DOI: 10.1007/s10535-018-0823-2 BIOLOGIA PLANTARUM 62 (4): 775-785, 2018

Salt oversensitivity derived from mutation breeding improves salinity tolerance in barley *via* ion homeostasis

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Abstract

Salt stress imposes a major environmental threat to agriculture, therefore, understanding the basic physiology and genetics of cell under salt stress is crucial for developing any breeding strategy. In the present study, the expression profile of genes involved in ion homeostasis including salt overly sensitive (*HvSOS1, HvSOS2*, *HvSOS3*), vacuolar Na^+/H^+ antiporter (*HvNHX1*), and H^+-ATPase (*HVA*) along with ion content measurement were investigated in two genotypes of *Hordeum vulgare* under 300 mM NaCl. The gene expressions were measured in the roots and shoots of a salt-tolerant mutant genotype M4-73-30 and in its wild-type cv. Zarjou by real-time qPCR technique. The critical differences between the salt-tolerant mutant and its wild-type were observed in the expressions of *HvSOS1* (105-fold), *HvSOS2* (24-fold), *HvSOS3* (31-fold), and *HVA* (202-fold) genes in roots after 6-h exposure to NaCl. The parallel early up-regulation of these genes in root samples of the salt-tolerant mutant genotype indicated induction of Na^+/H^+ antiporters activity and Na^+ exclusion into apoplast and vacuole. The earlier up-regulation of *HvSOS1*, *HVA,* and *HvNHX1* genes in shoot of the wild-type genotype corresponded to the relative accumulation of Na^+ which was not observed in salt-tolerant mutant genotype because of efficient inhibitory role of the root in Na^+ transport to the shoot. In conclusion, the lack of similarity in gene expression patterns between the two genotypes with similar genetic background may confirm the hypothesis that mutation breeding could change the ability of salt-tolerant mutant genotype for efficient ion homeostasis *via* salinity oversensitivity response.

Additional key words: *Hordeum vulgare*, NaCl, Na^+/H^+ exchanger, salt overly sensitive.

Introduction

Salinity is known as one of the most important stresses resulting in crop productivity reduction. Barley is a model salt-tolerant species and has great economic importance in salt-affected regions of the world. Therefore, investigation of salt tolerance in barley has been an interesting research for a long time and has resulted in an extensive data from studies using physiological, genetic, and cytogenetic approaches (Walia *et al.* 2006). Salinity induces osmotic stress and ion (mainly Na^+) specific toxicity (Blumwald 2000, Blumwald *et al.* 2000, Munns and Tester 2008). The conservation of intracellular ionic homeostasis by higher K^+ fluxes and lower Na^+ fluxes, resulting in a sufficient K^+/Na^+ ratio, is critical to the cell metabolism (Wang *et al.* 2013). It can be considered as a key component of salinity tolerance in plants (Tester and Davenport 2003, Volkov *et al.* 2004, Chen *et al.* 2007). Moreover, the improvement of Na^+ exclusion or K^+/Na^+ selectivity might represent a good opportunity for further enhancing the salinity tolerance of barley (Shavrukov *et al.* 2010). Along with retention of cytosolic K^+, the accumulation of excessive amounts of Na^+ in the cytosol should be prevented to keep an optimal intracellular K^+/Na^+ ratio in the presence of salinity (Zhonghua *et al.* 2007).

Several types of Na^+-transporters have been shown to play important roles in Na^+ homeostasis during salinity stress (Apse and Blumwald 2007, Hauser and Horie 2010). Ion homeostasis is mediated mainly by the

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Submitted 29 December 2017, *last revision* 30 March 2018, *accepted* 3 April 2018.

Abbreviations: CBL - calcineurin B-like protein; CIPK - CBL-interacting protein kinase; HVA - H^+-ATPase; NHX1 - Na^+/H^+ exchanger1; qPCR - quantitative PCR; SOS - salt overly sensitive.

Acknowledgements: The authors would like to acknowledge the financial support received from the Faculty of Plant Production, Department of Plant Breeding and Biotechnology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. The authors would like to thank the Seed and Plant Improvement Institute, Karaj, Iran, for the provision of seed material.

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salt overly sensitive (SOS) signalling pathway, which consists of three main salt overly sensitive genes (*SOS1*, *SOS2*, and *SOS3*) (Oh *et al.* 2007). *SOS1* [identified as Na^+/H^+ exchanger7, (NHX7)] encodes a plasma membrane Na^+/H^+ antiporter, playing a critical role in transporting of sodium into the apoplastic region and in controlling long distance Na^+ transport from roots to shoots (Shi *et al.* 2000, 2002, Yang *et al.* 2009). Gene *SOS2* [identified as calcineurin B-like protein (CBL) interacting protein kinase (*CIPK24*)] encodes a serine/threonine type protein kinase, which is activated by *SOS3* (Halfter *et al.* 2000) to activate *SOS1* (Yang *et al.* 2009). The *SOS1* gene has been isolated from several plants like *Arabidopsis*, rice, wheat, tomato, *Thellungiella salsuginea*, *etc*. (Yamaguchi *et al.* 2013). SOS3 [identified as calcineurin B-like protein 4 (CBL4)] is a myristoylated Ca^{2+} sensor belonging to the recovering-like family of SOS-like Ca^{2+} sensor/binding proteins. After Ca^{2+} binding, SOS3 undergoes dimerization to enhance the protein kinase activity of SOS2. Moreover, SOS3 recruits SOS2 to the plasma membrane to achieve efficient interaction with SOS1. The lack of them in either SOS2 or SOS3 in mutant plants results in the salt-sensitive phenotype of SOS1 plants (Martinez-Atienza *et al.* 2007). SOS3/SOS2 complex activates the expression and activity of ion transporters such as NHX1 in tonoplast (Chinnusamy *et al.* 2006). *AtNHX1* accumulates more K^+ that improves salt tolerance in transgenic poplar (Yang *et al.* 2017). The plant cells under high salinity have to keep a higher K^+/Na^+ ratio in the cytoplasm but also control the osmotic balance of the cell with the environment by accumulating Na^+ in the vacuoles. One of the strategies in this process to reduce excess cytosolic Na^+ is to compartmentalize Na^+ from the cytoplasm to the vacuoles. Thus, the vacuolar Na^+/H^+ antiporter1 (NHX1) plays a critical role in the salt-tolerance of a wide variety of plants (Blumwald *et al.* 2000, Fukuda *et al.* 2004). Researchers confirmed that the overexpression of *AtNHX1* increases salt-tolerance in *Arabidopsis thaliana*, *Lycopersicon esculentum*, and *Brassica napus* (Zhang and Blumwald 2001, Zhang *et al.* 2001, Yang *et al.* 2009). The driving force for Na^+ transport is provided by two main H^+-pumps; vacuolar H^+-ATPase (V-ATPase) and vacuolar H^+-inorganic pyrophosphatase (V-PPase) and so they have essential roles in the response to salt stress (Hasegawa *et al.* 2000, Fukuda *et al.* 2004, Horie and Schroeder 2004). The V-ATPase is the dominant H^+-pump at cellular membranes of most plants and it establishes and maintains an electrochemical proton gradient across the tonoplast (Lüttge *et al.* 2001). Under different stresses such as salinity and drought, the survival of the cells depends strongly on maintaining the activity of the V-ATPase (Dietz *et al.* 2001).

