Impact of ancestral wheat sodium exclusion genes *Nax1* and *Nax2* on grain yield of durum wheat on saline soils

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Abstract. *Nax1* and *Nax2* are two genetic loci that control the removal of Na^+ from the xylem and thereby help to exclude Na^+ from leaves of plants in saline soil. They originate in the wheat ancestral relative *Triticum monococcum* L. and are not present in modern durum or bread wheat. The *Nax1* and *Nax2* loci carry *TmHKT1;4-A2* and *TmHKT1;5-A*, respectively, which are the candidate genes for these functions. This paper describes the development of near-isogenic breeding lines suitable for assessing the impact of the *Nax* loci and their performance in controlled environment and fields of varying salinity. In young plants grown in 150 mM NaCl, *Nax1* reduced the leaf Na^+ concentration by 3-fold, *Nax2* by 2-fold and both *Nax1* and *Nax2* together by 4-fold. In 250 mM NaCl, *Nax1* promoted leaf longevity and greater photosynthesis and stomatal conductance. In the uppermost leaf, the Na^+-excluding effect of the *Nax* loci was much stronger. In the field, Na^+ in the flag leaf was reduced 100-fold by *Nax1* and 4-fold by *Nax2*; however, *Nax1* lines yielded 5–10% less than recurrent parent (cv. Tamaroi) in saline soil. In contrast, *Nax2* lines had no yield penalty and at high salinity they yielded close to 25% more than Tamaroi, indicating this material is suitable for breeding commercial durum wheat with improved yield on saline soils.

Additional keywords: HKT, osmotic stress, salinity, salt tolerance, turgor.

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Introduction

Soil salinity is a major issue in the more arid regions of the world, where soil and environmental factors contribute to the accumulation of salts to a level that adversely affects crop production. Salts can arise from weathering of rocks and from the ocean via wind or rain. Application of poor quality irrigation water and capillary rise of shallow saline groundwater can all contribute to the salinisation of the upper soil layers. Seawater intrusion onto land, due to global warming causing rising sea levels and more violent storms, can deposit a large amount of salts in soils of coastal lands. These particular processes contributing to salinisation, combined with the influence of other climatic and landscape features and the effects of human activities, determine where the salt accumulates in the landscape (Rengasamy 2010). The type of vegetation also determines how far below the soil surface salts accumulate (Rengasamy 2002).

Durum (tetraploid) wheat (*Triticum turgidum* L. ssp. *durum* (Desf.)) can attract a significantly higher price on the international market than hexaploid (bread) wheat (*Triticum aestivum* L.); however, it is poorly adapted to salt-affected soils (Zubaidi *et al.* 1999a). Its yield is more affected by salinity than bread wheat

(Francois *et al.* 1986; Maas and Grieve 1990). Durum wheat is less able to exclude Na^+ (Zubaidi *et al.* 1999b; Munns and James 2003), a trait associated with salt sensitivity in the Triticeae (Gorham *et al.* 1990; Dvořák *et al.* 1994). Durum wheat lacks the Na^+-excluding locus *Kna1* which enables hexaploid wheat to maintain lower leaf Na^+ and a greater K^+ to Na^+ ratio than durum wheat (Dvořák *et al.* 1994; Dubcovsky *et al.* 1996).

A search of an international collection of durum-related genotypes revealed the durum derivative 'Line 149' (AUS17045), which was found to have two genetic loci named *Nax1* and *Nax2* (Munns *et al.* 2003). Both loci had originated in the diploid wheat *Triticum monococcum* L. and had been serendipitously crossed into 'Line 149' by a breeder crossing genes for rust resistance in *T. monococcum* into durum wheat (The 1973; James *et al.* 2006a). As the durum wheat cultivar used for this cross (cv. Marrocos) was not adapted to Australian field conditions, further crossing was made into an Australian durum cultivar, Tamaroi. *Nax1* was located on chromosome 2A (Lindsay *et al.* 2004). *Nax2* was located on chromosome 5A (Byrt *et al.* 2007). *Nax1* removes Na^+ from the xylem in roots and the lower parts of leaves, the leaf sheaths,

whereas *Nax2* removes Na$^+$ from the xylem, only in the roots (James *et al.* 2006*a*). This is a novel source of Na$^+$ exclusion, not present in durum or hexaploid wheat (Huang *et al.* 2008). Both loci confer a reduced rate of Na$^+$ transport from roots to shoots by retrieving Na$^+$ from root xylem (Davenport *et al.* 2005; James *et al.* 2006*a*). Fine mapping of the *Nax1* locus indicated that the candidate gene was *TmHKT1;4-A2* (Huang *et al.* 2006), which likely encodes for a Na$^+$-specific transporter located on the plasma membrane of cells surrounding xylem vessels (Munns *et al.* 2012). An *HKT1;5*-like gene was proposed as the candidate for both *Nax2* and *Kna1* (Byrt *et al.* 2007).

This paper describes the pre-breeding work that led to the development of near-isogenic lines with and without *Nax1* and *Nax2* and the results with *Nax1* in comparison to *Nax2* on the performance of durum wheat when grown in a farmer's fields of varying salinity. Recently we showed that the presence of *Nax2* could increase the grain yield of wheat on salt-affected farmers' fields by up to 25% (Munns *et al.* 2012).

There has been little evaluation of salt tolerance traits on performance in the field, especially for wheat, but also for most other species; yet, scaling up from the level of the gene to yield of a crop is essential to test the validity of a trait for useful purposes (Passioura 2010). Recombinant durum lines with and without the *Kna1* locus for K$^+$/Na$^+$ discrimination introgressed from hexaploid wheat were evaluated in saline and non-saline fields by Dvořák *et al.* (1994). The *Kna1* lines had a similar biomass and yield to the *kna1* lines on fields of low salinity and a 12–17% increase in biomass on saline fields. Grain yields on saline fields were increased by 9–15% by *Kna1*, but this was not statistically significant. However, the differences in Na$^+$ exclusion controlled by the *Kna1* locus in this genetic background were small. The durum lines containing *Kna1* had a K$^+$:Na$^+$ ratio only twice that of the *kna1* lines, with Na$^+$ and K$^+$ concentrations being not significantly different. It is likely that larger differences in exclusion would give larger differences in yield on saline soil.

