The Na^+ transporter, TaHKT1;5-D, limits shoot Na^+ accumulation in bread wheat

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SUMMARY

Bread wheat (*Triticum aestivum* L.) has a major salt tolerance locus, *Kna1*, responsible for the maintenance of a high cytosolic K^+/Na^+ ratio in the leaves of salt stressed plants. The *Kna1* locus encompasses a large DNA fragment, the distal 14% of chromosome 4DL. Limited recombination has been observed at this locus making it difficult to map genetically and identify the causal gene. Here, we decipher the function of *TaHKT1;5-D,* a candidate gene underlying the *Kna1* locus. Transport studies using the heterologous expression systems *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes indicated that TaHKT1;5-D is a Na^+-selective transporter. Transient expression in *Arabidopsis thaliana* mesophyll protoplasts and *in situ* PCR indicated that *TaHKT1;5-D* is localised on the plasma membrane in the wheat root stele. RNA interference-induced silencing decreased the expression of *TaHKT1;5-D* in transgenic bread wheat lines which led to an increase in the Na^+ concentration in the leaves. This indicates that *TaHKT1;5-D* retrieves Na^+ from the xylem vessels in the root and has an important role in restricting the transport of Na^+ from the root to the leaves in bread wheat. Thus, *TaHKT1;5-D* confers the essential salinity tolerance mechanism in bread wheat associated with the *Kna1* locus via shoot Na^+ exclusion and is critical in maintaining a high K^+/Na^+ ratio in the leaves. These findings show there is potential to increase the salinity tolerance of bread wheat by manipulation of *HKT1;5* genes.

INTRODUCTION

Soil salinity is a major constraint on crop yield. It currently affects >20% of irrigated and 8% of rain fed agricultural land and these figures are rising due to natural and anthropogenic processes (FAO 2000; Pitman and Läuchli 2002; Pimentel et al. 2004; Rengasamy 2006; Hasegawa 2013). Consequently, genetic improvements in the salt tolerance of wheat are essential if we are to sustain increases in global food production. Bread wheat grain yield is influenced by many abiotic stresses including salinity, and can be associated with specific traits for salt tolerance. For example, leaf sodium (Na^+) exclusion was found to be the primary sub-soil constraint linked trait that was associated with genetic variation in wheat grain yield under Australian field conditions (McDonald et al. 2013). Bread wheat (*Triticum aestivum* L., genome AABBDD) is a relatively salt tolerant cereal species, especially compared to durum wheat (*T. turgidum* L. ssp. durum [Desf.], genome AABB), largely due to its superior ability to exclude Na^+ from the leaf (Dvorak et al. 1994). Uptake of $^{22}Na^+$ into bread wheat and durum wheat roots was found to be similar, but $^{22}Na^+$ transfer from the roots to the shoots in bread wheat was six times less than in durum wheat (Gorham et al. 1990). Under conditions of abiotic stress, such as saline soil conditions, membrane transporters are critical in maintaining plant productivity (Schroeder et al. 2013). In particular, leaf Na^+ exclusion is a trait that can be altered significantly by a single membrane transporter (Munns et al. 2012). However, the mechanism underpinning the major leaf Na^+ exclusion trait in bread wheat has not been characterised at a molecular level.

Kna1 is the major locus controlling leaf Na^+ exclusion in bread wheat and is critical in maintaining a high K^+/Na^+ ratio in the leaves in this major staple crop (Gorham et al. 1987; 1990). In saline conditions durum wheat recombinant inbred lines introgressed with *Kna1* produced higher grain yield and more biomass than lines without *Kna1* (Dvořák et al. 1994).

Kna1 has been mapped to a large region on chromosome 4DL (Dvořák and Gorham 1992). Identification of the location of *Kna1* was achieved by transferring the locus into durum wheat using a mutant line lacking the *Ph1* locus, a strategy which enabled recombination between homoeologous chromosomes that is normally supressed. Analysis of Na^+ exclusion in the resulting recombinant lines where chromosomes 4D and 4B were recombined indicated that *Kna1* is conferred by a single locus. Limited recombination at the *Kna1* locus has restricted attempts to employ fine mapping to determine the identity of the candidate gene for *Kna1* (Gorham et al. 1987; Dvořák and Gorham 1992; Dubcovsky et al. 1996; Luo et al. 1996; Milla and Gustafson 2001; Byrt et al. 2007). The segment of the chromosome containing *Kna1* spans 14% of the chromosome arm and it is difficult to eliminate genes within this region as candidates for this important salinity tolerance locus. For example, there are three QTL that impact salt tolerance during seed germination that all map to this region on 4DL (Ma et al. 2007).

In addition to *Kna1*, two other loci conferring Na^+ exclusion traits have been identified in wheat: *Nax1* (*Na*$^+$ *exclusion 1*) and *Nax2* (*Na*$^+$ *exclusion 2*) (James et al. 2006; Lindsay et al. 2004; Munns et al. 2003). Resources such as fine mapping populations, recombinant inbred lines and chromosome deletion lines were employed to determine the identity of the genes that determine the *Nax1* and *Nax2* shoot Na^+ exclusion phenotype (Huang et al. 2006; Byrt et al. 2007). *HKT* genes (high-affinity potassium (K^+) transporter) of the Class 1 type, which are predicted to transport Na^+ and not K^+ (Mäser et al. 2002; Platten et al. 2006), were found in both cases to map to the Na^+ exclusion loci; these were named *TmHKT1;4-A2* (*Nax1*) and *TmHKT1;5-A* (*Nax2*) based on their phylogeny and the chromosome on which they reside. *TmHKT1;4-A2* and *TmHKT1;5-A* are not ordinarily present in modern wheat as they are derived from the A genome of *Triticum monococcum* (James et al. 2006). The *T. monococcum* genome is

homologous to the A genome of modern polyploid wheat but not a direct ancestor (Huang et al. 2008). These genes were transferred into a cultivar of durum wheat by the way of a cross with a *T. monococcum* accession (James et al. 2006). Transfer of *TmHKT1;4-A2* and *TmHKT1;5-A* into durum wheat was found to increase leaf Na^+ exclusion (James et al. 2006). Furthermore, the presence of *TmHKT1;5-A* increased grain yield in durum wheat under saline conditions by 25% compared to near-isogenic lines without *TmHKT1;5-A* (Munns et al. 2012). Extensive crossing to introduce *TmHKT1;5-A* into different varieties of bread and durum wheat, and comparison of the accumulation of Na^+ in the leaf of near-isogenic lines with and without *TmHKT1;5-A* indicated that this was likely to be the candidate gene encoded by the Na^+ exclusion locus *Nax2* (Byrt et al. 2007; James et al. 2011, 2012; Munns et al. 2012). For the *Nax1* locus, recombination events either side of *TmHKT1;4-A2* made it possible to fine map and eliminate other possible candidate genes at the *Nax1* locus, providing convincing evidence that *TmHKT1;4-A2* was the Na^+ exclusion gene *Nax1* (Huang et al. 2006).

It was suggested previously that *Kna1* and *Nax2* may be homoeologous genes (Byrt et al. 2007). This was considered plausible because *Kna1* is located on chromosome 4DL and *Nax2* on 5AL, and the distal part of the 5AL chromosome ancestrally corresponds to the distal part of 4AL. The similarity in the phenotype conferred by *Kna1* and *Nax2* supported this suggestion. Durum wheat lines with *Nax2* had 2.5 times less leaf Na^+ than lines without *Nax2*, and bread wheat cytogenetic chromosome deletion lines with *Kna1* had four times less leaf Na^+ than lines lacking the *Kna1* region (Byrt et al. 2007). A homeologous gene of the candidate gene for *Nax2* (*TmHKT1;5-A*) was observed at the *Kna1* locus (*TaHKT1;5-D*). This observation indicated that *TaHKT1;5-D* may be the casual gene for *Kna1* but did not provide any direct evidence of this assertion.

To determine whether *TaHKT1;5-D* is in fact *Kna1*, we investigated the transport properties and localisation of TaHKT1;5-D. The encoding gene was expressed in *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes, *YFP::TaHKT1;5-D* was expressed in *Arabidopsis* mesophyll protoplasts and transcripts of *TaHKT1;5-D* were localised in bread wheat by *in situ* PCR. We also took a plant transformation approach, developing two constructs designed to specifically target and silence the expression of *TaHKT1;5-D*. These constructs were introduced into bread wheat and the impact on leaf ion accumulation was tested. These data indicate that the function and phenotypes associated with *TaHKT1;5-D* are consistent with this gene being responsible for, *Kna1*, the major leaf Na^+ exclusion locus of bread wheat.

RESULTS

TaHKT1;5-D is a plasma membrane Na^+ transporter

Yeast (*Saccharomyces cerevisiae* strain InvSc2) transformed with *TaHKT1;5-D* or an empty vector control plasmid were grown in liquid media, and on agar plates with and without additional NaCl in two types of growth media (Arginine Phosphate (AP) medium, Fig. 1A and Synthetic Complete medium minus uracil (SC-Ura) medium, Figure S1). Growth of yeast transformed with *TaHKT;5-D* was similar to that of the empty vector control when grown in liquid AP media, which contained less than 8 μM Na^+. Agar contains 6 mM Na^+, therefore, even without additional Na^+ supplementation there was approximately 6 mM Na^+ in the AP agar media and 10 mM Na^+ in the in SC-Ura agar media (Munns et al. 2012). This concentration of Na^+ slightly suppressed the growth of *TaHKT1;5-D* expressing yeast compared to the empty vector controls and the addition of 100 mM and 200 mM NaCl significantly inhibited the growth of *TaHKT1;5-D* expressing yeast (Fig. 1A, Figure S1). The reduced growth phenotype observed for

TaHKT1;5-D expressing yeast was similar to that observed for yeast expressing an *Arabidopsis thaliana* Na^+ transporter gene, *AtHKT1;1*, as demonstrated previously by Uozumi et al. (2000), and repeated here as a positive control. From these results it was apparent that the expression of *TaHKT1;5-D* causes a Na^+ hypersensitivity phenotype in yeast indicating that TaHKT1;5-D is likely to function as a Na^+ permeable transporter in a similar fashion to AtHKT1;1 and TmHKT1;5-A (Uozumi et al. 2000; Munns et al. 2012).

