DOI: 10.1007/s10535-012-0259-z BIOLOGIA PLANTARUM 57 (1): 113-120, 2013

Characterization of phospholipase D from *Chorispora bungeana* callus in response to freezing stress

N. YANG[1,2], X.L. YUE[1], H. ZHANG[1], G.F. WU[2], F.X. DING[2], T.G. ZHANG[2], and L.Z. AN[1]*

Key Laboratory of Arid and Pasture Agroecology of Ministry of Education, School of Life Science, Lanzhou University, Lanzhou 730000, P.R. China[1]
School of Life Science, Northwest Normal University, Lanzhou 730070, P.R. China[2]

Abstract

The influence of freezing on phospholipase D (PLD) was studied in *Chorispora bungeana* Fisch. & C.A. Mey. which is a naturally cold-tolerant species. During the freezing treatment (-4 °C), PLD activities in both microsomal and mitochondrial membranes increased at day 3, remained at a high level at day 6 and then declined to a moderate level. The RT-PCR analyses showed that PLD activity partially corresponded to the *CbPLD* gene transcript level. The freezing treatment resulted in increases in the K_m and V_{max} for microsomal and mitochondrial PLD, respectively. Freezing injury, as measured by electrolyte leakage and malondialdehyde content, peaked at day 6 and then gradually decreased. Alleviation of freezing injury was related to a decreased content of membrane-associated Ca^{2+}. We suggest that the specific mechanism of cold resistance of *C. bungeana* is linked with PLD.

Additional key words: calcium, electrolyte leakage, malondialdehyde, microsomal and mitochondrial membranes, RT-PCR.

Introduction

Chorispora bungeana is a representative alpine-subnival herbaceous plant that shows great resistance to low temperatures, high radiation, and strong wind. It grows up to 3900 m a.s.l. at subfreezing temperature during the growth period from June to September (An *et al.* 2000). *C. bungeana* shows extraordinary resistance not only to low temperatures but also to frequent temperature fluctuations. This species possesses no special morphological characteristics that would help it survive under such environments (Ayitu *et al.* 1998). Therefore, molecular and physiological mechanisms were assumed to account for its adaptability to low temperature.

Phospholipase D (PLD) is a ubiquitous enzyme that hydrolyzes structural phospholipids at the terminal phosphate diester bond, leading to the formation of phosphatidic acid and choline in the case of phosphatidylcholine (PC; Zhang *et al.* 2003). In plants, PLD could be involved in phospholipid turnover to maintain cell viability and homeostasis (Paliyath and Droillard 1992). Recent studies have demonstrated that PLD has critical roles in cell signaling cascades (Navari-Izzo *et al.* 2006, Russo *et al.* 2007, Sgherri *et al.* 2007). PLD is modulated by phospholipids, hormones, calcium, pH, and many other compounds (Wang 2002). The activity and gene expression of this enzyme might be also affected by phosphate starvation, cold, drought, salinity, wounding, pathogenic infection, and abscisic acid (Bargmann *et al.* 2006). Therefore, PLD is thought to be involved in plant responses to biotic and abiotic stresses, and to have multiple functions during plant growth and development (Paliyath and Thompson 1987, Pinhero *et al.* 2003).

Previous studies have demonstrated that the response of phospholipid catabolism to low temperatures is linked with PLD (Pinhero *et al.* 1998). An increase in PLD activity has been observed in response to chilling stress in cucumber and maize (Mao *et al.* 2007, Paliyath *et al.*

Received 17 February 2011, *accepted* 16 May 2011.
Abbreviations: BA - benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; MAD - malondialdehyde; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid; PC - phosphatidylcholine; PLD - phospholipase D; RT-PCR - reverse transcriptase - polymerase chain reaction.
Acknowledgments: This work was supported by the Major Project of Cultivating New Varieties of Transgenic Organisms (2009ZX08009-029B, 2008ZX08009-003), the National Basic Research Program of China (973 Program) (2007CB108902), the National Science Foundation of China (No. 31160087, 30900172 and 30960065), Gansu Natural Science Project (No. 0803RJZA038), and Project of Gansu's Department of Education (No. 1101-06).
* Corresponding author; fax: (+86) 931 891 2561, e-mail: lizhean@lzu.edu.cn

1999). Novotna *et al.* (1999) showed that the activity of rape seed PLD depends strictly on the presence of Ca^{2+}, and the optimal pH range was 5.5 - 6.0. Khatoon *et al.* (2007) demonstrated that PLD from mustard seeds was highly pH and temperature tolerant but not Ca^{2+} dependent. Yuan *et al.* (2005) demonstrated that PLD kinetic parameters were dependent on its spatial orientation in the membrane. They also found that PLD followed Michaelis-Menten kinetics and showed increased activity during development of strawberry fruits. Nevertheless, analysis of substrate-velocity relationships under *in vitro* conditions may provide insights into the kinetic properties of PLD localized in different compartments.

Although PLD has been studied in many plants, no examinations of its activity in response to freezing stresses in an alpine-subnival plant have been undertaken. In this study, we investigated the response of PLD to freezing stress and the effect of freezing treatment on the catalytic process in *C. bungeana*.