Several key factors differ between controlled environments and the field. Differences include light quality and quantity, unlimited rooting volume and large diurnal changes in temperature that cannot be supplied by a controlled environment facility. The other is that soil is a very different ionic medium from solution culture. A clay soil can influence Na$^+$ and Cl$^-$ availability and can result in different growth responses (Tavakkoli *et al.* 2010). There is, therefore, the need to confirm the usefulness of results found in controlled environments in the typical soils and ambient conditions that occur in grain production fields. To do this, the genes or trait of interest must be crossed into adapted germplasm, that is, into a cultivar or advanced breeding lines of commercial breeders.

The many steps required in the process of trait breeding have been summarised by Richards *et al.* (2010). This starts with a genotype chosen because it holds a desirable trait and finishes with a commercial product. The trait is crossed into a current cultivar or advanced breeder's line that is adapted to the target environment with the appropriate phenology, disease resistance and grain quality. A number (3–6) of backcrosses need to be made, with the trait selected in each generation. Out of this several possible lines are bulked up for testing. These lines undergo the following analysis:

(i)   evaluation of the trait under controlled environment conditions, or selection based on DNA markers (if reliable markers have already been established for the trait);

(ii) evaluation of breeding lines in non-saline field conditions; and

(iii) measurement of yield on well characterised saline field sites.

The present work presents results of breeding lines containing *Nax* loci in the commercial cultivar Tamaroi. First, we evaluated the lines in a controlled environment by measuring the effect on Na$^+$ exclusion and on rates of photosynthesis. Second, we evaluated the yield in the field in non-saline soil, with unexpected results. Third, we measured yield in a saline field, chosen in the middle of the durum growing area of northern New South Wales, Australia. Key field results with *Nax2* have recently been published (Munns *et al.* 2012). Here we compare the field performance of both *Nax1* and *Nax2* and describe the breeding program that developed the advanced breeding lines and also circumvented the yield penalty found with earlier material containing *Nax1*.

Materials and methods

Germplasm development

Backcrossed lines homozygous for *Nax1* and *Nax2* were developed from a cross between durum wheat (*Triticum turgidum* L. ssp. *durum*) Line 149 (Australian Winter Cereals Collection catalogue number AUS17045) and the Australian durum cultivar Tamaroi, with Tamaroi the recurrent parent for backcrosses (James *et al.* 2006*a*). Early stages of the breeding process were completed using phenotypic selection of F_2 populations, where F_2 plants with a leaf Na$^+$ concentration as low as Line 149 were selected for subsequent backcrossing to Tamaroi. A population of 100 BC_4F_2 seedlings were initially screened for leaf Na$^+$ concentration and also genotyped for the presence of *Nax1* (*TmHKT1;4-A2*), using a co-dominant marker *gwm312* developed by Lindsay *et al.* (2004). Following further selfing and subsequent seed multiplication, BC_4F_4 families were also assayed for the presence of *Nax2* (*TmHKT1;5-A*), initially using flanking dominant molecular markers *gwm410*, *gwm291* as described in Byrt *et al.* (2007). Later, a linked co-dominant marker, *cslinkNax2*, was used for validation of BC_4F_4 families homozygous for *Nax2*. Selected lines for evaluation were given the annotation Tamaroi[+]*Nax2*. Primer sequences and PCR protocols are described by James *et al.* (2011).

Initial field trials of a selected set of 60 BC_4F_4 families indicated that most lines containing *Nax1* carried a yield penalty of 10–15% on non-saline soils (data not shown). One BC_4 line (5020), which was homozygous for *Nax1* and heterogeneous for *Nax2*, was found to consistently yield similarly to recurrent parent Tamaroi, indicating that it did not carry the yield penalty typically associated with *Nax1*. Consequently, a new set of BC_4F_5 sibling lines were developed from Line 5020 that were either homozygous for *Nax1* or homozygous for both *Nax1* and *Nax2*. These lines were given the annotations Tamaroi[+]*Nax1* or Tamaroi[+]*Nax1,2* respectively.

Germplasm used for field trials at Yanco, Yuluma and Moree

A total of 15 Tamaroi-derived BC_4 *Nax* breeding lines were evaluated for yield potential under non-saline field conditions at Yanco in 2007. This set included six BC_4 *Nax1* lines (5020–7, 5020–8, 5020–11, 5020–27, 5020–29, 5020–43), four BC_4 *Nax2* lines (5004, 5042, 6096, 6139) and five BC_4 lines fixed for both *Nax1* and *Nax2* (5020–3, 5020–18, 5020–20, 5020–26, 5020–30). For field trials on saline soils at Yuluma (2009) and Moree (2008, 2009) a subset from the BC_4 *Nax* lines listed above were used for intensive evaluation and comparison to recurrent parent Tamaroi. This subset consisted of two BC_4 *Nax1* lines (5020–7, 5020–27), two BC_4 *Nax2* lines (5004, 5042) and two BC_4 lines fixed for both *Nax1* and *Nax2* (5020–20, 5020–26).

Measurement of Na^+ uptake into durum wheat leaves in 150 mM NaCl

The effect of *Nax1* and *Nax2* separately and the potential additive effect of *Nax1* and *Nax2* together on Na^+ accumulation in durum wheat leaves was evaluated on Tamaroi-derived BC_4 lines containing *Nax1* (4 lines), *Nax2* (4 lines) and *Nax1* plus *Nax2* (4 lines) and compared with Tamaroi. Plants were grown in supported hydroponics in two 40-L trays as described previously (James *et al.* 2008). At ~8 days after emergence and just before the appearance of leaf 3, 25 mM NaCl was added twice daily to a final concentration of 150 mM. Supplemental Ca^{2+} was also added as $CaCl_2$ to give a final $Na^+ : Ca^{2+}$ of 15 : 1. Plants were grown in a controlled environment chamber with a 12 h photoperiod and a PPFD of 800 $\mu mol\ m^{-2}\ s^{-1}$ at 24°C during the day and 18°C during the night. After 10 days in 150 mM NaCl, the blade of leaf 3 was harvested and dried at 70°C for 2 days, 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, Vic.).

