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Na^+ transporter HKT1;2 reduces flower Na^+ content and considerably mitigates the decline in tomato fruit yields under saline conditions

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RUNNING TITLE: *HKT1;2* gene protects flowers from sodium toxicity in tomato

ABSTRACT

Genes encoding HKT1-like Na^+ transporters play a key role in the salinity tolerance mechanism in Arabidopsis and other plant species by retrieving Na^+ from the xylem of different organs and tissues. In this study, we investigated the role of two *HKT1;2* allelic variants in tomato salt tolerance in relation to vegetative growth and fruit yield in plants subjected to salt treatment in a commercial greenhouse under real production conditions. We used two near-isogenic lines (NILs), homozygous for either the *Solanum lycopersicum* (NIL17) or *S. cheesmaniae* (NIL14) allele, at *HKT1;2* loci and their respective RNAi-*Sl/ScHKT1;2* lines. The results obtained show that both *ScHKT1;2*- and *SlHKT1;2*-silenced lines display hypersensitivity to salinity associated with an altered leaf Na^+/K^+ ratio, thus confirming that *HKT1;2* plays an important role in Na^+ homeostasis and salinity tolerance in tomato. Both silenced lines also showed Na^+ over-accumulation and a slight, but significant, reduction in K^+ content in the flower tissues of salt-treated plants and consequently a higher Na^+/K^+ ratio as compared to the respective unsilenced lines. This altered Na^+/K^+ ratio in flower tissues is associated with a sharp reduction in fruit yield, measured as total fresh weight and number of fruits, in both silenced lines under salinity conditions. Our findings demonstrate that Na^+ transporter HKT1;2 protects the flower against Na^+ toxicity and mitigates the reduction in tomato fruit yield under salinity conditions.

Key-words index: Na^+ flower content; fruit yield; *HKT1.2* gene; K^+ and Na^+ homeostasis; *Solanum lycopersicum* and *S. cheesmaniae*; tomato; salinity

1. Introduction

Soil salinity, which is a major threat to global food security, affects 2.1 % of dry agricultural land and up to 20% of irrigated land, accounting for one third of the world's food production (FAO, 2019). The losses in agricultural production worldwide due to salinity are estimated at 27 billion dollars per year (Munns and Gilliham, 2015). Worldwide, tomato is the most important horticultural crop, with a total production and cultivated area estimated at 164 Mt and 4.76 Mha, respectively (FAOSTAT, 2017). Tomato crops are grown mainly in areas with an arid or semi-arid Mediterranean climate. As tomato requires a large amount of water for its growth (Romero-Aranda et al., 2001; Reina et al. 2005), in areas with a shortage of good-quality water for irrigation, farmers often use low-quality water from wells and groundwater aquifers containing high concentrations of soluble salts, mainly NaCl. In these areas, electrical conductivity (EC) frequently exceeds 3 dS/m (equivalent to 2 g/L NaCl) in soils and irrigation water, which causes a sharp decrease in tomato production (Maas y Grieve, 1990; Romero-Aranda et al., 2002; Reina et al., 2005). All this highlights the necessity of seeking solutions to improve the salt tolerance of the tomato crop (Cuartero et al., 2006). An important strategy to reduce the impact of salinity on tomato plants involves exploiting the halotolerant genetic potential of wild relative species as donors of tolerance, whose beneficial gene alleles, identified by QTL analysis, could be introgressed into elite cultivated tomato (Cuartero et al., 2006). The identification of genes functionally underlying QTLs makes for more efficient marker-assisted selection in genetic improvement programs and facilitates their manipulation through genetic engineering (Arzani and Ashrarf, 2016).

For most crop plants, particularly tomato, excessive Na^+ accumulation in the aerial part can have highly negative effects on growth and development, as high concentrations of Na^+ competitively inhibit K^+ uptake systems and negatively affect many K^+-dependent physiological plant functions (Kronzucker et al., 2013). Although this Na^+ accumulation is considered to be an adaptive advantage in halotolerant accessions of some tomato wild-related species given that Na^+ is a biologically inexpensive osmolyte that contributes to maintaining an optimal water balance, disturbances in Na^+/K^+

concentrations inside the cell can adversely affect plant development in tomato cultivars (Kronzucker et al., 2013; Munns et al., 2016). Thus, maintaining a balanced cytosolic Na^+/K^+ ratio has become a key salinity tolerance mechanism in tomato (Cuartero et al., 2006). Na^+/K^+ homeostasis at the whole plant level is a complex mechanism involving multiple genes that participate in a network of transport processes which control absorption, extrusion through the plasma membrane, salt compartmentalization in cellular vacuoles and ion recirculation through the plant organs, thus enabling osmotic adjustment and the maintenance of a high K^+/Na^+ ratio in the plant cytosol (Pardo and Rubio, 2011). As in Arabidopsis, rice and other crop plants (Pardo and Rubio, 2011; Kronzucker et al., 2013), tomato genes encoding Na^+ transporter SOS1 and its regulatory proteins (Olías et al., 2009; Huertas et al., 2012), HKT1-like proteins (Asins et al., 2013; Jaime-Pérez et al., 2017) and certain NHX-type antiporters (Gálvez et al., 2012; Huertas et al.; 2013) are mainly involved in regulating Na^+ concentrations in various tissues of tomato plants. HKT1-like transporters, which are responsible for removing Na^+ from the xylem at root level, preventing its accumulation in the aerial part and indirectly improving K^+ accumulation, play an important role in salt tolerance (Ren et al., 2005; Davenport et al., 2007; Munns et al., 2012; Byrt et al., 2014; Suzuki et al., 2016). In addition, in the aerial part, HKT1-like proteins are involved not only in removing Na^+ from the xylem, which indirectly boosts the vacuolar accumulation of Na^+ in stems and leaves in collaboration with other transporters, but also possibly in its redistribution to other sink tissues via the phloem (Munns et al., 2016). HKT1-like transporters must therefore be related to energy-efficient osmotic adjustments by Na^+ ions and to the maintenance of turgor potential in above-ground tissues (Munns et al., 2019). Several *HKT1*-like genes have been identified as directly associated with QTLs responsible for Na^+ and K^+ concentrations in aerial parts of monocots such as rice, corn, barley and wheat (Ren et al., 2005; Munns et al., 2012; Zhang et al., 2017) and dicots including Arabidopsis and grapevine (Baxter et al., 2010; Henderson et al., 2018). Similarly, using candidate gene analysis of tomato, we identified two closely linked genes encoding HKT1-like transporters, *HKT1;1* and *HKT1;2*, as candidate genes for a major QTL associated with shoot Na^+/K^+

homeostasis using two populations of recombinant inbred lines (RILs) derived from *S. lycopersicum* cv. Cerasiform and the salt-tolerant wild tomato species *S. pimpinellifolium* and *S. cheesmaniae* (Asins et al., 2013). Under growth chamber conditions, we evaluated different transgenic lines derived from two near-isogenic lines (NILs) of *S. lycopersicum* cv. cerasiform differing in terms of their *HKT1;1/HKT1;2* alleles from *S. lycopersicum* and *S. cheesmaniae*, in which each of these genes is silenced by stable transformation. We found that the Na^+ transporter-encoding gene *HKT1;2* functionally underlies the major QTL controlling shoot Na^+/K^+ homeostasis and plays an important role in tomato salt tolerance in terms of vegetative growth, while HKT1;1 alleles had little effect (Jaime-Pérez et al., 2017).

Given that tomato yield is considered to be the ultimate criterion for salt tolerance, we decided to evaluate plant behaviour under natural greenhouse conditions following standard commercial cultural procedures in order to follow up the previous experiments carried out under controlled growth chamber conditions (Jaime-Perez et al., 2017). We also investigated the role of two *HKT1;2* alleles, *lycopersicum* and *cheesmaniae,* in salt tolerance in terms of plant physiological parameters, biomass production, as well as fruit yield and quality, by using both NILs homozygous for either the *S. lycopersicum* allele (NIL17) or the *S. cheesmaniae* allele (NIL14) at both *HKT1* loci, as well as transgenic lines derived from these NILs in which both *S. lycopersicum* and *S. cheesmaniae HKT1;2* alleles had been silenced by stable transformation. These new findings could potentially be very useful in future strategies for improving tomato productivity under Mediterranean greenhouse conditions.

2. Materials and methods

2.1. Plant material

The two tomato near-isogenic lines (NILs), NIL157-14 (NIL14) and NIL157-17 (NIL17), used in this study were obtained as described elsewhere (Asins et al., 2013). Briefly, they were developed by selfing a segregating F6 line (RIL B157), which itself was obtained after five selfing generations of an F_1 progeny from a cross between a salt-sensitive genotype of *S. lycopersicum*, var. Cerasiform, as female parent and a salt-tolerant genotype of *S. cheesmaniae* (L. Riley) Fosberg as male parent (Villalta et al.,

2007, 2008). NIL14 is homozygous for the *S. cheesmaniae* allele at both *HKT1;1* and *HKT1;2* loci (CC), while NIL17 is homozygous for the *S. lycopersicum* alelle at both *HKT1;1* and *HKT1;2* loci (LL). With regard to other genes involved in Na^+ homeostasis in this study, both NILs are homozygous for the *S. cheesmaniae* allele at *SOS1* and *NHX4*. We also used two homozygous transgenic lines derived from these NILs in which both *lycopersicum* and *cheesmaniae HKT1;2* alleles were silenced by stable transformation using RNAi, as described elsewhere (Jaime-Perez et al., 2017). As controls for comparative purposes, we used the non-silenced NIL14 and NIL17 lines, which were also subjected to whole gene transformation without RNAi constructs (Jaime-Perez et al., 2017).

2.2. Tomato growth conditions and salt treatments

The sterilized seeds were sown individually in seedbeds containing vermiculite, maintained in a growth chamber at 24ºC in darkness and irrigated with water until the cotyledons emerged after 5-7 days. The germinated seeds were cultivated in a growth chamber at 24°C/18ºC 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 initially irrigated with a ¼ strength Hoagland nutrient solution (Hoagland & Arnon, 1950) until 3-4 true leaves appeared. At this developmental stage, eleven-week-old individual tomato plants were transplanted to 17-L plastic pots filled with vermiculite. Pots in the greenhouse were arranged in rows 1 m apart, with two plants per meter in the rows, corresponding to a density of two plants per square meter. Two extra plants at the beginning and end of each row and two rows of plants before and after the first and final experimental rows were positioned to avoid border effects. Plants were grown under natural light conditions without temperature control during the autumn-spring season (November to May) in a 900 m^2 polyethylene greenhouse in the La Mayora Estación Experimental (IHSM-CSIC) in Malaga in Southern Spain (36º45'20.78"N 4º02'28.72"W) (Supporting Information Fig. S1). Pollination was performed manually from flowering start to fruit set. Pest and disease control was carried out according to standard commercial practices. All axillary vegetative buds were removed from the main stems which were trained vertically using plastic nets attached with string to the greenhouse ceiling. The plants were

supplied with a standard Hoagland nutrient solution adapted to tomato (5,9 mM N, 1 mM P, 6.6 mM K^+, 4.5 mM Ca^{2+}, 2 mM S 2 mM Mg^{2+}, and microelements) from the transplanting stage to the end of the experiment using an automatic drip irrigation system, with one dripper per plant discharging 2.3 L h^{-1}. Irrigation was computer-controlled to provide all plants with the same volume of nutrient solution. Twenty plants per genotype were irrigated for 180 d with Hoagland nutrient solution supplemented with either 0 or 80 mM NaCl. Electrical conductivity (EC, dS m^{-1}) and pH of the irrigation solution provided by the drippers and from pot lixiviates were measured weekly to confirm the stability of the irrigation conditions throughout the test period (Supporting Information Fig. S2). Two replicates of this experiment were carried out in two successive years during the same period of time (November to May). The data shown are representative of one of the two experiments.

