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ORIGINAL PAPER

Evaluation of salinity tolerance and analysis of allelic function of *HvHKT1* and *HvHKT2* in Tibetan wild barley

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Abstract Tibetan wild barley is rich in genetic diversity with potential allelic variation useful for salinity-tolerant improvement of the crop. The objectives of this study were to evaluate salinity tolerance and analysis of the allelic function of *HvHKT1* and *HvHKT2* in Tibetan wild barley. Salinity tolerance of 189 Tibetan wild barley accessions was evaluated in terms of reduced dry biomass under salinity stress. In addition, Na^+ and K^+ concentrations of 48 representative accessions differing in salinity tolerance were determined. Furthermore, the allelic and functional diversity of *HvHKT1* and *HvHKT2* was determined by association analysis as well as gene expression assay. There was a wide variation among wild barley genotypes in salt tolerance, with some accessions being higher in tolerance than cultivated barley CM 72, and salinity tolerance was significantly associated with K^+/Na^+ ratio. Association analysis revealed that *HvHKT1* and *HvHKT2* mainly control Na^+ and K^+ transporting under salinity stress, respectively, which was validated by further analysis of gene expression. The present results indicated that Tibetan wild barley offers elite alleles of *HvHKT1* and *HvHKT2* conferring salinity tolerance.

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Introduction

Soil salinity poses a serious threat to agricultural production, as more than 800 million hectares of land in the world have been salt affected, which account for 6% of the total land area (Munns and Tester 2008). Moreover, the amount is increasing due to unreasonable irrigation. It is the most effective approach to develop salt tolerance crop cultivars for improving productivity of salt-stressed soils (Yamaguchi and Blumwald 2005).

Among cereal crops, barley is relatively tolerant to high concentration of NaCl, but salinity stress is still a restricting factor in barley production. So it is imperative to enhance salt tolerance of barley through genetic improvement. On the other hand, modern cultivated barley (*Hordeum vulgare* L.) lost many allelic variations during its domestication (Russell et al. 2004). Thus, gene pools of cultivated barley exhibit limited genetic diversity in comparison with their wild ancestors, which impedes the development of adaptive cultivars. This has sparked an interest in studying wild relatives of cultivated barley (Saghai Maroof et al. 1990; Ellis et al. 2000; Yan et al. 2008; Feuillet et al. 2008; Shavrukov et al. 2010). It was reported that there is a wider genetic variation for the populations habiting in stressed environments (Nevo et al. 1997). The Qinghai-Tibet Plateau, known as the "ridge of the world" and well known for its harsh environment, is one of the original centers of cultivated barley, and rich in genetic diversity. In the past three decades, intensive investigation was done on the collection and evaluation of wild barley (*Hordeum vulgare* ssp. *spontanum*) growing in the Qinghai-Tibet Plateau. It was shown that there was a wide biochemical, morphological and physiological diversity for the barleys (Xu 1993). Sun and Gong (2009) identified some accessions with elite performance in

maturity, plant height, yield potential and disease resistance and developed some barley cultivars from the crosses of the wild barley and cultivated ones. However, little has been known about its salinity tolerance although it is considered as a useful genetic source of novel allelic variation for abiotic tolerance in view of its original environment.

