

## Coordinate Up-regulation of Vacuolar Pyrophosphatase and V-Na<sup>+</sup>/H<sup>+</sup> Antiporter to Early Salt Stress in Halophytic Monocot *Leptochloa fusca* Roots

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### ABSTRACT

Vacuolar H<sup>+</sup>-translocation pyrophosphatase (V-PPase) and Na<sup>+</sup>/H<sup>+</sup> antiporter (V-Na<sup>+</sup>/H<sup>+</sup>) are important transporters in plant cells and have essential roles against abiotic stresses. In this study, the effects of salt stress were surveyed on the transcription of V-PPase pump and Na<sup>+</sup>/H<sup>+</sup> antiporter genes at early times of NaCl treatment in roots of halophytic grass *Leptochloa fusca* through the Real Time PCR. The first results showed that *L. fusca* has high ability for accumulating Na<sup>+</sup> under saline conditions. Real-Time PCR analysis demonstrated that the expression level of V-PPase and V-Na<sup>+</sup>/H<sup>+</sup> increased in response to different levels of NaCl treatment, but the transcriptional increase of V-Na<sup>+</sup>/H<sup>+</sup> gene was higher than that of the V-PPase gene, indicating that V-Na<sup>+</sup>/H<sup>+</sup> had a crucial role in salt tolerance in this plant. These results indicated that coordinate up-regulations of V-PPase expression and Na<sup>+</sup>/H<sup>+</sup> antiporter expression were correlated with Na<sup>+</sup> sequestering into vacuoles of *L. fusca*. It was also shown that increase in the expression level of V-Na<sup>+</sup>/H<sup>+</sup> and V-PPase was correlated with the accumulation of sodium in roots, suggesting a physiological role for these antiporters and pump in Na<sup>+</sup> compartmentation during adaptation to high salinity.

**Keywords:** Na<sup>+</sup> accumulation, Real Time PCR, Transcript.

### INTRODUCTION

Environmental abiotic stresses, particularly salinity and drought, are currently the major factors that reduce crop yields worldwide (Vicente *et al.*, 2004). The total world area of salt-affected soils, including saline and sodic soils, is estimated to be as large as about 831 million hectares, extending over all the continents (Rengasamy, 2006). Hence, developing salt-tolerant cultivars of crops is essential (Boyer, 1982; Owens, 2001). Genetic engineering for the improvement of resistance to high salinity conditions requires a clear understanding of the

complex mechanisms of salt stress tolerance (Vicente *et al.*, 2004).

The most important crops are glycophytic monocots and, therefore, they are the best materials for studying the molecular basis of salt tolerance and exploiting the genes for improving crop performance. *Leptochloa fusca* L. Kunth also known as *Diplachne fusca* is a perennial salt tolerant C4 grass (Qureshi *et al.*, 1982) that has been reported by Lazarides (1970) and Kumar (1988). *Leptochloa fusca* is an attractive model plant to study the mechanism of salt tolerance mainly due to its characteristics as a typical euhalophyte and having both accumulating and excreting properties (Abdullah *et al.*, 1990).

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Research conducted during the last two decades has shown that the vacuole is an essential compartment for maintaining cytosolic pH and ion homeostasis under salt stress condition. Proton-translocation inorganic pyrophosphatase (V-PPase) and  $\text{Na}^+/\text{H}^+$  antiporter are two primary solutes transporters in vacuole. The  $\text{H}^+$  electrochemical gradient across vacuolar membranes generated by V-PPase provide the driving force for the accumulation of ions and other solutes into the vacuoles by  $\text{Na}^+/\text{H}^+$  antiporter (Sze *et al.*, 1992). Therefore, one possible strategy in response to salt stress is the changing of the transcript level of these transporters. The changing of  $\text{Na}^+/\text{H}^+$  antiporter transcript level under salt stress has been provided by several studies (Fukuda *et al.*, 2004). But, the effect of salt treatment on the transcript levels of V-PPase is unknown. Also, there are no reports about the simultaneous analysis of the effect of salt stress on the transcript levels of the  $\text{H}^+$ -pumps and  $\text{Na}^+/\text{H}^+$  antiporter in the halophytes.

