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Influence of low temperatures on photosystem II photochemistry and expression of the NADPH:protochlorophyllide oxidoreductase in the alpine, subnival perennial, *Chorispora bungeana*

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Abstract

Low temperature significantly influences chloroplast development and chlorophyll (Chl) biosynthesis, so effect of coldness on Chl content and Chl fluorescence characteristics was investigated in *C. bungeana* (*Chorispora bungeana* Fisch. & C.A. Mey). The levels of transcript and protein of an enzymatic step during Chl biosynthesis in response to chilling (4°C) and freezing (−4°C) were also examined in this work. Significant reduction in total Chl content was observed, but the reduction was much less at 4°C than that at −4°C. Moreover, the maximal quantum efficiency of photosystem II (PSII) photochemistry, indicated by F_v/F_m, decreased in the first 12 h, but then started to increase and reached higher levels than the control at 24 h and 48 h at 4°C, but decreased continuously at −4°C. Whereas quantum yield of PSII (Φ_{PSII}) showed no significant difference between the chilling-stressed and the control seedlings, at −4°C, Φ_{PSII} was markedly reduced with the prolonged treatment. In general, there were no significant responses of photochemical quenching (q_P) and non-photochemical quenching (NPQ) to cold treatment. Meanwhile, the full-length cDNA of NADPH:protochlorophyllide oxidoreductase (POR, EC 1.3.1.33) was isolated and termed *CbPORB* (GenBank Accession No. FJ390503). Its transcript and protein content only slightly declined at 4°C, but dramatically reduced at −4°C with the time. These results strongly suggest that *Cb*PORB possesses certain resistant characteristics and is a major player in Chl biosynthesis process involved in plant growth and development of *C. bungeana* under cold environmental conditions.

Additional key words: chlorophyll biosynthesis; chlorophyll fluorescence; *Chorispora bungeana*; low temperature; NADPH: protochlorophyllide oxidoreductase.

Introduction

It is well known that low temperature is one of the most important environmental stress factors that limit the distribution of native flora and reduce plant productivity (Yang *et al.* 2005, DeRidder and Crafts-Brandner 2008). When plants are exposed to low temperatures, Chl biosynthesis and chloroplast development are disturbed (Tewari and Tripathy 1998, 1999), the greening process is strongly inhibited (Van Hasselt and Strikwerda 1976, Mohanty *et al.* 2006) and the photosynthetic efficiency is greatly reduced (Sonoike 1996, DeRidder and Crafts-Brandner 2008). Therefore, it is important to understand the mechanisms of modulation of Chl biosynthesis and chloroplast development by a low temperature. This information can contribute to the generation of novel crop genotypes resistant to cold stress. The greening process is strongly inhibited under cold-stress conditions, indicating that impaired Chl biosynthesis is one of the first and determining processes in plastids affected by low-temperature stress (Van Hasselt and Strikwerda 1976, Tewari and Tripathy 1998, 1999). Reduced accumulation

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Abbreviations: Chl – chlorophyll; F_0, F_m, and F_v – minimal, maximal, and variable fluorescence after dark-adaptation; F_m' – maximal fluorescence after light-adaptation; F_v/F_m – maximal quantum efficiency of photosystem II; NPQ – non-photochemical quenching; POR – NADPH:protochlorophyllide oxidoreductase; q_P – photochemical quenching; PSII – photosystem II; Φ_{PSII} – quantum yield of PSII; RACE – rapid amplification of cDNA ends; RT-PCR – reverse transcription polymerase chain reaction.
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of Chl in cold-stressed plants could be due to reduced Chl synthesis and/or its more rapid degradation.

In angiosperms, the NADPH:protochlorophyllide oxidoreductase (POR, EC:1.3.1.33) (Griffiths 1978, Apel *et al.* 1980), catalyzing the photoreduction of protochlorophyllide to chlorophyllide, plays a key role in Chl biosynthesis and chloroplast development. POR is one of the major proteins localized in the prolamellar body of etioplasts with molecular mass of 36 to 37 kDa (Apel *et al.* 1980, Forreiter *et al.* 1991, Teakle and Griffiths 1993). While in mature chloroplasts, POR is localized in envelope and thylakoid membranes (Joyard *et al.* 1990, Pineau *et al.* 1993). A lot of attention and interest have gone towards unraveling the regulation mechanism and function of POR in angiosperms. So far, three different isoforms of POR have been identified and named PORA, PORB, and PORC, respectively. Previous research showed that PORA is active only in etiolated seedlings at the beginning of illumination, while PORB operates throughout the greening process and in light-adapted mature plants (Armstrong *et al.* 1995, Holtorf *et al.* 1995, Reinbothe *et al.* 1995). PORC was isolated from *Arabidopsis* and *PORC* mRNA was found to be light-inducible and is predominantly expressed in green tissues (Oosawa *et al.* 2000). In general, previous studies have focused on the effects of light conditions (Su *et al.* 2001, Masuda *et al.* 2002), circadian and diurnal rhythms (Holtorf *et al.* 1995, Kuroda *et al.* 2000), phytohormones (Kuroda *et al.* 1996, Kuroda *et al.* 2001, Seyedi *et al.* 2001a), developmental stage (Frick *et al.* 2003, Masuda *et al.* 2003) and on POR activity, transcript and protein abundance. Only limited studies dealing with the effect of low temperature on POR activity, transcript and protein abundance have been reported (Yang *et al.* 2005, Mohanty *et al.* 2006). In higher plants, POR is the only enzyme involved in catalysing the one light-dependent step during Chl synthesis (Griffiths 1978, Apel *et al.* 1980) and it is essential to initiate the transformation from etioplast into chloroplast. Thus, POR plays a pivotal regulatory role in Chl synthesis and leaf greening. In cold-stressed *Arabidopsis* seedlings, suppression of *POR* gene alone is enough to inhibit Chl biosynthesis and leaf greening (Yang *et al.* 2005). However, few experiments have addressed the physiological adaptations of alpine subnival plants to the rigors of their natural habitat and the cold-hardening response, and little effort has been made to employ the Chl fluorescence technique to monitor low-temperature stress in alpine subnival plants. Understanding how low temperature affects POR expression and Chl biosynthesis, and modulates the growing process may have a profound impact in the generation of improved crop varieties that are resistant to cold stress.

C. bungeana is a rare and representative alpine subnival plant species that can survive and flourish under frequent temperature fluctuations and even freezing temperatures. This plant is a perennial herb in the family of Brassicaceae. It grows in the freeze-thaw tundra around the border of glaciers where almost no other flowering plants are able to grow. *C. bungeana* mainly distributes in an ice-free cirque (altitudes 3,800–3,900 m) beside Glacier No. 1 in the source area of Urumqi River in Tianshan Mountains, Xinjiang, China. The annual average temperature therein is usually lower than 5°C in daytime and −4°C at night. The temperature conditions also undergo an acute fluctuation, which ranges from 4 to –10°C with occasional snow or hail storm even during the favorable growth season from June to September. It does not posses specific morphological characteristics helping it survive in cold environment (Ayitu *et al.* 1998, An *et al.* 2000). Over the years, *C. bungeana* has been proved to retain some stable variations of physiological and genetic characteristics in response to environmental stresses (Chang *et al.* 2006, Fu *et al.* 2006, Zhang *et al.* 2006). It is an ideal plant species for studying the inter-relationship between enzymatic activity, gene expression and protein abundance on Chl biosynthesis under cold environmental conditions.

In summary, POR plays a very important role in Chl biosynthesis and photosynthesis in plants. *C. bungeana* can survive freezing temperatures and blossom even embedded in snow, suggesting that it has an extraordinary cold-defensive system. In order to examine whether *Cb*PORB is the main factor in this system in *C. bungeana*, a POR gene was isolated and termed *CbPORB* (Accession No. FJ390503), and its expression patterns were investigated by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and western blot under cold stress.

Materials and methods

Plant material and cold treatment: Seeds of *C. bungeana* were collected from an ice-free cirque (43°05′N, 86°49′E, with an altitude 3,800–3,900 m) near the No.1 glacier in the source area of Urumqi River in Tianshan Mountains, Xinjiang, China. The regenerated seedlings derived from mature seeds of *C. bungeana* were obtained as described by Fu *et al.* (2006). Seedlings were grown in solid MS (Murashige and Skoog 1962) medium with a 16-h photoperiod provided by cool white fluorescent light (120 μmol m^{-2} s^{-1}) in a growth chamber with the temperature maintained at 22 ± 2°C and relative humidity 55 ± 5%. The seedlings were subcultured every three weeks. When the regenerated seedlings were approximately 5 cm tall, they were divided into three groups. One was allowed to remain at 22 ± 2°C (control temperature), and the other two were exposed to 4°C and −4°C for cold treatment under the same intensity of light and photoperiod. The leaves were collected from these plants

at various time points during the cold treatment. All samples were analyzed immediately or immersed rapidly in liquid N_2 for subsequent experiments.

