BIOTECHNOLOGY

Genome-wide analysis of key salinity-tolerance transporter (*HKT1*;5) in wheat and wild wheat relatives (A and D genomes)

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Received: 15 February 2012 / Accepted: 30 October 2012 / Published online: 18 December 2012 / Editor: J. Forster © The Society for In Vitro Biology 2012

Abstract Exclusion of sodium ions from cells is one of the key salinity tolerance mechanisms in plants. The highaffinity cation transporter (HKT1;5) is located in the plasma membrane of the xylem, excluding Na⁺ from the parenchyma cells to reduce Na⁺ concentration. The regulatory mechanism and exact functions of HKT genes from different genotypic backgrounds are relatively obscure. In this study, the expression patterns of HKT1;5 in A and D genomes of wheat were investigated in root and leaf tissues of wild and domesticated genotypes using real-time PCR. In parallel, the K⁺/Na⁺ ratio was measured in salt-tolerant and salt-sensitive cultivars. Promoter analysis were applied to shed light on underlying regulatory mechanism of the HKT1:5 expression. Gene isolation and qPCR confirmed the expression of HKT1;5 in the A and D genomes of wheat ancestors (Triticum boeoticum, AbAb and Aegilops crassa, MMDD, respectively). Interestingly, earlier expression of HKT1;5 was detected in leaves compared with roots in response to salt stress. In addition, the salt-tolerant genotypes expressed *HKT1*:5 before salt-sensitive genotypes. Our results suggest that HKT1;5 expression follows a tissue- and genotypespecific pattern. The highest level of HKT1;5 expression was observed in the leaves of Aegilops, 6 h after being

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School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, Australia e-mail: esmaeil.ebrahimie@adelaide.edu.au subjected to high salt stress (200 mM). Overall, the D genome allele (*HKT1*;5-*D*) showed higher expression than the A genome (*HKT1*;5-*A*) allele when subjected to a high NaCl level. We suggest that the D genome is more effective regarding Na⁺ exclusion. Furthermore, *in silico* promoter analysis showed that *TaHKT1*;5 genes harbor jasmonic acid response elements.

Keywords Salt tolerance $\cdot \operatorname{Na}^+$ transporter \cdot Wheat \cdot Wheat ancestors

Introduction

Approximately one third of irrigated lands are affected by salinity (Demidchik and Tester 2002), therefore understanding the mechanisms for salt tolerance plays a key role in improving crop productivity (Huang et al. 2006). One of the vital tolerance mechanisms in cells is maintaining a low concentration of cvtosolic Na⁺ under stress (Kader et al. 2006), with the plasma membrane being the most likely site for selective regulation of ion transport (Kader et al. 2006). Under high levels of Na⁺, due to similarities in their ion hydration energies and ionic radii, Na⁺ competes with K⁺ for uptake through membrane transport systems (Plett and Moller 2010). Because K^+ plays an essential role in many enzymatic functions, a high Na^+/K^+ ratio retards the function of many enzymes (Kader et al. 2006). The uptake of Na⁺ into cells takes place through multiple Na⁺-permeable cation channels/transporters, such as outward- and inward-rectifying K⁺-selective channels, particularly nonselective cation channels in the plasma membrane (Amtmann and Sanders 1999). Loading of xylem vessels with Na⁺ results in its transportation upward in the plant via the transpiration system (James et al. 2006). This transport triggers ion toxicity when the cytoplasmic concentration of Na⁺ rises above a threshold level (Kader et al. 2006).

Interestingly, the degree of salt tolerance is not similar among different plant tissues; specifically, shoots are generally more sensitive than roots to Na^+ concentrations (James et al. 2006). Salt tolerance, therefore, can be also related to a reduction in the transport of sodium from roots to shoots, and the ability to exclude Na^+ from cells to prevent the accumulation of high Na^+ levels in leaves (James et al. 2006).

High-affinity potassium transporters (*HKTs*) are a large superfamily of transporters in plants, bacteria, and fungi. It has been suggested that these transporters play crucial roles in salinity tolerant via removal of Na⁺ from the xylem during salinity stress (James et al. 2006). In general, little is known about Na⁺ excluding genes in plants, particularly in wild genotypes, or their degree of genetic diversity. In wheat, group I *HKT* genes confer salt tolerance through sodium exclusion mechanisms in leaves (Huang et al. 2006). *TaHKT1;5-D* (*Kna1*) is a specific Na⁺ transporter in *Triticum aestivum* (an allohexaploid, AABBDD genome) that plays the same role as *TmHKT1;5-A* (*Nax2*) in *Triticum monococcum* (diploid, AA genome). While the predicted amino acid identity between *TmHKT1;5-A* and *TaHKT1; 5-D* is 94% (Byrt et al. 2007).

Wild genotypes represent a potential source for discovering significant novel genes and promoters. Furthermore, erosion of genetic diversity has been suggested to be the main cause of salt sensitivity in modern crops (Dvorak and Akhunov 2005). Iran, as the center of the origin and genetic diversity of wheat, presents rich gene pools for wheat and its wild relatives, such as Triticum and Aegilops species (Tabatabaei and Maassoumi 2001). Recent studies have hypothesized that at least some accessions of diploid wheat varieties with the A genome (i.e., *T. boeoticum* and *T. monococcum*) are effective Na⁺ excluders (Shavrukov et al. 2009). It has been reported that *TmHKT1*; 5-A decreases Na⁺ concentrations in leaf blades and sheaths to a greater extent than TmHKT1;4-A (Nax1; James et al. 2006). Additionally, high levels of Na⁺ exclusion and salinity tolerance have been found to be associated with the D genome, such as in Aegilops and bread wheat (Shavrukov et al. 2009). *TaHKT1*;5-D plays a significant role in salt tolerance via Na⁺ exclusion from leaves and controlling xylem loading in roots. (Byrt 2008). Identifying new HKT alleles in wild relatives of wheat may provide an opportunity to achieve higher salt tolerance than is associated with the currently known alleles.

In silico promoter analysis can produce valuable information about the function and response of a gene to various cues. Regulatory elements on promoter region of a gene can be more determinant in conferring gene function than its protein structure (Deihimi et al. 2012). We believe that the superiority of an *HKT* homologue to other homologues can actually be related to its superior promoter structure, rather than its gene structure. Regarding the unknown role of *HKT* promoters, it appears that promoter analysis of *HKT* genes may be able to assist in solving many unanswered questions. The regulatory elements in promoters, such as transcription factor binding sites (TFBs; or *cis*-regulatory elements), are organized into distinct modules that control expression of many genes in systems biology. Thus, the identification of regulatory elements can be a first step in recognition of gene expression patterns (Mariño-Ramírez et al. 2009).

As the *HKT* gene family encodes one of the most significant Na⁺ transporters among plants, increasing the expression of key family members has been considered as a viable strategy for improving salinity tolerance (Plett et al. 2010). However, the physiological functions, gene networks, and signaling pathways related to *HKT* transporters have not been completely elucidated (Hauser and Horie 2010). In addition, the similarities and differences in the expression patterns of *HKT* genes in different tissues (such as shoots and roots) are relatively unknown. Moreover, while *HKT* genes such as *HKT1*;5 can originate from the D, B, or A genomes (Byrt 2008), the effects of the genomic background on the expression and activity of *HKTs* has not been thoroughly investigated.

In the present study, a comprehensive expression analysis of HKT1;5 under different salinity concentrations using saltsensitive and salt-tolerant varieties of bread wheat (*T. aestivum*) and wild wheat ancestors (*T. boeoticum* and *Aegilops crassa*) with different genomic backgrounds (A or D genomes, respectively) were carried out. In addition, because the main role of HKT1;5 is to prevent transport of Na⁺ from shoots to roots, the K⁺/Na⁺ ratio was measured in the leaves of salt-sensitive and salt-tolerant wheat cultivars under different salt stress concentrations. The promoter region of HKT1;5-D (*T. aestivum*) and HKT1;5-A (*T. monococcum*) were also analyzed *in silico*.