Over the past several decades, intensive research has been focused on the mechanism of salinity tolerance and understanding of the response to salt stress in plants (Munns et al. 1995; Zhu 2003; Møller and Tester 2007). To counter the two components of salinity stress (osmotic stress and ionic stress), glycophyte such as barley has evolved three strategies to adapt (Munns and Tester 2008). Firstly, the accumulation or synthesis of compatible solutes is often regarded as a basic strategy (Ueda et al. 2007; Hattori et al. 2009). However, Chen et al. (2007a) found that hyper-accumulation of some major compatible solutes in barley did not contribute to salt tolerance, but rather, might be a symptom of salt susceptibility. Secondly, the strategy is antioxidant protection. Witzel et al. (2009) emphasized that the proteins involved in reactive oxygen species (ROS) detoxification is key to salinity tolerance of barley. Nevertheless, the opposite results were found in *Arabidopsis* mutants (Miller et al. 2007) and cowpea (*Vigna unguiculata* L.) (Cavalcanti et al. 2004), indicating the plasticity of ROS regulatory pathway. The third strategy is the regulation of ion homeostasis. In recent times, more attention has been paid to study the mechanics of homeostasis maintenance and the interaction between sodium and potassium ions (Essah et al. 2003; Zhu 2003; Shabala and Cuin 2007; Kronzucker et al. 2008). In subsequence, the genes controlling ion homeostasis are concentrated in the relevant study. Especially, the genes encoding high-affinity K^+ transporters (HKT) are considered as candidates for salinity tolerance improvement (Berthomieu et al. 2003; Horie and Schroeder 2004; Rodríguez-Navarro and Rubio 2006). Schachtman and Schroeder (1994) isolated *HKT* from wheat roots, and found that the transporter confers to the acquisition of K^+, and might contribute to environmental alkali metal toxicities, including Na^+. Moreover, Rubio et al. (1995) reported that the transporter acted as a high-affinity K^+-Na^+ co-transporter. In *Arabidopsis thaliana*, Uozumi et al. (2000) identified *AtHKT1*. And it has been demonstrated that *AtHKT1* is a determinant for salinity tolerance by reducing Na^+ accumulation in shoots (Berthomieu et al. 2003; Davenport et al. 2007; Mäser et al. 2002; Møller et al. 2010; Sunarpi et al. 2005). *AtHKT1* is expressed in the vasculature and mediates Na^+ removal from the xylem sap (Davenport et al. 2007; Horie et al. 2006; Mäser et al. 2002; Møller et al. 2010; Sunarpi et al. 2005). In rice, two types of HKT transporters differing in properties of Na^+ and K^+ transporter were identified (Horie et al. 2001), and a quantitative trait locus *SKC1* controlling rice salt tolerance was found to encode a member of HKT-type transporter (Ren et al. 2005). Similarly, HKT is also crucial for salt tolerance in wheat (Byrt et al. 2007). In barley, the expression of *HKT1* regulated by K^+ was studied (Wang et al. 1998), and Haro et al. (2005) cloned the *HvHKT1* (AM000056), which mediates Na^+ uniport in roots. Consequently, the HKT families involving in Na^+-K^+ homeostasis are expected importance in salinity tolerance.

It is assumed that salinity tolerance in barley is a complex quantitative trait controlled by multiple major or minor genes. Traditionally, linkage analysis is applied to dissect and map these QTLs, and provides useful information of genetic loci (Mano and Takeda 1997; Xue et al. 2009), but the limited number of recombination events would lead to poor resolution of quantitative trait. Currently, association mapping based on re-sequencing of candidate functional gene is receiving considerable attention in plant genetics for its potential to validate candidate genes and identify elite alleles (Tian et al. 2009; Fricano et al. 2009).

Accordingly, the present study was conducted with the following objectives: (1) to evaluate the salinity tolerance of Tibetan wild barley; (2) to examine the significance of maintaining sodium and potassium homeostasis in salt tolerance; (3) to determine the function of genes encoding sodium transporter in salinity tolerance.

Materials and methods

Materials and hydroponic culture

For evaluation of salinity tolerance, 189 Tibetan wild barley accessions (genotypes) and a tolerant cultivated barley CM72 (Chen et al. 2005) were used. The experiment was carried out in 2009 at Huajiachi campus, Zhejiang University, Hangzhou, China. Seeds of all genotypes were surface sterilized in 3% H_2O_2 for 20 min, rinsed thoroughly with distilled water five times and germinated in moist sand in an incubator (22/18°C, day/night). When seedlings grew the second leaf (10 days old), they were selected for uniformity and transplanted onto a 35-L rectangular container, which was painted black and covered with a plastic lid with evenly spaced holes, where two plants were held using flexible pieces of foam. The containers were placed in a greenhouse. The composition of the nutrient solution at full strength was (mg L^{-1}): $(NH_4)_2SO_4$ 48.2, $MgSO_4$ 154.88, K_2SO_4 15.9, KNO_3 18.5, KH_2PO_4 24.8, $Ca(NO_3)_2$ 86.17, Fe-citrate 7, $MnCl_2{\cdot}4H_2O$ 0.9, $ZnSO_4{\cdot}7H_2O$ 0.11, $CuSO_4{\cdot}5H_2O$ 0.04, HBO_3 2.9, and H_2MoO_4 0.01. In order to discriminate clearly salt

difference among genotypes and to reduce the effect of osmotic shock, NaCl was added to the basic nutrient solution to form two levels at 10 days after transplanting: (1) 0 (control); (2) 300 mM NaCl, which was reached within 3 days with the initial concentration being 100 mM and gradual increasing by 100 mM every day. Meanwhile no supplementary Ca^{2+} was added according to Garthwaite et al. (2005). Then the treatment was maintained for 17 days. The nutrient solution in the containers was continuously aerated with pumps and renewed every 7 days throughout the experiment. The experiment was laid out as a completely randomized design with three replications and four plants per replication.

