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Journal of Experimental Botany, 2011; 62(8):2939-2947

Originally published at:
http://doi.org/10.1093/jxb/err003

Journal of Experimental Botany, Vol. 62, No. 8, pp. 2939–2947, 2011
doi:10.1093/jxb/err003 Advance Access publication 28 February, 2011
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Major genes for Na^+ exclusion, *Nax1* and *Nax2* (wheat *HKT1;4* and *HKT1;5*), decrease Na^+ accumulation in bread wheat leaves under saline and waterlogged conditions

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Received 19 October 2010; Revised 4 January 2011; Accepted 7 January 2011

Abstract

Two major genes for Na^+ exclusion in durum wheat, *Nax1* and *Nax2*, that were previously identified as the Na^+ transporters *TmHKT1;4-A2* and *TmHKT1;5-A*, were transferred into bread wheat in order to increase its capacity to restrict the accumulation of Na^+ in leaves. The genes were crossed from tetraploid durum wheat (*Triticum turgidum* ssp. *durum*) into hexaploid bread wheat (*Triticum aestivum*) by interspecific crossing and marker-assisted selection for hexaploid plants containing one or both genes. *Nax1* decreased the leaf blade Na^+ concentration by 50%, *Nax2* decreased it by 30%, and both genes together decreased it by 60%. The signature phenotype of *Nax1*, the retention of Na^+ in leaf sheaths resulting in a high Na^+ sheath:blade ratio, was found in the *Nax1* lines. This conferred an extra advantage under a combination of waterlogged and saline conditions. The effect of *Nax2* on lowering the Na^+ concentration in bread wheat was surprising as this gene is very similar to the *TaHKT1;5-D* Na^+ transporter already present in bread wheat, putatively at the *Kna1* locus. The results indicate that both *Nax* genes have the potential to improve the salt tolerance of bread wheat.

Key words: HKT7, HKT8, K/Na ratio, salinity, waterlogging.

Introduction

Increases in salinity tolerance for the world's two staple crops, wheat and rice, are an important goal as the world's population is increasing more quickly than the area of agricultural land to support it (FAO, 2010). Urban spread has reduced the area of prime land available for agriculture, so productivity must increase to maintain global food supply. Meanwhile, rising water tables due to land clearing or irrigation are causing salinization and desertification of previously productive land globally (Rengasamy, 2006). Food production is limited by this human-induced salinity, together with the natural and complex salinity found in soils of most semi-arid regions of the world (Rengasamy, 2010).

Salinity reduces the rate of leaf expansion, and closes stomates and thereby reduces photosynthesis, through the soil water deficit caused by the osmotic stress (Rahnama *et al.*, 2010). Over time, soil salinity causes toxic concentrations of Na^+ to accumulate in leaves (Munns, 2002). This imposes an additional limitation to growth by reducing the longevity of photosynthetic tissues (Munns, 2002). The control of Na^+ transport and its effective exclusion from the mesophyll cells of leaves is therefore an important requirement for salinity tolerance.

Na^+ exclusion from leaves is associated with salt tolerance in cereal crops including rice (Asch *et al.*, 2000; Ul Haq *et al.*, 2010), durum wheat (Munns and James, 2003), bread wheat (Cuin *et al.*, 2009, 2010), barley (Shavrukov *et al.*, 2010), pearl millet (Krishnamurthy *et al.*, 2007), and wild relatives such as *Hordeum* species (Garthwaite *et al.*, 2005), tall wheatgrass (Colmer *et al.*, 2006), and *Triticum tauschii* (Schachtman *et al.*, 1991).

Durum (pasta) wheat (*Triticum turgidum* L. ssp *durum* [Desf.]) is more salt sensitive than bread wheat (*T. aestivum* L.), due partly to its poor Na^+ exclusion (Gorham *et al.*, 1990; Munns and James, 2003). To improve the salt tolerance of durum wheat, a search was made of an international collection of durum and durum-related genotypes, and a novel source of Na^+ exclusion was found in an unusual genotype named Line 149. Genetic analysis showed that Line 149 contained two major genes for Na^+ exclusion, named *Nax1* and *Nax2* (Munns *et al.*, 2003). *Nax1* was located on chromosome 2A by quantitative trait locus (QTL) analysis (Lindsay *et al.*, 2004) and was identified by fine mapping as an Na^+ transporter of the *HKT* gene family *HKT7* (*HKT1;4*) (Huang *et al.*, 2006). *Nax2* was located on chromosome 5A and identified as *HKT8* (*HKT1;5*) (Byrt *et al.*, 2007). The *Nax* genes are not present in modern wheat (Huang *et al.*, 2008). Both *Nax* genes appeared to originate from a wheat relative, *Triticum monococcum* (C68-101) that was crossed with a durum wheat to transfer rust resistance genes, and this cross inadvertently transferred the *Nax* genes into Line 149 as well (James *et al.*, 2006*a*). These genes were therefore named *TmHKT7* (*TmHKT1;4-A2*) and *TmHKT8* (*TmHKT1;5-A*) to recognize their origin in *T. monococcum*.

