RESEARCH ARTICLE

Expression pattern of salt tolerance-related genes in *Aegilops cylindrica*

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Abstract *Aegilops cylindrica*, a salt-tolerant gene pool of wheat, is a useful plant model for understanding mechanism of salt tolerance. A salt-tolerant USL26 and a salt-sensitive K44 genotypes of *A. cylindrica*, originating from Uremia Salt Lake shores in Northwest Iran and a non-saline Kurdistan province in West Iran, respectively, were identified based on screening evaluation and used for this work. The objective of the current study was to investigate the expression patterns of four genes related to ion homeostasis in this species. Under treatment of 400 mM NaCl, USL26 showed significantly higher root and shoot dry matter levels and K⁺ concentrations, together with lower Na⁺ concentrations than K44 genotype. *A. cylindrica* *HKT1;5* (*AecHKT1;5*), *SOS1* (*AecSOS1*), *NHX1* (*AecNHX1*) and *VP1* (*AecVP1*) were partially sequenced to design each gene specific primer. Quantitative real-time PCR showed a differential expression pattern of these genes between the two genotypes and between the root and shoot tissues. Expressions of *AecHKT1;5* and *AecSOS1* was greater in the roots than in the shoots of USL26 while *AecNHX1* and *AecVP1* were equally expressed in both tissues of USL26 and K44. The higher transcripts of *AecHKT1;5* in the roots versus the shoots could explain

both the lower Na⁺ in the shoots and the much lower Na⁺ and higher K⁺ concentrations in the roots/shoots of USL26 compared to K44. Therefore, the involvement of *AecHKT1;5* in shoot-to-root handover of Na⁺ in possible combination with the exclusion of excessive Na⁺ from the root in the salt-tolerant genotype are suggested.

Keywords *HKT1;5* · *NHX1* · Salinity · Sodium exclusion · *SOS1*

Introduction

Increasing soil salinity due to human activities, climate changes as well as natural mineral weathering and secondary salinization is one of the major abiotic constraints affecting agricultural productivity worldwide. Increased salinization of arable land is estimated to result in land loss (by up to 30%) with devastating global consequences by 2050, when world population is projected to be 9.1 billion (FAO 2011). The conventional method of introgressing genes from related wild germplasm to a cultivated species encounters difficulty due to incompatibility barriers. Therefore, an alternative way to transfer alien genes to a crop species is transgenic technology (Arzani and Ashraf 2016). However, the molecular marker-aided traditional breeding and transformation techniques of gene transfer from salt-tolerant wild species require candidate genes to be identified whose expression is associated with salt tolerance.

The genetic basis of sodium and potassium selectivity has been investigated in relation to salt tolerance at the molecular, cellular and whole plant levels (Arzani and Ashraf 2016; Schachtman and Munns 1992; Shabala et al. 2013). One key mechanism of salt tolerance is the ability of

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a plant to regulate Na^+ transport at both the cellular and tissue levels, either by minimizing the amount of Na^+ entering the plant through its roots, or by secreting Na^+ into organelles, cells and tissues where it can do far less harm (Apse and Blumwald 2007; Munns and Tester 2008). Sodium exclusion does not allow the accumulation of Na^+ to toxic levels within leaves and that leads to a high K^+/Na^+ discrimination, has frequently been described as an important mechanism involved in salt tolerance in cereal crops such as bread wheat (Gorham 1990; Schachtman and Munns 1992), durum wheat (Houshmand et al. 2005), barley (Garthwaite et al. 2005; Wei et al. 2003) and rice (Zhu et al. 2001). The plasma membrane Na^+/H^+ SOS1 (salt overly sensitive) antiporters, the high-affinity potassium transporters (HKTs) and the intracellular Na^+/H^+ antiporters (NHXs) are known to be involved in the Na^+ homeostasis (Arzani and Ashraf 2016; Hamamoto et al. 2015; Shi et al. 2002). For instance, the key role that *HKT1;5* (previously *HKT8*) plays in salt tolerance through sequestering Na^+ to the non-photosynthetic reservoir, leaf sheaths and roots has been reported in wheat (Byrt et al. 2007) and rice (Platten et al. 2013). In addition, the role of the vacuolar *NHX1* gene has been well documented as contributing to the cytoplasmic Na^+ extrusion into the vacuole (Bassil and Blumwald, 2014). Vacuolar H^+ -pyrophosphatase (*VPs*) genes are also required to power the Na^+/H^+ antiporter through the electrochemical gradient for H^+ across the tonoplast (Zhao et al. 2007). In this regard, earlier reports showed that transgenic plants co-overexpressing the H^+ -PPase *VPI* and vacuolar Na^+/H^+ antiporter *NHX1* have a far greater tolerance to high concentrations of NaCl (Brini et al. 2007; Zhao et al. 2007). Nevertheless, the plant ability to discriminate between Na^+ and K^+ in soil solution and to preferentially exclude Na^+ and accumulate K^+ has also been described as a crucial salt tolerance mechanism in the Triticeae tribe (Arabbeigi et al. 2014; Arzani and Ashraf 2016; Dvorak et al. 1994; Shabala et al. 2013).

