

# Tonoplast H<sup>+</sup>-ATPase in response to salt stress in *Populus euphratica* cell suspensions

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## Abstract

A salt-tolerant stable cell suspension culture from *Populus euphratica*, which is the foremost tree to survive and form forests in the deserts of western China, has been established from calli generated from plantlets. We have characterized the tonoplast H<sup>+</sup>-ATPase activity and studied the responses of this enzyme to the salt stress. The tonoplast H<sup>+</sup>-ATPase from *P. euphratica* was sensitive to the same inhibitors (NEM, DCCD, Nitrate, Bafilomycin A<sub>1</sub>) as the tonoplast H<sup>+</sup>-ATPases from other plants. The kinetics of ATP-driven proton pumping by tonoplast vesicles from salt free and NaCl treated cell suspension cultures were compared. It was found that NaCl stimulated the proton pumping activity. Immunodetection of the enzyme showed that the increased activity could be due to increases in protein amount in the tonoplast induced by NaCl. In addition, the proton pumping was dependent upon the divalent cation and Mg<sup>2+</sup> was essential for enzyme activity. The tonoplast H<sup>+</sup>-ATPase from *P. euphratica* also showed substrate specificity to ATP. Results, to our knowledge, provide the first direct evidence for the existence of a tonoplast H<sup>+</sup>-ATPase from cell suspensions of *P. euphratica*, which plays an important role in the salt tolerance of this plant. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** *Populus euphratica*; Vacuolar; V-ATPase

## 1. Introduction

A vacuole of higher plant cells is an important component of cellular physiology, acting as a storage compartment to maintain the cellular metabolism due to its role in the long-term storage of toxic ions, in the long- or short-term storage of minerals or organic acids and in cytoplasmic pH and Ca<sup>2+</sup> homeostasis [1,2].

The vacuolar H<sup>+</sup>-ATPase, or V-ATPase, is a primary active pump located at the vacuolar membrane (tonoplast) of plant cell [3–5]. This multisubunit enzyme consists of two distinct domains: a transmembrane

proton channel (V<sub>0</sub>) and an attached catalytic complex (V<sub>1</sub>). The subunit composition of the enzyme may be varied with different plant species, but all contain at least four subunits exhibiting apparent molecular masses of ≈ 67 kDa (subunit A), 57 kDa (subunit B), 16 kDa (subunit c) and 100 kDa [6–8]. V-ATPase is characterized by specific inhibitors, e.g. DCCD [9,10], NO<sub>3</sub><sup>-</sup> [11] and bafilomycin A<sub>1</sub> [12]. The V-ATPases pump protons from the cytoplasm to the lumen of the vacuole using the energy released by ATP hydrolysis, thereby creating an electrochemical H<sup>+</sup>-gradient that is the driving force for a variety of transport events of ions and metabolites [13]. Anions, such as Cl<sup>-</sup> and NO<sub>3</sub><sup>-</sup>, are found at higher concentration in vacuoles than that in the cytoplasm from red beet. The accumulation of Cl<sup>-</sup> and NO<sub>3</sub><sup>-</sup> into vacuoles is driven by the electrical potential across the tonoplast [14].

Because of the importance of the V-ATPase in plant vacuole, it can be expected that the activity of the V-ATPase be modulated to cope with environmental and metabolic changes [15–18]. Many studies demonstrated

*Abbreviations:* BSA, bovine serum albumin; DCCD, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; MS, Murashige and Skoog; NAA, Naphthylene acetic acid; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride.

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that the salinity was able to induce an increase in V-ATPase activity [19–22]. It was reported that the expressional response to salinity of the V-ATPase was regulated tissue and cell specifically [23]. However, the activity was actually related to total tonoplast proteins and thus did not represent the true specific activity of the enzyme. The salinity-induced change in the V-ATPase amount was only available for very few species, e.g. *M. crystallinum* [19].

*P. euphratica* is the foremost tree to survive and form forests in the deserts of western China. *P. euphratica* is a halophytic plant and tolerates salt and drought stress. It is *P. euphratica* that maintains the ecological balance in western China [24]. Clearly, it is of great interest to understand the responses of *P. euphratica* to salt and drought stress. It has been found that the vacuoles of *P. euphratica* store higher concentration of  $\text{Cl}^-$  than in the cytoplasm. It was suggested that the accumulation of  $\text{Cl}^-$  into vacuoles be driven by the electrical potential across the vacuolar membrane [25].