Among various quantitative methods of measuring gene expression, real-time qPCR is the most flexible and sensitive method that can be used to compare precisely the amount of mRNAs in different samples, to characterize patterns of mRNA expression, and to discriminate between mRNAs closely related. The aim of current study was to follow the content of Na^+ and K^+ and also K^+/Na^+ ratios in salt-tolerant mutant genotype and its wild-type exposed to 300 mM NaCl. Further, to determine the gene expression patterns of *HvSOS1, HvSOS2, HvSOS3*, *HvNHX1*, and *HVA* in early and late time points of exposure to salt stress.

Materials and methods

Plants and growth conditions: Barley (*Hordeum vulgare* L.) seeds of a salt-tolerant mutant genotype M4-73-30 and its wild-type cv. Zarjou were obtained from the Seed and Plant Improvement Institute, Karaj, Iran (Kiani *et al.* 2017). M4-73-30 was produced by γ-irradiation approach and was evaluated for salt tolerance in farm trials. It has been introduced as a salt-tolerant line Roodasht by the Seed and Plant Improvement Institute (Mahluji *et al.* 2007). Previous studies showed that M4-73-30 has higher tolerance to salt stress than its wild-type according to the physiological response to salinity (Mahluji *et al.* 2007, Kiani *et al.* 2017). Uniform seeds of both genotypes were selected and sterilized using 5 % (m/v) sodium hypochlorite for 5 min and then rinsed 3 times with sterile water for 10 min. At least 150 seeds of each genotype were selected and germinated on moistened paper in glass Petri dishes in an incubator at a temperature of 22 ± 1 °C under the dark for 4 d. After germination, all seeds were transferred into a half-strength Hoagland solution with double iron concentration. By the addition of KOH, the pH of the solution was adjusted to 5.5 - 6.5. The plants were grown in controlled conditions at day/night temperatures 25/22 °C, a photoperiod of 12-h, an irradiance of 150 μmol m^{-2} s^{-1}, and an air humidity of 70 % in a greenhouse at Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. Afterwards, 12-d-old seedlings (two-leaf stage) were stressed by adding NaCl at a concentration of 300 mM to the nutrient solution. To maintain Na^+/Ca^{2+} ratio of 10:1 on a molar basis, $CaCl_2$ was also included. The roots and shoots were harvested separately after 3, 6, 12, 24, 48, 72, and 96 h of exposure to the salt treatment. Non-stressed control plants were grown concurrently and harvested at the same time. Samples were quickly frozen in liquid nitrogen and stored at -80 °C.

To determine Na^+ and K^+ content of the shoots and roots of the control (0) and the salt-stressed seedlings for 3, 6, 12, 24, 48, 72, and 96 h (three replicates per time point and five plants per replicate), the samples were washed with deionized water and dried at 70 °C in an oven for 24 h. Then they were ground into fine powder and their dry mass was recorded. The dried shoot and root samples were digested in 10 cm^3 of 2 M HCl at 95 °C for 1 h. The content of Na^+ and K^+ in the digested samples were measured using a flame photometer (*Jenway*, Stafford, UK) (Williams and Twine 1960).

RNA extraction and cDNA synthesis: Total RNA was extracted separately from approximately 70 mg of frozen samples of the shoots and the roots by *Biozol* method (*BioFuax*, Hangzhou, China) (Kiani *et al.* 2017). To confirm the RNA quality, the RNA was electrophoresed on 1 % (m/v) agarose gel. Afterwards, RNA was treated by *DNase* I to remove DNA contamination before cDNA synthesis according to manufacture′s instructions (*Thermo Fisher*, Massachusetts, USA). First strand cDNA synthesis was performed using the oligo (dT) primer and diethyl pyrocarbonate (DEPC)-treated water, followed by incubating at 70 °C for 5 min. The tubes were placed on ice before adding the cDNA synthesis master mix which consisted of 4 mm^3 of cDNA reaction buffer, 2 mm^3 of dNTP (10 mM 4 dNTP mix), 20 U RNase inhibitor, and DEPC-treated water to final volume of 19 mm^3. Then, tubes were incubated at 37 °C for 5 min and 200 U of *Revert Aid* enzyme (*MMLV-RT*) were added and reaction mixture was incubated at 42 °C for 60 min. Finally, the tubes were placed in *Thermo Fisher* incubator set at 70 °C for 5 min to stop the reaction (Kiani *et al.* 2017).