2.3. Measurement of physiological parameters, plant biomass and ion analyses

2.3.1. Photosynthesis and stomatal conductance

Net assimilation of CO_2 (A, µmol CO_2 $m^{-2}s^{-1}$) and stomatal conductance (gs, mmol $m^{-2}s^{-1}$) were determined using an LI-6400 portable infrared gas analyser (LI-COR Inc., Lincoln, NE, USA). The uppermost fully developed leaves and those fully exposed to the sun were selected. Six replicate leaves per genotype and salt treatment were measured. The measurements were conducted between 09:00 and 12:00h in the morning under natural air-temperature conditions at 370-400 µmol mol^{-1} CO_2 at a PPFD of 700 µmol m^{-2} s^{-1} provided by a 6400-02B LED Light Source (Li-COR).

2.3.2. Xylem sap collection, sap flow rate and sap ion analysis

After 34 days of salt treatment, the shoots of six plants per genotype and saline treatment were severed at 1.5 cm above the base of the stems where a silicone tube was inserted to collect the xylem sap exudate. The volume of xylem sap collected during the first 10 minutes was discarded to rule out possible contamination from damaged cells. The volume of xylem sap collected during 20-60 min was recorded to calculate the sap flow rate (Netting et al., 2012). After the xylem sap was collected, the

roots were washed and then oven-dried to determine root dry weight. Root hydraulic conductivity (Lpr, µl min^{-1} g^{-1}) was determined as Lpr = volume of root sap exudate (µl)/time (min) x root DW (g). Xylem sap, previously digested in a HNO_3:$HClO_4$ (2:1, v/v) solution, was analyzed for ion composition using a Varian 720-E inductively coupled plasma-optical emission spectrometer (ICP-OES; Scientific Instrumentation Service, EEZ, CSIC, Granada, Spain).

2.3.3. Plant dry biomass and ion analyses

Number of leaves, plant height and stem diameter were recorded weekly throughout the experimental period. After 85 days of salt treatment, six plants per genotype and saline treatment were sampled and divided into aerial parts (stems, leaves and flowers) and roots. The material was identified, placed in paper bags and dried at 65 °C for 72h in a forced-air oven, followed by measurement of dry mass. Shoot biomass was equal to the sum of aerial vegetative plant parts (leaves + stems +flowers). The root to shoot ratio was calculated by dividing root dry weight by the shoot dry weights. For Na^+ and K^+ analysis, the 9th leaf counted from the apex of the plant, and that of the 4th inflorescence (including peduncles, pedicels and flowers), sampled at d 34 and d 85 of salt treatment, respectively, were chosen based on a previous characterization of the saline performance of many tomato genotypes, which were developed in the same greenhouse and under cultural practices similar to those used in the present study (Romero-Aranda and Longuenesse 1995; Romero-Aranda et al., 2001, 2002). Na^+ and K^+ content was determined using an inductively coupled plasma spectrophotometer (Varian 720-E, Scientific Instrumentation Service, EEZ, CSIC, Granada, Spain).

2.3.4. Determination of yield, fruit quality, antioxidant compounds and antioxidant activity

Fruits from the first and second truss were harvested while maturing from the beginning of March up to the beginning of May. Number of fruits and fruit weight were determined for six plants per genotype and salt treatment. Total yield per plant was calculated by adding up the weight of fruits from all harvests. Fruits were washed and ground to a homogeneous liquid paste in a blender (Braun, Kronberg,

Germany). Subsamples of the homogenates obtained were immediately frozen at -20ºC and stored for further analysis. Total soluble solids (TSS), titrable acidity (TA), lycopene, β-carotene, total phenolic compounds, as well as antioxidant activity, were determined. TSS and TA were measured in the supernatant of the 5 g- frozen fruit tissue homogenate centrifuged at 6000 rpm for 20 min. TSS (ºBrix) was measured using a hand-held CR-400/410 Chroma Meter. TA, expressed as a percentage of citric acid, was determined as described by Rouphael et al. (2008). Lycopene and β-carotene were determined by spectrophotometric analysis according to the method developed by Fish et al. (2002). Total phenolic compound (TPC) content was determined according to the Folin–Ciocalteau spectrophotometric method as reported by Rousseaux et al (2005). Antioxidant activity was measured using the ferric-reducing antioxidant power (FRAP) reagent as described by Benzie and Strain (1996).

2.4. Gene expression analysis by qRT-PCR

Tissue samples were collected at the following times throughout the experimental period of this study: 34 days after the beginning of salt treatment in the case of roots and 9th leaf tissues and after 85 d in the case of floral tissue (Fig. 1). Three independent biological samples, with one plant per line and treatment each, were used for the analysis. Total RNA was isolated from the root, 9th leaf and 4th floral inflorescence, including peduncle, pedicel and flower, using the Aurum™ total RNA mini kit (Bio-Rad Laboratories, S.A.), together with RNAse-free DNase in-column treatment (Promega Biotech Ibérica, SL), as well as RNAsecure™ resuspension solution (Ambion Europa Ltd, Austin, TX, USA) for elution and resuspension according to the 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.) using the oligo-dT and random hexamer primer blend provided according to the supplier's protocol. As described elsewhere (Asins et al., 2013; Jaime-Perez et al., 2017), RT-qPCR was carried out using 1 µl of undiluted cDNA mixed with *iQ SyBr Green Supermix* (BioRad) and 0.45µM of forward and reverse primers (Supporting Information Table S1) in a BioRad MyiQ2 iCycler. Relative expression was 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 housekeeping gene. *LeEF1-α,* was highly stable regardless of genotype, tissue or treatment (Supporting Information Fig S3). The relative expression level was calculated with the aid of the 2EXP-[$\Delta\Delta C_t$] equation (Livak and Schmittgen, 2001) using the expression level of each gene in each tissue from non-silenced and non-salt-treated NIL17 as the calibrator sample.

2.5. Statistical analysis

Data were analyzed by two-way ANOVA with the factors genotype (G), salt treatment (E) and their interaction (G × E) using Statgraphics Centurion software (2009). The Tukey test ($P < 0.05$) was used to separate means of all treatments when the interaction was significant.

3. Results

3.1. Physiological and phenotypic evaluation: Sl/ScHKT1;2–RNAi silenced lines showed a salt-hypersensitive phenotype at the vegetative stage.

The gene expression patterns for *Sl/ScHKT1;2* allelic variants were analysed in root, leaf and flower tissues (Fig. 1). *SlHKT1;2* transcript levels in NIL17 roots were considerably higher than those of *ScHKT1;2* in NIL14 roots, while, in shoots (mainly leaves), their expression followed an opposite trend. As expected, *HKT1;2* gene expression of each *Sc/SlHKT1;2*-silenced line was much lower than that of the respective non-silenced NIL plants, regardless of tissue and salt treatment (Fig. 1). Salinity clearly increased *HKT1;2* transcript levels in NIL17 roots, while those in the leaves and flowers of both NILs remained unchanged. Salinity did not significantly affect *HKT1;2* expression in NIL 14 roots.

Growth of NIL17 and *SlHKT1;2*-silenced lines measured as plant height and stem diameter was significantly more vigorous than that of NIL14 and *ScHKT1;2*-silenced lines at d 34 of treatment with 0 and 80 mM NaCl (Table 1). However, stem diameter decreased in both *Sc/SlHKT1;2*-silenced plants under saline treatment. This salt treatment for 34 d did not modify stomatal conductance or photosynthesis rates, which, however, did significantly decrease in the salt-treated *ScHKT1;2*–silenced

line as compared to the control. On the other hand, salt treatment affected root hydraulic conductivity, which sharply decreased in all silenced and non-silenced lines (Table 1). Longer salt treatment of 66 d, and even 85 d, had little negative effect on plant height and dry weight biomass, but significantly reduced stem diameter in both *Sc/SlHKT1;2*-silenced lines (Table 1). Interestingly, after 85 d of salt treatment, *Sc/SlHKT1;2*-silenced plants showed total leaf necrosis in all expanded leaves, except in young leaves emerging from the plant apex, while non-silenced plants showed green leaves with no necrotic symptoms along the whole plant axis (Fig. 2, A-D). Most flowers from basal floral inflorescences of silenced plants showed necrotic sepals and petals and even lost their reproductive organs, with only receptacles attached to pedicels remaining (Fig. 2, E-L).

3.2. Sl/ScHKT1;2–RNAi silenced lines showed Na^+ overaccumulation in leaf and floral tissues

Regardless of genotype, Na^+ concentrations in xylem sap after 34 d of salt treatment were significantly higher in all salt-treated plants than in non-salt-treated plants (Fig. 3). Na^+ content was considerably higher in *Sc/SlHKT1;2*-RNAi silenced plants than in the respective non-silenced plants. Despite the upward trend, no significant change in xylem sap K^+ content caused by salt treatment was observed in any genotypes (Fig. 3). We then analysed the effect of silencing each Sc/Sl*HKT1,2* allelic variant on the pattern of Na^+ and K^+ accumulation in roots, leaves and flowers. In non-silenced plants salt treated for 34 d, NIL14 had a higher leaf Na^+/K^+ ratio than NIL17 due to higher Na^+ and lower K^+ concentrations in leaves (Fig. 4). However, both *SlHKT1;2*- and *ScHKT1;2*-RNAi lines showed higher levels of Na^+ accumulation and lower levels of K^+, and consequently, higher Na^+/K^+ ratios in leaves than their respective non-silenced NILs (Fig. 4). This is associated to a salt-hypersensitive phenotype, mainly characterized by total necrosis in all the basal expanded leaves (Fig. 2). Na^+ and K^+ content in roots behaved in an opposite fashion to that of leaves, with a higher root Na^+/K^+ ratio in NIL 17 than in NIL 14, mainly due to higher root Na^+ content (Fig. 4). After salt treatment for 85 d, floral tissues showed higher Na^+ content in both *SlHKT1;2*- and *ScHKT1;2*-RNAi lines (Fig. 5), while, in non-silenced plants, Na^+ and K^+ content remained virtually unchanged. The increase in Na^+ in floral tissues was slightly higher in the

SlHKT1;2-RNAi than in the *ScHKT1;2*-RNAi line, with no changes in K^+ content being observed, which led to an increased Na^+/K^+ ratio in flowers from salinized *SlHKT1;2*-RNAi as compared to *ScHKT1;2*-RNAi silenced plants (Fig 5).

3.3. Sl/ScHKT1;2 silencing dramatically affected tomato yield and quality under saline conditions

Tomato yield measured as the total fresh weight and total number of fruits is presented in Fig. 6. As expected, salinity reduced yield regardless of genotype. Under non-saline conditions, production was higher in non-silenced NIL17 plants, which also showed the highest root hydraulic conductivity, although it needs to be pointed out that over 95% of tomato fresh weight is related to its water content. The yields of non-silenced NIL17 and NIL 14 lines cultivated under saline conditions decreased by around 20 and 50%, respectively, as compared to the respective non-salinized controls. This salt-induced reduction in yields was even more dramatic in both *SlHKT1;2*- and *ScHKT1;2*-RNAi plant lines, some of which produced no fruit at all.

Larger amounts of total soluble solids (ºBrix) were found only in fruits from NIL17 and NIL14 plants grown under saline growth conditions. However, these TSS did not significantly increase in either *SlHKT1;2*- or *ScHKT1;2*-RNAi lines (Table 2). Significant differences were found in all antioxidant compounds studied and in antioxidant activity. Under non-saline conditions, antioxidant activity was similar in fruits from NIL17 and NIL14 and slightly higher, but not significantly, in fruits from *SlHKT1;2*- and *ScHKT1;2*-RNAi lines. As expected, antioxidant activity increased along with salinity in fruits from silenced lines which experienced a decrease in total fruit yield. Interestingly, salinity increased fruit lycopene in NIL 14 but not in NIL 17.

