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Additional Information

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Heterologous expression of the yeast *HAL5* gene in tomato enhances salt tolerance by reducing shoot Na^+ accumulation in the long term

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For salt tolerance to be achieved in the long-term plants must regulate Na^+/K^+ homeostasis over time. In this study, we show that the salt tolerance induced by overexpression of the yeast HAL5 gene in tomato (*Solanum lycopersicum*) was related to a lower leaf Na^+ accumulation in the long term, by reducing Na^+ transport from root to shoot over time regardless of the severity of salt stress. Furthermore, maintaining Na^+/K^+ homeostasis over time was associated with changes in the transcript levels of the Na^+ and K^+ transporters such as SlHKT1;2 and SlHAK5. The expression of SlHKT1;2 was up-regulated in response to salinity in roots of transgenic plants but down-regulated in the roots of WT plants, which seems to be related to the lower Na^+ transport rate from root to shoot in transgenic plants. The expression of the SlHAK5 increased in roots and leaves of both WT and transgenic plants under salinity. However, this increase was much higher in the leaves of transgenic plants than in those of WT plants, which may be associated with the ability of transgenic leaves to maintain Na^+/K^+ homeostasis over time. Taken together, the results show that the salt tolerance mechanism induced by HAL5 overexpression in tomato is related to the appropriate regulation of ion transport from root to shoot and maintenance of the leaf Na^+/K^+ homeostasis through modulation of SlHKT1 and SlHAK5 over time.

Abbreviations – FW, fresh weight; IAA, indole-3-acetic acid; NIL, near isogenic line; QTL, quantitative trait locus; RIL, recombinant isogenic line; WT, wild type.

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Introduction

One of the most detrimental effects on crop productivity is high concentrations of salts in soil since numerous species of agronomic interest are sensitive to salinity. There are two main components under salt stress in plants: an osmotic stress and an ion toxicity effect due to the accumulation of toxic ions which negatively affects cellular metabolism. Normally, during the first stages of salinity and at low stress levels the main induced effect is hyperosmotic stress, whereas at high stress levels and in long-term periods of saline conditions the main effect is the activation of mechanisms of ionic resistance. Nevertheless, in some cases ion toxicity already takes place during the first stages of the salt stress and the osmotic stress may persist during longer periods of salt stress (Munns and Tester 2008, Muñoz-Mayor et al. 2008). This is due to the fact that the exploit of organic solutes to maintain osmotic balance is energetically much more expensive for the plants than to make use for the same purpose of saline ions (De Costa et al. 2007, Shabala et al. 2010, Plett and Moller 2010). But it is necessary to take into account that given the limited Na^+ sequestration ability in leaf mesophyll elevated xylem Na^+ levels cannot be advantageous for a long time and, therefore, the tolerance mechanism may depend on the stress levels and exposure time (Pineda et al. 2012). Na^+ and other ions are absorbed by the roots and translocated by the transpiration stream to the shoot where they are accumulated. This accumulation negatively affects the plant cells metabolic and physiological operations and, if it surpassed certain levels, could even lead to cell death (Munns and Tester 2008, Hasegawa 2013). So, plant salt stress tolerance includes a combined action of several pathways to regulate the Na^+ homeostasis. Furthermore, for tolerance to be achieved the management of Na^+ ions must be organized on the basis of protecting K^+ ion uptake and distribution, as keeping under control these processes is a key requirement for plant growth (Shabala and Cuin 2008, Wu et al. 2013). These processes are mediated by ion transporters, and therefore manipulating the activity of this class of proteins has enormous potential to affect plant performance under saline conditions (Dreyer and Uozumi 2011, Hasegawa et al. 2013). However, concerning salt stress plant responses, knowledge acquired by molecular studies in this area has still not sufficiently progressed in order to develop crops with enhanced tolerance in natural non-laboratory environments. Therefore, research in induction of stress tolerance in plants should be focused on producing stress tolerant crops that maintain high growth rates and yields instead of developing crops that can survive isolated extreme stress events (Bechtold et al. 2010).

The kinetics and energetic facets of the transport of ions in most fungi and plant species share some common features that are not found in animals (Rodriguez-Navarro 2000). A set of halo-tolerance *HAL* genes have been identified in *Saccharomyces cerevisiae* and it was observed that the overexpression of these improves yeast growth under salt stress (Gaxiola et al. 1992, Serrano et al. 1994, Perez-Valle et al. 2007). Moreover, the overexpression of some of these genes enhanced halo-tolerance in higher plants, as observed in tomato plants overexpressing the *HAL1* gene (Gisbert et al.

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2000, Rus et al. 2001, Safdar et al. 2011). However, when the Na^+ exclusion capacity was very high in transgenic tomato plants the high energetic cost because of increased use of organic solutes for recovering osmotic homeostasis negatively affected growth and eventually fruit yield (Muñoz-Mayor et al. 2008). In this research work is described the behavior of transgenic tomato plants overexpressing yeast *HAL5* gene. This gene encodes a protein kinase involved in the activation of high-affinity K^+ transporters, TRK1 and TRK2, which improved K^+ (and Na^+) homeostasis and, subsequently, yeast salt tolerance (Mulet et al 1999, Perez-Valle et al. 2007, Casado et al. 2010). Homologs of these yeast TRK transporters have also been identified in plants and named HKT transporters (Very and Sentenac 2003). Unlike their yeast homologs, HKT transporters, particularly those belonging to class I, the only type existing in dicots (Platten et al 2006), are critical elements involved in Na^+ unloading from xylem vessels to other cells in the stele, which further lowers ion concentration in the transpiration stream (Hasegawa 2013). As with TRK transporters in yeast, genetic evidence suggests a complex regulation of HKT1-like transporters (Asins et al. 2013). In fact, the specific mechanisms by which *HAL5* kinase may stabilize and/or activate protein membrane transporters remain unknown.

