A sodium transporter for salt tolerance in durum wheat

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A sodium transporter (HKT7) is a candidate for \textit{Nax1},
a gene for salt tolerance in durum wheat\textsuperscript{1}

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Durum wheat is more salt sensitive than bread wheat. A novel source of Na⁺ exclusion conferring salt tolerance to durum wheat is present in the durum wheat Line 149 derived from *T. monococcum* C68-101, and a QTL contributing to low Na⁺ concentration in leaf blades, *Nax1*, mapped to chromosome 2AL. In this study, we used the rice genome sequence and data from the wheat EST deletion bin mapping project to identify markers and construct a high resolution map of the *Nax1* region. Genes on wheat chromosome 2AL and rice chromosome 4L had good overall co-linearity, but there was an inversion of a chromosomal segment which includes the *Nax1* locus. Two putative sodium transporter genes (*TmHKT7*) related to *OsHKT7* were mapped to chromosome 2AL. One *TmHKT7* member (*TmHKT7*-A1) was polymorphic between the salt tolerant and sensitive lines, and co-segregated with *Nax1* in the high resolution mapping family. The other *TmHKT7* member (*TmHKT7*-A2) was located within the same BAC contig of approximately 145 kb as *TmHKT7*-A1. *TmHKT7*-A1 and -A2 showed 83% amino acid identity, with *TmHKT7*-A2 but not *TmHKT7*-A1 being expressed in roots and leaf sheaths of the salt tolerant durum wheat Line 149. The expression pattern of *TmHKT7*-A2 was consistent with the physiological role of *Nax1* in reducing Na⁺ concentration in leaf blades by retaining Na⁺ in the sheaths. *TmHKT7*-A2 could control Na⁺ unloading from xylem in roots and sheaths.
Soil salinity causes a significant reduction in agricultural production (Pitman and Läuchli, 2002). More than 6% of the world’s arable land is affected by either salinity or sodicity and a significant proportion of agricultural land has become saline because of land clearing or irrigation (Munns, 2005). To meet this challenge, it is important to understand mechanisms of salt tolerance for further improving salt tolerance of crops, by either traditional breeding or gene manipulation.

In the Triticeae, sodium exclusion is one of the major mechanisms conferring salt tolerance (Gorham et al., 1990; Munns et al., 2006). Bread wheat (*Triticum aestivum* L., AABBDd) has a low rate of Na\(^+\) transport to the shoot and maintains a high ratio of K\(^+\)/Na\(^+\) in leaves (Gorham et al., 1990). The *Kna1* locus, contributing to a higher K\(^+\)/Na\(^+\) ratio and salt tolerance in bread wheat, was mapped to the distal region of chromosome 4DL (Dubcovsky et al., 1996). Durum wheat (*Triticum turgidum* L. ssp. *durum* [Desf.], AABB) is more salt sensitive than bread wheat (Gorham et al., 1990; Rawson et al., 1988) and production suffers when grown on saline soil (Francois et al., 1986; Maas and Grieve, 1990). A new source of Na\(^+\) exclusion was found in a durum wheat, Line 149, which had low Na\(^+\) concentrations and high K\(^+\)/Na\(^+\) ratios in the leaf blade similar to bread wheat (Munns et al., 2000). Line 149 was derived from a cross between *Triticum monococcum* L. (AA) accession C68-101 and the durum cultivar Marrocos (The, 1973). *Triticum monococcum* C68-101, not Marrocos, is the source of the *Nax1* gene in Line 149 (James et al., 2006). Genetic studies indicated that two major loci controlled leaf blade Na\(^+\) accumulation in Line 149 (Munns et al., 2003). A gene named *Nax1* which accounted for 38% of the phenotypic variation for low Na\(^+\) concentration, was mapped to the long arm of chromosome 2A (Lindsay et al., 2004). Physiological studies indicated that net xylem loading and leaf sheath sequestration in Line 149 interacted to control leaf blade Na\(^+\) concentration (Davenport et al., 2005). Using near-isogenic lines, it was found that the major role of *Nax1* in conferring salt tolerance was through greater removal of Na\(^+\) from the xylem in the roots and in the leaf sheath, thereby reducing Na\(^+\) concentrations in the leaf blade (James et al., 2006). Some members of the HKT family (High-affinity K\(^+\) Transporter) function as sodium transporters (Rodríguez-Navarro and Rubio, 2006) and they play an important role in regulation of Na\(^+\) transport in rice and *Arabidopsis* (Ren et al., 2005; Rus et al., 2004). HKT transporters appear important in control of Na\(^+\) transport in bread wheat (Laurie et al., 2002) and may also transport sodium and contribute to salt tolerance in durum wheat.

