

# Wheat grain yield on saline soils is improved by an ancestral Na<sup>+</sup> transporter gene

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**The ability of wheat to maintain a low sodium concentration ([Na<sup>+</sup>]) in leaves correlates with improved growth under saline conditions<sup>1,2</sup>. This trait, termed Na<sup>+</sup> exclusion, contributes to the greater salt tolerance of bread wheat relative to durum wheat<sup>3,4</sup>. To improve the salt tolerance of durum wheat, we explored natural diversity in shoot Na<sup>+</sup> exclusion within ancestral wheat germplasm. Previously, we showed that crossing of *Nax2*, a gene locus in the wheat relative *Triticum monococcum* into a commercial durum wheat (*Triticum turgidum* ssp. *durum* var. Tamaroi) reduced its leaf [Na<sup>+</sup>] (ref. 5). Here we show that a gene in the *Nax2* locus, *TmHKT1;5-A*, encodes a Na<sup>+</sup>-selective transporter located on the plasma membrane of root cells surrounding xylem vessels, which is therefore ideally localized to withdraw Na<sup>+</sup> from the xylem and reduce transport of Na<sup>+</sup> to leaves. Field trials on saline soils demonstrate that the presence of *TmHKT1;5-A* significantly reduces leaf [Na<sup>+</sup>] and increases durum wheat grain yield by 25% compared to near-isogenic lines without the *Nax2* locus.**

Salinity limits crop yield in arid and semi-arid areas, owing to irrigation with saline water, rising water tables resulting from land clearing or natural subsoil salinity<sup>6</sup>. Global food requirements are expected to increase by 70–110% by 2050 (ref. 7 and references therein), and as land degradation, urban spread and seawater intrusion are increasing over time, gains in agricultural productivity must come from saline soils. Wheat (*Triticum* spp.) and rice (*Oryza sativa*) constitute the world's major staple food crops, but accumulation of high concentrations of foliar Na<sup>+</sup> inhibits leaf function<sup>2</sup>. Therefore, improving the shoot Na<sup>+</sup> exclusion capacity of these cereals would be useful for improving salt tolerance<sup>1,8</sup>.

Domesticated wheats, through millennia of hybridization and crossing of selected plants, have become polyploid, that is, they have more than one set of paired chromosomes. This has resulted in an extremely complex genome that confers many favorable traits, but also the loss of others, such as salt tolerance. Durum wheat (*Triticum turgidum* ssp. *durum*) used for making pasta, couscous, bulgur and, in some instances, bread, is a tetraploid wheat with genomes A and B.

It is more salt sensitive than bread wheat (*Triticum aestivum*, a hexaploid with genomes A, B and D) as it lacks the Na<sup>+</sup>-excluding locus *Kna1* found on the D genome. The *Kna1* locus enables bread wheat to maintain lower leaf Na<sup>+</sup> and a greater K<sup>+</sup>/Na<sup>+</sup> ratio than durum wheat<sup>3,9</sup>. A source of Na<sup>+</sup> 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<sup>5</sup>. 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<sup>10</sup>.

The *Nax2* locus confers a reduced rate of Na<sup>+</sup> transport from roots to shoots by retrieving Na<sup>+</sup> from the root xylem<sup>5,11</sup>. *Nax2* was introgressed from *T. monococcum* into a modern durum cultivar, Tamaroi, by means of durum derivative line 149, and near-isogenic lines with and without *Nax2* were developed<sup>5</sup>. 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<sup>+</sup> exclusion were identified by screening for genes that confer similar phenotypes in other species<sup>12</sup>.

HKT (high-affinity potassium transporter) genes are known to be important in the regulation of Na<sup>+</sup> and K<sup>+</sup> transport in higher plants<sup>1,8,13</sup>. Proteins encoded by group 1 HKT genes of *Arabidopsis thaliana* and rice, *AtHKT1;1* and *OsHKT1;5*, reduce transport of Na<sup>+</sup> to shoots<sup>14–19</sup> and increase plant salinity tolerance as determined by measurement of leaf biomass under controlled conditions<sup>16,18</sup>. An *OsHKT1;5*-like gene was considered a possible *Nax2* candidate<sup>12</sup> because *OsHKT1;5* bestowed a similar reduced rate of Na<sup>+</sup> transport from roots to shoots in rice<sup>17</sup>. A probe was designed from a partial wheat *HKT1;5*-like expressed sequence tag (EST) sequence and this segregated with the low-Na<sup>+</sup> leaf phenotype of *Nax2* in segregating near-isogenic lines<sup>12</sup>. Further analysis of these near-isogenic lines showed that leaf Na<sup>+</sup> was reduced by at least 50% when the *HKT1;5*-like gene was present, which makes it a strong gene candidate for the Na<sup>+</sup>-excluding ability of *Nax2* (**Supplementary Fig. 1**).

