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Response to temperature stress of reactive oxygen species scavenging enzymes in the cross-tolerance of barley seed germination*

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Abstract: A number of studies have shown the existence of cross-tolerance in plants, but the physiological mechanism is poorly understood. In this study, we used the germination of barley seeds as a system to investigate the cross-tolerance of low-temperature pretreatment to high-temperature stress and the possible involvement of reactive oxygen species (ROS) scavenging enzymes in the cross-tolerance. After pretreatment at 0 °C for different periods of time, barley seeds were germinated at 35 °C, and the content of malondialdehyde (MDA) and the activities of ROS scavenging enzymes were measured by a spectrophotometer analysis. The results showed that barley seed germinated very poorly at 35 °C, and this inhibitive effect could be overcome by pretreatment at 0 °C. The MDA content varied, depending on the temperature at which seeds germinated, while barley seeds pretreated at 0 °C did not change the MDA content. Compared with seeds germinated directly at 35 °C, the seeds pretreated first at 0 °C and then germinated at 35 °C had markedly increased activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR). The SOD and APX activities of seeds germinated at 35 °C after 0 °C-pretreatment were even substantially higher than those at 25 °C, and GR activity was similar to that at 25 °C, at which the highest germination performance of barley seeds was achieved. These results indicate that low-temperature pretreatment can markedly increase the tolerance of barley seed to high temperature during germination, this being related to the increase in ROS scavenging enzyme activity. This may provide a new method for increasing seed germination under stress environments, and may be an excellent model system for the study of cross-tolerance.

Key words: Barley seed, Cross-tolerance, Germination/growth, Reactive oxygen species (ROS) scavenging enzyme, Pretreatment at low temperature, Temperature stress

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1 Introduction

Germination of seeds is a vital stage in the life cycle of plants. Of the many ecological factors that influence seed germination, temperature is one of the most important factors, in that it determines the capacity and rate of germination and breaks primary and/or secondary dormancy (Baskin and Baskin, 1998; Brándel, 2004). High- or low-temperature stress is also a very common kind of stress for plant growth and development (Iba, 2002). In many plants, gradual changes in the environmental conditions induce tolerance to the extreme situations. Often, exposure to one particular type of stress at moderate levels can enhance the resistance to multiple other stresses. This acclimation response is defined as cross-tolerance (Sabehat *et al*., 1998).

It has been reported that salt stress induces/increases cold hardiness in potato and spinach seedlings (Ryu *et al*., 1995); heat stress decreases heavy-metal toxicity (Orzech and Burke, 1988), increases the tolerance to salt stress (Kuznetsov *et al*., 1993), induces water-stress tolerance (Bonham-Smith *et al*., 1987), and reduces chilling injury in chilling-sensitive species, such as tomato fruits, mung bean hypocotyls,

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and cucumber cotyledons (Jennings and Saltveit, 1994; Collins *et al*., 1995). During germination of wheat seeds, the tolerance to salt stress can be increased by pretreatment at 33 ℃ for different periods of time, and the tolerance to heat stress can be increased by pretreatment in −0.8 MPa NaCl for different periods of time (Song *et al*., 2005). Mei and Song (2008) reported that for the germination of barley seeds, pretreatment at 30 °C for 8 h or in a solution of NaCl at 100 mmol/L for 24 h could both improve tolerance to subsequent heat stress (35 °C), and the improvement effect of pretreatment in a solution of NaCl at 100 mmol/L for 24 h (61% germination) was better than that at 30 °C for 8 h (26% germination).

