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Relation of several antioxidant enzymes to rapid freezing resistance in suspension cultured cells from alpine *Chorispora bungeana* ☆

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Abstract

Chorispora bungeana Fisch. & C.A. Mey (Crucifer) is a rare alpine subnival plant surviving sudden snowstorms. In this paper, we have attempted to explore possible roles of autoxidation rate (AR) and the antioxidant enzymes associated with cryoprotective mechanisms in the plant cells. The results showed that when the suspension cultures growing at 25 °C were suddenly exposed to −8 °C for 15 days, 2,3,5-triphenyltetrazolium chloride reduction was not affected within 9 days and AR remained at a low level in comparison with controls. This indicated that the cells maintained considerable amounts of soluble protein and the integrity of the cell membranes was intact during the whole freezing test. Furthermore, on average, the activity of antioxidant enzymes such as superoxide dismutase, dehydroascorbate reductase, ascorbate peroxidase and glutathione reductase were prominently enhanced in the freezing-stressed cells. Peroxidase activity significantly increased soon after freezing, possibly to make up for the early decrease of catalase activity in the cells. Statistical analysis showed negative correlations between resistance to rapid freezing and antioxidant enzyme activity in the cultured cells after exposure from 25 to −8 °C, indicating that the reduction of cell viability with freezing activates a combination of antioxidant enzymes that results in intact cells. All of these findings suggest a synergy between these antioxidant enzymes, leading to a low autoxidation rate that contributes to the protection of the cell membranes and plays an important role in the resistance of suspension cultured cells of *C. bungeana* to sudden freezing.

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Sudden freezing is one of the most important environmental stress factors that limit the distribution of native flora and reduce agricultural productivity. Selection and breeding to find freezing-resistant clones are vital to reduce yield losses and improve survival rates in northern countries [36].

Chorispora bungeana Fisch. & C.A. Mey (*C. bungeana*) is a typical alpine subnival herbaceous plant that can blossom even under acute freezing temperatures at altitudes over 3800 m and their roots live through all the asperity of northern winters [3]. Rapid cold hardening, as initially defined in insects, was used to describe any rapid change in cold tolerance in arthropods [33]. However, rapid freezing resistance (RFR) is interpreted more broadly here where it means the process whereby plant cells survive sudden freezing temperatures. Therefore, RFR might be expected in alpine plant cells, where it would be caused by physiological, biochemical and molecular cryoprotective mechanisms.

Like other stressful conditions, freezing damage in plant cells is thought to involve attack by active oxygen species (AOS), causing oxidative stress [29]. The ability of higher plant cells to scavenge the AOS seems to be a very important determinant of their tolerance to environmental stress. Along with analysis of mRNA, determination of enzymatic activity is also important, because enzymatic activity is directly responsible for the physiological and biochemical effect [25]. Plant tissue cultures are widely used as model systems to study how cells respond and acclimate to freezing, salinity and heat [21]. Cultured plant cells are also a good system for the study of antioxidant mechanisms [24].

However, to our knowledge, the study of rapid freezing resistance in alpine subnival plants has not involved antioxidant mechanisms so far. In this paper, we have attempted to explore the possible role of antioxidant enzymes in cryoprotective mechanisms in the rare subnival plant cells that adapt to rapid freezing. For this, the RFR properties, lipid peroxidation and several antioxidant enzymes were studied comparatively by exposing suspension cultures that were growing at 25 °C directly to −8 °C for 15 days. This freezing temperature enabled us to observe a severe freezing event during the most favorable growth season for this alpine plant.

Materials and methods

A wild species of *C. bungeana*, a perennial and herbaceous crucifer, was collected in an ice-free foreland (43°05′N, 86°49′E, with an altitude of 3800–3900 m) near the No. 1 glacier in the source area of the Urumqi River in Tianshan Mountains, Xinjiang, China. The alpine subnival plant undergoes an acute temperature fluctuation from nearly 4 to −10 °C during its most favorable growth season (from June to September) [3]. The plant leaf changes in morphology with growth and development from an oblong shape without serrate margins after burgeoning to a long-oblong shape with serrate margins before blossoming [10].

Preparation of suspension-cultured cells of Chorispora bungeana

Chorispora bungeana suspension cultures were maintained and used for experiments as previously described [10]. Briefly, the leaves (which are directly affected by environmental chilling) of *C. bungeana* without serrate margins were selected for callus induction. The sterile leaves were cut into 10 mm segments, with margins finely cut off, and cultured on MS basal medium containing 4.0 mg/L gibberellin acid (GA_3), 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.2 mg/L α-naphthalene acetic acid (NAA) with a 12-h long light period (4000 LX) at 25 °C. The medium was refreshed every 2 weeks. Suspension cultured cells of *C. bungeana* were cultivated in liquid MS basal medium containing 0.2 mg/L 2,4-D, 0.2 mg/L NAA, 0.2 mg/L 6-benzylaminopurine (6-BA), and 0.2 mg/L kinetin (KT) on a rotary shaker (120 rpm). After five or more subcultures, the synchronous steady cultures were collected and used in the following experiments.

