Grafting onto pumpkin rootstock is an efficient alternative to improve melon tolerance to NaCl stress

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Summary

The objective of this study was to determine whether salt tolerance of *Cucumis melo* L. 'Baimi' could be improved by grafting onto pumpkin rootstock *Cucurbita maxima* Duchesne × *Cucurbita moschata* Duchesne 'Jingxin 3'. Both self-rooted and grafted plants were cultivated in a nutrient solution with or without 150 mM NaCl. Salt treatment significantly reduced melon plant growth and productivity, leading to a series of physiological and biochemical changes. Melon plants grafted onto pumpkin rootstock showed much less inhibition of growth and photosynthesis by salt stress compared with the self-rooted plants. Stress-induced activities of superoxide dismutase, peroxidase, and catalase were considerably higher (24.61%, 100.69%, and 26.27%) in the rootstock-grafted plants. Leaves of these plants also had higher concentrations of total soluble sugars (28.11%), higher activities of sucrose synthetase (16.77%) and sucrose phosphate synthase (90.70%). The pumpkin rootstock significantly mitigated the negative effects of salt stress by reducing Na uptake, enhancing K uptake, and maintaining a higher K/Na ratio (19.01 for grafted plants, 8.77 for self-rooted plants) in the leaves and a lower K/Na ratio (0.42 for grafted plants, 1.44 for self-rooted plants) in the roots. Quantitative RT-PCR analysis showed that transcript levels of candidate genes, *CmSOS1*, *CmNHX1* and *CmGnT* were significantly higher in the grafted plants than in the self-rooted plants under salt stress. These results demonstrated that the salt tolerance of melon plants could be significantly improved by using pumpkin as a rootstock.

Keywords

Cucumis melo, gene expression, physiological and biochemical responses, salt tolerance

Introduction

Salinity in the soil or water is a major abiotic stress that reduces plant growth and crop productivity worldwide. Approximately 6% of the world's total land area is affected by salt stress (Munns et al., 2012). Moreover, salinity in the surface soils of greenhouses is increased by changes in the soil water balance, which results from both a lack of leaching by rainfall as well as from excess evaporation of soil water and high rates of fertilizer application (Darwish et al., 2005). The main consequences of plant exposure to salt stress are

Significance of this study

What is already known on this subject?

- Salinity is one of the major abiotic stresses that reduce plant growth and productivity in many areas of the world. Vegetable grafting is an important agricultural technique widely used to improve plant tolerance to soil-borne diseases and abiotic stresses.

What are the new findings?

- The salt tolerance of melon plants under high salt stress level (150 mM NaCl) could be significantly improved by using pumpkin as a rootstock on the agronomical and physiological responses. Up-regulated expressions of *CmSOS1*, *CmNHX1*, *CmHKT1* and *CmGnT* in grafted plants indicated that these genes played a significant role in conferring salt tolerance.

What is the expected impact on horticulture?

- The use of pumpkin rootstock is a valid strategy in increasing melon salt tolerance by reducing sodium toxicity. Commercial cultivars grafting onto appropriate rootstocks could be a promising tool for growers to improve plant growth and yield under salt stress.

water deficits due to relatively high solute concentrations in the soil or excessive ions resulting from altered K⁺/Na⁺ ratios and changes in Na⁺ and Cl⁻ ion concentrations (Chen et al., 2003). These adverse effects inhibit growth and can contribute to plant death (Serrano et al., 1999).

Plants employ various strategies to tolerate salinity. Excessive Na⁺ accumulations in the symplast can be prevented via three mechanisms that function cooperatively: 1) restriction of Na⁺ influx, 2) active Na⁺ extrusion at the root–soil interface, and 3) vacuolar sequestration of Na⁺ (Tester and Davenport, 2003). Transport systems that control net Na⁺ uptake into the root xylem vessels and regulate Na⁺ movement to the shoots are also determinants of salt tolerance (Shi et al., 2002; Munns et al., 2012). As a consequence of these primary effects, one common plant response is overproduction of different types of compatible organic solutes, e.g., soluble sugar and proline, which accumulate in many salt-stressed crops (Munns and Tester, 2008). Salt stress reduces the rate of photosynthesis, thereby limiting the supply of CO₂ to the leaf. This leads to an over-reduction of the photosynthetic electron transport chain and results in the production of ROS, including superoxide, hydroxyl radicals, hydrogen per-

oxide, and singlet oxygen, which cause significant damage to cell structures (Ashraf, 2009). Multiple antioxidant enzyme systems, in particular SOD, POD, and CAT, were believed to play an important role in ROS-scavenging, thus protecting cells from oxidative damage (Zhen et al., 2010).

