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不同生境下木麻黄脯氨酸含量和质膜ATPase活性的研究*

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摘要 采集生长于恶劣环境和中生环境的普通木麻黄(*Casuarina*)小枝，超速离心提取粗质膜制剂后，用两相系统法纯化得到质膜微囊，研究不同生境下木麻黄的质膜ATPase活性，并测定木麻黄小枝的游离脯氨酸含量。实验结果表明：同一生境中的木麻黄ATPase活性相对一致，而同一树种木麻黄不同生境下质膜微囊H^+-ATPase、Ca^{2+}-ATPase、K^+-ATPase活性有显著差异，表现出以渗透胁迫为主的恶劣环境下的木麻黄质膜微囊ATPase活性和木麻黄细胞内游离脯氨酸明显高于中生环境下生长的木麻黄。说明普通木麻黄在干旱和盐胁迫下能调整生理生化过程来提高其质膜ATPase活性和增加细胞内脯氨酸含量提高渗透调节能力以保证其在恶劣环境的正常生长。

关键词：木麻黄 质膜微囊 ATP酶 环境胁迫

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CHANGES OF PLASMA MEMBRANE ATPase ACTIVITIES AND PROLINE ACCUMULATION IN TWIGS OF *CASUARINA* UNDER DIFFERENT GROWTH CONDITIONS*

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ABSTRACT Twigs of *Casuarina* were collected from Dongshan and Xiamen Island, China. Crude plasma membrane preparation was obtained from *Casuarina* twigs by ultra-centrifugation. Sealed plasma membrane vesicles were isolated with aqueous PEG3350-Dextran T500 two phase partitioning. We studied the proline accumulation in twigs and the ATPase activities of plasma membrane of *Casurina* under different growth conditions. The results showed that free proline content and the PM ATPase activities from unfavorable growth conditions were obviously higher than those from favorable environment. This suggested that *Casuarina* responded to osmotic stress by increasing osmotic material content and the proton transportation activities of PM ATPase.

Key words: *Casuarina*. Plasma membrane vesicles. ATPase. Environmental stress

Casuarina, the woody tree grown widely in tropic and subtropics, can grow in seashore and salt-alkali soil to form shelterbelt. With its azotification, growth efficiency, good adaptability, strong salt tolerance, fine wind-preventing ability and sand-fixing ability, the species can not only offer lignum but also protect crops in agroforestry system by breaking wind in sandy coast. In order to find out the relationship between the growth of *Casuarina* and the seashore environment, one should study their eco-physiological characteristics, but the researches on the cellular and

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molecular biological characteristics of *Casuarina* were rarely reported, and the chief of previous studies on *Casuarina* was cultivation and forestation. For these reasons, this paper discussed the ATPase activities of plasma membrane vesicles of *Casuarina* under different conditions to disclose the mechanism of salt tolerance of this species.

Plasma membrane vesicles ATPase is regarded as the "master enzyme", because it can regulate many important physiological processes including cell division and elongation[1], as well as metabolism proceedings to resist stress such as osmoregulation, ions compartmentation[2] and so on, it is indispensable to plant cell under adversities and stresses including salt stress[3], freeze stress and drought stress[4]. For the difficulty of distilling plasma membrane vesicles from plant cell, especially in woody plant, the previous studies of ATPase laid great emphasis on the radicle and foliage of the herbaceous plant, there were few researches on the relationships between stress and ATPase activities in woody species. The materials utilized in this study were twigs of *Casuarina* (because their leaves were degenerated), so that this research would provide a new method to reveal the acclamatory responses of woody plant cell.

Proline accumulation, regarded as an index of osmotic stress of tolerant plant, can reflect the level of stress in a certain extent. Accordingly the free proline content of the *Casuarina* twigs was measured to uncover the relation between salt resistance of this species and osmotic stress.

1 MATERIALS AND METHODS

1.1 Field samples

1.1.1 Geographical situation

Dongshan Island shelter forest was located on 117° 18′E and 23° 3′N in Fujian Province. Xiamen Arboretum was located on 118°15′E and 24°25′N.

1.1.2 Climate condition

It was south subtropical oceanic climate in Xiamen, where its annual average air temperature was 20.8℃, with average annual precipitation of 1555.5 mm and average annual evaporation of 1651.3mm. In contrast, it was south subtropical coastal climate in Dongshan shelter field, where its average annual precipitation was 945.3 mm with average annual evaporation of 1656.1 mm and average air temperature of 20.8℃. It can be assumed that *Casuarina* in Dongshan Island was under environmental stress for the dry wind occurring from September to November.

