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Activity and Isozyme Profile of Antioxidative Enzymes at Booting Stage of Rice Treated with Cold Water

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ABSTRACT: This study was carried out to investigate the antioxidative enzymes and isozymes between chilling-tolerant and -susceptible varieties at the booting stage under cold water stress (13°C) in japonica rice. Total SOD, CAT, POX, and GR activities on the basis of protein were found to be important factors to defend cold water stress. Especially, SOD and CAT activities showed distinctive differences between chilling-tolerant and -susceptible varieties. Chilling-tolerant varieties were higher than chilling-susceptible varieties for SOD and CAT activities. One of eight isozyme bands for SOD was a inducible isoform. Three isozymes for CAT and one isozyme for POX were closely correlated with defense to cold water stress. Total GR activities except Stejaree 45 on the basis fresh weight and POX were increased by cold water stress, but there was no difference between chilling-tolerant and -susceptible varieties.

Keywords: rice, leaf, antioxidative enzymes, isozymes, booting stage, cold water stress

Responses of plant to environmental stress such as low temperature, drought, and high salinity have been studied intensively (Thomashow, 1999; Xiong *et al.*, 2002).

Membrane systems of the cell are the primary site of freezing injury in plants (Steponkus, 1984) Plants has evolved to stabilize membrane under low temperature stress conditions (Thomashow, 1999).

Environmental stress induces reactive O_2 species (ROS) in plant tissues (Alscher & Hess, 1993). ROS is produced as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) during metabolic processes. These ROS injure the cellular components of proteins, membrane lipids, and nucleic acids (Scandalios, 1993). To control the level of ROS and to protect cells under stress conditions, plant tissues contain several enzymes scavenging ROS [superoxide dismutase (EC; 1 15 1 1), catalase (EC; 1.11.1.6), peroxidases (EC; 1.11.1.7), and glutathione peroxidase] (Blokhina *et al.*, 2003). Both enzymic and nonenzymic mechanism have evolved to overcome oxygen toxicity (Alscher & Hess, 1993). These include antioxidants such as glutathione, ascorbate, and -tocopherol. Catalase (CAT) and peroxidases (POX) remove H_2O_2 and superoxide dismutase (SOD) converts superoxide to hydrogen peroxide (Blokhina *et al.*, 2003). These antioxidants compounds and antioxidative enzymes may play important roles to remove of toxic oxygen compounds (Prasad, 1997; Alscher *et al*, 2002; Kawano, 2003). Although chilling-tolerant plants has been shown to more increase antioxidants and antioxidative enzymes than chilling-susceptible ones, the various antioxidant enzymes and isozymes are differently compartmentalized and responded in the tissues(Anderson *et al.*, 1995; Saruyama *et al.*, 1995, Doulis *et al.*, 1997; Kuk *et al.*, 2002).

The objective of this study is to compare the antioxidative enzymes response between chilling-tolerant and -susceptible varieties of japonica rice at the booting stage and to examine whether these enzyme can be used to screen chilling-tolerant rice

MATERIAL AND METHODS

Plant material and treatment conditions

Four Japonica rice varieties selected through cold water treatment in 2002 (Table 1) were used to examine antioxidative enzymes response at the booting stage under cold water stress. Chilling-susceptible rice varieties (HR19621-AC6 and Sambaegbyeo) and chilling-tolerant rice varieties (Hitomebore and Stejaree 45) were transplanted in 1/5000 pot at 20 days after seeding. Fertilizers were applied at the rate of 11, 7.7 and 9 3 kg/10a in N, P_2O_5 and K_2O, respectively. Nitrogen fertilizers were split into two times of 60 and 40% as basal fertilizer and top dressing. The samples were selected at the booting stage of rice on the basis of the auricle distance (the distance between auricles of flag leaf and the previous leaf of the main culm). These plants with an

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Table 1. Comparison of fertility indices among varieties under cold water stress

Varieties	Fertility Indices		
	Inlet	Outlet	Mean
HR19621-AC6	4 9±6.9	65 6±7.8	35.2
Sambaegbyeo	16.4±8 3	57 2±3.9	36.8
Hitomebore	52 8±5.2	92 9±1 6	72 8
Stejaree 45	80 4±4 9	96 0±0 4	88 2
Mean	38 6	77.9	58.3
LSD at 5% level			
Varieties(V)	9 1		
Treatment(T)	6 4		
V*T+	**		

$$\text{Fertility Indices} = \frac{\text{Are Sine } \sqrt{\text{Treatment}}}{\text{Are Sine } \sqrt{\text{ Control Fertility}}} \times 100$$

+Comparison among varieties within treatment.

auricle distance ranging from -5 cm to 0 cm were exposed until flag auricle using cold water (13°C) by 3 days and 7 days, respectively. Flag leaf was used to examine antioxidative enzymes and their isozymes.