In durum wheat, these genes enhanced removal of Na^+ from the xylem, leading to low Na^+ concentrations in leaves (James *et al.*, 2006*a*). *Nax1* removes Na^+ from the xylem in roots and the lower parts of leaves, the leaf sheaths, while *Nax2* removes Na^+ from the xylem only in the roots (James *et al.*, 2006*a*). *Nax1* has a unique phenotype of a high sheath:blade ratio of Na^+ concentration. *Nax2* has the same phenotype as *Kna1*, the QTL for Na^+ exclusion and enhanced K^+/Na^+ selectivity in bread wheat, *T. aestivum* (Dvořák *et al.*, 1994). *Nax2* was shown to be homoeologous to *Kna1* in *T. aestivum*, namely *TaHKT8* (*TaHKT1;5-D*) (Byrt *et al.*, 2007).

The HKT gene family encodes transporters in the plasma membrane that mediate the uptake of Na^+ or K^+ from the apoplast (reviewed by Hauser and Horie, 2010). They are important for cellular Na^+ and K^+ homeostasis, and, if expressed in the stele, particularly in the xylem parenchyma cells lining the xylem vessels, they can retrieve Na^+ from the transpiration stream and so contribute to Na^+ exclusion from leaves (Munns and Tester, 2008; Hauser and Horie, 2010). Transporters in subfamily 1 are highly selective for Na^+ over K^+; those in subfamily 2 are either K^+ selective or transport both ions. *AtHKT1;1* is an Na^+ transporter that limits Na^+ transport from root to shoots by retrieving Na^+ from the xylem (Davenport *et al.*, 2007). Overexpression of this gene with a stele-specific promoter reduced the rate of transport of Na^+ to the leaves and improved the salt tolerance of *Arabidopsis* (Møller *et al.*, 2009). The orthologue in rice, *OsHKT1;5*, is also an Na^+-specific transporter and also functions to restrict Na^+ accumulation in leaves (Ren *et al.*, 2005). It has been proposed that *TmHKT1;5* and *TaHKT1;5* function similarly in wheat (Byrt *et al.*, 2007).

Both *Nax* genes appear to be confined to the diploid wheat relative, *T. monococcum*, and to be absent in modern wheat (Huang et al., 2008). They were introduced into bread wheat cultivars by conventional hybridization using a durum wheat (tetraploid) bridge crossed with the bread wheat (hexaploid) and the F_1 (pentaploid) was backcrossed to the bread wheat. Hexaploid progeny that contained one or both *Nax* genes were selected. *Nax* genes were thus transferred into four different Australian wheat cultivars. Lines with and without both *Nax* genes were analysed for their Na^+ excluding ability, their partitioning of Na^+ between the sheath and blade (the fingerprint of *Nax1*), and their photosynthetic performance in 150 mM NaCl. Saline soils are often waterlogged during the period of early vegetative growth (Colmer *et al.*, 2005). As *Nax1* operates in the shoot as well as the root, lines containing *Nax1* were tested under the combined stresses of salinity and waterlogging. This paper reports the effect of the *Nax* genes on Na^+ transport in to bread wheat leaves and shows their potential to improve the yield of bread wheat on saline soil.

Materials and methods

Plant material

The original source of the major genes *Nax1* and *Nax2* (later identified as *TmHKT1;4-A2* and *TmHKT1;5-A*) was a diploid (AA) *T. monococcum* accession C68-101 (James *et al.*, 2006*a*). This had been previously crossed with the tetraploid (AABB) durum wheat (*T. turgidum* L. ssp *durum* Desf.) cultivar Marrocos and the genes *Nax1* and *Nax2* inadvertently introduced in a selection programme for rust resistance. Line 149 containing *Nax1* and *Nax2* was discovered in a screen of tetraploid germplasm from the Australian Winter Cereals Collection and labelled Line 149 (Munns *et al.*, 2003). Line 149 was crossed with the Australian durum wheat cultivar Tamaroi, and a BC_1F_3 line (P01819) containing both genes was selected (Munns *et al.*, 2003). Parental material for the (hexaploid, AABBDD) bread wheat cross used in crossing, and in subsequent Na^+ uptake experiments, were the Australian bread wheat cultivars Westonia, Carnamah, Yitpi, and Sunstate. These were chosen as representative of the genetic backgrounds of bread wheats currently grown across the Australian wheat belt.

Interspecific crosses for transferring Nax genes into hexaploid wheat

The Na^+ exclusion genes *Nax1* and *Nax2* were introduced from the tetraploid BC_1F_3 line into representative hexaploid wheat by conventional crossing for different species with homologous chromosomes. Initial crosses were made between the tetraploid BC_1F_3 (male) and the hexaploid Westonia (female). The resulting F_1 pentaploids were backcrossed with Westonia (male) to produce BC_1F_1 plants, and the progeny then selfed. BC_1F_2 seedlings were genotyped using the *gwm12* marker for the Na^+ exclusion *Nax1* allele from the tetraploid parent as described by Lindsay *et al.* (2004). Three heterozygous *Nax1* lines were identified and were selfed to produce BC_1F_3 seed. Hexaploid ears were selected first on the basis of fertility and their hexaploid status was confirmed using a D-genome-specific marker, Dgas (McNeil *et al.*, 1994). These hexaploid BC_1F_3 selections homozygous for *Nax1* were backcrossed again into Westonia and also top-crossed to current Australian bread wheat cultivars Sunstate, Yitpi, and Carnamah. Further backcrosses and top-crosses were completed without selection on BC_2F_1 plants. BC_3F_2 populations were screened using the *gwm312* marker, thus generating BC_3F_3 homozygous families containing *Nax1*.

