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Research article

Overexpression of LeNHX2 and SlSOS2 increases salt tolerance and fruit production in double transgenic tomato plants

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ABSTRACT

Transgenic tomato plants (*Solanum lycopersicum* L. cv. MicroTom) overexpressing both the K^+,Na^+/H^+ antiporter LeNHX2 and the regulatory kinase SlSOS2 were produced by crossing transgenic homozygous lines overexpressing *LeNHX2* and *SlSOS2*. *LeNHX2* expression was enhanced in plants overexpressing *LeNHX2* but surprisingly even more in plants overexpressing *SlSOS2* with and without *LeNHX2*. All transgenic plants showed better NaCl tolerance than wild type controls and plants overexpressing both *LeNHX2* and *SlSOS2* grew better under saline conditions than plants overexpressing only one of these genes. Yield related parameters indicated that single and above all double transgenic plants performed significantly better than wild type controls. All transgenic plants produced fruits with a higher K^+ content than wild-type plants and plants overexpressing *SlSOS2* accumulated more Na^+ in fruits than the rest of the plants when grown with NaCl. Roots, stems and leaves of transgenic plants overexpressing *LeNHX2* showed a higher K^+ content than wild type and single transgenic plants overexpressing *SlSOS2*. Na^+ content in stems and leaves of NaCl treated plants was higher in *SlSOS2* overexpressing plants than in wild type and *LeNHX2* single transgenic plants. All transgenic lines showed a higher leaf relative water content and a higher plant water content and water use efficiency than wild type controls when both were grown in the presence of NaCl. Results in this work indicate that the joint overexpression of *LeNHX2* and *SlSOS2* improves growth and water status under NaCl stress, affects K^+ and Na^+ homeostasis and enhances fruit yield of tomato plants.

1. Introduction

Salinity is a major abiotic stress that imposes severe detrimental effects on plant growth and crop productivity. Improving salt tolerance of crops is a scientific challenge to ensure crop protection to high soil salinity (Bartels and Sunkar, 2005; Flowers, 2004; Munns, 2002; Munns and Gilliham, 2015; Roy et al., 2014; Rozema and Flowers, 2008). Plant tolerance to salinity is a multigene trait that involves several stress associated genes. In spite of its multigene complexity, it has been reported in several cases that introduction of one single key gene involved in salt tolerance can successfully enhance tolerance to NaCl in transgenic plants (Bartels and Sunkar, 2005; Zhu, 2001).

In the coastal areas of the Mediterranean countries, tomato is an economically important crop usually cultivated in greenhouses located in arid or semi-arid lands. In these locations and due to water scarcities, tomato plants are often irrigated with poor quality water containing high salt concentrations that affect both fruit production and quality (Reina-Sánchez et al., 2005; Romero-Aranda et al., 2002). In this scenario, development of cultivars with increased salt tolerance could improve the yield of this important horticultural crop. Development of tomato salt stress tolerant varieties by traditional breeding still remains a difficult task. To overcome this limitation a transgenic approach could be adopted to improve salinity tolerance by introducing candidate genes controlling salt tolerance traits. In this respect enhanced salt tolerance of transgenic plants overexpressing genes having a role in maintenance of ion homeostasis through ion uptake and compartmentalization has been demonstrated (Munns, 2005; Pardo et al., 2006; Rodríguez-Rosales et al., 2009).

While Na^+ is toxic for most plants, except halophytes, K^+ is an essential nutrient for plants, since it induces the osmotic gradient driving solute transport through the xylem and stimulates the transport of photo-assimilates as sugars and aminoacids from sources to sinks

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through the phloem (Lalonde et al., 2003; Mengel, 1997; Schobert et al., 1998). Moreover, K^+ is important for photosynthetic enzyme activities and stomata opening and therefore for regulation of transpiration (Cherel et al., 2014). Keeping a high K^+/Na^+ ratio in the cytosol is considered a fundamental process for salinity tolerance. This process involves the SOS pathway together with ion transporters responsible for intracellular K^+ and Na^+ compartmentalization belonging to the NHX family (Apse and Blumwald, 2007; Horie et al., 2009; Ren et al., 2005; Rodriguez-Rosales et al., 2009; Sunarpi et al., 2005; Venema et al., 2003; Zhang et al., 2001; Zhang and Blumwald, 2001).

In previous works we have identified in tomato several Na^+ and K^+ transporters and regulatory proteins and demonstrated their relevance for K^+ and Na^+ homeostasis (Gálvez et al., 2012; Huertas et al., 2012, 2013; Olías et al., 2009; Rodríguez-Rosales et al., 2008). Among these transporters we have identified four NHX isoforms and determined their expression level in the salt sensitive cultivated species *Solanum lycopersicum* L. cv. Volgogradskij and the salt tolerant wild species *Solanum pimpinelifolium* L. in response to salt stress (Gálvez et al., 2012). The higher salt tolerance of the wild tomato species was found to be associated to a NaCl induction of the LeNHX3 and LeNHX4 isoforms and correlated with the accumulation of Na^+ in aerial parts, thus supporting a role of NHX genes as determinants of salt tolerance in tomato. We have also reported regulation of Na^+ and K^+ homeostasis by the Ca^{2+}-dependent SOS pathway in tomato (Huertas et al., 2012; Olías et al., 2009). The genes coding for the plasma membrane Na^+/H^+ antiporter SlSOS1, and the kinase SlSOS2 were identified in tomato as functional homologous of AtSOS1 and AtSOS2 by heterologous expression in yeast and their involvement in NaCl tolerance was demonstrated by gene silencing and overexpression in tomato (Huertas et al., 2012; Olías et al., 2009). Moreover, we demonstrated that transgenic tomato plants overexpressing either *LeNHX2* (Huertas et al., 2013) or *SlSOS2* (Huertas et al., 2012) grow better in the presence of NaCl than the untransformed controls. We have also shown that overexpression of *SlSOS2* increases not only the level of expression of *SlSOS1* as expected, but also the expression levels of *LeNHX2* and *LeNHX4* (Huertas et al., 2012). Related to the above finding, we have shown a higher Na^+/H^+ exchange at the plasma membrane and an enhanced $Na^+,K^+/H^+$ exchange at the internal membranes as a result of *SlSOS2* overexpression in tomato. All these results indicated that SlSOS2 regulates the expression and activity of LeNHX2 in tomato leading us to hypothesize that likely the overexpression of both *LeNHX2* and *SlSOS2* could improve even more the salt tolerance of double transgenic plants relative to plants overexpressing only one of these genes. To check this possibility, tomato plants overexpressing either *LeNHX2* or *SlSOS2* were used to produce double transgenic lines overexpressing both genes and these plants were evaluated for salt tolerance.

