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Antioxidant Enzymes and DPPH-Radical Scavenging Activity in Chilled and Heat-Shocked Rice (*Oryza sativa* L.) Seedlings Radicles

HO-MIN KANG AND MIKAL E. SALTVEIT*

Mann Laboratory, Department of Vegetable Crops, University of California, One Shields Avenue, Davis, California 95616-8631

Chilling whole rice seedlings at 5 °C significantly increased the time needed to recover linear growth and reduced the subsequent linear rate of radicle growth. Subjecting nonchilled seedlings to a 45 °C heat shock for up to 20 min did not alter subsequent growth, whereas a 3 min heat shock was optimal in reducing growth inhibition caused by 2 days of chilling. The activity of five antioxidant enzymes [superoxide dismutase (EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2), and guaiacol peroxidase (GPX; EC 1.11.1.7)] and DPPH (1,1-diphenyl-2-picrylhydrazyl)-radical scavenging activity were measured in heat-shocked and/or chilled radicles. Heat shock slightly increased the activity of CAT, APX, and GR and suppressed the increase of GR and GPX activity during recovery from chilling. Increased CAT, APX, GR, and DPPH-radical scavenging activity and protection of CAT activity during chilling appear to be correlated with heat shock-induced chilling tolerance.

KEYWORDS: Ascorbate peroxidase; catalase; DPPH-radical scavenging activity; glutathione reductase; guaiacol peroxidase; *Oryza sativa*; superoxide dismutase

INTRODUCTION

Chilling injury is a physiological disorder that occurs in sensitive plants when they are exposed to nonfreezing temperatures below 12 °C (*1*, *2*). Symptoms include stunted growth, reduced photosynthetic capacity, necrosis and discoloration, abnormal ripening, and increased disease susceptibility. The production of activated oxygen species (AOS), such as superoxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radical, may contribute to the development of chilling injury symptoms (*3*). AOS are highly reactive and damage membrane lipids, proteins, and nucleic acids, thereby disrupting the homeostasis of the organism (*4*). Although the generation of AOS is a common event in growth and developmental processes, it increases under abiotic stress, such as chilling, heat, drought, pollutants, and UV radiation.

Plants have both enzymatic and nonenzymatic antioxidant systems to prevent or alleviate the damage from AOS. Several enzymes can efficiently detoxify AOS. Superoxide radicals are disproportionately detoxified by superoxide dismutase (SOD), and hydrogen peroxide is destroyed by catalase (CAT) and different kinds of peroxidases such as guaiacol peroxidase (GPX), etc. A major hydrogen peroxide-detoxifying system in plants is the ascorbate−glutathione cycle that includes ascorbate peroxidase (APX) and glutathione reductase (GR) (*5*). The synthesis of lipid and water soluble antioxidants, such as ascorbic acid, glutathione, α-tocopherol, flavonols, carotenoids, and other phenolic compounds, may be part of a complex mechanism of chilling tolerance involving both avoidance of the production of AOS and protection from AOS produced (*6*).

There appears to be a relationship between antioxidant enzyme activity and chilling tolerance. Chilling-tolerant cultivars of several crops have a higher antioxidant enzyme activity than susceptible cultivars (*7*−*9*). Some chemical treatments that induce chilling tolerance (e.g., paclobutrazol and salicylic acid) also enhance antioxidant enzyme activity (*10*, *11*).

Temperature preconditioning and acclamation at near chilling temperatures increases the chilling tolerance of many crops (*1*, *2*). For example, the protective effect of preconditioning zucchini squash against chilling has been associated with increased antioxidant enzymes activity (*12*−*14*). Chilling tolerance can be induced in cucumber and rice seedlings and tomato pericarp disks by exposure to heat shocks (*15*). The mechanism by which heat shock increases chilling tolerance is not fully understood. However, there is increasing evidence that heat shock produces an oxidative stress that induces genes and promotes the synthesis of enzymes involved in oxidative stress defense (*16*). Elevated levels of SOD, CAT, and APX in cucumber seedling radicles appear to be correlated with the development of heat-shocked-induced chilling tolerance (*17*). The heat shock treatment could induce oxidative stress, which then induces an increase in the antioxidant capacity of the tissue (*18*).