Chl measurements: Chl *a* and *b* were extracted with 80% chilled acetone from fresh *C. bungeana* leaves. The pigment content was determined spectrophotometrically according to the method of Arnon (1949). To prevent light-induced pigment degradation, all procedures were carried out in the dark and on ice.

Chl fluorescence analysis: Chl fluorescence was measured using a portable pulse modulation fluorometer (*PAM-2100*, Heinz Walz GmbH, Germany). Before each measurement, leaves were dark-adapted for 30 min. The minimal fluorescence (F_o) was recorded under weak modulated λ_{650}-irradiation (<0.1 μmol m^{-2} s^{-1}), which was too small to induce significant physiologic changes in the plant (Schreiber *et al.* 1986). The value stored was an average taken over a 1.6-s period. Maximal fluorescence (F_m) was obtained by applying a λ_{730} saturating pulse of white light (8,000 μmol m^{-2} s^{-1}) for 0.8 s. Maximal variable fluorescence (F_v) was calculated as $F_m - F_0$, and the maximal quantum efficiency of PSII photochemistry was calculated as ratio $F_v/F_m = (F_m - F_0)/F_m$. This ratio of variable to maximal fluorescence correlates with the number of functional PSII reaction centres, and dark-adapted values of F_v/F_m can be used to quantify photoinhibition (Maxwell and Johnson 2000). After dark measurements, the leaves were exposed to an actinic light light with the intensity of 120 μmol m^{-2} s^{-1}. Steady-state fluorescence yield (F_s) was achieved after exposure to the actinic light for 10 min. Saturating pulses (0.8 s) of white light (8,000 μmol m^{-2} s^{-1}) were applied to determine maximum fluorescence in the light-adapted state (F_m'). Using fluorescence parameters determined in both light- and dark-adapted states, the following ones were calculated: quantum yield of PSII [$\Phi_{PSII} = (F_m' - F_s)/F_m'$] (Genty *et al.* 1989); photochemical quenching [$q_P = (F_m' - F_s)/(F_m' - F_0')$, where F_0' corresponds to open reaction centre traps in the light-acclimated state, and F_0' was estimated using far-red light]; and non-photochemical quenching [$NPQ = (F_m - F_m')/F_m'$]. These fluorescence parameters, including F_v/F_m, Φ_{PSII}, q_P and NPQ were automatically calculated by fluorometer according to the above equations.

Cloning of the *CbPORB* gene from *C. bungeana*: Total RNA was extracted from the leaves of *C. bungeana* using TRIZOL reagent (*Invitrogen*, Carlsbad, CA). A pair of degenerate primers, 5′-TCACB(GTC)GGV(GAC)GCK (GT)TCN(ATGC)TCH(ATC)GG-3′ and 5′-TTGCTR (AG)TCY(CT)TTRTAHGCCTT-3′, designed based on the internal conserved parts of *Arabidopsis* (Accession No. NP_194474), pea (Accession No. Q01289) and cucumber (Accession No. Q41249) POR amino acid sequences, were used in a RT-PCR to obtain the fragment of partial *CbPORB*. Afterward, 5′- and 3′-rapid amplification of cDNA ends (RACE) were conducted with the SMART RACE cDNA Amplification kit protocol (*Clontech*, USA) to isolate the full-length cDNA of *CbPORB*. The full-length cDNA sequence of *CbPORB* was obtained by comparing, aligning and combining the sequences of 5′-, 3′-RACE and the middle region products. Amino acid sequence analysis and multiple alignments were performed with DNAMAN software. Signal sequence predictions used the IpSORT Program (Bannai *et al.* 2002; http://hc.ims.u-tokyo.ac.jp/ iPSORT/). Prediction of secondary structure was carried out by SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin).

Semi-quantitative RT-PCR analysis: To investigate the expression patterns of *CbPORB* under cold stress, total RNA was extracted from *C. bungeana* leaves. Aliquots of 1 μg total RNA of each treatment were used as the template in RT-PCR reactions. For RT-PCR, the specific primers P1 (5′-TGCCTAAAGGGAGTTACACGG-3′) and P2 (5′-TGGCAACGCCTGTGAAACT-3′) were used to obtain a fragment of partial *CbPORB*. Primers P3 (5′-GGAGCTGAGAGATTCCGTTGC-3′) and P4 (5′-GAAGCATTTCCTGTGGACAATCGA-3′), designed according to the conserved regions of the *C. bungeana* housekeeping gene (*ACTIN*; Accession No. AY825363), were used as an internal control to verify equal loading of RNA. PCR products (5 μl) were separated on 1% agarose gels stained with ethidium bromide. The RT-PCR reactions were repeated at least three times. Band intensity of the PCR products was quantified by *Quantity One-4.6*, *Gene Tools* software (*Bio-Rad*).

Antibody preparation and western blot: The methods of expressing and purifying of *C. bungeana* hexahistidine-tagged-PORB in *coli* Rosetta™ (DE3) cells were described previously (Pattanayak and Tripathy 2002). The purified hexahistidine-tagged-PORB protein was injected subcutaneously into rabbits to produce antibody for following western blot experiments. Total protein was isolated from 0.2 g of *C. bungeana* leaves and lysed in RIPA (150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulphate, 0.5% sodium deoxycholate, and 1% NP-40, 1 mM phenylmethylsulfonyl fluoride) buffer. The homogenate was centrifuged at 15,000 × *g* for 15 min at 4°C. The supernatant was collected and the protein concentrations were determined using BCA protein assay kit. Samples were run on 12% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE), transferred onto polyvinylidene fluoride membranes and incubated with primary polyclonal *Cb*PORB antibody for 2 h at 20°C with gentle agitation. Blots were then incubated with peroxidase-conjugated secondary antibodies for 1 h at 20°C, and visualized by enhanced chemiluminescence reagent (*Applygen Technologies Inc*., Beijing, China).

Statistical analysis: All data reported in this work are subjected to a one-way analysis of variance (*ANOVA*) followed by a post-hoc least significant difference (LSD) test. Pearson coefficients were calculated to assess correlation between different variables. Comparisons with $P<0.05$ were considered significantly different. The results presented in all the figures are the means ± SD. All these statistical analyses were conducted on *SPSS* software (*Ver. 15.0*, *SPSS*, Chicago, USA).

Results

Chl content: In general, both Chl *a* and Chl *b* (and hence total Chl) contents decreased with time of cold treatments at both 4°C and −4°C. The Chl *a*/*b* ratio was not significantly changed (data not shown). However, the reduction was much less at 4°C than that at −4°C (Fig. 1). Moreover, a correlation between the Chl *b* content and the levels of *Cb*PORB transcript and protein was found in chilling-stressed ($R = 0.506$, $P<0.05$ and $R = 0.539$, $P<0.05$, respectively) and freezing-stressed ($R = 0.913$, $P<0.01$ and $R = 0.452$, $P>0.05$, respectively) *C. bungeana* seedlings (Table 1). Meanwhile, the correlation between the content of Chl *a* and the levels of *Cb*PORB transcript and protein was observed in response to freezing treatment ($R = 0.913$, $P<0.01$ and $R = 0.538$, $P<0.05$, respectively). Additionally, there was no difference observed in the appearance of leaves between the chilled and the unstressed control seedlings. However, after exposure to −4°C, most of freezing-treated *C. bungeana* leaves showed wilted appearance after a 24-h period of treatment, which indicated that freezing caused severe damage to cells of *C. bungeana* (photos not presented).

Chl fluorescence parameters: Ratios of F_v/F_m in *C. bungeana* decreased significantly upon exposure to 4°C and −4°C in the first 12 h. Subsequently, it decreased greatly during the prolonged treatment at −4°C, and the reduction resulted mainly from a decrease in F_m (data not presented). On the contrary, F_v/F_m started to increase and reached higher levels than that of the control at 24 and 48 h respectively at 4°C (Fig. 2*A*). Similarly, Φ_{PSII} decreased markedly under −4°C conditions. However, Φ_{PSII} values did not show significant difference between the chilling-treated and the control seedlings during the experiment ($P>0.05$; Fig. 2*B*). There were, however, no significant responses of q_P or NPQ to chilling and freezing treatments in *C. bungeana* seedlings ($P>0.05$; Fig. 2*C*,*D*).