Westonia BC_1F_3 selections not containing *Nax1* were also screened for the presence of *Nax2*, using dominant molecular markers [*gwm410* and *gwm291* as described in Byrt *et al.* (2007)]. Later, a co-dominant marker, *cslinkNax2*, was developed, evaluated, and used for selection of *Nax2* lines in segregating populations. The bacterial artificial chromosome (BAC) sequence, made available by Jorge Dubcovsky, was used to design primers flanking microsatellite repeats, and amplified markers were tested on a range of germplasm with and without *Nax2* as indicated by the phenotype. One BC_1F_3 family which segregated for *Nax2* was identified and, with further backcrosses, Westonia BC_3F_3 homozygous families containing both *Nax1* and *Nax2* were developed.

Linked molecular markers for Nax *genes in hexaploid wheat*

The primer sequences for *Nax1* flanking the simple sequence repeat (SSR) marker *gwm312* were described by Röder *et al.* (1998). Primer sequences for the linked *Nax2* marker *cslinkNax2* were as follows: forward primer 5′ TCTCCATCATTCAACATCAATCG; and reverse primer 5′ TGTAGCTCGTCGGGGTGTGTTGC. Amplifications were performed in 20 µl aliquots containing 200 µM dNTPs, 10× PCR buffer, 0.5 µM of each primer, 1 U of *Taq* DNA polymerase (Hotstar®, Qiagen, Victoria Australia), and 100 ng of genomic DNA. The PCR was conducted under standard conditions with the following cycling protocol: 95 °C for 15 min; five cycles of 94 °C for 1min, 58 °C for 1 min, 72 °C for 1 min; and then 30 cycles of 94 °C for 30s, 58 °C for 30 s, 72 °C for 50 s. Product sizes amplified by *gwm312* and *cslinkNax2* markers were determined by DNA fragment analysis using an ABI3700 DNA analyser (Applied Biosystems).

Validation of molecular markers in the bread wheat background

To test the usefulness of these markers in identifying the Na^+ exclusion trait in a breeding programme, 18 Australian bread wheat cultivars of different genetic backgrounds were selected and screened with markers *gwm312* (*Nax1*) and *cslinkNax2* (*Nax2*) (Table 1). These markers proved to be informative in all wheat cultivars tested, distinguishing between the donor and recipient alleles particularly for *Nax2*, where the difference in product was typically in the order of 60 bp. In contrast to the clear distinction of alleles for *Nax2*, the PCR fragments for the *Nax1* marker were only slightly separated, typically 8–10 bp apart, and for two cultivars, Carnamah and Sunstate, a size difference of only 4 bp (Table 1).

Table 1. DNA fragment analysis of the gwm312 marker (Nax1) and cslinkNax2 marker (Nax2) in durum wheat Nax donor landrace, Line 149, and 18 diverse current Australian bread wheat cultivars

Cultivar/line	*Nax1* marker (*gwm312*) fragment size (bp)	*Nax2* marker (*cslinkNax2*) fragment size (bp)
Durum donor (Line 149)	199	171
Babbler	209	231
Batavia	238	231
Baxter	209	231
Cadoux	207	229
Camm	209	190
Carnamah	203	233
Chara	209	233
EGA Hume	238	231
Janz	209	231
Kukri	209	217, 231
Rees	207	213
Strzlecki	238	231
Sunco	240	231
Sunlin	207	219
Sunstate	203	215
Sunvale	209	231
Westonia	207	233
Yitpi	209	193

A complete *Nax* marker diagnostic kit, including background information, PCR protocols, and examples of marker application is available on writing to the corresponding author.

Phenotypic screens for the evaluation of Nax *genes in bread wheats*

Growth conditions: Plants were grown in supported hydroponics in four 40 L trays as described previously (James *et al.*, 2008). At ~6 d after emergence, and coinciding with the appearance of leaf 3, 25 mM NaCl was added twice daily to a final concentration of 150 mM, and $CaCl_2$ was added to give a final concentration of 10 mM. Plants were grown in a controlled environment chamber with a 10 h photoperiod and a photosynthetic photon flux density (PPFD) of 800 µmol m^{-2} s^{-1} at 25 °C during the day and 18 °C during the night.

After 10 d in 150 mM NaCl, the blade of leaf 3 and the sheath and blade of leaf 2 were harvested and dried at 70 °C for 2 d, weighed, extracted in 500 mM HNO_3 at 80 °C for 1.5 h, and analysed for Na^+ and K^+ by an inductively coupled plasma-atomic emission spectrometer (Vista Pro, Varian, Melbourne, Australia).

Nax1 in four Australian bread wheats: To evaluate the effect of *Nax1* on Na^+ accumulation in bread wheat, four separate experiments were completed on sets of between six and 10 homozygous BC_3F_3 [+]*Nax1* lines. Each set was derived from one of four current Australian bread wheat cultivars, Westonia, Sunstate, Yitpi, and Carnamah. Between three and five BC_3F_3 'null' sister lines (not containing *Nax1*; [–]*Nax1*) were included in each screening experiment together with the recurrent parent. All experiments were set up as a randomized block design, with 4–6 reps per line, where the blade of leaf 3 was harvested after 10 d in 150 mM NaCl

Nax1 and *Nax2 in bread wheat cultivar Westonia*: To evaluate the effect of *Nax1* and *Nax2* separately, and the possible additive effect of *Nax1* and *Nax2* together on Na^+ accumulation in bread wheat, a fifth experiment was completed using six homozygous BC_3F_3 [+]*Nax1* lines, six homozygous BC_3F_3 [+]*Nax2* lines, and six homozygous BC_3F_3 [+]*Nax1,2* lines. All lines were derived from bread wheat Westonia, which was also included in the experiment. The experiment was set up as an augmented Latinized spatial design, with eight replicates per line and 16 replicates for recurrent parent Westonia in 150 mM NaCl, and leaves were harvested 10 d after emergence.