The salt overly sensitive (SOS) signaling pathway has been proposed to mediate cellular signaling under salt stress, maintaining ion homeostasis and conferring salt tolerance. The Na⁺ transporter SOS1 is a Na⁺-effluxing plasma membrane-located Na⁺/H⁺ antiporter that is involved in controlling long-distance Na⁺ transport in plants from roots to shoots. Expression of SOS1 in *Arabidopsis thaliana* cells indicates that this transporter has a role in controlling Na⁺-loading of the vascular system (Shi et al., 2002). Sequestration of excess Na⁺ inside root or stem cell vacuoles is another strategy used by many plants to survive under salt stress (Yamaguchi et al., 2013). Plant Na⁺/H⁺ antiporter exchanger (NHX) genes, localized in the vacuolar membrane, are involved in this compartmentalization of sodium ions into the vacuole (Apse and Blumwald, 2007). This protects essential enzymatic reactions in the cytoplasm from excess Na⁺ levels while contributing to salt tolerance and ion homeostasis within the cells (Hasegawa et al., 2000). Some members of the high-affinity potassium transporter (HKT) protein family are thought to play major roles in Na⁺ uptake and recirculation within salt-stressed plants (Apse and Blumwald, 2007). The HKTs are divided into two subgroups based on their transport selectivity. Those in Group 1 are Na⁺ uniporters while proteins within Group 2 enable Na⁺ and K⁺ transport as well as Na⁺ uniport at high Na⁺ concentrations. The HKT genes from dicot species occur within the major subfamily HKT1 in Group 1 (Platten et al., 2006). N-acetylglucosaminyltransferase III (GnT-III) participates in the branching of N-glycans, catalyzing the formation of a unique sugar chain structure-bisecting GlcNAc. This enzyme is a key glycosyltransferase in the N-glycan biosynthetic pathway. N-acetylglucosaminyltransferase played a positive role in cellular survival in response to stress condition (Zachara et al., 2004; Zachara and Hart, 2006).

Melon (*Cucumis melo* L.), one of the most commercially important vegetable crops, is sensitive to soil salinity. Plant grafting is widely used to manage the probability of soil-borne diseases (Crinò et al., 2007). This technique is also now applied for improving crop responses to abiotic stresses such as salinity, heavy metals, nutrient deficiencies, and alkalinity (Colla et al., 2006a, b; Rouphael et al., 2008; Colla et al., 2010; King et al., 2010; Savvas et al., 2010; Zhang et al., 2016). In the case of salt stress, grafting has been tested with *Cucumis sativus* L. (cucumber; Huang et al., 2013), *Solanum lycopersicum* L. (tomato; Di Gioia et al., 2013), *Citrullus lanatus* L. (watermelon; Goreta et al., 2008) and *Cucumis melo* L. (melon; Colla et al., 2006b). For example, when exposed to greater salinity, reductions in shoot weight and leaf area were lower for grafted watermelon than for ungrafted plants (Goreta et al., 2008). Grafting of tomato or citrus crops also decreased concentrations of Na⁺ and Cl⁻ in the xylem and diminished accumulations of those ions in the leaves (Fernández-García et al., 2002). Our research objective was to determine whether grafting onto a pumpkin rootstock could provide an effective way to increase salt tolerance of melon and also explore possible links between the expression levels of candidate genes (*CmSOS1*, *CmNHX1*, *CmHKT1*, and *CmGnT*) and salt tolerance in grafted melon.