Increased salt tolerance of plants overexpressing regulatory genes in signalling pathways together with ion transporters was demonstrated in transgenic Arabidopsis co-overexpressing AtNHX1 and the plasma membrane Na^+/H^+ antiporter SOS1 (Pehlivan et al., 2016). Also in Arabidopsis, the joint overexpression of NHX1 and SOS3 was demonstrated to further improve salt tolerance relative to plants overexpressing only NHX1 (Yang et al., 2009). However, to our knowledge this is the first report evaluating salt tolerance of a plant species overexpressing both an endosomal NHX-type ion transporter and a SOS2 regulatory kinase. Since overexpression of *SlSOS2* in tomato is also related to an increase in fruit production in salt-treated plants (Huertas et al., 2012), the ion content and amount of fruits produced by double transgenic plants were also analysed in this work. Our results show that compared to single transgenic plants overexpressing *LeNHX2* or *SlSOS2*, the combined overexpression of the two genes improves salt tolerance and, above all, fruit production by plants cultivated either in the presence or the absence of NaCl.

2. Materials and methods

2.1. Generation of LeNHX2SlSOS2 double transgenic plants

Transgenic *Solanum lycopersicum* L. cv. MicroTom plants homozygous for the transgenes *LeNHX2* and *SlSOS2* obtained as described in Huertas et al. (2012, 2013) were used to generate *LeNHX2SlSOS2* double transgenic plants by cross-pollination. Homozygous single transgenic plants were grown up in sterile vermiculite/peat-moss (1/1) in a growth chamber at 24/20 °C, 16 h light (140 $\mu mol\,m^{-2}s^{-1}$) and 8 h darkness and irrigated with tap water for 6–8 weeks. The flowers of the *SlSOS2* overexpressing plants from lines L-74 and L-82 were used as female parents and those from the *LeNHX2* overexpressing plants from lines L-452 and L-932 were used as male parents. For the female parent, flowers in which the tips of the petals were just visible were selected. In these flowers sepals, petals and anthers were removed with a fine forceps, and only carpels remained intact. For the male parent, pollen of the open flowers was collected and placed on the stigmatic surface of the exposed carpels of the female parent to allow cross-pollination. The crosses were L-452 x L-74, L-452 x L-82, L-932 x L-74 and L-932 x L-82. In order to identify the fruits resulting from the cross, the flowers of the female parent that underwent cross pollination were labelled. After crossing, plants were grown under the conditions described above until mature fruits were obtained. Seeds were removed from mature fruits derived from cross pollinated flowers and stored at 4 °C until used for further experiments.

2.2. Molecular characterization of transgenic plants

Seeds from wild type and single transgenic plants overexpressing *LeNHX2* and *SlSOS2* as well as seeds derived from the crosses between *LeNHX2* and *SlSOS2* homozygous transgenic lines were germinated and plants grown under the conditions described above. The plants were checked for the integration of the transgenes by PCR using primers amplifying a fragment of the 35S promotor and the coding regions of *LeNHX2* or *SlSOS2* (Supplemental material, Table 1).

The level of expression of *LeNHX2* and *SlSOS2* genes in leaves of untransformed and transgenic plants was assessed by real time PCR using gene-specific primers (supplemental material, Table I) as previously described (Huertas et al., 2012, 2013). Leaves used for this determination were detached from plants cultivated in hydroponics for four weeks in ¼ Hoagland nutrient solution (Hoagland and Arnon, 1950) as indicated below.

2.3. Seed germination, plant growth and phenotypic analysis of plants

Plants were cultivated in hydroponics. For this purpose, seeds were surface sterilized in ethanol 70% for 1 min followed by 50% commercial bleach for 5 min and 3 washes with sterile distilled water. Seeds were then cultivated in polystyrene boxes containing quartz sand, watered for one week with one-tenth strength Hoagland nutrient solution (Hoagland and Arnon, 1950) and for another two weeks with one-fourth strength of the same solution. Seedlings with four leaves were then transferred to pots for hydroponic cultivation. Typically, plants were grown for four weeks in a 2.5 L pot containing an aerated one-fourth strength Hoagland nutrient solution. Hydroponic cultivation was performed in a growth chamber at 24/20 °C day/night, under an illumination of 140 $\mu mol.m^{-2}.s^{-1}$ (photoperiod of 16 h light and 8 h darkness) and 40–50% relative humidity. Salt treatment was applied by adding NaCl to the nutrient solution to a final concentration of 120 mM after 1 week of cultivation in hydroponics.

Alternatively, tomato seeds were sown in seedbeds containing sterile vermiculite/peat-moss (1/1). The seedbeds were kept in a growth chamber under the conditions described above for hydroponics cultivation and plants were irrigated with distilled water. After three weeks, plants were transferred to 1.2 L pots (1 plant per pot) containing sterile vermiculite/peat-moss (1/1) and grown under the conditions described above. Plants were irrigated with 120 mL distilled water every day for 1 week and then with either 120 mL distilled water or 120 mL of 75 mM NaCl three times every week for 8 weeks.

Phenotypic analysis consisted in the determination of growth parameters such as fresh and dry weight of shoots and roots and the evaluation of yield parameters such as production and weight of fruits by plants grown in hydroponics or in vermiculite/peat-moss.

2.4. Determination of ion content

Ion content was measured in untransformed and transgenic tomato plants grown in the conditions described above. Plant material was separated in roots, stems, leaves and fruits, dried for 48 h at 80 °C, milled to powder and digested in a concentrated $HNO_3/HClO_4$ (2/1, v/v) solution. K^+ and Na^+ concentrations were determined in the digested material by inductively coupled plasma spectrometry (Varian ICP 720-ES).

2.5. Determination of parameters related to plant water status

Plants were cultivated in hydroponics as previously described. Plant water content was calculated as the difference between fresh and dry weights after four weeks cultivation. To calculate leaf relative water content, leaves were harvested and their fresh weight (FW) determined immediately after harvest. Next, leaves were floated on deionized water at 4 °C for 24 h to record the turgescent weight (TW) and then dried at 80 °C to measure dry weight. Relative water content (RWC) was calculated as [(FW-DW)/(TW-DW)] X 100. To determine water use efficiency, the nutrient solution consumed by plants was measured and the solution replaced three times per week. Water use efficiency (WUE) was determined on the basis of solution consumption (L) relative to the fresh weight of plants (g) after four weeks cultivation.

2.6. Statistics

All data in this report were obtained from at least three independent experiments with three or four replicates each. For data analysed with Student's *t*-test the differences between treatments and plant genotypes were considered as significant when $P < 0.05$ in a two-tailed analysis.