Research reported in this paper was conducted to investigate the influence of heat shock treatments that induced chilling

* Corresponding author. Tel: 530-752-1815. Fax: 530-752-4554. E-mail: mesaltveit@ucdavis.edu.

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tolerance on the activity of selected antioxidant enzymes and DPPH (1,1-diphenyl-2-picrylhydrazyl)-radical scavenging activity in rice seedling radicles. We show that increased CAT, APX, GR, and DPPH-radical scavenging activity and protection of CAT activity during chilling are correlated with heat shock-induced chilling tolerance in rice seedling radicles.

MATERIALS AND METHODS

Plant Material. Rice (*Oryza sativa* L., cv. M202) seeds were obtained from a local vendor. Seeds (ca. 2 g) were imbibed in 1 L of aerated tap water overnight at 20 °C. Imbibed seeds were transferred to moist paper toweling overlying capillary cloth that was sandwiched between two 15 cm × 30 cm Plexiglas plates (6 mm thick) that were held together with rubber bands. The plates were held in a vertical position at 25 °C in a humid, dark, ethylene-free atmosphere for about 48 h, or until the radicles were about 16 mm long.

Germinated seeds with 16 ± 1 mm long radicles were removed from the large Plexiglas sandwich and gently transferred to moist paper toweling overlying capillary cloth and sandwiched between 7 cm × 13 cm Plexiglas plates (3 mm thick) as before. Each smaller plate held 6−7 seedlings and was treated as a unit of replication. The plates were positioned vertically in 20 cm × 26 cm × 14 cm tall white translucent plastic tubs and loosely covered with aluminum foil. The trays were either held at 25 °C for the initial measurements of radicle growth or chilled at 5 °C in the dark before being moved to 25 °C for the growth measurements.

Application of Heat Shock Treatments. Each small plate of rice seedlings with 16 ± 1 mm long radicles was placed in a plastic bag and immersed in water at 25 or 45 °C for 0−120 min. It took around 30 s to heat the seedling radicles from 25 to 45 °C and to cool them from 45 to 25 °C (*19*). The 25 °C treatment was considered the nonheat shock control. The top of the bags was left open to allow adequate gas exchange so that internal carbon dioxide did not exceed 0.1% during this phase of the experiment. The bagged plates were then held for 30 min in water at room temperature (ca. 20 °C) before removing them from the bags and placing them in plastic tubs lined with wet capillary cloth.

Measurement of Chilling Injury. The extent of chilling injury of the rice seedlings was measured as the subsequent linear growth of the radicle after chilling (*20*) by a method modified from that previously described (*21*). Radicle lengths for each seedling were regressed over time, and the slope (i.e., rate of growth) and correlation coefficient were calculated. The growth of radicles from nonstressed, control seedlings was linear during the duration of the experiments. To eliminate seedlings exhibiting abnormal growth from the study, only data from seedlings in which the growth rate had R^2 values greater than 0.99 were used in the analysis.

Preparation of Enzyme Fraction. Radicle tips (16 ± 3 mm in length) were excised from seedlings subjected to combinations of 3 min heat shock at 45 °C, chilling at 5 °C, and growth at 25 °C. Specifically, radicle tips were excised 2 h after the control or heat shock treatment (not chilled); 5, 18, and 48 h after chilling at 5 °C; and after growth at 25 °C for 48 and 96 h following 48 h of chilling. The 0.3 g fresh weight (ca. 150 radicle tips) was homogenized at 4 °C in 2.7 mL of extraction buffer (50 mM Tris-HCl buffer, pH 7.5, 3 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, and 1.5% w/v PVPP) with a mortar and pestle. The homogenate was then centrifuged at 25 000*g* for 20 min, and the supernatant was used as the crude extract in the five antioxidant enzyme assays.