Materials and methods

Plant materials and growth conditions

The experiment was conducted during the spring season of 2014 in the greenhouse at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing. As the scion, the commercial *Cucumis melo* L. 'Baimi' (oriental melon, obtained from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China) was grafted onto the commercial 'Jingxin3' pumpkin rootstock (*Cucurbita maxima* Duchesne × *Cucurbita moschata* Duchesne, obtained from Beijing Vegetable Research Center, Beijing, China). Plants were grown under natural light conditions. The greenhouse was maintained at daily temperatures between 17 and 33°C, and day/night relative humidities of 55/85%. Rootstock of pumpkin seeds were sown (on 15 April, 2014) 3 days before those of the scion ('Baimi', on 18 April) in 50 seedling plug trays filled with a 1:1:1 (v/v/v) mixture of peat: vermiculite: perlite. Seven days later (from 18 April), grafting was performed using the 'insertion grafting' procedure described by Lee (1994). Ungrafted 'Baimi' was used as the control. High relative humidity (RH) was maintained by covering the newly grafted plants with a layer of transparent plastic film and shading them for the first 72 h. Afterward, the plastic film was opened slightly each day and removed entirely on Day 7. Then the seedlings (with uniform growth) were transferred to 20-L plastic containers containing full strength Hoagland's solution. All nutrient solutions were prepared using deionized water. The nutrient solutions were refreshed at 5-d intervals and continuously aerated by an air pump (Huang et al., 2010; Lei et al., 2014). The pH of the nutrient solution was maintained close to 6.5 by adding H₂SO₄ or KOH. At the three-true-leaf stage, grafted and control plants were subjected to the following four treatments: SC, self-rooted 'Baimi', non-stressed (0 mM NaCl, EC 1.90 dSm⁻¹); SN, self-rooted 'Baimi' exposed to 150 mM NaCl (EC 15.16 dSm⁻¹); RC, rootstock-grafted 'Baimi', non-stressed; RN, rootstock-grafted 'Baimi' exposed to 150 mM NaCl. The salt-stress experiments ran for two weeks (Huang et al., 2010), because evident growth differences were observed among the treatments. Each treatment was replicated three times, with 10 plants per replicate, in a completely randomized design.

Plant growth parameters

Plant height, stem diameter and leaf areas of 3 plants per treatment were measured after two weeks of salt treatment, then the plants were divided into leaves, stems, and roots to determine separate fresh weight (FW) values before the tissues were heated to 105°C for 30 min. After oven-drying at 75°C for 48 h, the tissues were re-weighed to obtain dry weight (DW) values.

Photosynthetic measurements

Five plants per replicate were selected randomly after two weeks of treatment and the third leaf from the top of each plant was used for determining photosynthesis. The net photosynthesis rate (P_n), stomatal conductance (g_s), concentration of intercellular CO₂ (C_i), and transpiration rate (E) for fully expanded leaves from fruit-bearing nodes in each treatment were measured with a portable photosynthesis system (Li-Cor 6400; Li-Cor Inc., Lincoln, NE, USA), between 9:00 am and 11:00 am. Conditions during the measurement period included constant irradiation (800 μmol m⁻² s⁻¹), a CO₂ concentration in the leaf chamber of 365 μmol mol⁻¹, air temperature of 25°C, and RH of 70%.

Evaluation of leaf pigments and antioxidant system

The fourth leaf from the top of 6 plants per treatment were collected for pigment and antioxidant activities analysis after two weeks of treatment. Each 0.2 g (for leaf pigment analysis) and 0.5 g (for antioxidant analysis) fresh samples were collected, samples were immediately frozen in liquid nitrogen and stored at -80°C for analysis. Leaf pigments in mature leaves were analyzed by extracting chlorophyll (Chl) in 80% chilled acetone (v/v) and then quantifying with a spectrophotometer (UVICON-930; Kontron Instruments, Zürich, Switzerland). In particular, Chl *a* was determined at wavelength 663 nm, Chl *b* at 646 nm, and carotenoids at 470 nm.

The crude enzymatic extracts from each tissue type were prepared in 0.05 mol L^{-1} phosphate buffer (pH 7.8) by grinding the samples with a pestle and mortar under liquid nitrogen. The homogenates were filtered through one layer of nylon and centrifuged at $12,000\times g$ for 10 min. The supernatants were then used for assaying SOD, POD, and CAT activities. All operations were performed at 4°C . Briefly, SOD activity was measured by the nitro blue tetrazolium method (Van Rossum et al., 1997). Activity by POD was determined as the change in absorbance of 470 nm due to guaiacol oxidation, based on the method described by Polle et al. (1994). For CAT, the decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm (Cakmak and Marschner, 1992).

