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Mechanisms of Chilling Tolerance in Relation to Antioxidative Enzymes in Rice

Yong In Kuk*‡, Ji San Shin, Tay Eak Whang**, and Ja Ock Guh****

**Biotechnology Research Institute, Chonnam National University, Gwangju 500-757, Korea*
***Faculty of Applied Plant Science, Chonnam National University, Gwangju 500-757, Korea*

ABSTRACT : In order to examine the mechanistic basis for differential sensitivities to chilling and subsequent recovery between two rice (*Oryza sativa* L.) cutivars, a chilling-tolerant japonica type (Ilpumbyeo) and a chilling-susceptible indica type (Taebaekbyeo), changes of physiological responses and antioxidant enzymes were investigated. Both cultivars at 3 leaf stage were exposed at a low temperature of 5°C for 3 days and subsequently recovered in a growth chamber at a 25°C for 5 days with 250 mmol m^{-2} s^{-1}. Physiological parameters such as leaf fresh weight, relative water content, cellular leakage, lipid peroxidation, and chlorophyll a fluorescence showed that the chilling tolerant cultivar had a high tolerance during chilling. However, the chilling-susceptible cultivar revealed severe chilling damages. The chilling-tolerant cultivar was also faster in recovery than the chilling-susceptible cultivar in all parameters examined. We analyzed the activity and isozyme profiles of four antioxidant enzymes which are: superoxide dismutase (SOD), caltalase (CAT), ascorbate peroxidase (APX), and glutathion reductase (GR). We observed that chilling-tolerance was due to a result of the induced or higher antioxidant enzyme system, CAT and APX in leaves and SOD, CAT, APX, and GR in roots. Especially, we observed the most significant differences between the chilling-tolerant cultivar and -susceptible cultivar in CAT and APX activity. Also in isozyme profiles, CAT and APX band intensity in the chilling-tolerant cultivar was distinctively higher than in the chilling-susceptible cultivars during chilling and recovery. Thus, the cold stability of CAT and APX are expected to contribute to a tolerance mechanism of chilling in rice plants. In addition, the antioxidative enzymes activity in roots may be more important than in that of leaves to protect chilling damage on rice plants.

Keywords **:** Antioxidative enzymes; chilling stress; cold acclimation; isozyme profile; rice (*Oryza sativa* L.)

Rice, an important crop in Asia, originated in the tropics and is a sensitive plant to low temperature (De Datta, 1981). Especially, indica type is known to be more sensitive to photoinhibition at chilling temperature than japonica type (Hetherington *et al.*, 1989). Therefore, to improve cold tolerance in rice plants, it is important to understand physiological and biochemical tolerance mechanisms.

Various mechanisms have been suggested to account for chilling injury or tolerance in plants (Basra *et al.*, 2001). Some of the changes related to low-temperature stress include alterations in gene expression, composition of proteins, lipids, and carbohydrate, membrane damage (lipid peroxidation), solute leakage, mitochondrial respiration, and photosysnthesis (Foyer *et al.*, 1994; Guy, 1990; Howarth & Ougham 1993; Markhart, 1986; Smirnoff, 1993; Thomashow, 1990; Wang, 1982). Since low temperatures induce oxidative stress in tissues, chilling damage was partly attributed to the effects of *in vivo*-generated reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), and singlet oxygen ($^1O_2$) in the cell (Hodgson & Raison 1991; Terashima *et al.*, 1994).

Plants have evolved antioxidant systems to protect cellular membranes and organelles from the damaging effects of ROS (Foyer *et al.*, 1991). Antioxidant enzymes, such as superoxide dismutase (SOD, EC1.15.1.1), catalase (CAT, EC1.11.1.6) and various peroxidases such as guiacol peroxidase (POX, EC1.11.17) and ascorbate peroxidase (APX, EC 1.11.1.11), can eliminate these ROS (Foyer *et al.*, 1991; Lee & Lee 2000; Oidaira *et al.*, 2000; Omran 1980; Prasad 1996, 1997; Scandalios 1993). In conjunction with these enzymes, antioxidant compounds such as ascorbate, glutathione, β-carotene and α-tocopherol can also play important roles in the removal of toxic oxygen compounds (Hodges *et al.*, 1997; Wise & Naylor, 1987).

In chilling-sensitive plants, the ability to defend against oxidative damage has been shown to be inhibited by a reduction in antioxidants such as ascorbate, glutathione, and α-tocopherol (Wise & Naylor, 1987), CAT (Fadzillah *et al.*, 1996; Omran, 1980), and SOD (Michalski & Kaniuga, 1982). Chilling tolerance improved when GSH, peroxidase, and CAT levels were enhanced (Upadhyaya *et al.*, 1989). Induced SOD and APX activity were correlated with increased chilling tolerance in paclobutrazol-induced chilling tolerance in chilling-sensitive maize inbred (Pinhero *et al.*, 1997). GR

‡Corresponding author: (Phone) +82-62-530-2052 (E-mail) yikuk@chonam.ac.kr <Received August 1, 2002>

has also been shown to be important in the protection against oxidative stress in plants (Aono *et al.*, 1993; Foyer *et al.*, 1991; Guy & Carter, 1984). The various antioxidant isozymes are differentially compartmentalized and, depending on the tissue, likely respond differently to chilling (Anderson *et al.*, 1995; Prasad, 1996, 1997; Scebba *et al.*, 1998).