Table 1. Pearson correlation coefficients (two-tailed) between chlorophyll (Chl) content and the levels of *Cb*PORB transcript and protein of *C. bungeana* seedlings under chilling (4°C) and freezing (−4°C) conditions for 0, 6, 12, 24, 48, and 72 h (three replications, $n = 6 \times 3$). * – correlation is significant at the 0.05 level. ** – correlation is significant at the 0.01 level.

Chl	Chilling Transcript	Protein abundance	Freezing Transcript	Protein abundance
Chl *a*	0.245	0.333	0.913**	0.538*
Chl *b*	0.506*	0.539*	0.913**	0.452
Total Chl	0.411	0.454	0.919**	0.520**

Cloning and characterization of *CbPORB*: The full-length cDNA sequence of *C. bungeana PORB*, obtained by 5′- and 3′-RACE PCR with degenerate primers and specific primers, showed high homology to POR cDNA sequences from related species, especially *Arabidopsis*

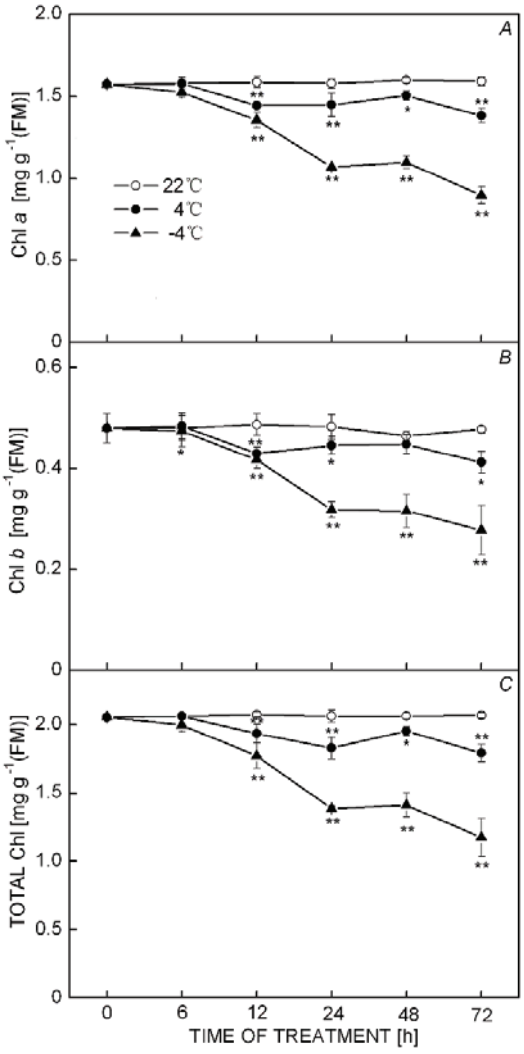

Fig. 1. (*A*) Chl *a*, (*B*) Chl *b* and (*C*) total Chl content in *C. bungeana* in response to cold treatment. Samples were measured at specific time points. Each data point was the average of three replicates. The error bar represented ± SD. *, ** – significant at $P<0.05$ and 0.01, respectively, as compared to the control.

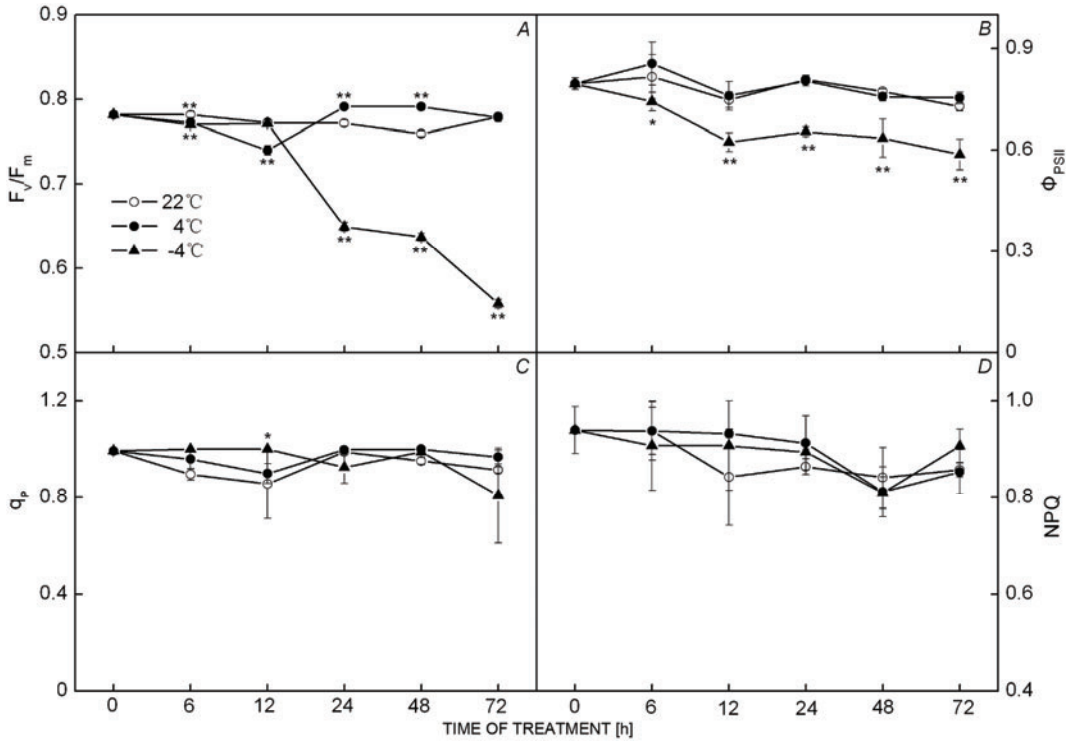

Fig. 2. Chl fluorescence analysis in dark-adapted *C. bungeana* leaves growing at 22 ± 2°C (control) and after cold treatment (4 and −4°C). (*A*) F_v/F_m − maximal quantum efficiency of PSII photochemistry. (*B*) Φ_{PSII} − quantum yield of PSII. (*C*) q_P − photochemical quenching. (*D*) NPQ − non-photochemical quenching. Measurements were made as described in Materials and methods. Values were means ± SD of five replicates. *, ** – significant at $P<0.05$ and 0.01, respectively, as compared to the control.

PORB (91.40%),pea (72.69%) and cucumber (73.28%). The full-length cDNA sequence of *CbPORB* is 1444 bp and contains a 54 bp of 5′- and 181 bp of 3′-untranslated region with a 27 bp poly (A) tail (GenBank Accession No. FJ390503). The full-length cDNA sequence and the deduced amino acid sequence of *Cb*PORB are presented in Fig. 3. The 1209-bp open reading frame encoded a predicted protein of 402 amino acid residues with a theoretical molecular mass of 43.37 kDa, and the isoelectric point was 9.43. In comparison with other available plant POR sequences at the protein level, *C. bungeana* PORB protein precursor is highly homologous to *Arabidopsis* PORB and we thus referred to it as *Cb*PORB (GenBank Accession No. ACJ12925). The deduced *Cb*PORB protein sequence showed high homology by sequence alignment with the sequences from *Arabidopsis* (93.78%), pea (82.13%), cucumber (83.08%) and barley (75.93%) (Fig. 4*B*). Most of the sequence divergence among POR proteins occurred at the N-terminal (Fig. 4*A*). Furthermore, a phylogenetic analysis showed that *Cb*PORB developed in the same branch of *At*PORB from *Arabidopsis* (Fig. 4*B*).

The *Cb*PORB protein deduced from the open reading frames contained all the characteristic domains found in other POR proteins, such as the IRAQ tetrapeptide (residues 65-68 in *Cb*PORB) (Fig. 4*A*). Most POR polypeptides in higher plants contain the IRAQ tetrapeptide, which constitutes the processing site for the cleavage of the plastid transit peptides (Reinbothe *et al.* 1995). The essential amino acid residues Tyr-277 and Lys-281 for POR catalytic activity and four highly conserved Cys residues at the amino acid positions 121, 173, 283, and 310 in mature *Cb*PORB protein have been compared with PORs from other plants and the comparison result indicated that the cDNA encoded *C. bungeana* PORB.

The secondary structure of the putative *Cb*PORB protein was analyzed by SOPMA and the result showed that the putative *Cb*PORB peptide contained 38.56% α-helix, 14.68% extended strand, 6.22% β-turn and 40.55% random coil (Fig. 5). The alpha helix and random coil constituted the interlaced domain of the main part of the secondary structure. From the above sequence analyses, *Cb*PORB was found to possess many characteristics that are common in plant PORs.