However, the physiological mechanisms of cross-tolerance in plants are poorly understood. One mechanism that may be involved in the resistance to many types of stresses is the activity of the reactive oxygen species (ROS) scavenging enzymes. These enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), as well as those of the ascorbate-glutathione cycle (Apel and Hirt, 2004). The increasing production of ROS scavenging enzymes under different kinds of stresses may suggest that they have a general role in the acquisition of stress tolerance by plants (Sabehat *et al*., 1998). Temperature pretreatment is one of the most convenient methods in the study of cross-tolerance and in the production of crop plants. However, little is known about whether low-temperature pretreatment can increase the tolerance to subsequent high-temperature stress and whether this cross-tolerance is associated with changes in ROS scavenging enzyme activity. Here we used a system of barley seed germination to investigate the cross-tolerance of low-temperature pretreatment to high-temperature stress and the possible involvement of ROS scavenging enzymes in the cross-tolerance.

2 Materials and methods

2.1 Plant materials

Barley (*Hordeum vulgare* L. cv. Pijiu) seeds were collected at maturity in Jianchuan County, Yunnan Province, China in May 2007. After collection, these seeds were dried to a water content of (0.135±0.002) g H_2O/g dry weight (DW) at 25 °C and 50% relative humidity, and were then kept at 10 °C until use.

2.2 Seed germination at different temperatures

Four replicates of 50 barley seeds each were germinated in perlite moistened with distilled water (1:3.3, w/w) in Petri dishes (9-cm diameter), at temperatures ranging from 5 to 40 °C and in darkness for 8 d. After imbibition, germination percentage of seeds was scored at 12-h intervals during 24 h, and at 24-h intervals during 24–192 h. The seeds showing radicle extension of 2 mm were scored as having germinated. Germination rate was expressed as a time course of germination and/or the time (h) taken to reach 50% of germinated seeds (T_{50}).

2.3 Test of cross-tolerance of low-temperature pretreatment to high-temperature stress during seed germination

After pretreatment at 0 °C for 1–4 d in the dark in perlite moistened with distilled water, barley seeds were transferred to 35 °C to germinate for another 5 d. After that, germination percentages of seeds and DWs of the radicles and shoots produced by germinating seeds were determined.

2.4 Effects of different temperatures on malondialdehyde (MDA) contents and ROS scavenging enzyme activities

Barley seeds were imbibed on two layers of filter paper moistened with 10 ml of distilled water at different temperatures and in the dark for 2 d, then ground to fine powder in liquid nitrogen, and kept at −80 °C until use.

MDA content was measured by the method of Hendry *et al*. (1993), where MDA was quantified from the second derivative spectrum against standards prepared from 1,1,3,3-tetraethoxypropane. The MDA content was expressed as μmol/g DW.

For extraction of SOD (EC 1.15.1.1), the fine powder mentioned above was ground in an extraction mixture composed of 50 mmol/L phosphate buffer solution (PBS, pH 7.0), 1.0 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% (w/v) polyvinylpolypyrrolidone (PVPP), and 1 mmol/L ascorbic acid (AsA). The homogenate was centrifuged at 16 000×*g* for 15 min (twice), and then the resultant supernatant was transferred to a new tube and kept at −80 °C for

subsequent assay of SOD activity. SOD was determined by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich, 1971). An illuminated blank without protein produced the maximum reduction of NBT, giving the maximum absorbance at 560 nm. SOD activity is described as $(1–A_s/A_b)\times100\%$, where A_s is the absorbance of sample, and A_b is the absorbance of blank. One unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50%. The specific activity of SOD was expressed as U/mg protein.

For extraction of APX, CAT, glutathione reductase (GR), and dehydroascorbate reductase (DHAR), the fine powder mentioned above was ground in 5 ml of 50 mmol/L Tris-HCl (pH 7.0) containing 20% (v/v) glycerol, 1 mmol/L AsA, 1 mmol/L dithiothreitol (DTT), 1 mmol/L EDTA, 1 mmol/L reduced glutathione (GSH), 5 mmol/L $MgCl_2$, and 1% (w/v) PVPP. The brei was centrifuged at 12000×*g* for 6 min and then at 26900×*g* for 16 min. The resultant supernatant was stored at −80 °C for subsequent assay of enzyme activity.

APX (EC 1.11.1.7) was measured according to the method of Nakano and Asada (1981), who assayed the decrease in absorbance at 290 nm [2.8 L/(mmol·cm)] due to AsA oxidation. Enzyme activity was expressed as μmol AsA/(mg protein·min).

