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Effects of freezing on plasma membrane H^+-ATPase of the callus from *Chorispora bungeana*

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Abstract

The influence of freezing treatment on plasma membrane (PM) H^+-ATPase was investigated using plasma membrane vesicles isolated from calluses from *Chorispora bungeana* Fisch. & C.A. Mey. by the discontinuous sucrose gradient centrifugation. Freezing treatment (-4 °C) for 5 d resulted in significant increases in the ATPase activity and the activity of *p*-nitrophenyl phosphate (PNPP) hydrolysis, decreases in the K_m for ATP hydrolysis and PNPP hydrolysis, and the shift of optimal pH from 6.5 to 7.0. Also, the activity PNPP hydrolysis was less sensitive to vanadate after freezing treatment compared to control, while the inhibition of ATP hydrolysis by hydroxylamine was more sensitive. In addition, freezing treatment also decreased the activation effects of trypsin on PNPP hydrolysis, but increased the activation effects of lysophosphatidylcholine on ATP hydrolysis. Taken together, these results suggested that PM H^+-ATPase might play an important role during adaptation to freezing and enhancing the frost hardness in *C. bungeana*.

Additional key words: alpine plant, kinase domain, *p*-nitrophenyl phosphate, phosphatase domain.

Introduction

The plasma membrane (PM) H^+-ATPase is a proton pump, which can pump protons from the cytoplasm to the apoplast and generate an electrochemical potential difference across the membrane (Morsomme and Boutry 2000). It plays important roles in the growth and development of plants, including nutrient uptake, phloem loading, stomatal opening, cell elongation and division (Serrano 1989, Briskin 1990, Michelet and Boutry 1995). This enzyme is a P-type ATPase, forming a phosphorylated intermediate during catalysis (Vara and Serrano 1983). Structural analysis indicates that P-type H^+-ATPase consists of a phosphatase domain, a transduction domain and a kinase domain. The kinase domain is the ATP-binding site that catalyses the formation of the phosphorylated intermediate, while the phosphatase domain catalyses the dephosphorylation of the phosphorylated enzyme (Serrano 1989).

The PM H^+-ATPase is encoded by a multigene family, and at least ten isoforms of the H^+-ATPase have been identified in plants (Morsomme and Boutry 2000). The expression of the enzyme seems to be dependent on the plant species and developmental stage (Michelet and Boutry 1995). The enzyme is modulated by phospholipids, Ca^{2+}, hormones, cGMP, light, 14-3-3 proteins, proton pump interactor 1 (Ppi1), *etc.* (Serrano 1989, Briskin 1990, Cooke and Burden 1990, Michelet and Boutry 1995, Suwastika and Gehring 1999, Morsomme and Boutry 2000, Yan *et al.* 2002). In addition, the ATPase activity and gene expression of this enzyme are affected by various environmental factors, such as drought, cold, salt, γ-radiation, iron deficiency and phosphate deficiency. Therefore, PM H^+-ATPase is thought to be involved in plant response to environmental stresses (Dong *et al*. 1994, Ahn *et al*. 1999, Rodriguez-Rosales *et al*. 1999, Dell'Orto

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Abbreviations: ATP - adenosine triphosphate; BSA - bovine serum albumin; DTT - dithiothreitol; EDTA - ethylenediaminetetraacetic acid; lyso-PC - lysophosphatidylcholine; Mes - 2-(*N*-morpholino)-ethanesulfonic acid, PM - plasma membrane; PMSF - phenylmethylsulfonyl fluoride; PNPP - *p*-nitrophenyl phosphate; Tris - *N*-tris(hydroxymethyl)-amino methane.
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et al. 2000, Morandini *et al.* 2002, Gong *et al*. 2003, Yang *et al*. 2004, Zhao *et al*. 2004, Chen *et al*. 2005).

Although there are some evidences suggesting that PM H^+-ATPase is sensitive to low temperature, the effects of freezing treatments on PM H^+-ATPase are controversial at present. For example, Zhao *et al.* (1995) showed that the PM H^+-ATPase activity decreased after several weeks of conditioning at 5 °C, but the activity of seedlings conditioned at 5 °C was less inhibited by freeze-thaw event (-5 or -10 °C). However, Iswari and Palta (1989) found that slight freezing treatments resulted in an increase in PM H^+-ATPase activity and a significant decrease in its K_m, while more severe freezing treatments resulted in decreases in both PM H^+-ATPase activity and K_m. Ahn *et al*. (1999) demonstrated that the gene expression of PM H^+-ATPase increased in roots of fig-leaf gourd plants under chilling soil temperature. However, there are only few reports concerning the changes of catalytic mechanism for the PM H^+-ATPase, and the alteration of the kinase domain and phosphatase domain of the enzyme under freezing condition. In order to study the effects of freezing treatment on the catalytic process of PM H^+-ATPase, we purified plasma membrane of the callus from *C. bungeana*.