The rice (*Oryza sativa* L.) genome sequence provides a useful reference for comparative genomics in the cereals (Yu et al., 2002). There are currently over 620,000 wheat ESTs (wESTs) in public databases, of which over 8,200 unique wESTs have been mapped to defined chromosome regions using deletion stocks of wheat (Qi et al., 2004;
Wheat chromosome group 2 contained co-linear regions with rice chromosomes 4 and 7, while the deletion mapping of wheat genes provides a tool to examine co-linearity with rice at the sub-chromosome level (Sorrells et al., 2003). The rice genome sequence has been successfully used as entry point for positional cloning of important agronomic traits in wheat (Griffiths et al., 2006; Yan et al., 2003). It is therefore feasible to use the rice genome sequence and data from wEST deletion bin mapping project to identify additional wheat markers for genetic mapping of \textit{Nax1}, and perhaps identify rice genes with close sequence relatedness to candidate genes for \textit{Nax1}.

The objective of this study was to use wESTs that were previously positioned in the physical deletion bins of wheat chromosome 2AL in conjunction with the rice genome sequence to define a detailed map position and clone a candidate gene for \textit{Nax1}. We provide evidence that a putative sodium transporter (closely related to OsHKT7) is a candidate gene for \textit{Nax1}, which may control Na\textsuperscript{+} unloading from xylem in roots and sheaths as indicated by its expression pattern and physiological role of Na\textsuperscript{+} partitioning into leaf sheaths.

\textbf{RESULTS}

\textbf{Exploiting wheat-rice synteny to identify markers and candidate genes}

\textit{Nax1}, a major gene for low Na\textsuperscript{+} concentration in leaf blades of durum wheat, was mapped as a QTL and linked to the microsatellite marker \textit{gwm312} on chromosome 2AL (Lindsay et al., 2004). Using a backcross-derived mapping family of 41 BC\textsubscript{5}F\textsubscript{2} lines, \textit{Nax1} was determined as a single genetic locus (Fig. 1). In this study, we used wESTs previously mapped to physical deletion bins in ‘Chinese Spring’ wheat as a source of potential markers. The microsatellite \textit{gwm312} marker was mapped to the deletion bin Flow Length (FL) 0.77-0.85 (Fig. 2). The position of \textit{gwm312} define the physical interval for \textit{Nax1} indicating that the gene was located somewhere between the centromere and distal breakpoint FL 0.85 on chromosome 2AL. 74 wESTs which had significant DNA sequence homology to rice genes in the syntenic region of 17.8 to 34.4 Mb on rice chromosome 4L had been mapped into the same physical region (http://wheat.pw.usda.gov/NSF/progress_mapping.html). The putative order for those 74 wESTs was inferred from the corresponding rice gene order and used as basis for developing RFLP markers in durum wheat (Table I). Initially, we selected three wEST markers (A, B and C) for mapping, because the related rice genes spanned the central region of rice chromosome 4L (24.1, 24.2 and 26.9 Mb; see Table I). Both A and B detected polymorphic markers between the donor Line 149 and the recurrent parent Tamaroi. However, only the Tamaroi alleles were present in the BC\textsubscript{4} parental line and in the BC\textsubscript{5}F\textsubscript{2} family suggesting that this chromosomal region was replaced by
Tamaroi during the process of backcrossing (Fig. 2). Marker C with sequence relatedness to a rice gene located at 26.9 Mb segregated in the BC$_3$F$_2$ family and mapped 7.3 cM from Naxl (Fig. 2). To identify additional markers, we therefore focused on wESTs that were closely related to rice genes located distal to 26.9 Mb on chromosome 4L.

Five additional markers (D, E, HAK11, F, SKOR) were developed which corresponded to rice genes located in the distal region of chromosome 4 (28.4 Mb to 32.5 Mb) (Fig. 2). Consistent with the physical location of corresponding rice genes, marker D was mapped as an RFLP proximal to Naxl (6.1 cM), while marker HAK11 co-segregated with Naxl (Fig. 2). Marker F corresponding to a rice gene near the distal end of chromosome 4L was also located on the distal side of Naxl co-segregating with gwm312 (Fig. 2). A break in co-linearity was observed with marker E; this marker co-segregated with gwm312, although its predicted map location was on the proximal side of Naxl. The genetic order of wEST markers was confirmed by their physical location within one of three deletion bins on chromosome 2AL including marker SKOR which was placed into the distal deletion bin (FL 0.85-1.00) consistent with the location of the corresponding rice gene (Fig. 2). These results suggest that an interstitial segment was re-arranged between wheat chromosome 2AL and rice chromosome 4L.

