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# 胡杨液泡膜微囊 $H^+$-ATPase 质子泵活性研究

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**摘要:** 将悬浮培养的胡杨(*Populus euphratica* Oliv.)细胞捣碎后,通过差速离心和不连续蔗糖密度梯度离心获得富集液泡膜的膜微囊。通过连续监测吖啶橙的荧光淬灭研究膜微囊上 $H^+$-ATPase 的质子转运特性。结果表明,质子转运依赖于 ATP,其表观米氏常数 $K_m$ 值为 0.65 mmol/L。质子泵活性受 pH 和温度的影响较大。测定液 pH 值为 7.5时,质子泵的活性最高(测定温度选定为 22 ℃)。一些二价阳离子可启动 $H^+$-ATPase 的质子转运,其中 $Mg^{2+}$ 的作用远高于 $Fe^{2+}$。在实验条件下,$Ca^{2+}$、$Cu^{2+}$ 和 $Zn^{2+}$ 均不能启动 $H^+$-ATPase 的质子转运。质子跨膜转运还可被一价阴离子激活,激活作用的顺序为:$Cl^- > Br^- > I^- > F^-$。质子泵活性受 NEM(乙基马来酰亚胺)、DCCD (二环己基碳二亚胺)、$NO_3^-$ 和 Bafilomycin $A_1$ 的强烈抑制,但对 $Na_3VO_4$ 和 $NaN_3$ 不敏感。这些性质说明胡杨液泡膜微囊上的 $H^+$-ATPase 属于囊泡型的 ATPase。

**关键词:** 胡杨;$H^+$-ATPase;质子转运活性

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# Studies on the Proton Pumping Activity of $H^+$-ATPase in Tonoplast Vesicles of *Populus euphratica*

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**Abstract:** Tonoplast-enriched vesicles were prepared from suspension-cultured *Populus euphratica* Oliv. cells by differential centrifugation and discontinuous sucrose density gradient centrifugation. The properties of the proton pumping activity of $H^+$-ATPases in tonoplast vesicles were studied by acridine orange fluorescent quenching measured at 22 ℃. The proton pumping activity of ATPase was ATP-dependent with apparent Michaelis-Menten Constant ($K_m$) for ATP about 0.65 mmol/L. The optimal pH for $H^+$-ATPases activity was 7.5. The proton pumping activity of $H^+$-ATPase could be initiated by some divalent cations, $Mg^{2+}$ being highly efficient, much more than $Fe^{2+}$; and $Ca^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ were inefficient under the experimental condition. The proton translocation could be stimulated by halide anions, with potencies decreasing in the order $Cl^- > Br^- > I^- > F^-$. The proton pumping activity was greatly inhibited by N-ethylmaleimide (NEM), N,N′-dicyclohexylcarbodiimide (DCCD), $NO_3^-$ and Bafilomycin $A_1$, but not by orthovanadate and azide. These results demonstrated that the $H^+$-ATPase in the tonoplast of *Populus euphratica* belonged to vacuolar type ATPase. This work was the first time that tonoplast-enriched vesicles were isolated from *Populus euphratica* cells.

**Key words:** *Populus euphratica*; $H^+$-ATPase; proton pumping activity

$H^+$-ATPases play an important role in the metabolism of eukaryotes and prokaryotes. At present, the $H^+$-ATPases known in nature are divided into three types: P, F and V types. In plant cells, P type $H^+$-ATPases are mainly distributed in plasma membrane, F types in mitochondria and chloroplasts; while V type

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$H^+$-ATPases are found in tonoplast, endoplasmic reticulum, lysosome, Golgi and clathrin coated vesicles[1].

Vacuolar (V type) $H^+$-ATPase is a kind of membrane bound mutimeric protein composed of more than 10 subunits. The protein complex contains two distinct sectors: the peripheral complex ($V_1$) and the membrane-associated complex ($V_0$). $V_1$, consisting of 5 – 8 subunits, can bind ATP, catalyze and regulate the ATP hydrolysis. $V_0$ is mainly composed of 16 kD subunits which form the proton channel[2]. Utilizing the energy released from ATP, V type $H^+$-ATPase can build up a proton gradient across the tonoplast to drive translocation of ions and metabolites. V type $H^+$-ATPase in the tonoplast plays crucial roles in metabolites translocation, ion balance, $Ca^{2+}$ signal transfer, crassulacean acid metabolism, plant resistance to salt stress and so on[3].