Therefore, the aim of this study was to evaluate the effect of salinity on vacuolar  $\text{Na}^+/\text{H}^+$  antiporter and vacuolar proton-pyrophosphatase transcript levels using Real Time PCR and physiological changes during salt stress.

## MATERIALS AND METHODS

### Plant Material

The seeds of *L. fusca* (L) were obtained from the Agricultural Biotechnology Research Institute of Iran (ABRII). The seeds were first sown in pots (five seeds per pot) containing sand and then irrigated with distilled water until germination. Seedlings were then transferred to hydroponic chambers in green house under condition of 16:8 hours (light: dark) photoperiod, at 25°C and fed with a half strength Hoagland's solution (Hoagland and Anon, 1950). Nine-week old plants were treated with five levels

of salinity (0, 100, 200, 300, 500 mM NaCl) in half strength Hoagland solution.

### Determination of $\text{Na}^+$ Contents in Roots

Plant materials were harvested after four different treatment sessions (6, 12, 24, and 48 hours) and then freeze-dried at -42°C for 24 hours, followed by digestion in 5-M  $\text{HNO}_3$ . The digested plant material was filtered, diluted with distilled water, and analyzed for  $\text{Na}^+$  concentrations using a Flame Photometry (Jenway PFP7).

### Isolation of mRNA and Synthesis of First-strand cDNA

Plants roots of the aforementioned treatments (6, 12, 24, and 48 hours) were briefly rinsed in distilled water, blotted dry, and were immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. Total RNA was extracted from about 50 mg of the frozen tissues of the root samples using the Fast Pure™ RNA Kit (Takara, Cat#9190). The quality of extracted RNA and its integrity were determined by running on agarose gel electrophoresis and nano drop spectrophotometer.

For removing DNA contamination, RNA samples were treated by DNase I (Fermentas, USA) for about 30 minutes. The first strand of cDNA was synthesized using Prime Script™ 1<sup>st</sup> strand cDNA Synthesis Kit (Takara, Cat#6110B) following the manufacturer's instructions.

### Real-Time PCR

Real-Time PCR assays were performed by ABI PRISM® 7300 Real-Time Amplification Thermal Cycling System. For the quantitative determination of *L. fusca* PPase and *L. fusca*  $\text{Na}^+/\text{H}^+$  in qPCR mixture, SYBR® Premix Ex Taq™ Perfect Real Time PCR (Takara, Cat#RR041A) was used. The pairs of primer for the V-PPase were designed according to

their sequence available in NCBI (Accession No. GQ387485.1) (Table1). In this experiment, 18S RNA was used as a house keeping gene.

Since the sequence of Na<sup>+</sup>/H<sup>+</sup> antiporter gene in *L. fusca* was not available in the database, the amplification of this gene was carried out by using degenerated primers, previously designed by Zhang *et al.* (2008) on the conserved regions of this gene in other plants.

The product of PCR amplification was first sequenced and the sense and antisense primers were designed for Real-Time PCR.

PCR reactions in a final volume of 20  $\mu$ l were initiated with a 30 seconds denaturation at 95 °C. The cycling program consisted of: denaturation at 95°C for 3 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 31 seconds repeated for up to 40 cycles.

Melting curves for every gene were obtained using SYBR-green fluorescence and did not show any redundant pick formation. Real Time PCR products were also checked on agarose gel electrophoresis to confirm the correct and single band.