Recently, Asins et al. (2013) identified two HKT1-like isoforms from tomato, *SlHKT1;1* and *SlHKT1;2*, which were classified on the basis of their phylogenetic analysis as class I HKT transporters, and should specifically transport Na^+ as they are involved in unloading Na^+ from xylem and perhaps in recirculating it to the roots (Platten et al 2006, Hauser and Horie 2010). Furthermore, Asins et al. (2013) also showed that *HKT1;1* and/or *HKT1;2* genes could be underlining a previously described major tomato QTL for the Na^+/K^+ homeostasis (Villalta et al. 2008), as indicated by candidate gene and expression analysis in tomato NILs coming from RILs derived from two salt-tolerant lines from wild tomato species *S. pimpinellifolium* and *S. cheesmaniae*. However, the connection between the allelic variants of HKT1 and salt tolerance in tomato is still unclear and depends mainly on salt tolerance criteria (Asins et al. 2013). In addition to HKTs, other carriers have been involved in Na^+ uptake. The regulatory kinase complex SOS2–SOS3 activates the plasma membrane Na^+/H^+ antiporter SOS1, which has been demonstrated to be responsible for maintaining Na^+ and K^+ homeostasis by retaining favorable K^+/Na^+ ratios in the cytoplasm and therefore it is involved in inducing salinity tolerance in *Arabidopsis*, rice and tomato (Martínez-Atienza et al. 2007, Olias et al. 2009, Pardo and Rubio 2011). SOS pathway role not only exerts a role in Na^+ efflux at the roots surface but it also intervenes in its redistribution throughout the plant by inducing the loading of the cation into the xylem (Shi et al. 2002, Olias et al. 2009, Huertas et al. 2012). K^+ contributes to the turgor and cellular growth and is a bounding factor for crop yield and quality (Dreyer and Uozumi 2011). Maintaining intracellular K^+ homeostasis is essential in many important cell processes including induction of numerous enzymatic activities, protein stabilization, pattern of membrane potential and maintenance of cytosolic pH. Thus, for plant growth under saline conditions it is critical the running of mechanisms controlling the K^+ level or K^+/Na^+ ratio homeostasis (Shabala 2013).

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Plants with different degrees of salt tolerance could have different regulatory mechanisms for the expression of genes encoding HAK-1 type transporters, or even different characteristics of these type of K^+-transporters influencing their activity (Rodriguez-Navarro and Rubio 2006, Brini and Masmoudi 2012). It has been observed in tomato plants that application of NaCl to the culture solution brings about a K^+ starvation effect that in its turn inhibits the expression of the tomato homolog *HAK*5, although the expression of this tomato gene was not related to the root K^+ concentration (Nieves-Cordones et al. 2007, 2008). HAK transporters could also be implicated in Na^+ transport (Corratgé-Faillie et al. 2010 and references therein, Benito et al. 2012), although, unfortunately, functional information on HAK transporters is still scarce and very complex to interpret (Aleman et al. 2011, Kronzucker and Britto 2011, Hasegawa 2013).

To date, much of the research on salt tolerance has been focused on the plant response measured over short periods of salt exposure, where multiple signaling pathways are activated in response mainly to osmotic stress induced by salinity. In this aspect, Shabala (2013) suggests that the 'ideal' scenario for a plant would be to rapidly transport the required Na^+ to the shoot in order to fulfill osmotic adjustment and to maintain the standard growth rate without yield penalties; but once the osmotic adjustment has been accomplished, Na^+ transport should be reduced to the absolute minimum required for driving cell turgor during growth and development of newly formed tissues. Therefore, experiments in the long term may be physiologically more relevant to disclose changes in Na^+ transport from roots to the shoot as the stress progresses (Munns and Tester 2008, Pineda et al. 2012). In order to gain an insight into mechanisms controlling salt tolerance in tomato in conditions of long-term salinity, the agronomical, physiological and molecular responses to salt stress were studied in transgenic tomato plants overexpressing the *HAL5* gene. Here we reported that *HAL5* overexpression enhances salt tolerance in tomato in the long-term on the basis of fruit yield, and transgenic plants are capable of maintaining lower leaf Na^+ accumulation over time by decreasing Na^+ transport to the shoot, as well as presenting higher ability to control the Na^+/K^+ ratio in leaves by regulating the expression of ion transporters such as *SlHKT1;2* in roots and *SlHAK5* in leaves.

Materials and methods

Tomato transformation and selection of transgenic lines

The yeast HAL5 coding sequence was subcloned into a plasmid vector with a duplicated CaMV 35S promoter and an AIMV RNA leader to enhance its expression (kindly provided by Prof R. Serrano, UPV-Valencia, Spain). This genetic construction was introduced into *Agrobacterium tumefaciens* and used for the transformation of *Solanum lycopersicum* cv. P-73 as described by Gisbert et al. (2000). The cotyledon explants used in the transformation by inoculation with the *Agrobacterium tumefaciens* formed buds twenty days after infection. In order to induce rooting the elongated shoots obtained after subculture of the buds were grown in Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 0.1 mg L^{-1} IAA and 50 mg L^{-1} kanamycin. Each tomato plant originated from an

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independent event of transformation (T_0 plants) – only a plant regenerated from a single poke was considered as being originated from an independent transformation event – were screened by a PCR analysis for the presence of *HAL5* gene using specific primers (see Appendix S1 in Supporting Information). The T_0 plants containing the *HAL5* sequence were transferred to soil and then they were grown under standardized greenhouse conditions (Estañ et al. 2005). The T_1 seeds lots of each T_0 plant were collected, and in their turn T_2 progenies from the resulting transgenic plants were obtained by selfing. The T_2 progenies were screened for kanamycin resistance and azygous and homozygous lines were identified according to results from this test (null plants showing resistance to the antibiotics and every plant exhibiting such resistance, respectively). Three T_2 lines (L2, L71 and L65) were selected for further studies and the *HAL5* expression level was analyzed by quantitative RT-qPCR, as described below, using primers for yeast *HAL5* (see Appendix S1 in Supporting Information).