Impact of Nax1 on CO_2 assimilation rate of salt-stressed durum wheat in 250 mM NaCl

To assess the effect of *Nax1* on photosynthesis of salt-stressed durum wheat, Tamaroi and a Tamaroi-derived BC_4 *Nax1* line (Line 5020–11) was grown in 250 mM NaCl for 30 days. Plants were grown hydroponically using an automatic subirrigation system as described above. Glasshouse air temperatures were maintained at 25°C during the day and 15°C during the night and daily PAR averaged 21.7 $mol\ m^{-2}\ day^{-1}$. The experiment was set up as a randomised block design, with six replications per genotype per harvest. Main stem leaf 3 emerged at the commencement of the salt treatment, when the seedling was 8 days old (8 days after emergence, DAE). After 20 days in the salt treatment, gas-exchange measurements, chlorophyll content and Na^+ concentration were determined on leaf 3. Gas-exchange measurements 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 1100 and 1500 hours 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 1000 $\mu mol\ m^{-2}\ s^{-1}$ with a red/blue light source, CO_2 was

maintained at 400 $\mu mol\ mol^{-1}$ and the leaf to air vapour pressure deficit (VPD) maintained between 1.2 and 1.4 KPa. Following each gas-exchange measurement the leaf was harvested and leaf chlorophyll content was estimated using a SPAD 502 m (Minolta, Osaka, Japan) and analysed for Na^+ and K^+ as described above. Additionally, main stem leaves 1–4 were analysed for Na^+ and K^+ from a remaining set of plants that were grown in 250 mM for a further 10 days (30 days in salt treatment).

Field trial sites, design and measurements

Yanco 2007

A field trial was conducted at the Yanco Research Station, Yanco, NSW, Australia (34.62°S, 146.43°E). The trial was sown on 10 June 2007 and harvested 11 December 2007. Seed was sown to give a sowing rate of 150 plants m^{-2} in plots that were ~10 m^2. Rainfall of 197 mm was supplemented by one irrigation event in August equivalent to ~40 mm rainfall. The trial was set out in a latinised 16-row × 6-column design. The latinisation of the design ensured an even distribution of the heavily replicated Tamaroi parent line across the trial and avoided replicating each remaining line in the same row or column.

Yuluma 2009

A field trial was conducted on a grower's (John Stevenson) property 'Airlie' near Yuluma, NSW, Australia (35.217°S, 146.495°E). The trial was sown on May 7, 2009 and harvested 24 November 2009. Seed was sown to give a sowing rate of 150 plants m^{-2} in plots that were ~10 m^2. Soil type was a grey vertosol and previously the paddock had been sown to barley (2007) followed by a long fallow (2008). The rainfall was 190 mm. The Yuluma 2009 trial was an 8 × 8 latin-square design. Recurrent parent Tamaroi was randomised to two of the latin square treatments and remaining *Nax* lines were randomised to one of the latin-square treatments each.

Moree 2008, 2009

Field trials were conducted on a grower's (Andrew and Jodie Crowe) property 'Sunbury' north of Moree, NSW, Australia (29.05°S, 149.78°E). The 2008 field trial was sown on a low salinity part of a large paddock on 23 June and harvested 1 December. The 2009 field trial was located in a more saline part of the same large paddock, ~2 km north of the 2008 trial. This trial was sown on 10 June and harvested on 5 November. The soil type for both trials was a black vertosol (pH 6.5–7.0) and the previous crop in each instance was barley followed by a summer fallow. Seed was sown to give a sowing rate of 100 plants m^{-2} in plots that were ~10 m^2. The Ashley 2008 trial comprised three rows of 50 plots each. Rows were treated as 'blocks' with lines randomised to each row, in such a way as to ensure minimum acceptable separations between the replicates of a line. This ensured a good spatial distribution of each line across the trial. The 2009 field trial was designed in three continuous blocks across an area where salinity ranged from low/moderate (Block 1) to high (Block 3). Each block contained 64 individual plots in an 8 × 8 latin-square design, with lines randomised separately to each block. Within each block, Tamaroi was randomised to two of the latin-square treatments so that

Tamaroi was doubly replicated compared with the other lines in the trial.

Na⁺ and K⁺ analysis of flag leaves from field trials

Flag leaves (3–5) from each field plot at all saline field trials were removed from individual mature wheat plants at mid grain fill stage. Fresh weights were measured on a subset of leaves for each genotype in order to calculate ion concentrations on a tissue water basis. Leaves were dried at 70°C for 3 days and combined samples each containing three leaves were analysed for Na⁺ and K⁺ as described above.

Mapping salinity and soil chemical analysis

Salinity levels at all saline field trial sites were characterised through mapping apparent electrical conductivity (ECa) using an EM38 electromagnetic induction meter (Geonics, Mississauga, Canada). ECa values covering the ECa range of the field site, were validated with chemical analysis (pH, soil moisture, $EC_{1:5}$ and Cl^- concentration) 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, USA) and converted to mM in the soil solution using the moisture content measured at the time of sampling.

Statistical analyses

Grain yield for each trial (and the separate blocks in the Ashley 2009 trial) was analysed independently using spatial mixed model methods in GENSTAT (VSN International, Hemel Hempstead, UK). 'Block', 'row' and 'column' design elements were included as random effects according to the design characteristics of the relevant trial and where found to be statistically significant. First order autocorrelated error structures were also included where found significant, as were large scale linear trends across row or columns. ECa (0–1.50 m depth) was measured for every field plot and subsequently included as a covariate in the Yuluma and Ashley 2009 analyses, with establishment counts included similarly in the Yanco 2007 analysis.

Results

Performance of selected breeding lines in a controlled environment

The presence of either *Nax1* or *Nax2* in the BC_4F_4 breeding lines greatly reduced the Na⁺ concentration in the blade of leaf 3 after 10 days in 150 mM NaCl (Table 1). *Nax1* was more effective than *Nax2* at reducing net Na⁺ uptake and accumulation in the leaf compared with recurrent parent Tamaroi. Leaf Na⁺ concentration was reduced on average by 66% by *Nax1* compared with 54% by *Nax2*. The K⁺ concentration was greatly increased by the presence of either *Nax1* or *Nax2* and the K⁺ : Na⁺ ratio of all *Nax* lines was much higher than Tamaroi (Table 1). Lines containing both *Nax1* and *Nax2* had the same Na⁺, K⁺ and K⁺/Na⁺ as Line 149 (Table 1).

