Plant, Cell & Environment

Plant, Cell and Environment (2013) **36**, 1171–1191 doi: 10.1111/pce.12051

Two closely linked tomato HKT coding genes are positional candidates for the major tomato QTL involved in Na^+/K^+ homeostasis

MARIA JOSÉ ASINS[1], IRENE VILLALTA[1], MOHAMED M. ALY[2]*, RAQUEL OLÍAS[2], PAZ ÁLVAREZ DE MORALES[2], RAÚL HUERTAS[2†], JUN LI[2‡], NOELIA JAIME-PÉREZ[2], ROSARIO HARO[3], VERÓNICA RAGA[1], EMILIO A. CARBONELL[1] & ANDRÉS BELVER[2]

[1]*Plant Protection and Biotechnology Center, Instituto Valenciano de Investigaciones Agrarias (IVIA), E46113 Valencia, Spain,* [2]*Department of Biochemistry, Molecular and Cellular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), E-18008 Granada, Spain and* [3]*Center for Plant Biotechnology and Genomics (UPM-INIA), Universidad Politécnica de Madrid, E28223 Madrid, Spain*

ABSTRACT

The location of major quantitative trait loci (QTL) contributing to stem and leaf [Na^+] and [K^+] was previously reported in chromosome 7 using two connected populations of recombinant inbred lines (RILs) of tomato. *HKT1;1* and *HKT1;2*, two tomato Na^+-selective class I-HKT transporters, were found to be closely linked, where the maximum logarithm of odds (LOD) score for these QTLs located. When a chromosome 7 linkage map based on 278 single-nucleotide polymorphisms (SNPs) was used, the maximum LOD score position was only 35 kb from *HKT1;1* and *HKT1;2*. Their expression patterns and phenotypic effects were further investigated in two near-isogenic lines (NILs): 157-14 (double homozygote for the *cheesmaniae* alleles) and 157-17 (double homozygote for the *lycopersicum* alleles). The expression pattern for the *HKT1;1* and *HKT1;2* alleles was complex, possibly because of differences in their promoter sequences. High salinity had very little effect on root dry and fresh weight and consequently on the plant dry weight of NIL 157-14 in comparison with 157-17. A significant difference between NILs was also found for [K^+] and the [Na^+]/[K^+] ratio in leaf and stem but not for [Na^+] arising a disagreement with the corresponding RIL population. Their association with leaf [Na^+] and salt tolerance in tomato is also discussed.

Key-words: *Solanum cheesmaniae*; *Solanum lycopersicum*; candidate gene analysis; HKT1-like genes; K^+ and Na^+ concentration.

Correspondence: A. Belver. Fax: +34 958129600; e-mail: andres.belver@eez.csic.es. M. J. Asins. Fax: +34 963424001; e-mail: mjasins@ivia.es

Andrés Belver and Maria Jose Asins contributed equally to this article.

*Current address: Agricultural Botany Department, Faculty of Agriculture, Cairo University, El-Gamaa St., 12613, Giza, Egypt.

†Current address: Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), C/ Ramiro de Maeztu 9, E-28040 Madrid, Spain.

‡Current address: Instituto de Agrobiotecnología (CSIC/UPNA/Gobierno de Navarra), Mutiloako etorbidea z/g, 31192 Mutiloabeti, Nafarroa, Spain.

INTRODUCTION

Ion homeostasis under salt stress conditions is essential for salt tolerance and involves a network of transport processes that regulates uptake, extrusion through the plasma membrane, compartmentation of salts into cell vacuoles and recirculation of ions through the plant organs, thus facilitating osmotic adjustment and maintenance of high K^+/Na^+ ratios in the cytosol of plants (Apse & Blumwald 2007; Pardo & Rubio 2011). The evidence accumulated indicates that cation transporters such as HKT, SOS1 and NHXs are candidate genes for salt tolerance by regulating internal concentrations of Na^+ in various tissues and also indirectly for K^+ homeostasis. Cation/H^+ antiporters belonging to the NHX family are involved in the accumulation of Na^+ in the vacuole, and their overexpression is used to enhance salt tolerance in different plant species (Apse & Blumwald 2007; Rodríguez-Rosales *et al*. 2009 and references therein). However, there is increasing evidence to show that the NHX antiporters regulate the homeostasis of K^+ and pH in intracellular membranes under normal and saline conditions (Venema *et al*. 2002, 2003; Leidi *et al*. 2010; Bassil *et al*. 2011; Barragán *et al*. 2012). In Arabidopsis and other plants like rice and tomato, the SOS signal transduction pathway is responsible for Na^+ and K^+ homeostasis and salinity tolerance by maintaining favourable K^+/Na^+ ratios in the cytoplasm through the action of the plasma membrane Na^+/H^+ antiporter SOS1 activated by the regulatory kinase complex SOS2-SOS3 (Zhu 2002; Martínez-Atienza *et al*. 2007; Olías *et al*. 2009a). Na^+ efflux carried out by the SOS pathway is not only restricted to the surface of roots but also to redistribution throughout the plant by the xylem loading process (Shi *et al*. 2002; Oh *et al*. 2009; Olías *et al*. 2009a,b; Huertas *et al*. 2012).

In addition to SOS1, HKT transporters from class I (also known as subfamily 1 or HKT1-like transporters) also play an important role in Na^+ and K^+ homeostasis (Rodríguez-Navarro & Rubio 2006; Horie, Hauser & Schroeder 2009). Although the role of HKT transporters is crucial for salt tolerance, this mostly depends on the species involved. HKTs are represented by a number of genes in different species, while the Arabidopsis genome contains a single gene. The

only member in Arabidopsis, *AtHKT1;1*, encodes a Na^+-selective transporter, localized at the plasma membrane of xylem parenchyma cells in roots and leaves (Uozumi *et al*. 2000; Xue *et al*. 2011). This determines salt tolerance in Arabidopsis by moving Na^+ from the xylem vessels to xylem parenchyma cells of leaves and roots and may indirectly promote vacuolar accumulation (Sunarpi *et al*. 2005; Davenport *et al*. 2007; Møller *et al*. 2009; Plett *et al*. 2010). The importance of HKT1-like transporters in relation to salt tolerance has been highlighted in studies where they have been directly associated with quantitative trait loci (QTL) responsible for Na^+ and K^+ concentrations in aerial parts. This is the case for *OsHKT1;5*, responsible for a QTL, *SKC1*, which determines the accumulation of K^+ in shoots over Na^+ in a variety of halotolerant rice (Ren *et al*. 2005). In durum wheat, HKT transporters have been described as candidate genes for major QTLs responsible for salt tolerance, *Nax1* and *Nax2*, which control Na^+ exclusion from leaves (James, Davenport & Munns 2006). The mapping of locus *Nax1* identified gene *TmHKT7-A2*, encoding the HKT1;4 transporter responsible for retrieval of Na^+ from the transpiration stream for storage in the leaf sheath tissue. HKT1;4 showed polymorphic differences between tolerant and sensitive lines (Huang *et al*. 2006; James *et al*. 2011). Although the *TmHKT1;5A* gene, underlying the *Nax2* locus, encoding HKT1;5, plays the same role, it functions primarily in the root tissue (Byrt *et al*. 2007). The inclusion of an ancestral HKT1;5 gene (Nax2) in commercial durum wheat varieties improved yield by 25% in field trials in a saline environment (Munns *et al*. 2012). In contrast with the salt tolerance mechanism based on Na^+ exclusion occurring in monocots, in Arabidopsis, high leaf Na^+ content in two coastal ecotypes (adapted to salinity) was due to reduced expression of an allelic variant of *AtHKT1;1* in roots (Rus *et al*. 2006; Baxter *et al*. 2010).

Wild tomato species, such as *Solanum cheesmaniae* and *S. pimpinellifolium*, have often been regarded as useful sources of salt tolerance genes for transfer to the cultivated tomato (Flowers 2004; Cuartero *et al*. 2006; Villalta *et al*. 2007). Up to now, the development, in production terms, of salt-tolerant varieties using traditional breeding techniques through the introgression of genes from wild species in crop varieties, has had limited or no success (Flowers 2004; Cuartero *et al*. 2006). Research into genes responsible for QTLs that control salt tolerance-related traits is envisaged in order to facilitate breeding for salt-tolerant crops (Asins 2002; Salvi & Tuberosa 2005; Yeo 2007). The candidate approach attempts to bypass cumbersome positional cloning procedures. Although the size of the QTL confidence interval could be a limitation because of the large number of candidate genes in a minimum of 10 cM, the position of the maximum significance for QTL detection might be an accurate indicator of the position of the responsible gene (Price 2006). In addition, the availability of a full-genome sequence is a helpful tool for filtering through genes in an interval, as the examination of annotation can often indicate which genes in the QTL interval might be likely candidates (Hansen, Halkier & Kliebenstein 2008).

In a previous search for candidate genes responsible for QTLs involved in salt tolerance, Villalta *et al*. (2008) studied the involvement of genes encoding for SlSOS1, SlSOS2, SlSOS3, LeNHX1 and LeNHX3 using two populations of recombinant inbred lines (RILs) derived from the cultivated species *S. lycopersicum* cv. Cerasiform and two salt-tolerant lines from the wild species *S. cheesmaniae* and *S. pimpinellifolium*. Only one of these candidates, LeNHX3, appeared to be responsible for a leaf Na^+ concentration QTL on chromosome 1 (*lnc1.1*). None of them mapped where highly significant and contributing QTLs (over 40%) located on chromosome 7 (Villalta *et al*. 2008). Such clusters included the following QTLs: *lkc7.1*, responsible for leaf potassium content; *tn7*, responsible for Na^+ transport to shoot; and *lkn7.1* responsible for the K^+/Na^+ ratio in leaf under saline conditions. Lines with the *S. lycopersicum* allele showed not only lower Na^+ and higher K^+ concentrations in stems and leaves but also a larger reduction in leaf area than lines with the allele of the wild salt-tolerant parent (Villalta *et al*. 2008). This could be explained by the fact that the only sodium leaf sensitivity QTL detected, *nls7.1*, located in this region too. This implies that in spite of an improved K^+/Na^+ ratio for the cultivated species allele at this genomic position, there is an associated larger reduction in the leaf area than for the wild allele; in other words, although the wild-type alleles increase Na^+ concentration, their leaves show higher tolerance. The research carried out on HKTs underlying similar QTLs in Arabidopsis, rice and wheat (Ren *et al*. 2005; Huang *et al*. 2006; Rus *et al*. 2006; Byrt *et al*. 2007) suggests that segregation at HKT1 loci might be responsible for the major QTLs associated with K^+ and Na^+ homeostasis on chromosome 7, as reported in relation to tomato by Villalta *et al*. (2008).

In our study, we identify and map the location of two tomato HKT genes (*HKT1;1* and *HKT1;2*) and their role as candidate genes for new QTL analyses of Na^+ and K^+ concentrations in leaf and stem using the same two populations of RILs previously evaluated for these traits by Villalta *et al*. (2008). The development and evaluation with regard to high-salinity tolerance of two near-isogenic lines (NILs), differing from the allele at both *HKT1;1* and *HKT1;2,* are described. These two NILs were also used in the HKT1 genomic structure (polymorphisms) and expression analyses.

MATERIALS AND METHODS

Isolation of *SlHKT1;1* and *SlHKT1;2* cDNA sequences

A partial *SlHKT1;1* cDNA sequence was first obtained by RT-PCR (Enhanced Avian HS RT-PCR kit, Sigma-Aldrich, Madrid, Spain) using tomato stem total RNA (RNeasy plant mini kit, Qiagen, Hilden, Germany) and specific primers (Supporting Information Table S1) based on the sequence of a potato EST clone (SGN-U294011, BioAtlantech, Canadian Potato Genomic Project, http://www.cpgp.ca), a close homolog of *AtHKT1;1*. Isolation of the full-length *SlHKT1;1* cDNA was carried out using 5 ′(3′) RACE (Smart RACE cDNA amplification kit, Takara Bio Europe/Clontech; Saint-Germain-en-Laye, France) according to the manufacturer's

instructions. The *SlHKT1;2* cDNA sequence was isolated by using a *S. lycopersicum* cv MicroTom EST clone homolog of AtHKT1;1 (clone LEFL1043BF05, Kazusa DNA Research Institute, Chiba, Japan). Finally, the open reading frames of *SlHKT1;1* and *SlHKT1;2* were obtained by RT-PCR using gene-specific primers and total RNA from *S. lycopersicum* cv. Moneymaker, subcloned in the pGEM-T Easy vector (Promega Biotech Iberica, Madrid, Spain), and were fully sequenced.

Candidate gene analysis in the P and C populations of RILs

Two populations of F_8 lines were developed from the salt-sensitive genotype *Solanum lycopersicum* var. Cerasiform as female parent (Villalta *et al*. 2007, 2008). Male parents were two salt-tolerant lines from *S. pimpinellifolium* L. for the P population and *S. cheesmaniae* (L. Riley) Fosberg for the C population. 142 F_8 P lines and 116 F_8 C lines were characterized for several salt stress-related traits under two saline regimes for the QTL analysis described by Villalta *et al*. (2008). They analysed the Na^+ and K^+ concentrations (mmol per kg of dry weight) in leaves (LNC, LKC) and stems (SNC, SKC) and the K^+/Na^+ ratio in leaves (LKN). They also evaluated the amount of total Na^+ content in the aerial part of the plant (TN) as the percentage of the theoretical Na^+ present in the volume of water absorbed by the plant (DLWxLNC + DSWxSNC in relation to the theoretical Na^+ absorbed by the plant, with DLW and DSW being the dried leaf weight and dried stem weight, respectively). This parameter was considered to be an indication of the Na^+ distribution within the plant (leaves and stems versus roots). The percentage of leaf area (LA) reduction (LAcontrol-LAsaline) × 100/LAcontrol) relative to the LNC was taken to be an estimation of sodium leaf sensitivity (NLS; Villalta *et al*. 2008). In our study, these traits, evaluated by Villalta *et al*. (2008), are subjected to QTL analysis using two new linkage maps of chromosome 7: one after genotyping both populations for SCAR (Sequence Characterized Amplified Region) markers corresponding to the candidate genes *HKT1;1* and *HKT1;2* from tomato, and another using 278 single-nucleotide polymorphisms (SNPs) from the SolCAP tomato panel (Illumina BeadChip WG-401–1004; Illumina Netherlands BV, Einhoven, Netherlands), which segregated in the P population of RILs at F_{10} (external genotyping service provided by Fundación Investigación Clínico, Valencia, Spain).