Although several studies on evidence for chilling-induced oxidative stress has been obtained in crops (Hodges *et al.*, 1997; Prasad *et al.*, 1994; Scebba *et al.*, 1998), little is known about the possibility of oxidative stress in chilling-susceptible plants of tropical origin such as rice. Therefore, the objectives of this research are to compare the physiological responses of chilling-tolerant and -susceptible rice plants during chilling and subsequent recovery, and to examine whether ROS-scavenging systems are related to the resistance to low temperature.

MATERIALS AND METHODS

Plant growth and treatment conditions

Seeds of two rice cultivars, a chilling-tolerant japonica type (Ilpumbyeo) and a chilling-susceptible indica type (Taebaekbyeo) were soaked in water for 4 days at 25°C and were sown in commercial soil in trays in a temperature-controlled greenhouse at 30 ± 3/25°C ± 3, day/night temperature. At 8 days after seeding, the roots of the seedlings were washed in the water and the seedlings were transferred to half-strength Hoagland's nutrient solution in a growth chamber. The conditions of the growth chamber were 70% relative humidity, 30/25°C (day/night), and light intensity of 250 μmol m^{-2} s^{-1} PAR with a 14 h photoperiod. The seedlings were grown in the growth chamber until they reached the 3-leaf stage. The seedlings were exposed to chilling at 5°C for 3 days. After the chilling treatment, all seedlings were allowed to recover for 5 days at 25°C.

Evaluation of chilling injury

The effect of the chilling and recovery between chilling-tolerant and -susceptible cultivars was examined by measuring the leaf fresh weight.

Chilling injury on leaves was evaluated by relative water content as calculated by a formula of (1-dry weight of leaf/fresh weight of leaf) × 100.

Chlorophyll was extracted and assayed according to the procedure of Hiscox and Israelstam (1979). The leaves of seedlings (0.5 g) from each treatment were soaked for 48 h in darkness in 10 ml of dimethyl sulfoxide at room temperature. Chlorophyll extraction was complete at this time. Total chlorophyll content in the extracts was determined spectrophotometrically. All treatments for each measurement were triplicated and values were expressed on a fresh weight basis.

Lipid peroxidation

Lipid peroxidation was estimated by the level of malondialdehyde (MDA) production using a slight modification of the thiobarbituric acid (TBA) method as previously described (Buege & Aust, 1978). The leaves (0.5 g) were harvested for each treatment period, and the tissues were homogenized with a mortar and pestle in 5 ml of a solution of 0.5% TBA in 20% trichloroacetic acid (TCA). The homogenate was centrifuged at 20,000×g for 15 min and the supernatant was collected. The supernatant was heated in a boiling water bath for 25 min and allowed to cool in an ice bath. Following centrifugation at 20,000×g for 15 min, the resulting supernatant was used for spectrophotometric determination of MDA. Absorbance at 532 nm for each sample was recorded and corrected for nonspecific turbidity at 600 nm. MDA concentrations were calculated using a molar extinction coefficient of 156 mM^{-1} cm^{-1}.

Chl a fluorescence and quantum yield measurements

In vivo Chl a fluorescence was measured at room temperature using a pulse amplitude modulation fluoremeter (PAM-2000, Walz, Effeltrich, Germany). Before measuring the fluorescence, all leaves were adapted in darkness for 5 min to allow relaxation of fluorescence quenching associated with thylakoid membrane energization (Krause *et al.*, 1983). Minimal fluorescence yield, F_0, was obtained upon excitation with a weak measuring beam from a pulse light-emitting diode. Maximal fluorescence yield, F_m, was determined after exposure to a saturating pulse of white light to close all reaction centers. The ratio of variable to maximum fluorescence (F_v/F_m) derived from the measurement was used as a measure of the maximum photochemical efficiency of PS II. The quantum yield of electron transport through PS II ($Y = DF/F'_m$) was calculated according to Genty *et al.* (1989).

Protein extraction

Frozen leaves or roots (0.5 g) were pulverized with a mortar and pestle using liquid N_2 and then resuspended with 3 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, 1% PVP-40, and 1 mM PMSF. For APX assay, the extraction buffer also contained 5 mM ascorbate. The suspension was centrifuged at 15,000×g for 20 min at 4°C. The resulting supernatant was directly used as an

enzyme source. For SOD assay, however, the supernatant was passed through a Sephadex G-25 M minicolumn (PD-10; Pharmacia, Uppsala, Sweden) at 4°C using 100 mM potassium phosphate buffer (pH 7.5) as the elution buffer to remove inhibitors of low molecular weight. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Enzyme assays

SOD activity was determined according to the procedure of Spychalla & Desborough (1990). The assay was performed in a 1-ml cuvette at 25°C with a spectrophotometer. The reaction mixture contained 50 mM Na_2CO_3/$NaHCO_3$ buffer (pH 10.2), 0.1 mM EDTA, 0.015 mM ferricytochrome C, and 0.05 mM xanthine. The assay was initiated by the addition of sufficient xanthine oxidase to produce a basal rate of ferricytochrome C reduction corresponding to an increase in absorbance at 550 nm of 0.025 units/min (V_1). After the verification of the V_1, the protein extract was added and the resulting velocity (V_2) was calculated. One unit of SOD was defined as the amount of enzyme which inhibited the rate of ferricytochrome C reduction by 50% (V_1/V_2=2) in a 1-ml assay volume. CAT activity was assayed by the method of Mishra *et al.* (1993) in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 11 mM H_2O_2, and the crude enzyme. The reaction was started by adding H_2O_2 solution and the activity was determined by monitoring the decrease of absorbance at 240 nm (ε = 36M cm^{-1}), as a result of the H_2O_2 consumption. APX activity was measured spectrophotometrically by monitoring the decline in absorbance at 290 nm as ascorbate (ε = 2.8 mM cm^{-1}) was oxidized, using the method of Chen & Asada (1989). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM ascorbate, and 0.2 mM H_2O_2. GR was assayed by monitoring the decline in absorbance at 340 nm as NADPH (ε = 6.2 mM cm^{-1}) was oxidized, as described by Rao *et al.* (1996). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, and 0.5 mM oxidized glutathione (GSSG). The reaction was initiated by the addition of GSSG solution.