1.1.3 Plant materials

Experimental materials were collected from Damao Mountain in Dongshan-Chishan shelter forest on October 23, 2001. There was a *Casuarina* windbreak with the length of 2000 meters and width of 300 meters near seashore. The first experiment zone was in the first forward position facing sea, each sample was gathered every 50m along the perpendicular coastline at random, 12 samples were obtained totally, each sprig of *Casuarina* came from the same orientation, the collected twigs were immediately stored in liquid nitrogen. In order to compare the *Casuarina* under stress with those in normal environment, at the same time we collected the twigs of this species in Xiamen botanic garden as the control, where the growth condition was favorable to plant. The differences of climate between Dongshan and Xiamen Island were showed in table 1[5].

1.1.4 Soil materials

Soil sampling and physical and chemical characteristics determining was carried out as described to "Methods for Soil Agricultural and Chemical Analysis"[6].

Table 1 Differences of climate between Xiamen and Dongshan Island in three months

Annual climate	Average temperature (℃)		Average wind speed (m/s)		Average precipitation (mm)		Average evaporation (mm)	
Month	Xiamen	Dongshan	Xiamen	Dongshan	Xiamen	Dongshan	Xiamen	Dongshan
Sep	26.7	26.6	3.5	6.2	119.3	97.2	178.2	205.7
Oct	23.2	23.6	4.1	8.6	42.7	42.8	192.8	249.5
Nov	19.2	19.7	4.0	8.8	38.5	30.6	145.9	193.6

1.2 Measurement of free proline content in twigs

Proline was extracted by grinding the fresh leaf tissue in a mortar with a pestle using 10 ml 3% (w/v) sulphosalicylic acid, then the ground tissue was heated with the acid in a sealed test tube at 100℃ for 30 minutes. Aliquots (2.0ml) of this extract were used for proline quantification following the acid ninhydrin procedure modified by Bates[7].

1.3 Separation and purification of plasma membrane vesicles and assay of ATPase hydrolysis activity

1.3.1 Extraction and fractionation of membrane

Plasma membrane vesicles were prepared referring to the technique described by Zheng H L with some modifications, being used in the determinations of protein content and ATPase activity[8]. Twigs were washed with cold deionized water and homogenized in extraction solution [Tris-Mes 15mmol/L (pH7.8), EGTA-Na_2 3mmol/L, Sucrose 0.25 mmol/L, 0.6% (v/v) PVP K-30, PMSF 1mmol/L]. PVP K-30 was added into homogenate solution to eliminate tannin and botanic hydroxybenzene from tissues of *Casuarina*. The crude membrane fractions were purified by two-phase partition[9]. The purified plasma membrane-enriched fractions were used immediately or frozen at -70℃ for later use.

1.3.2 Determination of H^+-ATPase hydrolysis activity of membrane vesicles

The PM H^+-ATPase hydrolysis activity was measured by methods of Zhang W H[10]. 20μl vesicle membrane preparations was added into 0.5ml reaction medium which contained $MgSO_4$ 3mmol/L, K_2SO_4 25mmol/L, Tritonx-100 0.02% (v/v), Tris-Mes 50 mmol/L (pH6.5), ATP-Na_2 3 mmol/L . The enzyme reaction was incubated at 37℃ for 30min and stopped by 20% (W/V)TCA. The activity of the enzyme was calculated by the amounts of Pi formed by hydrolysis in the reactions.

1.3.3 Determination of PM H^+-ATPase hydrolysis activity

The PM H^+-ATPase hydrolysis activity was quantified by method of Li M R[11]. 0.5ml reaction system contained Tris-Mes 36 mmol/L (pH6.5), $MgSO_4$ 3 mmol/L, ATP-Na_2 3 mmol/L, NH_4MoO_4 1 mmol/L, NaN_3 1 mmol/L, $NaNO_3$ 50 mmol/L, Tritonx-100 0.02% (v/v), and 20μl vesicle preparations. Chemical reaction was kept at 37℃ for 15min and stop by 20% TCA. The amounts of Pi formed by hydrolysis in solution were measured.

