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Cross-Tolerance and Responses of Antioxidative Enzymes of Rice to Various Environmental Stresse

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ABSTRACT In order to examine the cross-tolerance of two chilling-tolerant cultivars (Donganbyeo and Heukhyangbyeo) and two chilling-susceptible cultivars (Hyangmibyeo and Taekbaekbyeo) to salt, paraquat, and drought, changes of physiological response and antioxidant enzymes were investigated. The seedlings were grown in a growth chamber until the 4-leaf stage. The seedlings were exposed to chilling at 5℃ for 3 days. For drought treatment, the seedlings were subjected to drought by withholding water from plants for 5 days. For paraquat study, plants were sprayed with 300 μM paraquat. For the salt stress, the seedlings were transferred to the Hoagland's nutrient solution containing 0.6% (w/v) NaCl for 4 days. Chilling-tolerant cultivars showed cross-tolerant to other stresses, salt, paraquat, and drought in physiological parameters, such as leaf injury, chlorophyll a fluorescence, and lipid peroxidation. The baseline levels of antioxidative enzyme activities, catalase (CAT) and peroxidase (POX) activities in chilling-tolerant cultivars were higher than in the chilling-susceptible cultivars. However, there were no differences in ascorbate peroxidase (APX) and glutathione reductase (GR) activities between chilling-tolerant and -susceptible cultivars in untreated control. CAT activity in chilling-tolerant cultivrs was higher than that in chilling-susceptible cultivars during chilling, salt, and drought treatments, but not during paraquat treatment. However, other antioxidative enzymes, APX, POX, and GR activities showed no significant differences between chilling-tolerant and -susceptible cultivars during chilling, salt, paraquat, and drought treatments. Thus, it was assumed that CAT contribute to cross-tolerance mechanism of chilling, salt, and drought in rice plants.

Keywords : antioxidative enzymes, cross-tolerance, environmental stress; rice (*Oryza sativa* L.)

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Envirionmental stress conditions, such as extreme emperature, drought, and high salt condition, are detrimental to plant growth and development and thus affect the productivity of various crops around the world. Many of the important food crops, such as rice, soybean, and corn, are often exposed to the environmental stresses in the fields. When plants are subjected to environmental stresses, the generation of a variety of toxic, 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) have been noted.

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, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and various peroxidases such as guaiacol peroxidase (POX, EC 1.11.17) 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; 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 ROS (Hodges *et al.*, 1997; Wise & Naylor, 1987).

In several herbaceous plant species, antioxidative systems were induced in response to drought stress, suggesting that water deficit required increased capacities of protective systems for stress compensation (Smirnoff, 1993). Perl-Treves and Galun (1991) reported a paraquat-mediated increase in the steady state of tomato cytosolic Cu, Zn SOD mRNA. In maize, paraquat exposure led to an increase in chloroplast and cytosolic Cu, Zn SOD activities (Matters & Scandalios, 1986). Resistance to paraquat was correlated

with the enhanced activity of antioxidant enzymes in *Conyza bonariensis* (Amsellem *et al.* 1993; Fuerst & Vaughn, 1990). 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 reduced glutathione (GSH), peroxidase, and CAT levels were enhanced (Upadhyaya *et al.*, 1989). Induced SOD and APX activities were correlated with increased chilling tolerance in paclobutrazol-induced chilling tolerance in chilling-sensitive maize inbred (Pinhero *et al.*, 1997).

Other mechanisms have also been proposed to explain the drought, chilling, salt, and paraquat injury or tolerance in plants (Basra, 2001). Some of the changes related to environmental stresses include alterations in gene expression, composition of proteins, lipids, and carbohydrate, membrane damage (lipid peroxidation), solute leakage, mitochondrial respiration, and photosysnthesis (Bishop *et al.*, 1987; Foyer *et al.*, 1994; Guy, 1990; Howarth & Ougham, 1993; Markhart, 1986; Smirnoff, 1993; Tanaka *et al.*, 1986; Thomashow, 1990; Wang, 1982).

Although there are extensive studies of salt, drought, low temperature, and paraquat effects on rice and other crops, it is not known whether tolerance to low temperature correlate with tolerance to other stresses in rice. Therefore, the objectives of this research are to examine cross-tolerance to other stresses such as salt, paraquat, and drought in chilling-tolerant rice cultivars and to examine whether ROS-scavenging systems are related to the tolerance to the stresses.

