The Na^+ transporter encoded by the *HKT1;2* gene modulates Na^+/K^+ homeostasis in tomato shoots under salinity

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ABSTRACT

Excessive soil salinity diminishes crop yield and quality. In a previous study in tomato, we identified two closely linked genes encoding HKT1-like transporters, *HKT1;1* and *HKT1;2*, as candidate genes for a major QTL (*lkc7.1*) related to shoot Na^+/K^+ homeostasis –a major salt tolerance trait –using two populations of recombinant inbred lines (RILs). Here, we determine the effectiveness of these genes in conferring improved salt tolerance using two near-isogenic lines (NILs) that were homozygous for either the *Solanum lycopersicum* allele (NIL17) or for the *S. cheesmaniae* allele (NIL14) at both *HKT1* loci; transgenic lines derived from these NILs in which each *HKT1;1* and *HKT1;2* had been silenced by stable transformation were also used. Silencing of *ScHKT1;2* and *SlHKT1;2* altered the leaf Na^+/K^+ ratio and caused hypersensitivity to salinity in plants cultivated under transpiring conditions, whereas silencing *SlHKT1;1/ScHKT1;1* had a lesser effect. These results indicate that *HKT1;2* has the more significant role in Na^+ homeostasis and salinity tolerance in tomato.

KEY-WORDS INDEX: *HKT1;1* and *HKT1;2*, K^+ and Na^+ homeostasis, posttranscriptional gene silencing, *Solanum lycopersicum* and *Solanum cheesmaniae*, tomato, salinity

INTRODUCTION

Approximately 7% of land throughout the world is affected by salinity, which encompasses ~30% of all irrigated agricultural land (Schroeder *et al.* 2013). Plant roots exposed to high salt concentrations causes both ionic and osmotic stress to most conventional crop plants (Munns & Tester 2008; Munns & Gilliham 2015). The major ionic stress associated with high salinity is due to sodium (Na^+) toxicity, which occurs when Na^+ is taken up by roots, transported to shoots in the transpiration stream and is accumulated in cells over time (Munns & Tester 2008). The accumulation of Na^+ in the cytosol negatively affects many plant physiological processes through as yet undetermined mechanisms (Hasegawa *et al.* 2000; Munns & Tester 2008). In addition, high external Na^+ concentrations reduces K^+ uptake and stimulates K^+ efflux, leading to insufficient cellular K^+ concentrations for enzymatic reactions and osmotic adjustment (Kronzucker *et al.* 2013; Hasegawa *et al.* 2013). To deal with this, plants have developed mechanisms to prevent the damage caused by cytosolic Na^+ accumulation, which includes the regulation of intracellular concentrations of Na^+ and K^+ (homeostasis), considered to be a key mechanism in saline stress tolerance (Kronzucker & Britto 2011; Roy *et al.* 2014).

Soil salinity adversely affects the yield of a wide variety of crops, including tomato, which, in economic terms, is the world's most important horticultural crop (Bergougnoux 2014). To reduce the impact of salinity on tomato, both technological and biological strategies have been implemented (Cuartero *et al.* 2006). The biological strategies, which are of great importance in sustainable agriculture, have involved using the genetic potential of crop varieties and related species for the identification of tolerance features, and their introgression into crops through plant breeding or their eventual manipulation by genetic engineering (Schroeder *et al.* 2013; Roy *et al.* 2014; Mickelbart *et al.* 2015). In tomato, genetic sources of variation for salt tolerance have been identified in some wild species, such as *Solanum*

pimpinellifolium and *S. cheesmaniae*, which could act as donors of this feature to tomato cultivars of commercial interest (Cuartero *et al.* 2006). Tomato species have a wide genotypic diversity for controlling Na^+ long-distance transport when cultivated under salt stress, where, in general, the more tolerant accessions accumulate more salt in stems and leaves and less in the roots compared to the more sensitive varieties (Cuartero & Fernandez-Muñoz; 1999; Cuartero *et al.* 2006). Indeed, tomato roots can, to a large extent, determine Na^+ concentrations reaching the aerial parts depending on the intensity of stress (Estañ *et al.* 2005; Asins *et al.* 2010, 2015). However, it must be noted that Na^+ accumulation in the leaves of the more salt-tolerant tomato plants differs with leaf age, with young leaves maintaining lower Na^+ concentrations than mature leaves (Cuartero & Fernandez-Muñoz, 1999). It appears that accumulation of Na^+ is particularly harmful for young leaves (Cuartero & Fernandez-Muñoz; 1999), so a Na^+ detoxification mechanism involving transporters that extrude Na^+ out of cells in these tissues could play a major role in tomato salt tolerance. In mature leaves, the main mechanism preventing Na^+ accumulation in the cytosol involves the combined action of transporters mediating Na^+ unloading from the root and leaf xylem and transporters promoting Na^+ and K^+ accumulation in vacuoles and endosomes. These systems facilitate the regulation of cytosolic Na^+, the maintenance of a high K^+/Na^+ ratio, and the use of Na^+ as a cheap osmoticum while alleviating its toxicity (Belver *et al.* 2012; Huertas *et al.* 2012, 2013).

In model plants such as Arabidopsis and rice grown under saline conditions, several transporters that influence Na^+ and K^+ homeostasis have been identified (Rus *et al.* 2005; Pardo *et al.* 2006; Pardo & Rubio 2011). The SOS1 antiporter, which extrudes Na^+ out to the external medium, allegedly also involved in directly loading Na^+ to the xylem as is expressed on xylem-xilem parenchima interface (Shi *et al.* 2000, 2002), while HKT1-like transporters are involved in Na^+ xylem unloading (Ren *et al.* 2005; Sunarpi et. *al*. 2005; Davenport *et al.* 2007; Møller *et al.* 2009; Plett *et al.* 2009), and NHX-like antiporters affect intracellular Na^+

and K^+ compartmentalization (Rodriguez-Rosales *et al.* 2009; Pardo & Rubio 2011; Bassil & Blumwald 2014). In tomato, these three transporter types have been implicated with important roles for salt tolerance. For instance, silencing (RNAi) of tomato *SOS1*, revealed that the plasma membrane antiporter Na^+/H^+ not only facilitates the extrusion of Na^+ out of the root, but also controls the distribution of this ion to other plant organs (Olias *et al.* 2009a,b). Constitutive overexpression of SlSOS2, one of the regulatory proteins of SlSOS1, increased salt tolerance, concurrent with an increase in *SlSOS1*, *LeNHX2* and *LeNHX4* transcript levels (Huertas *et al.* 2012; Belver *et al.* 2012). Interestingly, constitutive overexpression of *LeNHX2* by itself, which encodes an endosomal class II K^+/H^+ antiporter, improved salt tolerance (Huertas *et al.* 2013). Furthermore, two tomato genes encoding class I HKT transporters SlHKT1;1 and SlHKT1;2 that have been shown to be Na^+ selective transporters (Asins *et al.* 2013; Almeida *et al.* 2014a,b) are proposed to underlie a major salt tolerance QTL in tomato, located on chromosome 7, identified using two RIL populations derived from *S. lycopersicum* x *S. pimpinellifolium* (P-RIL) and *S. lycopersicum* x *S. cheesmaniae* (C-RIL) (Villalta *et al.* 2007, 2008, Asins *et al.* 2013, 2015). We hypothesise that, as with the HKT1-like transporters from mono- and dicotyledonous species that underlie other salt tolerance QTL (Ren *et al.* 2005; Møller *et al.* 2009; Plett *et al.* 2010; Munns *et al.* 2012; Byrt *et al.* 2014; Suzuki *et al.* 2016), these tomato transporters are responsible for unloading Na^+ from the xylem, thus preventing Na^+ accumulation in aerial parts and indirectly improving K^+ homeostasis.

Given the tight linkage between *HKT1;1* and *HKT1;2* in tomato (Asins *et al.* 2013), a reverse genetic strategy based on loss of gene function is necessary to determine which HKT1 transporter, if any, plays the main role in regulating Na^+/K^+ shoot concentration when cultivated under saline conditions. Here, we apply this reverse genetic strategy to two near isogenic lines (NILs) that vary in the allele at the *HKT1* loci from *S. lycopersicum* or *S.*

cheesmaniae. Conceptually, silencing a *HKT1* locus that leads to a decrease in the level of halotolerance in both NILs, would indicate that this specific *HKT1* locus has an important role in the salt tolerance mechanism in tomato. Therefore, different transgenic lines derived from the above NILs were generated, in which a particular allele (from *S. lycopersicum* or *S. cheesmaniae*) at *HKT1;1* or *HKT1;2* locus was silenced by stable gene transformation, and the phenotype for each genotype (6 in total) was evaluated in relation to salt tolerance. The results obtained provide a basis for future research on improving salt tolerance in the tomato and other horticultural crops.

MATERIALS AND METHODS

Plant material

Two tomato NILs differing in their *HKT1;1* and *HKT1:2* alleles were developed by selfing a segregating F6 line (RIL B157), which itself was obtained after 5 selfing generations of an F_1 progeny from a cross between a salt sensitive genotype of *S. lycopersicum*, var. Cerasiform as the female parent and a salt tolerant genotype of *S. cheesmaniae* (L. Riley) Fosberg as a male parent (Villalta *et al.* 2007, 2008). NIL157-14 (NIL14) is homozygous for the *S. cheesmaniae* allele at both *HKT1;1* and *HKT1*;2 while NIL157-17 (NIL17) is homozygous for the *S. lycopersicum* alelle at both *HKT1;1* and *HKT1;2* (Asins et al 2013). Regarding other genes involved in Na^+ homeostasis, both NILs have the same allele for *SOS1*, *SOS2*, *NHX2* and *NHX4* (erroneously named *NHX3* in Villalta *et al.* 2008). NILs are homozygous for the *S. cheesmaniae* allele at *SOS1* and *NHX4*, and for *S. lycopersicum* allele at *SOS2* and *NHX2* (M.J: Asins & A. Belver, unpublished results). Therefore, this study involves a particular set of genotypes where genetic differences among them are minimal: NIL14 and NIL17 are distinguished by the presence of either *S. cheesmaniae* or *S. lycoperscum* alleles at *HKT1;1* and *HKT1;2* loci respectively. These *HKT* alleles represent two tightly linked loci that could

correspond to locus duplication in tandem. Four additional lines were obtained by silencing each locus in each NIL, which have made segregation at the *HKT1* loci possible.

RNAi silencing of *S. lycopersicum* and *S. cheesmaniae* alleles at *HKT1* loci

Stable gene silencing via transformation with *Agrobacterium tumefaciens* was carried out using a pKANNIBAL/pART27 vector system (Wesley *et al.* 2001), which was used to produce a hairpin RNAi construct for each *HKT1;1/HKT1;2* allelic variant from *S. lycopersicum* and *S. cheesmaniae*. Two PCR fragments of either 597-bp or 477-bp, encoding either 199 or 159 amino acids of tomato HKT1;1 and HKT1;2, respectively were obtained (Supporting Information Fig. S1A) and then cloned in pKANNIBAL as previously described (Olías *et al.* 2009a) using appropriate forward and reverse primers (Supporting Information Table S1). The nucleotide sequences of *SlHKT1;2* and *ScHKT1;2* were identical, while the *ScHKT1;1* sequence in NIL157-14 showed a single SNP (G658C) causing a substitution in the predicted amino acid sequence (V222L) in the $M1_B$ helix region as compared with that of SlHKT1;1 in NIL157-17 (Supporting Information Fig. S1A, Asins *et al.* 2013). Alignment of both tomato *HKT1;1* and *HKT1;2* PCR fragments show overall 39.7% nucleotide identity and 65 % identity in coincident nucleotide sequences (Supporting Information Fig. S1B). The whole *NotI* cassette from pKANNIBAL bearing both RNAi constructs was subcloned into the corresponding site of the binary vector pART27, under the control of the CAMV35S promoter, which was introduced into *Agrobacterium tumefaciens* strain LBA4404 cells and used for plant transformation of both NIL157-17 and NIL157-14 from *S. lycopersicum* var Cerasiform, as described in Gisbert *et al.* (2000).

Analysis of transgenic plants

At least 10 independent primary transformants per each RNAi construct were obtained from NIL14 and NIL17 and their ploidy level analyzed according to Ellul *et al.* (2003). In order to

detect the presence of RNAi constructs, only diploid tomato plants from each independent transformation (T_0) event were used to obtain genomic DNA obtained from tomato leaves and this was screened by PCR analysis using pKANNIBAL-specific primers flanking the cDNA sense fragment and *nptII* gene-specific primers (Supporting Information Table S1) (Gen Elute^{TM} Plant Genomic DNA miniprep kit, Sigma-Aldrich, Spain). Only plants showing PCR bands for both sets of primers were considered as transgenic. Several T_0 transgenic plants were selected to study *Sc/SlHKT1;1* and *Sc/SlHKT1;2* expression patterns by qRT-PCR, using primers for tomato *HKT1;1/HKT1;2* (Supporting Information Table S2) as described below, and total RNA isolated from three different biological samples (roots and leaves of regenerated *in vitro* plants, as well as leaves of acclimated T_0 plants grown in pots with cocopeat as inert substrate, in a greenhouse under environmental conditions described below). T_0 lines, with reduced expression for each *HKT1;1/HKT1;2* allelic variant and their respective T_1 seeds obtained by self-pollination, were chosen for phenotypic analysis and collected for further phenotype assessment under saline conditions. Several independent T_1 lines with only one RNAi construct insertion for each *HKT1* allelic variant, were selected for further studies on the basis of kanamycin resistance segregation according to a monogenic and dominant inheritance pattern typical of this reverse genetic strategy (3RNAi-Kan^R:1WT) (Wesley *et al.* 2001; Olías *et al.* 2009a) (i.e. their progeny segregated as 1/4 homozygous, 2/4 hemizygous, both bearing the RNAi constructs, and 1/4 azygous WT plants). Azygous plants from the T_1 progeny were removed following identification with FNTPII and RNTPII specific primers through diagnostic PCR from DNA obtained from germinating tomato seedling cotyledons following a method for rapid genomic DNA preparation for PCR (Kasajima *et al.* 2004) (Supporting Information Table S1, Fig. S2).

Tomato plant growth conditions

Phenotypic evaluation of T_1 lines (devoid of azygous plants) plants was performed using seedlings grown in medium solidified with agar in Petri plates (under non-transpiring conditions), as well as plants grown in hydroponics and in pots (under transpiring conditions). As controls, we used the non-silenced NIL14 (NIL14C) and NIL17 (NIL17C) lines, which were also subjected to the whole gene transformation process without RNAi constructs.

Petri plate culture

The tomato seeds were surface-sterilized and germinated in Petri plates (10 x 10 cm) containing ¼ Hoagland medium (Hoagland & Arnon 1950).. Cultivation was performed 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}. The seedlings were kept under these conditions for 5 days, after which they were transferred in sterile conditions to new plates (24 x 24 cm) containing ¼ Hoagland medium supplemented with 175 mM NaCl for an additional 7 days. The aerial parts and roots were obtained separately for fresh and dry weight determination.

Pot culture

The sterilized seeds were sown in pots containing cocopeat as an inert substrate, maintained in a culture chamber at 24ºC in darkness, and irrigated with water until the emergence of the cotyledons (5-7 days). The plants were then transferred to a greenhouse with natural light irradiation supplemented with artificial light of 122 µmoles m^{-2} s^{-1}, with a photoperiod, temperature and humidity of 16/8 hours, 24°C/18°C and 40/55%, day/night, respectively. Watering was applied 2-3 times a week with a ¼ Hoagland solution (Hoagland & Arnon 1950). When plants were at the 6-leaf vegetative stage, saline treatment was applied using a ¼ Hoagland nutrient solution containing 100 mM NaCl for 15 days. Six pots per line containing one plant per pot were used, three of which received the saline treatment and the other three

only a nutrient solution (control treatment). Growth analysis was monitored determining the fresh and dry weight of the stem and leaves.

