Molecular Biology Reports (2020) 47:4145–4153
https://doi.org/10.1007/s11033-020-05499-z

ORIGINAL ARTICLE

Overexpression of *LeNHX4* improved yield, fruit quality and salt tolerance in tomato plants (*Solanum lycopersicum* L.)

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Received: 2 March 2020 / Accepted: 3 May 2020 / Published online: 13 May 2020

Abstract

The function of the tomato K^+, Na^+/H^+ antiporter LeNHX4 has been analyzed using 35S-driven gene construct for overexpressing a histagged LeNHX4 protein in *Solanum lycopersicum* L. Compared to wild-type plants, the expression of *LeNHX4* was enhanced in most of plants transformed with a gene construct for *LeNHX4* overexpression although some plants showed a decreased *LeNHX4* expression. Overexpression of *LeNHX4* was associated to an increased fruit size while silencing of this gene was related to a decreased fruit size. We have investigated the effect of *LeNHX4* overexpression on fruit production and quality and we have also evaluated salt tolerance in two different overexpression lines by measuring proline, protein and glucose concentrations in tomato leaves grown either under control (0 mM NaCl) or saline (125 mM NaCl) conditions. Plants overexpressing *LeNHX4* showed a higher amount of fruits than WT plants and accumulated higher contents of sugars and cations (Na^+ and K^+). The application of 125 mM NaCl, affected negatively fruit production and quality of WT plants. However the transgenic lines overexpressing *LeNXH4* increased fruit quality and yield. In relation to salt tolerance, overexpression lines showed higher levels of leaf proline, glucose and proteins under NaCl treatment. The overexpression of *LeNHX4* in tomato plants, improved salinity tolerance and increased fruit yield and quality under both normal and salinity stress conditions.

Keywords *Solanum lycopersicum* · Proline · Soluble sugars · Protein · Salinity tolerance · LeNHX4 antiporter

Introduction

Soil salinity is the most severe factor limiting plant growth and crop productivity. This problem increases in arid and semi-arid regions. According to the report of FAO, over 6% of the world's land is affected by either salinity or sodicity which accounts for more than 800 million ha of land throughout the world [1].

High levels of salinity may cause both ionic and osmotic stress effects, which lead to a decline of turgor, membrane damage, the inhibition of water and essential ions uptake, disordered metabolism, altered levels of growth regulators, enzymatic inhibition and metabolic dysfunction, including photosynthesis which ultimately leads to plant death [2–6]. On the other hand, potassium (K) is an essential element required for various physiological and biochemical processes that influence plant growth and development. In plants K^+ is also a key player in the maintenance of osmotic adjustment and cell turgor [7] and plays an important role in enzyme activation, photosynthesis and respiration, assimilate transport, protein metabolism and stomatal regulation [8]. Due to similar physicochemical properties, Na^+ and K^+ ions have the potential to compete each other for uptake by plant-root cells. Under salt stress, the uptake of Na^+ is increased drastically, causing a decrease in the absorption and accumulation of K^+ and subsequent deficiency of this element [9–11].

Plant cell adaptation to salinity involves avoiding sodium toxicity and potassium loss (CITAS Adecuadas [12, 13]).

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11033-020-05499-z) contains supplementary material, which is available to authorized users.

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Therefore, keeping a high K^+/Na^+ ratio in the cytosol is considered a fundamental process for salinity tolerance [14, 15]. For this purpose, plant cells have developed mechanisms of restriction of Na^+ influx, active Na^+ efflux, and compartmentalization of Na^+ in the vacuole or other intracellular compartments [16]. Both Na^+ efflux and Na^+ compartmentalization need the operation of Na^+/H^+ antiporters located at the plasma membrane or intracellular membranes [17].

In previous works we have identified in tomato several Na^+, K^+/H^+ antiporters belonging to the NHX family of proteins 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 [18]. This study showed a higher expression of *LeNHX4* in the wild tomato and an accumulation of Na^+ in aerial parts of these plants. Interestingly, results from these studies showed that unlike other NHXs isoforms, tomato *LeNHX4* is highly expressed in reproductive tissues and thus may have a role in fruit production and quality.