Enzyme Assays. The activity of SOD, CAT, APX, GPX, and GR and the tissue protein content were assayed as previously described (*17*).

Measurement of the DPPH-Radical Scavenging Activity. Radicle tips (0.2 g fresh weight; ca. 100 tips) were ground in a mortar and pestle at 4 °C in 2.0 mL of absolute ethanol. One-half milliliter of the solution was mixed with 0.25 mL of 0.5 mM DPPH in ethanol solution and 0.5 mL of 100 mM acetate buffer (pH 5.5). The absorbance of the mixture was measured at 517 nm after 30 min (*22*).

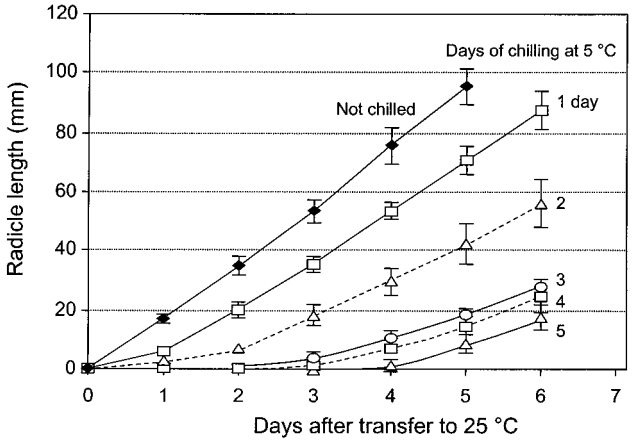

Figure 1. Length of whole rice seedling radicles grown at 25 °C following chilling at 5 °C for 0–5 days. Values are the mean ± SE (n = 10).

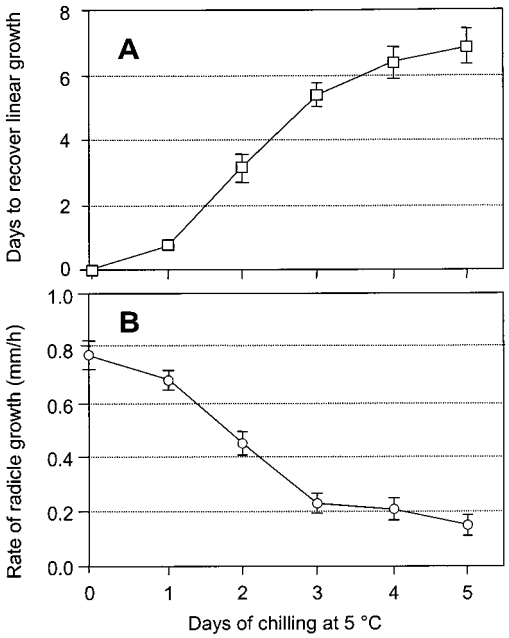

Figure 2. Time at 25 °C needed for radicles of whole rice seedlings to recover from 0 to 5 days of chilling at 5 °C and resume linear growth (A) and the rate of the linear portion of radicle growth following chilling and recovery (B). Values are the mean ± SE (n = 10).

RESULTS AND DISCUSSIONS

Determining Experimental Conditions. The growth of nonchilled rice seedling radicles was linear (radicle length = hours × 0.77 mm/h; R^2 = 0.94) for the duration of the experiment (**Figure 1**). Chilling at 5 °C for 1−5 days progressively increased the time it took the seedlings to recover and resume linear rates of radicle growth (**Figure 2A**) and reduced the subsequent rate of radicle growth at 25 °C (**Figure 2B**). The days to recover linear growth increased to 0.8 ± 0.4 days for seedlings chilled for 1 day and then rapidly increased to 2.9 ± 0.4 and 5.6 ± 0.4 days following 2 and 3 days of chilling, respectively (**Figure 2A**). The rate of linear radicle growth declined from 0.78 ± 0.11 mm/h for the nonchilled controls to 0.64 ± 0.08, 0.42 ± 0.07, and 0.19 ± 0.05 mm/h for seedlings chilled for 1−3 days, respectively. Because the steepest part of both slopes (**Figure 2A**,**B**) occurred around 2 days of chilling (i.e., subsequent radicle growth exhibited its greatest sensitivity to slight changes in chilling), we selected this duration of chilling for all subsequent experiments.