3. Results

3.1. Molecular characterization of transgenic plants

SlSOS2 and *LeNHX2* transgene integration in the genome of the F1 tomato plants generated by crossing SlSOS2 and LeNHX2 homozygous transgenic lines is shown in Supplemental Material (Supplemental Figs. 1 and 2). Analysis by real time PCR of F1 plants harbouring the constructs for *SlSOS2* and *LeNHX2* overexpression showed a higher expression of both genes in leaves of transgenic lines than in WT plants (Fig. 1). As expected, expression levels of *SlSOS2* were found to be higher in single and double transgenic lines overexpressing *SlSOS2* than in WT and *LeNHX2* overexpressing lines, which showed similar expression of *SlSOS2*. Expression of *LeNHX2* was induced in all single and double transgenic lines expressing either *LeNHX2* or *SlSOS2* or both. Surprisingly, LeNHX2 transcript levels were higher in plants expressing *SlSOS2* alone or in combination with *LeNHX2* than in plants overexpressing only *LeNHX2*. The double transgenic line obtained from the cross of lines L-932 and L-82 showed the highest expression level of *LeNHX2* and *SlSOS2* of all plants analysed.

3.2. Salt stress tolerance and fruit production

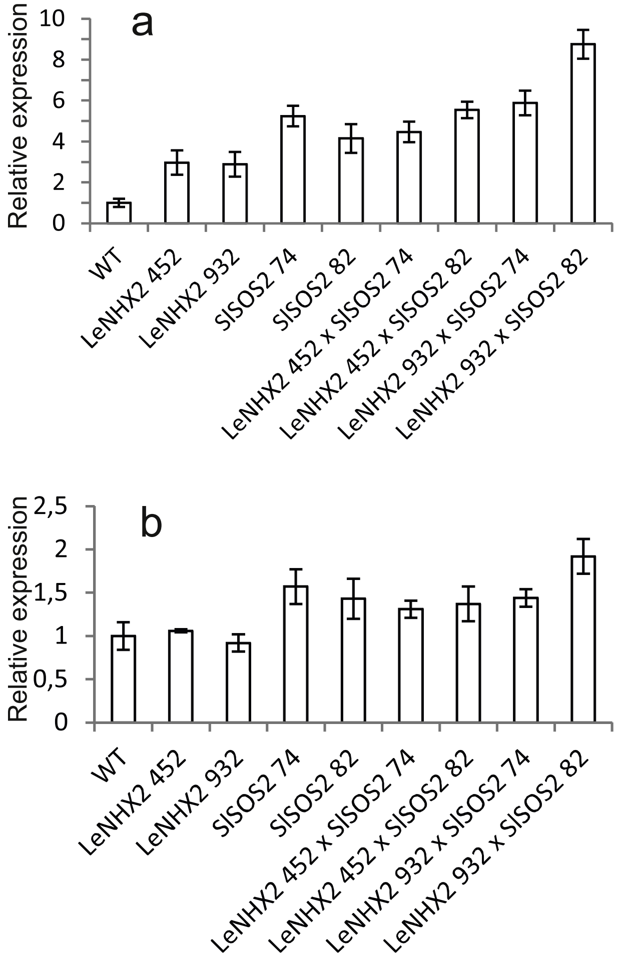

Fig. 1. Transcript levels of *LeNHX2* (a) and *SlSOS2* (b) in leaves of single and double transgenic plants overexpressing *LeNHX2* and *SlSOS2*. Plants were cultivated in hydroponics for 4 weeks. The results show the expression of *LeNHX2* and *SlSOS2*, quantified by real-time qPCR and expressed as increase in transcript levels relative to the level in leaves of WT plants, to which value 1 is assigned. Values are means ± standard deviation of three independent experiments with three replicates each.

Plant growth was evaluated in terms of fresh and dry weight of shoots and roots from plants cultivated in hydroponics (Fig. 2a and Supplemental Fig. 3). When grown for 4 weeks in hydroponics under control conditions (no NaCl added to the nutrient solution) shoot and root fresh weights of double transgenic plants were higher than those of single transgenic and WT plants, whereas all transgenic lines showed a higher root dry weight than WT plants with double transgenic lines showing the highest root dry weight values. Adding NaCl to the hydroponic nutrient solution to a final concentration of 120 mM for the last 3 weeks of cultivation inhibited growth of all plants, but double transgenic plants performed better than the rest of the plants (Fig. 2b). Under the latter cultivation condition, the fresh weights of shoots from single and, above all double transgenic plants, were higher than that of WT (Fig. 2a). Shoot to root ratio calculated from FW values was found to be higher in transgenic than in wild type plants cultivated with NaCl (Fig. 2c). The decrease of plant dry weight induced by NaCl was calculated as percentage relative to dry weight of plants grown under control conditions. Results in Fig. 2d show the lowest percentage values corresponded to double transgenic plants.

Fruit production was measured in plants cultivated for 12 weeks in vemiculite/peat-moss and irrigated with distilled water supplemented or not with NaCl as indicated in the material and methods section. Transgenic plants grown with no NaCl added to the irrigation solution showed a higher fruit production and fruit fresh weight than WT plants (Fig. 3a, b and c). Under this condition the highest fruit production and