An HKT-like gene cloned from the *Nax2* EST derived from *T. monococcum* was named *TmHKT1;5-A*, as its gene product shares

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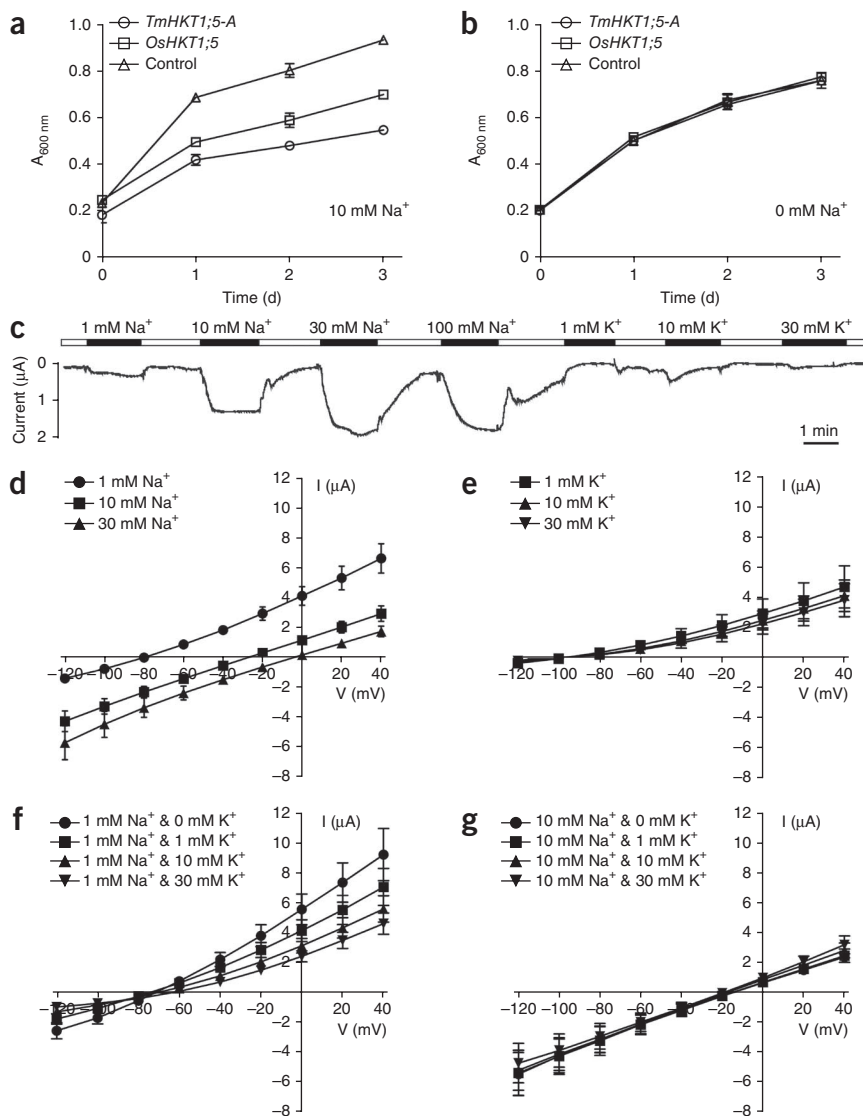
66% amino acid identity with that of *OsHKT1;5*, a gene that encodes a plasma membrane  $\text{Na}^+$  transporter expressed in cells surrounding xylem tissue<sup>17</sup>. *TmHKT1;5-A* was given the A designation as it is present on genome A and so distinguishes it from *TaHKT1;5-D*, the putative candidate gene for the *Knal* locus on genome D, with which it shares 94% amino acid identity<sup>12</sup>. Although *HKT1;5-A* is present in *T. monococcum*, it has not been found in any accession of *Triticum urartu*, which is the ancestral donor of genome A for modern durum and bread wheat<sup>10</sup>. Therefore, *TmHKT1;5-A* is likely to be absent in all modern wheat and as such we propose that *Nax2/TmHKT1;5-A* encodes a transporter that could provide a source of  $\text{Na}^+$  exclusion in durum wheat.