1.3.4 Determination of PM K^+-ATPase hydrolysis activity

PM K^+-ATPase hydrolysis activity was determined in term of the methods of Yi Y and Tang Z C[12]. 0.5ml reaction system contained sucrose 0.25 mol/L, Tris-Hepes 25 mmol/L (pH 7.5), NaN_3 1 mmol/L, $NaNO_3$ 50 mmol/L, NH_4MoO_4 0.1 mmol/L, EDTA 0.1 mmol/L, Tritonx-100 0.05% (v/v), KCl 3 mmol/L, ATP-Na_2 3 mmol/L, and 20μl vesicle preparations, the chemical condition was the same as the former one.

1.3.5 Determination of PM Ca^{2+}-ATPase hydrolysis activity

PM K^+-ATPase hydrolysis activity was determined referring to the methods of Li X M[13]. 1.1ml reaction system contained imidazole 10 mmol/L, $MgCl_2$ 5 mmol/L, $CaCl_2$ 50μmol/L, ATP-Na_2 3 mmol/L (pH7.0), and added 20μl membrane preparations. The enzyme reaction was the same as the of H^+-ATPase activity.

1.3.6 Determination of Pi

Determination of Pi was carried out referring to Ohnishi[14], and protein content was measured by using Coomassie Brilliant Blue G-250 referring to Bradford[15]. All the data came from the average value of three measure repetitions.

2 RESULTS

2.1 Results of sampling

The collected samples were showed in table 2, CK came from Xiamen botanic garden, $1^{\#}$ – $12^{\#}$ samples came from Dongshan shelter field. (Distance was indicated distance from sea; Age was indicated the years old of tree; DBH was indicated diameter at breast height of tree).

2.2 Physical and chemical characteristics of soil

Table 3 showed the physical and chemical characteristic of the soil collected from Dongshan Island shelter field. These figures demonstrated that soil of

Table 2 Circumstance of 12 samples from Dongshan shelter field and CK from Xiamen arboretum

	CK	1	2	3	4	5	6	7	8	9	10	11	12
Age	30	38	38	34	38	38	39	38	30	10	23	38	38
Distance	5km	10m	50m	80m	100m	100m	120m	150m	200	230	300m	300m	330m
Forest	nature	shelter	shelter	pure	pure	pure	pure	pure	pure	pure	pure	mixed	mixed
DBH(cm)	14.0	6.8	7.0	7.6	9.0	8.2	8.6	8.5	8.5	7.8	8.9	8.0	8.1
Height(m)	13.0	5.5	6.0	7.5	8.5	7.0	8.0	8.0	8.0	7.5	8.0	8.0	8.0
Growth	good	weak	weak	young	normal	normal	normal	normal	normal	normal	normal	shaded	shaded

Table 3 Characteristics of Dongshan Island shelter soil samples

Silver and powder sand (<0.15cm)	pH	Total l nitrogen	Total phosphorus	Total Potassium
80% - 95%	5.0 - 5.6	0.015% - 0.047%	0.024% - 0.074%	0.74% - 1.43%

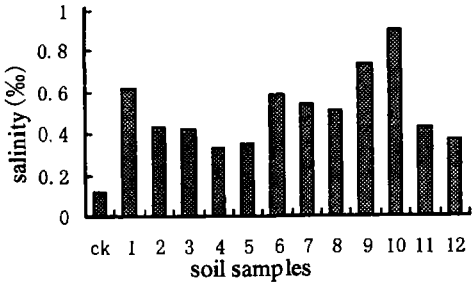

Fig. 1 Soil salinity of 12 samples came from Dongshan shelter field (1# - 12#) and Xiamen(CK)

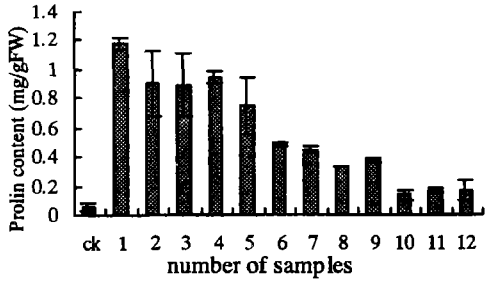

Fig. 2 Free proline content in twigs of Casuarina came from Dongshan shelter field (1# - 12#) and Xiamen(CK)

Dongshan Island was quite infertile. The Fig. 1 showed that the soil salinity of Xiamen garden was 0.12‰ and the Dongshan soil salinity ranged from 0.33‰ to 0.74‰.

