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Characterisation of two HKT1;4 Transporters from *Triticum monococcum* to elucidate the Determinants of the Wheat Salt Tolerance *Nax1* QTL

Running head: Insights into the wheat salt tolerance *Nax1* QTL

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Abbreviations: C_T, cycle threshold; *EF1α*, *Elongation factor 1α* gene, HKT family, High-affinity K⁺ Transporter family; I-V, current-voltage; ORF, open reading frame; qRT-PCR, quantitative (real-time) reverse transcription-PCR; QTL, quantitative trait locus; SE, standard error of the mean

Abstract

TmHKT1;4-A1 and *TmHKT1;4-A2* are two Na⁺ transporter genes that have been identified as associated to the salt tolerance *Nax1* locus found in a durum wheat (*Triticum turgidum* L. subsp. *durum*) line issued from a cross with *T. monococcum*. In the present study, we were interested in getting clues on the molecular mechanisms underpinning this salt tolerance QTL. By analysing the phylogenetic relationships between wheat and *T. monococcum* *HKT1;4*-type genes, we found that durum and bread wheat genomes possess a close homologue of *TmHKT1;4-A1*, but no functional close homologue of *TmHKT1;4-A2*. Furthermore, performing real-time RT-PCR experiments, we showed that *TmHKT1;4-A1* and *TmHKT1;4-A2* are similarly expressed in the leaves but that *TmHKT1;4-A2* is strongly more expressed in the roots, which would enable it to contribute more to the prevention of Na⁺ transfer to the shoots upon salt stress. We also functionally characterised the *TmHKT1;4-A1* and *TmHKT1;4-A2* transporters by expressing them in *Xenopus* oocytes. The two transporters displayed close functional properties (high Na⁺/K⁺ selectivity, low affinity for Na⁺, stimulation by external K⁺ of Na⁺ transport), but differed in some quantitative parameters: Na⁺ affinity was 3-fold lower and the maximal inward conductance was 3-fold higher in *TmHKT1;4-A2* than in *TmHKT1;4-A1*. The conductance of *TmHKT1;4-A2* at high Na⁺ concentration (>10 mM) was also shown to be higher than that of the two durum wheat *HKT1;4*-type transporters so far characterised. Altogether, these data support the hypothesis that *TmHKT1;4-A2* is responsible for the *Nax1* trait and provide new insight into the understanding of this QTL.

Key words: *HKT1;4*, *Nax1* salt tolerance QTL, qRT-PCR, sodium transport, *Triticum monococcum*, *Xenopus* oocyte.

Introduction

Salinity stress limits the growth and productivity of agricultural crops in many regions of the world (François *et al.*, 1986; Rengasamy, 2010). Na^+ ions can easily enter into root tissues of higher plants and then be transported throughout plant organs. Over-accumulation of sodium causes osmotic stress and inhibition of biochemical and physiological processes (Pardo *et al.*, 2006; Munns and Tester, 2008; Kronzucker and Britto, 2011; Nieves-Cordones *et al.*, 2016).

The two main cultivated wheats, durum wheat (*Triticum turgidum* subsp. *durum*) and bread wheat (*Triticum aestivum*), widely grown and consumed over the world and of major economic importance, display different sensitivity to salinity stress. Durum wheat is the most salt-affected. It is endowed with a lower aptitude, as compared to bread wheat, to exclude Na^+ , a trait associated with salt sensitivity in Triticeae (Zubaidi *et al.*, 1999; Munns *et al.*, 2006). In particular, durum wheat (constituted of A and B genomes) lacks the *Kna1* locus of Na^+ exclusion from the leaves (present in the D genome of the hexaploid bread wheat), which allows the maintenance of high K^+/Na^+ ratios in the leaves upon salt stress (Gorham *et al.*, 1987; Gorham *et al.*, 1990). An *HKT1;5*-type Na^+ transporter gene was demonstrated to be a likely candidate for the *Kna1* QTL (Byrt *et al.*, 2007; Byrt *et al.*, 2014). Besides, crossing the more salt tolerant wheat relative *Triticum monococcum* (Colmer *et al.*, 2003) with durum wheat, allowed to evidence two other loci of Na^+ exclusion from the leaves (or blades) originating from *T. monococcum*, *Nax1* (Na^+ exclusion 1) and *Nax2* (Na^+ exclusion 2), which were likewise found to correspond to *HKT* genes (respectively, of *HKT1;4* and *HKT1;5* types; Lindsay *et al.*, 2004; Huang *et al.*, 2006; Byrt *et al.*, 2007).

High-Affinity K^+ Transporter (HKT) genes encode Na^+ and/or K^+ transport systems, active at the cell plasma membrane (Corratgé-Faillie *et al.*, 2010; Almeida *et al.*, 2013; Véry *et al.*, 2014). While weakly represented in dicot species genomes (e.g., 1 single *HKT* gene in *Arabidopsis* and poplar), the *HKT* family comprises more members displaying a large functional diversity in monocots, including in cereals. For instance, rice (*Oryza sativa*) possesses 9 *HKT* genes (Garcia-deblás *et al.*, 2003), and barley (*Hordeum vulgare*) and wheat have been deduced from Southern blot analyses to possess 5 to 11 *HKT* genes per genome (Huang *et al.*, 2008). Based on phylogenetic and functional analyses, plant *HKT* genes have been divided into two subfamilies (Platten *et al.*, 2006). Subfamily 1 *HKT* (present in all higher plant species) encode Na^+ -selective transporters, while subfamily 2 ones (monocot specific) encodes systems permeable to both Na^+ and K^+ (Jabnourne *et al.*, 2009; Munns *et al.*, 2012; Oomen *et al.*, 2012; Sassi *et al.*, 2012; Ben Amar *et al.*, 2014; Byrt *et al.*, 2014; Suzuki *et al.*, 2016).

Na^+ transporters from the HKT subfamily 1 have been shown to play crucial roles in salinity tolerance in different plant species (Horie *et al.*, 2009; Munns *et al.*, 2012; Asins *et al.*, 2013). *AtHKT1;1*, which is expressed in vascular tissues (xylem parenchyma and phloem cells) of all *Arabidopsis* organs, has been shown to contribute to Na^+ retrieval from the ascending xylem sap and to Na^+ loading into the descending phloem sap upon salt stress, reducing the accumulation of Na^+ in shoots (Berthomieu *et al.*, 2003; Sunarpi *et al.*, 2005). In rice, the role of *OsHKT1;5*, deduced from the analysis of the salt tolerance *SKC1* (*shoot K^+ content*) trait to which this *HKT* gene is associated and from its tissue expression pattern, is quite similar (Ren *et al.*, 2005): the Na^+ transporter *OsHKT1;5* is expressed in vascular tissues (essentially in root xylem parenchyma), and retains Na^+ in roots upon salt stress thus enabling shoot tissues to maintain K^+ homeostasis. The bread wheat *TaHKT1;5-D* homologue (which corresponds to the *Kna1* QTL) is essentially expressed in root vasculature like *OsHKT1;5*, and has been proposed, similarly, to be involved in the control by the root of Na^+ transfer to the shoot in high salinity conditions (Byrt *et al.*, 2007; Byrt *et al.*, 2014).