C. bungeana is a representative alpine subnival plant which can survive frequent temperature fluctuations and freezing temperatures (A *et al.* 1998, An *et al.* 2000). It does not possess special morphological characteristics to adapt to freezing environment (A *et al*. 1998). Therefore, the changes of physiological and molecular mechanisms probably contribute to the adaptation to freezing environment in *C. bungeana.* Recently, it has been shown that the distribution and accumulation of Ca^{2+} could play an important role in the active cold-hardness in *C. bungeana* (Fu *et al.* 2005). In this study, we used the callus from *C. bungeana* to study the adaption to freezing environment and investigated the role of PM H^+-ATPase under freezing environment.

Materials and methods

Embryogenic calluses, derived from mature seeds of *Chorispora bungeana* Fisch. & C.A. Mey., were obtained as described by Fu *et al.* (2005). After sub-cultured several times, the calluses were divided to two groups, one was cultured under 25 °C with a 16-h photoperiod (irradiance of 80 μmol m^{-2} s^{-1}) as control and the other was cultured under -4 °C with the same photoperiod for 5-d as freezing treatment.

The plasma membranes were prepared by discontinuous sucrose gradient centrifugation methods as described by Qiu and Su (1998) with modifications. All steps were carried out at 4 °C. *C. bungeana* calluses were ground into power with liquid N_2 and homogenized in the isolation medium (1:2, m/v) containing 0.25 M sucrose, 10 % glycerol, 0.5 % bovine serum albumin (BSA), 3 mM EDTA, 1 mM dithiotreitol (DTT), 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.5 % polyvinylpyrrolidone (PVP-40) and 25 mM Tris-Mes, pH 7.6. The homogenate was filtered through four layers of cotton gauze and centrifuged at 15 000 *g* for 30 min. The supernatant was then centrifuged for 30 min at 80 000 *g* to obtain a microsomal pellet (microsomal membranes) which was resuspended in a buffer containing 0.25 M sucrose, 0.2 % BSA, 1 mM DTT and 2 mM Tris-Mes, pH 7.2. The microsomal membranes were carefully layered onto a previously prepared discontinuous gradient of sucrose solutions which consisted of 34 % (m/v) sucrose layered over 41 % sucrose. Both sucrose solutions contained 1 mM DTT and 2 mM Tris-Mes, pH 7.2. The mixture was then centrifuged at 100 000 *g* for 80 min. The fraction at the interface of 34 - 41 % sucrose solution was collected, diluted 3- to 5-fold with suspension buffer, and then centrifuged at 80 000 *g* for 30 min, after which the pellet was collected.

The purity of the plasma membrane was estimated according to Widell and Larsson (1990). The enzyme activity was inhibited by vanadate over 70 %, and reduced by azide, molybdate and nitrate less than 2.5 %, showing that the high purity of plasma membranes was obtained.

ATPase activity was determined by measuring the release of Pi from ATP according to Qiu and Zhang (2000). The reaction medium contained 3 mM ATP, 3 mM $MgSO_4$, 50 mM KCl, 1 mM NaN_3, 50 mM $NaNO_3$, 0.1 mM Na_2MoO_4, 0.02 % Triton X-100, 25 mM Tris-Mes (pH 6.5), and 10-15 μg PM proteins.

Hydrolysis of *p*-nitrophenyl phosphate (PNPP) was determined by measuring the release of Pi from PNPP according to Qiu and Zhang (2000). The reaction medium contained 20 mM PNPP, 50 mM KCl, 1 mM NaN_3, 50 mM $NaNO_3$, 0.1 mM Na_2MoO_4, 0.02 % Triton X-100, 25 mM Tris-Mes (pH 7.0), and 45-50 μg PM proteins.

Trypsin treatment of plasma membrane vesicles was performed as described by Qiu and Zhang (2001). Lysophosphatidylcholine (lyso-PC) treatment of plasma membrane vesicles was performed according to the method of Palmgren *et al*. (1988).

Protein content was determined as described by Bradford (1976).