Materials and methods

Plant material and low-temperature stress treatment: Embryogenic callus, derived from mature seeds of *Chorispora bungeana* Fisch. & C.A. Mey., was obtained as described previously with some modifications (Fu *et al.* 2006). Briefly, the hard seed coats were removed, rinsed in 70 % ethanol for 30 s and in 1 % hypochlorite solution + 0.1 % *Tween-20* for 20 min for surface sterilization and then washed three times with sterile water. Cotyledons were cut off and placed on Murashige and Skoog (MS) medium containing 1 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg dm^{-3} 6-benzyladenine (BA), then cultured at temperature of 25 °C and 16-h photoperiod (irradiance of 80 μmol m^{-2} s^{-1}). After 20 d, the embryogenic callus was collected for subculturing on MS medium containing 0.5 mg dm^{-3} 2,4-D, 0.1 mg dm^{-3} BA, and 0.5 mg dm^3 α-naphthaleneacetic acid (NAA). After subculture for about six times, the callus from the same medium was cultured at 25 °C or -4 °C for 6 or 12 d for measurement of PLD activity and freezing injury.

Electrolyte leakage was determined according to Walker *et al.* (2010). The calli (0.2 g) were quickly washed three times and then incubated in Petri dishes containing 5 cm^3 of distilled deionized water at 25 °C for 2 h. The conductivity in the bathing solution was determined (C1). Then, the samples were heated at 80 °C for 2 h and conductivity was read again (C2). Conductivity was measured using a conductance bridge (*DDS-11A*, *Yamei Electron Instrument Factory*, Wuxi, China). Electrolyte leakage, EL [%], was calculated as C1/C2 × 100.

Malondialdehyde (MDA) content was determined by thiobarbituric acid reaction (Sun *et al.* 2010). In brief, the calli (0.2 g) were homogenized in 2 cm^3 of 10 % (m/v) trichloroacetic acid. The homogenate was centrifuged at 4 000 *g* for 10 min. To a 1 cm^3 aliquot of the supernatant, 1 cm^3 of 0.6 % (m/v) thiobarbituric acid was added. The mixture was heated at 95 °C for 15 min and cooled immediately and the absorbance was read at 450, 532 and 600 nm. The MDA content was calculated as $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$.

Determination of membrane-bound calcium: The calli were prepared for Ca^{2+} measurements according to the method of Yapa *et al.* (1986) with some modifications. The calli (5 g) were homogenized in 15 cm^3 of extraction buffer containing 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 3 mM EDTA, 3 mM β-mercaptoethanol, and 1 g of polyvinylpyrrolidone. The homogenate was centrifuged at 1 500 *g* for 15 min. The supernatant was decanted and centrifuged at 15 000 *g* for 30 min to yield a pellet of membranes derived from the mitochondria and plastid fractions (Edwards and Gardestrom 1987). The supernatant was decanted and recentrifuged at 105 000 *g* for 60 min to yield a pellet of microsomal membranes and the cytosol (Paliyath and Thompson 1987). The resulting pellet was resuspended in buffer containing 0.125 M sucrose, 1 mM Tris-Mes (pH 7.2), and 3 mM β-mercaptoethanol. HCl was added to each suspension to a final concentration of 0.1 M and the mixture was shaken for 60 min. Ca^{2+} was extracted by centrifugation at 15 000 *g* for 10 min and the concentration of Ca^{2+} was measured with an inductive coupled plasma emission spectrometer (*Labtam 8410*, Melbourne, Australia).

PLD extraction and activity: As described by Mao *et al.* (2007), calli (0.2 g) were ground to a fine powder in liquid N_2. The powder was mixed with 2 cm^3 of 10 mM HEPES (pH 7.0) containing 0.32 M sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM ethyleneglycol-bis-N,N-tetraacetic acid. The resulting homogenate was centrifuged at 1 000 *g* for 15 min to remove the cell debris. The supernatant was decanted and centrifuged at 15 000 *g* (*Allegra TM64R* centrifuge, *Beckman*, Coulter, USA) for 30 min. Previously, the pellet yielded by the above centrifugation has been identified to consist of membranes derived from the mitochondria and plastid fractions. The supernatant was decanted and recentrifuged at 105 000 *g* (*L8-60M* ultracentrifuge, *Beckman*) for 1 h to yield a pellet of microsomal membranes and the cytosol. The resulting pellet was resuspended in 100 mM 3,3-dimethylglutaric acid (DMG, pH 6.5) for PLD extraction.

PLD activity was determined by measuring the

release of choline using an enzyme coupling colorimetric method (Huang *et al*. 1997). The basic assay mixture (0.2 cm^3) contained 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 mM DMG buffer (pH 6.5), 5 mM linoleic acid, 12 mM phosphatidylcholine (PC), and 0.02 cm^3 of enzyme extracts. The mixture was incubated at 30 °C for 30 min and reaction was terminated in a boiling water bath for 15 min. Then 0.8 cm^3 of chromogenic liquid containing 45 mM Tris-HCl (pH 8.0), 0.8 U of choline oxidase, 2.4 U of horseradish peroxidase, 0.24 mg of oxidized 4-amino-antipyrine, and 0.16 mg of phenol were added and the mixture was incubated at 30 °C for 90 min. The amount of choline released during the reaction was determined by mixing with 1 cm^3 of Tris-HCl containing 2 g dm^{-3} of *Triton X-100* (pH 8.0). After the color was stable, contaminating soluble proteins were removed by filtration through a 0.22 μm membrane filter and the absorption of the supernatant was read at 500 nm.