MATERIALS AND METHODS

Plant growth and treatment conditions

Seeds of four rice cultivars, two chilling-tolerant cultivars (Donganbyeo and Heukhyangbyeo) and two chilling-susceptible cultivars (Hyangmibyeo and Taekbaekbyeo) were soaked in water for 4 days at 25℃ and were sown in commercial soil (NP·KO, Korea) in trays (15×10 cm) in a temperature-controlled greenhouse at 30±3/25℃ ± 3℃, day/night temperature. The conditions of the growth chamber were 70% relative humidity, 30/25℃ (day/night), and light intensity of 250 μmol m^{-2} s^{-1} with a 14 h photoperiod. The seedlings were grown in the growth chamber until reached the 4-leaf stage. The seedlings were exposed to chilling at 5℃ for 3 days. For drought treatment, one group of 4-leaf stage plants was maintained under optimal irrigation (control) and the other group was exposed to drought by withholding water from plants for 5 days. For paraquat study, plants were sprayed with 300 μM paraquat. Treated plants were kept in the dark for 4 h to improve herbicide absorption. The plants were then returned to the growth chamber where they were exposed to light conditions (250 μmol m^{-2} s^{-1}) for 2 days. For the salt stress, 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 until the 4-leaf stage. The seedlings were transferred to the Hoagland's nutrient solution containing 0 and 0.6% (w/v) NaCl for 4 days.

The levels of leaf injury by environmental stresses were visually assessed each time after respective stress treatment on a scale of 0 to 100, where 0 equals no injury and 100 equals complete death. The conditions of chilling, salt, praquat, and drought were selected from preliminary experiment.

Lipid peroxidation

Lipid peroxidation was estimated by quantifying the amount of malondialdehyde (MDA) production using a slight modification of the thiobarbituric acid (TBA) method as previously described by Buege and Aust (1978). Each leaf (0.5 g) was harvested for each treatment period, and the tissue was homogenized using a mortar and pestle, in 5 mL of 0.5% TBA in 20% trichloroacetic acid. The homogenate was then 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 was allowed to cool in an ice bath. Following additional centrifugation at 20,000 ×g for 15 min, the resulting supernatant was used for the spectrophotometric determination of MDA. Absorbance was recorded at 532 nm for each sample and corrected for non-

specific turbidity at 600 nm. MDA concentrations were calculated using a molar extinction coefficient of 156 mM^{-1} cm^{-1}.

Chlorophyll (Chl) a fluorescence

In vivo Chl a fluorescence was measured at room temperature, using a pulse amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany). Before measuring fluorescence, the leaves were adapted to darkness for 10 min to minimize the amount of fluorescence quenching associated with thylakoid membrane electron excitation (Krause *et al.*, 1983). A minimal fluorescence yield, F_0, was obtained upon excitation of the leaves with a weak measuring beam of 0.12 μmol m^{-2} s^{-1} from a pulse light-emitting diode. A maximum fluorescence yield, F_m, was determined after exposure to a saturating pulse of white light to reduce all reaction centers. The ratio of variable to maximum fluorescence (F_v/F_m) derived from the measurement was used as an estimate of the maximum photochemical efficiency of photosystem (PS) II (Butler, 1978).

Protein extraction

The frozen leaves (0.5 g) were pulverized in liquid N_2 using a mortar and pestle, and then resuspended in 3 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM ethylenediaminetetraacetic acid, 1% polyvinylpyrrolidone -40, and 1 mM phenylmethylsulfonyl fluoride. For APX assay, the extraction buffer also contained 5 mM ascorbate. The suspension was centrifuged at 15,000×g for 20 min, at 4℃, and the resulting supernatant was used directly as an enzyme source. Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Enzyme assays

All the enzymes were assayed in a 1-mL cuvette, at 24 to 26℃. The activity was linear in respect to time and the enzyme concentrations. Spectrophotometric determinations were carried out with UV/Visual wavelength spectrophotometer (Ultrospec 4000, Pharmacia Biotech, England) operated in the split beam mode.