Transcript level of *CbPORB*: The expression pattern of the *CbPORB* mRNA in response to low temperatures was studied by semi-quantitative RT-PCR analysis of total RNA extracted at different times after the initiation of the cold treatment. The expression level of *CbPORB* in *C. bungeana* grown at 22 ± 2°C was used as control and referred to as 100%. When *C. bungeana* was exposed to 4°C, the transcript levels of *CbPORB* decreased slightly

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1       GACATCGATTTCGACAGTCTTCTTCTTCCATCGCTTTAGATATCTCTCTCAGCAATGACCCTTCAAGCTGCTTCT
1                                                       M  T  L  Q  A  A  S
76      TTGGTCTCCTCTGCTTTCTCTGTCCGAAAAGATGGAAAATTGAACGCTTCTTCATCATCCTTTAAGGATTCGAGT
8       L  V  S  S  A  F  S  V  R  K  D  G  K  L  N  A  S  S  S  S  F  K  D  S  S
151     CTATTCGGTGTCTCCATTACCGACCTAATCAAATCCGAACATGGATCTTCCTCAATAAGATTCAAGAGAGAACAT
33      L  F  G  V  S  I  T  D  L  I  K  S  E  H  G  S  S  S  I  R  F  K  R  E  H
226     AACTTGAGGAATGTAGCGATCCGAGCCCAAACCGCTGCGACTTCAAGCCCTTCCGTCACAAAATCTTCCGGTGAA
58      N  L  R  N  V  A  I  R  A  Q  T  A  A  T  S  S  P  S  V  T  K  S  S  G  E
301     GGGAAGAAAACGTTGAGGAAAGGAAATGTGGTAGTCACGGGAGCCTCGTCTGGGTTAGGCTTAGCCACGGCTAAA
83      G  K  K  T  L  R  K  G  N  V  V  V  T  G  A  S  S  G  L  G  L  A  T  A  K
376     GCTCTAGCCGAGACAGGGAAATGGCACGTGATAATGGCGTGCAGAGACTTCCTCAAAGCAGAGAGAGCCGCTAAA
108     A  L  A  E  T  G  K  W  H  V  I  M  A  C  R  D  F  L  K  A  E  R  A  A  K
451     TCCGCAGGGATGCCTAAAGGGAGTTACACGGTTATGCATTTAGACTTAGCCTCGTTGGACAGTGTGAGACAGTTC
133     S  A  G  M  P  K  G  S  Y  T  V  M  H  L  D  L  A  S  L  D  S  V  R  Q  F
526     GTTGATAACTTCAGGAGATCAGAGATGCCTCTTGATGTTTTGGTTTGCAATGCTGCGGTTTACTTTCCGACAGCT
158     V  D  N  F  R  R  S  E  M  P  L  D  V  L  V  C  N  A  A  V  Y  F  P  T  A
601     AAAGAGCCGACTTACAGTGCAGAAGGGTTTGAGCTTAGCGTCGGGACGAACCATTTGGGACATTTTCTTCTCTCA
183     K  E  P  T  Y  S  A  E  G  F  E  L  S  V  G  T  N  H  L  G  H  F  L  L  S
676     AGGTTGTTGCTTGATGACTTGAAGAAATCTGATTACCCTTCCAAGCGTCTCATTATCGTCGGATCCATTACCGGG
208     R  L  L  L  D  D  L  K  K  S  D  Y  P  S  K  R  L  I  I  V  G  S  I  T  G
751     AACACGAATACATTGGCTGGTAATGTACCACCGAAGGCGAATCTCGGAGATTTGAGGGGATTAGCGGGCGGATTG
233     N  T  N  T  L  A  G  N  V  P  P  K  A  N  L  G  D  L  R  G  L  A  G  G  L
826     AACGGTTTAAACAGCTCGGCTATGATTGACGGAGGAGATTTCGATGGTGCAAAGGCTTACAAAGCCAGTAAAGTA
258     N  G  L  N  S  S  A  M  I  D  G  G  D  F  D  G  A  K  A  Y  K  A  S  K  V
901     TGCAATATGTTGACGATGCAGGAGTTTCACAGGCGTTGCCATGAAGAAACCGGAGTCACATTCGCATCGCTTTAC
283     C  N  M  L  T  M  Q  E  F  H  R  R  C  H  E  E  T  G  V  T  F  A  S  L  Y
976     CCCGGTTGTATCGCCTCCACGGGTTTATTCAGAGAGCCCATTCCGCTCTTCCGTTTCCTCTTCCCTCCATTCCAA
308     P  G  C  I  A  S  T  G  L  F  R  E  P  I  P  L  F  R  F  L  F  P  P  F  Q
1051    AAATATATCACTAAAGGATATGTTTCCGAGACAGAGTCTGGCAAAAGACTTGCTCAGGTGGTGAGTGACCCGAGC
333     K  Y  I  T  K  G  Y  V  S  E  T  E  S  G  K  R  L  A  Q  V  V  S  D  P  S
1126    TTGACGAAATCAGGGGTTTATTGGAGCTGGAACAAGGCTTCAGCTTCTTTTGAGAACCAGTTGTCACAAGAAGCA
358     L  T  K  S  G  V  Y  W  S  W  N  K  A  S  A  S  F  E  N  Q  L  S  Q  E  A
1201    AGCGATGTTGAGAAGGCTCGTAAAGTGTGGGAGATCAGTGAGAAGCTCGTTGGCTTGGCCTAATAAAAACTCGAA
383     S  D  V  E  K  A  R  K  V  W  E  I  S  E  K  L  V  G  L  A  *
1276    CCTAGAGAACTTTCGGGAGTTTAGATGTCTGATTTTATTGCGCAATTTTCCGTTGAGTGTTTGTTGTTTGTAGAT
1351    TTTCTTGGCGAGTAGCCCCCAAGAAAGAAATAAAGGTTATCAATCTTACGCTTTTTTTGCTCTGTCCAAAAAAAA
1426    AAAAAAAAAAAAAAAAAA
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Fig. 3. The full-length cDNA sequence and deduced amino acid sequence of *C. bungeana* NADPH:protochlorophyllide oxidoreductase (*CbPORB*) gene (GenBank Accession No. FJ390503). The nucleotides and amino acid residues are numbered on the left, amino acids are indicated with single-letter abbreviation.

in the first 6 h and then maintained at approximately 85% of the control level up to 72 h after following an increase at 12 h (Fig. 6*A*). After exposure to −4°C, the transcript levels of *CbPORB* decreased progressively, which were different from those observed at 4°C. The *CbPORB* transcript approximately remained at 70% of the control level after 12 h of freezing treatment (Fig. 6*B*).

Protein abundance of *Cb*PORB: To investigate *Cb*PORB protein level in response to low temperatures, total protein was isolated from *C. bungeana* leaves at various times after the initiation of the cold treatment. The bands were analyzed on an *Image Quant RT ECL* (GE Healthcare, GE, USA). In each sample a polypeptide of an apparent molecular mass of approximately 36 kDa was recognized specifically by the antiserum, which was lower than the polypeptide that has been deduced from the *Cb*PORB open reading frame. This polypeptide had the same apparent molecular mass as the PORs in other plant species (Ikeuchi and Murakami 1982, Häuser *et al.* 1984, Teakle and Griffiths 1993). When *C. bungeana* was exposed to 4°C, the *Cb*PORB protein abundance was approximately inhibited by 15% compared to the control (0 h). There was no significant difference in *Cb*PORB protein abundance from 24 h to 72 h (Fig. 7*A*). In general, the change patterns of *Cb*PORB protein abundance were consistent with the *CbPORB* transcript under chilling stress. However, when exposed to −4°C, the *Cb*PORB protein abundance decreased substantially and reached the minimum value at 72 h, although an increase occurred at 24 h (Fig. 7*B*).

