Grain yield of modern wheat on saline soils is improved by ancestral HKT gene

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The ability of wheat to maintain low leaf sodium concentrations ([Na⁺]) correlates with improved growth under saline conditions¹,². This trait, termed Na⁺-exclusion, is considered important for the greater salt tolerance of bread wheat relative to durum wheat³,⁴. To improve salt tolerance of durum wheat we explored natural diversity in shoot Na⁺-exclusion within ancestral wheat germplasm. A gene locus that confers shoot Na⁺-exclusion, Nax2, was identified in wheat relative Triticum monococcum and crossed into commercial durum wheat (Triticum turgidum ssp. durum var. Tamaroi)⁵. A gene underlying the Nax2 locus, TmHKT1;5-A, encodes a Na⁺-selective transporter located on the plasma membrane of root cells surrounding xylem vessels – so is ideally localised to withdraw Na⁺ from the xylem and reduce transport of Na⁺ to leaves. Field trials on saline soils confirms that the presence of TmHKT1;5-A significantly reduces leaf Na⁺ content and improves grain yield by 25% compared to near-isogenic lines without TmHKT1;5-A.

Salinity limits crop yield in arid and semi-arid areas, due either to irrigation with saline water, rising water tables resulting from land clearing, or natural subsoil salinity⁶. Global food requirements are expected to increase by 70% by 2050⁷, and as land degradation, urban spread and seawater intrusion are increasing over time, gains in agricultural productivity need to come from saline soils to meet demand.
Wheat (Triticum spp.) and rice (Oryza sativa) constitute the world's major staple food crops, but high concentrations of Na\(^+\) are detrimental to leaf function\(^2\). Therefore, improving shoot Na\(^+\) exclusion capacity of these cereals represents a useful tool for improving salt tolerance and food supply\(^1,8\). Durum wheat (Triticum turgidum ssp. durum), which is largely used for making pasta, couscous and unleavened bread, is a tetraploid wheat with genomes A and B, and is particularly sensitive to salinity as it has a limited ability to exclude Na\(^+\) from leaves. Durum wheat is more salt-sensitive than hexaploid bread wheat (Triticum aestivum containing A, B and D genomes), as it lacks the Na\(^+\) excluding locus Kna1 found on the D genome. The Kna1 locus enables bread wheat to maintain lower leaf Na\(^+\) and a greater K\(^+\) to Na\(^+\) ratio compared to durum wheat\(^3,9\). A novel source of Na\(^+\) exclusion (Nax2) not present in durum or bread wheat was found in a diploid ancestral wheat relative Triticum monococcum, which contains only the A genome\(^5\). The A genome of T. monococcum is homologous to the A genome of durum and bread wheat, but has evolved separately to contain many genes not present in durum and bread wheat\(^10\).

The Nax2 locus confers a reduced rate of Na\(^+\) transport from roots to shoots by retrieving Na\(^+\) from root xylem\(^5,11\). Nax2 was introgressed from T. monococcum into a modern durum cultivar, Tamaroi, via durum derivative Line 149, and near-isogenic lines (NIL) with and without Nax2 were developed\(^5\). Due to the lack of recombination in the chromosomal region of the Nax2 locus, fine mapping was not possible. Therefore, candidate genes that could impart Na\(^+\) exclusion were identified by screening for genes that confer similar phenotypes in other species\(^12\).

HKT genes are known to be important in regulation of Na\(^+\) and K\(^+\) transport in higher plants\(^1,8,13\). Proteins encoded by group 1 HKT genes of Arabidopsis and rice, AtHKT1;1 and OsHKT1;5, reduce transport of Na\(^+\) to shoots\(^14-17\) and increase plant salinity tolerance as determined by measurement of leaf biomass under controlled conditions\(^18,19\). An HKT1;5-like gene was considered a possible Nax2 candidate\(^12\) because OsHKT1;5 bestowed a similar reduced rate of Na\(^+\) transport from roots to shoots in rice\(^17\). A probe was designed from a partial wheat HKT1;5-like EST sequence and this co-segregated with the low Na\(^+\) leaf phenotype of Nax2 in segregating NILs\(^12\). Further analysis of these NILs show that leaf Na\(^+\) was reduced by at least 50% when the HKT1;5-like gene was present, which makes it a strong gene candidate for the Na\(^+\) excluding ability of Nax2 (Supplementary Figure 1).