CAT activity was assayed according to the method of Mishra *et al.* (1993) by monitoring the decline in absorbance at 240 nm (ε = 36 M^{-1} cm^{-1}) as a result of H_2O_2 degradation. The APX activity was estimated with the method of Chen and Asada (1989) by monitoring the decline in absorbance at 290 nm as ascorbate (ε = 2.8 mM^{-1} cm^{-1}) was oxidized. POX activity was determined specifically with guaiacol at 470 nm following the method of Egley *et al.* (1983). GR was measured with the method of Rao *et al.* (1996) by monitoring the decline in absorbance at 340 nm as nicotinamide adenine dinucleotide phosphate (NADPH) (ε = 6.2 mM^{-1} cm^{-1}) was oxidized. Data were expressed as percentages of the control values.

RESULTS AND DISCUSSION

Physiological responses of chilling-tolerant and -susceptible rice cultivars to various environmental stresses Based on visual observations, the common symptoms of low temperature, salt, drought, and paraquat included wilting, yellowing of leaves, and inhibition of growth. Therefore, we visually investigated leaf injury during various environmental stresses (Fig. 1). Chilling-tolerant cultivars (Donganbyeo and Heukhyangbyeo) showed less leaf injury than chilling-susceptible cultivars (Hyangmibyeo and Taebaekbyeo) during chilling, salt, paraquat, and drought treatments. Although the levels of tolerance based on leaf injury were different among cultivars and kinds of stress, chilling-tolerant cultivars were also tolerant to other stresses, salt, paraquat, and drought.

Similar to leaf injury, chl a fluorescence (F_v/F_m) values in chilling-tolerant cultivars were higher than in chilling susceptible cultivars (Fig. 2) during chilling, salt, paraquat, and drought treatments. This result means that chilling-tolerant cultivars were cross-tolerant to other stresses, salt, paraquat, and drought.

The earliest symptom of chilling stress is observed in the photosynthetic apparatus in which CO_2 fixation is reduced and chlorophyll a fluorescence patterns are changed (Walker *et al.*, 1991). This means that the most immediate chilling injury is due to the photooxidation and reduced photosynthetic efficiency. The negative effect of cold treatment on

photosynthetic capacity and chlorophyll content is linked to the changes in lipid and protein components of the thylakoid membrane (Mostowska, 1997). For instance, exposing pea (*Pisum sativum* L.) to a chilling temperature (5℃) caused ultrastructural damage to the inner membrane of chloroplasts (Wise & Naylor, 1987). Due to chloroplast damage, cold stress results in the loss of chlorophyll, and lipid peroxidation results in the damage of cell membranes which subsequently causes the leakage of cell contents and loss of water (Koscielnak, 1993; Wilson, 1976). Therefore, visual symptoms of chilling damage include yellowing, wilting, inhibition of plant growth, and acceleration of senescence (Salveit &

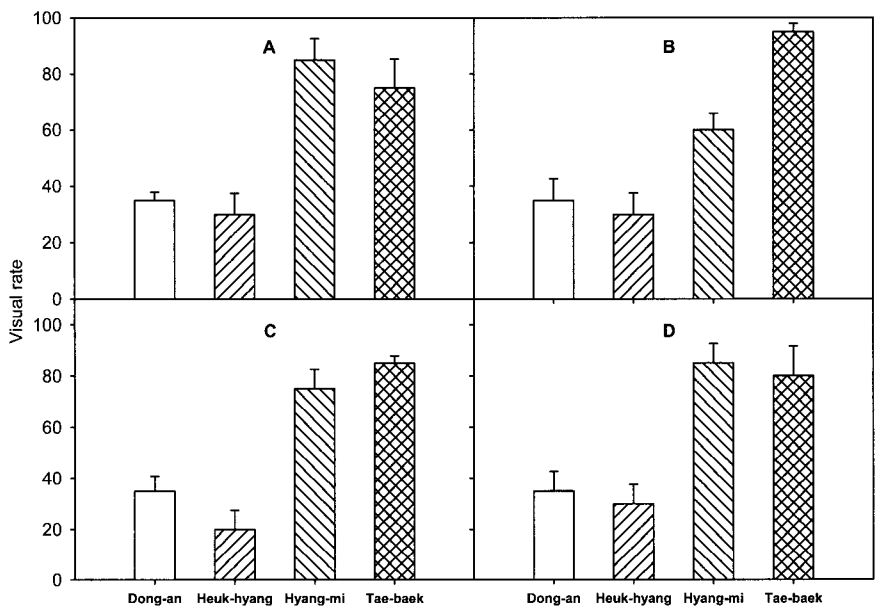

Fig. 1. Effect of low temperature (A), salt (B), paraquat (C), and drought (D) on leaf injury in rice. Visual rate (0-100, 0, no injury; 100, complete killed). Values are the mean±SE of three replicates.

