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Analysis of salinity tolerance of *Vitis vinifera* 'Thompson Seedless' transformed with *AtNHX1*

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Summary

Several transgenic plant species expressing *AtNHX1*, coding for a vacuolar Na^+/H^+ antiporter from *Arabidopsis thaliana*, have shown their ability to cope with salinity. The aim of this study was to analyze the response of *Vitis vinifera* cv. 'Thompson Seedless' transformed with *AtNHX1* to salt stress, using soil substrate or hydroponic media, and to compare the response with untransformed 'Thompson Seedless' and allegedly tolerant 'Criolla' cultivars: 'Pedro Giménez' and 'Criolla Chica'. 'Thompson Seedless' embryogenic calli were transformed with *Agrobacterium tumefaciens* carrying *AtNHX1* under the control of CaMV 35S promoter. Transgenic and untransformed plants were grown in a greenhouse under hydroponics or in pots with soil, and were subjected to increasing concentrations of sodium chloride (NaCl) up to 150 mM for a period of 7 weeks. Growth and toxicity symptoms were less affected in transgenics as compared to the untransformed grapevines, and transgenic lines had higher shoot length, leaf area and dry weights at the end of the experiment. Root concentrations of Na in transgenics were similar or lower than that observed in untransformed genotypes. Growth impairment and toxicity symptoms were observed in all genotypes under both conditions, but effects were more severe in plants growing with hydroponic culture. Potassium content and shoot to root dry weight ratio decreased with NaCl in hydroponics but not in pots. 'Criolla' cultivars grew less than the other genotypes, although 'Pedro Giménez' always exhibited highest shoot/root ratios.

K e y w o r d s : sodium chloride, potassium, grape, transgenic, hydroponics.

Introduction

Salinization is the accumulation of soluble salts in the soil to the extent that its fertility is severely reduced. Salinization of irrigated soils in arid areas is particularly critical, especially where periodic leaching is not or cannot be practiced. As a consequence, salinization of cultivated land increases every year and losses up to 50 % of arable land by 2050 have been forecast (Wang *et al.* 2003). In Argentina, salinity issues are present in the arid regions of the West (Vallone *et al.* 2007), including Cuyo, the country's largest wine producing region, with approx. 200,000 ha of vineyards (http://www.inv.gov.ar).

Types of physiological damage caused by salinity in plants are osmotic, nutritional and toxic (Greenway and Munns 1980). The effect of salt stress on grapevines is well documented and includes reduced photosynthetic rate, decreased vigour, lower yields, necrosis of leaf margin, leaf death and eventually death of the vine (Downton *et al.* 1990, Prior *et al.* 1992a, 1992b, Stevens and Harvey 1995, Fisarakis *et al.* 2001, Walker *et al.* 1997, 2002, Shani and Ben-Gal 2005). Approximately 25 % of yield decrease occurs at 2.7 $dS \cdot m^{-1}$ and 50 % at 4.5 $dS \cdot m^{-1}$ (Mass and Hoffmann 1976, Rhoades 1992, Grattan 2002). The two main strategies to minimize the deleterious effects of salinity in agriculture are agronomic practices and genetic improvement. The first consists of leaching irrigated soils with good water quality. These practices are used with success in some areas, but have the disadvantage of being expensive and insufficient at higher salinity levels or inappropriate soil types. The second strategy is the breeding of salt tolerant plants. The use of salt tolerant rootstocks has helped to improve crop growth on saline soils. The beneficial effect of rootstocks can be attributed to their ability to exclude ions and the increased vigour they confer to the scion. Zhang *et al.* (2002) found that '1103 Paulsen' and 'Ramsey', which are moderate chloride (Cl) excluders and confer strong vigor to the scion, were among the most salt tolerant when studying *Vitis vinifera* 'Thompson Seedless' grafted on seven rootstocks. Cl anions play an important role in grapevine toxicity because they accumulate at higher levels than sodium (Na) (Ehlig 1960); for this reason, current rootstock breeding efforts have focused on chloride exclusion (Tregeagle *et al*. 2006, Gong *et al.* 2011, Fort *et al.* 2013, 2015). 'Criolla' cultivars, which are autochthonous *V. vinifera* cultivars of Argentina (Martínez *et al.* 2003), are also regarded as more tolerant to drought and salinity. Kaiser (2003) found that these cultivars exhibited higher shoot dry weight and shoot/

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root ratio in response to water stress, while Cavagnaro *et al.* (2006) found that *in vitro* cultured 'Criolla' cultivars 'Pedro Giménez', 'Criolla Chica', 'Cereza', and 'Torrontés Riojano' grew better than European cultivars 'Malbec' and 'Chardonnay' in the presence of sodium chloride (NaCl). Currently, nurseries in Argentina are recommending the use of 'Criollas' as rootstock because they are considered tolerant to nematodes and salinity (DiFilippo 2008).

