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The influence of cold acclimation on antioxidative enzymes and antioxidants in sensitive and tolerant barley cultivars

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Abstract

In order to better understand the role of cold acclimation in alleviating freezing injury, two barley cultivars with different cold tolerance, *i.e.* a sensitive cv. Chumai 1 and a tolerant cv. Mo 103, were used. The freezing treatment increased leaf soluble protein content more in the tolerant cultivar than in the sensitive one. Cold acclimation increased H_2O_2 content of the two cultivars during freezing treatment, especially in Mo 103. Glutathione and ascorbate contents during freezing and recovery were significantly higher in cold-acclimated plants than in non-acclimated ones. Activities of peroxidase, ascorbate peroxidase and glutathione reductase were also higher in cold-acclimated plants than non-acclimated plants during freezing treatment. However, there was no significant difference between cold-acclimated plants and the control plants in catalase activity. It may be assumed that cold acclimation induced H_2O_2 production, which in turn enhanced activities of antioxidative enzymes and synthesis of antioxidants, resulting in alleviation of oxidative stress caused by freezing.

Additional key words: ascorbate peroxidase, catalase, glutathione reductase, *Hordeum vulgare*, hydrogen peroxide, low temperature, peroxidase, reactive oxygen species, recovery.

Introduction

Abiotic stresses, including low temperature, salinity and drought may induce production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$) (Okuda *et al.* 1991, Foyer *et al.* 1994a, Alscher *et al.* 1997). These ROS are capable of causing oxidative damage to proteins, DNA, and membrane lipids, even the cell death (Kendall and McKersie 1989, Apel and Hirt 2004). On the other hand, plants have evolved several enzymatic and non-enzymatic antioxidant systems against ROS. Thus, excess H_2O_2 can be removed by catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (POD, EC 1.11.1.7) and the ascorbate-glutathione cycle (AsA-GSH cycle) (Horvath *et al.* 2007, O'Kane *et al.* 1996, Prasad *et al.* 1994, Cakmak and Marschner 1992). The AsA-GSH cycle also provides photoprotection (Mittler 2002, Pang *et al.* 2005, Noctor and Foyer 1998) and ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.5.4) are the key enzymes in the AsA-GSH cycle in chloroplasts (Noctor and Foyer 1998). It was reported that ROS not only caused oxidative damage to cells, but also played an important role as signals in the plants exposed to abiotic stress (Gupta and Luan 2003, Mittler 2002, Mittler *et al.* 2004).

One of the most important agronomic traits in barley (*Hordeum vulgare* L.) is winter-hardiness or freezing tolerance, which can be enhanced by cold acclimation (Kosová *et al.* 2008, Giorni *et al.* 1999, Suzuki and Mittler 2006). The expression of cold-responsive genes (Kosová *et al.* 2007, 2008, Thomashow 1999) and antioxidant enzymes (Baek and Skinner 2003), which contribute to freezing tolerance of plants, could be induced by cold acclimation, thus improving tolerance to ROS (Scebba *et al.* 1998, 1999, Kuk *et al.* 2003). Shortly, cold acclimation may protect the integrity of cellular membranes, enhance the antioxidative mechanisms, and finally improve freezing stress tolerance (Mahajan and Tuteja

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbate; CAT - catalase; DTNB - 5,5'-dithio-bis-(2-nitrobenzoic acid); DTT - dithiothreitol; EDTA - ethylenediaminetetraacetic acid; GR - glutathione reductase; GSH - glutathione; HEPES - (N-2-hydroxyethyl) piperazin-N'-(2-ethanesulfonic acid); POD - peroxidase; PVP - polyvinyl polypyrrolidone; ROS - reactive oxygen species; SP - soluble protein; TP - total protein content.
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2005). However, little information is available on the effect of cold acclimation on content of H_2O_2 and ROS-scavenging systems in barley leaves during recovery after freezing.

The present experiments were aiming at determination of changes in contents of H_2O_2 and antioxidants and activity of antioxidant enzymes during cold acclimation, freezing and recovery, and elucidation of the role of cold acclimation in alleviation of freezing stress and improvement of recovery.