Discussion

It is well known that Chl content is susceptible to various stresses and usually continuously declines with stress treatment (Gajewska *et al.* 2006). When plants are exposed to low-temperature conditions, Chl biosynthesis is affected. There are a few reports that demonstrate that low temperature reduces Chl biosynthesis. Illumination

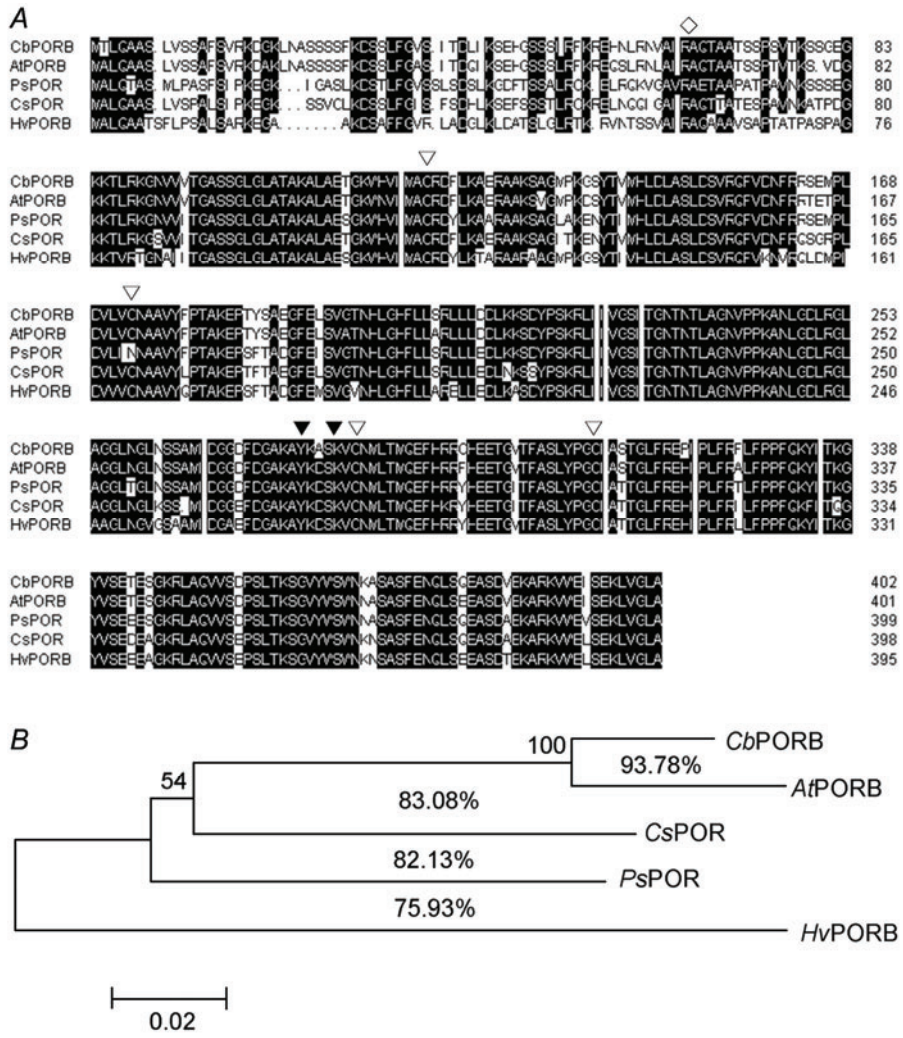

Fig. 4. Analysis of deduced amino acid sequences of *C. bungeana* *Cb*PORB. (*A*) Multiple sequence alignment of the *C. bungeana* *Cb*PORB amino acid sequences with POR from four other higher plants, the sources are *Cb*PORB (Genbank Accession No. ACJ12925); *At*PORB, *A. thaliana* PORB (Accession No. NP_194474); *Ps*POR, pea (Accession No. Q01289); *Cs*POR, cucumber (Accession No. Q41249); *Hv*PORB, barley (Accession No. Q42850). The amino acids identical among more than four PORs are shaded in black. The conserved cysteines are marked by (□), the putative N-terminal transit peptides cleavage site is indicated with a square (□). Essential amino acid residues Tyr-277 and Lys-281 of *Cb*PORB for POR catalytic activity are indicated by (▼). (*B*) The phylogenetic tree based on deduced protein sequences of POR-encoding genes and obtained by neighbor joining analysis.

of cucumber seedlings (Tewari and Tripathy 1998) and etiolated maize seedlings (Hodgins and van Huystee 1986a) at low temperature resulted in reduced Chl accumulation, and impairment of Chl biosynthesis was paralleled by an aberrant development of the thylakoid membranes, thus leading to poor photosynthetic performance and inhibition of leaf development. In this study, the decreases in Chl *a*, Chl *b*, and total Chl content were much less at 4°C than those at −4°C in *C. bungeana* seedlings (Fig. 1), which revealed that Chl content decreased with the reduction in the temperature and the duration of cold exposure. It is highly probable that chilling injury to the cells of *C. bungeana* is reversible, but the injury caused by freezing is irreversible at least during the time of the experiment. The reduction in Chl content due to cold stress could be the result of destabilization and degradation of the peripheral part of the antenna complex. Furthermore, as POR is a nuclear-encoded protein, its transcript and post-translational import into chloroplast may be impaired in cold stress (Dutta *et al.* 2009). Inhibition pattern of Chl *b* biosynthesis in chilling- and freezing-stressed *C. bungeana* seedlings matched well that of *Cb*PORB transcript and protein abundance (Figs. 1, 6, 7). Additionally, impaired Chl biosynthesis is not only due to down-regulation of protein expression of *Cb*PORB, it may be due to decline of gene and protein expression and enzymatic activities of several other enzymes, such as 5-aminolevulinic acid dehydratase, Mg-chelatase, and Mg-protoporphyrin IX monoester cyclase involved in Chl biosynthesis pathway

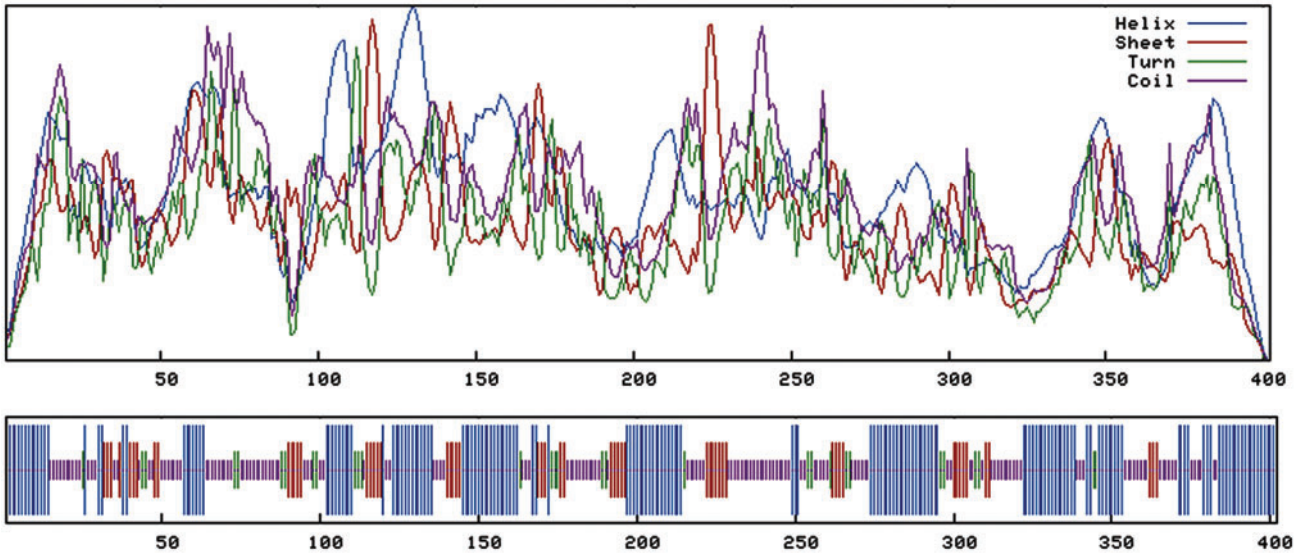

Fig. 5. Prediction of the secondary structure of *Cb*PORB. The helix, sheet, turn and coil were indicated respectively with blue, red, green, and purple lines.

in *C. bungeana* seedlings.

