# Salinity Tolerance and $Na^+$ Exclusion in Wheat: Variability, Genetics, Mapping Populations and QTL Analysis

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**Abstract**: A wide range of variability in both $Na^+$ exclusion and salinity tolerance was shown in *Triticum dicoccoides* and the best performing genotype, from Getit, was identified for further study and for crossing. In bread wheat, plants $BC_6F_1$ from the cross Chinese Spring/line SQ1 showed less variability, but the line 1868 was identified as a potential source of tissue tolerance to salinity. Two Afghani durum landraces were identified among 179 screened, with approximately 50% lower $Na^+$ accumulation in shoots. Genetic analysis of $F_2$ progenies between landraces and durum wheat showed clear segregation indicating on the single, major salinity tolerance gene in the landraces. Further genetic and molecular analysis of the candidate gene and its localization is in the progress. QTL analysis of two non-pedigree related mapping populations of bread wheat, Cranbrook × Halberd and Excalibur × Kukri, showed one QTL in each population on the same region of chromosome 7AS, independent of year or growing conditions (both supported hydroponics and field trials), and a novel gene is expected to be associated with this QTL.

**Keywords**: genetics; mapping populations; $Na^+$ exclusion; QTL analysis; salinity tolerance; variability

Salinity is a major abiotic stress and is likely to increase in severity as a consequence of global warming. Three primary components determine salinity tolerance (ST): osmotic tolerance, $Na^+$ exclusion and tissue tolerance. All three components are important, but contribute differently to overall ST.

In order to learn more about the genetics of and genes controlling ST, two major studies need to be undertaken: firstly, screening and identification of the most appropriate accessions, lines or genotypes for further crossings; and secondly, identification of potential candidate genes using mapping populations and QTL analysis. This two-step approach is referred to as 'Forward Genetics', employing the classical methods of screening, genetic analysis and molecular mapping.

We previously reported (Shavrukov *et al.* 2006, 2009) that wild relatives of wheat from the genus *Triticum* have much greater variation in $Na^+$ exclusion and ST compared to cultivated wheats. In contrast, cultivated durum and bread wheats have very little variability in these traits (Munns *et al.* 2000). This is mainly related to the erosion of genetic diversity through a consequence of two 'bottlenecks' of gene pool exploitation in the development of cultivated wheat: firstly, between two diploid species, progenitors of the A and B

genomes, and, secondly, between the tetraploid progenitor and a third diploid species, the progenitor of the D genome (Huang *et al.* 2002; Dvorak & Akhunov 2005). The domestication of tetraploid durum wheat and, later, bread wheat, particularly through the modern agricultural practices of pure breeding, resulted in a further loss of genetic diversity (Nevo 2004).

Genetics, mapping and identification of candidate genes using QTL analysis for ST in wheat, has not yielded many significant outcomes, despite a long history of ST research. This probably reflects the complexity of the ST trait. Usually, QTL analysis in saline hydroponics reports several chromosomes associated with ST (eg. Ma *et al.* 2007), while QTLs on chromosomes 5B and 5D were identified in a field experiment with saline irrigation (Quarrie *et al.* 2005). The *Kna1* gene was identified on chromosome 4D controlling $Na^+$ and $K^+$ accumulation in the shoot (Dubcovsky *et al.* 1996). More recently, the *Nax1* and *Nax2* genes for $Na^+$ exclusion were identified, originating from *T. monococcum* and corresponding to QTLs on chromosomes 2A and 5A, respectively (James *et al.* 2006). In this study, we present the results of our screenings and identification of the best accessions for ST, and our genetic and QTL analyses for further identification of novel candidate genes for ST in wheat.

## MATERIAL AND METHODS

### Plant material

Seeds of different wild species of the genus *Triticum*, and cultivars of durum and bread wheat, were supplied from the Australian Winter Cereals Collection, Tamworth (Australia) and the Vavilov Research Institute of Plant Industry, St.Petersburg (Russia). Wild emmer genotypes, *T. dicoccoides*, were provided by the Institute of Evolution, University of Haifa, Israel. DH lines from the cross Chinese Spring × line SQ1 were received from Steve Quarrie, University of Newcastle, UK. The mapping population Cranbrook × Halberd was distributed through the Australian Winter Cereals Collection, Tamworth (Australia). The mapping population Excalibur × Kukri was produced and provided by the Australian Centre for Plant Functional Genomics, Adelaide (Australia).