An HKT-like gene cloned from the ‘Nax2’ EST derived from T. monococcum was named TmHKT1;5-A as its gene product shared 66% amino acid identity with that of OsHKT1;5, a gene that encodes a plasma membrane Na\(^+\) transporter expressed in cells surrounding xylem tissues\(^17\). TmHKT1;5-A was given the A designation as it is present on the
A genome and so distinguishes it from TaHKT1:5-D, the putative candidate gene for the
Kna1 locus on the D genome, with which it shares 94% amino acid identity12. Significantly,
although HKT1;5-A is present in T. monococcum it has not been found in any accession of T.
urartu, which is the ancestral donor of the A-genome for modern durum and bread wheat10.
Therefore, TmHKT1;5-A is likely to be absent in all modern wheat and as such we proposed
that Nax2/TmHKT1;5-A encodes a transporter that could provide a novel source of Na+
exclusion in durum wheat11.

To test whether the Nax2 candidate TmHKT1;5-A encodes a Na+ permeable
transporter, it was expressed in Saccharomyces cerevisiae grown in a liquid culture containing
10 mM NaCl. OsHKT1;5 was used as a positive control17. Yeast transformed with
TmHKT1;5-A grew slower in 10 mM NaCl compared to OsHKT1;5-expressing yeast, and
both these strains grew slower than the empty-vector control (Figure 1a; P < 0.01). All strains
grew equally in liquid media lacking supplemental NaCl (Figure 1b). These results are
consistent with a reduction in growth of yeast by TmHKT1;5-A catalysing Na+ flux into yeast
cells and not by protein overexpression. To confirm that Na+ is transported through
TmHKT1;5-A, Xenopus laevis oocytes were injected with TmHKT1;5-A cRNA. TmHKT1;5-A
induced Na+ accumulation such that oocytes contained approximately double the [Na+], and
half the [K+], of water-injected oocytes (Supplementary Figure 2). Furthermore, when the
plasma membrane (PM) of TmHKT1;5-A cRNA injected oocytes was voltage clamped at
negative membrane potentials an inward current was induced in the presence of external Na+
but not K+ (Figure 1c–e), with a $K_m$ for Na+ of 0.88 ± 0.18 mM s.e (n=4) (Supplementary
Figure 3) indicating TmHKT1;5-A formed a Na+-, and not K+-permeable channel. To our
knowledge transport affinities for HKT1;5-like gene products have not previously been
reported, but compared to other HKT proteins19 the affinity of TmHKT1;5-A for Na+
transport is relatively high. This would allow considerable retrieval of Na+ from the xylem at
physiologically relevant concentrations in saline and non-saline conditions20.

Maintenance of high K+ and high K+/Na+ ratio in leaves is an important component of
salinity tolerance8. Thus, the transport properties of TmHKT1;5-A injected oocytes in
differing external K+ and Na+ concentrations ([x]ext) were examined. The reversal potential
($E_{rev}$) for TmHKT1;5-A cRNA induced-currents shifted positive with increasing [Na+]ext,
consistent with the predicted change in equilibrium potential for Na+ ($E_{Na}$), but did not change
with increasing [K+]ext (Figure 1d,e; Supplementary Figure 4A). This indicates that
TmHKT1;5-A acts as a Na+-selective uniporter, in an identical mode to other HKT1;5-like
gene products. Interestingly, conductance of TmHKT1;5-A cRNA injected oocytes decreased
from 61 ± 12 μS to 20 ± 4 μS (mean ± s.e.) in the presence of 1 mM [Na+]ext, when [K+]ext
was increased from 0 mM to 30 mM, though this did not occur in the presence of 10 mM Na$^+$ with conductance constant at 50 ± 10 µS (Figure 1f,g; Supplementary Fig. 4B). Although TmHKT1;5-A was not blocked by [K$^+$]$_{\text{ext}}$ at high [Na$^+$]$_{\text{ext}}$, a similar block of Na$^+$ transport through TaHKT2;1 and OsHKT2;1 by external K$^+$ has been observed in both low and high [Na$^+$]$_{\text{ext}}$. This was proposed to occur via binding of K$^+$ to Na$^+$-binding sites within the pore region. Neither phenomena have been previously observed from expression of AtHKT1;1 or OsHKT1;5 in X. laevis oocytes$^{17,23}$ suggesting differences in sensitivities of all these proteins to [K$^+$]$_{\text{ext}}$. Whether block of Na$^+$ transport by [K$^+$]$_{\text{ext}}$ plays a physiological role is yet to be determined. However, as [K$^+$] has been measured at 5 mM in salt-stressed wheat encoding Nax2$^5$, and would increase in low transpiring conditions e.g. night, any resulting depolarisation below E$_{\text{Na}}$ would result in leakage of Na$^+$ back into the xylem unless transport through TmHKT1;5-A was blocked.