Native PAGE and activity staining

Isoforms of CAT, SOD, APX, and GR were separated on nondenaturating polyacrylamide gels by using the slightly modified procedures of Laemmli (1970). Equal amounts of the protein extracts, with the addition of bromophenol blue and glycerol to a final concentration of 12.5%, were loaded on 7% (CAT) or 10% (SOD, APX, and GR) polyacrylamide gels. Electrophoretic separation was performed at 4°C for 3 h with a constant current of 30 mA. For APX, however, ascorbate at a concentration of 2 mM was added to the electrode buffer and the gel was pre-run for 30 min before the sample loaded (Mittler & Zilinskas, 1993). After completion of electrophoresis, the gels were stained separately for the activities of the individual antioxidative enzymes.

SOD activity staining of the gel was performed according to the method of Rao *et al.* (1996). The gels were stained by incubation in a solution containing 2.5 mM nitroblue tetrazolium in darkness for 25 min, followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM riboflavin and 28 mM tetramethyl ethylene diamine (TEMED) in darkness for 20 min. The gels were then exposed to dim light for 25 min at room temperature. In some experiments, the gels were incubated in 50 mM potassium phosphate buffer (pH 7.8) containing 3 mM KCN or 5 mM H_2O_2 for 30 min prior to the staining for SOD activity to visualize KCN- and H_2O_2sensitive isoforms as before (Britton et al., 1978). CAT activity staining of the gel was performed by the procedure of Anderson et al. (1995). The gels were soaked in 3.27 mM H_2O_2 for 25 min, rinsed twice in distilled water, and stained in a freshly prepared solution consisting of 1% (w/v) potassium ferricyanide and 1% (w/v) ferric chloride. APX isoforms were stained by incubating gels in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min (Rao *et al.*, 1996). The gels were incubated in the same buffer containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min, and then soaked in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM nitroblue tetrazolium for 15 min with gentle agitation (Rao *et al.*, 1996). GR activity was detected by incubating gels in 50 mL of 0.25 M Tris-HCl buffer (pH 7.5) containing 10 mg of 3-(4,5-dimethylthiazol-2-4)-2,5-diphenyl tetrazolium bromide, 10 mg of 2,6-dichlorophenolindophenol, 3.4 mM GSSG, and 0.5 mM NADPH in darkness for 1 h (Rao *et al.*, 1996).

Each staining reaction for the antioxidative enzyme activities was stopped with 7.5% glacial acetic acid. The gels in plastic boxes containing the 7.5% acetic acid solution were stored at 4°C until being photographed.

RESULTS

Growth and physiological responses of chilling-tolerant and -susceptible rice cultivars during chilling and subsequent recovery

We selected a chilling-tolerant and a chilling-susceptible rice cultivar from our preliminary experiment. Since the typical symptoms of chilling injury are wilting, yellowing of

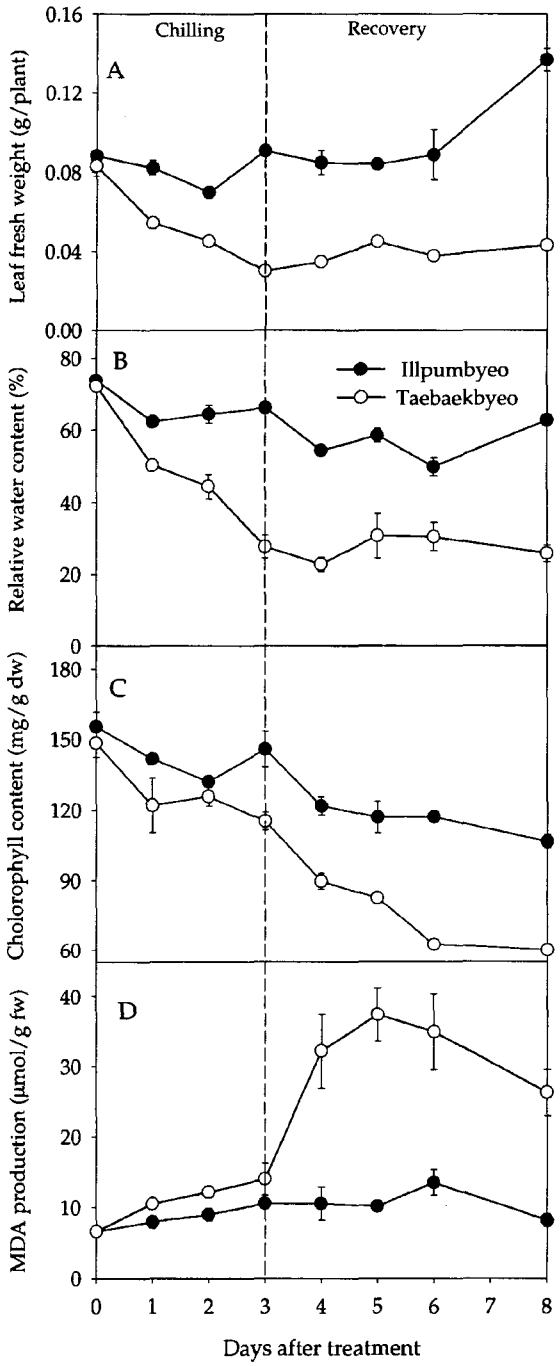

Fig. 1. Changes in (A) leaf fresh weight, (B) relative water content, (C) chlorophyll content, and (D) lipid peroxidation in the chilling-tolerant and -susceptible rice cultivars during chilling and subsequent recovery. The plants were chilled for 3 days at 5°C (chilling), and allowed to recover for 5 days at 25°C (recovery). Values are the mean SE of three replicates. In some cases, the error bar is obscured by the symbol.