Carbohydrate extraction and analysis

After the photosynthetic data were collected, the first fully expanded leaves from the top of 6 plants per treatment were sampled after two weeks of treatment. Leaf samples (each 1 g FW) were immediately frozen in liquid nitrogen and stored at -80°C for analysis. Leaf samples were finely ground and then extracted three times in 5 mL of 80% (v/v) ethanol for 30 min at 80°C . The extracts were combined and dried in a centrifugal evaporator. The residues were re-dissolved in 1 mL of distilled water and passed through a $0.45\text{ }\mu\text{m}$ filter. Afterward, 10 μL of each sample was injected into a high performance liquid chromatograph (HPLC) that consisted of a Sugar-Park column ($6.5\text{ mm} \times 300\text{ mm}$; Waters) that operated isocratically at 75°C with water as an eluant (0.5 mL min^{-1}), plus a refractive index detector. These eluted sugars were identified and quantified based on their retention times and peak heights for sugar standards, as described by Miron and Schaffer (1991).

Extraction and assays of enzymes for sugar metabolism

After the photosynthetic data were collected, the first fully expanded leaves of 6 plants per treatment were sampled after two weeks of treatment. Fresh samples (1 g) of

each replicate were stored in liquid nitrogen immediately for analysis. The leaf samples were finely ground in liquid nitrogen using a chilled mortar and pestle. Approximately 1 g of the powder was suspended in 1 mL of ice-cold extraction buffer containing 50 mM HEPES-NaOH buffer (pH 7.5) plus 10 mM MgCl_2 , 1 mM EDTA, 2.5 mM DDT, 0.1% (w/v) BSA, and 0.05% (v/v) TritonX-100. The homogenate was centrifuged at $13,000\times g$ for 20 min. The resulting supernatant was dialyzed with 20 mM HEPES-NaOH buffer (pH 7.0) containing 20 mM 2-mercaptoethanol for 16 h. Sucrose phosphate synthase (SPS, E.C.2.4.1.14), sucrose synthase (SS, E.C.2.4.1.13), and acid and neutral invertases (AI and NI, E.C.3.2.1.26) were assayed according to the procedures of Hu et al. (2009).

Determination of ion concentrations

After two weeks of treatment, 6 plants per treatment were harvested and divided into leaf (first fully expanded) and root portions. After measuring their dry weights, powders (0.5 g) of the same type were digested in a mixture of $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2$ (volume ratio 5:1) to measure the following ion concentrations (Na, P, K, Ca, Mg and Fe) with atomic absorption spectrometry (Shimadzu AA-6300; Shimadzu, Tokyo, Japan).

Total RNA extraction and real-time quantitative RT-PCR analysis

Young leaf samples (about 0.2 g) were collected after two weeks of treatment, frozen them immediately in liquid nitrogen and stored at -80°C until use for RNA extraction. Total RNA was extracted from the leaves as described in the TRI reagent protocol (Takara Bio Inc., Japan). For all samples, the total RNA (1 μg) was converted to cDNA using PrimeScript™ 1st Strand cDNA Synthesis Kits (Takara) according to the manufacturer's instructions. Primers were designed from the peptide sequences obtained after mass analysis, according to the NCBI and Melon databases (melonomics.net). Gene-specific primers used for real-time quantitative RT-PCR are shown in Table 1. All reactions were performed with SYBR PrimeScript™ RT-PCR Kits (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Quantitative RT-PCR was conducted with a LightCycler® 96 Instrument (Roche, Mannheim, Germany).

Statistical analysis

Data presented are the mean values of three replicates. Statistical assays were conducted via ANOVA, using the SPSS software package (SPSS 10 for Windows, 2001). Duncan's multiple range tests were then performed. For each measured variable, values were considered significantly different among treatments at $P < 0.05$.

TABLE 1. Sequences of the primers used to amplify the target genes using real-time quantitative polymerase chain reactions (RT-qPCR).