3.4. Relative expression levels of other ion homeostasis-related genes

ScSOS1 and *ScNHX4* gene expression was analysed in the same tissues and at the same developmental stage of silenced and non-silenced lines as those of *HKT1;2 (*Fig. 7). In roots, leaves and floral tissue from non-silenced and silenced genotypes, *ScSOS1* expression levels appeared to be

similar with or without NaCl. Salinity only increased *ScNHX4* transcript abundance in non-silenced NIL17 roots, with no apparent change in leaf or floral levels observed. In roots, *SlHKT1;2,* but not *ScHKT1;2,* silencing was associated with a significant increase in *ScNHX4* transcript abundance under salinity conditions. Thus, the genotype (*S. lycopersicum* or *S. cheesmaniae* allele, silenced or non-silenced) at the *HKT1;2* locus affects the transcription behaviour of the *S. cheesmanieae* allele at locus *NHX4* in roots.

4. Discussion

4.1. SlHKT1;2 and ScHKT1;2 Na^+ transporters prevent Na^+ over-accumulation in leaf tissues

The tomato gene *HKT1;2* encodes a Na^+-selective class I HKT transporter expressed in the vascular system, particularly in the xylem and possibly in the phloem of tomato leaves and roots (Asins et al., 2013; Almeida et al., 2014; Jaime-Perez et al., 2017). Under commercial greenhouse (natural light and no temperature control) and salinity conditions, *HKT1;2* gene expression in non-silenced NILs was very similar to expression patterns observed in previous studies (Fig. 1; Asins et al., 2013; Jaime-Perez et al., 2017). We also found that Na^+ levels in leaves were significantly higher in NIL14 than in NIL17 (Fig. 4). This result phenotypically reproduced the major QTL, controlling Na^+/K^+ homeostasis in tomato shoots. In this trait, the *cheesmaniae HKT1;2* allele is associated with larger amounts of Na^+ and smaller amounts of K^+ to be stored in the aerial part of NIL14 plants, while the *lycopersicum HKT1;2* allele has a converse effect (Villalta et al., 2008; Asins et al., 2013). This closely corresponds to much lower *ScHKT1;2* gene expression in roots and higher gene expression in NIL14 leaves, unlike *SlHKT1;2* gene expression in NIL17 leaves under salinity conditions (Fig 1; Asins et al., 2013; Jaime-Perez et al., 2017). In roots, the low transcription levels of *ScHKT1;2* in NIL14 (hypoallelic in roots) could mean that less Na^+ was retrieved from the xylem and that more Na^+ was transported via the transpiration stream to the aerial part as compared to the higher expressed *SlHKT1;2* in NIL17. In addition, the increased expression of *ScHKT1,2* in leaves from NIL14 (hyperallelic in leaf) may increase the withdrawal of Na^+ from the leaf xylem, thus promoting its intracellular accumulation in the mesophyll cells of expanding

leaves (Fig. 1; Asins et al., 2013; Jaime-Perez et al., 2017). This physiological mechanism provided by *HKT1;2* wild alleles involved in retrieving Na^+ from the xylem, which is hypoallelic in roots and hyperallelic in leaves, could result in improved and more inexpensive osmotic adjustments in leaves if safely stored in vacuoles. It would also explain why salt-tolerant tomato varieties and species accumulate more sodium in aerial parts than sensitive varieties (Cuartero and Fernandez-Muñoz, 1999, Cuartero et al., 2006).

Under saline conditions, the silencing of the *Sl/ScHKT1,2*-encoding Na^+ transporter caused a sharp increase in xylem sap Na^+ concentrations, with no change observed in concentrations of K^+, which is indicative of an increase in Na^+ translocation from roots to shoots (Fig. 3). Function loss in Sl/ScHKT1,2 resulted in higher Na^+ levels in both tomato leaves from *Sc/SlHKT1;2*-silenced lines than in their respective non-silenced lines. These results are very similar to those obtained in a previous study at the vegetative stage under controlled environmental conditions (Jaime-Perez et al., 2017). In a previous study, changes caused by salt treatment (100 mM NaCl for 15d) in the leaf Na^+/K^+ ratio in both *Sc/SlHKT1;2*-silenced lines cultivated in hydroponic pots under controlled environmental conditions were associated with a salt-hypersensitive phenotype characterized by a drastic reduction in shoot and root growth (Jaime-Perez et al 2017). However, in the present study, carried out under more moderate salinity conditions in a commercial greenhouse, both *Sc/SlHKT1;2*-silenced lines behaved in a similar fashion, with no reduction observed in the growth parameters studied (Table 1). However, both *Sc/SlHKT1;2*-silenced lines exhibited total necrosis in all basal expanded leaves following 2 to 3 months of salt treatment (1 to 2 months after sampling tissues for ion analysis following 34 days of salt treatment), with non-silenced plants showing no necrotic symptoms in green leaves along the plant axis (Fig 2). The appearance of this late phenotype could be due to the experiment having been carried out in the autumn-winter period in a greenhouse with natural illumination and no temperature control. Under these conditions, evaporative demand is very low as compared to that usually recorded in a greenhouse at other times of the year (Cuartero and Fernandez-Muñoz, 1998; Romero-Aranda et al. 2001, 2002).

This factor, together with the reduction in root hydraulic conductivity and transpiration rates observed under salt treatment (Table 1), may have considerably reduced the entry of salt into the plant despite the higher rates of Na^+ accumulation in leaves in *Sc/SlHKT1;2*-silenced lines after 34 days of salt treatment (Fig. 4). This could improve osmotic adjustments to some extent, thus enabling the maintenance of foliar turgor and subsequent ion dilution in the new foliar tissue (Cuartero and Fernandez-Muñoz, 1998). The toxicity threshold associated with foliar salt accumulation is probably delayed over time, which delays its negative effect on photosynthesis and biomass production rates (Table1). The salt-hypersensitive phenotype associated with *Sc/SlHKT1;2*-silenced lines was similar to that of the Arabidopsis *hkt1;1* mutant, which is characterized by hyperaccumulation of Na^+ and lower K^+ content in shoots under transpiring conditions (Berthomieu et al., 2003; Sunarpi et al., 2005; Davenport et al., 2007), again indicating that tomato HKT1;2 plays a role similar to that of AtHKT1;1 (Asins et al. 2013, Jaime Perez et al., 2017).

Apart from HKT1-like transporters, tomato genes encoding a Na^+ transporter like SOS1 and its regulatory proteins (Olías et al., 2009; Huertas et al., 2012), as well as some NHX-type antiporters such as vacuolar Na^+/H^+ antiporter NHX4 (Galvez et al 2012), are mainly involved in regulating Na^+ concentrations in various tomato plant tissues. The expression levels of *ScNHX4* in leaves were similar in both *ScHKT1;2*- and *SlHKT1;2*-silenced lines in response to salt stress (Fig. 7). In addition, neither Na^+ loading in xylem roots nor unloading from mesophyl cells via SOS1 are likely to be involved in these outcomes as no change in SOS1 expression was observed (Fig. 7). However, this possibility cannot be completely ruled out, as SOS1 function not only depends on *SOS1* gene transcription but also on the regulation of protein activity. It is worth noting that *ScNHX4* root transcription levels in both non-silenced NIL17 and silenced *SlHKT1;2* lines increased under salinity conditions, while *ScNHX4* root transcription levels of the *ScHKT1;2*-silenced line remained as high as those of the non-silenced NIL14 line (Fig. 7). This Na^+ detoxification capacity in roots could be regarded as part of the salt tolerance strategy of the Na^+ exclusion mechanism in NIL17 which is based on Na^+ sequestration in the root

vacuole together with Na^+ retrieval from the root xylem by the *HKT1;2 lycopersicum* allele (Asins et al., 2013).

4.2. Sl/ScHKT1;2 Na^+ transporters mitigate reductions in fruit yields by reducing floral sodium content

Salinity severely limits the reproductive growth and yield of tomato (Cuartero and Fernandez-Muñoz, 1998). Several factors, including the accumulation of toxic ions, such as Na^+, K^+ deficiency, plant hormone imbalance and reduced carbon supply, can negatively affect reproductive tissue development induced by salinity (Cuartero and Fernández-Muñoz, 1998; Albacete et al., 2014). We found that silencing both *ScHKT1;2 and SlHKT1;2* alleles in tomato NILs induces a Na^+ accumulation pattern in the 4th floral truss similar to that in leaves after 85 d of salt treatment (Fig. 5). There was a sharp increase in concentrations of Na^+, no change in K^+, as well as a subsequent sharp increase in the Na^+/K^+ ratio, while salt-induced Na^+ accumulation in flowers of both non-silenced NIL lines was negligible. This is in line with the almost totally silenced transcript levels of both alleles in *Sc/SlHKT1;2*-silenced NILs (Fig. 1C). The altered Na^+/K^+ ratios in flower tissues of both *ScHKT1;2*- and *SlHKT1;2*-silenced lines due to *ScHKT1;2* and *SlHKT1;2* function loss are associated with a phenotype typically found in basal floral trusses, where most flowers showed necrotic areas in sepals and petals and even lost their reproductive organs, with only the receptacles attached to pedicels remaining (Fig 2, E-L). However, both NIL14 and NIL17 were effective at limiting sodium flux to flowers under salinity conditions (Fig. 5). Na^+ levels in flower trusses were slightly higher in *SlHKT1;2*- than in *ScHKT1;2*-silenced lines (Fig. 5), concomitantly with a higher *ScHKT1;2* gene expression in NIL14 flowers than *SlHKT1;2* in NIL17 flowers under control and salinity conditions (Fig. 1). These data show that ScHKT1;2 and SlHKT1;2 Na^+ transporters can play an important role in limiting Na^+ flux to flower in order to protect this organ against Na^+ toxicity.

According to Cuartero and Fernández-Muñoz, (1998), the reduction in the tomato yields of commercial cultivars caused by low-to-moderate salinity levels (25–75mM NaCl) is primarily due to decreased fruit weight, while, at high salinity levels (>75–100mM NaCl), the reduced number of fruits is responsible for lower yields. In this study, total weight and number of fruits per plant were reduced by

salt treatment in both NILs and were reduced to a minimum in both *ScHKT1;2*- and *SlHKT1;2*-silenced lines (Fig. 6). This dramatic reduction in the fruit yields of ScHKT1;2- and *SlHKT1;2*-silenced lines under salinity conditions was associated with the accumulation of Na^+ in flower tissues (Fig. 5). Also, in chickpea, reduced crop yields under high salinity conditions were mainly due to Na^+ accumulation in reproductive structures (Samineni et al., 2011). Reduced tomato yields have also been attributed to decreased flowering under salty conditions which reduces pollen (Grunberg et al., 1995) and ovule abortion (Sun et al. 2004). Our data suggest that Na^+ retrieval by Sl/ScHKT1;2 from the xylem prevents Na^+ overaccumulation in reproductive organs, which appears to be crucial with regard to mitigating the reduction in fruit yields under salinity conditions. In other studies, OsHKT1;5 appears to mediate Na^+ exclusion in the phloem to protect young leaf blades and reproductive tissues against salt toxicity in rice (Kobayashi et al. 2017). Also, a hyper-functional *AtHKT1;1* allele in shoots drives the adaptation of Arabidopsis natural accessions to salinity by reducing floral Na^+ and increasing fecundity upon salt stress (An et al., 2017). These authors concluded that Na^+ levels in flowers, but not in leaves, are positively associated with saline adaptability, suggesting that floral Na^+ content is more suited to assessing plant salt tolerance than leaf (or shoot) Na^+ content.