CO_2 assimilation: To assess the effect of *Nax1* on photosynthesis of bread wheat, Westonia and a Westonia-derived BC_3F_3 *Nax1* line (P05901) and Sunstate and a Sunstate-derived *Nax1* line (P07110) were grown in 150 mM and 250 mM NaCl. Plants were grown hydroponically according to the method described above in a glasshouse, where air temperatures were maintained at 25 °C during the day and 15 °C during the night, and daily photosynthetically available radiation (PAR) averaged 21.7 mol $m^{-2}d^{-1}$. The experiment was set up as a randomized block design, with four replicates per genotype per treatment and with two hydroponics trays per salt treatment. All measurements were conducted over 3 d on seedlings that were 32–34 d old and had been in the salinity treatments for 23–25 d.

Gas exchange measurements, i.e. the CO_2 assimilation rate (A_N) and stomatal conductance (g_s), were taken from the mid portion of leaf 3 using a LI-6400 portable gas exchange system (LI-COR, Lincoln NE, USA). All measurements were taken between 11:00 h

and 15:00 h on sunny well-lit days. LI-6400 settings were chosen to approximate glasshouse conditions; leaf temperature was maintained at 25 °C, light intensity was set at 850 μmol m^{-2} s^{-1} with a red/blue light source, CO_2 was maintained at 400 μmol mol^{-1}, and the leaf to air vapour pressure deficit (VPD) was maintained between 1.2 kPa and 1.3 kPa. Following each gas exchange measurement the leaf was harvested, dried at 70 °C for 2 d, weighed, and analysed for Na^+ and K^+ as described above.

Waterlogging and salinity treatments: To assess the effect of *Nax1* and *Nax2* on Na^+ accumulation in the leaves of waterlogged and salt-stressed bread wheat, Westonia, the Westonia-derived BC_3F_3 *Nax1* line (P05917) and the Westonia-derived BC_3F_3 *Nax2* line (P05913) were grown in two treatments; a high salinity treatment (150 mM NaCl) and a waterlogged high salinity treatment (150 mM NaCl + waterlogging). Plants were grown hydroponically in a controlled growth chamber according to the method and conditions described above. The waterlogged treatment commenced with filling the hydroponic tray and allowing the solution to remain and naturally deplete O_2 levels, which were monitored daily using a Syland SIMPLAIR–S O_2 meter (Syland Scientific, Heppenheim, Germany). Within 3 d O_2 levels had fallen from 6.0 ppm to 2.3 ppm. After 6 d, O_2 levels had fallen further to 1.7 ppm and remained at this level over the duration of the experiment. Water levels were topped-up with de-oxygenated deionized water as required. After emergence for 10 d in 150 mM NaCl, the blades of leaf 3 were harvested, dried at 70 °C for 2 d, and analysed for Na^+ and K^+ as described above.

Statistical analysis

Data were analysed via a linear mixed model fitted using the REML procedure in Genstat (Genstat Release 11.1; VSN International Ltd, Hertz, UK). Genotype means and least significant differences (LSDs) at the 5% level of significance were obtained from the analysis.

Results

Phenotypic expression of Nax1 *in four different bread wheat cultivars*

Nax1 was introgressed into four diverse Australian bread wheats, Westonia, Yitpi, Sunstate, and Carnamah. Sets of homozygous BC_3 [+]*Nax1* lines, together with null BC_3 sister lines ([–]*Nax1*) and recurrent parents were grown in 150 mM NaCl for 10 d in four separate experiments to evaluate the possible effect of *Nax1* on Na^+ accumulation in the leaves.

In all four backgrounds tested, *Nax1* significantly decreased the Na^+ concentration in the leaves (Table 2). The response varied from a reduction in leaf Na^+ of 38% in Carnamah through to a 65% reduction in Yitpi. Average Na^+ concentrations in the null sister lines were generally similar to that of their respective recurrent parent, with the exception of the Yitpi and Westonia null lines, where the average Na^+ concentration was ~35% and 15% lower, respectively, than their recurrent parent.

Nax1 has previously been distinguished by preferential accumulation of Na^+ in the leaf sheath compared with the leaf blade, therefore resulting in a high sheath to blade ratio for Na^+ (Davenport *et al.*, 2005; James *et al.*, 2006*a*). Evidence for this 'signature' physiological characteristic of *Nax1* was apparent in the four diverse bread wheat backgrounds that were evaluated (Fig. 1). The mean sheath to blade ratio for Na^+ in the BC_3 [+]*Nax1* sets of lines ranged from between 1.7 and 3.1 times higher than their respective recurrent parents, whereas there was no effect measured in the BC_3 [–]*Nax1* (null) lines.

Phenotypic expression of Nax1 *and* Nax2 *in Westonia*

The presence of either *Nax1* or *Nax2* significantly reduced the Na^+ concentration in the leaf blade of the bread wheat Westonia after 10 d in 150 mM NaCl (Table 3, Fig. 2). *Nax1* was more effective than *Nax2* at reducing Na^+ accumulation in the leaf. The combination of both *Nax* genes together appeared to have even greater impact on reducing leaf Na^+ accumulation than either *Nax* gene individually. Na^+ concentration was reduced on average by 60% with the combination of both *Nax1* and *Nax2*, compared with a reduction of 45% by *Nax1* and of 26% by *Nax2* (Table 3, Fig. 2).