Materials and Methods

Plant materials. Seeds of wheat (*T. aestivum*) cultivars Mahuti and Alamut and wild wheat ancestors (*Triticum boeoticum* or *T. monococcum* subsp *aegilopoides* and *A. crassa*) were provided by Shiraz and Ilam Universities, Iran. Mahuti is a salt-tolerant cultivar, while Alamut is saltsensitive. The seeds were soaked in water for 24 h at 4°C on moist filter paper in wrapped Petri dishes. The seedlings were then transferred to hydroponic tanks containing halfstrength Hoagland's solution, pH 6.0 (Genc et al. 2007). The solutions were changed weekly. The plants were grown in a controlled glasshouse with day/night temperatures of 25/ 21 C under 16 h of light (300 μ molm⁻²s⁻¹).

Salinity stress experiment. For each of the four genotypes (*T. aestivum* cv. Mahuti and Alamut, *T. boeoticum*, and *A. crassa*), a salinity experiment was conducted based on a

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randomized complete block design (RCBD) with four NaCl concentrations as treatments, six sampling times, and with two biological replications. Seventeen days after germination, the plants were stressed by adding NaCl at concentrations of 0, 50, 100, or 200 mM. Sampling of leaves was carried out at 0 h (before stress), and at 3, 6, 10, 24, and 72 h after initiation of the stress treatments in all genotypes for all treatments. In addition, sampling of root tissue was performed 6, 10, 24, and 72 h after the initiation of salt stress from salt-tolerant (cv. Mahuti) and salt-sensitive (cv. Alamut) wheat cv. Nonstressed control plants of each genotype were grown concurrently and harvested at the same time as a control. Leaf and root tissues were collected during sampling, snap frozen in liquid nitrogen, and immediately stored at -80° C until further analysis was conducted.

Isolation of partial HKT1;5-A and HKT1;5-D sequences. As *HKT1*;5 sequence from *T. boeoticum* (*T. monococcum* subsp aegilopoides) and Aegilops had not been published, in order to conduct real-time PCR experiments, it was necessary to first isolate HKT1;5 alleles and confirm its expression in the above mentioned genotypes. Thus, primers for the amplification of TbHKT1;5-A (the HKT1;5 isoform in T. boeoti*cum*) were designed based on the protein coding sequence (CDS) of this gene in T. monococcum and for AeHKT1;5-D (HKT1;5 isoform in Aegilops) according to the CDS of this gene in bread wheat (TaHKT1;5-D) available in GenBank, using Vector NTI Suite 9. The following primer sequences were used for amplification of HKT1;5-A and HKT1;5-D: forward, 5'-CTATCACGTGGTGGTGCACC-3', and reverse, 5'-CGTGCGGCATGACTAGGAGCA-3'. These primers could amplify the partial CDS of HKT1;5 in T. boeoticum and Aegilops. Then, RNA was isolated from the leaves of T. boeoticum (AbAb) and A. crassa (MMDD) for RT-PCR using RNX[™] (-Plus) buffer followed by DNase digestion; RNA purification and synthesis of first-strand cDNA from the total RNA were performed according to the manufacturer's instructions (Fermentas, Ontario, Canada). cDNA was amplified using RT-PCR Master Mix in a Bioer thermocycler. The PCR conditions were as follow: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 59°C for 45 s, 2 min at 72°C, and final extension of 10 min at 72°C. PCR product of the partial cDNA sequences from A. crassa and T. boeoticum were sequence verified by Macrogen (http://www.macrogen.com/).

RNA extraction and cDNA synthesis for real-time RT-PCR. After confirming expression of the *HKT1*;5 gene in all four genotypes, Mahuti and Alamut, *T. boeoticum*, and *A. crassa*, qRT-PCR expression analysis was performed by isolating total RNA from the leaf and root tissues and synthesizing cDNA according to the above mentioned method. For quantitative real-time PCR, 2.5 μ l of cDNA was used as a template. The cDNA was amplified using SYBR Green PCR Master Mix (Takara SYBR premix EX-Tag II) in a Bioer thermocycler (Applied Bioer, LineGeneK, Hangzhou, China). Elongation factor α (elf- α) was selected as a reference gene to normalize the expression data for HKT1;5. The following primer sequences were used for elf- α : 5'-TTTCACTCTTGGAGTGAAGCAGAT-3' and 5'-GACCTCCTTGACAATTTCTTCATAA-3'. To analyze the expression of HKT1;5-D and HKT1;5-A, the forward and reverse primers sequences were: 5'-CTATCAC GTGGTGGTGCACC-3' and 5'-ACGGAGAAGGTGTG CAGGCTG-3'. The PCR conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 59°C for 15 s, and 72°C for 30 s. Each experiment was repeated independently twice for each genotype as a biological replicate. Standard determination curves were generated using serial dilutions of 10,000, 1,000, 100, and 10 ng cDNA in each well for every experiment. Two independent replicates were performed for each genotype.

Real-time RT-PCR data analysis. Normalization of the target genes (*HKT1*;5-*A* and *HKT1*;5-*D*) was carried out based on reference to an endogenous standard (*elf*- α). The Pfaffl formula (ratio= $2^{-\Delta \Delta Ct}$) was used to calculate relative expression (Pfaffl 2001), where $\Delta \Delta Ct = (\Delta Ct \text{ sample} - \Delta Ct \text{ control})$; ΔCt sample=(ΔCt target- ΔCt ref) for all sampling times and NaCl concentrations; and ΔCt control= (ΔCt target- ΔCt ref).

Statistical analysis. The experiments were conducted based on a RCBD with two biological replications, four NaCl concentrations, and four genotypes per treatment. Six different times were applied as blocks following the initiation of the stress treatment. To compare the differences in expression levels of *HKT1*;5 between genotypes, a 2×2 paired *t* test at the 0.05 significance level was applied using MINI-TAB 14 software for the data from leaf and root tissues.

Sodium and potassium analysis. The K^+/Na^+ ratio is a reliable index of salt tolerance in plants (Rush and Epstein 1976). This index was determined in leaf tissues from the salt-tolerant (Mahuti) and salt-sensitive (Alamut) bread wheat cv. at four salinity concentrations, 4 wk after the initiation of stress using the Flame-photometry method. This compared the salt-tolerant and salt-sensitive genotypes with respect to the association of *HKT* genes with the specific ability of the plant to exclude Na⁺ from leaves and maintain a stable K⁺/Na⁺ ratio.

In silico promoter analysis to identify major HKT1;5 elements Triticum genotypes. As there is currently no available database for promoter identification in wheat or wild wheat relatives, a study published by Byrt (2008) was used in this case. The putative promoter sequences of *HKT1*;5 genes of *T. aestivum* and *T. monococcum* were compared with known *cis*-regulatory elements in the collection of the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al. 2002). The *cis*-regulatory elements were noted and counted for each promoter.

Results and Discussion

Isolation of partial HKT1:5 sequences. Although bread wheat, T. aestivum, is an allohexaploid with an AABBDD genome, the HKT1;5-A gene is not present in this species (Byrt et al. 2007). However, as the sequence of the region where the TaHKT1;5-D allele is located is highly similar to TaHKT1;5-B, it was possible that the B genome isoform could be amplified by PCR (Byrt 2008). Primers designed to amplify HKT1;5 in two cultivars of bread wheat (Mahuti and Alamut) was performed based on the highly conserved coding region, but sequencing the cDNA products showed that only the TaHKT1;5-D allele was amplified. However, we were successfully able to amplify, for the first time, partial CDS of an HKT1;5 gene from T. boeoticum (T. monococcum subsp aegilopoides) and A. crassa. Analysis of the results showed that HKT1;5-A and HKT1;5-D are present in these genotypes, respectively, and are expressed under salt stress in leaf tissues. The sequences amplified for HKT1;5-like genes from T. boeoticum (T. monococcum subsp aegilopoides), A. crassa, and T. Aestivum cv. Mahuti are available from GenBank (accession numbers JQ677810, JQ677811, and JQ677812, respectively).

