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Up-regulation of plasma membrane H^+-ATPase under salt stress may enable *Aeluropus littoralis* to cope with stress

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A B S T R A C T

Plasma membrane H^+-ATPase is a major integral membrane protein with a role in various physiological processes including abiotic stress response. To study the effect of NaCl on the expression pattern of a gene encoding the plasma membrane H^+-ATPase, an experiment was carried out in a completely random design with three replications. A pair of specific primers was designed based on the sequence of the gene encoding plasma membrane H^+-ATPase in *Aeluropus littoralis* to amplify a 259 bp fragment from the target gene by PCR. A gene encoding actin was used as reference gene to normalize the expression level of the target gene. A pair of specific primers was designed to amplify a 157 bp fragment from the actin gene by PCR. Plants were treated with different concentrations of NaCl, 0, 50, 100, 150, 200, 250, 500 and 1000 mM, for two days. Our results showed that the expression level of the plasma membrane H^+-ATPase gene increased dramatically at 500 mM and then decreased with increasing concentrations of NaCl. The results also indicated that the leaves of plants, were treated with high concentrations of NaCl changed morphologically, but those grown under low concentrations of NaCl as well as the control plants did not show morphological changes in their leaves. Our results suggest a relation between morphological changes of treated plants and the expression level of the plasma membrane H^+-ATPase gene in *Aeluropus littoralis*.

Key words: Gene expression; Gouan, NaCl; Proton pump; Real-time PCR

INTRODUCTION

World agriculture faces many problems and challenges, mostly caused by biotic and abiotic stresses such as salt stress. Concerns about the effect of salt stress on plants have come to dominate those regarding the environment [1, 2]. Much of the injury to plant

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cells caused by salt stress is associated with oxidative damage and ion homeostasis disruption during stress [3].

Plasma membrane (PM) H^+- ATPases are major species of plasma membrane ion pumps [4], which are integral membrane proteins that use chemical energy to transfer protons to the extracellular environment by a primary active transport [4, 5]. PM proton pump is involved in many physiological processes in plants like salt tolerance [6, 7]. Recently, a number of studies have demonstrated that the expression of the PM H^+-ATPase gene increased in response to salt stress [8, 9]. The way salt stress up-regulates the expression of PM H^+- ATPase may be revealed by comparing halophytic and ordinary plants.

Gouan, *Aeluropus littoralis*, is a monocotyledonous angiosperm that thrives in salt areas [10]. Several studies have been carried out on salt-resistance in gouan but little is known about the mechanism. In other halophytes, the activity of PM H^+-ATPase has been shown to increase under salt stress [11]. It has also been shown that PM H^+-ATPase activity in *Zostera marina*, a marine halophytic plant, was not inhibited by NaCl treatment, but PM ATPase activities were inhibited in rice or freshwater grass [12]. Mature leaves of halophytes have also been reported to have protoplasts with high ATPase activity, which increases the capacity of ion flux between the inside and outside of the cell [13, 14]. These results indicate that salt tolerant ATPase activity in the plasma membrane must play a critical role in the regulation of ion homeostasis under salt stress in halophytic plants.

The aim of the present work was to examine the effect of salt on the expression pattern of PM H^+-ATPase gene in halophyte *A. littoralis*. The correlation between morphological changes in leaves and the expression pattern of PM proton pump gene were also studied.

MATERIALS AND METHODS

Plant materials: *A. littoralis* seeds were obtained from Pakan Seed Research Center, Isfahan, Iran. The seeds were surface sterilized by soaking in 1% (v/v) sodium hypochlorite for 20 min and rinsed several times with distilled water. They were then put in Petri dishes containing 2 moist filter paper sheets and germinated in an incubator at 25 ºC. The germinated seeds were transplanted into pre-compressed Jiffy pots soaked in water, and incubated in a growth chamber (with 25:16 ºC day:night temperature, 16 h light/8 h dark photoperiod) and irrigated daily with ½ MS nutrient solution [15].

NaCl treatment: After two months, plants were treated with NaCl at different concentrations for 48 h. To prevent osmotic shocks, NaCl was added gradually (50 mM every day) to plants up to the final concentrations of 0, 50, 100, 150, 200, 250, 500 or 1000 mM. The experiment was carried out in a completely randomized design with 3 replicates.