To test whether the *Nax2* candidate gene *TmHKT1;5-A* encodes a  $\text{Na}^+$  transporter, we expressed it in *Saccharomyces cerevisiae* grown in a liquid culture containing 10 mM NaCl. *OsHKT1;5* was used as a positive control<sup>17</sup>. 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 (Fig. 1a;  $P < 0.01$ ). All strains grew equally well in liquid media lacking supplemental NaCl (Fig. 1b). These results are consistent with a reduction in growth of yeast by *TmHKT1;5-A* catalyzing  $\text{Na}^+$  flux into yeast cells and not by protein overexpression.

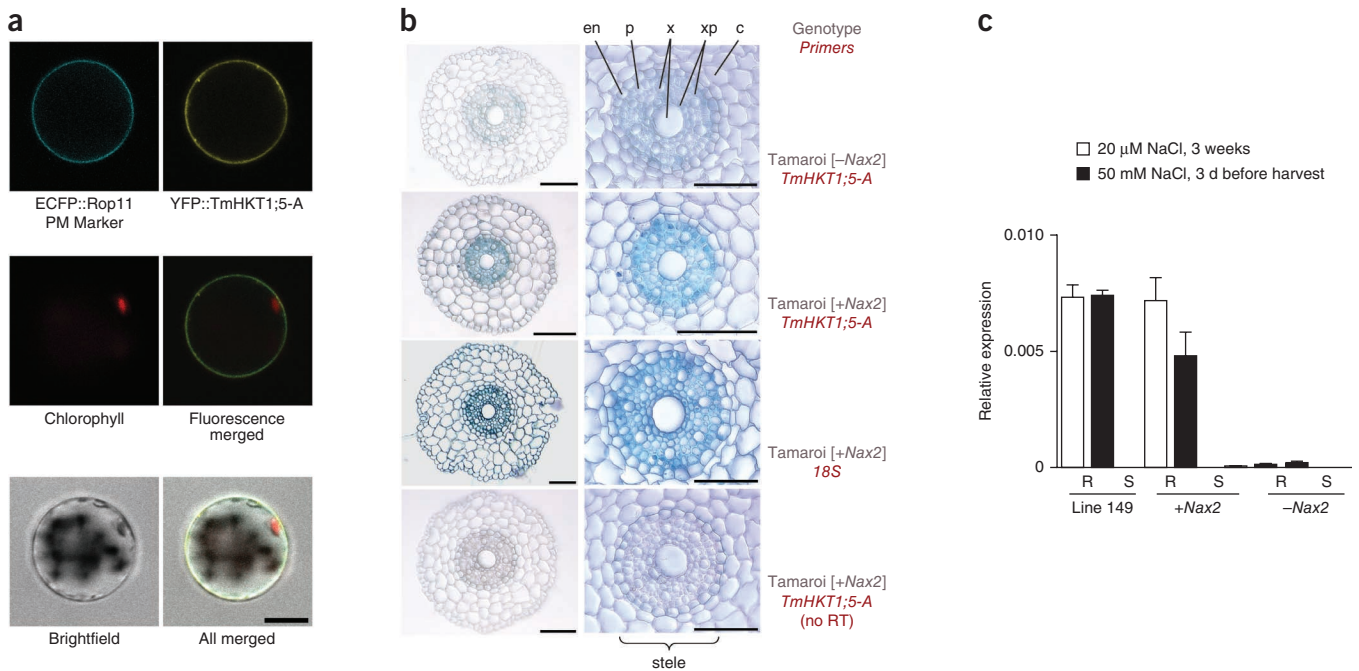
To confirm that  $\text{Na}^+$  is transported through *TmHKT1;5-A*, we injected *Xenopus laevis* oocytes with *TmHKT1;5-A* cRNA. The oocytes accumulated  $\text{Na}^+$ , containing approximately double the  $[\text{Na}^+]_{\text{ext}}$  and half the  $[\text{K}^+]_{\text{ext}}$  of water-injected control oocytes (Supplementary Fig. 2). Furthermore, when the plasma membrane of oocytes injected with *TmHKT1;5-A* cRNA was voltage clamped at negative membrane potentials, an inward current was induced in the presence of external  $\text{Na}^+$  but not  $\text{K}^+$  (Fig. 1c–e), with a  $K_m$  for  $\text{Na}^+$  of  $0.88 \pm 0.18$  mM (mean  $\pm$  s.e.m.;  $n = 4$ ) (Supplementary Fig. 3), indicating that *TmHKT1;5-A* formed a  $\text{Na}^+$ - but not  $\text{K}^+$ -permeable transporter. To our knowledge, transport affinities for *HKT1;5*-like gene products have not previously been reported; however, compared to other HKT proteins<sup>20</sup> the affinity of *TmHKT1;5-A* for  $\text{Na}^+$  transport is relatively high. Such a  $K_m$  would allow considerable