Tonoplast-enriched vesicles were isolated from suspension-cultured *P. euphratica* cells for the first time and their proton pumping properties systematically studied. These experiments have established the basis for investigating the function of V type $H^+$-ATPase in stress perception and salt resistance of *P. euphratica*.

# 1 Materials and Methods

## 1.1 Suspension-culture of *Populus euphratica* Oliv. cells

*P. euphratica* calli were induced from plantlets and subcultured in MS culture media containing 0.5 mg/L NAA, 0.25 mg/L BA, 30 g/L sucrose and 5 g/L agar (pH 5.8)[4]. Fast growing calli were inoculated into liquid MS culture media (of the same composition as the above, but without agar) and cultured in the dark on a shaker rotating at 100 r/min. Suspension-cultured *P. euphratica* cells were subcultured once after 15 – 20 d.

## 1.2 Isolation of tonoplast-enriched vesicles

Tonoplast-enriched vesicles were isolated from *P. euphratica* cells according to the procedure of Liu *et al*[5]. The suspension-cultured cells were filtrated on a 0.1 mm mesh nylon screen and homogenized in a medium containing 30 mmol/L MES-Tris (pH 8.0), 250 mmol/L sucrose, 5 mmol/L ethylenediamine tetracetic acid (EDTA), 10% glycerol (V/V), 500 μL/L β-mercaptoethanol, 2 mmol/L 1,4-dithiothreitol (DTT), 0.1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 0.1% (BSA), and 1 g insoluble polyvinylpolypyrrolidone (PVP)/100 g cell. The medium to cell ratio was 2 mL/g.

The slurry was filtered through 4 layers of cheesecloth. The filtrate was centrifuged at 10 000*g* (JLA-10.5) for 25 min, the pellet was discarded, and the supernatant was recentrifuged at 80 000*g* (Beckman 45Ti, rmax) for 45 min. After decanting the supernatant, the pellet was resuspended in 10 mmol/L MES-Tris (pH 7.5), 250 mmol/L sucrose, 5% glycerol (V/V), 1 mmol/L EDTA, 1 mmol/L DTT, 0.1 mmol/L PMSF. The suspension was overlaid on each 2 mL of 25% and 50% (W/W) sucrose dissolved in 5 mmol/L MES-Tris (pH 7.5), 1 mmol/L EDTA, 1 mmol/L DTT, 0.1 mmol/L PMSF, then centrifuged at 100 000*g* (Beckman SW 55Ti, rmax) for 2 h. The vesicles on the 0 – 25% sucrose interface were collected and diluted five fold with the above resuspension buffer. The vesicles were recentrifuged at 160 000*g* (Beckman 70Ti, rmax) for 45 min. The pellet was resuspended in the resuspension buffer and stored at – 80 ℃.

All the above procedures were carried out at 4 ℃.

## 1.3 Assay for proton transport

The proton transport activity of $H^+$-ATPase was determined through continuously monitoring acridine orange fluorescence quenching with a Hitachi 4010 spectrofluorometer (excitation = 495 nm, emission = 525 nm)[6]. The measurement solution contained 10 mmol/L MES-Tris (pH 6.0 – 8.5), 250 mmol/L sorbitol, 5 μmol/L acridine orange, 50 mmol/L KCl, 3 mmol/L $MgSO_4$, 20 – 140 μg membrane protein, 0.1 – 3 mmol/L ATP in the final volume of 2 mL. The reaction was initiated by adding ATP. The measurement temperature was 22 ℃ unless otherwise stated. To test the effect of various monovalent anions on the proton pumping activity of $H^+$-ATPase, KCl was replaced by the same amount of KBr or KI or KF. $MgSO_4$ was substituted by $CuSO_4$ or $FeSO_4$ or $ZnSO_4$ or $CaSO_4$ to study the response of proton pumping activity of $H^+$-ATPase to various divalent cations. To evaluate inhibition of proton pumping activity of $H^+$-ATPase by various inhibitors, 50 mmol/L nitrate, 0.6 mmol/L azide, 0.4 mmol/L vanadate, 10 – 100 μmol/L DCCD, 1.0 – 50.0 μmol/L N-ethyl-maleimide (NEM) or 5 – 100 nmol/L bafilomycin $A_1$ was separately added into the measurement solution.