Hydroponic culture

Sterilized tomato seeds were germinated in plastic boxes containing sterile quartz sand (inert support) for 5-7 days in darkness and at 24°C. Germinated seeds were cultivated in a growth chamber, at 24°C/18ºC, day/night, in a 16-h light/8-h dark cycle, with irradiation of 140 μmol m^{-2} s^{-1} and 40-50 % relative humidity. Seedlings were watered for one week with a 1/10 dilution of Hoagland nutrient solution and for an additional week with a ¼ dilution of the same nutrient solution. Four-leaf plants were transferred to 2.5-L pots (three plants per pot) and grown in a greenhouse under the same conditions indicated for pots, in a hydroponic system for 15 days in an aerated ¼ dilution of Hoagland solution that was renewed every three days to avoid contamination. Ten days after hydroponic culture initiation, salt treatment was applied by adding 100 mM NaCl, 50 mM initially, and another 50 mM after 3 h in order to prevent an osmotic shock to the new ¼ dilution nutrient solution, with the plants growing for 6 additional days. Two pots with 3 plants per line were used, two of which received the saline treatment and the others only the nutrient solution (control treatment). Growth analysis was carried out as for pot culture.

Determination of fresh and dry tissue weight and Na^+ and K^+ content

Tissue samples from leaves, stems and roots were collected from each plant after treatment, washed four consecutive times in deionized water to eliminate salt adhering to the surface of the tissues and dried with filter paper. Tissue samples were weighed to determinate fresh weight, oven-dried at 70°C for 48 hours between filter paper and weighed to obtain dry weight. The dry material was digested in a HNO_3:$HClO_4$ (2:1, v/v) solution, and the content of Na^+ and K^+ was determined using inductively coupled plasma spectrometry (Varian ICP

720-E, Instrumental Technical Services, Estación Experimental del Zaidín, CSIC, Granada, Spain).

Gene expression localization in tomato tissues by *in situ* PCR

Untransformed tomato NIL14 and NIL17 were cultivated in hydroponics as described above and treated with salt for three days by adding 100 mM NaCl to the nutrient solution to promote gene expression of tomato *HKT1* and *SOS1* (Olías *et al.* 2009, Asins *et al.* 2013). After treatment, tissue samples from roots, stems and leaves were collected and embedded in agarose following the protocol previously described (Athman *et al.* 2014). Primers used for the cDNA synthesis step (reverse only) and PCR (both forward and reverse) were the same as those used for quantitative RT-PCR as indicated in Supporting Information Table S2.

Gene expression analysis by qRT-PCR

Tomato seeds were cultivated in hydroponics as described above. Salt treatment was applied by adding 100 mM NaCl, 50 mM initially and an additional 50 mM after 3 h. Tissue samples were collected at day 3 with 100 mM NaCl in hydroponic cultures. Three pots with 3 plants per line were used for the analysis (three independent biological samples). Total RNA was isolated from the root, stem and leaf tissues using the *Aurum™ Total RNA plant mini kit* (Bio-Rad Laboratories, S.A.) which included an in-column treatment with RNAse-free DNase (Promega Biotech Ibérica, SL) and resuspension in *RNAsecure™ resuspension solution* (Ambion Europa Ltd) 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 Laboratories, S.A.) 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 previously described (Huertas *et al.* 2012, Asins *et al.* 2013) using 1 µl of undiluted cDNA mixed with *iQ SyBr Green Supermix* (BioRad) and 0.45µM of forward and

reverse primers (Supporting Information Table S2) in a BioRad iCycler MyiQ2. 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. 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 each tissue from non-silenced and non salt-treated NIL17 as the calibrator sample.

Statistical analysis

A two-way analysis of variance (ANOVA) was used to test for the effect of genotype and treatment on the transcript levels (relative gene expression), growth (measured as fresh weight) and leaf contents of Na^+ and K^+. Post hoc comparisons of the mean were made using a Tukey HSD test. Statistical significance was considered at the conventional 5% level ($P \leq 0.05$). All calculations were performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, Inc. The Infostat statistical package (Balzarini *et al.* 2004) was used to study the variability among the 6 genotypes by Principal Component Analysis

RESULTS

Localization of *HKT1* expression

The tissue-specific expression of both transporters in tomato was investigated by using *in situ* PCR. The expression of both *HKT1;1* and *HKT1,2*, could be detected in cells of the vascular bundles of the main and secondary veins of tomato leaf while only *HKT1;2* could be detected in the stelar cells of root tissues (Fig.1). *HKT1;1* expression was undetectable in roots using this localization technique. These results indicate that both transporters are likely to be localized in the xylem parenchyma cells, and possibly, phloem-associated cells.

Silencing of *S. lycopersicum* and *S. cheesmaniae* alleles at *HKT1* loci

Independent primary diploid transformants (T_0) generated in NIL14 and NIL17 bearing each silencing construct of *HKT1;1* or *HKT1;2* were selected by diagnostic PCR (Supporting information Fig. S2). Gene expression analysis by RT-qPCR using total RNA isolated from three different biological samples from primary transformants confirmed that the selected lines exhibited a reduced gene expression for each *HKT1* locus as compared to that of the respective non-silenced NIL plants transformed and regenerated plants without silencing constructs (NIL14C and NIL17C) (Supporting information Fig. S3). Finally, those lines with *HKT1;1* or *HKT1;2* gene expression consistently reduced in the three biological samples were selected for phenotypic evaluation. Lines 14.1 and 34.1, silenced in NIL17 for *SlHKT1;1* and *SlHKT1;2*, respectively, and lines 1.2 and 47.1, silenced in NIL14 for *ScHKT1;1 and ScHKT1;2*, respectively, were considered to be appropriate for phenotypic evaluation purposes. All these lines had one copy of the silencing RNAi construct as indicated by segregation in the kanamycin resistance test (3:1) in the segregant population (T_1) (not shown).

Reducing *Sl/ScHKT1,2* gene expression caused a salt-hypersensitive phenotype in respective NIL-RNAi lines and altered their leaf Na^+/K^+ ratio

The gene expression patterns for *Sl/ScHKT1*-like allelic variants were analyzed in different tissues of the T_1 progeny of each *Sc/SlHKT1;1/HKT1;2*-RNAi line grown hydroponically and treated for 3 days with 0 and 100 mM NaCl. *HKT1*-like gene expression in non-silenced NILs was very similar to the expression pattern previously obtained (Asins *et al.* 2013), where the transcript levels of *SlHKT1;2* in the roots of NIL17 were considerably higher than those of *ScHKT1;2* in NIL14, while, in shoots (mainly leaves), their expression followed an opposite trend. The expression levels of *ScHKT1;1* in NIL14 were much higher in leaves and roots than those of *SlHKT1;1* in NIL-17 (Fig. 2). Also, salinity clearly increased the level of

HKT1;2 transcripts in the roots of NIL17 and reduced it in leaves and stems of both NILs. With respect to *HKT1;1*, gene expression generally decreased during saline treatment in both NILs, except in the roots of NIL17 which showed an increase in gene expression. Irrespective of experiment, treatment and tissue, gene expression at each *HKT1* locus of the respective T_1 progeny of each *Sc/SlHKT1;1/HKT1;2*-RNAi line was strongly reduced as compared to that of the respective non-silenced NIL plants (Fig. 2).

To first test the effect of *HKT1* silencing on salt tolerance at very early stages of tomato development, *HKT1*-silenced seedlings lines were grown *in vitro* on Petri plates in ¼ Hoagland medium supplemented with 175 mM NaCl for 5 additional days under non-transpiring and sterile conditions. The growth, measured as the fresh weight of aerial parts and roots, of all lines was similarly affected by salt treatment, regardless of the silenced *HKT1* locus, with growth being more affected in root than shoot under non-transpiring conditions (Supporting information Fig. S4). In addition, plants were cultivated under transpiring conditions in a greenhouse either using hydroponics in aerated nutrient solution with 100 mM NaCl for 6 days or in pots with cocopeat as inert substrate and irrigated with the same NaCl-containing nutrient solution for 15 days. This brought about a sharp reduction in the growth of the aerial part of *ScHKT1;2*- and *SlHKT1;2*-RNAi lines, measured as fresh weight, as compared to their respective non-silenced plants; this reduction was significantly higher in *ScHKT1;2*- than in *SlHKT1;2*-RNAi plants (Fig. 3 and 4). Root growth in hydroponics was also negatively affected by salt stress only in *ScHKT1;2*- and *SlHKT1;2*-RNAi lines (Fig. 4). Notably, plants of the *ScHKT1;1*-RNAi line grown without NaCl under transpiring conditions showed significantly higher fresh weight of leaf, shoot, stem and roots than its control, NIL14C (Figs. 3 and 4).

To examine the effect of silencing each *HKT1* locus on shoot Na^+/K^+ in each NIL grown under salinity conditions, Na^+ and K^+ leaf concentration was analysed under

transpiring (pots and hydroponics) (Fig. 5) and non-transpiring conditions (Petri dishes) (Supporting information Fig. S5). Under non-transpiring conditions in ¼ Hoagland medium, there were no differences among lines (Supporting information Fig. S5). Under transpiring conditions, in non-silenced NIL14 and NIL17 grown under salinity conditions, the previously observed trend for *S. cheesmaniae* and *S. lycopersicum* alleles for Na^+ and K^+ leaf concentration was reproduced (Asins *et al.* 2013): NIL14 had a higher leaf Na^+/K^+ ratio than NIL17 under salinity due to a higher Na^+ and lower K^+ concentration in leaves, although it was not statistically significant (Fig. 5). *SlHKT1;2*- and *ScHKT1;2*-RNAi lines, which exhibited a salt-hypersensitive phenotype, showed similarly high levels of Na^+ accumulation and lower K^+, and consequently higher Na^+/K^+ ratios in leaves than their respective non-silenced NILs (Fig. 5). In contrast, silencing of *ScHKT1;1* in NIL 14 and *SlHKT1;1* in NIL17, which respectively, had less or no significant effect on growth under saline conditions, scarcely affected the leaf Na^+/K^+ ratio as compared to their respective non-silenced NIL lines under salinity conditions (Fig. 5).

ScSOS1, *LeNHX2* and *ScNHX4* gene expression was analyzed in different tissues of each *Sc/SlHKT1;1/HKT1;2*-RNAi line subjected to 100 mM NaCl for 3 days in hydroponics (Fig. 6). In all tissues from non-silenced NIL17, *ScSOS1* expression levels appeared to be similar with or without NaCl, while in non-silenced NIL14 salt treatment increased *SOS1* expression in the aerial part (stems and leaves) and decreased it in roots. This behaviour was not observed in NIL14 following silencing of any *HKT1* allele. However, silencing *SlHKT1;1* (in NIL17) was accompanied by a significant increase in *SOS1* expression in leaves under saline treatment as occurred in non-silenced NIL14 (Fig.6A). Silencing each *HKT1* allelic variant had little effect on *LeNHX2* expression regardless of the tissue and NIL involved (Fig. 6B). Salinity induced similar changes in *ScNHX4* transcript abundance in stem or root (no change) of non-silenced NILs but differences in leaf levels. Notably, the behaviour of

SlHKT1;1-RNAi was similar to that of non-silenced NIL 14 in leaf, i.e. both genotypes increased transcription of *ScNHX4* under salinity (Fig. 6C). In the root, silencing *SlHKT1;2* was associated with a significant increase in *ScNHX4* transcript abundance under salinity, while in the absence of NaCl, *ScHKT1;1*-RNAi plants showed a reduced level of *ScNHX4* transcript. Therefore, the genotype at the *HKT1* loci (*S. lycopersicum* or *S. cheesmaniae* alleles, silenced or not) affects the transcription behaviour of the *S. cheesmanieae* alleles at *SOS1* and *NHX4* loci.

Finally, the complex relationship among traits (i.e. the transcription of genes involved in Na^+ homeostasis, vegetative growth and the Na^+ and K^+ concentration in different plant tissues) in the different genotypes was studied by means of principal component analysis (Fig 7). Without NaCl, the closest genotypic responses were *SlHKT1;2*-RNAi and *ScHKT1;1*-RNAi lines, whilst NIL 14 had the most disparate response. This variation is best explained by component 1 (CP1 in Fig 7A): stem *SOS1* expression (S_sos1_c) and root *NHX4* expression (R_nhx4_c). Under salinity, NIL 17 was placed at the right side of Fig 7B near to its silenced line at *HKT1;1*, while both NILs silenced at *HKT1;2* are placed at the left side. Traits contributing most to the first component in Fig. 7B, were leaf fresh weight (LFW_s), stem fresh weight (SFW_s), leaf sodium concentration (LNC_s), and root *NHX4* expression (R_nhx4_s).

DISCUSSION

Tomato *HKT1;1* and *HKT1;2* genes are localized to vascular bundles

Tomato *HKT1;1* and *HKT1;2* genes encode Na^+-selective class I-HKT transporters (Asins *et al.* 2013, Almeida *et al.* 2014a,b). Previous gene expression analysis revealed that *HKT1;1* and *HKT1;2* were ubiquitously expressed in all complex tissues analyzed (Asins *et al.* 2013). Here, using an *in situ* PCR protocol (Athman *et al.* 2014), we have found that tomato *HKT1;2*

was expressed in the vascular system, including xylem and phloem cells of tomato leaves and roots, while expression of *HKT1;1* was detected in the same cell types only in leaves (Fig 1). *HKT1;1* expression in roots was undetectable using *in situ* PCR, probably due to its very low expression (Asins *et al.* 2013, Almeida *et al* 2014a, Supporting information Fig S6). In a previous study, Arabidopsis plants transformed with *SlHKT1;2prom::GUS* showed strongly stained cells adjacent to the xylem and phloem vascular tissues of both leaves and roots (Almeida *et al.* 2014a). Like other members of class I HKT transporters from dicots and monocots characterized up to now, tomato HKT1;1 and HKT1;2 are therefore likely to be responsible for unloading Na^+ from the xylem, thus preventing Na^+ accumulation in shoots (Sunarpi *et al.* 2005; Ren *et al.* 2005; Munns *et al.* 2012; Byrt *et al.* 2014). Our localisation also suggests in addition to xylem Na^+ unloading, HKT1;1 and/or HKT1;2 might be involved in Na^+ loading into the phloem sieves. This would suggest their involvement in Na^+ redistribution towards sink organs and tissues, as previously hypothesized for *AtHKT1;1* (Maser *et al.* 2003; Berthomieu *et al.* 2003; Sunarpi *et al.* 2005), even though this functional role has been seriously questioned (Davenport *et al.* 2007). Nevertheless, there is circumstantial evidence to show that tomato HKT1;1 and/or HKT1;2 could be involved in Na^+ loading into the phloem sap in leaves and unloading in sink organs, such as fruit and roots (Asins *et al.* 2015).

HKT1;1 gene expression plays a minor role in Na^+/K^+ homeostasis in the aerial part of tomato

In previous studies, genetic, molecular and physiological data provided strong evidence that natural genetic variation at closely linked loci *HKT1;1* and/or *HKT1;2* could explain the major QTL in chromosome 7 (*lkc7.1*) governing shoot Na^+/K^+ homeostasis in two RIL populations derived from *S. lycopersicum* and two salt tolerant accessions from the wild species *S. cheesmaniae* and *pimpinellifolium* (Villalta *et al.* 2008; Asins *et al.* 2013, 2015).