Based on these results, Naxl was located within a 7 cM genetic interval and was flanked by marker D and F. This genetic interval corresponded to a physical interval between 28.4 and 31.3 Mb on rice chromosome 4L. To identify rice genes which may be related to candidate genes for Naxl, the 3 Mb interval (28.4-31.3 Mb) was searched for genes encoding putative potassium or sodium transporters (http://www.gramene.org). Beside OsHAK11, three additional rice genes were identified with homology to putative potassium transporter (AL817940, OsHAK15) and sodium transporters (BE604162, OsHKT7; BJ472463, OsHKT4) in wheat and barley (Table II). A high resolution mapping family was developed to resolve the position of candidate genes relative to Naxl.

**Map position of candidates relative to Naxl**

To produce a high resolution mapping family, tightly linked flanking PCR-based markers were required for screening a large number of F$_2$ lines. To investigate the possibility of marker HAK11 and gwm312 flanking Naxl, we developed a cleavage amplification polymorphism sequence (CAPS) marker from CK205077 (Fig. S1) and screened 100 lines with both markers. Three recombinant F$_2$ individuals were identified and phenotyped for Na$^+$ accumulation. Based on these results, the most likely position for Naxl was in between HAK11 and gwm312. The markers were subsequently used to screen a larger number of F$_2$ lines to identify 22 F$_2$ lines that
incorporated recombination events within the HAK11- gwm312 interval (from a total of 864 F2 lines screened).

The high resolution family of 22 F2 lines were used to separate markers (HAK11 and HAK15) derived from putative potassium transporter genes from Nax1 by recombination, ruling them out as candidate genes (Fig. 3). Furthermore, a probe derived from the barley EST BJ472463 closely related to a putative sodium transporter gene (OsHKT4), failed to hybridise to genomic DNA of T. monococcum C68-101 (AA), the donor of Nax1 in Line 149, using a range of 5 restriction enzymes (EcoRI, EcoRV, HindIII, NcoI and XbaI; see example in Fig. 4). This result indicated that A genome of Line 149 had no HKT4-like gene. However, this probe hybridised to at least one and two gene members in tetraploid and hexaploid wheats indicating that the B and D genome could have HKT4-like members (Fig. 4).

Marker HKT7 co-segregated with Nax1 in the high resolution mapping family (Fig. 3) suggesting that a HKT7-like gene is a strong candidate for Nax1. The HKT7 probe hybridised to at least two putative gene members in T. monococcum C68-101 (Fig. 5). Line 149 contained both RFLP markers, but only one (HKT7-A1) was polymorphic between Line 149 and Tamaroi and co-segregated with Nax1. The second marker (HKT7-A2) was monomorphic between parents with a range of restriction enzymes but was present in the same deletion bin (FL 0.27-0.77) as HKT7-A1 (Fig. 5). It is possible that the marker HKT7-A2 was part of another candidate gene for Nax1. The HKT7 probe also hybridised to at least 4 bands in tetraploid (AABB) and six bands in hexaploid (AABBDD) wheat, suggesting that the B and D genomes also carry 2 copies of HKT7-like genes, respectively (Fig. 5).

**Inversion of interstitial region on chromosome 2AL**

The genetic order of HAK11, HAK15, HKT7, E and F was supported by their physical positions in deletion bins on chromosome 2AL (Fig. 3). The three proximal markers HAK11, HAK15 and HKT7 were also located in the proximal deletion bin FL 0.27-0.77, while marker E and F from the distal part of the map were present in the distal deletion bin FL 0.77-0.85 (Fig. 3). The physical order of rice genes corresponding to HAK11, HAK15, HKT7 and E was inverted suggesting that the chromosomal segment between 29.4 and 30.9 Mb was re-arranged between wheat and rice. The D and F markers corresponding to rice genes located at 28.4 and 31.3 Mb respectively were predicted to flank this interstitial inversion event (Fig. 3).
Isolation of full length HKT7-like candidate genes