To understand the molecular properties of the tonoplast  $\text{H}^+$ -ATPase from *P. euphratica*, we established a stable cell suspension culture from calli generated from plantlets and studied the activity of the tonoplast  $\text{H}^+$ -ATPase regulated by salt stress. In this communication, we show that salt-stress stimulates the proton translocation across the vacuolar membrane.

## 2. Materials and methods

### 2.1. Plant material

*P. euphratica* calli were induced from plantlets, as described in detail [26] and subcultured in Murashige and Skoog (MS) medium [27] supplemented with 0.5 g/l naphthylene acetic acid (NAA), 0.25 mg/l benzylamino-purine (BA), 30 g/l sucrose and 5 g/l agar at 25 °C in the dark. After 4 weeks, health calli were transferred into liquid MS supplemented medium without agar and incubated at 25 °C, with continuous shaking (100 rpm), in the dark. Treatments with different levels of NaCl were made by adding appropriate amounts to the liquid media prior to autoclaving (120 °C, 20 min). In all experiments, cells were used 10 days after transfer into the appropriate fresh medium.

### 2.2. Isolation of tonoplast

Cells ( $\approx 200$  g) of *P. euphratica* were filtered onto 0.1 mm mesh nylon screen and homogenized with a blender (Philips) in a medium containing 30 mM Mes-Tris (pH 8.0), 250 mM sucrose, 5 mM EDTA, 10% glycerol (v/v), 2 mM DTT, 0.1 mM PMSF, 0.1% BSA, 2 g polyvinylpyrrolidone (PVP)-40 using a medium to cell ratio of 2.0 ml/g at 4 °C. The slurry was strained through

cheesecloth and the homogenate was centrifuged for 25 min at  $10,000 \times g$ . The supernatant was centrifuged at  $80,000 \times g$  for 45 min. The pellet was resuspended in 10 ml of resuspension buffer (10 mM Mes-Tris (pH 7.5), 250 mM sucrose, 5% glycerol (v/v), 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF), layered on a 25/50% sucrose step gradient made up in gradient buffer (5 mM Mes-Tris (pH 7.5), 1 mM EDTA, 1 mM DTT and 0.1 mM PMSF). After 2 h of centrifugation at  $100,000 \times g$  in a Beckman SW-28 rotor, the 0/25% interface containing tonoplast-enriched membranes was recovered, diluted with 4 vol of resuspension buffer and pelleted at  $160,000 \times g$  (Beckman 70Ti) for 45 min. The tonoplast-enriched membranes were resuspended in resuspension buffer at a final concentration of 2 mg of membrane protein per milliliter. All operations were carried out at 4 °C.

### 2.3. Proton pumping assays

Proton pumping by tonoplast vesicles was monitored by the quenching of Acridine orange fluorescence, as described by Müller et al. [15]. The reaction mix (2 ml) contained 10 mM Mes-Tris, pH 7.5, 250 mM sorbitol, 50 mM KCl, 3.0 mM ATP and 5  $\mu\text{M}$  Acridine orange at 22 °C. Membrane proteins (30  $\mu\text{g}$ ) were typically used and the reaction was usually started with 3.0 mM  $\text{MgSO}_4$ . Fluorescence quenching (495 nm excitation, 525 nm emission) was measured in a Hitachi 4010 fluorescence spectrophotometer. Proton pumping activity was expressed as % quench/mg/min.

### 2.4. ATPase assays

Tonoplast vesicles were added to a reaction mix containing 3.0 mM ATP, 3.0 mM  $\text{MgSO}_4$ , 50 mM KCl, in 25 mM Mes-Tris buffer, pH 7.5. The total reaction volume was 500  $\mu\text{l}$ . After 30 min at 37 °C, the reaction was stopped by adding 0.1 ml of 10% SDS. The release of Pi from ATP was determined essentially as described by Ames [28] and the absorbance measured at 770 nm.

### 2.5. Immunoblotting

SDS-PAGE of partially purified enzymes was performed as described by Laemmli [29] on 12% polyacrylamide gels. The proteins from SDS gels were electrotransferred to nitrocellulose filters (100 V for 2 h) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The blots were blocked with 3% BSA in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 h before being incubated for the primary antibody to the 67 and 57 kDa subunit of the red beet V-ATPase (a gift from Professor M.F. Manolson, Hospital for Sick Children, Toronto, Ont., Canada M5G 1X8). The blots were then

washed in TBS and visualized by alkaline phosphatase-linked anti-rabbit goat IgG using 5-bromo-4-chloro-3-indolyl phosphate *p*-Toluidine salt and nitroblue tetrazolium as color development reagents.

