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Vacuolar proton pumps regulation during development of *Vigna unguiculata* seedlings under salt stress

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Abstract Global climatic changes as high temperatures and low precipitation contribute to increase cultivated areas affected by high salt soil content. Soil salinity is well known to reduce the ability of plants to take up water and this quickly causes reduction in their growth rate. V-ATPase (EC 3.6.3.14) and V-PPase (EC 3.6.1.1) hydrolytic and proton transport activities, and gene expression were evaluated in hypocotyls of 3-, 5-, 7-day-old *Vigna unguiculata* (L.) Walp cv. Vita 3 germinated in 100 mM NaCl in order to highlight their differential regulation and activity modulation under salt stress. Semi-quantitative RT-PCR revealed that both genes were up-regulated by salt stress in all salt exposition times studied. Up-regulation was correlated with the increase in protein content at 5 and 7-day-old seedlings. Co-expression between A and E V-ATPase subunits was also observed. The hydrolytic and proton transport activities showed that these enzymes presented a differential modulation of their activities in the presence of 100 mM NaCl. These results suggest that V-ATPase and V-PPase activities are modulated by salt stress and a multi-step regulation is exerted in order to re-establish homeostasis.

Keywords Cowpea · Proton pump · Salinity stress · Vacuolar membrane

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Abbreviations

ACMA	9-Amino-6-chloro-2-methoxyacridine
BTP	Bis-tris-propane [1,3-bis(tris(hydroxyl-methyl)methylamino)-propane]
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
PMSF	Phenylmethyl-sulfonyl fluoride
V-ATPase	Vacuolar H^+-ATPase (EC 3.6.3.14)
V-PPase	Vacuolar H^+-PPase (EC 3.6.1.1)

1 Introduction

Among the changes expected due to the climate change, higher frequency of drought episodes and augmentation of soil salinity are between the more severe ones. Drought and salinization of arable soils are becoming major threats to agriculture, food security and environment in the next decades (Koyro et al. 2012). Accumulation of salts in irrigated soil is a primary factor reducing crop yield and its the most negative effect is the disruption of the subsistence agriculture chain in arid regions of developing countries (Zhu 2001; Brini et al. 2007). Among the factors contributing to the excess of salt in the rooting zone of arable land are the use of poor quality of irrigation water, poor soil drainage, and the movement of soluble salts from deep to top layers of the soil profile due to evapotranspiration (Chinnusamy et al. 2005; Rozema and Flowers 2008).

To survive stresses imposed by environmental fluctuations, plants have evolved a wide spectrum of molecular programs to sense and rapidly change their metabolism, adapting itself to suboptimal conditions. Aside osmotic adjustment, a key factor conferring salt tolerance is the control of intracellular ion homeostasis, involving net intracellular Na^+ and Cl^- uptake and subsequent vacuolar compartmentalization without toxic ion accumulation in the cytosol. Accumulation of ions in the vacuole avoids ionic strength increase in the cytoplasm as well as increases cellular osmolarity to counter-act osmotic stress (Sairam and Tyagi 2004). Most cultivated plants are glycophytes and can not tolerate salt stress due to osmotic stress, injurious effects of toxic Na^+ and Cl^- ions and nutrient imbalance. It is suggested that Na^+ in glycophyte plants is not readily sequestered into vacuoles, as observed in halophytes, thus causing growth inhibition

and development delays (Blumwald et al. 2000; Dietz 2001; Sairam and Tyagi 2004; Marques et al. 2013).