Materials and methods

Two winter barley (*Hordeum vulgare* L.) cultivars Chumai 1 and Mo 103 were used. The previous results showed that Mo 103 had higher tolerance to low temperature than Chumai 1 (Dai *et al.* 2008). Seeds of both cultivars were sown in plastic pots (170 × 220 mm) filled with peat. Plants were grown in a greenhouse at Zhejiang University, China. When the 3rd leaf was fully expanded, plants were transferred to growth chambers (PRX-450D, *Safe*, Ningbo, China). The normal growth conditions were as follows: a 12-h photoperiod, photosynthetic photon flux density of 225 ± 25 μmol m^{-2} s^{-1} at plant height, relative humidity of 75 %, and day/night temperature of 20/15°C. The freezing treatment was conducted according to Bosco *et al.* (2003) with some modifications. The plants were incubated at -2 °C for 4 h, and then the temperature was reduced to -8 °C at a rate of 2 °C per hour. After 18 h dark treatment, temperature was gradually returned to -2 °C at a rate of increasing 2 °C per hour and then incubated for 15 h. Thereafter, the plants were returned to normal conditions, with the temperature being gradually increased at a rate of 1 °C per hour. Cold acclimation was performed in a growth chamber with day/night temperature of 3/1 °C for 5 d. Because freezing stress was conducted in the dark condition, the plants were placed into growth chambers with normal conditions for light adaptation after freezing treatment.

Leaf samples for protein assay were collected at the end of freezing treatment from the both plants of the control (without freezing treatment) and freezing treatment. Barley leaves (0.5 g) were frozen in liquid nitrogen and ground into fine powder, homogenized in 6 cm^3 soluble protein extraction buffer (50 mM Tris-HCl, pH 7.8, containing 0.5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT). Supernatant was collected after centrifugation at 12 000 *g* and 4 °C for 20 min. The residue was extracted for non-soluble protein with 3 cm^3 1 M NaOH, and incubated at a 90 °C water-bath for 20 min. The supernatants were collected after centrifugation at 4 000 *g* for 10 min. Protein contents were measured according to Bradford (1976) with bovine serum albumin (BSA) as a standard.

Leaf samples for antioxidative enzymes, antioxidants and H_2O_2 assays were collected from control plants (without freezing treatment) and at 2, 24, and 72 h during recovery after freezing treatment of the both non-acclimated and cold-acclimated plants. For antioxidative enzyme activity assay, 0.5 g barley leaves were frozen in liquid nitrogen and ground into fine powder, then homogenized in 8 cm^3 25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA and 1 % (m/v) PVP. The supernatant for enzyme analysis was collected after centrifugation at 12 000 *g* and 4 °C for 20 min. All operations were performed on an ice-bath. Activities of catalase (CAT) and peroxidase (POD) were assayed according to Cakmak and Marschner (1992). The activity of ascorbate peroxidase (APX) was measured according to Nakano and Asada (1981). The activity of glutathione reductase (GR) was assayed according to Rao *et al.* (1992).

For antioxidant and H_2O_2 assay, 0.5 g barley leaves were frozen in liquid nitrogen and ground into fine powder, then homogenized in 6 cm^3 5 % trichloroacetic acid (TCA) solution. The extraction was centrifuged at 12 000 *g* and 4 °C for 15 min, the supernatant was collected for following analysis. Ascorbate (AsA) and dehydroascorbate (DAsA) were measured according to Law *et al.* (1983). DAsA content was calculated from total ascorbate minus AsA. Total glutathione (GSH) was determined according to Griffith (1980). H_2O_2 content was determined according to Patterson *et al.* (1984) with some modifications: 2 cm^3 supernatant was added to a test tube with 0.7 g activated charcoal, and stirred, centrifuged at 15 000 *g* and 2 °C for 5 min. After adjustment of pH to 8.4 with 17 M ammonium solution, the supernatant was immediately filtered under pressure from a syringe to remove any precipitated material. The filtrate (1.2 cm^3) was mixed with 0.6 cm^3 of colorimetric reagent [(0.9 mM 4-(2-pyridylazo) resorcinol and 0.9 mM potassium titanium oxalate]. The mixture was incubated at 45 °C for 60 min, and then cooled on an ice bath and determined at wavelength of 508 nm by spectrophotometer (UV-2450，Shimadzu Corporation, Kyoto, Japan).

Each biochemical measurement was carried out in 3 replications. The data were analyzed using general linear models (*PROC GLM*) of *SAS V8.0* (SAS Institute, Cary, NC, USA) to compare the difference between the treatments.