Expression of TaHKT1;5-D in Mahuti leaf. In leaf tissue from Mahuti plants (the salt-tolerant bread wheat cv.), the expression of TaHKT1;5-D varied over time and at different NaCl concentrations (Fig. 1A). Mean separation values indicated that the highest expression of TaHKT1;5-D (approximately two times higher than control plants not subjected to salt stress) occurred at a concentration of 200 mM Na⁺ (Fig. 1A). The strongest upregulation of the TaHKT1;5-D transcripts was observed in this cultivar 3 h after the initiation of stress treatment compared with other concentrations tested. The primary induction of some HKT transporters can result from a number of factors, such as posttranscriptional or protein conformational changes in salt-tolerant cv., including Mahuti (Kader et al. 2006). It appears that due to continuing the application of salt stress, other mechanisms may have been initiated in the leaf; thus, the expression of the TaHKT1;5-D gene was decreased from 10 to 72 h.

t test analyses confirmed that there was no significant difference in *HKT1*;5 expression prior to salinity stress (at 0 h before stress) at p=0.05. Although the level of

expression at 0 h was very low so that it was near to control (0 mM treatment), Mahuti leaves exhibited the strongest expression at that time, though it was not significant (data not shown). This result showed that salt stress induced HKT1;5 in all genotypes.

Expression of TaHKT1;5-D in Mahuti root. The strongest induction of *TaHKT1;5-D* was nine times higher than in controls of Mahuti root tissue (Fig. 1*B*), at 100 mM, occurring 72 h after initiation of the salt stress treatment. The later induction of *TaHKT1;5-D* in roots likely causes Na⁺ to increase in upper parts of the plant, such as the leaves. Thus, upregulation of *TaHKT1;5-D* was induced earlier in leaves. On the other hand, since the induction of *HKT1;5* in the Mahuti roots was more severe than leaves, it seems this gene may act to exclude Na⁺ from absorption by the roots preferentially.

Expression of TaHKT1;5-D in Alamut leaf. The highest TaHKT1;5-D transcript levels were observed in leaves of the salt-sensitive cv. Alamut at 200 mM, which were 35 times higher than in controls after 10 h of treatment, with the higher salt concentrations showing the greatest response (Fig. 1C). Studies have indicated that in salt-sensitive rice cultivars, K⁺-selective channels (in addition to nonselective cation channels) also contribute to Na⁺ uptake (Kader and Lindberg 2005). It may be that the upregulation of TaHKT1;5-D in the salt-sensitive cv. could also induce strong induction of some Na⁺ influx transporters that led to an increase of the Na⁺ concentration in the plant cells, especially the xylem, resulting in greater toxicity to the plant. The reduced expression after 72 h could be related to the toxic effects of the salt treatment on the plant. Alamut exhibited stronger TaHKT1;5-D expression than the salttolerant, but this difference was not significant (Table 1).

Expression of TaHKT1;5-D in Alamut root. The peak induction of *TaHKT1;5-D* was approximately two and a half times higher than in controls not significantly (Fig. 1*D*) In this saltsensitive cv., Na⁺ uptake occurs via two pathways (Kader and Lindberg 2005). This process can induce root *TaHKT1;5-D* activity under low salt conditions, such as at a concentration of 50 mM NaCl. It is probable that the lower *TaHKT1;5-D* induction at higher NaCl concentrations, including 200 mM, in roots of the salt-sensitive cv. Alamut results in higher Na⁺ concentrations in leaves. Thus, upregulation occurred under high Na⁺ conditions in leaves (Table 2).

According to our result, this gene was upregulated late (72 h) in roots, and this late induction provided conditions resulting in earlier and higher *TaHKT1*;5-*D* expression in leaves. Previous studies have suggested that the *HKT* expression pattern is poorly understood and differs among tissues and genotypes (James et al. 2011). Higher expression of *TaHKT1*;5-*D* was observed in the salt-tolerant cv. Mahuti

Figure 1. Expression analysis of *HKT1*;5 by real-time RT-PCR; amplification of RNA from leaf and root tissues of wheat cv. Mahuti (salt tolerant) and Alamut (salt sensitive) and leaves of species *T. boeoticum* and *A. crassa* (*A*–*F*). Sampling was carried out after 3, 6, 10, 24, and 72 h of growing plants under salt stress conditions (50, 100, and 200 mM NaCl). Real-time data were normalized in relation to 0 mM NaCl.



than the salt-sensitive cv. Alamut in root tissue, as confirmed by a *t test* (Table 1). This result confirmed that *HKT1*;5 exhibited higher expression in the D genome. Although the expression of this gene in *T. boeoticum (HKT1*; *5-A*) was higher than in Alamut and Mahuti (including *HKT1*;5-D), Mahuti is an exceptional cultivar regarding salt tolerance, and Alamut is a salt-sensitive cv.; thus, this result is reasonable.

Comparison of HKT1;5 expression patterns between leaf and root tissues. HKT1;5 is a remarkable gene for decreasing Na⁺ concentration in plants. When comparing the expression

of *TaHKT1*;5-*D* in the different tissues, it was found to be higher in roots than in leaves in the salt-tolerant cv. Mahuti (p=0.05; Table 1). Therefore, it seems that the main activity for *HKT1*;5 occurs in the roots of this cultivar which may be related to its exceptional activity with respect to salt tolerance as based on other studies (Ghavami et al. 2004), or is due to varying levels of expression in different tissues (James et al. 2011). Analysis of the K⁺/Na⁺ ratio re-confirmed this result where this index was lower in Mahuti leaves than Alamut leaves. Consequently, the Na⁺ concentration was probably higher in the leaves of Mahuti cultivar than its roots; this may be because of tissue- specificity activity of the *HKT1*;5.

Table 1. Comparison of differences in the expression of <i>HKT1</i> ;5 (A and D genomes) in wheat genotypes	Tissue	Genotypes	Aegilops crassa	Triticum boeoticum	Mahuti	Alamut
wheat genotypes	Leaf	Alamut (<i>Triticum aestivum</i>) Mahuti (<i>T. aestivum</i>)	0.048* 0.032*	0.283 ns 0.039*	0.107 ns	
		T. boeoticum A. Crassa	0.076 ns	0.057		
Using paired <i>t</i> tests, differences in the expression of <i>HKT1</i> ;5	Root	Alamut Mahuti			0.035*	
were compared 2 by 2 for all genotypes <i>ns</i> nonsignificant * <i>p</i> =0.05, level of significance	Root and leaf K ⁺ /Na ⁺	Alamut Mahuti Alamut			0.149 ns 0.023* 0.018*	

Table 2.	Elements	present in the	promoter region	of TaHKT1;5	and TmHKT1;5	according	to the Plant	CARE database
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Cis-element	TmHKT1;5	TaHKT1;5	Function
CAAT box	12	7	Common cis-acting element in promoter and enhancer regions
Circadian	2	2	Cis-acting regulatory element involved in circadian control
CATT motif	2	2	Part of a light-responsive element
AE box	0	1	Part of a module for light response
G box	2	1	Cis-acting regulatory element involved in light responsiveness
MRE	0	1	Myb binding site involved in light responsiveness
LTR	2	2	Cis-acting element involved in low-temperature responsiveness
TC-rich repeats	1	1	Cis-acting element involved in defense and stress responsiveness
ARE	1	0	Cis-acting regulatory element essential for the anaerobic induction
Box 4	1	1	Part of a conserved DNA module involved in light responsiveness
Box I	1	1	Light-responsive element
ERE	1	1	Ethylene-responsive element
HSE	1	2	Cis-acting element involved in heat stress responsiveness
Skn-1_motif	1	1	Cis-acting regulatory element required for endosperm expression
TATA box	22	18	Core promoter element approximately -30 of the transcription start
ACE	1	1	Cis-acting element involved in light responsiveness
I box	2	0	Part of a light-responsive element
MNF1	1	1	Light-responsive element
O ₂ site	1	0	Cis-acting regulatory element involved in zein metabolism regulation
ATCT motif	1	1	Part of a conserved DNA module involved in light responsiveness
CCAAT box	1	1	MybHv1 binding site
CGTCA motif	2	1	Cis-acting regulatory element involved in the MeJA-responsiveness
Sp1	2	3	Light-responsive element
TGA element	1	1	Auxin-responsive element
TGACG motif	2	1	Cis-acting regulatory element involved in the MeJA responsiveness
GARE motif	1	1	Gibberellin-responsive element
CCGTCC box	0	1	Cis-acting regulatory element related to meristem-specific activation
A box	0	1	Cis-acting regulatory element

For Alamut (salt-sensitive wheat), on the other hand, the expression of *TaHKT1*;5-*D* in the leaves was not significantly different to the roots (p=0.05; Table 1), except at the 10 h time-point and high salt treatment where expression in Alamut leaves was very high, far exceeding what was observed for Mahuti leaf tissues. In contrast, in root tissue of Mahuti (tolerant cultivar), the expression of this gene was higher than sensitive cultivar (Alamut) (Fig. 1*A*–*D*) This supports a previous conclusion that *HKT* gene expression pattern exhibits different activity levels in diverse genotypes (James et al. 2011).