Vacuole and tonoplast are suggested as having an important role during plant acclimation, setting up a durable tolerance to salt stress. Vacuolar membrane has several components, such as proton-pumps (V-ATPase and V-PPase) and active transporters of sugars and inorganic ions (Maeshima et al. 1996). It is well known that these vacuolar enzymes play essential roles in the plant development and during the metabolic responses to environmental changes such as salt stress. Vacuolar proton pumps are vital to the plant development, as they promote cellular growth and turgor pressure for cell wall expansion, regulation of cytosol pH and ionic homeostasis maintenance (Colombo and Cerana 1993). Furthermore, the electrochemical proton gradient promoted by V-ATPase and V-PPase is the driving force for the accumulation of ions and other solutes in the vacuole as it is used to power vacuolar secondary transporters (Fukuda and Tanaka 2006). The effect of NaCl stress on V-ATPase and V-PPase activities and protein turnover has been explored over the past years. Genetic engineered plants for higher vacuolar proton pump activity has been proven as a successful strategy to increase salt tolerance. Transgenic plants of Arabidopsis (Gaxiola et al. 2001; Brini et al. 2007), tobacco (Gao et al. 2006; Duan et al. 2007), tomato (Park et al. 2005) and cotton (Asad et al. 2008; Lv et al. 2008) overexpressing V-PPase were more tolerant to salt stress (Li et al. 2010). Nevertheless, the regulation of both proton pumps V-ATPase and V-PPase by salt stress is not clear yet. Many reports have shown controversial results and a full understanding of the regulation of the vacuolar proton pumps role should target regulation mechanisms, gene expression control, post-translational regulation, protein turnover, and modulation of the vacuolar proton pumps activities (Nakamura et al. 1992; Binzel 1995; Ballesteros et al. 1996; de Oliveira Otoch et al. 2001; Silva and Gerós 2009).

It was previously reported that two cultivars of *Vigna unguiculata* (L.) Walp have different levels of drought and salt tolerance, Vita 3 being more tolerant than Vita 5 (Fernandes de Melo et al. 1994). Otoch et al. (2001) showed that V-ATPase and V-PPase of 7-day-old seedling hypocotyls of Vita 5 have a distinct activity profile for both enzymes and V-ATPase was suggested as being the responsive proton pump. In this work we investigated the differential regulation of

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Table 1 Primers and RT-PCR cycling conditions

Gene	Primers	Annealing/cycles
VuVHA-A	Forward: 5′ GCCTCCTGATGCCATGGGA 3′	62.5 °C/25 cycles
	Reverse: 5′ CGCATCATCCAAACAGACT 3′	
VuHVP	Forward: 5′ ACTGGTTATGGTCTCGGTGGGT 3′	55 °C/25 cycles
	Reverse: 5′ CCAGGGCATCAGTTCTCTCACG 3′	
Actin	Forward: 5′ GCGTGATCTCACTGATGCC 3′	55 °C/25 cycles
	Reverse: 5′ TCGCAATCCACATCTGTTGG 3′	

V-ATPase and V-PPase in response to salt stress in 3-, 5- and 7-day-old seedling hypocotyls from *V. unguiculata* cv. Vita 3 by assaying transport and hydrolysis activities and gene expression.

2 Materials and methods

2.1 Plant material

Seeds of *V. unguiculata* (L.) Walp cv. Vita 3 were soaked in distilled water for 1 h and germinated in filter paper for 3, 5 or 7 days at 26 °C in darkness. They were grown in the absence (control condition) or in the presence of 100 mM NaCl (salt stress condition).

2.2 Proton pumps gene expression analysis by RT-PCR

Manipulation of nucleic acids was performed using standard protocols. Total RNA was extracted from hypocotyls using RNA Plant Minikit (QIAGEN GmbH, Hilden, Germany), following manufacturer's instructions. The RT-PCR reactions were carried out using specific primers (Table 1). *VuVHA-A* (V-ATPase subunit A) and *VuHVP* (V-PPase) primers were obtained from the respective *V. unguiculata* sequences (accession numbers: DQ056751 and DQ056749). *Actin* was used as reference gene (Costa et al. 2004). The RT-PCR amplifications were analyzed in 1.5 % agarose electrophoresis using ethidium bromide staining, and image analysis was employed to compare the level of expression of genes against *Actin*, using the software Scion Image—Release beta 3b software (Scion Corporation—USA).