For TmHKT1;5-A to be directly involved in retrieval of Na$^+$ from the xylem – thus limiting Na$^+$ transport from root to shoot and reducing shoot Na$^+$ accumulation, it should be targeted to the plasma membrane (PM) in cells within the stele of the root, and preferentially expressed within cells lining the xylem. Previously, AtHKT1;1 was shown to be located on the PM of Arabidopsis$^{24}$ and OsHKT1;5::GFP was identified on the PM of X. laevis oocytes$^{17}$. Similarly, we detected TmHKT1;5-A::YFP on the PM through transient expression in Arabidopsis mesophyll cell protoplasts (Figure 2a). In roots, in situ PCR indicated that expression of TmHKT1;5-A was predominantly within the stele, particularly within xylem parenchyma and pericycle cells adjacent to early metaxytem vessels of Tamaroi [+] Nax2/TmHKT1;5-A (the NIL containing Nax2/TmHKT1;5-A) but was absent in Tamaroi [-] Nax2/TmHKT1;5-A (the NIL without Nax2/TmHKT1;5-A) (Figure 2b). TmHKT1;5-A transcripts were abundant in roots of Line 149 and Tamaroi [+ ] Nax2/TmHKT1;5-A, but below reliable detection limits in shoots; no TmHKT1;5-A transcripts were detected in Tamaroi [-] Nax2/TmHKT1;5-A (Figure 2c). As no induction of TmHKT1;5-A transcript in either root or shoot was detected in any of germplasm tested (Figure 2c), the expression of TmHKT1;5-A differs from that of OsHKT1;5 in rice, where transcripts were observed in roots and shoots, and transcript levels increased in response to salt treatment$^{17}$. This indicates that TmHKT1;5-A could confer a root-specific constitutively-active mechanism of Na$^+$ retrieval leading to the lower Na$^+$ leaf content in saline and non-saline conditions as seen in lines containing Nax2 (Supplementary Figure 1; Figure 3a). Previously, increased vacuolar [Na$^+$] was measured within pericycle and xylem parenchyma in mature seminal roots of Line 149 (the donor of Nax2/TmHKT1;5-A) when compared to Tamaroi when both were grown in 150
mM NaCl; we now provide a direct link between the cell-types that express *TmHKT1;5-A* and those that accumulate additional Na$^+$. In summary, *TmHKT1;5-A* encodes a high-affinity (for a HKT), Na$^+$-specific transporter located on the PM (Figure 1 and 2). It is present within cells adjacent to the xylem of wheat roots containing the *Nax2* locus and this results in a reduced accumulation of Na$^+$ in seedling leaves by at least 50% (Supplementary Figure 1). This provides very strong evidence that *TmHKT1;5-A* is the gene responsible for the shoot Na$^+$ exclusion phenotype controlled by the *Nax2* locus.$^5,11,12$

Leaf Na$^+$ exclusion correlates with enhanced shoot growth in salt-stressed wheat grown in controlled environments$^{25-27}$, but there is no evidence that it enhances grain yield under field conditions. We therefore evaluated the impact of the Na$^+$ excluding gene *TmHKT1;5-A* on durum wheat grown in saline fields of the Australian wheat belt. A map of the apparent electrical conductivity (ECa) across a large commercially farmed field shows the typically variable nature of soil salinity (Figure 3a). The ECa was related to salinity with soil cores taken to a depth of 0.8 m, and three blocks of contrasting salinity were selected for the yield trial (Figure 3b).