To further investigate the transport properties of its encoded protein, *TaHKT1;5-D* cRNA was injected into *Xenopus laevis* oocytes. The oocytes injected with *TaHKT1;5-D* cRNA accumulated Na^+; 3 d post-injection they contained approximately double the Na^+ concentration and one-third less K^+ relative to water-injected and non-injected control oocytes (Fig. 1B). *TaHKT1;5-D* cRNA-injected oocytes were also assayed by two-electrode voltage-clamp. Significant inward currents were observed for *TaHKT1;5-D* expressing oocytes in the presence of Na^+, but not K^+ (Fig. 1C; Figure S1B). When external Na^+ was replaced with 100 mM of other monovalent cations (K^+, Cs^+, Li^+, Rb^+, $TRIS^+$) no significant inward currents were detected (Fig. 1D). A 10-fold increase in $[Na^+]_{ext}$, from 1 mM to 10 mM, gave rise to a 48.7 ± 4.8 mV increase in the reversal potential (E_{rev}) with a further 20.1 ± 1.6 mV increase resulting from a 10 mM to 30 mM increase in $[Na^+]_{ext}$ (Fig. 1E). These are close to the predicted shifts in membrane potential based on Nernst potential calculations for Na^+ (E_{Na}; 59 and 26 mV, respectively). Taken together this evidence suggests that the inward and outward currents in Figure 1D (and 1E) are likely to be constitute Na^+ influx and efflux from the oocyte, respectively. As such, these results are consistent with TaHKT1;5-D forming a Na^+-selective uniporter. However, as clearly illustrated in Figure 1F, there is an inhibition of Na^+ transport through TaHKT1;5-D when $[K^+]_{ext}$ is elevated. We observed a reduction in the magnitude of the inward currents, but not outward

currents in the presence of 10 mM external K^+. We also quantified this effect and found that the affinity for transport for Na^+ was reduced by the presence of elevated external [K^+]; the K_m for Na^+ transport for TaHKT1;5-D with 0 mM $[K^+]_{ext}$ was 4.01 ± 0.5 mM, but in the presence of 10 mM K^+ it was 11.3 ± 1.5 mM (Fig. 1G). Therefore, TaHKT1;5-D is a Na^+-selective uniporter that is blocked by the presence of external K^+.

TaHKT1;5-D is expressed at the plasma membrane of stelar cells in roots of bread wheat

To confirm that TaHKT1;5-D was capable of transporting Na^+ into or out of plant cells its membrane localisation was investigated in a plant system by expressing YFP::TaHKT1;5-D in *Arabidopsis* mesophyll protoplasts. YFP::TaHKT1;5-D was detected on the plasma membrane, as indicated by its co-localisation with the plasma membrane marker ECFP::Rop11 (Munns et al., 2012) (Fig. 2A).

Previously, *TaHKT1;5-D* mRNA expression has been observed in the roots, but not the leaves, of bread wheat (Byrt et al. 2007). The tissue specific expression of *TaHKT1;5-D* in roots was investigated by *in situ* PCR. The expression of *TaHKT1;5-D* was predominantly within the stele, particularly within xylem parenchyma and pericycle cells adjacent to the xylem vessels (Fig. 2B).

Transformation of bread wheat to introduce two _TaHKT1;5-D_ RNAi constructs

Two constructs were generated, RNAi1 and RNAi2, which encoded hairpin *TaHKT1;5-D* fragments. The fragments differed by 14 bp at the 5' end and 27 bp at the 3' end and had no significant similarity to other HKT-type genes. Callus from 500 embryos was co-bombarded with the RNAi constructs and a plasmid for antibiotic selection. The plants generated from this

callus that survived selection in tissue culture were grown to maturity and T1 seed was harvested. The genotype of the transgenic plants was confirmed by a diagnostic PCR. T1 seed from two independent transgenic plants, one T0 RNAi1 and one T0 RNAi2, was harvested. This seed was planted and the T1 plants tested for leaf Na^+ and K^+ accumulation and for the presence or absence of the constructs (Figure S2A, B). As expected, the T1 seed was segregating for the presence of the RNAi constructs. Seed was collected from five different RNAi1 T1 plants and screening of the T2 plants grown from this seed indicated that three were positive for the RNAi1 construct and two were null lines. Seed was collected from four RNAi2 T1 plants; screening of the respective T2 plants grown from this seed indicated that one was positive and three were still segregating. The genotype of the transgenic lines was tested by PCR in four consecutive generations and in total 197 progeny of the T0 RNAi1 transgenic plant and 167 progeny of the T0 RNAi2 transgenic plants were tested. Of these segregating lines 98 plants tested positive for the RNAi1 construct and 99 null RNAi1 lines were identified, 112 plants tested positive for the RNAi2 construct and 55 null RNAi2 lines were identified (Table SI).

Reducing *TaHKT1;5-D* transcripts *in planta* increases leaf Na^+ concentration

The mean transcript abundance of *TaHKT1;5-D* in the roots of transgenic lines containing an RNAi construct was lower than that of wild type and the respective null lines over multiple generations (Fig. 3A, 4A and Figure S3A). There was no detectable difference in the transcript level of the *HKT1;5-B1* or *HKT1;5-B2* genes in Bob White, the transgenic lines or their relative null lines (Figure S4A,B). The expression of the *HKT1;5-B* genes is already very low, particularly relative to the expression the *HKT1;5-D* gene (Figure S4C). To examine what effect a reduction of *TaHKT1;5-D* expression would have on leaf Na^+ and K^+ accumulation in

the transgenic lines they were grown in saline conditions. The lines with the RNAi constructs had higher leaf Na^+ concentrations and lower leaf K^+/Na^+ ratios than their respective null lines (Fig. 3 B and C and Figure S2, C and D). This trend was evident in all generations, although it was not statistically significant in every case (Fig. 3, 4 and Figure S2 and Table SII). Higher leaf Na^+ was observed whether the NaCl concentration was minimal (Figure S2, C and D), 50 mM (Fig. 3), 100 mM (Fig. 4) or 150 mM (Figure S2, A and B), with the exception of the T4 plants grown in media with minimal NaCl, where there was no significant difference in leaf Na^+ concentrations or K^+/Na^+ ratios between the lines (Figure S3, B and C).

In the T2 generation, where plants were grown in standard hydroponic media with an additional 50 mM NaCl, the lines with the RNAi2 constructs had significantly higher leaf Na^+ and significantly lower leaf K^+/Na^+ ratios than that of the null RNAi2 lines (Fig. 3, B and C). In the T3 generation, where plants were grown in soil with no additional NaCl, both the lines with the RNAi1 and the lines with the RNAi2 constructs had significantly higher leaf Na^+ and significantly lower leaf K^+/Na^+ ratios than that of their respective null lines (Figure S2, C and D). In the T4 generation, where plants were grown in standard hydroponic media with an additional 100 mM NaCl, the lines with the RNAi1 constructs had significantly higher leaf Na^+ and significantly lower leaf K^+/Na^+ ratios than that of the null RNAi1 lines (Fig. 4, B and C).

DISCUSSION

Kna1 is a major locus contributing to salinity tolerance in bread wheat where it controls leaf Na^+ exclusion, but the gene responsible for this phenotype has remained unresolved. In bread wheat, cytogenetic chromosome deletion lines lacking *Kna1* were shown previously to have four times more leaf Na^+ than the cultivar with *Kna1* (Byrt et al. 2007). In that study it was

noted that a putative HKT transporter, *TaHKT1;5-D*, was present within the *Kna1* locus. However, there are 682 ESTs associated with chromosome 4D and the *Kna1* locus encompasses 14% of chromosome 4DL (Qi et al. 2004). Therefore, the number of expressed genes co-located in the *Kna1* locus is likely to be in the order of 100. Previous studies have proposed the role of *HKT1;5*-like genes based on the phenotype of near-isogenic lines generated through conventional breeding approaches. These studies cannot rule out the possible effects of other genes also contained within the, often sizable, recombined chromosomal fragment (Byrt et al. 2007; Munns et al. 2012). To distinguish the role of *TaHKT1;5-D* from that of the other genes in the *Kna1* locus genetic transformation of bread wheat is used to specifically alter *TaHKT1;5-D* expression.

Here, we present direct evidence that *TaHKT1;5-D* is the causal gene for *Kna1*. We established that *TaHKT1;5-D* is expressed in the stele in wheat roots and that TaHKT1;5-D is present on the plasma membrane (Fig 2). We then demonstrate that knock-down of *TaHKT1;5-D* transcription in transgenic bread wheat lines results in higher leaf Na^+ content (Fig. 3 and Fig. 4). The elevated leaf Na^+ phenotype was observed in multiple generations of transgenic wheat lines derived from two independent plant transformation events and the phenotype was observed in both non-saline and saline growing conditions. This indicates that the mechanism by which *TaHKT1;5-D* limits the accumulation of Na^+ in the leaves is by removing Na^+ from the root stelar apoplast. The phenotype conferred by *TaHKT1;5-D* in the transgenic wheat lines described here is consistent with the phenotype associated with *Kna1*; that is, lines with *Kna1* have lower Na^+ levels in the leaves than recombinant lines without *Kna1* (Dvořák et al. 1994). This indicates that *TaHKT1;5-D* confers the major Na^+ exclusion trait *Kna1* and demonstrates the impact of a single *HKT* gene on the accumulation of Na^+ in the shoot of bread wheat.