The wild relatives of cultivated plants, which include their ancestors and more or less distantly related species, perform superior under adverse environments, and hence constitute a valued gene pool for tolerance to abiotic stress (Arzani and Ashraf 2016; Saha et al. 2016). *Aegilops cylindrica* Host., commonly known as Jointed goat grass, is an amphidiploid ($2n = 4x = 28$; CCDD) originated from the ancestors of *A. markgrafii* (Greuter) Hammer ($2n = 2x = 14$; CC) and *A. tauschii* Coss. ($2n = 2x = 14$; DD) species and represents a valuable source of salt tolerance genes (Arabbeigi et al. 2014; Arzani and Ashraf 2016; Colmer et al. 2006; Kiani et al. 2015). Our previous research identified a hyper-salt tolerant genotype originated from the Uremia Salt Lake shores (North-West Iran) with the ability to exclude high Na^+ ions (Arabbeigi et al. 2014;

Kiani et al. 2015). The observations for the germplasm exploration conducted in the West Iran showed that *A. cylindrica* is the sole member of **Triticeae tribe** that grows in the presence of high concentrations of salt in salinized shore area of Uremia Salt Lake (Arzani and Ashraf 2016). Knowledge about the sequences and expression profiles of the genes responsible for Na^+ exclusion would contribute towards better understanding of the mechanisms leading to hyper-salt tolerance of *A. cylindrica*. The aim of this work was, therefore, to investigate the expression profiles of some of the most important Na^+ transporter related genes (namely, *AecHKT1;5*, *AecSOS1*, *AecNHX1* and *AecVPI*) in the root and shoot tissues of both salt tolerant and salt sensitive genotypes of *A. cylindrica*.

Materials and methods

Plant material, growth conditions and treatments

A hyper-salt tolerant genotype (USL26, originating from Uremia Salt Lake shores Northwest Iran) and a salt sensitive genotype (K44, originating from Kurdistan province, West Iran) were selected based on our previous studies (Arabbeigi et al. 2014; Kiani et al. 2015) and used in this study. Data on growth parameters and physiological measurements of the roots and shoots of 44 genotypes under control and 400 mM NaCl treatments (4 weeks) were presented in this study. All the details related to salt tolerance evaluation experiment are given in our previous publication (Arabbeigi et al. 2014). The whole set of *A. cylindrica* genotypes (88) were also assessed for salt tolerance at a lower stress level (350 mM NaCl) due to the poor growth and survival rates of the genotypes originated from non-saline habitat (Kiani et al. 2015; for the list of the genotypes see its Supplementary Table online at: doi:<https://doi.org/10.1007/s11738-015-1881-0>).

For gene expression analysis, seeds were germinated for 4 days at 4 °C on moist filter paper in Petri dishes. Germinated seeds were planted into 60 × 40 cm plastic trays containing gravel, under greenhouse conditions. The excess solution passing through the drainage holes at the bottom of each tray was collected by an underneath plate. Seedlings were watered initially with tap water, and then irrigated with half-strength Hoagland nutrient solution 3 days after emergence. At second leaf emergence, the nutrient solution was replaced with full strength Hoagland nutrient solution to which was added NaCl in three gradual steps of irrigation until final concentrations reached 400 mM ($\text{EC} \approx 40 \text{ ds m}^{-1}$). Greenhouse air temperature ranged from 25 °C (day) to 17 °C (night). Treatments were replicated four times with ten plants per replication. The experiment was carried out based on a factorial design with

three factors; NaCl treatments (0 and 400 mM NaCl at 24 h and 400 mM at 7 days), two genotypes and two tissues as well as two biological and two technical replications. Shoot and root tissues of each genotype were collected during sampling, snap frozen in the liquid nitrogen, and immediately stored at -80°C until use.

Na⁺ and K⁺ concentrations

The root and shoot samples were dried at 80°C for 48 h to determine the dry matter (DM). One gram of the ground shoot and root dried sample was further dried to ash at 550°C for 4 h; mixed with 2 M hot HCl, filtered, and brought to a final volume of 50 ml with distilled water. The Na⁺ and K⁺ concentrations of the solutions were analyzed using flame photometry (Jenway PFP7, UK).

RNA extraction and cDNA synthesis

In total, 100 mg of leaf and root tissues were ground into a fine powder using liquid nitrogen. Total RNA was isolated from 50 mg of the ground tissue using the Spectrum Plant RNA Kit (Sigma-Aldrich). Tissues were mixed thoroughly in 1 ml of G1 buffer in an RNase-free microfuge tubes and incubated at room temperature for 5 min. The tubes were then centrifuged at $12,000\times g$ for 10 min at 4°C . After transferring into a new tube, the supernatant from each sample was mixed with 200 μl of chloroform and the tube was incubated at room temperature for 3 min. After centrifugation at $12,000\times g$ for 15 min at 4°C , the supernatant was precipitated into a new tube with half the volume of isopropanol and the same volume from G2 buffer for 10 min at room temperature. The RNA was pelleted by centrifugation at $10,000\times g$ for 10 min at 4°C and washed using 70% ethanol. After brief drying, RNA was re-suspended in 30 μl of RNase-free water. The purity of the total RNA was quantified by Eppendorf AG22331 BioPhotometer Spectrophotometer (Hamburg, Germany). DNase treatment of total RNA was carried out using RQ1 (RNA Qualified) RNase-Free DNase Kit (Promega, USA) according to the manufacturer's instructions. Synthesis of cDNA from 1 μg RNA was carried out using ImProm-IITM Reverse Transcription System Kit (Promega, USA) as per the manufacturer's instructions in a 20 μl final volume.

Sequencing genes for salt tolerance proteins and primer design

In order to develop *HKT1;5*, *SOS1*, *NHX1*, and *VPI* gene-sequence specific primers for *A. cylindrica*, nucleotide sequences of these genes from wheat, rice, maize and Arabidopsis were aligned by ClustalW tool. A pair of primers was designed from the conserved aligned

sequences for each gene and the details of the primers used for screening the genes of interest are provided in Table 1. Fragments of the *AecHKT1;5*, *AecSOS1*, *AecNHX1* and *AecVPI* genes were amplified following the PCR conditions reported earlier (Saha and Blumwald 2014) and sequenced from Macrogen (South Korea). Sequences were subjected to the basic local alignment search tool using a nucleotide query (BLASTn) of the National Center for Biotechnology Information (NCBI) for similarity search. The partial sequences of the genes were deposited in NCBI (*AecHKT1;5* and *AecSOS1*) and EMBL (*AecNHX1* and *AecVPI*) databases.