Rapid freezing stress

The freezing test was conducted according to the method of Ishikawa et al. [21]. Briefly, cultures initiated with 0.5 g fresh weight (FW) of cells from the 7-day-old stock cultures, were grown for 1 day and were divided into two groups on the following

day. One group were controls that remained at 25 °C on a rotary shaker (120 rpm) with a 12-h long light period (4000 LX) for 0–15 days. Another group experienced freezing stress by direct exposure to −8 °C under the same conditions as the controls except for the temperature in the freezing test chamber. The duration (0–15 days) and the temperature (−8 °C) in the test were selected by referencing to the natural local severe freezing event that may occur during the favorable growth season of the alpine plant.

Following the treatments, cells were harvested and briefly washed with 15 mL of sterilized distilled water for a few seconds to remove medium residues. This was done by filtration through a 400-screen mesh according to the method of Ishikawa et al. [21] and the cells were used for RFR determination immediately. The cells weighed 0.250 g (FW) and were quickly stored at −70 °C for determination of lipid peroxidation and enzyme activities. To avoid the recovery of cells from freezing induction and to keep their actual state, we conducted all assays for freeze-stressed cells under 0.5 °C except when using the freezing test method of Janda et al. [22].

Determination of rapid freezing resistance

Rapid freezing resistance was measured by the TTC reduction test [21,20] following the treatments, 0.050 g of FW samples was incubated in 4 mL of 0.08% TTC in phosphate buffer (pH 7.5) in the dark at 23 °C for 24 h. The TTC solution was removed by vacuum aspiration and washed with 9 mL of distilled water. The reduced TTC was extracted with 5 mL of 95% ethanol at 60 °C in the dark for 10 min and absorbance read at 485 nm (UV 7501/Vis spectrometer). TTC reduction was expressed as the ratio of the extracted red formazan concentration per gram of treated cells to that of untreated cells.

Assay of lipid peroxidation

Lipid peroxidation was determined in three random replicates by measuring the malondialdehyde (MDA) equivalents [15]. The samples were homogenized in a mortar with 8 mL of 50 mM phosphate buffer (pH 7.0). The uniform homogenate was divided equally into two cuvettes. The first one was immediately centrifuged at 3000*g* for 10 min at 4 °C. The supernatants were pooled and 2 mL was added to a test tube with an equal volume of the solution comprising 0.65% thiobarbituric acid (TBA) in 20% (w/v) trichloroacetic acid (TCA). Samples were heated at 95 °C for 25 min and quickly cooled to room temperature. Absorbance was read at 450, 532, and 600 nm after centrifuged at 3000*g* for 10 min again. The other cuvette of homogenate was exposed to air and incubated with mild shaking at 25 °C for 5 h. MDA was determined at the designated time [19]

$$\text{Malondialdehyde } (\mu\text{mol/L}) = [6.45(\text{OD}_{532} - \text{OD}_{600}) - 0.56\,\text{OD}_{450}]. \quad (1)$$

The autoxidation rate (AR) was calculated as the average increased rate of MDA in the two cuvettes [26].

Assay of the activity of antioxidant enzymes

All determinations for enzymes were performed at 4 °C. The samples were homogenized in liquid nitrogen with 5 mL extract buffer containing 0.2 mM ethylenediaminetetraacetic (EDTA) and 2% polyvinylpyrrolidone (PVP) in 25 mM PBS buffer (pH 7.8) [9]. The homogenate was centrifuged at 15,000*g* for 20 min and the supernatant was used for the protein and enzyme analysis. Protein concentration was determined by reading absorbance at 260 and 280 nm (UV 7501/Vis, Japan) [37].

$$\text{Protein } (\text{mg/mL}) = [1.55\,\text{OD}_{280} - 0.76\,\text{OD}_{260}]. \quad (2)$$

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed photochemically, using the assay system consisting of 50 mM Na-phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 2.0 mM riboflavin, and 0.1 mL of the enzyme extract [17]. Riboflavin was added last and the reaction was initiated by placing the tubes under two 15 W fluorescent lamps; it was terminated after 20 min by removing the light source. Non-illuminated and illuminated reactions without supernatant served as calibration standards. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction at 560 nm.

A modified method of Abassi et al. [1] was employed for the assay of catalase (CAT, EC 1.11.1.6) by monitoring the decrease in absorbance at 240 nm. Enzyme extract (0.1 mL) was added to 1.9 mL of the buffer that consisted of 12.5 mM H_2O_2 in 50 mM Na-phosphate buffer (pH 7.0).

The enzyme solution containing 50 mM Na-phosphate buffer (pH 7.0) was used as a control ($\varepsilon = 39.4$/mM/cm).

Peroxidase (POD, EC 1.11.1.7) activities were determined by the increase in absorbance at 470 nm due to guaiacol oxidation in a reaction mixture containing 25 mM Na-phosphate buffer (pH 7.0), 0.05% guaiacol and 10 mM H_2O_2 ($\varepsilon = 26.6$/mM/cm) [9].

Ascorbate peroxidase (APX, EC 1.11.1.11) was assayed by the decrease in absorbance at 290 nm [12] in an assay mixture containing 50 mM Na-phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbic acid (AsA), 0.1 mM H_2O_2, and enzyme extract ($\varepsilon = 2.8$/mM/cm). Corrections were made for non-enzymatic and H_2O_2 independent oxidation.