Regulation of osmotic adjustment under salt stress is also an important determinant of salt tolerance. In many plant species, the accumulation of compatible osmolytes (i.e., proline and sugars) is an important strategy leading to stress tolerance [19]. These molecules regulate the osmotic adjustment in plants grown under salt stress and enables plants to reestablish the water and osmotic homeostasis [20]. Proline is accumulated preferentially in leaves in order to maintain chlorophyll level and cell turgor to protect photosynthetic activity under salt stress [21]. In addition, soluble sugars are highly accumulated in vacuoles and produce high turgor pressure affecting osmotic potentials [22–25]. Interestingly, a relationship between *NHX* gene expression and sugar and proline accumulation has been reported [26–30].

Based on all the above, in this work we have studied whether the overexpression of the vacuolar Na^+, K^+/H^+ antiporter LeNHX4 affect tomato fruit yield and quality under unstressed and NaCl stress cultivation conditions.

Materials and methods

Transformation and molecular characterization of transgenic plants

For stable expression of the LeNHX4 protein in tomato (*S. lycopersicum* L. cv. MicroTom), the LeNHX4 coding sequence to which the sequence for a C-terminal RGS(H)10 tag was added, was cloned under control of the 35S promoter in the pCAMBIA1303 plant expression vector as described. The plasmid pCAMBIA 1303 carrying the LeNHX4-RGS(H)10 fragment was transferred in the LB4404 *Agrobacterium tumefaciens* strain [31] and used for tomato cotyledon transformation as has been described by Ellul et al. [32].

The presence of the LeNHX4-RGS(H)10 construction in selected tomato transgenic lines was assessed by polymerase chain reaction (PCR) analysis using specific primers to amplify a 468 bp fragment of the hygromicin resistance gene harboured in the plant expression cassette (Forward: 5′-GATGTTGGCGACCTCGTATT-3′, Reverse: 5′-GTG CTTGACATTGGGGAGTT-3′) and DNA obtained from tomato leaves following a method by Edwards et al. [33].

The level of expression of *LeNHX4* in leaves of untransformed and transgenic plants was assessed by real time PCR using gene-specific primers as previously described [18].

Plant material and growth conditions

Plants used for molecular characterization and fruit yield and quality determinations were cultivated in hydroponics for 4 weeks in 1/4 Hoagland nutrient solution [34]. For this purpose, seed 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 1 week with one-tenth strength Hoagland nutrient solution [34] and another 2 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 4 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 µmol/m^2/s (photoperiod of 16 h light and 8 h darkness) and 40–50% relative humidity.

Alternatively, and in order to extend the growing period, tomato seeds were sown in seedbeds containing peat-moss. The seedbeds were kept in a greenhouse and irrigated with tap water for 7 weeks. 50-day-old tomato plants were transferred to 1.2 L pot (1 plant per pot) containing peat-moss and kept in a greenhouse. These plants were irrigated with tap water for 1 week and then with either tap water or 125 mM NaCl three times ever week for 16 weeks.

Determination of fruit quality parameterss

Ion content was measured in untransformed and transgenic tomato plants grown in hydroponics for 4 weeks in growth chamber under the conditions described above. Fruits were 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).

Plant water content was calculated as $[(FW - DW)/FW)] \times 100$.

Sugar analysis by GC–MS was carried out by a method modified from Schauer et al. [35]. Five tomato fruits were taken at postharvest stage to perform the GC–MS determinations.

Fruits were harvested and the fleshy, edible tissue was frozen in liquid nitrogen and crushed to powder with a grindomixer (Retsch, Haan, Germany). Samples were stored at − 80 °C until further analysis. For each individual fruit, frozen tomato tissue (150 mg) was extracted with 700 µL MeOH solution containing ribitol (9 µg ribitol/mL MeOH) as an internal standard. The mixture was extracted for 15 min at 70 °C and mixed vigorously with 700 µL of distilled water. To separate polar and non-polar metabolites, 325 µL chloroform was added to the mixtures. After centrifugation at 14,000 rpm for 5 min, the upper methanol/water phase (500 µL) was taken and dried overnight under vacuum.

The residue was re-dissolved in 40 µL of 20 mg/mL methoxyamine hydrochloride in pyridine and derivatized at 37 °C for 90 min followed by a 30 min treatment with 60 µL MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamine) at 37 °C. Sample volumes of 1 µL were then injected into the GC column.