The K^+ concentration in the leaf blade was significantly higher due to the presence of either *Nax1* or *Nax2*, and further enhanced with *Nax1* and *Nax2* together (Table 3). The combination of enhanced K^+ accumulation and Na^+

Table 2. The effect of Nax1 on leaf Na^+ accumulation in four diverse Australian bread wheat backgrounds

Values are means (n=4–6). Seedlings were grown at 150 mM NaCl and leaf 3 was harvested 10 d after emergence.

Category	Leaf Na^+ concentration (μmol g DW^{-1})			
	Westonia	Yitpi	Sunstate	Carnamah
Recurrent parent	195	369	358	248
BC_3 [+]*Nax1*	110	130	170	155
BC_3 [–]*Nax1*	164	244	351	278
$LSD_{(0.05)}$	19	28	45	77

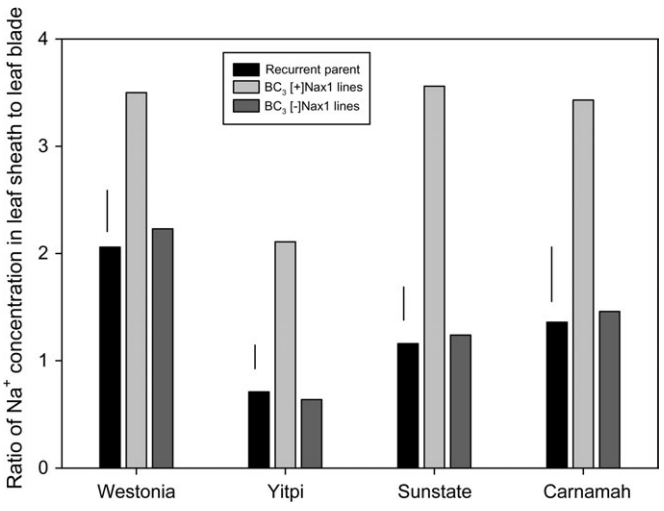

Fig. 1. The effect of *Nax1* on the ratio of Na^+ concentration in the leaf sheath to the leaf blade (leaf 2) in four diverse Australian bread wheat backgrounds grown at 150 mM NaCl. Values are means (n=4–6). Bars indicate $LSD_{(0.05)}$.

exclusion resulted in higher K^+:Na^+ ratios in the *Nax* lines compared with the recurrent parent Westonia.

The ratio of Na^+ in the leaf blade to the leaf sheath was unchanged due to the presence of *Nax2*. In contrast, preferential partitioning of Na^+ into the leaf sheath was ~3 times higher in both lines containing *Nax1* (Table 3).

Relationship between CO_2 assimilation and Na^+ accumulation

In general, CO_2 assimilation rates (A_N) did not vary significantly between *Nax1* lines and their recurrent parents, at either high (150 mM NaCl) or very high (250 mM NaCl) salinities (Table 4). Any small genotypic variation in A_N within a salt treatment could be explained by variation in stomatal conductance (g_s) and the resulting C_i:C_a ratio. For example, genotypes with the highest A_N in the 150 mM NaCl treatment (e.g. Westonia and Sunstate) also had the highest g_s and C_i:C_a ratios. Similar trends were evident in the 250 mM NaCl treatment.

CO_2 assimilation rates typically decreased by 10–15% when seedlings were grown in 250 mM NaCl, compared with 150 mM NaCl (Table 4). Consistent with the above, a decrease in g_s and a concurrent decrease in the C_i:C_a ratio indicated that the decline in A_N was due entirely to stomatal factors and not due to non-stomatal (e.g. photosynthetic capacity) factors. This observation is also supported by the Na^+ and K^+:Na^+ data. While the leaf blade Na^+ concentrations were 2–3 times higher in both Westonia and Sunstate compared with the *Nax1* lines, these concentrations were only intermediate and, together with K^+:Na^+ ratios >1.0, were not likely to threaten the integrity of the photosynthetic apparatus. There was some evidence for an ameliorating impact of *Nax1* on salt-induced leaf death in the Westonia background. Chlorophyll content (indicated by SPAD readings) in leaf 1 of the Westonia-derived *Nax1* line was 50% higher than in Westonia and, similarly in leaf 2, was 15% higher than Westonia (data not shown).

Table 3. The effect of Nax1 and Nax2 on leaf 3 Na^+ and K^+ accumulation (μmol g DW^{-1}) and the ratio of Na^+ concentration in the leaf sheath to the leaf blade, of Westonia and Westonia-derived BC_3F_3 lines (n=6; see Fig. 2) containing Nax1 and Nax2

Seedlings were grown in 150 mM NaCl for 10 d. Values are means (*n*=8).