To locate HKT genes, a total of 159 markers genotyped for the P population and 137 markers for the C population were subjected to linkage analysis (Villalta *et al*. 2005, 2007, 2008) using Joinmap 3.0 software for Windows (Van Ooijen & Voorrips 2001). A minimum logarithm of odds (LOD) of 3 was set as a threshold to create linkage groups using a recombination fraction of 0.5 for linkage analysis. Kosambi's mapping function (Kosambi 1944) was used to order the markers and to estimate interval distances. The P population was additionally genotyped for 7720 SNPs from the SolCAP tomato panel and 278 were included in chromosome 7 at LOD ≥10 by using Joinmap 4 software for Windows (Van Ooijen 2006).

QTL analyses were carried out using Interval Mapping (IM) and Multiple QTL Mapping (MQM) procedures in MapQTL® 6 (Van Ooijen 2009) and selecting linkage groups P7 and C7 in the P- and C-RIL populations, respectively. Permutation tests were used to determine the LOD scores corresponding to an overall experiment-wise significance level of 5%. These critical values depended on the trait and the population (1.6–1.7 in the C population and 1.7–1.9 in the P population). When linkage map P7 based on SNPs was used, critical values increased slightly (1.9–2.3 depending on the trait).

Development and phenotyping of NILs differing for alleles *at HKT1;1* and *HKT1;2*

Several NILs were derived from RIL 157 of the C population through self-pollination given that this RIL segregated at F_6 in the genomic region between SSRW356_900 and SSRW244_550 on C7 chromosome containing *HKT1;1* and *HKT1;2*. Plants of the next selfing generation, homozygous for the alternative alleles (*L* and *C* for *S. lycopersicum* and *S. cheesmaniae* alleles, respectively), were chosen to develop *CC* and *LL* NILs at *HKT1;1* and *HKT1;2*. Markers on other genomic regions (C2a, C11 and C4) also segregated at that RIL. After several selfing generations, a pair of highly homogeneous NILs, 157-14 (*CC*) and 157-17 (*LL*), were obtained where most plants were still heterozygote for marker SSRW66_200 on C2a and markers TG43_750, SSR31_130, SSRW306_310, and SSR94_190 on C4. Other NILs derived from RIL 157 targeting the same region on C7 were fixed for different alleles at other regions of chromosomes C4 and C2a, thus enlarging their genetic differences.

Fourteen plants from both NIL 157-14 and NIL 157-17 were genotyped for markers at C7, C2a and C11a (Villalta *et al*. 2007) to select six homogeneous plants from each NIL, differing only at C7. These plants were individually potted on sand and irrigated with 150 mL of half-strength Hoagland solution three times a week for 15 d and grown under controlled temperature and humidity conditions in a greenhouse during the summer of 2011 in Valencia (Spain). Three marker-selected plants per NIL were irrigated with 150 mL of half-strength Hoagland (Hoagland & Arnon 1950) solution plus 68 mM NaCl (6.5 dS m^{-1}, pH 7.5) as control or 145.5 mM NaCl (15 dS m^{-1}, pH 7.5) for 74 d. This high-salinity treatment was carried out in two stages during a period of 1 week. Fruit yield (number of fruits, FN, total fruit weight, TFW and mean fruit weight, MFW) of plants was evaluated for 7 weeks after the first week of the fruit harvest. At the end of the salinity experiment, plants were harvested and evaluated for vegetative traits and their ionomic profile in the root (R), stem (S) and leaves (L) of each plant. Cations were determined using inductively coupled plasma spectrometry in ppm (Varian ICP 720-E, Instrumental Technical Services, Estación Experimental del Zaidín, CSIC, Granada, Spain). Tissue Cl^- concentration (mg L^{-1}) was measured, as described by Gilliam (1971), using a Sherwood chloride analyser (model 926; Sherwood Scientific Ltd, Cambridge, UK).

The vegetative traits were fresh and dry weights (in grams) of plant (PFW, PDW), stem (SFW, SDW), root (RFW, RDW), leaf (LFW, LDW) and the water content of plant (PWC), stem (SWC), root (RWC) and leaf (LWC) as the difference between the corresponding fresh and dry weights. The line (genotype), treatment (salinity level) and interaction effects were studied using analysis of variance (ANOVA) for all evaluated traits and some derivatives such as Na^+/K^+ ratios and differences in cation concentration between aerial (leaves plus stems) and root systems (L + S-R).

cDNA and genomic sequence analyses of *HKT1;1* and *HKT1;2* allelic variants from tomato NILs

Plant genomic DNA was extracted from young leaves using the GenElute™ Plant Genomic DNA Miniprep Kit following the manufacturer's instructions (Sigma-Aldrich, Spain). Total RNA was isolated from plant tissues using the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. Open reading frames (ORFs) and introns of *HKT1;1* and *HKT1;2* allelic variants from NIL157-14 and 157-17 were obtained by RT-PCR from total RNA and by PCR from genomic DNA, respectively, and several sets of primer pairs were designed according to *SlHKT1;1* and *SlHKT1;2* full cDNA sequences (Supporting Information Table S1). 5′-upstream untranslated sequences containing promoter regions of *SlHKT1;1* and *SlHKT1;2* allelic variants were obtained by PCR using several combinations of primer pairs based on the promoter sequences obtained *in silico* by comparing *HKT1* cDNA sequences against the draft of the entire sequence of tomato species *S. lycopersicum* cv. Heinz 1706 (International Tomato Genome Sequencing Consortium, http://solgenomics.net). PCR reactions were performed using genomic DNA from both NILs and the Expand High Fidelity^{PLUS} PCR System, dNTPack (Roche, Spain), or Pfu-Ultra II Fusion HotStart DNA Polymerase (Agilent Technology, Madrid, Spain) following the manufacturer's instructions. For SNP and indel analysis, several independent PCR products and several clones of each allelic variant previously subcloned in the pGEM-T® Easy vector system were fully sequenced. *Cis*-regulatory elements in promoter regions were analysed *in silico* with PLant Cis-Acting regulatory DNA Elements (PLACE; Higo *et al*. 1999) and PlantCARE (Lescot *et al*. 2002) and NSITE-PL (http://linux1.softberry.com) databases and tools. The presence of CpG islands was checked by the CpG Islands Searcher web tool using the program's default settings (Takai & Jones 2002).

Expression analysis of *HKT1;1* and *HKT1;2* of NILs

Seeds from NIL157-14 (*CC*) and NIL157-17 (*LL*) were surface sterilized and incubated in the dark at 4 °C overnight for the stratification stage and germinated on Petri dishes over moistened filter paper at 26 °C in the dark for 3 d. Germinated seeds were cultivated in polystyrene boxes containing quartz sand, watered for 1 week with one-tenth Hoagland nutrient solution and for another 2 weeks with a one-fourth dilution of the same solution. Seedlings with four leaves were then transferred to a 2.5 L pot containing an aerated one-fourth Hoagland nutrient solution and cultivated in an environmentally controlled chamber at 24 °C/18 °C, day/night and a 16-h light/8-h dark cycle with irradiation of 140 μmol m^{-2} s^{-1} and 40–50% relative humidity. After 10 d of hydroponic cultivation, salt treatment was carried out by adding 100 mM NaCl, 50 mM initially and another 50 mM after 3 h for 10 additional days in order to prevent osmotic shock to the nutrient solution.

Tissue samples were collected at day 0, 1, 3 and 10 after treatment with 100 mM NaCl in hydroponic cultures. Three pots with three plants per NIL (NIL157-14 and NIL157-17) and per day of treatment were used for the analysis (three independent biological samples). Total RNA was isolated from root, stem and leaf tissues using the RNeasy plant mini kit, which included an in-column treatment with RNAse-free DNase (Qiagen), and resuspension in RNAsecure™ resuspension solution (Ambion Europa Ltd, Austin, TX, USA) according to the respective manufacturer's instructions. First-strand cDNA synthesis from 1 μg of total RNA was performed with iScript™ Reverse T Supermix for RT-qPCR (Bio-Rad, Hercules; CA, USA) according to the supplier's protocol using the oligo-dT and random hexamer primer blend provided. Quantitative real-time RT-qPCR was carried out as described by Huertas *et al*. (2012) using 1 μL of undiluted cDNA mixed with iQ SyBr Green Supermix (Bio-Rad) and 0.45 μM of forward and reverse primers (Supporting Information Table S2) in a Bio-Rad iCycler MyiQ2. Serial dilutions of cDNA were used to obtain a standard curve to optimize amplification efficiency (Supporting Information Table S2). All reactions were performed in triplicate. No template controls were included. The specificity of RT-qPCR amplification was confirmed by the presence of a single peak in the melting temperature curve analysis and a single band on an agarose gel. Relative expression data were calculated from the difference in the threshold cycle (ΔC_t) between the genes studied and DNA amplified by specific primers for the tomato Elongation Factor 1α (*LeEF1-α*, acc. AB061263) as a housekeeping gene. *LeEF1-α* expression was very stable and did not vary between plant lines and treatments (Supporting Information Fig. S1). The relative expression level was calculated with the aid of the equation $2EXP[\Delta\Delta C_t]$ (Livak & Schmittgen 2001) using the expression level of each gene in roots from NIL157-17 at day 0 of NaCl treatment as the calibrator sample.

Cation uptake experiments in yeast cells

For yeast expression, the open reading frames of *SlHKT1;1* and *SlHKT1;2* were cloned as a *XbaI-KpnI* fragment amplified by PCR (Supporting Information Table S1) in the yeast shuttle vector pYPGE15 under the *PGK1* promoter (Brunelli & Pall 1993). The yeast strain WΔ6 (*Mat* a *ade2 ura3 trp1 trk1*Δ::*LEU2 trk2*Δ::*HIS3*), deficient in the endogenous K^+ uptake systems TRK1 and TRK2, was used for functional

complementation and transport assays (Haro & Rodríguez-Navarro 2003). For yeast growth experiments at low K^+ concentrations, serial dilution drops of yeast cells were inoculated on arginine phosphate (AP) medium (Rodríguez-Navarro & Ramos 1984) supplemented with different K^+ concentrations. For Na^+ uptake experiments in yeast cells, K^+-starved cells were previously obtained by transferring actively growing cells in 50 mM K^+ AP medium to K^+-free AP medium and then incubating them for 4 h. The K^+-starved cells were suspended in testing buffer (10 mM MES-Ca^{2+}, pH 6.0) supplemented with 2% glucose, and after the addition of Na^+, samples were taken at intervals. Na^+ uptake was carried out using the depletion procedure (Haro & Rodríguez-Navarro 2003; Haro *et al*. 2005). Na^+ was determined by atomic emission spectrophotometry. All experiments were repeated several times.

Sequence data in this article can be found in the EMBL/GenBank data libraries under accession numbers HE962483, HE962484, HE962485 and HE962486.

RESULTS

Isolation of *SlHKT1;1* and *SlHKT1;2* cDNA sequences and functional analysis in yeast

SlHKT1;1 and *SlHKT1;2* complete ORFs from *S. lycopersicum* cv. Moneymaker were 47% identical in terms of their amino acid sequence (Fig. 1, Supporting Information Table S3). In order to investigate structural differences, the deduced amino acid sequences of tomato HKT1;1 and HKT1;2 were aligned with the AtHKT1;1 sequence, both of which were 47% identical (Fig. 1, Supporting Information Table S3). The positions of transmembrane and pore segments were predicted according to the model proposed for the AtHKT1;1 topology (Durell & Guy 1999; Kato *et al*. 2001) based on the four-MPM structural model (transmembrane segment, pore, transmembrane segment, Fig. 1). The alignment of the two HKT1 tomato isoforms with other known amino acid sequences of HKT from plants revealed that both are similar to those of *Messembryanthemum*, *Suaeda*, *Eucalyptus*, *Arabidopsis*, *Vitis* and other genera, showing sequence identities ranging from 62 to 48% (Supporting Information Table S3). All belong to class I HKT transporters, typical of dicotyledonous plants (Fig. 2), whose members are characterized by the presence of a Ser rather than a Gly at the corresponding position in the first P-loop region (P_A on Fig. 1).

In order to assess their ion specificity and transport mode, SlHKT1;1 and SlHKT1;2 were expressed heterologously in the WΔ6 yeast mutant lacking major endogenous K^+ transporters (TRK1 and TRK2). Both isoforms were unable to complement the growth of the yeast mutant at a low K^+ (Supporting Information Fig. S2). However, yeast cells expressing SlHKT1;1 were able to deplete external Na^+. This Na^+ depletion was unaffected by the presence of K^+, thus indicating that SlHKT1;1 was a Na^+-selective transporter (Fig. 3). No transport activity for Na^+ or K^+ was detected for SlHKT1;2 (results not shown).