#### Data Analysis of Real-Time PCR

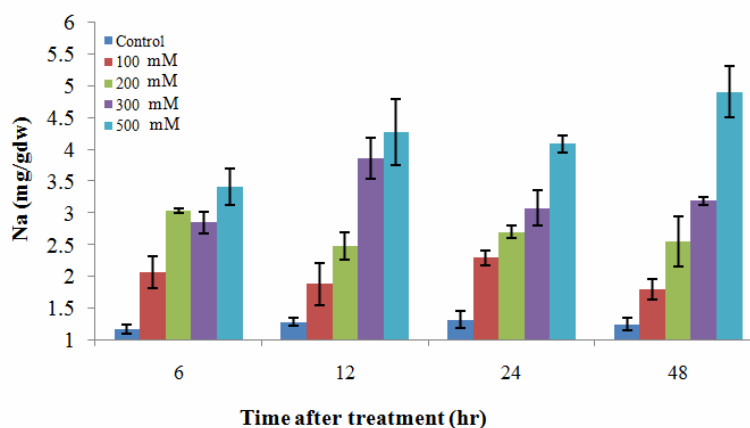
The expression level of the

proton-pyrophosphatase was quantified using the relative quantification method. The threshold cycle values ( $C_T$ ) of all products were compared with those of the 18S RNA gene as a house-keeping gene which was not affected by the treatment. The delta delta  $C_T$  ( $\Delta\Delta C_T$ ) values were calculated by subtracting the  $dC_T$ 's from none treated and treated samples, and fold changes were calculated using the  $2^{-\Delta\Delta C_T}$  method (Livak and Schmittgen, 2001). Which  $\Delta\Delta C_T = (C_{T,target} - C_{T,18S})_{salt\ treated} - (C_{T,target} - C_{T,18S})_{untreated}$ . SAS program was used for data analysis.

## RESULTS

### Sodium Accumulation

The Na<sup>+</sup> content (mg per g of dry weight) accumulated in roots when the NaCl concentration was increased (Figure 1). However, the Na<sup>+</sup> content in 500 mM NaCl treatment was markedly higher than those in other levels of salinity (Figure 1). At all NaCl levels, there was no significant difference among the time courses, but different Na<sup>+</sup> content were observed between levels of salinity (Figure 1).



**Figure 1.** Accumulation of Na<sup>+</sup> content in roots of *Leptochloa fusca* in response to different levels of salinity in different time of salinity treatment.



### Relative Expression of the Vacuolar Na<sup>+</sup>/H<sup>+</sup> Antiporter

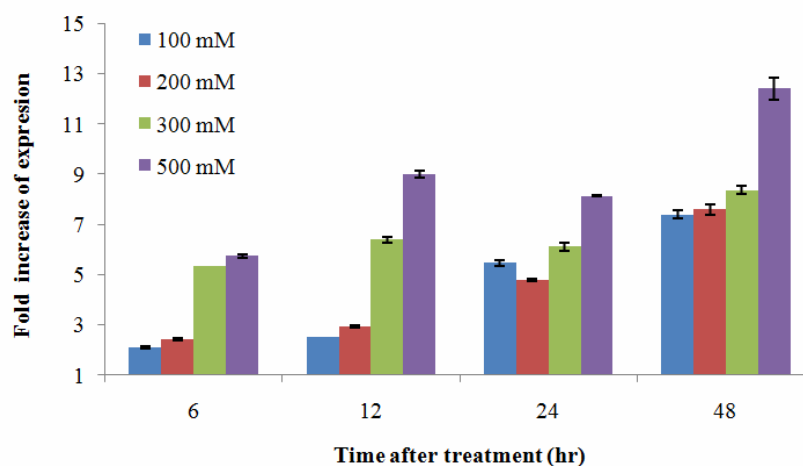
The analysis clearly showed an induction of the expression of *Lf Na<sup>+</sup>/H<sup>+</sup>* in roots by NaCl treatment (Figure 2). When plants were treated with 100 mM NaCl, the expression level of *Lf Na<sup>+</sup>/H<sup>+</sup>* gene was 2 folds in comparison with the control 6 hours after treatment, while in 200, 300 and 500 mM NaCl treatments, the expression levels were, respectively, 2.5, 5, and 6 fold the control. In 48 hours after the treatments, the changes for 100, 200, 300 and 500 mM NaCl were 7, 7.5, 8, and 12 fold, respectively. Comparison of all treatments revealed that the maximum level of the expression of Na<sup>+</sup>/H<sup>+</sup> antiporters in roots occurred in 500 mM NaCl treatment after 48 hours, which was a 12-fold change (Figure 2).