## 2.6. Protein concentration

Estimates of protein concentrations were carried out routinely by a simplified Lowry method after trichloroacetic acid precipitation [30].

## 3. Results

### 3.1. Tonoplast $H^+$ -ATPase activities

Fig. 1 shows a typical kinetics of a proton pumping by tonoplast vesicles monitored by the quenching of Acridine orange fluorescence. Each proton pumping reaction was started with  $MgSO_4$ . After equilibrium was reached, the pH gradient was collapsed with  $4 \mu M$  gramicidin D and the fluorescence was restored. This result indicated that the fluorescence quenching was caused by the proton translocation, not the proteoliposomes leaking. The proton pumping activities of the tonoplast vesicles from salt-free and 50 mM NaCl treated cell suspensions were compared by Acridine orange fluorescence quenching (Fig. 1). When the

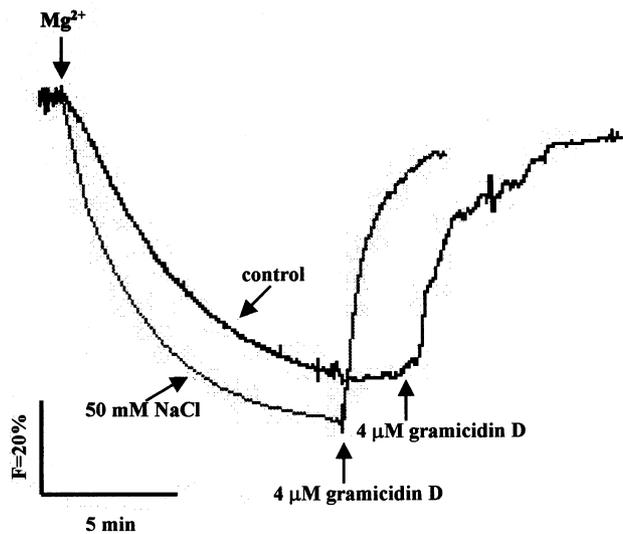


Fig. 1. ATP-dependent proton pumping and membrane permeability of tonoplast vesicles isolated from control (no salt) and 50 mM NaCl-treated *P. euphratica* cell suspension cultures. The proton translocation across vesicle membrane was monitored by the quenching of Acridine orange fluorescence as described in Section 2 in the presence of tonoplast vesicles (30  $\mu g$  of membrane protein) in this representative experiment. Salt-treated cell suspension cultures were obtained by transferring health calli into liquid MS supplemented medium containing 50 mM NaCl for 10 days. The reaction was started with  $MgSO_4$ . After equilibrium was reached, the residual pH gradient was collapsed with  $4 \mu M$  gramicidin D.

tonoplast vesicles from salt treated cell suspensions were diluted to give the same initial rate of  $H^+$  pumping as that from salt-free cell suspension, they developed a greater pH gradient (total quench). We conclude that the tonoplast  $H^+$ -ATPase from the salt-treated cell suspensions behaves at a higher proton pumping activity than that from salt-free cell suspensions.

The ATP hydrolysis activities of the tonoplast vesicles from salt-free and salt-treated cell suspensions were also compared and represented by nitrate-sensitive activities. It was found that the tonoplast vesicles from cell suspensions treated with 50 mM NaCl are able to hydrolyze more ATP ( $62 \pm 6 \mu mol$  Pi/min/mg protein) than that from NaCl free cell suspensions ( $22 \pm 4 \mu mol$  Pi/min/mg protein). This result indicates that the proton translocation across the tonoplast membrane is correlated with the ATP hydrolysis and suggests that the tonoplast vesicles from *P. euphratica* cell suspension responds to the salt by increasing their  $H^+$ -ATPase activities.