In this study, silencing of either *SlHKT1;1* or *ScHKT1,1* allelic variants did not significantly inhibit the growth or alter the Na^+/K^+ ratio of plants grown in hydroponics or in pots under salinity conditions (Figs. 3 and 4). Therefore, the *HKT1;1* gene, although expressed in the same type of vascular cells as *HKT1;2* (Fig. 1), seems to play a minor role in Na^+ transport and Na^+/K^+ homeostasis in the aerial part of the plant. In fact, the expression of *HKT1;1* was always much lower than that of *HKT1;2*, irrespective of the NIL and tissue considered (Asins *et al.* 2013, Almeida *et al* 2014a, Supporting information Fig. S6). The *ScHKT1;1* allele had a single substitution in the amino acid sequence (V222L, Val222Leu) in the M1B helix region as compared to the allele *SlHKT1;1* (Asins *et al.* 2013). However, this substitution did not correspond to substitutions reported to influence salt tolerance, K^+ selectivity or other functional properties of HKT transporters when expressed in heterologous systems (Corratgé-Faillie *et al.* 2010; Asins *et al.* 2013; Ali et *al.* 2016, and references therein). Whether such a substitution provides different kinetic properties inducing a physiological effect on Na^+/K^+ homeostasis in tomato is still uncertain. In fact, the kinetic parameters of *SlHKT1;1/ScHKT1;1* allelic variants (in addition to *SlHKT1;2/ScHKT1;2*) in yeast mutants defective in endogenous K^+ transporters *(Δtrk1* and *Δtrk2*) were analyzed in our laboratory following procedures described elsewhere (Haro *et al.* 2005; Asins *et al.* 2013); however, the data on the kinetic parameters (Km and Vmax) of SlHKT1;1/ScHKT1;1 allelic variants obtained were highly variable, which prevented us from carrying out a reliable statistical analysis of their different kinetic properties (not shown). A previous study was unable to record any transport activity in oocytes expressing *HKT1;1* from *S. lycopersicum* or *S. pennellii* (Almeida *et al.* 2014a). This could be due to a number of pitfalls that occurs when HKT1 proteins are expressed in heterologous systems (Garciadeblas et al 2003; Haro et al. 2005). However, the pattern of expression of *ScHKT1;1* in NIL14 greatly differed from that of *SlHKT1;1* in NIL 17. *ScHKT1;1* gene expression in leaves and roots of NIL 14 was higher

than that of *SlHKT1;1* in NIL 17 (Fig. 2). Like for *HKT1;2*, differences found in the frequency of specific *cis*-elements in their respective promoter of sequences may account for this differential expression (Asins *et al.* 2013). Moreover, data on transcription levels of *HKT1;1* in leaves of NIL14 suggest that *S. cheesmaniae* is similar to *S. pimpinellifolium* in the sense that, *HKT1;1* expression occurs in leaves of wild species contrary to *S. lycopersicum* (Supporting information Fig S6). Therefore, cultivated tomato species have diverged from both wild species regarding the regulation of *HKT1;1* expression. The loss of leaf (and, perhaps, root) *HKT1;1* expression and the fixation of a hyperactive *HKT1;2* allele have occurred during the domestication of tomato.

NIL14 and NIL17 differed in the transcriptional changes that occurred between control and salinity treatments for root *ScNHX4*, and *ScSOS1* in leaf, stem and root tissues (Fig. 6), and these differences disappeared for *ScHKT1* silenced lines. Whether or not these regulatory changes occurred during domestication and are responsible for a loss of adaptability to environmental variability in NaCl and nutrient concentrations is difficult to assess without additional experiments. Interestingly, there was a significant increase in the plant growth of *ScHKT1;1*-silenced NIL 14 with respect to the non-silenced genotype (Figs. 3 and 4) making *ScHKT1;1*-RNAi plants as large as NIL17 plants under the absence of NaCl, which is unlike the natural growth habitat of *S. cheesmaniae*.

Na^+/K^+ homeostasis in the aerial part of tomato is mainly regulated by the Na^+ transporter encoded by the *HKT1;2* gene

The results obtained in this study provide strong evidence that Na^+/K^+ homeostasis in tomato leaves is mainly regulated by the *HKT1;2* locus regardless of allele (*S. cheesmaniae* or *S. lycopersicum*). The growth of *HKT1;2*-silenced NILs 14 and 17, particularly under transpiring conditions, showed greater sensitivity to salinity compared to their respective non-silenced plants (Figs. 3, and 4), and this explains their association in the principal component

analysis under salinity (Fig 7B). Both silenced lines showed similarly high levels of Na^+ and lower levels of K^+ and consequently increased Na^+/K^+ ratios (Fig. 5). The increased sensitivity to salt stress in *ScHKT1;2*- and *SlHKT1;2*-silenced lines may therefore be a consequence of altered Na^+/K^+ ratios due to loss of function of *ScHKT1;2* and *SlHKT1;2*, respectively. This salt-hypersensitive phenotype was very similar to that of the Arabidopsis *hkt1;1* mutant, which was characterized by a hyperaccumulation of Na^+ and a reduction in K^+ in shoots under transpiring conditions (Mäser *et al.* 2002; Berthomieu *et al.* 2003; Sunarpi *et al.* 2005; Davenport *et al.* 2007; Supporting information Fig. S7), indicating that tomato *HKT1;2* plays a similar role to *AtHKT1;1*, particularly in roots, as suggested in a previous study (Asins *et al.* 2013). It was also reported by Almeida *et al.* (2014c) that Na^+ concentrations in both leaves and stems were positively correlated with *HKT1;2* expression in the roots of 23 tomato accessions.

S. cheesmaniae (or *pimpinellifolium*) alleles at the major QTL, *lkc7.1*, enable the storage of more Na^+ and less K^+ in the aerial part of the plant, while *S. lycopersicum* alleles have the opposite effect (Villalta *et al.* 2008). This trait could be explained if the main function of HKT1;2, which is localized in xylem associated cells (and possibly in phloem-associated cells) from leaves and roots (Fig. 1), is to retrieve Na^+ from the xylem in roots in accordance with HKT1-like transporters in dicots and monocots (Hauser & Horie 2010; Su *et al.* 2015). Although the ion transport kinetics of SlHKT1;2 and ScHKT1;2 have not been measured, given the identical nucleotide-encoding sequences (Asins *et al.* 2013), distinct Na^+ transport rates due to differential affinities for Na^+ by these transporters, as reported for SlHKT1;2 and *S. pennelli* HKT1;2 (Almeida *et al.* 2014a), can be ruled out. Therefore, differences in tomato leaf Na^+ content (and K^+ content) is probably mainly influenced by *HKT1;2* transcript abundance. In fact, differences found in the frequency of specific *cis*-elements in their respective promoter sequences may account for the lower expression of

ScHKT1;2 in the roots of NIL14 as compared to that of *SlHKT1;2* in NIL17 (Asins *et al.* 2013). In roots, low transcription of *ScHKT1;2* in NIL14 (fixed for the *HKT1;2* hypoallele) would imply lower Na^+ retrieval from the xylem, and consequently higher Na^+ transport via the transpiration stream to the aerial part as compared to the higher expressed (*HKT1;2* hyperallele) *SlHKT1;2* in NIL17. At the same time, increased expression of *ScHKT1,2* and, to some extent, of *ScHKT1;1*, in leaves from NIL14 (Fig. 2) might increase the withdrawal of Na^+ from the leaf xylem, thus promoting its intracellular accumulation in the mesophyll cells of expanding leaves. Similarly to a previous study (Asins *et al.* 2013) and despite the apparent trend towards a higher accumulation of Na^+ in NIL14 leaf and, consequently a higher Na^+/K^+ ratio than that of NIL17, there was no statistically significant difference in leaf Na^+ and K^+ concentrations between non-silenced NIL14 and NIL17, when plants were subjected to saline treatment under transpiring conditions (Fig 5).

Salt-induced leaf damage is thought to be caused by salt accumulation in the cytoplasm or apoplast compartments, when the rate of Na^+ export from roots to leaves exceeds that of Na^+ delivery across the plasma membrane of leaf cells or when vacuolar Na^+ storage capacity is saturated (Munns & Tester 2008). In Arabidopsis, the hypersensitivity to salt stress in the *athkt1;1* (*sas2*) mutant was due to an excessive rate of Na^+ accumulation in shoots, especially when plants transpired considerably, and to a reduction in shoot K^+ (Mäser *et al.* 2002; Berthomieu *et al.* 2003). Moreover, *AtHKT1;1* loss of function has been reported to negatively affect, though indirectly, tissue vacuolar loading (Davenport *et al.* 2007). Therefore, the salt hypersensitivity of leaves in *Sc/SlHKT1;2*-silenced plants, may be due to combined *HKT1;2* loss of function in roots, increasing the rate of Na^+ export from roots to leaves, and *HKT1;2* loss of function in the cells of leaf vascular bundles, preventing Na^+ delivery across the plasma membrane and subsequent compartmentation into vacuoles. This explanation was also proposed for the salt hypersensitive Arabidopsis *sas1* mutant (Nublat *et*

al. 2001). Interestingly, the reduction in growth of the aerial part caused by salinity was even higher when the *ScHKT1;2* hypoactive allele was silenced in NIL14 than when the *SlHKT1;2* hyperactive allele was silenced in NIL17 (Figs. 3, 4), although both types of *HKT1*-silenced lines showed a similar increase in Na^+ concentration and decrease in K^+, and consequently, a similar increase in Na^+/K^+ ratios in leaf (Fig. 5). This effect may be partly due to the severe reduction in *HKT1;2* expression in the roots as well as the usually high *HKT1;2* expression in leaves in *ScHKT1;2*-silenced NIL14 (Fig. 2), which may decrease the unloading of Na^+ from the xylem in leaves. Also, phloem loading and redistribution to roots might be affected. This could allow its accumulation in the apoplast of mesophyll cells of expanding leaves which might negatively affect Na^+ intracellular accumulation in vacuoles. In tomato, K^+,Na^+/H^+ antiporters from endosomal class II NHX2 and vacuolar class I NHX4 prevent Na^+ toxicity at the cellular level through the efficient sequestration of this cation into subcellular compartments (Venema *et al.* 2013; Galvez *et al.* 2012; Huertas *et al.* 2012, 2013). No differences in the expression of these genes between NIL17 and NIL14 would be expected due to genomic differences because both NILs share the same alleles at both loci (Asins *et al.* 2013). However, there could be differences in endosomal and vacuolar Na^+/H^+ antiporter activities due to differences in Na^+ concentrations in cytosol or differences in transcript levels between the NILs. In this study, while the expression of *LeNHX2* in leaves was slightly, but significantly, enhanced in *SlHKT1;2*- compared to *ScHKT1;2*-silenced lines by salt treatment, the expression of *ScNHX4* in leaves was more reduced in *ScHKT1;2*- than in *SlHKT1;2*-silenced lines in response to salt stress (Fig 6). Therefore, the capacity for Na^+ detoxification in leaves, based on sequestration into the leaf vacuole, could be reduced in *ScHKT1;2*-silenced lines when the rate of Na^+ import into the leaf is excessively high due to both the combined reduced expression of *ScHKT1;2* in roots and leaves, as well as of *ScNHX4* in the leaves. It is worth noting that the root *ScNHX4* transcription level in the *SlHKT1;2*-silenced

line significantly increased under salinity in comparison to non-silenced NIL17 while root *ScNHX4* transcription level of the Sc*HKT1;2*-silenced line remains as high as that from non-silenced NIL14 (Fig. 6). It is important to take into account that root *NHX4* expression under salinity (R_nhx4_s in Fig. 8B) is inversely related to fresh weight traits under salinity (salt tolerance) and provides a major contribution to the first factor of principal component analysis to explain variability among the six genotypes under study. An additional explanation for the higher salt-hypersensitivity of *ScHKT1;2*-silenced lines as compared to *SlHKT1;2*–silenced lines could be provided by the usually highly expressed *HKT1;1* in leaves from *ScHKT1;2*-silenced NIL14 (Fig. 2). This may increase the unloading of Na^+ from the xylem in leaves, thus allowing its accumulation to toxic levels in the mesophyll cell cytosol of expanding leaves, particularly, taking into account that the capacity for Na^+ detoxification in leaves and salt tolerance being based on sequestration into the leaf vacuole, could be reduced due to a lower expression of *ScNHX4* in *ScHKT1;2*-silenced lines..

Apart from HKT1-like transporters, the plasma membrane Na^+/H^+ antiporter, SlSOS1, is also involved in long-distance Na^+ transport in tomato, (Olías *et al.* 2009a). It has been suggested that the transport function of SOS1 in xylem loading in roots is coordinated with that of HKT1-like transporters in xylem unloading in leaves for long-distance transport of Na^+ (Pardo *et al.* 2006, Belver *et al.* 2012). Accordingly, a perturbation in either system could alter long-distance Na^+ transport and the appropriate partitioning of Na^+, resulting in a salt-sensitive phenotype. Therefore, SOS1 may also be directly or indirectly involved in altering the Na^+/K^+ leaf ratio in *HKT1;2*-silenced plants. *SOS1* maps to chromosome 1 (Villalta et al 2008), and both NIL 17 and NIL 14 have the same *S. cheesmaniae* alleles (Asins et al 2013). As for NHX transporters, differences in the expression of *SOS1* gene between NIL17 and NIL14 were not expected due to genomic differences. A recent study has shown that *Nax* loci, *Nax*1, functionally supported by TmHKT1;4-A2 (Huang *et al.* 2006), and *Nax*2, supported by

TmHKT1;5-A (Byrt *et al.* 2007), negatively regulate the activity and expression levels of a *SOS1*-like Na^+/H^+ exchanger in the xylem tissue of wheat (Zhu *et al.* 2015). These authors suggest that *Nax* loci confer two highly complementary mechanisms, both of which contribute to reducing xylem Na^+ content. One enhances the retrieval of Na^+ back into the root stele via HKT1;4 or HKT1;5, whilst the other reduces the rate of Na^+ loading into the xylem via SOS1. However, in this study, *ScSOS1* expression only decreased in the root of NIL14, and concurrently increased in the aerial part (leaf and stem) in response to salt treatment, but appeared to be unaffected in stem and root of NIL17. In accordance with the above mentioned hypothesis (Zhu *et al.* 2015), it is possible that hypoactive *ScHKT1;2* in NIL14 roots enables the storage of more Na^+ and less K^+ in the aerial part of the plant, thus rapidly achieving full osmotic adjustment while maintaining normal growth. Once osmotic adjustment is achieved, it would be advantageous for NIL14 plants to prevent excess Na^+ accumulation in photosynthetically-active leaf tissues by reducing the rate of xylem Na^+ loading by ScSOS1 to an absolute minimum to maintain cell turgor in growing tissues. On the other hand, in the aerial part of NIL14 treated with salt, increased expression of *ScSOS1* could mediate Na^+ efflux through the plasma membrane of mesophyll cells into the xylem, mainly in younger leaves, to prevent Na^+ toxicity in less vacuolated cells with no efficient ion compartmentation mechanism (Olias *et al.* 2009a, 2009b). Such changes in the expression of *ScSOS1* are suppressed in *ScHKT1;*2-RNAi line (Fig. 6), indicating some kind of functional relationship between the two types of transporters in tomato. Some additional evidence of this functional relationship has been obtained using *SlSOS1*-silenced tomato plants (*S. lycopersicum* cv. Moneymaker). These plants, which also displayed a salt-hypersensitive phenotype, a Na^+ distribution root-to-leaf gradient and a reduced capacity to accumulate Na^+ in stems (Olías *et al.* 2009a, 2009b), showed a dramatic increase in the expression levels of

SlHKT1;1 in all plant tissues, especially under salt stress, and a concomitant reduction in *SlHKT1;2* transcript levels after 3 d of salt treatment (Supporting information Fig. S8).

It is worth noting that under non-transpiring conditions, growth of all non-silenced and silenced lines was similarly affected by salinity (Supporting Information Fig. S4), and displayed similar increases in leaf Na^+ and reductions in leaf K^+ contents (Supporting Information Fig. S5). Similar results were obtained with Arabidopsis *hkt1;1* mutants cultured in Petri dishes under non-transpiring conditions (Supporting information Fig. S7). In the absence of transpiration, the salt tolerance mechanism in tomato seedlings probably depends on Na^+ extrusion to the root external medium and/or Na^+ accumulation in root vacuoles rather than on long-distance transport and unloading of Na^+ from the xylem by the HKT1 system (Shi et al. 2002; Berthomieu *et al.* 2003; Huertas *et al.* 2012).