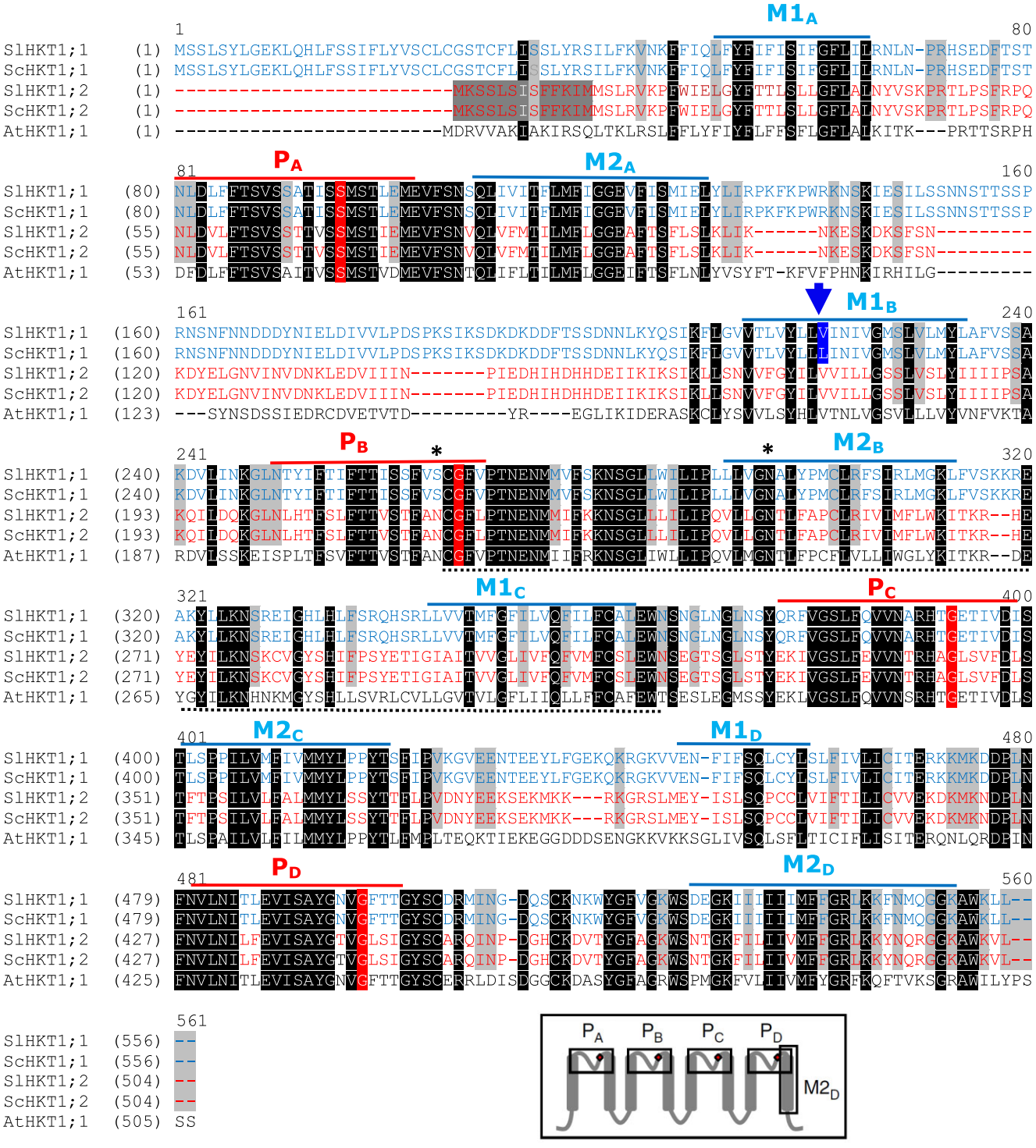
Genetic location of *SlHKT1;1* and *SlHKT1;2* in chromosome 7 in C and P populations. QTL analyses

Tomato *HKT1;1* and *HKT1;2* markers co-localized in linkage groups P7 and C7 at LOD ≥4.0 and 6.0, respectively (Fig. 4). No recombination between both genes was observed in either RIL population. The MQM procedure and cofactors automatically selected by the software showed that the position of tomato *HKT1;1* and *HKT1;2* corresponded to the position of the maximum LOD scores of QTLs for traits SNC, LNC, LKC, LKN, SKC, TN and NLS under salinity conditions and LNC, SNC and LKN under control conditions in the P population (Table 1 and Fig. 5), and for traits SNC, LNC, LKC, LKN, SKC, TN and NLS under salinity conditions in the C population (Table 1 and Fig. 6). Na^+- and K^+-related traits were not evaluated in this population under control conditions. With respect to the gene effects under salinity conditions, the wild *HKT1* alleles (*pimpinellifolium* and *cheesmaniae*) are associated with higher values (negative additive values in Table 1) of TN, LNC and SNC, while the *lycopersicum* allele is associated with higher values of LKC, SKC, LKN and NLS. In the P population under control conditions (no salinity), higher values for LNC and SNC are again associated with the wild *HKT1* allele.

To test the physical proximity of tomato *HKT1;1* and *HKT1;2* to the position of the maximum LOD scores of the above mentioned QTLs, the linkage map of chromosome 7 based on 278 SolCAP SNPs was used for QTL analysis (MQM methodology) of Na^+- and K^+-related traits. The main results for significant and adjacent markers are shown in Table 2. Only three SNPs were significant and the maximum LOD scores were located at Solcap_snp_sl_57007 (Cytochrome P450; 40.805 cM, 5 056 490 bp) for LNC, LKC, LKN, SNC, SKC, TN and NLS under salinity and LKN and SNC under control conditions. LNC under control conditions showed maximum LOD at the other two SNPs, co-located at 40.271 cM: Solcap_snp_sl_57002 (ADP-ribosylation factor, 5 035 786 bp) and Solcap_snp_sl_56997 (Isoamylase isoform 1, 4 925 909 bp). Tomato *HKT1* genes are only 35 kb from Solcap_snp_sl_57007, since *HKT1;2* is located at a chromosome position of 5 091 851 bp and *HKT1;1* at 5 103 988 bp (called Solyc07g014680.2.1 and Solyc07g014690.2.1, respectively, in the sequenced tomato genome of cv. Heinz 1709, according to ITAG2.40, http://solgenomics.net/, Table 3).

Sequence analyses of *HKT1;1* and *HKT1;2* alleles of tomato NILs

The ORFs of *HKT1;1* and *HKT1;2* allelic variants in NIL157-17 (homozygote for the *lycopersicum* alleles at the genomic region containing *HKT1;1* and *HKT1;2*) and NIL157-14 (homozygote for the *cheesmaniae* alleles at the same genomic region containing *HKT1;1* and *HKT1;2*) were cloned and sequenced. The sequence analysis revealed that the amino acid sequences of SlHKT1;2 (NIL157-17) and ScHKT1;2 (NIL157-14) were identical. The sequence analysis of *ScHKT1;1* in NIL157-14 showed a single SNP (G658C)



causing a single substitution in the predicted amino acid sequence (V222L) in the $M1_B$ helix region as compared with the SlHKT1;1 in NIL157-17 (Fig. 1).

Each *HKT;1* and *HKT1;2* genomic sequence contained two introns, as predicted for all members of HKT1-like transporters from dicotyledonous plants (Platten *et al.* 2006). The first intron of the tomato *HKT1;1* genomic sequence is almost identical to the second, 626 bp and 691 bp, respectively, while the length of the first intron of tomato *HKT1;2*, was much longer than the second, 3795 bp and 363 bp, respectively. An additional putative intron is wrongly predicted by ITAG2.40 (http://solgenomics.net/) for the genomic sequence of *SlHKT1;2* (Solyc07g014680.2.1; Fig. 1). The data provided in our study demonstrates that this putative intron encodes a

Figure 1. Alignment of SlHKT1;1 amino acid sequence with SlHKT1;2. Sequences were aligned using Clustal W program (Thompson, Higgins & Gibson 1994). Identical residues in all sequences are highlighted in black. Identical residues in tomato HKT sequences are highlighted in light grey. Blue arrowhead indicates the position of residue substitution of Val in the HKT1;1 sequence from the *S. lycopersicum* allele (NIL157-17) by Leu in the HKT1;1 allele introgressed from *S. cheesmaniae* (NIL157-14; both highlighted in blue). Amino acid sequence of SlHKT1;2 (NIL157-17) and ScHKT1;2 (NIL157-14) are identical. Positions of transmembrane and pore segments were predicted according to the model proposed for the topology of the AtHKT1 protein, based on the four-MPM structural model (transmembrane segment, pore, transmembrane segment inset; Durell *et al*. 1999; Kato *et al*. 2001). The conserved Gly residues in the K^+ channel selectivity filter GYG of the P-loop-like domains (highlighted in red) determine the K^+ selectivity of HKT transporters (Mäser *et al*. 2002). The presence of Gly in the P_A-loop is conserved in K^+ permeable HKT transporters (class II), while the presence of Ser instead of Gly is conserved in Na^+ permeable HKT transporters (class I) (Mäser *et al*. 2002; Platten *et al*. 2006). For tomato HKT1;2 sequences (Solyc07g014680.2), location of a putative 5′UTR is highlighted in dark gray and an additional putative intron is indicated by a dotted line underneath, both predicted by ITAG 2.40 (http://solgenomics.net/). Asterisks define positions of Asp residues (D) shown to be essential determinants for K^+ transport activity in TsHKT1;2 (Ali *et al*. 2012).

region highly homolog to that of SlHKT1;1 and AtHKT1;1 amino acid sequences, spanning parts of the pore B and $M2_B$ and $M1_C$ transmembrane domains (Durell & Guy 1999; Kato *et al*. 2001). In *ScHKT1;1* (NIL157-14), the nucleotide sequence of intron 1 was 2 bp shorter than that of *SlHKT1;1* (NIL157-17) and displayed three SNPs compared with the later sequence, while intron 2 displayed only one SNP but identical in size (Supporting Information Fig. S3). Intron 2 of *HKT1;2* was the same size in all allelic variants and showed no polymorphism (not shown). Isolation of the longest intron 1 of *HKT1;2* was unsuccessful probably due to difficulties in PCR amplification. Intron sequences of *S. lycopersicum* Cv. Heinz *HKT1;1* were used as reference (http://solgenomics.net/).

The upstream 5′ untranslated sequences containing the promoter regions of the allelic variants of *HKT;1* and *HKT1;2* were isolated and identified by PCR amplification. Only the first 1.319 kb for *HKT1;1* (Fig. 7) and the 814 bp for *HKT1;2* (Fig. 8) upstream sequences from the translation start codon from both tomato NILs 157-14 and 157-17 were cloned and sequenced. No CpG islands were predicted for any promoters of *HKT1;1* or *HKT1;2* allelic variants. This may suggest that the possible regulation mechanism of both genes is not associated with epigenetic regulation. In addition to a great number of common *cis*-acting elements in promoter and enhancer regions, such as TATA and CAAT boxes, and polyadenylation signals, such as POLASIG boxes, a search for *cis*-elements identified sequences putatively related to tissue-specific, light-, hormone- and circadian-regulated expression as well as to several known stress-responsive elements, including defence and anaerobic induction elements, the MYB-binding site involved in drought induction, heat stress-responsive and hypo-osmolarity-responsive elements (Supporting Information Tables S4 and S5). The promoter regions of *ScHKT1;1* and *SlHKT1;1* revealed more polymorphisms (including SNPs and indels) than promoters of *HKT1;2* alleles (Figs 7 & 8). Consequently, differences in the promoter sequence of *ScHKT1;1*/*SlHKT1;1* affected more predicted *cis*-elements than those of *ScHKT1;2*/*SlHKT1;2* (Tables 4 and 5).

Salt tolerance phenotypes and gene expression of *HKT1;1* and *HKT1;2* of NILs 157-17 and 157-14

NILs 157-14 (*CC*) and 157-17 (*LL*) differed significantly in terms of all vegetative traits and total fruit weight (TFW), with 157-17 being considerably more vigorous and slightly more productive than 157-14 (Supporting Information Table S6). High salinity significantly affected all vegetative traits and mean fruit weight. Both NILs behaved in a similar fashion when compared under control and high-salinity conditions by increasing mean fruit weight and decreasing all growth and water content parameters except for root growth-related traits, which showed significant NIL × Treatment interactions. High salinity affected RDW and RFW only slightly and consequently PDW of NIL 157-14 in comparison with 157-17 (Table 6). 157-14 could therefore be regarded as tolerant to high salinity.

With regard to Na^+-K^+ homeostasis (Table 6), a significant difference between NILs was found for K^+ concentration and the Na^+/K^+ ratio in all tissues but not for Na^+ concentration. Root Na^+ concentration was only significant in NIL 17 when comparing control and high salinity. Leaf Na^+/K^+ was significantly higher in NIL14 than in NIL17 under high salinity (Table 6). High salinity significantly increased Na^+ concentration and the Na^+/K^+ ratio in all tissues and markedly reduced K^+ concentration in leaves. It is worth noting that both lines behaved differently (significant NIL × Treatment interaction) in relation to root K^+ and Na^+ concentration. Although NILs did not significantly differ in terms of leaf and stem Na^+ concentrations, NIL 157-17 sharply increased root Na^+ while NIL 157-14 did not do so. Both NILs differed in terms of root K^+ under control (low salinity) conditions, with the *cheesmaniae* allele recording a higher level. Nevertheless, as in the QTL analysis (with the whole RIL population), the *cheesmaniae* allele in the NIL is associated with lower K^+ concentration than the *lycopersicum* allele in the aerial parts of the plant (leaf and stem). Both NILs also clearly differed in terms of their K^+ capacity to translocate to the aerial part (K_S + L-R). 157-17 (*LL*) showed a high level of K^+ translocation capacity but a low level for P, particularly under high-salinity conditions (Supporting Information Table S6).