Category	[Na^+] blade	[K^+] blade	K^+:Na^+ ratio blade	[Na^+] ratio sheath:blade
Westonia	219	1010	5.2	1.75
Westonia [+]*Nax1*	121	1100	12.1	4.69
Westonia [+]*Nax2*	161	1076	8.0	1.52
Westonia [+]*Nax1,2*	88	1146	15.4	5.37
$LSD_{(0.05)}$	35	38	3.3	0.45

Effect of Nax1 *and* Nax2 *on Na^+ accumulation under waterlogging and salinity*

Figure 3 summarizes the changes in leaf Na^+ concentration and K^+:Na^+ ratio in Westonia and Westonia-derived *Nax* lines when grown in a high salt treatment (150 mM NaCl) compared with a combined high salt and waterlogged treatment. Comparably with that reported above (Table 3), *Nax1* and *Nax2* decreased Na^+ accumulation in leaf 3 of salt-stressed Westonia by 50% and 25%, respectively (Fig. 3.). Similarly, the K^+:Na^+ ratio increased significantly by 2.5-fold in the [+]*Nax1* line compared with the recurrent parent Westonia; there was only a modest increase in the K^+:Na^+

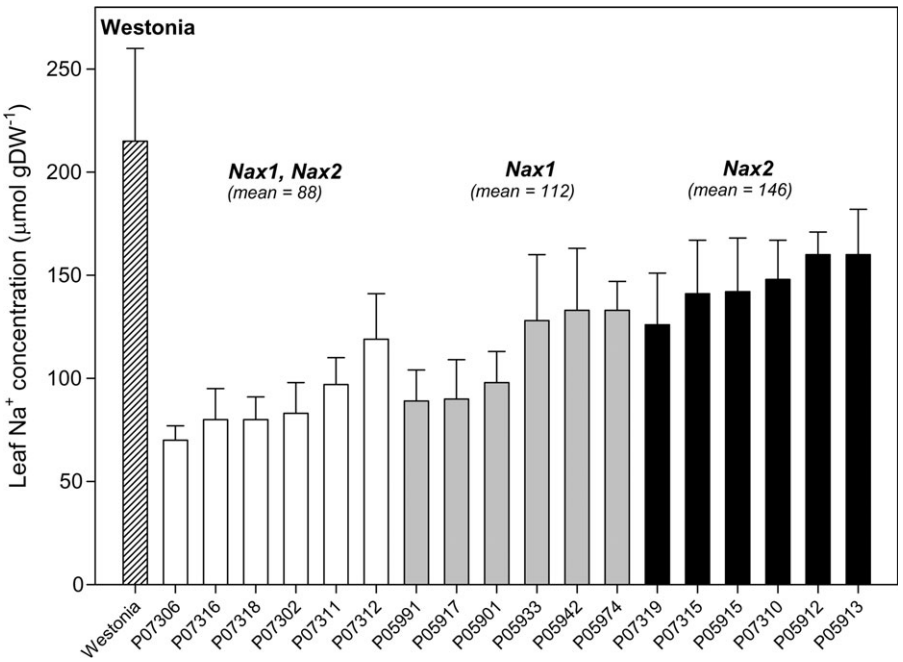

Fig. 2. Na^+ accumulation in leaf 3 of Westonia and Westonia-derived BC_3F_3 lines containing *Nax1* and *Nax2* grown in 150 mM NaCl for 10 d. Values are means ±SE (*n*=8).

Table 4. CO_2 assimilation rate (A_N), stomatal conductance (g_s), C_i:C_a ratio, Na^+ concentration, and K^+:Na^+ ratio in leaf 3 of Westonia and Sunstate and derived BC_3 Nax1 lines after 23 d in either 150 mM or 250 mM NaCl

Treatment	Genotype	A_N (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	C_i:C_a	[Na^+] (μmol g DW^{-1})	K^+:Na^+ ratio
150 mM NaCl	Westonia	17.3	0.204	0.62	460	1.16
	Westonia [+]*Nax1*	14.6	0.144	0.53	180	4.32
	Sunstate	16.6	0.204	0.63	481	1.20
	Sunstate [+]*Nax1*	14.9	0.171	0.61	255	3.33
250 mM NaCl	Westonia	15.1	0.122	0.46	450	1.54
	Westonia [+]*Nax1*	17.3	0.158	0.52	197	3.90
	Sunstate	14.4	0.125	0.51	711	1.10
	Sunstate [+]*Nax1*	13.9	0.119	0.50	262	4.07
$LSD_{(0.05)}$	[Genotype×treatment]	2.3	0.044	0.08	136	1.78

ratio of ~10% in the [+]*Nax2* line. The waterlogging/salinity interaction significantly increased the leaf Na^+ concentration and decreased the leaf K^+:Na^+ ratio in all three lines. However, the ability of *Nax1* to exclude Na^+ was largely maintained despite hypoxia, and the K^+:Na^+ ratio was at a intermediate level of ~3.0, compared with 1.3 in Westonia (Fig. 3.). In contrast, the ability of *Nax2* to prevent Na^+ accumulation in the leaf was completely removed by hypoxia. Leaf Na^+ concentration increased by ~4-fold in the Westonia [+]*Nax2* line to 830 μmol g DW^{-1}, similar to that in Westonia, but increased by only 2.5-fold in the Westonia [+]*Nax1* line. The impact of the waterlogging treatment was also evident in the growth of seedlings after 4 weeks, as shoot biomass decreased by ~35% relative to the (non-waterlogged) salinity treatment (data not shown).