Primer designing and real-time qPCR analysis: Primers were designed using *Primer 3* software (www.embnet.sk/cgi-bin/primer3_www.cgi) based on 3' untranslated region (3'-UTR). The primer names, the sequences and the accession Nos, as well as housekeeping gene α-tubulin are given in Table 1 Suppl. For qPCR, the quantity of mRNA corresponding to each gene was measured by *SYBR Green*. The PCR mixture consisted of 9.9 mm^3 of 2× *SYBR Bio Pars* (*SBB*, town?, Iran) PCR master mix, 0.5 mm^3 of dimethylsulphoxide, 0.5 mm^3 of each gene-specific primer (10 pM), 0.2 mm^3 of *Taq* enzyme, and 3 mm^3 of cDNA diluted with double distilled water to a final volume of 17 mm^3. All the PCRs were performed under the following conditions: 1 cycle at 95 °C for 5 min, 40 cycles at 95 °C for 10 s, at 60 °C for 10 s, and at 72 °C for 20 s, and 1 cycle at 80 °C for 5 min. The specificity of amplicons was verified by the melting curve analysis (at 55 °C for 10 s) after 81 cycles. The relative expression was calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$) using α-tubulin as an internal reference gene (Livak and Schmittgen 2001). Expression pattern analysis of *SOS1, SOS2, SOS3, NHX1*, and *HVA* genes was carried out by real time qPCR in shoots and roots after different treatments. Each PCR was performed in three technical and biological replicates for each sample. Relative expression was computed using the formula which presented Pfaffl and Hegeleit (2001). The ratio between the amount of target gene and the housekeeping reference genes was analyzed by *REST* software (Pfaffl *et al.* 2002). Melting curves were used to check a specificity of the primers.

Data analysis: Statistical analysis was carried out using the *SAS 9.1.3* (Chicago, USA) software. The comparisons of means between the M4-73-30 and wild-type at the same treatment time, and also at different time points with the control were carried out by either Student's *t*-test or least significant difference (LSD) test at a significance level of 0.05 or 0.01.

Results

The Na^+ content generally increased until 96 h in both root and shoot samples of the salt-tolerant mutant genotype M4-73-30 and also its wild-type cv. Zarjou after the salt stress treatment (Fig. 1). However, there were significant differences in root samples in some time points (3, 12, and 24 h) between two genotypes, and also in Na^+ transport from the root to the shoot. The Na^+ was accumulated approximately twice more in the shoot samples of the wild-type than in the mutant because the salt-tolerant mutant transported fewer Na^+ from the root to the shoot and had obvious tendency to accumulate Na^+ in its root.

The K+ content and the K^+/Na^+ ratio gradually decreased till 96 h in the roots of both genotypes under salt stress. However, the significant differences between the two genotypes were observed in the shoot K^+ content after 24 and 48 h. The higher K^+ content and lower Na^+ content in the salt-tolerant mutant than in the wild type led to the higher K^+/Na^+ ratio (particularly in shoots of mutant) in all time points (Fig. 1).

The expression patterns of *HvSOS1 (HvNHX7)*, *HvSOS2* (*HvCIPK24*), *HvSOS3* (*HvCBL4*), *HvNHX1*, and *HVA* genes in the shoot and the root samples of the salt-tolerant mutant and the wild-type were evaluated by real-time qPCR (Fig. 2). The *HvSOS3* and *HvSOS2* were significantly up-regulated at 3 and 6 h of exposure to the salt stress in the root sample of the mutant. However, the maximum induction of *HvSOS1* in the root of this genotype was at 3 h. The gene expressions were approximately 15-fold for *HvSOS2* and *HvSOS3* at 3 and 6 h and 130-fold for *HvSOS1* at 3 h of salt stress (Fig. 2). Practically the same increase in transcriptions of *HvSOS1, HvSOS2*, and *HvSOS3* was observed in the shoot samples of the mutant at 6, 24, and 96 h of the salt stress.

As concern the wild type, the increase of *HvSOS1* transcription was higher in the shoot than in the shoot of the salt-tolerant mutant (Fig. 2) which is in correspondence with enhanced Na^+ transport and accumulation in the shoot of the wild type genotype in comparison with the mutant (Fig. 1).

The expression of *HvNHX1* gene in the roots of both genotypes was already induced by NaCl treatment for 3 h, and the highest expression was reached after 24 h (Fig. 2). The highest expression of *HvNHX1* gene in the shoot of the salt-tolerant mutant was at 96 h (approximatley 110-fold higher than in the shoot of the wild-type) and in the shoot of wild-type it was at 48 h (almost 30-fold higher than in that of the salt-tolerant mutant).