Total soluble solid (TSS) was measured as °Brix in a few drops of the juice using a digital refractometer (ATAGO Co., Ltd., Tokyo, Japan).

Tests for pH were performed on fruit homogenate using a portable pH meter.

Glucose, proline and protein determination in leaves

For glucose and proline determination samples of 0.5 g of plant materials were used by following the methods described by Irigoyen et al. [36] and Paquin and Lechasseur [37], respectively.

Fresh leaves were used for the determination of soluble proteins using Bradford G-250 reagent [38], and expressed as mg/g of fresh weight.

Statistics

All data in this report were obtained from at least three independent experiments with three or four replicates each. Analysis of variance was used to assess difference between treatments and plant genotypes were considered as significant when $P \leq 0.05$. Significant differences according to the Duncan's multiple range test (DMRT) are indicated with different letters in the figures and tables.

Results

Molecular characterization of transgenic plants

The cDNA corresponding to *LeNHX4* gene was expressed under the cauliflower mosaic virus 35S gene promoter in tomato (*S. lycopersicum* L. cv MicroTom). T3 homozygous plants with a single insertion of the transgene were selected on the basis of PCR amplification of hygromicin resistance gene and the analysis of hygromicin resistance segregation after seed germination in selective media containing the antibiotic (Supporting Information Fig. S1). From these plants, three independent lines, L-2, L-3 and L-5, were further characterized. Real-time RT-PCR analysis showed a higher expression level of *LeNHX4* in leaves of L-3 and L-5 transgenic lines than in those of untransformed controls (Fig. 1a). Surprisingly, the expression level of *LeNHX4* in plants from line L-2 was half of that in WT plants, which indicate the silencing of *LeNHX4* in this line (Fig. 1a).

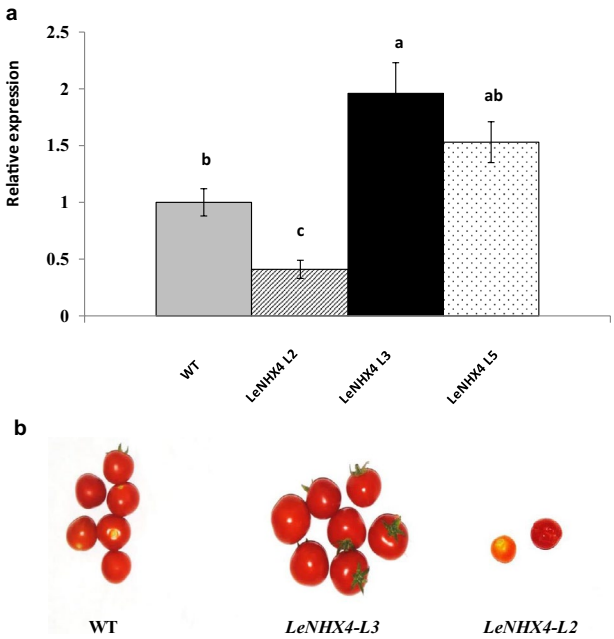

Fig. 1 **a** Transcript levels of *LeNHX4* in leaves of WT and transgenic plants. Plants were cultivated in hydroponics for 4 weeks. The results show the expression of *LeNHX4*, quantified by real-time qPCR and expressed as increase in transcript levels relative to the level in leaves of WT plants, to which value 1 is assigned. Values are means ± standard deviation of three independent experiments with three replicates each. Means that have different letters at the top of each bar are significantly different at $P \leq 0.05$. **b** Image of the fruits obtained from WT and transgenic plants (lines L-3 and L-2). It can be observed that relative to WT plants, the low expression of *LeNHX4* in L2 causes a lower fruit size while, while the high expression of the gene in L3 line provokes an increase of fruit size

Characterisation of fruits

Since *LeNHX4* is highly expressed in fruits [18] we tested whether overexpression of *LeNHX4* have an effect on fruit yield. Fruit size was related to the level of expression of *LeNHX4* in the transgenic lines (Fig. 1b). Plants ovexpressing *LeNHX4* (lines 3 and 5) showed the highest fruit size, while plants in which *LeNHX4* (line 2) was silenced showed the lowest fruit size. Fruit production was measured in untransformed and transgenic tomato plants overexpressing *LeNHX4* grown in hydroponics for 4 weeks in Hoagland nutrient solution (Fig. 2a). Our data demonstrate that overexpression of *LeNHX4* lead to a significant increase in fruit production, while the lowest production was reached in WT plants. Fruit fresh weight (Fig. 2b) showed trends similar to those of fruit production, reaching high values in transgenic lines L-3 and L-5, exceeding WT by 61% and 88% respectively.