Expression of TbHKT1;5-A in T. boeoticum leaves. HKT1;5- A in *T. boeoticum* reduces Na⁺ concentrations in sheaths and blades of leaves and, thus, leads to increases in K⁺ concentrations in leaves and induces Na⁺ removal from the xylem (Byrt 2008). It has been documented that *HKT1;5-A* is not present in bread wheat (Byrt et al. 2007). In this study, the strongest upregulation of *TbHKT1;5-A* at 100 mM NaCl was

approximately 50 times higher than control levels (Fig. 1E). Although previous studies have shown that TmHKT1;5-A (in *T. monococcum*) removes Na^+ from the xylem only in the roots (James et al. 2006), the present study shows that TbHKT1;5-A can be highly expressed in leaf tissue of T. boeoticum. The expression of TbHKT1;5-A was low for all time-points of the 200 mM NaCl treatment. Based on our data, there are two likely reasons: TbHKT1;5-A is not effective under high Na⁺ concentrations, and *TbHKT1*;5-A expression is different among various tissues (James et al. 2011). For example, upregulation of this gene in roots or shoots may prevent Na⁺ uptake in leaves, resulting in a decrease in TbHKT1;5-A expression in leaves. However, further verification will require further study; we assume that the first explanation is more probable. Other studies have shown that wheat lines carrying HKT1;5-A exhibit a 3.6 times greater leaf K^+/Na^+ ratio, and their leaf Na^+ levels are approximately 2.5 times lower than those of lines without HKT1;5-A (Huang et al. 2006). Clearly, the Na⁺

reduction associated with HKT1;5-A is lower compared with HKT1;5-D. Thus, the effect of HKT1;5-D may be stronger than that of HKT1;5-A with respect to the enhancing salt tolerance. HKT1;5-A decreases the Na⁺ loading and uptake from roots to shoots (Byrt et al. 2007). However, in 2011, James showed that TmHKT1;4-A is more effective than TmHKT1;5-A at reducing Na⁺ in leaf tissue. The upregulation of HKT1;5-A was higher in T. boeoticum than the salt-tolerant cv. Mahuti, which was confirmed by a *t test* (Table 1). However, the stronger induction of this gene in T. boeoticum was

Alamut based on a *t test*. The relative expression of *TbHKT1*;5-*A* was higher 10 h after the induction of stress than at other times. This expression is apparently lower than that of the D genome (*HKT1*;5-*D*). It appears that *HKT1*;5-*D* carries regulatory elements that allow a rapid response to salinity stress.

not significantly different than that in the salt-sensitive cv.

Expression of AeHKT1;5-D in A. crassa leaves. Real-time PCR analysis showed that AeHKT1;5-D expression was maximally upregulated in A. crassa leaf tissue at 200 mM NaCl, to levels 250 times higher than control levels (Fig. 1F) The three highest expression levels for HKT1;5 was detected in A. crassa at 200, 100, and 50 mM NaCl, respectively. According to real-time PCR, HKT1;5 exhibited higher activity in this wild species compared with cultivated plants under all stress conditions. The D genome is known to contain many genes conferring salt tolerance (Gorham et al. 1990), thus providing useful genetic variation related to Na⁺ exclusion in each wild species and subspecies (such as Aegilops). There was high expression of AeHKT1;5-D 6 h after stress was initiated. Thus, the earlier and stronger upregulation of AeHKT1;5-D gives raises the question whether the high salinity tolerance of Aegilops led to a high Na^+ concentration in its leaves that forces strong *AeHKT1*; 5-D upregulation. We proposed two possibilities: firstly, AeHKT1;5-D is not expressed in lower Aegilops tissues, such as roots and shoots. Second, Na⁺ accumulates in Aegilops leaf vacuoles via AeHKT1;5-D or other transporters. Although this function of HKT genes has not yet been observed (as the role of these genes is relatively unknown), this function may occur via AeHKT1;5-D. We prefer that the first hypothesis because Byrt showed that the TaHKT1;5-D gene is expressed in Chinese Spring roots, but not shoots, and thus, this gene can show tissue specific expression. The fact that the highest expression of HKT1;5 was observed in A. crassa compared with other wheat species studies (Table 1) presents new options for improving salinity tolerance in wheat. We previously showed that TaSOS1 and TaSOS4, as two important genes in salt tolerance, have higher expression in A. crassa comparing to the other genotypes (Ramezani et al. 2012). Here, t test analyses confirmed the expression of *AeHKT1*;5-*D* was higher in *Aegilops* than the salt-sensitive and salt-tolerant cv. Alamut and Mahuti at p=0.05 while the increase in the expression of *HKT1*;5 between *Aegilops* and *T*. *boeoticum* was not significant (Table 1).

 K^+/Na^+ ratio. Although many researchers agree that K^+/Na^+ Na⁺ homeostasis is key in plant salinity tolerance (Genc et al. 2007), our results indicated that the Na^+ content in the leaves of salt-tolerant cv. (Mahuti) and salt-sensitive cv. (Alamut) increased due to increasing the NaCl concentration in parallel with decreasing the K^+ level in both cultivars (Fig. 2). Unexpectedly, tolerant wheat showed a lower $K^+/$ Na^+ ratio than the salt-sensitive cultivar. t test results showed that the K^+/Na^+ ratio was significant in Mahuti and Alamut at p=0.05 (Table 1). Although Mahuti exhibits high tolerance to salinity, this cultivar presents a low K^+/Na^+ ratio compared with the other cultivars. Thus, it is possible that HKT transporters, or possibly genes involved in the modification of the K^+/Na^+ ratio are not particularly effective in Mahuti leaves. In agreement with this finding, some reports have noted that Na⁺ exclusion and tissue tolerance vary independently (Genc et al. 2007). In the present study, because the expression of TaHKT1;5-D was lower in the leaves of the salt-tolerant cv. Mahuti than in the salt-sensitive cv.

It should be noted that TaHKT1;5-D expression and K^+/Na^+ were also low in the Mahuti leaves than Alamut. In contrast, in Mahuti, the most significant expression for HKT1;5 occurred in the roots. We believe that Mahuti is one



Figure 2. The K^+/Na^+ ratio of dry leaf tissues of wheat cv. Mahuti (salt tolerant) and Alamut (salt sensitive) under different concentrations of NaCl (0–control, 50, 100, and 200 mM).

Figure 3. The sequences and the predicted ciselements of the putative promoter region of TaHKT1;5-D in T. aestivum (A) and TmHKT1;5-A in T. monococcum (B). The various colored sequences are related to different putative cis-elements in the promoter regions that are identified by PlantCARE database. HSE heat stress responsiveness, LTR lowtemperature responsiveness, MYB light responsiveness, MeJA MeJA responsiveness, auxin, gibberellin, TC-rich, and ethylene response elements.