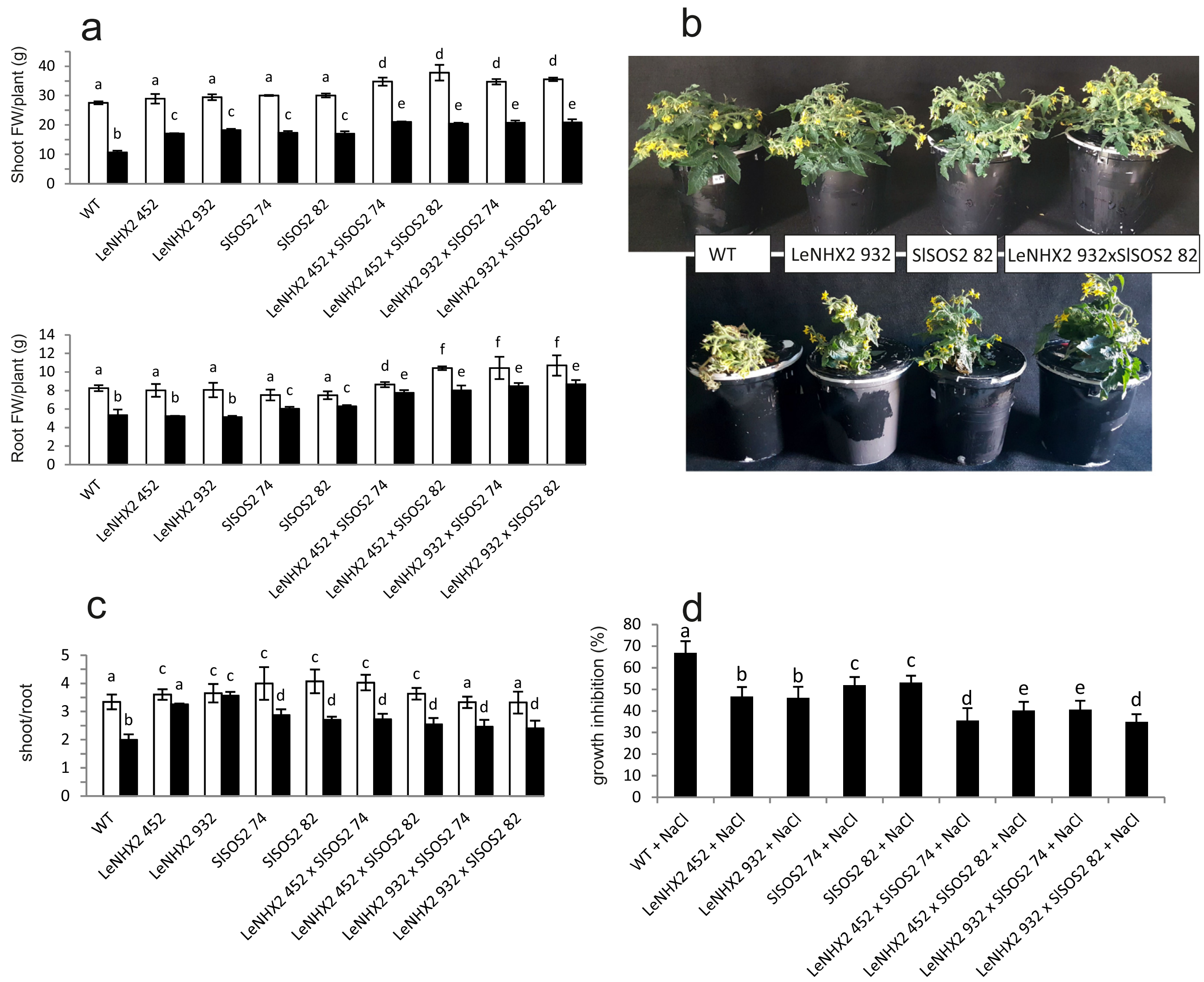

Fig. 2. Effect of *LeNHX2* and *SlSOS2* overexpression on growth of tomato plants. (a) Fresh weights of shoots and roots were determined in plants cultivated in hydroponics for 1 week in 1/4 Hoagland nutrient solution followed by 3 additional weeks in the same solution supplemented with 0 (empty bars) or 120 (black bars) mM NaCl. (b) Image of a representative experiment showing WT, *LeNHX2, SlSOS2* and *LeNHX2SlSOS2* overexpressing plants grown in the absence (upper panel) or the presence (lower panel) of 120 mM NaCl. (c) Shoot/root ratio obtained from tissue fresh weight values. (d) Percentage of decrease in dry weight of NaCl grown plants relative to their dry weight in the absence of NaCl. Values are means ± standard deviation of three independent experiments with three replicates each. Values with different letters are significantly different (Student's *t*-test, $P \leq 0.05$).

fresh weight corresponded to double transgenic plants (Fig. 3a and b). NaCl treatment of plants cultivated under the above condition decreased the amount and fresh weight of fruits in all plant types; however under this cultivation condition all transgenic lines produced a higher amount of fruits than WT plants with the double transgenic lines showing the highest amount of fruit produced under saline conditions. It should be noted that fruits produced by transgenic lines and, above all by double transgenic lines, showed a higher fresh weight than those produced by WT plants under saline conditions (Fig. 3a and b).

3.3. K^+ and Na^+ contents in roots, stems, leaves and fruits

K^+ and Na^+ contents were determined in leaves, stems and roots of WT and transgenic plants cultivated in hydroponics for 4 weeks. Results in Fig. 4a show a higher K^+ content in roots, leaves and stems of single and double transgenic plants overexpressing *LeNHX2* than in WT and single transgenic plants overexpressing *SlSOS2* when they were cultivated in the absence of NaCl. Plants cultivated without NaCl showed very low Na^+ content in all tissues analysed with no significant differences of Na^+ content between them (Fig. 4b). Treatment with NaCl provoked a decrease of K^+ and an increase of Na^+ contents in all plant tissues (Fig. 4a and b). In these conditions the aerial parts of single and double transgenic plants overexpressing *LeNHX2* showed a higher K^+ content than WT and *SlSOS2* overexpressing lines, while roots of all transgenic plants showed a higher content of this cation than WT controls with a slightly higher value in single transgenic plants overexpressing *LeNHX2*. When plant were grown in the presence of NaCl, Na^+ content was higher in leaves and stems of transgenic plants overexpressing *SlSOS2* alone or with *LeNHX2* than in the rest of the plants (Fig. 4b). Na^+ content after NaCl treatment for 3 weeks was similar in roots of WT and single transgenic plants. Double transgenic plants showed the lowest root Na^+ content of all plants analysed (Fig. 4b).

Ionic composition of fruits was determined in ripe red fruits of plants grown in vermiculite/peat moss for 12 weeks (Fig. 5). Relative to WT plants, fruits of all transgenic lines analysed accumulated more K^+