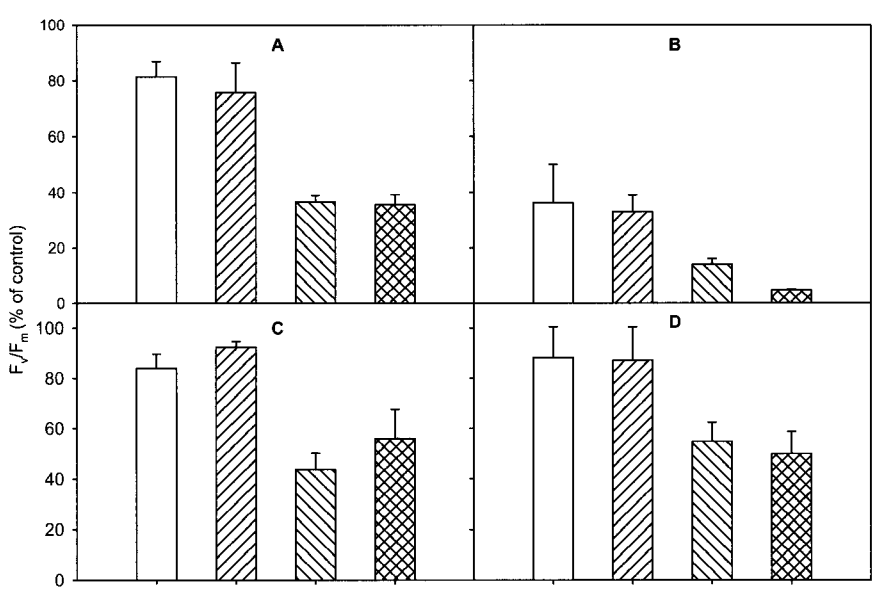

Fig. 2. Effect of low temperature (A), salt (B), paraquat (C), and drought (D) on Chl a fluorsencence (F_m/F_v) in rice. Values are the mean±SE of three replicates. In some cases, the error bar is obscured by the symbol.

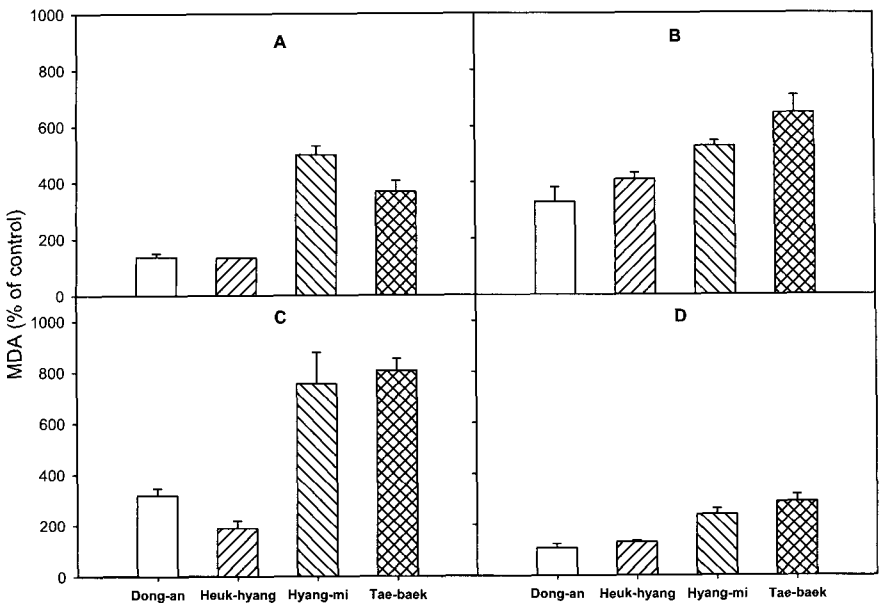

Fig. 3. Effect of low temperature (A), salt (B), paraquat (C), and drought (D) on MDA production in rice. Values are the mean±SE of three replicates. In some cases, the error bar is obscured by the symbol.