In conclusion, the present study indicates that *HKT1;2* plays an important role in Na^+ (and K^+) homeostasis and in salinity tolerance of tomato; silencing of *HKT1;2* altered the leaf Na^+/K^+ ratio and increased salt hypersensitivity, unlike *HKT1;1*. This confirms our previous hypothesis that the *HKT1;2* gene is responsible for the major QTL involved in Na^+ and K^+ homeostasis in tomato (Villalta *et al.* 2008 Asins *et al.* 2013). Furthermore, the greater effect of silencing the *S. cheesmaniae HKT1;2* allele compared to the *S. lycopersicum* allele on growth of tomato NILs under salinity, suggests a more potent role for the *S. cheesmaniae HKT1;2* allele in salt tolerance. The combined action of this transporter and other Na^+ transporters, like SOS1 and NHX4, are required to regulate internal concentrations of Na^+ in various tissues, and also indirectly for K^+ homeostasis, through extrusion through the plasma membrane, compartmentation of salts into cell vacuoles and distribution of ions through the plant organs.

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FIGURE LEGENDS

Figure 1. Tissue localization of tomato *HKT1;1* and *HKT1;2* by i*n situ* PCR. The blue stain indicates the presence of transcript. Expression of elongation factor-1α (*SlEF-1α* is seen in all cell types) is shown as positive control while a no RT (reverse transcription) in NIL14 is used as negative control to show lack of genomic DNA contamination. **A)** Shows the expression of *HKT1;1* and *HKT1;2* in the vascular bundle of NIL 14 leaf sections (midvein in the left panels). **B)** Vasculature-specific expression of *HKT1;2* in NIL17 root sections. **C)** Diagram of a leaf cross-section (top panel) and a root cross-section (lower panel) showing the different tissues. Images on the right are magnifications on their respective left images. Scale bars represent 100μm

Figure 2. Transcript levels of *HKT1* and *HKT1;2* in root, stem and leaf tissues of different silenced lines of *HKT1;1/HKT1;2* allelic variants from *lycopersicum* and *cheesmaniae* in two tomato NILs. NIL17C and NIL14C lines are NIL17 and NIL14, respectively, transformed and regenerated without a silencing construct. Total RNA was purified from the leaf, stem and root of five-week-old T_1 transgenic plants cultivated for 24 days on hydroponics and treated for 3days with 0 (dark bars) and 100 mM NaCl (clear bars). The tomato elongation factor gene (*LeEF-1α*) was used as the reference gene. The results show the expression of each *HKT1* gene as an increase or decrease in their transcript levels relative to those in the roots, stems and leaves of untransformed plants cultivated in the absence of stress, to which value 1 is assigned. Each value is the mean ± the standard error of the mean (SEM) from nine repeats for roots, stems and leaves (three biological and three technical repeats). Significant differences are indicated by different letters according to Tukey's test ($p<0.05$).

Figure 3. Effect of NaCl treatment on growth, measured as fresh weight of leaves and stems in different silenced lines of *SlHKT1* (blue bars) and *ScHKT1* (red bars) grown in pots. A) Five-week-old T_1 transgenic plants cultivated in cocopeat in pots and irrigated with 1/4x Hoagland solution in a greenhouse. **B)** Fresh weight of leaves and stems. Plants were treated with 0 mM NaCl (dark bars) and 100 mM NaCl (clear bars) for 15 days. Each value is the mean of 3 replications (3 different pots) ± SEM. Significant differences ($P < 0.05$) are indicated by different letters according to Tukey's test

Figure 4. Effect of NaCl treatment on growth, measured as fresh weight of aerial part and roots in different silenced lines of *SlHKT1* (blue bars) and *ScHKT1* (red bars) grown in hydroponics. A) Plants were cultivated for 24 days on hydroponics with an aerated 1/4x Hoagland solution in a greenhouse, and treated for 6 days with 0 (dark bars) and 100 mM NaCl (clear bars). **B)** Fresh weight of shoots and roots. Each value is the mean of 3 replications (3 different buckets) ± SEM. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test.

Figure 5. Leaf contents of Na^+ and K^+ in control and salt-treated plants of *SlHKT1;1/HKT1;2* and *ScHKT1;1/HKT1;2*-silenced NIL lines grown in pots and in hydroponics. Leaf content of Na^+ and K^+ in control (dark bars) and salt-treated (clear bars) from non-silenced and silenced NIL 17 (*SlHKT1* alleles) and NIL 14 lines (*ScHKT1* alleles). Tomato plants were grown in pots and in hydroponics as indicated in the legends for figures 3, 4 and 5, respectively. Values represent the mean ± SEM of three different samples. Significant differences ($P < 0.05$) are indicated by different letters according to Tukey's test

Figure 6. Transcript levels of *ScSOS1*, *LeNHX2* and *ScNHX4* in root, stem and leaf tissues of different silenced lines of *HKT1;1/HKT1;2* allelic variants from *lycopersicum* and *cheesmaniae* in two tomato NILs. NIL17C and NIL14C lines are NIL17 and NIL14, respectively, transformed and regenerated without a silencing construct. Total RNA was purified the leaf **(A)**, stem **(B)** and root **(C)** of five-week-old T_1 transgenic plants cultivated for 24 days in hydroponics and treated for 3days with 0 (dark bars) and 100 mM NaCl (clear bars). The tomato elongation factor gene (*LeEF-1α*) was used as the reference gene. The results show the expression of each gene as an increase or decrease in their transcript levels relative to those in roots, stems and leaves of untransformed plants cultivated in the absence of stress, to which value 1 is assigned. Each value is the mean ± SEM from nine repeats for roots, stems and leaves (three biological and three technical repeats). Significant differences are indicated by different letters according to Tukey's test ($p<0.05$).

Figure 7. Graphic representation (biplot) of principal component analysis of variability found among 6 closely related genotypes: 1 (NIL17C), 2 (*SlHKT1;1*-RNAi), 3 (*SlHKT1;2*-RNAi), 4 (NIL14C), 5 (*ScHKT1;1*-RNAi) and 6 (*ScHKT1;2*-RNAi) under control condition (A) and salinity (B) for evaluated traits (expression of genes, and physiological and vegetative plant traits.

SUPPORTING INFORMATION

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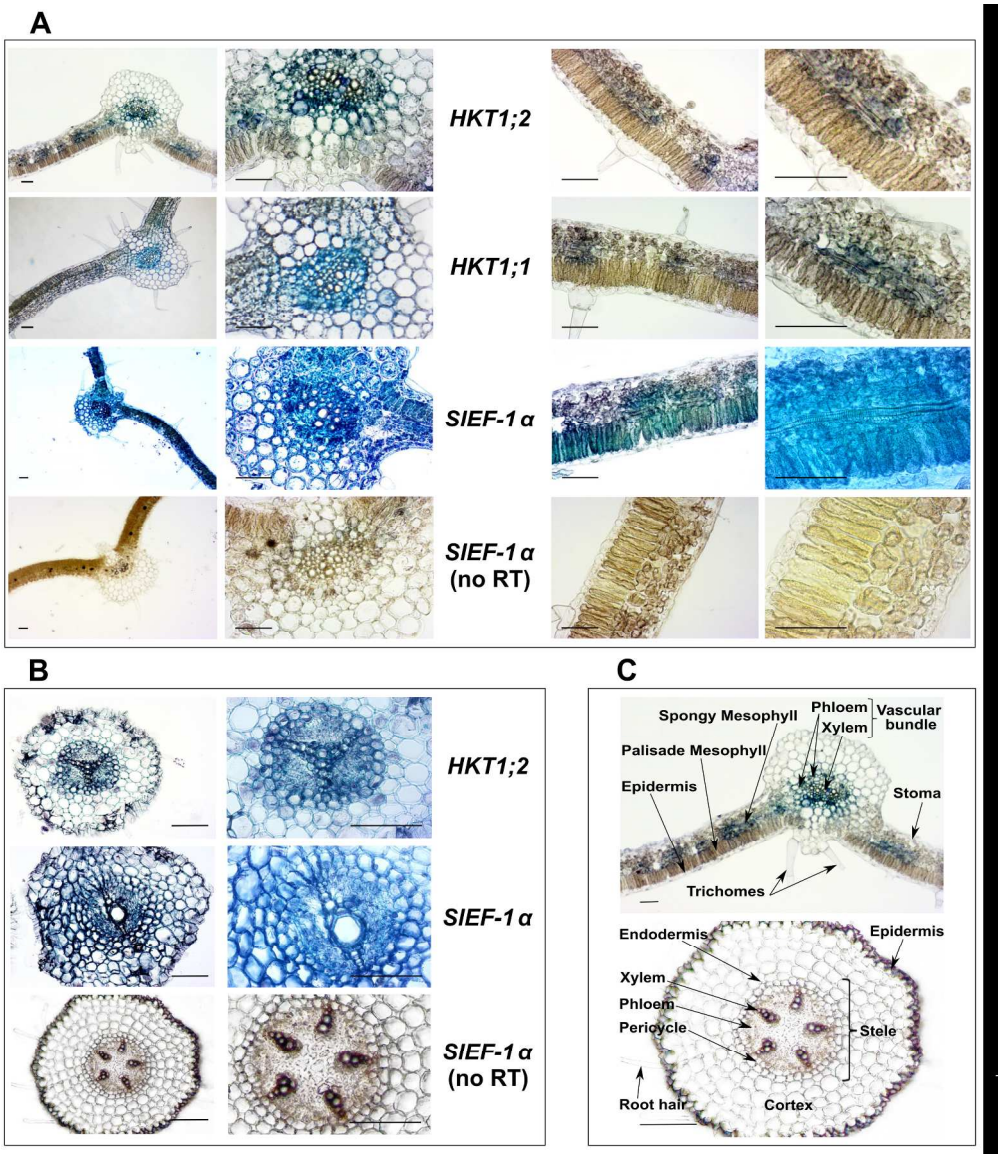
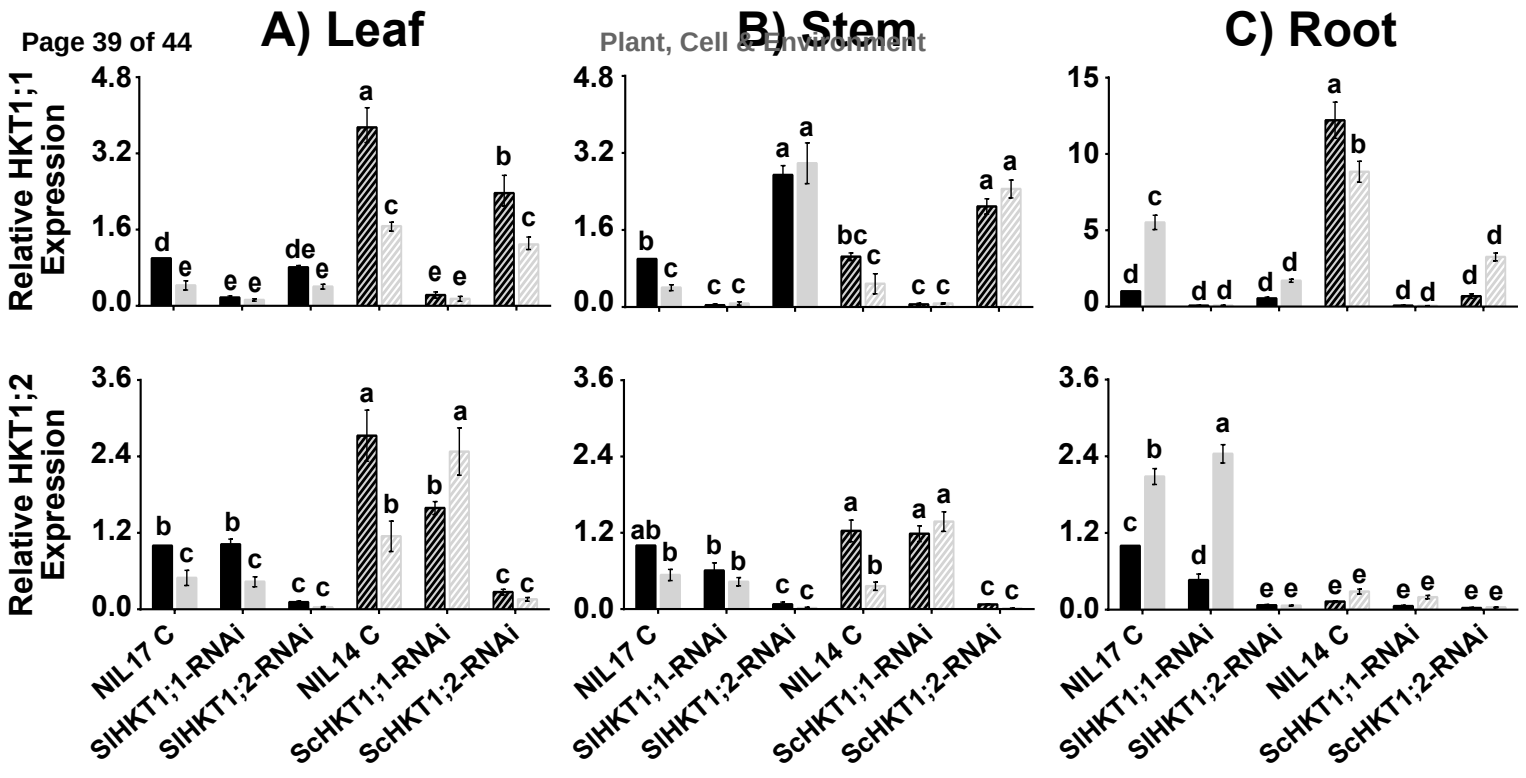
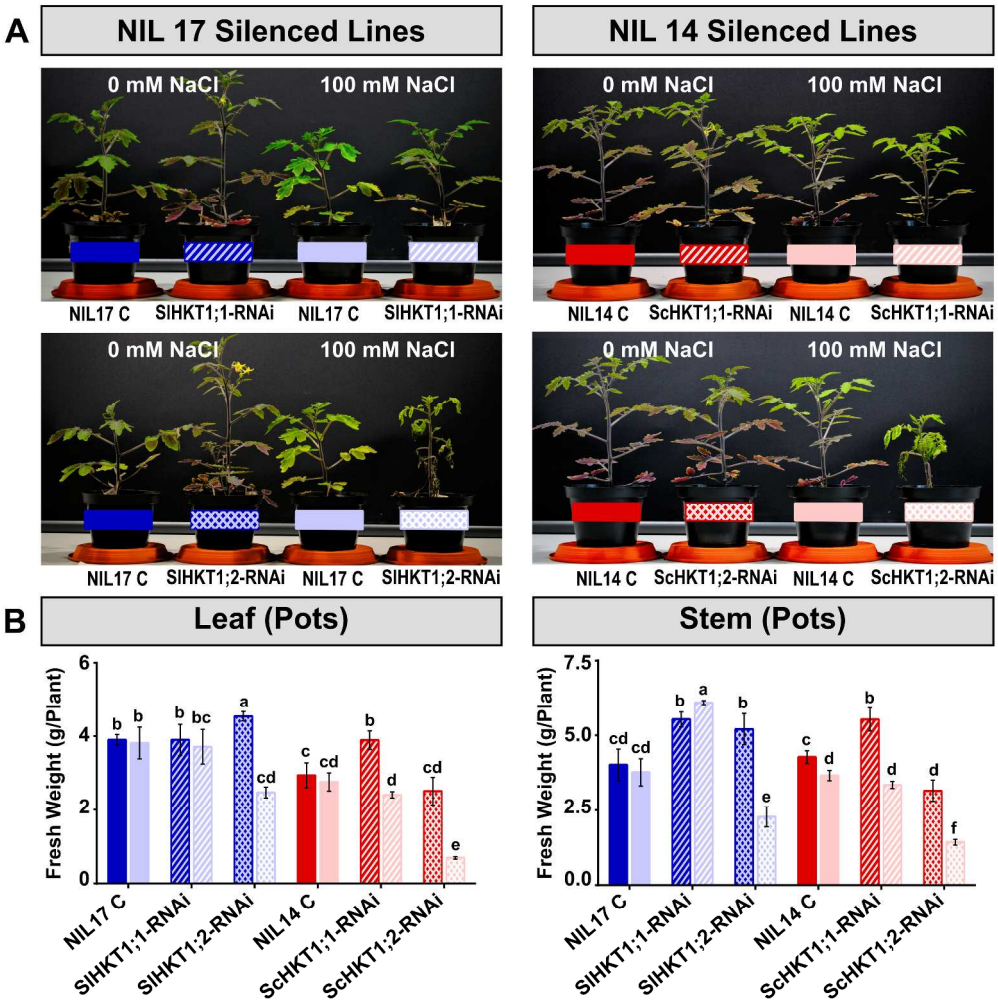
Figure S6. Expression data of *SlHKT1;1* (Solyc07g014690) and *SlHKT1;2* (Solyc07g014680) in different tissues of *Solanum lycopersicum* Cv Heinz obtained from the *eFP tomato Browser.*