Quantitative real-time PCR

A pair of primers from each gene was developed using Oligo 7 software (Molecular Biology Insights, Inc., USA) for quantitative real-time PCR (qRT-PCR). The qRT-PCR was carried out in a 10- μl volume containing 5 μl of 2x Fast SYBR Green PCR Master Mix (Applied Biosystems), 2 μl of 1 μM each of reverse and forward primers, 2 μl of diluted cDNA and 1 μl of RNase free water following the procedure described by Saha and Blumwald (2014). The qRT-PCR was performed in an optical 96 well plate (Applied Biosystems, Foster City, CA) using a StepOnePlusTM real time PCR system (Applied Biosystems). The PCR reactions were conducted with following the cycling conditions: 50°C for 2 min, 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. After 40 cycles, a melting curve analysis was performed ($60\text{--}95^{\circ}\text{C}$) to verify the specificity of amplicons. All amplification reactions were carried out in duplicate and reverse transcriptase negative controls were included to test for genomic DNA contamination. The *18S rRNA* and *Gapdh* reference genes were used as an endogenous control to normalize gene expression and the fold expression changes of target mRNAs were determined using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001). The data are presented as the fold change of mRNA expression in plant tissues (roots and shoots) relative to normal tissues after normalization to an endogenous control.

Phylogenetic analysis of salt tolerance genes

Related nucleotide sequences of the *AecHKT1;5*, *AecSOS1*, *AecNHX1* and *AecVPI* genes were obtained from NCBI GenBank using BLASTn search. Multiple sequence alignments of all homologous sequences for each gene from closely related grass species were performed in ClustalW (Thompson et al. 1994) and phylogenetic analysis was conducted using the maximum likelihood (ML) method with 1000 bootstrap replication in the MEGA v. 5.2.1 software (Tamura et al. 2011).

Table 1 Sequences of primers used for genomic DNA amplification and qRT-PCR in this study

Primer ^a	Sequence	Amplicon length (bp)	T _m (°C)
<i>HKT1</i> ;5-G	F: CATCTCCTTCTTCGGTTTGG R: GATCATCCCTTCGTTGTTCG	610	55
<i>HKT1</i> ;5-R	F: GCAAACCAGCACCATCATCT R: CGCTCAGGTAGACCAGCATC	198	60
<i>SOS1</i> -G	F: CAAGGATTTTGTCCCTCAG R: CTTCTGGTTCGCTTCCACAT	804	58
<i>SOS1</i> -R	F: CCATTGAGCTTGGGCATAAT R: TGCTCTAGCCTCCACCTGAT	197	60
<i>NHX1</i>	F: ATTCTTCACCAGCACCGTTC R: TCAGATCCAGCAGCATTGAC	158	60
<i>VPI</i>	F: GGAGCCATCACATCTCTGGT R: TGACAACAAGAAGCCCATGA	150	60
<i>18S</i>	F: GAATTGACGGAAGGGCACCACCAG R: ACTAAGAACGGCCATGCACCACCA	149	60

^aG denotes amplification of genomic DNA and R denotes qRT-PCR

Statistical analysis

Analysis of variance (ANOVA) was carried out to examine the effects of NaCl treatments (0, 400 mM at 24 h and 400 mM at 7 days) on the gene expressions of plant tissues (shoot and root) in salt tolerant and salt sensitive genotypes using factorial ANOVA in SAS version 9.3 (SAS Institute 2011). Assumptions of the normality of distribution and the homogeneity of variances were assessed with the Kolmogorov–Smirnov test and Bartlett’s test, respectively, before being subjected to ANOVA. Data were analyzed as a factorial design, where salt stress treatments were used as a fixed factor while genotype, tissue and their interactions as random effects using the PROC GLM procedure of SAS. Mean comparisons were conducted using the Fisher’s least significant differences test (LSD_{0.05}).

The data obtained from the physiological response of the 44 *A. cylindrica* genotypes to salt stress (400 mM NaCl) were subjected to principal component analysis using PROC FACTOR (method = prin) using the Kaiser’s criterion (i.e., Eigenvalue ≥ 1.0 in SAS 9.3) (SAS Institute 2011). A genotype-by-trait biplot was then constructed using the first two principal components (PC1 and PC2).

A backward stepwise regression analysis was also performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, United States). The backward procedure first calculated the statistics for a model that included DM as the dependent variable and Na⁺, K⁺, K⁺/Na⁺, *AecHKT1*;5, *AecSOS1*, *AecNHX1* and *AecVPI* as the independent ones.

Results

Root and shoot growth and physiological traits

Salt tolerance of 44 *A. cylindrica* genotypes collected from the Uremia Salt Lake shores and Kurdistan was evaluated using such physiological traits as shoot H₂O₂, MDA, carbohydrate content, Na⁺ and K⁺ concentrations, K⁺/Na⁺ ratio and DM as well as root Na⁺ and K⁺ concentrations, K⁺/Na⁺ ratio and DM under 0 and 400 mM NaCl in greenhouse conditions. Genotype-by-trait biplot was constructed as a tool to visualize discrimination among the 44 *A. cylindrica* genotypes on the basis of multiple traits (Fig. 1). The experimental results, except root data, accompanied by molecular markers associated with salt tolerance were described in our earlier article (Arabbeigi et al. 2014). The overall shoot and root data of our trial presented in Fig. 1 revealed that USL26 is the most salt-tolerant genotype collected from saline Uremia Salt Lake shore area, while K44 is the most salt-sensitive genotype originated from non-saline Kurdistan province. The hyper-salt tolerant genotype USL26 not only survived completely the whole experimental period but also showed superior root and shoot DM as well as physiological traits compared to other genotypes (Fig. 1).