Gene	Forward primer	Reverse primer
<i>CmNHX1</i>	TCTGCCTGGTGTGAAGATAAT	GCCAAATAGTAATGGACAGAACG
<i>CmSOS1</i>	TGCCCTTCAATCAGATGACAAT	TTTCTGCTTCTGTGGTGGTCTA
<i>CmGnT</i>	CCTGGGAATTGCATACGAGA	AAGACCAACACGGTTGACTG
<i>CmHKT1</i>	TCCTCTGCCATCTTGATTCTG	TTCCCTTTCTTCTTCTTCTCTCT
<i>Actin</i>	TCGTTCTTCTTCTTCTTCTTCT	AGCCTTCACCATTCAGTTC

CmNHX1 = Vacuolar membrane-located Na^+/H^+ antiporter gene of melon (gene ID: 103492731).

CmSOS1 = Plasma membrane-located Na^+/H^+ antiporter gene of melon (gene ID: 103504147).

CmGnT = N-Acetylglucosaminyltransferase III gene of melon (gene ID: 103488964).

CmHKT1 = High-affinity potassium transporter gene of melon (gene ID: 103494214).

TABLE 2. Effects of NaCl treatment (150 mM) on plant growth, photosynthesis parameters and antioxidant activities of self-rooted and rootstock-grafted melon plants.

Parameters/Treatments	SC	SN	RC	RN
Plant height (cm)	29.50±1.06b	16.00±1.20d	33.40±2.29a	21.33±1.05c
Stem diameter (mm)	3.04±0.52b	2.80±0.16b	5.77±0.72a	5.43±0.30a
Leaf area (cm ²)	49.75±3.30b	26.61±0.93d	51.52±3.77a	35.26±2.78c
Shoot dry mass (g)	2.86±0.12a	0.47±0.01c	3.05±0.15a	1.56±0.02b
Root dry mass (g)	0.26±0.04a	0.10±0.02c	0.25±0.09a	0.16±0.01b
Pn (μmol m ⁻² s ⁻¹)	8.67±0.11b	3.88±0.05d	9.66±0.01a	6.60±0.27c
g _s (mol H ₂ O m ⁻² s ⁻¹)	0.69±0.01a	0.11±0.01c	0.61±0.01a	0.26±0.00b
C _i (μmol mol ⁻¹)	181.17±0.35a	141.49±1.62c	189.24±0.19a	152.73±0.69b
E (mmol H ₂ O m ⁻² s ⁻¹)	6.01±0.02a	2.00±0.00c	6.26±0.01a	3.89±0.02b
Chl a+b (mg g ⁻¹)	2.16±0.01a	1.70±0.01b	1.78±0.01b	1.72±0.02b
SOD (U g ⁻¹ FW)	88.54±6.89a	68.96±5.68c	73.28±4.52b	85.93±5.87a
POD (U g ⁻¹ FW min ⁻¹)	27.22±1.25b	18.80±2.58c	28.40±2.98b	37.73±4.20a
CAT (U g ⁻¹ FW min ⁻¹)	57.65±3.58b	53.94±4.23b	57.12±3.21b	68.11±4.15a

Plant growth and the biomass data referred to the same plants. Means followed by different letters within a column indicate significant differences at $P < 0.05$ by Duncan's multiple range test. Data are mean $\pm$ SE ($n=3$).

SC = self-rooted 'Baimi' exposed to 0 mM NaCl; SN = self-rooted 'Baimi' exposed to 150 mM NaCl; RC = rootstock-grafted 'Baimi' exposed to 0 mM NaCl; RN = rootstock-grafted 'Baimi' exposed to 150 mM NaCl; Pn = Net photosynthetic rate; g_s = stomatal conductance; C_i = intercellular CO₂ concentration; E = transpiration rate; SOD = superoxide dismutase; POD = peroxidase; CAT = catalase.

Results

Plant growth, photosynthesis, and activities of antioxidant enzymes

In both rootstock-grafted and self-rooted plants, values for all growth parameters decreased in response to NaCl treatment (Table 2). However, grafted 'Baimi' (RN) plants were significantly taller and had larger stem diameters and leaf areas when compared with the self-rooted plants (SN). Salt stress was also associated with lower shoot and root biomasses, although samples from grafted plants were less affected.

Reductions in the net photosynthesis rate (Pn), stomatal conductance (g_s), concentration of intercellular CO₂ (C_i), and transpiration rate (E) in response to NaCl treatment were significantly smaller for grafted plants (31.68% for Pn, 57.38% for g_s, 19.29% for C_i, and 37.86% for E) when compared with the stressed control plants (respectively decreases of 55.25%, 84.06%, 21.90%, and 66.72%). No significant differences were found in chlorophyll concentrations between grafted and self-rooted plants under salinity conditions.