Plant growth conditions and salt tolerance experiments

To evaluate the salt tolerance in the long-term, experiments were performed in greenhouse. Seeds of WT and transgenic lines were germinated in darkness, in a 2:1:1 (v/v) mixture of peat:perlite:siliceous sand, at 28°C temperature and 90% relative humidity. Seedlings were maintained in a controlled growth chamber until transplantation to the greenhouse. During this period, plants were irrigated daily with half-strength Hoagland solution (Hoagland and Arnon 1950). The environmental conditions applied were the optimal ones for tomato seedlings growth, varying temperature between 18 and 25°C and relative humidity between 50 and 80%, with a photoperiod of 16 h light/8 h dark. A photosynthetic photon flux (400–700 nm) of 345 μmol m^{-2} s^{-1} at the plant level was provided by fluorescent tubes (Osram Lumilux daily-light 58 W and Fluora 58 W). At the seven-true-leaves stage, at least eight plants per treatment of WT and of transgenic lines were transferred to a polyethylene greenhouse and grown on cocoa peat, using a drip irrigation system, with 3 L h^{-1} drippers. The fertirrigation solution (Hoagland solution) was prepared in 2000 liter tanks with local irrigation water (Electrical Conductivity, EC = 0.9 dS m^{-1}). For the salt treatments, the required amounts of NaCl were added to reach 75 and 100 mM concentration into the tanks. Treatments started after 10 days of transplantation and they lasted until the end of the experiment. Ripe fruits were collected each week during 2 months, corresponding approximately to the 40–80 day period of salt treatment and the fruit number and weight were recorded.

To analyze the physiological response, experiments were fulfilled in a controlled growth chamber under the same temperature, relative humidity and light conditions as described above. When the seedlings had developed two-true leaves, they were transferred to hydroponic culture (aerated half-strength Hoagland solution). Salt treatments (0, 100 and 200 mM NaCl) were initiated after 7 days of acclimation of plants into hydroponic culture. The required NaCl was added to the nutrient solution in two-steps operation to avoid the possible osmotic effect caused by a single addition.

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Nutrient solution was frequently changed according to the growth stage of the plants (from 6 days at the beginning, to 2 days at the end of the experiment), to ensure plants were in a nutritional steady state. During the experiments, plants were harvested on several days between day 0 and day 28 of salt treatments to study the time-course of Na^+ and K^+ contents. In each harvest, a part of the plant material was weighed and then dried to determine ion content (six replicates per line). The other part was immediately frozen into liquid nitrogen for posterior molecular analysis. Whole plants were also harvested just before applying the salt treatment (day 0), and at 14 and 28 days after the final salt concentration was reached (six replicates per line in each harvest). At the end of the experiments, fresh weights of shoot and root were determined.

Physiological analysis

Physiological analyses were carried out on plant material constituted by the third fully developed leaf. The concentrations of Na^+ and K^+ were measured in plant material dried during 48 h at 80°C, milled to powder and digested in a concentrated HNO_3:$HClO_4$ (2:1 v/v) solution. Na^+ and K^+ were analyzed by inductively coupled plasma spectrometry (ICP) (Ionomic Service of CEBAS-CSIC, Murcia, Spain). Net translocation rate of Na^+ from root to shoot (Na^+-TR) and flux of Na^+ to the leaf (Na^+-FL) were calculated as described by Muñoz-Mayor (2008).

For analysis of sugars, plant material (leafs) was placed in 5-ml pipette tips containing a glass wool filter in the tip and immediately frozen with liquid nitrogen. When the material was thawed, leaf sap was obtained by centrifugation and stored at –80°C until analysis. The extract was filtered through a Durapore 0.45 mm HV (Millipore Corporation, USA) membrane disk and then passed through a C18 Plus Sep-Pak cartridge (Waters Corporation, Massachusetts, USA). Quantification was carried out as described by Sanchez-Bel (2011) using a Shimadzu HPLC equipment (Kyoto, Japan).

Molecular analysis

For gene expression analysis, plant material was sampled, immediately frozen in liquid N_2 and stored under –78°C until analysis. Total RNA was isolated from roots and leaves of WT and transgenic plants; frozen tissue was ground with liquid N_2 in a mortar and 100 mg of this material was used for total RNA extraction with RNeasy kit (Qiagen). Contaminating DNA was removed with RNAse-free DNase (DNA-free kit, Ambion) and RNA quality was assessed by electrophoresis on a denaturing agarose gel. Total RNA was quantified in a GeneQuant II spectrophotometer (Pharmacia Biotech) and 5 µg were used for cDNA synthesis with the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific).

The expression level of *HAL5*, *SlSOS1*, *SlHKT1;2* and *SlHAK5* was assessed by quantitative real-time qPCR using 1 µl of undiluted cDNA mixed with iQ SyBr Green Supermix (BioRad), and 0.45 µM of forward and reverse primers using assay conditions as previously described (Asins et al. 2013) (see Appendix S1 in Supporting Information). Serial dilutions of cDNA were used to make a standard

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curve to optimize amplification efficiency. No template controls were included. All reactions were performed in triplicate. The presence of a single band on an agarose gel electrophoresis, and of a single peak in the melting temperature curve confirmed the specificity of RT-qPCR amplification. Relative expression data were calculated as described by Asins (2013) using the tomato elongation factor 1α (LeEF1α, acc. AB061263) as housekeeping gene. The expression level was calculated from $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001), using the expression level of each gene from non-treated untransformed tissue as the calibrator sample.