Dehydroascorbate reductase (DHAR, E.C. 1.8.5.1) was assayed in a reaction mixture containing 25 mM Na-phosphate buffer (pH 7.0), 2 mM reduced glutathione (GSH), 0.2 mM dehydroascorbate (DHA) and enzyme extract by monitoring the increase in absorbance at 265 nm ($\varepsilon = 14$/mM/cm) [23]. DHA must be freshly prepared and kept on ice until it is added to the reaction mixture in the cuvette to prevent its rapid oxidation.

Glutathione reductase (GR, EC 1.6.4.2) activity was determined by following the rate of NADPH oxidation as measured by the decrease in the absorbance at 340 nm [16]. The assay mixture contained 0.025 mM extract buffer (pH 7.8), 0.12 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and the enzyme extract ($\varepsilon = 6.2$/mM/cm).

The specific enzyme activity for all enzymes was expressed as units/mg protein according to the method of Kubo et al. [25]. Standard NADPH, EDTA, DHA, GSH, and GSSG were from Sigma. Other chemicals were of no less than analytical grade.

Statistical analysis

The results of the enzyme activity data are the means of three measurements. The significance of differences among means of treatments was tested by ANOVA (Univariate) and Duncan's multiple range test ($P < 0.05$). Pearson correlation (two-tailed) was used to assess the relationship between enzyme activity and RFR, measured by TTC reduction in each temperature case ($n = 9 \times 3$). All these statistical analyses were conducted on SPSS11.5 software (USA).

Results

Rapid freezing resistance measured as TTC reduction

After 15 days at −8 °C the suspensions were not completely frozen and the cultured cells looked fresh with yellow and green colouration that was similar to the controls, indicating that the cells were in an ultracold state with retarded extracellular ice formation. Fig. 1 shows the changes of TTC reduction after holding at −8 or 25 °C for 15 days. The result showed that the survival of suspension cultures that were rapidly cooled to −8 °C in was not significantly different from that of the synchronized controls during 9 days, indicating that the freezing did not affect the ability of cells to reduce TTC during this period. The activity decreased gradually after 9 days but still remained over 50% of the controls before treatment compared with the end of the test ($P < 0.01$) (Fig. 1).

The recovered protein

Changes in the relative amount of protein recovered after 0 and 5 h at 25 °C, following freezing at −8 °C are given in Fig. 2. The relative protein content changed with the duration of the freezing treatment. It changed a great deal with the recovery time following 1–9 days of freezing but altered very little after freezing for more than 10 days. The protein content decreased following 1, 7 or 9 days of freezing (the average decreased was 27.0%, $P < 0.01$). However, the protein did significantly increase after a recovery period of 5 h at 25 °C when compared with their synchronized controls (The average

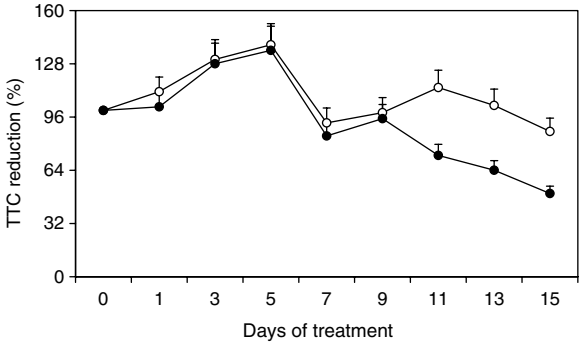

Fig. 1. The changes of TTC reduction in suspension cultured cells of *C. bungeana* exposed to −8 °C (●) and 25 °C (○) for 15 days. Points and error bars indicate means ± SD ($n = 3$).

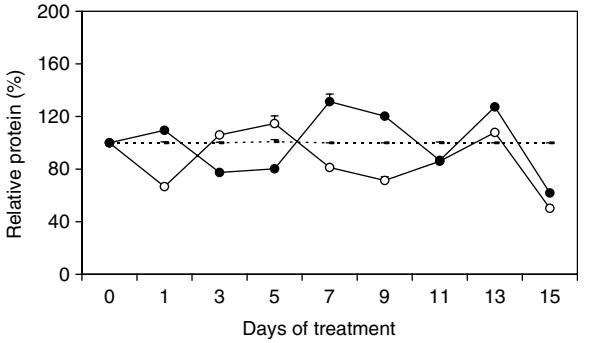

Fig. 2. The changes of the amount of protein measured as a percentage of the unfrozen synchronized control (−) in suspension-cultured cells of *C. bungeana* after recovery for 0 h (○) and 5 h (●) following 0–15 days of rapid freezing at −8 °C. Points and error bars indicate means ± SD ($n = 3$).

increase was 20.4%, $P < 0.01$). The result was the reverse after sudden freezing for 3–5 days. The amount of protein declined to a minimum after rapid exposure to −8 °C for 15 days and the level recovered from 50.2% ($P < 0.01$) to 61.9% ($P < 0.01$) in 5 h under normal conditions, although it failed to equal its control value (Fig. 2).