There are two groups of HKT transporters. These vary in their permeability to K^+ and Na^+, and in their role in controlling ion transport within plants (Platten et al. 2006; Hauser and Horie 2010). *TaHKT1;5-D* is a member of *HKT* sub-family 1, a group which includes other genes encoding Na^+ transporters associated with shoot Na^+ exclusion such as *TmHKT1;5-A* (Ren et al. 2005; Horie et al. 2009; Hauser and Horie 2010; Plett et al. 2010; Munns et al. 2012). TaHKT1;5-D is 94% identical to TmHKT1;5-A; both proteins show a high selectivity for Na^+ transport and little permeability to K^+, furthermore they are blocked by the presence of external K^+ (present results versus Munns et al. 2012). Previous studies indicate that Na^+ transport by OsHKT2;1 from rice (*Oryza sativa* L.), a group 2 HKT, is also blocked when external K^+ concentration is above a certain threshold. The mechanism for this phenomenon is unknown but could involve binding of K^+ to an allosteric site of the pore which results in an alteration in the transport properties of the protein (Gassmann et al. 1996); this is a matter for further investigation as is the physiological significance of this functional property. Interestingly, the affinity for Na^+ transport for TaHKT1;5-D is about 4.5-fold lower than that of TmHKT1;5-A in the absence of external K^+ (present results versus Munns et al. 2012). This may explain in part why introducing *TmHKT1;5-A* into bread wheat, which already has endogenous *TaHKT1;5-D*, can further increase the plants ability to exclude leaf Na^+ (James et al. 2011; 2012). This also indicates that salinity tolerance in wheat can be further improved by adding different *HKT1;5* alleles.

The expression of *TaHKT1;5-D* did not differ when there was minimal (6 mM) Na^+ in the hydroponic media relative to when 100 mM NaCl was added. This indicated that the high concentrations of external NaCl may not trigger a significant increase in the expression of *TaHKT1;5-D* in cv. Bob White (Fig. 4A and Figure S3A). Similarly, increasing external Na^+

concentration did not increase the expression of *TmHKT1;5-A* in a durum wheat, Line 149 (Munns et al., 2012). However, there appears to be a basal level of expression of these genes that is clearly important for Na^+ exclusion. In this study, a reduction in the transcript level of *TaHKT1;5-D* was observed in transgenic RNAi lines and this was associated with an increase in the Na^+ concentration in the leaves (Fig. 3 and 4). In the current study and that by Munns et al. (2012), it appears likely that there is a threshold level of *HKT1;5* expression maintained regardless of variation in external NaCl concentration.

In the case of *Nax2* (*TmHKT1;5-A*) and *Kna1* (*TaHKT1;5-D*) it was observed that a Na^+ selective transporter, expressed in stelar root cells, can limit the amount of Na^+ that is transported in the xylem to the leaf tissues (Munns et al. (2012) and current study). Retrieval of Na^+ into the leaf sheath, a trait conferred by *Nax1* (*TmHKT1;4-A2*) is also effective in limiting Na^+ accumulation in the leaves. The ion transport characteristics of TmHKT1;4-A2 are yet to be described, but a recent report has shown that similar alleles from durum wheat *TdHKT;4-1* and *TdHKT1;4-2* encode Na^+ selective transporters (Amar et al. 2013). Interestingly, in contrast to the K^+ block of Na^+ transport for TaHKT1;5-D and TmHKT1;5-A, a K^+-stimulation of Na^+ transport was reported for TdHKT1;4-1 and TdHKT1:4-2 when expressed in *Xenopus laevis* oocytes (Amar et al. 2013). From combining our observations with that of others, it is clear that there is significant variation in the Na^+ transport activity of different wheat HKT1;4/1;5 proteins and there is also variation in the tissues in which *HKT1;4/1;5* genes are expressed and the level of *HKT1;4/1;5* mRNA expression. A clear opportunity now presents itself to determine the factors that underpin all of these properties, using these closely related genes, and this will assist in our understanding of how the regulation in gene expression and structural determinants of selectivity combine to determine leaf Na^+ exclusion (Waters et al. 2013).

Subtle differences in the activity of different transporters can have a profound effect on plant phenotype. In rice, leaf Na^+ exclusion is influenced by *OsHKT1;5* transcript abundance, protein structural features within the selectivity filter for OsHKT1;5 and the abundance of correctly spliced *OsHKT1;4* transcript in the leaf sheath (Cotsaftis et al. 2012). Transgenic rice containing the Nona Bokra *OsHKT1;5* allele had higher shoot K^+ concentrations compared with control lines in saline conditions. The functional differences between the *OsHKT1;5* allele from the salt-tolerant rice variety, Nona Bokra, and an allele from the salt-sensitive variety, Koshihikari, were attributed to six nucleotide substitutions which led to four amino-acid substitutions (Ren et al. 2005). Fifteen different *OsHKT1;5* alleles have been described in rice and there is a strong association between different *OsHKT1;5* alleles and leaf Na^+ concentrations (Negrão et al. 2013; Platten et al. 2013). Therefore, allelic variation in the sequence of *HKT1;5* and variation in the activity of HKT1;5 in cultivated wheat and its ancestors should be determined and could be a useful resource for further increasing leaf K^+/Na^+ ratio and wheat salinity tolerance.

Salinity tolerance and Na^+ exclusion mechanisms in different plant species are quite distinct. In barley loss of *HvHKT2;1* function in a mutant plant resulted in less Na^+ influx and reduced growth in saline conditions and overexpression of *HvHKT2;1* increased leaf Na^+ and salt tolerance (Horie et al. 2007; Mian et al. 2011). In wheat, *HKT1;5* expression within the stele contributes to Na^+ exclusion in the leaves and salt tolerance (Munns et al. 2012 and present results). In Arabidopsis, another HKT sub-family 1 Na^+ transporter, AtHKT1;1, unloads sodium from xylem vessels to xylem parenchyma cells, thereby reducing the Na^+ content in the leaves (Mäser et al. 2002; Sunarpi et al. 2005). Overexpression of *AtHKT1;1* in the root pericycle of plants lacking expression of endogenous *HKT1;1* increased Na^+ influx into xylem parenchyma

cells which led to increased shoot Na^+ exclusion and increased salinity tolerance (Møller et al. 2009). Interestingly, Na^+ exclusion in transgenic rice was increased by overexpression of *AtHKT1;1* in the mature root cortex in rice (Plett et al. 2010), the mechanism by which this phenotype occurs is yet to be determined but may involve up-regulation of endogenous *HKT1;5* in the stele.

The present data indicates that reducing the transcript levels of a group 1 *HKT* gene by gene silencing in wheat roots results in a significant increase in leaf Na^+ and a reduction in leaf K^+. These data will inform future strategies to make significant improvements in the salinity tolerance in bread and durum wheat. By further investigating the impact of *HKT1;4/HKT1;5* variants on the control of Na^+ and K^+ transport *in planta* it may be possible to identify allelic variants that could be introduced into commercial wheat lines to further increase their ability to exclude Na^+ and improve salinity tolerance.

EXPERIMENTAL PROCEDURES

Growth inhibition assays in *Saccharomyces cerevisiae*

Growth inhibition assays of *TaHKT1;5-D* were performed in *S. cerevisiae* strain INVSc2 (MATa, his3-D200, ura3-167, Invitrogen) and expression was induced using the GAL1 promoter in the pYES-DEST52 vector (Invitrogen) following Munns et al. (2012). The plasmids containing *TaHKT1;5-D* cDNA (from Byrt et al. 2007), *AtHKT1;1* (from Plett et al. 2010) and an empty vector were transformed into yeast using the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl 2007). The transformants were selected on Synthetic Complete minus uracil medium (SC-Ura) [2% (w/v) Glucose, 0.67% (w/v) Difco™ Yeast Nitrogen Base and 1.67% (w/v) Difco™ Bacto agar (BD, USA) pH 5.6]. The growth inhibition study of yeast expressing

TaHKT1;5-D was performed on two media: SC-Ura [2% (w/v) Galactose, 1.67% (w/v) agar, pH 5.6], which contains 4 mM Na^+ and Arginine Phosphate (AP) [2% (w/v) Galactose, 1.67% (w/v) agar, 0.06% (w/v) histidine, and pH 6.5], which contains less than 8 µM Na^+ (Rodríguez-Navarro and Ramos 1984). Agar contains 6 mM Na^+. Overnight-cultured yeast was adjusted to the optical density $OD_{(600)}$ nm of 1.0 using a spectrophotometer (BioRad, Smart Spec^{TM} 3000), from which four serial dilutions were made; $OD_{(600)}$ nm of 0.1, 0.01, 0.001 and 0.001. 15 µL of each dilution of yeast containing different constructs was spotted on the plates and incubated at 30^{o}C for 4 days.

Characterisation of TaHKT1;5-D in *Xenopus laevis* oocytes

pGEMHE-DEST containing *TaHKT1;5-D* was linearised using *Sbf*I-HF (New England Biolabs); cRNA was synthesised using the mMESSAGE mMACHINE T7 Kit (Ambion) following manufacturer's instructions. 46 nL of cRNA (23 ng) or RNA-free water were injected into oocytes with a Nanoinject II microinjector (Drummond Scientific). Oocytes were incubated for 48 h and electrophysiology carried out following Roy et al. (2008) and Munns et al. (2012). Membrane currents were recorded in HMg solution (6 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM MES and pH 6.5 adjusted with TRIS base) ± Na^+ glutamate and/or K^+ glutamate as indicated. The osmolarity of all solutions was adjusted using mannitol 240–260 mOsmol kg^{-1} (Vapor pressure osmometer, Wescor). The moles of Na^+ and K^+ in the oocytes was measured three days after injection of cRNA or water, control oocytes were not injected. Oocytes were kept in modified Barth's solution containing 96 mM Na^+ and 2 mM K^+ with horse serum and antibiotics following Zhou et al. (2007). Three oocytes per sample were homogenized in 1 mL of 1% nitric acid and incubated at 75°C for 1 hr. An aliquot was diluted in 1% nitric acid and the moles of Na^+ and K^+ were measured relative to standards using a flame photometer (M410, Corning).