retrieval of  $\text{Na}^+$  from the xylem at physiologically relevant concentrations in saline and nonsaline conditions<sup>21</sup>.

Maintenance of high  $[\text{K}^+]_{\text{ext}}$  and a high  $\text{K}^+/\text{Na}^+$  ratio in leaves is an important component of salinity tolerance<sup>8</sup>. Therefore, the transport properties of *TmHKT1;5* cRNA-injected oocytes in differing external  $\text{K}^+$  and  $\text{Na}^+$  concentrations ( $[\text{x}^+]_{\text{ext}}$ ) were examined. The reversal potential ( $E_{\text{rev}}$ ) for *TmHKT1;5-A* cRNA-induced currents shifted positive with increasing  $[\text{Na}^+]_{\text{ext}}$ , consistent with the predicted change in equilibrium potential for  $\text{Na}^+$  ( $E_{\text{Na}}$ ), but did not change with increasing  $[\text{K}^+]_{\text{ext}}$  (Fig. 1d,e and Supplementary Fig. 4a). This indicates that *TmHKT1;5-A* acts as a  $\text{Na}^+$ -selective uniporter, in an identical mode to other HKT1;5-like gene products. Notably, conductance of *TmHKT1;5-A* cRNA-injected oocytes decreased from  $61 \pm 12 \mu\text{S}$  to  $20 \pm 4 \mu\text{S}$  (mean  $\pm$  s.e.m.) in the presence of 1 mM  $[\text{Na}^+]_{\text{ext}}$  when  $[\text{K}^+]_{\text{ext}}$  was increased from 0 mM to 30 mM, although this did not occur in the presence of 10 mM  $\text{Na}^+$  with conductance constant at  $50 \pm 10 \mu\text{S}$  (mean  $\pm$  s.e.m.) (Fig. 1f,g and Supplementary Fig. 4b). Similarly, an inhibition of  $\text{Na}^+$  transport through *TaHKT2;1* and *OsHKT2;1* by external  $\text{K}^+$  has been observed, but in both low and high  $[\text{Na}^+]_{\text{ext}}$ . This inhibition was proposed to occur through association of  $\text{K}^+$  to  $\text{Na}^+$ -binding sites within the pore region<sup>20,22,23</sup>. Neither phenomenon has been previously observed from expression



**Figure 1** *TmHKT1;5-A* expression in yeast (*S. cerevisiae*) and *X. laevis* oocytes. (a) Growth of yeast expressing *TmHKT1;5A*, *OsHKT1;5* and empty-vector control in AP media<sup>31</sup> with 10 mM NaCl. (b) Growth of yeast without added NaCl. (c) Currents elicited by *TmHKT1;5-A* cRNA-injected oocyte when bathed in  $\text{Na}^+$ - or  $\text{K}^+$ -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  $\text{Na}^+$  ( $n = 5$ ) (d),  $\text{K}^+$  ( $n = 4$ ) (e), 1 mM  $\text{Na}^+$  plus different  $\text{K}^+$  ( $n = 5$ ) (f) and 10 mM  $\text{Na}^+$  plus different  $\text{K}^+$  (g). Average currents from water-injected controls at each voltage have been subtracted in all cases.