### 3.2. Inhibitor sensitivity of the tonoplast $H^+$ -ATPase

#### 3.2.1. Azide and vanadate

The phosphate analogue vanadate is extremely effective at inhibiting P-type ATPase, with  $IC_{50}$  values in the submicromolar range. V-ATPase is also sensitive to vanadate, but at much higher concentrations ( $IC_{50}$  of 0.5–0.6 mM) [15,31]. Thus, the sensitivity to vanadate has been used to discriminate between V- and P-type ATPase.  $F_0F_1$ -ATP synthase (F-type ATPase) is specifically inhibited by azide. This property has been used to discriminate between V- and F-type ATPase [32]. At 0.4 mM vanadate and 0.6 mM azide, the proton pumping activity of the tonoplast  $H^+$ -ATPase from *P. euphratica* was 93 and 92%, respectively, of the control (data not shown), indicating that very few P- and F-type ATPase were contaminated in our tonoplast vesicles.

#### 3.2.2. Nitrate

Inhibition of V-ATPase activity by nitrate is attributed to the dissociation of peripheral  $V_1$  subunits from the membrane and the oxidizing effect of nitrate [11]. Thus, nitrate-sensitive ATPase activity has been used to characterize V-ATPase activity. Fig. 2(A) shows that 50 mM  $KNO_3$  completely abolishes proton pumping activity of the tonoplast  $H^+$ -ATPase from *P. euphratica*.

#### 3.2.3. NEM

Because of the presence of cysteine residues close to the active site of  $V_1$ , the inactivation of ATP-hydrolysis activity arises through a steric effect of NEM adduct to the cysteine residues, subsequently effects on the proton pumping activity [33,34]. In our experiments, it was observed that NEM inhibited proton pumping activity

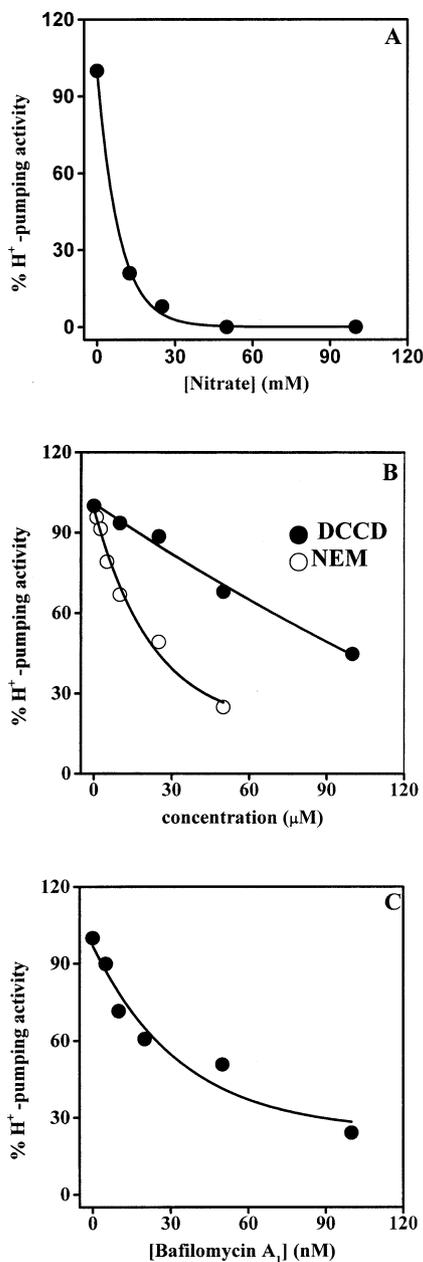


Fig. 2. Inhibition of ATP-dependent H<sup>+</sup>-pumping activity by KNO<sub>3</sub> (A), NEM (B), DCCD (B) and bafilomycin A<sub>1</sub> (C) in tonoplast vesicles from *P. euphratica* cell suspension cultures. Inhibitors at the concentrations indicated and 30 μg of membrane protein were added to 2 ml of the reaction mix (composition as in Section 2). Each reaction was started with 3.0 mM MgSO<sub>4</sub> and the initial rates of Acridine orange fluorescence quenching are measured. The reported results are the means of three independent measurements for ATP-dependent proton pumping activity of the tonoplast H<sup>+</sup>-ATPase at 22 °C. Results are expressed as a percentage of the activity of control (without inhibitors).

of the tonoplast H<sup>+</sup>-ATPase with IC<sub>50</sub> = 20.3 ± 2.7 μM (Fig. 2B).

