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盐胁迫对豌豆根液泡膜 H^+-ATPase 活性及含量的影响

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摘要： 为了阐明液泡膜 H^+-ATPase 在盐胁迫下的作用和适应性调节机制，对豌豆(*Pisum sativum* L.)植株进行不同盐浓度和不同盐胁迫时间(1～3 d)的处理后，分别测定液泡膜 H^+-ATPase 的 H^+ 转运活性、水解活性和蛋白含量(A 亚基)的变化。结果表明，100 mmol/L 和 200 mmol/L NaCl 处理 1 d H^+-ATPase 的水解活性没有变化，而 250 mmol/L NaCl 处理 1 d 引起水解活性降低约 25%。100 mmol/L NaCl 处理 2 d 内水解活性没有变化，而第 3 天活性下降约 20%。但是上述盐胁迫均能提高液泡膜 H^+-ATPase 的质子转运活性，说明盐胁迫后 H^+-ATPase 的水解活性和质子转运活性的变化不成比例，盐胁迫可能导致偶联比率的改变。Western blot 研究发现，上述盐胁迫对液泡膜 H^+-ATPase(A 亚基)的含量基本无影响，仅 100 mmol/L NaCl 处理 3 d 后 A 亚基的量略有下降。这些结果证明，盐胁迫能刺激提高豌豆根液泡膜 H^+-ATPase 的 H^+ 泵效率，且泵效率的提高是源于偶联比率的改变，而不是由于 ATP 水解活性的提高和蛋白含量的增加。

关键词： H^+-ATPase；液泡膜；盐胁迫；豌豆根
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Effects of Salt Stress on the Activity and the Amount of Tonoplast H^+-ATPase from Pea Roots

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Abstract: To study the function and adaptive mechanism of tonoplast H^+-ATPase under salt stress, pea (*Pisum sativum* L.) seedlings were treated with different concentrations of salt (100－250 mmol/L NaCl) and with 100 mmol/L NaCl for different days (1－3 d). The ATP hydrolytic activity and the proton transport activity and the changes of the amount of tonoplast H^+-ATPase (subunit A) were measured. ATP hydrolytic activity of H^+-ATPase prepared from plants treated with 250 mmol/L NaCl was reduced by about 25% compared to that of control plants, but that of stressed plants treated with 100 mmol/L and 200 mmol/L NaCl was unchanged. The activity from plants treated with 100 mmol/L NaCl for up to 3 d was lower than that of control plants by 20%. But the proton transport activity was increased under the same salt stresses as above. These results showed that the changes of the hydrolytic activity and the proton transport activity were not in proportion and salt stress may cause the change of the coupling ratio of H^+-transport activity to ATP hydrolysis. The protein amount kept unchanged and reduced a little only when pea was treated with 100 mmol/L NaCl for 3 d. These results indicated that salinity stimulated the increase of the pump efficiency of the V-ATPase from pea roots, which was due to the change of the coupling ratio, but not due to the increase of ATP hydrolysis and the amount of V-ATPase.

Key words: H^+-ATPase; tonoplast; salt stress; pea roots

Much work has shown that plant growth declines when exposed to saline conditions. The deleterious effects of salinity are thought to come from water stress, ion toxicity, nutritional imbalance, or a combination of these

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factors[1]. The adaptation of plant cells to salt requires osmotic adjustment and ion compartmentalization and/or exclusion of sodium ions, which are driven by H^+ gradient across the membranes[1,2]. The plant vacuole is very important in response to salinity. Osmotic adjustment and ion compartmentalization both require the accumulation of ions and solutes in the vacuole[2]. One of the most important enzymes involved in the formation of proton gradient across tonoplast is vacuolar H^+-ATPase (V-ATPase)[3]. The V-ATPase pumps proton from the cytoplasm to the lumen of the vacuole using the energy released by ATP hydrolysis, functioning in cellular pH homeostasis as well as in providing the driving force for a wide range of secondary active and passive transport processes[4]. It is a complicated protein complex composed of at least eight subunits, among which the three ubiquitous subunits A, B and C are the major ones[4]. Subunit A is responsible for binding and hydrolyzing ATP, and subunit C is responsible for proton transport. The enzyme is sensitive to nitrate but is insensitive to vanadate in distinction to P-ATPase, and also insensitive to azide and oligomycin, the inhibitors of F-ATPase. Thus V-ATPase is characterized by its azide- and vanadate- resistant, nitrate- sensitive activity for ATP hydrolysis and its ATP-dependent activity for H^+-transport[4]. V-ATPase is not only extraordinarily important as a house-keeping enzyme, but also functions as a stress response enzyme under environmental stresses[4]. Recently, the effects of salt stress on V-ATPase have been studied in many plants and it has been found that the proton transport activity of V-ATPase increases under salt stress[5-11]. But due to the complexity of the enzyme, the mechanism of how to increase the proton transport under salt stress is still unclear. To elucidate the mechanism of regulation, the present work is undertaken to study the effects of salt stress on the ATP hydrolysis activity, proton transport activity and the amounts of V-ATPase in pea.