When the fluorescence became stable at the end of each assay, 2.5 μg/mL gramicidin D was added into the measurement solution to test that the fluorescence quench was caused by proton translocation, because gramicidin D can collapse the proton gradient across the membrane. The proton pumping activity was expressed as % quench·$min^{-1}$·$mg^{-1}$ protein. $\Delta F/F$(%) indicated the percent of acridine orange fluorescence quenching ($F$, fluorescence intensity; $\Delta F$, decrease of fluorescence intensity). The apparent Michaelis-Menten constant ($K_m$) for ATP of

$H^+$-ATPase was calculated by Hanes-Wolf plot.

### 1.4 Protein assay

After being solubilized with sodium deoxycholic acid and precipitated by trichloroacetic acid, the membrane protein was quantified by the Lowry *et al*[7] method using bovine serum albumin as standard.

## 2 Results and Discussion

### 2.1 ATP-dependent proton transport activity of $H^+$-ATPase and its apparent $K_m$

As shown in Fig. 1, the fluorescence of acridine orange started to quench after ATP was added into the measurement solution. Furthermore, the initial slope and maximum extent of fluorescence quenching increased with ATP concentration increasing. The fluorescence was restored after adding gramicidin D into the measurement solution (Fig. 1A). This means the quenching of fluorescence was caused by proton translocation, and the proton pumping was ATP-dependent. Calculated by Hanes-Wolf plot, the apparent $K_m$ value for ATP of $H^+$-ATPase was about 0.65 mmol/L (Fig. 1B).

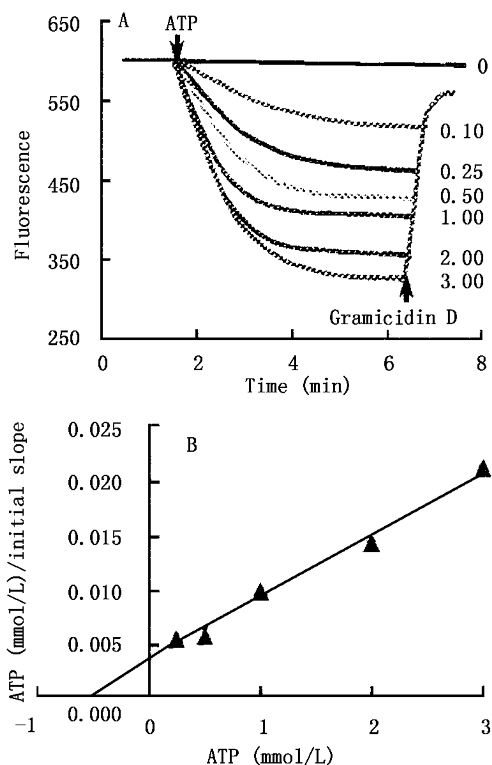


**Fig.1.** ATP concentration-dependent proton pumping activity of tonoplast $H^+$-ATPase.
A. ATP-dependent fluorescence quenching of Acridine orange. B. Hanes-Woolf Plot. Values on the right of the Fig.1A are the ATP concentration (mmol/L) in the measurement solution.

### 2.2 Effect of membrane protein dosage on the acridine orange fluorescence quenching

The initial slope and maximum extent of fluorescence quenching increased with increasing membrane protein content in the measurement solution (Fig. 2A). At low protein concentration range, the relationship between protein content and initial slope of fluorescence quenching was almost linear, but became hyperbolic at higher concentration of protein (Fig. 2B). Wang *et al*[8] obtained similar results using the tonoplast vesicles isolated from soybean hypocotyl. These results suggest that there may exist an optimal ratio between the contents of protein and acridine orange.