Severe increase in expression of *Lf Na<sup>+</sup>/H<sup>+</sup>* in 100 and 200 mM NaCl began 24 hours after the NaCl treatment, whereas in 300 and 500 mM NaCl, increase in *Lf Na<sup>+</sup>/H<sup>+</sup>* transcript level began 12 hours after treatment. This might be related to Na<sup>+</sup> content in the culture medium and in

the root tissues.

### Relative Expression of the Vacuolar PPase Antiporter

As shown in Figure 2, the expression level of *Lf PPase* in salt-treated plants exhibited the following patterns: in comparison with the control, treatment with 100 mM NaCl caused 1.5-, 1.5-, 2.1- and 3.1-fold increase in the expression of *Lf PPase* in roots, respectively, 6, 12, 24 and 48 hours after the treatment. Also, transcript level of *Lf PPase* in 200 mM NaCl treatment increased by 1.3-, 1.7-, 2.1- and 4.5-folds, respectively, in the four aforementioned time-intervals. Changes of *Lf PPase* transcript at the other levels of salinity are shown in Figure 2. Comparison of *Lf PPase* expression in the four treatments of NaCl showed that the maximum increase in the level of *Lf PPase* transcript was 6 folds and was recorded in 500 mM NaCl treatment. Also, we observed that by increasing the time exposure to NaCl, the *Lf PPase* transcript levels were increased.



**Figure 2.** Increasing of the expression level of putative Na<sup>+</sup>/H<sup>+</sup> antiporter in *Leptochloa fusca* upon salt stress. Transcript levels were determined by Real-Time PCR. Data represent mean

## DISCUSSION

### Na<sup>+</sup> Accumulation

Since enzymes of halophytes are as sensitive to salinity as those of glycophytes, the maintenance of Na<sup>+</sup> homeostasis becomes even more crucial (Glenn *et al.*, 1994). To prevent the growth cessation or cell death, the excessive amount of Na<sup>+</sup> should be extruded or compartmentalized in the vacuole (Zhu *et al.*, 2003). Higher concentration of Na<sup>+</sup> in roots after NaCl treatment compared to the control (Figure 1) indicate an effective sequestration of Na<sup>+</sup> into the vacuoles, resulting in the tolerance of this plant to high cytoplasmic Na<sup>+</sup> concentration. Moreover, previous studies have shown that the uptaking of Na<sup>+</sup> by shoots are one of the major strategies of this plant for tolerating high salinity (Jeschke *et al.*, 1995). This study showed that the accumulation of Na<sup>+</sup> in root vacuoles may be important. Since the halophytes usually use Na<sup>+</sup> and Cl<sup>-</sup> as a cheap osmolytes sequestering them into the vacuoles (Greenway and Munns, 1980).

### Relationship of V-Na<sup>+</sup>/H<sup>+</sup> Antiporter and V-PPase Pump with Na<sup>+</sup> Accumulation and Salt Resistance

In order to determine the importance of Na<sup>+</sup>/H<sup>+</sup> antiporter in Na<sup>+</sup> compartmentalization, its transcript level was analyzed by Real Time PCR in roots. Although Na<sup>+</sup>/H<sup>+</sup> is the first and most intensively studied vacuolar antiporter to date (Blumwald *et al.*, 2000; Saqib *et al.*, 2005), the regulation of its gene expression in response to salinity condition, especially in halophytes, is largely unknown. Conflicting data regarding the expression *AtNHX1* in Arabidopsis under salinity condition has been reported (Gaxiola *et al.*, 1999; Apse *et al.*, 1999).