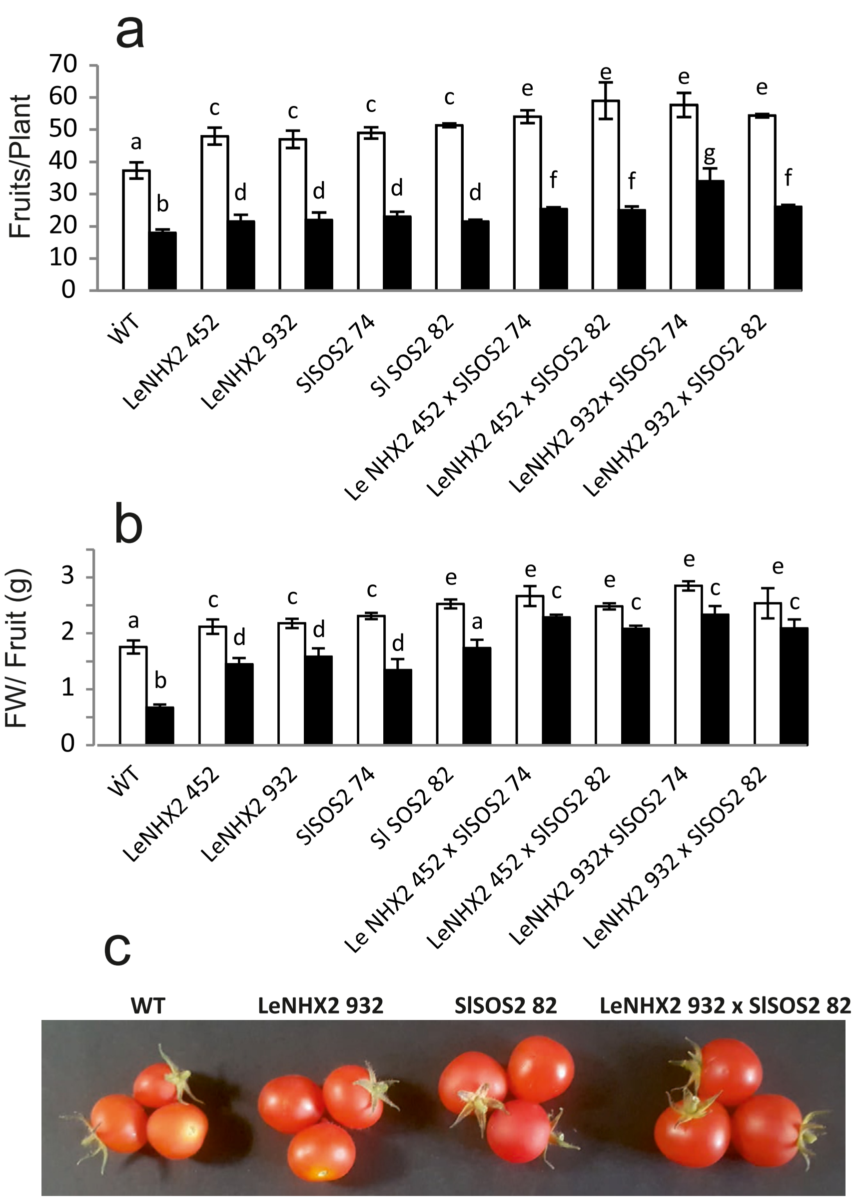

Fig. 3. Effect of *LeNHX2* and *SlSOS2* overexpression on fruit production (a) and fruit weight (b) of tomato plants. Wild-type, single and double transgenic plants overexpressing *LeNHX2* and *SlSOS2* were cultivated in vermiculite/peat-moss for 12 weeks in the presence (black bars) or the absence (empty bars) of NaCl. A representative image of fruits from WT, single and double transgenic plants is included (c). Values are expressed as number of fruits per plant and fresh weight per fruit and are means ± standard deviation of three independent experiments with 5 plants per line. Values with different letters are significantly different (Student's *t*-test, $P \leq 0.05$).

irrespective of the cultivation conditions (Fig. 5a). When NaCl was added to the irrigation solution fruits of transgenic lines overexpressing *SlSOS2* or *SlSOS2* and *LeNHX2* accumulated more Na^+ than those of WT and *LeNHX2* overexpressing plants (Fig. 5b).

3.4. Plant water content, leaf relative water content and water use efficiency

Parameters related to plant water status as plant water content, leaf RWC and WUE are shown in Fig. 6. In the absence of NaCl in the cultivation media, double transgenic plants showed a higher water content than the rest of the plants. Treatment with NaCl decreased water content in all plants, being the decrease lower in transgenic than in WT plants. No significant differences of leaf RWC were found between plants grown in the absence of NaCl. Adding NaCl to the cultivation media decreased leaf RWC of all plants, being the decrease higher in WT than in transgenic plants. Water use efficiency was higher in transgenic than in WT plants grown either in the presence or the absence of NaCl. Relative to control conditions, treatment with NaCl provoked a decrease of WUE in WT plants but did not significantly change WUE of transgenic plants.

4. Discussion

It is well known that salt stress signalling is mediated by the SOS pathway (Zhu, 2002, 2003; Chinnusamy et al., 2005) and that salt tolerance involves Na^+ and/or K^+ intracellular accumulation mediated by NHX transporters (Apse et al., 1999; Apse and Blumwald, 2007; Jiang et al., 2010). Related to this, overexpression of either *NHX* or *SOS* genes was shown to improve salt tolerance in several plant species (Rodríguez-Rosales et al., 2009; Roy et al., 2014; Shi et al., 2003; Yang et al., 2009). The SOS pathway is made up of three components: the plasma membrane Na^+/H^+ antiporter SOS1, the calcium interacting protein kinase SOS2 and the calcium binding protein SOS3 (Quintero et al., 2002; Qiu et al., 2002). When activated by SOS3 the SOS2 protein can phosphorylate SOS1, thereby activating the Na^+/H^+ antiport activity on the plasma membrane (Liu et al., 2000; Quintero et al., 2002). SOS2 can also phosphorylate AtNHX1 on vacuolar membranes and activate the $Na^+,K^+/H^+$ antiport activity at this membrane (Qiu et al., 2002; Zhu, 2003). Therefore, SOS2 is a regulator for SOS1 and NHX1, linking the two main mechanisms of salt tolerance (Na exclusion and Na^+ or K^+ intracellular accumulation). In spite of the above findings only few reports in the literature show the effect on plant salt tolerance

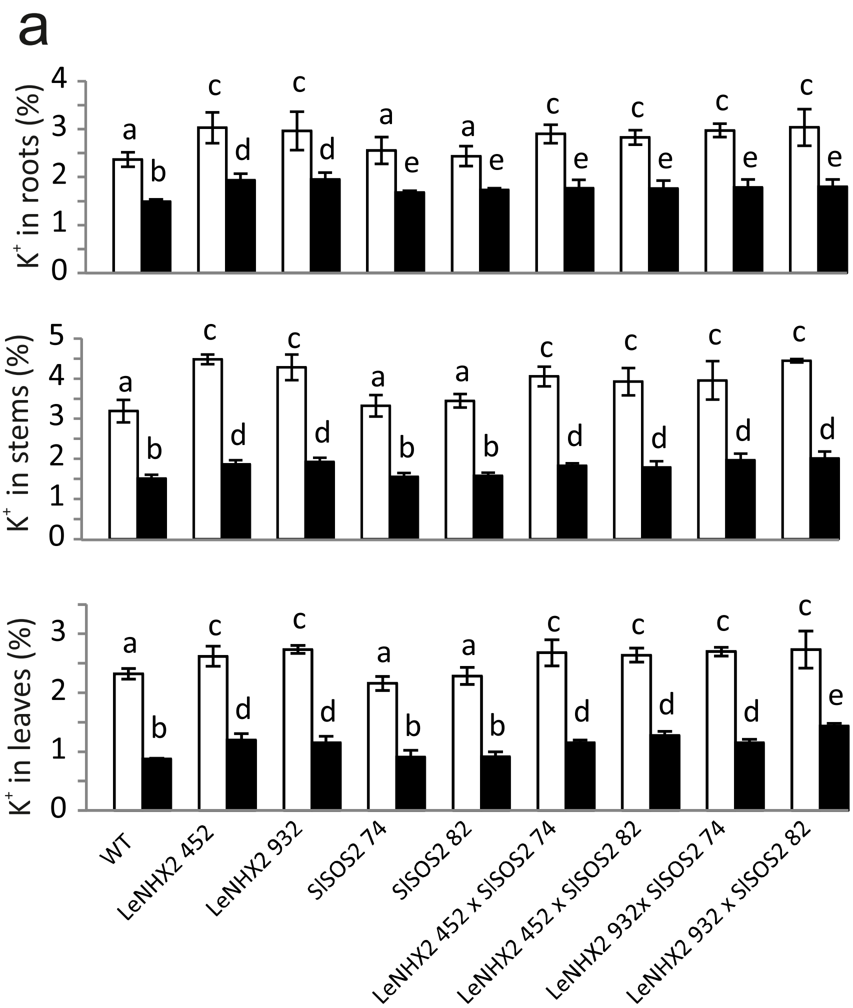

Fig. 4. K^+ (a) and Na^+ (b) contents in roots, stems and leaves of wild-type and single and double transgenic tomato plants overexpressing *LeNHX2* and *SlSOS2*. Plants cultivated for one week in in 1/4 Hoagland nutrient solution were treated with 0 (empty bars) and 120 (black bars) mM NaCl for three additional weeks. Values, expressed as percentages relative to dry weight, are means ± standard deviation of three independent experiments with four replicates each. Values with different letters are significantly different (Student's *t*-test, $P \leq 0.05$).