Moderate salinity levels improve the quality of tomato fruits, whose total soluble solids and antioxidant compounds typically increase under these conditions (Cuartero and Fernandez-Muñoz, 1999; Cuartero et al.; 2006). As expected, total soluble solids (ºBrix) increased only in fruits from NIL17 and NIL14 plants grown under saline conditions. This could be related to the smaller amounts of water in fruit, which induces an increase in sugar concentrations (Romero-Aranda et al., 2001; Reina et al., 2005), an increase was not, however, detected in either *SlHKT1;2*- or *ScHKT1;2*-RNAi lines (Table 2). Salt stress has been reported to negatively affect plant growth and development partly due to oxidative damage caused by increased production of reactive oxygen species (ROS). In the absence of a protective mechanism in plants, ROS can cause serious damage to different aspects of cell structure and function, such as lipid peroxidation, as well as to DNA, proteins and other small molecules (Gill and

Tuteja, 2010). ROS-mediated membrane damage has been demonstrated to be a major cause of cellular toxicity in salt-stressed tomato (Mittova et al., 2002). Antioxidant activity is associated with compounds such as lycopene and phenolics which act through several chemical mechanisms to prevent oxidative damage to plant tissues (Zhao et al., 2015). The data in Table 2 show that salt treatment only increased antioxidant activity and related compounds (lycopene and total phenolics) in silenced genotypes. Although we did not measure ROS production or Na^+ content in fruits, the massive entry of Na^+ via the xylem due to Sc/SlHKT1;2 silencing may have increased ROS production in fruits and subsequently antioxidant activity.

It is still unclear why differences in *HKT1;2* gene expression observed between the *cheesmaniae* and *lycopersicum* alleles did not translate into salt tolerance differences, even when tomato fruit production is used as an important criterion of salt tolerance. In a previous study, under salinity conditions higher than those used in the present study, NIL17, which is more vigorous and slightly more productive than NIL14 under normal conditions, appears to be more salt-tolerant despite the sharper reduction in shoot growth as compared to NIL14 (Asins et al., 2013). Interestingly, Jaime-Perez et al. (2017) found that silencing the *cheesmaniae* HKT1;2 had a more pronounced negative effect on plant growth under salinity than silencing the *lycopesicum* HKT1;2, which agrees with our findings. Thus, a higher foliar Na^+/K^+ ratio was observed in the *ScHKT1;2*-RNAi line than in the *SlHKT1;2*-RNAi line (Fig.4), although the *ScHKT1;2*-RNAi line showed a slightly less pronounced increase in the flower Na^+/K^+ ratio than that of the *SlHKT1;2*-RNAi line (Fig. 5). This indicates that Na^+ retrieval from the xylem by the ScHKT1;2 transporter in the aerial part may play a role in Na^+/K^+ homeostasis equally as important as that of the *SlHKT1;2* transporter in roots. Nevertheless, silencing *ScHKT1;2* and *SlHKT1;2* caused a visible salt hypersensitivity phenotype in leaves and flowers as a result of Na^+ over accumulation in these tissues (Figs. 2, 4 and 5). Given the localisation of HKT1;2 in the vascular system, including the xylem and phloem (Jaime-Perez et al.2017), this salt hypersensitivity phenotype may be due to the combined effect of *HKT1;2* function loss in roots, which increases Na^+

export from roots to leaves and flowers, and *HKT1;2* function loss in the cells of leaf vascular bundles, preventing Na^+ delivery through the plasma membrane and subsequent compartmentation into large mesophyll cell vacuoles from expanded leaf. This could lead to a sharp increase in Na^+ accumulation in the apoplast, which has a hyperosmotic effect on the mesophyll cells of expanding leaves and flowers, causing damage to these cells due to water leakage (physiological drought). A similar explanation for the salt hypersensitivity of Arabidopsis mutant *sas1* has also been proposed (Nublat *et al.* 2001). The differential expression of *Sl/ScHKT1;2* allelic variants in the root and aerial parts of NIL17 and NIL14 undoubtedly makes it difficult to clarify the role of tomato HKT1-like transporters in salt tolerance (Fig 1; Asins et al., 2013; Jaime-Perez et al., 2017). *ScHKT1;2* expression levels are much lower in roots and much higher in leaves and flowers, unlike *SlHKT1;2*, whose expression follows a converse pattern. It is therefore important to determine in which tissue the function loss of each allelic variant has the greatest impact on tomato salt tolerance. It is also important to determine the relative contribution of each allelic variant in the removal of Na^+ from the xylem stream at root level with respect to that of the aerial part in each NIL. Finally, we will need to investigate the role of tomato HKT1-like transporters in phloem loading and their impact on Na^+ content in sink tissues, such as those of young leaves and fruits, as well as on Na^+ redistribution to roots.

In conclusion, this study indicates that Na^+ transporter HKT1;2 protects flowers from Na^+ toxicity and mitigates the reduction in tomato fruit yields under salinity conditions. The results obtained provide basic data for future research on reciprocal grafting experiments to improve the fruit yield and quality of tomato and other horticultural crops under salinity conditions.

Conflicts of interests

The authors declare no potential or actual conflict of interest.

Contributions

MRRA and AB conceived and designed the research; PGF, JRPT; MRLD, JE, EG, BP, BGS, JAT, MRRA and AB conducted the experiments and analysed the data; MRRA, MJA, VM and AB wrote the manuscript and supervised the design and interpretation of the experiments..

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Appendix A. Supplementary data

Table S1. Primers used for quantitative real-time PCR.

Figure S1. Overview of crop development under commercial greenhouse conditions.

Figure S2. Electrical conductivity (EC) and pH of irrigation solutions from drippers and in pot lixiviates recorded at different times during the experimental period of the study**.**

Figure S3. Stability of tomato EF1-*a* gene expression, expressed as cycle threshold (Cts), in response to salt stress treatment in root (A), leaf (B) and flower (C) tissues of non-silenced, *SlHKT1;2*- and *ScHKT1;2*-silenced tomato NIL lines

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Table 1. Means ± standard deviation and p-values of traits corresponding to several growth and physiological parameters of NILs 14 and 17 and their respective silenced lines *ScHKT1;2* and *SlHKT1;2*, measured on different days under control (C, 0 mM NaCl) and saline (S, 80 mM NaCl) conditions. Growth traits: PH (plant height, centimeters), SD (stem diameter, millimeters), RDW (root dry weight, grams), SDW (shoot dry weight - stem and leaf - grams). Physiological parameters: *gs* (stomatal conductance, mol H_2O $m^{-2}s^{-1}$), Lpr (root hydraulic conductivity, µl $m^{-1}g^{-1}$ RDW), A (photosynthesis, µmol CO_2 $m^{-2}s^{-1}$), E (transpiration, mmol m^{-1} s^{-1}). *P*-values in the mixed model analysis, with significant p-values ($p<0.05$) in red. For traits showing significant G × E interaction, means with the same letter do not significantly differ according to Tukey's test ($P < 0.05$).

Days in salt/ Trait	17_C	SlHKT1.2_C	17_S	SlHKT1.2_S	14_C	ScHKT1.2_C	14_S	ScHKT1.2_S	G	E	GxE
34 d											
Lpr	38.8±8.0 c	14.5±2.4 b	1.9±0.2 a	2.7±0.8 a	16.3±3.5 b	22.3±1.6 b	1.1±0.4 a	3.3±0.6 a	0.0000	0.0000	0.0000
A	15.7±1.1 b	15.9±2.3 b	16.2±1.1 b	11.1±1.2 ab	12,0±5.0 ab	15.7±1.5 b	14.9±2.1 ab	8.8±0.8 a	0.0754	0.0453	0.0062
gs	0.28±0.06	0.20±0.03	0.25±0.03	0.18±0.03	0.13±0.07	0.16±0.07	0.20±0.04	0.13±0.01	0.0030	0.9335	0.3304
E	4.7±0.8 b	3.8±0.2 ab	4.3±0.3 b	3.3±0.5 ab	2.6±1.2 a	3.9±0.1 ab	3.7±0.6 ab	2.6±0.2 a	0.0040	0.2585	0.0249
PH	44.5±3.2	49.8±5.0	46.0±7.4	49.2±3.2	34.3±6.5	37.2±4.07	32.67±6.22	41.33±5.79	0.0000	0.5977	0.5783
SD	5.3±0.8	5.6±0.6	5.3±0.6	4.7±0.3	4.5±0.9	5.2±0.5	4.1±0.6	4.4±0.8	0.0035	0.0109	0.2538
66 d											
PH	116.2±15.0 c	105.9±7.0 bc	88.7±7.3 ab	103.1±8.5 bc	104.7±8.7 bc	92.5±4.3 ab	83.1±3.3 a	91.8±6.7 ab	0.0114	0.0001	0.0015
SD	8.2±0.5 cde	7.4±0.4 bc	7.1±0.3 b	5.4±0.3 a	8.8±0.8 de	8.8±0.7 e	7.4±0.7 bcd	5.5±0.3 a	0.0000	0.0000	0.0000
85 d											
SDW	4,0±1.1 ab	6.2±0.4 b	5.8±1.4 b	4.3±0.5 ab	3.3±1.6 ab	4.2±1.5 ab	2.5±0.8 a	4.3±1.7 ab	0.0092	0.4474	0.0034
RDW	0.44±0.25	0.45±0.17	0.45±0.22	0.39±0.19	0.40±0.29	0.4±0.16	0.20±0.12	0.21±0.17	0.5536	0.0667	0.4059

Table 2. Antioxidant activity (µmol Trolox kg^{-1} FW), lycopene (ug/g FW), β-carotene (ug/g FW), total phenolics (mg GAE/100g FW), sugars (ºBrix) and titrable acidity (% citric acid) of fruits from NILs 17 and 14 and their respective silenced lines *SlHKT1;2* and *ScHKT1;2* grown under control (C, 0 mM NaCl) and saline (S, 80 mM NaCl) conditions. *P*-values in the mixed model analysis, with significant p-values (p<0.05) in red). For traits showing significant G × E interaction, means with the same letter do not significantly differ according to Tukey's test (P < 0.05).

	17_C	SlHKT1.2_C	17_S	SlHKT1.2_S	14_C	ScHKT1.2_C	14_S	ScHKT1.2_S	G	E.	GxE
Antioxidant activity	1122±237 a	1481±235 a	1678±236 a	2548±611 b	1251±281 a	1315±289 a	1709±384 a	3098±440 b	0.0000	0.0000	0.0022
Lycopene	23.1±8.0 a	25.4±12.1 a	20.5±9.1 a	50.3±11.1 b	23.0±3.5 a	27.5±7.0 a	36.7±14.6 b	52.8±11,0 b	0.0002	0.0000	0.0044
β-carotene	39.3±8.2	64.5±30.9	34.0±12.3	51.7±14.9	56.3±7.5	45.4±13.7	56.0±11.6	59.0±18.3	0.0290	0.3847	0.4553
Total carotenoids	62.5±15.4	81.3±26.5	54.5±21.1	102.0±24.5	79.2±10.4	72.9±20.5	90.1±20.4	111.9±28.7	0.0083	0.0143	0.2043
Total phenolics	55.8±5.2 ab	54.1±10.2 ab	71.8±9.9 ab	131.1±22.7 c	50.0±6.3 a	50.1±8.6 a	76.4±10.4 b	133.1±18.2 c	0.0000	0.0000	0.0000
ºBrix	7.7±0.8 ab	9.4±1.2 b	13.4±1.7 c	8.9±1.8 b	6.1±0.4 a	7.9±0.5 ab	14.9±2.0 c	9.5±1.6 b	0.0023	0.0000	0.0000
Titrable acidity	0.54±0.06	0.51±0.07	0.55±0.10	0.56±0.08	0.37±0.03	0.57±0.05	0.51±0.08	0.55±0.12	0.0021	0.0583	0.0858

Figure Legends

Figure 1. Transcript levels of *HKT1;2* in root (A), leaf (B) and flower (C) tissues of non-silenced, *SlHKT1;2*- and *ScHKT1;2*-silenced tomato NIL lines. Non-silenced and silenced NIL 17 lines contain the SlHKT1;2 allele (blue bars), while non-silenced and silenced NIL 14 lines contain the ScHKT1;2 allele (red bars). Total RNA was purified from roots, the 9th leaf of tomato plants was treated for 34 d and the fourth floral inflorescence (peduncle, pedicel and flowers) of tomato plants was treated for 85d with 0 mM NaCl (dark bars) and 80 mM NaCl (light bars). The tomato elongation factor gene, LeEF-1α, was used as the reference gene. The results show an increase or decrease in the transcript levels of each *HKT1;2* gene in relation to the levels for roots, leaves and flowers of non-silenced NIL17 plants cultivated in the absence of stress, to which the value 1 is assigned. Each value is the mean ± the standard error of nine replicates for roots, stems and leaves (three biological replicates and three technical replicates).