2.3 Preparation of tonoplast-enriched membrane vesicles

Tonoplast vesicles were isolated from homogenates of hypocotyls by a combination of differential and sucrose gradient centrifugations (Mariaux et al. 1994), with modifications. Hypocotyls were homogenized in a mortar with with buffer 100 mM Tris–HCl pH 8.0, containing 600 mM mannitol, 3 mM $MgSO_4$, 5 mM EDTA, 0.5 % (w/v) PVP 40, 1 mM DTT, 1 mM PMSF and 1 mM benzamidine. The homogenate was filtered through cheesecloth and centrifuged at $4,000 \times g$ for 10 min at 4 °C and the supernatant was layered over a 25 % (w/v) sucrose cushion containing 5 mM Hepes-Tris pH 7.5, 1 mM EDTA, 1 mM benzamidine, 2 mM DTT and 1 mM PMSF and then centrifuged at $150,000 \times g$ for 90 min at 4 °C. The tonoplast vesicles were banded upon the interface between the supernatant and sucrose cushion. Tonoplast vesicles were collected, diluted with one volume of a medium containing 50 mM MOPS-KOH, pH 7.0, 3 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 1 mM benzamidine, and then pelleted at $150,000 \times g$ for 50 min. The resulting pellet was solubilized in 10 mM MOPS-KOH, pH 7.0, 40 % (v/v) glycerol, 3 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine. All steps were performed at 4 °C and vacuolar membranes were stored in liquid nitrogen for later use.

Protein content in vacuolar membranes was quantified by the method of Lowry et al. (1951), using BSA as standard.

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2.4 SDS-PAGE and immunoblotting

Primary antibodies were prepared against subunits A and E from mung bean vacuolar V-ATPase (Matsuura-Endo et al. 1992; Kawamura et al. 2000, 2001) and the purified V-PPase (Maeshima and Yoshida 1989). Antigens were visualized with phosphatase-conjugated goat antiserum to rabbit IgG and color development reagents (Ward and Sze 1992). The levels of antigens on the nitrocellulose membrane were quantified using Scion Image—Release beta 3b software (Scion Corporation—USA).

2.5 Hydrolysis assay

Hydrolysis of adenosine triphosphate (ATP) and pyrophosphate (PPi) was assayed in a 0.5 mL reaction medium containing 25 mM Tris–HCl pH 7.2, 2.5 mM $MgSO_4$, 1 mM ATP-BTP or 1 mM PPi-MES, 0.2 mM $NaMoO_4$, 2 mM NaN_3, 250 M Na_3VO_4 and 70 mM KCl. The reaction was started by addition of 40 g·mL^{-1} of vacuolar membranes at 37 °C and after 30 min the amount of Pi released was determined (Fiske and Subbarow 1925). ATPase activity was measured in the presence and absence of 100 mM KNO_3, and the nitrate-sensitive activity was determined as the V-ATPase (O'neill et al. 1983). The activity induced by 70 mM KCl was determined as the V-PPase (Wang et al. 1986) and calculated as half the rate of Pi released from PPi (µmoles PPi consumed per unit of time). All reaction media for V-ATPase contained inhibitors for non-specific phosphatases (molybdate), for plasma membrane ATPases (vanadate), and for mitochondrial ATPases (azide). These inhibitors excluded ATP hydrolysis by phosphatases and ATPases other than V-ATPase.

2.6 H$^+$ transport assay

ATP- and PPi-dependent H$^+$-transport across tonoplast-enriched membrane vesicles were measured as the initial rate of fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) at 32 °C. The reaction medium for V-ATPase contained 50 mM Tris–HCl pH 7.2, 1 mM ATP-BTP, 2.5 mM $MgSO_4$, 0.1 mM Na_3VO_4, 2 mM NaN_3, 1 M ACMA, 100 µg of membrane vesicles and 70 mM KCl. The reaction medium for V-PPase contained 0.1 mM PPi-MES instead of ATP-BTP provided with inhibitors as indicated in the V-ATPase hydrolysis assay. The reaction was started by ATP-BTP or PPi-MES addition.

Fluorescence was monitored with a Hitachi F-2000 Fluorescence Spectrophotometer at excitation and emission wavelengths of 415 and 485 nm, respectively.

2.7 Statistical analysis

The differences between treatments were tested through the analysis of variance followed by Tukey post hoc test ($\alpha = 0.05$). The experiments were independently repeated at least six times, with three technical replicates for each sample. Data are presented as means $\pm$ STD.

3 Results

3.1 Vacuolar proton-pumps expression

The transcript levels of *VuVHA-A* and *VuHVP* in 3-, 5- and 7-day-old hypocotyls from seedlings of *V. unguiculata* cv. Vita 3 germinated in 100 mM NaCl were analyzed by RT-PCR (Fig. 1). Both *VuVHA-A* and *VuHVP* had their transcript levels increased in the salt treated seedlings. *VuVHA-A* expression increased 80, 40 and 275 %, and *VuHVP* increased 110, 40 and 25 % in 3-, 5- and 7-day-old seedling hypocotyls respectively, when compared to control ones.