The data in all analyses were obtained from one experiment with three to five replicates and at least two experiments performed on independent PM preparations. All mean comparisons were done using *t*-test and the confidence was set at 0.05.

Results and discussion

It has been demonstrated that cell membranes are directly involved in cold acclimation and freezing tolerance (Steponkus 1984) and that plasma membranes are the primary sites of freezing injury (Arora and Palta 1991). Within the cell membrane, H^+-ATPase activity has been implicated in the ability of plants to survive freezing temperature (Hellergren *et al*. 1983). Furthermore, lots of studies have demonstrated that PM H^+-ATPase responded to low temperature at pre-transcriptional, transcriptional, and post-transcriptional levels. However, little is known about the detailed changes in the catalytic mechanism of this enzyme.

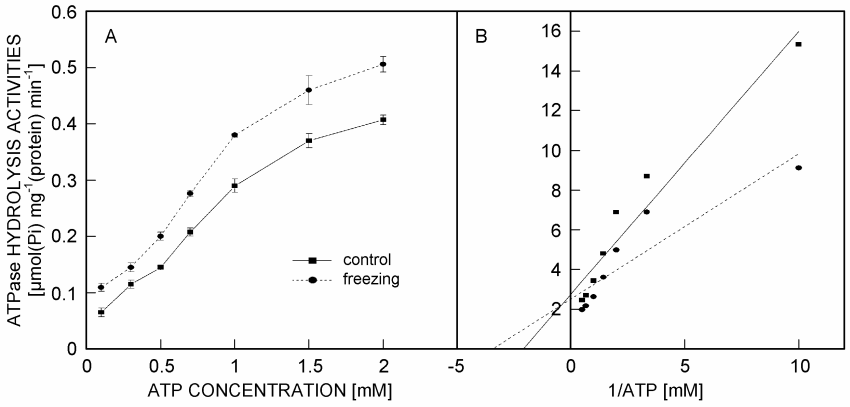

Fig. 1. Changes of the plasma membrane H^+-ATPase activity under freezing treatment (*A*). The enzyme activities were assayed as described in Materials and methods. 0.1, 0.3, 0.5, 0.7, 1.0, 1.5 or 2.0 mM ATP was added. Values are means of three to five replicates ± SE. Kinetics analysis was shown (*B*).

In the present work, we studied the changes of catalytic mechanism for the PM H^+-ATPase of the callus from *C. bungeana*. The ATP hydrolysis activity of the PM H^+-ATPase increased under freezing stress compared to that of control (Fig. 1). Similar results also have been found previously. Iswari and Palta (1989) found that slight freezing treatments resulted in an increase in PM H^+-ATPase activity and a significant decrease in its K_m, while more severe freezing treatments resulted in decreases in both PM H^+-ATPase activity and K_m. Ahn *et al*. (1999) demonstrated that gene expression of PM H^+-ATPase increased in roots of fig-leaf gourd plants under chilling soil temperature. Therefore, freezing treatment altered the PM H^+-ATPase activity, suggesting that this enzyme might be involved in the response to freezing stress in plants. Kinetic studies indicated that the K_m values for ATP hydrolysis changed after freezing treatment, decreased from 0.480 ± 0.021 to 0.292 ± 0.015 mM (Fig. 1). These results implied that the catalytic mechanism of PM H^+-ATPase was altered by freezing treatment. Also, freezing treatment changed the optimal pH value of the PM H^+-ATPase, from 6.5 to 7.0 (Fig. 2). Since the plant PM H^+-ATPase has an optimal PH value of 6.5 (Briskin 1990), the alteration of optimal pH value suggested that the catalytic mechanism was altered by freezing treatment.

There are some evidences suggesting that the PM H^+-ATPase from soybean hypocotyls had PNPP hydrolysis activity and that it is the phosphatase domain of this enzyme that catalysed PNPP hydrolysis (Qiu 1999). In present study, we also found that PM H^+-ATPase of the callus from *C. bungeana* had PNPP hydrolysis activity. Further studies showed that freezing treatment significantly increased PNPP hydrolysis activity of the PM H^+-ATPase (Fig. 3). Kinetic studies indicated that the K_m values for PNPP hydrolysis was changed after freezing treatment, shifted from 3.14 ± 0.13 to 2.41 ± 0.11 mM.