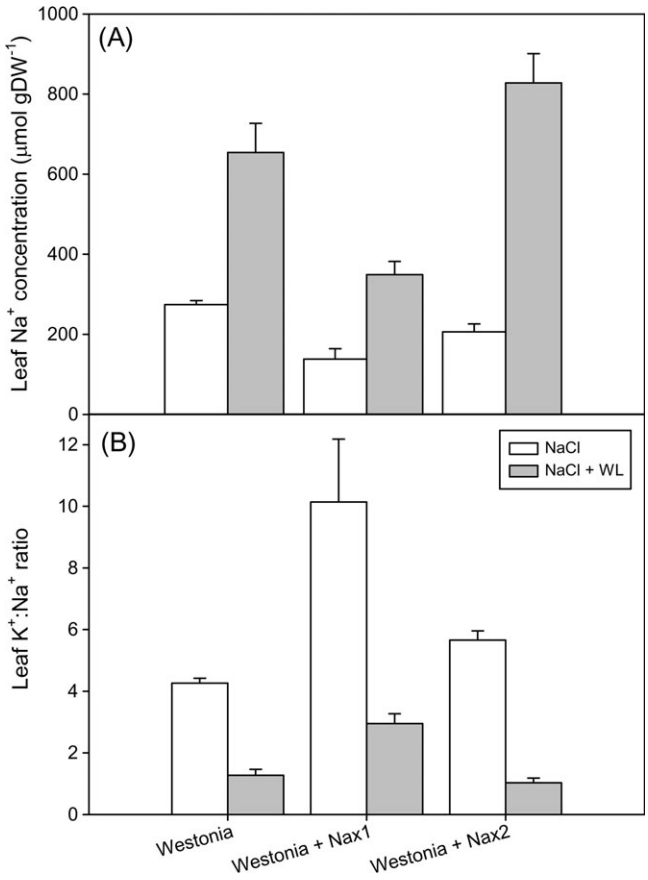

Fig. 3. The effect of salinity (150 mM NaCl) and the interaction of salinity and waterlogging after 10 d treatment on the (A) Na^+ concentration and (B) the K^+:Na^+ ratio in leaf 3 of Westonia and Westonia-derived BC_3F_3 lines containing *Nax1* and *Nax2*. Values are means ±SE (n=4).

Discussion

New genes for salt tolerance in bread wheat

The *Nax* genes originate from an ancestral diploid wheat relative, *T. monococcum* (James *et al.*, 2006*a*), and are not present in modern wheat. When transferred into the durum wheat cultivar Tamaroi, these genes were found to enhance removal of Na^+ from the xylem, leading to low Na^+ concentrations in leaves (James *et al.*, 2006*a*).

Nax1 (*TmHKT1;4-A2*) removes Na^+ from the xylem in roots and the lower parts of leaves, the leaf sheaths. *Nax1* has a unique phenotype of a high sheath:blade ratio of Na^+ concentration. When transferred into bread wheat via the durum wheat 'bridge' from the original *T. monococcum*, the phenotype of a low blade Na^+ accompanied by a high sheath:blade Na^+ ratio was found in all four bread wheat parents. Comparison between the four Australian bread wheats with and without the addition of *Nax1* showed that Westonia had a particularly low leaf Na^+ concentration, presumably due to minor genes for Na^+ exclusion. The nulls in two cases had a lower Na^+ concentration than the recurrent parent. This is probably because Westonia was the 'bridge' into the other varieties and a minor gene was transferred as well as *Nax1*. Minor genes for Na^+ exclusion have been found in bread wheat on chromosome 7A (Ogbonnaya *et al.*, 2008). The family of HKT genes are not the only genes controlling Na^+ transport to the shoot. Studies with Na^{22} showed that durum wheat lacking the *Nax* genes was able to withdraw ~50% of the Na^{22} flowing to the shoot (Davenport *et al.*, 2005). The presence of either *Nax1* or *Nax2* increased this withdrawal from the xylem to ~90%.

Nax2 (*TmHKT1;5-A*) removes Na^+ from the xylem only in the roots (James *et al.*, 2006*a*). *Nax2* had not been expected to be effective in bread wheat, as it is homoeologous to *Kna1* (*TaHKT1;5-D*; 94% sequence similarity) and

has the same phenotype as *Kna1* (Byrt *et al.*, 2007). Nevertheless, introduction of *Nax2* lowered the leaf blade Na^+ concentration in bread wheat, although not as much as *Nax1*. It is likely that the A and D genome *HKT1;5* gene family members have different expression patterns or different levels of activity, and so the A genome member complements the function of the D genome member in reducing the total transport of Na^+ from root to shoot.

Modern dwarf rice is less tolerant of salinity than wheat, but the original landraces Pokkali and NonaBokra are relatively tolerant. This is due largely to the presence of an orthologue of *TmHKT1;5-A* namely *OsHKT1;5* (Ren *et al.*, 2005). However, the functional expression of an orthologue of *TmHKT1;4* has not been indicated: no QTL for Na^+ exclusion has been found on chromosome 4, the syntenic region to wheat chromosome 2A containing the *Nax1* gene. Yet the gene sequence for *HKT1;4* is present on the rice genome (Huang *et al.*, 2006). It is possible that this gene is silenced in rice, or for whatever reason its expression is prevented, leading to the interesting idea that transformation of rice with *TmHKT1;4* might increase its salt tolerance. This might be particularly beneficial when the soil is flooded so that insufficient oxygen reaches the roots, despite the aerenchyma, to maintain ion transport activity in roots.