Membrane Localisation

Arabidopsis mesophyll protoplast isolation and transformation were adapted from Yoo et al (2007) where W2 solution (4 mM MES, 0.4 M mannitol, 15 mM KCl, 10 mM $CaCl_2$ and 5 mM $MgCl_2$, adjusted to pH 5.7 with KOH) was used to take the place of WI and W5 solution. Arabidopsis plants that were 5-6 weeks old and grown as described by Conn et al (2013) were used for protoplast isolation. In total, 10-20 leaves, consisting of 3-4 leaves from each plant, were cut into 1 mm strips and transferred by dipping both side of the strips into 10 mL of enzyme solution (20 mM MES, 1.5% (w/v) Cellulase R10, 0.4% (w/v) Macerozyme R10, 0.4M mannitol, 20 mM KCl, 10 mM $CaCl_2$, 0.1% (w/v) BSA, pH = 5.7 by KOH). The enzyme solution with leaf strips was vacuum-infiltrated for 30 min using a desiccator and incubated in the dark at room temperature. After 3 hr incubation the incubation media was mixed with ice-cold 10 mL W2 solution to stop the reaction. Then transferred and filtered using a 75 µm nylon mesh into a new 50 mL falcon tube. The protoplast solution was centrifuged at 150 × g at 4°C for 2 min followed by one wash in 10 mL ice-cold W2 buffer. Protoplast cells were collected again by centrifugation at 150 × g at 4°C for 2 min to remove the excess enzyme solution and resuspended in ice-cold W2 solution. Protoplasts (100 µL) were mixed with 10 µg plasmid DNA and 110 µL PEG solution (30% (w/v) PEG 4000, 0.2 M mannitol and 100 mM $CaCl_2$) and incubated for 5 min at room temperature for protoplast transformation. The transformation reaction was stopped by adding 400 µL W2 solution and collected by centrifugation by 200 × g at room temperature for 4 min. The cell pellet was gently resuspended in 500 µL W2 solution, transferred into a 12-well plate (Iwaki) and incubated in the dark at room temperature for 16-24 hr to allow the gene of interest to be expressed in protoplasts before imaging. The fluorescence of fluorescent proteins transiently-expressed in Arabidopsis mesophyll protoplasts was imaged by a confocal

laser scanning microscope equipped with a Zeiss Axioskop 2, LSM5 PASCAL and an argon laser (Carl Zeiss). Sequential scanning and laser excitation was used to capture fluorescence via the LSM5 PASCAL from YFP (excitation = 514 nm, emission Band Pass (BP) = 570-590 nm), CFP (excitation = 458 nm, emission BP = 470-500 nm) and chlorophyll autofluorescence (excitation = 543 nm, emission Long-Pass = 560 nm).

RNA *in situ* PCR

Followed Munns et al. (2012) with the following modifications: 1.5U Phusion DNA polymerase (New England Biolabs) was used instead of *Taq* DNA polymerase. The primers used for the cDNA synthesis step (R only) and PCR (both F and R) were TaHKT8F CGACCAGAAAAGGATAACAAGCAT and TaHKT8R AGCCAGCTTCCCTTGCCAA; the product amplified by these primers was sequenced to confirm that they are specific to *TaHKT1;5-D*. Cycling parameters were as follows: initial denaturation at 98°C for 30 s, then 27 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 7 s and a final extension at 72°C for 10 min.

Generation and characterisation of transgenic plants containing RNAi constructs

The vector used to generate the RNAi constructs, pSTARGATE, contains the maize ubiquitin promoter and introns and is similar to pSTARLING (Christensen et al. 1996; Rooke et al. 2000) with the exception that it is enabled with Gateway™ recombination sites. The constructs express a self-complementary RNA for which the sense and antisense sequences hybridise to form a hairpin RNA fragment. Two independent RNAi constructs with homology to *TaHKT1;5-D* were generated, RNAi1 and RNAi2. The *TaHKT1;5-D* fragments incorporated in RNAi1 were 600 bp in size and started at nucleotide 1082 and the fragments incorporated in RNAi2 were 559 bp in size and started at nucleotide 1096; *TaHKT1;5-D* sequence data is available in the GenBank data base (accession DQ646342; Byrt 2008). A Basic Local Alignment Search Tool (BLAST)

comparison of these fragments to the cDNA sequence for the next most closely related HKT-type gene, *HKT1;4*, indicated that there was no significant similarity. There are no A genome *HKT1;5* genes in bread wheat but there are three B genome *HKT1;5* genes in wheat (Huang et al. 2008). One is truncated (DQ646335), the other two (DQ646336 and DQ646337) are full length genes; the protein sequences are 46.9%, 86.5% and 91.9% identical to *TaHKT1;5-D*, respectively. *Triticum aestivum* cv. Bob White scutella 1 mm in length were co-bombarded with the circular RNAi constructs and pNEO1 plasmid DNA, containing the *npt*II gene which confers resistance to the antibiotic geneticin. Particle bombardment methods were as described by Pellegrineschi et al. (2002). Plants generated from the callus that survived selection in tissue culture were grown to maturity and T1 seed was harvested. The quality of DNA isolated from T0 plants that survived tissue culture and T1 plants was checked by amplifying a fragment of the *Actin* gene by PCR under standard conditions with the primers 5'-GGCACACTGGTGTCATGG-3' and 5'-CTCCATGTCATCCCAGTT-3', courtesy of Dr Linda Tabe (CSIRO Plant Industry, Australia). Transgenic plants were identified by PCR under standard conditions using primers (5'-GCTTGGCCATCTTCATCGCCGTG-3' and 5'-GGCCACAGCTGTACCCGGTGCTG-3') complementary to the transgene. The reaction amplified one or two fragments depending on whether plants were negative or positive for the presence of an RNAi construct, respectively. An 322 bp fragment from the native *TaHKT1;5-D* gene was amplified from all lines; in those transgenic lines containing an RNAi construct an additional 147 bp fragment was amplified from the transgene. Four generations (T1, T2, T3, T4) of transgenic plants were assayed for leaf Na^+ and K^+ accumulation because the numbers of plants available for analysis were limited initially by the number of fertile grain produced and later by the need to select plants that had germinated at a similar time and so reached a similar developmental stage. T1 plants were grown in a

supported flood and drain hydroponic system as depicted in Figure S5; NaCl was gradually added over two days following Munns et al. (2000). Leaf tissue was harvested from T1 plants after ten days growing in 150 mM NaCl with supplemental calcium ($Ca(NO_3)_2$, added to maintain a Na^+ to Ca^{2+} ratio of 15:1) and leaf Na^+ and K^+ concentration was measured on a leaf dry weight basis (μmol g^{-1} DW) using Inductively Coupled Plasma (ICP) analysis following Byrt et al. (2007). The hydroponics screen for the T2 was similar and followed Genc et al. (2007); the hydroponics media had 50 mM NaCl and supplemental $CaCl_2$ (0.825 mM). The T3 generation were grown in coco peat soil mixture; the growth conditions, DNA extraction and PCR methods were as described by Krishnan (2013). For the T3 and T4 generation the leaf Na^+ and K^+ content in the plant sap (tissue water basis) was measured following Shavrukov et al. (2010); this involved recording leaf fresh and dry weights, digesting leaves in 1% HNO_3 at 85°C for 4 h and measuring the Na^+ and K^+ in the digest using a flame photometer (Sherwood, UK, model 420) by comparing the concentration in the digest to known standards [(total volume of digest) ÷ (fresh — dry weight of leaf sample)]. The hydroponics methods and DNA extraction methods for the T4 generation, and additional Bob White plants followed Shavrukov et al. (2010). In the T2 generation the same plants were used for analysis of both leaf Na^+ and K^+ accumulation in leaf tissues and *TaHKT1;5-D* transcript levels in root tissues whereas in the T4 generation tissue from independent sets of plants was harvested for these analyses. RNA from the T2 material was extracted as described by Krishnan (2013). RNA extractions, cDNA synthesis and quantitative PCR followed Burton et al. (2004) with exceptions: qPCR data was normalised to four genes; *TaActin*, *TaEFalpha* (*Elongation Factor alpha*), *TaGAPdh* (*Glyceraldehyde-3-phosephate dehydrogenase*) and *TaCycl* (*Cyclophilin*) in the T2 generation whereas in the T4 generations data was normalised to the first three genes, respectively. The relative amount of different

HKT1;5 gene transcripts in Bob White and T4 cDNA samples (RNAi1, RNAi1 null, RNAi2 and RNAi2 null) was investigated by semi-quantitative Reverse Transcription-PCR (RT-PCR). The RT-PCR amplified transcripts of *HKT1;5-B1*, *HKT1;5-B1* and *HKT1;5-B2 (B1/B2)* and *HKT1;5-D* for 32 cycles; and *Actin* for 28 cycles using Phire Hot Start II DNA Polymerase (Thermo Scientific); the primers amplified 252 bp fragment specific for *TaHKT1;5-D* (5'-CTGCGGCTTCGTCCCGA-3' and 5'- CGCTAGCACGAACGCCG-3'), a 111 bp fragment specific to *TaHKT1;5-B1* (5'-CTGCATGTCTCCTGCAGTA-3' and 5'-GATGGTTACAAAGTACAAGAAATGGA-3'), and a 95 bp product from both *TaHKT1;5-B1* and *TaHKT1;5-B2* (5'- CGCGCAGAAGCTCGTCA-3' and 5'-CAACGGCTGACGACACC-3'). The *HKT1;5-B1*, *HKT1;5-B1* and *HKT1;5-B2 (B1/B2)* products were visible after 45 PCR cycles; these fragments were sequenced to confirm the specificity of the primers to the respective *HKT1;5* B genes. The five wheat *HKT1;5* gene sequences were mapped using publically available RNA-Seq data from the International Wheat Genome Sequence Consortium (IWGSC (2014); https://urgi.versailles.inra.fr/files/RNASeqWheat/): the undirected 2x199bp paired-end data from duplicate root tissues from Chinese Spring isolated from three developmental stages (Zadok scale Z10, first leaf through coleoptile; Z13, three leaves unfolded; and Z39, flag leaf ligule just visible) using BioKanga (v.2.95.0), allowing for up to three mismatches and requiring the best alignment to have at least one fewer mismatches than the next best alignment. Fragments Per Kilobase Of Exon Per Million Fragments (FPKM) were calculated using the total number of concordant paired-end reads mapped to the 99386 IWGSC chromosomal survey sequences which had protein-coding gene models developed by MIPS (v2.1). Graphpad Prism was used for statistical analysis and supplementary Table SII includes a summary of P values. The sequence of *TaHKT1;5-D* referred to in this paper is in the GenBank data base (DQ646342).