Statistical analysis

Data were statistically analyzed using the SPSS 13.0 software package by one-way ANOVA and LSD test (P <0.05). All data are given as mean ± SE (n = sample size). Significant differences between means were denoted by different lower case letters or asterisks.

Results

HAL5 overexpression in tomato results in increased salt tolerance on fruit yield basis

Twenty-nine transgenic plants were generated by insertion of the yeast *HAL5* gene into the tomato cultivar P73, in independent transformation events. Transformants with only one copy of the overexpressing *HAL5* genetic construction were selected by segregation for kanamycin resistance (3:1) in the segregant populations (T_1). Three lines (L2, L71 and L65) were initially selected to evaluate the salt tolerance to long term in the T_1 population, by using non-transformed plants (WT) as control. The lines L2 and L71 had significantly higher expression levels of *HAL5* gene than L65 (Fig. 1A). When the plants were grown at 100 mM NaCl, no significant differences were found between the fruit yield of WT and L2 plants; in L71, there was a tendency to increase fruit yield, which was reflected in a significant increase in fruit number, respect to WT. This response may correspond to the tendency to decrease the expression level of *HAL5* gene in L71 (45 fold) with respect to L2 (56 fold), although no significant differences were achieved between the expression levels of both lines (Fig. 1A). In the L65 (with lower expression level), fruit yield and fruit number increased significantly with respect to those of both WT and the other two transgenic lines, which demonstrates that the lower the expression level of *HAL5*, the higher the salt tolerance reflected in the transgenic tomato plants. On the basis of these results, the subsequent experiments were carried out with transgenic lines L71 and L65 in order to corroborate whether the higher salt tolerance is associated to lower expression level of the *HAL5* gene.

T_2 homozygous plant lines from L71 and L65 and lines without the transgene (azygous line) were obtained and the phenotypic and agronomic responses were analyzed under control conditions. The results obtained indicate that *HAL5* overexpression does not affect plant growth and fruit yield in the absence of salt stress, as all the plants assayed showed similar phenotype and fruit yield values (see Appendix S2 in Supporting Information). As no differences in plant growth and fruit yield were

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found between the untransformed WT, non-transgenic azygous and transgenic homozygous plants growing under control conditions, only WT was used as untransformed control in the experiments to evaluate the salt response of homozygous transgenic plants.

Next, we confirmed the agronomic response to the salinity of the T2 homozygous lines, L71 and L65, and analyzed their physiological responses in the long term. Again, transgenic lines showed similar fruit yields to those of WT when the plants were grown under control conditions over the long term (Fig. 2A). Under salinity, L65 showed higher fruit yield than WT at 75 and 100 mM NaCl, while fruit yield did not increase significantly in L71, with respect to WT (Fig. 2A), with this line again showing a tendency to increase its fruit yield with respect to WT, similar to that found in T_1 plants. These results corroborate the salt tolerance of the L65 on the basis of fruit yield. In this experiment, the physiological response was also examined by analyzing Na^+, K^+, Na^+/K^+, and sugar concentration in the third fully developed leaf of plants grown for 50 days under salinity. In the absence of stress, the solute contents were similar in all plants analyzed (Fig. 2B). Cultivation in the presence of NaCl increased leaf Na^+ content in all types of plants, although WT plants showed higher (P <0.05) Na^+ accumulation than transgenic plants, especially at 100 mM NaCl. It is worth pointing out that the most salt-tolerant line (L65) showed the lowest accumulation of Na^+ in leaf, while L71 showed intermediate levels between WT and L65 (Fig. 2B). With respect to K^+ and sugar concentrations, WT and transgenic plants showed similar contents under both saline conditions. According to the lower Na^+ accumulation in leaves of transgenic lines, both transgenic lines showed a lower leaf Na^+/K^+ ratio (P <0.05) than WT plants and among both transgenic lines, with L65 displaying a significantly lower Na^+/K^+ ratio (Fig. 2B). These results suggest that the salt tolerance induced by *HAL5* overexpression is due to decreased accumulation of toxic Na^+ in leaves to long term.

The salt tolerance induced by *HAL5* overexpression is related to lower rates of Na^+ transport to the shoot and lower Na^+/K^+ ratio in leaves

In order to gain an insight into processes responsible for lower Na^+ accumulation in transgenic plants, Na^+ partitioning was analyzed in different organs of the WT and L65, the transgenic line showing higher salt tolerance and lower Na^+ accumulation. When plants were grown at 100 mM NaCl for 28 days, there were two clear phases (Table 1): The first phase was characterized by significant and similar increases of Na^+ concentration in the three plant organs analyzed (root, stem and leaf), in both transgenic and WT plants, which could correspond to the osmotic phase. A second phase that may basically correspond to the ionic phase was observed between 14 and 28 days of salt treatment, where differences in Na^+ partitioning between WT and transgenic plants were clearly observed. Thus, leaf and stem Na^+ concentration remained at a steady level only in the homozygous *HAL5*-overexpressing line and not in WT plants, where Na^+ accumulation in both plant organs continued to increase during the two last weeks. The net balance between cation fluxes from root to shoot, as well as among the different organs in a time interval were measured by the formulas of the Na^+ translocation rate from

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root to shoot (Na^+-TR) and Na^+ flux to the leaves (Na^+-LF). The different accumulation patterns of Na^+ in WT and transgenic plants are clearly shown between 14–28 days (Table 1), as Na^+-TR and Na^+-LF decreased significantly in L65 during this time period. Thus, transgenic plants are able to regulate Na^+ transport from the root to the shoot as well reducing the Na^+ accumulation in leaves over time.