The production of transgenic plants can be a useful alternative to traditional breeding methods and has been considered as a means of achieving salt tolerance. Na^+/H^+ antiporters exchange protons for Na^+ ions when moving Na^+ across membranes and are particularly active in the vacuoles of plants, algae and fungi. Genetic transformation with vacuolar antiporter Na^+/H^+ *AtNHX1* induced salt tolerance in transgenic arabidopsis, tomato and brassica (Apse *et al.* 1999, Zhang and Blumwald 2001, Zhang *et al.* 2001). Similiar results have been obtained in many other species (Yang *et al.* 2005, Tian *et al.* 2006, He *et al.* 2007, Li *et al.* 2010, Banjara *et al.* 2012). It has been proposed that in salt-tolerant plants, the compartmentation of Na into vacuoles, through the operation of a vacuolar Na^+/H^+ antiport, provides an efficient mechanism to avert the deleterious effects of Na in the cytosol while maintaing osmotic balance by using Na (and Cl) accumulated in the vacuole to drive water into the cells. However, *AtNHX1* functions cannot be solely explained by Na accumulation in the vacuole since NHX proteins also have a role in potasium (K) exchange, pH regulation and ion homeostasis (Leidi *et al.* 2010, Reguera *et al.* 2014). In order to study this response in grapevines, transgenic plants of 'Thompson Seedless' were produced *via Agrobacterium tumefaciens* mediated transformation with a gene encoding *AtNHX1* under the control of the constitutive CaMV 35S promoter. Preliminary results showed that some of the lines exhibited greater tolerance to salinity and lower leaf Na content than non-transformed plants (Agüero *et al.* 2005). The objective of this study was to conduct an in-depth analysis of NaCl stress in four *AtNHX1* transgenic lines growing in: 1) an experimental hydroponic system to impose salinity stress isolated from the effects of soil, and 2) pots with soil substrate, as an intermediate step before testing the plants in the field. Additionally, this work aimed to compare the response of transgenic genotypes to salinity with Argentinian 'Criolla' cultivars 'Pedro Giménez' and 'Criolla Chica'.

Material and Methods

Plant material: 'Pedro Giménez' (PG) and 'Criolla Chica' (CC), two 'Criolla' cultivars, and 4 independent transgenic lines of *V. vinifera* cv. 'Thompson Seedless' (A425, B540, C646 and D694), PCR-positive for the presence of *AtNHX1*, were multiplied *in vitro* along with untransformed 'Thompson Seedless' plants (UN), and acclimated to greenhouse conditions at EEA Mendoza INTA, Luján de Cuyo, Mendoza, Argentina. Transgenic 'Thompson Seedless' were produced by transformation of embryogenic calli as previoulsy described (Agüero *et al.* 2006) with *A. tumefaciens* strain LBA4404 containing binary plasmid pBX1 carrying *AtNHX1* under the control of constitutive promoter CaMV 35S and nos terminator (suppl. Fig. 1). Light intensity at mid-day was approximately 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, temperature and relative humidity values varied between 15-30 °C and 40-70 % respectively.

Hydroponic culture: A425, B540, C646 and D694 transgenic lines, UN and PG were transplanted to 20L styrofoam containers when their main shoot had 4-5 fully expanded leaves. Containers were 52 cm long, 32 cm wide, and 20 cm high and contained Long Ashton solution (Hewitt and Smith 1975) pH 6-6.5, aerated every 15 min. with an aquarium air pump. Nutrient solution was renewed every 10 d. One plant of each genotype was installed in 10 containers. In 5 containers, the NaCl concentration was increased to 150 mM at a rate of 25 mM every 10 d, starting 15 d after transplant to hydroponic conditions. This experiment consisted of a 2 x 6 factorial with a split-plot design with randomized complete block main plots, where NaCl was the whole plot factor and genotype was the split-plot factor.

Culture in pots: A425, B540, C646 and D694 transgenic lines, UN, PG and CC were transplanted to 3 L pots when their main shoot had 4-5 fully expanded leaves. Soil substrate composition consisted of a mix of 7/3 parts of pomace and sand. Plants were watered with 1.5 $g \cdot L^{-1}$ KSC II Phytactyl (Timac Agro – USA) nutrient solution (suppl. Tab. 1), until water drained freely from the drainage holes at the bottom of each pot, on alternate days. The concentration of NaCl in the nutrient solution used to water half of the pots, was increased to 150 mM at a rate of 25 mM every 10 d, starting 15 d after transplant. This experiment consisted on a 2 x 7 factorial organized in a randomized complete block design.