CAT (EC 1.11.1.6) was assayed as described by Aebi (1983), who directly determined the decomposition of H_2O_2 at 240 nm [0.04 L/(mmol·cm)]. Enzyme activity was expressed as μmol H_2O_2/(mg protein·min).

GR (EC 1.6.4.2) was tested according to Halliwell and Foyer (1978), as the decrease in absorbance at 340 nm [6.2 L/(mmol·cm)] due to the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH). Enzyme activity was expressed as μmol NADPH/(mg protein·min).

DHAR (EC 1.8.5.1) was determined by the method of Hossain and Asada (1984), who measured the formation of AsA at 265 nm [14 L/(mmol·cm)]. Enzyme activity was expressed as μmol AsA/(mg protein·min).

2.5 Determination of MDA contents and ROS scavenging enzyme activities in cross-tolerance of low-temperature pretreatment to high-temperature stress

After pretreatment at 0 °C in the dark on two layers of filter paper moistened with 10 ml of distilled water for 2 d, barley seeds were then transferred to 35 °C to germinate continuously for another 2 d. The seeds (and seedlings) were ground to fine powder in liquid nitrogen, and then kept at −80 °C until use. MDA contents and ROS scavenging enzyme activities were assayed as described in Section 2.4.

2.6 Assay of protein content

Protein content in the extract was assayed by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

2.7 Statistical analysis

All data were analyzed using a one-way analysis of variance (ANOVA) model from the SPSS 11.0 package for Windows (SPSS Inc., Chicago, Illinois).

3 Results

3.1 Response of seed germination on different temperatures

The germination percentage and germination rate of barley seeds were obviously influenced by temperature ($P\leq0.001$, Fig. 1). The optimal temperature was 5–20 °C for germination percentage (Fig. 1), 20–25 °C for germination rate (Fig. 1b), and 25 °C for seedling growth following germination as measured by DWs of radicles and shoots (Fig. 1a). The time taken to reach 50% of germinated seeds (T_{50}) at 5, 10, 15, 20, and 25 °C was about 70, 24, 15, 8, and 8 h, respectively. It was noted that the germination percentage of the seeds was 44% at 30 °C, 4.7% at 35 °C, and 0 at 40 °C (Fig. 1), respectively.

Interestingly, barley seeds were able to reach a germination of 87% when germinated at 0 °C for 192 h and had a T_{50} of about 132 h (Fig. 1).

3.2 Cross-tolerance of low-temperature pretreatment to high-temperature stress during seed germination

To test the cross-tolerance of low-temperature pretreatment to subsequent high-temperature stress, barley seeds were first pretreated at 0 °C for 1–4 d, and were then transferred to 35 °C to germinate continuously. Barley seeds pretreated at 0 °C for 1–4 d

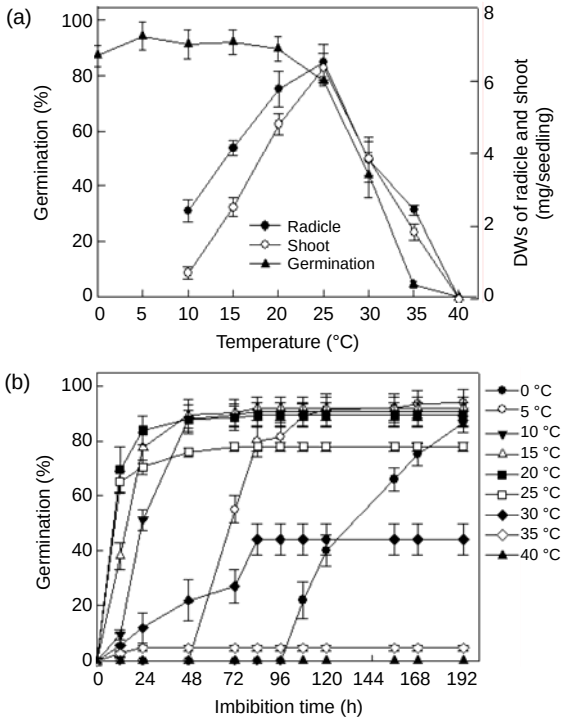

Fig. 1 Effects of temperature on germination percentage of barley seeds, DWs of radicles and shoots produced by germinating seeds (a), and time courses of germination (b)