The chilling tolerance of *C. bungeana* plants was further confirmed by measuring changes in Chl fluorescence at 4°C. The ratio of F_v/F_m, which is a measure of the intrinsic efficiency of PSII photochemistry in the dark-adapted state, was used to estimate the quantum yield of PSII (Strasser and Butler 1977). Environmental stresses that damage the efficiency of PSII resulted in decreases in the F_v/F_m ratio (Artus *et al.* 1996). A decrease in F_v/F_m indicates the extent of photoinhibition caused by various environmental stresses (Krause 1994). In our study, the F_v/F_m ratio started to decline in the first 12 h of chilling treatment, thereafter, it began to increase and reached higher levels than the control (Fig. 2*A*). The F_v/F_m ratios were higher in chilling-stressed seedlings than in control ones, which suggested that *C. bungeana* seedlings did not experience photoinhibition during this time at 4°C. Furthermore, there was no evidence that chilling affected the integrity or function of the photochemical apparatus in *C. bungeana*, although Chl *a* and *b* content decreased somewhat. On the contrary, the F_v/F_m ratio significantly decreased in freezing-stressed *C. bungeana* seedlings, although a temporary recovery was observed at 12 h at −4°C, which indicated that a large level of photoinhibition was induced by freezing stress. Photoinhibition is caused by damage to photosynthetic components, and this effect can be short-term and reversible (dynamic photoinhibition) or long-term and irreversible (chronic photoinhibition; Werner *et al.* 2002). In the case of *C. bungeana*, photoinhibition was mainly dynamic at 4°C, since the F_v/F_m ratio recovered completely at 24 and 48 h in chilling-stressed seedlings. Otherwise, photoinhibition was chronic at −4°C, since the F_v/F_m ratio decreased continuously and was much lower than that of the control. This was probably due to the damage on the photochemical (PSII) apparatus and the synthesis of photosynthetic pigments was irreversible with increased time of freezing in *C. bungeana*. However, Hormaetxe *et al.* (2007) suggested that chronic photoinhibition plays a photoprotective role *via* reducing the efficiency of light energy capture.

In addition, Φ_{PSII} showed no significant difference in the chilling-stressed and the control seedlings throughout the time of experiment (Fig. 2*B*). However, Φ_{PSII} was significantly lower than that of the control in freezing-stressed *C. bungeana* seedlings. This could be explained by the higher photoinhibition (lower F_v/F_m) recorded during this freezing treatment. q_P, which provides an indication of the proportion of PSII open reaction centres (Maxwell and Johnson 2000), showed no significant responses to cold treatment (Fig. 2*C*). It demonstrated that coldness did not markedly reduce the proportion of open reaction centres in *C. bungeana* seedlings. NPQ works as a defense system, producing an increase in thermal dissipation in the PSII antennae, to ameliorate the effects of excess light energy (Müller *et al.* 2001). Usually, Φ_{PSII} decreases as a consequence of the decrease in q_P and an increase in NPQ, which indicates that plants dissipate light as heat, thus protecting leaves from light-induced damage (Maxwell and Johnson 2000). Therefore, an increase in NPQ would be expected under cold stress as a result of a decrease in the utilization of light energy due to cold-induced reduction in PSII efficiency and the quantum yield. Several studies have reported stress-induced increases in the value of NPQ (Bilger *et al.* 1987, Horton and Ruban 1992). However, in the present study, there were no significant differences in NPQ between the cold-stressed and the control seedlings (Fig. 2*D*). Similarly, no significant responses of q_P and NPQ to a wide range

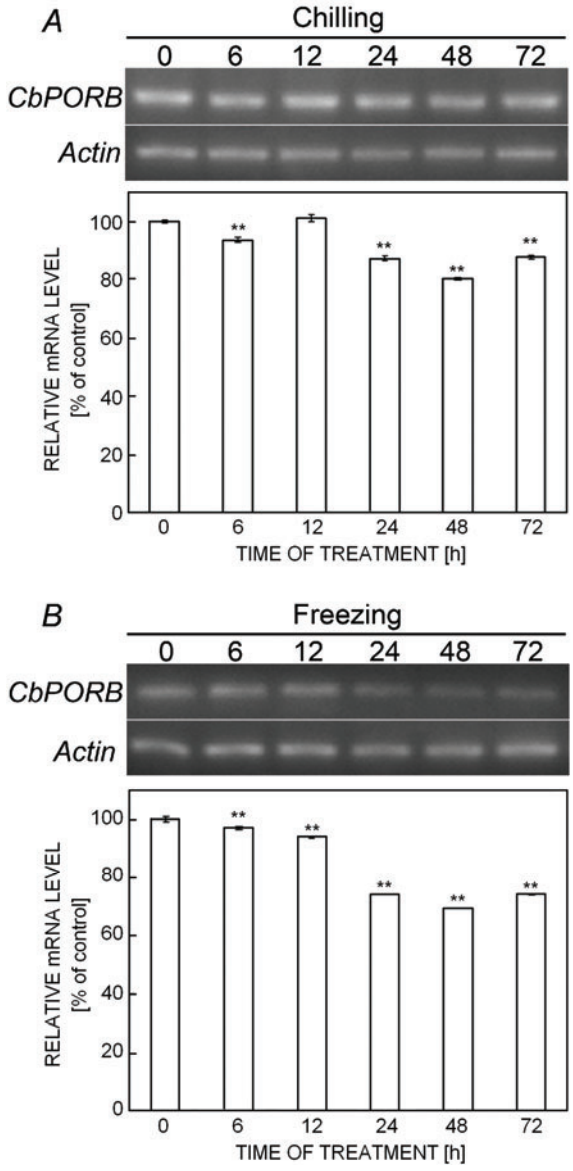

Fig. 6. Expression patterns and relative mRNA levels of *CbPORB* following exposure to chilling (4°C) (*A*) and freezing (−4°C) (*B*). Total RNA was isolated at 0, 6, 12, 24, 48, and 72 h after cold treatment, the *actin* gene was used as an internal control (*lower panel*). Relative transcript levels were calculated with reference to the controls (taken as 100%). The results were given as the mean ± SD of three independent experiments. *, ** – significant at $P<0.05$ and 0.01, respectively, as compared to the control.

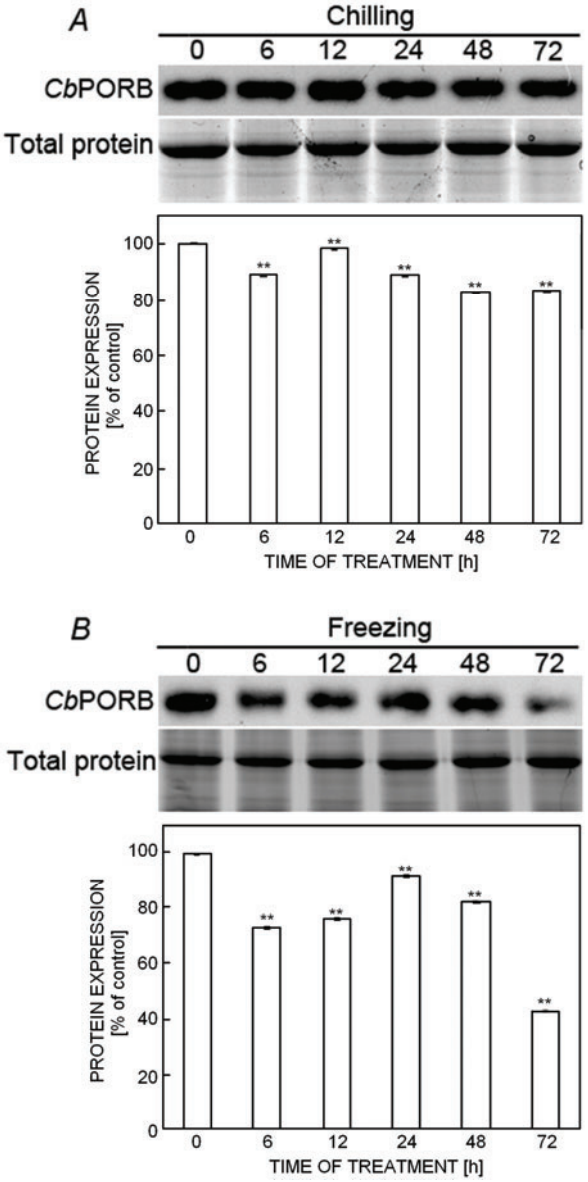

Fig. 7. Western blot analysis of *Cb*PORB protein expression levels in response to cold treatment. (*A*) Chilling (4°C), (*B*) freezing (−4°C). Total protein was isolated at 0, 6, 12, 24, 48, and 72 h after cold treatment. Coomassie blue-stained SDS-PAGE gels were also shown to control for loading variations (*lower panel*), equal amounts of total protein (10 μg) was loaded into each lane for all examined samples. Relative protein levels were calculated with reference to the controls (taken as 100%). The results were given as the mean ± SD of three independent experiments. *, ** – significant at $P<0.05$ and 0.01, respectively, as compared to the control.

of salinities have been reported in an extreme halophyte, *Arthrocnemum macrostachyum* (Redondo-Gómez *et al.* 2010). Redondo-Gómez *et al.* (2006) suggested that photorespiration and cyclic electron transport could be mechanisms to protect *Sarcocornia fruticosa* against excess radiation under high salinities. Both pathways can lead to additional consumption of reducing equivalents and can thus function as sinks for excess excitation energy (Asada 1996). It indicated that these two physiological processes could be relevant mechanisms to protect *C. bungeana* against excess radiation under cold conditions, therefore, as in the case of *Sarcocornia fruticosa*, the relatively stable NPQ in cold-stressed *C. bungeana* seedlings suggested that cold stress did not cause an increase in thermal dissipation in PSII antennae.