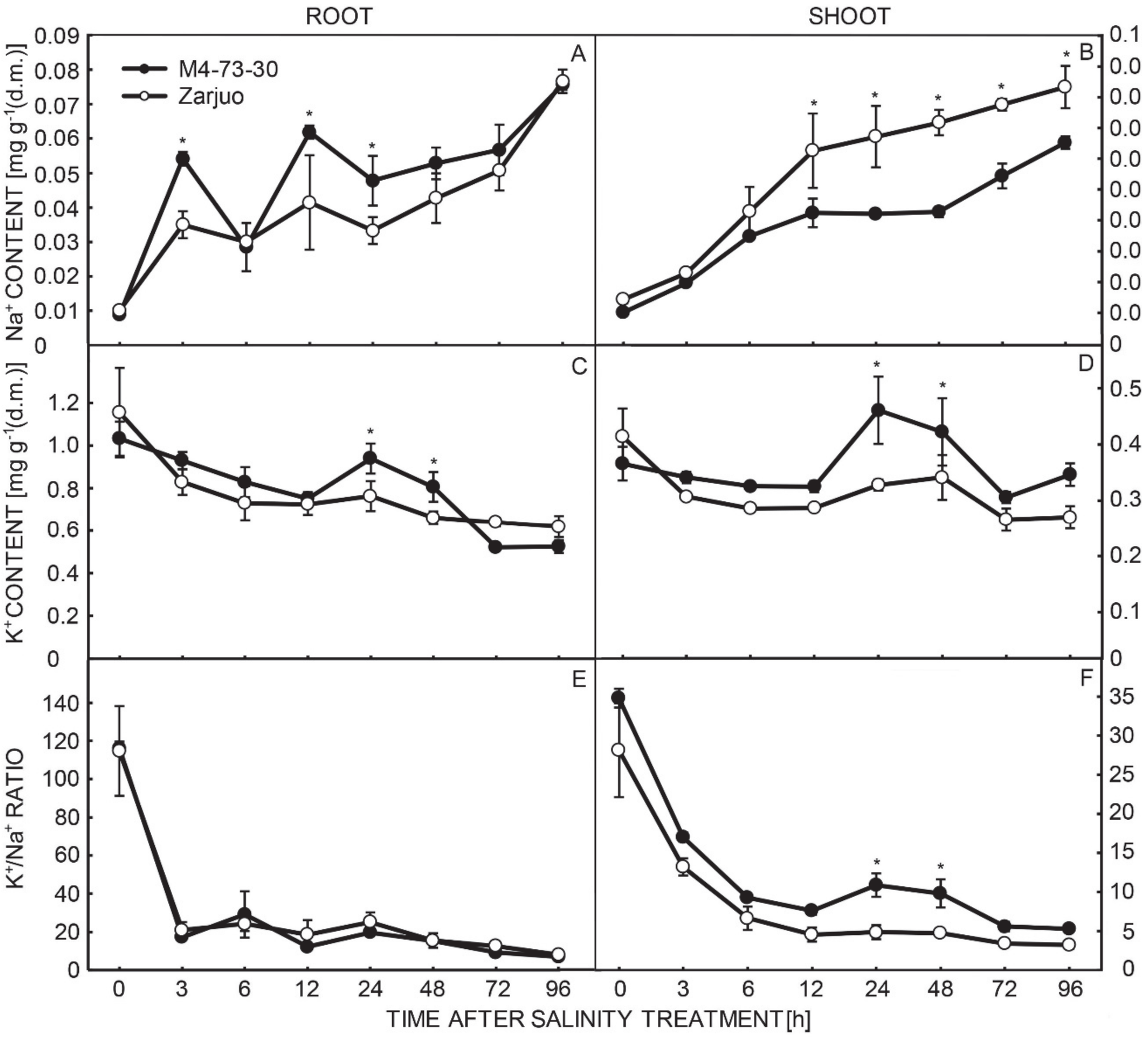

Fig. 1. Na^+ content (*A,B*) and K^+ content (*C,D*) and K^+/Na^+ ratio (*E,F*) in the roots and shoots of the barley salt-tolerant mutant M4-73-30 (*closed circles*) and its wild-type cv. Zarjou (*open circles*) in different time points (0, 3, 6, 12, 24, 48, 7,2 and 96 h of exposure to 300 mM NaCl). Means ± SE, $n = 3$, * and ** indicate significant differences between the M4-73-30 and wild-type at $P \leq 0.05$ and 0.01, respectively.

The expression pattern of *HVA* gene was similar to that of *HvNHX1*; the highest *HVA* expressions in the shoots of the salt-tolerant mutant and the wild-type were at 96 h and 48 h, respectively. Moreover, the transcription of *HVA* in the root of the wild-type increased at 3 and 24 h (more than 30-fold in comparison to the control). However, a different trend was seen in roots of the mutant, where the highest transcript abundance was observed already after 6 h (about 50-fold in comparison to the control; Fig. 2).

The expressions of *HvSOS3*, *HvSOS2*, *HvSOS1*, and *HVA* genes were more increased in the salt-tolerant mutant genotype in comparison to the wild-type at 6 h of exposure to the salt stress.

Discussion

Researchers believe that the plant capability to maintain K^+/Na^+ homeostasis is crucial for salinity tolerance (Cuin and Shabala 2006, Volkov and Amtmann 2006). K^+ plays an important role in maintaining cell pressure potential in leaf and shoot samples. The amount of K^+ in barley roots under NaCl treatment is positively correlated with tolerance to salt stress (Chen *et al.* 2007). Our results showed increased Na^+ content in both roots and shoots of salt-tolerant mutant and its wild-type genotype during the salt stress, however, this increase was lower in the shoot of salt-tolerant mutant compared to its wild genotype. Higher Na^+ content was observed in the root samples of the salt-tolerant mutant in comparison with the wild type (Fig. 1). Therefore, the salt-tolerant mutant could keep the higher amount of Na^+ content in its roots, leading to lower Na^+ transport from the roots to the shoots.