3.2 Immunoblot analysis

Immunoblot analysis performed with antibodies raised against subunits A and E of V-ATPase and V-PPase
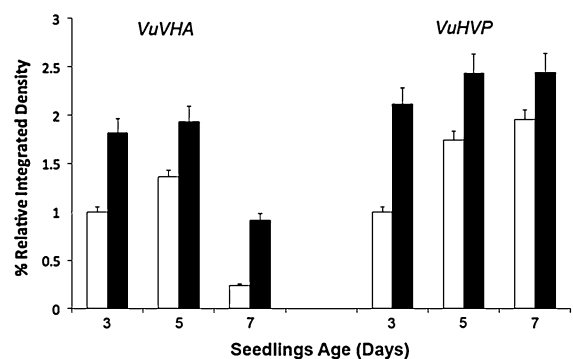

Fig. 1 Semi-quantitative expression of V-ATPase subunit A (*VuVHA*) and V-PPase (*VuHVP*), normalized with *Actin* cDNA and expressed as % of the 3-day-old control plants. *White columns* identify control treatment and *black columns* identify 100 mM NaCl treatment. *Vertical lines* indicate standard deviation
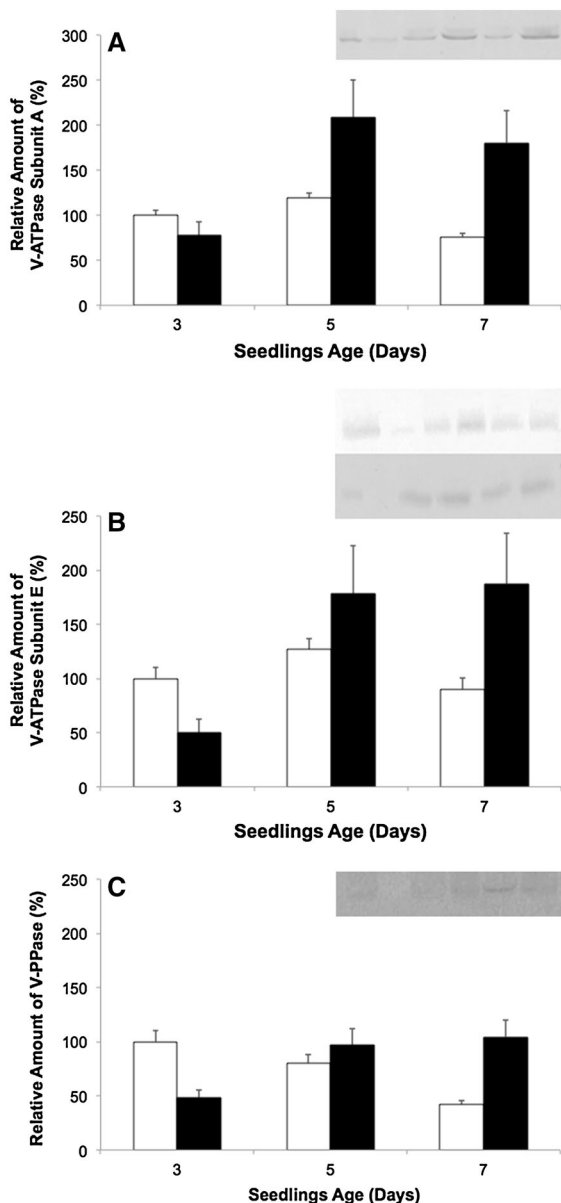
Fig. 2 Relative amount (columns) of V-ATPase subunit A (**A**), V-ATPase subunit E (**B**) and V-PPAse (**C**) detected by Western blotting and nitrocellulose membranes. From left to right in the nitrocellulose membrane lanes: 3-day-old control seedlings, 3-day-old salt stressed seedlings, 5-day-old control seedlings, 5-day-old salt stressed seedlings, 7-day-old control seedlings and 7-day-old salt stressed seedlings. *White columns* identify control treatment and *black columns* identify 100 mM NaCl treatment. *Vertical lines* indicate standard deviation