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SUPPORTING INFORMATION:

Table SI Numbers of plants tested for leaf Na^+ and K^+ accumulation and for the presence or absence of an RNAi construct in each generation.

Table SII Summary of statistical significance.

Figure S1 TaHKT1;5-D functions as a Na^+ transporter in *Saccharomyces cerevisiae* and currents over time for water injected oocytes in solutions with varying Na^+ and K^+ concentrations.

Figure S2 Transgenic lines with constructs designed to reduce *TaHKT1;5-D* transcripts by RNAi induced gene silencing have increased leaf Na^+ concentration relative to control lines in multiple generations.

Figure S3 *TaHKT1;5-D* transcripts and leaf Na^+ concentration in the leaves of T4 transgenic lines grown in minimal NaCl.

Figure S4 Relative expression of the *HKT1;5* genes in bread wheat and the transgenic lines.

Figure S5 Transgenic wheat lines (T1) growing in a supported flood and drain hydroponics system.

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FIGURE LEGENDS

Figure 1 *TaHKT1;5-D* heterologous expression in *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes.

A. *TaHKT1;5-D* functions as a Na^+ transporter in *S. cerevisiae*; images of the growth of *S. cerevisiae* strain INVSc2 expressing *AtHKT1;1* as a positive control, *TaHKT1;5-D*, and a negative (empty vector) control. Five serial dilutions were spotted on AP media (Rodríguez-Navarro & Ramos 1984) with 2% (w/v) Gal, which contained 6 mM Na^+ and additional either zero or 200 mM NaCl. AP media is low in Na^+ (< 8 μM) and Agar contains 6 mM Na^+.

B. Ion concentration of *X. laevis* oocytes injected with *TaHKT1;5-D*-cRNA. Internal Na^+, K^+ and K^+/Na^+ ratio of *X. laevis* oocytes expressing *TaHKT1;5-D*. The concentration of Na^+, K^+ and the K^+/Na^+ ratio of oocytes expressing *TaHKT1;5-D*, control oocytes (injected with water) and

oocytes injected with an empty vector was determined by flame photometer after 3 d in modified Barth's solution containing 96 mM Na^+. Data are means ± SE (control n = 11; water n = 8; TaHKT1;5-D n = 10) from three batches of oocytes. Different letters indicate a significant difference ($P < 0.001$) using Tukey's test following a one-way ANOVA.

C. TaHKT1;5-D functions as a Na^+ transporter in *X. laevis* oocytes; currents over time for oocytes injected with *TaHKT1;5-D*-cRNA and controls, 2 d after injection, in the presence of varying external concentrations of Na^+ or K^+.

D. Current-voltage (*I-V*) curves for *TaHKT1;5-D*-expressing *X. laevis* oocytes clamped in a series of monovalent cations: 100 mM $TRIS^+$, Rb^+, Cs^+, K^+, Na^+ or Li^+. Data are means ± SE of currents in n = 4-7.

E. TaHKT1;5-D conductance in varying external concentrations of Na^+; current-voltage (*I-V*) curves for *TaHKT1;5*-cRNA injected oocytes in the presence of external 1, 10 or 30 mM Na^+ with or without additional 10 mM K^+.Data are means ± SE of currents in n = 4.

F Currents of *TaHKT1;5*-cRNA injected oocytes clamped at 40 mV or -140 mV in serial Na^+ or Na^+ and K^+ solutions as indicated, plotted from figure 1E (*** represents $P < 0.001$, and **** $P < 0.0001$).

G. Na^+ flux kinetics of TaHKT1;5-D in *X. laevis* oocytes; *TaHKT1;5-D*-cRNA injected oocytes were irrigated in external Na^+-glutamate concentrations of 0.01 mM, 0.02 mM, 0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM, 5 mM, 10 mM, 30 mM with or without 10 mM K^+. Inward current at −140 mV was used from Fig. 1E and additional data. The apparent half-saturation constant (K_M) for Na^+ was determined by the Michaelis-Menten equation, $K_M = 4.01 \pm 0.5$ mM with no K^+ and $K_M = 11.3 \pm 1.5$ mM with 10 mM K^+.

Figure 2 Membrane and cell-type localisation of TaHKT1;5-D.

A. Plasma membrane (PM) localisation of YFP::TaHKT1;5-D in *A. thaliana* mesophyll protoplasts; ECFP::Rop11 (Molendijk et al. 2008) was used as a plasma membrane marker. Scale bar, 10 μм. Images were captured using the following wavelengths: YFP (excitation, 514 nm; emission, 525-538 nm), CFP (excitation, 405 nm; emission, 450-490 nm) and chlorophyll auto fluorescence (excitation, 448 nm; emission, 640-740 nm).

B. Tissue localisation of *TaHKT1;5-D* by *in situ* PCR; 21-d-old roots grown in 2 мм NaCl; cells in which transcript is present stain blue. *TaHKT1;5-D* was detected in the stele of bread wheat variety Bob White, *18S* rRNA was used as a positive control to show presence of cDNA in all cell-types; a no RT (reverse transcription) control was included to show lack of genomic DNA contamination; c, cortex; en, endodermis; p, pericycle; x, xylem; xp, xylem parenchyma; scale bars, 100 μm.

Figure 3 T2 transgenic lines with reduced *TaHKT1;5-D* transcripts have increased Na^+ in the leaves and lower leaf K^+/Na^+ than their control lines.

A. Quantitative RT-PCR analysis of *TaHKT1;5-D* transcript levels in root tissues relative to four control genes following Burton et al. (2004).

B. Leaf four Na^+ concentration was increased in the transgenic lines with reduced *TaHKT1;5-D* transcripts relative to the null lines and wild type.

C. K^+/Na^+ ratio in leaf four of transgenic lines with reduced *TaHKT1;5-D* transcripts was reduced relative to the null lines and wild type.

Values are means ± SE. (A, B, C: WT n = 6; RNAi1 n = 5; RNAi2 n = 16; null RNAi1 n = 9; null RNAi2 n = 5). Different letters indicate a significant difference ($P < 0.05$) using Tukey's

test following a one-way ANOVA. T2 plants were grown in aerated hydroponics following Genc et al. (2007) with 50 mM NaCl and supplemental $CaCl_2$ (0.825 mM).

Figure 4 T4 transgenic lines with reduced *TaHKT1;5-D* transcripts have increased Na^+ in the leaves and lower leaf K^+/Na^+ than their control lines.

A. Quantitative RT-PCR analysis of *TaHKT1;5-D* transcript levels in root tissues in 100 mM NaCl relative to control genes following Burton et al. (2004).

B. Leaf four Na^+ concentration was increased in the transgenic lines with reduced *TaHKT1;5-D* transcripts relative to the null lines in 100 mM NaCl.

C. K^+/Na^+ ratio in leaf four of transgenic lines with reduced *TaHKT1;5-D* transcripts was reduced relative to the null lines in 100 mM NaCl.

Lines were grown in aerated hydroponics following Shavrukov et al. (2010). Values are means ± SE (A: RNAi1 n = 12; RNAi2 n = 12; null RNAi1 n = 10; null RNAi2 n = 9; B, C: RNAi1 n = 9; RNAi2 n = 10; null RNAi1 n = 8; null RNAi2 n = 10). Different letters indicate a significant difference ($P < 0.05$) using Tukey's test following a one-way ANOVA.