1 Materials and Methods

1.1 Plant material

Pea (*Pisum sativum* L.) seeds were soaked in running tap water overnight. Seeds were placed on the plastic plate with pores to grow for 7 days at 25 ℃. Water was supplied four times everyday. Then plants were exposed to aerated NaCl solutions of different concentrations for 1 day or 100 mmol/L NaCl for different days. Roots were collected. Control plants were treated with water.

1.2 Isolation and purity test of tonoplast-enriched vesicles (TEM)

Vesicles were prepared by a modification of the methods of our laboratory[6]. All operations were the same, except a different homogenate medium containing 250 mmol/L Sucrose, 1 mmol/L DTT, 1 mmol/L PMSF, 3 mmol/L EGTA, 0.2% (W/V) BSA, 3 mmol/L $MgSO_4$, 1.5% (W/V) PVP, 10% (V/V) glycerol, 50 mmol/L Hepes-Tris (pH 7.6) was used. The vesicles prepared were used immediately or frozen in liquid N_2 and stored at −70 ℃ for later use.

The purity of the isolated vesicles was tested by measurement of the activity of the marker enzymes[12] and the formation of ATP-dependent H^+ gradient across vesicle membranes (see **1.4** in detail), the difference between the maximum fluorescence quenching of acridine orange (AO) without KNO_3 and with KNO_3 represented the proton transport capability of V-ATPase inhibited by KNO_3.

1.3 Assay of ATP hydrolysis activity

The hydrolysis activity of nitrate-sensitive ATPase was measured by the method described by Ratajczak *et al*[12].

1.4 Assay of proton transport activity

The H^+ transport activity was measured by method of Hassidim *et al*[13]. The rate of acridine orange fluorescence quenching was measured with a Hitachi 4010 spectrofluorophotometer. The wavelength of excitation and emission light was 495 nm and 530 nm, respectively. The reaction medium consisted 250 mmol/L Sucrose, 33 mmol/L Hepes-Tris (pH 7.0), 50 mmol/L KCl, 3.5 mmol/L $MgSO_4$, 5 μmol/L AO (acridine orange), 50 μg TEVs. The reaction mixture was incubated at 25 ℃ for 5 min and the reaction was initiated by adding ATP (final concentration 3.5 mmol/L) and the rate of quenching was monitored and recorded. When maximum quench was reached 10 mmol/L NH_4Cl was added to the reaction medium. The recovering of the fluorescence to the level near the initial value, implied that fluorescence quenching was caused by proton transport across tonoplast. The proton transport activity of V-ATPase was represented by the initial rate of fluorescence quenching estimated by calculating the slope of a line drawn with data in approximately the first 10 s (quench·mg^{-1} protein·min^{-1}).

1.5 Western blot Analysis

The TEVs were solubilized in sample buffer containing 4% SDS, 10% glycerol, 0.25% mercaptoethanol, and 60 mmol/L Tris (pH 6.8) and 20 μg of protein were separated by SDS-PAGE with Bio-Rad Apparatus[14]. Western blot was conducted according to Towbin[15]. After electrophoresis, proteins were transferred to nitrocellulose filter at 80 V for 3 h. The filter blots were incubated

with 1∶500 dilution of antibodies against subunit A of V-ATPase for 2 h and the bands were visualized using a goat anti-rabbit alkaline phosphatase conjugate with 1∶30 000 dilution.

1.6 Protein determination

Protein in the membrane vesicles was quantified by the method of Bradford[16] with BSA as the standard.

2 Results

2.1 Purity of membrane vesicles

Fig. 1 shows the pumping activity of V-ATPase, that is the time-course of quenching of AO fluorescence. The maximum quenching of fluorescence without KNO_3 was taken as 100%. Fig. 1 shows that KNO_3 inhibited the ATP-dependent proton transport activity by 70%, suggesting that the vesicles were tonoplast-enriched. Fig. 1 also shows that when fluorescence did not decrease any more, addition of NH_4Cl destroyed the proton gradient across the membrane and fluorescence density recovered to near initial value, suggesting that fluorescence quenching rises from transport of H^+ into the vesicles.