Stress-induced activities of SOD, POD, and CAT in grafted leaves increased by 24.61%, 100.69%, and 26.27% respectively when compared with the self-rooted plants (SN).

Soluble sugars and enzymes

In response to salt treatment, glucose, fructose, and stachyose concentrations decreased in both grafted and self-rooted plants while levels of sucrose and raffinose increased (Table 3). Leaves from grafted melon plants accumulated more soluble sugars, such as sucrose and glucose under salt stress. Concentrations of total soluble sugars were 28.11% higher in stressed grafted plants than in the stressed self-rooted plants (Table 3).

The induction of salt stress was associated with significant increase in SS and SPS activities, and decrease in AI and NI activities (Table 4). Compared with SN treatment, the activities of SS, SPS, AI, and NI of RN treatment increased by 16.77%, 90.70%, 47.27% and 57.48%, respectively (Table 4).

Concentrations of mineral elements in leaves and roots under NaCl stress

Salinity treatments generally increased the levels of Na in tissue samples, with rootstock-grafted plants showing a lower concentration (decreased by 46.47%) in the leaves but a higher concentration (increased by 115.15%) in the roots when compared with the self-rooted control. Although exposure to NaCl also elevated K concentrations in the leaves of rootstock-grafted plants (increased by 16.03%), roots from those plants had lower K concentrations (decreased by

TABLE 3. Effects of NaCl treatment (150 mM) on soluble sugar concentrations of self-rooted and rootstock-grafted melon leaves.

Treatments/Sugars	SC	SN	RC	RN
Glucose (mg g ⁻¹ FW)	1.98±0.08b	0.48±0.08d	2.49±0.08a	1.21±0.02c
Fructose (mg g ⁻¹ FW)	0.73±0.03a	0.25±0.05c	0.71±0.04a	0.35±0.04b
Sucrose (mg g ⁻¹ FW)	1.19±0.02c	4.94±0.45b	1.28±0.08c	6.15±0.15a
Raffinose (mg g ⁻¹ FW)	0.32±0.03b	0.49±0.02a	0.33±0.02b	0.53±0.03a
Stachyose (mg g ⁻¹ FW)	1.40±0.02a	0.92±0.02b	1.28±0.07a	0.83±0.03b
Total soluble sugars	5.62±0.04c	7.08±0.12b	6.09±0.06c	9.07±0.05a

Means followed by different letters within a column indicate significant differences at $P < 0.05$ by Duncan's multiple range test. Data are mean $\pm$ SE ($n=3$).

TABLE 4. Effects of NaCl treatment (150 mM) on the activities of carbohydrate enzymes of self-rooted and rootstock-grafted melon leaves.

Treatments/Enzymes	SC	SN	RC	RN
SS (mg g ⁻¹ FW h ⁻¹)	1.50±0.42c	6.38±1.05b	1.45±0.23c	7.45±0.98a
SPS (mg g ⁻¹ FW h ⁻¹)	1.46±0.28c	2.15±0.25b	2.38±0.54b	4.10±0.51a
AI (mg g ⁻¹ FW h ⁻¹)	32.17±3.27c	24.50±4.80d	39.17±3.06a	36.08±1.32b
NI (mg g ⁻¹ FW h ⁻¹)	11.61±0.57b	7.22±0.38c	15.36±1.69a	11.37±2.78b

Means followed by different letters within a column indicate significant differences at $P < 0.05$ by Duncan's multiple range test. Data are mean $\pm$ SE ($n=3$). SS = sucrose synthetase; SPS = sucrose phosphate synthase; AI = acid invertase; NI = neutral invertase.

TABLE 5. Effects of NaCl treatment (150 mM) on the concentrations of mineral elements (Na, K, P, Ca, Mg, Fe) of self-rooted and rootstock-grafted melon leaves.