Figure 2. Effect of NaCl treatment on growth of tomato NILs and *Sl/ScHKT1;2-RNAi* lines grown under commercial greenhouse conditions. Plants were grown under natural light conditions without temperature control during the autumn-spring season (November to May). Tomato plants were cultivated in 17-L pots filled with vermiculite as substrate and irrigated for 85 d with Hoagland solution supplemented with 0 mM NaCl (C) and 80 mM NaCl (S). Control lines of NIL17 and NIL 14 (A), salt-treated lines NIL17 and NIL 14 (B), control lines of *SlHKT1;2-RNAi* and *ScHKT1;2-RNAi* (C), salt-treated lines of *SlHKT1;2-RNAi* and *ScHKT1;2-RNAi* (D). Magnified images of *SlHKT1;2-RNAi* and *ScHKT1;2-RNAi* control lines (E, F) and SlHKT1;2-RNAi and ScHKT1;2-RNAi salt-treated lines (G, H). Detail of inflorescence and a flower from *SlHKT1;2-RNAi* (I, J) and *ScHKT1;2-RNAi* salt-treated lines (K, L).

Figure 3. Na^+ and K^+ content in xylem sap from control and salt -treated non-silenced, *SlHKT1;2* and *ScHKT1;2*-silenced tomato NIL lines. Root exudate content of Na^+ and K^+ from non-silenced and silenced NIL 17 contains the *SlHKT1;2* allele (blue bars) and NIL 14 lines contain the *ScHKT1;2* allele (red bars). Eleven-week-old tomato plants were cultivated in vermiculite and irrigated with 1x Hoagland solution in a commercial greenhouse and treated for 34 d with 0 mM NaCl (dark bars) and 80 mM NaCl (light bars). Values represent the mean ± standard error of five different samples. Data were analyzed by two-way ANOVA using genotype (G), salt treatment (E) and their interaction (G x E) as sources of variation. Their statistical significance was evaluated (*P*-value; ns, not significant; **P*<0.05, ***P*<0.01; ****P*<0.001). Significant differences are indicated by different letters according to Tukey's test (*P*<0.05).

Figure 4. Na^+ and K^+ content and Na^+/K^+ ratio of 9th leaf (A) and root (B) in control and salt-treated non-silenced, *SlHKT1;2* and *ScHKT1;2*-silenced tomato NIL lines. Non-silenced and silenced NIL 17 contains the *SlHKT1;2* allele (blue bars) and NIL 14 lines contain the *ScHKT1;2* allele (red bars). Eleven-week-old tomato plants were cultivated in vermiculite and irrigated with 1x Hoagland solution in a commercial greenhouse and treated for 34 d with 0 mM NaCl (dark bars) and 80 mM NaCl (light bars). Values represent mean ± standard error of five different samples. Data were analyzed by two-way ANOVA using genotype (G), salt treatment (E) and their interaction (G x E) as sources of variation. Their statistical significance was evaluated (*P*-value; ns, not significant; **P*<0.05, ***P*<0.01; ****P*<0.001). Significant differences are indicated by different letters according to Tukey's test (*P*<0.05).

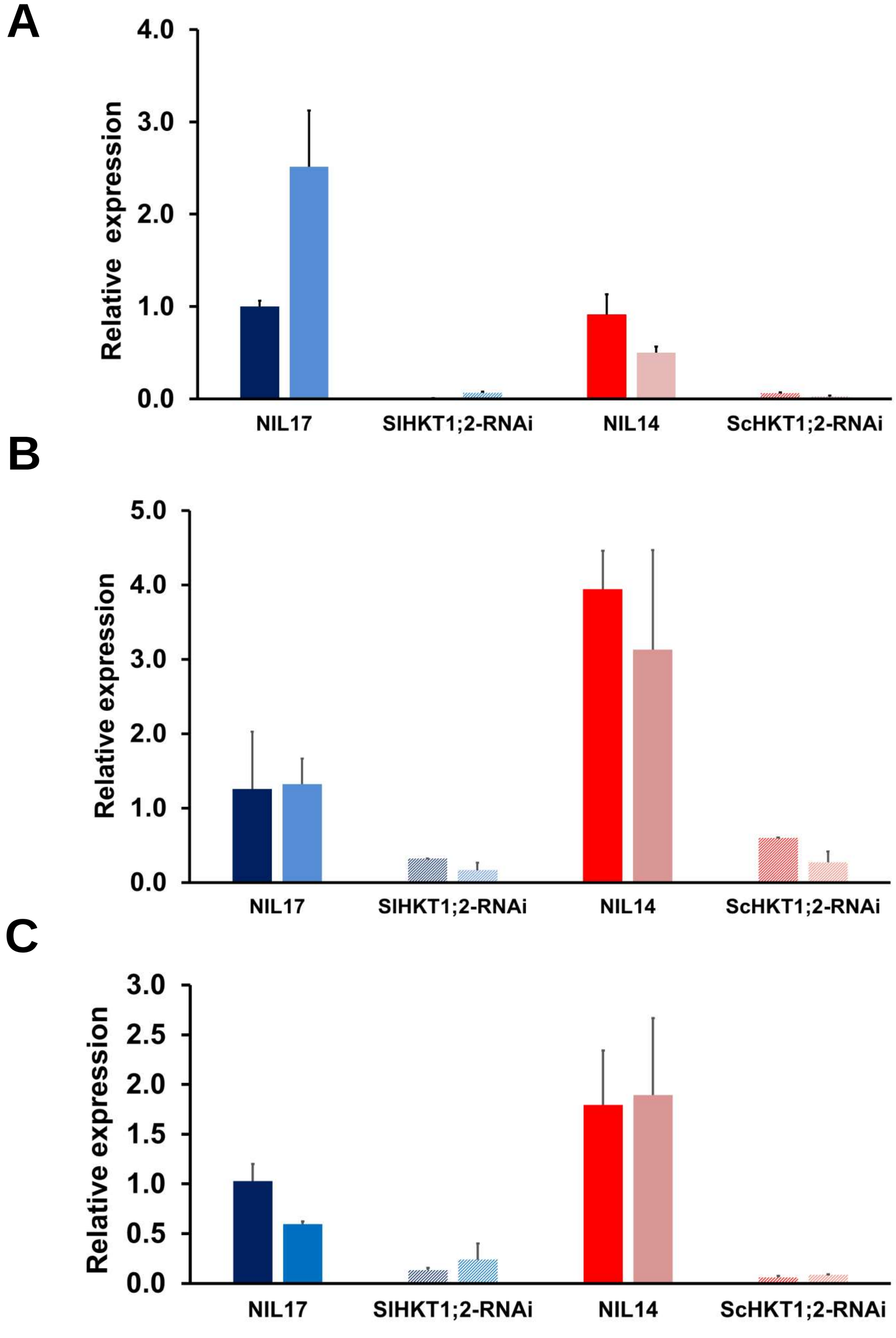
Figure 5. Flower Na^+ and K^+ content and Na^+/K^+ ratio in control and salt-treated non-silenced, *SlHKT1;2*- and *ScHKT1;2*-silenced tomato NIL lines. Na^+ and K^+ content in the 4th floral inflorescence, including peduncle, pedicel and floral receptacle, from non-silenced and silenced NIL 17 (*SlHKT1;2* allele -blue bars-) and NIL 14 lines (*ScHKT1;2* allele -red bars-). Eleven-week-old tomato plants were cultivated in vermiculite, irrigated with 1x Hoagland solution in a commercial greenhouse

and treated for 85 d with 0 mM NaCl (dark bars) and 80 mM NaCl (light bars). Values represent mean ± standard error of five different samples. Data were analyzed by two-way ANOVA using genotype (G), salt treatment (E) and their interaction (G x E) as sources of variation. Their significance was evaluated (P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001). Significant differences are indicated by different letters according to Tukey's est (P<0.05).

Figure 6. Fruit yield measured as total weight per plant (A) and number of fruits per plant (B) of NILs 14 and 17 and their respective *ScHKT1;2*- and *SlHKT1;2*-silenced lines grown under control and saline conditions. Eleven-week-old tomato plants were cultivated in vermiculite pots, irrigated with 1x Hoagland solution in a commercial greenhouse and treated for 180d with 0 mM NaCl (dark bars) and 80 mM NaCl (light bars). Fruits were harvested from the first and second trusses in a period of time between 120 and 180 d of salt treatment. Values represent mean ± standard error of six different samples. Data were analyzed by two-way ANOVA using genotype (G), salt treatment (E) and their interaction (G x E) as sources of variation. Their statistical significance was evaluated (P-value; ns, not significant; *P<0.05, **P<0.01; ***P<0.001). Significant differences are indicated by different letters according to Tukey's test (P<0.05).

Figure 7. Transcript levels of *ScNHX4* and *ScSOS1* in root (A), leaf (B) and flower tissues (C) of non-silenced, *SlHKT1;2*- and *ScHKT1;2*-silenced tomato NIL lines. Non-silenced and silenced NIL 17 contains the SlHKT1;2 allele (blue bars) while non-silenced and silenced NIL 14 lines contain the ScHKT1;2 allele (red bars). Total RNA was purified from roots, the 9th leaf of tomato plants was treated for 34 d and floral inflorescence (peduncle, pedicel and floral receptacle) of tomato plants was treated for 85d with 0 mM NaCl (dark bars) and 80 mM NaCl (light bars) after eleven weeks of growth in vermiculite and irrigated with 1x Hoagland solution in a commercial greenhouse. The tomato elongation factor gene (LeEF-1α) was used as the reference gene. The results show an increase or decrease in

transcript levels relative to those in roots, leaves and flowers of non-silenced plants cultivated in the absence of stress, to which value 1 is assigned. Each value is the mean ± the standard error (SE) from nine replicates for roots, stems and leaves (three biological replicates and three technical replicates).



