**.** Functional analysis of water channels in barley roots, Plant and Cell Physiology **43,** 885–893.

Katsuhara M, Koshio K, Shibasaka M, Hayashi H, Hayakawa T, Kasamo K. 2003. Over-expression of a Barley Aquaporin Increased the Shoot/ Root Ratio and Raised Salt Sensitivity in Transgenic Rice Plants, Plant and Cell Physiology **44,** 1378–1383.

Leonova TG, Goncharova EA, Khodorenko AV, Babakov AV. 2005. Characteristics of salt-tolerant and salt-susceptible cultivars of barley. Russian Journal of Plant Physiology **52,** 774–778.

Lian HL, Yu X, Ye Q, Ding XS, Kitagawa Y, Kwak SS, Su WA, Tang ZC. 2004. The role of aquaporin RWC3 in drought avoidance in rice. Plant and Cell Physiology **45,** 481-489.

Ligaba A, Katsuhara M. 2010. Insights into the salt tolerance mechanism in barley (*Hordeumvulgare*) from comparisons of cultivars that differ in salt sensitivity. Journal of Plant Research **123,** 105–118.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25,** 402-408.

Maathuis FJM, Amtmann A. 1999. K+ nutrition and Na+ toxicity: the basis of cellular K+/Na+ ratios. Annals of Botany **84,** 123-33.

Mahajan S, Tuteja N. 2005. Cold, salinity and drought stresses: an overview. Archives of Biochemistry and Biophysics **444,** 139-58.

Maurel C, Chrispeels MJ. 2001. Aquaporins: a molecular entry into plant water relations. Plant Physiology**1251,** 135–138.

Munns R, Tester M. 2008. Mechanisms of salinity tolerance. Annual Review of Plant Physiology **59,** 651-681.

Munns R. 2001. Avenues for increasing salt tolerance of crops. In: Horst J *et al.* (eds) Plant nutrition: food security and sustainability of agro-ecosystems. Kluwer Dordrecht, 370–371.

Pardo JM, Cubero B, Leidi EO, Quintero FJ. 2006. Alcalication exchangers: roles in cellular homeostasis and stress tolerance. Journal of Experimental Botany**57,** 1181–1199.

Qiu QS, Guo Y, Quintero FJ, Pardo JM, Schumaker KS, Zhu JK. 2004. Regulation of vacuolar Na^+/H^+ exchange in Arabidopsis thaliana by the Salt-Overly-Sensitive (SOS) pathway. The Journal of Biological Chemistry **279,** 207–215.

Rezaei Mashaeia M, Nematzadeha GA, Askarib H, Mozaffari Nejadc AS, pakdind A. 2014. Quantitative gene expression analysis of some sodium ion transporters under salinity stress in *Aeluropuslittoralis*. Saudi Journal of Biological Science **21,** 394–399.

Rodriguez-Rosales MP, Galvez FJ, Huertas R, Aranda MN, Baghour M, Cagnac O, Venema K. 2009. Plant *NHX* cation/proton antiporters. Plant Signaling and Behavior **4,** 265– 276.

Royo A, Aragüe´s R, Playa´n E, Ortiz R. 2000. Salinity-grain yield response functions of barley cultivars assessed with a drip injection irrigation system. Soil Science Society of America Journal **64,**

359–365.

Royo A, Aragüe´s R. 1999. Salinity-yield response functions of barley genotypes assessed with a triple line source sprinkler system. Plant and Soil **209,** 9–20.

Sakurai J, Ishikawa F, Yamaguchi T, Uemura M, Maeshima M. 2005. Identification of 33 rice aquaporin genes and analysis of their expression and function. Plant and Cell Physiology **46,** 1568–1577.

Tan WK, Lin Q, Lim TM, Kumar P, Loh CS. 2013. Dynamic secretion changes in the salt glands of the mangrove treespecies *Avicenniaofficinalis* in response to a changing saline environment. Plant, Cell and Environment**36,** 1410-1422.

Tester M, Davenport R. 2003. Na^+ tolerance and Na^+ transport in higher plants. Annals of Botany**91,** 503–527.

Wu CA, Yang GD, Meng QW, Zheng CC. 2004. The cotton *GhNHX1* gene encoding a novel putative tonoplast Na^+/H^+antiporter plays an important role in salt stress. Plant, Cell and Physiology **45,** 600-607.

WidodoPJH, Patterson JH, Newbigin E, Tester M, Bacic A, Roessner U. 2009. Metabolic responses to salt stress of barley (*Hordeumvulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. Journal of Experimental Botany **60,** 4089-4103.

Xu HX, Jiang XY, Zhan KH, Cheng XY, Chen XJ, Pardo JM, Cui D. 2008. Functional characterization of a wheat plasma membrane Na^+/H^+antiporter in yeast. Archives of Biochemistry and Biophysics **473,** 8–15.

Xue ZY, Zhi DY, Xue GP, Zhang H, Zhao YX, Xia GM. 2004. Enhanced salt tolerance of transgenic wheat (*Triticumaestivum* L.) expressing a vacuolar Na^+/H^+antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na^+. Plant Science**167,** 849–859.

Yang Q, Chen ZZ, Zhou XF, Yin HB, Li X, Xin XF, Hong XH, Zhu JK, Gong Z. 2009. Overexpression of SOS (Salt Overly sensitive) genes increases salt tolerance in transgenic Arabidopsis. Molecular Plant **2,** 22-31.

Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM. 2002. Differential expression and function of *Arabidopsis thaliana NHX* Na^+/H^+antiporters in the salt stress response. Plant Journal **30,** 529-39.

Zhang GH, Su Q, An LJ, Wu S. 2008. Characterization and expression of a vacuolar Na^+/H^+antiporter gene from the monocot halophyte *Aeluropuslittoralis*. Plant Physiology and Biochemistry**46,** 117-26.