The overexpression of *LeNHX4* increased the fruit water content respect to the value in WT plants (Fig. 2c). The concentrations of glucose, fructose and sucrose were determined in fruits of WT plants and plants overexpressing *LeNHX4* in order to study the effect of the overexpression of *LeNHX4* on the quality of tomato fruits (Fig. 2d). Fructose and glucose levels were strongly increased in transgenic plants in comparison to WT plants. However, the concentration of sucrose was not significantly different between WT and transgenic plants.

Figure 3 shows the contents of Na^+ and K^+ in fruits of WT plants and plants overexpressing *LeNHX4*. The highest Na^+ contents were found in the *LeNHX4* overexpressing lines, with values of 0.10 and 0.06% of dry weight, respectively, while the WT fruits reached the lowest value. Similar results were obtained for fruit K^+ content, with transgenic lines L-3 and L-5 showing 14 and 18% higher K^+ content than WT fruits.

The effect of *LeNHX4* overexpression in fruit yield and quality was also studied in plants grown in the presence or the absence of NaCl for longer time periods. Our results demonstrate that plants overexpressing *LeNHX4* showed higher fruit production that WT plants when both were irrigated either with or without NaCl (Fig. 4a). Compared to untreated plants salt-treated wild type plants reduced fruit production by 33%, while fruit production by *LeNHX4* overexpressing plants was slightly increased as a result of salt treatment (Fig. 4a).

Fruit quality characteristics such as total soluble solids (TSS% or °Brix), and pH are shown in Table 1. Under normal conditions (without 125 mM NaCl), transgenic lines overexpressing *LeNHX4* showed a significant reduction in TSS. In relation to pH, we have not found significant differences between WT and both transgenic lines. However,