In the current study, these two contrasting genotypes were further characterized for root and shoot Na⁺ and K⁺ concentrations as well as expressions of genes related to ion homeostasis under 0 mM (control) 400 mM for 24 h and 7 days of NaCl treatments. The results revealed that shoot and root DM of the genotypes decreased under salt stress (400 mM NaCl) compared to control, while the salt

Fig. 1 Biplot of 44 genotypes of *A. cylindrica* (1–44) and physiological traits (vectors) affected by 400 mM NaCl for 4 weeks. The PC1 and PC2 explain 53.4 and 31.5% of the total variations, respectively. Traits abbreviations: STi: salt tolerance index, rNa: root

Na^+ , rK: root K^+ , rK/Na: root K^+/Na^+ , sNa: shoot Na^+ , sK: shoot K^+ , sK/Na: shoot K^+/Na^+ , rDW: root dry matter, sDW: shoot dry matter; MDA: shoot malondialdehyde, H_2O_2 : shoot hydrogen peroxide and Car: shoot carbohydrate

tolerant genotype USL26 showed threefold higher DM compared to salt-sensitive genotype K44 (Table 2).

The Na^+ and K^+ content as well as K^+/Na^+ ratio in the shoot and root tissues showed that not only salt stress significantly altered the ionic concentrations of both the genotypes, but also that USL26 and K44 responded significantly differently to NaCl treatments by differential accumulation of the ions in their tissues (Table 2). Although a significant increase was observed in Na^+ content of both the root and shoot tissues in the salt treated plants compared to those of the control, the USL26 possessed lower Na^+ content and higher K^+ content and K^+/Na^+ ratio in the roots and shoots (Table 2). The comparison of Na^+ content in the roots and shoots between the treatment periods showed that Na^+ concentration was

much lower at 24 h than 7 days of salt treatment. In addition, a higher Na^+ accumulation was occurred in the roots than in the shoots at both the treatment periods.

Sequence homology and diversity of salt tolerance genes

The PCR amplified the 580, 782, 158 and 150 bp amplicons of *AecHKT1;5*, *AecSOS1*, *AecNHX1* and *AecVPI* genes respectively, and were gel eluted and sequenced. The sequences were deposited to the NCBI GenBank database (accession numbers KR051076–KR051077 for *AecHKT1;5* and *AecSOS1*) and EMBL/GenBank database (accession numbers LN834336 for *AecNHX1* and LN834335 for *AecVPI*). BLAST analysis of nucleotide sequences

Table 2 Mean comparisons of root and shoot ionic concentrations affected by salt treatments in two contrasting *A. cylindrica* genotypes

Treatment/tissue (mM NaCl)	Tolerant genotype (USL26)				Sensitive genotype (K44)			
	Na^+ (mM gDM $^{-1}$)	K^+ (mM gDM $^{-1}$)	K^+/Na^+	DM (g/plant)	Na^+ (mM gDM $^{-1}$)	K^+ (mM gDM $^{-1}$)	K^+/Na^+	DM (g/plant)
Control (0)/root	1.46 h ^a	1.04 e	0.62 c	0.11 c	1.31 h	0.99 f	0.54 c	0.08 c
24 h (400)/root	3.45 de	3.58 c	1.03 b	0.07 cd	6.36 b	1.48 e	0.23 e	0.03 d
7 d (400)/root	5.65 c	5.25 b	0.92 b	0.03 d	8.53 a	2.93 cd	0.34 d	0.01 d
Control (0)/shoot	1.22 h	0.99 f	0.81 bc	0.45 a	1.26 h	1.12 e	0.63 c	0.40 a
24 h (400)/shoot	1.99 g	3.88 c	1.94 a	0.4 a	4.23 cd	1.32 e	0.31 d	0.24 b
7 d (400)/shoot	2.89 f	6.95 a	2.05 a	0.18 bc	6.63 b	2.23 d	0.33 d	0.06 cd

^aMeans followed by the same letter in each column are not significantly different at $P < 0.05$

revealed that *AecHKT1;5* gene had a homology of 99% with *TaHKT1;5-D*, 97% with *TmHKT1;5*, 94% with *TdHKT1;5-B2* and *TaHKT1;5-B2*, 94% with *TdHKT1;5-B3*, 87% with *HvHKT1;5* and 86% with *TaHKT1;5-B1* (for details see Table 3). A relatively lower identity (80–81%) was observed between *AecHKT1;5* and *OsHKT1;5*. The comparison of nucleotide sequences for the consensus sequences of the *AecHKT1;5* gene from USL26 and K44 revealed their identity. A motif search using Pfam and NCBI CDD (http://www.genome.jp/tools-bin/search_motif_lib/) was performed. The predicted polypeptide (150 aa) from the *AecHKT1;5* partial sequence indicated the presence of three domains including a conserved domain TrKH (cation transport protein), an ISK_channel (slow voltage-gated potassium channel) and the QueT (QueT transporter).

Phylogenetic analysis of nucleotide sequences of the *AecHKT1;5*, *AecSOS1*, *AecNHX1* and *AecVP1* genes with homologous sequences from closely related species are presented in Fig. 2. The phylogenetic tree for *HKT1;5* gene revealed three distinct clusters, where *AecHKT1;5* remained in a group with highly similar two *HKT1;5* genes (*TaHKT1;5* and *TmHKT1;5*) from wheat species. In another cluster *OsHKT1;5* (*HKT1;5* from *Oryza sativa*) stayed at the center close to the root, while the third cluster included distinctly related *HvHKT1;5* and *BdHKT1;5L* from barley and *Brachypodium distachyon*, respectively (Fig. 2a). Phylogenetic analysis of *SOS1* gene demonstrated that *AecSOS1* was mostly related to *TaSOS1* and *AetSOS1*, while poorly related to *LpSOS1* (from *Lolium perenne*), *BdSOS1*, *OsSOS1*, *DsSOS1* (from *Distichlis spicata*) and *SiSOS1* (from *Setaria italica*) (Fig. 2b). The *NHX1* tree revealed *AecNHX1* and *TaNHX1* were closely related, whereas the phylogenetic analysis of *VP1* clustered *AecVP1*, *TaVP* and *HvVP* together indicating their close relationship in the evolutionary process (Fig. 3d).