Measurements: Shoot length, total number of leaves, number of leaves with symptoms and transpiration were measured every 10 d. Mid-day transpiration was measured with a Decagon SC-1 porometer on the 5th leaf from the apex. Leaf area was measured using a LI3000A meter, and root and shoot dry weights were measured at the end of the experiments, e.g. 10 d after NaCl concentration was increased to 150 mM. Na and K contents were measured using flame photometry and Cl contents with the Mohr's method (AOAC 1990, Sadzawka *et al.* 2007).

Gene expression: Total RNA was extracted from young expanding leaves using the CTAB method (Blanco-Ulate *et al.* 2013). RNA pellet was further purified using the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from the prepared RNA using M-MLV Reverse Transcriptase (Promega). qRT-PCR was performed on a StepOnePlus PCR System using Fast SYBR Green Master Mix (Applied Biosystems). Primer sequences were AtNHX1 Forward: CTACCTATTACCGCACCAGAACG and AtNHX1 Reverse: CTCAATGAACGAGTCTTGGTCC (Bassil *et al.* 2011). All qRT-PCR reactions were performed as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), using the grape actin gene as reference (Jones *et al.* 2014), was applied to analyze the expression levels of the transgenes.

Statistical analysis: Data were analyzed with two-way analysis of variance (ANOVA) followed by LSD

Fisher for mean separation. Some variables were transformed by the natural logarithm function. Data that violated assumptions of parametric tests were analyzed using the non-parametric test of Friedman. Statistical analyses were performed using the InfoStat software program, version 2.0 (DiRienzo *et al.* 2013).

Results

Hydroponic culture. Growth parameters: At the end of the experiment, electrical conductivity (EC) of saline solutions (150 mM NaCl) reached a value of 17 $dS \cdot m^{-1}$, while EC of non saline solutions was lower than 4 $dS \cdot m^{-1}$. Salt treatment drastically affected vegetative growth, and all variables analyzed by ANOVA were significantly different between 0 and 150 mM NaCl (Figs 1 and 2). Growth curves showed that NaCl treatment started to affect plant height at 50 mM in PG, 75 mM in UN and 100 mM to 125 mM in transgenic lines (suppl. Fig. 2). At 150 mM, UN plants were more than 50 % shorter than their control counterparts, while transgenics were taller than UN and PG. The interaction between NaCl and genotype was statistically significant (Fig. 1). Total number of leaves decreased from 31-36 in the controls to 17-31 at 150 mM NaCl. For this variable, PG was the most affected genotype and D694 was the least. The first leaf symptoms appeared at 100 mM NaCl, but no statistical differences were found among genotypes or in the interaction (data not shown). Leaf symptoms were more severe in adult leaves and consisted of necrotic margins followed by leaf flaccidity and wilting due to over-accumulation of Na and Cl. Leaf area was reduced 4 times by NaCl, with PG having significantly less leaf area than the rest of the genotypes either in the presence or abscence of NaCl. Leaf area of the transgenics was greater, although not statistically significant, than UN (Fig. 2). Transpiration rates of all genotypes declined 60 to 70 % at 150 mM (suppl. Tab. 2).

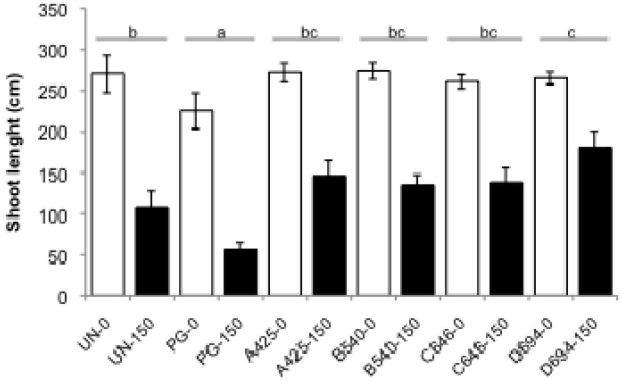

Fig. 1: Shoot length of untransformed 'Thompson Seedless' (UN), 'Pedro Giménez' (PG) and transgenic lines A425, B540, C646 and D694 growing in liquid nutrient solution with 0 or 150 mM NaCl. Significant differences were found at NaCl, genotype and NaCl*genotype levels with 2-way ANOVA ($p < 0.05$). Different letters indicate significant differences among genotypes according to Fisher LSD test ($\alpha = 0.05$). Data are shown as means ± SE, n = 5 for each combination of genotype and NaCl treatment.