revealed that the relative amount of protein of vacuolar membranes from hypocotyls of *V. unguiculata* varied among 3-, 5- and 7-day-old plants (Fig. 2). V-ATPase subunit A and E showed an

increased in 5- and 7-day-old seedlings submitted to salt treatment when compared to control condition (Fig. 2A, B). The increase for subunit A was c.a. 80 and 145 % for 5- and 7-day-old plants, respectively (Fig. 2A) while subunit E enhancement was 40 and 109 % also when comparing 5- and 7-day-old plants (Fig. 2B). The amount of protein in 3-day-old seedlings was lower than the control for both subunits, being subunit E the lowest, which corresponds to 50 % of the control. The V-PPase protein amount showed an increase of 25 and 140 % in 5- and 7-day-old seedlings subjected to salt stress, respectively, when compared to control treatment (Fig. 2C). In contrast, the amount of protein was lower and similar to V-ATPase subunit E in 3-day-old seedlings.

3.3 Hydrolysis and H^+ transport assays

The vacuolar proton-pumps hydrolytic activities of *V. unguiculata* hypocotyls showed different profiles at 3-, 5- and 7-day-old seedlings germinated on salt (Fig. 3). V-ATPase hydrolysis activities (Fig. 3A) were lower in 7-day-old salt treated seedlings when compared to control one. Concerning V-PPase (Fig. 3B), salt treated seedlings showed an increase from 3- to 7-day-old, while a decrease occurred in the control seedlings.

The vacuolar ATPase- and PPase-dependent proton transport were also evaluated in *V. unguiculata* hypocotyls (Fig. 4). The V-ATPase transport activity was lower in 3- and 5-day-old salt stressed seedlings, becoming similar in 7-day-old control and treated seedlings (Fig. 4A). Compared to the control seedlings, the V-PPase transport activity showed an increase of 43 and 115 % in 5- and 7-day-old salt-treated seedlings, respectively (Fig. 4B).

4 Discussion

One of the most important strategies adopted by plants to tolerate salt stress is the compartmentalization of ions in the vacuole, in order to maintain low NaCl concentration in the cytoplasm (Niu et al. 1995; Hasegawa et al. 2000; Mimura et al. 2003; Schnitzer et al. 2011). Thus, the control of ion-movement across tonoplast is dependent on the electrochemical gradient generated by proton-pumps, V-ATPase and V-PPase. As a consequence, the regulation of the activities of
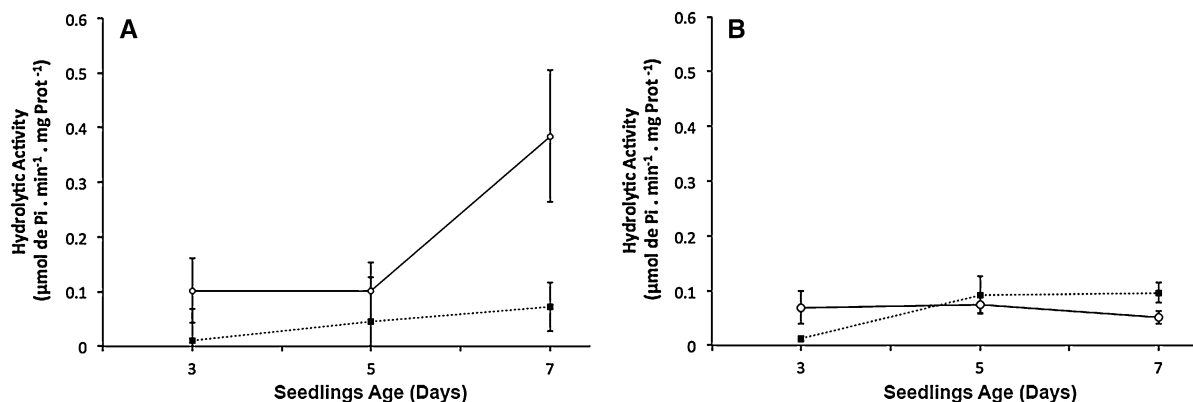
Fig. 3 V-ATPase (**A**) and V-PPase (**B**) hydrolytic activities in tonoplasts isolated from hypocotyls of *Vigna unguiculata*. Control seedlings are identified by *open circles* and salt treated seedlings by *solid squares*. *Vertical lines* indicate standard deviation