Protein extraction

Frozen leaves (0.3 g) were pulverized with a mortar and pestle using liquid N_2, and then resuspended with the extraction buffer contained 100 mM sodium phosphate buffer (pH 7.0), 10 mM sodium ascorbate and 5 mM diethylene triaminepentaacetic acid (DTPA) for SOD (Spychalla *et al.*, 1990), and with the extraction buffer contained 100mM sodium phosphate buffer (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethysulfonyl floride (PMSF), and 10 mg/m*l* polyvinylpolypyrrolidone (PVPP) (Anderson *et al.*, 1995) for catalase and peroxidase, and with the extraction buffer contained 50 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 1 mM acrobate, and 1% (w/v) PVPP (O'Kane *et al.*, 1996) for GR. The suspension was centrifuged at 12,000 g for 20 min at 4 °C. The resulting supernatant was directly used as an enzyme source The protein levels were determinated by the methods of Bradford (1976) with bovine serum albumin as a standard.

Enzyme assays and isozyme analysis

SOD activity was determined by the xanthine oxidase-nitro blue tetrazolium (NBT) assay (Oberly & Spitz, 1984). The reaction mixture (1 m*l*) contained 50 mM potassium phosphate (pH 7.8), 0.1 mM xanthine, 0 056 mM NBT, 1 mM diethylenetriamine pentaacetic acid (DETAPAC), and 1 unit catalase. 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 the absorbance at 560 nm of 0.017 units/min. SOD isozymes were analyzed after separating proteins on 10% polyacrylamide gels as described (Yun & Lee, 1994).

CAT activity was assayed by determining the rate of change in the absorbance at 240 nm in a reaction mixture (1 m*l*) that contained 50 mM potassium phosphate (pH 6.9), 100 mM H_2O_2 and 10 mM DTT at 25°C (Beers & Sizer, 1952). Catalase isozyme patterns were detected on a 7% polyacrylamide gel by incubating the gel in the substrate (3.27 mM H_2O_2) and developing (1% potassium ferricyanide and 1% ferric chloride) solutions (Woodbury *et al.*, 1971) In the loading buffer for catalase, 60 mM DTT was substituted for β-ME.

Peroxidase activity was measured spectrophotometically by monitering the decline in the absorbance at 470 nm in a reaction mixture that consisted of 50 mM potassium phosphate (pH 6.4), 10 mM guaiacol, and 100 mM H_2O_2 (Chance *et al*, 1955). Peroxidase isozymes were detected on a 10% polyacrylamide gel by incubating 100 mM sodium acetate buffer (pH 4.5) contained 2 mM benzidine (Rao *et al.*, 1996).

GR (EC; 1.6.4.2) activity was assayed by monitering the decline in the absorbance at 340 nm as NADPH was oxidized (O'kane *et al.*, 1996). The reaction mixture was consisted of 100 mM Tris-HCl (pH 7.8), 2 *n*M EDTA, 50 *u*M reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), and 0.5 *n*M oxidized L-glutathione (GSSG).

GR isozymes bands were analysed on a 10% polyacrylamide gel by staining buffer composed of 0.24 mM 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenylteyrazolium bromide (DPIP), 0.4 mM NADPH, 0.34 mM 2,6-dichloropheolindphenol (MTT), and 250 *n*M Tris (pH7.8) contained 3.6 mM GSSG in the dark (Anderson *et al*, 1995).

RESULTS AND DISCUSSION

Total SOD, CAT, POX, and GR activities were assayed with the chilling-tolerant and β-susceptible japonica rice varieties under cold water stress (13°C) at the booting stage. SODs are metalloproteins that catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen (Takahashi & Asada, 1983). This enzyme is ubiquitous in aerobic organisms where it plays a major role in defense generation of reduced oxygen radical-mediated toxicity. SODs have been proposed to be important in plant stress tolerance (Mckersie *et al.*, 1993, 1999; Gupta *et al*, 1993).