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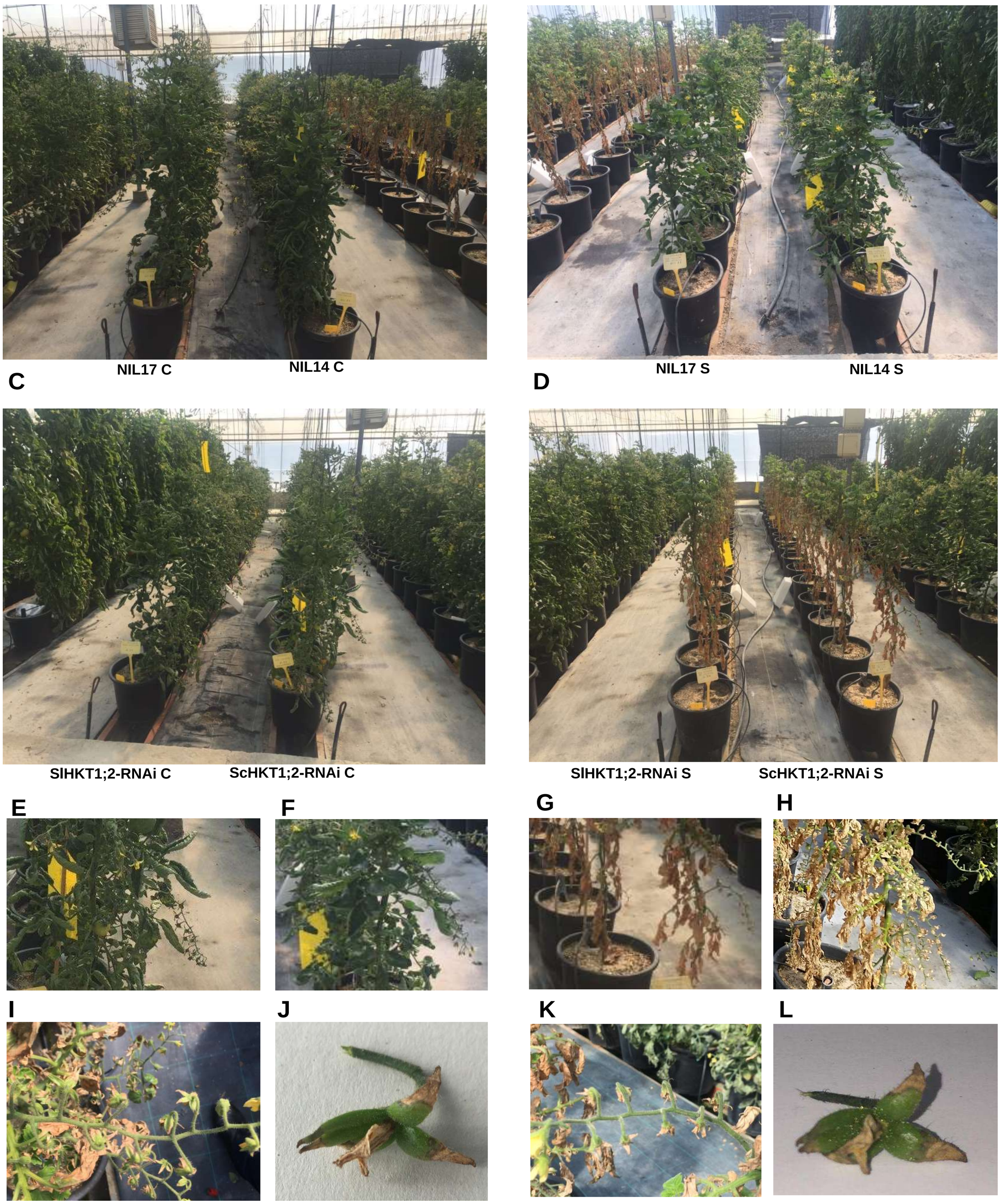

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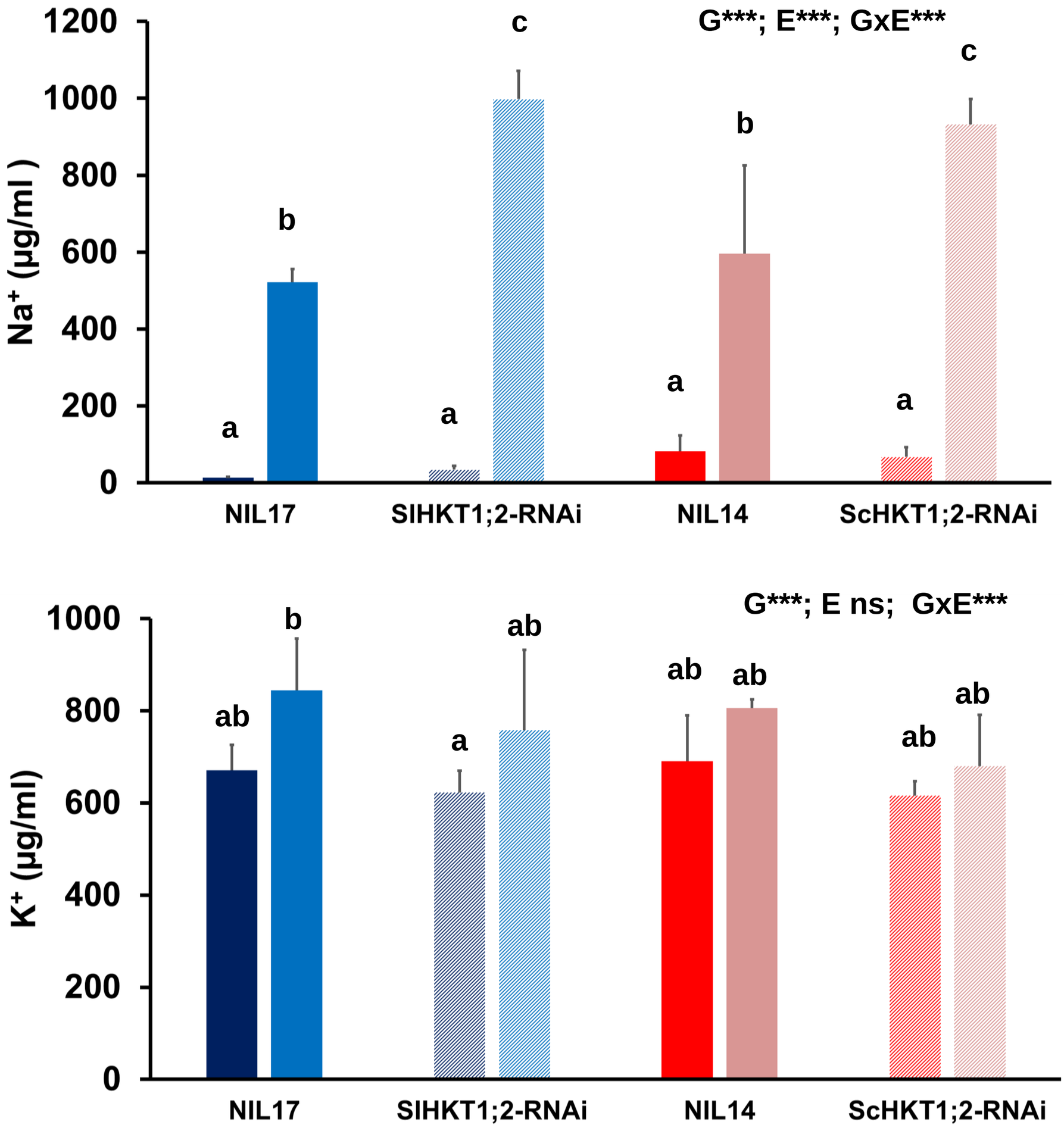

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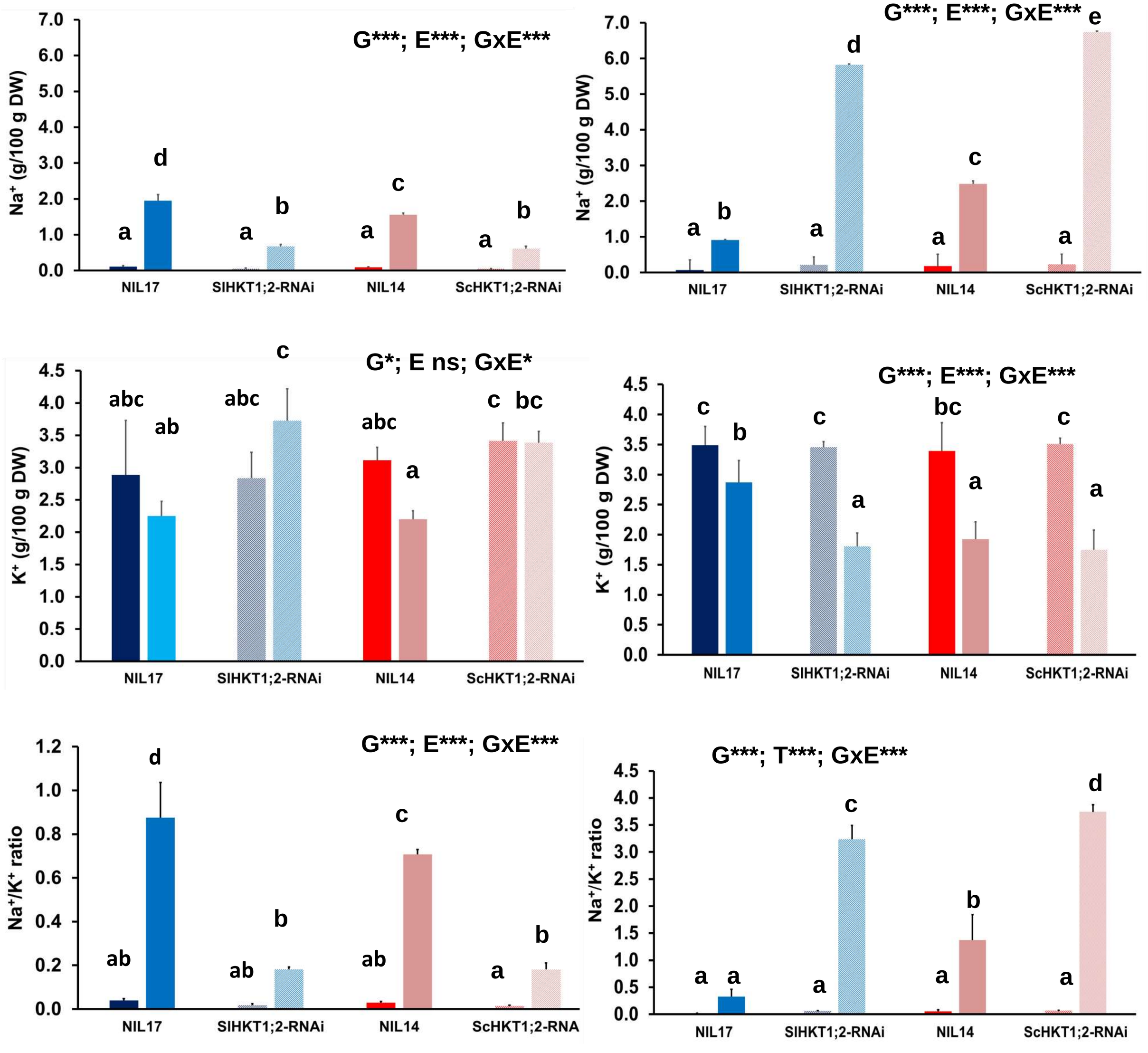