A T. monococcum DV92 BAC library (Lijavetzky et al., 1999) was screened using wEST BE604162 as probe to isolate full-length sequences corresponding to both TmHKT7-A1 and -A2 gene members. The T. monococcum DV92 had the same low Na⁺ concentration in leaf blades as T. monococcum C68-101 (data not shown). Nine positive BAC clones were isolated and separated into two groups using a TmHKT7-A1 intron specific probe (see Materials and Methods). Similar fingerprints following digestion with HindIII suggested that BAC clones containing TmHKT7-A1 and TmHKT7-A2 were overlapping (Fig. S2). The approximate physical distance between TmHKT7-A1 and TmHKT7-A2 was less than 145 kb based on the estimation of sizes of BAC clone inserts and overlapping fragments (Fig. S2). Two open reading frames (ORF) corresponding to TmHKT7-A1 and TmHKT7-A2 were identified by direct DV92 BAC clone sequencing. The predicted ORF (1692 bp) of TmHKT7-A1 with two introns shared 88% identity with the predicted ORF of TmHKT7-A2 (1665 bp) which contained only one intron (Fig. 6). This result was confirmed by the isolation of full-length cDNA of TmHKT7-A2 from T. monococcum C68-101 using RT-PCR. Furthermore, the sequences of TmHKT7-A1 and TmHKT7-A2 amplified from T. monococcum C68-101 and Line 149 were 100% identical to those from DV92. At amino acid sequence level, the TmHKT7-A1 and TmHKT7-A2 were 70% and 72% identical to OsHKT7, respectively (Fig. 7). Further amino acid sequence comparisons revealed that TmHKT7-A2 had nine fewer amino acids than TmHKT7-A1 while the OsHKT7 sequence was shorter by 46 amino acids at the N terminus (Fig. 7). A filter serine in P-loop A was present in all three HKT7 transporters (Fig. 7), indicating that they may function as a Na⁺ transporter (Mäser et al., 2002). Furthermore, TmHKT7-A1 and A2 shared very similar topological structure except in the N-terminal hydrophilic region (Fig. S3). Compared with TmHKT7-A1 and A2, OsHKT7 lacked the N-terminal hydrophilic tail and had a slight difference in topological structure (Fig. S3).

Expression of TmHKT7-A1 and TmHKT7-A2

Using gene specific primers that were flanking introns for RT-PCR analysis, no cDNA product was detected corresponding to TmHKT7-A1 in roots, leaf sheaths or blades of T. monococcum C68-101, Line 149 or Tamaroi (Fig. 8). This result was confirmed by another pair of specific primers spanning a large intron region (data not shown). However, for TmHKT7-A2 the expected cDNA product was detected in roots and leaf sheaths of T. monococcum C68-101 and Line 149 but not in Tamaroi (Fig. 8). TmHKT7-A2 was not expressed in leaf blades of T. monococcum C68-101 or Line 149, consistent with the physiological role of Nax1 in reducing the
Na⁺ concentration in blades by retaining Na⁺ in the sheaths (James et al., 2006). Therefore, *TmHKT7-A2* is proposed to be the candidate gene for *Nax1*.

**DISCUSSION**

We used the rice genome sequence and wESTs mapped in deletion bins to identify markers that assisted in the detailed mapping of *Nax1*. Comparative mapping results showed that the *Nax1* region on wheat chromosome 2AL showed a high level of gene order co-linearity with rice chromosome 4L (Fig. 2) and that the rice sequence was useful in identifying candidate gene(s) for *Nax1*. In another study, good co-linearity was found for at least 12 genes in the region containing the vernalization gene *Vrn-A1* on chromosome 5AL and the syntenic rice chromosome 3 (Yan et al., 2003). However, co-linearity may be interrupted, as observed here by an inversion between wheat 2AL and rice 4L, and as reported previously (Brunner et al., 2003; Guyot et al., 2004). Therefore, the success of map-based gene cloning in wheat using a syntenic rice chromosome as reference is dependent on the particular chromosome location of the target gene.

We have developed co-dominant wEST RFLP markers between Line 149 and Tamaroi for mapping. In all cases, the polymorphic band in Line 149 was the same size as a band in *T. monococcum* C68-101, while the allelic band in Tamaroi was the same size as the corresponding band in hexaploid Chinese Spring (see an example in Fig. 5). These results were consistent with our hypothesis that chromosome segment of A genome in Line 149 originate from *T. monococcum* C68-101 (The, 1973). The A genome in Tamaroi may be more closely related to the A genome in *T. urartu* (AA). Other studies have found A genome specific markers from tetraploid wheat that were not in *T. monococcum* but were present in *T. urartu* (Khlestkina and Salina, 2001). The A genomes of tetraploid (AABB) and hexaploid wheat (AABBDD) may share a common ancestor, and *T. urartu* is considered to be the closest diploid ancestor surviving today (Dvořák et al., 1988).

The individual *HKT* gene member in wheat appears to be different from that reported in rice (Garcia-deblás et al., 2003). The gene corresponding to *OsHKT4* was absent in the A genome but is likely to be present in the B and D genome of wheat (Fig. 4). In rice, *OsHKT4* is mainly expressed in shoots (Garcia-deblás et al., 2003). There is one copy of *OsHKT7* present in the rice genome, but two copies of *HKT7*-like member in each genome of wheat (Fig. 5). *TmHKT7-A2* is unique as it only contains one intron (Fig. 6), while all HKT genes from rice and *Arabidopsis* have two introns (Garcia-deblás et al., 2003; Uozumi et al., 2000). The fact that two *HKT7*-like genes with different numbers of intron are located close to each other may indicate that they diversified following duplication to have different functions. *TmHKT7-A2* was identified as a candidate gene for *Nax1*, because it is expressed in root and leaf sheaths but not in leaf blades (Fig. 8). In rice, *OsHKT7* was
mainly expressed in shoots (Garciadeblás et al., 2003). A barley HKT7-like gene (BQ739876) was also expressed in the leaves of drought-stressed plants (Ozturk et al., 2002). The wEST BE604162 matching OsHKT7 was isolated from a drought stressed wheat leaf cDNA library indicating it was also expressed in the leaf tissues (NCBI database). BE604162 could belong to a HKT7 member from the B genome in wheat, because the partial nucleotide sequence amplified from Line 149 and Tamaroi were 100% identical to BE604162 (data not shown). It would be interesting to investigate any expression of other HKT7-like genes in roots like TmHKT7-A2 (Fig. 8).