### 3.2.4. DCCD

The proteolipid-binding inhibitor, *N,N'*-dicyclohexylcarbodi-imide (DCCD), covalently binds to c subunits

of V<sub>0</sub> [9,10]. Although DCCD inhibition is generally very effective with an IC<sub>50</sub> = 3–4 μM, the inhibition is less pronounced to the tonoplast H<sup>+</sup>-ATPase from *P. euphratica* with IC<sub>50</sub> = 88 ± 4 μM (Fig. 2B).

### 3.2.5. Bafilomycin A<sub>1</sub>

Bafilomycin A<sub>1</sub>, a macrolide antibiotics, inhibits proton translocations of tonoplast H<sup>+</sup>-ATPase by binding to V<sub>0</sub> in nanomolar concentrations [12]. Fig. 2(C) shows the typical inhibition of proton pumping activity by bafilomycin A<sub>1</sub> (IC<sub>50</sub> = 35.6 ± 7.9 nM), indicating that the tonoplast H<sup>+</sup>-ATPase from *P. euphratica* is highly sensitive to the bafilomycin A<sub>1</sub>, which is similar to most of the tonoplast H<sup>+</sup>-ATPases.

### 3.3. Ion effect on the tonoplast H<sup>+</sup>-ATPase from *P. euphratica*

Divalent cation e.g. Mg<sup>2+</sup>, is required for the tonoplast H<sup>+</sup>-ATPase activity. Therefore, Mg<sup>2+</sup> was normally used to start reactions of proton translocation. Here, we examined the effect of a series of divalent cations on the proton pumping activity of the tonoplast H<sup>+</sup>-ATPase. As revealed in Table 1, Cu<sup>2+</sup>, Zn<sup>2+</sup> and Ca<sup>2+</sup> were unable to start the proton translocation, whereas Fe<sup>2+</sup> was capable of initiating this proton translocation, but with lower activity (24% of the activity of Mg<sup>2+</sup> starting the reaction).

### 3.4. pH Optima, Km and Vmax

As shown in Fig. 3, the pH optima for the proton pumping activity of the tonoplast H<sup>+</sup>-ATPase is ≈ 7.5. The Km and Vmax values based on proton pumping activity were also determined. By measuring the initial rates of proton pumping, the Km and Vmax values were calculated by Hanes-Woolf linearizations of the obtained first order kinetics, which were 0.65 mM and 238% quench/min/mg.

Table 1

Effect of divalent cations on the proton pumping activity of the tonoplast H<sup>+</sup>-ATPase

Divalent cation (3 mM)	Relative activity (% of Mg <sup>2+</sup> starting reaction)
Cu <sup>2+</sup>	0
Zn <sup>2+</sup>	0
Ca <sup>2+</sup>	0
Fe <sup>2+</sup>	24.0 ± 2
Mg <sup>2+</sup>	100

Measurements of the proton pumping activity were obtained as described in Section 2. Divalent cation (3.0 mM) was added into assay mix to start the reaction. The reported results are the means of three independent measurements for divalent dependent proton pumping activity of the tonoplast H<sup>+</sup>-ATPase at 22 °C. Results are expressed as a percentage of the activity of Mg<sup>2+</sup> starting the reaction.

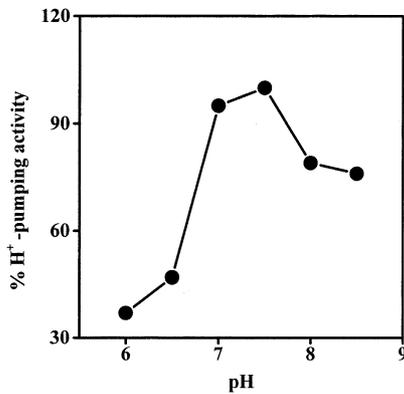


Fig. 3. Determination of the pH optima for H<sup>+</sup>-pumping by the tonoplast H<sup>+</sup>-ATPase from *P. euphratica* cell suspension cultures. Membrane protein (30 μg) were assayed for ATP-dependent proton pumping in a reaction mix (2 ml) containing 250 mM sorbitol, 50 mM KCl, 3.0 mM ATP and 5 μM Acridine orange. The mix was buffered with 10 mM Mes-Tris to the indicated pH. The reaction was started with 3.0 mM MgSO<sub>4</sub> and the initial rates of Acridine fluorescence quenching are measured. The reported results are the means of three independent measurements for ATP-dependent proton pumping activity of the tonoplast H<sup>+</sup>-ATPase at 22 °C. Results are expressed as a percentage of the activity at pH 7.5.

