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BRIEF COMMUNICATION

Expression of Na^+/H^+ antiporter gene in response to water and salinity stress in grapevine rootstocks

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Abstract

Grapevine rootstocks belonging to different species of *Vitis* differ in their response to stress. Vines of rootstocks 110R and 1613C were subjected to different salt and water stresses individually and in combination. Expression of *VvNHX1*, a Na^+/H^+ antiporter gene, was analyzed at 7 and 21 d of stress treatment. In 110R, the expression of *VvNHX1* gene increased in response to both salinity and water stress already after 7 d. Under salinity and combined stress enhanced expression of this gene was observed also after 21 d whereas expression decreased under water stress. In 1613C, expression of this gene did not increase under salinity stress. There was delayed response to water and combined stress and expression increased several fold after 21 d of stress. The stressed vines of 110R maintained lower sodium content and higher K^+/Na^+ ratio as compared to rootstock 1613C.

Additional key words: potassium. sodium, *Vitis vinifera*, *VvNHX1* gene.

Soil salinity is one of the major abiotic factors limiting agriculture productivity. It is estimated that about 6 % of the world's land is affected by salinity (Yeo 1999). Plant responses to salinity occur in two phases: a rapid, osmotic phase and a slower, ionic phase. High sodium ion content affect enzyme activity, cell membrane integrity and other plant physiological functions. Prolonged exposure to high salinity results in growth inhibition and subsequently death of plant (Munns and Tester 2008). Salt-tolerant plants sequester and accumulate salt into the cell vacuoles, controlling the salt content in the cytosol and maintaining a high cytosolic K^+/Na^+ ratio. In plants, the movement of Na^+ across membranes is facilitated by Na^+/H^+ antiporter which transports Na^+ from cytoplasm to vacuoles using electrochemical H^+ gradient (Blumwald *et al.* 2000). In plants, the first antiporter gene was cloned in *Arabidopsis* (Gaxiola *et al.* 1999). Vacuolar Na^+/H^+ exchangers (NHXs) have been cloned from rice (Fukuda *et al.* 1999), *Atriplex gmelini* (Hamada *et al.* 2001), beet root (Xia *et al.* 2002), barley (Fukuda *et al.* 2004), wheat (Brini *et al.* 2005), alfalfa (Yang *et al.* 2005), *Suaeda salsa* (Li *et al.* 2007) and grapevine (Hanana *et al.* 2007). Improved tolerance to NaCl was obtained by over expressing *AtNHX1* in transgenic *Arabidopsis thaliana* (Apse *et al.* 1999) and tomato (Zhang and Blumwald 2001).

Grapevine (*Vitis vinifera* L.) faces several biotic and abiotic stresses during its life span. It tolerates water stress to some extent without any adverse effect on the fruit yield. However, high salinity inhibits shoot growth and affects berry properties considerably (Walker *et al.* 2002). Majority of the world vineyards are raised on rootstocks which belong to either a North American *Vitis* species or an interspecific hybrid. Besides resistance to biotic factors, use of rootstocks to overcome abiotic stresses like high soil pH, alkalinity, salinity and drought is increasingly important. Grape rootstocks vary in their response to soil salinity in terms of maintaining scion growth and sustaining yield (Southey and Jooste 1992, Walker *et al.* 2002). Salinity tolerance may also involve exclusion of chloride (Walker *et al.* 2002) and sodium ions (Sharma and Upadhyay 2008). 110R, an interspecific hybrid of *V. berlandieri* × *V. rupestris* shows tolerance to water stress and salinity (Sharma and Upadhyay 2004). A less tolerant rootstock 1613C is of multi species origin .

Understanding the environmental stress response at molecular level will help in improvement in grape production. Transcript profiling of the response of grapevine to water and salinity stress revealed distinct

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difference between water deficit and salinity (Cramer *et al.* 2007). Hanana *et al.* (2007) reported cloning of vacuolar Na^+/H^+ antiporter gene, *VvNHX1* and its increased expression during berry ripening. However, its expression during abiotic stress has not been studied. In this study, we used two rootstocks which have shown differential performance under stress in field conditions to study the expression of *VvNHX1* gene in response to salt, water and combined stresses.