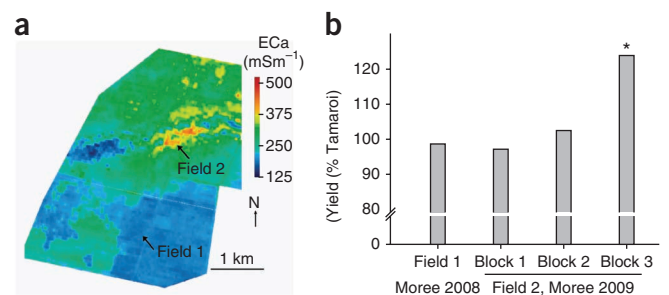


**Figure 2** Localization of *TmHKT1;5-A* and its encoded protein, and its transcriptional regulation by salt. **(a)** Plasma membrane (PM) localization of YFP::TmHKT1;5-A in *Arabidopsis* mesophyll protoplasts; ECFP::Rop11 (ref. 32) was used as plasma membrane marker. Scale bar, 10  $\mu$ m. Images 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 localization of *TmHKT1;5-A* using *in situ* PCR on 21-d-old roots grown in 2 mM NaCl; cells in which transcript is present stain blue. From top to bottom, *TmHKT1;5-A* primers in Tamaroi [-] *Nax2* (the near-isogenic line without *Nax2*/*TmHKT1;5-A*) to confirm absence in lines without *Nax2*; *TmHKT1;5-A* primers in Tamaroi [+] *Nax2* (the near-isogenic line with *Nax2*/*TmHKT1;5-A*), demonstrating the stelar localization 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; c, cortex; en, endodermis; p, pericycle; x, xylem; xp, xylem parenchyma; scale bars, 100  $\mu$ m. **(c)** *TmHKT1;5-A* is highly expressed in roots of line 149 (derived from a cross of *T. monococcum* and durum wheat to contains *Nax2*) and Tamaroi [+] *Nax2* compared to Tamaroi [-] *Nax2*, and shoots of all genotypes after 3 weeks in 20  $\mu$ M NaCl (clear bars). Additionally, in 3-week-old roots expression of *TmHKT1;5-A* was not found to be inducible by increasing  $[Na^+]_{ext}$  from 20  $\mu$ M to 50 mM for 3 d before harvest in the roots or shoots of any germplasm tested (filled bars). R, roots; S, shoots. Data presented as mean  $\pm$  s.e.m. ( $n = 6$ ; three biological replicates per treatment with each replicate comprising two pooled plants, with qPCR performed in triplicate).

of *AtHKT1;1* or *OsHKT1;5* in *X. laevis* oocytes<sup>17,23,24</sup>, suggesting differences in sensitivities of all these proteins to  $[K^+]_{ext}$ . Whether an inhibition of  $Na^+$  transport by  $[K^+]_{ext}$  plays a physiological role is yet to be determined. However, as xylem  $[K^+]_{ext}$  has been measured at 5 mM in salt-stressed wheat containing *Nax2* (ref. 5) and would increase in low-transpiring conditions (e.g., at night), such high  $[K^+]_{ext}$  would depolarize the membrane potential of stelar cells below  $E_{Na}$  and would result in leakage of  $Na^+$  back into the xylem unless transport through *TmHKT1;5-A* was prevented.

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 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 plasma membrane of *Arabidopsis*<sup>19</sup> and *OsHKT1;5::GFP* was identified on the plasma membrane of *X. laevis* oocytes<sup>17</sup>. Similarly, we detected YFP::TmHKT1;5-A on the plasma membrane through transient expression in *Arabidopsis* mesophyll cell protoplasts (Fig. 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 the xylem vessels of Tamaroi with [+] *TmHKT1;5-A* but was absent in Tamaroi without [-] *TmHKT1;5-A* (Fig. 2b). *TmHKT1;5-A* transcripts were abundant in roots of line 149 and Tamaroi [+] *TmHKT1;5-A*, but below reliable detection limits in shoots; no *TmHKT1;5-A* transcripts

were detected in Tamaroi [-] *TmHKT1;5-A* (Fig. 2c). As no induction of *TmHKT1;5-A* transcript in either root or shoot was detected in any germplasm tested (Fig. 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<sup>17</sup>. 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 nonsaline conditions as seen in



**Figure 3** Variation in salinity across a commercially farmed field, and the relative increase in grain yield due to the presence of *TmHKT1;5-A*. **(a)** Apparent electrical conductivity (Eca) of a salinity-affected field near Moree in northern New South Wales, Australia. Numbers indicate location of field trial site in 2008 (1) and 2009 (2). **(b)** Relative increase in grain yield of Tamaroi [+] *TmHKT1;5-A* compared to Tamaroi [-] *TmHKT1;5-A*. \*,  $P < 0.05$ .