The effect of *TmHKT1;5-A* on Na$^+$ exclusion and on grain yield was evaluated by comparing Tamaroi [+] *TmHKT1;5-A* with parent cultivar Tamaroi (lacking *TmHKT1;5-A*) on the three blocks. Na$^+$ concentration in the flag leaf (the uppermost and last fully formed leaf below the grain head) was measured during grain fill, a developmental stage when a constraint on photosynthetic capacity of the flag leaf may limit eventual grain yield. The flag leaf [Na$^+$] increased in all genotypes with increasing soil salinity, however, [Na$^+$] in Tamaroi [+] *TmHKT1;5-A* was 4-12 fold lower than in Tamaroi (Figure 3b). Even at high soil salinity (Block 3), the presence of *TmHKT1;5-A* kept flag leaf [Na$^+$] below 100 mM (whole tissue) compared to 326 mM in Tamaroi (Figure 3b). Previously we determined in durum wheat that below a leaf [Na$^+$] of 250 mM (whole tissue), vacuolar compartmentation of Na$^+$ in mesophyll cells is effective in minimising any increase in [Na$^+$] within the cytoplasm or chloroplast.$^{28}$ At higher leaf [Na$^+$], such as those that occur in Tamaroi, chloroplastic and cytoplasmic [Na$^+$] increases and photosynthesis is impaired. This would limit the carbon assimilation needed for initiation and growth of florets, and starch formation in the developing grain. We would therefore expect that the Na$^+$ exclusion due to *TmHKT1;5-A* to become important in preventing Na$^+$ toxicity in leaves only at a critical salinity level.

Grain yield of all genotypes declined substantially with increasing salinity, due to the osmotic effect of the salt outside the roots (Figure 3a). At the low to moderate salinity levels occurring in Block 1, grain yields of both Tamaroi and Tamaroi [+] *TmHKT1;5-A* were about
2.5 tonnes per hectare (t ha$^{-1}$), a typical and profitable yield for broad-acre rainfed (non-irrigated) wheat grown in semi-arid regions of the world. At the highest salinity level (Block 3), yields of Tamaroi decreased by 50%, however Tamaroi [+]$TmHKT1;5$-A were much less affected, with yields reduced by only 36%. The net result was that lines containing $TmHKT1;5$-A yielded significantly more than Tamaroi in high salinity, namely 0.3 t ha$^{-1}$ or 24% more. This was due to more grains per plant rather than larger grains, as the grain weights of Tamaroi [+]$TmHKT1;5$-A were marginally less than Tamaroi (Supplementary Table 1). Therefore, the substantial decrease in leaf Na$^+$ concentration in the $TmHKT1;5$-A lines was associated with a grain yield improvement of 24% in the highly saline soils.

It is significant that lines containing $TmHKT1;5$-A had similar yields to the recurrent parent Tamaroi in less saline soil, that is, there was no 'yield penalty' associated with presence of $TmHKT1;5$-A. This can be observed in the least saline block in the main trial in 2009, or in a neighbouring field with very low salinity in 2008 (Figure 3). These two years of field trials were preceded by four years of preliminary field trials at ten different sites in very different soil types and climates in south-eastern Australia. In all sites with non-saline soil, lines with $TmHKT1;5$-A had the same height, tiller density, seed weight and harvest index as Tamaroi [-]$TmHKT1;5$-A and the parent cultivar Tamaroi (e.g. Supplementary Table 2). In all sites, the grain yield in Tamaroi [+]$TmHKT1;5$-A was the same or greater than Tamaroi [-]$TmHKT1;5$-A or the parent cultivar Tamaroi (Supplementary Table 3). As most salt-affected fields are not uniformly saline (e.g. Figure 3a) it is important that an introduced gene brings no penalty on the best soil as most of the grain yield comes from the least saline patches$^{29}$. Optimal yield in both saline and non-saline soil is an important factor that will influence the adoption of these lines by farmers.