	-	TGCAGATGTT ACGTCTACAA	CGCATACACT GCGTATGTGA	CAACCATAAG GTTGGTATT <mark>C</mark>	AATGCATGCA TTACGTACGT	CACACACA <mark>CT</mark> GTGTGTGTGA	CCTACTAAAT GGATGATTTA	GCACATCG <mark>CC</mark> CGTGTAGCGG	A
	+	LTR GAAAGGCCTG CTTTCCGGAC	AAATGAATGC TTTA <mark>CTTACG</mark>	HSE AAGAAAATGC TTCTTTTACG	GACCACCAGT CTGGTGGTCA	GTCAAGTCTA CAGTTCAGAT	GAACTTGAAC CTTGAACTTG	CCTGGTGGGT GGACCACCCA	
2	+	TATTTCCATC ATAAAGGTAG	ACAAGCAACC TGTTCGTTGG	TAACCATTTG ATTGGTAAAC	AGTTACCCTC TCAATGGGAG	AGCTCGCTAT TCGAGCGATA	G <mark>CCAACATTA</mark> CGGTTGTAAT Ethylene	ATTAACAATA TAATTGTTAT & HSE	
3	+	GCAAACTTGT CGTTTGAACA	TTCAC <mark>DAWA</mark> T AAGTGATATA	ТТАТСА <mark>ТААТ</mark> ААТА G ТАТТА	<mark>АТАА</mark> ТТТСТА ТАТТАААGAT	G <mark>ATATAT</mark> AGT CTATATATCA	СА <mark>АААТААТТ</mark> GTTTTATTAA	TCAAATATTT AGTTTATAAA	
	+	ATGAATGAAG TACTTACTTC	GGAGCACCAT CCTCGT <mark>GGTA</mark>	GCTATGG <mark>PAA</mark> CGATACCATT	TATAGATGCA ATATCTACGT	TTACTTTGGA AATGAAACC <mark>T</mark>	GGAGCTAGTT CCTCGATCAA	GTAGGTAGCT CATCCATCGA	
	+	CTAAACATGT GATTTGTACA	ATTTTCATAC TAAAAGTATG	ТТТСТААТТТ АААGАТТААА	TTGGCATGTA AACCGTACAT	TTTTCTATCT AAAAGATAGA	TCTATGTG <mark>PA</mark> AGATACACAT	TATCTTTTTC ATAGAAAAAG LTR	
	+	GGTCTGTATG CCAGACAT <mark>AC</mark>	TATATGTG <mark>TA</mark> ATAT <mark>ACACAT</mark>	TATGTACTTT ATACATGAAA	TCGTTGCACT AGCAACGTGA	TAGTACAACA ATCATGTTGT MY	CAAGTCAGGT GTTCAGTCCA B	GGTTGCCCTG CCAACGGGAC	
	+	AG <mark>CTCC</mark> TTCT TCGAGGAAGA	CTTCATGATG GAAGTACTAC MYB	CCACGCTCAC GGTGCGAGTG	ACCCTACGAT TGGGATGCTA	ACATATC <mark>CAA</mark> TGTATAGGTT	CGGAGCGGGG GCCTCGCCCC	CATCGCACCC GTAGCGTGGG	
	+	GGTGGGCAC <mark>C</mark> C <mark>CACCCGTGG</mark>	AACTGACTCT TTGACTGAGA	TGTTCGTTAC ACAAGCAATG	CGGTGATACG GCCACTATGC	G <mark>ACGTGGA</mark> AC CTGCACCTTG	TTATCACTCA AATAGTGAGT	CCCGCAAAAA GGGCGTTTTT MeJA & TC rich	
	+	AAAAAGTTAT TTTTTCAATA	CACTCGATTC GTGAGCTAAG	CATTGTTTCT GTAACAAAGA	TCCACAAGTC AGGTGTTCAG	TGCTCTCTTG ACGAGAGAAC	TAGGAGTACC AT <mark>CCTC</mark> ATGG	TA <mark>ATTTTCGT</mark> ATTAAAA <mark>SCA</mark>	
1	+	CATATGATAT GIATACTATA	GCCTCGCAAA CGGAGCGTTT	AAAGATATGC TTTCTATACG	CTCCCACGAG GAG <mark>GGTGC</mark> TC	CTCCCATTGT GAGGGTAACA	GCGCTAGCTT CGCGATCGAA	TTGCGATTAG AAC <mark>GCTAATC</mark>	
1	+	ATTCAGTAAT <mark>TAA</mark> GTCATTA	TAAGACAC	ATTACAGCAA ATTACAGCAA Auxin	CGTCCCTCAT	AAGCAACATG TTCGTTGTAC	GAAAAAGAAA CTTTTTCTTT	A TTAGAGATTT AATCTCTAAA	
	+	TCTTTGTAGT AGAAACATCA	GGCAGGCAAA	GTCTAGCATT CAGATCGTAA Gibberellin	TTTGCGTCCA AAACGCAGGT	CCCCCCTTTT GGGGGGGAAAA	TTGGG <mark>TATA</mark> A AACCCATATI	A TAATCCATTA ATTAGGTAAT	
	+	GTCTCTGATT CAGAGAC <mark>TAA</mark>	GCCTCCAACA CGGAGGTTGT	AAACAGACCA TTTGTCTGGT	AGAAGTCTCT TCTTCAGAGA	ACACAACTTA TGTGTTGAAT	CAGTAGA GTCATCT		
+		AGATGTTCGC ICTACAAGCG	ATACACTCAA TATGTGAGTT	CCCTAAGAAT GGGATTCTTA	GCCTGCACAC CGGACGTGTG LTR	ACACACACAC TGTGTGTGTG	ACACACACAC TGTGTGTGTGTG	ACACACACAC TGTGTGTGTGTG	B
4 - 4 -	+ 1	AGATGTTCGC ICTACAAGCG ACACACACAC IGTGTGTGTGTG	ATACACTCAA TATGTGAGTT A <mark>CTCC</mark> TACTA TGAGGATGAT	CCCTAAGAAT GGGATTCTTA AAGGCACATC TTCCGTGTAG	GCCTGCACAC CGGACGTGTG LTR GCCGAAAGGC CGGCTTTCCG	ACACACACAC TGTGTGTGTGTG CT <mark>CAAAT</mark> GAA GAGTTTACTT	acacacacac tgtgtgtgtgtg tgcaagaaaa acgttctttt TC-rich	ACACACACAC TGTGTGTGTGTG CACGACCATC GTGCTGGTAG	B
+ - +		AGATGTTCGC ICTACAAGCG ACACACACACC IGTGTGTGTGTG AATGTCAAG <mark>T</mark> ITACAGTTCA	АТАСАСТСАА ТАТСТАСТАСТА АСТССТАСТА ТGAGGATGAT СТАGAACTTG GATCTTGAAC	CCCTAAGAAT GGGATTCTTA AAGGCACATC TTCCGTGTAG AATCCTGGTG TTAGGACCAC	GCCTGCACAC CGGACGTGTG LTR GCCGAAAGGC CGGCTTTCCG GGTTATTTCC CCAATAAAGG	ACACACACAC TGTGTGTGTG CTCAAATGAA GAGTTTACTT ATCACAAACA TAGTGTTTGT	ACACACACAC TGTGTGTGTG TGCAAGAAAA ACGTTCTTTT TC-rich AAGAAACCAT TTCTTTGCTA	ACACACACAC TGTGTGTGTGTG GTGCTGGTAG TTGAGTTACC AACTCAATGG	B
+ - + - + -	H 1 H - H - H - H - H - H - H - H - H -	AGATGTTCGC TCTACAAGCG ACACACACACA TGTGTGTGTGTG AATGTCAAG <mark>T</mark> TTACAGTTCA CTCAGTTCGC GAGTCAAGCG	АТАСАСТСАА ТАТСТАСТА АСТССТАСТА ТСАСБАТСАТА СТАСБАСТТС САТСТТСААС ТАТСССААС АТАСССТАСТА АТАСССТАСТА АТАСССТАСТА АТАСССТАСТА АТАСССААС НЕСКАТА	CCCTAAGAAT GGGATTCTTA AAGGCACATC TTCCGTGTAG AATCCTGGTG TTAGGACCAC AATTAATGA AATTAATGT Ethylene	GCCTGCACAC CGGACGTGTG LTR GCCGAAAGGC CGGCTTTCCG GGTTATTTCC CCAATAAAGG ATAGCAAACT TATCGTTTGA	АСАСАСАСАС ТGTGTGTGTG CT <mark>САААТ</mark> GAA GAGTTTACTT АТСАСАААСА ТAGTGTTTGT TGTTTCATTA АСАААGTAAT	ACACACACAC TGTGTGTGTGT ACGTTCTTTT TC-rich AAGAAACCAT TTCTTTGCTA TATATACAGTA	АСАСАСАСАС ТGTGTGTGTGTG GTGCTGGTAG TTGAGTTACC ААСТСААТGG ААТАТААТТТ ТТАТАТТААА	B
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4 - 4 - 4 - 4 - 4 - 4 - 4		AGATGTTCGC TCTACAAGCG ACACACACAC TGTGTGTGTGTG TTACAGTTCA CTCAGTTCGC GAGTCAAGCG CTAAATATAT GATTTATATA GCATTACTTT CGTAATGAAA GTATTTTCTA CATAAAAGAT	АТАСАСТСАА ТАТСТАСТАСТА ТСАССТАСТА СТАССАСТАСТА СТАСААСТТС СТАСААСТТС АТАТСССААСА АТАСССТАСТА АСССААСА АСССААСТА ССТССТАТСА ССТССТССАТ ССТССТАТСА	СССТААGААТ GGGATTCTTA ААGGCACATC TTCCGTGTAG AATCCTGGTG TAGGACCAC TAATTAACA AATTAATTAA AATTAATTAA CAACATCCAT GTTGTAGGTA CAACATCCAT GTTATACTAT GTTATACTAT CAACATCCAT	GCCTGCACAC CGGACGTGTG LTR GCCGAAAGGC CGGCTTTCCG GGTTATTTCC CCAATAAAGG ATAGCAAACT TATCGTTTGA TTTATGAACC AAATACTTGG GCTCTAAACA CGAGATTTGT TTCAGGATTC AAGTCCTAAG	АСАСАСАСАС ТСТСАЛАТСАА САСТТТАСТТ АТСАСАЛАСА ТАСТСТТТСАТТА СТАСТСТТСАТТА АСАЛАСТААТ ААСССАСССС ТСТАТТТСА АСАТАЛААСТ ТССТССТСАТС ТССТСТСАТА АСАСАСАТАТ	ACACACACAC TGTGTGTGTGT ACGTTCTTTT TC-rich AAGAAACCAT TTCTTTGGTA TATTTGTCAT ATAAACAGTA CGTGCTACGG GCACGATGCC TAGTTTATAA ATCAAATATT TGTGTATATAG ACACATATAC	ACACACACACAC TGTGTGTGTGTG GTGCTGGTAG GTGCTGGTAG AACTCAATGG AACTCAATGG AATATAATTT TTATATTAAA TAACATACAT ATTGTATGTA TTTTCGGCAT AAAAGCCGTA LTR TACTTTTCGT ATGAAAAGCA	В