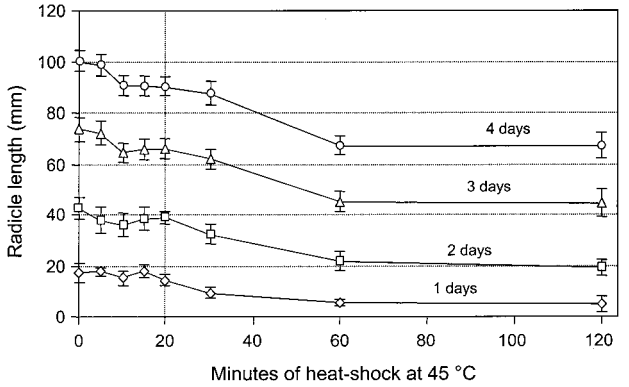

Figure 3. Length of whole rice seedling radicles measured after 1–4 days at 25 °C following 0–120 min of heat shock at 45 °C. Values are the mean ± SE ($n = 10$).

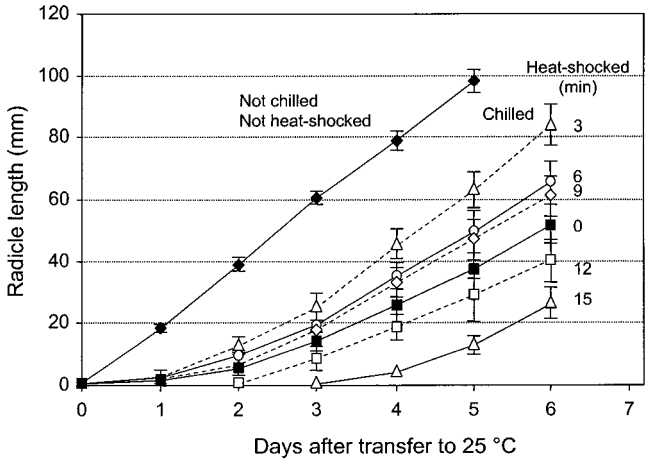

Figure 4. Length of whole rice seedling radicles after transfer to 25 °C following 0–15 min of heat shock at 45 °C and 2 days of chilling at 5 °C. Values are the mean ± SE ($n = 10$).

Heat shock treatments at 45 °C for up to 20 min did not significantly reduce subsequent radicle growth, but exposures for 30−120 min resulted in a reduction in the rate of subsequent radicle elongation (**Figure 3**). As the radicles grew at 25 °C for 1−4 days after the heat shock, it became apparent that exposure to more than 10 min resulted in a slightly slower rate of growth. Over the 4 days of growth at 25 °C, the growth of radicles receiving 0 or 5 min of heat shock was 10.5% greater than seedlings receiving 10−20 min of heat shock (1.05 ± 0.06 vs 0.95 ± 0.05 mm/h). To further refine treatment conditions, seedlings were heat-shocked at 45 °C for 3−15 min and then chilled for 2 days at 5 °C. (**Figure 4**). Subsequent radicle growth at 25 °C was severely reduced by the 2 day chilling treatment. Growth of the chilled radicles after 2 and 4 days at 25 °C was only 15 and 33%, respectively, of the nonchilled, nonheat-shocked controls. A 3 min heat shock was optimal in reducing growth inhibition caused by 2 days of chilling. Chilling inhibition of growth was reduced from 85 to 65% and from 67 to 42% for seedlings grown at 25 °C for 2 and 4 days, respectively. Chilling for 2 days reduced the linear rate of radicle growth from 0.82 ± 0.06 to 0.53 ± 0.11 mm/h. After recovery, the rate of radicle growth of the 3 min heat-shocked seedlings was 0.80 ± 0.10 mm/h. Once recovered from the initial effect of chilling, the linear rate of radicle growth was still significantly less in the chilled seedlings as compared to the nonchilled controls, whereas it was similar to the nonchilled rate for seedlings that had been heat-shocked for 3 min prior to chilling. Obviously, the heat shock treatment was making the seedlings more resistant to chilling. Apart from the known induction of protective hsps (*23*, *24*), the protective effects of the heat shock treatment could also include modifications of the antioxidant capacity of the tissue (*17*, *25*).