The result of lipid peroxidation

In the suspension cultured cells at −8 °C for 15 days, the MDA level gradually increased and reached a peak of 3.78 μmol/g FW ($P < 0.01$) on the first day; then it decreased to 2.31 μmol/g FW on the 15th day, which was significantly different when compared with its synchronized control ($P < 0.05$) but was not significant compared with the initial (zero day) level (2.33 μmol/g FW). When the cultures were growing at 25 °C, the MDA level decreased gradually and reached its nadir at 1.35 μmol/g FW on the 7th day. After that it fluctuated (Fig. 3A). These results indicate that rapid freezing resulted in some AOS formation leading to lipid peroxidation in the suspension cultures because MDA is an end product of lipid peroxidation. However, the MDA accumulation could be held back when freezing persisted. This capability to retard lipid peroxidation was also in evidence in the changes in autoxidation rate (Fig. 3B). After sudden exposure to −8 °C, the AR in the cultured cells exhibited a low and steady trend in comparison with the controls. AR represents the average increased rate of MDA after freezing removal, so it can better reveal the repair capability of the cell membranes.

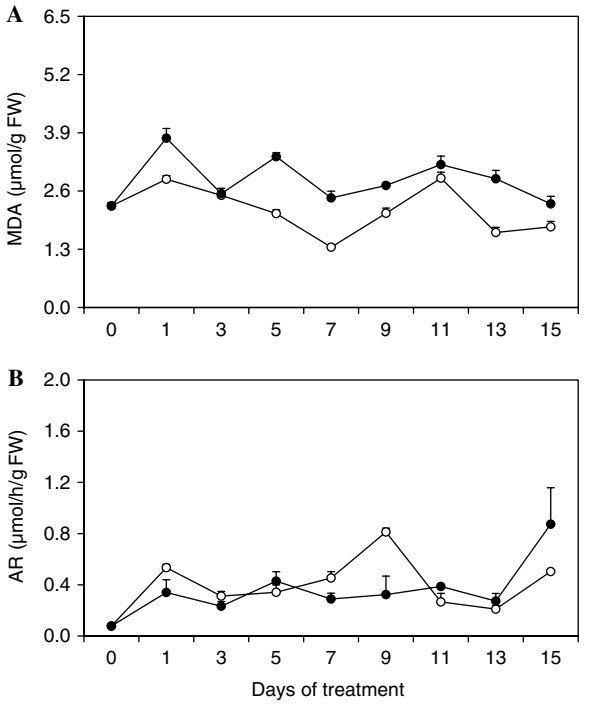

Fig. 3. The changes of lipid peroxidation measured as MDA content (A) and autoxidation rate (B) in suspension cultured cells of *C. bungeana* both at −8 °C (●) and at 25 °C (○) for 15 days. Points and error bars indicate means ± SD ($n = 3$).

The result of antioxidant enzyme activities

Within 1 day, cells that had been freezing-stressed at −8 °C showed significant increases in SOD, CAT, POD, and APX, but thereafter the activities significantly declined to reach a minimum on the 3rd day. The minimum was delayed till the 5th day for DHAR and GR activity. Afterwards, the cells that had been exposed to −8 °C showed another significant increase in the combined activity of the antioxidant enzymes. This was more efficient in the frozen cells than in the controls, especially with respect to the activity of APX, DHAR, GR, and SOD. On the average, during the whole freezing test lasting 15 days, the activity of these four enzymes increased by 96.5% ($P < 0.001$), 38.9% ($P < 0.001$), 24.4% ($P < 0.01$) and 20.5% ($P < 0.01$), respectively, when compared with the controls.

CAT and POD activities also demonstrated a rather similar trend but with a little alteration. A big 'age-dependent' increase in CAT activity was apparent after 3 days, both at 25 °C and at −8 °C. CAT activity in the cells that were freezing-stressed

at −8 °C did not exceed its control until 7 days when it stayed at that higher level until the end of the test following that day. On the 9th day the freezing increased CAT activity by 57.6% ($P < 0.001$) as compared with its synchronized control (Fig. 4). POD activity was greatest among all the enzymes that were assayed both after exposure to −8 and 25 °C. The enzyme activity significantly increased with early freezing, which increased by 77.1% ($P < 0.001$) during the first 5 days.

A correlation between the rapid freezing resistance measured as TTC reduction and the activity of antioxidant enzymes such as SOD ($R = -0.711$, $P < 0.001$), CAT ($R = -0.795$, $P < 0.001$), POD ($R = -0.489$, $P < 0.01$), APX ($R = -0.715$, $P < 0.001$) and GR ($R = -0.765$, $P < 0.001$) was found in rapid freezing stress at −8 °C but not in the controls at 25 °C. However, the correlation did not exhibit in DHAR activity in both cases (Table 1).