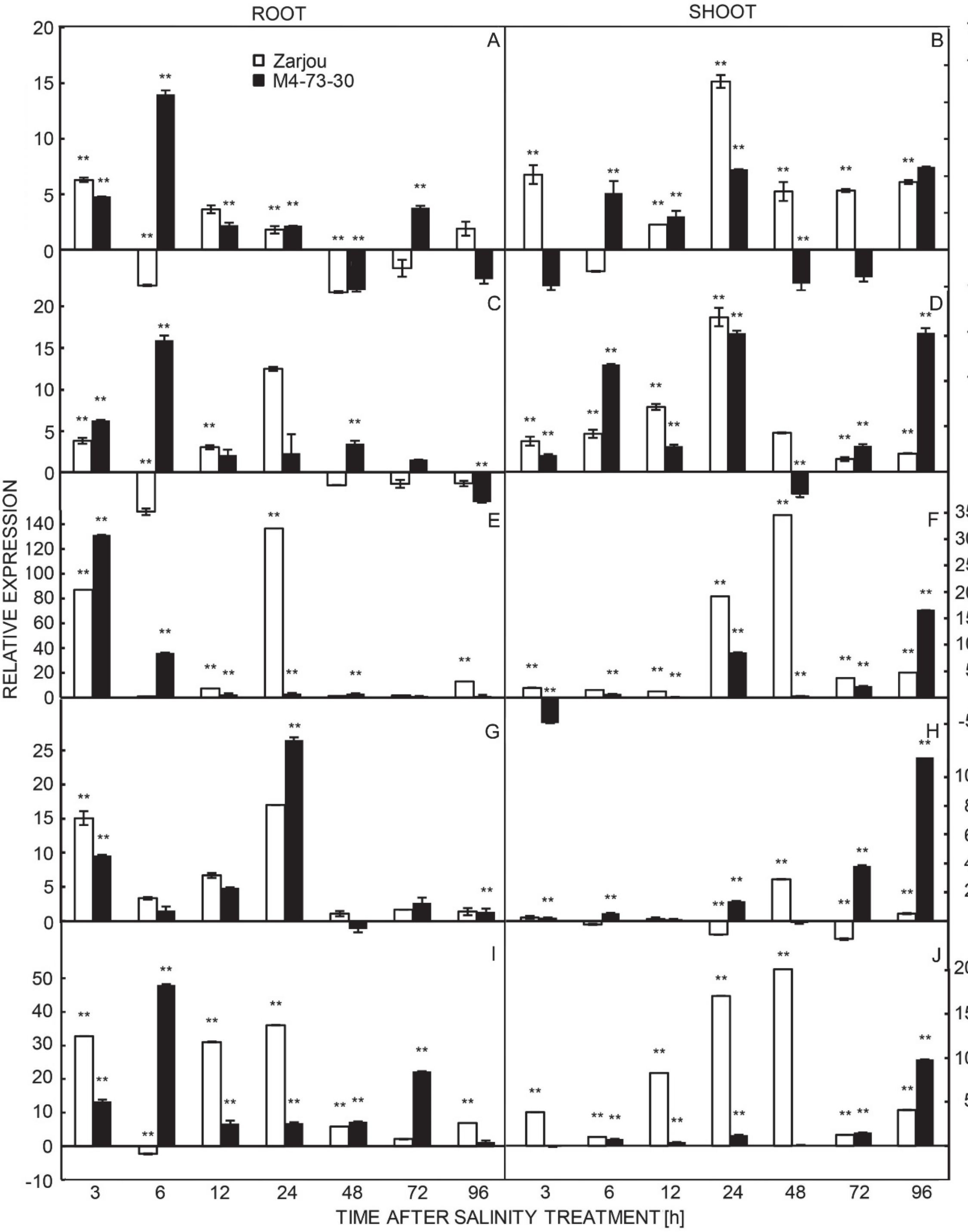

Fig. 2. The expression patterns of *HvSOS3* (*A,B*)*, HvSOS2* (*C,D*), *HvSOS1* (*E,F*), *HvNHX1* (*G,H*), and *HVA* (*I,J*) genes in the roots and shoots in salt-tolerant mutant M4-73-30 and its wild-type cv. Zarjou at 3, 6, 12, 24, 48, 72, and 96 h of salt stress in comparison with the control (0 h). Means ± SE, *n* = 3,* and ** indicate significant differences at $P \leq 0.05$ and 0.01, respectively.

Munns and Tester (2008) showed that salt tolerant plants have a Na$^+$ exclusion mechanism to keep a low content of Na$^+$ in the leaves during salt stress. When Na$^+$ enters into roots, it can potentially be excluded (back to the soil) by Na$^+$/H$^+$ exchangers located in the plasma membrane such as SOS1, or sequestered into the vacuole by Na$^+$/H$^+$ exchangers like NHX proteins located in the tonoplast (Apse and Blumwald 2002, 2007, Yokoi *et al.* 2002, Qiu *et al.* 2003, Hasegawa 2013). Moreover, Na$^+$ can be translocated and redistributed to different parts of the plant through the vascular system. Salt redistribution plays a critical role in salt tolerance, as demonstrated by studies on two Na$^+$ transporters (AtHKT1 and SOS1) in *Arabidopsis* (Shi *et al.* 2002, Apse *et al.* 2003, Berthomieu *et al.* 2003, Qiu *et al.* 2004, Rus *et al.* 2004). Apse *et al.* (2003) reported that *Arabidopsis atnhx1* mutant showed markedly decreased vacuolar Na$^+$/H$^+$ antiporter activity and displayed sensitivity to Na$^+$, emphasizing the function of vacuolar NHXs in Na$^+$ accumulation under salinity stress. In this study, the maximal expressions of *HvSOS1* and *HvNHX1* genes in the root of the salt-tolerant mutant were at 3 and 24 h of salt treatment, respectively (Fig. 2). These results show that salt-tolerant mutant in order to maintain the cell homeostasis uses Na$^+$/H$^+$ antiporter (SOS1) at early time point of salt stress. Afterwards, the expression of vacuolar Na$^+$/H$^+$ antiporter gene *NHX1* increased to sequester Na$^+$ into the root vacuoles and declined the Na$^+$ content in the cytoplasm and so ameliorated possible damage due to excess of Na$^+$. It was reported by other researchers that the up-regulation of *HvNHX1* expression declined Na$^+$ translocation from roots to shoots through Na$^+$ accumulation in the root vacuoles (Fukuda *et al.* 2004, Zhang *et al.* 2008, Wang *et al.* 2011) which was in agreement with our results. In addition, Garbarino and DuPont (1988) showed that also in barley, the Na$^+$ is stored in the vacuoles of roots, and so the transport of Na$^+$ to the shoot is declined. But after a long-term exposure of a plant to salinity, Na$^+$ is translocated from the roots to shoots (Maser *et al.* 2002). It can be seen from our data that the sharp rise in the amount of *HvNHX1* transcripts in the shoots of two genotypes was after 48 h of salt stress in wild-type and after 96 h in the salt tolerant mutant.