Other genes belonging to the HKT family have been studied in wheat. TaHKT1 was the first HKT gene cloned from higher plants, showing expression in cortical cells (Schachtman et al., 1994). The down-regulation (by an antisense construct) of HKT1 in wheat increased shoot fresh weight by 50-100% in 200 mM NaCl under conditions of K+ deficiency (Laurie et al., 2002). Following the down-regulation of HKT1, transgenic wheat had smaller Na+-induced depolarisations in root cortical cells than the control and low 22Na+ influx, indicating HKT1 in wheat mediated Na+ influx (Laurie et al., 2002). Further evidence using a root uptake system and a yeast transformation system also supported that TaHKT1 and HvHKT1 (91% identity at amino acid level) functioned as a Na+ uniport (Haro et al., 2005). In Arabidopsis, there is only one sodium transporter AtHKT1 (Uozumi et al., 2000). AtHKT1 plays a critical role in regulation of Na+ homeostasis (Rus et al., 2004), but its mechanism in conferring salt tolerance is still not clear, as shown by contradictory models for Na+ recirculation (Berthomieu et al., 2003; Sunarpi et al., 2005). In rice, Ren et al. (2005) suggested that OsHKT8 contributed to the maintenance of high shoot K+ and low Na+ accumulation under salt stress in a salt tolerant cultivar by controlling the unloading of Na+ from the root xylem. TmHKT7-A2, the best candidate for Nax1, could control Na+ unloading from xylem in roots and sheath of durum wheat. This hypothesis is supported by the expression of TmHKT7-A2 in roots and sheath of Line 149 (salt-tolerant) but not of Tamaroi (salt-sensitive) (Fig. 8) and is consistent with the Na+ concentration gradient along the sheath of near-isogenic lines containing Nax1, indicating a retrieval from the sheath xylem, and the greater retrieval of Na+ from the root xylem (James et al., 2006). The removal of Na+ from the xylem resulted in a nearly four-fold difference in blade Na+ concentration between the low Na+ parental line and Tamaroi (Fig. 1).

In summary, one of two HKT7-like genes (TmHKT7-A2) was identified as a candidate for Nax1. The expression of the TmHKT7-A2 gene in root and leaf sheath tissue of T. monococcum and Line 149 was consistent with the physiological role of Nax1. Functional analysis of TmHKT7-A2 as a sodium transporter using a yeast transformation system is under way. Future work will determine if TmHKT7-A2 is functioning as a sodium transporter in cereals and contributing to salt
tolerance by unloading sodium from the xylem in roots and leaf sheaths and by preventing it from accumulating to toxic concentrations in the blade.

MATERIALS AND METHODS

Plant material and mapping families

To generate a low resolution mapping family, Line 149 (salt-tolerant) was crossed with the Australian durum cultivar Tamaroi (salt-sensitive) and backcrossed to produce a homozygous low Na⁺ BC₄F₃ line that was used as the parent in an additional backcross (James et al., 2006). The difference in leaf 3 Na⁺ concentration between low Na⁺ BC₄F₃ parent line and Tamaroi was nearly four-fold (Fig. 1). In this low resolution mapping family of 41 BC₄F₂ individuals, the segregation of the sodium exclusion trait fitted the expected ratio for a single major gene (Nax1) (Expected 10:21:10; Observed: 11:21:9; χ² =0.22, Pₓ0.05=6.00) (Fig 1). Subsequently, a high resolution mapping family was generated by screening 864 BC₅F₂ half seeds without embryos (equivalent to 1728 gametes) with flanking gwm312 marker and a CAPS marker derived from wEST CK205077 (HAK11) (Fig. S1). Twenty two F₂ lines that contained recombination events in the marker interval gwm312 – HAK11 constituted the high resolution mapping family. At least 8 F₃ individuals from each F₂ plant were phenotyped for Na⁺ concentration to confirm the phenotypic scores obtained at the F₂ generation.