leaves, and inhibition of growth, the relative water content, chlorophyll content, and leaf fresh weight in the leaves were examined during chilling and subsequent recovery (Fig. 1). The leaf fresh weight was different during chilling and subsequent recovery between chilling-tolerant and -susceptible cultivar (Fig. 1A). The chilling-tolerant cultivar was little reduced in leaf fresh weight during chilling and was almost recovered 1 day after transfer to 25°C. However, in the chilling-susceptible cultivar, the reduction of leaf fresh weight progressed rapidly with increasing the chilling duration and was not recovered during recovery.

One of the chilling injuries is wilting of leaves; therefore, we investigated relative water content during chilling and subsequent recovery (Fig. 1B). There was no difference in relative water content of control plants between the chilling-tolerant and -susceptible cultivars. Although the relative water content was decreased during chilling in both cultivars, the level of decreasing was much less in the chilling-tolerant cultivar than in the chilling-susceptible cultivar. On the other hand, the relative water content in the chilling-tolerant cultivar was higher than in the chilling-susceptible cultivar during recovery.

Although the chlorophyll content also decreased in both the chilling-tolerant and -susceptible cultivars during chilling and subsequent recovery, the chlorophyll content in the chilling-tolerant cultivar was higher than in the chilling-susceptible cultivar (Fig. 1C).

Changes in lipid peroxidation

The level of MDA production was measured to determine as an estimate of lipid peroxidation during chilling and subsequent recovery (Fig. 1D). There was a big difference in lipid peroxidation between the chilling- tolerant and -susceptible cultivars during chilling and subsequent recovery. In the chilling susceptible cultivar, lipid peroxidation increased with increasing the chilling duration. The level of lipid peroxidation increased further following 2 days after the recovery and then decreased thereafter. However, little or no lipid peroxidation occurred in the chilling-tolerant cultivar during chilling and subsequent recovery.

Chl a fluorescence during chilling and subsequent recovery

Chl a fluorescence (F_v/F_m) values in the chilling-tolerant cultivar were almost the same as those in the chilling-susceptible cultivar during growing at 25°C (Fig. 2A). However, the subsequent chilling rapidly reduced Chl a fluorescence in the chilling-susceptible cultivar, whereas the Chl a fluorescence in the chilling-tolerant cultivar exhibited similar to that of control leaves values during chilling. The Chl a fluorescence in the chilling-tolerant cultivar was fully restored

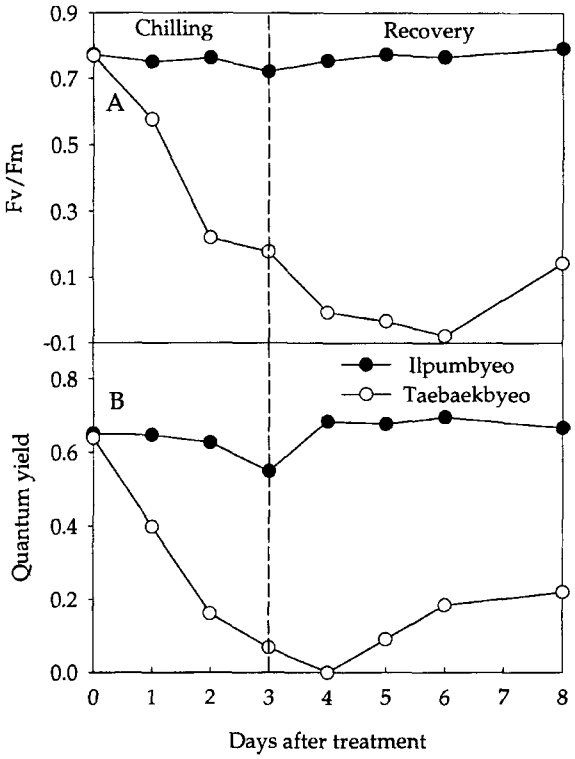

Fig. 2. Changes in (A) F_v/F_m ratio and (B) quantum yield in the chilling-tolerant and -susceptible rice cultivars during chilling and subsequent recovery. The plants were subjected to the same treatments as in Fig. 1. Values are the mean ± SE of three replicates. In some cases, the error bar is obscured by the symbol.

during recovery. Whereas, the Chl a fluorescence of the chilling-susceptible cultivar was not restored during recovery.

Similar to the Chl a fluorescence, the quantum yield in the chilling-susceptible cultivar leaves dropped rapidly during chilling and was not restored during recovery. Whereas, the quantum yield of the chilling-tolerant cultivar leaves only reduced 3 days after chilling and was restored 1 day after recovery.