In our experiment, the *Lf Na<sup>+</sup>/H<sup>+</sup>* transcript level was up-regulated by NaCl. In 500 mM salinity, the relative expression of the *Lf Na<sup>+</sup>/H<sup>+</sup>* antiporter in the roots was 29% higher than the controls (Figure 2). This high expression may indicate the sequestration of more Na<sup>+</sup> into the vacuoles, so the cytoplasm should be safe from the toxic effects of Na<sup>+</sup>. These data are also in agreement with previous studies, which have shown that the higher expression of the Na<sup>+</sup>/H<sup>+</sup> antiporters in transgenic plants can help avoid Na<sup>+</sup> stress (Apse *et al.*, 1999, Zhang *et al.*, 2001, Fukuda *et al.*, 2004).

In one study, the expression of Na<sup>+</sup>/H<sup>+</sup> (Fukuda *et al.*, 1999) was two-fold in both shoots and roots of treated rice plant with 100 mM NaCl in comparison with control plants. Using whole plants of *A. thaliana* (ecotype Columbia), mRNA of *AtNHX1* was found to increase by 4.2- and 2.8-folds after 6 hours of treatment by 250mM NaCl or KCl, respectively (Gaxiola *et al.*, 1999). In spite of previous studies, Apse *et al.* (1999) and Cosentino *et al.* (2010) analyzed the root mRNAs of wild type *A. thaliana* and *Mesembryanthemum crystallinum* at different times after NaCl treatment, but they could not detect any increase in *NHX* transcript levels. However, our result showed an increase in the transcript level of *Lf Na<sup>+</sup>/H<sup>+</sup>* in roots at different time intervals i.e. 6, 12, 24, 48 hours (Figure 2). Also, our results showed a high correlation between Na<sup>+</sup> concentration of tissue and Na<sup>+</sup>/H<sup>+</sup> expression, which agrees with the study of Fukuda *et al.* (1999). However, an increase of transcript levels of *Lf Na<sup>+</sup>/H<sup>+</sup>* in roots suggests that this antiporter plays a major role in Na<sup>+</sup> loading of the xylems in roots in *L. fusca*. The activity of proton pumps such as V-pyrophosphatase could influence salt tolerance through affecting the membrane potential and proton gradient.

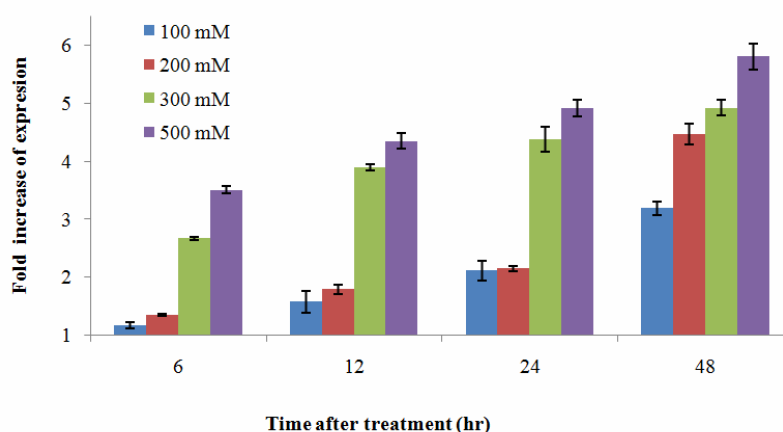
Both factors can have an effect on the uptaking, exclusion, and sequestration of Na<sup>+</sup> and other ions, which greatly impact



salt tolerance. Fukuda *et al.* (2004) showed that PPase transcript increased in response to salt stress in barley. In our experiment, salt stress also caused an increase in the transcript level of *Lf PPase* in *L. fusca* roots (Figure 3). Similar results have been reported in *B. vulgaris* and *N. tabacum* (Kim *et al.*, 1994; Lerch *et al.*, 1995).