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		AGATGTTCGC TCTACAAGCG ACACACACAC TGTGTGTGTGTG TTACAGTTCA CTCAGTTCA CTCAGTTCGC GAGTCAAGCG CTAAATATAT GATTTATATA GCATTACTTT CGTAATGAAA GCATTACTTT CGTAATGAAA ACGATCCATA ACGGTCCATA ACGGTCCGTGC TACCTGGACC CAAGTCTGCT	ATACACTCAA TATGTGAGATT TGAGGATGAT CTAGAACTTG GATCTTGAAC TATGCCAACA ATACGGTGA ATACGGTGA ATACGGTGA ATACGGTGA AGTCAAAATA TCAGTTTTAT GGAGGAGCTA CCTCCTCGAT ACAACACACAGT TGTTGTGTGCA ACCAATGGAG AGGTTACCTC CCTCTGGGAG CTTCTGGGAG	CCCTAAGAAT GGGATTCTTA AAGGCACATC TTCCGTGTAG AATTCCTGGTG AATTCAGACA AATTAATGA AATTAATGA CAGGTGGTA GTTGTAGGTA GTTGTAGGTA CAACATCCAT CAAGGCATCG GTCCACCACA CAAGGCATCG GTCCCCTACCAT	GCCTGCACAC CGGACGTGTG LTR GCCGAAAGGC CGGCTTTCCG GGTTATTTCC CCAATAAAGG ATAGCAAACT TATCGTTTGA TTATGGAACC AAATACTTGG GCTCTAAACA CGGGACTCGAG CCCTGAGCTC GGGACTCGAG CCCTGAGCTC GGGACTCGAG CACCCGGTGG CACCCGGTGG CACCCGTAG CACCCGTAG CACCCGTAG CACCCGTAG CACCCGTAG CACCCGTAG CACCCGTAG CACCCGTAG CACCCGTAG CACCATATACA	ACACACACAC TGTGTGTGTGTG GAGTTTACTT ATCACAAACA TAGTGTTTACTT GTTTCATTA AAGGGAGCAC TTCCCTCGTG TGTATTTTCA ACATAAAAGT TGTGTGTGTATA ACACACATAT CTTCTCTTCA GAACACAACG GAACAACCG CGCCTATCACT TGAATAGTGA TATGCCTCGC	ACACACACAC TGTGTGTGTGT TGCAAGAAAA ACGTTCTTTT TC-rich AAGAACCAT TTCTTTGGTA TATTTGTCAT ATATACAGTA CGTGCTACGG GCACGATGCC TAGTTTATAA ATCAAATATT TGTGTATATG ACACATATAC CGATGCCACG GCTACGGTCGT GCTACGGTCGT GCTACGGTACAT GCTAAGGTAA AAAAAAGATA	ACACACACAC TGTGTGTGTGTG GTGCTGGTAG TTGAGTTACC AACTCAATGG AATTCAATGG AATTATAATTT TTATATTAAA TAACATACAT ATTGTATGTA TATGTATGTA LTR TACTTTCGGCAT AGAAAAGCA CTCACACCCT ATGCCCCCAC	В
		AGATGTTCGC TCTACAAGCG ACACACACAC TGTGTGTGTGTG AATGTCAAGT TTACAGTTCA CTCAGTTCGC GAGTCAAGCG CTAAATATAT GATTATATAT GATTATATAT GCATTACTTT CGTAATGAAGAT CGTAATGAAGAT ACGATGCATACATA TGCACGTAGCATA ATGCACGTGCACC CAAGTCTGCT GTTCAGACCATA	ATACACTCAA TATGTGAGATT ACTCCTACTA TGAGGATGAT CTAGAACTTG GATCTTGAAC TATGCCAAC ATACGGTTG HSE & AGTCAAAATA TCAGTTTTAT GGAGGAGCTA CCTCCTCGAT ACAACACACAGT TGTTGTGTGTCA ACCAATGGAG AGGTTACCAC AGGTTACCAC TCCAATGGAG GAAGACCCTC TGTGCGCGCAG	CCCTAAGAAT GGGATTCTTA AAGGCACATC TTCCGTGTAG AATCCTGGTGG AATCCTGGTG AATTAATAAT AATTAATAAT CAACTACATCAT GTTGTAGGTA GTTGTAGGTA GTGTAGGTAG GTGTACATCCAT CAAGGCATCG GTTCCGTAGG CCAAGGCATCG GTTCCGTAGC CAAGGCATCG GTTCCGTAGC TCACACGCAA AGTGTGCGCT TACCTAATTT ATGGATTAAA	GCCTGCACAC CGGACGTGTG LTR GCGAAAGGC CGGCTTTCCG GGTTATTCC CCAATAAAGG ATAGCAAACT TATCGTTTGA TTTATGAACC AAATACTTGG GCTCTAAACA CGAGATTTGT TTCAGGATTC AAGTCCTAAG CACCCGGTGG GTGGGCCACC AAGAAAAAA TCTTTTTT MEJA CGTCATATGA GCACTATAGA CAGTATACT	ACACACACAC TGTGTGTGTGTG GAGTTTACTT ATCACAAACA TAGTGTTTGT TGTTTCATTA ACAAAGTAAT AAGGGAGCAC TTCCCTCGTC TGTATTTTCA ACATAAAAGTAAT CTTCTCTTCA GAAGAGAAAGT GCACCAACCG CGTGGTTGGC ACTTATCACT TGAATAGTGG AATTAAGACA	ACACACACAC TGTGTGTGTGTG ACGTTCTTTT TC-rich AAGAACCAT TTCTTTGGTA TATATACAGTA CGTGCTACGG GCACGATGCC TAGTTTATAA ATCAAATATT TGTGTATATG CGATGCCACG GCTACGGTGC ACTCGTTCGT TGAGCAAGCA CGATTCCATT GCTAAGGTAA AAAAAGATA TTTTTTCTAT	ACACACACACAC TGTGTGTGTGTGTG GTGCTGGTGGTGGTG AATATAATTA TTAAATAAATTT TTATATTAAAA AATACATAC	В
		AGATGTTCGC TCTACAAGCG ACACACACAC TGTGTGTGTGTG AATGTCAAGT TTACAGTTCAC CTCAGTTCGC GAGTCAAGCG CTAAATATAT GATTTATATA GCATTACTTT CGTAATGAACA ACGATCCATA TGCACGTAGTAT ACGGACGTGC TACCTGCACCA CAAGTCTCCAT GTAAGCACCA GAGCTCCCAT GTAAGCACC CATTCCGTG	ATACACTCAA TATGTGAGATT CTAGAGATGAT GATCTTGAACTTG GATCTTGAAC TATGCCAAC HSE & ATACGGTTGT HSE & AGTCAAAATA TCAGTTTTAT GGAGGAGCCAA CCTCCTCGAT TCTTCTATGT TCTTCTATGT AGAACACACGT CCTCCTCGGAG AGGTTACCTC FACTTATCAC TGTAGGGAC GAAGACCCTC TGTGGGCGAG ACACGCGCAC ACCACGGAC ACCACGCAC	CCCTAAGAAT GGGATTCTTA AAGGCACATC TTCCGTGTAG AATCCTGGTG TTAGGACCAC IAATTAATGA AATTAATGA ATTCAAATA AATTAATGT TAAAGTTTAA CATCTAAGAAA CATCTACATC CAAGCACCCAAC CAAGGCATCG GTCCCCTAACTT TACCTAATTT AAGGATTAAA CTTTTGCGAT AAATTTTAC	GCCTGCACAC CGGACGTGTG LTR GCCGAAAGGC CGGCTTTCCC CCAATAAAGG ATACCAAACT TATCGTTTGA TTACGAACC AAATACTTGG GCTCTAAACA CGAGATTGT TTCAGGATTC GGGACTCGAG CCCTGAGCTC GGGACTCGAG CACCCGGTGG GTGGGCCACC AAGAAAAAAA TTCTTTTTTT MeJA CGTCATATGA GCAGTATACT MeJA	ACACACACAC TGTGTGTGTGTG GAGTTTACTT ATCACAAACA TAGTGTTTACT TGTTTCATTA AAGGGAGCAC TTCCCTCGTG TGTATTTTCA AAGGGAGCAC TTCCCTCGTG GCACCAACCG CGTGGTTGGC ACTTATCACT TGAATAGTGA TATGCCTCGC AATTAAGACA AATTAAGACA	ACACACACAC TGTGTGTGTGTG ACGTTCTTTT TC-rich AAGAACCAT TTCTTTCGTA TATATACACAT CGTGCTACGG GCACGATGCC TAGTTTATAA ATCAAATATT TGTGTATATAC CGATGCCACG GCTACGTCGT CGATGCCACG CGATTCCATT GCTAAGGTAA AAAAAAGATA TTTTTTCTAT	ACACACACACA TGTGTGTGTGTG GTGCTGGTAG GTGCTGGTAG TTGAGTTACC AACTCAATGG AATATAATTT TTATATATAAA TAACATACAT ATTGTATGTA ATTGTATGTA LTR TACTTTTCGT ATGAAAAGCA CTCACACCCT CAGGTGTGGGA TACGGGTGAT ATGCCCACTA TTTTCTTCCA AAAAGAAGGT TGCCTCCCAC ACGGAGGGTG ACGGAGGGTG ACGGAGGGTG CTTACAGGGA ACGATAAACT	В
		AGATGTTCGC TCTACAAGCG ACACACACAC TGTGTGTGTGTG AATGTCAAGT TTACAGTTCA CTCAGTTCAC CTCAGTTCGC GAGTCAAGCG CTAAATATAT GATTTATATA GCATTACTTT CGTAATGAACA ACGACTTAGT ACGTGAATCA ACGACCACATA ACGACCACATA ACGACCACACA CAAGTCTGCACC CAAGTCTGCACC CAAGTCTGCACA GTAAAGAATTC ATTTCTTAAG	ATACACTCAA TATGTGAGATT ACTCCTACTA TGAGGATGAT GATCTTGAAC TATGCCAACA ATACGGTGAT HSE & AGTCAAAATA TCAGTTTTAT GGAGGAGCTA CCTCCTCGAT TCTTCTATGT ACAACACAGAG ACACACACAG AGTATACCAC CTCCTGGGAG GAAGACCCTC TGTGCGCGATC ACACGCGATC ACACGCGATC ACACGCGAC TACTACCGAC ACACGCGAC ACACGCGAC ACACGCGAC ACACGCGAC	CCCTAAGAAT GGGATTCTTA AAGGCACATC TTCCGTGTAG AATCCTGGTG TAGGACCAC AATTAATAA AATTAATGA AATTAATGA AATTCAAATA CAGGTGGATA GTTGTAGGTA GTCCACCACAA CAAGGCATCG GTCCCGTAGC TCACACGCAA AGTGTGCCGT AAATTTTAC TTTAAAAATG TTTAAAAATG TCGAGTCCGT ACATCAGGCA	GCCTGCACAC CGGACGTGTG LTR GCCGAAAGGC CGGCTTTCCG GGTTATTTCC CCAATAAAGG ATAGCAAACT TATCGTTTGA TTATGGAACC AAATACTTGG GCTCTAAACA CGAGATTCG CCCTGAGCTC GGGACTCGAG CCCCTGAGCTC GGGACTCGAG CACCCGGTGG GTGGCCACC AAGAAAAAAA TTCTTTTTTT MeJA TAGATTCACT AGACCTCACG TCTGGAGTGC CGTCTGTCTA GCAGACAGAT	ACACACACAC TGTGTGTGTGTG GAGTTTACTT ATCACAAACA TAGTGTTTACTT GTTTCATTA AAGGGAGCAC TTCCCTCGTG TGTATTTTCA AAGGGAGCAC TTCCCTCGTG TGTATTTTCA ACACACATAT CTTCTCTCTCA GCACCACCG CGCCTACCGC ACTTATCACT TGAATAGTCA TATGCCTCGC AATTAAGACA TTAATCTGT GGATGGGCTG CCTACCCACAC ACTTTTCG GCATTTCG CCATTTCG CCATTTCG CCATTTCG CCATTTCG CCATTTCG CCATTTCG CCATTTCG CCATTTCG CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCATTTCC CCCCCCC CCCCCACACCC CCCCCCCC	ACACACACAC TGTGTGTGTGTG ACGTGTGTGTGT TC-rich AAGAAACAT TTCTTTGGTA TATTTGTCAT TATTTGTCAT ATAAACAGTA CGTGCTACGG GCACGATGCC TAGTTTATAA ATCAAATATT TGTGTATATG CGATGCCACG GCTACGGTGC ACTCGTTCGT TGGGCAAGCA ACTCGTTCCATT GCTAAGGTAA AAAAAAGATA TTTTTTCTAT CGTAGCACGA ACTCGTCGTCT GATATTA	ACACACACAC TGTGTGTGTGTG GTGCTGGTAG GTGCTGGTAG AATATAATTT TTAAATATAT TTAAATATAATTT TTAAATATATAA TAACATACAT	В