2.3 Free proline accumulation in twigs of *Casuarina*

The experimental results showed in Fig. 2 that the free proline content of CK twigs was much lower than that of the Dongshan *Casuarina* (1# - 12#), the closer to the seashore, the higher the proline content was. With the increasing environmental stress level, the free proline accumulated vastly in twigs of *Casuarina*, for example, the twigs proline content of sample 1# was the highest among these samples, which was 20 times higher than that of CK, in that sample 1# was collected from seashore where trees were damaged by salt frog and typhoon most directly and violently. The proline content of 10#, 11# and 12# sample was lower than 1# - 9# sample for their locating in the other side of shelterbelt where environmental stress was relatively mild. This result was consistent with the previous researches that proline biosynthesis was activated by salt stress[16].

2.4 H^+-ATPase hydrolysis activity of membrane vesicles of *Casuarina*

The F-type ATPase existing in mitochondria and chloroplast could be inhibited by azide, V-type ATPase existing in vacuole, could be inhibited by nitrate. In order to measure the H^+-ATPase activity of membrane preparations, the majority of which were plasma membrane, but maybe contained chloroplast and vacuole membrane, so NaN_3 or $NaNO_3$ was not added into this reaction medium. Experimental result (Fig. 3-a) showed that the membrane vesicles H^+-ATPase hydrolysis activity of all the Dongshan samples was higher than CK, which illuminated that environment stress can promote H^+-ATPase activity to adapt itself to unfavorable conditions. H^+-ATPase activity of 1# and 2# *Casuarina* was the lowest of all, possibly because they came from the frontline of the shelter forest, damaged by typhoon and salt frog most violently and directly. The H^+-ATPase activity of sample 4# reached maximum, presumably appro-

priate stress could stimulate ATPase activities to the whole hog. Moreover the data of $5^{\#}$ – $12^{\#}$ sample were similar, probably they all grew in the center of community and the attacks of sea wind were less. The results were in accord with the previous reports that claimed that stressful growth conditions could stimulate H^+-ATPase activity of halophyte[17].

2.5 PM H^+-ATPase hydrolysis activity of *Casuarina*

NaN_3 and $NaNO_3$ were added into the reaction system to inhibit the activities of F-ATPase and V-ATPase. The H^+-ATPase was activized by Mg^{2+} ions in solution. This result (Fig. 3-b) was similar to the former outcome (Fig. 3-a), but MV (membrane vesicles) H^+-ATPase activity was a little higher than PM H^+-ATPase, presumably the membrane vesicles contained partly chloroplast or mitochondria membrane besides plasma. PM H^+-ATPase activity of CK was lower than that of all those from Dongshan samples. The sample $1^{\#}$ obtained from seashore was straightly attacked by salt frog, thus its PM H^+-ATPase activity was the highest one. Comparatively the discrepancies of PM H^+-ATPase activity of sample $2^{\#}$ – $12^{\#}$ were not obvious. Furthermore PM H^+-ATPase activity of $11^{\#}$ and $12^{\#}$ sample was relatively lower than that of all the others, for they were farther away from the sea, or shaded by *Eucalyptus*, Acacia and Pinus in mixed-forest, which would reduce their photosynthesis efficiency.

2.6 PM K^+-ATPase activity of *Casuarina*

It was indicated that some primary proton transportation proteins do not need Mg^{2+}, Mg^{2+} ions were indispensable to H^+-ATPase activity, so when determining K^+-ATPase activity, the magnesium ions were not added into the reaction medium. This result (Fig. 3-c) showed that PM K^+-ATPase activity of Dongshan *Casuarina* was about ten times that of Xiamen garden, so it was an example to illustrate that K^+-ATPase activity could be promoted under environmental stress, which was consistent with the already known results under osmotic stress[12].

2.7 PM Ca^{2+}-ATPase activity of *Casuarina*

In order to inhibit another type ATPase, imidazole was added into reaction medium[13]. The results was the same as the former ones, PM Ca^{2+}-ATPase activity of the control was lower than that of Dong-