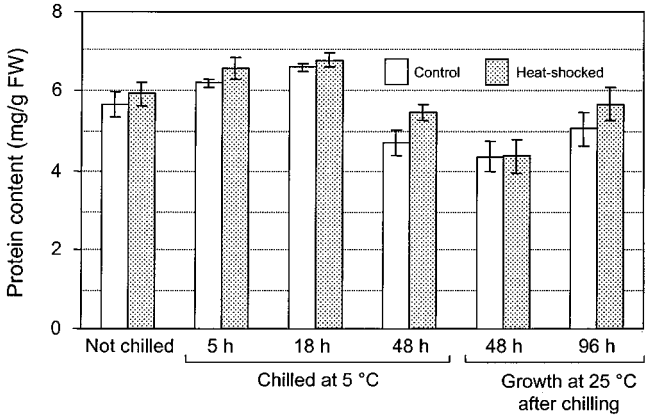

Figure 5. Protein content of the apical 1 cm of whole rice seedling radicles subjected to combinations of 3 min of heat shock at 45 °C and chilling at 5 °C. The seedlings were assayed 2 h after the control or heat shock treatment (not chilled); 5, 18, and 48 h after chilling at 5 °C; and after growth at 25 °C for 48 and 96 h following 48 h of chilling. Values are the mean ± SD ($n = 4$).

The heat shock-induced reduction in chilling sensitivity, as measured by the subsequent growth of rice seedling radicles, was further studied by measuring how these treatments affected the antioxidant activity of the radicle. It has been suggested that plants suffer from post-chilling oxidative stress when they are abruptly transferred from chilling to nonchilling conditions (*8*). Shen et al. (*9*) suggested that the higher antioxidant enzymes activity in chilling-tolerant cultivars than in sensitive cultivars during the warming period could provide a defense against post-chilling oxidative stress.

Antioxidant Enzyme and DPPH-Radical Scavenging Activity. The protein content of the 1 cm apical section of the rice seedling radicle was relatively unaffected by the heat shock treatment over all chilling and growth regimes (**Figure 5**). Overall, heat shock increased the protein content 7.2 ± 5.6% (range 2.0−16.2%). Protein content increased 10 and 15% over the nonchilled controls during the first 5 and 18 h of chilling, respectively, and then declined to levels 12.4% below the nonchilled controls as chilling continued for 48 h. This low level dropped to levels 25% below the nonchilled controls during the first 48 h of growth at 25 °C and then increases until it was only 7.3% below the nonchilled controls by 96 h.

The heat shock treatment significantly increased DPPH-radical scavenging activity under all conditions (**Figure 6**). DPPH activity in HS tissue was 23% greater than in non-HS tissue in nonchilled tissue assayed 2 h after the heat shock, 16% greater in tissue assayed after 48 h of chilling, and 14% greater in tissue assayed after an additional 48 h at 25 °C. Activity in both control and heat-shocked seedlings declined 23 and 27%, respectively, after 48 h of chilling. The level of DPPH activity in nonheat-shocked radicles was still 13% below the nonchilled control after an additional 48 h at 25 °C, while it had increased to the nonchilled control level (i.e., 100%) but was still 19% lower than the nonchilled heat-shocked tissue.