In conclusion, durum wheat is particularly sensitive to salinity compared to bread wheat, due largely to poorer Na$^+$ exclusion capability. Here we present the functional characterisation of $TmHKT1;5$-A, which is a novel gene for Na$^+$ exclusion obtained from an ancestor of modern wheat that is not present in commercial wheat genotypes$^{10}$. This study demonstrates the great potential for the genetic diversity inherent in non-domesticated germplasm for improving plant productivity and stress tolerance. Furthermore, by use of marker-assisted breeding to introduce this gene into durum wheat this crop is not classified as transgenic and will not be subject to the restrictions associated with genetically-modified material. The introduction of $TmHKT1;5$-A into durum wheat resulted in significantly reduced Na$^+$ uptake into leaves leading to an improvement in grain yield by as much as 25% when grown in saline soil. This is the first field study to demonstrate a grain yield improvement in saline soils by improving shoot Na$^+$-exclusion through the transfer of a root-localised $HKT$
gene into a commercial wheat variety. To emphasise the potential impact of this approach to food security in general, incorporation of Nax2 (TmHKT1;5-A) into bread wheat was recently shown to increase leaf Na\(^+\) exclusion when grown in the greenhouse\(^{30}\). Interestingly, greater leaf Na\(^+\) exclusion is observed in bread wheat despite the presence of the Na\(^+\)-excluding locus Kna1\(^{30}\); field trials in commercial bread wheat varieties containing both Nax2 and Kna1 are underway.

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**ACCESSION NUMBERS**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ646332 (TmHKT1;5-A mRNA) and HQ162137 (OsHKT1;5 cv Pokkali).

**AUTHOR CONTRIBUTIONS**

R.M, R.A.J., M.T., M.G., D.P. conceived the project and planned experiments. R.M. and M.G. supervised the research. B.X. performed all Xenopus, yeast and protoplast experiments, R.A.J. performed field research. R.A.H. assisted with original identification of Nax2. C.S.B. performed wheat genotyping. S.D.T. assisted with electrophysiology experiments. S.J.C., A.A and C.J. performed in situ PCR and qPCR. M.G., D.P., R.A.J, R.M. wrote the manuscript. All authors made comment on the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.
FIGURE LEGENDS

Figure 1. TmHKT1;5-A expression in yeast (Saccharomyces cerevisiae) and Xenopus laevis oocytes.
(a) Growth of yeast expressing TmHKT1;5-A, OsHKT1;5 and empty-vector control in AP<sup>31</sup> media with 10 mM NaCl. (b) Growth of yeast without added NaCl. (c) Currents elicited by TmHKT1;5-A-cRNA injected oocyte when bathed in Na<sup>+</sup>- or K<sup>+</sup>-glutamate solution clamped at −120 mV; in water injected oocytes no inward currents were detected. (d-g) Current-voltage (I-V) curve of TmHKT1;5-A-injected oocytes exposed to different concentrations of Na<sup>+</sup> (n = 5), K<sup>+</sup> (n = 4), and Na<sup>+</sup> plus K<sup>+</sup> (n = 5). Average currents from water-injected controls at each voltage have been subtracted in all cases.

Figure 2. (a) Plasma membrane localisation of TmHKT1;5-A::YFP in Arabidopsis mesophyll protoplasts; ECFP::ROP11<sup>32</sup> was used as plasma membrane marker (see online methods); scale bars = 10 µM. Images were captured using the following wavelengths YFP (excitation = 514 nm, emission = 525–538 nm), CFP (excitation = 405nm, emission = 450–490nm) and chlorophyll auto fluorescence (excitation = 448, emission = 640–740 nm). (b) Tissue localisation of TmHKT1;5-A using in situ PCR on 21 day old roots grown in 2 mM NaCl; cells in which transcript is present stain blue. From left to right, TmHKT1;5-A primers in Tamaroi [−] Nax2 (to confirm absence in lines without Nax2), TmHKT1;5-A primers in Tamaroi [+] Nax2 (to show stelar localisation of TmHKT1;5-A), 18S rRNA in Tamaroi [+] Nax2 (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; scale bars = 100 µM. (c) TmHKT1;5-A is highly expressed in roots of Line 149 and Tamaroi [+] Nax2 compared to Tamaroi [−] Nax2 and shoots of all genotypes after 3 weeks in 20 µM NaCl (clear bars). Additionally, in 3 week-old roots expression of TmHKT1;5-A was not found to be inducible by increasing [Na<sup>+</sup>]<sub>ext</sub> from 20 µM to 50 mM for 3 days prior to harvest in the roots or shoots of any germplasm tested (filled bars). Expression was normalised using TaActin, TaCyclophilin and TaEF-α as per online methods. Data presented as mean ± s.e. (n=6; three biological replicates per treatment with each replicate comprising of two pooled plants, with qPCR performed in triplicate).