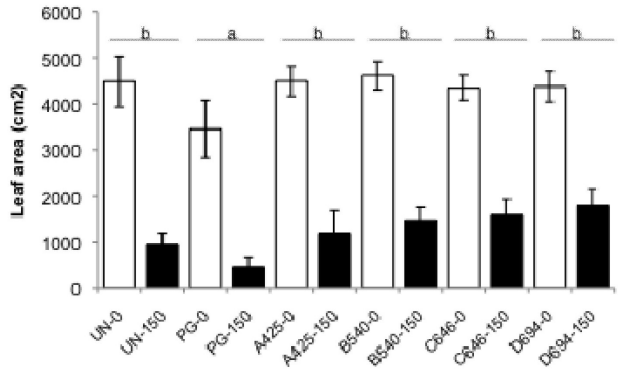

Fig. 2: Leaf area of untransformed 'Thompson Seedless' (UN), 'Pedro Giménez' (PG) and transgenic lines A425, B540, C646 and D694 growing in liquid nutrient solution with 0 or 150 mM NaCl. Significant differences were found at NaCl and genotype levels with 2-way ANOVA ($p < 0.05$). Different letters indicate significant differences among genotypes according to Fisher LSD test ($\alpha = 0.05$). Data are shown as means ± SE, n = 5 for each combination of genotype and NaCl treatment.

Root, shoot and whole plant dry weight were also severely affected. Under salt stress dry weight of the transgenics was significantly higher than PG in all instances. A425, C646 and D694 dry weights were also significantly higher than UN at the whole plant level (Fig. 3). Salinity treatment reduced shoot dry weight to a greater extent than root dry weight. This fact was reflected in the shoot/root ratio, which decreased approximately from 5 to 3 at 150 mM, with the exception of PG that maintained the ratio and was significantly higher than UN, B540 and C646 (Tab. 1). A425 and D694 exhibited higher ratios than UN at 150 mM NaCl.

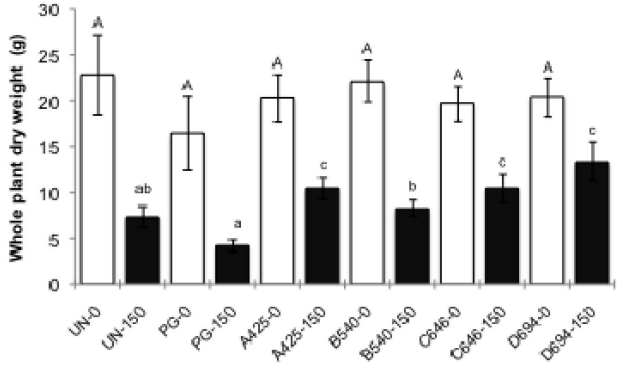

Fig. 3: Whole plant dry weight of untransformed 'Thompson Seedless' (UN), 'Pedro Giménez' (PG) and transgenic lines A425, B540, C646 and D694 growing in liquid nutrient solution with 0 or 150 mM NaCl. Different letters indicate significant differences in whole plant DW among genotypes according to non parametric Friedman test ($\alpha = 0.05$). Data are shown as means ± SE, n = 5 for each combination of genotype and NaCl treatment.

Ionic content: Values in roots surpassed values in shoots for the three elements (Na, Cl and K) either in the absence or presence of NaCl (Tab. 2).

Sodium: Under salinity, Na tissue concentration increased 30x in shoots and 50x in roots, leading to 1:+2 shoot:root ratio (Tab. 2). Lower, but not significant, shoot Na contents were found among transgenics, while an interaction between genotype and salinity treatments was found in roots probably due to lower levels that accumulated in PG.

Table 1

Shoot/root dry weight ratio of untransformed 'Thompson Seedless' (UN), 'Pedro Giménez' (PG) and 'Criolla Chica' (CC) and transgenic lines A425, B540, C646 and D694 growing in hydroponics or pots treated with 0 or 150 mM NaCl. Different letters indicate significant differences according to non parametric Friedman test ($\alpha = 0.05$), ns indicates not significant.

		Hydroponics			Pots	
		0	150		0	150
UN	n = 10	4.76	2.76 a	n = 14	2.61 abc	2.40
PG	n = 10	5.87	5.50 d	n = 14	5.17 f	4.76
CC				n = 14	3.11 cde	2.80
A425	n = 10	4.97	3.48 bcd	n = 14	3.03 bcd	2.51
B540	n = 10	4.84	3.09 abc	n = 14	2.17 ab	2.69
C646	n = 10	4.72	3.07 ab	n = 14	2.03 a	3.30
D694	n = 10	4.61	3.60 cd	n = 14	3.52 cde	2.96
p-value genotype		ns	< 0.01		< 0.01	ns

Table 2

Na, K, and Cl content expressed as w/w percentage and K/Na ratio in shoots and roots of untransformed 'Thompson Seedless' (UN), 'Pedro Giménez' (PG) and transgenic lines A425, B540, C646 and D694 growing in liquid nutrient solution with 0 or 150 mM NaCl. Different letters indicate significant differences according to Fisher LSD test ($\alpha = 0.05$), ns indicates not significant, n = 5 for each combination of genotype and treatment