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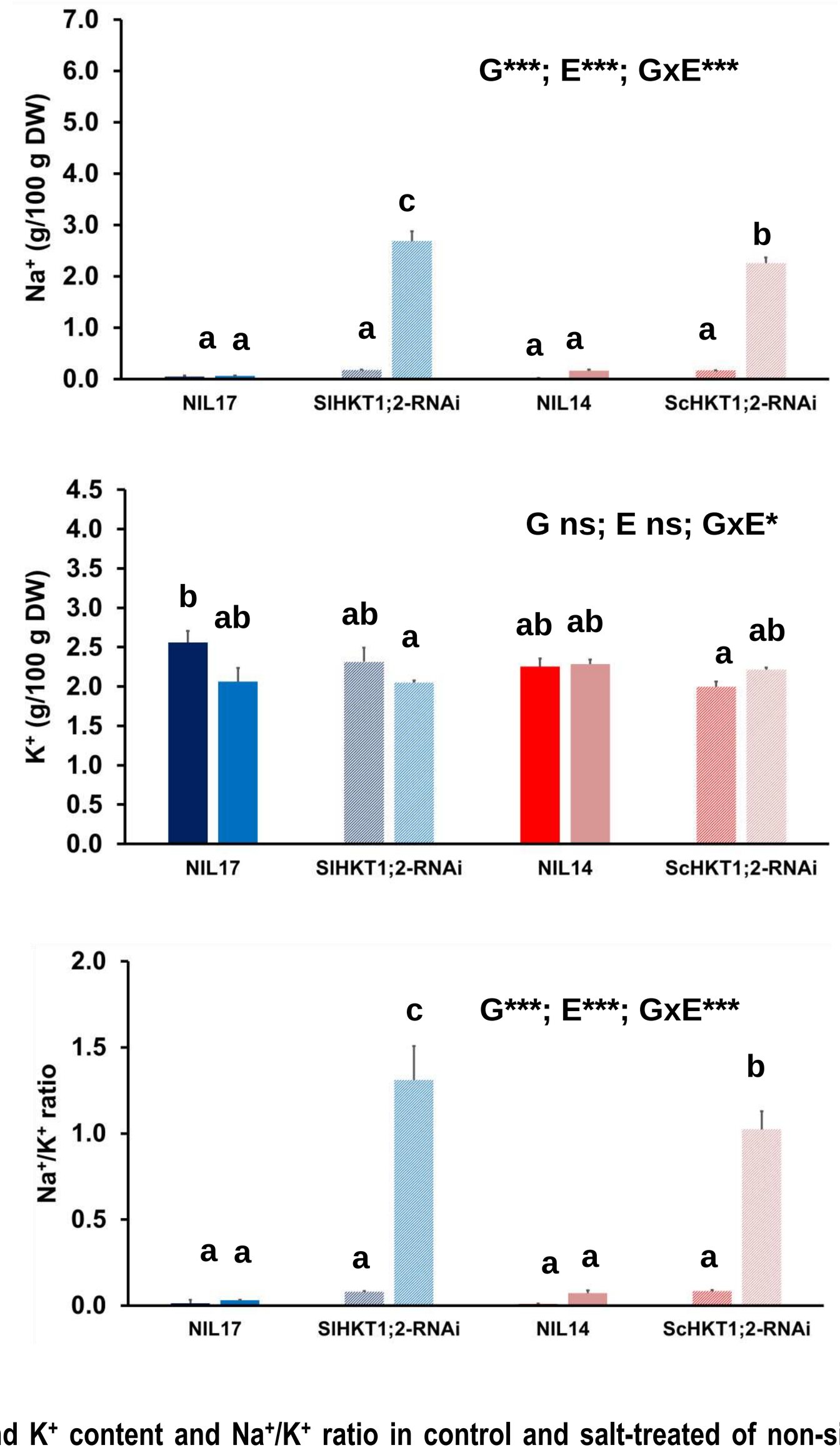

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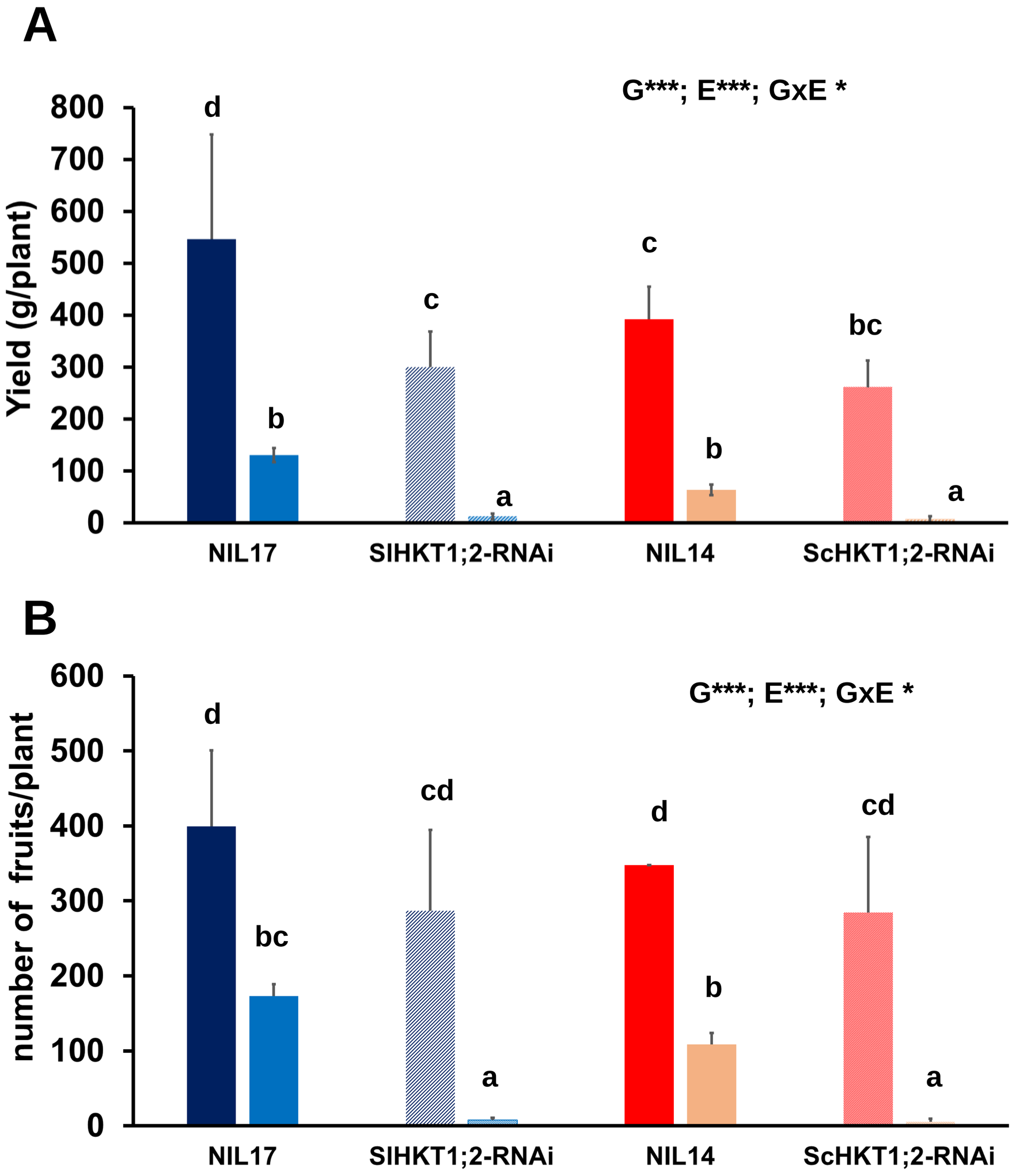

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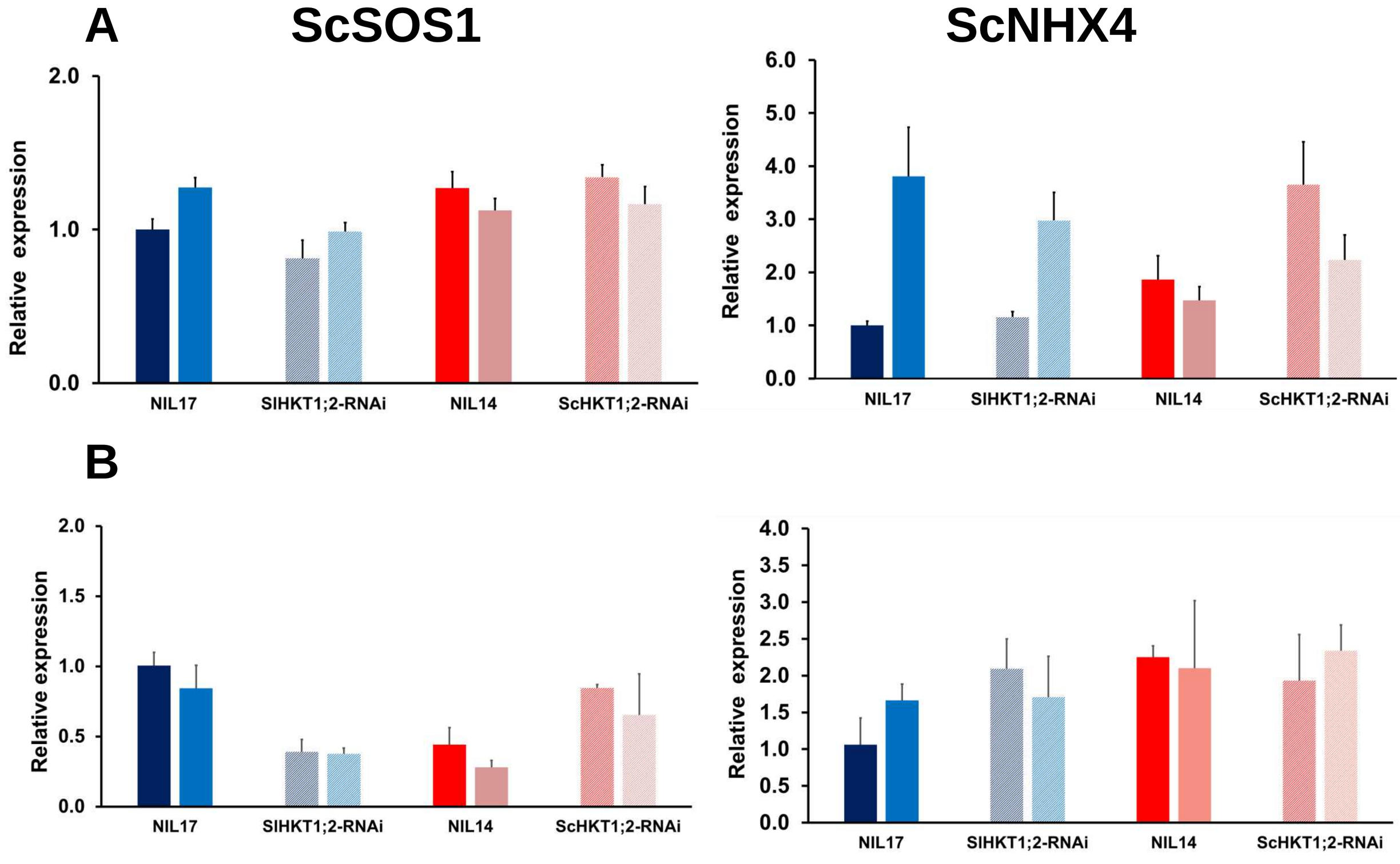

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Appendix A. Supplementary data

Table S1. Primers used for quantitative real-time PCR.

Figure S1. General view of crop development under commercial greenhouse conditions

Figure S2. Conductivity and pH of nutrient solution going out of the dripper of each row of pots and those of their respective drain liquid

Figure S3. Stability of tomato EF1-*a* gene expression (expressed as cycle threshold- Cts-) in response to salt stress treatment in root (A), leaf (B) and flower (C) tissues of non silenced, *SlHKT1;2*- and *ScHKT1;2*-silenced tomato NIL lines.