of the joint overexpression of *NHX* and *SOS* pathway genes. Pehlivan et al. (2016) reported that overexpression of *AtNHX1* or *SOS1* could improve salt tolerance in transgenic plants, but the improved salt tolerance is limited to NaCl concentrations below 200 mM. Co-overexpression of *AtNHX1* and *SOS1* could further improve salt tolerance, making transgenic Arabidopsis able to tolerate up to 250 mM NaCl (Pehlivan et al., 2016). Yang et al. (2009) found that transgenic Arabidopsis overexpressing *AtNHX1* and *SOS3* showed higher salt tolerance than plants overexpressing only *AtNHX1* but similar salt tolerance to plants overexpressing *SOS3* alone. The transport activity of NHXs require the H^+ motive force generated by the vacuolar ATPase or PPase and in accordance, an improved salt tolerance of tomato, rice, wheat and Arabidopsis plants was found by overexpressing NHXs simultaneously with the vacuolar pyrophosphatase (Bhaskaran and Savithramma, 2011; Brini et al., 2007; Gouiaa et al., 2012; Zhao et al., 2006). An increased salt tolerance of Arabidopsis and tomato plants overexpressing *LeNHX2* or *SlSOS2* individually and a regulation of LeNHX2 expression and activity by SlSOS2 were reported in our previous papers (Huertas et al., 2012, 2013; Rodríguez-Rosales et al., 2008). We have now generated double transgenic tomato plants overexpressing both *LeNHX2* and *SlSOS2* together and compared their salt tolerance and fruit production with those of WT and single transgenic plants. To our knowledge the effect of the joint overexpression of SOS2 kinases and NHX-type ion transporters has not yet been reported in the literature.

Homozygous transgenic tomato lines overexpressing *LeNHX2* and *SlSOS2* were crossed and the presence of both transgenes was confirmed in the plants resulting from the crosses (Supplemental material, Figs. 1 and 2). As expected the expression levels of *LeNHX2* and *SlSOS2* were higher in transgenic than in WT plants (Fig. 1). Expression of *LeNHX2* was higher in single and double transgenic plants overexpressing *SlSOS2* than in plants overexpressing only *LeNHX2* (Fig. 1), thus confirming our previous finding on the regulation of *LeNHX2* expression by the kinase SlSOS2 in tomato.

Data of fresh weight of tomato plants cultivated in media lacking NaCl show an improvement of plant growth as a result of the joint overexpression of *LeNHX2* and *SlSOS2* (Fig. 2a). Gouiaa et al. (2012) also found that co-overexpressing the Na^+/H^+ antiporter TNHXS1 and H^+- pyrophosphatase TVP1 genes from wheat in tobacco results in seedlings with higher fresh and dry weight when cultivated in NaCl-free medium. Opposite to these results, Arabidopsis transgenic plants overexpressing SOS1 + AtNHX1, SOS3 + AtNHX1, SOS3 + SOS2 and SOS3 + SOS1 + SOS2 showed no differences in growth compared to control plants cultivated under non stressful conditions (Yang et al., 2009). In tomato the inhibitory effect of NaCl on plant growth was lower in double transgenic plants overexpressing *LeNHX2* and *SlSOS2* than in the rest of the plants analysed (Fig. 2b), highlighting that under saline conditions the joint overexpression of *LeNHX2* and *SlSOS2* causes

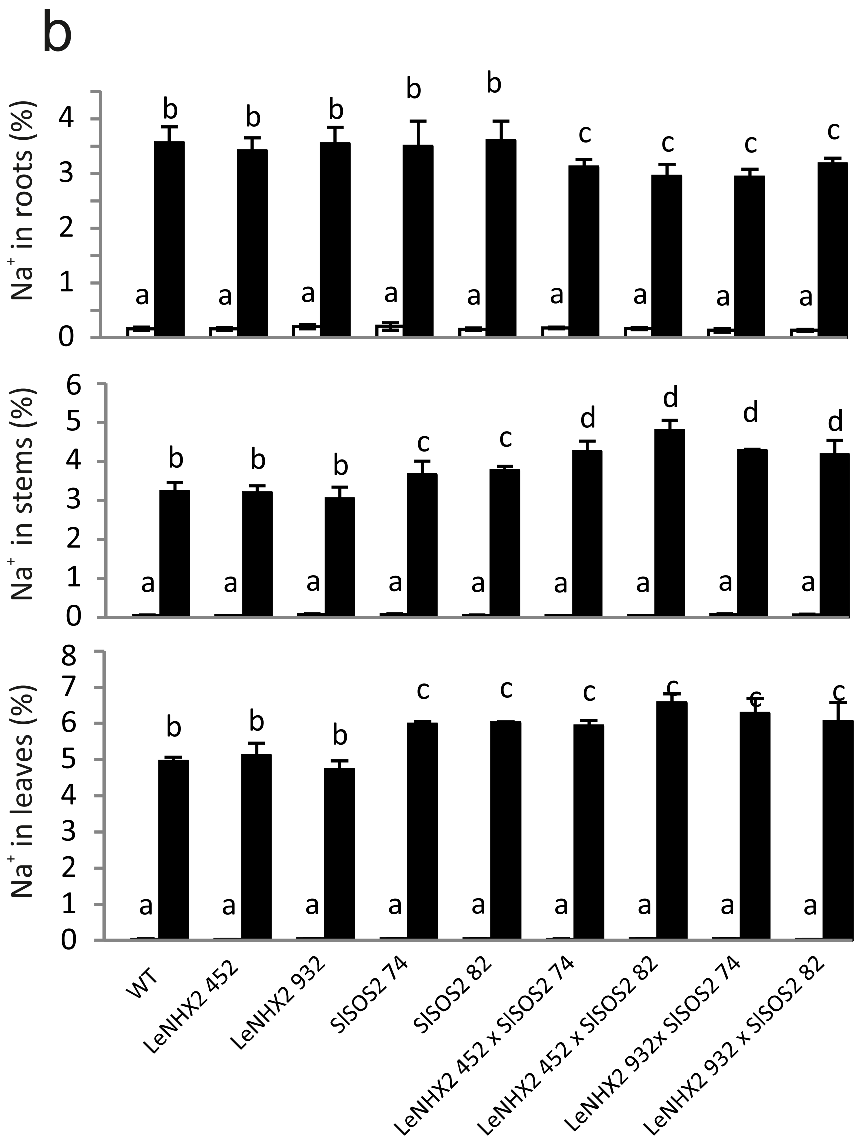

Fig. 4. (*continued*)