Primer design: Gene coding sequences for PM H^+-ATPase (accesstion number: AB686268) and actin (accesstion number: FJ603097) were obtained from the NCBI website. Two-pair primers were designed based on the sequences of these genes using Vector NTI (Version 9) program (Table 1).

Table 1. The sequences of primers used to amplify the genes encoding a plasma membrane H^+-ATPase (target gene) and actin as reference gene in Q-PCR.

Gene	Name of Primer	Sequence	TM (°C)
target gene	PMH1RTPF	5'- ACCTGAGAAGACCAAGGAGTCT-3'	62.15
	PMH1RTPR	5'- TACAGGAAGTGCTTCAAGTGTAG-3'	60
actin gene	ActinAlF	5'- CGTACAACTCCATCATGAAGTG-3'	61.96
	ActinAlR	5'- CAAACACTGTACTTTCTCTCCG-3'	60.35

RNA extraction and cDNAs synthesis: Total RNA was isolated from *A. littoralis* leaves using RNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's instructions. The quality of RNA was assessed by electrophoresis on agarose gel. cDNAs were reverse transcribed from the total RNA extracted from leaves using SuperScript II Kit (Invitrogen, USA) with oligo(dt) primer [16].

RT-PCR: cDNAs were amplified by PCR using a specific primer for the gene encoding plasma membrane proton pump. PCR reactions were carried out in a final volume of 20 µl reaction mixture containing 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µl dNTPs, 0.3 µM of each primer and 1 unit of ExTaq DNA polymerase (Takar, Japan) under following conditions: 5 min 94ºC followed by 30 cycles at 94ºC for 30s, 60ºC for 1 min, and 72ºC for 5 min, with a final extension at 72ºC for 15 min.

Quantitative real-time PCR: PCR reactions were carried out in a final volume of 10 µl containing 5 µl of SYBR Premix ExTaq (Takar, Japan), 1 µl cDNAs and 0.3 µM of each primer (Table 1) under the following conditions: 1 min at 94ºC, 45 cycles at 94ºC for 15s, 60ºC for 15s, and 72ºC for 30s. At the end of the program, the specificity of the primers' set was confirmed by melting curve analysis (65-95ºC with a heating rate of 0.5ºC/min). The copy numbers of the genes' mRNAs were estimated by comparing the results of real-time PCR with serial dilutions (10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies/µl) of the plasmid containing amplified fragments of each gene. The gene which encodes actin was used to normalize the expression ratio of the target gene.

RESULTS AND DISCUSSION

The quality of the extracted RNA was determined by electrophoresis on 1% agarose gel. The OD260/OD280 ratio of the extracted RNA was 1.8. Bands corresponding to 18S and 28S rRNA were distinctly visible on the gel, indicating high quality, non-degraded RNA (Fig. 1).

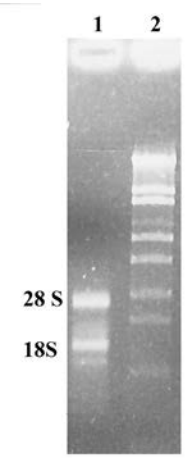

Figure 1: Total RNA extracted from leaves of *A. littoralis* was separated by 1% agarose gel electrophoresis and stained with ethidium bromide. 1) Total RNA extracted from sample and 2) size marker λ/*Sty*I.

Since RT-PCR is a reaction sensitive to the presence of inhibitors or the degradation of RNA, cDNAs prepared from RNA were used as template in a standard PCR reaction to test RNA quality. A 259 bp fragment was amplified with a specific primer for the PM proton pump gene (Fig. 2) which indicated the high quality of the prepared cDNAs.

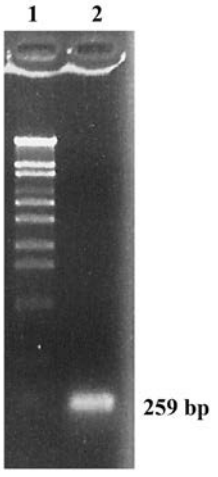

Figure 2: Agarose gel electrophoretic analysis of RT-PCR-amplified cDNA of a plasma membrane proton pump gene. 1) Size marker λ/*Sty*I and 2) A 259 bp fragment was amplified from cDNAs prepared from RNA of leaves.