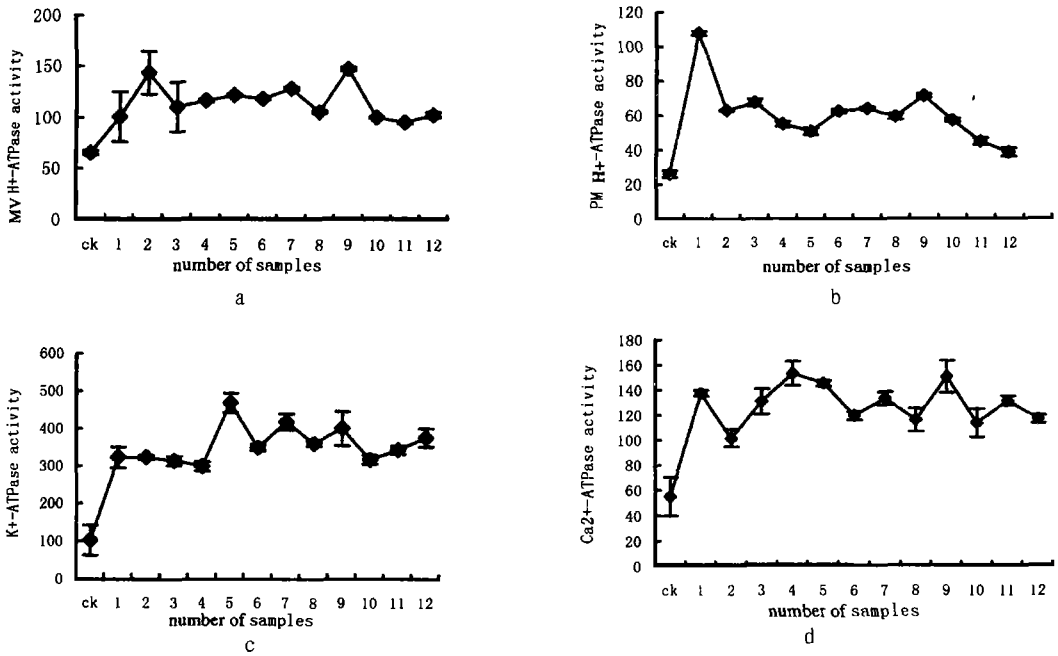

Fig. 3 ATPase activities of *Casuarina* under different growth condition

a. Membrane vesicles H^+-ATPase activity; b. PM H^+-ATPase activity;
c. PM K^+-ATPase activity; d. PM Ca^{2+}-ATPase activity.
The unit of ATPase activity is μ molPi·mg^{-1} protein^{-1}·h^{-1}.

shan, and the data of $1^{\#} - 12^{\#}$ sample were close to each other. There were reports interpreting why Ca^{2+}-ATPase transcription activity would be enhanced under stress, in lack of salt and phosphorus, Ca^{2+}-ATPase can pump Ca^{2+} from cytoplasm to outer-cytoplast and into cell organelles, and balance of Ca^{2+} content in cytoplasm can be kept in this way[18].

3 DISCUSSION

Dongshan Island was surrounded by sea, with environmental stresses including drought, salt, typhoon and infertile soil, for this reason, it was described as "There are nine droughts in ten year", where stressful growth condition was very unfavorable to plants. But *Casuarina* can grow well there because of their salt tolerance, anti-drought ability, leanness endurance and wind resistance. This experiment studied ATPase called "Master Enzyme", the conclusion could be drawn that ATPase activity would be increased under certain stresses. Binzle etc had proved that plasma membrane ATPase could balance the osmosis potential of cytoplasm to keep moisture of cell and improve its abilities to resist environment stress in plant cells[19]. In summary, increasing PM-ATPase activity played important role for *Casuarina* to adapt itself to the stressful growth conditions.

Salt stress may cause ionexcess, iondeficits and osmotics stress. To keep osmotic balance in cell, free proline was synthesized vastly in *Casuarina* under environmental stress. In order to mitigate salt stress, *Casuarina* should gain more energy to produce osmotic substances such as proline by improving H^+-ATPase and K^+-ATPase activities. It can be conjectured that to avoid excessive Na^+ which would damage halophyte cell, some available ions such as K^+ would be accumulated in cytoplasm and osmotic agents would be synthesized to increase osmotic value, also excessive Na^+ ions would be comparted in vacuole or transported into old tissues. These processes of salt tolerance were all based on ATP working as their direct energy, consequently PM ATPase activity of *Casuarina* in Dongshan must be heightened to resist environmental stresses. In contrast, *Casuarina* of Xiamen grew better than that of Dongshan apparently, because the growth condition in Xiamen was favorable to *Casuarina*, there was no need to consume extra energy to resist stress, so the PM ATPase activity was lower than that of Dongshan.

REFERENCES

[1] Serrano, R., 1989, Structure and function of plasma membrane ATPase. *Annu. J. Rev. Plant Physiol. Plant Mol. Biols.*, **40**: 61－69.