Figure 3. (a) Electrical conductivity of a saturated soil extract, EC<sub>e</sub> (0 – 0.8 m), mean soil NaCl concentration (0 – 0.8 m), flag leaf Na<sup>+</sup> concentration and grain yield of durum wheat cultivar Tamaroi and Tamaroi [+] TmHKT1;5-A, grown in Field 1 at Moree (2008) and in three separate trial blocks in Field 2 at Moree (2009). Values are adjusted means with range given in brackets. (b) Apparent electrical conductivity (ECa) of a salinity–affected field near Moree in northern NSW, Australia. Numbers indicates location of field trial site in 2008 (1) and 2009 (2). (c) Relative increase in grain yield of Tamaroi [+] TmHKT1;5-A compared to Tamaroi. * indicates significant difference (P<0.05).
**ONLINE METHODS**

*Germplasm development, genotyping, and phenotyping*

Line 149 was derived from a cross between durum wheat cultivar Marrocos and *Triticum monococcum* accession C68-101. Line 149 was backcrossed with durum wheat cultivar Tamaroi to produce two independent BC$_4$ lines (5004 and 5042) lines containing Nax2 (*TmHKT1;5-A*)$^{5,12}$.

For phenotyping, seedlings were grown in supported hydroponics as described previously$^{27}$. After 10 d in 150 mM NaCl, the blade of leaf 3 was harvested, and Na$^+$ concentration measured$^5$. For genotyping, seedlings were screened for the presence of *TmHKT1;5-A*, using dominant molecular markers (*gwm410, gwm291*)$^{12}$. A linked codominant marker, *cslinkNax2*, developed from a BAC sequence, was used for selection and validation of BC$_4$F$_4$ families fixed for *TmHKT1;5-A* (Supplementary Table 4). PCR amplifications were performed in 20 µL- aliquots containing 200 µM dNTPs, 10X PCR buffer, 0.5 µM each primer, 1 unit of *Taq* DNA polymerase (Hotstar*, Qiagen), and 100 ng of genomic DNA with the following cycling protocol: 95°C for 15 min; 5 cycles of 94°C for 1 min; 58°C for 1 min; 72°C for 1 min; and then 30 cycles of 94°C for 30 s; 58°C for 30 s; 72°C for 50 s.

**Isolation of TmHKT1;5-A and plasmid construction**

*TmHKT1;5-A* was amplified from cDNA isolated from 3 week-old Line 149 root tissue grown in ¼ Hoagland solution using Phusion™ Hot Start High-Fidelity DNA polymerase (Finnzymes) with primers described in Supplementary Table 4, cloned into the Gateway® entry vector PCR8/GW/TOPO (Invitrogen) and transformed into *E.coli* (Qiagen). Clones of correct sequence were recombined into Gateway® destination vectors pYES2-DEST52, pGEMHE-DEST and pBS (35S::YFP-attR) for expression in *S. cerevisiae*, *X. laevis* oocytes and for membrane localisation, respectively. A plasma membrane marker was constructed by replacing 35S::YFP-attR within pBS with 35S::Rop11::eCFP using *PstI.*

**Growth inhibition assay of TmHKT1;5A in S. cerevisiae**

*TmHKT1;5-A* and *OsHKT1;5* were transformed into a *S. cerevisiae* strain without mutated transport characteristics, InvSc2 (*MATa, his3-D200, ura3-167*, Invitrogen), using the LiAc/SS carrier DNA/PEG method$^{33}$. Growth inhibition assays of 10 mL AP media$^{31}$ ± NaCl aliquots at 30°C were quantified using 0.3 mL from each sample at OD$_{600}$ (nm) every 24 h for 3 d (BioRad, Smart Spec™ 3000).
Characterisation of TmHKT1;5-A in X. laevis oocytes

pGEMHE-DEST containing TmHKT1;5-A was linearised using SbfI-HF (New England BioLabs); cRNA was synthesized using mMESSAGE mMACHINE® T7 Kit (Ambion) following manufacturer’s instructions. 46 nL/23 ng of cRNA or equal volumes of RNA-free water were injected into oocytes with a Nanoinject II microinjector (Drummond Scientific). Oocytes were incubated for 48 hours and electrophysiology performed as previously described\(^3\). 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. All solution osmolarities were adjusted using mannitol 240-260 mOsmol kg\(^{-1}\) (Vapour pressure osmometer, Wescor).