a significant improvement of plant growth in comparison to plants overexpressing only one of these genes. Similar results were obtained by Pehlivan et al. (2016) in Arabidopsis plants overexpressing SOS1 and AtNHX1, whereas Yang et al. (2009) found that NaCl tolerance of Arabidopsis overexpressing AtNHX1 + SOS3 was higher than that of plants overexpressing only NHX1 but not significantly different from that of plants overexpressing SOS3 alone. According to this, not in all cases co-overexpressing genes of the SOS signalling pathway with NHX transporters results in an additive effect on plant salt tolerance. Results in this work showing a higher shoot to root ratio in NaCl-treated transgenic plants relative to WT plants (Fig. 2c) suggests that transgenic tomato plants grown in the presence of NaCl are able to allocate more resources to leaves and thus improve the potential for photoassimilate production.

Data on Na^+ and K^+ content in roots, leaves and stems of WT and single transgenic tomato plants (Fig. 4) confirm the results obtained in our previous studies (Huertas et al., 2012, 2013) and point to an intracellular accumulation of K^+ by plants that overexpress *LeNHX2* alone or with *SlSOS2* when cultivated under non stressful conditions. Overexpression of *LeNHX2* in tomato was shown to modulate *LeHAK5* expression (Huertas et al., 2013). An increased *LeHAK5* expression was demonstrated to be related to a higher K^+ uptake by tomato roots (Huertas et al., 2013; Leidi et al., 2010), which could explain the higher K^+ content found in single and double transgenic plants that overexpress *LeNHX2* when compared to the rest of the plants (Fig. 4a). As expected, cultivation in media supplemented with 120 mM NaCl for 3 weeks provoked a decrease of K^+ and an increase of Na^+ content in all plants analysed (Fig. 4a and b). The higher stem and leaf Na^+ contents in single and double transgenic tomato plants overexpressing *SlSOS2* compared to WT and single transgenic plants overexpressing *LeNHX2* (Fig. 4b), suggest that the overexpression of *SlSOS2* induces intracellular accumulation of Na^+ in the aerial parts of NaCl grown plants. As SOS1 is involved in long distance Na^+ transport from root to shoot (Olías et al., 2009), SOS1 activation in *SlSOS2* overexpression lines would likely increase Na^+ translocation form root to shoots (Olías et al., 2009). We previously showed that tomato plants overexpressing *SlSOS2* show increased expression of the vacuolar Na^+,K^+/H^+ antiporter LeNHX4 as well as a stimulation of the Na^+/H^+ antiport activity in tonoplast vesicles, which would ultimately lead to enhanced accumulation of Na^+ in vacuoles in the aerial parts of the plant (Huertas et al., 2013).

We have demonstrated in a previous study that NaCl-treated tomato plants overexpressing *SlSOS2* show higher relative growth rates, earlier flowering and higher fruit production compared to non-transformed controls (Huertas et al., 2012). In this work we extended the study of fruit yield to double transgenic plants, determining the amount of fruits

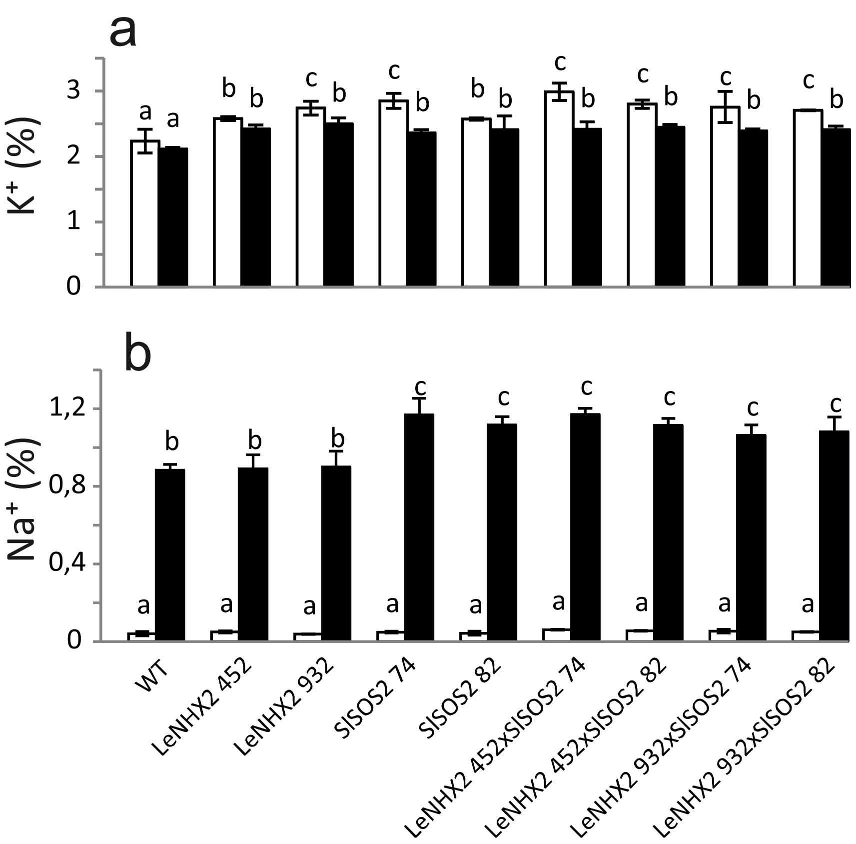

Fig. 5. K^+ (a) and Na^+ (b) contents in ripe red fruits of wild-type and single and double transgenic tomato plants overexpressing *LeNHX2* and *SlSOS2*. Plants were cultivated in vermiculite/peat-moss for 12 weeks in the presence (black bars) or the absence (empty bars) of NaCl. Values, expressed as percentages relative to dry weight, are means ± standard deviation of two independent experiments with four replicates each. Values with different letters are significantly different (Student's *t*-test, $P \leq 0.05$).