### Growth conditions, experimental design and salt treatment

Seeds were germinated for four days at room temperature on moist filter paper before being transferred to a supported hydroponics setup as previously described (Shavrukov *et al.* 2006,

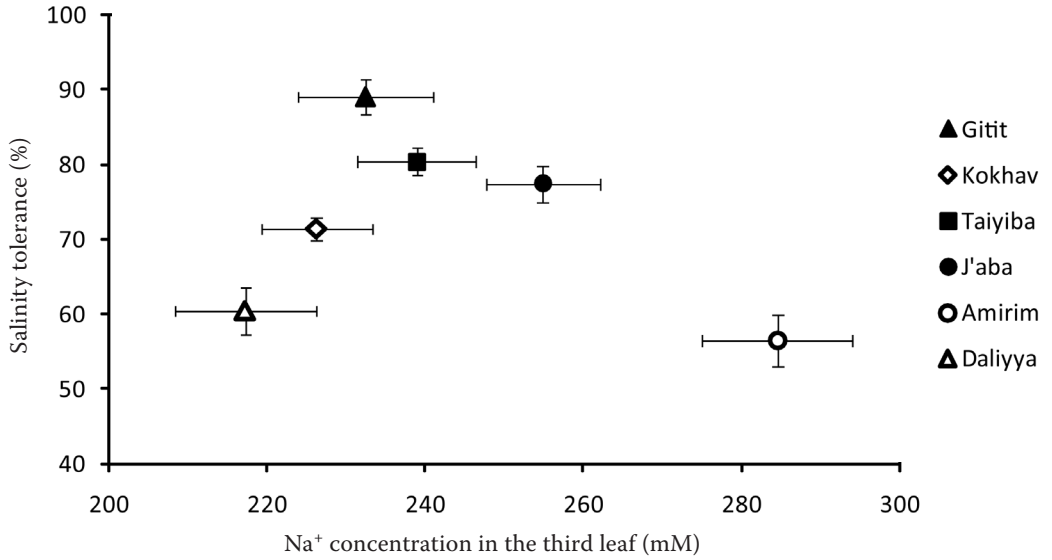


Figure 1. $Na^+$ accumulation and salinity tolerance (ST) in six populations of wild emmer, *Triticum dicoccoides*; plants were grown in supported hydroponics in the presence of 200mM NaCl for 10 days, and $Na^+$ concentrations were measured in the third leaf; shoot dry weights were recorded for both salt-treated and control plants, and ST was calculated as a ratio; each value is the mean of six replicates ± standard error

2009). Numbers of biological replicates varied between four and six in the different experiments. Salt stress was also varied: 100mM NaCl (durum wheat, *T. turgidum* ssp. *durum*), 100–150mM NaCl (bread wheat, *T. aestivum*) and 200mM NaCl (wild emmer, *T. dicoccoides*), with exposure to salt for either 10 or 20 days depending on the experiment. In order to maintain constant calcium activity across NaCl treatments additional $CaCl_2$ was applied to the growth solutions as necessary.

The field experiments were both grown at Roseworthy, South Australia and have previously been described (Edwards *et al.* 2008).

### Measurements and statistical analysis

Concentration of $Na^+$ was measured in the third leaf after 10 days or in the fourth leaf after 20 days growth in the presence of NaCl, as previously described (Shavrukov *et al.* 2009). ST was calculated as the ratio between the average values of shoot dry weight (SDW) for each accession: SDW (salt treatment)/SDW (control) × 100%. The data for genotype analysis represent means ± standard error, with 4–6 replicates in different experiments. The Chi-square test was used to test differences related to theoretical distributions. Standard Excel program software was used for the calculation of averages and standard errors.

## RESULTS

### Screening, variability and identification of the best accessions. The example of wild emmer, *Triticum dicoccoides*

We screened 54 genotypes of wild emmer wheat, *T. dicoccoides*, from nine geographic populations from Israel. The populations showed enormous variability in both $Na^+$ exclusion and ST (Shavrukov *et al.* 2010) and six wild emmer genotypes were identified for further study on the basis of plant growth, $Na^+$ exclusion and ST. Four of the nine populations (Gitit, Kokhav-Hashahar, Taiyiba and J'aba) are characterized by environments strongly affected by drought and salinity (xeric regions), while two contrasting populations (Amirim and Daliyya) come from environments with relatively high rainfall and low soil salinity (mesic regions) (Figure 1).