Changes in antioxidantive enzymes in leaf

The results of the analysis of antioxidant enzymes (SOD, CAT, APX, and GR) activity in the chilling-tolerant and -susceptible cultivars during chilling and subsequent recovery are summarized in Fig. 3. In general, no differences were found in SOD activity between the chilling-tolerant and -susceptible cultivars during chilling and recovery (Fig. 3A). However, the SOD activity in the chilling-tolerant cultivar was higher than in the chilling-susceptible cultivar 3 days after chilling. In contrast, the SOD activity in the chilling-susceptible cultivar was higher than

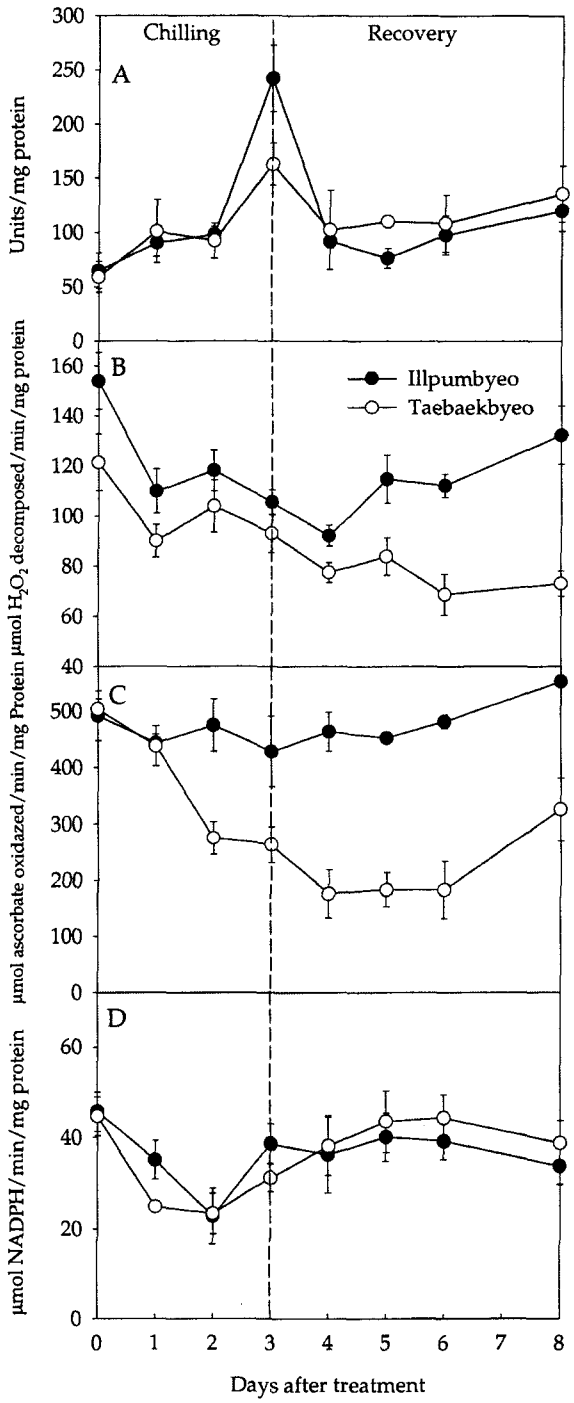

Fig. 3. Changes in (A) SOD, (B) CAT, (C) APX, and (D) GR activities in leaves of the chilling-tolerant and -susceptible rice cultivars during chilling and subsequent recovery. The plants were subjected to the same treatments as in Fig. 1. Values are the mean ± SE of three replicates. In some cases, the error bar is obscured by the symbol.

in the chilling-tolerant cultivar 2 days after recovery.

CAT activity in the control plants was higher in the chill-

ing-tolerant cultivar than in the chilling-susceptible cultivr (Fig. 3B). Although CAT activity was decreased in both the cultivars compared with control plants, the CAT activity in the chilling-tolerant cultivar was higher than in the chilling-susceptible cultivar 1 day after chilling. On the other hand, CAT activity in the chilling-susceptible cultivar was more decreased with increasing the recovery duration compared with chilling duration and control plants. However, CAT activity in the chilling-tolerant cultivar was increased with increasing recovery duration except for 1 day after recovery. Thus, the CAT activity in the chilling-tolerant cultivar 5 days after recovery was almost the same as those of control plants.

APX activity in the chilling-tolerant cultivar was higher than in the chilling-susceptible cultivar over 2 days after chilling and during recovery (Fig. 3C). In addition, APX activity in the chilling-tolerant cultivar was almost the same as the control plants, or rather increased by chilling and recovery. However, APX activity in the chilling-susceptible cultivar was increased with increasing the chilling duration. The level of APX activity decrease in the chilling-susceptible cultivar decreased further following 3 days after recovery and then increased thereafter.

Unlike SOD, CAT, and APX, GR activities had no differences between the chilling-tolerant and -susceptible cultivars during chilling and recovery (Fig. 3D).

Changes in antioxidantive enzymes in root.

Changes in antioxidative enzyme activities of SOD, CAT, APX, and GR in the chilling-tolerant and -susceptible cultivar during chilling and subsequent recovery are summarized in Fig. 4. In the chilling-susceptible cultivar, SOD activity was higher than in the chilling-tolerant cultivars in control plants and 2 and 3 days after chilling. In contrast, SOD activity in the chilling-tolerant cultivar was higher than in the chilling-susceptible cultivar over 2 days after recovery. In addition, SOD activity in the chilling-tolerant cultivar was slowly decreased by chilling and recovery, but the SOD activity in the chilling-susceptible cultivar was decreased rapidly with increasing recovery duration compared with control plants or chilling-tolerant cultivar.