The test of the activity of specific marker enzymes indicated that the membrane fractions isolated were tonoplast-enriched membrane vesicles (data not shown).

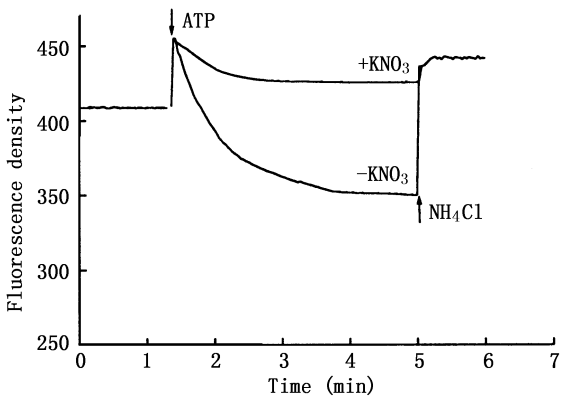

Fig. 1. Proton pumping activity of tonoplast H^+-ATPase from pea root.

2.2 Effects of salt stress on the hydrolytic activity of V-ATPase

The effects of treatment with different concentration of NaCl and with 100 mmol/L NaCl for different days on the hydrolytic activity of V-ATPase are shown in Figs. 2, 3, respectively. As shown in Fig. 2, ATP hydrolytic activity of H^+-ATPase prepared from plants treated with 250 mmol/L NaCl was reduced by about 25% compared to that of control plants, but that of stressed plants treated with 100 mmol/L and 200 mmol/L NaCl was unchanged. The activity from plants treated with 100 mmol/L NaCl for up to 3 d was lower than that of control plants by 20% (Fig. 3).

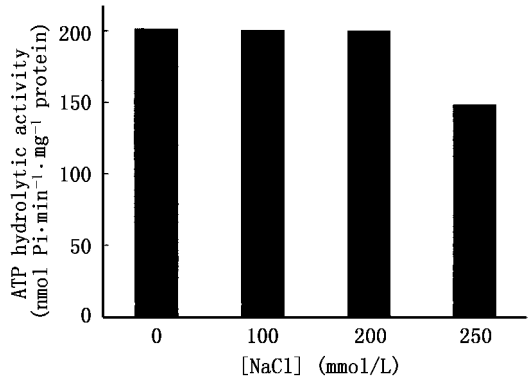

Fig. 2. Effects of treatment with different concentrations of NaCl for 1 day on the hydrolytic activity of tonoplast H^+-ATPase from pea roots.

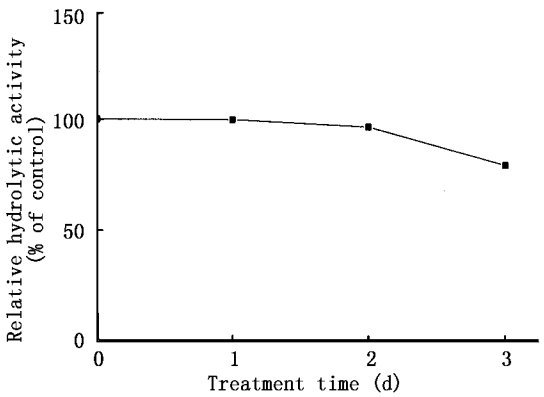

Fig. 3. Effects of treatment with 100 mmol/L NaCl for different days on the hydrolytic activity of tonoplast H^+-ATPase from pea roots (the hydrolytic activity before treatment with NaCl was taken as 100%).

2.3 Effects of salt stress on proton transport activity of V-ATPase

As shown in Fig. 4, after treatment with different concentrations of NaCl, the initial rate of proton transport and the maximum transport amount were both higher than those of control plants. The activity also increased when pea seedling were treated with 100 mmol/L NaCl for 1, 2 and 3 d. These results indicated that salt stress stimulated the ATP-dependent proton transport.

2.4 Effects of salt stress on the amount of V-ATPase protein

The TEVs of control and salt-treated pea seedlings were analyzed by SDS-PAGE and Western blot using a polyclonal antibody directly against subunit A of V-ATPase from mung bean. The protein contents of subunit A showed little variation and reduced a little only when pea was treated with 100 mmol/L NaCl for 3 d (Figs. 6, 7). In every lane, there were two blot bands, one was subunit A and the other was subunit B characterized by their molecular weight. The appearance of two blot bands may be due to the using of the polyclonal antibody.