For gene expression assay, eight representative varieties (cultivated barley CM72 and Gairdner Tibetan wild barley T16, T61, T113, T115, T169 and T178) were used. Twenty-day-old seedlings growing in the above basic nutrient solution were stressed by adding NaCl at a final concentration of 300 mM, which was reached within 2 days with the initial concentration being 150 mM. The whole root tissues were sampled at five different time points between 0 and 48 h, and immediately frozen in liquid nitrogen.

Salt tolerance evaluation

Twenty-seven days after transplanting, the plants of each treatment were harvested and then separated into roots and shoots, dried in an oven at 105°C for 3 h, and then 80°C for 48 h, and weighted. Salt tolerance (ST) was estimated as treatment/control × 100% based on shoot and root dry matter weight.

Determination of sodium and potassium concentrations in plant tissues

Shoots and roots of the sampled plants were ground to a fine powder in a ball mill and approximately 0.1 g of sample was ashed in a muffle furnace, and dissolved with 10 ml HNO_3:H_2O (1:1). Na^+ and K^+ concentrations were determined by an atomic absorption spectroscope (Shimadzu, Japan).

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from barley seedlings using Universal Genomic DNA Extraction Kit Ver.3.0 (TaKaRa, Japan) following the manufacturer's instructions. Based on the sequences of AK249167 (*Hordeum vulgare* ssp. *vulgare* cDNA clone: FLbaf23c09, mRNA sequence) and AM000056.1 (*Hordeum vulgare* mRNA for high-affinity sodium transporter), PCR primers were designed with the Primer 5.0 (Table S1). Each 25 μl amplification reaction consisted of 2.5 μl 10× *TransTaq* HiFi Buffer I (200 mM Tris–HCl (pH 8.4), 200 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$), 2 μl 2.5 mM dNTPs, 2 μl 10 μM primers, 0.5 μl 5 unit μL^{-1} of *TransTaq* polymerase High Fidelity (Beijing TransGen Biotech Co., Ltd. China), and 1 μl 50 ng of genomic DNA. All amplifications were performed on a DNA Engine Dyad thermal cycler (Bio-Rad, Inc.) under the following conditions: 5 min at 94°C, followed by Touch-down PCR steps, 30 s at 94°C, 30 s at 60°C, decreased 0.2°C every cycle, and 30 s at 72°C for 30 cycles, and 10 min at 72°C for a final extension. After the PCR product was purified, DNA sequencing was performed on an ABI 3100 automated sequencer following the manufacturer's instructions (Applied Biosystems, Inc.). The rare SNPs or indels were re-sequenced with additional independent PCR products.

RNA extraction and cDNA synthesis for real-time PCR

Total RNA was isolated using TRIzol reagent following the manufacturer's recommended procedure, and the residual genomic DNA was removed with amplification grade DNase I (Invitrogen USA). First-strand cDNA synthesis and real-time PCR reaction were carried out according to the manufacture of SYBR Prime Script RT–PCR Kit II (TaKaRa, Japan). The primers of housekeeping gene *GAPDH* and specific genes *HvHKT1* and *HvHKT2* were shown in Table S1. The amplifications were performed on a CFX96 Real Time System (Bio-Rad, Inc.) and the cycling protocol consisted of: pre-denaturation at 94°C for 30 s, then, denaturation at 94°C for 5 s, annealing at 60°C for 30 s, plate reading, and repeated for up to 45 cycles. The relative expression was calculated by comparative Ct (2-ΔΔ Ct) method (Livak and Schmittgen 2001).

Statistical analysis

The growth parameters, such as shoot and root dry weight, the tissue concentration of Na^+ and K^+ and association between them were subjected to statistical analysis using software of SAS V8. The sequences were assembled using the software of DNAStar. The nucleotide diversity (π, the average number of nucleotide difference per site between two sequences), were estimated using DNASP 4.0. Phylogenetic analysis was performed using MEGA 4.1. The association analysis between nucleotide polymorphisms and different component traits of salinity tolerance were performed with TASSEL (Trait analysis by association, evolution and linkage) Version tassel2.1_standalone software. The significance (P value) and the rate of total variation explained (R^2) were calculated for each marker by applying general linear model (GLM).