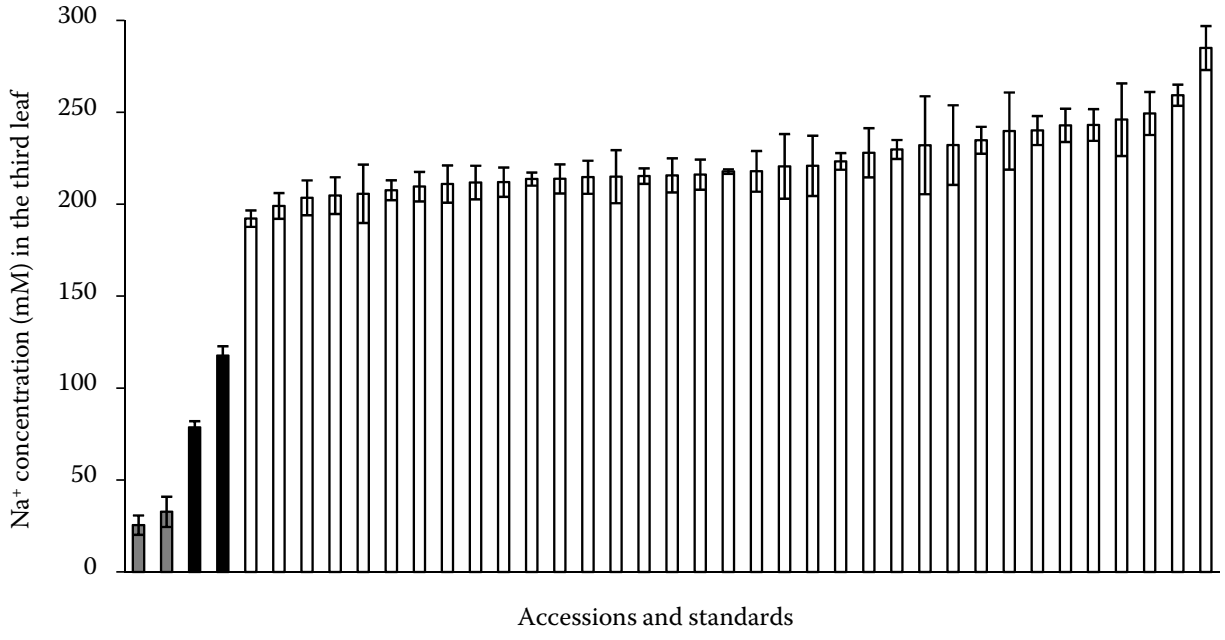


Figure 2. $Na^+$ accumulation in 37 accessions of *Triticum turgidum* ssp. *durum*; two bread wheat cultivars, Krichauff and Kharchia-65, were used as standards and are represented by the shaded bars; two durum landraces, lines 752 and 740, are represented by the black bars; a further 35 durum accessions are depicted as clear bars; plants were grown in supported hydroponics in the presence of 100mM NaCl for 10 days, and $Na^+$ concentrations were measured in the third leaf; each value is the mean of four replicates ± standard error

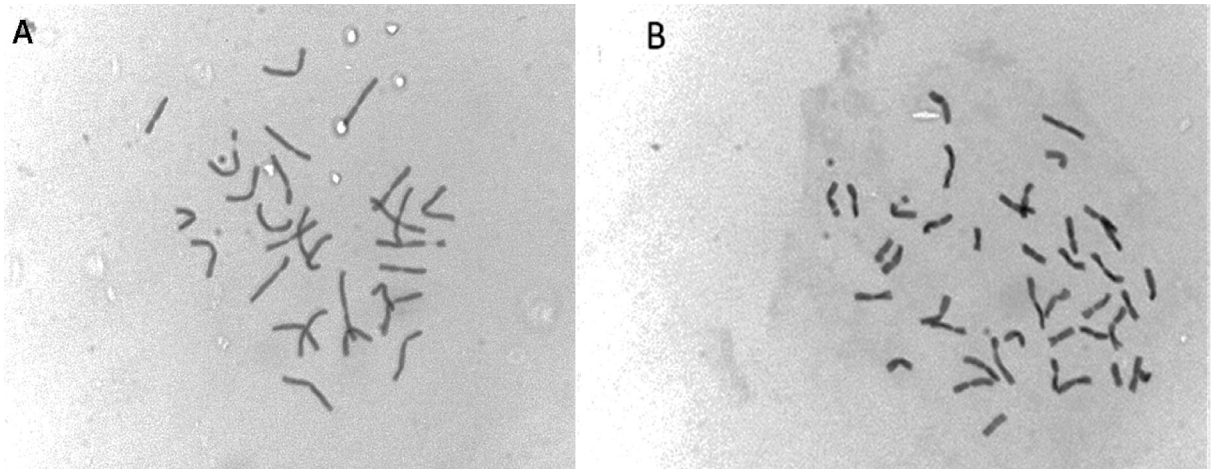


Figure 3. Metaphase plates of chromosomes in: (A) durum line 740 with $2n = 28$; and (B) bread wheat, control, with $2n = 42$