To further test whether there is a connection between the accumulation of Na^+ and K^+ and the tomato HKT1 genes, their expression patterns were analysed in different tissues of tomato NILs. A preliminary RT-PCR analysis indicated that *SlHKT1;1* and *SlHKT1;2* were ubiquitously expressed in all tissues analysed from cultivated tomato (leaves, stems, roots, flowers and fruits) grown under normal conditions (Supporting Information Fig. S4). RT-qPCR analysis revealed a complex pattern of expression for tomato *HKT1;1* and

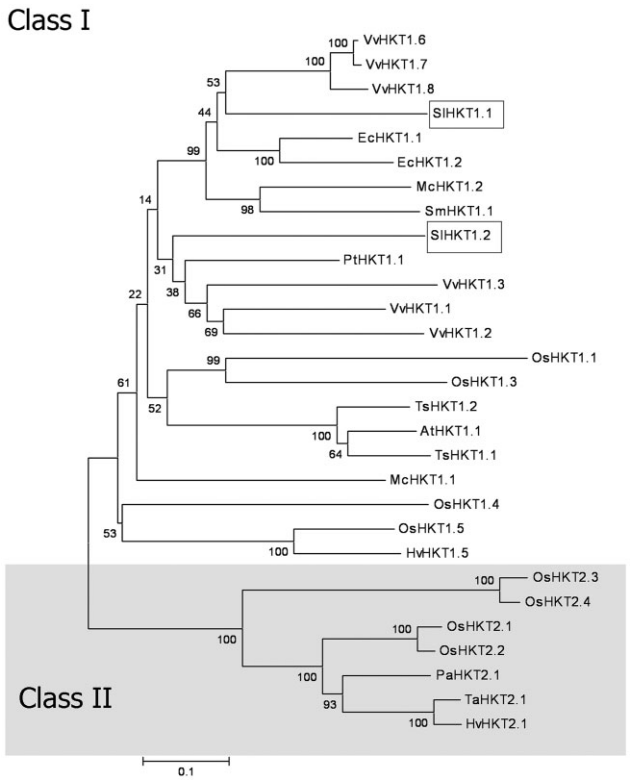


Figure 2. Phylogenetic relationship between *S. lycopersicum* HKT1-like proteins and other plant HKT transporters. Unrooted minimum-evolution phylogenetic tree was constructed using full polypeptide sequences aligned with Clustal W program (Thompson *et al*. 1994) with 10 000 bootstrap replicates, using MEGA4 software (Tamura *et al*. 2007). The scale bar corresponds to a distance of 0.1 substitutions per site. Bootstrap values are indicated adjacent to the corresponding node. The protein accession numbers as listed in the GeneBank database are: AtHKT1;1 NP_567354; EcHKT1;1 AAF97728; EcHKT1;2 AAD53890; HvHKT1;5 ABK58096; HvHKT2;1 CAJ01326; McHKT1;1 AAK52962; McHKT1;2 AAO73474; OsHKT1;1 CAD37183; OsHKT1;3 CAD37185; OsHKT1;4 CAD37197; OsHKT1;5 BAB93392; OsHKT2;1 BAB61789; OsHKT2;2 BAB61791; OsHKT2;3 CAD37187; OsHKT2;4 CAD37199; PaHKT2;1 BAE44385; PtHKT1;1 XP_002325229.1; SmHKT1;1 AAS20529.2; TaHKT2;1 AAA52749; TsHKT1;1 JQ063120; TsHKT1;2 BAJ34563; VvHKT1;1 CAO64083; VvHKT1;2 CAO64075; VvHKT1;3, CAO64081; VvHKT1;6 CAO64069; VvHKT1;7, CAO6407; VvHKT1;8, CAO64071. At, *Arabidopsis thaliana*; Ec, *Eucalyptus camaldulensis*; Hv, *Hordeum vulgare*; Mc, *Mesembryanthemum crystallinum*; Os, *Oryza sativa*; Pa, *Phragmites australis*; Pp, *Physcomitrella patens*; Pt, *Populus trichocarpa*; Sl, *Solanum lycopersicum*; Sm, *Suaeda maritima*; Ta, *Triticum aestivum*; *Ts*, *Thellungiella salsuginea* (or *Eutrema salsugineum*), Vv, *Vitis vinifera*.

HKT1;2 that was essentially tissue and NIL dependent and in some cases treatment dependent (Fig. 9). Salinity clearly increased the level of *SlHKT1;2* transcripts in the roots of NIL157-17, while in NIL157-14, the expression of *ScHKT1;2* was induced only 10 d after beginning salt treatment. In leaves, SlHKT1*;2* expression was reduced by salt treatment. Similar behaviour was observed for *ScHKT1;2* (transcript levels decreased 1 d after treatment). The transcript levels of *HKT1;2* in the roots of NIL157-17 were considerably higher than those of *HKT1;2* from NIL157-14. The expression of the *lycopersicum* allele in leaves was negligible, while the *cheesmaniae* allele was expressed at levels similar to those in roots. In stems, expression of both *HKT1;2* alleles was approximately 1/4 the level of the calibrator, and no difference in relation to the NIL was observed. With respect to *HKT1;1*, the general trend was a decrease in expression during saline treatment in stems and leaves in both NILs. In stems, expression of both *HKT1;1* alleles showed an analogous pattern, with a level similar to that for the calibrator, although it decreased by half with salt treatment. The expression level of *HKT1;1* in NIL157-14 was much higher in roots, and particularly in leaves, than that of *HKT1;1* in NIL157-17 at any day of salt treatment, reaching about 20 and 25 times, respectively, the expression level of the calibrator sample (*lycopersicum* allele in NIL157-17 roots at day 0). However, it decreased during salt treatment in roots. In relation to the

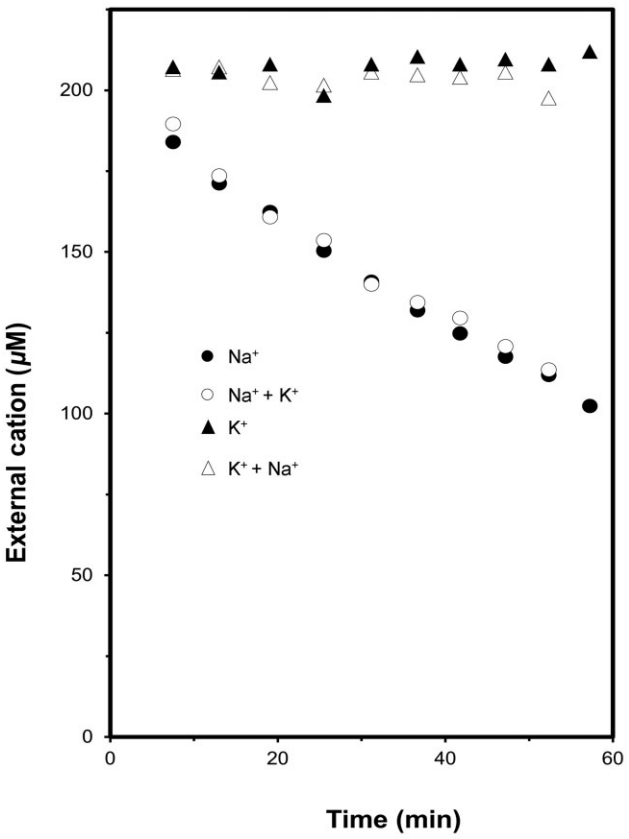


Figure 3. Time course of Na^+ and K^+ depletion in WΔ6 yeast cells expressing *SlHKT1;1*. K^+-starved cells were suspended in testing buffer (10 mM MES-Ca^{2+}, pH 6.0) supplemented by 2% glucose and, after the addition of NaCl (200 μM) or KCl (200 μM), samples were taken at intervals. Depletion of K^+ and Na^+ was determined by atomic emission spectrophotometry. The changes in Na^+ concentration (circles) in the absence (closed circles) or presence of K^+ (open circles) and changes in K^+ concentration in the absence (closed triangles) or presence of Na^+ (open triangles) were recorded. All experiments were repeated several times.

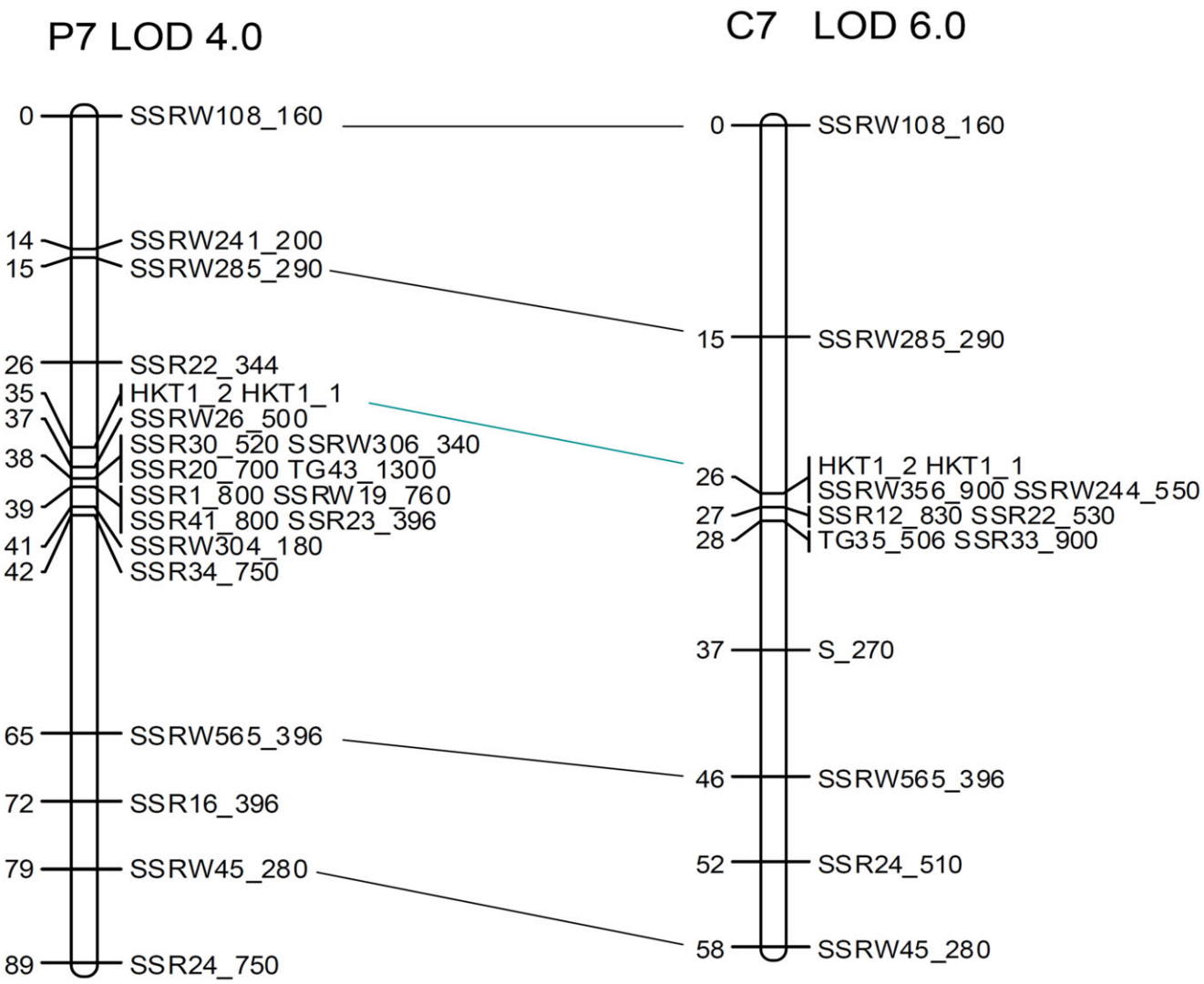


Figure 4. Linkage maps of chromosome 7 obtained from P and C populations of RILs. Tomato *HKT1;1* and *HKT1;2* markers located at chromosome 7 when using a total of 159 markers genotyped for the P population and 137 for the C population at LOD ≤ 4.0 and 6.0, respectively.

two HKT1 isoforms, *HKT1;2* was always considerably more expressed than *HKT1;1*, irrespective of treatment time, NIL and tissue (Supporting Information Fig. S5).

DISCUSSION

SlHKT1;1 and *SlHKT1;2* are two tomato Na^+-selective class I-HKT transporters

HKT transporters are crucial for salt tolerance in plants since, along with other transporters, they are responsible for ion homeostasis and Na^+ distribution within the plant (Rodríguez-Navarro & Rubio 2006; Hauser & Horie 2010; Pardo & Rubio 2011). We isolated two *HKT1*-like isoforms, *SlHKT1;1* and *SlHKT1;2*, from tomato. Following sequence and phylogenetic analysis, both are classified as class I HKT transporters (Fig. 2, Platten *et al*. 2006). In our view, after carrying out a more in-depth sequence analysis, these two isoforms could be Na^+ transporters. HKT proteins in vascular plants can be divided into two subfamilies with putatively distinct ion selectivities (Platten *et al*. 2006; Hauser & Horie 2010). It has been suggested that a conserved Gly residue in the K^+ channel selectivity filter (GYG of the P-loop-like domains) determines the ion selectivity of HKT transporters (Mäser *et al*. 2002). In subfamily class II HKT transporters, the presence of Gly in the first P-loop region is conserved, determining a robust K^+ permeability. However, some members of this group are clearly involved in primary Na^+ uptake in roots under particular external conditions such as K^+ deficiency (Horie *et al*. 2007). The presence of Ser instead of Gly in the P_A-loop, as predicted for SlHKT1;1 and SlHKT1;2 (Fig. 1), seems to be a key feature that determines preferential Na^+ conductance in class I HKT transporters (Mäser *et al*. 2002; Rodríguez-Navarro & Rubio 2006; Horie *et al*. 2009; Corratgé-Faillie *et al*. 2010; Hauser & Horie 2010).