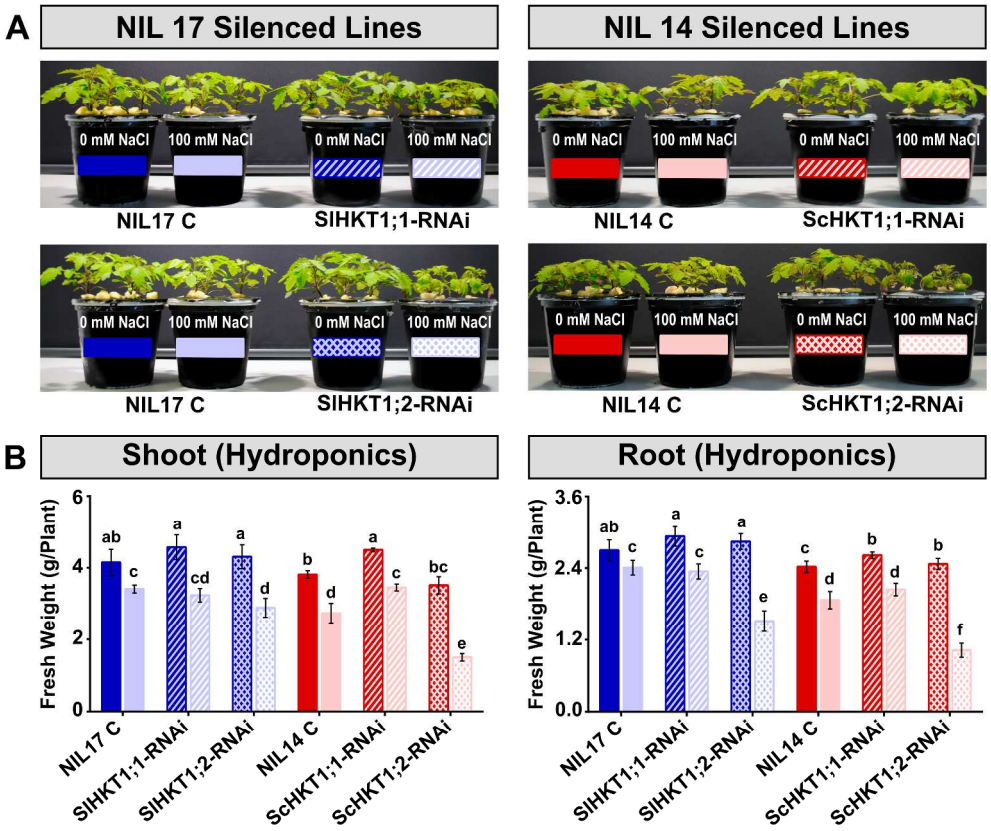
Figure S7. Effect of NaCl treatment on growth, Na^+ and K^+ content in different *athkt1;1* mutant lines grown under transpiring and non- transpiring conditions.

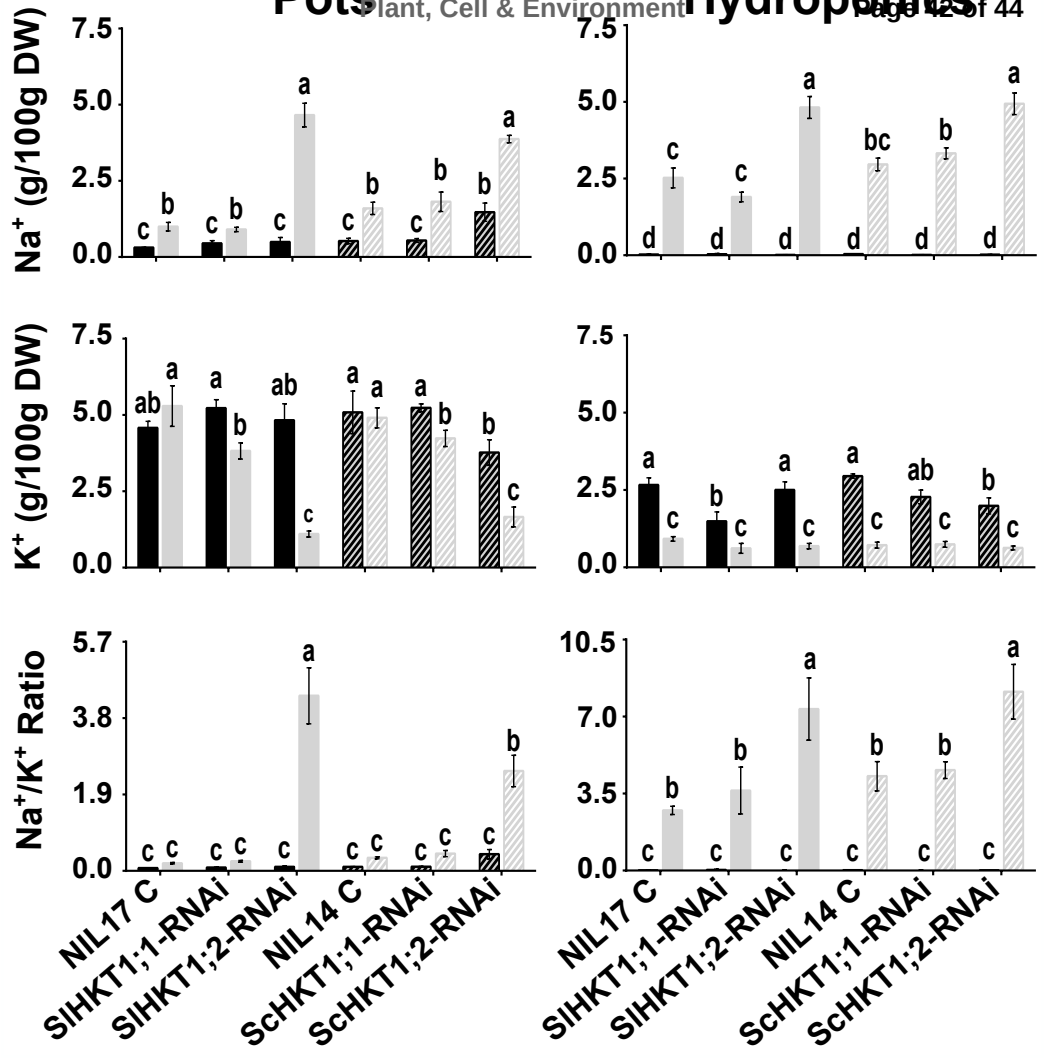
Figure S8. Gene expression analysis of *SlSOS1*, *SlHKT1;1* and *SlHKT1;2* in response to salt stress in wild type and an homozygous T3 *SOS1*-silenced line of tomato (*S. lycopersicum* var.. Moneymaker).

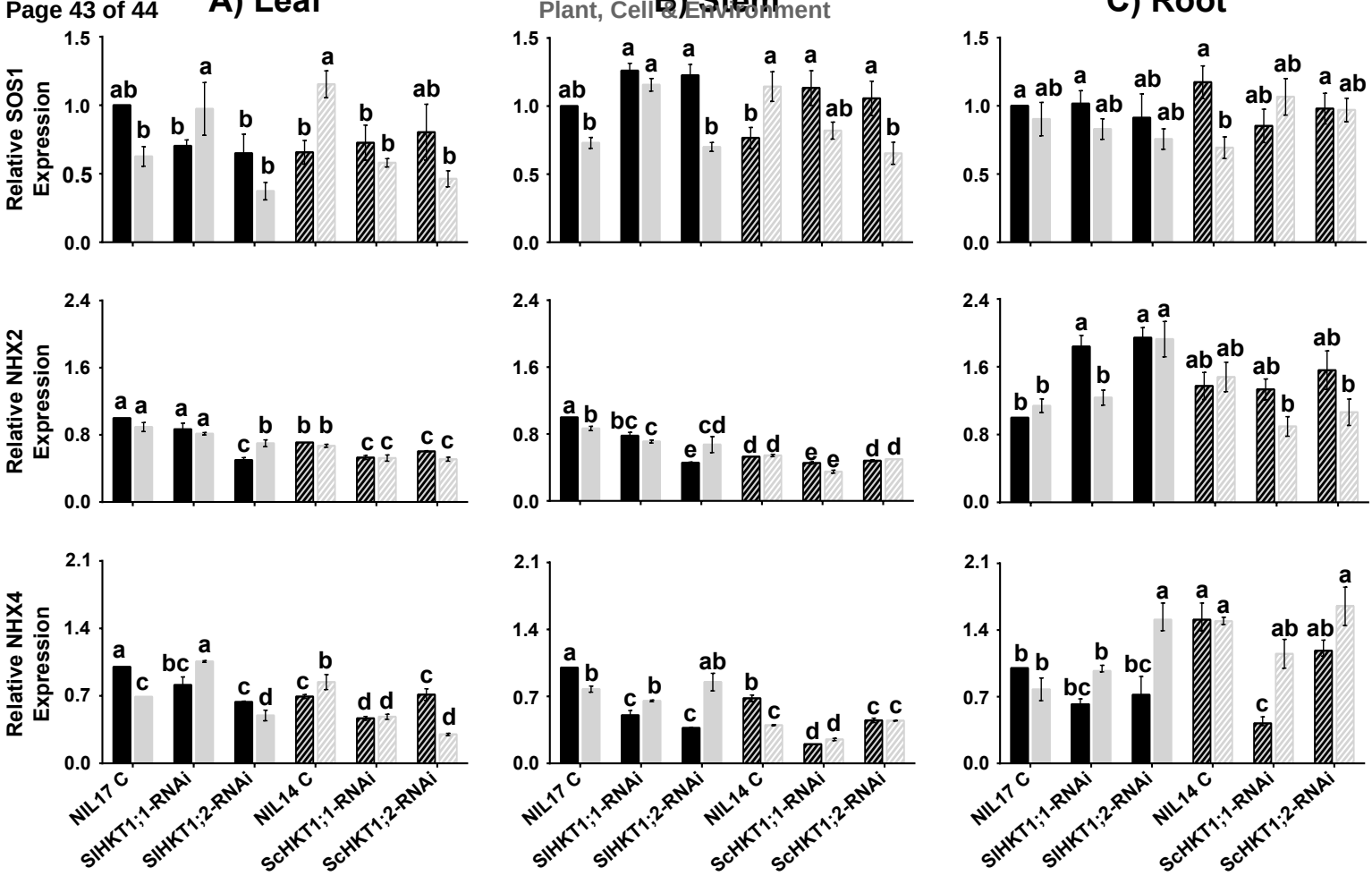

A
HKT1;2
HKT1;1
SIEF-1 α
SIEF-1 α
(no RT)
B
HKT1;2
SIEF-1 α
SIEF-1 α
(no RT)
C
Spongy Mesophyll
Phloem
Xylem
Vascular
bundle
Palisade Mesophyll
Epidermis
Stoma
Trichomes
Endodermis
Epidermis
Xylem
Phloem
Pericycle
Stele
Root hair
Cortex

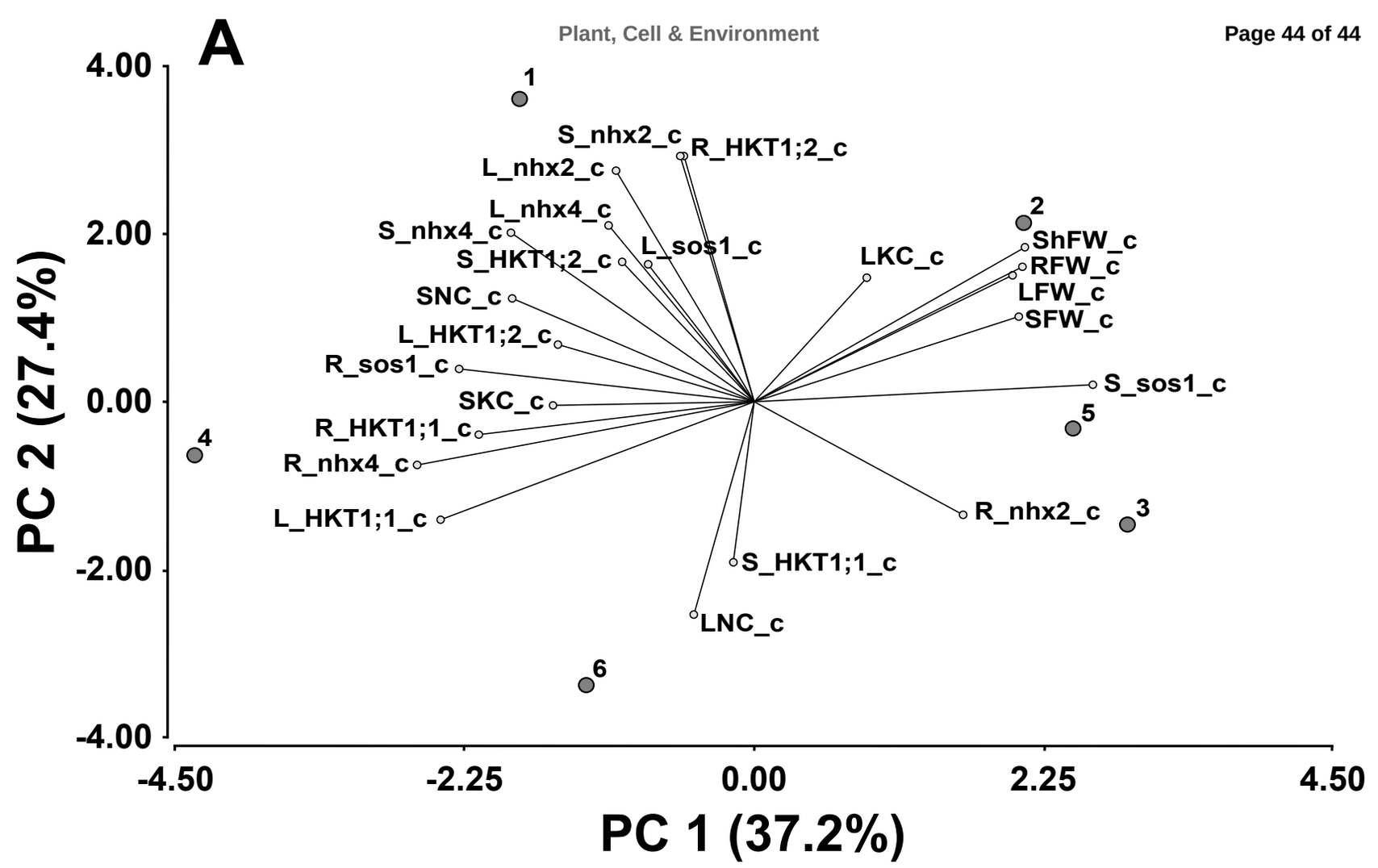

A) Leaf
B) Stem
C) Root
Relative HKT1;1 Expression
Relative HKT1;2 Expression
NIL17 C
SlHKT1;1-RNAi
SlHKT1;2-RNAi
NIL14 C
ScHKT1;1-RNAi
ScHKT1;2-RNAi