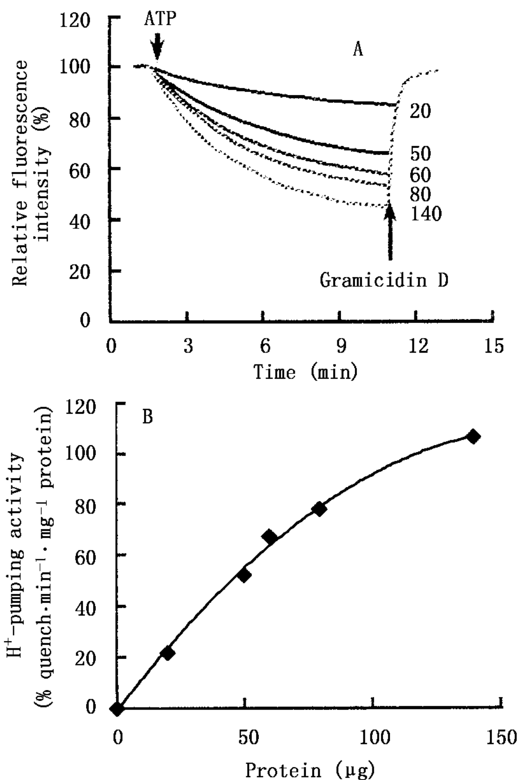


**Fig.2.** Membrane protein dosage-dependent acridine orange fluorescence quenching.
A. Protein dosage-dependent fluorescence quenching of Acridine orange. B. Protein dosage-dependent proton pumping activity of $H^+$-ATPase. Values on the right of Fig. 2A are the protein content (μg) in the measurement solution.

### 2.3 Optimal measurement temperature and pH of measurement solution

The effect of temperature on proton transport was complex. Under the experimental conditions, the initial slope of fluorescence quenching was 66.81% quenching·$min^{-1}$·$mg^{-1}$ protein at 15 ℃, while it rose to 188.64% at 37 ℃ (Fig. 3B). Proton pumping activity increased about 182.4% between 15 ℃ and 37 ℃. But the maximum extent of acridine orange quenching ($\Delta F/F$) rapidly decreased at the same time (Fig. 3A). The maximum quenching of acridine orange fluorescence ($\Delta F/F$) was approximately 45.3% at 15 ℃, while it decreased to 6.8% at 37 ℃, possibly due to reduction in the intactness of membrane vesicles at high temperatures. The

measurement temperature was decided to be 22 ℃ in the following investigations.

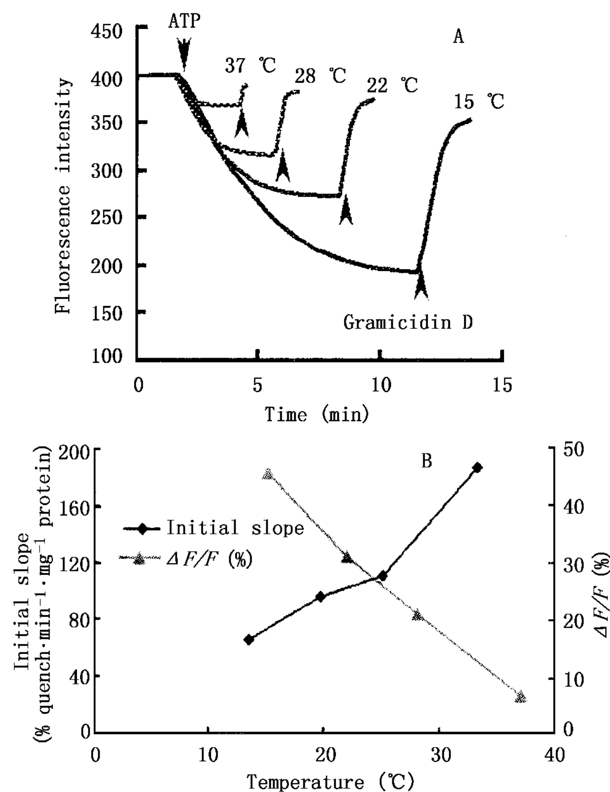


**Fig. 3.** Effect of temperature on the proton pumping activity of $H^+$-ATPase.
A. Effect of temperature on fluorescence quenching of Acridine orange. B. Effect of temperature on proton pumping activity of $H^+$-ATPase.