Although subfamily 1 HKT transporters seem to share expression in vascular tissues and are therefore likely to be all involved in control of root to shoot Na^+ transfer (Hauser and Horie, 2010; Almeida *et al.*, 2013; Véry *et al.*, 2014), differences among members can be found in the organ where they are expressed and in functional properties (affinity for Na^+ , sensitivity to K^+ , direction of transport, ...), leading to different expected contribution to the control of Na^+ translocation and accumulation. In rice, for instance, *OsHKT1;1*, *OsHKT1;4* and *OsHKT1;5*, which contribute to Na^+ exclusion from leaf blades upon salt stress, are reported to be expressed mainly in roots for *OsHKT1;5*, mainly in leaf sheaths and panicles for *OsHKT1;4*, and in the whole leaf *OsHKT1;1* (Ren *et al.*, 2005; Cotsaftis *et al.*, 2012; Wang *et al.*, 2015; Suzuki *et al.*, 2016). In wheat, the analysis of the *Nax1* and *Nax2* sources of leaf Na^+ exclusion, which correspond to *TmHKT1;4* type and *TmHKT1;5*-type genes, also suggests that these *HKT*-type genes display distinctive expression patterns, root and leaf sheath for the former, or root only for the latter (James *et al.*, 2006).

Here, we were interested in further analysing the bases of the *Nax1* QTL. Fine mapping had localised two *T. monococcum* *HKT1;4* genes, *TmHKT1;4-A1* and *TmHKT1;4-A2*, at the *Nax1* locus (Huang *et al.*, 2006). The characterisation of the two *T. monococcum* genes had just been initiated, providing RT-PCR detection of *TmHKT1;4-A2* in roots and leaf sheath (Huang *et al.*, 2006), a pattern compatible with *Nax1* role in leaf blade desalinization (James *et al.*, 2006). On the other hand, functional data were available on two *TmHKT1;4* relatives from durum wheat, *TdHKT1;4-1* and *TdHKT1;4-2* (Ben Amar *et al.*, 2014), but not on the *T. monococcum* *HKT1;4* transporter(s) responsible for the trait of improved salt tolerance. In the present study, we

have performed a detailed characterisation of the two *T. monococcum* *HKT1;4* genes mapped to the *Nax1* locus. Real-time PCR experiments allowed to compare the expression patterns of both *TmHKT1;4* genes. We have also analysed the functional properties of the two *TmHKT1;4* transporters, heterologously expressed in *Xenopus laevis* oocytes, in order to compare them with each other and with their previously characterised durum wheat close homologues. Altogether, these analyses enabled to shed light on the molecular determinants of the *Nax1* trait of salt tolerance.

Materials and methods

Plant material and growth conditions

Seeds of *Triticum monococcum* (cv Turkey) were provided by the ICARDA (Genetic resources Unit, Syria). They were treated with 10% potassium hypochlorite solution for 15 min, thoroughly washed three times with sterile water and germinated on a sheet of Whatman filter paper placed in a Petri dish. The dishes were stored in a growth room at 23°C and 65% hygrometry, under light/dark conditions of 16 h light at 250 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ /8 h dark.

For quantitative real-time PCR analysis, seven days after germination on filter paper moistened with sterile water, the seedlings were transferred to hydroponic culture conditions (same growth chamber as for germination in Petri dishes) onto half-strength Hoagland solution (Davenport *et al.*, 2005). The growth solution was changed every week. After 18 days, salt stress treatments were applied. NaCl concentration of the culture medium was increased to a final concentration of 100 or 200 mM. Progressive increases in salt concentration were performed by addition of 50 mM NaCl twice within 2 days (for 100 mM treatment) or four times within 3 days (for 200 mM treatment). Control plants were kept in half-strength Hoagland medium. Plant tissue samples (whole shoot and roots) were collected after 6, 24, 48, 72, 96 or 192 h of final salt treatments, immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation and expression analyses.

Molecular cloning of the TmHKT1;4 cDNAs

Total RNA were isolated from seven-day-old *Triticum monococcum* seedlings germinated on filter paper moistened with 100 mM NaCl, using the RNeasy plant mini kit (Qiagen). To remove the remaining genomic DNA, seven microgrammes of RNA were treated with DNase (Promega). First strand cDNA were synthesized from 3 μg of total RNA, using the SuperScript®III Reverse transcriptase kit (Invitrogen), according to the

manufacturer's protocol. For PCR amplification, specific primers were designed for *TmHKT1;4-A1* (forward 5'-ATGGCCGGAGCTCGTCATTAAGGTC-3', and reverse 5'-CTAACTAAGCTTCCAGGCTTT-3') and *TmHKT1;4-A2* (forward 5'-ATGGCCGGAGCTCATCATTAAAGGTC-3', and reverse 5'-CTAACTAAGCTTCCAGGCTTT-3'). The PCR protocol consisted in an initial denaturation at 94°C for 5 min followed by 35 cycles comprising a first step at 94°C for 30 s, an annealing step at 55°C for 30 s and an elongation at 72°C for 1 min, and final 10 min extension at 72°C. Purified amplified products were cloned in pGEM-T Easy vector and sequenced.

Real-time PCR

Total RNA, extracted using the RNeasy plant mini kit (Qiagen), were treated using the "DNase I" kit (Invitrogen). First strand cDNA synthesis was performed from 2.5 µg of DNase-treated RNA using the "SuperScript III Reverse transcriptase" kit (Invitrogen). Real-time PCR was performed in 384-well plates with the Light Cycler® 480 Real-Time PCR System (Roche) using SYBR Green I (Roche). Primers, designed using Primer 3 software for the two *TmHKT1;4* genes as well as for the actin and *EF1α* housekeeping genes, were as follows: *TmHKT1;4-A1* (forward 5'-GCACGCTTCTGTCACAACCTC-3', reverse 5'-CTGACGACTTCGACGAGGAT-3'), *TmHKT1;4-A2* (forward 5'-GGAGACACAGGGTATCAGGC-3', reverse 5'-AGCTTCTCCCTCTCGGTGAC-3'), *actin* (forward 5'-TGCATAGAGGGAAAGCACG-3', reverse 5'-AACCCAAAAGCCAACAGAGA-3'), *EF1α* (forward 5'-ATGGTTGTTACCTTTGGCCC-3', reverse 5'-CAACATTGTCACCAGGAAGC-3'). PCR reactions were performed in a 10 µl final volume containing 3 µl cDNA (obtained from 40 ng of DNase-treated RNA), 0.5 µl of each primer (at 10 µM), 5 µL 2x SYBR Green I master mix and 1 µl of RNase-free water (Sigma). The reaction consisted in an initial denaturation at 94°C for 10 min followed by 45 cycles composed of 10 s at 94°C, 10 s at 60°C, and 15 s at 72°C, then a melting curve (5 s at 95°C, 1 min at 65°C and 5 min with temperature increasing from 65°C to 97°C). Three biological repetitions were performed for each experimental condition, with 3 technical repetitions for each sample. Absolute numbers of cDNA molecules were determined using standard curves obtained from dilution series of known amounts of fragments from the corresponding cDNA. Rough expression values of *TmHKT1;4* genes were slightly corrected using a normalization factor obtained by geometric averaging of *actin* and *EF1α* expression values using geNORM v.3 software (Vandesompele *et al.*, 2002).