Because the salt tolerance mechanism of plants may vary as a function of salt stress levels, we then attempted to determine whether transgenic plants are able to maintain ion homeostasis when severe stress treatment was applied. At 200 mM NaCl, transgenic plants displayed enhanced growth and higher (P <0.05) root and shoot biomass, compared to WT plants, after 28 days of treatment (Fig. 3A). It is well known that excessive Na^+ concentrations in leaf lead to a degradation of chlorophyll and subsequent chlorosis, as was observed in WT plants but not in transgenic plants (Fig. 3B). In order to determine the exposure time necessary for transgenic plants to show lower leaf Na^+ accumulation when grown under severe stress, the differences in Na^+ distribution between WT and transgenic plants were measured at different times (Fig. 3C). In roots, a significant increase in Na^+ content was observed in transgenic plants with respect to WT after 22 and 28 days of 200 mM NaCl, which suggests that transgenic plants try to prevent Na^+ transport to the shoot through its accumulation in roots when high stress is applied. Thus, lower Na^+ accumulation was found in the stems of transgenic plants from 22 days of salt treatment and in leaves from 14 days of salt treatment. In spite of the differences found in Na^+ accumulation according to the stress levels (100 and 200 mM NaCl), similar reduction percentages of Na^+ TR from root to shoot were found in the transgenic plants at the end of the experiments (Table 1 and Fig. 3D), as compared with WT plants, which demonstrates that the salt tolerance mechanism operating in *HAL5*-overexpressing plants is independent of the salt stress level. Salt tolerance induced by *HAL5* overexpression could also be reached by increasing K^+ uptake and subsequently decreasing Na^+ uptake. After 100 mM NaCl treatment, K^+ levels in WT and transgenic plants were similar (Table 1), whereas when 200 mM of NaCl was applied, K^+ content in the leaves of transgenic plants tended to increase compared to WT plants, although these differences were only significant during the last week of salt treatment (Fig. 3E). The Na^+ content reduction in the leaves of transgenic plants, together with the increase in K^+ resulted in a sharp decrease in the leaf Na^+/K^+ ratio in transgenic plants from 14 days to the end of the experiment, with respect to WT plants (Fig. 3E).

HAL5-overexpressing plants up-regulate the expression of _SlHKT1;2_ and _SlHAK5_ transporters involved in Na^+/K^+ homeostasis

Since physiological evidence indicates that *HAL5* overexpression reduces the Na^+ transport rate from root to shoot, we subsequently analyzed the expression of *SlSOS1* and *SlHKT1;2* genes, which are the main transporters identified until now involved in long-distance Na^+ transport in tomato (Olias et al. 2009, Asins et al. 2013). In roots, the *SlSOS1* expression increased in WT from 7 days of 100 mM

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NaCl, with this increase being maintained at longer time (14 days), while the *SlHKT1;2* expression was significantly reduced at these times (Fig. 4A). Contrarily, the *SlHKT1;2* expression increased significantly at 100 mM NaCl in transgenic roots, while the *SlSOS1* expression remained constant along the treatment period. The next question to clarify was whether this mechanism is similar when a severe stress level is applied. At 200 mM NaCl, the *SlHKT1;2* expression increased again in roots of transgenic plants (Fig. 4A), with an amount of increased transcript 2-fold higher at 14 days than at 7 days of 200 mM NaCl. Moreover, a significant increase in the *SlSOS1* expression was found in the transgenic roots after 14 days of 200 mM NaCl treatment. These results indicate that the main Na^+ transporter increasing during salt treatment in transgenic roots was *SlHKT1;2*. In leaves, however, the expression pattern of *SlHKT1;2* was more similar in WT and transgenic plants (Fig. 4B). With respect to the relative *SlSOS1* expression, significant increases were found in both WT and transgenic leaves of the plants grown at 100 and 200 mM NaCl, with the increases in the *SLSOS1* expression being generally lower in transgenic leaves than in WT leaves.

Could there be other transporters regulating the Na^+ and/or K^+ homeostasis in the leaves of the transgenic plants to long term? Taking into account, on the one hand, that transgenic plants had higher K^+ concentration in leaves after 21 days of 200 mM NaCl and, on the other hand, that the HAK5 transporter in tomato seems to be involved in K^+ homeostasis (Nieves-Cordones et al. 2007, 2008), the changes induced by salinity in the expression of *SlHAK5* gene were analyzed in roots and leaves after 14 days at 100 and 200 mM NaCl. In roots, similar increases were induced by salinity in WT and transgenic plants (Fig. 5), with significantly higher *SlHAK5* expression at lower salt levels (100 mM NaCl) than at 200 mM of NaCl. In leaves, it is interesting to point out the important increases in the *SlHAK5* expression induced by salinity in both WT and transgenic plants, although the differential response between WT and transgenic plants depends on the stress level; at 100 mM NaCl, the increases were similar in WT and transgenic plants. However, at 200 mM NaCl, the *SlHAK5* expression increased 16-fold and 43-fold in leaves of the WT and transgenic plants, respectively, corresponding the very high *SlHAK5* expression with the increased K^+ concentrations found in the leaves of the transgenic plants over time.