Fig. 4 V-ATPase (**A**) and V-PPase (**B**) H$^+$-transport activities in tonoplast of hypocotyls from *Vigna unguiculata*. Control seedlings are identified by *open circles* and salt treated seedlings by *solid squares*. *Vertical lines* indicate standard deviation
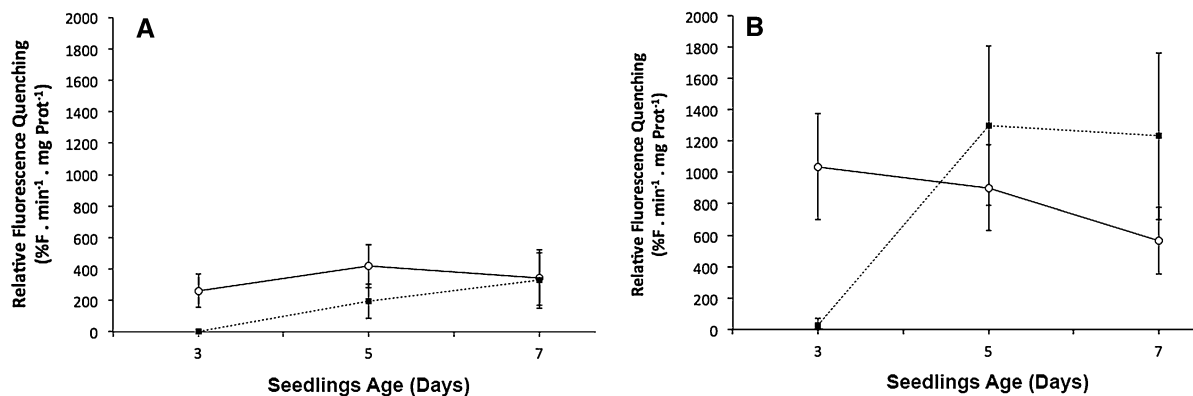
these enzymes plays an important role in salt tolerance. Although the regulation of both vacuolar H$^+$-ATPase and vacuolar H$^+$-PPase activities by salt is well reported in the literature, to date no clear correlation pattern has been found for activation or deactivation of both proton pumps in response to salinity (Silva and Gerós 2009).

It has been reported that salt stress strongly induces V-ATPase activities in tomato (Binzel 1995), cucumber (Janicka-Russak and Kłobus 2007) and *Broussonetia papyrifera* (Zhang et al. 2012). On the other hand, there are contradictory results concerning the effect of salinity in V-ATPase activity, since induction (Reuveni et al. 1990; Nakamura et al. 1992; Binzel 1995), no effect (Colombo and Cerana 1993), and induction of H$^+$-transport but not hydrolysis activity (Ballesteros et al. 1996; Löw and Rausch 1996) have

been reported. Such results could be partially explained by the genomic diversity of the plants so far studied as well as by the complexity of a multisubunit endomembrane proton pump such as V-ATPase. Here, our results highlight that the vacuolar proton pumps regulation in *V. unguiculata* is exerted at gene and protein activity levels.

We chose *V. unguiculata* as an experimental model since cultivars with different degrees of salt/drought tolerance are available, such as Vita 3 (more tolerant) and Vita 5 (less tolerant). The use of Vita 3 hypocotyls in this study was based on a previous work by Fernandes de Melo et al. (1994), who showed that Vita 3 does not transport salt to the leaves while Vita 5 does. Probably, the distinct physiological behavior of both cultivars concerning salt tolerance could be correlated to differential

vacuolar proton-pumps activity. Indeed, the salt tolerance is attributed to the maintenance of K^+ and Na^+ homeostasis and consequently depends on K^+ and Na^+ transport systems located not only in plasma membrane but also in vacuolar membrane, and further sequestration in the vacuole (Blumwald 2000; Zahran et al. 2007).