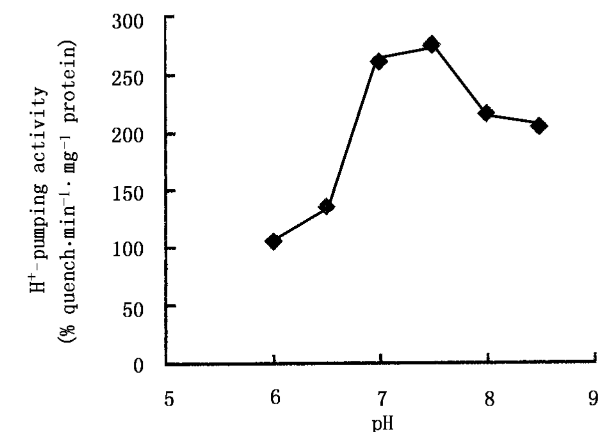


**Fig. 4.** Effect of the pH on the proton pumping activity of $H^+$-ATPase.

The proton pumping activity of $H^+$-ATPase in neutral and weak alkaline media was higher than that in weak acidic media, it reached the maximum at pH 7.5 (Fig. 4), which was consistent with the results of Zhang *et al*[9].

## 2.4 Effect of divalent cations on the proton pumping activity of $H^+$-ATPase

Divalent cations are necessary for ATP hydrolysis and proton pumping activity of $H^+$-ATPase. The reaction can be initiated by $Mg^{2+}$[8]. Results in Table 1 indicated that $Fe^{2+}$ and $Mg^{2+}$ could both start the reaction. But the efficiency of $Mg^{2+}$ was 3.2 folds higher than that of $Fe^{2+}$. Under the experimental conditions, $Cu^{2+}$, $Zn^{2+}$ and $Ca^{2+}$ could not initiate the reaction.

**Table 1** Effect of divalent cations on the proton pumping activity of $H^+$-ATPase

| Divalent cation (3 mmol/L) | Proton pumping activity (% quench·min$^{-1}$·mg$^{-1}$ protein) |
|---|---|
| $Cu^{2+}$ | 0.0 |
| $Zn^{2+}$ | 0.0 |
| $Ca^{2+}$ | 0.0 |
| $Fe^{2+}$ | 37.2 |
| $Mg^{2+}$ | 154.8 |

## 2.5 Stimulation of monovalent anions to the proton pumping activity of $H^+$-ATPase

Monovalent anions can stimulate the proton pumping activity of $H^+$-ATPase for two reasons: 1) the permeable anions accumulated into the vesicles are driven by the proton gradient across the membrane to neutralize the positive electric charge in the vesicles; and 2) the anions could combine with the enzyme to change its conformation and stimulate its activity[10].

Fig. 5 showed that $Cl^-$ and $Br^-$ strongly and $F^-$ slightly stimulated the $H^+$ translocating activity of $H^+$-ATPase, and the effect of $Cl^-$ was greater than that of $Br^-$. $I^-$ was found greatly reduced the fluorescence of acridine orange. Furthermore, gramicidin D only restored 11.4% of the quenched fluorescence, but 79.4% of the acridine orange fluorescence was quenched after the addition of ATP.

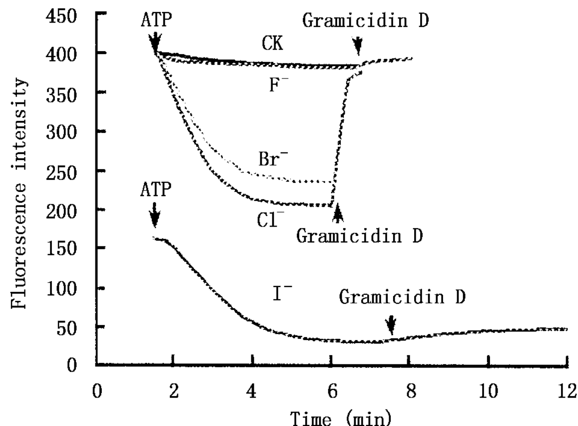


**Fig. 5.** Effect of monovalent anion on the proton pumping activity of $H^+$-ATPase.

**Table 2** The sensitivity of proton pumping activity of $H^+$-ATPase to N，N′-dicyclohexylcarbodiimide (DCCD)，N-ethylmaleimide (NEM) and Bafilomycin $A_1$

| DCCD | | NEM | | Bafilomycin $A_1$ | |
|---|---|---|---|---|---|
| Concentration (μmol/L) | Relative $H^+$-pumping activity (%) | Concentration (μmol/L) | Relative $H^+$-pumping activity (%) | Concentration (nmol/L) | Relative $H^+$-pumping activity (%) |
| 0 | 100.0 | 0 | 100.0 | 0 | 100 |
| 10 | 93.6 | 1.0 | 95.7 | 5 | 89.9 |
| 25 | 88.6 | 2.5 | 91.4 | 10 | 71.6 |
| 50 | 68.0 | 5.0 | 79.2 | 20 | 60.7 |
| 100 | 44.7 | 10.0 | 66.8 | 50 | 50.7 |
| | | 25.0 | 49.2 | 100 | 24.1 |
| | | 50.0 | 24.9 | | |