The seeds showing radicle extension of 2 mm were scored as having germinated. When seedling age was 8 d, the DWs of the radicles and shoots were measured. Values are expressed as mean±SD (n=4) of 50 seeds each

had dramatically increased subsequent germination percentage (P≤0.001) and growth of radicle (P≤0.001) and shoot (P≤0.001) at 35 °C (Fig. 2). For example, compared with seeds directly germinated at 35 °C, the germination percentage, radicle and shoot DWs of seeds pretreated at 0 °C for 2 d increased by 77%, 57%, and 112%, respectively (Fig. 2). However, the optimum pretreatment period of time at 0 °C was 3 d for seed germination, and 4 d for growth of radicle and shoot following germination, respectively (Fig. 2).

3.3 Effect of different temperatures on MDA contents and ROS scavenging enzyme activities

The MDA content of barley seeds (and seedlings) decreased with increasing temperature within the range of 0–10 °C, and increased with rising temperature from 10 to 40 °C, being the lowest at 10 °C (P≤0.001, Fig. 3a).

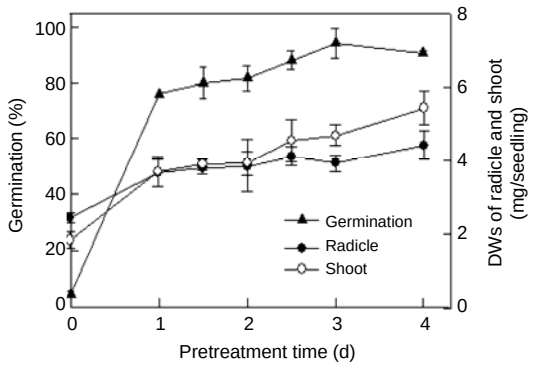

Fig. 2 Effects of pretreatment at 0 °C on barley seed germination and seedling growth at 35 °C

After pretreatment at 0 °C for an indicated period of time, seeds were germinated at 35 °C for another 5 d. The seeds showing radicle extension of 2 mm were scored as having germinated. Values are expressed as mean±SD (n=4) of 50 seeds each

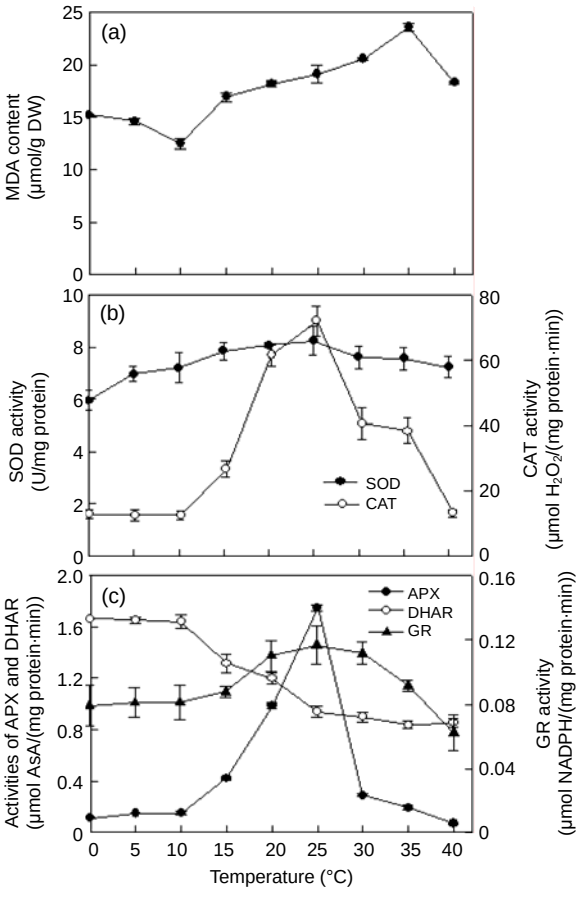

Fig. 3 Effects of temperature on MDA contents (a) and SOD (b), CAT (b), APX (c), GR (c), and DHAR (c) activities of barley seeds