Table 1. Significant LOD scores, percentage of explained variance (PEV) and additive values (*a*) by using MQM procedure for traits that presented maximum LOD at *HKT1* genes in both the P and C population of RILs

	P-Pop.			C-Pop			
Trait	LOD at *HKT1*	PEV	*a*	LOD at *HKT1*	PEV	*a*	Trait
logLNC_S	13.69	37.00	–0.08	9.32	33.50	–305.75	LNC_S
LKC_S	4.03	12.70	59.71	7.14	26.90	0.65	LKC_S
sqLKN_S	14.15	37.90	0.17	10.34	36.40	0.39	LKN_S
logSNC_S	10.77	30.40	–0.08	10.75	37.60	–493.25	SNC_S
SKC_S	2.57	8.30	92.04	5.75	22.30	0.61	SKC_S
TN_S	13.31	36.10	–0.32	6.48	24.70	–0.52	TN_S
NLS_S	3.24	10.80	7.70	4.52[a]	25.00	11.56	NLS_S
NLC_C	6.40	19.40	–17.74				
LKN_C	7.24	21.60	4.17				
SNC_C	6.15	18.70	–29.79				

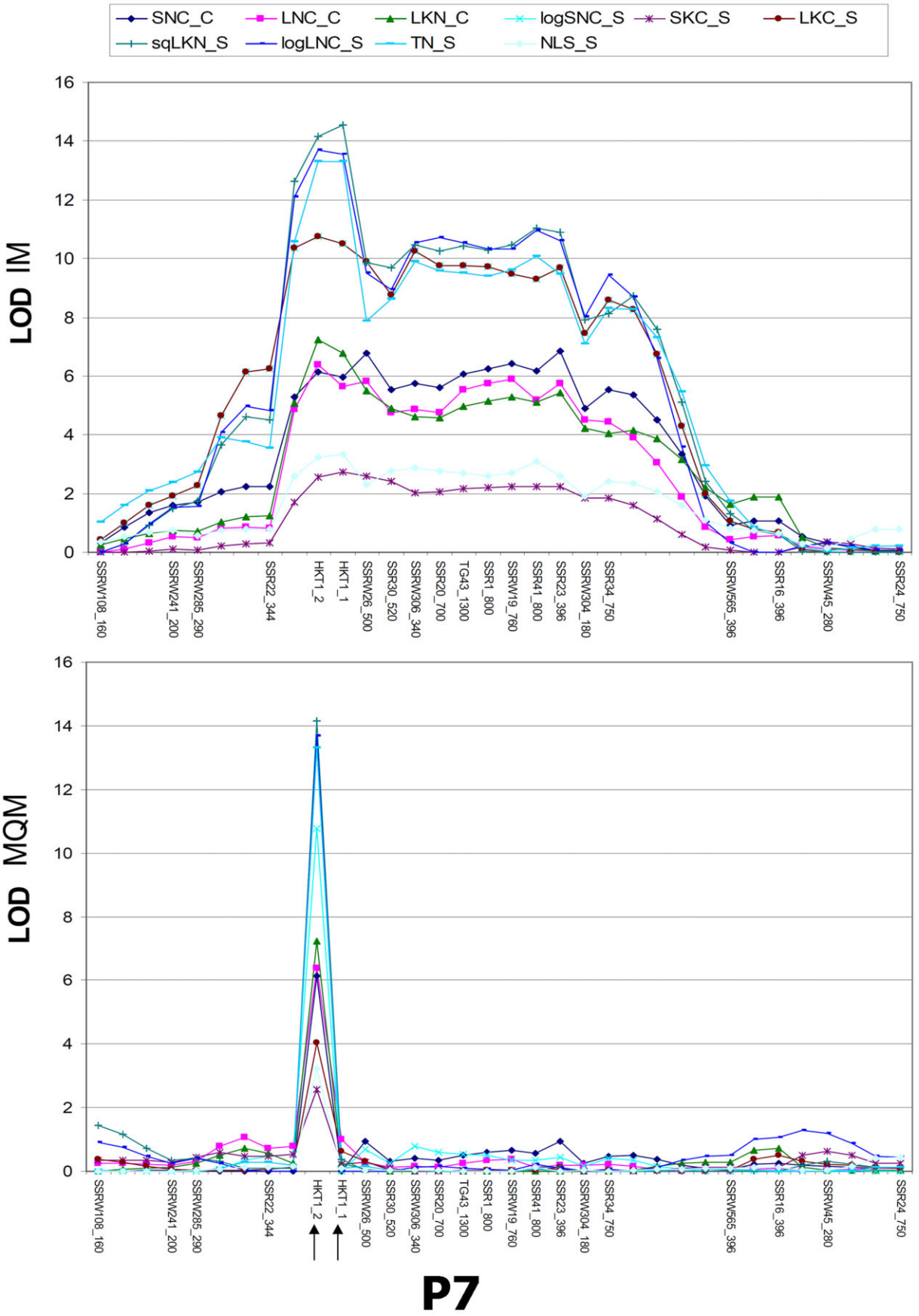
Negative *a* values correspond to the wild allele.
[a]Another significant QTL in C7 is located at marker TG35_506 for NLS (LOD = 2.4, PEV = 9.6, *a* = –8.89).

The members of this class, AtHKT1;1, OsHKT1;1, OsHKT1;5 and TmHKT1;5-A, characterized so far, have been described as low affinity and specific Na^+ transporters located in the plasma membrane of parenchyma cells surrounding the xylem vessels, responsible for unloading Na^+ from the xylem, thus preventing Na^+ accumulation in shoots (Uozumi *et al*. 2000; Ren *et al*. 2005; Sunarpi *et al*. 2005; Davenport *et al*. 2007; Xue *et al*. 2011; Munns *et al*. 2012). As with class II transporters, some exceptions have been observed in relation to the transport activity of class I transporters, especially when these transporters were expressed in heterologous systems (Fairbairn *et al*. 2000; Su *et al*. 2003). However, these exceptions in terms of ion selectivity were much more complex than expected. In the halophytic *Thelungiella salsuginea*, one of the two isoforms found to belong to class I, TsHKT1;2, showed strong K^+ transporter activity and selectivity over Na^+ (Ali *et al*. 2012). K^+ transport capacity was attributed to the presence of two aspartic residues, D207 and D238, in the transmembrane (M_{2B}) and pore (P_B) domains. In both these locations, Asn (N) residues were found in Arabidopsis and all other known plant sequences (Ali *et al*. 2012). These residues are not present in the sequence of any tomato isoforms, indicating that both are closer in sequence to typical Na^+ transporters (Fig. 1). Following expression experiments in yeast mutant cells defective in endogenous K^+ transporters (TRK1 and TRK2) and cation uptake experiments, we can conclude that tomato HKT1;1 is a Na^+-selective transporter unaffected by K^+ (Fig. 3), as observed in the case of AtHKT1;1 (Uozumi *et al*. 2000). With respect to SlHKT1;2, ion selectivity and transport activity are not clear as none of the transport experiments in yeast cells were positive. Similar results were obtained with some rice HKT isoforms when expressed heterologously in this type of yeast mutant (Garciadeblás *et al*. 2003). The lack of activity might be explained by a 5′-UTR sequence predicted in the genomic sequence of *SlHKT1;2* annotated by ITAG2.40 (http://solgenomics.net/), affecting the first thirteen amino acids of its putative ORF (see Fig. 1), a possibility that requires further investigation. Other differences in sequence have been found, such as 22 extra amino acids in SlHKT1;2 between the M_{2A} and M_{1B} transmembrane domains, which were absent in both SlHKT1 and AtHKT1;1 sequences. However, its effect on protein structure and consequently on protein function and/or activity is probably limited because of the poor homology shown in this region as compared with *S. lycopersicum* HKT1-like proteins and other plant HKT transporters (Fig. 2, Supporting information Fig. S6).

HKT1;1 and/or *HKT1;2* genes could underlie a major tomato QTL for Na^+/K^+ homeostasis as indicated by candidate gene analysis in RIL populations and expression analysis in tomato NILs

Salt tolerance is a quantitative trait in plants. The identification of quantitative trait loci (QTLs) controlling this characteristic is of great importance in order to breed salt-tolerant crops (Flowers 2004; Cuartero *et al*. 2006). In a previous study, using both P-RIL (*S. lycopersicum* × *S. pimpinellifolium*), and C-RIL populations (*S. lycopersicum* × *S. cheesmaniae*), a cluster of major QTLs controlling Na^+ and K^+ concentrations in the aerial part of the plant under salinity conditions was identified in chromosome 7, although no Na^+ transporters or regulatory proteins involved in Na^+/K^+ homeostasis tested as candidate genes in that study located at this genomic region (Villalta *et al*. 2008). Taking into account the importance of HKT1-like encoding genes in other species determining Na^+ concentration QTLs and salt tolerance (Ren *et al*. 2005;

Figure 5. LOD function using Interval Mapping (IM) and Multiple QTL Mapping (MQM) for 10 characters showing significant QTLs (LOD > 2) on chromosome 7 in P population: the Na^+ and K^+ concentrations in leaves (LNC, LKC) and stems (logSNC, SKC), the K^+/Na^+ ratio in leaves (sqLKN), the transported Na^+ (TN) and Na^+ leaf sensitivity (NLS). Trait suffixes _C and _S stand for control and salinity conditions, respectively. SlHKT1 markers are indicated with arrows.


SNC_C
LNC_C
LKN_C
logSNC_S
SKC_S
LKC_S
sqLKN_S
logLNC_S
TN_S
NLS_S
LOD IM
LOD MQM
0
2
4
6
8
10
12
14
16
SSRW108_160
SSRW241_200
SSRW285_290
SSR22_344
HKT1_2
HKT1_1
SSRW26_500
SSR30_520
SSRW306_340
SSR20_700
TG43_1300
SSR1_800
SSRW19_760
SSR41_800
SSR23_396
SSRW304_180
SSR34_750
SSRW565_396
SSR16_396
SSRW45_280
SSR24_750
P7

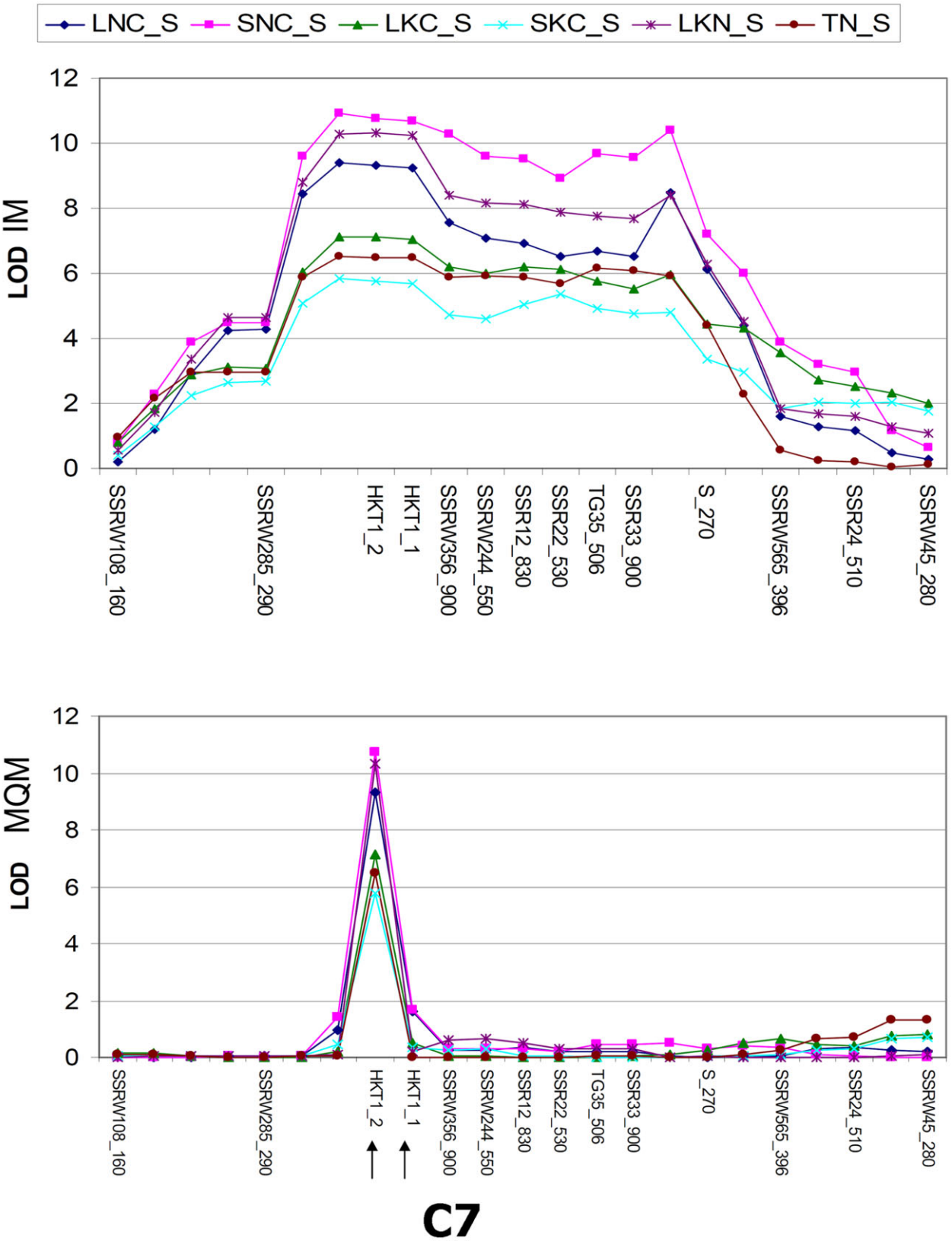


Figure 6. LOD function using Interval Mapping (IM) and Multiple QTL Mapping (MQM) for six characters showing significant QTLs (LOD > 2) on chromosome 7 in C population: the Na^+ and K^+ concentrations in leaves (LNC, LKC) and stems (SNC, SKC), the K^+/Na^+ ratio in leaves (LKN) and the transported Na^+ (TN). Trait suffixes _C and _S stand for control and salinity conditions, respectively. *HKT1* markers are indicated with arrows.