Mineral	Leaf (mg g ⁻¹ DW)				Root (mg g ⁻¹ DW)			
	SC	SN	RC	RN	SC	SN	RC	RN
Na	1.42±0.02b	3.40±0.23a	0.99±0.06c	1.82±0.17b	5.48±0.94d	16.37±0.94b	10.65±1.31c	35.22±1.67a
K	25.21±2.97c	29.81±0.10b	28.51±0.39b	34.59±3.82a	28.80±3.89a	23.51±1.70b	19.22±2.38c	14.68±2.25d
P	3.10±0.53bc	3.67±0.86b	2.86±0.38c	4.97±0.13a	3.82±0.21c	5.34±0.41b	4.14±0.24c	6.54±0.57a
Ca	30.85±3.10a	23.30±3.08c	31.36±2.66a	26.42±2.23b	12.89±2.84b	10.80±2.31c	14.32±2.18a	12.23±1.67b
Mg	7.56±1.00b	6.83±0.83b	8.86±2.02a	7.56±0.30b	5.05±1.07b	3.76±0.43c	6.26±0.84a	4.92±0.73b
Fe	1.41±0.27a	0.77±0.10b	1.16±0.50a	0.93±0.11b	6.96±1.60b	3.39±0.72d	8.53±3.19a	5.00±0.80c

Means followed by different letters within a line (respectively in leaf and root) indicate significant differences at $P < 0.05$ by Duncan's multiple range test. Data are mean $\pm$ SE ($n=3$).

37.56%) than roots from the self-rooted control (Table 5). The pumpkin rootstock had a significant influence on P concentrations in stressed plants, with values being higher in their leaves and roots than in tissues from self-rooted plants. Similarly, Ca levels were higher in both the leaves and roots of rootstock-grafted plants than in corresponding samples from the self-rooted plants. Finally, concentrations of Mg and Fe in roots were significantly affected by the pumpkin rootstock, being 30.85% higher for Mg and 47.49% higher for Fe when compared with the self-rooted control, whereas no significant differences among treatments were observed on Mg and Fe concentrations in leaves.

Expression of candidate genes for salt tolerance

Quantitative RT-PCR analysis showed that transcripts of candidates *CmSOS1*, *CmNHX1*, *CmHKT1* and *CmGnTIII* were more abundant in stressed rootstock-grafted tissues than in their unstressed counterparts (Figure 1). Under salinity conditions, expressions of *CmSOS1*, *CmNHX1*, and *CmGnT* in leaves from grafted plants were 2.1-fold, 2.2-fold and 2.1-fold higher, respectively, than in leaves from the self-rooted plants.

Discussion

Growth can be inhibited by salinity because stressed plants often show a decrease in their photosynthetic capacity. High salt concentrations strongly limit CO₂ fixation, as reflected in net photosynthesis rates, stomatal conductance, and intercellular CO₂ concentrations (Rouphael et al., 2012). At a treatment level of 150 mM NaCl, rootstock-grafted plants showed higher values for Pn, g_s, Ci and E, which clearly demonstrated that this practice moderated the effects of salt stress for this crop. The pumpkin rootstock can directly influence growth and photosynthesis through interactions among the following processes: increases in the uptake of water and nutrients due to a more vigorous root system (Lee, 1994), enhanced production of endogenous hormones (Zijlstra et al., 1994), or improvements in scion vigor (Colla et al., 2006a, b). Similar results have been reported on *Citrus* spp. and *Prunus* spp. (Massai et al., 2004), and *Cucumis sativus* and *Cucumis melo* (Rouphael et al., 2012) when elite cultivars were grafted onto salt-tolerant rootstocks.

The development of salt tolerance in plants requires a net increase in the quantity of osmotically active solutes in tissues (Huang et al., 2010). Soluble sugars serve as osmotic

FIGURE 1. Analysis of relative expression for putative *CmSOS1*, *CmNHX1*, *CmHKT1* and *CmGnT* by qRT-PCR in leaves from self-rooted and rootstock-grafted plants. Tissues were sampled after plants were exposed to 150 mM NaCl for two weeks. For each gene, expression was normalized to the level measured in plants of SC, for which it was assigned a value of '1'. The data for each variable were analyzed by ANOVA first and then followed by Duncan's multiple range test at $P < 0.05$. Data are mean $\pm$ SE ($n=3$). SC = self-rooted 'Baimi' exposed to 0 mM NaCl; SN = self-rooted 'Baimi' exposed to 150 mM NaCl; RC = rootstock-grafted 'Baimi' exposed to 0 mM NaCl; RN = rootstock-grafted 'Baimi' exposed to 150 mM NaCl.