Six months old potted vines of rootstocks 110R (*V. berlandieri* × *V. rupestris*) and 1613C (cv. Solonis × cv. Othello) were used for the experiment. To obtain the uniform growth, the vines were pruned and allowed to grow for 12 weeks. Plants were irrigated on alternate days using full strength Hoagland's nutrient solution. Water stress was imposed by irrigating vines to 50 % of water holding capacity (WHC). Control vines were irrigated to 100 % of WHC. Salinity stress was imposed by irrigating the vines with saline water. The electric conductivity (EC) of irrigation water was adjusted either to 2 or 4 dS m^{-1} by adding NaCl. The control plants were irrigated with normal water (EC ~ 0.7 dS m^{-1}). Combined stress was imposed by irrigating the vine with saline water of 2 dS m^{-1} and 4 dS m^{-1} to 50 % of WHC.

Young leaves were sampled at 7 and 21 d of stress, frozen in liquid nitrogen and stored at -80 °C till use. Total RNA was extracted from 70 - 80 mg leaf tissue using *Spectrum* plant RNA extraction kit (*Sigma-Aldrich*, St. Louis, MO, USA) according to manufacturer's instructions. On-column DNAse (*Sigma-Aldrich*) treatment was included to degrade genomic DNA. The content of RNA was estimated by measuring absorbance at 260 nm and quality by A_{260}/A_{280} ratio (> 1.8) and A_{260}/A_{230} ratio (> 2.0) using nano-spectrophotometer (*Implen*, *Schatzbogen*, München, Germany). The integrity of RNA was confirmed on 1 % agarose gel electrophoresis. The RNA samples were stored at -80 °C till use. Total RNA (1 µg) was used to synthesize first strand cDNA using *oligo* $(dT)_{20}$ primer and *Omniscript* cDNA kit (*Qiagen*, Valencia, CA, USA) according to manufacturer's instructions. The volume of first strand cDNA was made up to 0.2 cm^3, and stored at -20 °C. A sequence partially covering last exon and 3' un-translated region of *VvNHX1* gene (gene ID: 100232960) was used to design primers for real time PCR using web based software *Primer3plus* (Rozen and Skaletsky, 2000). The primer sequences were 5'-GGATCACCCACTGAACCAAA-3' (VvNHX1F) and 5'-TTCACACAGAAAGCCGATTG-3' (VvNHX1R).

The real time PCR reaction in 20 mm^3 contained 2 mm^3 diluted cDNA, 1× *Maxima SYBR Green* mix (*Fermentas*, Ontario, Canada) and 0.5 µM each of forward and reverse primers. The PCR was performed in *Rotor Gene Q* (*Qiagen*, Valencia, CA, USA) with the following temperature profile: initial denaturation at 95 °C for 10 min, 45 cycles of step 1, 95 °C for 20 s; step 2, 60 °C for 20 s and step 3, 72 °C for 20 s, hold at 72 °C for 5 min. At the end, a melt was performed at 50 - 95 °C with increase of 1 °C at each step, held 45 s for first step and 5 s for subsequent steps. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and *actin* were used as reference genes for the data normalization. The standard curve was used to estimate reaction efficiency of the primers. Cq values and reaction efficiency were estimated using software *Rotor Gene Q v. 1.7* (*Qiagen*). Relative expression of target gene was calculated using relative expression software tool *REST v. 2.1.13* (Pfaffl *et al.* 2002). The software utilizes reaction efficiency to calculate the absolute concentration and the gene of interest concentration is divided by the reference gene value to obtain a relative expression level. A pair-wise fixed reallocation randomization test is used to determine whether the difference in expression is significant.

The residual leaves from treated and control vines were harvested and used for nutrient analysis at the end of the experiment. Leaves were detached from petiole, washed with distilled water and oven dried at 70 °C. Samples were ground in *Cyclotec* sample mill (*Foss Tecator*, Hillerod, Denmark) followed by digestion in block digester with H_2SO_4:H_2O_2 mixture. The digested samples were analyzed on *Analyst 100* atomic absorption spectrophotometer (*Perkin Elmer*, Waltham, MA, USA) for the estimation of Na^+ and K^+. The K^+/Na^+ ratio was calculated. The means were compared using Duncan's multiple range test using *SAS* software (*SAS Institute*, Cary, NC, USA).