Expression analysis of salt tolerance genes

In this study, the results of ANOVA for the gene expression data revealed a significant effect of salt stress on the expression profiles of four genes in the root and shoot tissues (Table 4). The results of real-time PCR showed that the transcript levels of the all genes were significantly different between the root and shoot tissues. The salt treatment up-regulated (3–5 fold) the expression of *AecHKT1;5* compared to control in both the genotypes. In the tissue level, the expression of this gene was 4-fold higher in the roots than in the shoots (Fig. 3). In addition, the accumulation of *AecHKT1;5* transcript was 2-fold higher at 24 h than 7 days of 400 mM NaCl treatment (Fig. 3a). The expression of *AecHKT1;5* in the root tissues was much higher in USL26 than K44, while their transcript level in the shoots indifferently upregulated under NaCl stress conditions (Fig. 3a, b). The expression analysis of *AecSOS1* gene indicated that overall expression level was relatively low compared to *AecHKT1;5*. The expression level of *AecSOS1* was significant upregulated in the roots and shoots of salt treated plants with 1.5-fold higher transcript levels in the roots than shoots (Fig. 4). The expression levels of *AecSOS1* were significantly surpassed by the salt-tolerant USL26 genotype in both the root and shoot tissues.

The expression patterns of gene expression analyses for *AecNHX1* and *AecVP1* showed similar results with 2-fold higher transcript levels of *AecNHX1* than *AecVP1* under the 400 mM NaCl stress (Figs. 5, 6). In comparison to control, a 8-fold and a 5-fold increase of *AecNHX1* transcript levels in roots and in shoots of USL26 was observed. In the control condition, no significant difference was observed between the salt-tolerant and salt-sensitive genotypes in the expression level of these genes either in root or the shoot tissues.

Table 3 Results of BLAST homology search of *AecHKT1;5* gene using NCBI BLASTn tool

Species	Gene	Accession number	Identity (%)
<i>Triticum aestivum</i>	<i>TaHKT1;5-D</i>	DQ646342.2	99
		DQ646338.1	
<i>T. aestivum</i>	<i>TaHKT1;5-B2</i>	DQ646341.1 DQ646337.1	94
<i>T. aestivum</i>	<i>TaHKT1;5-B1</i>	DQ646340.1 DQ646336.1 DQ646333.1	86
<i>T. monococcum</i>	<i>TmHKT1;5</i>	DQ646339.2	97
		DQ646332.1	
<i>T. turgidum</i> subsp. <i>durum</i>	<i>TdHKT1;5-B2</i>	DQ646334.1	94
<i>T. turgidum</i> subsp. <i>durum</i>	<i>TdHKT1;5-B3</i>	DQ646335.1	94
<i>Hordeum vulgare</i> subsp. <i>vulgare</i>	<i>HvHKT1;5</i>	DQ912169.1	87
<i>Oryza sativa</i>	<i>OsHKT1;5</i>	JQ695816.1	80

Fig. 2 Phylogenetic tree based on nucleotide sequences, showing the relation of **a** *AecHKT1;5*, **b** *AecSOS1*, **c** *AecNHX1* and **d** *AecVP1* to the correspondences of members of the genus *Triticum* spp. and the species *Aegilops tauschii*, *Hordeum vulgare*, *Oryza sativa*, *Zea mays*, *Brachypodium distachyon*, *Setaria italica* and other

Association of ion content and ion-related transcripts to DM

Regression analysis was employed to define the combination of the explanatory variables that best predicted DM under salt stress conditions. Results showed that Na^+ and *AecHKT1;5* were the strongest predictors of DM under salt stress (Table 5). Clearly, the regression model including these two variables explained 51% of the variability in DM. Among these two explanatory variables which

Fig. 3 Expression analysis of the *AecHKT1;5* gene in **a** root and **b** shoot tissues of *A. cylindrica* tolerant (USL26) and sensitive (K44) genotypes. Bars represent means $\pm$ SE and bars headed by the same letter represent not significantly different at $P < 0.05$

significantly affected salt tolerance, plant Na^+ concentration ($R^2 = 0.82$; $r = -0.90^{**}$) contributed the most towards the variation in DM.

Discussion

Over the past decades, molecular candidates for voltage-gated channels of K^+ , Na^+ and Ca^{2+} ions, as well as ion transporters have been identified and biochemically characterized using model plants and crop species (Arzani and Ashraf 2016; Hasegawa 2013). However, the molecular and physiological identities of transporters involved in high salt tolerance in *A. cylindrica* are still elusive. Byrt et al. (2007) reported that *TaHKT1;5-D* and *TdHKT1;5* are likely candidates for the major QTLs responsible for Na^+ exclusion of in durum wheat (*Nax2*) and in bread wheat (*Kna1*), respectively. However, compelling evidence have been presented recently (Zhu et al. 2016) suggesting that *Nax* loci contributes in the reduction of Na^+ content in xylem by two mechanisms, one that retrieves Na^+ back into the root stele via *HKT1;4* or *HKT1;5*, while the other leading to reduction in the rate of Na^+ loading into the xylem via *SOS1*. HKT transporters can be classified into at