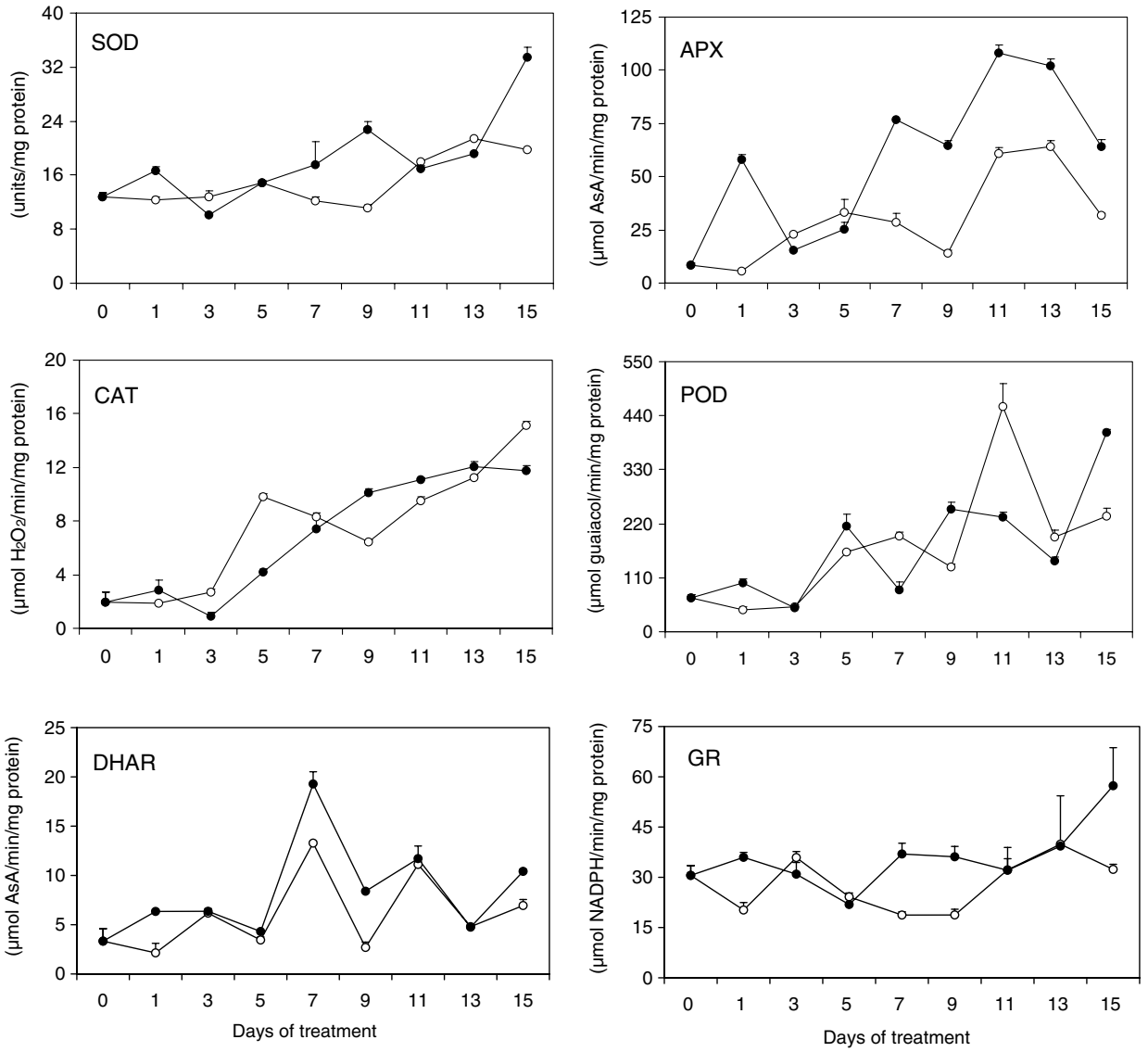

Fig. 4. The changes of the activity of antioxidant enzymes such as superoxide dismutases (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) in suspension cultured cells of *C. bungeana* both at −8 °C (●) and at 25 °C (○) for 15 days. Points and error bars indicate means ± SD ($n = 3$).

Table 1
Pearson correlation coefficients (two-tailed) between rapid freezing resistance measured as TTC reduction and antioxidant enzyme activities in suspension cultured cells of *C. bungeana* at 25 and −8 °C for 0, 1, 3, 5, 7, 9, 11, 13, and 15 days (three replications, $n = 9 \times 3$)

Enzymes	25 °C	−8 °C
SOD	−0.126	−0.711***
CAT	−0.253	−0.795***
DHAR	−0.220	−0.377
POD	−0.109	−0.509**
GR	0.170	−0.765***
APX	0.047	−0.715***

* Correlation is nonsignificant at the 0.05 level without labeling.
** Correlation is significant at the 0.01 level.
*** Correlation is significant at the 0.001 level.

Discussion

The membrane stability and strong restored ability associated with the RFR

Species can escape freezing damage by freeze-hardening or freeze-resistance [34], producing a complex, multifaceted process in plants. The plasma membranes play an important role in freezing resistance, for they are most sensitive to freezing stress [36]. TTC reduction shows similar trends to the intact cell ratio, which is used for judging the freezing resistance [20]. The amount of soluble proteins may protect cellular membranes and enzymes from irreversible damage caused by freezing [35]. By these techniques, freezing resistance can be described quantitatively.

Chorispora bungeana is a rare alpine subnival plant growing near the No. 1 glacier, the origin of Urumqi River on the Tianshan Mountains in China. The results showed that the suspension-cultured cells from the leaves of the wild alpine plant exhibited great freezing resistance when suddenly exposed to −8 °C, which means that it has a germplasm that has adapted well to provide rapid freezing resistance in nature. This is supported by the following the evidence. First, the TTC-reducing ability was not affected when rapidly exposed to −8 °C for 9 days and the activity remained over 50% until the end of this freezing test (Fig. 1). This result revealed that the alpine plant cells remained intact under severe freezing conditions. Further, the freezing and thawing could induce considerable amounts of soluble protein, which might mitigate structural damage in the plasma membranes (Fig. 2). Finally, the suspensions were not totally frozen at −8 °C, indicating that the cells were in a supercooled state due to the retardation of extracellular ice formation, probably by inducing physiologically active substances. To our knowledge, the RFR of suspension-cultured cells from *C. bungeana* far exceeds that of most other plant cells.