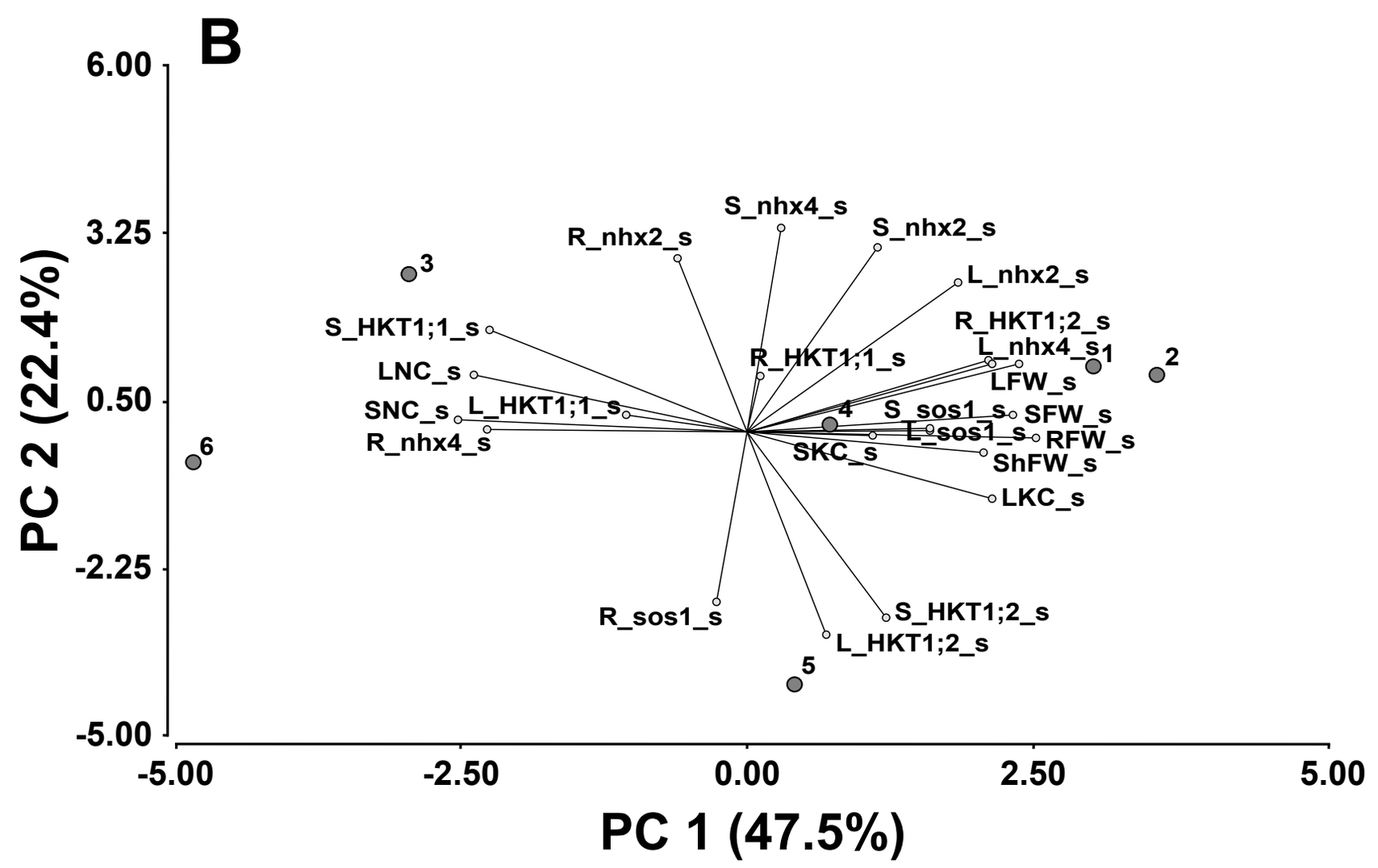

A
NIL 17 Silenced Lines
NIL 14 Silenced Lines
0 mM NaCl
100 mM NaCl
NIL17 C
SlHKT1;1-RNAi
NIL17 C
SlHKT1;1-RNAi
NIL14 C
ScHKT1;1-RNAi
NIL14 C
ScHKT1;1-RNAi
NIL17 C
SlHKT1;2-RNAi
NIL17 C
SlHKT1;2-RNAi
NIL14 C
ScHKT1;2-RNAi
NIL14 C
ScHKT1;2-RNAi
B
Leaf (Pots)
Stem (Pots)
Fresh Weight (g/Plant)
NIL17 C
SlHKT1;1-RNAi
SlHKT1;2-RNAi
NIL14 C
ScHKT1;1-RNAi
ScHKT1;2-RNAi


A
NIL 17 Silenced Lines
NIL 14 Silenced Lines
0 mM NaCl
100 mM NaCl
NIL17 C
SlHKT1;1-RNAi
NIL14 C
ScHKT1;1-RNAi
SlHKT1;2-RNAi
ScHKT1;2-RNAi
B
Shoot (Hydroponics)
Root (Hydroponics)
Fresh Weight (g/Plant)
NIL17 C
SlHKT1;1-RNAi
SlHKT1;2-RNAi
NIL14 C
ScHKT1;1-RNAi
ScHKT1;2-RNAi


Pots
Hydroponics
Na+ (g/100g DW)
K+ (g/100g DW)
Na+/K+ Ratio
7.5
5.0
2.5
0.0
5.7
3.8
1.9
10.5
7.0
3.5
NIL17 C
SlHKT1;1-RNAi
SlHKT1;2-RNAi
NIL14 C
ScHKT1;1-RNAi
ScHKT1;2-RNAi


A) Leaf
B) Stem
C) Root
Relative SOS1 Expression
Relative NHX2 Expression
Relative NHX4 Expression
NIL17 C
SlHKT1;1-RNAi
SlHKT1;2-RNAi
NIL14 C
ScHKT1;1-RNAi
ScHKT1;2-RNAi


A
4.00
2.00
0.00
-2.00
-4.00
PC 2 (27.4%)
-4.50
-2.25
0.00
2.25
4.50
PC 1 (37.2%)
1
2
3
4
5
6
S_nhx2_c
R_HKT1;2_c
L_nhx2_c
L_nhx4_c
S_nhx4_c
L_sos1_c
S_HKT1;2_c
LKC_c
ShFW_c
RFW_c
LFW_c
SFW_c
SNC_c
L_HKT1;2_c
R_sos1_c
S_sos1_c
SKC_c
R_HKT1;1_c
R_nhx4_c
L_HKT1;1_c
R_nhx2_c
S_HKT1;1_c
LNC_c


B
6.00
3.25
0.50
-2.25
-5.00
PC 2 (22.4%)
-5.00
-2.50
0.00
2.50
5.00
PC 1 (47.5%)
1
2
3
4
5
6
S_nhx4_s
S_nhx2_s
R_nhx2_s
L_nhx2_s
R_HKT1;2_s
S_HKT1;1_s
L_nhx4_s
R_HKT1;1_s
LNC_s
LFW_s
SNC_s
L_HKT1;1_s
S_sos1_s
SFW_s
L_sos1_s
RFW_s
R_nhx4_s
SKC_s
ShFW_s
LKC_s
R_sos1_s
S_HKT1;2_s
L_HKT1;2_s

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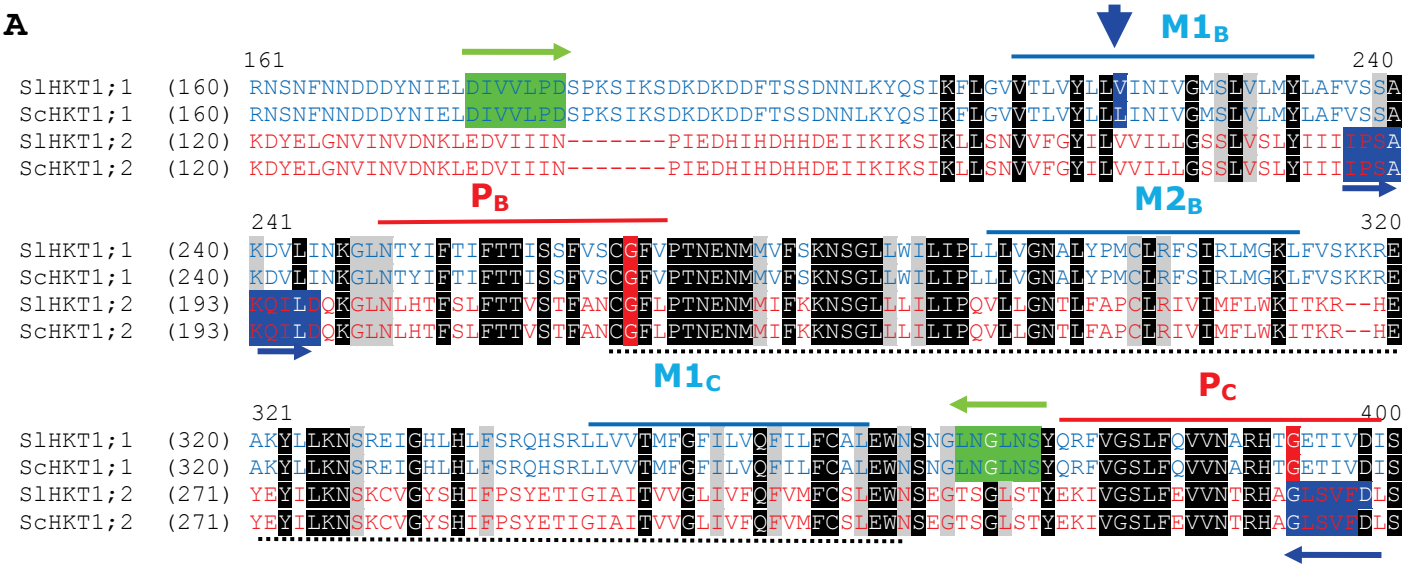
Gene Primer	Direction/ Restriction site	Plasmid/ purpose	Sequence 5'-3'
Sl/ScHKT1;1			
FHKT1-BXi	F, *BamHI, XhoI*	pGEM-T/ pKANNIBAL RNAi fragment cloning	GGATCCTCGAGACATTGTTGTTTTACCTGATTC
RHKT1-HKi	R, *HindIII KpnI*	pGEM-T/ pKANNIBAL RNAi fragment cloning	AAGCTTGGTACCTATTTAGTCCATTGAGACCATT
Sl/ScHKT1;2			
FHKT2-BXi	F, *BamHI, XhoI*	pGEM-T/ pKANNIBAL RNAi fragment cloning	GGATCCTCGAGATCCCTAGCGCCAAACAAATC
RHKT2-HKi	R, *HindIII KpnI*	pGEM-T/ pKANNIBAL RNAi fragment cloning	AAGCTTGGTACCAAATACAGATAGACCAGCATGCC
Vector			
FNPTII	F	pKANNIBAL/ marker gene, *NPTII* (Kan^R)	CCGCAACTTCTTTACCTATTTCC
RNTPII	R	pKANNIBAL/ marker gene, *NPTII* (Kan^R)	GAACTCGTCAAGAAGGCGATA
FpKB35S	F	pKANNIBAL/flanking cDNA sense fragment	GTTCATTTCATTTGGAGA
RpKBintr	R	pKANNIBAL/ flanking cDNA sense fragment	CGTCTTACACATCACTTG

Table S2. Primers used for quantitative RT-PCR

Primers	Sequence 5'-3'	Size	Reference
SlHKT1.1 forward	TCTAGCCCAAGAAACTCAAAT	178 bp	Asins et al. (2013)
SlHKT1.1 reverse	CTAATGTTACAACTCCAAGGAATT		
SlHKT1.2 forward	TGAGCTAGGGAATGTAATAAACG	188 bp	Asins et al. (2013)
SlHKT1.2 reverse	AGAGAGAAACTAACGATGAACC		
SlSOS1 forward	TCGAGTGATGATTCTGGTGG'	129 bp	Huertas et al. (2012)
SlSOS1 reverse	ATCACAGTGTGGAAAGGCT'		
LeNHX2 forward	CCTTTGAGGGGAACAATGG'	173 bp	Huertas et al. (2012)
LeNHX2 reverse	CATCTTCATCTTCGTCTCC'		
LeNHX4 forward	TGGTGGGCAGGTTTGATGAGAG	165 bp	Huertas et al. (2012)
LeNHX4 reverse	TGTGGTGGCAGCAGGAGACTTA		
LeEF1α forward	GACAGGCGTTCAGGTAAGGA	119 bp	Asins et al. (2013)
LeEF1α reverse	GGGTATTCAGCAAAGGTCTC		

A



B

```
SlHKT1;1-RNAi fragment  (523) ACATTGTTGTTTTACCTGATTCTCCAAAATCTATTAAGTCAGATAAAGAT
SlHKT1;2-RNAi fragment  (564) --------------------------------------------------

SlHKT1;1-RNAi fragment        AAAGATGATTTTACGTCATCAGATAATAATCTCAAGTACCAATCTATTAA
SlHKT1;2-RNAi fragment        --------------------------------------------------

SlHKT1;1-RNAi fragment        ATTCCTTGGAGTTGTAACATTAGTTTATCTTCTACTCATCAACATTGTTG
SlHKT1;2-RNAi fragment        --------------------------------------------------

SlHKT1;1-RNAi fragment        GTATGTCACTAGTTCTAATGTATTTAGCCTTTGTTTCAAGTGCAAAAGAT
SlHKT1;2-RNAi fragment        ---------------------------------ATCCCTAGCGCCAAACAA

SlHKT1;1-RNAi fragment        GTA-TTAATAAACAAGGGGCTAAAT-ACCTACATTTTCACGATTTTTACA
SlHKT1;2-RNAi fragment        ATCCTTGACCAAAAAGGCCTTAATTTACATACTTTTTCACTATTC--ACC
                              251                                         300
SlHKT1;1-RNAi fragment        ACCATTTCATCCTTTGTTAGCTGTGGATTTGTTCCAACAAACGAAAACAT
SlHKT1;2-RNAi fragment        ACAGTATCAACTTTTGCAAATTGTGGTTTTTTACCTACAAATGAAAACAT

SlHKT1;1-RNAi fragment        GATGGTGTTTAGCAAGAATTCAGGTCTTCTTTGGATTCTCATCCCTCTAC
SlHKT1;2-RNAi fragment        GATGATTTTCAAGAAAAATTCAGGTCTTCTTCTCATTCTTATCCCTCAAG

SlHKT1;1-RNAi fragment        TTCTTGTTGGGAACGCGTTATATCCAATGTGTTTGAGATTTTCGATTAGG
SlHKT1;2-RNAi fragment        TCCTTCTAGGGAACACTTTGTTTGCTCCTTGTTTACGCATCGTTATAATG

SlHKT1;1-RNAi fragment        TTGATG-GGAAAATTATTTGTGTCTAAGAAAAGAGAAGCTAAGTATTTGT
SlHKT1;2-RNAi fragment        TTCTTATGGAAAATCA-------CAAAGAGACATGAGTATGAGTATATTT

SlHKT1;1-RNAi fragment        TGAAGAATTCAAGAGAAATAGGACATTTACATTTGTTTTCAAGACAACAT
SlHKT1;2-RNAi fragment        TGAAGAACTCAAAATGTGTTGGATATTCACATATTTTTCCAAGTTATGAA

SlHKT1;1-RNAi fragment        TCAAGATTGTTGGTGGTTACTATGTTTGGTTTCATTTTGGTGCAATTCAT
SlHKT1;2-RNAi fragment        ACAATTGGTATTGCTATTACTGTTGTGGGATTAATAGTATTTCAATTTGT

SlHKT1;1-RNAi fragment        ATTGTTTTTGTGCTTTGGAGTGGAACTCTAATGGTCTCAATGGACTAAATA
SlHKT1;2-RNAi fragment        TATGTTTTGTTCATTGGAGTGGAATTCTGAAGGTACTTCTGGATTAAGTA

SlHKT1;1-RNAi fragment        --------------------------------------------------
SlHKT1;2-RNAi fragment        CTTATGAGAAGATTGTGGGATCTTTGTTTGAAGTTGTGAATACAAGGCAT

SlHKT1;1-RNAi fragment        ------------------ (1120)
SlHKT1;2-RNAi fragment        GCTGGTCTATCTGTATTT (1041)
```

Figure S1. Sequence fragments used for generating RNAi constructs to silence by PTGS the respective allelic variants of *HKT1;1* and *HKT1;2* from S. *lycopersicum* and *cheesmaniae*. Panel A displays a section of the alignment of the amino acid sequence fragments of Sl/ScHKT1;1 and Sl/ScHKT 1;2, spanning the first and a part of the second M-P-M domain as previously described (see Fig. 1 in Asins et al 2013 –here, the Arabidopsis HKT1;1 sequence has been removed from that original Fig 1, so that identical residues are highlighted in black and gray). Green and blue arrows indicate the amino acid sequences (also highlighted) to which forward and reverse primers were designed to obtain their corresponding PCR fragments used for RNAi constructs. Panel B shows the alignment of respective PCR fragments of *SlHKT1;1* and *SlHKT1;2* used in RNAi constructs. Identical residues are highlighted in black.