Differences were detected in total SOD activity between chilling-tolerant and -susceptible varieties. SOD activity was

more increased in chilling-tolerant varieties than in chilling-susceptible ones under cold water treatments. Especially, SOD activity of Stejaree 45 was more improved than that of any other variety after treatments. SOD activity of Hitomebore increased until 3 days, but deceased more or less at 7 days after treatments. However, SOD activity in chilling-tolerant varieties was more improved in the treatment than control. In chilling-susceptible varieties, HR19621-AC6 and Sambaegbyeo, there was no difference between controlled and treated plants for SOD activity (Fig. 1A). Plants have multiple isozymes of SOD. The existence of SOD isozymes in plants and their genetic basis was first demonstrated in maize (Baum & Scandalios, 1979, 1982). Two Mn-SOD isozymes were known to exist in the chilling-tolerant and -susceptible cultivars during the chilling and the recovery period at the rice seedling stage (Kuk *et al.*, 2002). Eight SOD isozymes were found in all varieties. There were no different isozymes between chilling-tolerant and -susceptible varieties, but "a" minor isozyme activity was improved after cold water treatments (Fig. 1B) Therefore, SOD was shown to be important to enhance chilling tolerance at the booting stage.

The intercellular level of H_2O_2 is regulated by a wide range of enzymes and one of the most important enzyme is catalase (Willekens *et al.*, 1995)

Our results indicated that differences of CAT activity on the unit protein basis were distinctive between chilling-tolerant and chilling-susceptible varieties. Total CAT activities of chilling-tolerant varieties were more enhanced than those of chilling-susceptible varieties after cold water treatments. CAT activities of HR19621-AC6 increased until 3 days, but, showed the same level in 7 days, compared with control There was no difference of CAT activities in Sambaegbyeo

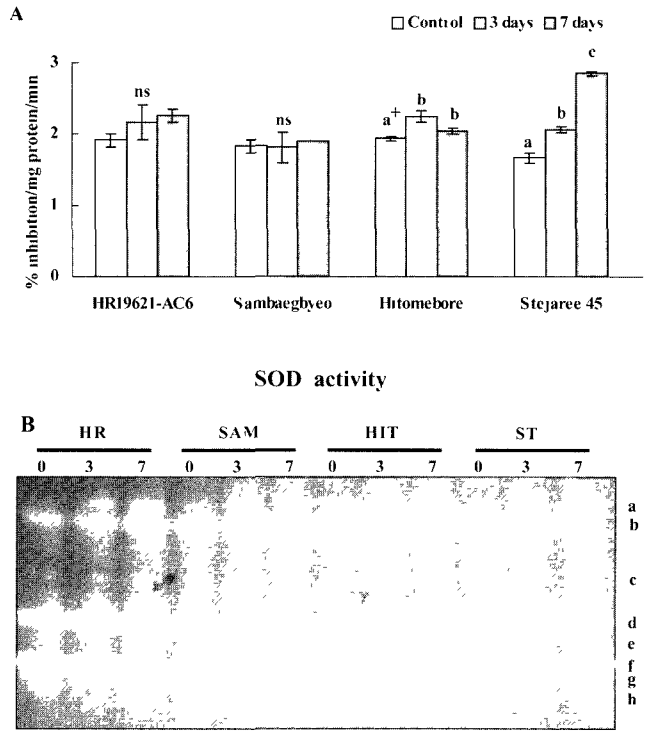

Fig. 1. Superoxide dismutase in rice booting stage leaves by cold water treatments A SOD activities by the unit protein Values represent means ± SE of the two replicates B. SOD isoforms in days after treatments of the four rice varieties, HR19621-AC6 (HR), Sambaegbyeo (SAM), Hitomebore (HIT), and Stejaree 45 (ST) Each letter indicates different isoforms. The same letters are not significantly different among treatments within a variety (+) at the 5% level by DMRT