The ability to retard lipid peroxidation associated with the RFR

Lipid peroxidation resulting from the failure of free radical scavenging is an integral feature of membrane deterioration, leading to cell death [11]. MDA is an end product of lipid peroxidation that is used as indicator of oxidative damage in membranes [32]. We observed that when the suspension cultured cells were suddenly exposed to −8 °C for 15 days, MDA content rapidly accumulated in the first day and then decreased. However, it decreased gradually in 7 days and then fluctuated when the cultures were growing at 25 °C (Fig. 3A). These results indicate that rapid freezing induced some AOS formation, leading to lipid peroxidation in the suspension cultures, but this lipid peroxidation could be retarded if the freezing was extended. Dat et al. [13] reviewed the dual action of AOS; high concentrations of AOS can lead to phytotoxicity whereas relatively low levels can be used for acclamatory signaling and cellular strengthening. Therefore, the controlled modulation of AOS levels in plant is extremely important and may in part explain complex behaviour such as cross tolerance. Our study showed that the freezing-induced AOS could be tightly controlled if freezing persisted, thus allowing signaling and strengthening in the freezing-tolerant alpine plant cells, for MDA is also a molecular messenger. The cell walls of the epiderm in wild *C. bungeana* leaf blades were thickened without lignification under natural alpine environmental conditions [5], which could further demonstrate the strengthening functions.

The great ability of tight controlling of AOS leading to lipid peroxidation was also evident in the changes of autoxidation rate at −8 °C for 15 days (Fig. 3B). After rapid exposure to the freezing stress, the cultured cells of *C. bungeana* exhibited low and steady AR compared with controls growing at 25 °C, which was in agreement with the result of Li et al. [27], who reported that the cold pretreated Korean pine seedlings exhibited low and steady AR whereas the unpretreated seedlings showed a little

change at −8 °C for 4 days. Korean pine can live through freezing temperature at −50 °C in winter [27]. The autoxidation rate, the average increased rate of MDA, was determined after 5 h of exposure to air at 25 °C meaning that the stress was removed, so that it could reveal the total scavenging ability of AOS and reflect the ability to keep and recover membrane integrity [27,26]. These results suggested that the alpine subnival plant cells possessed remarkable repair ability and the total AOS detoxifying capability. The regeneration capacity of vegetation for the alpine plant after the removal of leaves was also found in a field test, further verifying the recovery function.

Based on these results, we conclude that the cultured cells of *C. bungeana*, a typical alpine subnival plant, possess an excellent ability to retard lipid peroxidation and to repair membranes under the stress of rapid freezing. The relatively low AOS induced by freezing functioned as a signaling and strengthening mechanism, and activated the expression of antifreeze genes, leading to the production of protective substances, resulting in a low autoxidation rate.

The association of the highly efficient antioxidant system with rapid freezing resistance

Exposure to low temperature may result in the increased generation of ROS in plants [2,31]. The AOS may play two very different roles: exacerbating damage or signaling the activation of defense responses. The capacity for AOS to serve as signals adds to the importance of antioxidants to specifically regulate different AOS in various cellular locations [13]. In the present study, the responsively combined activity of antioxidant enzymes provided the biochemical evidence for AOS signaling, leading to rapid-freezing resistance of the alpine plant cells (Fig. 4).

SOD activity modulates the relative amounts of $O_2^{\cdot -}$ and H_2O_2, the two Haber–Weiss reaction substrates and decreases the risk of $OH^{\cdot}$ radical formation, which is highly reactive and may cause severe damage to membranes, protein and DNA [7]. In this work, freezing increased SOD activity conspicuously, which on average increased by 20.5% ($P < 0.01$) in the suspension cultured cells during 15 days, suggesting that the alpine plant cells have a better $O_2^{\cdot -}$ radical scavenging ability. It has been shown that freezing increases SOD activity in cold pretreated Korean pine seedlings more than in un-pretreated seedlings with a freezing stress of −8 °C for 4 days [27]. In addition, SOD enhanced the tolerance of freezing stress in transgenic alfalfa [28].

The product of SOD activity is H_2O_2, which is still toxic and must be eliminated by conversion to H_2O in a subsequent reaction that is catalyzed by various enzymes [6]. Uncontrolled accumulation of H_2O_2 may perturb the cellular redox state and lead to damage to membrane lipids and DNA, finally cause cell death [30]. Our results showed that the cultured cells enhanced CAT activity with persisting freezing. On the contrary, POD activity increased prominently early during freezing, possibly to make up for the early decrease of CAT activity. It was also noticed that POD, in contrast to other enzymes, was the highest throughout the experiment, both at 25 and −8 °C. This enzyme is very important in the formation of the metabolic response of plants to different stress factors [6]. Developing such a high POD activity was a better strategy to tolerate rapid freezing, which coincided with the result of Janda et al. [22] in freezing tolerant cereals. In addition to this, POD was also reported to participate in lignin biosynthesis, building up a physical barrier against stresses and involving the degradation of IAA affecting growth of the plant [18,4]. This might explain why the alpine plant cells had such a strong recovery capability from freezing (low AR, see Fig. 3B).