Chilling either had no effect or slightly depressed the activity of the 5 antioxidant enzymes (**Figures 7** and **8**). The heat shock treatment had no significant effect on SOD, GR, or GPX activity during chilling, but it did maintain higher levels of CAT and APX activity. All antioxidant enzymes increased in their

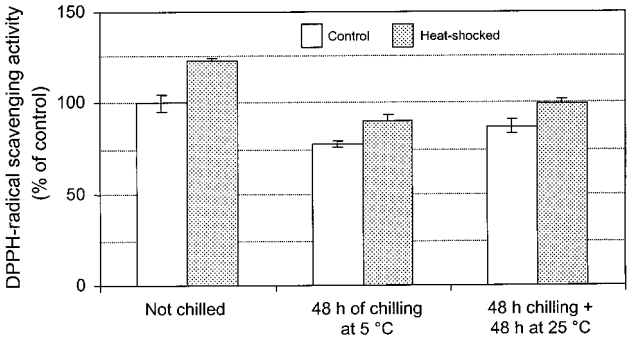

Figure 6. DPPH-radical scavenging activity of the 1 cm apical section of rice radicles. The seedlings were assayed 2 h after the control or heat shock treatment (not chilled); 48 h after chilling at 5 °C; and after growth at 25 °C for 48 h following 48 h of chilling. Values are the mean ± SD ($n = 4$).

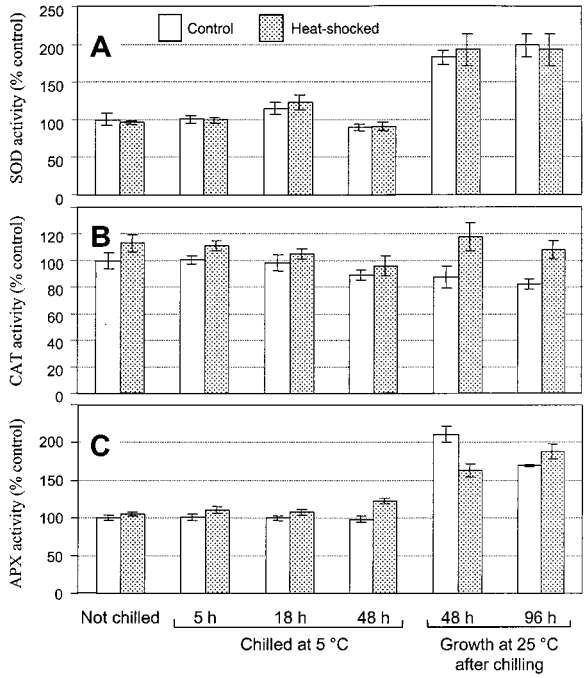

Figure 7. Activity of (A) SOD, (B) CAT, and (C) APX in the apical 1 cm of whole rice seedling radicles subjected to combinations of 3 min heat shock at 45 °C and chilling at 5 °C. The seedlings were assayed 2 h after the control or heat shock treatment (not chilled); 5, 18, and 48 h after chilling at 5 °C; and after growth at 25 °C for 48 and 96 h following 48 h of chilling. Values are the mean ± SD ($n = 4$).

activities after the chilled rice seedlings were transferred to the 25 °C growth temperature. However, only heat-shocked radicles showed an increase in CAT activity upon warming. The increase during growth at 25 °C was most pronounced for the nonheat-shocked radicles: GPX (8.2-fold increase), SOD (1.8-fold), APX (2.1-fold), and GR (1.7-fold). The increase was much smaller in heat shock tissue: GPX (5.1-fold), APX (1.6-fold), and GR (1.2-fold). There was no difference in the increase of SOD activity between the control and heat shock treatments.

Some researchers report that high-temperature treatments enhanced SOD activity, but most of these measurements were of SOD activity in leaves, not roots. The dismutation of O_2^- by SOD may be a primary step in the defense against chilling injury. SOD activity appears to be chilling-tolerant. However, it cannot be ruled out that the activity of some SOD isozymes may be susceptible to deactivation by chilling, since isozymes of SOD exhibit different characteristics and intercellular localization (*26*). Increases in SOD mRNA levels after low-temperature stress were observed in *Nicotiana plumbaginifolia* (*27*). Raising the temperature from 5 to 25 °C might have stimulated the production of O_2^- that trigger SOD synthesis. An abrupt increase in H_2O_2 levels with cold treatment was reported in wheat seedlings (*28*). The increased activity of SOD after the tissue was transferred from 5 to 25 °C could have been induced by an increase in the production of H_2O_2.