**Table 1** Field trials of Tamaroi [ $\pm$ ] *TmHKT1;5-A* on soils that vary in salinity

Category	Genotype	Moree 2008		Moree 2009, Field 2		
		Field 1	Block 1	Block 2	Block 3	
ECe (dS m <sup>-1</sup> )		2.3 (1.9–2.9)	12.9 (11.7–14.0)	11.4 (7.2–15.4)	14.8 (12.1–20.6)	
Mean [NaCl] in soil (mM)		6 (1–11)	42 (23–63)	75 (35–98)	169 (114–310)	
Flag leaf [Na <sup>+</sup> ] (mM)						
	Tamaroi	81	258	297	326	
	Tamaroi [+] <i>TmHKT1;5-A</i>	7	33	51	87	
	LSD <sub>(0.05)</sub>	17	30	28	43	
Grain yield (t/ha)						
	Tamaroi	3.27	2.57	2.08	1.30	
	Tamaroi [+] <i>TmHKT1;5-A</i>	3.24	2.51	2.14	1.61*	
	LSD <sub>(0.05)</sub>	0.27	0.16	0.15	0.16	

Electrical conductivity of a saturated soil extract, ECe (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. \*,  $P < 0.05$ .

lines containing *Nax2* (**Supplementary Fig. 1** and **Fig. 3a**). Previously, increased vacuolar [Na<sup>+</sup>] was measured within pericycle and xylem parenchyma cells of mature seminal roots in line 149 when compared to Tamaroi, when both were grown in 150 mM NaCl<sup>21</sup>; we now provide a direct link between the cell types that express *TmHKT1;5-A* and those that accumulate additional Na<sup>+</sup>.

In summary, *TmHKT1;5-A* encodes a high-affinity (for a HKT), Na<sup>+</sup>-specific transporter located on the plasma membrane (**Figs. 1** and **2**). It is present within cells adjacent to the xylem vessels of wheat roots containing the *Nax2* locus and this results in at least a 50% reduction in Na<sup>+</sup> accumulation within seedling leaves (**Supplementary Fig. 1**). This provides compelling evidence that *TmHKT1;5-A* is the gene responsible for the shoot Na<sup>+</sup>-exclusion phenotype controlled by the *Nax2* locus<sup>5,11,12</sup>.

Leaf Na<sup>+</sup> exclusion correlates with enhanced shoot growth in salt-stressed wheat grown in controlled environments<sup>25–27</sup>, but there is no evidence that it enhances grain yield under field conditions. We therefore evaluated the impact of the Na<sup>+</sup>-excluding gene *TmHKT1;5-A* on durum wheat grown in saline fields of the Australian wheat belt. The apparent electrical conductivity (ECa) mapped across a large commercially farmed field illustrates the typically variable nature of soil salinity (**Fig. 3a**). The ECa was related to salinity after analysis of soil cores taken to a depth of 0.8 m, and field blocks of contrasting salinity were selected for the yield trials (**Table 1** and **Fig. 3a**).

The effect of *TmHKT1;5-A* on Na<sup>+</sup> exclusion and on grain yield was evaluated by comparing Tamaroi [+]*TmHKT1;5-A* with parent cultivar Tamaroi (lacking *TmHKT1;5-A* within the *Nax2* locus). [Na<sup>+</sup>] in the flag leaf (the uppermost 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<sup>+</sup>] increased in all genotypes with increasing soil salinity, however, [Na<sup>+</sup>] in Tamaroi [+]*TmHKT1;5-A* was 4- to 12-fold lower than in Tamaroi (**Table 1**). Even at high soil salinity (field block 3 in 2009), the presence of *TmHKT1;5-A* kept flag leaf [Na<sup>+</sup>] below 100 mM (whole tissue) compared to 326 mM in Tamaroi (**Table 1**). Previously, we determined in durum wheat that below a leaf [Na<sup>+</sup>] of 250 mM (whole tissue), vacuolar compartmentation of Na<sup>+</sup> in mesophyll cells was an effective salinity tolerance mechanism<sup>28</sup>. At higher leaf [Na<sup>+</sup>], such as those that occur in Tamaroi, we calculated that cytoplasmic [Na<sup>+</sup>] increased and hypothesized that this was the reason for the observed impairment in photosynthesis<sup>28</sup>. This would limit the carbon assimilation needed for initiation and growth of florets and starch formation in the developing grain. We would

therefore expect Na<sup>+</sup> exclusion due to *TmHKT1;5-A* to become important in preventing Na<sup>+</sup> toxicity in leaves only when they would otherwise accumulate critical [Na<sup>+</sup>] (approximately >250 mM).