Morris, 1990).

The level of MDA production was measured to estimate the lipid peroxidation during stresses (Fig. 3). Similar to leaf injury and chl a fluorescence (F_v/F_m), there was a big difference in lipid peroxidation between the chilling-tolerant and -susceptible cultivars during chilling. This result means that the levels of lipid peroxidation in chilling-susceptible cultivars were more increased than those in chilling tolerant cultivars during chilling. The levels of lipid peroxidation in chilling-susceptible cultivars were also higher than in chilling-tolerant cultivars during other stresses, salt, paraquat, and drought treatments. Thus, this result suggests that chilling-tolerant cultivars were corss-tolerant to other stresses, salt, paraquat, and drought.

Lipid peroxidation, as a measure of cellular injury from various environmental stresses, increased by over two-fold in stress-treated leaves compared to nonstress-treated ones during stress. This indicates that stress conditions caused damage to cell membranes, which resulted in leakage of cell contents and significant dehydration of leaves in stressed rice plants. In maize, lipid peroxidation also increased by about two-fold in nonacclimated seedlings compared to control or cold-acclimated seedlings during and after chilling (Prasad, 1996).

Although the levels of tolerance in chilling-tolerant cultivars were different among stresses, the chilling-tolerant cultivars appeared cross-tolerant to other environmental stresses. Other studies have demonstrated the existence of cross-tolerance to various oxidative stress-generating factors, biotic or abiotic. Cotton (*Gossypium hirsutum* L.) plants that were deprived of water were found to be more resistant to paraquat than water-replete plants, and ozone-tolerant tobacco varieties showed cross-tolerance to paraquat (Burke *et al.*, 1985; Shaaltiel *et al.*, 1988). Inbred varieties of paraquat-tolerant maize also showed higher resistance to drought stress (Malan *et al.*, 1990). In pea plants, resistance to sulphur dioxide was correlated with resistance to paraquat (Madamanchi *et al.*, 1994). In addition, tolerance to paraquat in cucumber leaves was correlated with low-temperature tolerance (Kuk & Shin, 2007). There is a universal systematic response to various stress factors that cause oxidative stress (Hughes & Dunn, 1996). Therefore, different stresses produce similar effects at the cellular level (Bowler *et al.*, 1992).

Antioxidant enzyme activities in chilling-tolerant and -susceptible rice cultivars to other stresses

In untreated plants, antioxidative enzyme activities, APX and GR were generally the same between chilling-tolerant (Donganbyeo and Heukhyangbyeo) and chilling-susceptible cultivars (Taekbackbyeo and Hyangmibyeo) except for one

cultivar (Taekbackbyeo) in APX during the nontreated stress condition (Fig. 4). However, the baseline levels of antioxidative enzyme activities, CAT and POX activities in chilling-olerant cultivars were higher than those in the chilling-usceptible cultivars. No differences were found in GR activity between the chilling-tolerant and -susceptible cultivars during chilling (Fig. 5). However, the CAT activity in chilling-tolerant cultivars was higher than that in chilling-susceptible cultivars during chilling. APX activity in the chilling-tolerant cultivars was higher than in the chilling-susceptible cultivar, Taekbackbyeo, but not in Hyangmibyeo during chilling. Donganbyeo, a chilling tolerant cultivar, showed higher POX activity than other cultivars regardless the degree of cold tolerance.

No significant change in APX activity was observed between chilling-tolerant and chilling-susceptible cultivars dur-