Results

SP content in freezing-treated Chumai 1 and Mo 103 plants was 16.4 and 56.0 % higher than that of the control, respectively (Fig. 1*A*). On the other hand, the effect of freezing treatment on total protein (TP) content was

significant only for Mo 103 (Fig. 1*B*). As a result, the SP/TP ratio was higher in the freezing plants than in the control (Fig. 1*C*).

Effect of freezing treatment on H_2O_2 content differed between cold-acclimated and non-acclimated plants, and also between cultivars (Fig. 2*A*). Cold acclimation significantly increased H_2O_2 content of both cultivars, and Mo 103 had higher H_2O_2 content than Chumai 1. During recovery after freezing treatment, the differences in H_2O_2 content induced by cold-acclimation or differences between cultivars gradually decreased. In contrast, H_2O_2 content gradually increased in the non-acclimated plants.

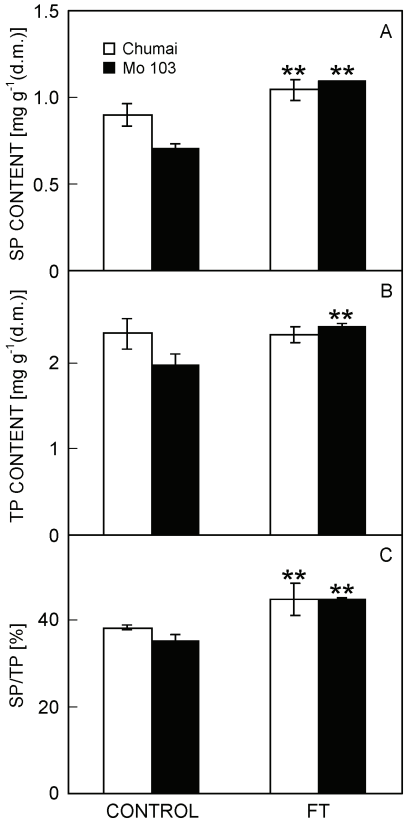

Fig. 1. Effects of freezing treatment on soluble protein content (SP), total protein content (TP) and SP/TP ratio in barley leaves after freezing treatment. Control, no freezing treatment; FT, in the end of freezing treatment (Means ± SD, n = 3, ** - significant at $P \leq 0.01$ as compared to the control).

In comparison with the non-acclimated plants, cold-acclimation increased GSH content by 85.7 and 111.2 % for Chumai 1 and Mo 103, respectively (Fig. 2*B*). The effect of cold-acclimation on GSH content after freezing treatment varied with cultivar and recovery time. After 2- and 24-h recovery, significantly increased GSH content in cold-acclimated plants relative to the control was observed. However, after 72-h recovery, the non-acclimated plants showed a significant increase, while cold-acclimated Mo 103 showed a significant reduction in GSH content.

Cold-acclimation increased AsA content (Fig. 2*C*) more in Mo 103 than in Chumai 1. The similar effect of cold-acclimation on AsA content was observed also after 2- and 24-h recovery after freezing in both cultivars, but after 72 h recovery only in Chumai 1. After 72-h recovery, the non-acclimated Mo 103 plants showed higher increase in AsA content than Chumai 1.