To examine the impact of Na⁺ exclusion on leaf function under high salinity, the cv. Tamaroi and a Tamaroi-derived BC_4 line with *Nax1* (Tamaroi [+]*Nax1*) were grown in the very high salinity (for durum wheat) of 250 mM for 30 days. After 20 days in the salt treatment, CO_2 assimilation rates and chlorophyll

Table 1.   Na⁺ concentration and K⁺ : Na⁺ ratio in the blade of leaf 3 of durum wheat cv. Tamaroi and *Nax* donor durum landrace Line 149 and near-isogenic BC_4F_5 Tamaroi-derived lines containing *Nax1* or *Nax2* or a combination of *Nax1* and *Nax2*, grown in 150 mM NaCl for 10 days
Values are means ($n = 5$)

Genotype	Na⁺ concentration	K⁺ concentration	K⁺ : Na⁺ ratio
	(μmol g DW⁻¹)		
Tamaroi (recurrent parent)	1186	604	0.51
Line 149 (*Nax1*, *Nax2* donor)	255	1187	4.76
Tamaroi [+]*Nax1* (4 lines)	403	914	2.56
Tamaroi [+]*Nax2* (4 lines)	549	863	1.59
Tamaroi [+]*Nax1,2* (4 lines)	208	1081	5.68
l.s.d.$_{(0.05)}$	66	59	1.02

Table 2.   Gas-exchange parameters, Na⁺ concentration and chlorophyll content of leaf 3 of durum wheat Tamaroi and Tamaroi [+]*Nax1* (BC_4 Tamaroi-derived line) grown in 250 mM NaCl for 20 days
Values are means ± s.e. ($n = 5$)

Parameter	Germplasm	
	Tamaroi	Tamaroi [+]*Nax1*
CO_2 assimilation rate (μmol m⁻² s⁻¹)	12.6 ± 0.6	19.6 ± 0.7
Stomatal conductance (mmol m⁻² s⁻¹)	122 ± 10	165 ± 13
C_i (μmol mol⁻¹)	216 ± 10	190 ± 6
$C_i : C_a$ ratio	0.55 ± 0.02	0.49 ± 0.01
Na⁺ concentration (μmol g DW⁻¹)	1625 ± 89	477 ± 62
Chlorophyll content (SPAD units)	36.0 ± 1.4	54.8 ± 1.0

contents were ~50% higher and leaf Na⁺ concentration was 3.5-fold lower in the Tamaroi [+]*Nax1* line compared with Tamaroi (Table 2). Assuming previously measured FW : DW ratio for leaf 3 of 5.0, the Na⁺ concentration in leaf 3 on a tissue water basis of Tamaroi [+]*Nax1* would be in the order of 100 mM, compared with 400 mM in Tamaroi. Leaf Na⁺ concentrations above 250 mM are considered to be toxic to photochemistry (James *et al.* 2002, 2006*b*). The higher intercellular CO_2 concentration (C_i) and resulting $C_i : C_a$ ratio (ratio of intercellular CO_2 concentration to ambient CO_2 concentration) in Tamaroi compared with the Tamaroi [+] *Nax1* line, notwithstanding the lower stomatal conductance (Table 2), indicates that the lower rates of CO_2 assimilation in Tamaroi were largely a result of impairment to the photosynthetic machinery, but possibly due to diffusional limitations as well.

After 30 days in 250 mM NaCl, Na⁺ concentrations were measured in main stem leaves 1–4, with leaf 4 being the most recent fully expanded leaf (Fig. 1). Leaf Na⁺ concentration was greater in the older leaves, probably reflecting the greater duration of exposure to salinity. Na⁺ concentration in the leaves of Tamaroi was always higher than in the *Nax1* line. The differences between the genotypes became more profound with each new leaf appearing. A similar trend was also found when these same genotypes were grown in 150 mM NaCl for 35 days (see Fig. S1, available as Supplementary Material to this paper).
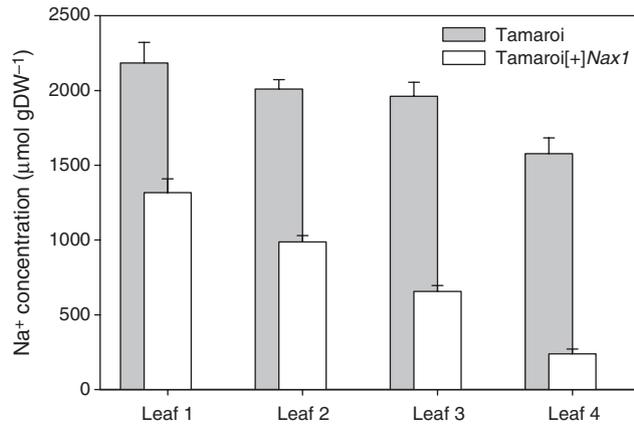
Fig. 1. Na$^+$ concentration in main stem leaves 1–4 of durum wheat cv. Tamaroi and Tamaroi [+]*Nax1* grown in 250 mM NaCl for 30 days in controlled environment. Bars indicate ±s.e. ($n = 6$).

Table 3. Grain yield and harvest details of durum cv. Tamaroi and Tamaroi-derived BC$_4$ lines with *Nax1* and *Nax2* at Yanco (2007)
Flowering score was recorded on 3 October

Genotype	Yield (t ha^{-1})	Height (cm)	Flowering score (Zadok's scale)	Harvest index
Tamaroi	1.71	69	60	0.39
Tamaroi[+]*Nax1*	1.67	69	62	0.39
Tamaroi [+]*Nax2*	1.75	63	63	0.41
Tamaroi [+]*Nax1,2*	1.79	67	62	0.40
l.s.d.$_{(0.05)}$	0.25	6	2	0.04

Evaluation of Nax *lines on a non-saline field trial site*

Advanced backcrossed (BC$_4$) *Nax* breeding lines were evaluated for grain yield and other agronomic features in a non-saline field site in 2007 (Table 3). All *Nax* lines yielded similarly to recurrent parent Tamaroi at ~1.7 t ha^{-1} in a season where low water availability late in the season substantially limited the grain yield. Final plant heights were comparable, except for *Nax2* lines, which were ~10% shorter than both Tamaroi and other BC$_4$ lines containing *Nax1*. This is likely to have impacted

on a marginally higher harvest index (grain mass as a proportion of total shoot mass) of 0.41 for the *Nax2* lines compared with Tamaroi at 0.39. There was only a small variation in flowering time recorded for all lines evaluated. On average *Nax* lines were slightly more advanced than Tamaroi, the flowering time being 1–2 days earlier than Tamaroi (Table 3).