APX, DHAR, and GR are the key enzymes of the ascorbate–glutathione cycle involved in scavenging H_2O_2. APX eliminates H_2O_2 by converting AsA to DHA [32] and the regeneration of AsA from DHA is catalyzed by GSH-dependent DHAR [38]. In our study, APX, DHAR, and GR activities were induced most strongly at −8 °C for 15 days (Fig. 4). The increases would result in the accumulation of AsA and GSH levels, which scavenge AOS in other pathways. It was reported that APX activity in freeze-hardened cereal plants was significantly higher in the tolerant group than in the sensitive group [22]. Transgenic tobacco plants overexpressing GR had both high levels of GSH and increased tolerance to oxidative stress [8].

It is noteworthy that the increased SOD activity in both control and freezing-stressed cells was accompanied by a greater increase in combined activity of CAT, POD, GR, and APX (see Fig. 4). These enzyme activities exhibited no correlation with TTC reduction at 25 °C (Table 1), coinciding with the result of Janda et al. [22] in freezing-unhardened cereal leaves. However, when the suspension cultures were suddenly exposed to −8 °C for

15 days, the enzyme activities did exhibit negative correlations with rapid freezing resistance measured as TTC reduction. Meanwhile, we found the correlation was positive under lethal freezing stress at −20 °C (data not shown). Several authors [14] also found a negative correlation between POD activity and salt tolerance in rice. The results revealed that H_2O_2 detoxifying enzyme activities coordinated with SOD activity play a key role in the AOS scavenging process, and the reduction of cell viability with extended freezing was correlated with an increase in the combined activity of antioxidant enzymes in intact cells. However, in freeze-damaged plant cells, the reduction of cell viability resulted from the denaturation or inactivation of antioxidant enzymes.

Thus, our results suggest that the suspension cultured cells of *C. bungeana*, a typical alpine subnival plant, could survive freezing temperatures and activate their antioxidant system with sequential and synergetic action to retard lipid peroxidation further and augment repair function molecules, resulting in low autoxidation under rapid freezing stress. The active improvement of these enzymes is related to freezing-induced oxidative stress tolerance in *C. bungeana* cultured cells. Further work is needed to investigate the precise molecular mechanisms responsible for induction of antioxidant enzyme activity in the alpine plant cells under rapid freezing conditions.

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References

[1] N.A. Abassi, M.M. Kushad, A.G. Endress, Active oxygen-scavenging enzymes activities in developing apple flowers and fruits, Sci. Horticult. 74 (1998) 183–184.
[2] R.G. Alscher, J.L. Donahue, C.L. Cramer, Reactive oxygen species and antioxidants: relationship in green cells, Physiol. Plant 100 (1997) 224–233.
[3] L.-Z. An, Y.-H. Liu, G.-N. Feng, H.-Y. Feng, G.-N. Feng, G.-D. Cheng, Studies on the characteristics of element contents of altifrigetic subnival vegetation at the source area of Urumchi river, Acta Bot. Boreal.-Occident. Sin. 20 (2000) 1063–1069.
[4] K. Asada, Ascorbate peroxidase-a hydrogen peroxide scavenging enzyme in plants, Physiol. Plant 85 (1992) 235–241.
[5] R. Ayi tu, D.-Y. Tan, Z.–J. Li, F. Yao, The relationship between the structure of vegetative organs in *Chorispora bungeana* and its environment, J. Xinjiang Agric. Univ. 21 (1998) 273–277.
[6] K.-H. Baek, D.Z. Skinner, Alteration of antioxidant enzyme gene expression during cold acclimation of near-isogenic wheat lines, Plant Sci. 165 (2003) 1221–1227.
[7] C. Bowler, M. Van Montagu, D. Inzé, Superoxide dismutase and stress tolerance, Annu. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 83–116.
[8] P. Broadbent, G.P. Creissen, B. Kular, A.R. Wellburn, P. Mullineaux, Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity, Plant J. 8 (1995) 247–255.
[9] I. Cakmak, H. Marschner, Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves, Plant Physiol. 98 (1992) 1222–1227.
[10] J. Chang, X. Fu, L. An, S. Xu, J. Wang, M. Zhang, H. Feng, T. Chen, Properties of cellular ubiquinone and stress-resistance in suspension-cultured cells of *Chorispora bungeana* during early chilling, Environ. Exp. Bot. (2005) in press.
[11] M. Dalal, K.-C. Renu, Lipid peroxidation is an early event in necrosis of wheat hybrid, Biochem. Biophys. Res. Commun. 262 (1999) 109–112.
[12] D.A. Dalton, F.J. Hanus, S.A. Russell, H.J. Evans, Purification, properties and distribution of ascorbate peroxidase in legume root nodules, Plant Physiol. 83 (1987) 789–794.
[13] J. Dat, S. Vandenabeele, E. Vranová, M. Van Montagu, D. Inzé, F. Van Breusegem, Dual action of the active oxygen species during plant stress responses, CMLS Cell. Mol. Life Sci. 57 (2000) 779–795.
[14] T. Demiral, İ. Türkan, Comparative lipid peroxidation, antioxidant defense systems and praline content in roots of two rice cultivars differing in salt tolerance, Environ. Exp. Bot. 53 (2005) 247–257.
[15] S.R. Dhindsa, W. Matowe, Drought tolerance in two mosses: correlated with enzymatic defence against lipid peroxidation, J. Exp. Bot. 32 (1981) 79–91.
[16] C.H. Foyer, B. Halliwell, The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism, Planta 133 (1976) 21–25.
[17] C.N. Giannopolitis, S.K. Ries, Superoxide dismutase, occurrence in higher plants, Plant Physiol. 59 (1977) 309–314.
[18] A. Hegedüs, S. Erdei, G. Horváth, Comparative studies of H_2O_2 detoxifying enzymes in green and greening barley seedlings under cadmium stress, Plant Sci. 160 (2001) 1085–1093.
[19] M.D. Hodges, J.M. Delong, C.F. Forney, R.K. Prange, Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds, Planta 207 (1999) 604–611.
[20] E. Ishikawa, S.-K. Bae, O. Miyawaki, K. Nakamura, Y. Shiinoki, K. Ito, Freezing injury of cultured rice cells analyzed by dielectric measurement, J. Ferment. Bioeng. 83 (1997) 222–226.
[21] M. Ishikawa, A.J. Robertson, L.V. Gusta, Comparison of viability tests for assessing cross-adaptation to freezing heat and salt stresses induced by abscisic acid in bromegrass (*Bromus inermis* Leyss) suspension cultured cells, Plant Sci. 107 (1995) 83–93.
[22] T. Janda, G. Szalai, K. Rios-Gonzalez, O. Veisz, E. Páldi, Comparative study of frost tolerance and antioxidant activity in cereals, Plant Sci. 164 (2003) 301–306.