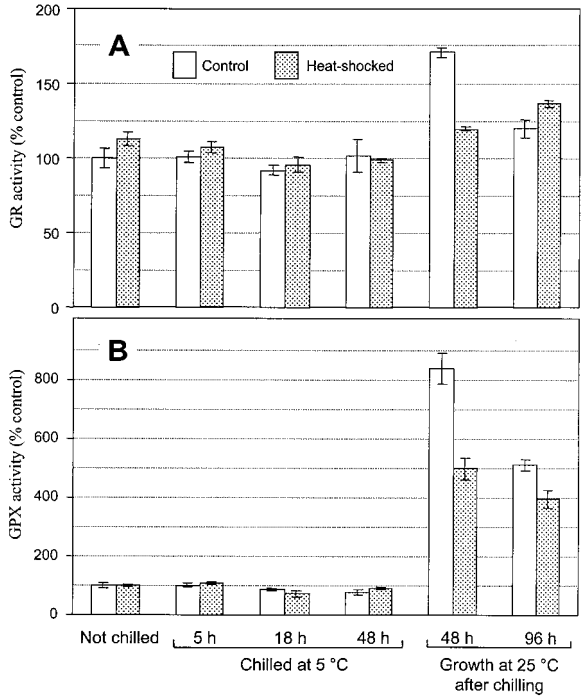

Figure 8. Activity of (A) GR and (B) GPX in the apical 1 cm of whole rice seedling radicles subjected to combinations of 3 min of heat shock at 45 °C and chilling at 5 °C. The seedlings were assayed 2 h after the control or heat shock treatment (not chilled); 5, 18, and 48 h after chilling at 5 °C; and after growth at 25 °C for 48 and 96 h following 48 h of chilling. Values are the mean ± SD ($n = 4$).

Hydrogen peroxide is detoxified by CAT, APX, and some peroxidases. The progressive decline in CAT activity that occurred during chilling continued when the tissue was warmed to 25 °C. Saruyama and Tanida (*8*) reported that CAT in chilling-sensitive cultivar was irreversibly damaged by exposure to chilling temperatures and that recovery of activity upon transfer to 25 °C was very low in embryos (33%) and roots (14%). The cessation of growth in chilling-sensitive cultivar at 25 °C could be attributed to the cold sensitivity of CAT and/or expression of the CAT gene. However, CAT activity of heat shock radicles remained higher than the nonheat-shocked seedlings through both chilling at 5 °C and growth at 25 °C. In fact, CAT activity increased significantly when the seedlings were moved to the growing conditions. The chaperone effect of heat shock protein can protect CAT. Both Hsp25 and α-crystallin efficiently suppressed the thermal aggregation of CAT (*29*).

The cold liability of CAT could be compensated for by an increase in the activity of another antioxidant enzyme with similar function (e.g., APX). APX appears to have an important role in increasing the oxidative tolerance of many plants, included rice seedlings (*8*, *25*, *30*). Increased APX activity of chilled radicles that had lower CAT activity after recovery shows the possible compensatory role of APX for CAT (**Figure 7**). This role for APX at a low temperature has been reported for many plants (*31*, *32*). Sato et al. (*25*) heat-stressed whole rice

plants at 42 °C for 1−24 h before chilling them at 5 °C for 7 days. The 24 h heat treatment was the most effective and almost completely prevented the development of chilling injury (i.e., wilting of leaves and no growth upon warming to 25 °C for 7 days). Their 24 h heat stress treatment significantly increased the level of APX activity in the leaves, had no effect on SOD activity, and decreased CAT activity. The level of *APXa* mRNA was doubled by the heat treatment. Chilling reduced it to the level of the nonheat treated, nonchilled control, while the level in the nonheat-stressed leaves declined to around 34% of the control. The heat shock-induced increase in APX activity was not as great in our seedling radicles as in the seedling leaves reported by Sato et al. (*8*), but these differences in magnitude could be accounted for by differences in the response of the cultivars and tissues used.