Discussion

Salt tolerance is considered to be a complex trait governed by multiple genes but many studies have shown that it may be enhanced by introducing effector protein-encoding single genes (Cuartero et al. 2010), which include genes that control Na^+ and K^+ homeostasis (Munns 2011 and references therein). Our results show that the overexpression of the yeast *HAL5* gene improves salt tolerance in tomato to long term on the basis of fruit yield. Moreover, the increased fruit yield was determined in both the segregating T_1 populations and the homozygous T_2 plants, in comparison with WT plants (Figs 1 and 2). This observation suggests that the introduced trait is functional and it is stable in transgenic tomato plants. In relation to the salt tolerance induced by *HAL5* gene in tomato, it is interesting to remark

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three aspects: i) The use of constitutive promoter 35S did not induce a pleiotropic effect in the plant growth and development of transgenic plants, as these plants showed fruit yields similar to those of non-transformed plants in absence of salt stress (Fig. 1 and Appendix S2 in Supporting Information), which makes it easier to discern whether the differences in plant growth and physiological response are due to salinity tolerance induced by gene overexpression. ii) Transgenic plants of the line with lower *HAL5* gene expression (L65) showed the highest salt tolerance on a fruit yield basis, which indicates that it is convenient to test initially transgenic lines with different expression levels of the candidate gene before elucidating the role of the interest gene. It remains to be seen whether the high *HAL5* expression does indeed affect some metabolic pathways or rather reflects a higher energetic cost of the plant. Moreover, above a critical threshold, the excess of transcripts could trigger RNases degradation mechanisms as well as hyper-methylation of the DNA homologous sequences of the degradation products (Kohli et al. 2003). iii) It is also remarkable that salt tolerance acquired by*HAL5* gene overexpression was associated with a higher fruit yield and lower Na^+ accumulation in leaves over the long term (Table 1, Fig. 2 and 3), especially taking into account that salt tolerance of the transgenic plants was evaluated in a greenhouse under high transpiration conditions, in which the transport of ions such as Na^+ through the plant was highly favored for the long timing of stress (Atkinson and Urwin 2012, Mittler and Blumwald 2010.

As most glycophytes, tomato plants must regulate Na^+ delivery to the shoot to long-term in order to avoid the Na^+ toxicity in leaves (Pineda et al. 2012). Our results show that transgenic plants only reduced the Na^+ transport rate to the shoot after a treatment period, and not during the first days of salt treatment (Table 1, Fig. 3), where Na^+ concentration does not seem to have reached toxic levels yet, and Na^+ may be used for leaf osmotic adjustment, a typical strategy used by salt-tolerant plants (Mian et al. 2011, Muñoz-Mayor et al. 2012). This first phase, marked by similar rates of Na^+ accumulation in WT and transgenic plants, suggests that the ability to reestablish osmotic homeostasis is not altered in *HAL5* transgenic plants. However, significant differences were found in the leaf Na^+ accumulation of WT and transgenic plants after a treatment period (2nd phase), which demonstrates the main salt tolerance mechanism induced by *HAL5* overexpression is the reduction in the Na^+ transport rate to the shoot avoiding or alleviating specific Na^+ damage in leaves. Furthermore, it is worth pointing out that the reductions in the Na^+ translocation rates from root to shoot were similar (about 30%) when the plants were grown at 100 and 200 mM NaCl, which suggests that the same salt tolerance mechanism is operating at both mild and high stress levels. Taken together, the salt tolerance of the transgenic plants is mainly due to their ability to regulate the Na^+ transport rate from root to the shoot over time, as the time-dependent regulation of the rate of Na^+ transport to the shoot appears to be critical for plant salinity tolerance (Maathuis 2013, Shabala 2013).

What is the mechanism operating in the reduction of the Na^+ transport to the shoot in the transgenic plants? It could be due to the different expression of the genes involved in Na^+ transport to long distance in tomato; the main genes known until now are *SlSOS1* and *SLHKT1;2* (Olias et al.

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2009, Asins et al. 2013), which probably work in a coordinated manner to achieve the Na^+ (and K^+) homeostasis (Shi et al. 2002, Martínez-Atienza et al. 2007, Olias et al. 2009, Tang et al. 2010). Here, the expression levels of both genes were analyzed in roots and leaves to relatively long term, as transporters contributing to Na^+ exclusion from aerial tissues may be involved in tolerance to salt stress in a different way depending on the stress levels and exposure time (Brini and Masmoudi 2012, Hasegawa 2013). *SlSOS1* expression increased mainly in WT roots but not in transgenic roots, which suggests that *SlSOS1* is not the main cause of the reduction in the Na^+ transport rate from root to shoot in transgenic plants. These results could also be a consequence of the prolonged salt exposure time up to the point that gene expression analysis was undertaken, although the only increase in the *SlSOS1* expression of the transgenic roots was observed after 14 days of severe salt stress (Fig. 4a). Interestingly, the expression pattern of *SlHKT1;*2 showed opposite responses in WT and transgenic roots, as its expression increased significantly with salinity in transgenic roots while it decreased in WT roots. These results suggest that *SlHKT1;2* Na^+ transporter is responsible for the reduction of the Na^+ transport rate to the shoot found in transgenic plants regardless of the stress level, while the Na^+ transport in WT roots would be mainly controlled by *SlSOS1*. Many studies have consistently concluded that any rise in plant tolerance to salt stress, achieved by means of genetic manipulation of HKT transport activity, is always associated with a decrease in leaf Na^+ content (Berthomieu et al. 2003, Rus et al. 2004, Sunarpi et al. 2005, Davenport et al. 2007, Moller et al. 2009, Plett et al. 2010, Hauser and Horie 2010). Asins et al. (2013) also found a correlation between plants with higher *SlHKT1;2* expression and lower shoot Na^+ under saline conditions. Assuming functional similarity between HKT1-like transporters in dicots (Hauser and Horie 2010), *SlHKT1;2* would control Na^+ xylem unloading in roots. Therefore, increased *SlHKT1;2* expression in the roots of transgenic plants implies higher Na^+ retrieval from the xylem in roots and consequently, less Na^+ transport via the transpiration stream to the aerial part as compared to WT. Furthermore, the finding that *SlHKT1* transporter is modulated in tomato by overexpression of the *HAL5* yeast gene, which regulates in yeast the homolog transporters (TRKs) to HKTs transporters in plants, poses important questions regarding the conservation of the regulatory network controlling ion homeostasis in fungi and plants. In this regard, it is interesting to point out that findings from studies on yeast expressing HvHAK1 reveal that HAK1 was modulated by HAL5 among other yeast genes (Fulgenzi et al. 2008), which constitutes a potential posttranslational regulatory mechanism for such transporters in plants. Moreover, it has been observed that heterologous expression of *SlSOS1* allowed for restoring halo-tolerance of yeast strain AXT3K lacking major Na^+ transporter systems (Olías et al. 2009). On the other side, it is evident that the ion transporters activities are regulated by phosphorylation, as it is the case of SOS2 (Ser/Thr protein-kinase belonging to the SnRK3/CIPK family of kinases) that regulates SOS1 activity (Chinnusamy et al. 2004).