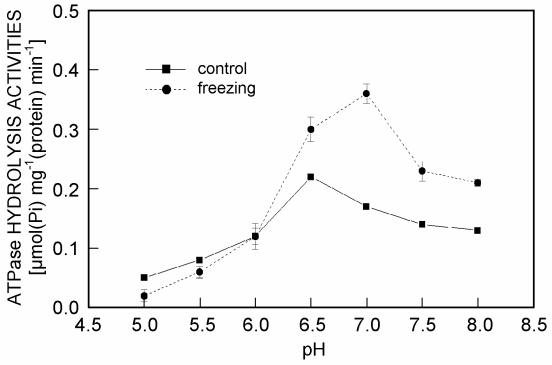

Fig. 2. Changes of the optimal pH of the PM H^+-ATPase under freezing treatment. The enzyme activities were assayed as described in Materials and methods, with pH values of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 or 8.0. Means of three to five replicates ± SE.

(Fig. 3). These results suggested that freezing treatment altered the catalytic activity of the phosphatase domain of the PM H^+-ATPase. Vanadate is a specific inhibitor of the PM H^+-ATPase by binding with the phosphatase domain of the enzyme (Briskin 1990, Michelet and Boutry 1995). Our results showed that freezing treatment decreased the

inhibitory effect of vanadate on PNPP hydrolysis of the PM H^+-ATPase (Fig. 4). So, the change in the vanadate effect also implied that the catalytic activity of the phosphatase domain of the PM H^+-ATPase was changed under freezing treatment.

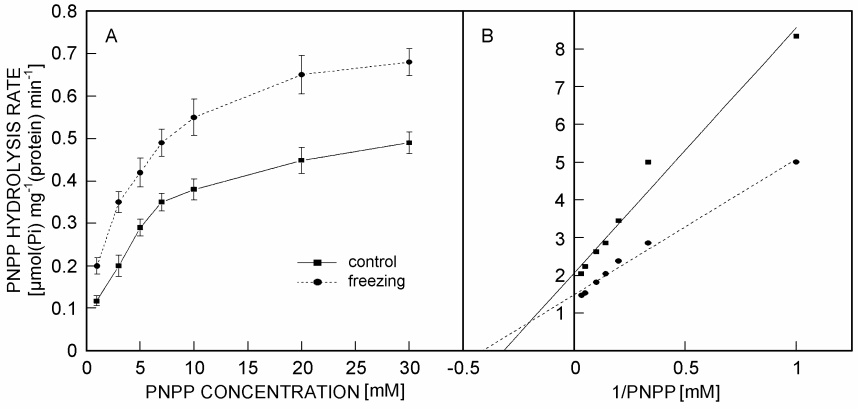

Fig. 3. Changes of the PNPP hydrolysis activities of the PM H^+-ATPase under freezing treatment (*A*). PNPP hydrolysis activities were assayed as described in Materials and methods and 1, 5, 10, 20 or 30 mM PNPP was added. Means of three to five replicates ± SE. Kinetics analysis was shown (*B*).

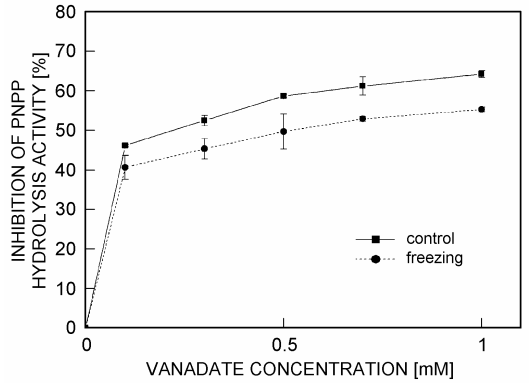

Fig. 4. Inhibitory effect of vanadate (0, 0.1, 0.3, 0.5, 0.7 or 1.0 mM Na_3VO_4) on PNPP hydrolysis of the PM H^+-ATPase. Means of three to five replicates ± SE.

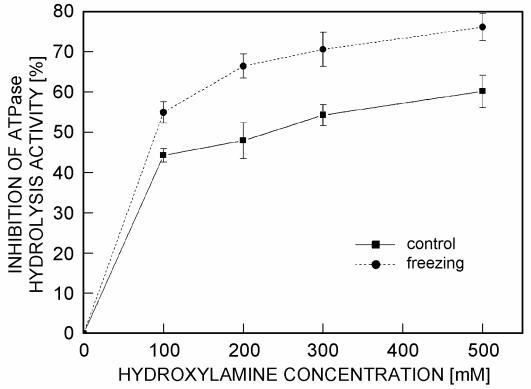

Fig. 5. Inhibitory effect of hydroxylamine on the PM H^+-ATPase (0, 100, 200, 400 or 500 mM hydroxylamine was added). Means of three to five replicates ± SE.