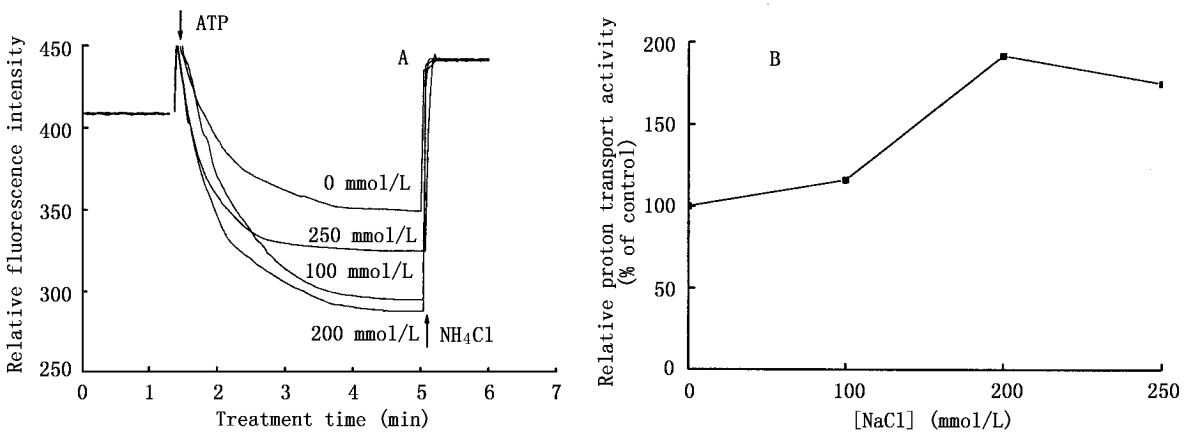

Fig.4. Proton transport activity of tonoplast H^+-ATPase from pea roots treated with different concentrations of NaCl for 1 d. A. Quenching of acridine orange fluorescence. B. Relative proton transport activity.

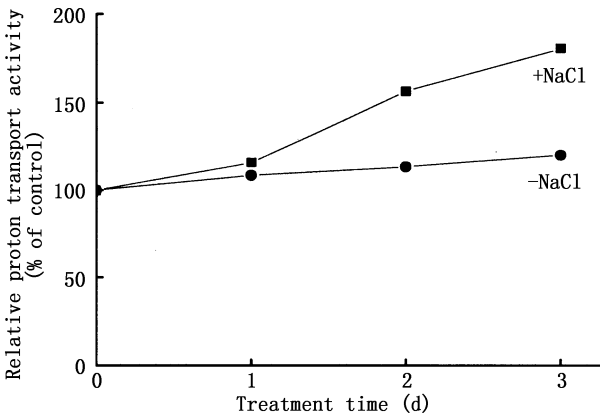

Fig.5. Effects of treatment with 100 mmol/L NaCl for different days on the proton transport activity of tonoplast H^+-ATPase from pea roots.

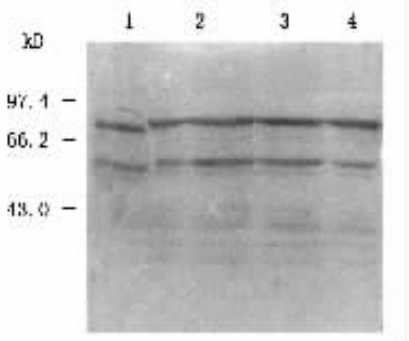

Fig.6. Western blot analysis of the tonoplast H^+-ATPase from pea roots treated with different concentrations of NaCl for 1 d. Lane 1, 0 mmol/L NaCl; Lane 2, + 100 mmol/L NaCl; Lane 3, + 200 mmol/L NaCl; Lane 4, + 250 mmol/L NaCl.