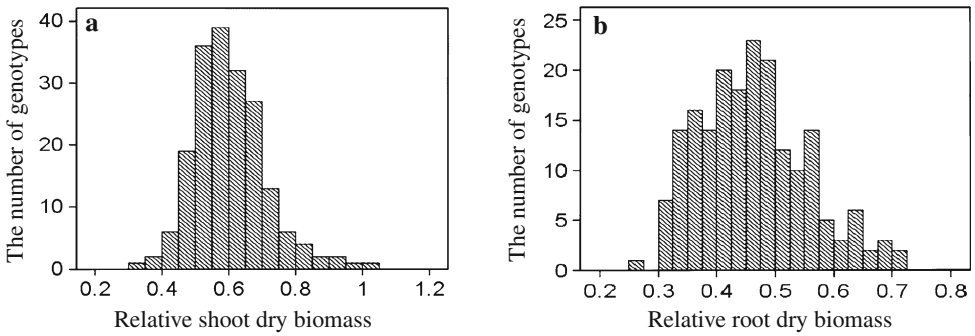

Fig. 1 The frequency ($n = 191$) of relative shoot (**a**) and root (**b**) dry biomass under 300 mM NaCl in the tested accessions

Results

Salt tolerance evaluation

As shown in Fig. 1, there was a wide difference in the response to salt stress among the 191 genotypes, indicating great difference in salinity tolerance among Tibetan wild barleys. On the whole the relative shoot dry weight under salt stress was 60.2% of the control, and ranged from 32% for T169 to 101% for T113 among the examined genotypes. However, the relative root dry weight under salt stress was only 46.1% of the control, and ranged from 27% for T174 to 72% for T38 (Table S2). In addition, the correlation coefficient between shoot and root dry weight is 0.82. It can be seen that root growth is more affected than shoot when plants are exposed to salt stress. For the cultivated barley CM72, the relative shoot and root dry weight was 53.4 and 39.9% of the control when exposed to salinity, both being lower than the average of the tested accessions.

Based on relative shoot dry weight, 48 genotypes were selected for further study, including 22 tolerant genotypes (ST > 70%), 15 moderate (50% < ST < 70%) and 11 sensitive ones (ST < 50%).

Sodium and potassium concentrations in plant tissues

In the solution without NaCl addition, Na^+ concentration in the shoots of the tested genotypes ranged from 2.09 to 4.29 mg g^{-1} DW, while root Na^+ concentration ranged from 1.93 to 7.39 mg g^{-1} DW (Table 1). Salt treatment remarkably increased Na^+ concentration in shoots and roots of all genotypes, being 29.76- and 13.36-fold of the control for shoots and roots, respectively. In addition, there were significant differences among accessions for the shoot Na^+ concentration, but not for that in the roots. Under the condition without salt stress, K^+ concentration in shoots and roots for all of accessions is 68.32 and 63.57 mg g^{-1} DW, on average, respectively (Table 1). NaCl addition into the culture solution significantly reduced K^+ concentrations in both shoots and roots. Moreover, there was no significant difference in the accessions for the K^+ concentration both in shoots and roots. For the ratio of Na^+/K^+ concentration, averaged over all genotypes, shoots and roots of the plants without salt stress were only 0.04 and 0.06, respectively. However, the values for the plants exposed to salt stress were 1.71 and 11.12, respectively (Table 1). Particularly, the Na^+/K^+ ratio in the shoots of all the tested Tibetan wild barely, on average, was lower than that in cultivated barley CM72 (Na^+ and K^+ concentrations in the shoot of CM72 were 92.52 and 48.01 mg g^{-1} DW under 300 mM NaCl, respectively), while the reverse was true for that in the roots, indicating that less movement of Na^+ from roots to the shoot might be attributed to salinity tolerance in wild barley.