produced per plant as well as fresh weight per fruit. To rule out the possibility that an enhanced fruit production of transgenic plants was due to an early flowering of transgenic lines relative to WT controls, plants were grown in vermiculite/peat-moss for 12 weeks to ensure that the flowering was over. Our data demonstrate that overexpression of *SlSOS2* alone or with *LeNHX2* provokes an increase in fruit production as well as in fruit fresh weight in plants cultivated under non-stressful conditions (Fig. 3a and b). Compare to plants overexpressing *NHX1* or *SOS1* individually, an increased seed yield was reported in Arabidopsis simultaneously overexpressing AtNHX1 and SOS1 grown in salt-free medium (Pehlivan et al., 2016). However, under the same cultivation condition no differences in silique number and seed weight between single and double transgenic plants overexpressing AtNHX1 and/or SOS1 were reported (Pehlivan et al., 2016). Also under NaCl stress the enhanced expression of *LeNHX2* or *SlSOS2* and, above all, of both genes induce in tomato higher fruit production and fruit fresh weight (Fig. 3a and b). An increased fruit production under NaCl conditions was also reported in tomato plants co-overexpressing the *Pennisetum glaucum* vacuolar Na^+/H^+ antiporter and Arabidopsis H^+-pyrophosphatase (Bhaskaran and Savithramma, 2011). Likewise, under salt stress an increased silique production was detected in double transgenic Arabidopsis plants overexpressing AtNHX1 and SOS1 (Pehlivan et al., 2016). These results from various laboratories suggest a relationship between fruit production and regulation of ion homeostasis by NHX and SOS proteins. Indeed it was shown that potassium plays a vital role in photoassimilate transport from source to sink (Lalonde et al., 2003; Mengel, 1997; Schobert et al., 1998) and thus improved potassium homeostasis could enhance fruit production. Contrary to the results obtained for roots, stems and leaves, no major changes were detected in K^+ content of tomato fruits as a result of NaCl treatments (Fig. 5a). NaCl-induced increase in Na^+ content was higher in fruits of plants overexpressing the kinase SlSOS2 than in the rest of the plants (Fig. 5b). This can be related to an effect of SlSOS2 upregulating the expression and activity of LeNHX4, since this vacuolar $Na^+,K^+/H^+$ antiporter is highly expressed in fruits and proved to be upregulated by SlSOS2 (Gálvez et al., 2012; Huertas et al., 2012).

Parameters related to plant water status (Fig. 6) suggest that transgenic tomato plants withstand better a shortage of water under NaCl stress. In relation to this, the more efficient regulation of water loss under salinity could also be related to the higher fruit fresh weight of transgenic plants relative to WT controls. Similar to our results in tomato, tobacco plants overexpressing wheat *TNHXS1* together with *TVP1* showed a higher RWC under NaCl stress than WT controls (Bhaskaran and Savithramma, 2011).

In conclusion, the results presented in this study indicate that double transgenic tomato plants overexpressing both *LeNHX2* and *SlSOS2* show an increased fruit yield and a better performance under NaCl stress than WT and single transgenic plants overexpressing only one of these genes. Under saline growth conditions, the joint overexpression of *LeNHX2* and *SlSOS2* improves water status and Na^+ and K^+ ion accumulation and partitioning in tomato plants. Results from this and other studies suggest that transgenic approaches contributing to a better knowledge of the role of proteins regulating ion homeostasis under salt stress can be important to enable biotechnological applications based on the genetic manipulation of processes involved in mineral nutrition in order to establish crops in areas that are not productive due to high salinity or low mineral nutrient content.

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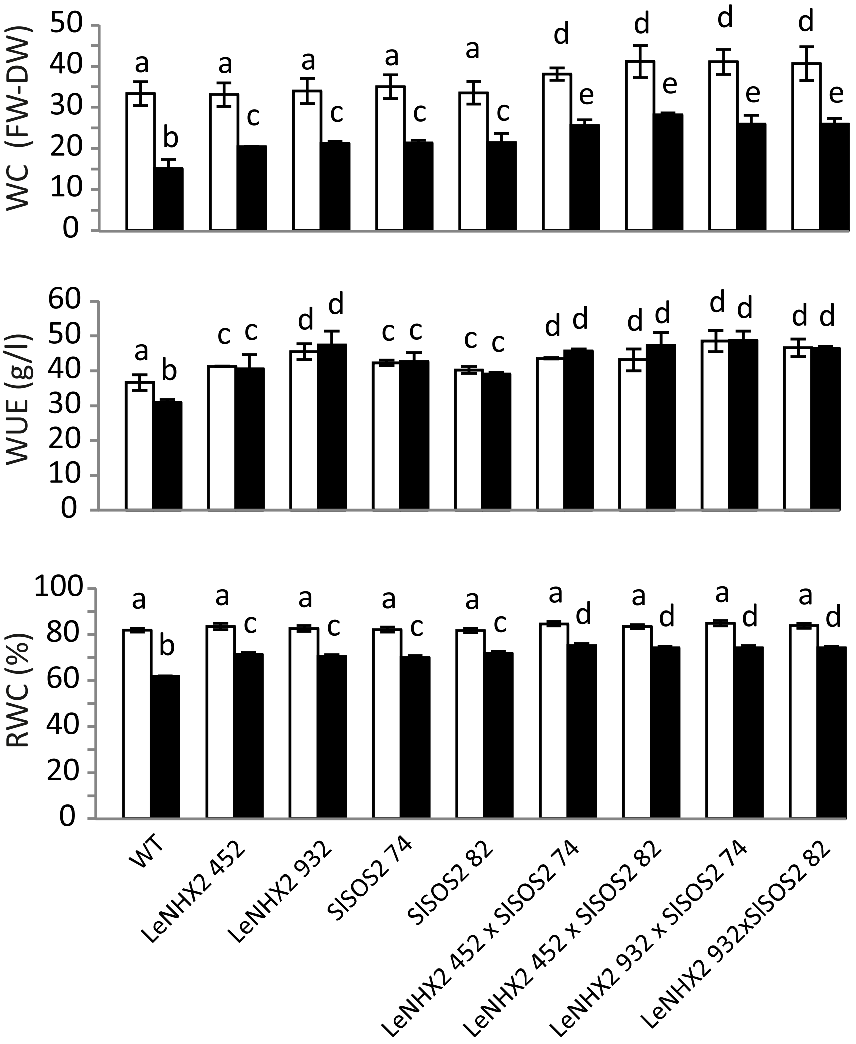

Fig. 6. Plant water content (WC) and water use efficiency (WUE) and leaf relative water content (RWC) of wild-type and single and double transgenic tomato plants overexpressing *LeNHX2* and *SlSOS2*. Plants cultivated for one week in in 1/4 Hoagland nutrient solution were treated with 0 (empty bars) and 120 (black bars) mM NaCl for two (WUE and RWC) or three (WC) additional weeks. Values, expressed as FW-DW (WC), g/l (WUE) or percentage (RWC), are means ± standard deviation of three independent experiments with four replicates each. Values with different letters are significantly different (Student's *t*-test, $P \leq 0.05$).

Conflicts of interest

Authors declare they have no potential or actual conflict of interest.

CRediT authorship contribution statement

Mourad Baghour: Formal analysis. **Francisco Javier Gálvez:** Formal analysis, Investigation. **M. Elena Sánchez:** Formal analysis, Investigation. **M. Nieves Aranda:** Formal analysis, Investigation. **Kees Venema:** Formal analysis, Writing - original draft. **M. Pilar Rodríguez-Rosales:** Formal analysis, Writing - original draft.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.11.028.

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