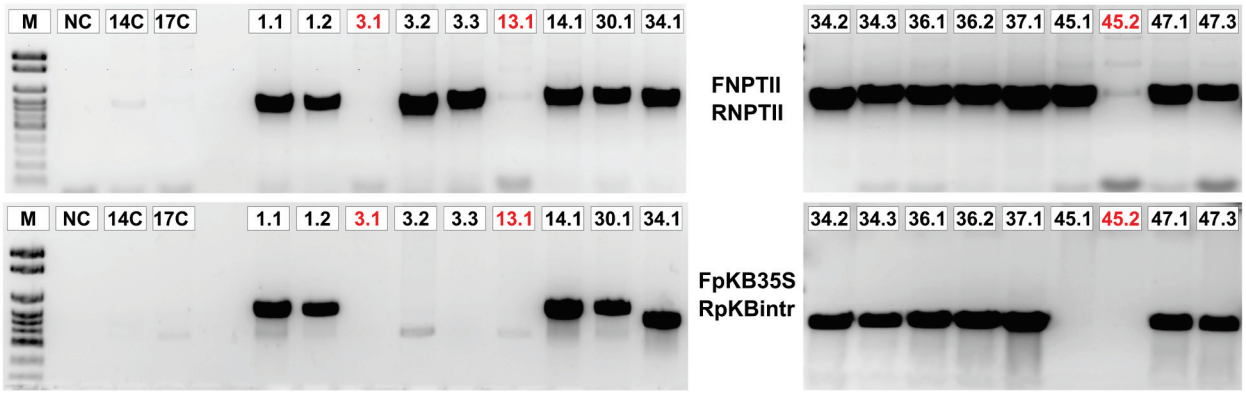


Figure S2. Diagnostic PCR to detect the presence of RNAi constructs bearing the *HKT1;1/HKT1;2* DNA fragments in primary transformants lines (T0 plants) in two tomato NILs. Transgenic plant were considered as positive when amplified PCR bands using genomic DNA and specific primers for NPTII gene was 800 bp (upper panel), in addition to the presence of an expected fragments of 802 bp for *HKT1;1* and 682 bp for *HKT1;2*, using pKANNIBAL-specific primers flanking the respective cDNA sense fragments (lower panel). Lanes 17C and 14C are NIL17 and NIL14 lines , respectively, transformed and regenerated without a silencing construct (WT phenotype). Lane M (marker) is a 100-2000bp ladder and lane NC is a PCR negative control.

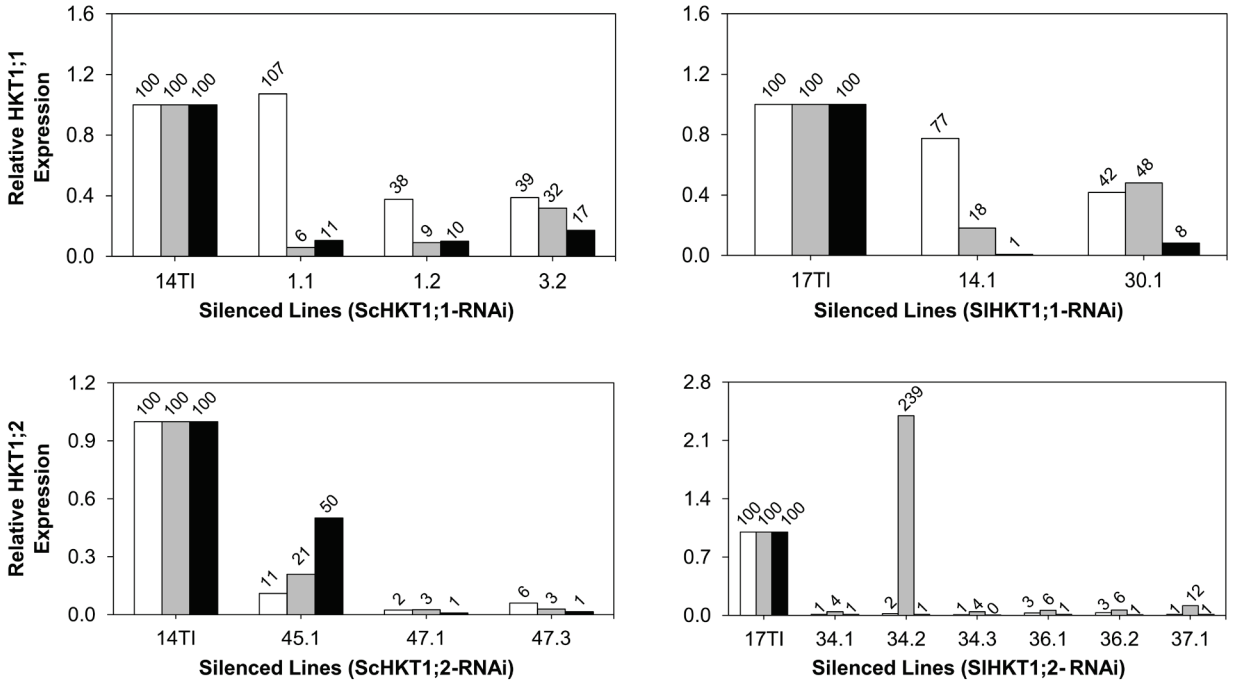


Figure S3. Gene expression determined by RT-qPCR in three different biological samples of different silenced lines of *HKT1;1/HKT1;2* allelic variants from *lycopersicum* and *cheesmaniae* in two tomato NILs. NIL17C and NIL14C lines are NIL17 and NIL14, respectively, transformed and regenerated without a silencing construct. Total RNA was isolated from root (white bars) leaf (gray bars) of regenerated primary transformants grown in vitro culture and leaf (black bars) of acclimated plants grown in pots in growth chamber. Numbers in top of bars represent % gene expression level respect to that of each non-silenced NIL17C and NIL14C.

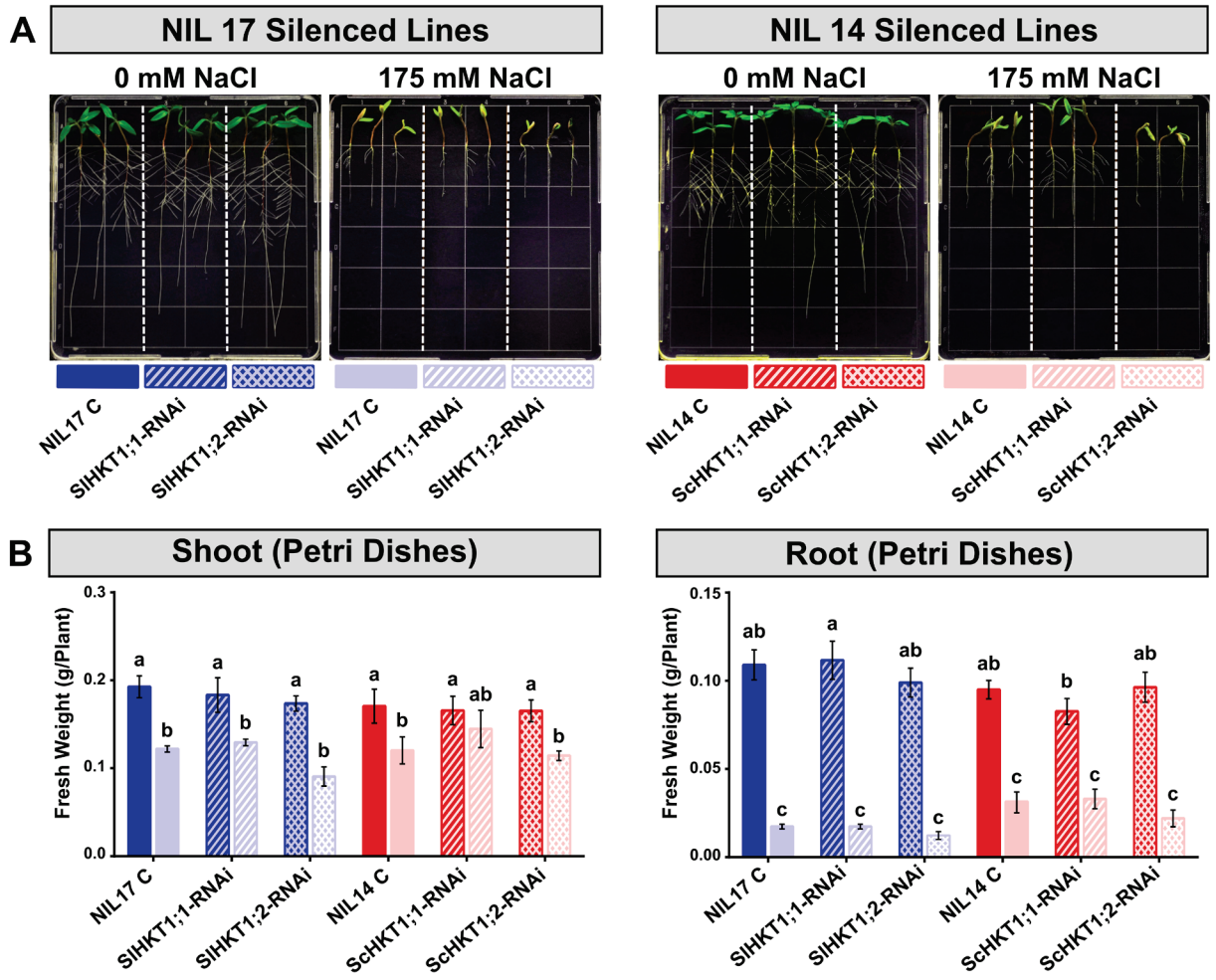


Figure S4. **Effect of NaCl treatment on growth, measured as fresh weight of shoot and root, in different silenced lines of SlHKT (blue bars) and ScHKT (red bars) grown in Petri dishes. A)** Four-day-old T1 transgenic seedlings cultivated on Petri dishes in ¼ x Hoagland medium were transferred to Petri dishes containing the same medium supplemented with 0 mM NaCl (dark bars) and 175 mM NaCl (clear bars) and grown on it for 5 additional days. **B)** Fresh weight of shoot and root. Each value is the mean of 3 replications (3 different plates) ± SEM. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test.

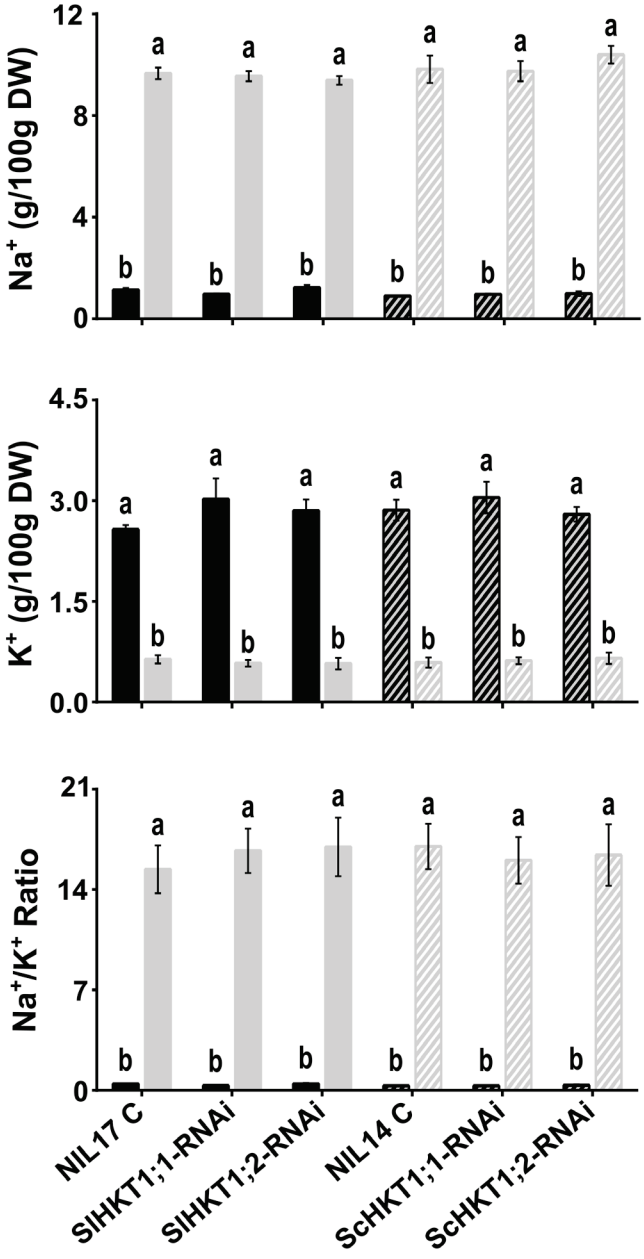
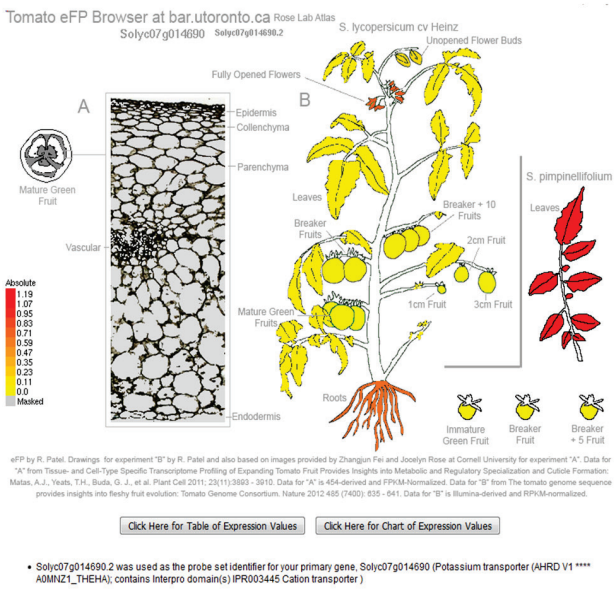
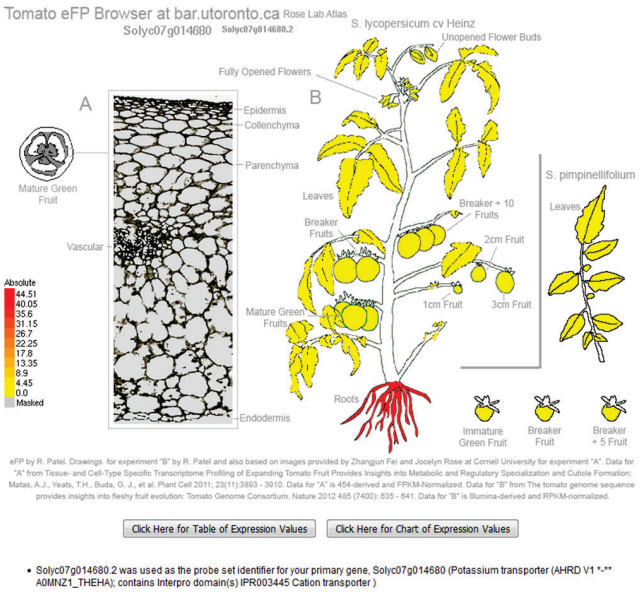