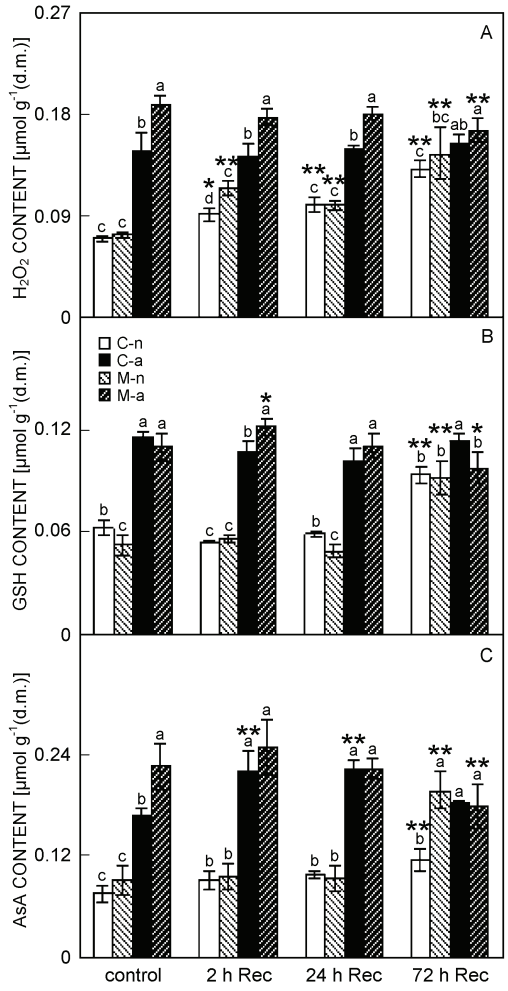

Fig. 2. Effects of freezing treatment on the content of H_2O_2 and antioxidants (GSH and AsA) in cold- and non-acclimated barley leaves and during recovery after freezing treatment. C-n: non-acclimated Chumai 1, M-n: non-acclimated Mo 103, C-a: cold-acclimated Chumai 1, M-a: cold-acclimated Mo 103, control: no freezing treatment, Rec: recovery. Means ± SD, n = 3, *,** - significant at $P \leq 0.05$ and 0.01, respectively, as compared to the control, the same letter on the top of column presents no significant difference between the treatments.

The effect of cold acclimation on POD activity was significant only in Chumai 1 (Fig. 3*A*). During recovery after freezing treatment, there was no significant difference between cold-acclimated and non-acclimated plants.

No significant difference was detected between cold acclimated and non-acclimated plants in CAT activity for both cultivars (Fig. 3*B*). However, the difference could be found during recovery after freezing treatment. For

instance, cold acclimated plants had significantly higher CAT activities than non-acclimated one after 24-h recovery for both cultivars. In comparison with the control, CAT activity significantly increased after 72-h recovery in the both cultivars.

APX activity was increased by 52.0 and 42.8 % in cold-acclimated leaves relative to that in non-acclimated leaves for Chumai 1 and Mo 103, respectively (Fig. 3*C*). The significant difference remained in the freezing-treated plants after 2 and 24 h recovery and disappeared after 72 h recovery. After 72 h recovery, APX activity decreased in cold-acclimated plants and increased in non-acclimated plants.

GR activity was increased by 89.4 and 87.7 % in cold-acclimated leaves relative to that in non-acclimated leaves for Chumai 1 and Mo 103, respectively (Fig. 3*D*). Moreover, the significant difference between cold-acclimation and non-acclimation in GR activity remained during recovery after freezing treatment. In comparison with the control, freezing treatment did not alter the enzyme activity after 2- and 24-h recovery. However, after 72 h recovery, the non-acclimated plants of Chumai 1 and Mo 103 increased GR by 34.3 and 55.6 % relative to the control, respectively.