Phenotyping

Plants were grown according to the method of Munns and James (2003). At 8 d after seedling emergence, 25 mM NaCl salt solution was added to the irrigation solution twice daily until a final concentration of 150 mM for low resolution mapping family and 50 mM for high resolution mapping family was reached. There was little difference in shoot Na⁺ concentration between 150 and 50 mM NaCl treatment (Husain et al., 2004). Additional CaCl₂ was added to give a final Na⁺:Ca²⁺ ratio of 15:1. A lower NaCl concentration was used to phenotype the high resolution mapping family, to reduce the stress on seedlings which were half-seed derived and less vigorous. Those half seeds containing embryos with recombination events were treated with the fungicide Thiram (1.4 g/L) and germinated on filter papers with Thiram in sealed sterile dishes at 4°C for 3 days and then at 25°C for 2 days before planting.

Na⁺ concentration in the blade of the third leaf, 10 d after emergence, was measured according to Munns et al., (2000). Leaves were dried at 70°C for 3 d, extracted in 500 mM HNO₃ at 80°C for 1.5 h, and Na⁺ concentration was measured by an Inductively Coupled Plasma-Atomic Emission Spectrometer (Varian Vista Pro, Melbourne, Australia).
DNA extraction

Plants were transplanted from the salt tanks into soil and allowed to grow for approximately 4 weeks before DNA was extracted as described by Lagudah et al. (1991). For selection of recombinants, a half seed extraction method according to Mago et al. (2005) was used to isolate DNA from the BC5F2 lines. Four microliter of extracted DNA was used to perform PCR. The embryo sections of recombinants were subsequently germinated for phenotypic analysis.

Flanking microsatellite and PCR markers

Primer sequences of flanking microsatellite marker gwm312 were described in Röder et al., (1998). Amplifications were performed in 20 µL aliquots containing 1.5 mM MgCl2, 2 µM of each primer, 200 µM dNTPs, 200 µM 1× PCR buffer, 2 units Taq DNA polymerase and 100 ng genomic DNA. The PCR program and gel running conditions were as described by Lindsay et al. (2004). Primer sequences for wEST CK205077 were as follows: Forward primer, 5’ACGTTCAGGAACCTGATTTGT; Reverse primer, 5’GTTAGAAGAATTTCCCCGCCTTC. PCR products amplified from both parents contained different RsaI restriction sites. This polymorphism was used to develop a CAPS marker (Fig. S1).

RFLP markers

DNA from Chinese Spring, 2AL deletion lines (Endo and Gill 1996), T. monococcum accession C68-101, parental lines and F2 lines was digested with six restriction enzymes (DraI, EcoRI, EcoRV, HindIII, NcoI and XbaI). DNA hybridisation analysis was conducted according to Seah et al. (1998).

Comparative analysis of wheat and rice sequences

The region containing the Nax1 locus on wheat chromosome 2AL is syntenic with chromosome 4 of rice (Conley et al., 2004). 347 wESTs previously mapped to the deletion bin 2AL 0.0-0.85 were downloaded from the Graingenes web site (http://wheat.pwusda.gov/NSF/progress_mapping.html). BlastN (E value ≤ 10^{-15} and the length of identity greater than 100 bp) was used to search the NCBI and Gramene databases (http://www.ncbi.nlm.nih.gov/; http://www.gramene.org). 74 of 347 wESTs detected closely related genes between 17.8 Mb and 34.4 Mb of rice chromosome 4L. Table I lists wESTs with close matches to rice genes, which were used to develop RFLP or PCR-based markers.
Cloning and sequencing of wESTs

Primers (Table SI) were designed on the basis of published wheat ESTs listed in Tables I and II. The amplified products were cloned using the pGEM T-Easy Vector system (Promega, USA) and confirmed by sequencing. DNA probes were amplified by PCR and labelled with $^{32}$P using Megaprime DNA Labelling System (Amersham Biosciences, UK). Because there was no matching wheat EST for OsHKT4 (a putative sodium transporter) in the database, a closely related barley EST (BJ472462) was isolated and used as DNA probe.

BAC clone screening and sequencing

High-density filters for the BAC library from T. monococcum accession DV92 (Lijavetzky et al., 1999) were screened with the probe matching wEST BE604162 as shown in Fig 5. Initially, the partial sequences (close to 3’ end) of TmHKT7-A1 and A2 were amplified using primers designed from BE604162. A 153 bp intron was present in TmHKT7-A1 but no intron in this region was present in TmHKT7-A2. Clones containing TmHKT7-A1 were separated by a probe matching TmHKT7-A1 intron. Contigs were assembled by BAC clone fingerprints after digestion with HindIII (Fig. S2). Direct BAC clone sequencing was used to determine full length sequence of TmHKT7-A1 and A2. BAC DNA template was purified with BACMAX DNA purification kit (Epicenter, USA).