regulators. Thus, a salinity-related rise in sugar concentrations in leaves from our grafted melon probably reflected an osmotic adjustment that was achieved through the enhanced synthesis of sugars. The pumpkin rootstock also significantly mitigated the negative effects of salt stress by reducing Na uptake, enhancing K uptake, and maintaining a higher K/Na ratio in the leaves and a lower K/Na ratio in the roots. The maintenance of K selectivity could be a strategy used by grafted plants for increasing their salt tolerance (Cayuela et al., 2001; Chen et al., 2003; Kaya et al., 2003; Estañ et al., 2005; Martinez-Rodriguez et al., 2008).

To explore the differential response to salt stress between self-rooted and grafted plants, we measured the expression of key genes *SOS1*, *NHX1*, *HKT1*, and *GnT*. Exposure to 150 mM NaCl induced the up-regulation of putative *SOS1* and *NHX1* expression in our grafted plants. *SOS1* had a critical role in controlling Na⁺ compartmentalization by mediating vacuolar integrity and pH homeostasis under salt stress (Zhu, 2002). The *NHX1* protein was localized in the vacuolar membrane and protected cells against the effects of salinity by sequestering toxic concentrations of sodium ion into the vacuole (Serrano et al., 1999). Nawaz et al. (2014) compared six *Brassicaceae* glycophytes and halophytes for salt tolerance and the expression levels of the candidate salt tolerance genes, high-level salt tolerance was associated with a strongly enhanced expression of the Na⁺ transporters, *NHX1* and *SOS1*. Similar salt tolerance has been seen in cucumber (Lei et al., 2014), tomato (Zhang and Blumwald, 2001), and rice (Fukuda et al., 2004).

HKT1 modulated Na⁺ influx into cells and was a key factor in determining salinity tolerance because it was involved in regulating the distribution of Na⁺ between roots and shoots by helping to retrieve Na⁺ from the transpiration stream (Sunarpi et al., 2005). Seven functional *OsHKT* genes were found to exist in a japonica rice (*Oryza sativa*) cultivar and similar composition of the *HKT* gene family was observed in wheat and barley (Hamamoto et al., 2015). Initial studies in the model plant *Arabidopsis thaliana* identified the *AtHKT1;1* transporter as a largely Na⁺ selective transporter and found that the *AtHKT1;1* gene provided a key mechanism for protecting leaves from Na⁺ over accumulation under salt stress (Rus et al., 2004; Sunarpi et al., 2005). Assaha et al. (2015) reported that salinity-induced expression of *HKT* may be crucial for Na⁺ exclusion in the leaf of huckleberry (*Solanum scabrum* Mill.), on the contrary, *SmHKT* was repressed in the leaf of eggplant (*Solanum melongena* L.) under salt stress. In our study, the expression of *CmHKT1* gene was a little higher in grafted melon leaves under salt stress; however, no significant differences were observed between self-rooted and grafted melon under salt stress.

Glycosyltransferases have critical roles in maintaining cell homeostasis, and in regulating plant growth and development. For example, the *Arabidopsis* glycosyltransferase gene *UGT85A5* was significantly induced by salt stress, indicating that it was involved in conferring salt tolerance. When this gene was over-expressed in *Nicotiana tabacum*, seeds from transgenic plants had higher germination rates, and salt-stressed seedlings exhibited better growth, decreased losses of chlorophyll, and more proline and soluble sugars (Wang et al., 2012; Sun et al., 2013). So, improved expressions by *CmSOS1*, *CmNHX1*, and *CmGnT* were necessary to the higher degree of salt tolerance by grafted melon.

Conclusions

Salinity significantly reduced plant growth and crop productivity, leading to a series of physiological and biochemical changes. Melon plants grafted onto pumpkin rootstock maintained higher biomass production exposed to excess NaCl, greater capacity for photosynthesis, stronger ability to remove oxide products, and better nutritional status. Higher Na concentrations in the roots of grafted plants meant that less of that ion was allocated to the shoots. Up-regulated expressions of *CmNHX1*, *CmSOS1*, and *CmGnT* in grafted plants indicated that these genes played a significant role in conferring salt tolerance. The salt tolerance of melon plants could be significantly improved by using pumpkin as a rootstock.

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