Table S1. Primers used for quantitative RT-PCR

Primers	**Sequence 5’-3’**	**Size**	**Reference**
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’		
LeNHX4 forward	TGGTGGGCAGGTTTGATGAGAG	165 bp	Huertas et al. (2012)
LeNHX4 reverse	TGTGGTGGCAGCAGGAGACTTA		
LeEF1α forward	GACAGGCGTTCAGGTAAGGA	119 bp	Asins et al. (2013)
LeEF1α reverse	GGGTATTCAGCAAAGGTCTC		

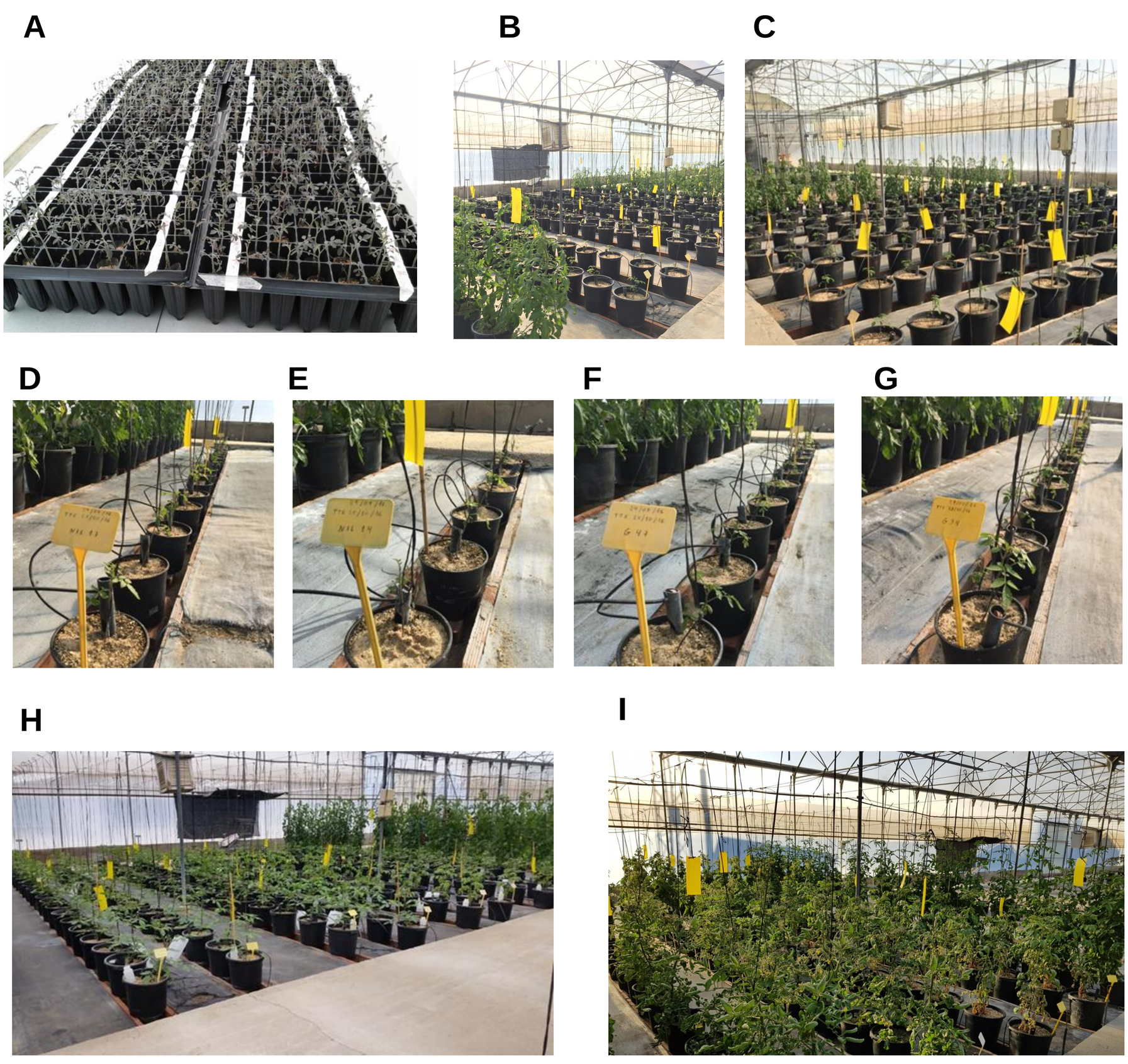


Figure S1. Overview of crop development under commercial greenhouse conditions . Plants were grown under natural light conditions without temperature control during the fall-spring campaign (November to May). A. 3-4 true-leaf seedlings before being transplanted. B and C. Plants were transplanted to 17 L pots filled with vermiculite, one seedling per pot. Pots were arranged in rows 1 m apart, with 2 plants per meter in rows (4 plants/m^2), amounting to 20 plants per row. Two extra plants at the beginning and end of every row and two rows before and after the first and final experimental rows were planted to avoid border effects. D,E, F and G. Lines NIL17, NIL 14, ScHKT1;2-RNAi and SlHKT1;2-RNAi at 0 d of salt-treatment. H and I. Aspect of plants salt-treated for 33 d and 85 d, respectively.

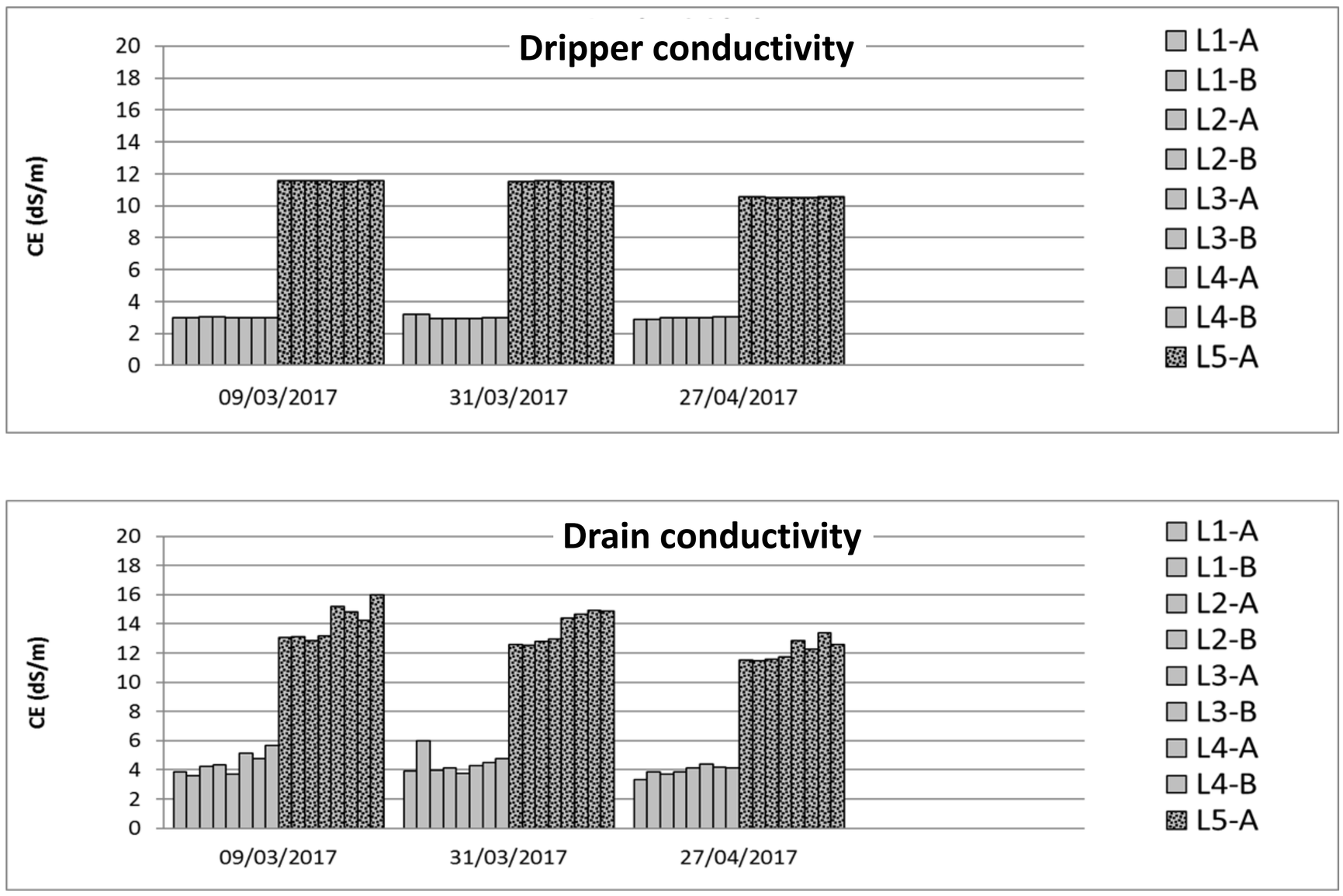
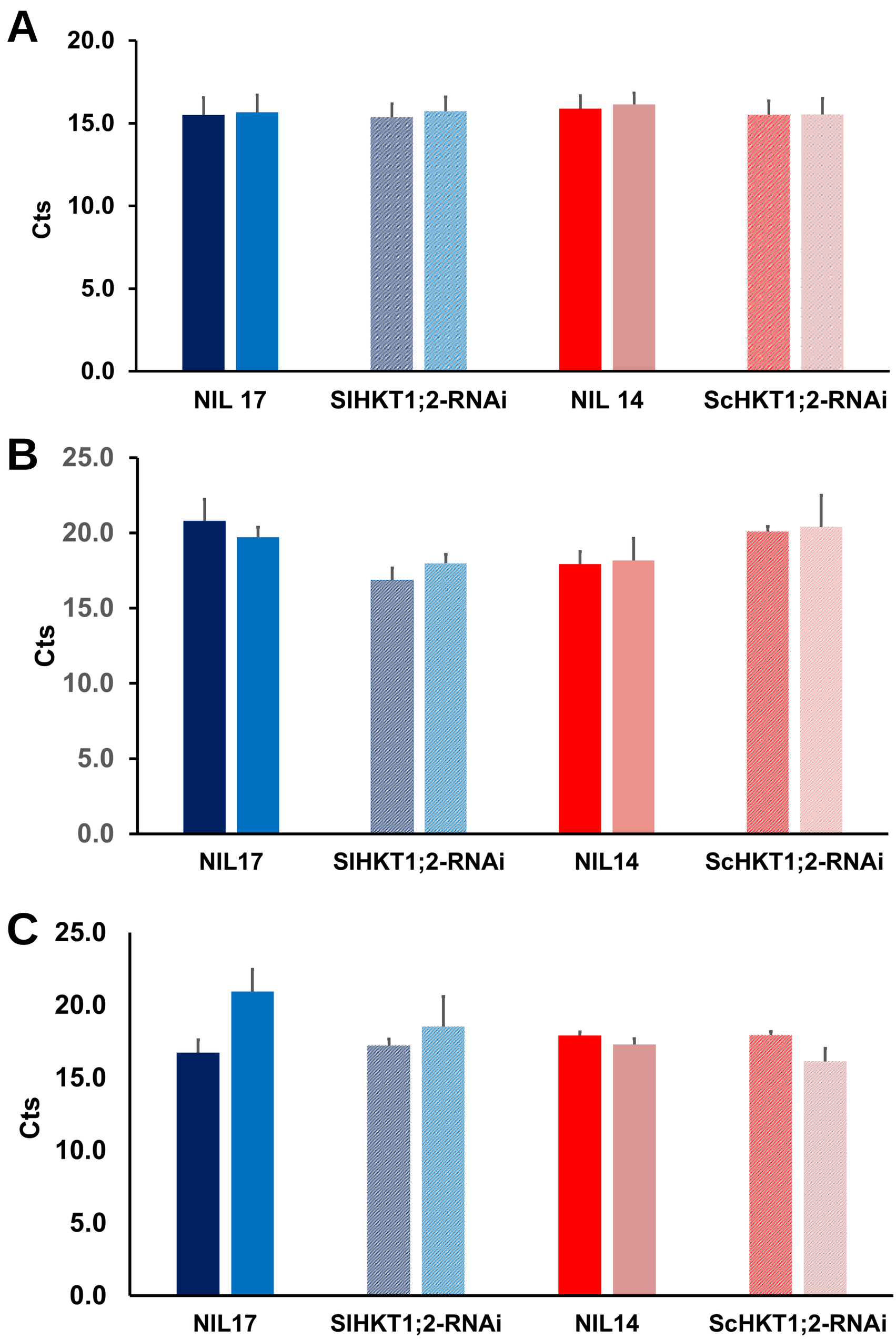


Figure S2. Electrical conductivity (EC) and pH of irrigation solutions from drippers and pot lixiviates recorded at different times during the experimental period of study. Values show that each pot in the control or salt row, received the same nutrient solution. LnB and LnA are rows whose drippers irrigated pots with nutrient solution with or without 80 mM NaCl, respectively.



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