Seeds were imbibed at an indicated temperature for 2 d, and then MDA contents and SOD, CAT, APX, GR, and DHAR activities were immediately assayed. Values are expressed as mean±SD (n=4) of 100 seeds each

The activities of SOD, CAT, APX, and GR of barley seeds (and seedlings) notably increased from 0 to 25 °C, and decreased at the temperature higher than 25 °C (P≤0.001, Figs. 3b and 3c). However, the DHAR activity gradually decreased with increasing germination temperature (P≤0.001, Fig. 3c).

3.4 Changes of MDA contents and ROS scavenging enzyme activities in cross-tolerance of low-temperature pretreatment to high-temperature stress

The MDA content of the seeds pretreated at 0 °C for 2 d and then transferred to 35 °C for another 2 d was a little lower than that of the seeds germinated directly at 35 °C (P=0.124), but was much higher than that of the seeds germinated directly at 0 or 25 °C for 2 d (P≤0.001) (Fig. 4).

Compared with seeds germinated directly at high-temperature stress (35 °C), the seeds pretreated at 0 °C for 2 d and then germinated at 35 °C for another 2 d notably increased the activities of SOD (P≤0.001), APX (P≤0.001), CAT (P=0.013), and GR (P≤0.001), and decreased DHAR activity (P≤0.001); while, compared with seeds germinated directly at the optimal temperature (25 °C), the seeds pretreated at 0 °C obviously increased SOD (P=0.001) and APX (P≤0.001) activities, decreased CAT (P=0.001) and DHAR (P≤0.001) activities, and had a similar activity of GR (P=0.893) (Fig. 5). It is noted that activities of SOD and APX of seeds pretreated at 0 °C plus germinated at 35 °C were much higher than those of seeds germinated directly at 0, 25, and 35 °C (Fig. 5).

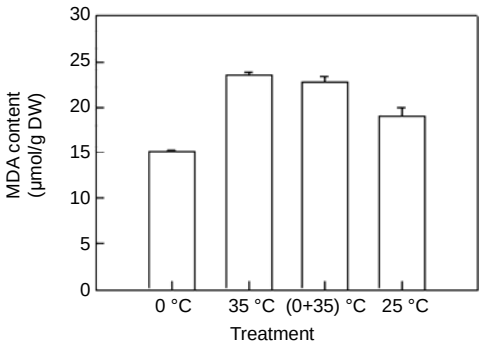

Fig. 4 Changes in MDA contents associated with cross-tolerance during germination of barley seeds
0, 35, and 25 °C: seeds were germinated directly at these temperatures for 2 d; (0+35) °C: after pretreatment at 0 °C for 2 d, seeds were then transferred to 35 °C to germinate for another 2 d. Values are expressed as mean±SD (n=4) of 100 seeds each