Grain yield of all genotypes declined substantially with increasing salinity, owing to the osmotic effect of the salt outside the roots<sup>2,8</sup>. At the low to moderate salinity levels occurring in field block 1 in 2009, grain yields of both Tamaroi and Tamaroi [+]*TmHKT1;5-A* were about 2.5 tonnes per hectare (t ha<sup>-1</sup>), a typical and profitable yield for broad-acre, rain-fed (nonirrigated) wheat grown in semi-arid regions of the world. At the highest salinity level (field block 3), yields of Tamaroi decreased by 50%; however, Tamaroi [+]*TmHKT1;5-A* was much less affected, with yields reduced by only 36% (**Table 1**). The net result was that lines containing *TmHKT1;5-A* yielded significantly more than Tamaroi in high salinity, namely 0.3 t ha<sup>-1</sup> or 24% more (**Fig. 3b**). This was because there were 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**).

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 the presence of *TmHKT1;5-A*. This can be observed in the least saline field block in the main trial in 2009 or in a neighboring field with very low salinity in 2008 (**Table 1**). These 2 years of field trials were preceded by 3 years of preliminary field trials at nine different sites in very different soil types and climates in southeastern Australia. In sites with nonsaline 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., **Fig. 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<sup>29</sup>. Optimal yield in both saline and nonsaline 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 leaf Na<sup>+</sup> exclusion capability. Here we present the functional characterization of *TmHKT1;5-A*—a gene that confers Na<sup>+</sup> exclusion—that was obtained from an ancestor of modern wheat and is not present in commercial wheat genotypes<sup>10</sup>. This study demonstrates the great potential for the genetic diversity inherent in nondomesticated 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 engineered material. The introduction of *TmHKT1;5-A* into durum wheat resulted in significantly reduced Na<sup>+</sup> uptake into leaves, leading to an improvement in grain yield of as much as 25% when grown in saline soil. To our knowledge, there has been no previous field study demonstrating a grain yield improvement in saline soils by improving shoot Na<sup>+</sup> exclusion through the transfer of a root-localized *HKT* gene into a commercial wheat variety. To emphasize the further potential impact of this approach to improving food security, incorporation of *Nax2* (*TmHKT1;5-A*) into bread wheat was recently shown to increase its capacity to exclude Na<sup>+</sup> from leaves when grown under greenhouse conditions<sup>30</sup>. This result was surprising as, unlike durum wheat, bread wheat already contains the Na<sup>+</sup>-excluding locus *Kna*<sup>3,9</sup>. Field trials in commercial bread wheat varieties containing *Nax2* are underway, to determine its impact upon yield.

## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturebiotechnology/>.

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

Note: Supplementary information is available on the Nature Biotechnology website.

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## AUTHOR CONTRIBUTIONS

R.M., R.A.J., R.A.H., M.T., D.P. and M.G. conceived the project and planned experiments. R.M. and M.G. supervised the research. B.X. performed all *Xenopus*, yeast and protoplast experiments and R.A.J. performed field research. 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. and R.M. wrote the manuscript. All authors commented on the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## 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 (ref. 5). Line 149 was backcrossed with durum wheat cultivar Tamaroi to produce two independent BC<sub>4</sub> lines (5004 and 5042) lines containing *Nax2* (*TmHKT1;5-A*)<sup>5,12</sup>.

For phenotyping, seedlings were grown in supported hydroponics as described previously<sup>27</sup>. After 10 d in 150 mM NaCl, the blade of leaf 3 was harvested, and Na<sup>+</sup> concentration measured<sup>5</sup>. For genotyping, seedlings were screened for the presence of *TmHKT1;5-A*, using dominant molecular markers (*gwm410*, *gwm291*)<sup>12</sup>. A linked co-dominant marker, *cslinkNax2*, developed from a BAC sequence, was used for selection and validation of BC<sub>4</sub>F<sub>4</sub> families fixed for *TmHKT1;5-A* (Supplementary Table 4). PCR amplifications were performed in 20 µl-aliquots containing 200 µM dNTPs, 10× 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 *M15* competent *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 localization, respectively. A plasma membrane marker was constructed by replacing 35S::YFP-attR within pBS with 35S::ECFP::Rop11 (ref. 31) using *Pst*I.