Significant correlation existed between tissue Na^+ or K^+ concentrations under salt stress and tissue biomass (Fig. 2). Hence, tissue Na^+ and K^+ concentrations were negatively

Table 1 Na^+, K^+ concentrations and Na^+/K^+ ratio in shoots and roots of 48 representative genotypes under control (0 mM NaCl) and salinity stress (300 mM NaCl) conditions

	Na^+ (mg g^{-1} DW)		K^+ (mg g^{-1} DW)		Na/K ratio	
	Control	Salinity	Control	Salinity	Control	Salinity
Shoot						
Mean	2.86	85.12	68.32	50.24	0.04	1.71
Min	2.09	64.95	38.75	41.12	0.03	1.14
Max	4.29	113.10	90.39	58.05	0.08	2.64
CV (%)	14.76	14.20	16.92	8.74	26.56	18.79
Treatment	**		**		**	
Genotype	**		NS		NS	
Root						
Mean	3.75	50.09	63.57	4.79	0.06	11.12
Min	1.93	18.15	29.96	2.09	0.03	2.68
Max	7.39	115.72	82.94	11.05	0.14	23.76
CV (%)	29.97	26.26	19.01	29.53	40.47	31.75
Treatment	**		**		**	
Genotype	NS		NS		NS	

NS non-significant

** Significant at 0.01

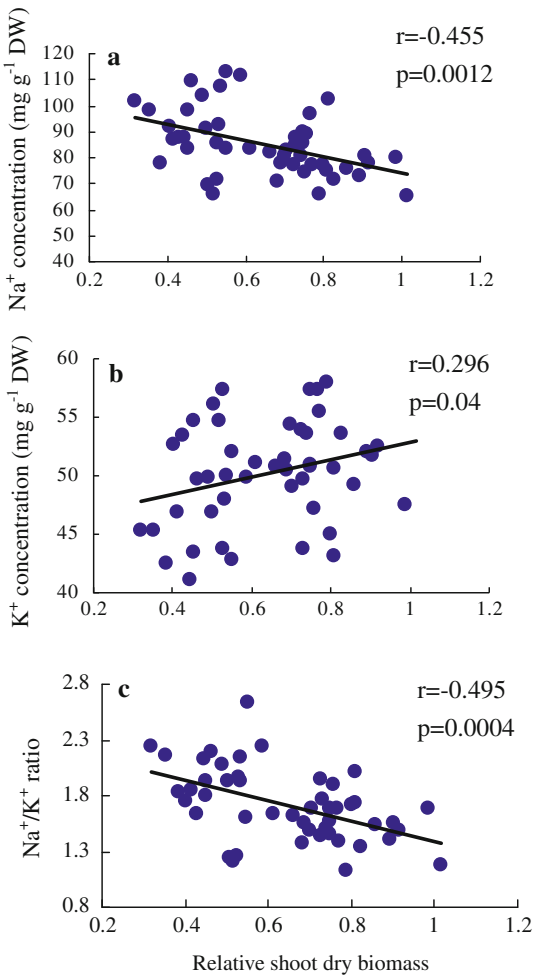

Fig. 2 The relationship between relative shoot dry biomass and sodium, potassium concentrations in shoot under salt stress. **a** Relative shoot dry biomass and sodium concentration, **b** relative shoot dry biomass and potassium concentration, **c** relative shoot dry biomass and the ratio of sodium and potassium concentration

and positively correlated with relative shoot biomass ($r = -0.455$, $P < 0.01$, $r = 0.296$, $P < 0.05$), respectively. In addition, ratio of Na^+/K^+ concentration was significantly and negatively correlated to relative biomass ($r = -0.495$, $P < 0.001$), indicating that maintenance of ion homeostasis is crucial for salinity tolerance.

The structure and nucleotide diversity of *HvHKT1* and *HvHKT2*

Sequence data of 40 barley genotypes were obtained from five DNA fragments amplified using separate PCR reactions. The whole length of 2,505 and 2,055 bp genomic DNA sequences for *HvHKT1* and *HvHKT2* were assembled, respectively (Table 2). The full length of the two coding regions was 1,758 and 1,593 bp, respectively, which consisted of three exons separated by two introns.

The polymorphism density including SNPs and Indels were found, with 1 SNP per 92.8 bp, 1 Indel per 835.3 bp for *HvHKT1*, and 1 SNP per 114.2 bp for *HvHKT2* (Table 2). The mean pair-wise nucleotide diversity (π) for the genomic region was 0.00389 and 0.0027 for *HvHKT1* and *HvHKT2*, respectively. In most case, nucleotide diversity was significantly lower in coding region than that in non-coding region, except for exon2 of *HvHKT2*. Meanwhile, diversity of non-synonymous substitution was lower than that of synonymous. Tajima's *D* test for selection indicated that those barley genotypes did not show significant deviation from the neutral expectation in the whole genomic region for the two candidate genes. Obviously, the neutral mutation can explain the DNA polymorphism in the whole region. However, DNA polymorphism in intron2 of the two genes could not be explained by the neutral mutation hypothesis, indicating that the SNP diversity in this region might be the result of natural selection.