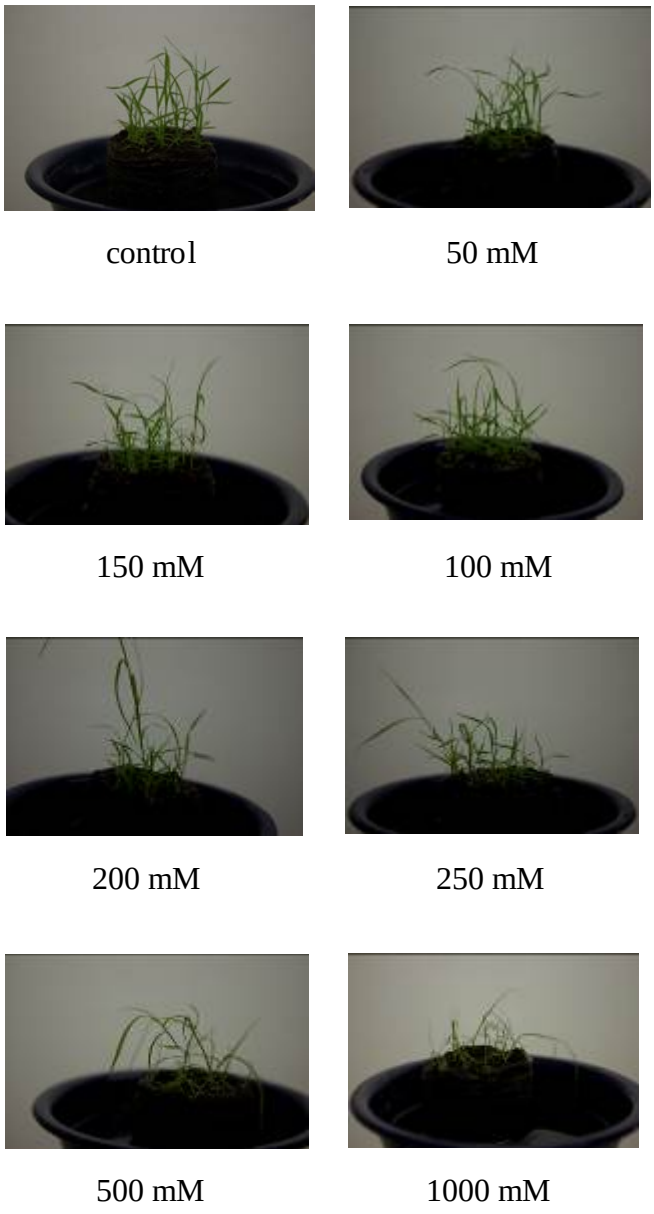

Figure 3: *A. littoralis* plants were treated by different concentrations of NaCl (control, 50, 100, 150, 200, 250, 500, 1nd 1000 mM).

The results also demonstrate a lack of significant changes in the expression level of the gene encoding PM H^+-ATPase in *A. littoralis* with increasing NaCl concentrations; however, this expression suddenly increased at 500 mM, followed by a dramatic decrease at 1000 mM (Fig. 4). It may be suggested that the cells of this plant, which must be salt tolerant through intrinsically cellular mechanisms, regulate the expression of the proton pump gene in response to NaCl to accommodate the solute accumulation

necessary for osmatic adjustment. Since the Na^+ ion is one of the solutes toxic to cytosolic metabolisms, it must be extruded out of the cell by a PM Na^+/H^+ antiporter.