Plants from all xeric geographic populations (Gitit, Kokhav-Hashahar, Taiyiba and J'aba) had values of $Na^+$ accumulation in the shoots in the range between 226 and 255mM NaCl, as well as high ST (71–89%). Plants from both mesic geographic populations (Amirim and Daliyya) performed more poorly with respect to ST (57–60%), but showed quite different $Na^+$ accumulation trends: 284 and 217mM NaCl, respectively. Genotypes from the Gitit population were chosen as the best for crossing with Australian durum wheat and are being investigated further. Additionally, a metabolomics experiment is underway.

**Durum wheat: screening and genetic analysis**

In durum wheat, two landraces originating from Afghanistan, lines 740 and 752, were identified as the best sodium excluders from a screen of 179 durum landraces (Figure 2 represents a selection of the screen and includes only 37 durum landraces).

While most of the durum lines studied accumulated between 200 and 250mM $Na^+$ in the third leaf during 10 days of growth in 100mM NaCl, lines 740 and 752 accumulated only 160 and 120mM $Na^+$, respectively. The tetraploid status of both accessions was confirmed using both molecular markers to show the absence of the wheat D genome, and cytologically, confirming $2n = 28$ (shown for line 740 in Figure 3).

We have now crossed the lines 740 and 752 with elite Australian durum cultivars and breeding lines, to introgress their ST traits into cultivated durum wheat. $F_2$ and $F_1B_1$ progenies of four cross combinations between the low $Na^+$ excluding lines and the Australian durum cultivars, Kalka and Jandaroi, as well as two breeding lines, 53380 and Zbl, have been analysed. Figure 4 represents results of the segregation in $F_2$ and $BC_1F_1$ in the cross Kalka × line 752.

Clear segregation has been found in all of the four $F_2$ progenies, with the $Na^+$ exclusion traits from lines 740 and 752 showing a dominance effect. Frequencies observed plants with low and high $Na^+$ accumulation in the cross Kalka × line 752 were not statistically different from the expected ratio (3:1) for simple monogenic segregation ($\chi^2 = 2.20 < \chi^2 = 3.84_{0.05}$), using a total number of 95 analysed $F_2$ plants. The analysis of backcross populations $BC_1F_1$ with Australian elite durum wheat as the recurrent parent is also showing similar results. Based on the observed segregation ratios, we hypothesize that there exists either one novel gene for $Na^+$ exclusion with two different alleles, or two distinct genes for $Na^+$ exclusion in the durum landrace lines 740 and 752. Detailed analysis of segregation types in the $F_2$ generation and of further progenies is currently underway.

The absence of the two known *T. monococcum* $Na^+$ exclusion genes, *Nax1* and *Nax2*, has been confirmed in all lines, with the exception of the breeding line Zbl which had been generated from a line that was crossed with *T. monococcum* (Figure 5).

Segregation for $Na^+$ exclusion in $F_2$ progeny from the cross Zbl × line 752 was more transgressive between both parents (Figure 6). This observation is suggestive of a possible interaction between at