Up to date, PORs have been identified from most oxygenic photosynthetic organisms to land plants (Armstrong 1998, Fujita *et al.* 1998, Masuda and Takamiya 2004). However, POR in *C. bungeana* has not been described so far. Therefore, in our report, cDNA encoding *CbPORB* was isolated from *C. bungeana* using degenerate primers designed according to conserved region of other PORs. Its identity was confirmed by structural data, functional analysis and its close homology with other PORs. Our results indicated that *Cb*PORB showed a high degree of similarity with PORs previously isolated from other species, such as *Arabidopsis*, cucumber and barley.

Until now, few studies have been reported on the effect of low temperature on POR gene expression and protein content. In our work, *Cb*PORB transcript and protein content were inhibited by approximately 15% up to 72 h at 4°C, as compared to the control (Figs. 6*A*,7*A*). It has been already confirmed that the expression behavior of *POR* gene in response to chilling stress matches with that of enzymatic activity observed in response to chilling stress (Tewari and Tripathy 1998), which means that the *Cb*PORB enzymatic activity also reduces to a similar extent of its protein content under cold stress. The changes of transcript level were not similar to that of Sonoike (1996), who found that chilling treatment induced drastic reduction in the *POR* mRNA level in cucumber seedlings. In addition, Yang *et al.* (2005) reported that chilling stress severely suppressed *POR* gene expression and led to serious inhibition of Chl synthesis in leaves of mung bean seedlings. Therefore, it is common that the decrease of mRNA and protein abundance of POR in plants can be induced by low temperature, but the extent of decrease in the level of *POR* mRNA and protein abundance varied depending on the plant species. To our knowledge, this is the first report of a plant *POR* gene of which the transcript and expression levels are slightly inhibited by chilling treatment for 3 days. It is proposed that in these chilled *C. bungeana* seedlings the synthesis of degrading *Cb*PORB enzymes were probably partially inhibited, thus resulting in non-destruction of *Cb*PORB and its accumulation.

The striking feature of *Cb*PORB was that certain transcript and protein levels at 4°C were only slightly lower than those in control seedlings grown at 22 ± 2°C, which may reveal that *C. bungeana s*eedlings could still efficiently phototransform protochlorophyllide to chlorophyllide and synthesize Chl molecules to meet the demands of growth and pigment turnover at 4°C. Besides, although the mRNA level and protein content of *Cb*PORB substantially declined during prolonged freezing treatment, some of cold-treated seedlings can survive for 72 h under −4°C conditions, since they can completely recover after being transferred to a warm temperature for several days. All these results strongly suggest that *C. bungeana* has evolved an efficient defense system to maintain the integrity of photochemical and photosynthetic apparatus, thus sufficing the biosynthesis of Chl and the growth demands to adapt to the cold environment.

To sum up, we have demonstrated now that *Cb*PORB possesses certain resistant characteristic and is a major player in Chl biosynthesis process involved in plant growth and development in *C. bungeana*. The present results combined with previous studies strongly suggest that *C. bungeana* is a plant with strong chilling tolerance. These data obtained in this study will be helpful for better understanding the physiological and molecular adaptations to cold environment in alpine subnival plants.

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.

Apel, K., Santel, H.J., Redlinger, T.E., Falk, H.: The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). Isolation and characterization of the NADPH : protochlorophyllide oxidoreductase. − Eur. J. Biochem. **111**: 251-258, 1980.

Armstrong, G.A.: Greening in the dark: light-independent chlorophyll biosynthesis from anoxygenic photosynthetic bacteria to gymnosperms. − J. Photochem. Photobiol. B Biol. **43**: 87-100, 1998.

Armstrong, G.A., Runge, S., Frick, G., Sperling, U., Apel, K.: Identification of NADPH:protochlorophyllide oxidoreductase A and oxidoreductase B. A branched pathway for light dependent chlorophyll biosynthesis in *Arabidopsis thaliana*. − Plant Physiol. **108**: 1505-1517, 1995.

Arnon, D.I.: Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. − Plant Physiol. **24**: 1-15, 1949.

Artus, N.N., Uemura, M., Steponkus, P.L., Gilmour, S.J., Lin, C.T., Thomashow, M.F.: Constitutive expression of the cold-regulated *Arabidopsis thaliana COR15a* gene affects both chloroplast and protoplast freezing tolerance. − Proc. Natl. Acad. Sci. USA **93**: 13404-13409, 1996.

Asada, K.: Radical production and scavenging in the chloroplasts. − In: Baker, N.R. (ed.): Photosynthesis and the Environment. Pp. 123-150. Kluwer Academic Publ., Dordrecht – Boston – London 1996.

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.]

Bannai, H., Tamada, Y., Maruyama, O., Nakai, K., Miyano, S.: Extensive feature detection of N-terminal protein sorting signals. − Bioinformatics **18**: 298-305, 2002.

Bilger, W., Schreiber, U., Lange, O.L.: Chlorophyll fluorescence as an indicator of heat induced limitation of photosynthesis in *Arbutus unedo* L. − In: Tenhunen, J.D., Catarino, F.M., Lange, O.L., Oechel, W.C. (ed.): Plant Response to Stress. Pp. 391-399. Springer-Verlag, Berlin – Heidelberg – New York – London – Paris –Tokyo 1987.

Chang, J.F., Fu, X.Y., An, L.Z., Xu, S.J., Wang, J.H., Zhang, M.X., Feng, H.Y., Chen, T.: Properties of cellular ubiquinone and stress-resistance in suspension-cultured cells of *Chorispora bungeana* during early chilling. − Environ. Exp. Bot. **57**: 116-122, 2006.

DeRidder, B.P., Crafts-Brandner, S.J.: Chilling stress response of postemergent cotton seedlings. − Physiol. Plant. **134**: 430-439, 2008.

Dutta, S., Mohanty, S., Tripathy, B.C.: Role of temperature stress on chloroplast biogenesis and protein import in pea. − Plant Physiol. **150**: 1050-1061, 2009.

Forreiter, C., van Cleve, B., Schmidt, A., Apel, K.: Evidence for a general light-dependent negative control of NADPH-protochlorophyllide oxidoreductase in angiosperms. − Planta **183**: 126-132, 1991.

Frick, G., Su, Q., Apel, K., Armstrong, G.A.: An *Arabidopsis porB porC* double mutant lacking light-dependent NADPH : protochlorophyllide oxidoreductases B and C is highly chlorophyll-deficient and developmentally arrested. − Plant J. **35**: 141-153, 2003.

Fu, X.Y., Chang, J.F., An, L.Z., Zhang, M.X., Xu, S.J., Chen, T., Liu, Y.H., Xin, 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.

Fujita, Y., Takagi, H., Hase, T.: Cloning of the gene encoding a protochlorophyllide reductase: the physiological significance of the co-existence of light-dependent and -independent protochlorophyllide reduction systems in the cyanobacterium *Plectonema boryanum*. − Plant Cell Physiol. **39**: 177-185, 1998.

Gajewska, E., Skłodowska, M., Słaba, M., Mazur, J.: Effect of nickel on antioxidative enzyme activities, proline and chlorophyll contents in wheat shoots. − Biol. Plant. **50**: 653-659, 2006.

Genty, B., Briantais, J.M., Baker, N.R.: The relationship between the quantum yield of photosynthetic electron-transport and quenching of chlorophyll fluorescence. − Biochim. Biophys. Acta **990**: 87-92, 1989.

Griffiths, W.T.: Reconstitution of chlorophyllide formation by isolated etioplast membranes. − Biochem. J. **174**: 681-692, 1978.

Häuser, I., Dehesh, K., Apel, K.: The proteolytic degradation *in vitro* of the NADPH-protochlorophyllide oxidoreductase of barley (*Hordeum vulgate* L.). − Arch. Biochem. Biophys. **228**: 557-586, 1984.

Hodgins, R., van Huystee, R.B.: Porphyrin metabolism in chill stressed maize (*Zea mays* L.). − J. Plant Physiol. **125**: 325-336, 1986a.