Table 2. LOD scores, percentage of explained variance (PEV) and additive values (*a*) for significant and adjacent SolCAP single-nucleotide polymorphisms (SNPs; http://solgenomics.net/) in chromosome P7 by using MQM procedure for traits that presented maximum LOD at *HKT1* genes in the P population of RILs (Table 1)

SolCAP SNP no.	45 591	57 007	57 002/56 997	56 998
LOD LogLNC_S	0.08	13.10	12.22	0.04
PEV	0.20	35.60	33.70	0.10
a	0.03	–0.08	–0.08	0.02
LOD LKC_S	0.01	4.89	4.77	0.01
PEV	0.00	15.20	14.80	0.00
a	11.76	68.71	68.15	12.78
LOD sqLKN_S	0.03	15.44	14.37	0.05
PEV	0.10	40.50	38.30	0.10
a	–0.04	0.18	0.18	–0.05
LOD logSNC_S	0.28	9.08	8.38	0.08
PEV	0.70	26.30	24.50	0.20
a	0.06	–0.08	–0.07	0.04
LOD SKC_S	0.11	3.29	3.06	0.02
PEV	0.30	10.50	9.80	0.10
a	100.78	108.92	105.56	–49.17
LOD TN_S	0.08	12.30	11.61	0.01
PEV	0.20	33.90	32.30	0.00
a	0.13	–0.32	–0.32	0.05
LOD NLS_S	0.04	3.84	3.65	0.00
PEV	0.10	12.60	12.10	0.00
a	–4.39	8.79	8.61	–1.17
LOD LNC_C	0.08	6.18	6.39	0.21
PEV	0.20	18.80	19.30	0.60
a	10.78	–18.37	–18.71	–17.20
LOD LKN_C	0.16	5.49	5.46	0.04
PEV	0.40	16.80	16.80	0.10
a	–3.35	3.87	3.88	1.77
LOD SNC_C	0.02	8.99	8.87	0.04
PEV	0.00	26.10	25.80	0.10
a	–8.20	–37.04	–36.94	–12.85

Negative *a* values correspond to the wild allele.

Huang *et al*. 2006; James *et al*. 2006, 2011; Byrt *et al*. 2007), we wanted to test HKTs as candidate genes for those QTLs reported in tomato by Villalta *et al*. (2008).

Interestingly, *HKT1;1* and *HKT1;2* were located together in chromosome 7 of both RIL populations (Fig. 4) in a genomic position overlapping with the position of maximum significance of the LOD value for the aforementioned QTLs (Table 1, Figs 5 & 6). The recent sequencing of tomato species *S. lycopersicum* cv Heinz 1709 has confirmed their tandem location in chromosome 7 at a distance of only 6 kb apart (Table 3, http://solgenomics.net/). Moreover, when the map with only the SolCAP SNPs was considered for QTL analysis, *HKT1;1* and *HKT1;2* were just 35 kb away from the SNP with the maximum LOD score for LNC, LKC, LKN, SNC, SKC, TN and NLS under salinity conditions, and LKN and SNC under control conditions, which matched the positional range where other genes responsible for QTLs were found (Price 2006). Other candidate genes in this genomic region might also be responsible for these QTLs, particularly *lkc7.1*. A search for other tomato genes encoding putative potassium or sodium transporters in the 10-Mb physical interval spanning *SlHKT1;1* and *SlHKT1;2* loci of chromosome 7 (tomato genomic sequence annotated by ITAG2.40, http://solgenomics.net/) identifies other functional candidates (Table 3). However, all are more than 3.5 Mbp away from Solcap_snp_sl_57007 (Cytochrome P450; 40.805 cM, 5 056 490 bp, Table 3) where the maximum LOD for QTLs controlling Na^+ and K^+ concentrations is located (Table 2). Given the 1-LOD confidence intervals in our QTL analysis shown in Table 2, the responsible gene(s) is (are) likely to be located within a physical interval of 1 113 481 bp (between SolCAP SNPs 45591 and 56998). Therefore, *HKT1;1* and/or *HKT1;2* are prime positional, and possibly functional, candidate genes for underlying the chromosome 7 QTL cluster related to Na^+ and K^+ traits.

To further determine whether the allelic variation in *HKT1;1* and *HKT1;2* functionally underlies the cluster of Na^+/K^+ QTLs in chromosome 7, NILs 157-14 (double homozygote for the *HKT1;1* and *HKT1;2* alleles from *S. cheesmaniae,* named *ScHKT1;1* and *ScHKT1;2*, respectively)

Table 3. Genes encoding putative ion transporters in 10 Mbp-regions (SL2.40ch07:0.10003161) spanning *SlHKT1;1* and *SlHKT1;2* loci in chromosome 7 of *S. lycopersicum* cv. Heinz tomato as annotated by ITAG2.40 (http://solgenomics.net/)

Chromosome position	Tomato gene	Annotation/predicted function
SL2.40ch07-90358.92771	Solyc07g005040.2.1	H^+- ATPase, ATPase-type plasma membrane proton-efflux
SL2.40ch07-339058.340332	Solyc07g005440.1.1	CIPK, CBL-interacting protein kinase
SL2.40ch07-408207.409532	Solyc07g005520.1.1	Potassium channel tetramerization domain-containing protein
SL2.40ch07-481459.487527	Solyc07g005590.2.1	Cyclic nucleotide-gated channel
SL2.40ch07-1194436.1196139	Solyc07g006370.1.1	Sodium calcium exchanger protein, homolog to AtCAX7
SL2.40ch07-1308919.1317697	Solyc07g006510.2.1	Cyclic nucleotide-gated ion channel, homolog to AtCNGC17
SL2.40ch07-2247006.2251786	Solyc07g007600.2.1	Pyrophosphate-energized proton pump, homolog to AtVHP1;1
SL2.40ch07-3079524.3097721	Solyc07g008320.2.1	Calcium-transportingATPase, homolog to AtACA10
SL2.40ch07-4107448.4119376	Solyc07g009130.2.1	ATPase, P-type, heavy metal-(Cd/Co/Hg/Pb/Zn)-translocating, homolog to ATHMA2
SL2.40ch07-5091851.5097815	**Solyc07g014680.2.1**	**Potassium transporter, homolog to AtHKT1;1, identical to SlHKT1;2**
SL2.40ch07-5103988.5106992	**Solyc07g014690.2.1**	**Potassium transporter, homolog to AtHKT1;1, identical to SlHKT1;1**
SL2.40ch07-5203700.5206540	Solyc07g014740.2.1	Homolog to sodium bile acid symporter family
SL2.40ch07-8095828.8102636	Solyc07g017780.2.1	ATPase P-type plasma membrane proton-efflux, homolog to: AtAHA2

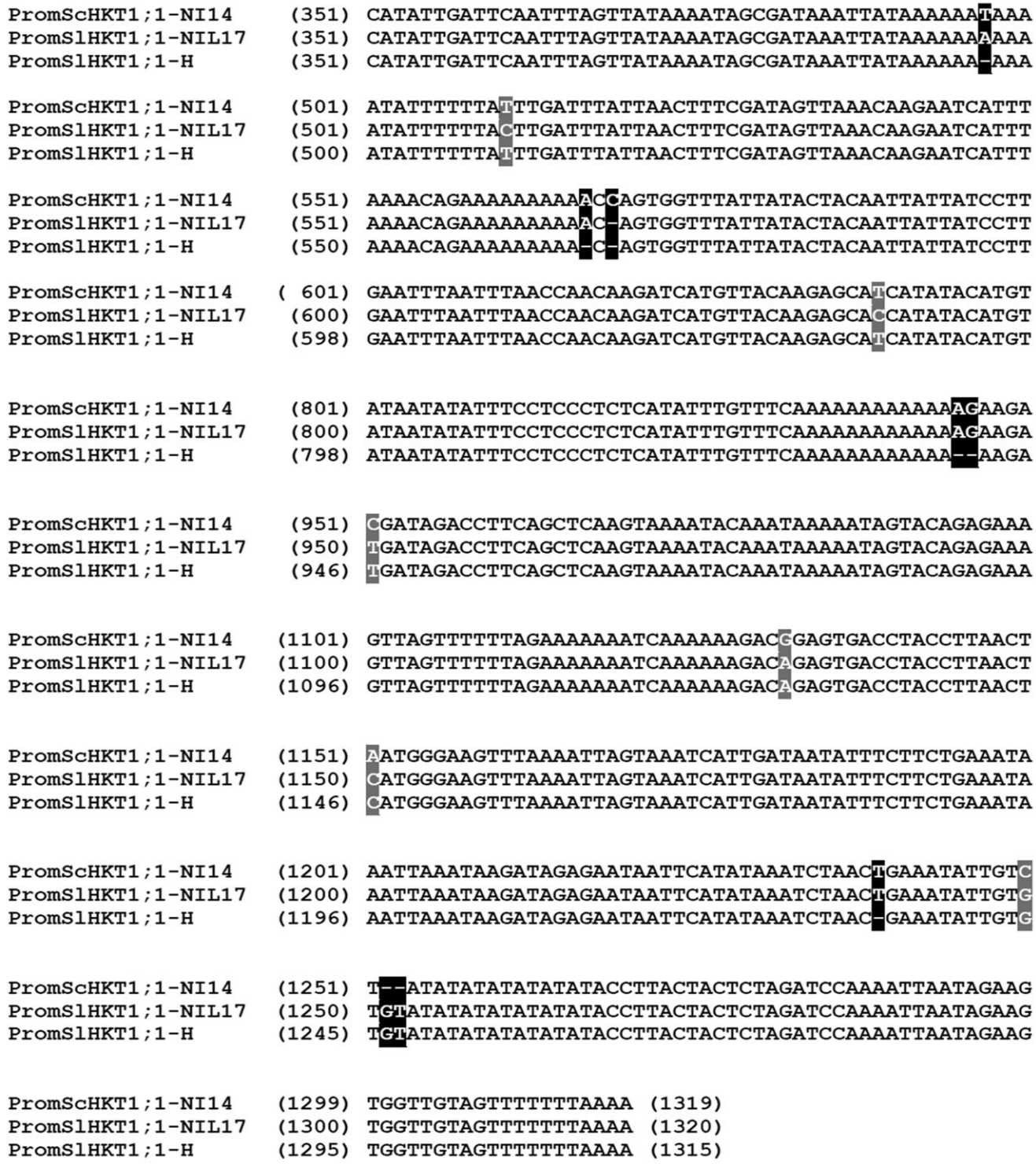


Figure 7. Sequence polymorphism in promoter region of *HKT1;1* allelic variants from tomato NIL157-14 (CC) and NIL157-17(LL). Single-nucleotide polymorphisms (SNPs; highlighted in grey) and indels (highlighted in black) in tomato *HKT1;1* promoter region up to 1300 bp upstream of the translation start site from genomic sequences of *S. licopersicum* cv. Cerasiform, NIL157-14 (ScHKT1;1-NIL14) and NIL157-17 (SlHKT1;1-NIL17). The *SlHKT1;1* (Solyc07g014690.2.1) genomic sequence of *S. lycopersicum*, cv Heinz 1709 (H) was obtained from genome tomato database (ITAG2.40, http://solgenomics.net/). Positions are counted from 5′end of the sequence and the last position refers to the base pair immediately upstream of the start codon.