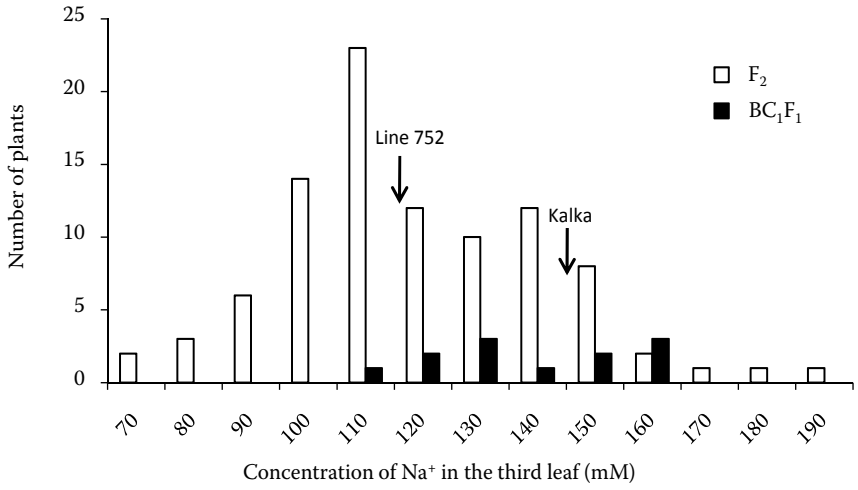


Figure 4. Segregation for $Na^+$ accumulation in the third leaf in $F_2$ (clear bars, 95 plants) and $BC_1F_1$ (black bars, 12 plants) progenies from a cross between the Australian durum cultivar Kalka and durum landrace line 752; $Na^+$ accumulation for both parents is shown with arrows, having been recorded as an average of six replicates in the same experiment

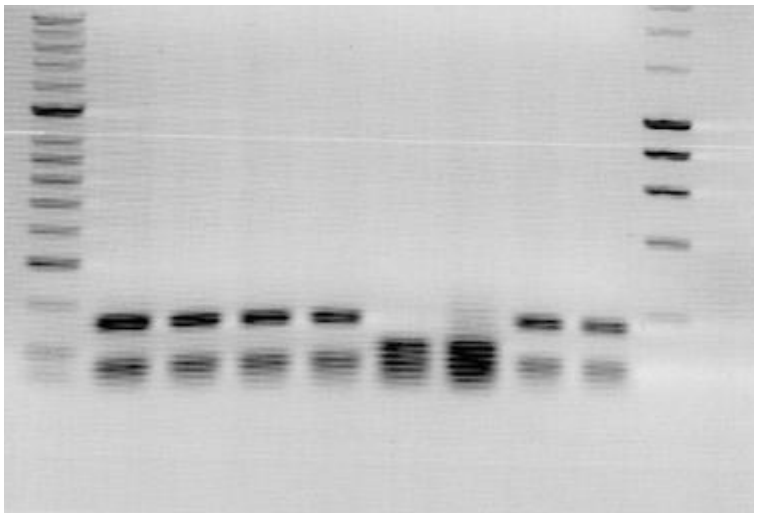

Figure 5. Separation of the products of CAPS marker CK-205377+*Rsa*I in 2.5% agarose gel; PCR was performed with primers CK-205377 (Huang *et al.* 2006), followed by digestion with the restriction enzyme *Rsa*I; first and last lanes represent lambda markers for 100 bp and 1 kb, respectively; eight samples of digested PCR products (lanes 2–9) represent: four durum cultivars, Tamaroi, Wollaroi, Kalka and Jandaroi (lanes 2–5); durum breeding line Zbl (lane 6); *Triticum monococcum* accession AUS-90382, containing *Nax1* gene (lane 7); and two durum landraces, lines 740 and 752 (lanes 8 and 9)

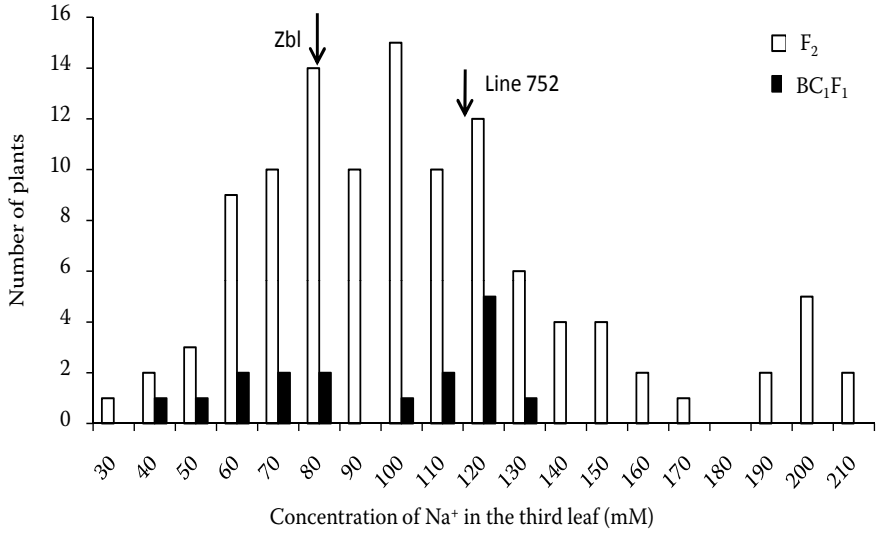


Figure 6. Segregation for $Na^+$ accumulation in the third leaf in $F_2$ (clear bars, 112 plants) and $BC_1F_1$ (black bars, 17 plants) progenies from a cross between durum breeding line Zbl, containing the *Nax1* gene, and durum landrace line 752; $Na^+$ accumulation for both parents is shown by arrows, having been recorded as an average for six replicates from the same experiment