CAT activity in the chilling-susceptible cultivar was higher than in the chilling-tolerant cultivar in the control plants, but CAT activity in the chilling-tolerant cultivar was higher than in the susceptible during chilling (Fig. 4B). Although CAT activity in both cultivars was decreased with increasing chilling duration, CAT activity in both cultivars was increased with increasing recovery duration compared with chilling duration. Thus, the CAT activity in both cultivars 5 days after recovery was almost the same or rather

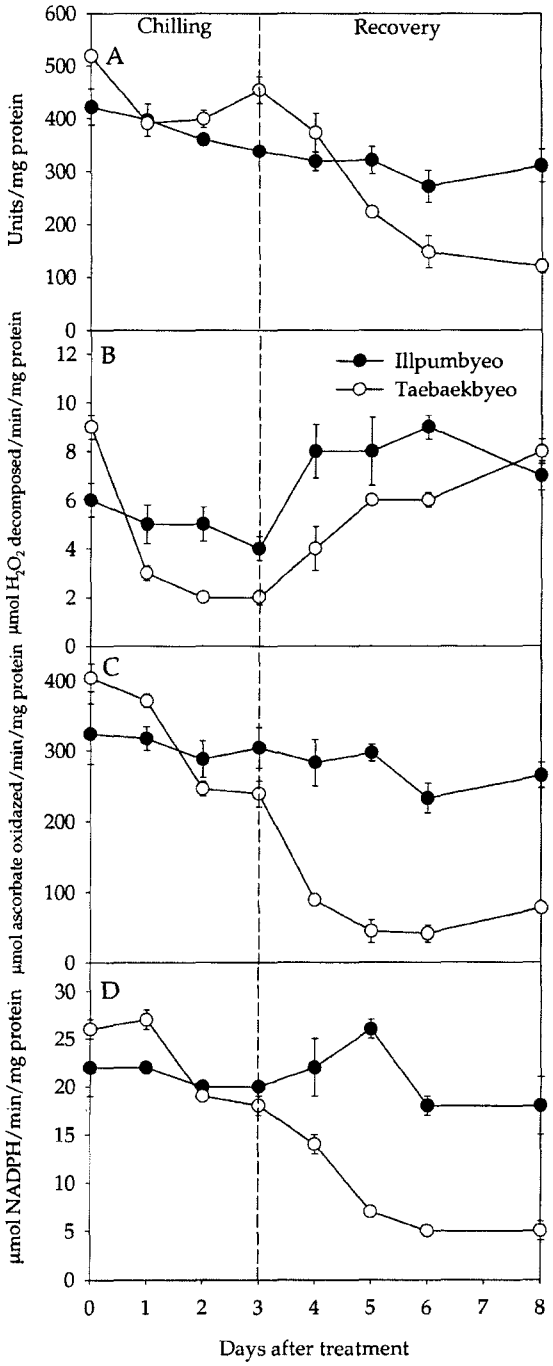

Fig. 4. Changes in (A) SOD, (B) CAT, (C) APX, and (D) GR activities in roots of the chilling-tolerant and -susceptible rice cultivars during chilling and subsequent recovery. The plants were subjected to the same treatments as in Fig. 1. Values are the mean ± SE of three replicates. In some cases, the error bar is obscured by the symbol.

increased as control plants. However, the CAT activity in the chilling-tolerant cultivar was much higher than in the chilling-susceptible cultivar during recovery except for 5 days

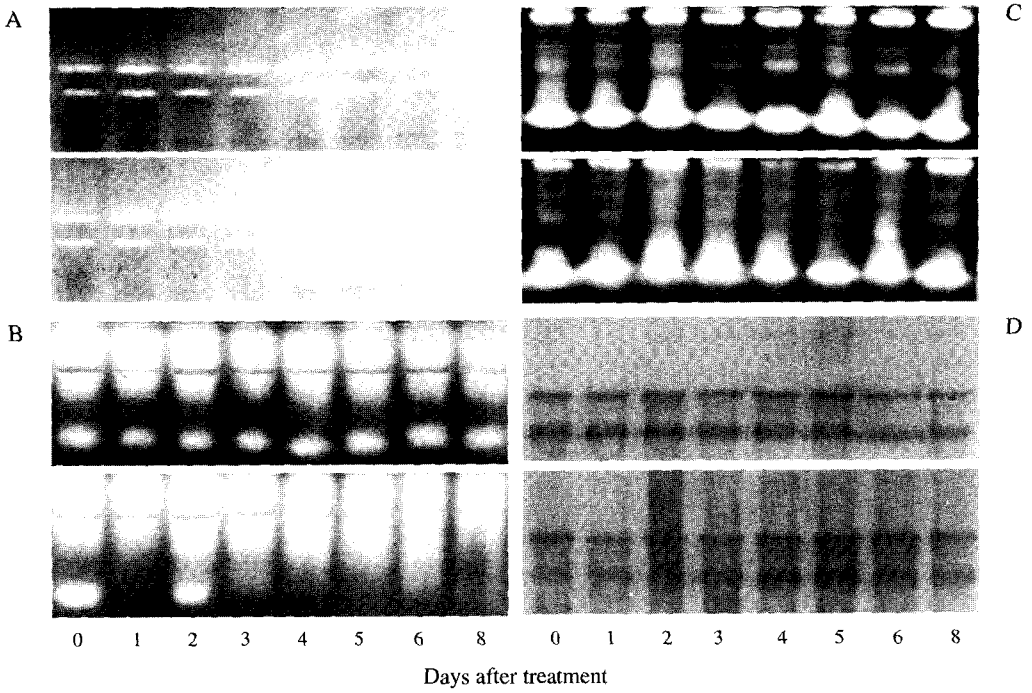

Fig. 5. Changes in (A) SOD, (B) CAT, (C) APX, and (D) GR isozyme profiles in leaves of the chilling-tolerant (upper) and -susceptible rice cultivars (below) during chilling (1-3 days), and recovery (4-8 days). The plants were subjected to the same treatments as in Fig. 1.

after recovery.