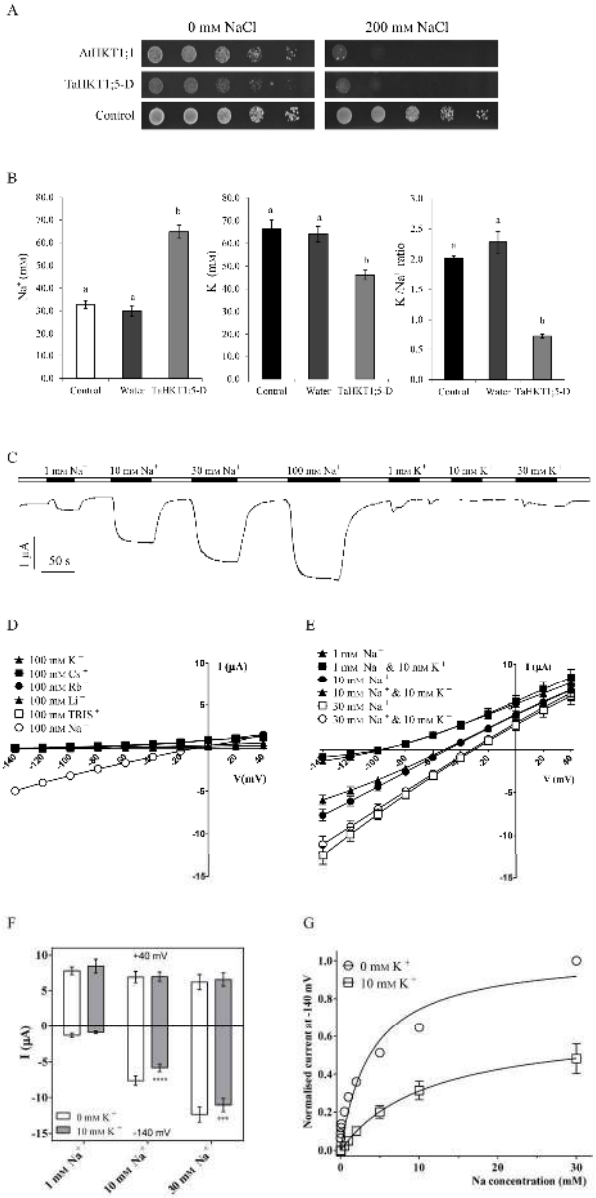

Figure 1
148x300mm (300 x 300 DPI)

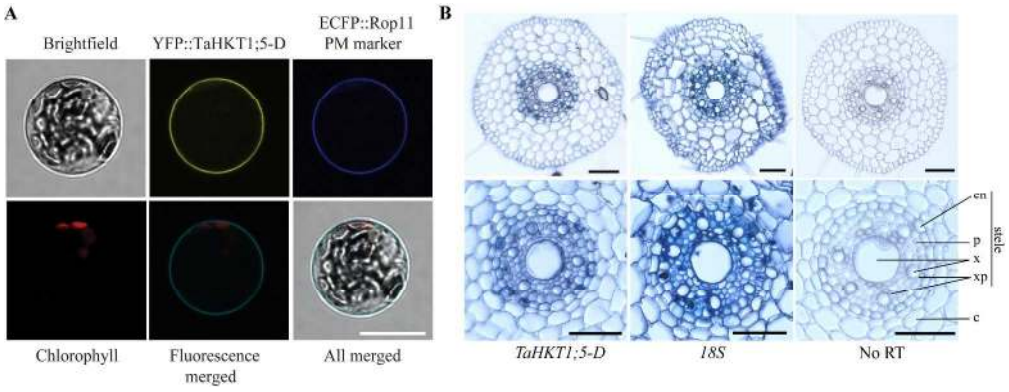

Figure 2
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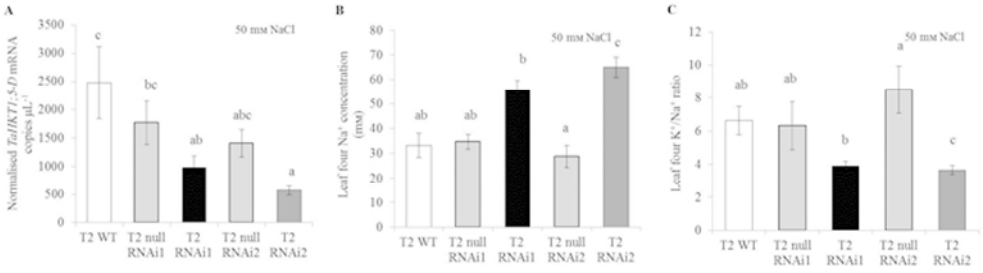

Figure 3
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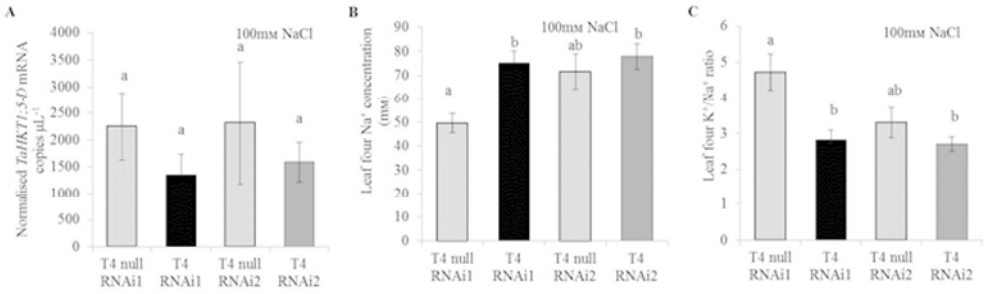


Figure 4
60x18mm (300 x 300 DPI)

SUPPORTING INFORMATION

Table SI Numbers of plants tested for leaf Na^+ and K^+ accumulation and for the presence or absence of an RNAi construct in each generation.

		Numbers of plants tested			
	Generation	[a]T1	T2	T3	T4
RNAi1	Leaf Na^+ and K^+ tested	14	14	35	153
	PCR (+:–)	3+	5+; 9-	23+;12-	67+;78-
RNAi2	Leaf Na^+ and K^+ tested	10	21	30	118
	PCR (+:–)	2+;1-	16+;5-	20+;10-	74+;39-

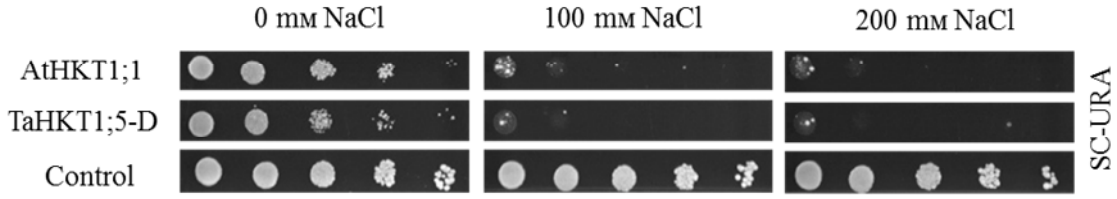
+ or – indicates presence or absence of an RNAi construct, respectively. Data was collected from multiple experiments and not all data is presented graphically.

[a]DNA from three T1 plants derived from a T0 plant positive for the RNAi1 construct was included in a PCR test and all three samples were positive for the RNAi1 construct, whereas when DNA from three T1 plants derived from a T0 plant positive for the RNAi2 construct was included in a PCR test two samples were positive for the RNAi2 construct and one was negative for the construct.

Table SII Summary of statistical significance. P value summary from Tukey's tests following one-way ANOVA using Graphpad Prism.

Figure 1B	A		B		C	
	Control vs. TaHKT1;5-D	****	Control vs. TaHKT1;5-D	***	Control vs. TaHKT1;5-D	****
	Water vs. TaHKT1;5-D	****	Water vs. TaHKT1;5-D	***	Water vs. TaHKT1;5-D	****
Figure 3	A		B		C	
	WT vs. RNAi1	*	WT vs. RNAi2	**	WT vs. RNAi2	**
	WT vs. RNAi2	***	Null RNAi1 vs. RNAi2	***	Null RNAi1 vs. RNAi2	**
	Null RNAi1 vs. RNAi2	*	RNAi1 vs. Null RNAi2	*	RNAi1 vs. Null RNAi2	**
			Null RNAi2 vs. RNAi2	***	Null RNAi2 vs. RNAi2	****
Figure 4	B		C			
	Null RNAi1 vs. RNAi1	*	Null RNAi1 vs. RNAi1	**		
	Null RNAi1 vs. RNAi2	*	Null RNAi1 vs. Null RNAi2	*		
			Null RNAi1 vs. RNAi2	**		
Figure S2	C		D			
	Null RNAi1 vs. RNAi1	****	Null RNAi1 vs. RNAi1	****		
	Null RNAi1 vs. RNAi2	****	Null RNAi1 vs. RNAi2	****		
	RNAi1 vs. Null RNAi2	****	RNAi1 vs. Null RNAi2	***		
	Null RNAi2 vs. RNAi2	***	Null RNAi2 vs. RNAi2	**		
KEY	**P Value summary**					
	< 0.0001	****				
	< 0.001	***				
	< 0.01	**				
	< 0.05	*				

A



B

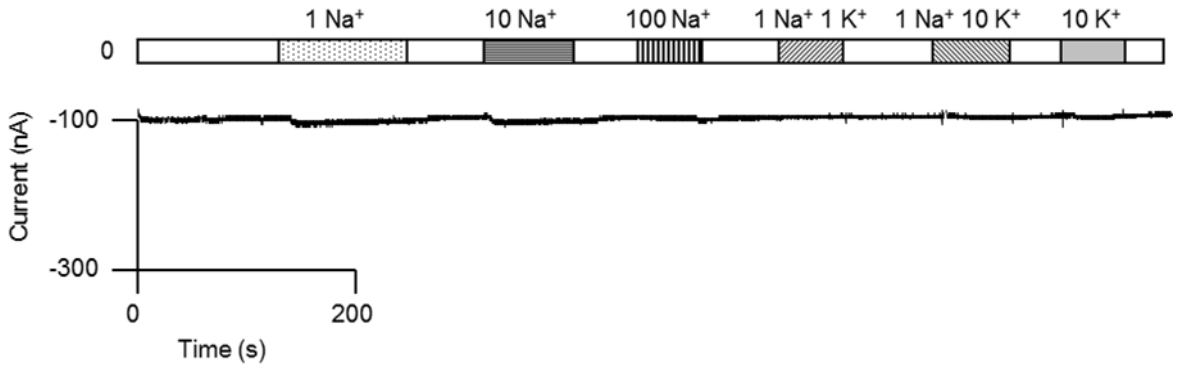


Figure S1 TaHKT1;5-D functions as a Na^+ transporter in *Saccharomyces cerevisiae* and currents over time for water injected oocytes in solutions with varying Na^+ and K^+ concentrations.