Association test of SNPs-traits

For *HvHKT1*, there were 17 SNP sites associated with root Na^+ concentration in saline environment (Table 3), and

Table 2 The structure and nucleotide diversity of *HvHKT1* and *HvHKT2*

Genes	Index	Genomic	Exon1	Intron1	Exon2	Intron2	Exon3
HvHKT1	Size	2,505	1,289	600	225	147	244
	SNP (Indels)	30 (3)	11	11 (3)	2	3	3
	Hd	0.705	0.672	0.664	0.05	0.586	0.619
	π	0.00389	0.00249	0.00670	0.00044	0.00972	0.00416
	Tajima's *D*	1.28928	0.73089	1.59287	−1.48662	2.23425*	0.95441
HvHKT2	Size	2,055	1,172	204	222	258	199
	SNP (Indels)	18	4	4	5	2	3
	Hd	0.824	0.754	0.458	0.653	0.522	0.622
	π	0.0027	0.00113	0.00450	0.00713	0.00385	0.00370
	Tajima's *D*	1.01442	0.95201	−0.05698	0.88287	2.10182*	0.09458

* Significant at 0.05

Table 3 Statistically associated SNPs with component traits of salinity tolerance in *HvHKT1*

Traits (mg g^{-1} DW)	SNP position	Means of SNP alleles (accession classes)	R^2 (variation explained)
Root Na^+ concentration	110	T:49.91 C:41.96	0.10*
	546	C:50.67 T:44.04	0.10*
	594	A:50.67 G:44.04	0.11*
	610	G:50.67 A:44.04	0.11*
	760	G:50.82 A:44.24	0.11*
	900	G:50.02 A:39.98	0.14*
	1399	C:52.18 T:44.04	0.18**
	1677	C:52.18 T:44.04	0.18**
	1681	G:51.95 A:42.79	0.22**
	1837	T:50.01 C:42.52	0.10*
	1841	C:50.01 G:42.52	0.10*
	1861	T:50.01 G:42.52	0.10*
	2135	C:50.91 T:42.92	0.11*
	2151	T:51.95 C:42.79	0.22**
	2166	T:51.95 C:42.79	0.22**
	2312	C:51.95 G:43.87	0.18**
	2498	G:50.01 A:42.52	0.10*
Root K^+ concentration	1302	C:4.49 T:5.52	0.15*
	1335	T:4.61 C:6.34	0.12*
Shoot K^+ concentration	164	G:51.23 C:48.04	0.12*
	546	C:51.65 T:48.15	0.18**
	594	C:51.65 T:48.15	0.18**
	610	C:51.65 T:48.15	0.18**
	760	G:51.95 A:47.84	0.26***
	900	G:51.16 A:46.84	0.16*
	1302	C:51.37 T:47.07	0.20**
	1677	C:51.83 T:48.90	0.14*
	2135	C:51.30 T:48.67	0.14*

* Significant difference between the two accession classes. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

they could explain for 10–22% of the total genetic variation and phenotypic means of the respective SNP alleles, indicating that this candidate gene might act as Na^+ transporter. In addition, nine polymorphism sites were detected to be associated with shoot K^+ concentration under salt stress. Among them, SNP_{760}, a non-synonymous substitution, where the isoleucine encoded by ATT was replaced by valine encoded by GTT, was closely associated with shoot K^+ concentration under salt stress ($P < 0.001$), and could explain for 26% of the total variation, suggesting that *HvHKT1* might also mediate shoot K^+ homeostasis indirectly.

Within the *HvHKT2* gene, the two SNPs (pos. 1637 and 1768) in intron2 of *HvHKT2* were significantly associated with both shoot and root K^+ concentration under salinity

Table 4 Statistically associated SNPs with component traits of salinity tolerance in *HvHKT2*