Expression of HKT1;4 transporters in Xenopus laevis oocytes

The complementary DNA of *TmHKT1;4-A1* and *TmHKT1;4-A2* were sub-cloned into a modified pGEMHE vector (Lebaudy *et al.*, 2010) between the *EcoRI* and *SpeI* restriction sites present in the vector. Capped and polyadenylated cRNA were synthesized *in vitro* from linearized vector using the mMESSAGE mMACHINE T7 kit (Ambion) following manufacturer's instructions. Oocytes were isolated as previously described (Véry *et al.*, 1995), injected with 20 ng of *HKT1;4* cRNA (in 20 nl of RNase-free water) or with 20 nl of RNase-free water for control oocytes, and kept at 18°C in ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 2.5 mM sodium pyruvate, and 5 mM HEPES-NaOH, pH 7.4) supplemented with 0.5 mg.l⁻¹ gentamycin, until voltage-clamp recordings. The electrophysiological measurements on injected oocytes were made 1 to 2 days following the injection as described by Mian *et al.* (2011). The perfusion solutions contained as background 6 mM MgCl₂, 1.8 mM CaCl₂, and 10 mM MES-1,3-bis[tris(hydroxymethyl) methylamino] propane, pH 5.5. Monovalent cations were added to the background as glutamate or chloride salts. The chloride concentration was constant in each set of solutions. The osmolarity of all solutions was adjusted when necessary using D-mannitol, being set to 220 to 240 mOsmol.l⁻¹. Data acquisition and analysis were performed using the pCLAMP10 program (Axon instruments) and Sigmaplot11 (Jadel Scientific) software. To extract HKT1;4-mediated currents from total oocyte currents, mean currents recorded in water-injected control oocytes from the same batch in the same ionic conditions were subtracted from those recorded in the HKT1;4-expressing oocytes. HKT1;4 current–voltage (I–V) relationships were constructed with transporter extracted currents.

Results

Phylogenetic relationships between Triticum monococcum and wheat HKT1;4 transporters

The putative Na⁺ transporter genes *TmHKT1;4-A1* and *TmHKT1;4-A2* from *Triticum monococcum* had been previously identified as co-localising with the salt tolerance *Nax1* locus in a durum wheat line derived from a cross with *Triticum monococcum* (Huang *et al.*, 2006; James *et al.*, 2006). Whereas durum wheat possesses 2 genomes (A and B), and bread wheat 3 genomes (A, B and D), the wheat relative *Triticum monococcum* has only a A genome. Southern blot analyses have suggested that *TmHKT1;4-A1* and *TmHKT1;4-A2* are the sole *TmHKT1;4*-type genes in *Triticum monococcum* (Huang *et al.*, 2006). In contrast, four or five *HKT1;4*-type genes in durum wheat and eight ones in bread wheat have been predicted by such analyses (Huang *et al.*, 2006; Huang *et al.*, 2008).

The coding regions of the *T. monococcum* *TmHKT1;4-A1* and *TmHKT1;4-A2* transporter genes were amplified by RT-PCR using specific primers based on full-length sequences retrieved from GenBank (NCBI) database (accessions EF062820.1 and EF062819.1, respectively). The genomic structure of *TmHKT1;4-A1* displays two introns like that of all rice *HKT* genes (Garcia-deblás *et al.*, 2003; Huang *et al.*, 2006), whereas that of *TmHKT1;4-A2* lacks the second intron (Huang *et al.*, 2006; Fig. 1A). The open reading frame of *TmHKT1;4-A1* is slightly longer than that of *TmHKT1;4-A2* (1692 and 1665 bp, respectively; Huang *et al.*, 2006; Fig. 1A), the translated sequences displaying 84% identity.

In order to further compare the two *T. monococcum* transporters with their wheat HKT1;4-type homologues, blast searches were performed on GenBank database and on released sequences from the (bread and durum) wheat genome sequencing projects (<http://wheat-urgi.versailles.inra.fr/Seq-Repository/Assemblies>; The International Wheat Genome Sequencing Consortium (IWGSC), 2014). A phylogenetic tree was constructed with translated protein sequences from identified genes/mRNAs. Rice HKT1;4 and HKT1;5 sequences were also included as HKT-type references (Garcia-deblás *et al.*, 2003) (Fig. 1B). Two durum wheat *HKT1;4*-type full-length mRNAs (*HKT1;4-1* and *HKT1;4-2*) had been already isolated (Ben Amar *et al.*, 2014). One more durum wheat *HKT1;4* member (*TdHKT1;4_2BL4*) was found in a durum wheat chromosome 2BL scaffold (n° 5397702; Fig. 1B), the already identified *TdHKT1;4-2* gene being present (ORF fully identical) in another 2BL scaffold (n° 2592336). Among the three identified *TdHKT1;4* transporters, *TdHKT1;4-1* was very similar to *T. monococcum* HKT1;4-A1 (97% identity, Fig. 1B), and none of them was closely related to *TmHKT1;4-A2* (only 75 to 84% identity).

Survey of the bread wheat genomic sequences allowed also to evidence HKT1;4 members corresponding (or highly similar) to the three identified durum wheat ones (all in chromosome 2BL scaffolds; Fig. 1B). An additional chromosome 2BL member (*TaHKT1;4_2BL5*, scaffold 8005063) displayed translated sequence closer to *TmHKT1;4-A2* (90% identity). Beside B genome *HKT1;4* members, these *in silico* searches also identified a sequence corresponding to the last two exons (25% of the coding sequence) of a *HKT1;4* gene in a D genome scaffold (2DL n° 9906616). At last, a A genome *HKT1;4* appeared as highly similar to *TmHKT1;4-A2* (2AL scaffold n° 6425420; 96 and 94% identity at the nucleic and amino acid levels, respectively). This wheat gene was however not predicted to encode a functional HKT1;4 transporter due to the presence of an early STOP codon (C/A substitution at 13% of the coding sequence), and additionally downstream, of two deletions affecting the reading frame.