Table 4 Results of analysis of variance for the effects of NaCl treatments (0, 400 mM at 24 h and 400 mM at 7 days) on relative gene expressions of the plant tissues (shoot and root) in the salt-tolerant and salt-sensitive *A. cylindrica* genotypes

Source of variations	Degree of freedom	Mean squares			
Salt stress (S)	2	4.89**	3.54**	2.89*	3.11*
Genotype (G)	1	3.18*	1.76*	3.09*	3.15*
Tissue (T)	1	3.19*	3.65**	1.01	0.09
S × G	2	3.35*	3.34**	4.65**	5.2**
S × T	2	2.36*	3.99**	3.01*	2.23*
G × T	1	3.27*	1.82*	2.92*	4.83**
S × G × T	2	2.32*	0.65	3.97**	1.94*
Residual	36	0.08	0.09	0.10	0.14

*, ** Significant at 0.05 and 0.01 probability levels, respectively

Fig. 4 Expression analysis of the *AecSOS1* gene in **a** root and **b** shoot tissues of *A. cylindrica* tolerant (USL26) and sensitive (K44) genotypes. Bars represent means $\pm$ SE and bars headed by the same letter represent not significantly different at $P < 0.05$

least two subfamilies, class I and class II (Horie et al. 2009). In contrast to the class II subfamily that in general shows Na^+ – K^+ co-transport activity, class I HKT transporters exhibit Na^+ -selective transport with poor K^+ permeability (Horie et al. 2009). *AtHKT1;1*, a class I transporter in *Arabidopsis thaliana*, was demonstrated to function in Na^+ transport (Uozumi et al. 2000); HKT1 transporter also operated as highly selective Na^+ channel (with no permeation for K^+) in *Dionea* glands (Böhm et al.

Fig. 5 Expression analysis of the *AecNHX1* gene in **a** root and **b** shoot tissues of *A. cylindrica* tolerant (USL26) and sensitive (K44) genotypes. Bars represent means $\pm$ SE and bars headed by the same letter represent not significantly different at $P < 0.05$

2015). Very recently, a novel function of *HKT1;5* in mediating Na^+ exclusion in the phloem was shown in rice (Kobayashi et al. 2017). The results of the current study revealed a high identity (over 90%) of *AecHKT1;5* with wheat *HKT1;5* genes, which suggest that is an orthologue of *TaHKT1;5-D*.

This study examined whether or not an interrelationship exists between the explanatory variables and DM in a

Fig. 6 Expression analysis of *AecVP1* gene in **a** root and **b** shoot tissues of *A. cylindrica* tolerant (USL26) and sensitive (K44) genotypes. Bars represent means $\pm$ SE and bars headed by the same letter represent not significantly different at $P < 0.05$

multivariate analysis. Based on our multiple regression analysis, *AecHKT1;5* is the only gene that could significantly contribute to salt tolerance, though its association was lower than that of plant Na^+ concentration (Table 5). Interestingly, it was found that incorporation of *TmHKT1;5-A* into bread wheat that already contains endogenous *TaHKT1;5-D*, can further improve the plant's ability to exclude leaf Na^+ (James et al. 2011). This may also show that salt tolerance in wheat could be further improved by incorporating *HKT1;5* genes from wild relatives, such as *AecHKT1;5*. An argument can be made for

the evolution of *HKT1;5* in *Triticum* spp. and *Aegilops* spp. from a single ancestor by the results obtained from phylogenetic tree analysis (Fig. 2a). Likewise, a non-allelic variation for the *HKT1;5-A* gene has been described in wheat genotypes with different levels of salt tolerance by Byrt et al. (2007). Adding to this line of evidence, in a phylogeny analysis of 115 rice accessions conducted by Platten et al. (2013), seven major and three minor alleles of *OsHKT1;5* have been categorized, one of which represented the highest Na^+ exclusion. The high homology of *AecHKT1;5* and *TaHKT1;5-D* coupled with the location of this gene on wheat 4D chromosome could suggest the assumption that *TaHKT1;5* is originated from the D genome of the common ancestor of wheat species such as *A. tauschii* and *A. cylindrica*.

The high expression of *AecHKT1;5* in roots than shoots observed in the current study was consistent with those of Byrt et al. (2007) and Byrt et al. (2014) who reported that the *TaHKT1;5-D* gene was expressed in the root rather than in the shoot. Zamani Babgohari et al. (2013) also reported that the *HKT1;5* expression followed a genotype- and tissue-specific pattern in wheat and its wild relatives. The *AecHKT1;5* may counteract Na^+ root to shoot transport in a different or complementary way to *SOS1* because, expression level of *AecHKT1;5* in root were higher than *AceSOS1* expression at 24 h NaCl treatment.

The high homology of *AecSOS1* with *TaSOS1* and *AetSOS1*, and that of *AecNHX1* with *TaNHX1* as well as *AecVP1* with *TaVP1* may suggest their close evolutionary relationships in the tribe Triticeae (Fig. 2). In spite of the hypothesis of similar ancestral lineage among the studied genes and those of bread wheat (*T. aestivum* L.), transcriptional and post-transcriptional regulations can be unique for each of the *A. cylindrica* and *T. aestivum* genes. The recent work by Nawaz et al. (2014) provided strong evidence in support to the specific role played by the operation of *SOS1* and *NHX1* genes in the halophytic and glycophytic *Brassicaceae* species.