Traits (mg g^{-1} DW)	SNP position	Means of SNP alleles (accession classes)	R^2 (variation explained)
Root Na^+ concentration	1267	G:18.15 C:51.35	0.13*
Root K^+ concentration	1637	T:5.21 A:4.51	0.10*
	1768	A:5.21 G:4.51	0.10*
Shoot Na^+ concentration	127	C:88.25 T:77.71	0.14*
	219	C:84.24 T:99.01	0.11*
	1012	T:87.39 A:78.34	0.10*
	1257	A:88.25 T:77.71	0.16*
	1258	A:88.25 T:77.71	0.16*
	1443	G:88.25 A:77.71	0.16*
	1575	A:88.25 G:77.71	0.16*
	1941	G:87.39 A:78.34	0.10*
Shoot K^+ concentration	1637	T:52.12 A:47.19	0.31***
	1768	A:52.12 G:47.19	0.30***

* Significant difference between the two accession classes. * $P < 0.05$ and *** $P < 0.001$

($P < 0.001$, $P < 0.05$), and could explain for 31 and 10% of the total variation, respectively (Table 4), revealing that the candidate gene might control K^+ transporting under saline condition directly. Additionally, there are one SNP (pos. 1267) and eight polymorphism sites associated with root and shoot Na^+ concentration under saline condition, respectively, and explained for 13% and 10–16% of the genetic variation, showing a significant difference between phenotypic means of the contrasting alleles. It can be seen that *HvHKT2* might also regulate Na^+ transporting when barley was exposed to saline environment.

Expression analysis of *HvHKT1* and *HvHKT2*

The expression patterns of *HvHKT1* and *HvHKT2* were investigated. For *HvHKT1* in the roots of the eight tested genotypes, the transcripts were all induced by salinity, showing nearly 50 times higher, on average, at 6 h after 150 mM NaCl treatment than the control. In addition, the degree of induction varied with time, showing that the relative expression was not changed significantly at 24 h compared with that at 6 h, while remarkably induced at 30 and 48 h when 300 mM NaCl was reached (Fig. 3). In terms of *HvHKT2*, the transcripts showed substantial reduction when the plants were exposed to salt stress (Fig. 3), with the decrease of about fivefold at 6 h compared with that of the control. At 24 h after 150 mM NaCl treatment, the transcripts of *HvHKT2* were significantly up-regulated in comparison with those at 6 h, although they were still lower than those of the control. However, when

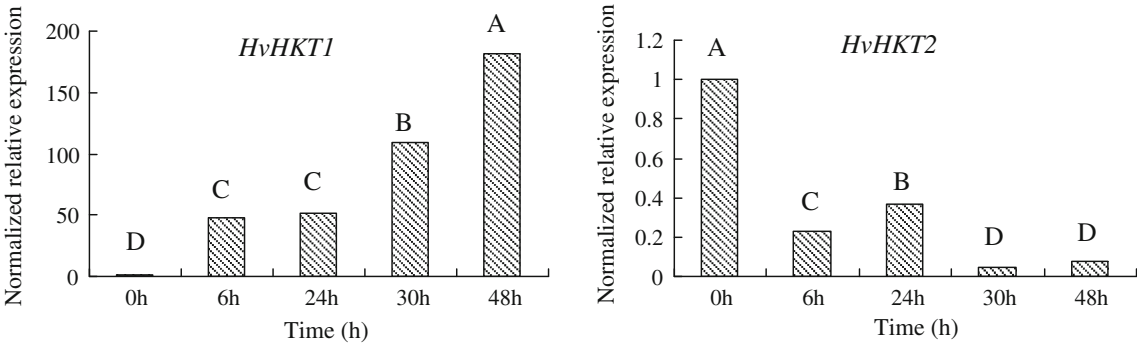

Fig. 3 Expression analysis of *HvHKT1* and *HvHKT2* by means of real-time RT–PCR amplification of RNA from root tissues of eight representative varieties (cultivated barley CM72 and Gairdner Tibetan wild barley T16, T61, T113, T115, T169, and T178). The number of hours (6, 24, 30, 48) elapsed after 150 mM NaCl treatment. *Capital letters* means statistical difference between treatments ($P < 0.01$)

plants were exposed to 300 mM NaCl, the expression of *HvHKT2* were down-regulated significantly, being reduced by around 20 times at 30 and 48 h compared with that of the control.