Evaluation of selected Nax lines on saline soil on a commercial farm

Site characterisation for saline field trial sites

The pattern of soil salinity at all saline field sites was undertaken by mapping the apparent electrical conductivity (ECa). At Moree, to identify a location with potentially high salinity for a salt tolerance field trial in 2009, a preliminary ECa survey of a large area (~16 km^2) on a 100 m grid was utilised and published by Munns *et al.* (2012) as fig. 3a. An area was initially identified with ECa ranging up to 500 mS m^{-1} and further characterisation of this area, undertaken by mapping ECa on a 5 m grid, verified a site where ECa varied between 280 and 500 mS m^{-1} (Fig. 2). This site (25 m × 280 m) was subsequently separated into three continuous blocks for the field trial and four soil cores from each block were extracted to a depth of 0.8 m and analysed for chloride. There was a strong positive relationship between ECa measured to depths of 0.75 and 1.50 m, respectively, with mean chloride concentration being indicative of NaCl salinity (Fig. 3). Analysis of soil cores into 0.2 m segments showed that chloride concentrations increased substantially with soil depth in all three blocks (Fig. 4). Chloride measured in a 1 : 5 soil water extract in the top soil (0–0.2 m) was low, ranging between 10 and 50 mM on a soil water basis. At depths of ~0.5 m, chloride concentrations increased substantially to high levels, particularly in Blocks 2 and 3. Chloride concentrations in the soil solution at a depth of 0.8 m in Block 3 were calculated to have been 250 mM, which is equivalent to about half sea water concentration. Over the course of the growing season in non-saline soils, roots would typically be expected to be found at or below a depth of 1 m.

Na$^+$ *concentration and K$^+$:Na$^+$ ratio in flag leaves*

The impact of the *Nax* loci on Na$^+$ concentration in flag leaves was measured at the mid-grain fill growth stage, a growth stage
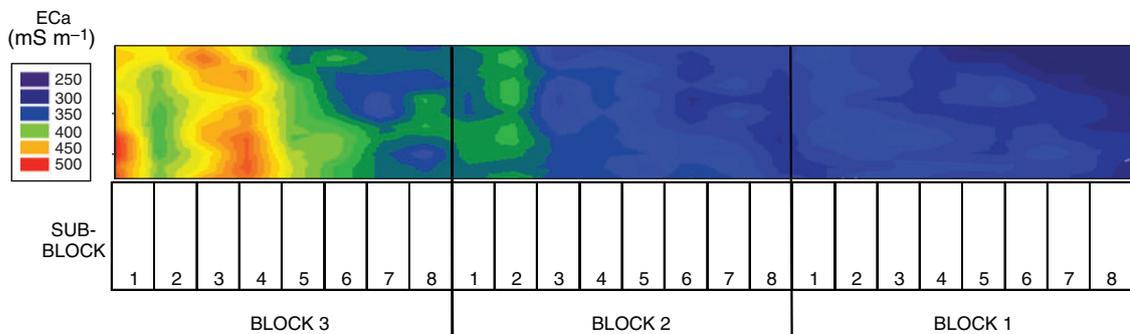

Fig. 2. Contour map of ECa (mS m^{-1}) and block location of the Moree 2009 saline field trial. Each block contains eight sub-blocks, with each sub-block containing eight individual 10 m^2 plots. ECa was measured in the middle of all plots to derive the contour map and also used as a covariate in the grain yield analysis.
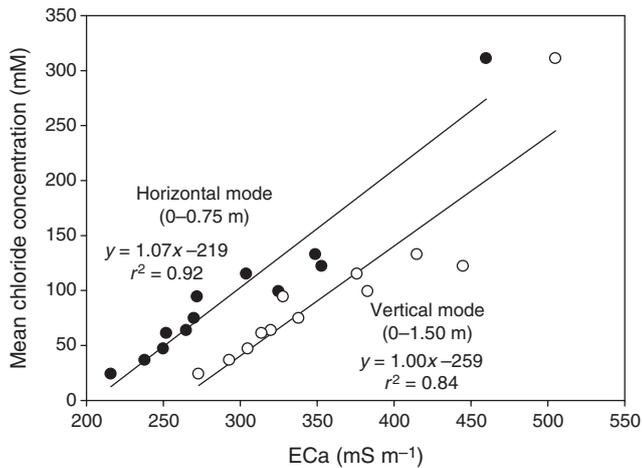
Fig. 3. Relationship between the apparent electromagnetic conductivity (ECa) measured in both vertical and horizontal modes using an EM38 and mean chloride concentration (mM) determined from soil cores taken to a 0.8 m depth from Moree 2009 field trial site.
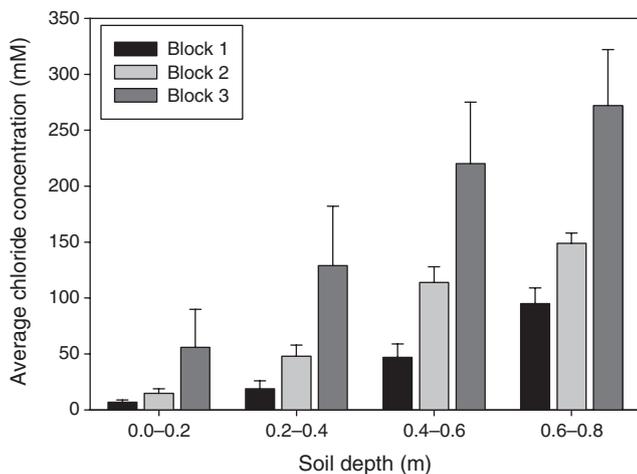

Fig. 4. Average chloride concentration in soil to the depth of 0.8 m in three experimental field trial blocks at Moree 2009. Chloride concentration (mM) was measured in a 1:5 soil:water extract and calculated on a soil water content basis.