3 Discussion

The quality of the isolated tonoplast vesicles is very important in this study. Fig.1 shows that protons could be transported into vesicles in an ATP-dependent manner and that the vesicles were tonoplast-enriched, being not leaky to protons . The test of the activity of specific marker

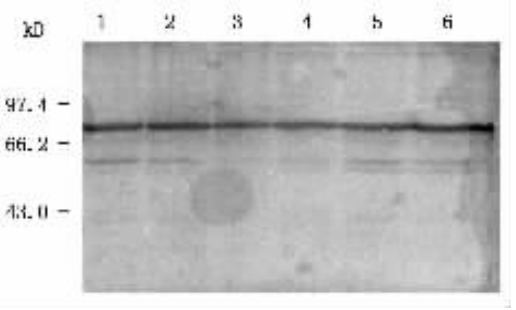

Fig.7. Western blot analysis of the tonoplast H^+-ATPase from pea roots treated with 100 mmol/L NaCl for different times. Lanes 1, 3, 5, represent control for 1 d, 2 d and 3 d, respectively; Lanes 2, 4, 6, represent NaCl treatment with 100 mmol/L NaCl for 1 d, 2 d and 3 d, respectively.

enzymes also showed that the vesicles were TEVs. These results suggested that vesicles prepared from pea roots were not only mostly vacuolar membranes being able to couple ATP hydrolysis to proton pumping, but also were sealed. Such high-quality vesicles can be used for further studies.

Evidence from this study indicates that salt stress results in obvious increase of the proton transport activity and maximum transport ability of V-ATPase (Figs.4,5). The increased proton transport activity of V-ATPase may provide stronger driving force for a wide range of secondary active and passive transport processes and stimulate the concentration of a series of ions and metabolic products in the vacuole, leading to osmotic adjustment and elimination of ion toxicity[2].

An interesting result is that salt stress increased H^+-transport activity of V-ATPase while the hydrolytic activity kept unchanged and even decreased by about 20% when pea is treated with low concentration of NaCl (100 mmol/L) for a long time (3 d) or with high concentration of NaCl (250 mmol/L) for 1 d (Figs.2,3). The immunoblotting data indicate that there was no significant change in protein content and the amount of protein

reduced only when pea was treated with 100 mmol/L NaCl for 3 d, and the extent of decrease was very little (Figs.6,7). These data indicate that the increase of proton transport activity was not due to the increase of hydrolytic activity of ATP and the amount of the protein of V-ATPase, but resulted from the alteration of the coupling ratio (moles of H^+ transported per moles of ATP hydrolyzed) of V-ATPase. Similar results have been obtained with vacuolar vesicles from *Daucus carota*[9], *Lycopersicon esculentum*[10] and *H. vulgare*[7] showing that salinity increased H^+-transport activity while ATP hydrolysis activity remained unchanged. The coupling ratio is thought usually to be 2. But using the patch-champ technique, Davies and his colleagues demonstrated that the coupling ratio was not a fixed parameter, it depended on not only the pH at both sides of the tonoplast but also the absolute difference of pH across the membrane, ranging between 1.75 and 3.28[3]. The increase in coupling ratio can be explained in several ways. Firstly, changes in lipid composition of tonoplast membrane occur as a result of exposure to salt that affects the structure and function of the V_0-complex, thereby indirectly stimulate the transport of H^+[17]. Secondly, the number of transport/binding sites for H^+ could be changed by post-translational modification of one or more V-ATPase subunits in salt-stress plants as compared to control plants[18]. Thirdly, the coupling ratio of V-ATPase could be modified by a change of the pump 's intramolecular disulphide bridges. It has been shown with V-ATPase of coated vesicles from bovine train that the pump could be reversely inactivated by oxidation, about 50% of the pumps in such vesicles were in the oxidized inactive state[19]. In such a mechanism, the H^+-transport should be more sensitive to redox state than ATP hydrolysis[19,20].

Our previous results with CAM (crassulaceae acid metabolism) plant *Crassula agenten* Thunb showed that the responses to salt stress were very different among different plant. Under salt stress, both the hydrolytic activity and the proton transport activity increased, but the coupling ratio kept unchanged, being around 2[6]. It was also found that the amount of protein increased[6]. We supposed that in *Crassula agenten* Thunb the increased proton transport activity might come from the increase of the amount of V-ATPase. It has been reported that in C_3/CAM intermediate plant *Mesembryanthemum crystallimum*, salt treatment of intact plants and cultured cells stimulates the increase of H^+-transport activity to a larger degree compared to ATP hydrolysis activity and also stimulates the increase of the amount of protein[12]. It is likely that due to the complexity of the subunit composition and the structure, V-ATPase is very sensitive to salt stress and highly variable in salt stress responses.

The results in this study indicate that salinity stimulates the increase of the pump efficiency of the V-ATPase from pea roots, not because of the increase of ATP hydrolytic activity and not the amount of V-ATPase, but due to the change of the coupling ratio. Further study should be carried out to elucidate the mechanism of how the coupling ratio is altered.

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