Treatments		Shoot % Na	Root % Na	Shoot % K	Root % K	Shoot % Cl	Root % Cl	Shoot K/Na	Root K/Na
UN	0	0.05	0.07	2.52	5.7	0.29	0.69	48.61	82.71
UN	150	1.50	3.98 b	2.18	2.54 b	2.54 cd	4.85 b	1.54	0.65
PG	0	0.06	0.16	2.90	3.83	0.30	0.50	49.97	44.20
PG	150	1.57	2.88 a	2.40	1.52 a	3.03 e	4.59 ab	1.59	0.50
A425	0	0.05	0.09	2.74	5.62	0.33	0.67	55.08	70.36
A425	150	1.25	4.08 b	2.04	2.50 b	1.93 b	6.47 c	1.90	0.62
B540	0	0.06	0.08	2.57	5.86	0.33	0.69	46.43	77.99
B540	150	1.31	3.17 a	2.01	2.47 b	2.14 bc	4.80 b	1.57	0.77
C646	0	0.05	0.07	2.74	5.20	0.31	0.76	56.78	76.48
C646	150	1.35	3.88 b	2.00	2.54 b	2.68 de	5.65 c	1.54	0.66
D694	0	0.06	0.07	2.82	5.44	0.32	0.65	49.52	80.19
D694	150	1.17	3.50 ab	1.86	2.84 b	1.41 a	3.71 a	1.87	0.70
p-value NaCl		< 0.01	< 0.01	< 0 .01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
p-value genotype		ns	< 0.01	ns	< 0.05	< 0.01	< 0.01	ns	ns
p-value NaCl*gen		ns	< 0.01	ns	ns	< 0.01	< 0.01	ns	ns

Potassium: At 0 mM, K content was at least 10 times higher than Cl and Na but it decreased at 150 mM, especially in roots. No differences were found among genotypes besides PG roots, which exhibited significantly lower K than UN and transgenics (Tab. 2).

Chloride: At 0 mM, Cl content was approximately 6x higher than Na (Tab. 2). Under salinity Cl in shoots and roots increased 7-8x and, although in lower proportion, it continued to surpass Na. Interactions between genotype and salinity treatments were found in shoot and root Cl since genotypes responded differently: under salinity stress D694 accumulated less Cl either in roots and shoots, while A425 and C646 accumulated more Cl in roots.

Potassium-sodium ratio: At 150 mM, the K/Na ratio decreased considerably in shoots and in roots (Tab. 2). Shoot K/Na ratio under salinity was higher in A425 and D694 but differences were not significant.

Culture in pots. Growth parameters: Substrate EC increased from 0.63 at the begining of the experiment to 1.31 and 7.8 $dS \cdot m^{-1}$ in the non saline and saline treatment, respectively, at the end of the experiment. The initial sodium adsorption ratio (SAR) of 3.1 decreased to 0.7 in the non saline treatment due to the presence of Ca and Mg in the nutrient solution, while the SAR of saline treatment increased to 35.8, which greatly exceeds values tolerated by most cultivated plants (RICHARDS 1954). EC of

the percolation water at the end of the experiment was 4 and 18 $dS \cdot m^{-1}$ in the non saline and saline treatment, respectively.

As in hydroponics, saline treatment drastically affected vegetative growth, and all studied variables were significantly different between 0 and 150 mM NaCl (Figs 4, 5 and 6). Growth curves showed that the NaCl treatment started to affect plant height at 75 mM in CC and B540, at 100 mM in UN, A425, C646 and PG, and at 125 mM in D694 (suppl. Fig. 3). At 150 mM, the height of most genotypes was reduced by half as compared to their untreated counterparts. Fig. 4 shows that transgenics were significantly taller than 'Criollas' with the exception of C646. UN was only significantly taller than PG. At the end of the experiment, total leaf number varied between 37 and 42 in the absence of NaCl, to 24 and 34 at 150 mM NaCl. 'Criollas' had a significant lower number than the rest of the genotypes which was reflected in a lower leaf area (Fig. 5). Leaf symptoms were first observed at 100 mM NaCl, but no statistical differences were found among genotypes. UN, PG and CC had a higher percentage of leaves with symptoms (data not shown). Leaf area decreased more than 50 % in saline treatements, due to a lower number of leaves and a decrease in their size. Leaf area was higher in transgenic lines but only A425 was statistically higher than UN. PG and CC exhibited the lowest leaf area; PG was statistically lower than UN and transgenics, while A425, B540, and D694 were significantly higher than CC (Fig. 5). NaCl treatment significantly decreased dry weight of shoots, roots and whole plants (Fig. 6); A425, B540 and D694 had highest whole plant weights and 'Criollas' the lowest, but their differences with UN were not significant. A425 and B540 also exhibited the highest levels of gene expression (Fig. 7). We assume similar expression levels in roots because *AtNHX1* is under the control of constitutive promoter CaMV 35S. Shoot/root ratio remained in the range of 2 to 3 with the exception of PG, which had values higher than 4.0, e.g. 5.17 at 0 mM and 4.76 at 150 mM (Tab. 1). Transpiration was reduced approximately 50 % by saline treatment but no differences were found among genotypes (suppl. Tab. 2).