APX activity in the chilling-tolerant cultivar was higher than in the chilling-susceptible cultivar 2 and 3 days after chilling (Fig. 4C). In contrast, APX activity in the chilling-susceptible cultivar was higher than in the chilling- tolerant cultivar in control plants and 1 day after chilling. During recovery, APX activity in the chilling-susceptible cultivar was rapidly decreased with increasing the recovery duration. However, APX activity in the chilling-tolerant cultivar was slowly decreased or the same as control plants during recovery.

Similar to SOD activity, GR activity in the chilling-susceptible cultivar was rather higher than in the chilling-susceptible cultivar in control plants and 1 and 3 days after chilling (Fig. 4D). GR activity in the chilling-tolerant cultivar was the same as or rather increased in control plants during recovery. However, GR activity in the chilling-susceptible cultivar was decreased with increasing the recovery duration.

Changes in antioxidant isozyme profiles

To relate changes in antioxidant isozyme patterns with chilling injury or tolerance in chilling-tolerant and -susceptible cultivars, we analyzed SOD, CAT, APX, and GR isozymes on native polyacrylamide gels. As shown in Fig. 5A, SOD isozymes were identified as Mn-SOD, by its insensitivity to 3 mM KCN and 5 mM H_2O_2, whereas Fe-SOD and Cu/Zn-SOD was not observed in all treatments. There were two Mn-SOD isozymes in the chilling-tolerant and -susceptible cultivars during chilling and recovery. As the total SOD activity showed not much difference in both cultivars, intensity of Mn-SOD isozymes were also not effected by chilling and subsequently recovery in chilling tolerance.

As total CAT activity indicated (Fig. 3B), we observed distinctive differences in the staining intensity of one isoform of two CAT isozymes between the chilling-tolerant and -susceptible cultivars during chilling and recovery. In the chilling-tolerant cultivar, the intensity of two bands was not changed during chilling and recovery. However, one isoform of two isozymes in the chilling-susceptible cultivar was disappeared 1 and 3 days after chilling and 1, 2, and 3 days after recovery and then dimly appeared 5 days after recovery.

Five isozymes of APX were visible on the activity gels (Fig. 5C); Two APX isozymes 2 and 3 (from bottom) were very faint, but APX isozyme 1, 4, and 5 (from bottom) were prominent. As there was significant difference in total APX activity between the chilling-tolerant and -susceptible cultivars, intensity of major APX bands was more induced in the chilling-tolerant cultivar than in the chilling-susceptible cultivar during chilling and recovery. That is, in the chilling-tol-

erant cultivar, APX isozymes (1, 4, and 5) bands were not changed or rather induced during chilling and recovery compared with control plants. Whereas, the APX isozymes the bands in the chilling-susceptible cultivar were less induced or decreased than in the chilling-tolerant cultivar. Especially, APX isoform 3 was decreased in the chilling-susceptible cultivar during recovery except for 5 days after recovery.

There were two GR isozymes in both the chilling-tolerant and -susceptible cultivars during chilling and recovery (Fig. 5D.). However, there was no difference in the isozyme band intensity between the chilling-tolerant and -susceptible cultivars during chilling and recovery.

DISCUSSION

To study the mechanisms of the chilling injury or tolerance, most studies have utilized comparisons of the metabolic difference between chilling-tolerant and -susceptible cultivars as model systems (Jahnke *et al.*, 1991; Pinhero *et al.*, 1997; Saruyama & Tanida, 1995; Walker & McKersie, 1993).

Once the plants has become established, the earliest symptom of chilling is the damage to the photosynthetic apparatus as evidenced by reduced CO_2 fixations and altered chlorophyll a fluorescence patterns (Walker *et al.*, 1991). Other symptoms, such as water loss and wilting (Koscielniak, 1993; Wilson, 1976), the closure of leaf stomata at chilling temperatures, along with increased water viscosity and reduced root permeability (Miedema, 1983), impairs the ability to transport water (Stamp, 1984) occur. Reduced accumulation of dry mass in roots and shoots (Stamp, 1984) are also common visible symptoms of chilling stress.

There were no differences in physiological parameters during growing at 25°C (Figs. 1, 2) between the chilling-tolerant and -susceptible cultivars. During chilling, however, the chilling-tolerant cultivar showed a higher tolerance to chilling stress than the chilling-susceptible cultivar with respect to the parameters of leaf fresh weight, relative water content, lipid peroxidation, F_v/F_m ratio, and quantum yield (Figs. 1, 2). The chilling tolerant-cultivar was also faster in recovery than the chilling-susceptible cultivar. These results suggest that chilling-tolerant cultivar diminishes oxidative damage during chilling and subsequent recovery. A similar phenomenon was demonstrated in other plant species, such as tomato and maize (Brûggemann *et al.*, 1999; Hodges *et al.*, 1997).