Quantification of ion-profile in oocytes

Oocytes were incubated at 18 °C in ND96 solution for 2 d. Three replications of 4 grouped oocytes were rinsed in HMg solution 3 times, homogenized in 1 mL 1% (w/v) nitric acid by vortexing, incubated at 75°C for 1 hour and stored at 4 °C overnight and ion-profiles quantified by flame photometry (Sherwood 420)\(^16\).

Membrane localisation of TmHKT1;5A in Arabidopsis mesophyll protoplasts

pBS vectors containing TmHKT1;5-A and Rop11::eCFP were co-transformed into Arabidopsis mesophyll protoplasts as described previously\(^31,35\) using 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) replacing WI and W5 solutions. Fluorescent signals were captured from co-transformed protoplasts by a confocal laser scanning microscope (Leica TCS SP5).

Quantitative reverse transcriptase PCR (qPCR)

Root RNA was isolated using Trizol™ reagent (Invitrogen) from 3 week-old Tamaroi and Line 149 grown in modified ¼ Hoagland solution (20 μM NaCl) ± 50 mM NaCl for 3 d. Genomic DNA was removed using Turbo DNA-free (Life Technologies), with 1 μg RNA reverse transcribed using Thermoscript™ RT (Invitrogen), omitting the RNase H step. qPCR was performed using KAPA SYBR® FAST Bio-Rad iCycler 2X qPCR master mix (KAPA Biosystems), with 250 nM of primer using the following cycling parameters: initial denaturation at 95 °C for 2 mins, then 40 cycles of 95 °C for 20 s, 55 °C for 20 s, 72 °C for 20 s, with DNA sequencing and melt curve analysis performed to confirm product identity. qPCR normalisation was performed using TaActin, TaEFA and TaCyclophilin as
normalisation genes (primers listed in Supplementary Table 4), presenting data as normalised transcript abundance.  

**In situ PCR**

Previous methods were followed with the following modifications. Fifty µm sections were obtained using a VT 1200 S Vibrating Microtome (Leica) and aspirated with a cut pipette tip into 100U of RNaseOUT in 100 µl of cold sterile water kept on ice. Genomic DNA was removed by treatment for 45 min at 37°C in 8U RNase-free DNase (Qiagen) in 1X Turbo DNase buffer I (Ambion), followed by addition of EDTA to a final concentration of 15 mM and heat inactivation at 75 °C for 10 min. cDNA synthesis was made using Thermoscript™ RT (Invitrogen), omitting the RNase H step, with a gene-specific primer (0.5 µM, Supplementary Table 4) used for reverse transcription. PCRs were carried out in a final volume of 50 µl containing 1X PCR buffer with 1.5 mM MgCl$_2$, 200 µM dNTPs, 0.2 nmol digoxigenin-11-dUTP (Roche), 0.5 µM of each primer, and 2 U Taq DNA polymerase (New England Biolabs). Cycling parameters were as follows: initial denaturation at 95 °C for 30 s, then 34 cycles of 95 °C for 10 s, 58 °C for 25 s, 72 °C for 5 s and a final extension at 72°C for 5 mins. Sections then were washed, incubated with 1.5 U alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Roche), and developed for 45 min in the dark with 50µl BM Purple AP-substrate (Roche, Cat. No. 11442074001). For negative control sections, reverse transcriptase was omitted, and 18S rRNA primers (listed in Supplementary Table 4) were included to check whether there was any amplification from genomic DNA.