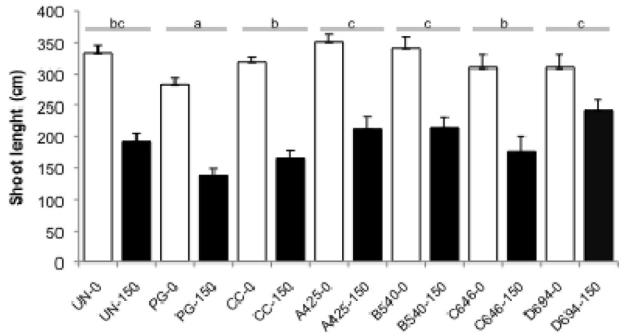

Fig. 4: Shoot lenght of untransformed 'Thompson Seedless' (UN), 'Pedro Giménez' (PG), 'Criolla Chica' (CC) and transgenic lines A425, B540, C646 and D694 growing in pots and irrigated with 0 or 150 mM NaCl. Significant differences were found at NaCl, and genotype levels with 2-way ANOVA ($p < 0.05$). Different letters indicate significant differences among genotypes according to Fisher LSD test ($\alpha = 0.05$). Data are shown as means ± SE, n = 7 for each combination of genotype and NaCl treatment.

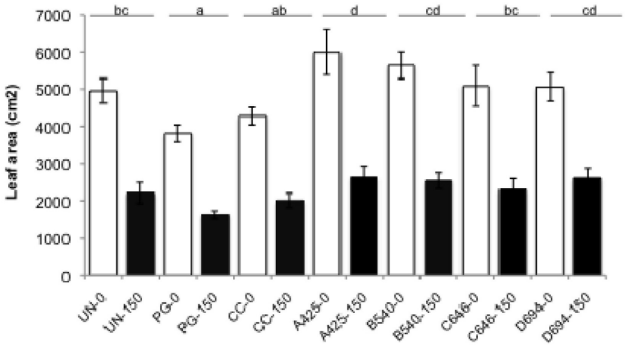

Fig. 5: Leaf area of untransformed 'Thompson Seedless' (UN), 'Pedro Giménez' (PG), 'Criolla Chica' (CC) and transgenic lines A425, B540, C646 and D694 growing in pots and irrigated with 0 or 150 mM NaCl. Significant differences were found at NaCl, and genotype levels with 2-way ANOVA ($p < 0.05$). Different letters indicate significant differences among genotypes according to Fisher LSD test ($\alpha = 0.05$). Data are shown as means ± SE, n = 7 for each combination of genotype and treatment.

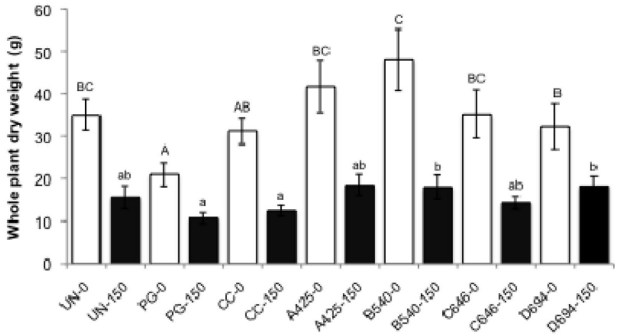

Fig. 6: Whole plant dry weight of untransformed 'Thompson Seedless' (UN), 'Pedro Giménez' (PG), 'Criolla Chica' (CC) and transgenic lines A425, B540, C646 and D694 in pots and irrigated with 0 or 150 mM NaCl. Different letters indicate significant differences in whole plant DW among genotypes according to non parametric Friedman test ($\alpha = 0.05$). Data are shown as means ± SE, n = 7 for each combination of genotype and treatment.

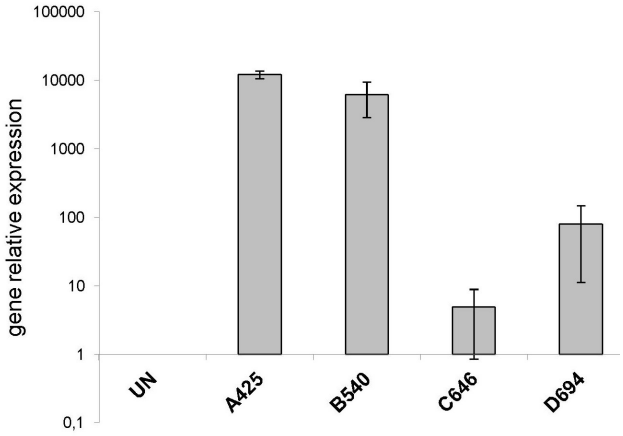

Fig. 7: *AtNHX1* expression in transgenic lines relative to untransformed 'Thompson Seedless' (UN). Error bars represent standard error of the mean (n = 3). UN: Untransformed 'Thompson Seedless'; A425, B540, C646, D694: transgenic lines.