Expression patterns of TmHKT1;4-A1 and TmHKT1;4-A2 in Triticum monococcum grown under different saline conditions

Previous analyses (by semi-quantitative RT-PCR) detected *TmHKT1;4-A2* transcripts both in roots and leaf sheaths, but no *TmHKT1;4-A1* transcripts in the corresponding tissues (Huang *et al.*, 2006). However, only plants treated for 48 h with 50 mM NaCl salinity stress were analysed in this report. Here, tissue expression of *TmHKT1;4-A1* and *TmHKT1;4-A2* was investigated using real-time RT-PCR in *T. monococcum* plants grown either in standard conditions or subjected to different salinity stresses (addition of 100 or 200 mM NaCl), the effect of 100 mM NaCl stress on both gene expression being followed over 8 days.

TmHKT1;4-A1 gene expression, in line with absence of detection in the previous report, appeared to be low in both roots and leaves of 3-4 week-old plants, either grown in control conditions or subjected to salt stress (Fig. 2A). *TmHKT1;4-A2* gene was much more expressed, especially in roots where its transcripts were 40 to 100-fold more abundant than those of *TmHKT1;4-A1*, both in plants grown in control or in salt stress conditions (Fig. 2B). *TmHKT1;4-A2* transcript accumulation was found to be predominant in roots (6 to 10-fold more than in leaves), while the level of *TmHKT1;4-A1* transcript accumulation was similar in roots and leaves.

The 100 mM NaCl salt stress had little effect on *TmHKT1;4-A2* expression (Fig. 2D). Only slight and late expression increases (by no more than 2-fold from 4-8 days after stress application; Fig. 2D) were indeed observed. In *TmHKT1;4-A1*, the 100 mM NaCl stress also weakly affected root expression (2-fold increase 8 days after application), but increased leaf expression from two days after application, by up to 4-fold after 8 days (Fig. 2C).

Functional properties of TmHKT1;4-A1 and TmHKT1;4-A2 expressed in Xenopus oocytes

In order to get precise information on TmHKT1;4-A1 and TmHKT1;4-A2 functional properties, the two transporters were expressed in *Xenopus laevis* oocytes and two-electrode voltage-clamp experiments were performed. Injections of 20 ng cRNA per oocyte of either of the *TmHKT1;4* genes allowed to record large non-endogenous currents (10 to >100 times larger than those of control water-injected oocytes) 1 to 2 days following the injection, demonstrating that TmHKT1;4-A1 and TmHKT1;4-A2 were efficiently expressed and targeted to the oocyte membrane (Fig. 3A, B).

TmHKT1;4-A1 and TmHKT1;4-A2 phylogenetically belong to the HKT subfamily 1 and could thus be predicted to be Na⁺-selective transporters (Mäser *et al.*, 2002; Platten *et al.*, 2006). The cation selectivity of both TmHKT1;4 transporters was precisely compared, performing monovalent cation (Na⁺, K⁺, Li⁺, Cs⁺ and Rb⁺)

exchanges in the solution bathing the oocytes (Fig. 3). The external cation concentration was 3 mM. Inward or outward TmHKT1;4 currents were recorded, depending on voltages applied, in the presence of the different cations. The reversal potential of both transporter currents and the macroscopic inward conductance of both transporters, were however strongly different when K^+ , Li^+ , Cs^+ or Rb^+ replaced Na^+ , as expected for Na^+ -selective transporters (Fig. 3A, B). The reversal potential of both transporter currents was close to -80 mV in the presence of 3 mM external Na^+ and was negatively shifted in the presence of K^+ , Li^+ , Cs^+ or Rb^+ at the same concentration, by about 35-40 mV in TmHKT1;4-A1 and 60 mV in TmHKT1;4-A2 (Fig. 3A-D). This indicated, based on Goldman-Hodgkin-Katz formalism, permeability ratios P_{Na}/P_X (X being either of the four other cations) close to 5 and 11, respectively, for TmHKT1;4-A1 and TmHKT1;4-A2. The macroscopic inward conductance in the presence of sodium, as compared to that in presence of the other cations, was about 2 times higher in TmHKT1;4-A1 and 3 to 4 times higher in TmHKT1;4-A2 (Fig. 3E, F). Altogether, these results confirm that both transporters are Na^+ -selective, but that the selectivity for Na^+ is slightly higher in TmHKT1;4-A2 than in TmHKT1;4-A1.

Several Na^+ -selective HKT transporters have been reported to be sensitive to the presence of K^+ in the external medium, K^+ either reducing or enhancing currents (Jabnourne *et al.*, 2009; Munns *et al.*, 2012; Ben Amar *et al.*, 2014). For instance, in *T. monococcum*, TmHKT1;5 was characterised as a Na^+ -selective uniporter blocked by external K^+ at low external Na^+ concentration (Munns *et al.*, 2012). In contrast, in durum wheat, the presence of external K^+ was reported to enhance Na^+ transport through TdHKT1;4-1 and TdHKT1;4-2 (Ben Amar *et al.*, 2014). The effect of external K^+ on Na^+ transport by both TmHKT1;4 transporters was examined at two external Na^+ concentrations, 0.1 or 3 mM, K^+ being added at 30 or 50 mM, respectively (Supplementary Fig. S1A, B). In both transporters, inward and outward currents were clearly enhanced in the presence of K^+ (1.5 to 2-fold increase in conductance in the presence of K^+ at both submillimolar and millimolar Na^+ concentrations; Supplementary Fig. S1A, B). Detailed analysis indicated that the increase in TmHKT1;4-A1 and TmHKT1;4-A2 currents in the presence of external K^+ was accompanied by small shifts of zero-current potential. These small shifts, which are in agreement with the much higher permeability to Na^+ than to K^+ of both transporters, suggested that the additional current in the presence of K^+ was mostly carried by Na^+ and not by K^+ , and hence that Na^+ transport activity is stimulated by external K^+ in both transporters.

To determine the affinity for Na^+ of TmHKT1;4-A1 and TmHKT1;4-A2, different concentrations of Na^+ in the external medium were used (0.1, 1, 10, 50 and 100 mM). TmHKT1;4-A1 and TmHKT1;4-A2 currents strongly responded to these changes in external Na^+ concentration, displaying positive shifts of zero-current

potential and increased inward conductance when the external Na^+ concentration was increased (Fig. 4A, B). The mean change in the reversal potential of currents was of 50 mV per 10-fold change in the external Na^+ activity in the Na^+ concentration range of 1 to 100 mM, in both transporters (Fig. 4C, D). This confirmed that Na^+ was the main ion passing through TmHKT1;4-A1 and TmHKT1.4-A2 in these conditions, the observed variation in reversal potential being close to the expected value for a purely Na^+ -selective system (58 mV per 10-fold change in the external Na^+ activity). Fitting the response of the inward conductance to increased external Na^+ concentrations with Michaelis–Menten hyperbolic functions allowed to compare the affinity for Na^+ of the two transporters (Fig. 4E, F). The concentration at which half saturation of the inward conductance occurred (apparent K_M) was determined to be 12 and 33 mM in TmHKT1;4-A1 and TmHKT1.4-A2, respectively. Thus, both transporters displayed low affinity for Na^+ transport, the affinity of TmHKT1.4-A2 being however 3-fold lower than that of TmHKT1;4-A1. Analysis of conductance saturation kinetics using the Michaelis–Menten formalism also suggested that the maximal inward conductance (equivalent to the V_{\max} parameter) was about 3-fold higher in TmHKT1;4-A2 than in TmHKT1;4-A1.