[2] Michel, O. S., U. Pick and A. Mordhay, 1988, Involvement of the plasma membrane ATPase in the osmoregulatory mechanism of alga Dunaliella salina. *Plant Physiol*, 1989, **89**:1258－1263.

[3] Reinhold, L., Y. Braun, M. Hassidim and H. Lerner, 1989, The possible role of various membrane transport mechanisms in adaptation to salinity. In: Environmental Stress in Plants. NATO SAI Series (J. H. Cherry ed), Berlin: Springer Verlag Verlin Heidelberg, Vol. G1 **9**:121－130.

[4] Arora, R. and J. P. Palta, 1991, A lose in the plasma membrane ATPase activity and its recovery coincides with incipient freeze-thaw injury and postthaw re-covery in onion bulb scale tissue. *J. Plant Physiol.*, **95**: 846－852.

[5] Song, D. Z. , 1996, Climate of Fujian Island. In: Cause of Climate Formation, (D. Z. Song and P. D. Wang), Beijing: Weather Publishing Company, 19－54.

[6] Lu, R. K., 2000, Measure of analyzing agricultural and chemical character of soil. In: Sampling and Measure of Analyzing Nutrient of Soil, (D. P. Yang, X. H. Fan and R. K. Lu), Beijing: Chinese Agriculture and Technology Publishing Company, 85－188.

[7] Bates, L. S., R. P. Waldren and I. D. Teare, 1973, Rapid determination of free proline for waterstress studies. *Plant and Soil*, **39**: 205－207.

[8] Zheng, H. L., Z. Q. Zhao, C. G. Zhang and X. J. Huang, 2000, Changes in lipid peroxidation, the redox system and ATPase activities in plasma membranes of rice seedling roots caused by lanthanum chloride. *BioMetals.*, **13**: 157－163.

[9] Zhu, M. J., J. Chen, Q. H. Zhu and X. C. Wang, 1997, Identification of plasma membrane of maize roots prepared by two-phase partition. *Chinese Biochemical Journal*, **13**(6): 686－690.

[10] Zhang, W. H., Y. L. Liu and B. J. Yu, 1997, Improvement of determination of ATP-Dependent proton pump in tonoplast vesicles from barley Roots. *J. Plant Physiology Commun.*, **33**(4): 282－285.

[11] Wang, J. M. and M. R. Li, 2000, Effect of Low Temperature on the Activity of Mg^{2+}-ATPase in Root Plasma Membrane Tonoplast Membrane of Rice Seedlings. *Journal of Hubei Agricultural College*, **20**(4): 295－305.

[12] Yi, Y. and Z. C. Tang, 1996, K^+-ATPase and its transport characterization of plasma membrane vesicles from

sorghum roots after osmotic stress. *Acta Phytophysiologica Sinica*, **22**(3): 231－236.

[13] Li, X. M. and J. Z. Ni, 1995, Effects of rare earths on the activity of sarcoplasmic reticulum Ca^{2+}-ATPase. *Chinese Biochemical Journal*, **11**(3): 281－284.

[14] Ohnishi, T., R. S. Gall and M. L. Mayer, 1978, An improved assay of inorganic phosphate in the presence of extralabile phosphate compounds: Application to the ATPase assay in the presence of phosphocreatine. *Anal Biochem*, **69**: 261－267.

[15] Bradford, M. M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**: 248－254.

[16] Zhao, F. G., C. Sun and Y. L. Liu, 2001, Ornithine pathway in proline biosynthesis activated by salt stress in Barley seedlings. *J. Acta Botanica Sinica*, **43**(1): 36－40.

[17] Guo, F. Q., H. Huang and Z. C. Tang, 1999, Effects of NaCl Stress on H^+-ATPase and H^+-PPase activities of tonoplast-enriched vesicles isolated from the roots of salt-tolerant mutant of wheat and its wild type. *J. Acta Phytosiologica Sinica*, **25**(4): 395－400.

[18] Muchihal, U. S., C. Liu and K. G. Raghothama, 1997, Ca^{2+}-ATPase is expressed differentially in phosphatestraved roots of tomato. *J. Plant Physiol*, **101**: 540－544.

[19] Binzel, M. L., 1995, NaCl-induced accumulation of tonoplast and plasma-membrane H^+-ATPase message in tomato. *J. Physiolgia Plantarum*, **94**: 772－728.