Ionic content. Sodium: The shoot Na levels increased by 7x and were significantly higher in the saline treatment (Tab. 3). Na content in transgenics was lower than in UN and significant differences were found at the root level.

Potassium: Shoot K remained unchanged with saline treatment, although there were differences among genotypes, with PG significantly higher than the rest of the 'Thompson Seedless' genotypes. At the root level, there was an interaction between genotype and saline treatment; due to the different response of 'Criollas' and UN and transgenics, the former with lower K at 150 mM NaCl (Tab. 3).

Chloride: Cl increased 2-3x in roots and shoots, respectively, but there was an interaction between genotype and saline treatment, with UN accumulating more Cl in shoots and roots (Tab. 3).

Potassium-sodium ratio: The K/Na ratio decreased with saline treatment and it was significantly higher in roots of B540, C646 and D694 (Tab. 3).

Discussion

The aims of this study were: 1) to study the effects of *AtNHX1* ectopic expression in grapevine and its response to salt stress; 2) to determine if transgenic AtNHX1 'Thompson Seedless' grapevines were more tolerant to NaCl than untransformed 'Thompson Seedless' and 'Criolla' cultivars 'Pedro Giménez' and 'Criolla Chica'; 3) to analyze the response through measurements of Na and Cl concentrations that accumulated in plant tissues; and 4) to compare the response using two culture systems: hydroponics and pots with soil substrate.

As expected, growth was affected by salinity in either hydroponic culture or pot-based soil media, however effects were more visible in hydroponics, probably due to the direct contact of roots with the nutrient and saline solution. Dry weight and leaf area were reduced by 70 % in hydroponics and 55 % in pots with soil, while the percentage of leaves with symptoms was 40 % in hydroponics and 13 % in pots with soil. Similar results were found in barley, where growth reductions were greater under hydroponic culture than in soil at same EC values (Tavakkoli *et al.* 2010). Salinity decreased shoot and root K content and shoot/root dry weight ratio in hydroponics but not in pot-based soil media. These differences could also be explained by the positive effect of trace elements and/or a reduced uptake of Na and Cl due to the buffering effect of soil. Under pot-based soil culture, a lower level of stress might have favored osmotic stress over ion effects (Shani and Ben-Gal 2005). In both salinity treatments, transgenics grew more than UN and 'Criollas', exhibiting increased shoot length, leaf area and dry weight. Symptoms were also more accute in UN and 'Criollas' than in transgenics. A425, B540 and D694 exhibited the highest levels of gene expression, which is consistent with their overall better performance, although a better correlation was found in pots. C646 performed as well as the UN control under the less stringent potted vine conditions. Several studies have examined the ectopic expression of *AtNHX1* - mostly in annual species - and have shown that Na and K contents in transgenic plants are higher than untransformed controls as the result of *AtNHX1* activity in the tonoplast (Asif *et al.*

Table 3

Na, K, and Cl content expressed as w/w percentage and K/Na ratio in shoots and roots of untransformed 'Thompson Seedless' (UN), 'Pedro Giménez' (PG), 'Criolla Chica' (CC) and transgenic lines A425, B540, C646 and D694 growing in pots and irrigated with 0 or 150 mM NaCl. Different letters indicate significant differences according to Fisher LSD test ($\alpha = 0.05$), ns indicates not significant, n = 7 for each combination of genotype and treatment