During early stages, *i.e.* 7 d after treatment, at low level of salinity stress (2 dS m^{-1}) the expression of *VvNHX1* gene in rootstock 110R increased only 1.3-fold. However, at higher salinity (4 dS m^{-1}), the expression increased about 4.5-fold indicating significant up-regulation of this gene in response to salinity (Table 1). In rootstock 1613C, reduction in the expression of *VvNHX1* gene was observed at early stages of stress at low as well as high salinity, indicating down-regulation of *VvNHX1* gene in 1613C as early response to salinity. After prolonged exposure (21 d) to salinity at both levels, the expression of this gene decreased marginally in 110R while expression increased in 1613C (Table 1).

Under water stress, the expression of *VvNHX1* in 110R increased 7.5-fold after 7 d. In contrast, the gene expression in water stressed vines of 1613C was only

Table 1. Expression of *VvNHX1* gene in two rootstocks in response to salinity and water stress. * - expression level in treated samples was different from control at $P < 0.001$.

Treatment	Expression level			
	Rootstock 110R		Rootstock 1613C	
	7 d	21 d	7 d	21 d
2 dS m^{-1}	1.34*	0.82*	0.81*	1.34*
4 dS m^{-1}	4.46*	0.93*	0.23*	1.29*
50 % WHC	7.52*	1.28*	0.18*	8.35*
2 dS m^{-1} + 50 %	2.05*	1.35*	0.22*	3.63*
4 dS m^{-1} + 50 %	1.87	1.92*	0.27*	5.54*
Control	1	1	1	1

Table 2. Nutrient content in the leaf blade of two rootstocks in response to salinity and water stress. Means ± SE, $n = 5$. Means with the same letter in columns are not significantly different at $P < 0.05$.

Treatment	Na^+ [µmol g^{-1}(d.m.)]		K^+ [µmol g^{-1}(d.m.)]		K^+/Na^+ ratio	
	110R	1613C	110R	1613C	110R	1613C
Control	27.7±2.1^{B}	31.6±3.3^{F}	250±19^{C,D}	187±13^{C}	9.1±1.1^{D}	6.0±0.8^{A}
2 dS m^{-1}	28.4±3.3^{B}	40.2±2.1^{E}	256±16^{C,D}	168±13^{D}	9.1±1.2^{D}	4.2±0.5^{B}
4 dS m^{-1}	19.7±2.7^{C}	57.4±3.0^{C}	303±15^{B}	146±12^{E}	15.7±2.5^{B}	2.5±0.2^{C}
50 % WHC	13.1±1.4^{D}	47.8±4.3^{D}	269±10^{C}	283±19^{A}	21.0±2.4^{A}	5.9±0.7^{A}
2 dS m^{-1} +50 %	30.4±2.7^{B}	76.2±6.6^{B}	376±14^{A}	279±12^{A}	12.4±0.9^{C}	3.7±0.4^{B}
4 dS m^{-1} + 50 %	35.9±1.5^{A}	87.0±5.3^{A}	364±13^{A}	232±10^{B}	10.2±0.7^{D}	2.7±0.2^{C}
$LSD_{0.05}$	3.1	4.5	19	17	2.1	0.7

0.18-fold of expression in control vines (Table 1). Thus at the early stage, water stress induced up-regulation of this gene in 110R and down-regulation in 1613C. This response to water stress at early stage was similar to response observed at high salinity (4 dS m^{-1}) in both the rootstocks. After 21 d of water stress, the expression of *VvNHX1* gene was found to increase only 1.28-fold in the leaves of 110R rootstock while 8,3-fold increase in 1613C (Table 1).

In 110R, the expression of *VvNHX1* gene enhanced about 2-folds when exposed to both levels of combined stress during early stage. The longer exposure resulted in marginal decrease in expression level at salinity 2 dS m^{-1} and 50 % WHC. Gene expression in 1613C was reduced significantly during the initial stage of combined stress. However, longer exposure resulted in up-regulation of *VvNHX1* gene in 1613C at both the levels of combined stress with increase being more pronounced at 4 dS m^{-1} salinity and 50 % WHC (5.5-fold) as compared to salinity 2 dS m^{-1} and 50 % WHC (3.6-fold; Table 1).