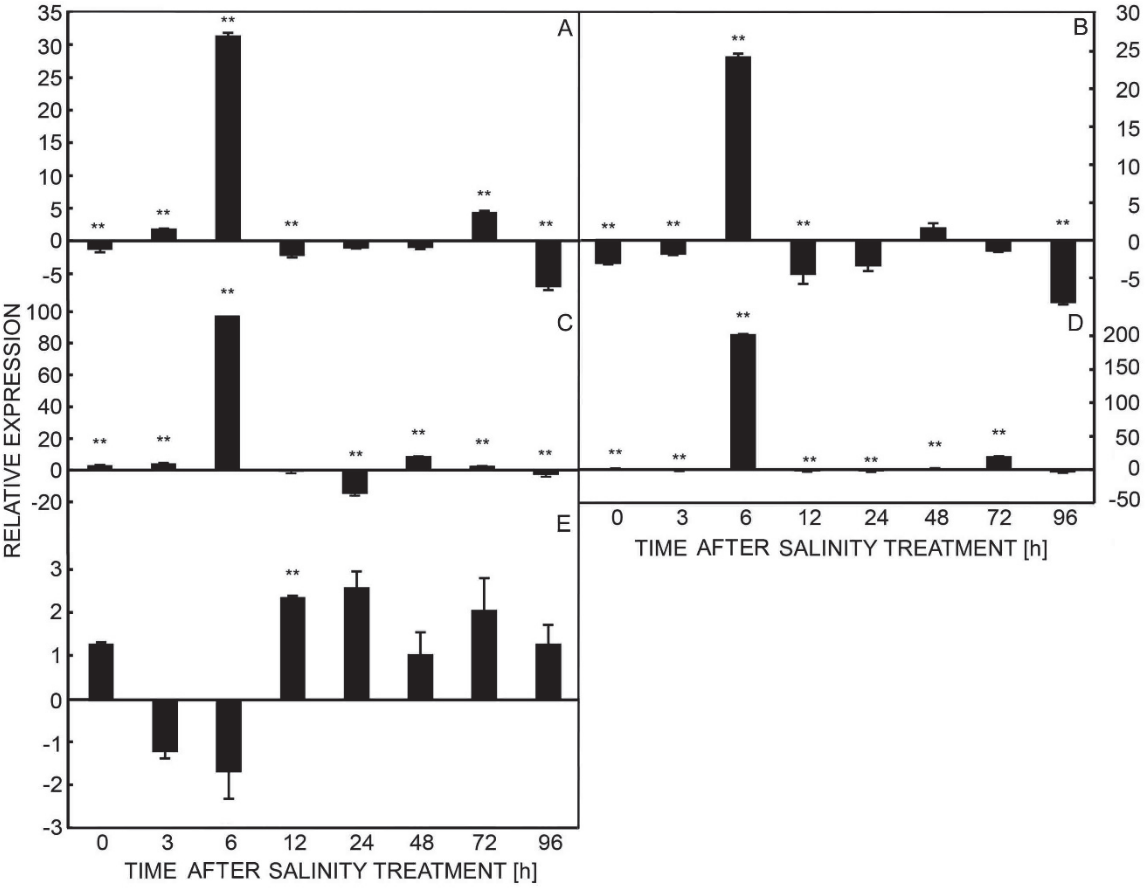

Fig. 3. The expression pattern of *HvSOS3* (*A*), *HvSOS2* (*B*), *HvSOS1* (*C*), *HVA* (*D*), and *HvNHX1* (*E*) genes under 300 mM NaCl at different time points (0, 3, 6, 12, 24, 48, 72, and 96 h) in the root of the salt-tolerant mutant M4-73-30 in comparison with the wild-type cv. Zarjou at the same treatment time. Means ± SEs, $n = 3$, * and ** indicate significant differences at $P \leq 0.05$ and 0.01, respectively.

The induction of *HvNHX1* in the root of the salt-tolerant mutant was delayed till 12 h that was due to Na^+ efflux in earlier time point using plasma membrane Na^+/H^+ antiporter (SOS1) to maintain cell homeostasis (Fig. 3). Afterwards, the expression of vacuolar Na^+/H^+ antiporter gene *NHX1* increased to sequester Na^+ into the root vacuoles and declined the Na^+ content in the cytoplasm and so ameliorate possible damage due to excess of Na^+. It was reported by other researchers that the up-regulation of *HvNHX1* expression declined Na^+ translocation from roots to shoots through Na^+ accumulation in the root vacuoles (Fukuda *et al.* 2004, Zhang *et al.* 2008, Wang *et al.* 2011) which was in agreement with our results. In addition, Garbarino and DuPont (1988) showed that also in barley, the Na^+ is stored in the vacuoles of roots, and so the transport of Na^+ to the shoot is declined. But after a long-term exposure of a plant to salinity, Na^+ is translocated from the roots to shoots (Maser *et al.* 2002). It can be seen from our data that the sharp rise in the amount of *HvNHX1* transcripts in the shoots of two genotypes was after 48 h of salt stress in wild-type and after 96 h in the salt tolerant mutant.