RNA extraction and RT-PCR assay

Plants were grown as described in the phenotyping section. RNA from roots, leaf sheaths and leaf blades of 8-d-old plants treated with 50 mM NaCl for 48 h was extracted using Trizol method (Invitrogen, Australia). RT-PCR procedures were performed using OneStep RT-PCR Kit (Qiagen, Australia) under the following condition: 50ºC for 30 min; 95ºC for 15 min; 35 cycle of 95ºC for 30 sec, 58ºC for 30 sec, 72ºC for 50 sec, and then 72ºC for 5 min, 25ºC for 1 min. The specific spanning intron primers to TmHKT7-A1 and A2 for RT-PCR analysis are listed in Table SII.

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**LITERATURE CITED**


Brunner S, Keller B, Feuillet C (2003) A large rearrangement involving genes and low copy DNA interrupts the microlinearity between rice and barley at the \textit{Rph7} locus. Genetics \textbf{164}:673-683


Francois LE, Maas EV, Donovan TJ, Youngs VL (1986) Effect of salinity on grain yield and quality, vegetable growth, and germination of semi-dwarf and durum wheat. Agron J \textbf{78}:1053-1058


filters determine potassium selectivity in four-loop-per-subunit HKT transporters from plants. Proc Natl Acad Sci USA 99:6428-6433


The TT (1973) Transference of resistance to stem rust from Triticum monococcum L. to hexaploid wheat. PhD thesis. University of Sydney, Sydney


**FIGURE CAPTIONS**

**Figure 1.** Frequency distributions for leaf Na⁺ concentrations of BC₅F₂ family, grown at 150 mM NaCl for 10 d. The black and white bars represent homozygous lines with low and high Na⁺ concentration in leaf. The grey bars represent heterozygous lines with medium Na⁺ concentration in leaf. Arrows indicate parental leaf Na⁺ concentration means (µmol g⁻¹DW, n=6). Line 149: 141 ± 14, P₁ (BC₄F₃): 233 ± 39, Tamaroi: 811 ± 31. (From Fig 1A of James et al., 2006).

**Figure 2.** Comparative map of wheat chromosome 2AL and rice chromosome 4 using the low resolution mapping family. **Left:** Physical map of rice chromosome 4 constructed from the sequence annotations of rice genes as shown on Gramene (http://www.gramene.org). The solid line connects non-collinear markers. **Middle:** Genetic map of wheat chromosome 2AL in the low resolution mapping family. The top region (grey highlight) represents Tamaroi chromatin. **Right:** Physical mapping of markers into deletion bins on wheat chromosome 2AL.

**Figure 3.** Comparative map of wheat chromosome 2AL and rice chromosome 4 using the high resolution mapping family. **Left:** Physical map of rice chromosome 4 constructed from the sequence annotations on Gramene (http://www.gramene.org). The solid lines highlight the rearrangement between wheat and rice. **Middle:** Genetic map of Nax1 region using the high resolution mapping family. **Right:** Physical mapping of markers into deletion bins of wheat chromosome 2AL. The broad grey arrow on the left indicates the interstitial inversion event.

**Figure 4.** DNA gel blot hybridised with barley BJ472463 corresponding to OsHKT4. The genomic DNA was digested by HindIII.

**Figure 5.** DNA gel blot hybridised with wEST BE604162 corresponding to OsHKT7. The genomic DNA was digested by EovRV. The arrows on the right side indicate polymorphic allelic bands between Line 149 and Tamaroi which co-segregated with Nax1 in the high resolution mapping family. The polymorphic and monomorphic alleles in A genome between Line 149 and Tamaroi were named as TmHKT7-A1 and TmHKT7-A2, respectively.

**Figure 6.** Gene structures of TmHKT7-A1, A2 and OsHKT7. The grey triangles represent intron region of the gene. The arrows indicate the primers designed for gene expression analysis (Table SII and Fig. 8).
**Figure 7.** The alignment of amino acid sequence from putative sodium transporters of TmHKT7-A1, A2 and OsHKT7. The highlighted black boxes indicate the identical amino acids and the highlighted grey boxes indicate the positive amino acids. The P-loop A regions are covered by an open box. The arrow indicates filter serine in P-loop A.