Discussion

HKT1;4-type genes in wheat display high diversity

Whereas a single *HKT1;4*-type gene is present in rice (Garcia-deblás *et al.*, 2003), several *HKT1;4*-type genes have been identified in the different wheat genomes based on Southern blot analyses (Huang *et al.*, 2006, Huang *et al.*, 2008): 2 in the A genome of durum and bread wheats, 2 in the B genome of durum wheat, and 3 in the B and D genomes of bread wheat. Thus, *HKT1;4*-type genes appears as the most duplicated (as compared to rice) *HKT* genes in wheat. For instance, *HKT1;1*- and *HKT1;3*-type genes are expected to display one copy at most in each wheat genome. Also, *HKT2;4* for which a close homologue exists in rice has been shown to be present as a single copy in each wheat genome (Garcia-deblás *et al.*, 2003; Huang *et al.*, 2008; Ariyaratna *et al.*, 2016).

Searching in genome and transcript (GenBank, wheat-urgi) databases confirmed the important number of *HKT1;4*-type genes in wheat: 3 genes found in durum wheat, and 6 genes in bread wheat including 2 clear counterparts of the durum wheat genes (Fig. 1B). This totalizes 75% of the expected number of genes of this type based on the initial Southern blot analyses (Huang *et al.*, 2006, Huang *et al.*, 2008). The presently available information also indicates that the genomic allocation of these genes would be slightly different from that predicted by the Southern blots. Most sequences (4 over 7) are present in the B genome (chromosome 2L; in durum wheat, *Td_2BL4* scaffold 5397702 and *TdHKT1;4-2* mRNA corresponding to *Td_2BL9* scaffold

2592336). Only 1 incomplete *HKT* sequence is found allocated to the D genome (chromosome 2L), and 1 to the A genome in bread wheat (chromosome 2L). This latter A genome *HKT1;4* (2AL2 scaffold 6425420) can be predicted to be not functional due to deletion-induced frame shifts and presence of early STOP codon. Thus, at least in durum wheat, the A genome is certainly the lowest contributor to the set of functional *HKT1;4* genes. Available information suggests that this is also the case for other wheat *HKT* types (Huang *et al.*, 2008; Ariyaratna *et al.*, 2014).

In addition to the large number of *HKT1;4*-type transporters in wheat, the level of sequence conservation among them is rather low, when compared with that in the other groups of *HKT* transporters. Indeed, while the levels of sequence identity among all *TaHKT2;1*- or *TaHKT2;4*-type members are $\geq 90\%$ (Ariyaratna *et al.*, 2014; Ariyaratna *et al.*, 2016), and those among the reported *TaHKT1;5* members are $\geq 88\%$ (Byrt *et al.*, 2014; Fig. 1B), those found among *TaHKT1;4* or *TdHKT1;4* members are between 74% and 90% (Fig. 1B). This suggests that the *HKT1;4*-type transporter group in wheat displays a higher functional diversity amongst its members than the other *HKT*-type transporter groups. Interestingly also, another source of diversity in this *HKT1;4*-type group is present in the wheat relative *Triticum monococcum* (Fig. 1B), which was at the basis of the *Nax1* salt tolerance QTL (see below “New insights into the understanding of the *Nax1* QTL”). Further diversity may exist in other wheat relatives (e.g., possessing D genome; Colmer *et al.*, 2006).

The physiological meaning of the large number of *HKT1;4* genes and their high level of sequence variability in wheat is still unclear. This may reflect a particular physiological importance for this *HKT* transporter type in several distinct functions. The significant contribution of this *HKT* group to wheat adaptation to salinity stress has been revealed by the discovery of the *Nax1* QTL (Huang *et al.*, 2006; James *et al.*, 2006; James *et al.*, 2012).

New insights into the understanding of the Nax1 QTL

TmHKT1;4-A1 and *TmHKT1;4-A2* both co-localise with the *Nax1* locus in a durum wheat line possessing A genome chromosome fragment(s) from *T. monococcum* (Huang *et al.*, 2006). The wheat A-genome progenitor is believed to be that of the wild relative *Triticum Urartu*, the genome of *Triticum monococcum* being less closely related (Khlestkina and Salina, 2001; Ling *et al.*, 2013). Southern blot analyses of the *Nax1* locus had led to the conclusion that a *TmHKT1;4-A2* counterpart is present in durum wheat at the respective locus but no *HKT1;4-A1* gene (Huang *et al.*, 2006). At least one close homologue of *TmHKT1;4-A1* is however present in durum wheat (*TdHKT1;4-1*, 98% identity in coding sequences; Ben Amar *et al.*, 2014). This suggests that transporters with

close functional features, and maybe also similar level of activity and location in planta, are present in both species (see also below). With respect to the *TmHKT1;4-A2* counterpart in wheat, this seems to be the A genome *HKT1;4_2AL2* pseudogene (98% identity in genomic coding sequences, outside of the deleted regions). *HKT1;4_2AL2* sequence has been so far identified only in bread wheat genomic database. However, the presence of this pseudogene in durum wheat is likely, owing to the large conservation of sequence observed between bread wheat and durum wheat (e.g., 99.8 to 100% identity for the coding sequences of the available *HKT1;4*-type and *HKT1;5*-type genes; Fig. 1B). Hence, it can be assumed that no functional counterpart of *HKT1;4-A2* exists in bread and durum wheats. Within the framework of this hypothesis, *HKT1;4-A2* is thus more likely responsible for the *Nax1* trait.

TmHKT1;4-A2 has also been proposed as candidate for *Nax1* based on compatible expression pattern (Huang *et al.*, 2006). Indeed, the analysis of *Nax1* trait suggested that the corresponding gene was involved in the retrieval of Na^+ from the xylem ascending sap in both the root and the leaf sheath (James *et al.*, 2006), and a preferential accumulation of *HKT1;4-A2* transcripts had been accordingly observed in roots and leaf sheaths (Huang *et al.*, 2006). The present real-time PCR data indicate that, in roots, the accumulation level of *HKT1;4-A2* transcripts is about 20 times higher than that of *HKT1;4-A1* (Fig. 2A and B). The expression level of *TmHKT1;4-A2* appears as weakly sensitive to salt stress, in leaves as well as in roots (Fig. 2), like that of the *TmHKT1;5-A* gene shown to be involved in the *Nax2* QTL (Munns *et al.*, 2012). It is however worth to note that substantial expression in leaves, beside roots, is a specificity of *Nax1*, as compared to the other salt tolerance QTL identified in cereals, which concern *HKT1;5*-type genes (Ren *et al.*, 2005; James *et al.*, 2006; Munns *et al.*, 2012; Byrt *et al.*, 2014). Since the difference between the expression levels of *HKT1;4-A1* and *HKT1;4-A2* is much lower in leaves than in roots (Fig. 2A and B) and *HKT1;4-A1* displays increased expression in leaves upon salt stress (Fig. 2C), it cannot be excluded that *HKT1;4-A1* brings some contribution to the *Nax1* trait in the leaves.