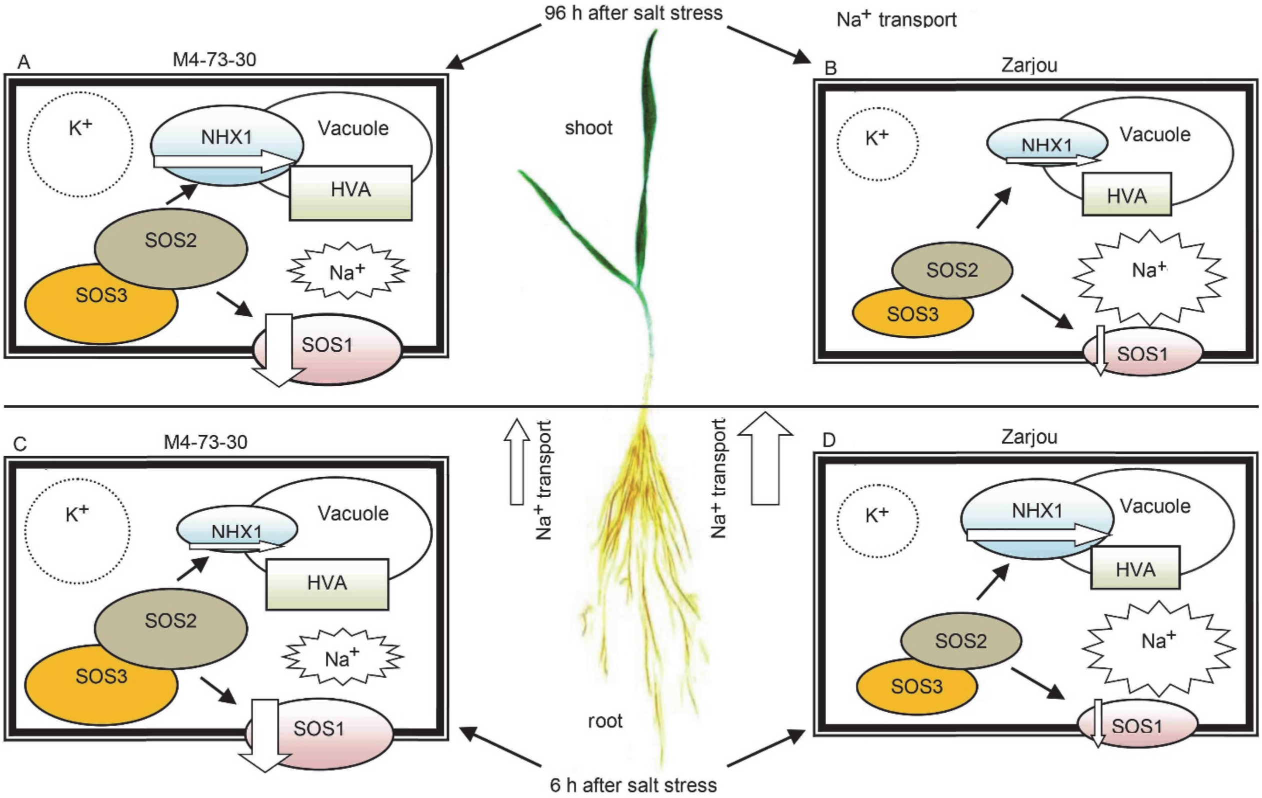

Fig. 4. The schematic diagram of the major differences between the shoots (96 h) (*A,B*) and roots (6 h) (*C,D*) of the salt-tolerant mutant M4-73-30 (*A,C*) and its wild-type cv. Zarjou (*B,D*) in response to 300 mM NaCl. Na^+ and K^+ content and expressions of salinity-related genes were indicated in the shoot and root samples of both genotypes. Sizes of shapes approximately correspond to changes induced by NaCl stress.

In general, the amount of K^+ and K^+/Na^+ ratio in this study slightly dropped in the roots and shoots of both genotypes during salt stress (Fig. 1). Also other researchers reported that the K^+/Na^+ ratio falls under salinity due to excessive Na^+ accumulation (Maathuis and Amtmann 1999, Zhu 2000, Leigh 2001, Tester and Davenport 2003). In addition, enhanced K^+ leakage from the cells resulting from NaCl-induced membrane depolarization under salinity (Cakirlar and Bowling 1981, Shabala *et al.* 2003) was proved. The K^+ transport to leaves can be affected by Na^+ content through its competition for K^+ binding sites of transport proteins. Therefore, the acquisition of K^+ may be a contributing factor to salinity tolerance (Blumwald *et al.* 2000, Munns and Tester 2008). The lower accumulation of Na^+ in the shoot of salt-tolerant mutant allowed higher K^+ transport from root to shoot. Hence the K^+/Na^+ ratio was higher in shoots of salt-tolerant mutant in comparison to its wild-type. The salt tolerance in wheat and many other monocot species is also closely associated with a low Na^+ accumulation in leaves and a high K^+/Na^+ ratio (Schachtman *et al.* 1992, Colmer *et al.* 2005, Kader and Lindberg 2005). Moreover, Shabala and Cuin (2008) showed that the salinity tolerance of a plant is determined not only by the leaf Na^+ content but also by the ability to maintain high cellular K^+ content. In this study, salt-tolerant mutant kept higher cellular K^+ content in the shoots during salt stress (maximum at 24 to 48 h). In addition, Shabala *et al.* (2010) reported that the tolerant barley genotypes are capable of maintaining higher xylem K^+/Na^+ ratios and efficiently sequester the Na^+ into vacuoles. Also Tavakoli *et al.* (2010) indicated that higher salt tolerance in barley genotype Afzal was correlated with a higher K^+/Na^+ ratio in the shoots compared with the salt sensitive genotype. Furthermore, Wang *et al.* (2013) indicated that keeping cellular K^+ content above a certain threshold level and maintaining a high cytosolic K^+/Na^+ ratio is critical for plant growth and salt tolerance. A salt tolerant barley mutant (Golden Promise) produced by γ-radiation shows the higher K^+/Na^+ ratio and lower Na^+ content in young expanding leaves compared to its wild-type (Maythorpe) (Wei *et al.* 2003). Moreover, Yang *et al.* (2017) showed that expression of Na^+/H^+ exchanger encoding genes, especially *AtNHX1*, accumulated more K^+ that improved salt tolerance in transgenic plants poplar.