Holtorf, H., Reinbothe, S., Reinbothe, C., Bereza, B., Apel, K.: Two routes of chlorophyllide synthesis that are differentially regulated by light in barley (*Hordeum vulgare* L.). − Proc. Natl. Acad. Sci. USA **92**: 3254-3258, 1995.

Hormaetxe, K., Becerril, J.M., Hernández, A., Esteban, R., García-Plazaola, J.I.: Plasticity of photoprotective mechanisms of *Buxus sempervirens* L. leaves in response to extreme temperatures. − Plant Biol. **9**: 59-68, 2007.

Horton, P., Ruban, A.V.: Regulation of photosystem II. − Photosynth. Res. **34**: 375-385, 1992.

Ikeuchi, M., Murakami, S.: Behavior of the 36,000-dalton protein in the internal membranes of squash etioplasts during greening. − Plant Cell Physiol. **23**: 575-583, 1982.

Joyard, J., Block, M., Pineau, B., Albrieux, C., Douce, R.: Envelope membranes from mature spinach chloroplasts contain a NADPH:protochlorophyllide reductase on the cytosolic side of the outer membrane. − J. Biol. Chem. **265**: 21820-21827, 1990.

Krause, G.H.: Photoinhibition induced by low temperatures. − In: Baker, N.R., Bowyer, J.R. (ed.): Photoinhibition of Photosynthesis from Molecular Mechanisms to the Field. − Pp. 331-348. BIOS Scientific Publ., Oxford 1994.

Kuroda, H., Masuda, T., Ohta, H., Shioi, Y., Takamiya, K.: Effects of light, developmental age and phytohormones on the expression of the gene encoding NADPH-protochlorophyllide oxidoreductase in *Cucumis sativus*. − Plant Physiol. Biochem. **34**: 17-22, 1996.

Kuroda, H., Masuda, T., Fusada, N., Ohta, H., Takamiya, K.: Expression of NADPH-protochlorophyllide oxidoreductase gene in fully green leaves of cucumber. − Plant Cell Physiol. **41**: 226-229, 2000.

Kuroda, H., Masuda, T., Fusada, N., Ohta, H., Takamiya, K.: Cytokinin-induced transcriptional activation of NADPH-protochlorophyllide oxidoreductase gene in cucumber. − J. Plant Res. **114**: 1-7, 2001.

Masuda, T., Fusada, N., Shiraishi, T., Kuroda, H., Awai, K., Shimada, H., Ohta, H., Takamiya, K.: Identification of two differentially regulated isoforms of protochlorophyllide oxidoreductase (POR) from tobacco revealed a wide variety of lightand development-dependent regulations of POR gene expression among angiosperms. − Photosynth. Res. **74**: 165-172, 2002.

Masuda, T., Fusada, N., Oosawa, N., Takamatsu, K., Yamamoto, Y.Y., Ohto, M., Nakamura, K., Goto, K., Shibata, D., Shirano, Y., Hayashi, H., Kato, T., Tabata, S., Shimada, H., Ohta, H., Takamiya, K.: Functional analysis of isoforms of NADPH:protochlorophyllide oxidoreductase (POR), PORB and PORC, in *Arabidopsis thaliana*. − Plant Cell Physiol. **44**: 963-974, 2003.

Masuda, T., Takamiya, K.I.: Novel insights into the enzymology, regulation and physiological functions of light-dependent protochlorophyllide oxidoreductase in angiosperms. − Photosynth. Res. **81**: 1-29, 2004.

Maxwell, K., Johnson, G.N.: Chorophyll fluorescence - a practical guide. − J. Exp. Bot. **51**: 659-668, 2000.

Mohanty, S., Grimm, B., Tripathy, B.C.: Light and dark modulation of chlorophyll biosynthetic genes in response to temperature. − Planta **224**: 692-699, 2006.

Müller, P., Li, X.P., Niyogi, K.K.: Non-photochemical quenching. A response to excess light energy. − Plant Physiol. **125**: 1558-1566, 2001.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. − Physiol. Plant. **15**: 473-497, 1962.

Oosawa, N., Masuda, T., Awai, K., Fusada, N., Shimada, H., Ohta, H., Takamiya, K.: Identification and light-induced expression of a novel gene of NADPH-protochchlorophyllide oxidoreductase isoform in *Arabidopsis thaliana*. − FEBS Lett. **474**: 133-136, 2000.

Pattanayak, G.K., Tripathy, B.C.: Catalytic function of a novel protein protochlorophyllide oxidoreductase C of *Arabidopsis thaliana*. − Biochem. Biophys. Res. Commun. **291**: 921-924, 2002.

Pineau, B., Gerard-Hirne, C., Douce, R., Joyard, J.: Identification of the main species of tetrapyrrolic pigments in envelope membranes from spinach chloroplasts. − Plant Physiol. **102**: 821-828, 1993.

Redondo-Gómez, S., Wharmby, C., Castillo, J.M., Mateos-Naranjo, E., Luque, C.J., de Cires, A., Luque, T., Davy, A.J., Figueroa, M.E.: Growth and photosynthetic responses to salinity in an extreme halophyte, *Sarcocornia fruticosa*. − Physiol. Plant. **128**: 116-124, 2006.

Redondo-Gómez, S., Mateos-Naranjo, E., Figueroa, M.E., Davy, A.J.: Salt stimulation of growth and photosynthesis in an extreme halophyte, *Arthrocnemum macrostachyum*. − Plant Biol. **12**: 79-87, 2010.

Reinbothe, S., Reinbothe, C., Holtorf, H., Apel, K.: Two NADPH:protochlorophyllide oxidoreductases in barley: evidence for the selective disappearance of PORA during the light-induced greening of etiolated seedlings. − Plant Cell **7**: 1933-1940, 1995.

Schreiber, U., Schliwa, W., Bilger, U.: Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorimeter. − Photosynth. Res. **10**: 51-62, 1986.

Seyedi, M., Selstam, E., Timko, M.P., Sundqvist, C.: The cytokinin 2-isopentenyladenine causes partial reversion to skotomorphogenesis and induces formation of prolamellar bodies and protochlorophyllide 657 in the lip1 mutant of pea. − Physiol. Plant. **112**: 161-272, 2001a.

Sonoike, K.: Photoinhibition of photosystem I: Its physiological significance in the chilling sensitivity of plants. − Plant Cell Physiol. **37**: 239-247, 1996.

Strasser, R.J., Butler, W.L.: Fluorescence emission spectra of photosystem I, photosystem II and the light-harvesting chlorophyll *a*/*b* complex of higher plants. − Biochim. Biophys. Acta **17**: 307-313, 1977.

Su, Q., Frick, G., Armstrong, G., Apel, K.: POR C of *Arabidopsis thaliana*: a third light- and NADPH-dependent protochlorophyllide oxidoreductase that is differentially regulated by light. − Plant Mol. Biol. **47**: 805-813, 2001.

Teakle, G.R., Griffiths, W.T.: Cloning, characterization and import studies on protochlorophyllide reductase from wheat (*Triticum aestivum*). − Biochem. J. **296**: 225-230, 1993.

Tewari, A.K., Tripathy, B.C.: Temperature-stress-induced impairment of chlorophyll biosynthetic reactions in cucumber and wheat. − Plant Physiol. **117**: 851-858, 1998.

Tewari, A.K., Tripathy, B.C.: Acclimation of chlorophyll biosynthetic reactions to temperature stress in cucumber (*Cucumis sativus* L.). − Planta **208**: 431-437, 1999.

Van Hasselt, P.R., Strikwerda, J.T.: Pigment degradation in discs of the thermophilic *Cucumis sativus* as affected by light, temperature, sugar application and inhibitors. − Physiol. Plant. **37**: 253-257, 1976.

Werner, C., Correia, O., Beyschlag, W.: Characteristic patterns of chronic and dynamic photoinhibition of different functional groups in a Mediterranean ecosystem. − Funct. Plant Biol. **29**: 991-1011, 2002.

Yang, M.T., Chen, S.L., Lin, C.Y., Chen, Y.M.: Chilling stress suppresses chloroplast development and nuclear gene expression in leaves of mung bean seedlings. − Planta **221**: 374-385, 2005.

Zhang, T.G., Liu, Y.B., Xue, L.G., Xu, S.J., Chen, T., Yang, T.W., Zhang, L.J., An, L.Z.: Molecular cloning and characterization of a novel MAP kinase gene in *Chorispora bungeana*. − Plant Physiol. Biochem. **44**: 78-84, 2006.