Maintaining a low Na^+ but a high K^+ in the cell cytosol as well as the coordinated long-distance transport of Na^+ in

Table 5 Results of multiple regression analysis with dry matter (DM) as the dependent variable under salt stress conditions

Independent variable ^a	Unstandardized coefficient (<i>B</i>)	Standard error	Standardized coefficient (β)	Adjusted R square ^b (R^2)	<i>P</i>
Intercept	0.215	0.030			0.0001
<i>AecHKT1;5</i>	0.039	0.021	0.375	0.347	0.0001
Plant Na^+	− 0.022	0.007	0.826	0.819	0.001

$R^2 = 0.51$ and $C_p = 2.1$

^aSum of root and shoot data for the dependent (DM) and independent variables used

^bCalculated by linear regression analysis

the xylem and phloem in plants are the key aspects in plant salt tolerance (Arzani and Ashraf 2016). The role of SOS1 in xylem loading/unloading, Na^+ export by roots, retention in stems, and the differential accumulation/distribution in old leaves may be even overtly manifested in plants with high levels of salt tolerance (Ji et al. 2013; Shi et al. 2000, 2002). However, SOS1 transcripts are also highly abundant at the xylem parenchyma (Shi et al. 2000), and it was shown both theoretically (Shabala 2013) and experimentally (Zhu et al. 2017) that SOS1 mediates xylem Na^+ loading under saline conditions. As we did not separated epidermal and stellar tissues for their PCR analysis, higher SOS1 transcript levels in tolerant genotype may potentially indicate their better ability to deliver Na^+ to the shoot for the purpose of osmotic adjustment (if expressed in the stele). Real time PCR analysis of *AecSOS1* expression revealed the differential expressions not only in the salt-sensitive and salt-tolerant genotypes but also in root and shoot tissues. The higher *AecSOS1* transcripts obtained for roots than for shoots in the two periods of salt stress treatments are consistent with those reported for the glycophytic *AtSOS1* (Shi et al. 2002) and halophytic *SbSOS1* genes (Yadav et al. 2012). In rice, Martinez et al. (2007) also reported salt treatment induced *OsSOS1* mRNA accumulation exclusively in the roots. The results of the current study revealed that *AecSOS1* transcripts were higher in the initial stage (24 h) than at a later stage (7 days) after salt treatment. Likewise, Ramezani et al. (2013) reported that *TaSOS1* transcript levels in wheat were found to be down regulated at a very early stage and up-regulated 24 h after salt treatment. However, Niu et al. (1995) noted that plants increase expression of transporters when they are adapting to new salt conditions, but that once acceptable ion concentrations have been attained under saline conditions they are retained by passive mechanisms.

Na^+ vacuolar sequestration is reportedly essential to prevent the toxic effects of Na^+ in the cytosol, and enhanced function of NHX tonoplast Na^+/H^+ exchangers has been accordingly exhibited to improve salt tolerance in plants (Bassil and Blumwald 2014). In the current study, we observed the remarkable induced expression of *AecNHX1* of the salt tolerant genotype (Fig. 5). These results are in agreement with our ion data of 2-fold Na^+ content in the roots than in the shoots in the 400 mM NaCl treatment of the salt-tolerant genotype. This in turn, indicates the essential role of *AecNHX1* in the sequestration of Na^+ into the root vacuoles to alleviate Na^+ toxicity in stems and leaves. The expression response in the current study was comparable with that reported in wheat (Yousfi et al. 2016), but somewhat more stringent than that of Adem et al. (2014), who found no change in the transcript level of the NHX1 in barley. The expression levels of the

AecNHX1 and *AecVP1* genes followed an identical pattern in both root and shoot tissues. These findings are consistent with the observations reported elsewhere that the expression of *SsNHX1* in *Suaeda salsa* was increased by NaCl treatment in the whole plant including roots and shoots (Ma et al. 2004). It is also believed that the compartmentalization of sodium ions into the vacuole occurs simultaneously in all tissues (Bartels and Dinakar 2013). On the other hand, *NHX1* has been found to be more strongly expressed in the shoots of the halophytic and glycophytic *Brassicaceae* species than in their roots (Nawaz et al. 2014). Our results may extend the findings from others suggesting that co-overexpression of *VP1/NHX1* results in higher salt tolerance levels in plants than either *VP1* or *NHX1* single-gene-overexpression (Shen et al. 2015). The highest expression increments upon 7 d exposure to 400 mM NaCl indicated that the synchronous function of these genes to sequester the excess Na^+ into the vacuole might occur with Na^+ accumulation in the cytosol.

The consistency between the results observed in the sequences and expression of the *AecHKT1;5*, *AecSOS1*, *AecNHX1* and *AecVP1* genes, on one hand, and Na^+ concentrations in the roots and shoots of the contrasting *A. cylindrica* genotypes, on the other, support the critical roles of these genes in salt tolerance. The lower Na^+ concentration in shoot tissues compared to that in root tissues in the salt-tolerant genotype and its significant lower level than in the salt-sensitive genotype are in coordination with the functions of the *AecSOS1* and *AecHKT1;5* genes. These findings are in support of our prior observations that Na^+ exclusion is a vital salt tolerance mechanism in the *A. cylindrica* species (Arabbeigi et al. 2014; Kiani et al. 2015). Colmer et al. (2006) suggested that Na^+ concentration in leaves could be used as an indicator of the relative ability to 'exclude' Na^+ within a species, or even within a genus. A functional link and possible interplay between *AtHKT1;1* and *AtSOS1* to achieve Na^+ (and K^+) homeostasis has been previously suggested (Rus et al. 2004). Our results follow a model in which HKT1 loads Na^+ into the shoot phloem where handover to roots occurs through the downward stream of phloem, whereas SOS1 mediate the transfer of Na^+ from the xylem parenchyma to xylem vessels, preferably in the roots. The coordinated functions of these transporters would eventually regulate the amount of Na^+ that is transferred to the shoots. It seems that despite the crucial role of the *AecSOS1* gene in ion homeostasis, *AecHKT1;5* gene plays a more important role in Na^+ exclusion from shoots to roots than does *SOS1* in the studied species. Similarly, Munns and Tester (2008) reported that both *HKT1;5* and *SOS1* genes had functions in Na^+ recirculation from shoots to roots, by loading Na^+ from the shoots into the phloem and then unloading it into the roots for efflux. Recently, in parallel to our findings,