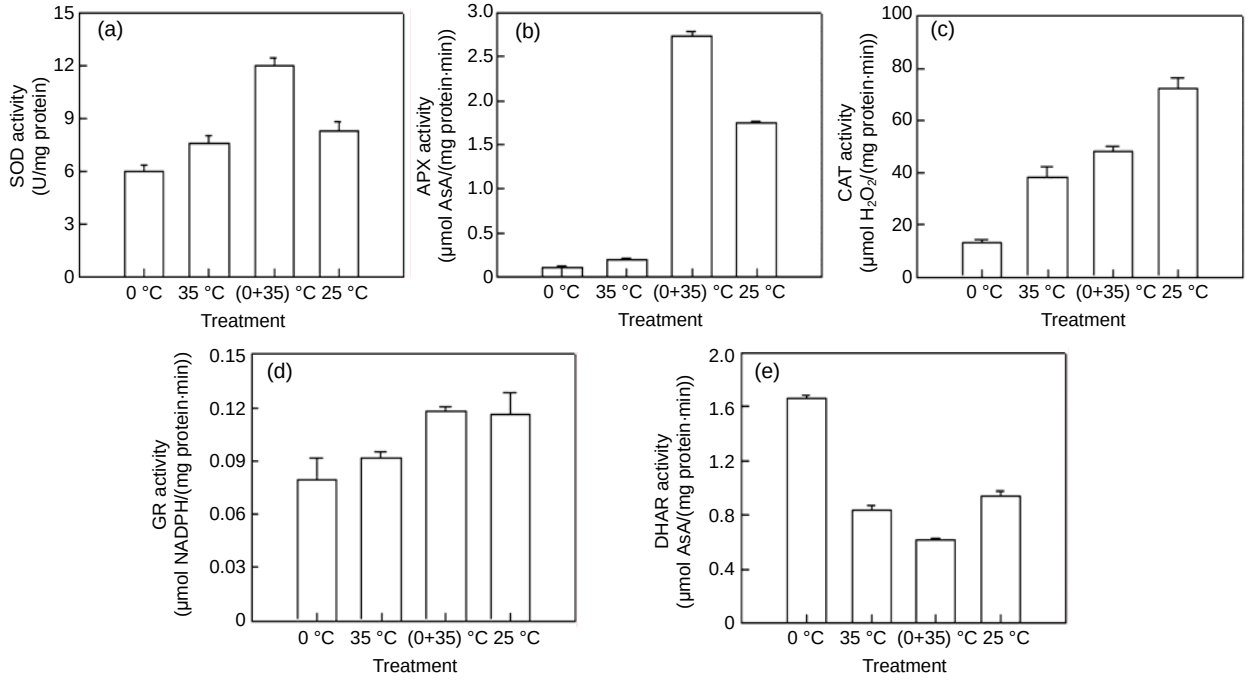

Fig. 5 Changes in activities of SOD (a), APX (b), CAT (c), GR (d), and DHAR (e) associated with cross-tolerance during germination of barley seeds
0, 35, and 25 °C: seeds were germinated directly at these temperatures for 2 d; (0+35) °C: after pretreatment at 0 °C for 2 d, seeds were then transferred to 35 °C to germinate for another 2 d. Values are expressed as mean±SD (n=4) of 100 seeds each

4 Discussion

Cross-tolerance has important ecological and agricultural implications, but little is known about its physiological mechanism. High-temperature stress is a common limiting factor for seed germination, especially for agricultural important crops including barley. Barley seeds often must be able to germinate under high temperatures, for instance, for the brewery industry. However, temperatures above 30 °C often inhibit the germinating ability of barley seeds. We designed these experiments to test whether low-temperature pretreatment could enhance the germination of barley seeds at high temperature and characterized some of the physiological processes associated with the effects of the low-temperature pretreatment.

The optimal temperature was 5–20 °C for germination percentage of barley seeds, 20–25 °C for germination rate, and 25 °C for seedling growth (Fig. 1). These results indicated that there was a clear temperature difference among germination percentage, germination rate, and seedling growth, and that the optimal temperature of seedling growth was higher than that of germination for barley seeds. Temperatures over 30 °C strongly inhibited seed germination (Fig. 1). Our experiments indicated that the germination ability of barley seeds at high temperature stress could be substantially enhanced by pretreatment at 0 °C for upto 4 d (Fig. 2). These results suggest that there was an obvious cross-tolerance for the germination of barley seeds, and the potential use of this system as a model to dissect the physiological and molecular bases of cross-tolerance during seed germination.