Nax1 *confers* Na^+ *exclusion in waterlogged saline conditions*

Flooding is common in many agricultural regions, and is likely to increase in frequency and severity with global climate change (Colmer and Voesenek, 2009). Most crops will not tolerate floods, as without specific traits to enhance oxygen transfer to the submerged roots, their energy production is limited and energy-consuming processes such as ion transport are disrupted (Colmer and Voesenek, 2009). One notable impact of the combined waterlogging and salinity treatment compared with the aerated salinity treatment was a significant increase in leaf Na^+ concentration and a decrease in the K^+:Na^+ ratio in all lines. Increases in leaf Na^+ concentrations due to a salinity–waterlogging combination have been found in a range of species including wheat (summarized by Barrett-Lennard, 2003). Oxygen depletion due to waterlogging is likely to impair both active and passive ion transport processes, as both are ultimately dependent on the maintenance of membrane potential differences by H^+-ATPase-generated H^+ gradients across the plasma membrane (Greenway and Gibbs, 2003). Depolarization of the plasma membrane in xylem parenchyma cells to –100 mV or less would result in reduced Na^+ retrieval from the xylem via the HKT1 subfamily transporters, increased passive flow of Na^+ into the xylem via non-selective outward rectifying channels (NORCs), and, consequently, increased Na^+ transport to the shoot (Colmer and Greenway, 2011, and references therein).

In the combined waterlogging and salinity treatment, the *Nax1* line had only half the Na^+ accumulation of Westonia or Westonia containing *Nax2*. Unlike *Nax2* which functions solely in the roots, the novel aspect of *Nax1* is that it also removes Na^+ from the xylem into leaf sheaths (James *et al.*, 2006*a*), a character which is not found in bread wheat. As the basal shoot tissues were not submerged in the present experiments, it is likely that the process of moving Na^+ out of the xylem and partitioning into sheath tissue was maintained in this *Nax1* line despite waterlogging, and, therefore, leaf Na^+ concentrations were lowered to about half that of Westonia.

This unique functioning of *Nax1* would help in waterlogged soils, and so be important in salinity caused by rising water tables where the crop is often waterlogged during the vegetative phase (Colmer *et al.*, 2005). Therefore, providing that the shoot can continue to grow in waterlogged and saline conditions, Na^+ retrieval from the xylem and storage in basal shoot tissues will continue to provide a mechanism for reducing Na^+ accumulation and the consequent rate of death of mature leaves.

Relationship between Nax1 *and rates of* CO_2 *assimilation*

The presence of *Nax1* had no significant effect on photosynthesis of wheat seedlings grown in high salinities for 5 weeks. This is probably because even in the parental bread wheats (Westonia and Sunstate), Na^+ concentrations were not very high and K^+:Na^+ ratios, while low at ~1.2, were not limiting. For example, leaf Na^+ concentrations for Westonia and Sunstate were in the range of 450–700 μmol g DW^{-1} (Table 4) corresponding to Na^+ concentrations of between 90 mM and 140 mM on a tissue water basis. Previously, it had been determined that Na^+ concentrations >200 mM and K^+:Na^+ ratios well below 1.0 in the leaves of durum wheat corresponded to a decline in photosynthetic capacity (James *et al.*, 2002, 2006*b*). In the current study, small variation in CO_2 assimilation rates between lines was attributed to stomatal factors and not due to any impairment of the photosynthetic apparatus which may have resulted from Na^+ toxicity.

Genotypic differences in the relationship between increasing leaf Na^+ concentration and decreasing rates of CO_2 assimilation are more likely to be evident in a moderate to highly saline field late in the growing season. As soil water availability decreases throughout the season, the salt concentration in the soil steadily rises and will concentrate to a large extent in the rhizosphere. The resulting high salinity levels, and subsequent high Na^+ accumulation in the leaves and the interaction with water stress, are most likely to cause premature leaf death. This will affect the photosynthetic performance of green and transpiring later leaves such as the flag leaf on a mature wheat plant. These later leaves and, most importantly, the flag leaf, provide carbon to the filling grain; therefore, the high Na^+ accumulation in the later leaves is more likely to affect grain size than grain number (Husain *et al.*, 2003).

Additionally, with the increased size of the sheath and stem 'storage reservoir' on a maturing wheat plant, the impact of *Nax1* on lowering leaf blade Na^+ concentrations

through greater removal of Na^+ from the xylem into sheath tissues should become increasingly profound. Good evidence for this comes from a comparison of flag leaf Na^+ concentrations of the durum wheat cultivar Tamaroi, and Tamaroi-derived *Nax1* isogenic lines grown in a moderately saline field trial. The *Nax1* isogenic lines showed a 100-fold decrease in Na^+ accumulation and a corresponding 50% increase in photosynthesis, compared with the recurrent parent Tamaroi (RAJ and RM, unpublished data).

Summary and conclusions

In conclusion, it has been shown that by introgressing *Nax* genes from *T.monococcum* into hexaploid wheat, the leaf Na^+ concentration is reduced and the proportion of Na^+ stored in leaf sheaths is increased. These extra Na^+-excluding genes have the potential to increase the salt tolerance of bread wheat. Information on the markers providing tools for breeders to make use of these genes is available to all interested.

These lines are being evaluated on saline sites.

Acknowledgements

The authors would like to thank Dr David Bonnett for advice on appropriate Australian bread wheat varieties for crossing, and John To and Karen Glover for genotyping Australian bread wheats. We thank Dr Jorge Dubcovsky for making available the BAC sequence used to design primers for *Nax2*, and we thank Dr Wolfgang Spielmeyer for supervision of much of the marker development and evaluation work. We are indebted to Lorraine Mason and Richard Phillips for ion analysis. We also acknowledge the Australian Centre for Plant Functional Genomics for providing a PhD scholarship to CB and the Grains Research and Development Council for funding and supporting this research.

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