Protein content in the extract was determined using the protein dye binding method and bovine serum albumin as the standard (Bradford 1976). All steps of the protein extraction procedure were carried out at 4 °C.

For the kinetic studies, desired quantities of PC were added to the reaction mixture to evaluate the effect of substrate concentration. To evaluate the effect of calcium, varying amounts of calcium chloride were added to the reaction mixture. To evaluate the effect of pH, PLD activity was determined at pH 5 - 8 using different buffers (0.1 M sodium acetate buffer, pH 5.0 and 5.5; 0.1 M sodium phosphate buffer, pH 6.0, 6.5, 7.0, and 7.5).

Semiquantitative RT-PCR analysis: To investigate the expression patterns of *CbPLD* under freezing stress, we extracted total RNA from 0.1 g of *C. bungeana* calli using *TRIzol* reagent. Aliquots (1 μg) of total RNA of each treatment were used as the template in reverse transcriptase polymerase chain reaction (RT-PCR) using the forward primer P_1 (5′-TTCATCTACGTTGAA AACCAGTA-3′) and reverse primer P_2 (5′-TCATCG TCAACAATCATCAT-3′) which were designed according to the *CbPLD* gene sequence (*GenBank* acc. No. HM756247), in a one-step RT-PCR kit (*TaKaRa*, Tokyo, Japan). Amplifications were performed at 94 °C for 5 min followed by 25 cycles of amplification (94 °C for 40 s, 54 °C for 40 s, and 72 °C for 60 s). PCR products were separated on 1 % agarose gels and stained with ethidium bromide. The signals of the PCR products were analyzed with *BandScan* software. RT-PCR reactions for actin (*GenBank* accession No. AY825362), using specific primers Act-FP (5′-GCTCCGTGTTGC CCCTGAAGA-3′) and Act-RP (5′-CTCGGCGGTGG TGGTGAACA-3′), were performed under the same conditions as described above. The RT-PCR reactions were repeated at least three times, and representative results from one reaction are shown in the figures.

Statistical analysis: All experiments were repeated at least three times. Analysis of variance and Duncan's multiple range tests were performed using *SPSS v. 13* software.

Results

During exposure to -4 °C, the electrolyte leakage increased, reached maximum at day 6 and then gradually decreased (Fig. 1*A*). MDA, one of the final products of stress-induced lipid peroxidation of polyunsaturated fatty acids, shared similar trends with electrolyte leakage. MDA content reached maximum 12.94 nmol g^{-1}(f.m.) at day 6 and then slowly declined to 10.56 nmol g^{-1}(f.m.) (Fig. 1*B*).

The amount of Ca^{2+} associated with membrane fractions in the callus of *C. bungeana* was measured after 12 d at -4 °C. Freezing treatment resulted in decrease of the membrane-binding calcium concentration compared to the control ($P < 0.05$) (Fig. 1*C*). When calluses were treated at -4 °C for 12 d, the Ca^{2+} content in mitochondrial fractions declined by 38 % and the Ca^{2+} content in microsomal membrane fractions declined by 39 %.

The accumulation of *CbPLD* mRNA in response to freezing treatment was studied by means of a semi-quantitative RT-PCR analysis of total RNA extracted at different times after initiation of the cold treatment. Following exposure to -4 °C, the *CbPLD* transcript levels progressively increased to maximum at day 6 and then decreased at day 9 (Fig. 2). Under freezing stress, PLD activities in both mitochondrial and microsomal membranes were significantly higher than in the control (25 °C) ($P < 0.05$), and in both fractions showed maximum at day 6 and then declined to a moderate level. The increases of PLD activity corresponded to the level of *CbPLD* mRNA. A correlation was apparent between the increase in PLD transcript levels and activities at different days of freezing treatment. These results suggested that PLD activity was dependent on *CbPLD* gene expression.

PLD activities of both mitochondrial and microsomal fractions increased with increasing substrate concentration from 1 to 4 mM at 25 °C and at a PC concentration above 4 mM, PLD activity decreased. By contrast, when calluses were treated at -4 °C, PLD activity in the microsomal fraction increased with increasing PC concentration ranging from 1 to 4.5 mM and decreased at 5 mM PC. PLD activity in the mitochondrial fraction was considerably lower and slightly increased with increasing substrate concentration, and maximum activity at 4.5 mM PC was 1.6-fold lower than the maximum activity of microsomal PLD.