### 3.5. Substrate specificity

The substrate specificity of the tonoplast H<sup>+</sup>-ATPase from salt-free and salt-stressed cell suspension was investigated. The results represented in Table 2 show that ATPase activity is highly specific to ATP as substrate and GTP was hydrolyzed at slower rate. We also measured the effect of these nucleotides on the proton pumping activity, indicating the similar substrate specificity to ATPase hydrolysis of ATP > GTP (data not shown). As indicated in Table 2, the tonoplast H<sup>+</sup>-ATPase from the salt-stressed cell suspension does not change the substrate specificity to the nucleotides.

Table 2  
Substrate specificity of ATPase activity of the tonoplast H<sup>+</sup>-ATPase

Substrate (3 mM)	Relative activity (% of the activity of ATPase substrate)	
	Salt-free	50 mM NaCl treated
ATP	100	100
GTP	84.0 ± 8	68.78 ± 6

Measurements of the ATP hydrolysis activity were obtained as described in Section 2. The tonoplast H<sup>+</sup>-ATPase was obtained from salt free and 50 mM NaCl treated suspension cells for 10 days. The assay mix contained 3.0 mM of substrate. The reported results are the means of three independent measurements for ATP hydrolysis activity of the tonoplast H<sup>+</sup>-ATPase at 37 °C. Results are expressed as a percentage of the activity of ATP as substrate.

### 3.6. Partial purification of the tonoplast H<sup>+</sup>-ATPase from *P. euphratica*

Most of the membrane proteins from the tonoplast-enriched membranes were first removed with 0.15% deoxycholic acid. Following centrifugation, the pellets were treated with 40 mM octylglucoside. The solubilized enzymes were subjected to density gradient centrifugation on a linear 10–30% sucrose gradient. The obtained partially purified enzyme showed double nitrate-sensitive ATPase activity of initial tonoplast membrane and SDS-PAGE appeared most of V-ATPase subunit bands, e.g. A, B, c, judged by comparison with reported SDS-PAGE of V-ATPase from mung beans [8] (data not shown). The partially purified enzyme was also subjected to Western blot analysis (Fig. 4A). As shown in Fig. 4(A), immunoblots probed with antibodies to A and B subunit of the red beet V-ATPase confirm the existence of V-ATPase in the tonoplast membrane from salt-free and salt-stressed cell suspension. Fig. 4(A) indicates that A and B band from 50 mM NaCl stressed cell suspensions are stronger than those from salt-free cell suspensions, although A band is less pronounced than B band, which may be due to the less affinity of A subunit to the antibody of red beet. Considering that the V-ATPase amount during the partial purification might vary, Western blot analysis was also performed with the tonoplast membranes. Similarly, B band from salt-stressed cell suspension was stronger (Fig. 4B). This result suggests that more tonoplast H<sup>+</sup>-ATPase were expressed from salt-treated cell suspensions.

## 4. Discussion

Plant tonoplast V-ATPases have been well documented. To our knowledge, however, very little is known about the tonoplast H<sup>+</sup>-ATPase from *P. euphratica* because of the difficulties of obtaining enough experimental materials. Therefore, we first established a stable cell suspension culture from calli generated from plantlets and expected to understand the salt tolerance of *P. euphratica* by studying the cell suspension culture.

*P. euphratica* is a halophytic plant and tolerates salt and drought stress, so that it is the foremost tree to survive and form forests in the deserts of western China. It was found that NaCl accumulated in the vacuole, suggesting the existence of a vacuolar ATPase, which served as a primary-active proton pump for the secondary transporter, such as Na<sup>+</sup>/H<sup>+</sup> antiporter. In this study, we isolated the tonoplast and provide direct evidence for the existence of the tonoplast H<sup>+</sup>-ATPase from the cell suspension of *P. euphratica*.