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PromScHKT1;2-NIL14    (251)  TTTAATAAGATTTCAGAGCAGGTTGAAATCTTGTGATCGGATCTCACTCC
PromSlHKT1;2-NIL17    (251)  TTTAATAAGATTTCAGAGCAGGTTGAAATCTTGTGATCGAATCTCACTCC
PromSlHKT1;2-H        (251)  TTTAATAAGATTTCAGAGCAGGTTGAAATCTTGTGATCGAATCTCACTCC

PromScHKT1;2-NIL14    (301)  ACTTATGTAAAAAGAATTTTCACATGCTTTGACCCATAAAAAATTTTTTG
PromSlHKT1;2-NIL17    (301)  ACTTATGTAAAAAGAATTTTCACATGCTTTGACCCATAAAATTTTTTTG
PromSlHKT1;2-H        (301)  ACTTATGTAAAAAGAATTTTCACATGCTTTGACCCATAAAATTTTTTTG

PromScHKT1;2-NIL14    (551)  AAGGAAATATCAATGATCATTTGGCAAGAATTATTATATTCTAACTCCTT
PromSlHKT1;2-NIL17    (551)  AAGGAAATATCAGTGATCATTTGGCAAGAATTATTATATTCTAACTCCTT
PromSlHKT1;2-H        (551)  AAGGAAATATCAGTGATCATTTGGCAAGAATTATTATATTCTAACTCCTT

PromScHKT1;2-NIL14    (801)  ATTTATTTTACTA (814)
PromSlHKT1;2-NIL17    (801)  ATTTATTTTACTA (814)
PromSlHKT1;2-H        (801)  ATTTATTTTACTA (814)
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Figure 8. Sequence polymorphism in promoter region of *SlHKT1;2* from NIL157-14 (CC) and NIL157-17(LL). Single-nucleotide polymorphisms (SNPs; highlighted in grey) in tomato *HKT1;2* promoter region up to 814 bp upstream of the translation start site from genomic sequences of *S. licopersicum* cv. Cerasiforme, NIL157-14 (ScHKT1;2-NIL14) and NIL157-17 (SlHKT1;2-NIL17). The *SlHKT1;2* (Solyc07g014680.2.1) genomic sequence of *S. lycopersicum*, cv Heinz 1709 (H) was obtained from genome tomato database (ITAG2.40, http://solgenomics.net/). Positions are counted from 5′end of the sequence and the last position refers to the base pair immediately upstream of the start codon.

and 157-17 (double homozygote for the *HKT1;1* and *HKT1;2* alleles from *S. lycopersicum*, named *SlHKT1;1* and *SlHKT1;1*, respectively) were used to study their expression pattern (Fig. 9) as well as differences in nucleotide sequence (SNPs and indels), particularly in the 5′ regulatory upstream region (Figs 1, 7, 8 and Supporting Information Fig. S3). A complex pattern of expression for tomato *HKT1;1* and *HKT1;2* was observed. The Sc*HKT1;2* transcript level in roots of NIL 157-14 was lower than *SlHKT1;2* in NIL 157-17, although expression of *ScHKT1;1* and *ScHKT1;2* in leaves was much higher at any day of salt treatment (Fig. 9). The expression level of HKT1-like transporters has been reported to be directly related to salt tolerance and Na^+ specific tissue distribution according to the plant source. In rice and wheat, whose more tolerant varieties accumulate less Na^+ in leaves (and have a high K^+/Na^+ ratio in leaves), highly expressed and/or hyperactive allelic variants of HKT1-like transporters support functionally similar QTLs (Ren *et al*. 2005; Huang *et al*. 2006; James *et al*. 2006; Byrt *et al*. 2007). In Arabidopsis, where the salt tolerance of some ecotypes is associated with higher Na^+ concentration in shoots, naturally occurring weak alleles (weakly expressed) of *AtHKT1;1* were typical of ecotypes adapted to coastal and saline soils in Europe (Rus *et al*. 2006; Baxter *et al*. 2010; Jha *et al*. 2010). Assuming the functional similarity between the HKT1-like transporters described in dicots (Hauser & Horie 2010) and bearing in mind that cellular/tissue location requires further study, it would be reasonable to expect that HKT1;2 controls xylem Na^+ unloading in roots. Reduced expression of *ScHKT1;2* in the roots of NIL-157-14 should imply lower Na^+ retrieval from the xylem in roots and consequently more Na^+ should be transported via the transpiration stream to the aerial part as compared to NIL157-17. At the same time, increased expression of *ScHKT1,2* and, to some extent, that of *ScHKT1;1*, in leaves might increase the withdrawal of Na^+ from the xylem, thus promoting its intracellular accumulation in the mesophyll cells of expanding leaves. It is possible to speculate that the contribution of the *HKT1;2* isoform to Na^+ movements in tomato could be greater than that of HKT1*;1* since the expression level of any allelic variant of *HKT1;2* is higher than that for *HKT1;1* when relative expression data are calculated using the *HKT1;1* expression level at each NIL, tissue and treatment day as the calibrator sample (Supporting Information Fig. S5). In addition, HKT1*;2* has an expression profile similar to that found in the literature for *AtHKT1;1* (high in the root, low in the shoot, which is unlike the pattern for *SlHKT1;1*) and the NIL with higher *HKT1;2* expression has a lower, though not significantly different, shoot Na^+. It is therefore possible to suggest that this isoform performs a role similar to that of AtHKT1;1, particularly in roots, while tomato HKT1;1 may function mainly in leaves.

The differences observed in the expression levels of *HKT1*-like genes (and eventually in transport activity) in tomato NILs might be explained by changes in their promoter sequences that alter the potential binding of regulatory elements. In different Arabidopsis ecotypes, the *cis*-regulatory allelic variation of *AtHKT1;1* and the subsequent effect on its expression has been linked to differential salinity tolerance (Rus *et al*. 2006; Baxter *et al*. 2010). A deletion in the promoter of NIL157-14 at approximately 1251 bp (Fig. 7) could potentially lead to an additional TATA box, not seen in NIL157-17 or Heinz (Table 2), just before transcription start. If this is the case, the addition of this TATA box could explain why NIL157-14 has a much higher expression level for *ScHKT1;1* than *SlHKT1;1* in NIL157-17 in root and leaf (Fig. 9). *ScHKT1;2*, having one less ARR1AT box than for the *lycopersicum* allele (Table 3), might be responsible for lower expression in the roots of NIL157-14 as compared with that of *SlHKT1;2* in NIL157-17 (Fig. 9) since ARR-related

Table 4. *Cis*-regulatory elements in 5′ UTR of *HKT1;1* genomic sequences of *S. lycopersicum* cv. Cerasiforme, NIL157-14 (CC) and NIL157-17 (LL) affected by sequence polymorphism indicated in Fig. 7

Regulatory element	H	NIL17	NIL14	Sequence	Function
ARE	1	1	2	TGGTTT	*Cis*-acting regulatory element essential for anaerobic induction
CAAT	27	27	28	CAAAT	Common *cis*-acting element in promoter and enhancer regions
CACTFTPPCA1	31	32	31	YACT	Tetranucleotide (CACT) is a key component of Mem1 (mesophyll expression module 1) found in the *cis*-regulatory element in the distal region of the phosphoenolpyruvate carboxylase (ppcA1) of the C4 dicot *F. trinervia*; Y = T/C
CIACADIANLELHC	4	3	4	CAANNNNATC	*Cis*-acting regulatory element involved in circadian control
MARTBOX	7	8	7	TTWTWTTWTT	T-Box; Motif found in SAR (scaffold attachment region; or matrix attachment region, MAR)
MYB1AT	2	2	3	WAACCA	MYB recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes in Arabidopsis.
MYB2AT	0	1	1	TAACTG	MYB-binding site involved in drought inducibility
MYB2CONSENSUSAT	1	2	2	YAACKG	MYB recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes in Arabidopsis; Y = C/T; K = G/T
MYBCORE	1	2	2	CNGTTR	Binding site for all animal MYB and at least two plant MYB proteins ATMYB1 and ATMYB2, both isolated from Arabidopsis; ATMYB2 is involved in regulation of genes responsive to water stress in Arabidopsis.
PREATPRODH	1	1	0	ACTCAT	PRE (Pro- or hypoosmolarity-responsive element) found in the promoter region of proline dehydrogenase (ProDH) gene in Arabidopsis; core of 9-bp sequence ACTCATCCT necessary for the efficient expression of ProDH in response to L-Pro and hypoosmolarity
PRECONSCRHSP70A	1	0	0	SCGAYNRNNNNNNNNNNNNNNHD	Consensus sequence of PRE (plastid response element) in the promoters of HSP70A in Chlamydomonas; involved in induction of HSP70A gene by both MgProto and light
SEF4MOTIFGM7S	6	6	5	RTTTTTR	SEF4 binding site; soybean (G.m.) consensus sequence found in 5′upstream region (–199) of beta-conglycinin (7S globulin) gene (Gmg17.1); binding with SEF4 (soybean embryo factor 4)
POLASIG	23	22	24	AATAAA/AATTAAA/AATAAT	Plant polyA signal; consensus sequence for plant polyadenylation signal.
SORLREP3AT	1	1	0	TGTATATAT	Sequences Over-Represented in Light-Repressed Promoters (SORLREPs) in Arabidopsis; computationally identified phyA-repressed motifs.
TATABOX	16	15	17	TATTAAT/TATATAA/TTATTT	TATA box, sequence and spacing of TATA box elements are critical for accurate initiation.
TCA-element	0	0	1	CCATCTTTTT	*Cis*-acting element involved in salicylic acid responsiveness

Sequence corresponding to *S. lycopersicum* cv. Heinz 1709 (H) was used as a reference (Solyc07g014690.2.1). Frequencies in each NIL/cultivar are indicated. Promoter sequences were searched against PLACE (Higo *et al*. 1999), PlantCare (Lescot *et al*. 2002) and NSITE-PL (http://linux1.softberry.com) databases and tools.

Table 5. *Cis*-regulatory elements in 5′ UTR of HKT1;2 genomic sequences of *S. lycopersicum* cv. Cerasiforme, NIL157-14 (CC) and NIL157-17 (LL) affected by sequence polymorphism indicated in Fig. 8

Regulatory element	H	NIL17	NIL14	Sequence	Function
ARR1AT	13	13	12	NGATT	ARR1-binding element' found in Arabidopsis; ARR1 is a cytokinin-regulated transcription factor.
CAAT	7	7	8	CAAAT	Common *cis*-acting element in promoter and enhancer regions.
CACTFTPPCA1	18	18	17	YACT	Tetranucleotide (CACT) is a key component of Mem1 (mesophyll expression module 1) found in the *cis*-regulatory element in the distal region of the phosphoenolpyruvate carboxylase (ppcA1) of the C4 dicot F. trinervia; Y = T/C.
HSE	1	2	1	AAAAAATTTC	*Cis*-acting element involved in heat stress responsiveness.
PRECONSCRHSP70A	1	1	2	SCGAYNRNNNNNNNNNNNNNHD	Consensus sequence of PRE (plastid response element) in the promoters of HSP70A in Chlamydomonas; involved in induction of HSP70A gene by both MgProto and light

Sequence corresponding to *S. lycopersicum* cv. Heinz 1709 (H) was used as a reference (Solyc07g014680.2.1). Frequencies in each NIL/cultivar are indicated. Promoter sequences were searched against PLACE (Higo *et al*. 1999), PlantCare (Lescot *et al*. 2002) and NSITE-PL (http://linux1.softberry.com) databases and tools.

transcription factors involved in cytokinin signalling have been shown to regulate *AtHKT1;1* gene expression in roots in response to cytokinin (Mason *et al*. 2010). It is not clear how differences in the frequency of other *cis*-elements (Tables 2 and 3) affect tomato *HKT1*-like gene expression. It is also possible that other allelic differences in the 5′ upstream region of the promoter sequences apart from those shown in this study might contain critical motives involved in the differential gene expression of NILs. Thus, the reduced root expression of *AtHKT1;1* observed in two coastal ecotypes of Arabidopsis has been attributed to a deletion in a tandem repeat sequence approximately between 5 kb and 3 kb upstream from the *AtHKT1;1* encoding sequence, which affected a dense cluster of small RNAs (Rus *et al*. 2006). To find out whether these affected *cis*-regulatory elements predicted by *in silico* analysis are responsible for the differences observed in the expression levels of each NIL will require further analysis. As pointed out by Jha *et al*. (2010), it is important to note that changes in gene expression are not necessarily linked to changes in protein abundance, nor can they elucidate the activity of the individual transporters. Nevertheless, there might be differences between genotypes in

Table 6. Means, their standard errors (E.E.) and *P*-values of Na^+ and K^+ related traits of NILs 157-14 and 157-17 under control (14_C and 17_C) and high salinity (14_S and 17_S) conditions

Trait	*P*-NIL	*P*-Treat.	*P*-Interact.	14_C	E.E.	14_S	E.E.	17_C	E.E.	17_S	E.E.
K_S	0.00087	0.36702	0.76791	25 419.67	1671.48	23 143.41	1621.67	34 164.84	2705.72	32 990.52	529.45
Na_S	0.84404	0.00099	0.43768	39 797.43	3240.22	51 866.98	3617.09	38 046.91	2916.32	54 776.99	684.35
Na/K_S	0.00064	0.00025	0.56162	1.58	0.17	2.24	0.09	1.11	0.02	1.66	0.02
K_R	0.00128	0.56156	0.00677	4611.10a	507.38	2984.09ab	357.56	1353.37b	220.82	2514.20b	398.12
Na_R	0.95968	0.00341	0.03153	5360.87ab	730.91	6727.77ab	720.39	2949.13b	200.36	9044.71a	1486.53
Na/K_R	0.00001	0.00001	0.39981	1.15	0.04	2.27	0.09	2.25	0.21	3.59	0.11
K_L	0.00665	0.02990	0.05692	29 559.96	1630.24	24 277.60	789.50	31 093.29	718.36	30 644.16	964.55
Na_L	0.07004	0.00010	0.64183	43 613.75	1062.20	68 125.76	4260.46	38 444.14	2957.13	59 845.67	3660.16
Na/K_L	0.00139	0.00002	0.03331	1.49 bc	0.10	2.80a	0.09	1.23c	0.07	1.96b	0.17
K_S + L-R	0.00004	0.04438	0.41466	50 368.53	576.37	44 436.92	1376.64	63 904.76	3186.08	61 120.47	1002.64
Na_S + L-R	0.24106	0.00012	0.74986	78 050.31	3468.77	113 264.97	5405.73	73 541.91	5603.38	105 577.95	4484.51
RFW	0.00004	0.00245	0.01564	8.97 bc	1.02	7.09c	0.61	25.09a	2.03	14.30b	1.72
RDW	0.00004	0.00081	0.00176	2.73b	0.20	2.42b	0.37	9.24a	0.69	4.28b	0.60
PDW	0.00007	0.00043	0.00865	12.81b	1.01	10.67b	1.09	23.03a	0.99	14.46b	0.54

First group of traits corresponds to cation concentration (given in ppm – $\mu g\ g^{-1}$ DW) in the stem (S), followed by the root (R), the leaf (L) and the difference between the aerial and root parts (S + L-R). The last group corresponds to important growth traits like fresh and dry weights (–FW and –DW) of plant (P), R and S tissues (given in grams). For traits showing significant NIL × Treatment interaction, means with the same letter are not significantly different.