The functional characterisation of *TmHKT1;4-A1* and *TmHKT1;4-A2* showed similarity in the transporter general properties (selectivity for Na^+ , bidirectional transport ability, low affinity for Na^+ , enhancement by external K^+ of Na^+ transport; Figs 3 and 4, Supplementary Fig. S1) but also revealed quantitative differences: the Na/K selectivity is ~2-fold higher in *TmHKT1;4-A2* than in *TmHKT1;4-A1* (Fig. 3, the affinity for Na^+ is 3-fold lower in *TmHKT1;4-A2* than in *TmHKT1;4-A1* (Fig. 4E, F), and the maximal transporter inward conductance in oocytes injected with same amount of transcripts is 3-fold higher in *TmHKT1;4-A2* (Fig. 4E, F). Overall, this suggests that *TmHKT1;4-A2* would be more efficient than

TmHKT1;4-A1 (if similarly expressed) at taking up Na^+ at high external Na^+ concentrations. The inward conductance of both transporters at different Na^+ concentrations was compared in Fig. 5: in all Na^+ concentrations in the 1 to 100 mM range, the conductance of TmHKT1;4-A2 was significantly higher than that of TmHKT1;4-A1, a ratio of at least 2 between the two conductances being observed from 50 mM Na^+ .

Two from the likely three (Fig. 1B; Huang *et al.*, 2008; discussion above) functional HKT1;4-type transporters existing in durum wheat, TdHKT1;4-1 and TdHKT1;4-2, have been previously characterised (Ben Amar *et al.*, 2014). Their functional features were very close to those of the two TmHKT1;4 transporters characterised in the present report. Even the enhancement by external K^+ of Na^+ transport, an unusual property among HKTs, was observed. Like the *T. monococcum* HKT1;4-type transporters, the durum wheat ones were shown to slightly differ in their affinity for Na^+ and their maximal inward conductance (Ben Amar *et al.*, 2014). For fine comparison, Fig. 5 displays conductance data concerning these two durum wheat transporters (obtained in the same conditions as those of the *T. monococcum* transporters) alongside those of the *T. monococcum* transporters. TmHKT1;4-A1 and TdHKT1;4-1, which display high sequence homology, have very close conductances in the 1 to 100 mM external Na^+ concentration range. Interestingly, TmHKT1;4-A2 conductance is the highest from Na^+ concentrations ≥ 10 mM. Although TdHKT1;4_2BL4 has still to be characterised in order to get a complete view of the durum wheat equipment in HKT1;4-type transporters, and comparative expression features of *T. monococcum* and durum wheat *HKT1;4* genes have to be obtained, this already suggests that TmHKT1;4-A2 could be more conductive than its durum wheat homologues in salt stress conditions.

Few data allowing to understand the different wheat salt tolerance QTL linked to *HKT1;5* transporter genes are available so far. The durum wheat *Kna1* QTL involving the bread wheat D genome *TaHKT1;5* gene could certainly be explained by the low level of expression of the three durum wheat *HKT1;5* genes (from B genome) as compared to *TaHKT1;5-D* (Byrt *et al.*, 2014). The *Nax2* QTL involving the *T. monococcum* *TmHKT1;5-A* gene might be similarly explained by higher expression of the *T. monococcum* *TmHKT1;5* gene as compared to durum wheat ones (Byrt *et al.*, 2007; Munns *et al.*, 2012; Byrt *et al.*, 2014). For both QTL, possible functional differences between HKT1;5 transporters from durum wheat *versus* bread wheat or *versus* *T. monococcum* were not addressed. Here, we show that the *Nax1* QTL could probably be explained by absence of functional *TmHKT1;4-A2* counterpart gene in durum wheat, a gene strongly expressed in roots and encoding a transporter displaying a particularly high Na^+ conductance, as compared to that of the durum wheat HKT1;4 transporters characterised so far, in the presence of high Na^+ concentrations.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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Legends to figures

Fig. 1 Gene structure (A) and phylogenetic relationships (B) of the *Triticum monococcum* HKT1;4-type transporters. (A) Open reading frame of *TmHKT1;4-A1* and *TmHKT1;4-A2* genes compared to that of the *Oryza sativa* *OsHKT1;4* gene. The gray rectangles represent exons. Triangles mark positions and sizes of introns. (B) Phylogenetic tree of *TmHKT1;4* transporters and their close homologues in wheat and rice. Polypeptide sequences were obtained from BLAST search on GenBank NCBI databases and, for HKT1;4 homologues, on genomic sequences recovered from the URGI website (<http://wheat-urgi.versailles.inra.fr/Seq-Repository/Assemblies>; v1 download 2AL-, 2BL-, 2DL-ab-k71-contigs.fa.longerthan_200.fa.gz, TGAC_WGS_durum_v1.fasta). The unrooted phylogenetic tree was constructed using full polypeptide sequences aligned with MUSCLE v3.8.31 (Edgar, 2004), and the neighbour-joining method with 1000 bootstrap replicates, using PhyML version 20131022 software (<http://phylogeny.lirmm.fr>). The tree was drawn using Dendroscope (Huson *et al.*, 2007). Bootstrap values (as percentages) are indicated at the corresponding nodes. The protein (GenBank) or genomic scaffold accession numbers are: *OsHKT1;4*, Q7XPF7.2; *OsHKT1;5*, Q0JNB6.1; *Ta_2BL4+2BL1*, scaffolds 7988520 and 7944580; *Ta_2BL5*, scaffold 8005063; *Ta_2BL7*, scaffold 8039827; *Ta_2BL9*, scaffold 8092286; *TaHKT1;5-B1*, ABG33943; *TaHKT1;5-B2*, ABG33944; *TaHKT1;5-D*, ABG33945; *Td_2BL4*, scaffold 5397702; *TdHKT1;4-1*, KF443078; *TdHKT1;4-2*, KF443079; *TmHKT1;4-A1*, ABK41858; *TmHKT1;4-A2*, ABK41857; *TmHKT1;5*, ABG33939. Os, *Oryza sativa*; Ta, *Triticum aestivum*; Td, *Triticum turgidum* subsp. *durum*; Tm, *Triticum monococcum*.

Fig. 2 Real-time RT-PCR analysis of *TmHKT1;4-A1* (A, C) and *TmHKT1;4-A2* (B, D) expression in tissues of *Triticum monococcum* grown under different saline conditions. (A, B) Absolute quantification of *TmHKT1;4-A1* and *TmHKT1;4-A2* transcript levels in roots and leaves of plants grown in standard conditions or subjected to 100 or 200 mM NaCl treatment for 72 h. (B, D) Evolution of *TmHKT1;4-A1* and *TmHKT1;4-A2* expression in roots (top panels) and leaves (bottom panels) after the addition of 100 mM NaCl for 6, 24, 48, 96 or 192 h.