A. Images of the growth of *S. cerevisiae* strain INVSc2 expressing *AtHKT1;1* as a positive control, *TaHKT1;5-D*, and a negative (empty vector) control. Five serial dilutions were spotted on agar plates with synthetic complete minus uracil medium with 2% (w/v) Gal, 0.06% (w/v) histidine, which contains 10 mM Na^+ and additional either zero, 100 mM or 200 mM NaCl. Agar contains approximately 6 mM Na^+ and SC-URA media contains 4 mM Na^+.

B. Currents over time for water injected oocytes in response to varying Na^+ and K^+ concentrations. Units for Na^+ and K^+ are mM; the membrane potential was held at -120 mV; 0 refers to HMg solution without additional Na^+ and K^+.

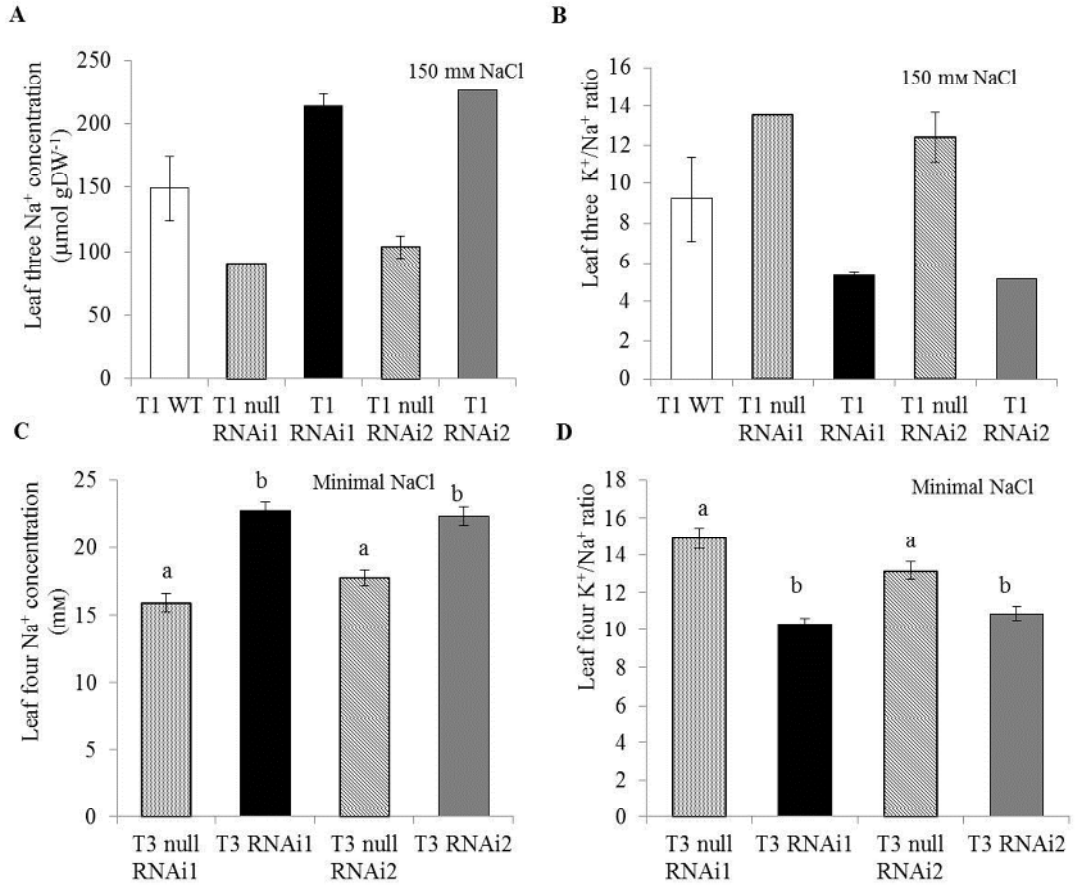


Figure S2 Transgenic lines with constructs designed to reduce *TaHKT1;5-D* transcripts by RNAi induced gene silencing have increased leaf Na^+ concentration relative to control lines in multiple generations.

A. Leaf Na^+ concentration in the T1 generation

B. Leaf K^+/Na^+ ratio in the T1 generation

C. Leaf Na^+ concentration in the T3 generation

D. Leaf K^+/Na^+ ratio in the T3 generation

The T1 generation of transgenic lines, respective null and wild type (WT = Bob White) controls, were grown in aerated hydroponics following Byrt et al. (2007) containing 150 mM NaCl and the fourth leaf was harvested after 10 d (RNAi1 n = 2; null RNAi1 n = 1; RNAi2 n = 1; null RNAi2

n = 2). The T3 generation and respective null controls were grown in coco peat with no additional NaCl (T3:RNAi1 n = 23; RNAi2 n = 20; null RNAi1 n = 14; null RNAi2 n = 10). Different letters indicate a significant difference ($P < 0.05$) using Tukey's test following a one-way ANOVA.

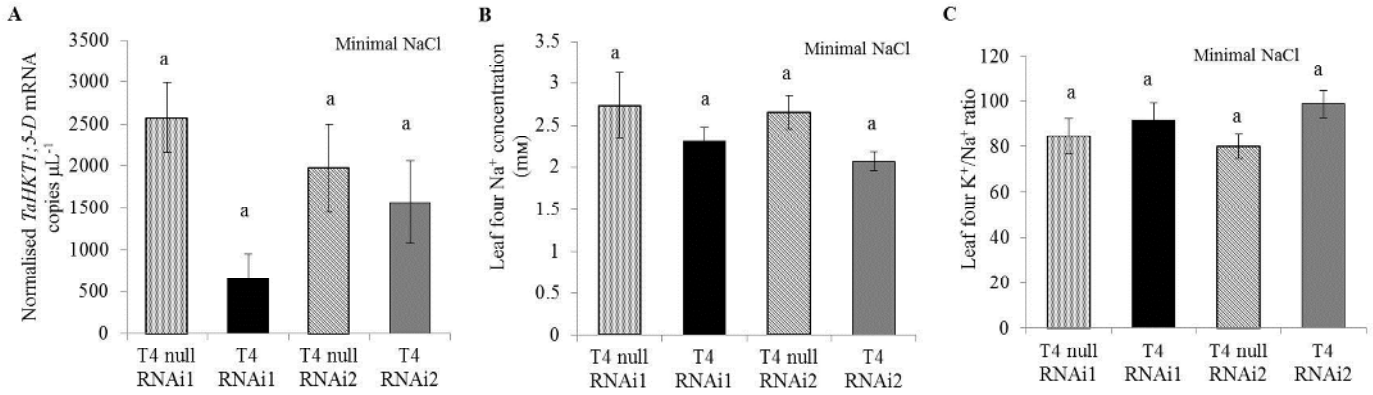


Figure S3 *TaHKT1;5-D* transcripts and leaf Na^+ concentration in the leaves of T4 transgenic lines grown in minimal NaCl.

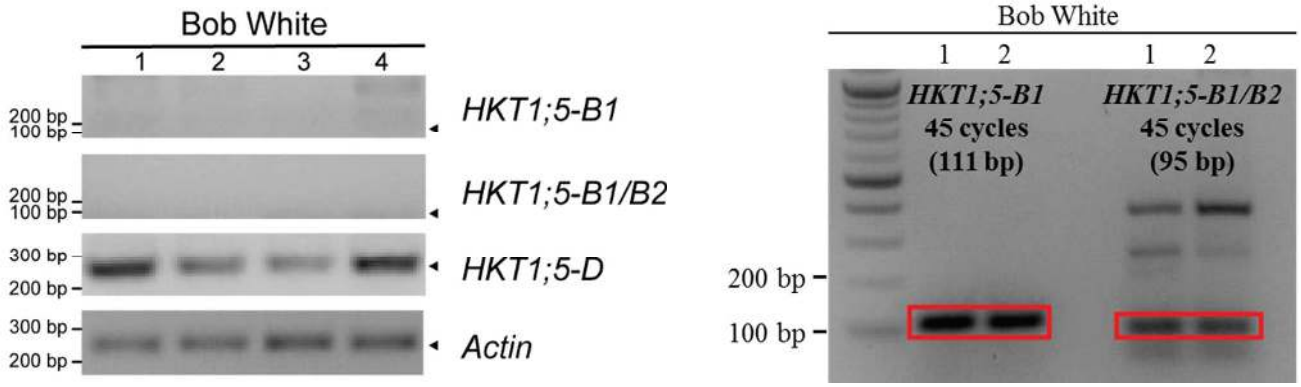
A. Quantitative RT-PCR analysis of *TaHKT1;5-D* transcript levels in root tissues in minimal NaCl relative to control genes following Burton et al. (2004).

B. Leaf four Na^+ concentration was increased in the transgenic lines with reduced *TaHKT1;5-D* transcripts relative to the null lines in minimal NaCl.

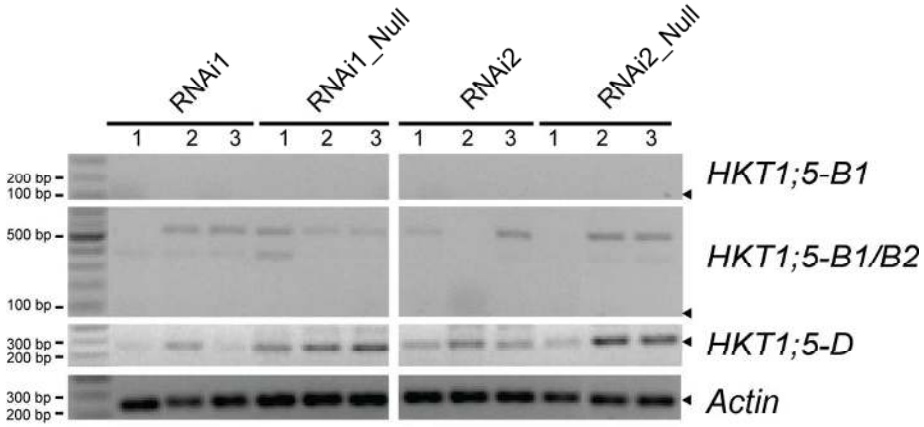
C. K^+/Na^+ ratio in leaf four of transgenic lines with reduced *TaHKT1;5-D* transcripts was reduced relative to the null lines in minimal NaCl.