Treatments		Shoot % Na	Root % Na	Shoot % K	Root % K	Shoot % Cl	Root % Cl	Shoot K/Na	Root K/Na
UN	0	0.11	0.34 c	1.84 ab	1.98	0.44	0.79	17.47	5.74 a
UN	150	0.89	1.53	2.09	1.85	1.67 d	2.71 e	2.33	1.25
PG	0	0.10	0.40 c	2.48 c	2.37	0.45	0.83	22.90	6.40 ab
PG	150	0.79	1.32	2.34	1.58	1.36 ab	2.21 de	2.06	1.24
CC	0	0.10	0.29 bc	1.88 bc	1.67	0.45	0.88	17.44	5.82 a
CC	150	0.77	1.14	2.14	1.29	1.39 abc	2.24 e	1.73	1.12
A425	0	0.11	0.37 bc	1.68 ab	1.74	0.47	0.87	16.83	7.67 abc
A425	150	0.84	1.24	1.96	2.10	1.34 abc	1.83 cd	2.61	1.71
B540	0	0.10	0.24 a	1.66 a	1.55	0.46	0.71	15.52	7.77 bc
B540	150	0.71	0.93	1.86	1.69	1.42 bc	1.68 a	2.50	2.74
C646	0	0.10	0.27 ab	1.91 ab	1.81	0.48	0.81	19.51	9.15 bc
C646	150	0.73	1.20	1.89	2.07	1.43 cd	1.65 ab	2.93	2.46
D694	0	0.11	0.28 ab	1.86 ab	2.14	0.43	0.74	22.36	12.97 c
D694	150	0.75	1.03	1.79	1.76	1.33 a	2.10 bc	2.44	2.10
p-value NaCl		< 0.01	< 0.01	ns	ns	< 0.01	< 0.01	< 0.01	< 0.01
p-value genotype		ns	<0.01	< 0.01	ns	< 0.01	< 0.01	ns	< 0.05
p-value NaCl*gen		ns	ns	ns	< 0.05	< 0.01	<0 .01	ns	ns

2011, Tian *et al.* 2011, Adem *et al.* 2015, Fan *et al.* 2015, Sahoo *et al.* 2016). However, in our reseach transgenic lines accumulated similar or lower levels of Na and K in shoots and roots than UN. Since we did not separate leaves from stems and petioles, we can't rule out the possible variation of ion distribution in different organs and tissues. We don't know whether *AtNHX1* has been correctly targeted in the grape cell either. In poplar, a perennial tree species, no obvious differences in Na concentrations were found between the transgenic and wild type (Jiang *et al.* 2011). Xue *et al.* (2004) found that leaves of transgenic wheat accumulated significantly less Na^+ than the non-transgenic control and suggested that the overexpression of the vacuolar Na^+/H^+ antiporter could cause a shift from some portion of the tonoplast to the plasmamembrane pathway. Alternatively, a higher dilution rate of the total Na^+ content could result from higher growth rates of transgenics.

Interestingly, the lower Na concentrations found in transgenics in this research were accompained by higher K/Na ratios in roots and lower Cl in shoots and roots of the plants growing in pots. Similar results were also obtained in poplar (Jiang *et al.* 2011), which highlights the complex role of NHX mediated salt tolerance (Rodríguez-Rosales *et al.* 2009, Leidi *et al.* 2010, Reguera *et al.* 2014). In grapevine, Cl is particulary relevant, since salinity symptoms are generally associated with an increase in Cl rather than Na concentrations (Ehlig 1960, Walker *et al.* 1997, DiFilippo 2008, Henderson *et al.* 2014). In our study, Cl content in roots and shoots was several times higher than Na content at 0 NaCl. This predominance was maintained at 150 mM NaCl, even though Na increased in higher proportion than Cl, and corroborates the importance of Cl in grapevines.

Taken together, these results suggest the potential benefit of *AtNHX1* expression in grapevines growing under saline conditions and the need of long term testing of these lines under field conditons, which should include evaluation of grafted vines, fruit quality and drought tolerance.

It has been reported that 'Criolla' cultivars exhibitied tolerance to salinity *in vitro* (Cavagnaro *et al.* 2006), but in our experiments PG and CC, which is one of the parents of PG (Durán *et al.* 2011), grew less than UN and transgenics, and were more affected by saline treatments, except by the fact that PG, but not CC, exhibited a higher shoot/root ratio than the rest of the genotypes in the presence of NaCl. Vila *et al.* (2016) found that 'Cereza', another 'Criolla' cultivar, was less salt tolerant than own rooted 'Malbec' growing in 20 L pots. It is possible that the outstanding vigor that characterizes 'Criolla' cultivars (Alcalde 1989) is the main factor contributing to salinity tolerance in the field.

Conclusions

As shown in other species, *AtNHX1* ectopic expression improved salinity tolerance in transgenic grapevine lines. This improved response was accompained by lower Na and Cl contents rather than Na accumulation. Hydroponics conditons were more stringent than when plants were tested in pots with soil media; the growth variables were more affected and Cl and Na tissue contents reached considerably higher levels than in plants growing in soil media. Therefore, hydroponic culture could represent an alternative method of evaluation using lower concentrations or shorter periods of time. The fact that Cl was found at higher concentrations than Na in roots and shoots at 0 and 150 mM NaCl confirms the important role of Cl in ion toxicity under NaCl stress. The salinity tolerance of 'Criolla' cvs. 'Pedro Giménez' and 'Criolla Chica' was not confirmed under the experimental conditions used in this study. Transgenic lines with highest expression levels will be tested under field conditions for a more comprehensive set of evaluations considering the major role that Na plays in fruit and wine quality.

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