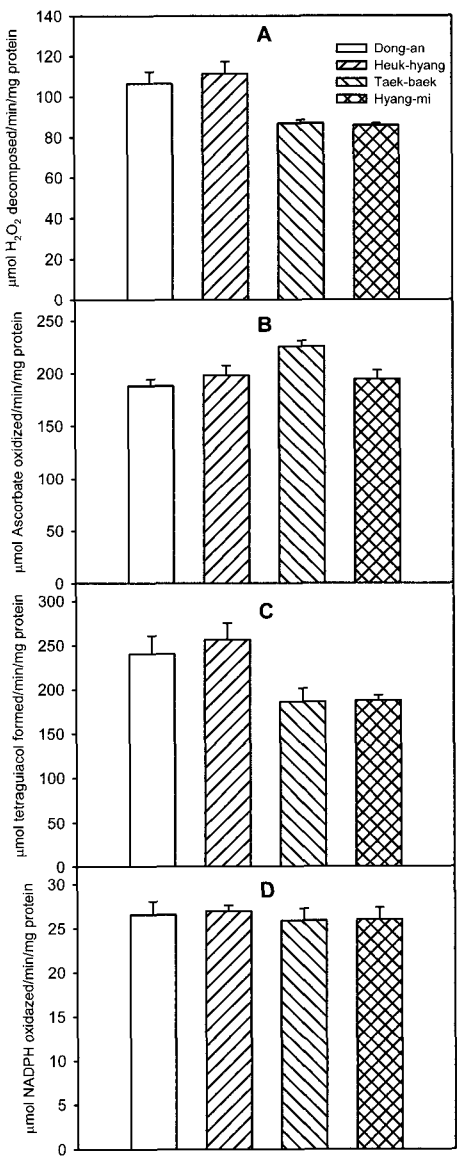

Fig. 4. CAT (A), APX (B), POX (C), and GR (D) activities in untreated rice cultivars. Enzyme activities are expressed as enzyme unit mg^{-1} protein h^{-1}. Values are the mean±SE of three replicates. In some cases, the error bar is obscured by the symbol.

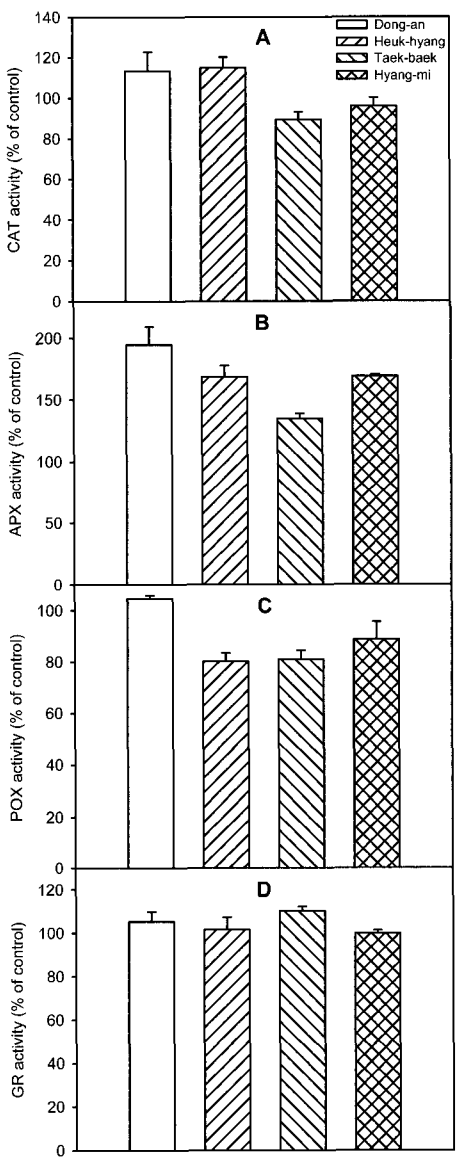

Fig. 5. Effect of low temperature on CAT (A), APX (B), POX (C), and GR (D) in rice. Enzyme activities are expressed as percentage of the untreated control (enzyme unit mg^{-1} protein h^{-1}). Values are the mean± SE of three replicates. In some cases, the error bar is obscured by the symbol.

ing salt treatment (Fig. 6). Although CAT activity was decreased in both chilling-tolerant and -susceptible cultivars compared with control plants, the CAT activity in the chilling-tolerant cultivars was higher than in the chilling-susceptile cultivars during salt treatment. In contrast, the POX activity in the chilling-susceptible cultivar (Taekbackbyeo) was higher than that in the chilling-tolerant cultivars and -susceptible cultivar (Hyangmibyeo) during salt treatment. GR activity in the chilling-tolerant cultivars and chilling-susceptible cultivar (Taekbackbyeo) was higher than that in the chilling-susceptible cultivar (Hyangmibyeo) during salt treatment.