The studies on salt tolerance have predominantly focused on traits related to Na^+ accumulation (Munns and Tester 2008, Plett et al. 2010, Cuin et al. 2011), although salt tolerance of crop species

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appears to be determined not only by Na^+ content but rather by the K^+/Na^+ ratio in the cytosol (Estañ et al. 2005, Shabala and Cuin 2008). Moreover, results were generally obtained in roots, even though ultimately the ability (or inability) of the plant to control the K^+/Na^+ ratio resides in photosynthetically active leaf tissues that determines its photosynthetic competence (and hence growth and yield) under saline conditions (Wu et al. 2013). In this study, the relative expression of *SlHAK5* transporter was analyzed after 14 days of salt stress (Fig. 5), as K^+ levels increased only at the end of the treatment period in the leaves of transgenic plants. Surprisingly, relative *SlHAK5* expression under severe stress (200 mM NaCl) was much higher in transgenic than in WT leaves, which suggests that the regulation of *SlHAK5* gene is contributing to superior leaf Na^+/ K^+ homeostasis and salt tolerance in transgenic plants. Recently, Wu et al. (2013) reported that K^+ retention in leaf mesophyll is an essential process to confer salinity tolerance in barley and wheat, and they even suggest that salt-induced damage to leaf lamina observed as necrotic spots on leaf tips in both species may be the result of mesophyll cells undergoing programmed cell death as a consequence of K^+ loss. In this study, the increase in K^+ content in leaves over time associated with high *SlHAK5* expression in the leaves of transgenic plants under severe stress, suggests that this transporter could be involved in maintaining K^+ homeostasis. Taken together, the salt tolerance mechanism induced by *HAL5* overexpression is related to an appropriate regulation of the Na^+ transport from root to shoot and maintenance of the Na^+/K^+ homeostasis in leaf through modulation of *SlHKT1* in roots and *SlHAK5* in leaves over time. These results may open up novel and previously unexplored possibilities to improve salinity tolerance in crops by regulating timing of xylem Na^+ loading, which have been not investigated to date by breeders.

Author contributions

JOGA, IE, PSB, MCB and FBF designed research, JOGA, IES, BP and BGS performed experiments, PSB, IE, AB, VM and MCB analyzed data, PSB, AB and MCB wrote the paper.

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Figure legends

Figure. 1. The salt tolerance degree induced by *HAL5* overexpression differs among the transgenic lines. (A) Relative expression of *HAL5* gene in three tomato transgenic lines (L2, L71 and L65) analyzed by real-time qPCR. B) Fruit yield and fruit number in plants grown at 100 mM NaCl for 80 days of non-transformed (WT) and the segregant population (T_1) of the three transgenic lines (L2, L71 and L65). Data are the mean ± SE (n = 10). Values with different letters are significantly different as determined by LSD ($P \leq 0.05$).

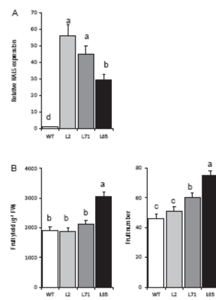

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Fig. 2. Effect of *HAL5* overexpression on agronomical and physiological responses of tomato plants grown under salinity treatment. (A) Non-transformed tomato plants (WT) and two T_2 homozygous lines overexpressing *HAL5* gene (L71 and L65) were grown under saline conditions (0, 75 and 100 mM of NaCl) for 80 days, and fruit yield was quantified by collecting ripe fruits for 2 months. (B) In these plants, Na^+, K^+, Na^+/K^+ ratio and sugars were measured in leaves (3rd true leaf) after 50 days of salt treatment. Data are the mean ± SE (n = 6). Values with different letters are significantly different as determined by LSD ($P \leq 0.05$).