Transformation of the substrate-velocity data through a Lineweaver-Burk plot provided further understanding

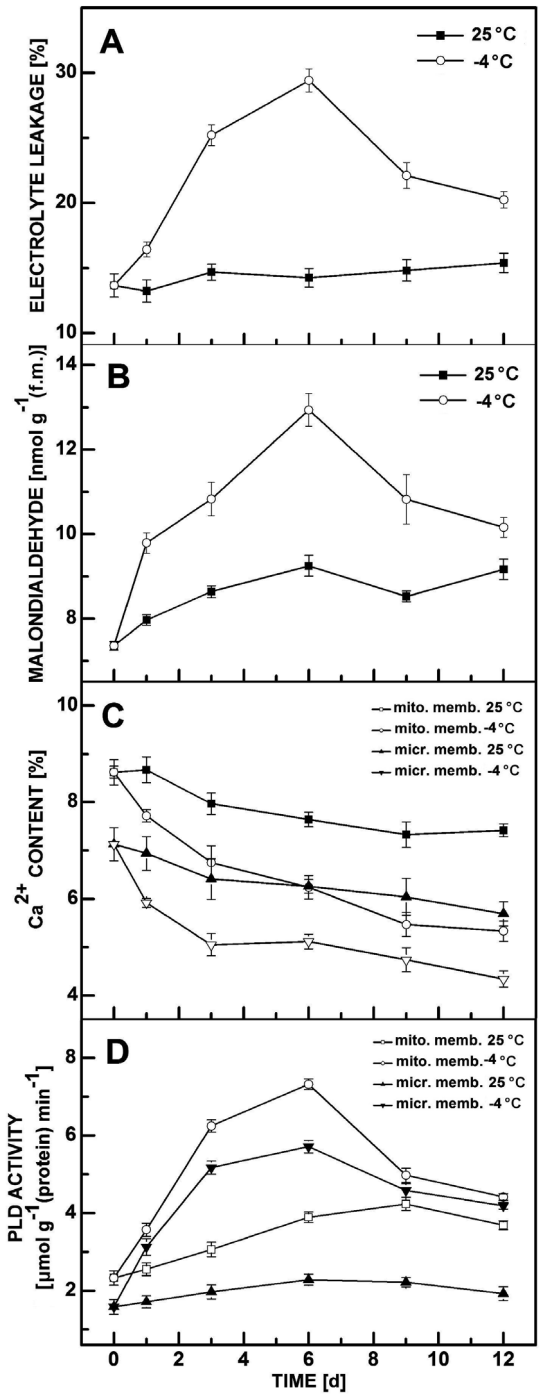

Fig. 1. Changes in electrolyte leakage (*A*), malondialdehyde content (*B*), membrane-bound calcium content (*C*), and activities of PLD in mitochondrial membranes (mito. memb.) and microsomal membranes (micr. memb.) in calluses of *C. bungeana* exposed to -4 °C and 25 °C for 12 d. Values are the mean ± SE of at least three independent experiments.

of the kinetic properties of PLD (Table 1). At 25 °C, V_{max} of microsomal PLD was about 1.6-fold higher than that of the mitochondrial PLD. The K_m values for the mitochondrial and microsomal PLD were 8.79 and 6.11 nM, respectively. After freezing treatment, K_m values for microsomal PLD increased to 40.33 nM and for mitochondrial PLD to 7.66 nM. These results implied that inherent differences exist in the kinetic properties of mitochondrial and microsomal PLD and the PLD kinetic properties were altered by freezing treatment.

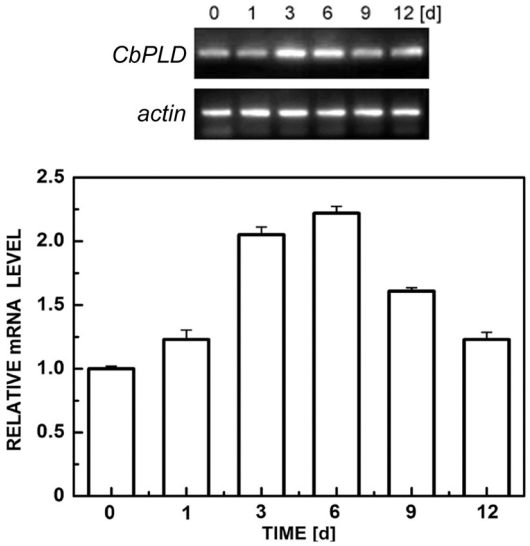

Fig. 2. Expression patterns and relative mRNA levels of the *CbPLD* gene following exposure to freezing treatment. Calluses were treated at -4 °C for 0, 1, 3, 6, 9, and 12 d. For all of these treatments, total RNA (1 µg per sample) was isolated from calluses and subjected to one-step RT-PCR amplification with 25 cycles. The *actin* gene was used as an internal control. The mRNA levels were quantified with *Bandscan* software and normalized to the actin gene. Relative transcript levels were calculated with reference to the controls (taken as 1). Values are the mean ± SE of at least three independent experiments.

Table 1. Kinetic parameters of phospholipase D, V_{max} [µmol g^{-1}(protein) min^{-1}] and K_m [nM], measured at 25 or -4 °C with different concentrations of PC as substrate.

	Microsomal PLD		Mitochondrial PLD	
	25 °C	-4 °C	25 °C	-4 °C
V_{max}	20.96	151.05	13.44	24.45
K_m	8.79	40.33	6.11	7.66
V_{max}/K_m	2.38	3.75	2.20	3.19

The pH profile is quite distinct between mitochondrial and microsomal PLD (Fig. 4*A*,*B*). The microsomal PLD showed optimal values at pH 7 both at 25 °C and -4°C. The mitochondrial PLD has optimum at pH values of 7 only at 25 °C but at pH values of 5.5 at -4 °C.