least two genes: *Nax1* and a novel ST gene from line 752. We hypothesize that plants with superior $Na^+$ exclusion compared to either parent contain both *Nax1* and novel $Na^+$ exclusion genes, but it has been difficult to clearly identify these progeny. Conversely, there was definite cluster of nine progeny with significantly higher shoot $Na^+$ accumulation (190–210mM $Na^+$), where we can hypothesise that these plants contain null alleles of both *Nax1* and a novel $Na^+$ exclusion gene. The frequency of two null alleles was not statistically different from the hypothetical value 1/16 for digenic segregation ($\chi^2 = 0.61 < \chi^2 = 3.84_{0.05}$), using a total number of 112 analysed plants.

Further genetic analysis and backcrossing is currently underway, for mapping and for identification of progenies with superior ST. We also expect to obtain results from an analysis of $BC_1F_2$ after self-pollination of the initial backcrosses, and $BC_2F_1$ after a subsequent round of backcrossing with the recurrent Australian durum wheat parent. These results will further increase the confidence in our understanding of the genetics of novel $Na^+$ exclusion genes originating from the exotic Afghani durum landraces. Ultimately, the best identified progenies will be tested in field trials for ST.

**Synthetic wheat reconstruction**

In cooperation with CYMMIT, the Afghani durum landrace lines 740 and 752 are now being crossed with several accessions of *Aegilops tauschii*, for reconstruction of hexaploid synthetic wheat. After several rounds of backcrossing with modern bread wheat, the hybrids will be evaluated for $Na^+$ exclusion and tested for ST in field trials.

**Bread wheat: identification of a breeding line with potential tissue tolerance to salinity**

Eight near-isogenic lines (NILs, $BC_6F_1$ progeny), originating from a cross made by Steve Quarrie between Chinese Spring and the inbred line SQ1 (Quarrie *et al.* 2005, 2006), were selected after a preliminary screening of 24 NILs. The selected NILs were tested for $Na^+$ accumulation and ST after 20 days growth in the presence of 150mM NaCl (Figure 7). Seven of the tested NILs showed intermediate $Na^+$ accumulation between both parents, and significantly lower ST. However, one NIL (1868) had both higher $Na^+$ accumulation and approximately two-fold greater levels of ST. The discovery of this

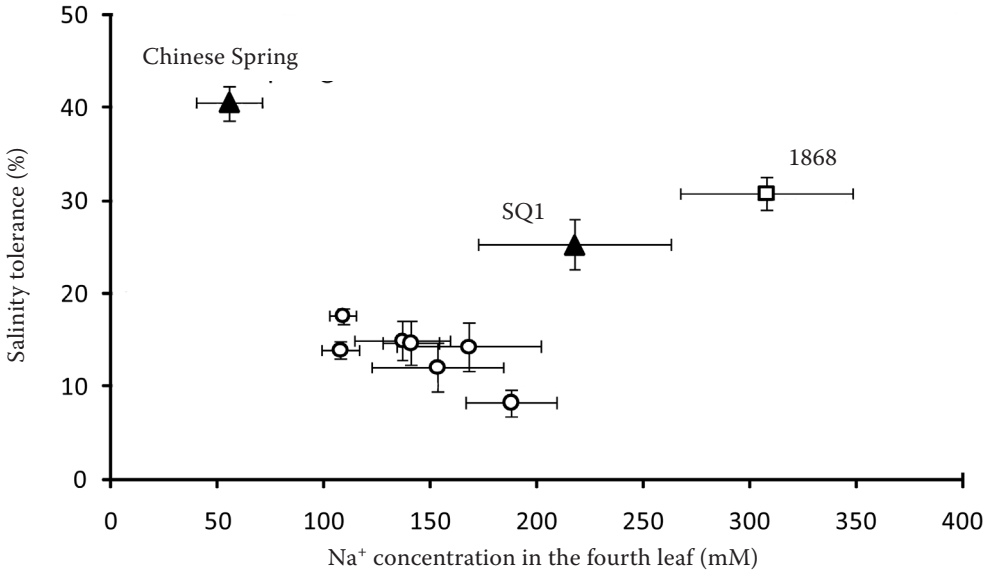


Figure 7. $Na^+$ accumulation and salinity tolerance (ST) in eight NILs (open circles and square) of bread wheat, originating from a cross between Chinese Spring and breeding line SQ1 (Quarrie *et al.* 2005, 2006); NIL 1868 is shown as a clear square; the two parents are represented as black triangles; plants were grown in supported hydroponics in the presence of 150mM NaCl for 20 days, and $Na^+$ concentrations were measured in the fourth leaf; shoot dry weight was recorded for both salt treatments and control, and ST was calculated as a ratio; each value is the mean of six replicates ± standard error