of the modern salt-tolerant bread wheat cultivars (Ghavami et al. 2004), it is possible that maybe its mechanisms do not admit to uptake Na^+ within its upper parts, so this gene should be active in roots; however the more study can help to understand it more precisely.

Comparative study of promoter regions between HKT1;5-A and HKT1;5-D. Using an in silico promoter analysis tool (Lescot et al 2002), the results of the PlantCARE analysis showed that the regulatory elements related to the stress and hormone response have some differences between TaHKT1;5-D (T. aestivum) and TmHKT1;5-A (T. monococcum). The cis-elements of the HKT1;5 promoter are shown in Fig 3. TATA box and CAAT elements were more frequent in the TmHKT1;5 promoter than that of TaHKT1;5. The jasmonate response element was two times more frequent in TmHKT1;5 than TaHKT1;5 (Fig. 3). Jasmonate is involved in plant adaptation to biotic and abiotic stresses and is accumulated transiently in response to osmotic/salt stress (Lehmann et al. 1995). The real-time PCR analysis revealed that HKT1;5 expression was low under 200 mM NaCl in T. boeoticum; thus, this genotype is active at lower salinity. However, verifying this hypothesis will require further study. It is likely that the regulatory elements of the TbHKT1;5 promoter are different compared with TmHKT1;5, however, the sequence of TbHKT1;5 is unknown at present.