No differences were found in CAT, APX, and GR activities between chilling-tolerant and -susceptible cultivars in the treatment of paraquat (Fig. 7). However, POX activity in chilling-susceptible cultivar, Hyangmibyeo was higher

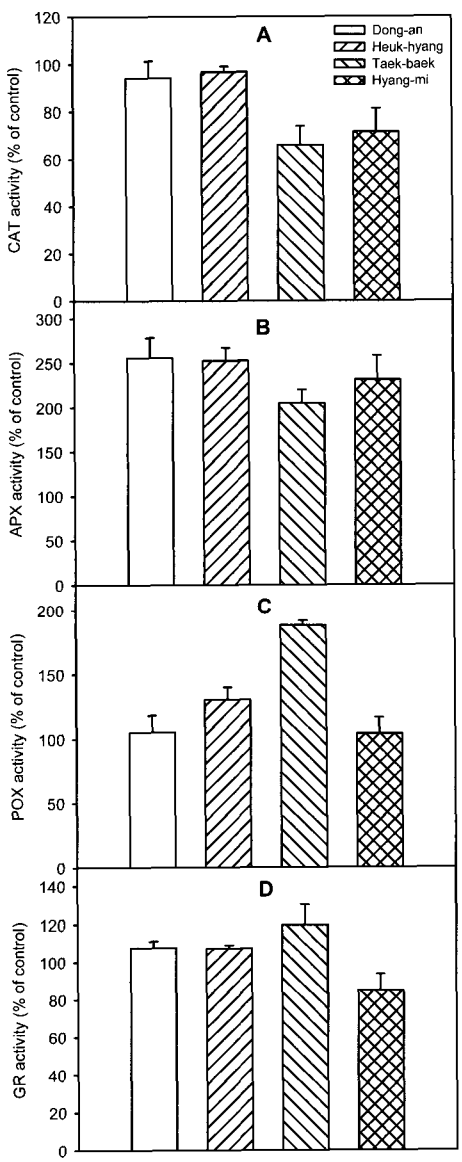

Fig. 6. Effect of salt on CAT (A), APX (B), POX (C), and GR (D) in rice. Enzyme activities are expressed as percentage of the untreated control (enzyme unit mg^{-1} protein h^{-1}). Values are the mean±SE of three replicates. In some cases, the error bar is obscured by the symbol.

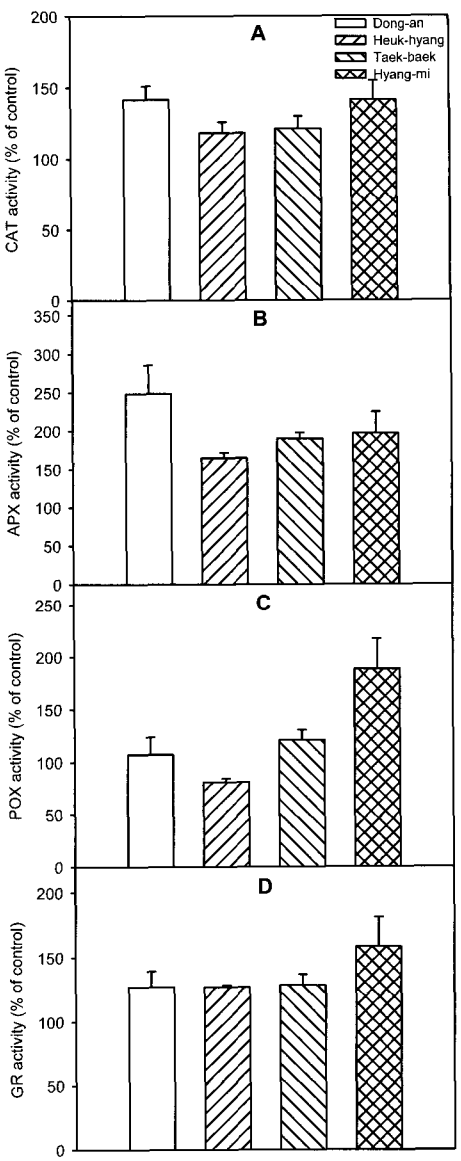

Fig. 7. Effect of paraquat on CAT (A), APX (B), POX (C), and GR (D) in rice. Enzyme activities are expressed as percentage of the untreated control (enzyme unit mg^{-1} protein h^{-1}). Values are the mean±SE of three replicates. In some cases, the error bar is obscured by the symbol.

than in the chilling-tolerant cultivars and -susceptible cultivar, Taekbackbyeo in paraquat treatment.