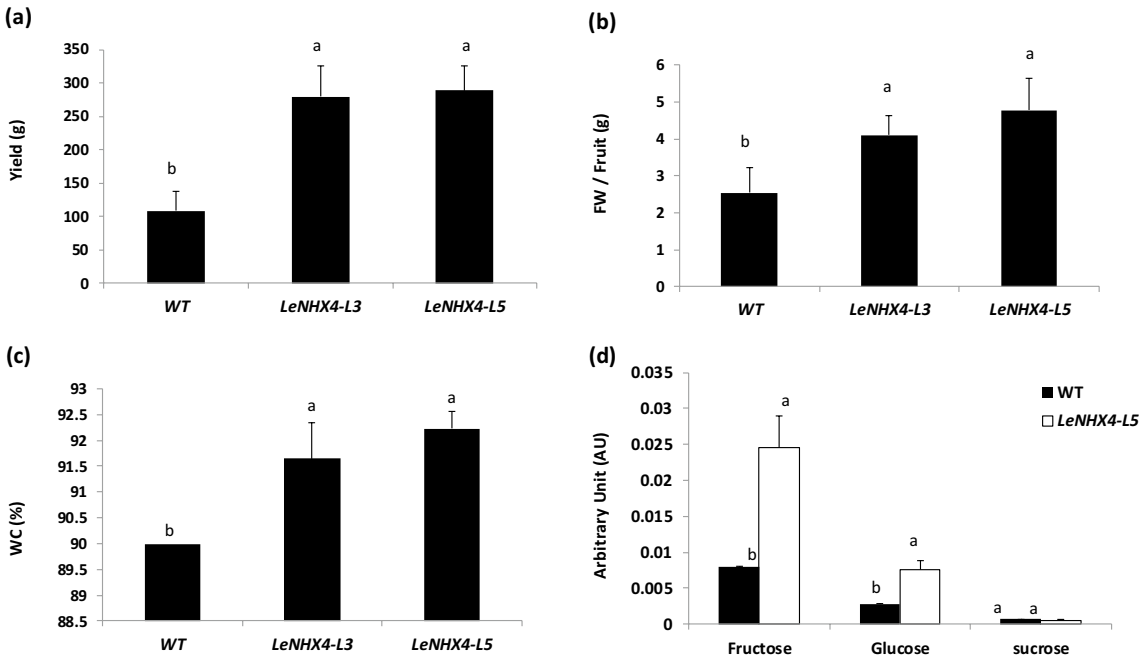

Fig. 2 Effect of *LeNHX4* overexpression on fruit production (**a**), fruit weight (**b**), water content (**c**) and soluble sugars (**d**) of tomato plants. WT and *LeNHX4* plants were cultivated in hydroponics for 4 weeks. Values are means ± standard deviation of three independent experiments with three replicates each. Means that have different letters at the top of each bar are significantly different at $P \leq 0.05$

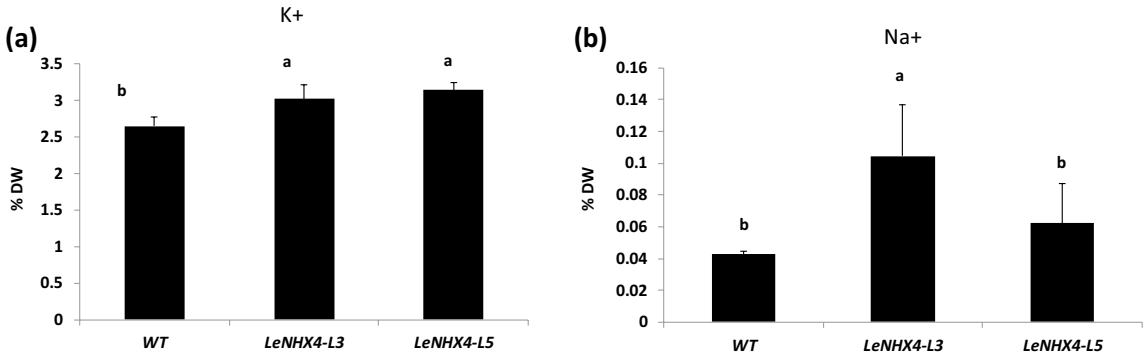

Fig. 3 K^+ (**a**) and Na^+ (**b**) contents in fruit of WT and transgenic tomato plants overexpressing *LeNHX4*. Plants were cultivated in hydroponics for 4 weeks. Values are means ± standard deviation of three independent experiments with three replicates each. Means that have different letters at the top of each bar are significantly different at $P \leq 0.05$