Table 4. Apparent electrical conductivity (ECa, 0–1.5 m), Na$^+$ concentration and K$^+$:Na$^+$ ratio in the flag leaves (mid grain fill) of durum wheat Tamaroi and Tamaroi–derived *Nax1* and *Nax2* BC$_4$ lines grown at Moree 2008, 2009 and Yuluma 2009

Note: nm, not measured

	Moree (2008)	Yuluma (2009)	Moree (2009) Block 1	Block 2	Block 3
ECa (mS m^{-1}) mean	200	236	309	334	400
ECa (mS m^{-1}) range	189–213	148–260	281–332	300–380	330–501
Na$^+$ concentration (mM)					
Tamaroi	81	179	258	297	326
Tamaroi [+]*Nax1*	1	2	2	2	2
Tamaroi [+]*Nax2*	7	36	33	51	87
Tamaroi [+]*Nax1,2*	1	2	2	2	2
l.s.d.$_{(0.05)}$	11	9	30	28	43
K$^+$:Na$^+$ ratio					
Tamaroi	nm	1	2	2	1
Tamaroi [+]*Nax1*	nm	100	275	346	315
Tamaroi [+]*Nax2*	nm	7	21	15	13
Tamaroi [+]*Nax1,2*	nm	97	293	349	324
l.s.d.$_{(0.05)}$	–	14	41	30	26

Table 5. Apparent electrical conductivity (ECa, 0–1.5 m) and grain yield of durum wheat variety Tamaroi and Tamaroi-derived BC$_4$ lines containing *Nax1*, *Nax2* and *Nax1,2* at Moree in 2008 and 2009 and Yuluma in 2009

Yield values are adjusted means. Yields significantly different from Tamaroi are indicated: *, $P < 0.05$

Parameter/line	Moree (2008)	Yuluma (2009)	Moree (2009) Block 1	Block 2	Block 3
ECa (mS m^{-1})					
Mean	200	236	309	334	400
Range	189–213	148–260	281–332	300–380	330–501
Yield (t ha^{-1})					
Site/block mean	3.25	2.82	2.43	2.02	1.36
Tamaroi	3.27	2.78	2.57	2.08	1.30
Tamaroi [+]*Nax1*	3.22	2.75	2.38*	1.95	1.26
Tamaroi [+]*Nax2*	3.24	2.88	2.51	2.14	1.61*
Tamaroi [+]*Nax1,2*	3.26	2.84	2.33*	1.94	1.24
l.s.d.$_{(0.05)}$	0.27	0.17	0.16	0.15	0.16

when a potential restraint on carbon production in the flag leaf could be an important determinant of grain yield. Increasing leaf Na$^+$ concentrations in Tamaroi from Moree 2008 through to Moree 2009 reflected increasing ECa (Table 4) and associated salt concentrations in the soil (Fig. 3).

Both *Nax* loci had a profound impact on Na$^+$ accumulation in the flag leaf (Table 4). *Nax1* reduced Na$^+$ concentration in the flag leaf by ~100 fold compared with Tamaroi across all sites and ECa range. Na$^+$ concentration in *Nax1* lines and also in lines containing both *Nax1* and *Nax2* was virtually unchanged at very low concentrations of between 1 and 2 mM across sites and all levels of salinity. *Nax2* also significantly reduced Na$^+$ uptake into the flag leaf, but not to the same extent as lines containing *Nax1* (Table 4). Whereas Na$^+$ concentrations in *Nax2* lines increased with salinity, Na$^+$ concentrations were maintained well below

100 mM, even at the highest salinity levels. This is in contrast to recurrent parent Tamaroi, where leaf Na$^+$ concentrations were at very high and potentially toxic concentrations at or above 300 mM in blocks 2 and 3 at Moree in 2009. Flag leaf K$^+$:Na$^+$ ratios in Tamaroi ranged between 1–2, which was ~100 times lower than in lines containing *Nax1* and 7–10 times lower than in lines containing only *Nax2* (Table 4). This large genotypic variation in flag leaf K$^+$:Na$^+$ ratio was mostly a result of variation in Na$^+$ concentration and to a lesser extent, variation in K$^+$ concentration. For example, average flag leaf K$^+$ concentration across trial blocks at Moree (2009) for Tamaroi was 480 μmol g DW^{-1}, which, although significant ($P < 0.05$),

were only marginally lower than 550 μmol g DW^{-1} in *Nax2* lines and 600 μmol g DW^{-1} in lines containing *Nax1* (data not shown).

Grain yield

Grain yield of all lines declined substantially with increasing ECa and salinity (Table 5). At low salinity levels such as those found in Moree 2008, average grain yields of all lines evaluated was 3.25 t ha^{-1}. At the highest salinity levels such as those present in Block 3 (Moree 2009), grain yields had decreased substantially to between 1.2 and 1.6 t ha^{-1}. It was also under these high salinity levels that the yields of the *Nax2* lines were found to be significantly less reduced than Tamaroi. Under these conditions, *Nax2* lines yielded 0.3 t ha^{-1} or 24% higher than recurrent parent Tamaroi. All four BC$_4$ lines containing *Nax1* were between 5 and 10% lower than Tamaroi in all three trial blocks at Moree 2009, which ranged from moderate to high salinity levels (Table 5; Fig. 5). At field sites with low salinity (Yuluma 2009, Moree 2008) or no salinity (Yanco 2007, Table 3) these lines were all yield-neutral compared with recurrent parent Tamaroi.

Discussion

Relationship between Na$^+$ exclusion and grain yield in saline fields

Salt tolerance in cereals depends upon mechanisms for maintaining photosynthetically-active leaf area, both the total green leaf area and a high rate of photosynthesis per unit leaf area. Both are important for the maintenance of an adequate supply of carbon to growing leaves and roots and reproductive structures. For crop plants such as wheat, this should result first, in the maintenance of the initiation and subsequent growth of tillers, second in the establishment of fertile florets and third, in the subsequent supply of assimilate to the developing grain. The toxic effect of high accumulations of Na$^+$ in leaves
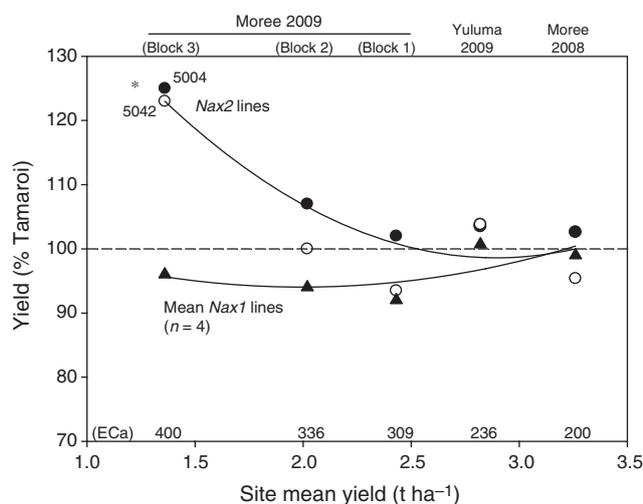