CAT activity in the chilling-tolerant cultivars was higher than that in the chilling-susceptible cultivars during drought treatment (Fig. 8). POX activity in the chilling-susceptible cultivars was higher than that in the chilling-tolerant cultivars during drought stress. However, there were no differences in APX and GR activities between chilling-tolerant and -susceptible cultivars during drought treatment.

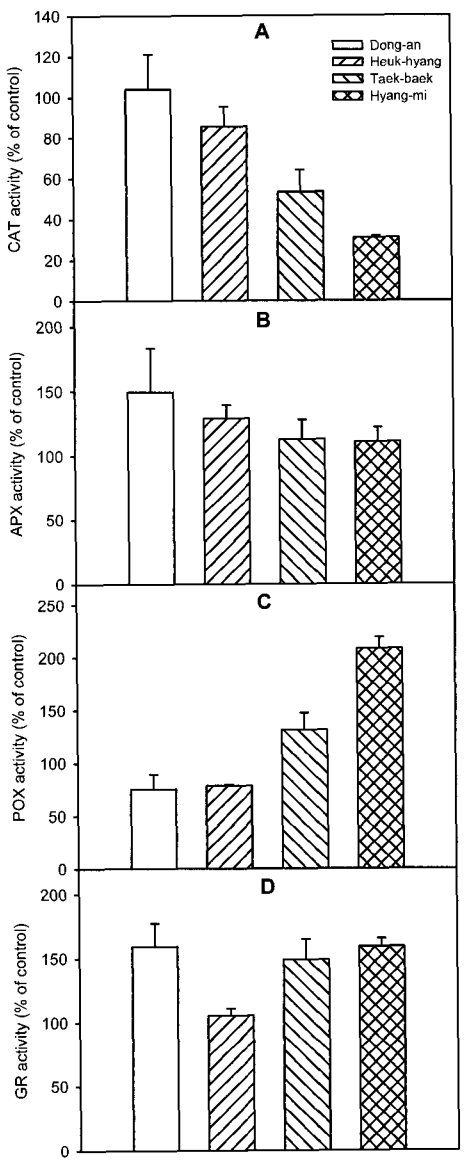

Fig. 8. Effect of drought on CAT (A), APX (B), POX (C), and GR (D) in rice. Enzyme activities are expressed as percentage of the untreated control (enzyme unit mg^{-1} protein h^{-1}). Values are the mean±SE of three replicates. In some cases, the error bar is obscured by the symbol.

Plants produce ROS in greater quantities when exposed to environmental stresses such as drought, salt, herbicides, metal, and low temperatures. The ROS trigger a series of deleterious effects such as lipid peroxidation and degradation of proteins and DNA damage in the cell (Fridovich, 1978; Halliwell & Gutteridge, 1986). Under normal conditions, the antioxidant system is usually sufficient in preventing damage (Hideg, 1997). However, stressful environmental conditions impare the plant's natural capacity to dissipate excess light energy and scavenge oxygen radicals.

In our study, CAT activity was decreased in both chilling-tolerant and -susceptible cultivars during salt and drought treatments. This confirms the high stress-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 during salt and drought. In other stress, CAT activity was increased in chilling tolerant cultivars during chilling treatment compared with the untreated control, while CAT activity was descreased in the chilling-susceptible cultivars. Also, CAT acitivity of chilling-tolerant cultivars in untreated control was higher than that of chilling-susceptible cultivars. This was partly due to the higher tolerance to stress.

APX, POX, and GR have also been shown to be important in protection from various environmental stresses in many plants (Aono *et al.*, 1993; Foyer *et al.*, 1991; Kuk *et al.*, 2003), but not in our rice cultivars.

In chilling-tolerant cultivars, increase in CAT activity appeared to play an important role in chilling, salt and drought tolerance; however, it could not fully account for the high level of the stress tolerance observed. A mechanism other than increased enzyme activity must be in place to explain the high levels of the stress tolerance observed. One such mechanism may be a more active role of nonenzymatic antioxidants, such as ascorbic acid and GSH.

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