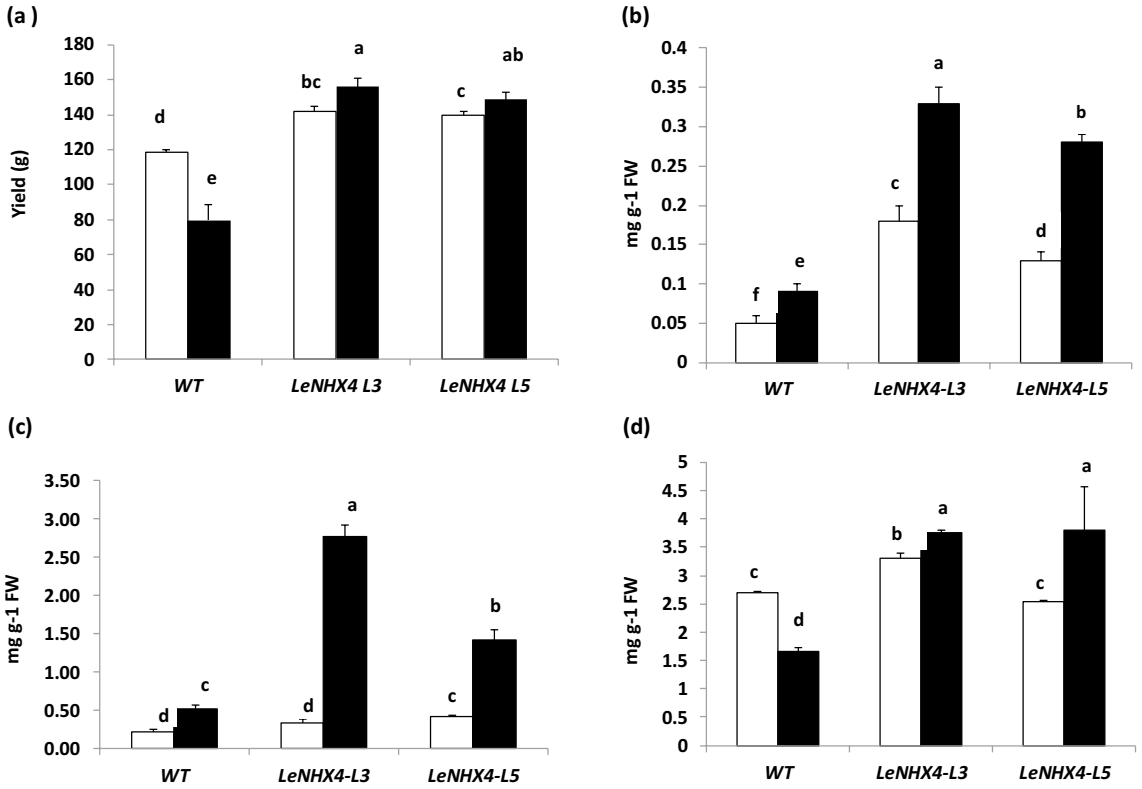

Fig. 4 Effect of *LeNHX4* overexpression on fruit production (**a**) and glucose (**b**), proline (**c**) and protein concentrations (**d**) of tomato plants. WT and *LeNHX4* plants were cultivated in peat-moss for 16 weeks in the absence (empty bars) or the presence (black bars) of 125 mM NaCl. Values are means ± standard deviation of three independent experiments with three replicates each. Means that have different letters at the top of each bar are significantly different at $P \leq 0.05$

the addition of 125 mM NaCl to the irrigation solution significantly increased total soluble solids in *LeNHX4* overexpressing lines and decreases it in WT plants. Similarly, the pH decreased in WT and increased in transgenic lines under salinity conditions.

Glucose, proline and proteins in leaves of WT and transgenic plants overexpressing *LeNHX4*

Figure 4b shows the glucose concentration in leaves of WT and the transgenic lines overexpressing *LeNHX4* (lines L-3 and L-5). The NaCl treatment increases the glucose level in all plants, above all in *LeNHX4* overexpressing plants.

Table 1 pH and solid solute content (°Brix) in fruit of wild-type and transgenic plants overexpressing *LeNHX4* grown under control conditions (0 mM NaCl) and 125 mM NaCl

	TSS (°Brix)		pH	
	0 mM NaCl	125 mM NaCl	0 mM NaCl	125 mM NaCl
WT	10.56 ± 0.36 a	9.58 ± 0.33 b	4.01 ± 0.13 a	3.43 ± 0.42 b
LeNHX4-L3	6.36 ± 0.74 b	11.3 ± 0.36 a	3.79 ± 0.41 a	4.92 ± 0.28 a
LeNHX4-L5	6.08 ± 0.28 b	10.84 ± 0.56 a	3.83 ± 0.41 a	4.53 ± 0.33 a

Plants were cultivated in peat-moss for 16 weeks in the absence or the presence of 125 mM NaCl. Values are means ± standard deviation of three independent experiments with three replicates each. Means followed by different letters are significantly different at $P \leq 0.05$

Our results show that NaCl treatment significantly increased the proline content in WT and *LeNHX4* overexpressing plants, being the increase more pronounced in the transgenic plants (Fig. 4c).

Figure 4d shows the effect of NaCl treatment on protein contents. Significant differences were found in the protein content between untreated and treated plants. Under NaCl stress, transgenic plants showed higher protein content than untreated plants, while the protein level in WT plants was negatively affected.

Discussion

Salt stress is a major abiotic factor limiting plant growth and productivity. Plants exhibit various physiological and biochemical responses to salt stress. An important mechanism to overcome salt stress is the exclusion of Na^+ from the cytoplasm, by the operation of Na^+/H^+ antiporters at the plasma membrane or tonoplast. Plant NHX antiporters play a key role in NaCl tolerance by the extrusion of Na^+ out of cytosol [18, 39].