[23] Y.-H. Jin, D.-L. Tao, Z.-Q. He, J. Ye, Y.-J. Du, H.L. Liu, Y.B. Zhou, Environmental stresses and redox status of ascorbate, Acta Bot. Sin. 45 (2003) 795–801.
[24] Y.-H. Kim, Y. Kim, E. Cho, S. Kwak, S. Kwon, J. Bae, B. Lee, B. Meen, G.-H. Huh, Alterations in intracellular and extracellular activities of antioxidant enzymes during suspension culture of sweet potato, Phytochemistry 65 (2004) 2471–2476.
[25] A. Kubo, M. Aono, N. Nakajima, H. Saji, K. Tanaka, N. Kondo, Differential responses in activity of antioxidant enzymes to different environmental stresses in *Arabidopsis thaliana*, J. Plant Res. 112 (1999) 279–290.
[26] B.-L. Li, H.-S. Mei, Relationship between oat leaf senescence and activated oxygen metabolism, Acta Phytophysiol. Sin. 15 (1989) 6–12.
[27] J. Li, X.-F. Yan, Y.-G. Zu, Generation of activated oxygen and change of cell defense enzyme activity in leaves of Korean pine seedling under low temperature, Acta Bot. Sin. 42 (2000) 148–152.
[28] B.D. Mckersie, Y.R. Chen, M.D. Beus, S.R. Bowler, D. Inzé, K.D. Halluin, J. Botterman, Superoxide dismutase enhances tolerance of freezing stress in transgenic alfalfa (*Medicago sativa* L.), Plant Physiol. 103 (1993) 1155–1163.
[29] L.S. Monk, K.V. Fagerstedt, R.M.M. Crawford, Oxygen toxicity and superoxide dismutase as an antioxidant in physiological stress, Physiol. Plant. 76 (1989) 456–459.
[30] G. Noctor, C.H. Foyer, Ascorbate and glutathione: keeping active oxygen under control, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 249–279.
[31] T. Okuda, Y. Matsuda, A. Yamanaka, S. Sagisaka, Abrupt increase in the level of hydrogen-peroxide in leaves of winter-wheat is caused by cold treatment, Plant Physiol. 97 (1991) 1265–1267.
[32] M.L. Salin, Toxic oxygen species and protective systems of the chloroplast, Physiol. Plant 72 (1987) 681–689.
[33] B.J. Sinclair, S.L. Chown, Rapid responses to high temperature and desiccation but not to low temperature in the freeze tolerant sub-Antarctic caterpillar *Pringleophaga marioni* (Lepidoptera, Tineidae), J. Insect Physiol. 49 (2003) 45–52.
[34] D. Taschler, G. Neuner, Summer frost resistance and freezing patterns measured in situ in leaves of major alpine plant growth forms in relation to their upper distribution boundary, Plant Cell Environ. 27 (2004) 737–746.
[35] F.R. Toman, H.L. Mitchell, Soluble proteins of winter wheat crown tissues and their relationship to cold hardiness, Phytochemistry 7 (1968) 365–373.
[36] V. Tsarouhas, W.A. Kenney, L.Z. Zsuffa, Application of two electrical methods for the rapid assessment of freezing resistance in *Salix eriocephala*, Biomass Bioenergy 19 (2000) 165–175.
[37] Z. Zhang, Experimental guidance to plant physiology, second ed., High Educational Press, Beijing, 2000 (in Chinese).
[38] J.-X. Zhang, M.B. Kirkham, Enzymatic responses of the ascorbate-glutathione cycle to drought in sorghum and sunflower plants, Plant Sci. 113 (1996) 139–147.