Zhang et al. (2017) tested the expression patterns of *PtHKT1;5*, *PtSOS1* and *PtNHX1* under low salt stress (25 and 150 mM NaCl) in a halophytic grass *Puccinellia tenuiflora* and suggested that these genes may play a synergistically crucial role in regulating Na⁺ homeostasis. Flowers (2004) argues for the hypothesis that manipulation of a single salt tolerance gene cannot be expected to be effective in improving a trait as complex as salt tolerance. To support his argument, he analyzed 13 species that had been transformed with nearly 40 genes between 1993 and 2003 to show that the majority of the experiments had used rice, tobacco and *Arabidopsis* for transformation. To mitigate this unpromising perspective it is clear that the majority of the over-expressed genes used so far originated from salt-sensitive species such as those listed by Flowers (2004). There is an example of successful introgression of the *T. monococcum* *TmHKT1;5* gene into a susceptible commercial durum wheat variety which led to significant increase in grain yield under saline field conditions, far exceeding the expected yield increase of up to 25% (Munns et al. 2012).

In the current study, a significant negative association was found between Na⁺ content and DM under the 400 mM NaCl stress treatment, indicating the vital role of Na⁺ ion exclusion mechanisms on salt tolerance in *A. cylindrica*. Of great importance, however, is the finding that the root and shoot K⁺ contents were profoundly higher in the salt-tolerant than in the salt-sensitive genotype under the 400 mM NaCl conditions. To date, there is no experimental evidence—to the authors' knowledge—for a significant role of HKT transporters in K⁺ transport in plants. It is clear, however, that not only are transport and sequestration of Na⁺ essential for avoiding the toxic effects of salt, but cytosolic K⁺ maintenance is also important for enhancing salt tolerance in plants, as also highlighted by Pottosin and Shabala (2014). Ion homeostasis under salt stress conditions is crucial in salt tolerance and implicates a network of transport processes controlling uptake, extrusion through the plasma membrane, recirculation of ions through the plant organs and compartmentalization of salts into cell vacuoles, thus regulating the osmotic adjustment and retention of high K⁺/Na⁺ ratios in plant cytosol (Apse and Blumwald 2007; Arzani and Ashraf 2016). Our results of higher potassium contents in the salt tolerant genotype coupled with the genotypically differential upregulation of the *AecHKT1;5* gene in the roots and the presence of conserved domain TrKH (cation transport protein) in this gene might also highlight yet another possible role in K⁺ uptake and transport in plant (Hamamoto et al. 2015). Low concentration of leaf Na⁺ and high K⁺/Na⁺ ratio lead to improved discrimination and preference of K⁺ over Na⁺ in their transport from the root to the shoot, while no effects have been suggested on root Na⁺ concentration as the

likely roles of *Nax2* and *Kna1* (Byrt et al. 2007; James et al. 2006). Our findings, therefore, lend support to the hypothesis that the capacity to discriminate between Na⁺ and K⁺ uptakes and to preferentially exclude Na⁺ and accumulate K⁺ is one of the key salt tolerance mechanisms in the tribe Triticeae (Arzani and Ashraf 2016; Dvorak et al. 1994; Kiani et al. 2015; Shabala et al. 2013). It is clear, hence, that not only are Na⁺ transport and sequestration needed, but cytosolic K⁺ maintenance is also essential for mediating salt tolerance in plants (Anschutz et al. 2014; Pottosin and Shabala 2014; Shabala and Pottosin 2014).

All of these evidences along with the inference of *A. cylindrica* species is the only member of Triticeae tribe coping hyper-saline conditions of shore land of Uremia Salt Lake could be explained on the grounds that polyploidy has the positive roles in the adaptive response to saline conditions (see the review by Arzani and Ashraf (2016) for further details on the effects of polyploidy on salt tolerance), C genome has impacted on salt tolerance (Kiani et al. 2015) and that the saline-habitat genetic resources perform better than normal-habitat genetic resources screened for salt tolerance (Arzani and Ashraf 2016). While this study illustrates neatly the importance of the D-genome originated salt related genes on Na⁺ alleviation in *A. cylindrica* plants, future molecular studies are required to determine the ion homeostasis genes associated with the C genome of this species. Thus, the analysis requires a sequenced genome as a prelude to enhanced analysis and understanding of salt tolerance genes in this grass species.

Conclusions

The results of our experiments suggest a functional link and a likely interplay between *AecHKT1;5* and *AecSOS1* to achieve Na⁺ (and K⁺) homeostasis in *A. cylindrica*. We observed lower Na⁺ and higher K⁺ concentrations in the roots/shoots in addition to higher levels of expression of these genes in the salt-tolerant genotype compared to its salt-sensitive counterpart. The higher Na⁺ concentration in the roots than in the shoots was in agreement with the transcript profiles of *AecHKT1;5* in these tissues. *AecHKT1;5* expression and Na⁺ concentration were identified as the strongest predictors of DM. Therefore, it appears that the involvement of *AecHKT1;5* in Na⁺ sequestration in the root tissue possibly in combination with the activity of Na⁺ exclusion from roots, resulted in salt-tolerant plants ability to retain Na⁺ in their roots and thereby avert it from reaching the photosynthetic tissues. Since about half the variation observed in the DM of the salt-tolerant genotypes can be explained with reference to

the root and shoot Na^+ concentrations and the *AecHKT1;5* gene that is likely to be located in the D genome, future work should focus on genes conferring the salt tolerance in the panel of the C genome of *A. cylindrica*. In this particular species, it appears likely that tolerance to high salt concentration must be dealt with the ability of plant to maintain low Na^+ concentration in the tissues, particularly in the shoots.

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