Transcript levels were given relative to the average ones in control plants (kept in standard conditions) at the same time. Data in (A-D) are means \pm SE of three biological replicates.

Fig. 3 Cation selectivity of TmHKT1;4-A1 (A, C, E) and TmHKT1;4-A2 (B, D, F). *Xenopus laevis* oocytes were successively bathed with external solutions composed of the standard background supplemented with either Na⁺, K⁺, Li⁺, Cs⁺ or Rb⁺ (as chloride salts), at 3 mM. The voltage-clamp protocol consisted of 12 pulses of 1 s, with a voltage increment of 15 mV between pulses. (A, B) Current-voltage (I-V) curves. Insets in (A) and (B) displays I-V relationships in water-injected control oocytes belonging to the same batch as HKT1;4-expressing ones. (C, D) Reversal potential of currents through the HKT1;4 transporters in the different solutions. (E, F) Macroscopic inward conductance of the HKT1;4 transporters (determined close to the reversal potential) in the presence of the different cations. Data are means \pm SE ($n=3$ in (A, C, E) and $n=5$ in (B, D, F)) and are representative of at least two experiments performed on different oocyte batches

Fig. 4 Effect of external Na⁺ on TmHKT1;4-A1 and TmHKT1;4-A2 currents. (A, B) I-V relationships in TmHKT1;4-A1 (A) and TmHKT1;4-A2-expressing oocytes (B) in bath solutions containing Na⁺ (as glutamate salt) at different concentrations (0.1, 1, 10, 50, and 100 mM). (C, D) Effect of external Na⁺ activity on the reversal potential of currents through TmHKT1;4-A1 (C) and TmHKT1;4-A2 (D). (E, F) Inward conductance dependency on external Na⁺ concentrations in TmHKT1;4-A1 (E) and TmHKT1;4-A2 (F). Macroscopic inward conductances were determined as the slopes of the I-V relationships between the three most negative imposed voltages. The concentration at which apparent half saturation of the conductance occurred (K_M) was determined with a hyperbolic fit (Michaelis-Menten equation). Fitted parameters were as follows: $K_M = 12 \pm 2$ mM and G_{max} (maximal HKT1;4 whole-oocyte conductance) = 289 ± 22 μ S for TmHKT1;4-A1; $K_M = 33 \pm 3.5$ mM and $G_{max} = 872 \pm 96$ μ S for TmHKT1;4-A2. Data are means \pm SE ($n=4$ in (A, C, E) and $n=5$ in (B, D, F)) and are representative of 3 experiments performed in different oocyte batches. These experiments were performed in parallel for TmHKT1;4-A1 and TmHKT1;4-A2 on same batches of oocytes.

Fig. 5 Comparison of TmHKT1;4 transporter conductances with those of durum wheat HKT1;4 homologues. Oocytes were injected with same amount of cRNA for *Triticum monococcum* and durum wheat (Ben Amar *et al.*, 2014) transporters and analysed in same conditions. Na-glutamate concentration in the bath solution was

successively 1, 10, 50 and 100 mM. Data are means \pm SE (same as in Fig. 4 (E, F) for TmHKT1;4 transporters, $n=4$ for TdHKT1;4-1, $n= 5$ for TdHKT1;4-2).