The PLD showed some activity in the absence of calcium. Therefore, calcium is not mandatory for the activity of PLD. However, PLD activity was stimulated by calcium (Fig. 4*C*,*D*). Freezing treatment changed the optimal calcium concentration of the microsomal PLD

from 0.8 to 0.4 mM and that of the mitochondrial PLD from 0.6 to 0.4 mM. Both microsomal and mitochondrial PLD are stimulated at micromolar concentrations of calcium.

Discussion

In recent years, the adaptation to cold temperature has been widely investigated both at physiological and molecular level (Zhang *et al*. 2010). Cell membrane stability has been used to characterize stress tolerance (Campos *et al.* 2003). Price and Hendry (1991) suggested that cell membranes are the first structures to be damaged when plant suffers temperature injury. Electrolyte leakage can indicate plasma membrane injury induced by cold stress (Zhang *et al*. 2010). MDA is used as an index of cell oxidative damage under environmental stresses (Leshem 1987). We expect that evaluation of electrolyte leakage and MDA production at 25 °C and -4 °C may give an indication of the ability of *C. bungeana* to cope with low temperature stress. After exposure to -4 °C for 6 d, the electrolyte leakage and MDA content of the *C. bungeana* callus increased rapidly but could be restored to a moderately low level with the prolongation of freezing treatment. Thus, the cell membranes were not degraded in the *C. bungeana* callus under the freezing temperature. Fu *et al*. (2006) also showed that the permeability could be restored in plant cells with a high cold hardiness.

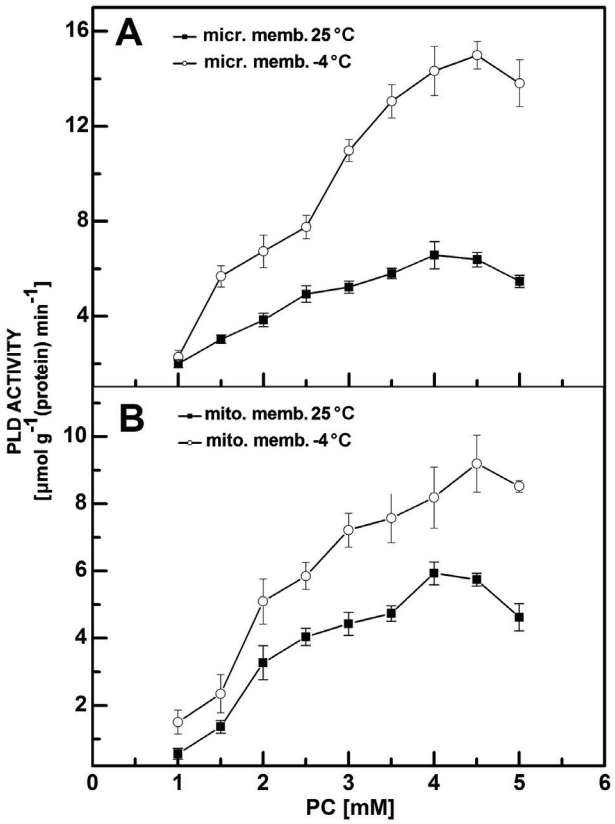

Fig. 3. Changes of PLD activities in microsomal (*A*) and mitochondrial (*B*) membranes under freezing treatment. The enzyme activities were assayed as described in the Materials and methods and 1~5 mM PC was added. Values are the mean ± SE of at least three independent experiments.

Many studies have shown that calcium plays a vital role in many processes by acting as a messenger in regulation of plant growth and development as well as in response and adaptation to environmental stresses (Bush 1995, Lachaud *et al.* 2010, Liu *et al.* 2011). Knight *et al.* (1996) reported that the increase in Ca^{2+} concentration played important role under low-temperature stress. Calcium has a profound effect on the activity of plant PLDs and PLD could be stimulated at Ca^{2+} content ranging from 1 to 40 μM (Ulbrich-Hofmann *et al.* 2005). Stimulation of PLD by Ca^{2+} might arise through several mechanisms including changes in membrane properties which can increase enzyme substrate interactions and substrate availability as well as potential activation of the enzyme itself. As observed in cabbage, the activation of PLD by Ca^{2+} was connected with a destabilization of the protein conformation (Younus *et al.* 2003). Translocation of soluble PLD to the membrane was suggested to be mediated by Ca^{2+} inducing a conformational change in the C2 domain of PLD (Zheng *et al.* 2000). The C2 domain of PLD contained two to three acidic motifs that might be involved in calcium binding (Qin *et al.* 1997). These structural features enable the stimulation of PLD activity at low Ca^{2+} concentrations. *In vivo*, PLD occurs in cytosol, plasma membrane, endoplasmic reticulum (ER), vacuole, and mitochondria. In addition, during fruit development there is increased binding of PLD to the ER and plasma membrane potentially in response to increased cytosolic calcium. Pinhero *et al.* (2003) reported that the membrane association of PLD is inherently linked to the elevation in cytosolic Ca^{2+} and the role of PLD in signal transduction events is linked to cytosolic Ca^{2+}. Mohapatra *et al.* (1989) provided evidence that low temperature stress caused an increase in cytosolic free Ca^{2+}. Fu *et al.* (2006) showed that free Ca^{2+} in mesophyll protoplasts directly corresponded to chilling induction. The free Ca^{2+} content in the mesophyll protoplasts cultured under chilling was higher than in non-chilled protoplasts. In this study, freezing treatment resulted in a decrease of the membrane-bound calcium concentration compared with the control in the *C. bungeana* calluses and PLD activation induced by freezing stress was related to the decreased content of Ca^{2+} associated with membrane fractions. Our previous