Discussion

Tibetan wild barley offers elite alleles for salt tolerance improvement of barley

Wild barley, mainly distributed in the Middle East's "fertile crescent" region and China's Qinghai-Tibet Plateau, is a primary gene pool of cultivated barley, since there is no reproductive isolation between them (Nevo 1992; Ceccarelli et al. 1995). Up to date, a number of Middle East types have been used in barley breeding programs (Ellis et al. 2000). Nevertheless, due to special geographical and social conditions, little study was done on Tibetan wild barley (Konishi 2001). In the current study, a hydroponic experiment was performed to evaluate salt tolerance of 189 Tibetan wild barleys. The results revealed a wide variation in salt tolerance among accessions. In particular, a lot of accessions, such as T16, T113, T115, and T178 exhibited stronger salt tolerance than CM72, which is commonly recognized as a salt-tolerant cultivar (Chen et al. 2005). It may be indicated that huge variation in salt tolerance existing in Tibetan wild barleys might offer elite alleles for salt tolerance improvement of barley.

Salt tolerance in Tibetan wild barley is mainly attributed to regulation of ion homeostasis

It was reported that the growth reduction during the first 'osmotic' phase of salinity is similar amongst genotypes of wheat or barley, while that in the second 'salt-specific' phase is different, which was attributed to difference in ability to avoid ion toxicity in the shoots (Munns et al. 1995). Additionally, Chen et al. (2005, 2007b, c) found that, on tissue level, the ability to maintain high K^+/Na^+ ratio (either acquisition of K^+ or exclusion of Na^+ from accumulating in leaves) is a key feature for salt tolerance in barley. Similarly, Garthwaite et al. (2005) found that salt tolerance in wild *Hordeum* species is associated with restricted entry of Na^+ and Cl^- into the shoots. The current results also showed that salt tolerance of Tibetan wild barley is mainly due to superior Na^+ exclusion and better maintenance of tissue K^+ concentration. In the present research, although on the whole a good correlation is seen between relative tissue biomass and ratios of the two ions, some genotypes did not observe the correlation. For an instance, genotypes T178 and T61 had Na^+ concentration of 102.1 and 65.8 mg g^{-1} DW, respectively, their relative shoot dry weights were 81 and 52%, respectively. The result suggested that tissue Na^+ concentration is not a single criterion for salt tolerance in Tibetan wild barley. The similar results were found in bread wheat (Genc et al. 2007), indicating that both exclusion and tissue tolerance should be paid attention for identification of genotypes with salinity tolerance.

HvHKT1 and *HvHKT2* acts as mediating Na^+ and K^+ homeostasis

In barley, Haro et al. (2005) first cloned the *HvHKT1* (AM000056), which was later renamed as *HvHKT2;1*. It belongs to Na^+-K^+ co-transporters, subfamily 2 (Platten et al. 2006). In the current study, we presumed that *HvHKT2* might mainly control K^+ transporter under saline condition, by association test. Furthermore, according to the results of expression analysis, the *HvHKT2* transcripts were substantially down-regulated after salt stress, corresponding to the decreased K^+ concentration in both shoots and roots of barley under salinity, confirming the function

of this candidate gene. In barley, subfamily 1 is considered as the sodium specific transporters, and there is only one copy of *HKT1*;1/2 in barley genome (Huang et al. 2008). The results in the present study validated the function of this candidate gene, showing *HvHKT1* is significantly associated with root Na^+ concentration under saline environment and up-regulated immediately after salt stress, the later is line with the significant increase of Na^+ concentration both in shoots and roots when the plant were exposed to salinity. Nevertheless, this study also detected several polymorphism sites in *HvHKT1* and *HvHKT2* associated with both K^+ and Na^+ concentration, indicating that the exact function of these two genes is complex. Therefore, more research is required to elucidate the function of these genes in the future.

Additionally, the allelic diversity was not associated with relative shoot biomass directly, which is used as an indicator of salt tolerance in the present paper. Therefore, it may be assumed that salinity tolerance is influenced by many additional factors, including other cation channels or transporters. These factors would contribute, to a different extent, to the expression of salinity tolerance. Additionally, as an indicator of salinity tolerance, the relative tissue weight has some disadvantages, for instance, the different plant growth rate would affect the phenotype under different environment, despite biomass production is a better predictor of yield compared with the ability to germinate in saline environment (Rawson et al. 1988; Munns 2002). Consequently, the other candidates, including gene-based and even genome-wide association analysis, should be further investigated in order to illustrate the genetic mechanisms of salt tolerance and develop the better barley cultivars.

In conclusion, association test and expression analysis revealed that the examined candidate genes have the function of regulating ionic homeostasis, which is the determinant trait of salinity tolerance. It may be indicated that Tibetan wild barley offers elite alleles of *HvHKT1* and *HvHKT2* conferring to salinity tolerance.

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