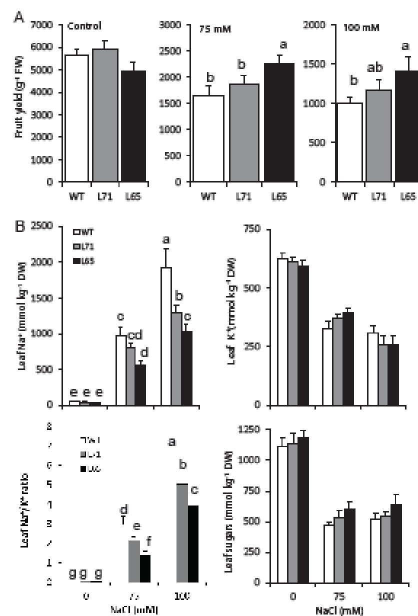

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Figure. 3. Salt tolerance induced by *HAL5* overexpression is related to the maintenance of the Na^+/K^+ homeostasis over time. Plants of WT and homozygous line overexpressing *HAL5* gene (L65) were transferred to hydroponic culture and grown in Hoagland solution supplemented with 200 mM NaCl for 28 days. (A) Root and shoot fresh weights at the end of the experiment and (B) image of leaves representative of WT and transgenic line at this time. (C) Time course of Na^+ accumulation in different plant parts of WT and transgenic line. (D) Na^+ translocation rate (Na^+-TR) from root to shoot (mmol Na^+ Kg^{-1} root FW) and Na^+ flux to the leaves (Na^+-FL, mmol Na^+ Kg^{-1} leaf FW) in the second phase of the saline response (14–28 days of 200 mM NaCl treatment). (E) Time course of leaf K^+ concentration and Na^+/K^+ ratio in WT and transgenic line. Data are the mean ± SE (n = 6). Asterisks indicate significant differences between WT and transgenic line ($P < 0.05$).

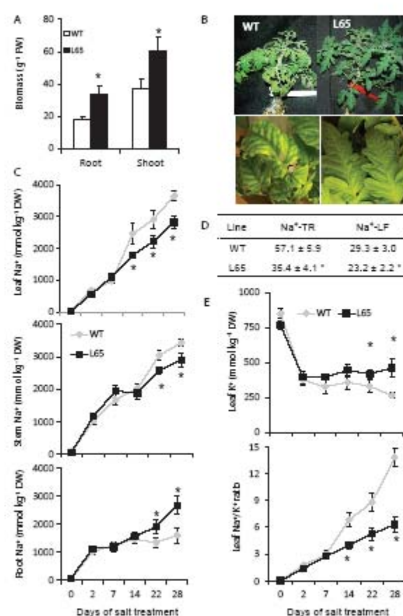

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Fig. 4. Relative expression of *SlSOS1* and *SlHKT1;2* quantified by real-time qPCR in roots (A) and leaves (B) of WT and homozygous line overexpressing *HAL5* gene (L65) plants after 7 and 14 days of 100 and 200 mM NaCl treatments. The relative expression level was calculated by using as the calibrator sample the expression level of each gene in roots or leaves of plants grown in absence of salt stress (equal to 1). Data are the mean ± SE (n = 6). Asterisks indicate significant differences between WT and transgenic line ($P < 0.05$).

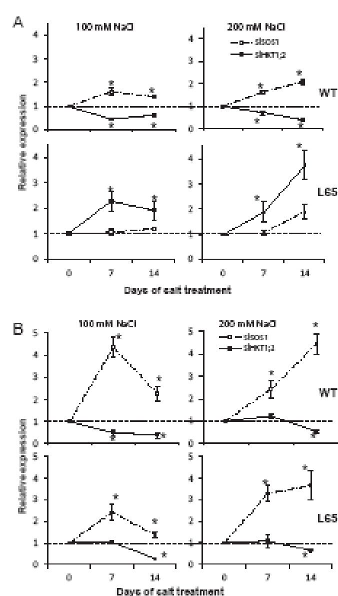

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Fig. 5. Relative expression of *SlHAK5* gene quantified by real-time qPCR in leaves of WT and homozygous line overexpressing *HAL5* gene (L65) plants after 14 days of 100 and 200 mM NaCl treatments. The relative expression level was calculated by using as the calibrator sample the expression level in leaves of WT plants in absence of salt stress (0 mM) (equal to 1). Data are the mean ± SE (n = 6). Asterisks indicate significant differences between WT and transgenic line ($P < 0.05$). The bottom figure represents the lower part of the graph (below the dashed line) with the y-axis expanded to better visualize the different relative expression values in roots.

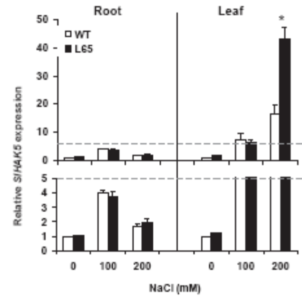

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Table 1. Effect of *HAL5* overexpression on Na^+ partitioning, translocation from root to shoot and flux to the leaves in plants treated for 28 days with 100 mM NaCl. (A) Na^+ concentrations (mmol Na^+ kg^{-1} DW) in roots, stems and leaves of WT and transgenic plants (L65). (B) Na^+ translocation rate (Na^+-TR) from root to shoot (mmol Na^+ Kg^{-1} root FW) and Na^+ flux to the leaves (Na^+-LF, mmol Na^+ Kg^{-1} leaf FW) in both phases of the saline response (from 0 to 14 days, and from 14 to 28 days of salt treatment). Values are means ± standard error of 6 replicates. Significant differences at $P < 0.05$ between lines were indicated with *.

A)		**Days of salt treatment**		
		0	14	28
Root	WT	59 ± 1	1134 ± 74	1147 ± 172
	L65	82 ± 3	1057 ± 70	1210 ± 57
Stem	WT	90 ± 9	1930 ± 104	2505 ± 147
	L65	71 ± 8	2273 ± 213	2024 ± 116*
Leaf	WT	28 ± 2	1129 ± 201	1562 ± 199
	L65	22 ± 3	1125 ± 193	1010 ± 215*

B)		**Time period**	
		0–14	14–28
Na^+-TR	WT	47.8 ± 5.0	32.1 ± 3.5
	L65	55.9 ± 6.3	22.3 ± 2.7*
Na^+-LF	WT	20.8 ± 2.4	14.2 ± 2.0
	L65	22.2 ± 1.9	9.0 ± 1.2*

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