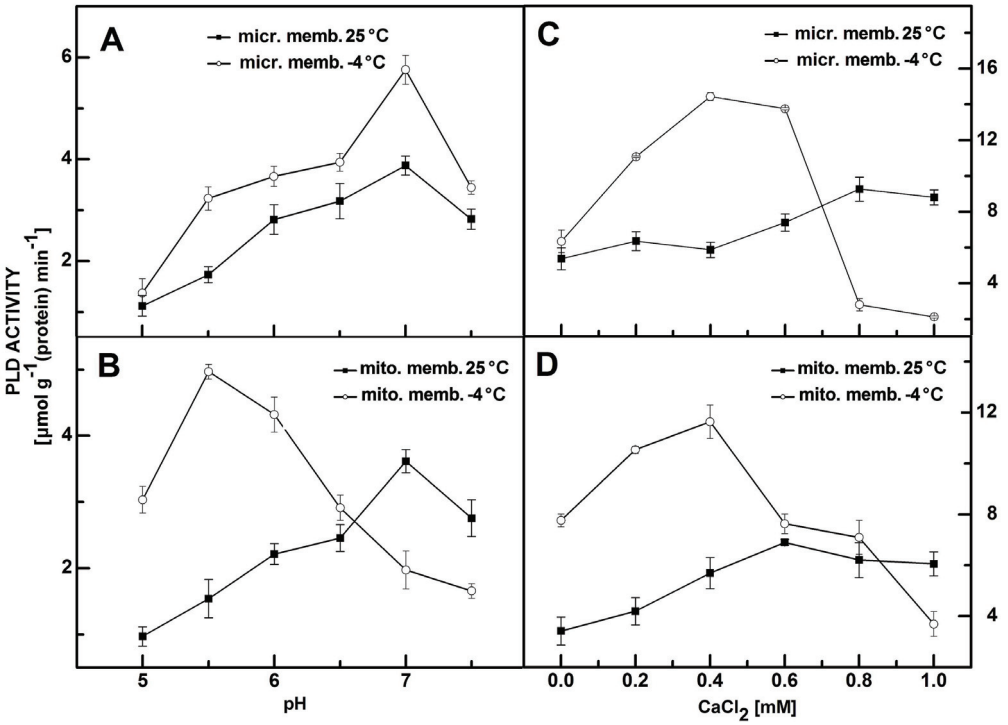

Fig. 4. Changes of the optimal pH and calcium ion concentration for PLD activity under freezing treatment. Changes of the optimal pH for PLD activity (*A* and *B*). The enzyme activities were assayed as described in the Materials and methods with pH values of 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5. Changes of the optimal calcium ion concentration for PLD activity (*C* and *D*). The enzyme activities were assayed as described in the Materials and methods at 0 - 1.0 mM calcium chloride. Values are the mean ± SE of at least three independent experiments.

study found that the membranes of *C. bungeana* undergo compositional change during low-temperature stresses (Shi *et al.* 2008). According to this, we believe that compositional change of membranes during low-temperature stresses might cause the decreased Ca^{2+} content in the membrane fractions. Low Ca^{2+} content in the membrane fractions probably leads to relatively higher content of cytosolic Ca^{2+}. In addition, increased membrane association of PLD has been observed in response to exposure to chilling stress in maize leaf and kernel membranes (Pinhero *et al.* 1998, Paliyath *et al.* 1999). Mao *et al.* (2007) demonstrated that chilling stress would initially activate PLD activity through induction of PLD gene expression in cucumber fruit. Li *et al.* (2004) found that the plasma membrane-bound PLD enhances freezing tolerance in *Arabidopsis thaliana*. Zhao and Wang (2009) showed that the *PLD* gene mediated signal transduction function of low temperature as well as it is involved in regulating proline content during the osmotic adjustment in *Arabidopsis thaliana*. In this study, the transcription level and activity of PLD could positively respond to freezing stress in *C. bungeana*. The increase of PLD activity corresponded to the level of *CbPLD* mRNA. These results indicated that the transcription level of *CbPLD* and activity of PLD correspond and thus that PLD activity was dependent on *CbPLD* gene expression. Yapa *et al.* (1986) showed that decreased PLD activity could be the result of membrane rigidification caused by degradation of phospholipids during the late period of low-temperature treatment. According to our study, the cellular membrane of *C. bungeana* calluses retained their integrity at freezing temperature, and the major reason causing the decrease of PLD activity during the late period of freezing stress in *C. bungeana* calluses might be down-regulation of the *CbPLD* gene. We also believed that the decrease of PLD activities during the later period of freezing stress relieves the damage on the cell by participating in the decrease of electrolyte leakage and MDA content in *C. bungeana* calluses.

The present study showed that the K_m values of PLD both in the mitochondrial fractions and microsomal fractions changed after freezing treatment. The microsomal PLD was found more efficient in responding to low-temperature stress than mitochondrial PLD. There are inherent differences in the kinetic properties of mitochondrial PLD and microsomal PLD. The significance of this observation is not clear but a less efficient mitochondrial PLD will help maintain the mitochondrial membrane structure and the energy production will not be affected during low-temperature stress.