Fig. 5. Relationship between mean grain yield of saline field trial sites or blocks and the grain yield of *Nax* lines as a percentage of recurrent parent Tamaroi at Yuluma and Moree. Mean ECa (0–1.5 m, mS m^{-1}) for each site/block is indicated in figure. Yields significantly different from Tamaroi are indicated: *, $P < 0.05$.

inhibits photosynthesis (Table 2) and causes premature senescence and leaf death.

Although several studies in crop species have shown that with greater Na$^+$ exclusion or enhanced K$^+$/Na$^+$ discrimination comes improved salt tolerance, these are based on early stage biomass in controlled environment studies and the relationship between grain yield and Na$^+$ exclusion capacity was rarely measured. Moderate correlations between grain yield and Na$^+$ exclusion from leaves have been shown in bread wheat in pot studies (Chhipa and Lal 1995; Ashraf and O'Leary 1996; Cuin *et al.* 2009), but other studies have shown no correlation, e.g. Genc *et al.* (2007). However, in that latter study the lack of correlation between yield and Na$^+$ exclusion was most likely due to the low stress applied (100 mM NaCl) and low concentrations of Na$^+$ in leaves and the relatively low genetic variation for Na$^+$ exclusion in the germplasm that was evaluated. Here we present data for the relationship between Na$^+$ exclusion in the leaves and grain yield in the field using near-isogenic lines differing significantly in leaf Na$^+$ concentrations.

Field performance of Nax1 *lines*

The yield penalty recorded for the initial set of BC$_4$ *Nax1* lines under non-saline field conditions was most likely due to 'linkage drag', that is the carrying of linked genes that reduce the yield potential. The introgression of the *Nax1* locus from *T. monococcum* is possibly large, carrying a large part of chromosome 2A from *T. monococcum* that does not readily recombine with the homologous chromosome in durum wheat (S. Huang, unpubl. data). A yield penalty is relatively common when wheat relatives or progenitors are used as a source of genetic variation in wheat breeding (Colmer *et al.* 2006) and particularly when used as a source of novel genes for disease resistance (The *et al.* 1988; Brown 2002).

We overcame this linkage drag issue by identifying what is likely to be a rare recombinant BC$_4$ line (5020) that appeared to show no yield penalty under non-saline conditions. From Line 5020 we developed a series of fixed sibling lines containing *Nax1*, with and without *Nax2* and subsequent field evaluation of these lines established their respective grain yield performances that appeared to validate their lack of yield penalty.

The poor yield performance of the 5020 lines containing *Nax1* under high salinity conditions in the field was therefore perplexing. *Nax1* lines that yielded identically to Tamaroi under low (Table 5) or non-saline field conditions (Table 3), yielded between 5 and 10% lower than Tamaroi in the moderate to high salinity blocks of the Moree 2009 trial (Fig. 5). This result was all the more surprising given the greater efficiency of *Nax1* at excluding Na$^+$ from the leaves compared with *Nax2*, which would lead to the expectation of a greater yield benefit compared with *Nax2*. The concentration of Na$^+$ in the flag leaves of *Nax1* lines grown in high salinities was kept astonishingly low at 2 mM, which was 25–40 times lower than *Nax2* lines and 100–150 times lower than Tamaroi (Table 4). Previously we had found that *Nax1* was responsible for the removal of Na$^+$ from the xylem, not only in the roots, but uniquely in sheath tissue as well (James *et al.* 2006*a*). Therefore as the wheat plant increases in size and tillers elongate, the size of the sheath also increases, thus effectively increasing the potential of *Nax1* to 'filter' Na$^+$ from the xylem

stream. Evidence for this is seen in the present work, in the large disparity in Na$^+$ concentrations between seedling stage leaves in a controlled environment compared with the flag leaf in the field, and also in glasshouse experiments by Husain *et al.* (2003). It is possible that the stem, which starts to elongate only while the later leaves are developing, also retains Na$^+$ by a similar mechanism as the sheath, but this idea has not been tested.

One possible explanation for this surprising yield result is that the removal of too much Na$^+$ from the xylem stream may have effectively deprived the upper leaves of a cheap source of osmoticum necessary for osmotic adjustment and turgor maintenance required to cope with severe osmotic stress. Boyer *et al.* (2008) found that an increase in total inorganic osmotica in durum wheat leaves was largely responsible for the increase in leaf osmotic potential and therefore osmotic adjustment, with increasing salinity levels. In that study, Na$^+$ accounted for ~30% of the increase in leaf osmotic potential and similar values have been reported previously (James *et al.* 2002; Rivelli *et al.* 2002; Cuin *et al.* 2009). The sum of K$^+$ + Na$^+$ was 20–25% lower in *Nax1* lines (625–631 mM) than Tamaroi (790–825) in the high salinity blocks, suggestive that the increase in K$^+$ may not have been sufficient to make up for the dramatic decline in the contribution of Na$^+$ to osmotic adjustment in *Nax1* lines.

Our conclusion from the present work is that *Nax1* may have been too effective at reducing Na$^+$ uptake into the upper leaves of wheat grown in high salinities, not improving yield, but instead causing a small reduction in relative yield. However, for wheat grown in waterlogged saline areas, the greater benefit of *Nax1* derived from aboveground Na$^+$ exclusion might outweigh any of the deleterious factors mentioned above (James *et al.* 2011). Oxygen depletion due to waterlogging would impair ion transport processes in the roots, resulting in high Na$^+$ accumulation in leaves. This process would be ameliorated to a degree by *Nax1*, through retrieval and storage of Na$^+$ in basal shoot structures, which would remain unaffected by waterlogging.