MDA is a final product of lipid peroxidation, and its amount can be used as a measure of lipid peroxidation (McDonald, 1999). In addition, MDA is poisonous to plant cells (Hendry *et al*., 1993). In plants, the MDA content increases in response to low (Doulis *et al*., 1997) and high (Iba, 2002) temperatures. Our data show that the MDA content of barley seeds was the lowest at 10 °C, and increased when temperatures were above and below 10 °C (Fig. 3). This shows that MDA content is a good indicator of temperature stress. However, we have not observed a significant difference in the MDA content between seeds germinated at 35 °C after pretreatment at 0 °C and seeds germinated directly at 35 °C (Fig. 4), suggesting that during germination of barley seeds, lipid peroxidation was not related to the high-temperature tolerance induced by low-temperature pretreatment, but depended on the temperature at which seeds were germinated, i.e., the level of lipid peroxidation did not affect the enhanced cross-tolerance during barley seed germination.

The superoxide radical and H_2O_2 are synthesized at very high rates in the cells even under optimal conditions (Apel and Hirt, 2004). Noctor and Foyer (1998) suggested that the major toxicity of superoxide radical and H_2O_2 is due to their ability to initiate a cascade of reactions that result in production of hydroxyl radicals and of other destructive species such as lipid peroxides. In plants, ROS scavenging enzymes include SOD, APX, and CAT. SOD can dismutate superoxide radical to produce H_2O_2, and act as the first line of defense against ROS (Møller, 2001), but the toxicity of H_2O_2 is equal to that of superoxide radical (Almeselmani *et al*., 2006). APX and CAT can detoxify H_2O_2, but APX requires an ascorbate and glutathione regeneration system. GR can regenerate reduced glutathione from oxidized glutathione using NAD(P)H as a reducing agent (McDonald, 1999; Apel and Hirt, 2004). The activities of SOD, APX, CAT, and GR of barley seeds germinated at 25 °C were the highest (Fig. 3), which was in accordance with the optimal temperature for germination rate and seedling growth produced by germinating seeds (Fig. 1), and decreased at temperatures lower or higher than 25 °C (Fig. 3). In addition, activities of SOD, APX, CAT, GR, and DHAR decreased with increasing germination time at 35 °C, which were consistent with the decrease in germination percentage and germination rate of barley seeds (data not shown).

It has been suggested that under different kinds of stresses, the acquisition of tolerance is closely related with ROS removal (Møller, 2001; Møller *et al*., 2007). Compared with seeds germinated directly at 35 °C, seeds pretreated at 0 °C notably increased the activities of SOD, APX, CAT, and GR; especially APX activity has increased 13 times (Fig. 5). The SOD and APX activities of seeds germinated at 35 °C after pretreatment were even much higher than those at 25 °C, and GR activity was similar to that at 25 °C, at which the highest germination rate and seedling growth were achieved for barley seeds. These results

showed that the increase in high-temperature tolerance caused by low-temperature pretreatment was a result of increasing ROS scavenging enzyme activities. It has also been shown that the enhanced ROS scavenging enzyme activity was implicated in the cross-tolerance of wheat seeds to salt stress induced by heat treatment at 33 °C (Lei *et al*., 2005). Wheat seeds pretreated in NaCl solution at −0.8 MPa also increased tolerance to subsequent temperature stress, which was also associated with the increased activities of SOD, APX, and CAT (Lei *et al*., 2005). Thus, increases in ROS scavenging enzyme activities appear to be a common component in cross-tolerance of seed germination.

Another mechanism for the cross-tolerance phenomenon that has been proposed is the effect of specific proteins. These proteins are induced by one type of stress and are involved in the protection against other types of stresses. For example, chilling resistance induced by water stress involves the activation of drought-regulated genes (Takahashi *et al*., 1994), and cold hardiness is stimulated by salt stress through the activation of cold- and ABA-responsive genes (Ryu *et al*., 1995). We are currently employing a proteomics approach to identify potential proteins associated with cross-tolerance of barley seed germination to high-temperature stress caused by low-temperature pretreatment.

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