PLD activity is highly regulated by pH (Yuan *et al.* 2005). In this study, freezing treatment changed the

optimal pH values of the mitochondrial PLD but did not affect the optimal pH value of the microsomal PLD (Fig. 4). As mentioned earlier, PLD exists as different isoforms. It is tempting to speculate that the different responses to pH value might be related to the different isoforms in different membrane fractions. At present, there is no evidence as to which isoforms play major roles in different membrane fractions. Further studies are required to address this question. We believed that PLD might be involved in the response to freezing stress through two aspects in *C. bungeana*: the first is altering PLD activity by changing the membrane-associated PLD and *CbPLD* gene expression; the second is altering the PLD catalytic mechanism.

To our knowledge, the kinetic properties of PLD associated with different subcellular fractions under freezing treatment have not been studied previously and this is the first report on the cold hardiness of *C. bungeana* associated with PLD.

References

An, L.Z., Liu, Y.H., Feng, G.N., Feng, H.Y., Chen, T., Cheng, G.D.: Studies on ecological properties of altifrigetic subnival vegetation at the source area of Urumqi River. - Acta bot. boreal-occident. sin. **20**: 98-105, 2000.

Ayitu, R., Tan, D.Y., Li, Z.J., Yao, F.: [The relationship between the structures of vegetative organs in *Chorispora bungeana* and its environment]. - J. Xinjiang Agr. Univ. **21**: 273-277, 1998. [In Chin.]

Bargmann, B.O., Munnik, T.: The role of phospholipase D in plant stress responses. - Curr. Opin. Plant Biol. **9**: 515-522, 2006.

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

Bush, D.S.: Calcium regulation in plant cells and its role in signaling. - Annu. Rev. Plant Physiol Plant mol. Biol. **46**: 95-122, 1995.

Campos, P.S., Quartin, V., Ramalho, J.C., Nunes, M.A.: Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants. - J. Plant Physiol. **160**: 283-292, 2003.

Edwards, G.E., Gardestrom, P.: Isolation of mitochondria from leaves of C_3, C_4, and crassulacean acid metabolism plants. - Methods Enzymol. **148**: 421-433, 1987.

Fu, X.Y., Chang, J.F., An, L.Z., Zhang, M.X., Xu, S.J., Chen, T., Liu, Y.H., Xing, H., Wang, J.H.: Association of the cold-hardness of *Chorispora bungeana* with the distribution and accumulation of calcium in the cells and tissues. - Environ. exp. Bot. **55**: 282-293, 2006.

Huang, Y., Wu, X.Z., Qureshi, I.A., Chen, H.L.: Determination of phosphatidyl choline specific phospholipase D using enzyme coupling colorimetric method and its application. - Acta Acad. Med. Shanghai **24**: 343-346, 1997.

Khatoon, H., Talat, S., Younus, H.: Identification and partial characterization of a highly active and stable phospholipase D from *Brassica juncea* seeds. - Int. J. biol. Macromol. **40**: 232-236, 2007.

Knight, H., Trewavas, A.J., Knight, M.R.: Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. - Plant Cell **8**: 489-503, 1996.

Lachaud, C., Silva, D.D., Cotelle, V., Thuleau, P., Xiong, T.C., Jauneau, A., Brière, C., Graziana, A., Bellec, Y., Faure, J.D., Ranjeva, R., Mazars, C.: Nuclear calcium controls the apoptotic-like cell death induced by D-*erythro*-sphinganine in tobacco cells. - Cell Calcium **47**: 92-100, 2010.

Leshem, Y.: Membrane phospholipid catabolism and Ca^{2+} activity in control of senescence. - Physiol. Plant. **69**: 551-559, 1987.

Li, W., Li, M., Zhang, W., Welti, R., Wang, X.: The plasma membrane-bound phospholipase D enhances freezing tolerance in *Arabidopsis thaliana*. - Nat. Biotechnol. **22**: 427-433, 2004.

Liu, D.C., He, L.G., Wang, H.L., Xu, M., Sun, Z.H.: Expression profiles of *PtrLOS2* encoding an enolase required for cold-responsive gene transcription from trifoliate orange. - Biol. Plant. **55**: 35-42, 2011.

Mao, L.C., Pang, H.Q., Wang, G.Z., Zhu, C.G.: Phospholipase D and lipoxygenase activity of cucumber fruit in response to chilling stress. - Postharvest Biol. Technol. **44**: 42-47, 2007.

Mohapatra, S.S., Wolfraim, L., Poole, R.J., Dhindsa, R.S.: Molecular cloning and relationship to freezing tolerance of cold acclimation-specific genes of alfalfa. - Plant Physiol. **89**: 375-380, 1989.

Navari-Izzo, F., Cestone, B., Cavallini, A., Natali, L., Giordani, T., Quartacci, M.F.: Copper excess triggers phospholipase D activity in wheat roots. - Phytochemistry **67**: 32-42, 2006.

Novotna, Z., Kas, J., Daussant, J., Sajdok, J., Valentova, O.: Purification and characterisation of rape seed phospholipase D. - Plant Physiol. Biochem. **37**: 531-537, 1999.