As exposure to low temperatures can induce an overproduction of ROS (Okuda *et al.*, 1991), the ROS trigger a series of deleterious processes, such as lipid peroxidation and degradation of proteins and DNA damage in the cell (Fridovich, 1978; Halliwell & Gutteridge, 1986; Scandalios, 1993). Lipid peroxidation as a measure of cellular injury was increased by over 50% and 180% in the chilling-susceptible cultivar, compared to control leaves or the chilling-tolerant cultivar, during chilling and recovery, respectively (Fig. 1D). Thus, we conclude that chilling and subsequent recovery conditions induce oxidative stress in rice plants. Correlative evidence from different studies also suggests that the chilling sensitivity of plants increases with increased lipid peroxidation (Shewfelt & Erickson, 1991; Prasad, 1996). On the other hand, all of these studies suggest that ROS-induced lipid peroxidation was, at least in part, responsible for increased chilling sensitivities in maize seedlings and, perhaps, all of the chilling-sensitive crop plants.

SODs catalyse the dismutation of O_2^- to H_2O_2 and O_2 (McCord & Fridovich, 1969) and high SOD activity has been associated with stress tolerance in plants where overproduction of O_2^- is involved (Bowler *et al.*, 1992). In this study, total SOD activity in leaves was not significantly different between the chilling-tolerant and -susceptible cultivars during chilling and recovery except for 3 days after chilling and 2 days after recovery (Fig. 3A). Total SOD activity in roots of the chilling-susceptible cultivar was rapidly decreased with increasing chilling and recovery duration, but the SOD activity in roots of the chilling-tolerant cultivar was almost the same or little decreased as control plants during chilling and recovery. Thus, it is possible that the maintenance of constant SOD levels in the chilling-tolerant cultivar could be more sufficient in comparison to the chilling-susceptible cultivar to ensure protection against the O_2^- produced during chilling and subsequent recovery because of the high SOD protein turnover (Scandalios, 1993). The existence of multiple molecular forms of SODs, their location within cells, tissues, or organelles and the changes observed, during plant development or in response to different stress imply a separate role for each of the SOD isozymes (Scandalios, 1993). We observed two SOD isozymes during chilling and recovery, respectively on non-denaturing polyacrylamide gels, but none of them was strikingly affected by chilling and recovery (Fig. 5A).

In our research, CAT activity was decreased with increasing chilling duration in both chilling-tolerant and -susceptible cultivars in leaves and roots (Figs. 3B, 4B). This confirms the high cold-sensitivity of CAT, as observed in wheat, cucumber and rice (Omran, 1980; Mishra *et al.*, 1993; Saruyama & Tanida, 1995). However, there was a significant difference between the chilling-tolerant and -susceptible cultivars in the CAT activity and the staining intensity of two CAT isoforms during chilling and recovery. The higher CAT activity and band intensity in the chilling-tolerant cultivar suggests a more efficient scavenging of

H_2O_2, which may result in better protection against this toxic molecule during chilling and recovery.

The cold lability of CAT in both rice cultivars and a corresponding increase in APX in the chilling-tolerant cultivar suggested the significance of the co-operative function of APX in compensating for the loss of CAT activity in those cultivars during chilling stress. The important role of APX in relation to the increase of oxidative tolerance has been reported for many plants (Feierabend *et al.*, 1992; Gupta *et al.*, 1993; Lee & Lee 2000). In this study, significant change in APX activity of leaves and root was observed between the chilling-tolerant and -susceptible cultivars during chilling and recovery (Figs. 3C, 4C). That is, APX activity of leaves and roots in the chilling-tolerant cultivar was almost the same as the control, or rather increased by the chilling and subsequent recovery. In contrast, the chilling-susceptible cultivar showed a decrease in activities by chilling and recovery. This difference also showed in the band intensity of APX isozymes (Fig. 5C). The higher band intensity of some isozymes and total activity in the chilling-tolerant cultivar, suggesting that these could be important for detoxification of H_2O_2. Unlike to our results, total APX activity was unaffected by chilling in mesocotyls of dark-grown maize seedling (Prasad *et al.*, 1994).

GR has also been shown to be important in protection from oxidative stress in many plants (Aono *et al.*, 1993; Foyer *et al.*, 1991; Guy & Carter, 1984). We observed three isozymes of GR in leaves. No significant changes in GR isozymes were observed in both cultivars (Fig. 5D). Similar to GR isozymes, GR activity in leaves was not different between the chilling-tolerant and -susceptible cultivars during chilling and recovery (Fig. 3D). However, GR activity in the roots of the chilling-tolerant cultivars was higher than in those of chilling-susceptible cultivars during recovery. In addition, the GR activity of leaves was found to be decreased in both the cultivars as compared with the control during chilling. Similar to our results, the GR activity in rice cultivars was decreased at 4°C (Fadzillah *et al.*, 1996). However, other researchers reported that GR activity was increased slightly by acclimation and examination of GR isozymes profiles revealed three isozymes that were greatly affected by acclimation in maize seedling. Exposure to low temperature has also resulted in altered GR isozyme profiles in pea (Edwards *et al.*, 1994) and spinach (Guy & Carter, 1984) that are correlated with cold tolerance.

We found clear differences between the chilling-tolerant and -susceptible rice cultivars. This was partly due to a result of the cold sensitivity of antioxidant enzyme system, SOD and APX of leaves and SOD, CAT, APX, and GR of roots, especially may involve up-regulating CAT and APX of leaves and roots. Thus, CAT and APX prevented the accumulation of ROS during chilling and subsequent recovery. However, ROS should be taken into consideration that the mechanism of protection from chilling stress is quite complicated and not only dependent on the activity of antioxidative enzymes, but also on other enzymes and on the production of several antioxidant molecules (Badiani *et al.*, 1997; Leipner *et al.*, 1997). Our results also indicate a differential genotype behaviour between chilling-tolerant and -susceptible cultivars, which may confer a more effective defence against ROS during chilling and subsequent recovery.

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