**Field trials**

Field experiments were conducted near Moree, NSW, Australia (29.05°S, 149.78°E). Soil type was a black vertosol (pH 6.5 – 7.0). Field trial sites were initially identified through mapping (Agis, Delta Data Systems, Mississippi, US) apparent electrical conductivity (ECa) using an EM38 electromagnetic induction meter (Geonics, Mississauga, Canada) in a 100 m grid (Figure 3b). Candidate sites were further characterised by fine ECa mapping (5 m grid) and validated with chemical analysis of soil cores down to 0.8 m. Chloride was measured on a filtered sub-sample of a 1:5 soil:water extract using a QC8500 automated ion analyser (Lachat, Loveland, CO). ECa measurements were taken by furrowing an EM38 meter into the soil so that it was flat and in contact with moist soil which was typically at a depth of 2 cm. Soil cores were taken in locations that reflected the range in ECa across each experimental ‘Block’. ECa was measured on every plot and four soil cores were taken covering the range in ECa within each Block. Soil cores were taken at the beginning of the season at sowing when soil moistures
were high. Additional soil cores were not taken mid or late season. Instead, flag leaves (the uppermost and last emerging leaf below the grain head) were removed at mid-grain fill and measured for Na⁺ concentration (as described above). Flag leaf Na⁺ concentrations of poor Na⁺ excluding lines such as Tamaroi, reflected the level of soil salinity experienced by the wheat plant over the life of the flag leaf (4 weeks). Field trials were conducted at two locations over two years. The 2008 field trial site (Field 1) with low/negligible salinity levels was sown in three randomised blocks. The trial was sown in June 2008 and harvested December 2008. The trial was not irrigated, but received 191 mm rainfall between sowing and harvesting. The 2009 field trial site (Field 2) with moderate/high salinity levels was sown in three blocks where salinity ranged from low/moderate (Block 1) to high (Block 3). Each block contained Tamaroi (16 replicate plots) and two independent Tamaroi[+] TmHKT1;5-A lines (8 replicate plots each), which were sown as part of an augmented Latin Square design (8 x 8), with each block containing 64 individual 10m² plots. The trial was sown in May 2009, harvested November 2009, and received 74 mm rainfall during that period.

Statistical Analysis
Grain yield data from the field trial were analysed as mixed models fitted via GenStat REML (VSNi), accounting for effects of block and allowing for spatial autocorrelation (lag 1) between results across rows and columns. ECa was recorded for every plot and used as a covariate in the analysis of yield data. Graphpad Prism was used for graphing data and all other analysis.
REFERENCES


**ONLINE METHODS REFERENCES**
Figure 1

(a) Comparison of OD at 600 nm (OD₆₀₀nm) for different treatments: TmHKT1;5-A, OsHKT1;5, and Control. Over 3 days, the graph shows a clear increase in OD for the treatments compared to the control.

(b) Same as (a), but for 0 mM Na. The graph indicates a different trend compared to 10 mM Na.

(c) Graph showing current (µA) over time (days) with different concentrations of Na (1 mM, 10 mM, 30 mM, 100 mM) and K (1 mM, 10 mM, 30 mM). The graph indicates a significant increase in current with higher concentrations of Na and K.

(d) Graph showing current (µA) vs. voltage (mV) for different Na concentrations (1 mM, 10 mM, 30 mM). The graph shows a linear relationship between current and voltage for each concentration.

(e) Similar to (d), but for K concentrations (1 mM, 10 mM, 30 mM). The graph also shows a linear relationship between current and voltage for each concentration.

(f) Graph showing current (µA) vs. voltage (mV) for different combinations of Na and K concentrations. The graph indicates a complex relationship between current and voltage depending on the combination of Na and K.

(g) Similar to (f), but for a different set of combinations. The graph shows a similar trend, indicating a complex relationship between current and voltage with varying Na and K concentrations.
Figure 2

(a) Brightfield, PM marker CFP, TmHKT1;5-A::YFP, Chlorophyll, Merged

(b) Line: Tamaroi [-Nax2], Tamaroi [+Nax2], Tamaroi [+Nax2], Tamaroi [+Nax2]
Primers: TmHKT1;5-A, TmHKT1;5-A, 18S, TmHKT1;5-A (no RT)

(c) Relative Expression

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## Figure 3

### Table: 

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<td>11.4 (7.2–15.4)</td>
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<td>42 (23–63)</td>
<td>75 (35–98)</td>
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<td>Flag leaf [Na⁺] (mM)</td>
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![Image](image1.jpg)

### (a) ECa map of Moree 2008 Field 1 and 2009 Field 2.

![Image](image2.jpg)

### (b) Yield map of Tamaroi in Moree 2008 Field 1 and 2009 Field 2.

![Image](image3.jpg)

### (c) Yield (% Tamaroi) in Moree 2008 Field 1 and 2009 Field 2.