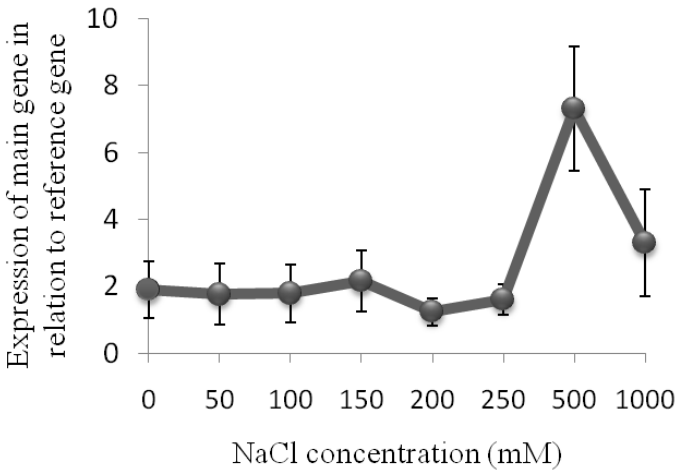

Figure 4: Quantitative Real-time PCR analysis. The relative expression level of a gene encoding plasma membrane proton pump in leaves of *A. littoralis* (normalized by the level of actin gene, as a housekeeping gene) is shown.

As mentioned above, morphological changes were also induced at 500 mM, suggesting that proton pump in *A. littoralis* has an important role in its response to salt stress and that a reduction of its expression can result in a wide variety of morphological changes. It has been previously shown that the expression of genes which encode H^+-ATPase pumps in different plants increased in response to salt stress [8, 21, 22]. Similarly, our results showed the expression pattern of H^+-ATpase gene to be affected by salt stress; yet, no other report has indicated the expression of the PM proton pump gene to increase markedly in high concentrations of NaCl (500 mM) or to decline under still higher concentrations (1000 mM). Although the expression of some genes such as *Tsvp*, a gene for H^+-pyrophosphatase in *Thellungiella halophila*, was shown to have increased during the first 16 h under high concentrations of NaCl (300 mM) and decreased after 16 h, the way the expression was regulated under high concentrations of NaCl was not investigated [23].

It can be suggested that *A. littoralis* cells regulate the expression of the plasma membrane proton pump gene in response to NaCl to generate a proton motive force (PMF). PMF is the source of energy for a variety of cellular proteins such as Na^+/H^+ antiporters [24]. Despite the fact that toxic ions such as Na^+ would be compartmentalized in the vacuole by tonoplast antiporters and pumps [25], extruding them out of the cell across the plasma membrane is critical to the maintenance of ionic homeostasis in the cytosol carried out by plasma membrane antiporters and pumps [24, 26]. The activity of PM Na^+/H^+ antiporters in *A. littoralis* cells lacking Na^+ ATPase

activities induces the ability of these cells to export Na^+ ions and greatly increase their resistance to salinity [27]. It has been shown that small changes in proton pump activities are important in plant growth and abiotic stress tolerance [28]. It can be thus suggested that salt stress triggers the expression of the PM proton pump to provide the driving force for Na^+ exclusion. In other words, PM H^+-ATPase and Na^+/H^+ antiporters may coordinate the regulation of salt tolerance.

It seems that the activity of this protein is essential for ion homeostasis in *A. littoralis* under 500 mM NaCl, but under a higher 1000 mM concentration when the plant needs more energy, the quantity of this protein decreases to minimize energy expenditure by proton pumps, thus increasing fitness. In addition, it is possible for cell membranes to be destroyed by exposure to high concentrations of NaCl, decreasing the necessity of proton pump and its expression. It is shown in figure 3 that plants were severely affected by salt stress at 1000 mM NaCl.

Enhanced expression of the PM proton pump gene was detected in *A. littoralis* leaves in 500 mM of NaCl, but not in the higher concentration (1000 mM). The high degree of NaCl induction of PM H^+-ATPase mRNA accumulation in *A. littoralis* leaves indicates a requirement for this pump in the cells during salt adaptation. Our data revealed that the halophyte *A. littoralis'* was fast to respond to NaCl by inducing the PM proton pump gene expression. This result is consistent with a previous report that showed a rapid turnover of the PM proton pump in maize [29].

On the basis of our results, a correlation between salt-induced morphological changes and the expression level of the PM proton pump gene in *A. littoralis* may be also suggested. The great capacity of halophyte *A. littoralis* to induce the PM proton pump gene in response to high concentrations of NaCl indicates the presence of aunique regulatory elements and/or transcription factors that are highly responsive to salt stress.

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Conflict of Interest: The authors declare no conflicts of interest.

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