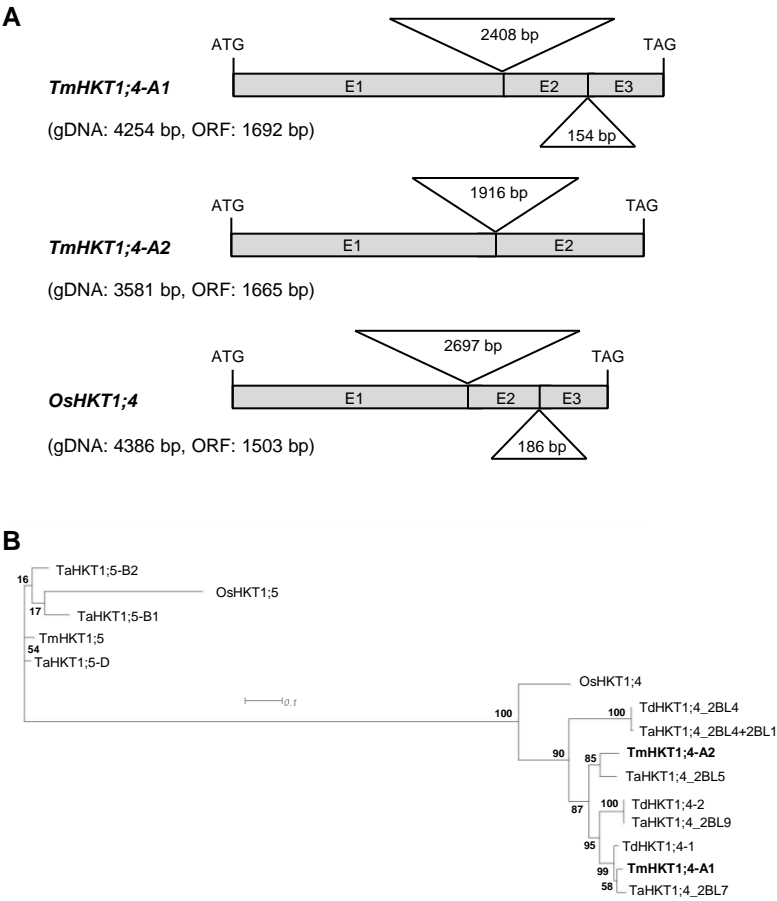


Fig. 1

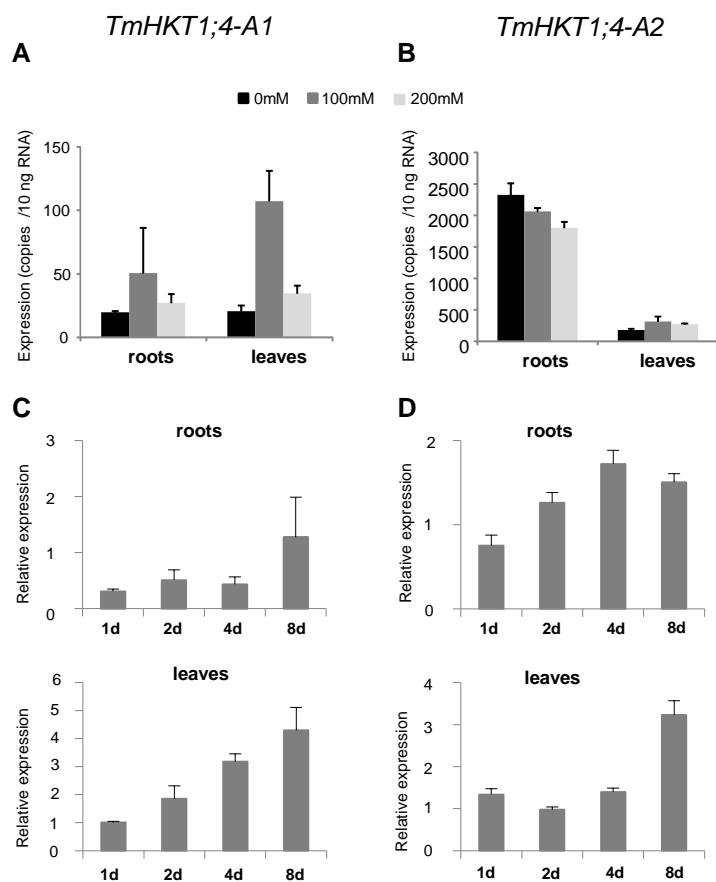


Fig. 2

TmHKT1;4-A1

TmHKT1;4-A2

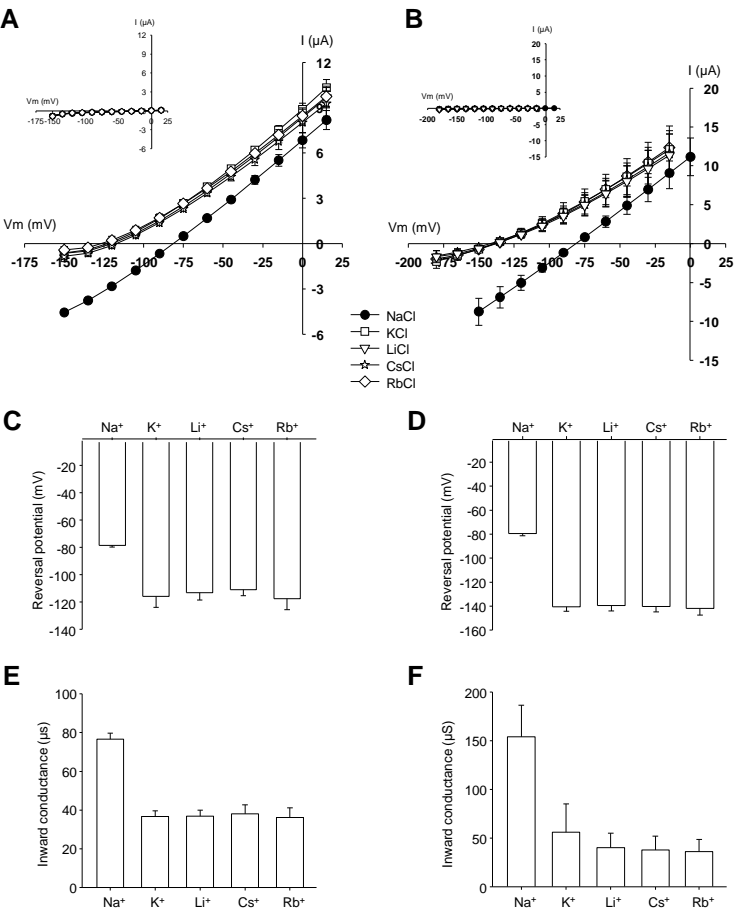


Fig. 3

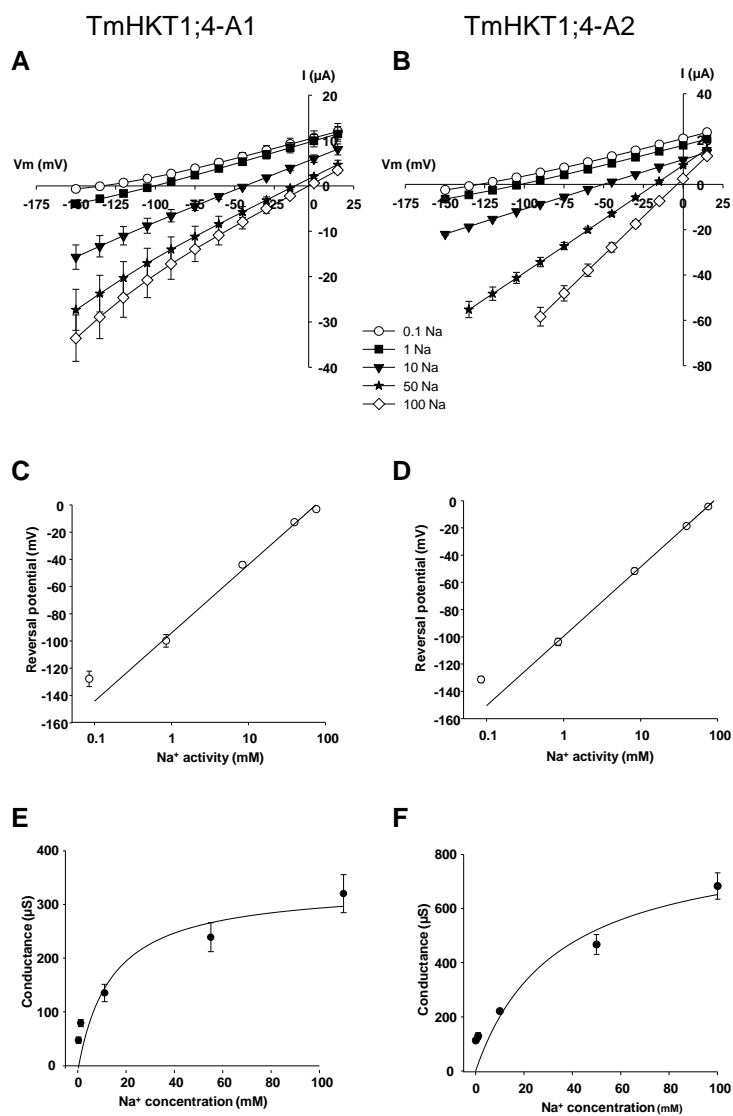


Fig. 4

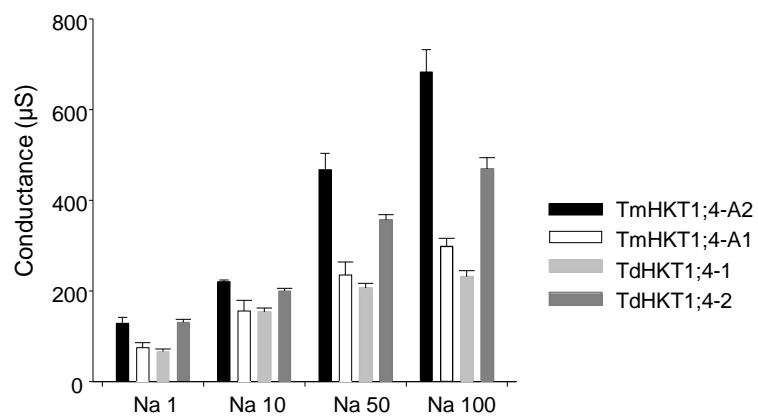


Fig. 5