Paliyath, G., Droillard, M.J.: The mechanisms of membrane deterioration and disassembly during senescence. - Plant Physiol. Biochem. **30**: 789-812, 1992.

Paliyath, G., Pinhero, R.G., Yada, R.Y., Murr, D.P.: Effect of processing conditions on phospholipase D activity of corn kernel subcellular fractions. - J. Agr. Food Chem. **47**: 2579-2588, 1999.

Paliyath, G., Thompson, J.E.: Calcium and calmodulin-regulated breakdown of phospholipids by microsomal membrane from bean cotyledons. - Plant Physiol. **83**: 63-68, 1987.

Pinhero, R.G., Almquist, K.C., Novotna, Z., Paliyath, G.: Developmental regulation of phospholipase D in tomato fruits. - Plant Physiol. Biochem. **41**: 223-240, 2003.

Pinhero, R.G., Paliyath, G., Yada, R.Y., Murr, D.P.: Modulation of phospholipase D and lipoxygenase activities during chilling. Relation to chilling tolerance of maize seedlings. - Plant Physiol. Biochem. **36**: 213-224, 1998.

Price, A.H., Hendry, G.A.R.: Iron-catalyzed oxygen radical formation and its possible contribution to drought damage in nine native grasses and three cereals. - Plant Cell Environ. **14**: 451-477, 1991.

Qin, W., Pappan, K., Wang, X.: Molecular heterogeneity of

phospholipase D (PLD): cloning of PLD gamma and regulation of plant PLD alpha, beta and gamma by polyphosphoinositides and calcium. - J. biol. Chem. **272**: 28267-28273, 1997.

Russo, M.A., Quartacci, M.F., Izzo, R., Belligno, A., Navari-Izzo, F.: Long- and short-term phosphate deprivation in bean roots: plasma membrane lipid alterations and transient stimulation of phospholipases. - Phytochemistry **68**: 1564-71, 2007.

Sgherri, C., Quartacci, M.F., Navari-Izzo, F.: Early production of activated oxygen species in root apoplast of wheat following copper excess. - J. Plant Physiol. **164**: 1152-60, 2007.

Shi, Y.L., An, L.Z., Zhang, M.X., Huang, C.H., Zhang, H., Xu, S.J.: Regulation of the plasma membrane during exposure to low temperatures in suspension-cultured cells from a cryophyte (*Chorispora bungeana*). - Protoplasma **232**: 173-181, 2008.

Sun, W.H., Duan, M., Li, F., Shu, D.F., Yang, S., Meng, Q.W.: Overexpression of tomato *tAPX* gene in tobacco improves tolerance to high or low temperature stress. - Biol. Plant. **54**: 614-620, 2010.

Ulbrich-Hofmann, R., Lerchner, A., Oblozinsky, L., Bezakova, M.: Phospholipase D and its application in biocatalysis. – Biotechnol. Lett. **27**: 535-543, 2005.

Walker, D.J., Romero, P., Correal, E.: Cold tolerance, water relations and accumulation of osmolytes in *Bituminaria bituminosa*. - Biol. Plant. **54**: 293-298, 2010.

Wang, X.M.: Phospholipase D in hormonal and stress signaling. - Curr. Opin. Plant Biol. **5**: 408-414, 2002.

Yapa, P.A.J., Kawasaki, T., Matsumoto, H.: Changes of some membrane-associated enzyme activities and degradation of membrane phospholipids in cucumber roots due to Ca^{2+} starvation. - Plant Cell Physiol. **27**: 223-232, 1986.

Younus, H., Schops, R., Lerchner, A., Rucknagel, K.P., Schierhorn, A., Saleemuddin, M., Ulbrich-Hofmann, R.: Proteolytic sensitivity of a recombinant phospholipase D from cabbage: identification of loop regions and conformational changes. - J. Protein Chem. **22**: 499-508, 2003.

Yuan, H.Y., Chen, L.G., Paliyath, G., Sullivan, A., Murr, D.P.: Characterization of microsomal and mitochondrial phospholipase D activities and cloning of a phospholipase D alpha cDNA from strawberry fruits. - Plant Physiol. Biochem. **43**: 535-547, 2005.

Zhang, W., Wang, C., Qin, C., Wood, T., Olafsdottir, G., Welti, R.: The oleate-stimulated phospholipaseD, PLDδ, and phosphatidic acid decrease H_2O_2-induced cell death in *Arabidopsis*. - Plant Cell **15**: 2285-2295, 2003.

Zhang, X.Y., Liang, C., Wang, G.P., Luo, Y., Wang, W.: The protection of wheat plasma membrane under cold stress by glycine betaine overproduction. - Biol. Plant. **54**: 83-88, 2010.

Zhao, P., Wang, D.L.: [The role of phospholipase Dβ on signal transduction of low temperature in *Arabidopsis thaliana*.] - Genomics appl. Biol. **28**: 901-907, 2009. [In Chin.]

Zheng, L., Krishnamoorthi, R., Zolkiewski, M., Wang, X.: Distinct Ca^{2+} binding properties of novel C2 domains of plant phospholipase Dα and β. - J. biol. Chem. **275**: 19700-19706, 2000.