In tomato, four LeNHX proteins were reported by Gálvez et al. [18]. From these proteins, LeNHX2 was studied in relation to its role in salt tolerance. Indeed, Huertas et al. [40] showed that tomato plants overexpressing *LeNHX2* grew better in the presence of 120 mM NaCl than untransformed controls and Baghour et al. [14] demonstrated that the joint overexpression of *LeNHX2* and *SlSOS2* increased even more the plant salt tolerance. In addition to the increased salt tolerance, an increased fruit production was found in plants overexpressing *LeNHX2*, *SlSOS2* or both [14, 41]. In relation to this, Bassil et al. [42] reported that *AtNHX1* and *AtNHX2* are required for growth and floral development in *Arabidopsis*, while *AtNHX3* and *AtNHX4* play role in seed development [43, 44]. More recently, Sharma et al. [45], showed that in *Triticum aestivum* the *TaNHX4* group genes were highly expressed in later development stages of leaf, spike and grain.

In previous work, we have demonstrated that *LeNHX4* is highly expressed in flowers and fruits of tomato plants, indicating specific roles of this isoform in the reproductive tissues [18]. In this work, data of fruit production of tomato plants cultivated in media lacking NaCl indicate an improvement of fruit yield in transgenic lines overexpressing the *LeNHX4* antiporter (Fig. 2a, b), suggesting a role of *LeNHX4* in fruit production. Fruit of tomato plants overexpressing *LeNHX4* showed a higher water content than those of WT plants (Fig. 2c), which together with the higher accumulation of sugars (Fig. 2d), K^+ and Na^+ (Fig. 3) in fruits of these plants under NaCl cultivation conditions could suggest that plants overexpressing *LeNHX4* withstand better a shortage of water under salinity conditions. In agreement with our results, Li et al. [46] showed that *Arabidopsis* transgenic plants overexpressing *RtNHX1* increased their capacity for osmotic adjustment and improved salinity tolerance due to a high relative water content and accumulation of solutes allowing osmoregulation.

The total soluble solids (TSS) and pH are important quality parameters, which plays a significant role in fruit selection. Reina-Sánchez et al. [47] recorded that an increase in fruit quality represented an increase of total soluble solids and titratable acidity. Salinity positively affect the levels of TSS, including sugars, organic acids, and amino acids in fruits [48–54]. Salinity stress enhances gluconeogenesis, as well as metabolic flow in ripe fruit, resulting in high-Brix fruit [54]. In relation to the role of NHX ion transporters on fruit quality Hanana et al. [55] reported that the NHX antiporters play a critical role in plant growth and development, and improve organoleptic characteristics of fruits as well as those related to fruit ripening [41]. Contrary to what was expected, TSS levels are lower in overexpressing lines than in wild type plants cultivate in the absence of NaCl. However the overexpression of *LeNHX4* gene improved the fruit quality in tomato by increasing the solid solute content and pH under salt stress (Table 1). As opposed to our results, Zhang and Blumwald [56] reported that overexpression in tomato of the *Arabidopsis* antiporter *AtNHX1* did not affect the total soluble solids content in plants grown under 200 mM NaCl.

The increase in the concentration of soluble sugars such as glucose enhances plant tolerance to several abiotic stresses, such as drought, salinity and cold [57] because of their involvement in osmotic adjustment. Our results in

tomato showed an increase of glucose and fructose content in fruits of transgenic plants under normal conditions (Fig. 2d) and suggest that the increased expression of *LeNHX4* could have a positive effect on glucose synthesis under non stressful conditions. Similar results were reported by Leidi et al. [26], who reported that transgenic tomato plants overexpressing *AtNHX1* accumulated higher level of free sugars even if they were not transferred to salt. NaCl treatment significantly increased the concentration of glucose in plants overexpressing *LeNHX4* in relation to WT plants (Fig. 4b). Lieu et al. [58] did not find any significant differences on the sugar contents of WT and transgenic *Beta vulgaris* plants overexpressing *AtNHX3*. However, with the application of high NaCl treatment (300 or 500 mM), a greater sugar accumulation was observed in transgenic plants, suggesting that the expression of *AtNHX3* in sugar beet improved sugar synthesis in transgenic plants and increased the plants salt tolerance by influencing the transcription of genes involved in the sugar synthesis pathways.