### 2.6 Inhibition of proton pumping activity of $H^+$-ATPase by various inhibitors

Specific inhibitors for F type $H^+$-ATPases are oligomycin and azide，for V type $H^+$-ATPases are bafilomycin $A_1$ and nitrate，and for P type $H^+$-ATPases is vanadate. But the sensitivities of these ATPases to the relevant inhibitors are different. These types of $H^+$-ATPases are distributed in different membranes，therefore the purity of the isolated membrane-bound proteins or membrane vesicles can be evaluated by their sensitivities to relevant inhibitors[11].

From Fig. 6，it can be found that，0.6 mmol/L azide and 0.4 mmol/L vanadate only reduced the proton pumping activity of $H^+$-ATPase by 8.7% and 7.5%，respectively. However，50 mmol/L nitrate almost completely inhibited this activity. These data indicated that the membrane preparation was enriched with tonoplast vesicles，and the content of plasmalemma，chloroplast and mitochodrial membrane was very small. The results also suggested that the proton pumping activity measured by acridine orange fluorescence quenching belonged mainly to V type $H^+$-ATPase.

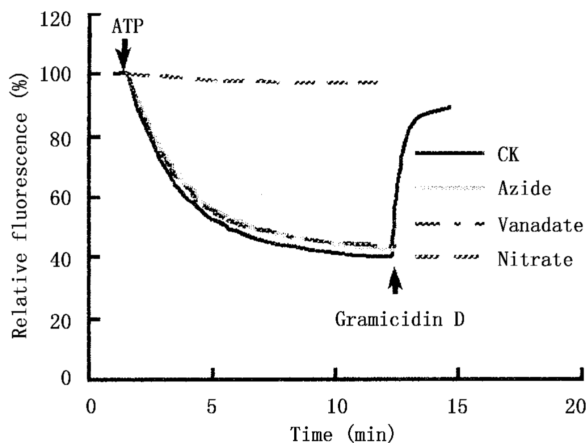


**Fig. 6.** The sensitivity of the proton pumping activity of $H^+$-ATPase to specific inhibitors.

N，N′-dicyclohexylcarbodiimide (DCCD) and NEM are two inhibitors of $H^+$-ATPase. DCCD can bind with the 16 kD subunits in the proteolipid section of $H^+$-ATPase to block proton translocation across the membrane[12]. NEM，a kind of alkylating reagent，could covalently modify the sulfhydryl group of cysteine in this protein[13]. The data in the Table 2 show that the proton pumping activity of $H^+$-ATPase in the tonoplast of *P. euphratica* was strongly inhibited by DCCD and NEM，and decreased rapidly with the concentration of inhibitors increasing. 100 μmol/L DCCD inhibited 55.3% of the proton pumping activity of $H^+$-ATPase，suggesting the existence of a kind of proton channel. The proton pumping activity was inhibited by 50 μmol/L NEM，suggesting that cysteine residue played an important role in $H^+$-ATPase.

Bafilomycin $A_1$，an antibiotic，can bind with $V_0$ section of this enzyme and block the proton channel. V type $H^+$-ATPase is very sensitive to this kind of antibiotic，the $IC_{50}$ was about $10^{-9}$ mol/L. However，F type $H^+$-ATPase is insensitive to bafilomycin $A_1$. So bafilomycin $A_1$ is a specific inhibitor of V type $H^+$-ATPase[14]. As shown in the Table 2，the proton pumping activity of $H^+$-ATPase decreased dramatically with increasing concentration of bafilomycin $A_1$. In the presence of 100 nmol/L bafilomycin $A_1$，about 75.9% of the activity of this enzyme was inhibited.