The tonoplast H<sup>+</sup>-ATPase from the cell suspension of *P. euphratica* has been characterized. We have conducted a systematic study of the inhibitor sensitiv-

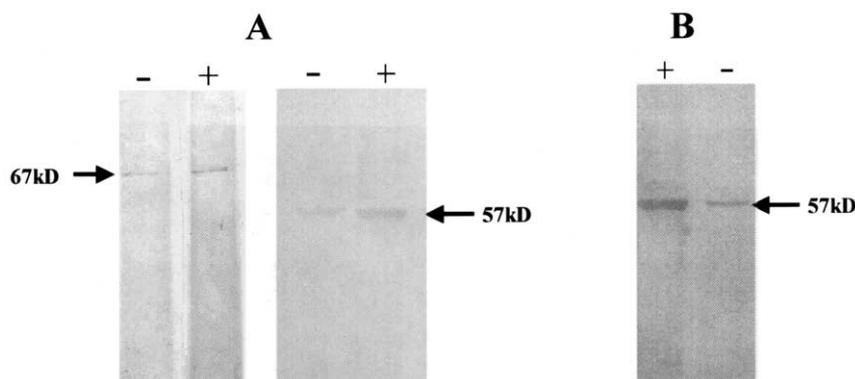


Fig. 4. Western blot analysis of the partially purified tonoplast  $H^+$ -ATPase (A) and tonoplast membrane (B) from *P. euphratica* cell suspension cultures without NaCl (–) and with 50 mM NaCl (+). The partially purified enzyme were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred onto nitrocellulose, probed with a polyclonal antibody raised against the 67 (A) or 57 kDa (B) subunit of the red beet V-ATPase and visualized by alkaline phosphatase-linked anti-rabbit goat IgG using 5-bromo-4-chloro-3-indolyl phosphate *p*-Toluidine salt and nitroblue tetrazolium as color development reagents. Partially purified tonoplast (20  $\mu$ g) from NaCl free (–) and 50 mM NaCl (+) treated cell suspension cultures were loaded on the gel. Similar experiments (but 50  $\mu$ g of tonoplast membranes used) against 57 kDa (B) subunit of the red beet V-ATPase were performed with tonoplast membranes (B).

ities of the enzyme. Azide and vanadate are specific inhibitors of F- and P-type ATPase, respectively. It was found that the tonoplast  $H^+$ -ATPase of *P. euphratica* was not sensitive to azide and vanadate, indicating that protons across the tonoplast membrane mainly contributed to the tonoplast  $H^+$ -ATPase. Similar to other vacuolar ATPases, the proton pumping activity of the tonoplast  $H^+$ -ATPase of *P. euphratica* was inhibited by the specific inhibitors of V-ATPase, i.e.  $NO_3^-$ , NEM, DCCD and bafilomycin  $A_1$  (Fig. 2). It should be noted that DCCD inhibition on the V-ATPase was normally with  $IC_{50} = 3\text{--}4 \mu\text{M}$ . Here, we find that it is  $\approx 80 \mu\text{M}$ , indicating that the tonoplast  $H^+$ -ATPase of *P. euphratica* is not very sensitive to DCCD. A similar result was also observed from the tonoplast  $H^+$ -ATPase of the juice sac membrane of lemon [15].

The behavior of the tonoplast  $H^+$ -ATPase of plants under salt-stress conditions has been investigated at different levels. It was found that the activity of the V-ATPase from most plants increased in response to the salt-stress. It should point out, however, that in those studies the activity of the enzyme was related to the total tonoplast protein and thus the salt induced increase of the activity of the enzyme could be due to changes of the amount of the enzyme and/or the specific activity of the enzyme. In this presentation, the activity of the tonoplast  $H^+$ -ATPase from the cell suspension culture of *P. euphratica* treated with different concentration NaCl and time were assayed (data not shown). We found that the optimum activity of the enzyme appeared at 50 mM NaCl for 10 days. Fig. 1 represents the typical activation of the proton pumping activity under the salt-stress condition. In order to explore how the salt-stress regulated the tonoplast  $H^+$ -ATPase, Western blot analysis was performed. An interesting finding was that more subunit A and B from the salt treated cell

appeared, which suggested that salt-stress induced an increase in the tonoplast  $H^+$ -ATPase.

In summary, the tonoplast  $H^+$ -ATPase from *P. euphratica* shows a number of similarities to other vacuolar  $H^+$ -ATPases. However, the enzyme is apparently activated under the salinity condition. A hypothesis on the regulation of V-ATPase from plant by salt was proposed with the incorporation of new subunits, albeit not found yet. Therefore, the subunit composition of the holoenzyme should be identified. Moreover, the behavior of the tonoplast  $H^+$ -ATPase from *P. euphratica* plant under different environment conditions should be studied.

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