The promoter analysis showed that HSEs (*cis*-acting elements involved in heat stress responsiveness) elements are more frequent in *TaHKT1*;5-*D* than *TmHKT1*;5; thus, it is likely that these *HKT*s are also expressed in response to high temperature. Because there was no significant difference between the number of *cis*-regulatory elements, such as auxin, ethylene, gibberellin and light response elements in the promoters of *TaHKT1*;5 and *TmHKT1*;5, we do not expect *T. monococcum* and *T. aestivum* to be different when developmental stages and ripening happen.

Conclusions

In conclusion, this study provides a comprehensive expression analysis of HKT1;5 transporters for four wheat genotypes under salt stress by real-time PCR. Because *A. crassa* showed the highest level of HKT1;5 expression, we predict that Na⁺ exclusion is the main salinity tolerance mechanism in this species. An important insight from this study was showing that the Mahuti the HKT1;5 gene has a different action compared with other genotypes, which is confirmed with K⁺/Na⁺ ratio analysis. However, to fully understand the precise HKT1;5 activity several different plants tissues should be analyzed and compared. According to our results, it is likely that, due to the differences in *HKT1*;5 function in different tissues, specific promoter elements may be differentially activated in different tissues.

Acknowledgments The authors would like to thank the Institute of Biotechnology for supporting this research and the Bioinformatics Research Group in the College of Agriculture (Shiraz University). We thank Dr. Mehrabi (Ilam University) for kindly supplying seeds of wild genotypes for this study. We thank Mr. Amin Ramezani for help in performing the real-time PCR experiments.

References

- Amtmann A, Sanders D (1999) Mechanisms of Na⁺ uptake by plant cells. Adv Bot Res 29:75–112
- Byrt CS (2008) Genes for sodium exclusion in wheat. Ph.D. thesis, University of Adelaide. Available from http://hdl.handle.net/ 2440/56208. Accessed 24 April 2012
- Byrt CS, Platten JD, Spielmeyer W, James RA, Lagudah ES, Dennis ES, Tester M, Munns R (2007) *HKT1*;5-like cation transporters linked to Na⁺ exclusion loci in wheat, *Nax2* and *Kna1*. Plant Physiol 143:1918–1928
- Deihimi T, Niazi A, Ebrahimi M, Kajbaf K, Fanaee S, Bakhtiarizadeh MR (2012) Finding the undiscovered roles of genes: an approach using mutual ranking of coexpressed genes and promoter architecture-case study: dual roles of thaumatin like proteins in biotic and abiotic stresses. SpringerPlus 1:30
- Demidchik V, Tester M (2002) Sodium fluxes through nonselective cation channels in the plasma membrane of protoplasts from *Arabidopsis* roots. Plant Physiol 128:379–387
- Dvorak J, Akhunov ED (2005) Tempos of gene locus deletions and duplications and their relationship to recombination rate during diploid and polyploid evolution in the *Aegilops–Triticum* alliance. Genetics 171:323–332
- Genc Y, McDonald GK, Tester M (2007) Reassessment of tissue Na⁺ concentration as a criterion for salinity tolerance in bread wheat. Plant Cell Environ 30:1486–1498
- Ghavami F, Malboobi MA, Ghanadha MR, Yazdi-samadi B, Mozaffari J, Jafar-Aghayi M (2004) Evaluation of salt tolerance of Iranian wheat cultivars at germination and seedling stages. Iranian Journal of Agricultural Sciences 35(2):453–464
- Gorham J, Win Jones RG, Bristol A (1990) Partial characterization of the trait for enhanced K⁺–Na⁺ discrimination in the D genome of wheat. Planta 180:590–597
- Hauser F, Horie T (2010) A conserved primary salt tolerance mechanism mediated by *HKT* transporters: a mechanism for sodium exclusion and maintenance of high K⁺/Na⁺ ratio in leaves during salinity stress. Plant Cell Environ 33:552–565
- Huang S, Spielmeyer W, Lagudah ES, James RA, Platten JD, Dennis ES, Munns R (2006) A sodium transporter (*HKT7*) is a candidate for *Nax1*, a gene for salt tolerance in durum wheat. Plant Physiol 142:1718–1727
- James RA, Blake C, Byrt CS, Munns R (2011) Major genes for Na⁺ exclusion, Nax1 and Nax2 (wheat HKT1;4 and HKT1;5), decrease Na⁺ accumulation in bread wheat leaves under saline and waterlogged conditions. J Exp Bot 62:2939–2947
- James RA, Davenport RJ, Munns R (2006) Physiological characterization of two genes for Na⁺ exclusion in durum wheat, *Nax1* and *Nax2*. Plant Physiol 142:1537–1547
- Kader MA, Lindberg S (2005) Uptake of sodium in protoplasts of saltsensitive and salt-tolerant cultivars of rice *Oryza sativa* L. determined by the fluorescent dye SBFI. J Exp Bot 56:3149–3158

- Kader MA, Seidel T, Golldack D, Lindberg S (2006) Expressions of OsHKT1, OsHKT2, and OsVHA are differentially regulated under NaCl stress in salt-sensitive and salt-tolerant rice (Oryza sativa L.) cultivars. J Exp Bot 57:4257–4268
- Lehmann J, Atzorn R, Brückner C, Reinbothe S, Leopold J, Wasternack C, Parthier B (1995) Accumulation of jasmonate, abscisic acid, specific transcripts and proteins in osmotically stressed barley leaf segments. Planta 197:156–162
- Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouzé P, Rombauts S (2002) PlantCARE, a database of plant *cis*acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. Nuc Acids Res 30:325–327
- Mariño-Ramírez L, Tharakaraman K, Bodenreider O, Spouge J, Landsman D (2009) Identification of *cis*-regulatory elements in gene co-expression networks using A-GLAM. Meth Mol Biol 541:3–22
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucl Acids Res 29:e45

- Plett DC, Moller IS (2010) Na⁺ transport in glycophytic plants: what we know and would like to know. Plant Cell Environ 33:612–626
- Plett D, Safwat G, Gilliham M, Moller IS, Roy S, Shirley N, Jacobs A, Johnson A, Tester M (2010) Improved salinity tolerance of rice through cell type-specific expression of *AtHKT1*;1. PLoS One 5: e12571
- Ramezani A, Niazi A, Abolimoghadam AA, Zamani Babgohari M, Deihimi T, Ebrahimi M, Akhtardanesh H, Ebrahimie E (2012) Quantitative expression analysis of TaSOS1 and TaSOS4 genes in cultivated and wild wheat plants under salt stress. Mol Biotechnol (in press)
- Rush DW, Epstein E (1976) Differences between salt-sensitive and salt-tolerant genotypes of the tomato. Plant Physiol 57:162–166
- Shavrukov Y, Langridge P, Tester M (2009) Salinity tolerance and sodium exclusion in genus *Triticum*. Breed Sci 59:671–678
- Tabatabaei SMF, Maassoumi TR (2001) *Triticum boeoticum* ssp *thaoudar* exist in Iran. Cereal Res Commun 29:121–126