Values are means ± SE Lines were grown in aerated hydroponics following Shavrukov et al. (2010). No additional NaCl was added to the hydroponic media, however the hydroponic media is not free of sodium; approximately 1 mM Na^+ is present in the media (Genc et al. 2007), thus it is referred to as minimal NaCl media. A: RNAi1 n = 12; RNAi2 n = 12; null RNAi1 n = 10; null RNAi2 n = 9; B, C: RNAi1 n = 9; RNAi2 n = 10; null RNAi1 n = 8; null RNAi2 n = 10. Different letters indicate a significant difference ($P < 0.05$) using Tukey's test following a one-way ANOVA.

A.


Bob White
1 2 3 4
200 bp
100 bp
HKT1;5-B1
HKT1;5-B1/B2
300 bp
200 bp
HKT1;5-D
Actin
Bob White
1 2 1 2
HKT1;5-B1
45 cycles
(111 bp)
HKT1;5-B1/B2
45 cycles
(95 bp)
200 bp
100 bp

B.


RNAi1
RNAi1_Null
RNAi2
RNAi2_Null
1 2 3 1 2 3 1 2 3 1 2 3
200 bp
100 bp
500 bp
HKT1;5-B1
HKT1;5-B1/B2
HKT1;5-D
Actin

C.

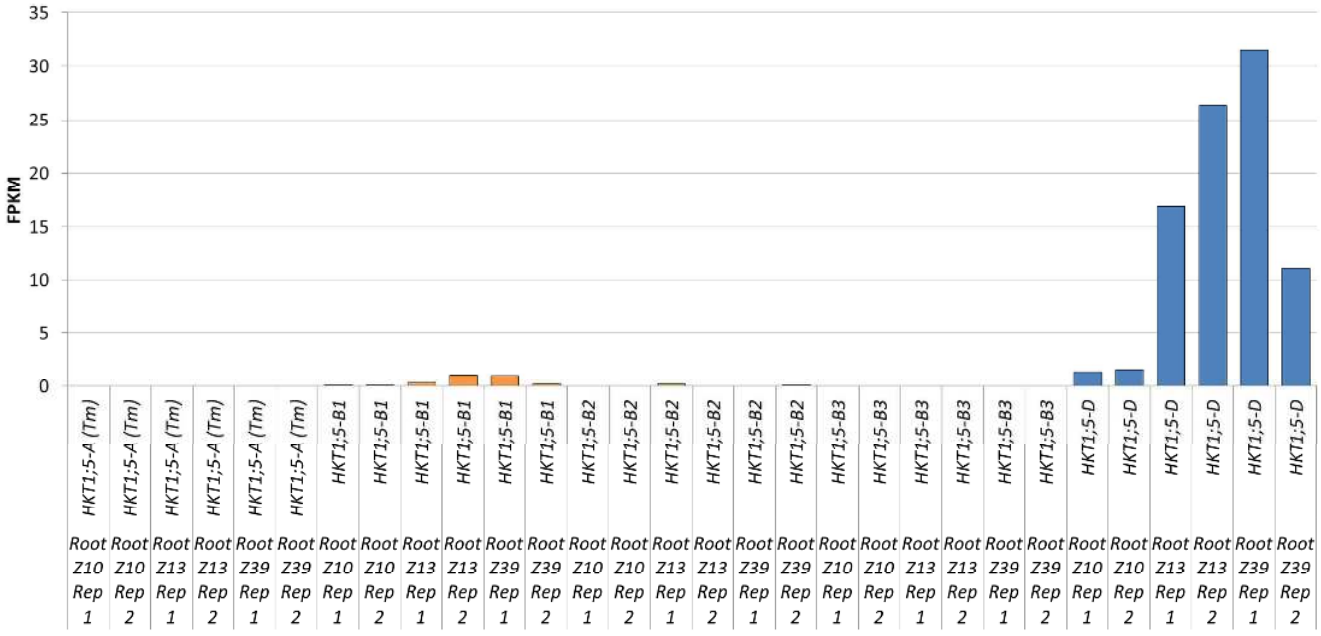

FPKM
35
30
25
20
15
10
5
0
HKT1;5-A (Tm) Root Z10 Rep 1
HKT1;5-A (Tm) Root Z10 Rep 2
HKT1;5-A (Tm) Root Z13 Rep 1
HKT1;5-A (Tm) Root Z13 Rep 2
HKT1;5-A (Tm) Root Z39 Rep 1
HKT1;5-A (Tm) Root Z39 Rep 2
HKT1;5-B1 Root Z10 Rep 1
HKT1;5-B1 Root Z10 Rep 2
HKT1;5-B1 Root Z13 Rep 1
HKT1;5-B1 Root Z13 Rep 2
HKT1;5-B1 Root Z39 Rep 1
HKT1;5-B1 Root Z39 Rep 2
HKT1;5-B2 Root Z10 Rep 1
HKT1;5-B2 Root Z10 Rep 2
HKT1;5-B2 Root Z13 Rep 1
HKT1;5-B2 Root Z13 Rep 2
HKT1;5-B2 Root Z39 Rep 1
HKT1;5-B2 Root Z39 Rep 2
HKT1;5-B3 Root Z10 Rep 1
HKT1;5-B3 Root Z10 Rep 2
HKT1;5-B3 Root Z13 Rep 1
HKT1;5-B3 Root Z13 Rep 2
HKT1;5-B3 Root Z39 Rep 1
HKT1;5-B3 Root Z39 Rep 2
HKT1;5-D Root Z10 Rep 1
HKT1;5-D Root Z10 Rep 2
HKT1;5-D Root Z13 Rep 1
HKT1;5-D Root Z13 Rep 2
HKT1;5-D Root Z39 Rep 1
HKT1;5-D Root Z39 Rep 2

Figure S4. Relative expression of the *HKT1;5* genes in bread wheat and the transgenic lines
A. Semi-quantitative comparison of *HKT1;5-B1*, *HKT1;5-B1* and *HKT1;5-B2* (*B1/B2*), and *HKT1;5-D* transcripts in Bob White (32 cycles); with *Actin* as a control gene (28 cycles). Black arrows indicated the expected product sizes: 111 bp (*HKT1;5-B1*); 95 bp (*HKT1;5-B1* and *HKT1;5-B2*); 252 bp (*HKT1;5-D*); and 234 bp (*Actin*) (n = 4). The *HKT1;5-B1* (111 bp) and *HKT1;5-B1* and *HKT1;5-B2* (95 bp) were visible when PCR cycling was increased to 45 cycles (n = 2); these products, indicated by red boxes, were sequenced to confirm the specificity of the reaction.
B. Semi-quantitative comparison of *HKT1;-5-B1*, *HKT1;-5-B1* and *HKT1;5-B2* (*B1/B2*), and *HKT1;5-D* transcripts in RNAi lines and their respective null lines (32 cycles); with *Actin* as a control gene (28 cycles). Black arrows indicated the expected product sizes: 111 bp (*HKT1;5-B1*); 95 bp (*HKT1;5-B1* and *HKT1;5-B2*); 252 bp (*HKT1;5-D*); and 234 bp (*Actin*) (n = 3).
C. International Wheat Genome Sequencing Consortium RNA-Seq data for *HKT1;5* genes. In bread wheat *HKT1;5-D* is 34× and 341× more highly expressed than *HKT1;5-B1* and *HKT1;5-B2*, respectively; *HKT1;5-B3* is not expressed. The *HKT1;5-A* gene is not present in most bread wheat; (*Tm*) = *Triticum monococcum HKT1;5-A* transcript sequence (Byrt et al. 2007). The five wheat *HKT1;5* gene sequences were mapped using publically available RNA-Seq data from the International Wheat Genome Sequence Consortium in *Triticum aestivum* cv. Chinese Spring (IWGSC (2014); https://urgi.versailles.inra.fr/files/RNASeqWheat/). Unidirected, 2x100bp paired-end data from Chinese Spring root tissue samples from three developmental stages (Zadok scale Z10, first leaf through coleoptile; Z13, three leaves unfolded; and Z39, flag leaf ligule just visible) were mapped using BioKanga. Fragments Per Kilobase Of Exon Per Million Fragments (FPKM) were calculated using the total number of concordant paired-end reads mapped to the 99386 IWGSC chromosomal survey sequences which had protein-coding gene models developed by MIPS (v2.1).

Figure S5 Transgenic wheat lines (T1) growing in a supported flood and drain hydroponics system.

Seed is germinated on Petri dishes prior to planting in gravel pots inside stacked tanks. The base tank contains half strength Hoadland's solution. A pump system floods the pots with solution and the solution then drains back into the bottom tank to prevent the plants from becoming waterlogged. Salt treatment commenced when the second leaf is half emerged. The NaCl concentration of the hydroponic solution is increased by 25 mM twice daily to reach a final concentration of 150 mM (in additional experiments the final NaCl concentration was 50 and 100 mM). Supplemental calcium ($Ca(NO_3)_2$) is added to achieve a Na^+ to Ca^{2+} ratio of 15:1. Leaf

three is harvested after 10 days growing in the saline conditions. The Na^+ and K^+ concentration in the leaves is measured; in this case using inductively coupled plasma analysis for the T1 generation (following Byrt et al. 2007); and using a flame photometer following Shavrukov et al. (2010) for the T2, T3 and T4 generations.