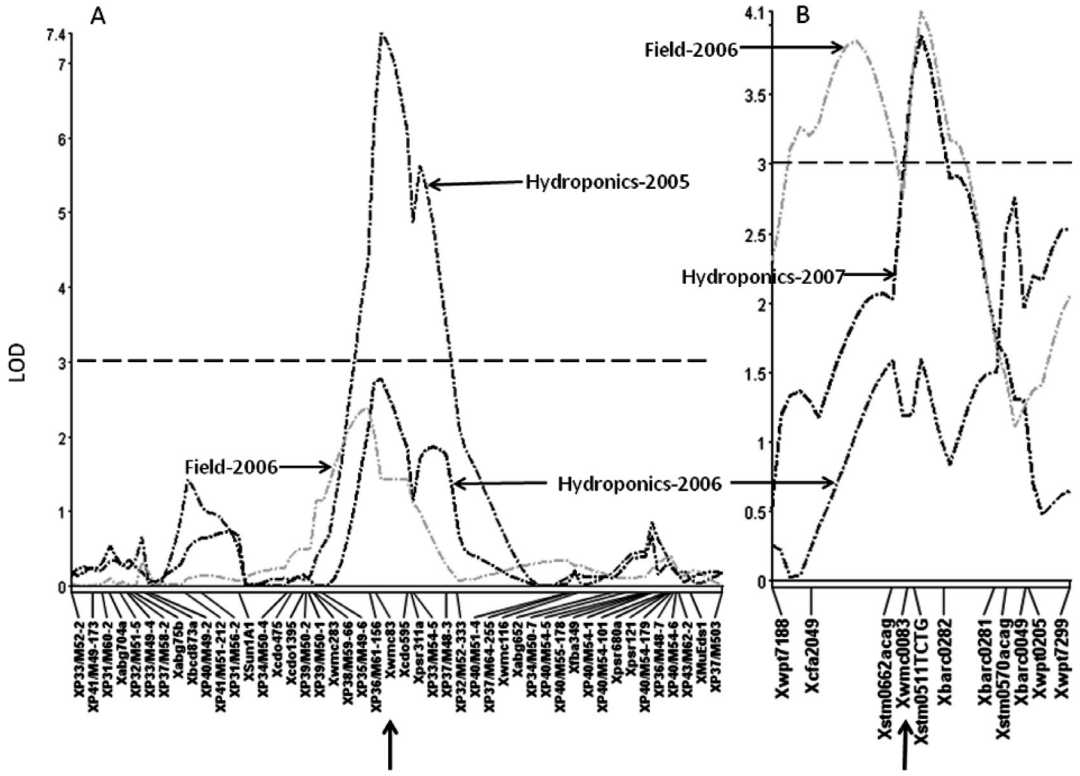


Figure 8. Composite interval mapping of $Na^+$ exclusion on the chromosome 7AS in (A) Cranbrook × Halberd and (B) Excalibur × Kukri DH mapping populations; each mapping population was used in three experiments: twice in hydroponics and once in field trials, as shown on the figure; the approximate 5% significance level on LOD scale is represented by a dashed line; both mapping populations include the SSR marker *Xwmc83*, as indicated on the *x*-axis of the molecular map by arrows

NIL may be extremely interesting, with a possible hypothesis being that the NIL 1868 contains an effective mechanism for tissue tolerance to salinity and, therefore, can be a potential source of novel gene(s) for ST.

### Bread wheat: QTL analysis for $Na^+$ exclusion in two mapping populations

In bread wheat, two $F_1$-derived doubled haploid mapping populations made from crosses Cranbrook × Halberd (160 lines), and Excalibur × Kukri (233 lines), were used to define the location of QTLs associated with $Na^+$ exclusion (Figure 8).