It has been shown that V-ATPase activities were enhanced under salt stress exposition while V-PPase activities were down regulated in *V. unguiculata* cv. Vita 5 (Otoch et al. 2001). It is also known that salt stress induced the H^+-transport activity of vacuolar pumps in other salt-tolerant (Hasegawa et al. 2000; Golldack and Dietz 2001) and salt-sensitive plants (Maeshima 2000). In this scenario, enhanced expression of these proteins should be followed by sequestration of ions into the vacuole (Gaxiola et al. 2001). Our results with *V. unguiculata* cv. Vita 3 showed that salt stress (100 mM NaCl) up-regulated the transcript levels and the amount of both enzymes (Figs. 1, 2). Further, the hydrolytic and transport activities of V-PPase (Figs. 3B, 4B) were in parallel with increases in gene expression and protein amount (Figs. 1, 2C). On the other hand, no increase in hydrolytic and transport activities was observed for V-ATPase (Figs. 3A, 4A) in spite of the induction of gene expression and protein amount (Figs. 1, 2A, B). Several works reported that salt treated plants induce the hydrolytic and H^+-transport activities of tonoplast proton pumps encompassing with the transcriptional and post-transcriptional level (Janicka-Russak and Kłobus 2007, and references therein). Furthermore, biochemical characterization of V-ATPases from different sources suggested various degrees of coupling between ATP hydrolysis and proton-transport, revealing an important way of regulation for V-ATPase, specifically a loss of coupling efficiency at high ATP concentrations (Kane 2006, and references therein).

Our results showed that hydrolytic and transport activities under salt stress were not dependent on changes in the expression of A subunit V-ATPase (Fig. 1). These results were in agreement with Kabala and Klobus (2008), which reported that hydrolytic and transport activities in cucumber V-ATPase under salt stress were also not dependent on changes in the expression of either A or c subunits supporting the idea that post-translation modification of V-ATPase protein are induced by salt stress.

Similar results were observed for potato cell cultures, where activities of V-ATPase and V-ATPase subunit A expression were not directly correlated (Queirós et al. 2009). Tavakoli et al. (2001) showed that ATP hydrolysis and proton pumping activity of this enzyme were inhibited by H_2O_2 supporting the hypothesis that ATPase activity could be mediated by redox control depending on the metabolic requirements. Finally, another possibility associated with the decrease in the V-ATPase hydrolytic and proton transport activities could be related with the complex nature of this enzyme compared with V-PPase. V-PPase is constituted by a single polypeptide and probably is more resistant to degradation induced during stressful conditions (Martinoia et al. 2007).

We found that A and E subunits from V-ATPase were co-expressed under salt stress. Kawamura et al. (2000) reported an interaction between both subunits involving a conformational change in the subunit E caused by the ATP analogue binding to subunit A. In establishing a catalytic function for A subunit the role of E subunit remains unclear (Ratajczak 2000; Kane 2006). Nevertheless, it is documented that over-expression of the E subunit produces more tolerant plants under salt stress in genetic modified *A. thaliana* (Zhao et al. 2009) and *B. papyrifera* (Zhang et al. 2012). The over expression of Na^+/H^+ antiporters and V-PPase showed an increase in tolerance of the transgenic lines to salt stress (Li et al. 2010). Nevertheless, our results highlight that an increase in the V-ATPase subunit A and E gene expression in the stressed plants was not followed by an increase in hydrolysis and H^+ transport activities of V-ATPase. Unlike V-ATPase activity, V-PPase hydrolytic and proton transport activities were higher under salt exposure in 5- and 7-day-old seedlings compared to control conditions (Figs. 3, 4). However, Fukuda et al. (2004) demonstrated that V-PPase proton transport activity in barley was inhibited while the transcript level was increased under 100 mM NaCl treatment. Additional findings support the relevance of vacuolar V-PPase in plant salt stress. The overexpression of V-PPase in *A. thaliana* and *Suaeda salsa* improves salt- and drought-stress tolerance (Gaxiola et al. 2001; Guo et al. 2006). Several efforts have been undertaken to enhance the salt tolerance of economically important plants by traditional plant breeding as well as by biotechnological approaches but are limited by the multigenic nature of the trait.

Our results suggest that the different proton-pumps activities in Vita 3 could be associated to a differential multi-step regulation between both pumps. In this context, *V. unguiculata* V-PPase of tolerant Vita 3 cultivar seems to play an important role in the adjustment to the salt stress adaptation.

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