**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<sup>33</sup>. Growth inhibition assays of 10 ml AP media<sup>31</sup> ± NaCl aliquots at 30 °C were quantified using 0.3 ml from each sample at OD<sub>600</sub> (nm) every 24 h for 3 d (Bio-Rad, Smart Spec<sup>T</sup> 3000).

**Characterization of *TmHKT1;5-A* in *X. laevis* oocytes.** pGEMHE-DEST containing *TmHKT1;5-A* was linearized using *Sbf*I-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 h and electrophysiology done as previously described<sup>34</sup>. Membrane currents were recorded in HMg solution (6 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 10 mM MES and pH 6.5 adjusted with TRIS base) ± Na<sup>+</sup> glutamate and/or K<sup>+</sup> glutamate as indicated. All solution osmolarities were adjusted using mannitol 240–260 mOsmol kg<sup>-1</sup> (Vapor pressure osmometer, Wescor).

**Quantification of ion-profile in oocytes.** Oocytes were incubated at 18 °C in ND96 solution for 2 d. Three replications of four grouped oocytes were rinsed in HMg solution 3 times, homogenized in 1 ml 0.1% (wt/vol) nitric acid by vortexing, incubated at 75 °C for 1 h and stored at 4 °C overnight and ion-profiles quantified by flame photometry (Sherwood 420)<sup>16</sup>. These concentrations were converted to ion activities using previously measured activity constants<sup>35</sup>.

**Membrane localization of *TmHKT1;5A* in *Arabidopsis* mesophyll protoplasts.** *Arabidopsis* mesophyll protoplasts were transformed with pBS vectors containing *TmHKT1;5-A* and *ECFP::Rop11* as described previously<sup>36</sup> using W2 solution (4 mM MES, 0.4 M mannitol, 15 mM KCl, 10 mM CaCl<sub>2</sub> and 5 mM MgCl<sub>2</sub>, adjusted to pH 5.7 with KOH) replacing WI and W5 solutions. Fluorescent signals were captured from 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 done using KAPA SYBR FAST Bio-Rad iCycler 2× qPCR master mix (KAPA Biosystems), with 250 nM of primer using the following cycling parameters: initial denaturation at 95 °C for 2 min, 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 done to confirm product identity. qPCR normalization was carried out using *TaActin*, *TaEFA* and *TaCyclophilin* as normalization genes (primers listed in Supplementary Table 4), presenting data as normalized transcript abundance<sup>37</sup>.

**In situ PCR.** Previous methods<sup>38</sup> were followed with the following modifications. We obtained 50-µm sections using a VT 1200 S Vibrating Microtome (Leica) and aspirated with a cut pipette tip into 100 U 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 1× 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 1× PCR buffer with 1.5 mM MgCl<sub>2</sub>, 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 min. 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 50 µl BM Purple AP-substrate (Roche). For negative control sections, reverse transcriptase was omitted and *TmHKT1;5-A* primers (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) apparent electrical conductivity (ECa) using an EM38 electromagnetic induction meter (Geonics) in a 100 m grid (Fig. 3a). Candidate sites were further characterized 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 subsample of a 1:5 soil/water extract using a QC8500 automated ion analyzer (Lachat). ECa measurements were taken by furrowing an EM38 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<sup>+</sup> concentration (as described above). Flag leaf Na<sup>+</sup> concentrations of poor Na<sup>+</sup> 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). The 2008 field trial site (Field 1) with low/negligible salinity levels was sown in three randomized 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 × 8), with each block containing 64 individual 10 m<sup>2</sup> plots. The trial was sown in May 2009, harvested November 2009 and received 74 mm rainfall during that period. Before the main trials, additional field trials with a larger number of lines were completed at ten locations in southern New South Wales, the Australian Capital Territory and South Australia between 2004 and 2006. For details of these sites see Supplementary Tables 2 and 3.

**Statistical analysis.** Grain yield data from the field trial were analyzed 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.

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