The salt stress-increased Ca^{2+} content may be sensed by Ca^{2+}-dependent protein kinases such as calcineurin B-like protein (CBL4, originally identified as SOS3) (Harmon *et al.* 2001). Although the affinity for Ca^{2+} binding this protein is unknown, an increase in cytosolic Ca^{2+} content likely causes the dimerization of SOS3 and the subsequent interaction with a CBL-interacting protein kinase (CIPK24, originally identified as SOS2). The CBL4/CIPK24 (SOS3/SOS2) complex is targeted to the plasma membrane through a myristoyl fatty acid chain covalently bound to CBL4/SOS3. Afterwards, it enables the phosphorylation and thus the activation of one of the plasma membrane bound Na^+/H^+ antiporter, SOS1 (Maughan *et al.* 2009). SOS1 causes the extrusion of Na^+ from the cytoplasm to the apoplast (Hasegawa 2013) and it is also involved in long distance Na^+ transport from roots to shoots (Shi *et al.* 2002). Although the increased expressions of *HvSOS1*, *HvSOS2*, and *HvSOS3* in the roots of both genotypes occurred at the early time points of salt stress, the amount of transcripts was higher in the salt-tolerant mutant than in the wild-type (Figs. 2 and 3). Results showed that an increase of Na^+ content in the shoot of the wild-type contributed to the highest gene expression at 24 h for *HvSOS2* and *HvSOS3* genes, which continued by 350-fold for *HvSOS1* at 48 h (Fig. 2). Overexpression and knockout studies of the *SOS1* gene highlight the involvement of this gene in salt tolerance; *e.g.*, Shi et al. (2002) observed that *Arabidopsis SOS1* mutant accumulated more Na^+ in the shoot and xylem than the wild type. Hence, they proposed that SOS1 could act in a Na^+ scavenging mechanism at the root xylem-symplast interface. Jannesar *et al.* (2014) showed that *alaSOS1* expression in *Aeluropus lagopoides* is hardly detectable in roots under control conditions and it is induced by 600 mM NaCl, so its expression is critical for osmotic regulation and nutrients uptake. In addition, Xu *et al.* (2008) reported that the expression of *TaSOS1* in the leaves of wheat plants exposed to 200 mM NaCl remained fairly constant at different points (0, 3, 6, 12, 24, 48 h), except for the transient two-fold increase at 9 h.

According to Fig. 3, the up-regulation of *HvSOS1* in the roots of the salt-tolerant mutant after 6 h of NaCl exposure caused a contemporary decline in the amount of Na^+ in the root of salt-tolerant mutant at this time (Fig. 3). *HvSOS1* may play an important role in the transport of Na^+ from the cytoplasm to the apoplast (Ding *et al.* 2010). Martinez-Atienza *et al.* (2007) also reported that salinity induced the accumulation of *OsSOS1* mRNA in rice roots, but not in shoots. The lower Na^+ accumulation in the shoots of salt-tolerant mutant may be the outcome of the higher expression of *HvSOS1* in the root. Similarly, Chen *et al.* (2007) reported that differential salt sensitivity among barley cultivars is connected with a higher Na^+/H^+ antiporter (possibly SOS1) activity in the root of salt-tolerant cultivars. It was often found that Na^+ accumulation in the shoots is significantly lower in salt-tolerant cultivars. Moreover, the parallel of up-regulation of *HvSOS3*, *HvSOS2*, *HvSOS1*, and *HVA* genes was identified in the root samples of the salt-tolerant mutant at 6 h of salt stress (Fig. 3). Therefore, *SOS1* gene expression under NaCl stress is controlled by the SOS3/SOS2 regulatory pathway. Moreover, the SOS3/SOS2 complex activates *SOS1* and *NHX1* and it is also essential in regulation of electrochemical proton gradient across tonoplast using *HVA* (Shi *et al.* 2000). The V-ATPase is a dominant H^+-pump at cellular membranes of most plant cells and critical for salt tolerance mechanisms and osmotic adjustment in plants because the increased H^+-ATPase expression would provide the proton gradient and the driving force for a Na^+/H^+ antiport (SOS1, NHX1, and other transporters on the vacuolar membranes; Shi *et al.* 2000, Hasegawa 2013). Under different stresses, such as salinity and drought, survival of the cells depends strongly on maintaining V-ATPase activity (Dietz *et al.* 2001, Rezaei Moshaei *et al.* 2014). The increased activity of the vacuolar H^+-pumps may acidify the vacuoles, which stimulated the activity of the *HvNHX1* protein, mainly functioned to accumulate Na^+ into the vacuoles in response to a salt stress. As explained by Fukuda *et al.* (2004), alkalization of vacuolar lumen might regulate the H+-pump gene expression and its acidification induces Na+/H+ antiporters (NHX1). In addition, several reports revealed salt stress based induction of *HVA* at mRNA and protein levels in various plants (Fukuda *et al.* 2004). In view of the critical role of H^+-ATPase in response to salt stress, these membrane proteins provide cells with a primary driving force for ion transport and homeostasis to cope with the stressed environment (Stevens *et al.* 1997, Palmgren 2001, Wu *et al.* 2014).

Our results indicated a significant difference between the roots and shoots of the salt-tolerant mutant M4-73-30 and of its wild-type (Fig. 4). The up-regulation of *HvSOS1*, *HvSOS2*, *HvSOS3*, and *HVA* genes were observed in the root sample of salt-tolerant mutant at early time points (6 h) of exposure to salt stress. Therefore, it resulted in higher Na^+ exclusion from root symplast to apoplast region *via HvSOS1* transporter which led to a delay in up-regulation of *HvNHX1* in the shoot, and also slower Na^+ transport from roots to shoots of the salt-tolerant mutant in comparison to the wild-type. Totally, the salt-tolerant mutant had higher ability to increase the evaluated genes via physiological response and ion homeostasis. Moreover, that led to keeping more Na^+ in the root, the higher content of K^+ and K^+/Na^+ ratio in the shoot and, finally, less damage in the salt-tolerant mutant compared to wild-type (Fig. 4). The differences in gene expression patterns between two genotypes with similar genetic background confirm the hypothesis that mutation breeding can be successfully utilized as an efficient tool for improving salt tolerance in crop plants. It can be applied in genomic, transcriptomic, and proteomic approaches to recognize and determine the candidate gene(s) that is associated with salt tolerance.

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