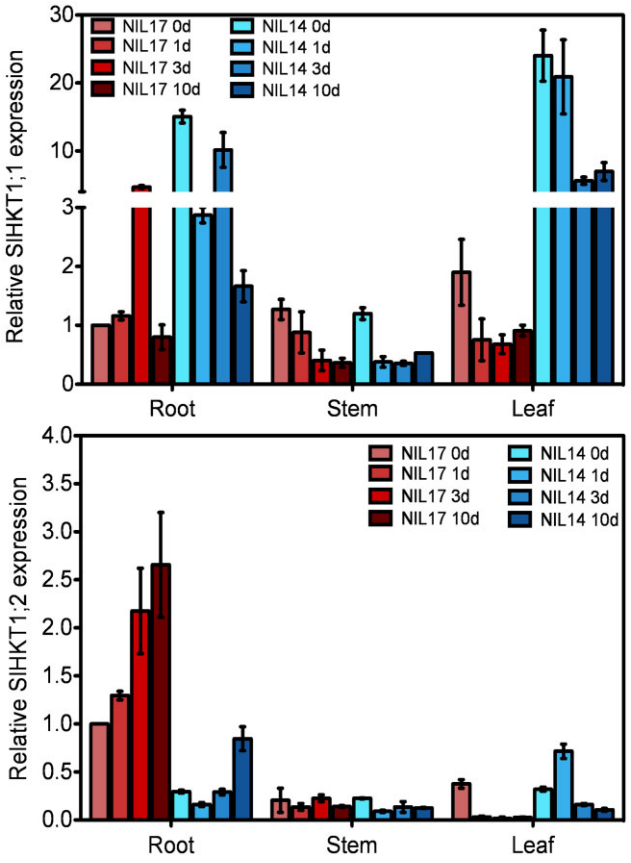

Figure 9. Gene expression of *SlHKT1;1* and *SlHKT1;2* in response to salt stress in NIL157-14 (CC) and NIL157-17(LL). Total RNA was purified from the leaf, stem and root of tomato plants treated with 100 mM NaCl for 0, 1, 3 and 10 d in hydroponic cultures. Transcript level was analysed by RT-qPCR using primers indicated in Supporting Information Table S2. The tomato elongation factor gene (*LeEF1-α*) was used as the reference gene. The relative expression level was calculated by using as the calibrator sample the expression level of each gene in roots of NIL157-17 at day 0 of NaCl treatment (equal to 1). Error bars indicate the SD from nine repeats for stems and leaves (three biological and three analytical repeats) and 12 repeats for roots (four biological and three analytical repeats).

terms of the effectiveness of the regulators of these proteins or, as our study discovered, the only amino acid substitution found in the allelic variant of *ScHKT1;1* affecting the $M1_B$ helix region (Fig. 1) could alter the activity of the transporter (discussed below).

Differences in the expression of *HKT1;1* and *HKT1;2* alleles should fit differences in genotypic means at QTLs for Na^+ and K^+ parameters on chromosome 7 of the C population of RILs obtained in a previous study, where RILs with (a) weak *cheesmaniae* allele(s) in roots were associated with higher Na^+ and lower K^+ concentrations in stems and leaves than RILs with the *lycopersicum* allele (Villalta *et al*. 2008). Contrary to expectations, significant differences between NILs have only been observed for leaf $[K^+]$ and the $[Na^+]/[K^+]$ ratio under salinity conditions but not for leaf $[Na^+]$ (Table 6). This means that the NILs (or our NIL experiment) do not precisely reproduce the allelic differences observed when using the whole population of RILs (Villalta *et al*. 2008). Nevertheless, NIL 157-17 accumulated significant levels of Na^+ in the root (see Na_R mean comparisons in Table 6). This would explain the significant reduction in the plant and root dry weight of NIL 157-17 (salt sensitive), while no significant differences in these parameters (RDW and PDW) were found in NIL 157-14 (salt tolerant) when comparing both salinity levels.

Most phenotyped plants from NIL 157-17 and 157-14 continued to be heterozygous at one marker on chromosome 2 and at several markers on chromosome 4 (chromosomes where neither $[K^+]$ nor $[Na^+]$ QTLs were previously detected by Villalta *et al*. 2008); the only genomic segments segregating among selected NILs derived from RIL 157. The tested lack of genomic differences between 157-17 and 157-14 (except for the target region of chromosome 7) rules out their putative responsibility for the differing results from the RIL and NIL experiments. An epistatic effect of the genetic background on these NILs over the stem and leaf $[Na^+]$ could explain these differences (Lecomte *et al*. 2004; Muir & Moyle 2009). Two other NILs 157-12 (double homozygote for the *lycopersicum* allele at *HKT1;1* and *HKT1;2*) and 157-9 (double homozygote for the *cheesmaniae* allele at *HKT1;1* and *HKT1;2*) were evaluated for leaf and root Na^+ and K^+ concentrations in a previous experiment, where plants were kept under high-salinity conditions for just 33 d (data not shown). In this experiment, both lines differed significantly ($P = 0.0004$) in relation to leaf Na^+ concentration, with the *cheesmaniae* allele being associated with the higher Na^+ concentration. NILs 157-12 and 157-9 are fixed for different alleles (*lycopersicum* and *cheesmaniae*, respectively) at those markers on chromosome 4 for which 157-17 and 157-14 are heterozygous. Thus, NILs 157-12 and 157-9 differed not only in relation to the target region on chromosome 7 but also in relation to another region on chromosome 4. Consequently, the presence of putative epistatic interactions between *HKT1;1* (or *HKT1;2*) and this region on chromosome 4 was studied in the C-RIL population to explain the variation observed in leaf Na^+ using data from Villalta *et al*. (2008). No such significant epistatic interaction was found. Therefore, the only factor that would appear to explain the failure to detect significant effects on leaf Na^+ concentration between NILs 157-14 and 157-17 is the duration of the salinity treatment: around 5 weeks for the C-RIL population and the experiment with NILs 157-9 and 157-12 referred to above, while the leaf Na^+ concentration of NILs 157-14 and 157-17 was measured after 10 weeks of salt treatment (including 7 weeks of fruit yield).

How might differences in *HKT1* alleles explain the differences observed in $[K^+]$ and $[Na^+]/[K^+]$ between NILs given that HKT1-like transporters seem to be Na^+ specific? Sequence analysis indicated that the amino acid sequences of HKT1;2 from both NILs were identical (Fig. 1). However, the allelic variant of HKT1;1 from *S. cheesmaniae* (salt tolerant) in NIL57-14, *ScHKT1;1* had a single substitution in the amino acid sequence (V222L, Val222Leu) in the M1B helix region as compared with the cultivated allele *SlHKT1;1* (Fig. 1). This

substitution did not affect any of the four substitutions reported to enhance salt tolerance in TaHKT1 (Rubio, Gassmann & Schroeder 1995), the additional conserved residues reported to be necessary for K^+ selectivity (Kato *et al*. 2007), other mutations reported to affect the functional properties of HKT transporters when expressed in heterologous systems (Corratgé-Faillie *et al*. 2010), and the substitution V395L in a rice variety suggested as responsible for less active Na^+ transport or ion selectivity for OsHKT1;5 (Cotsaftis *et al*. 2012). A functional analysis of *SlHKT1;1/ScHKT1;1* allelic variants in yeast mutants, defective in endogenous K^+ transporters (*Δtrk1* and *Δtrk2*), will be carried out to test whether such a substitution provided different kinetic properties that account for its physiological contribution to QTL *lkc7.1*. However, allelic differences in the gene expression of tomato HKT1-like transporters could explain the reported behaviour of NIL and RIL populations for K^+ concentration in leaves. Other HKT1-like transporters, like AtHKT1;1 or OsHKT1;5 and TmHKT1;5A, that did not mediate K^+ transport (Uozumi *et al*. 2000; Ren *et al*. 2005; Munns *et al*. 2012), have been reported to maintain high shoot K^+ and low Na^+ under saline conditions by retrieving Na^+ from the root xylem (Rus *et al*. 2004; Ren *et al*. 2005; Sunarpi *et al*. 2005; Byrt *et al*. 2007). Moreover, root stele-specific overexpression of AtHKT1;1 resulted not only in a reduction in shoot Na^+ but also in a high K^+ concentration in shoots compared with controls in Arabidopsis and rice (Møller *et al*. 2009; Plett *et al*. 2010). The mechanisms by which HKT1-like transporters may affect K^+ homeostasis are still unknown. Physiological evidence suggests that this effect is indirect since Na^+ competes with K^+ for uptake, with one ion being capable of affecting the transport and accumulation of the other ion (Ren *et al*. 2005; Rus *et al*. 2005). It has been suggested that uptake of Na^+ via HKT1 could depolarize xylem parenchyma cells, which, in turn, may activate K^+ efflux channels such as *K^+-outwardly rectifying channels* (KORC), resulting in the release of K^+ into the xylem sap (Sunarpi *et al*. 2005 and references therein). Thus, the higher retention of K^+ found in NIL157-14 roots under low salinity might be a consequence of a weak *ScHKT1;2* allele in roots because of an indirect reduction in the K^+ loading of xylem vessels (see K_R mean comparisons in Table 6). In contrast, high expression of *ScHKT1;2* alleles in leaves should indirectly lead to a reduction of K^+ content in leaves and a subsequent reduction in K^+/Na^+.

The connection between the allelic variants of tomato *HKT1;1* and *HKT1;2* and salt tolerance is still unclear and mostly depends on salt tolerance criteria. Assuming that plant survival at the end of the salinity experiment is a salt tolerance criterion (Rus *et al*. 2006), only one plant from NIL 157-17 was close to death, even though it had one fruit. If salt tolerance is defined in terms of growth reduction, as some authors do (Qiu *et al*. 2011), NIL 157-14 (a homozygote for wild alleles), showing less root transcriptional activity for *ScHKT1;2* and no significant accumulation of Na^+ in its root, would be regarded as more salt tolerant than NIL 157-17, whose considerable root Na^+ accumulation might be the cause of the sharp reduction in its root growth as compared to NIL 157-14). Nevertheless, 157-17 is superior to 157-14 in relation to all traits under salinity conditions regardless of its larger reduction in growth (Supporting Information Table S6). It is important to point out that the more vigorous nature of 157-17 might be due to epistasis, as two significant epistatic interactions ($P = 0.02$) have been detected in the C-RIL population under salinity between either *HKT1;1* or *HKT1;2* and two markers in two linkage groups still segregating in RIL 157: SSRW223_800 on C11a in relation to fresh plant weight and SSR66_200 on C2a in terms of sodium leaf sensitivity.

Although stable silencing of *HKT1;1* and *HKT1;2* genes in both NILs is being carried out to show that these genes are responsible for the major QTL involved in Na^+ and K^+ homeostasis in tomato, current findings regarding their proximity to the genetic and physical position with a maximum LOD score as well as expression analysis support this hypothesis.

ACKNOWLEDGMENTS

We would like to thank Prof. Alonso Rodríguez Navarro for his critical reading of the manuscript, Dr. A. Granell and Dr. A.J. Monforte for comprehensive access to information on SolCAP SNPs, María Isabel Gaspar Vidal and Elena Sánchez Romero for technical assistance, the Instrumental Technical Service at EEZ-CSIC for DNA sequencing and ICP-OES mineral analysis and Michael O'Shea for proof-reading the text. This work was supported by ERDF-co-financed grants BIO2009-07655, AGL2010-17090 (A.B.) and RTA04-075-C2, AGL2008-00197/AGR (M.J.A.) from the Spanish Ministry of Science and Innovation. M.M.A. was supported by an ESF-cofinanced JAE-Predoctoral Program from CSIC, R.H. by grant AGR2005-436, P.A.D.M. by a postgraduate I3P grant from CSIC, N.J-P. by a F.P.I. Program and J.L. by a grant from the Spanish Ministry of Science and Innovation.

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Received 11 July 2012; accepted for publication 28 November 2012

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Stability of tomato EF1-*a* gene expression (expressed as cycle threshold -Ct-) in response to salt stress treatment in NIL157-14 (CC) and NIL157-17(LL).

Figure S2. Drop-test analysis of K^+-deficient phenotype complementation of tomato *HKT1*-like allelic variants from NIL157-14 (ScHKT1;1/ScHKT1;2) and NIL157-17 (SlHKT1;1/SlHKT1;2) in the WΔ6 yeast strain defective in K^+ transporters (Δ*trk1*, Δ*trk2*).

Figure S3. Sequence polymorphism in tomato *HKT1;1* Intron 1 and 2 regions from genomic sequences of *S. lycopersicum*, cv Heinz (H) and *S. lycopersicum* cv. Cerasiforme, NIL157-14 (CC) and NIL157-17(LL).

Figure S4. Gene expression of *SlHKT1;1* and *SlHKT1;2* in different tissues from tomato.

Figure S5. Relative gene expression of tomato HKT1*;1* and *HKT1;2* in response to salt stress in NIL157-14 (CC) and NIL157-17(LL).

Figure S6. Detail of the multiple alignments of *S. lycopersicum* HKT-like proteins and other plant HKT transporters used to construct phylogenetic tree in Fig. 2 showing the amino acid sequence region between transmembrane domain $M2_A$ and $M1_B$.

Table S1. Primers used for cloning and sequencing.

Table S2. Primers used for quantitative real-time PCR.

Table S3. Homology of tomato HKT1-like protein sequences with other plant HKT transporters.

Table S4. Known cis-elements in 5′ UTR of *HKT1;1* genomic sequences of *S. lycopersicum* cv. Cerasiform, NIL157-14 (CC) and NIL157-17 (LL) up to 1300 bp upstream of the translation start site.

Table S5. Known cis-elements in tomato *HKT1;2* promoter regions up to 814 bp upstream of the translation start site.

Table S6. Means, their standard errors (E.E.) and *P*-values of significant traits of NILs 157-14 and 157-17 under control (14 °C and 17 °C) and high salinity (14_S and 17_S) conditions.