The data of Table 2 indicated that the tonoplast-enriched vesicles isolated from suspension-cultured *P. euphratica* cells by differential centrifugation and discontinuous sucrose gradient centrifugation showed high proton pumping activity. Through continuously monitoring the acridine orange fluorescence quenching，it was found that the proton translocation was ATP-dependent and could be started with $Mg^{2+}$ or $Fe^{2+}$ and stimulated by $Cl^-$ and $Br^-$. Conversely，the proton pumping activity could be strongly inhibited by DCCD，NEM，$NO_3^-$ and bafilomycin $A_1$，and insensitive to azide and vanadate. Therefore the

$H^+$-ATPase in the tonoplast of *P. euphratica* was one kind of V type ATPase.

## References:


[1] Wang Y-Z (王延枝), Xu X-Z (许献忠). The progress in the study of vacuolar-ATPase. *Prog Biochem Biophys* (生物化学与生物物理进展), 1993, **20**: 19-23. (in Chinese with English abstract)

[2] Stevens T H. Structure, function and regulation of the vacuolar $H^+$-ATPase. *Annu Rev Cell Dev Biol*, 1997, **13**: 779-808.

[3] Xia Z-H (夏朝晖), Chen J (陈珈). V-type $H^+$-ATPase in responses to stress. *Plant Physiol Commun* (植物生理学通讯), 1998, **34**: 168-174. (in Chinese)

[4] Gu R-S (谷瑞升), Jiang X-N (蒋湘宁), Guo Z-S (郭仲琛). Organogensis and plantlet regeneration *in vitro* of *Populus euphratica*. *Acta Bot Sin* (植物学报), 1999, **41**: 29-33. (in Chinese with English abstract)

[5] Liu Q-L (刘群录), Zhang X-J (张旭家), Li Y (李义), Wang S-S (王沙生), Huang Y-G (黄有国), Jiang X-N (蒋湘宁). Purification of tonoplast vesicles from *Populus euphratica* and its proton pumping activity. *Chin J Biochem Mol Biol* (中国生物化学与分子生物学报), 2000, **16**: 372-376. (in Chinese with English abstract)

[6] Müller M L, Irkens-kiesecker U, Rubeistein B, Taiz L. On the mechanism of hyperacidification in lemon. *J Biol Chem*, 1996, **271**: 1916-1924.

[7] Lowry O H, Rosebrough N J, Farr A L, Randall R J. Protein measurements with the folin phenol reagent. *J Biol Chem*, 1951, **193**: 265-275.

[8] Wang H (王欢), Zhu X-W (祝雄伟), Wang Y-Z (王延枝). Fluorescence measurement of proton pumping of tonoplast $H^+$-ATPase. *Prog Biochem Biophys* (生物化学与生物物理进展), 1999, **26**: 184-187. (in Chinese with English abstract)

[9] Zhang W-H (章文华), Liu Y-L (刘友良), Yu B-J (於丙军), He P-Q (贺平清). Improvement of determination of ATP-dependent proton pump in tonoplast vesicles from barley roots. *Plant Physiol Commun* (植物生理学通讯), 1997, **33**: 282-285. (in Chinese)

[10] Churchill K A, Sze H. Anion-sensitive, $H^+$-pumping ATPase in membrane vesicles from oat roots. *Plant Physiol*, 1983, **71**: 610-617.

[11] Widell S, Larsson C. A critical evaluation of markers used in plasma membrane purification. Larsson C, Mllфer I. The Plant Plasma Membrane-structure, Function and Molecular Biology. Berlin: Springer-Verlag, 1990.

[12] Kaestner K K, Randall S K, Sze H. N, N′-dicyclohexylcarbodiimide-binding proteolipid of the vacuolar $H^+$-ATPase from oat roots. *J Biol Chem*, 1988, **263**: 1282-1287.

[13] Feng Y, Forgac M. Inhibition of vacuolar $H^+$-ATPase by disulfide bond formation between cysteine 254 and cysteine 532 in subunit A. *J Biol Chem*, 1994, **269**: 13224-13230.

[14] Yoshimori T, Yamamoto A, Moriyama Y, Futai M, Tashiro Y. Bafilomycin $A_1$, a specific inhibitor of vacuolar-type $H^+$-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J Biol Chem*, 1991, **266**: 17707-17712.


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