Shoot sodium accumulation was measured in both supported hydroponics and in field trials at Roseworthy, South Australia. Hydroponics experiments were conducted twice and the field trial once for each population. A QTL located on chromosome 7AS was present in both environments (hydroponics and field trials) and in both populations. This QTL was suggestive (LOD = 2.9 and 3.0) and accounted for approximately 7% of the total phenotypic variation in both populations (a range of 3% to 41%, depending on the sampling environment, population and population size), with the favourable ($Na^+$ exclusion) allele originating from Cranbrook and Excalibur. The QTL is of potential interest as it has been detected here in two unrelated populations and in both a controlled growing environment (supported hydroponics) and under field conditions.

The QTL links to a related interval on rice chromosome 8 and potential candidate genes are currently under investigation. *HKT*, *SOS* and *NHX* genes have been shown to contribute to ST in plants. Members of these gene families have been mapped in wheat, but none have been located to the short arm of group 7 chromosomes.

Further field evaluation is required to validate whether the effect of this QTL on $Na^+$ exclusion has a significant positive effect on grain yield in high salinity environments and that it does not

have a deleterious effect on yield in the broader environments targeted by wheat breeding programmes.

## DISCUSSION

Multiple strategies are necessary to study and fully understand plant tolerance to abiotic stresses such as salinity. With respect to salinity stress tolerance, it is first necessary to assess overall ST and key components of the process: tolerance to osmotic stress, $Na^+$ exclusion and tissue tolerance to salinity. Different species and sometimes different genotypes within one species can have different reactions to salinity stress. For example, a strong positive correlation between $Na^+$ exclusion and ST in durum wheats has been clearly demonstrated (Munns & James 2003), while in bread wheat, an ability to exclude $Na^+$ from the shoot does not play an important role in ST (Genc *et al*. 2007) and perhaps tissue tolerance could be a more important component of ST.

Tests of variability for ST and its components among accessions and lines is a very important second step in studying plant ST. Usually, the variability is very limited among cultivated wheats, both in durum and bread wheat types. In contrast, variability to ST is much higher amongst the wild species, including different diploid and tetraploid species (Shavrukov *et al.* 2006, 2009). In this step, identification of an exotic genotype, accession or line with superior $Na^+$ exclusion, osmotic stress or tissue tolerance to salinity is extremely important for further genetic studies and breeding programmes. For example, we identified a genotype of wild emmer wheat, *T. dicoccoides*, originating from Gitit, showing excellent performance for $Na^+$ exclusion and ST (Figure 1). These characteristics have not previously been described in emmer wheat and, therefore, we included the wild emmer genotype from Gitit in our breeding program. Similarly, we identified an unusual bread wheat line (NIL 1868) with potentially high tissue tolerance to salinity (Figure 7). Such a line has not previously been described. This NIL might be a source of novel trait for ST and can be interesting for further investigation.

The discovery of two exotic Afghani landraces, lines 740 and 752, with approximately two-fold lower $Na^+$ accumulation in shoots (Figure 2) and potentially very high ST, may open a new page for our wheat genetic and breeding programmes. Clear segregation in $F_2$ progenies from crosses with Australian elite durum wheats, including Kalka (Figure 4), suggests the existence of a novel, single major gene controlling $Na^+$ exclusion and ST. It will be of interest to evaluate hybrids with a combination of the novel $Na^+$ exclusion gene with the known *Nax1* gene. We hope to study the interaction processes between these two genes (Figure 6) and to identify interesting genotypes/progenies with superior ST, that also show no 'linkage drag' of accompanying deleterious traits from wild landraces. Further localization and mapping, and identification of potential candidates for the novel gene, is underway. The novel ST gene will also be amenable for reconstruction of synthetic hexaploid wheat, which could facilitate rapid progress towards improving ST in bread wheat.

The process of mapping and identification of candidate gene(s) for ST is a very complicated one, requiring lots of resources, time and expense. However, in this case, we were able to use two existing mapping populations. The Cranbrook × Halberd DH mapping population, which was produced many years ago, is publicly available and widely used. A relatively new mapping population of RILs from the cross Excalibur × Kukri was designed at ACPFG for drought studies, but it has also proven to be successful for analysis of $Na^+$ exclusion and ST (Figure 8). We hope to map and identify a new ST gene on the 7AS chromosome, using the comparison between these two non-pedigree related mapping populations as a basis.

As with other abiotic stresses, tolerance to salinity stress involves a very complex reaction by plants where tens, and possibly hundreds, of genes are involved. To better understand this process, to identify the key genes involved, and to thoroughly investigate the genetic basis of all of the components of ST, will require a great investment of resources, but these will be important steps for the improvement of ST in modern wheats.

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