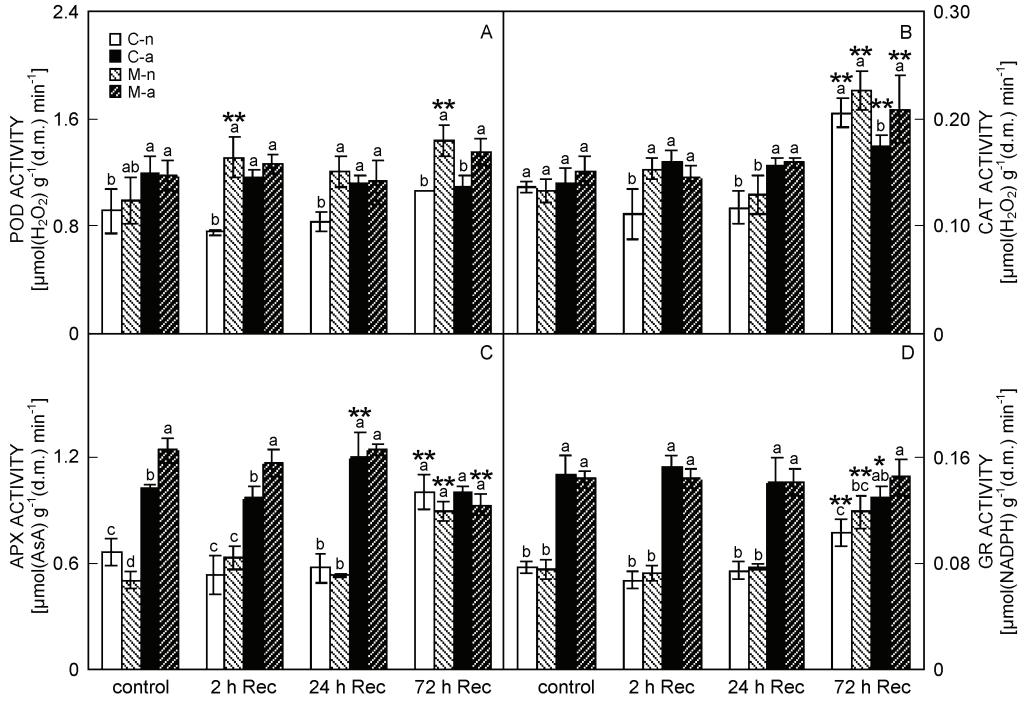

Fig. 3. Effects of freezing treatment on the activities of antioxidative enzymes (POD, CAT, APX and GR) in cold- and non-acclimated barley leaves and during recovery after freezing treatment. C-n: non-acclimated Chumai 1, M-n: non-acclimated Mo 103, C-a: cold-acclimated Chumai 1, M-a: cold-acclimated Mo 103, control: no freezing treatment, Rec: recovery. Means ± SD, $n = 3$, *,** - significant at $P \leq 0.05$ and 0.01, respectively, as compared to the control, the same letter on the top of column presents no significant difference between the treatments.

Discussion

Freezing stress increased soluble protein (SP) content in barley leaves, and moreover a cold tolerant cv. Mo 103 showed higher increase (Fig. 1*A*). It may be assumed that increase in SP content under freezing condition was a ubiquitous response of the plants to the stress, favourable for improving freeze tolerance.

Cold acclimation and recovery after freezing stress were very complicated processes and involve a lot of physiological and metabolic changes (Kosová *et al*. 2008). Freezing stress was shown to induce H_2O_2 accumulation in plant cells (O'Kane *et al.* 1996, Prasad *et al.* 1994, Okuda *et al.* 1991) and enhance activities of ROS-scavenging enzymes. In this study, H_2O_2 content in freezing-stressed barley leaves was significantly increased due to cold acclimation, but did not show further increase during freezing treatment and recovery (Fig. 2*A*). However, for the non-acclimated plants, H_2O_2 content showed a significant increase during freezing and recovery. Similarly, Okuda *et al.* (1991) reported that H_2O_2 content increased rapidly in wheat leaves during freezing. Mo 103 had significantly higher POD activity than Chumai 1 after 72-h recovery in cold-acclimated plants (Fig. 3*A*). The results indicated that the tolerant barley cultivar is characterized by higher capacity of scavenging H_2O_2 than

the sensitive one.

Cold acclimation increased the activities of APX and GR (Fig. 3*C*,*D*). Similarly, elevated activities of APX and GR were found to be associated with increased freezing tolerance during cold acclimation in pine trees (Tao *et al*. 1998). It has been reported that APX and GR played the most important role in the fine regulation of H_2O_2 content in cells (Horvath *et al.* 2007, Wllekens *et al.* 1995, Noctor and Foyer 1998). Hence, high APX and GR activities induced by cold acclimation can prevent plant cells from injury brought by high content of H_2O_2. Meanwhile, cold acclimation also increased AsA and GSH contents (Fig. 2*B*,*C*). The increase in GSH synthesis and/or GR activity is necessary for preventing H_2O_2 accumulation to toxic level during cold acclimation (Kocsya *et al.* 2001). Moreover, Kuk *et al.* (2003) deduced that CAT and APX were the most important for chilling tolerance, and these two enzymes as well as other enzymes were operated in concert to scavenge H_2O_2 (Foyer *et al.* 1994b). For example, the activity of CAT was not increased until 72 h after recovery in the present study, but APX was dramatically increased after cold acclimation. On the other hand, after 72-h recovery, the activity of CAT dramatically increased for both cultivars, irrespective of cold-acclimation, but the APX activity of Mo 103 decreased for cold-acclimated plant. It was found in the current study that tolerant culti- var Mo 103 showed higher increase in APX activity than the sensitive one, Chumai 1 after cold acclimation (Fig. 3*C*), indicating that APX is very important in detoxification of H_2O_2 for barley plants under freezing stress.

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