One typical property of the P-type ATPase is the generation of phosphorylated intermediates during hydrolysis (Vara and Serrano 1983). It was found that hydroxylamine could break the acyl-phosphate bond forming during the phosphorylation catalyzed by the kinase domain (Briskin 1990). The inhibitory rate of 500 mM hydroxylamine on ATP hydrolysis increased from 60.1 to 76.2 % after freezing treatment (Fig. 5). The significant changes showed that kinase domain of the PM H^+-ATPase is sensitive to freezing and its catalytic activity was altered under freezing treatment.

The C-terminal end is an autoinhibitory domain of the PM H^+-ATPase: the enzyme is activated through removal of its C-terminal domain by trypsin or lyso-PC treatment (Palmgren *et al*. 1990, Palmgren 1991). It might regulate the phosphatase domain and kinase domain of the enzyme (Zhang and Qiu 2000, Qiu and Zhang 2001). Zhang and Qiu (2000) found that PNPP hydrolysis activity of PM H^+-ATPase is stimulated by trypsin, implying that the phosphatase domain might be regulated by the C-terminal end of this enzyme. In order to examine whether freezing treatment influenced the regulatory effects of C-terminal end on the phosphatase domain, we measured the PNPP hydrolysis of PM H^+-ATPase under control and freezing treatment after trypsin treatment. The results indicated that freezing treatment decreased the stimulation effect of trypsin on PNPP hydrolysis activity (Fig. 6), implying that freezing treatment might change the characteristic of C-terminal end, therefore affecting the phosphatase domain. In addition, Lyso-PC treatment stimulated the ATP hydrolysis, but did not affect the PNPP hydrolysis and inhibitory effect of vanadate, implying that the kinase domain might be an action site or regulatory region of C-terminal end in plant PM H^+-ATPase (Qiu and Zhang 2001). In our present study, we observed that freezing

treatment increased the stimulatory effect of lyso-PC on ATP hydrolysis of PM H^+-ATPase (Fig. 6), implying freezing treatment might change the characteristic of C-terminal end, thus affecting the kinase domain.

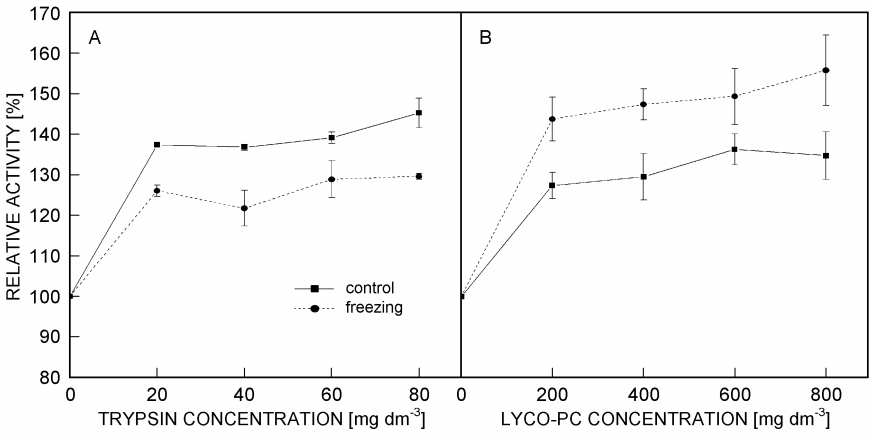

Fig. 6. Effect of trypsin on the PNPP hydrolysis activity of PM H^+-ATPase (*A*). Effect of lyso-PC on the ATP hydrolysis activity of PM H^+-ATPase (*B*). Trypsin or lyso-PC treatment of plasma membrane vesicles and determination of ATP and PNPP hydrolysis activity were as described in "Materials and methods". Values are means of three to five replicates ± SE, and expressed as percent of the activity without trypsin and lyso-PC (100 %)

To sum up, the present results gave first indication, to our knowledge, that freezing treatment altered not only the catalytic processes of kinase domain but also phosphatase domain, therefore affected the PM H^+-ATPase activity. It also suggested the changes of kinase domain and phosphatase domain might be dependent on the alteration of characteristic of C-terminal end of this enzyme. Furthermore, above results suggested that PM H^+-ATPase might play an important role during adaptation to freezing and enhancing the frost hardness in *C. bungeana*.

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