Field performance of Nax2 lines

The effect of *Nax2* on Na$^+$ exclusion and grain yield was evaluated by comparing Tamaroi [+]*Nax2 lines* with parent cv. Tamaroi across three field sites over 2 years. The data for *Nax2* lines at Moree have recently been published (Munns *et al.* 2012). Here we present the additional data at the Yuluma site in NSW in a contrasting environment to confirm the universality of the results. At the low to moderate salinity levels such as those at the Moree 2008, Yuluma 2009 and Block 1 (Moree 2009) field sites, grain yields of both Tamaroi and Tamaroi [+]*Nax2* were virtually identical. This provides further evidence, consistent with earlier trials in SA and NSW in 2004 and 2005 that there was no yield penalty associated with the presence of *Nax2* (Munns *et al.* 2012). This is very important, as farmers will adopt a new wheat variety only if the yield is the same or better than existing varieties where salinity is not limiting biomass or grain yield.

It was only at the highest salinity levels such as those present in Block 3 (Moree 2009) that lines containing *Nax2* yielded significantly more than Tamaroi in high salinity, namely 0.3 t ha^{-1} more or 24% more. This corresponded to very high (toxic) Na$^+$ concentrations in the flag leaves of Tamaroi

(>300 mM), whereas flag leaf Na$^+$ concentrations in *Nax2* lines were maintained at low (non-toxic) levels (<100 mM). These results show that the Na$^+$ exclusion provided by *Nax2* increased grain yields of wheat grown in high salinity levels in the field.

Candidate genes

The most likely candidate gene for *Nax2* is *TmHKT1;5-A* which encodes a Na$^+$-specific transporter located on the plasma membrane of root cells surrounding xylem vessels (Munns *et al.* 2012). HKT (high-affinity potassium transporter) genes are known to be important in the regulation of Na$^+$ and K$^+$ transport in wheat and other species (Schachtman and Schroeder 1994; Munns and Tester 2008; Horie *et al.* 2009). The group 1 HKT transporters of *Arabidopsis* (AtHKT1;1) and rice (OsHKT1;5) reduce transport of Na$^+$ to shoots by unloading Na$^+$ from xylem vessels to xylem parenchyma cells in roots (Mäser *et al.* 2002; Ren *et al.* 2005; Sunarpi *et al.* 2005; Davenport *et al.* 2007) and increase plant salinity tolerance as determined by the effect of cell-specific expression on shoot growth of *Arabidopsis* (Moller *et al.* 2009; Plett *et al.* 2010). The likely candidate gene for *Nax1* is *TmHKT1;4-A2* (Huang *et al.* 2006) although as yet we do not have proof of its function in higher plants as a Na$^+$-specific transporter.

Impact of the osmotic stress on grain yield in a saline field

It is widely appreciated that growth of salt-stressed plants is limited mostly by the osmotic effects of salinity. The osmotic potential of the salt solution in soil, like soil water deficit due to drought, causes a decrease in soil water potential, which induces a reduction in leaf expansion and stomatal conductance and thereby photosynthesis (Munns 2002). Additional growth limitations caused by the accumulation of excessive salt concentrations in mature leaves occurs at a later stage of growth.

Field experiments conducted at close locations with soils of different salinity allow us to estimate the osmotic effect of the salt solution – when soil volume and light are not limiting – as typical of controlled environments and when climatic conditions are identical. The experiment described here at Moree in 2009 with three field blocks of different salinity adjacent to each other and so having identical ambient conditions but with varying soil salinity, allow us to quantify osmotic versus salt-specific effects on yield. Grain yield of all genotypes declined significantly with increasing salinity (Table 5) and we propose that this decline in site mean yield from 2.43 to 1.36 t ha^{-1} is largely due to the osmotic effect of salts in soil solution. The flag leaf Na$^+$ concentration of *Nax2* lines (Table 4) was well below that which would be considered critical, which is greater than 250 mM (James *et al.* 2006*b*). In the high salinity block yield of Tamaroi decreased by 50% whereas *Nax2* lines decreased by only 36%. The decline in yield of *Nax2* lines was entirely due to osmotic stress as Na$^+$ concentrations in leaves were kept at low non-toxic levels, so the additional 14% decline in Tamaroi was due to Na$^+$ toxicity.

A similar conclusion can be drawn for durum wheat lines containing the *Kna1* locus for K$^+$/Na$^+$ discrimination introgressed from hexaploid wheat (Dvořák *et al.* 1994). In moderate salinities in the field yield in *Kna1* lines decreased by 30%, most likely due

to the osmotic effect of soil solution, as Na^+ concentration in leaves was low, ~50 mM (calculated from Dvořák *et al.* 1994). Lines lacking *Kna1* had an additional 10% reduction in both biomass and yield.

The dominance of soil moisture or interaction of water stress and salt stress is important in the field and the relative impact of osmotic stress vs. salt-specific stress on yield deserves more attention. Schubert *et al.* (2009) grew maize in very large pots through to grain maturity and found that increases in yield were associated only with a combination of Na^+ exclusion and osmotic stress tolerance whereas hybrids with just Na^+ exclusion were insufficient.

Conclusion

The Na^+ exclusion phenotype of *Nax1* lines looked the most promising at early seedling stage in controlled environment conditions, however, the more moderate Na^+ exclusion capacity of *Nax2* proved to be more successful in the field. This study illustrates the effectiveness of the field environment for revealing difference in plant growth and grain yield that cannot be seen in controlled environments. The study also showed the importance of well characterised field sites to account for natural variation in salinity. Salinity varied even across small areas, at a scale of metres and the impact of this on grain yield could be accounted for only by measuring soil salinity under each plot and using this data as a covariate in statistical analysis of the trial.

Both *Nax1* and *Nax2* have been crossed into hexaploid wheat, as described by James *et al.* (2011) and field trials in several commercial bread wheat cultivars revealed some promising *Nax2* lines, as well as *Nax1* lines without a yield penalty, thus indicating the potential for additional genetic gains for improving salt tolerance in bread wheat.

The *Nax2* lines produced in this study are in the background of Tamaroi, a cultivar that has been recently superceded by new varieties with improved quality attributes. The lines together with molecular markers are available for breeders to cross into their advanced breeding lines adapted to specific regions, to ensure that the 25% yield advantage on saline soils can be achieved in other salt-affected regions in the world.

Acknowledgements

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