**Figure 8.** Expression of *TmHKT7*-A1 and A2 in roots, sheaths and blades of *T. monococcum*, Line 149 and Tamaroi using specific intron spanning primers (A1F/A1R and A2F/A2R; see Fig. 5 and Table SII). The expected sizes of genomic DNA (gDNA) and cDNA of *TmHKT7*-A1 are 292 and 138 bp respectively. The expected sizes of gDNA and cDNA of *TmHKT7*-A2 are 2361 and 451 bp respectively. There was no amplification of gDNA of *TmHKT7*-A2 due to spanning a large intron (Fig. 6).
Table I. BlastN search results of selected wESTs located in wheat deletion bin 2AL (0.0-0.85) with rice chromosome 4.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Wheat EST</th>
<th>BlastN</th>
<th>Locus</th>
<th>Distance (Mb)</th>
<th>BlastX</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>BE498441</td>
<td>5E-15</td>
<td>LOC_Os04g41040</td>
<td>24.14</td>
<td>oj991113_30.8 [Oryza sativa (japonica cultivar-group)]</td>
</tr>
<tr>
<td>B</td>
<td>BM137419</td>
<td>5E-27</td>
<td>LOC_Os04g41200</td>
<td>24.21</td>
<td>oj991113_30.22 [Oryza sativa (japonica cultivar-group)]</td>
</tr>
<tr>
<td>C</td>
<td>BF474590</td>
<td>7E-16</td>
<td>LOC_Os04g45800</td>
<td>26.89</td>
<td>putative sphingosine kinase [Oryza sativa]</td>
</tr>
<tr>
<td>D</td>
<td>BE403863</td>
<td>9E-44</td>
<td>LOC_Os04g48130</td>
<td>28.42</td>
<td>membrane protein; protein id: At5g07250.1, supported by cDNA: gi_16648761, [Arabidopsis thaliana]</td>
</tr>
<tr>
<td>E</td>
<td>BG262791</td>
<td>2E-57</td>
<td>LOC_Os04g49570</td>
<td>29.35</td>
<td>ligand-gated ion channel, putative; protein id: At1g42540.1 [Arabidopsis thaliana]</td>
</tr>
<tr>
<td>HAK11</td>
<td>BE423738*</td>
<td>5E-58</td>
<td>LOC_Os04g52390</td>
<td>30.91</td>
<td><strong>putative potassium transporter</strong> [Oryza sativa (japonica cultivar-group)]</td>
</tr>
<tr>
<td>F</td>
<td>BE403217</td>
<td>3E-61</td>
<td>LOC_Os04g52900</td>
<td>31.29</td>
<td>MRP-like ABC transporter [Oryza sativa (japonica cultivar-group)]</td>
</tr>
</tbody>
</table>

* BE423738 and CK205077 matched the same rice gene of putative potassium transporter (OsHAK11). A co-dominant CAPS marker was developed based on sequence of CK205077 (Fig. S1).
Table II. List of candidate genes for *Nax1* on rice chromosome 4L*.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Wheat or Barley EST</th>
<th>E-value</th>
<th>Locus</th>
<th>Distance (Mb)</th>
<th>BlastX</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>HKT4</em></td>
<td>BJ472463</td>
<td>2E-43</td>
<td>LOC_Os04g51820</td>
<td>30.51**</td>
<td>Putative Na⁺ transporter</td>
</tr>
<tr>
<td><em>HKT7</em></td>
<td>BE604162</td>
<td>3E-67</td>
<td>LOC_Os04g51830</td>
<td>30.52</td>
<td>Putative Na⁺ transporter</td>
</tr>
<tr>
<td><em>HAK15</em></td>
<td>AL817940</td>
<td>3E-97</td>
<td>LOC_Os04g52120</td>
<td>30.73</td>
<td>Putative K⁺ transporter</td>
</tr>
<tr>
<td><em>HAK11</em></td>
<td>CK205077</td>
<td>1E-79</td>
<td>LOC_Os04g52390</td>
<td>30.91</td>
<td>Putative K⁺ transporter</td>
</tr>
<tr>
<td><em>SKOR</em></td>
<td>CA498418</td>
<td>2E-99</td>
<td>LOC_Os04g55080</td>
<td>32.53</td>
<td>Cyclic nucleotide and calmodulin-regulated K⁺ channel</td>
</tr>
</tbody>
</table>

* The *HKT* and *HAK* families in rice were systematically named by García-de Blás et al. (2003) and Bañuelos et al. (2002), respectively.

** Based on search results from Gramene database (http://www.gramene.org), *OsHKT4* and *OsHKT7* located side by side separated by ~ 3Kb on chromosome 4.
Chinese Spring

*T. monococcum*

Line 149

Tamaroi
Chinese Spring
2AL 0.85-1.00
2AL 0.77-0.85
2AL 0.27-0.77
*T. monococcum*
Line 149
Tamaroi
**TmHKT7-A1**

- 5' region: 1268bp
- 3' region: 2408bp
- 154bp

**TmHKT7-A2**

- 5' region: 1244bp
- 3' region: 1916bp
- 421bp

**OsHKT7**

- 5' region: 1082bp
- 3' region: 2697bp
- 199bp
genomic DNA (292bp)
A1-cDNA (138bp)
A2-cDNA (451bp)

RNA (EtBr)

T. monococcum
Line 149
Tamaroi