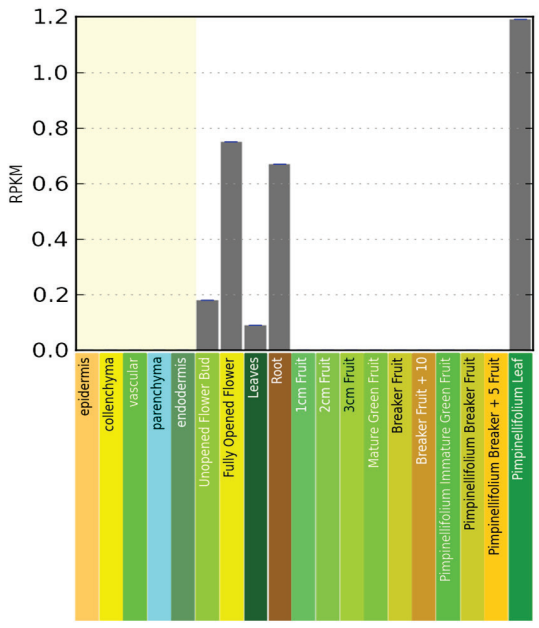
Figure S5. Leaf contents of Na^+ and K^+ in control and salt-treated plants of different *SlHKT1;1/HKT1;2* and *ScHKT1;1/HKT1;2*-silenced NIL lines grown in Petri dishes. Leaf contents of Na^+ and K^+ in control (dark bars) and salt-treated (clear bars) from non silenced and silenced NIL 17 (SlHKT1 alleles) and NIL 14 (ScHKT1 alleles). Tomato plants were grown in Petri dishes, as indicated in legend of figure S4. Values represent the mean ± SEM of three different samples. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test.

SlHKT1;1



SlHKT1;2





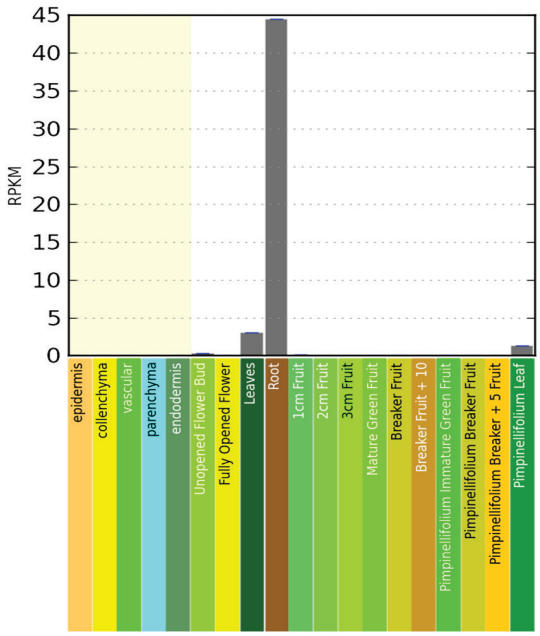


Figure S6. Expression data of *SlHKT1;1* (Solyc07g014690) and *SlHKT1;2* (Solyc07g014680) in different tissues of *Solanum lycopersicum* Cv Heinz obtained from the *eFP tomato Browser*. Expression data in leaves of *S. pimpinellifolium* is included. Rose_Lab_Atlas was used as data resource (Bio-Analytic Resource –BAR- of University of Toronto, Winter et al, 2007, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

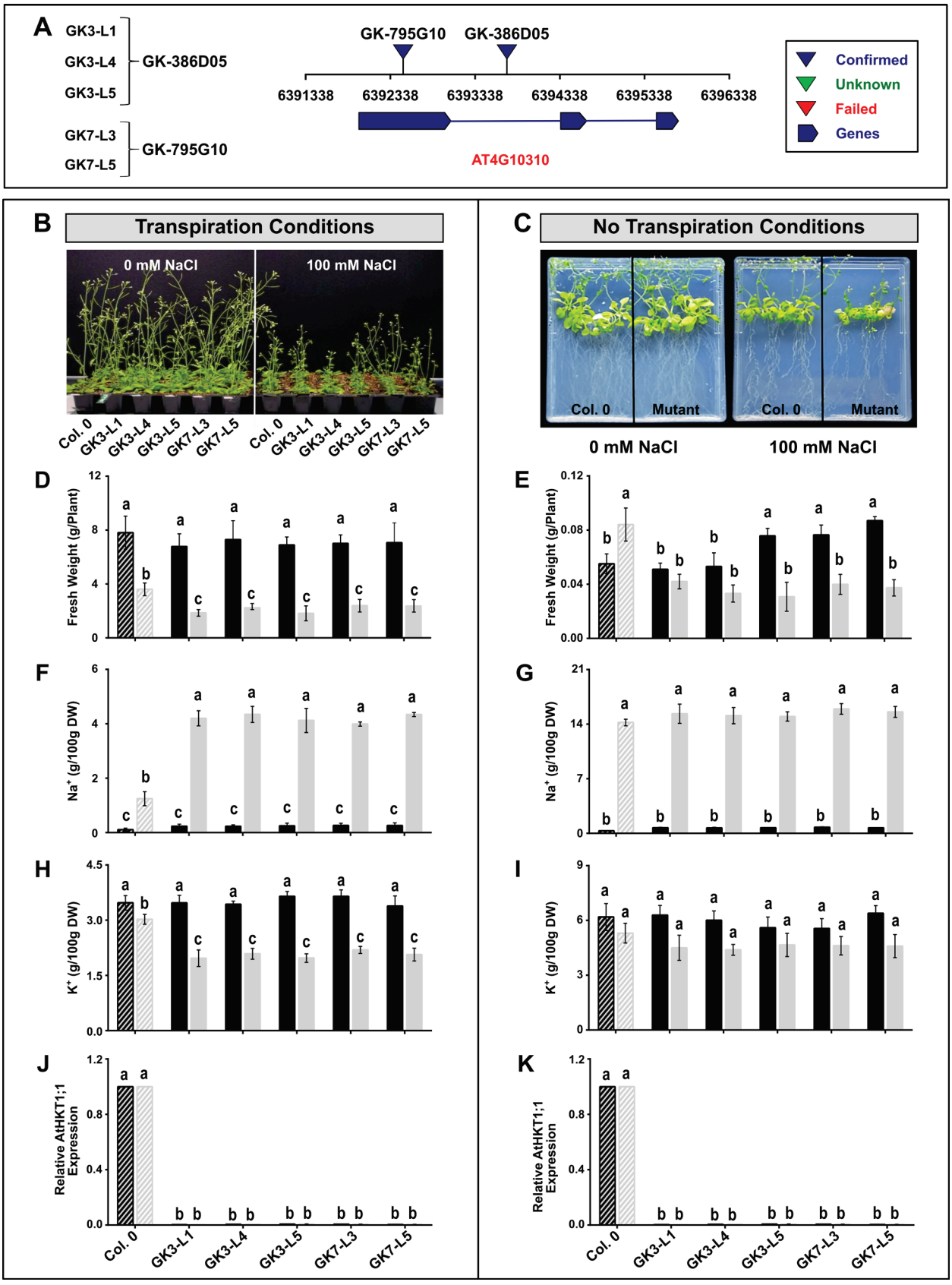

A
GK3-L1
GK3-L4
GK3-L5
GK-386D05
GK7-L3
GK7-L5
GK-795G10
GK-795G10
GK-386D05
6391338
6392338
6393338
6394338
6395338
6396338
AT4G10310
Confirmed
Unknown
Failed
Genes
B
Transpiration Conditions
0 mM NaCl
100 mM NaCl
Col. 0
GK3-L1
GK3-L4
GK3-L5
GK7-L3
GK7-L5
C
No Transpiration Conditions
Col. 0
Mutant
Col. 0
Mutant
0 mM NaCl
100 mM NaCl
D
Fresh Weight (g/Plant)
E
Fresh Weight (g/Plant)
F
Na+ (g/100g DW)
G
Na+ (g/100g DW)
H
K+ (g/100g DW)
I
K+ (g/100g DW)
J
Relative AtHKT1;1 Expression
K
Relative AtHKT1;1 Expression
Col. 0
GK3-L1
GK3-L4
GK3-L5
GK7-L3
GK7-L5

Figure S7. Effect of NaCl treatment on growth, Na^+ and K^+ content in different *athkt1;1* mutant lines grown under transpiring and non- transpiring conditions. **A**. Three and two different homozygous lines from two distinct *athkt1;1* mutants in genetic background col.0, respectively (GK-386D05 and GK-795G10) from GABI-Kat collection were used (purchased from NASC). **B**. Growth of wild type (col. 0) and five different homozygous lines from GABI-Kat collection for 3 weeks in peat-moss:vermiculite 1:1 at 22/20ºC, 16 h light (120 µmol/m^2s^1) /8 h darkness and treated with 0 mM and 100 mM NaCl for 7 days under transpiring conditions. **C**. Growth of wild type (col 0) and GK3-L1 mutant in minimal medium subjected to 0 mM and 100 mM salt treatment for 40 days under non-transpiring conditions (Mäser *et al.,* 2002). **D**. Fresh weight of complete aerial part of Arabidopsis plants grown in seedbeds (transpiring conditions) and treated with 0 mM (dark bars) and 100 mM NaCl (clear bars) for 7 days. **E**. Fresh weight of complete aerial part of Arabidopsis plants grown in Petri dishes (non-transpiring conditions) with minimal medium, subjected to 0 mM (dark bars) and 100 mM NaCl (clear bars) for 40 days (Mäser *et al.,* 2002). **F**. Aerial part contents of Na^+ in control (dark bars) and salt-treated plants (clear bars) grown under transpiring conditions. **G**. Aerial part contents of Na^+ in control (dark bars) and salt-treated plants (clear bars) grown under non-transpiring conditions. **H**. Aerial part contents of K^+ in control (dark bars) and salt-treated plants (clear bars) grown under transpiring conditions. **I**. Aerial part contents of K^+ in control (dark bars) and salt-treated plants (clear bars) grown under non-transpiring conditions. **J, K**. Gene expression analysis of *AtHKT1;1* in wild type (col 0) and different mutant lines of *athkt1;1*. Total RNA was isolated from whole Arabidopsis 3-week-old plants grown under control conditions. Transcript level was analyzed by RT-qPCR using primers described elsewhere (Mason *et al*. 2010) Tubuline-3 (dark bars) and actine-2 (clear bars) were used as the reference genes. Each value is the mean of 5 replication (5 different plants) ± SEM. Significant differences ($P < 0.05$) are indicated by different letters, according to Tukey's test.

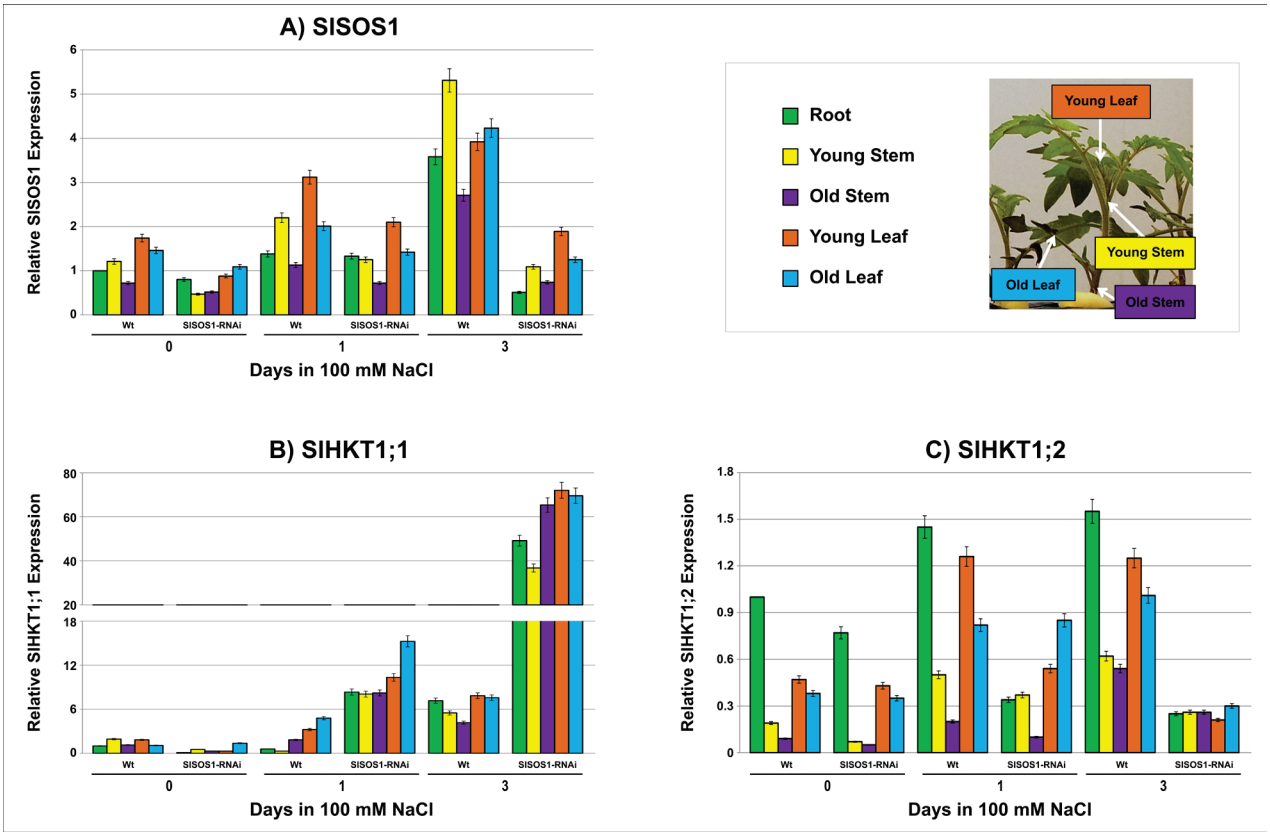


Figure S8. Gene expression analysis of *SlSOS1*, *SlHKT1;1* and *SlHKT1;2* in response to salt stress in wild type and an homozygous T3 *SOS1*-silenced line of tomato (*S. lycopersicum* var. Moneymaker). Total RNA was isolated from tissues of tomato plants treated with 100 mM NaCl for 0, 1, 3 days in hydroponic cultures. Transcript level was analyzed by RT-qPCR using primers described in Supporting Information Table S2. The tomato elongation factor gene (*LeEF1-α*) was used as the reference gene. The relative expression level was calculated using the equation 2EXP[ΔΔCt] using the expression level of each gene in roots from wt at day 0 of NaCl treatment as the calibrator sample (equal to 1)

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References

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V., & Provart, N. J. (2007). An "electronic fluorescent pictograph" Browser for exploring and analyzing large-scale biological data sets. *PLoS ONE*, *2*(8).