The Na^+ content in control vines of both the rootstocks was *on a par*. At high salinity, the leaves of 1613C accumulated more Na^+. Na^+ content was almost three times higher than that in 110R (Table 2). In 110R, the K^+ content in the leaves of control vines and treated at 2 dS m^{-1} did not vary significantly and increased at 4 dS m^{-1} indicating that K^+ homeostasis was maintained in this rootstock also at high salinity level. On the other hand, K^+ content decreased with increasing salinity in 1613C (Table 2). K^+/Na^+ ratio was higher in salt stressed vines of 110R as compared to 1613C.

Under water stress, the Na^+ content in the leaves of 110R was less than in the control vines whereas accumulation of Na^+ was observed in the leaves of 1613C when exposed to water stress. The K^+ content in water stressed vines of 110R was not significantly different from the control vines. However K^+ content increased significantly in water stressed vines of 1613C as compared to the control vines. The K^+/Na^+ ratio in the water stressed vines of 110R was significantly higher than the K^+/Na^+ ratio in water stressed vines of 1613C (Table 2).

The Na^+ accumulation pattern in the leaves of 110R and 1613C under combined salinity and water stress followed same pattern as salinity stress alone. In 110R, the K^+ concentration was higher than in 1613C at all the levels of combined stress. The K^+/Na^+ ratio was maintained higher in the vines of 110R as compared to 1613C at both the levels of combined stress.

These results suggested that as compared to rootstock 1613C, rootstock 110R has better ability to exclude Na^+ and/or to compartmentalize it into vacuoles. Na^+/H^+ antiporter play a major role in sodium efflux as well as long distance Na^+ transport from root to shoot. The involvement of Na^+/H^+ antiporters and H^+ pumps in Na^+ extrusion has been established at tissue and cellular levels in bean (Shabala and Newman 2000) and poplar (Sun *et al.* 2009). These results are in accordance with several reports on stress induced higher expression of Na^+/H^+ antiporter in salt tolerant cultivars. Tang *et al.* (2010) reported up-regulation of Na^+/H^+ antiporter gene in *Trifolium* in response to salt stress. Ligaba and Katsuhara (2010) observed higher expression of Na^+/H^+ antiporter genes in tolerant cultivars of barley as compared to the sensitive cultivars, which also accumulated more Na^+ in shoots. Ding *et al.* (2010) reported abundance of genes of Na^+/H^+ antiporters in tolerant poplar species.

Though *VvNHX1* gene is known to be involved in the transport of Na^+ ions, its expression was found to increase in response to water stress as well suggesting that this gene might be part of stress response pathway network and triggered by a common stress signal and play important role in maintaining cell homeostasis under conditions of stress.

Salinity stress has two phases of effects on plants (Munns and Tester 2008), osmotic stress followed by ionic stress. The high expression observed in water stressed vines of 110R at early stages suggests that higher expression of antiporter gene in presence of high salinity in growth medium may be due to osmotic stress. Combined water and salinity stress results in higher level of osmotic stress even at low salinity resulting in several folds increase in gene expression in both the rootstocks. Increased levels of Na^+/H^+ antiporter gene transcript in response to osmotic stress was also observed in barley (Fukuda *et al*. 2004) and *Zygophyllum* (Wu *et al.* 2011).

The tolerance to salt is usually correlated with the higher uptake of K^+ and higher K^+/Na^+ ratio in the

presence of higher Na^+ in growing media. Higher K^+/Na^+ ratio maintain cell homeostasis in presence of stress (Glenn *et al.* 1999). In 110R, the exposure to high salinity did not decrease leaf K^+ content; however in 1613C there was significant decrease in K^+ content even at low level of salinity. The K^+/Na^+ ratio was also maintained in stressed vines of 110R while K^+/Na^+ ratios decreased with increasing stress levels in 1613C. Alizadeh *et al.* (2010) also reported decreased K^+/Na^+ ratio in salt sensitive rootstocks in response to salt stress. These properties of rootstocks influence the field performance of scion cultivars. Maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in soils with a high NaCl concentration and it is expected that besides Na^+/H^+ antiporter, the involvement of other ion channels play an important role in maintaining the cell homeostasis which needs further in-depth analysis.

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