A large number of studies show a positive correlation between proline accumulation and plant stress and proline concentration has been shown to be generally higher in salt tolerant than in salt sensitive plants. The accumulation of osmotic compounds such as proline is a common response of plants to high salinity to combat osmotic stress [59, 60]. Proline is accumulated preferentially in leaves in order to maintain chlorophyll level and cell turgor to protect photosynthetic activity under salt stress [21]. Plants overexpressing *NHXs* show an enhanced capacity for osmotic adjustment and improved salinity tolerance due to a better relative water content and accumulation of proline [26–30]. Our data showed that under control conditions, the proline content in leaves was slightly increased in the transgenic lines (Fig. 4c). However, when exposed to saline conditions, the level of this aminoacid dramatically increased in plants overexpressing *LeNHX4*. Recently, Wang et al. [61] reported that the overexpression of *Ipomoea batatas IbNHX2* significantly increased proline content, superoxide dismutase (SOD) activity, and photosynthetic rate in sweet potato. Similarly, transgenic *Arabidopsis* plants overexpressing the Na^+/H^+ antiporter *RtNHX1* from *Reaumuria trigyna* accumulated more proline than WT and *nhx1* mutant plants under 200 mM NaCl treatment [46]. The increase of proline during salt stress has been associated to the up-regulation of proline biosynthesis genes (AtP5CS1 and AtP5CS2) in transgenic plants [46, 61].

There are many evidences that proteins could have a role in osmotic adjustment [62–64]. Plants are also able to respond and adapt to salt stress through the synthesis of specific proteins, and the synthesis of stress-induced proteins is part of that stress tolerance mechanism [65]. Our results show that the NaCl treatment affected negatively the protein content in WT plants (Fig. 4d). However, the overexpression of *LeNHX4* increased the protein content in transgenic lines L-3 and L-5. These results are consistent with those reported by Tester and Davenport [66], who suggested that the disruption in protein synthesis appears to be an important cause of damage caused by Na^+. Comparing the total soluble protein content between the salt-tolerant cultivar (Rio Grande) and the salt-sensitive one (Heinz-2274), Kahlaoui et al. [67] found that the protein levels significantly increased more in the salt-tolerant tomato cultivar when irrigated with saline water. The accumulation of these proteins under saline conditions may provide a storage of nitrogen that is re-utilized when stress is over [68] and may play a role in osmotic adjustment [67]. However, there are also studies reporting no differences in protein content as a result of NaCl tolerance. Ashraf and Fatima [69] found that salt-tolerant and salt-sensitive safflower accessions did not differ significantly in soluble leaf proteins.

In conclusion, the results reported in this study indicate that overexpression of *LeNHX4* in tomato increases size, sugar (glucose and fructose) and ion (Na^+ and K^+) contents of fruits. Moreover, relative to WT plants, plants overexpressing *LeNHX4* show an enhanced fruit yield under both non-stress and salinity stress conditions as well as higher levels of osmoregulatory compounds (proline and proteins) in leaves.

Acknowledgements This work was supported by Grants from Consejería de Economía, Innovación, Ciencia y Empresa, Junta de Andalucía, Spain (CVI-7558 to MPRR), Spanish Ministry of Economy and Competitiveness and Agencia Estatal de Investigación (BIO2015-65056-P, BIO2016-81957-REDT/AEI and Programa I-COOPB+2013 Ref. COOPB20053). National Centre for Scientific and Technical Research and Minister for Higher Education, Scientific Research and Executive Training (Morocco).

Author contributions Study concepts: MB, KV, MPRR. Study design: MB, KV, MPRR. Literature research: MM, MB, MPRR. Experimental studies: MM, FJG, MES, MNA. Data analysis/interpretation: MM, MB, MA, FJG, KV, MPRR. Statistical analysis: MM, MB. Manuscript preparation: MM, MB, MPRR. Manuscript revision: MB, MPRR.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This manuscript does not imply human participants or studies on animals.

Informed consent Not required.

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