GR is necessary for the regeneration of ascorbate, which is required for the activity of APX. In addition, the importance of GR in relation of oxidative stress has been reported for many plants. In this study, the change of GR activity was similar to that of APX. Upon growth at 25 °C for 48 h, GR activity of chilled radicles was significantly higher than heat-shocked radicles, but this relationship was reversed as growth continued to 96 h (**Figure 8**). Chilling temperature also increased the activities of GR in *Arabidopsis thaliana* (*33*). Transcript levels of GOR2 (a GR cDNA) increased during the recovery (post-stress) phases of both drought and chilled pea plants by about 10- and 3-fold, respectively (*34*). Kaminaka et al. (*35*) suggested that expression of the rice cytosolic GR gene was regulated via an ABA-mediated signal transduction pathway during environmental stresses, such as chilling, drought, and salinity. GR activity also increased during chilling and recovery in maize seedlings (*36*).

Some researches report that high-temperature treatments cause an increase in peroxidase activity (*37*). However, peroxidase activity and lignin levels were reduced by the heat treatment of tomato seedling roots (*38*), and heat shock treatments decreased GPX activity in iceberg lettuces (*39*). Peroxidase genes were highly expressed upon chilling at 4 °C in suspension cultures of sweet potato, but both expression and chilling injury were reduced by prior acclimation at 1 °C (*40*). GPX activity was also increased 55% as compared to the control in chilled *Coffea arabica* roots (*41*).

Effects of Heat Shock Treatments. Heat shock treatments have a number of physiological effects on plant tissues. Their most studied effect is on the induction of protective heat shock proteins (hsps), which are thought to increase chilling tolerance (*23*, *24*). However, the induction of the synthesis of hsps is also accompanied by the greatly diminished synthesis of other proteins. For example, a heat shock prevents phenylammonia lyase (PAL) from being synthesized from wound-induced PAL mRNA in lettuce tissue (*42*). The diversion of protein synthesis away from the synthesis of some specific proteins after chilling to the production of innocuous hsps could be just as important in the ability of heat shock to reduce the development of chilling injury symptoms as is its ability to promote the production of protective hsps before chilling (*2*). This theory is supported by the observation that chilling injury was reduced in cucumber and rice seedlings and in tomato pericarp disks by heat shocks applied after chilling (*15*).

Plants respond to some biotic stresses with an increased production of AOS that retards the proliferation of the biotic stress. Because plants have a limited repertoire of responses to stress, an abiotic stress such as chilling may also induce the production of "protective" AOS. Like trauma-induced tissue inflammation in animals, the production of chilling-induced AOS may be more damaging than the actual inducing stress. Heat shock treatments could reduce chilling damage by diverting protein synthesis away from chilling-induced proteins to hsps and thereby reducing the synthesis of chilling-induced enzymes in pathways responsible for the increased production of AOS.

A third possibility is that the heat shock treatment can induce an increase in the antioxidant capacity of the tissue; this idea is supported by data presented in this paper and other recently published papers (*17*, *25*). We have shown that a mild heat shock of 3 min at 45 °C enhances DPPH-radical scavenging activity and CAT, APX, and GR activity and sustains CAT activities during recovery. Previously, we showed that a heat shock treatment increased the chilling tolerance and antioxidant capacity of cucumber seedling (*17*). Our work confirms the findings of others that one stress (e.g., heat shock) can increase the plant's tolerance to other stresses (e.g., chilling). The exact mechanism by which heat shock increases chilling tolerance is unknown, but three possibilities exist as follows: induced synthesis of hsps, diverted protein synthesis, and increased antioxidant capacity. Increased CAT, APX, GR, and DPPH-radical scavenging activity and protection of CAT activity during chilling appear to be correlated with heat shock-induced chilling tolerance.

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