We conclude that increasing *Lf PPase* transcript can bring about a significant

degree of salt resistance. Also, our experiment showed that there was a high correlation between the expression of V-PPase and *Lf Na<sup>+</sup>/H<sup>+</sup>* in different NaCl treatments and also different times after treatments started (Table 2). The authors suggest that *Lf PPase* complemented the activity of *Na<sup>+</sup>/H<sup>+</sup>* antiporter in halophytic plants under abiotic stress conditions.



Values±SD (n= 3)

**Figure 3.** Quantification of *Leptochloa fusca* vacuolar PPase expressions in response to 100, 200, 300 and 500 mM NaCl in different times after treatment.

**Table 1.** Primer sequences used in Real time PCR reactions.

Primers	Forward	Reverse	Product size
V-PPase	5'-ATCTTCGGTCTTGCTCTTGG-3'	5'-CTCATTCCAGCCATCTCAGC-3'	210 bp
V- <i>Na<sup>+</sup>/H<sup>+</sup></i>	5'-TTCTGGATTGCTCAGTGCTT-3'	5'-CAGCCAGCATGTAAGAGAGG-3'	110 bp
18S rRNA	5'-ATGATAACTCGACGGATCGC-3'	5'-CTTGGATGTGGTAGCCGTTT-3'	170 bp

**Table 2.** Correlation between the content of *Na<sup>+</sup>*, the expression levels of Vacuolar *Na<sup>+</sup>/H<sup>+</sup>* antiporter and Vacuolar PPase genes in response to salt stresses

	<i>Na<sup>+</sup></i>	PPase	<i>Na<sup>+</sup>/H<sup>+</sup></i>
<i>Na<sup>+</sup></i>	1.0000	0.6104*	0.6743**
PPase	0.6104*	1.0000	0.9279**
<i>Na<sup>+</sup>/H<sup>+</sup></i>	0.6743**	0.9279**	1.0000

\*: Significant at 5 % level, \*\*: Significant at 1 % level.

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### بیان هماهنگ پمپ پروتونی PPase و آنتی پورتر واکوئلی $Na^+/H^+$ در واکنش به تنش های کوتاه مدت در گیاه هالوفیت تک لپه ای *Leptochloa fusca*

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#### چکیده

شوری، از تنش های غیر زیستی مهم به خصوص در مناطق خشک و نیمه خشک می باشد. به نظر می رسد که گیاهان با مکانیسم های پیچیده ای در سطح کل گیاه، سطح سلولی و یا مولکولی به تنش پاسخ می دهند. از جمله این پاسخ ها تغییر در بیان ژن های ترانسپورترهای یونی درگیر در جذب و انتقال آب و یون ها می باشد. لذا در این تحقیق اثرات شوری (۰، ۱۰۰، ۲۰۰، ۳۰۰، ۵۰۰ میلی مولار NaCl) کوتاه مدت (۶، ۱۲، ۲۴، ۴۸ ساعت) بر میزان بیان آنتی پورتر واکوئلی  $Na^+/H^+$  و پمپ واکوئلی  $H^+$  در گیاه هالوفیت تک لپه ای *Leptochloa fusca* با استفاده از روش Real time PCR مورد بررسی قرار گرفت. نتایج نشان داد که اولاً این گیاه دارای توانایی بالایی در تجمع سدیم در ریشه خود می باشد نیز آنالیزهای مولکولی حاکی از افزایش بیان این ترانسپورترها در تمام تیمارهای شوری دارد. بطوریکه تغییرات بیان آنتی پورتر واکوئلی  $Na^+/H^+$  بیشتر از  $H^+$ -PPase بوده که نشانگر نقش مهمتر این آنتی پورتر در مقاومت به شوری در این گیاه می باشد. نیز تجزیه و تحلیل داده ها حاکی از وجود همبستگی بالای بین بیان آنتی پورتر واکوئلی  $Na^+/H^+$  و پمپ پروتونی  $H^+$ -PPase و تجمع سدیم در پاسخ به تنش شوری می باشد.