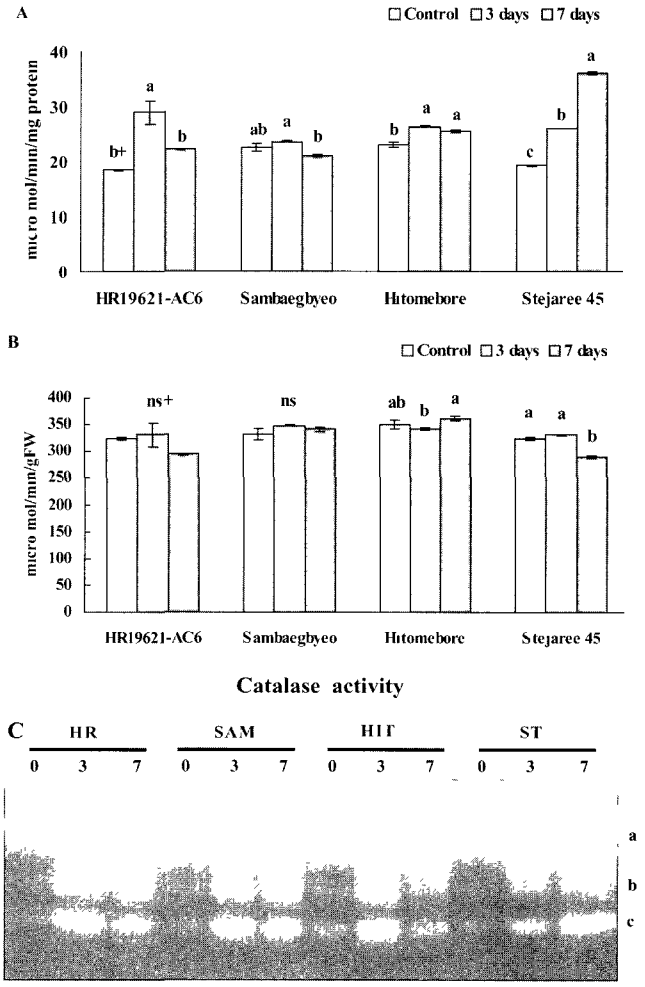

Fig. 2. Catalase in rice booting stage leaves by cold water treatments. A CAT activities by the unit protein B CAT activities by the fresh weight Values represent means ± SE of the two replicates C CAT isoforms in days after treatments of the four rice varieties, HR19621-AC6 (HR), Sambaegbyeo (SAM), Hitomebore (HIT), and Stejaree 45 (ST) Each letter indicates different isoforms The same letters are not significantly different among treatments within a variety (+) at the 5% level by DMRT

according to the days after treatment. In chilling-tolerant plant, Hitomebore and Stejaree 45, CAT activity on the unit protein basis was more increased in treatments than control (Fig. 2A). There was not different in CAT activity on fresh weight basis between control and treatment in chilling-susceptible varieties, while there was significant different in chilling-tolerant varieties, and CAT activity of stejaree 45 decreased at 7 days compared with control (Fig. 2B).

As total CAT activity indicated, we observed clearly differences in the staining of three isozymes consisting of one major isozyme band and two minor isozyme band patterns (Fig. 2C). Four japonica varieties at the booting stage had "a" isozyme in the control condition. After cold water treatments, "a" isozyme activity increased intensively in all varieties. But, "b and c" were shown to be inducible isozymes expressed after cold water treatments. Two CAT isozymes were reported in the seedling stage, and one of two isozymes in the chilling susceptible varieties disappeared from 1 day after chilling to 3 days after recovery and thereafter, appeared dimly again in 5 days after recovery (Kuk *et al.*, 2002). These results indicated that CAT activity and isozymes were important to screen chilling- tolerant rice varieties.

Plant peroxidases are heme-containing glycoproteins and are usually classified as acidic, neutral, or basic, according to their isoelectric points. POXs participate in various physiological processes, such as lignification, suberization, auxin catabolism, wound healing and defense mechanisms against pathogen infection (Hiraga *et al.*, 2001) Total POXs activity were examined under cold water stress at the booting stage (Fig. 3A). There was significant difference in POXs activities on the unit protein and fresh weight basis between control and treatments in all varieties. POXs activities in all varieties were higher in the treatments than in the control But, no difference of POXs activities was found between chilling-tolerant and -susceptible varieties as compared with fertility indices (Table 1). Total POX activities were showed to be distinctive differences in the staining of one isozyme. "d" isoform was a isozyme induced after cold water stress (Fig 3C).

Glutathione (*r*-glutamyl cysteinyl Gly) is a versatile regulator of cell metabolism and function (Rennenberg, 1982) It has been found in all cell compartments, such as cytosol, endoplasmic reticulum, vacuole and mitochondria (Jimenez *et al.*, 1998).

The enzyme glutathione reductase is important for the function of the glutathione system in eukaryotic cells (Noctor *et al.*, 1998). This enzyme catalyzes the reduction of GSSG to GSH in a NADPH-dependent reaction(May *et al.*, 1998). GR has a pivotal role in maintaining GSH within the cellular under the stress environment. Chilling sensitivity in maize leaves has been related to antioxidant status of the

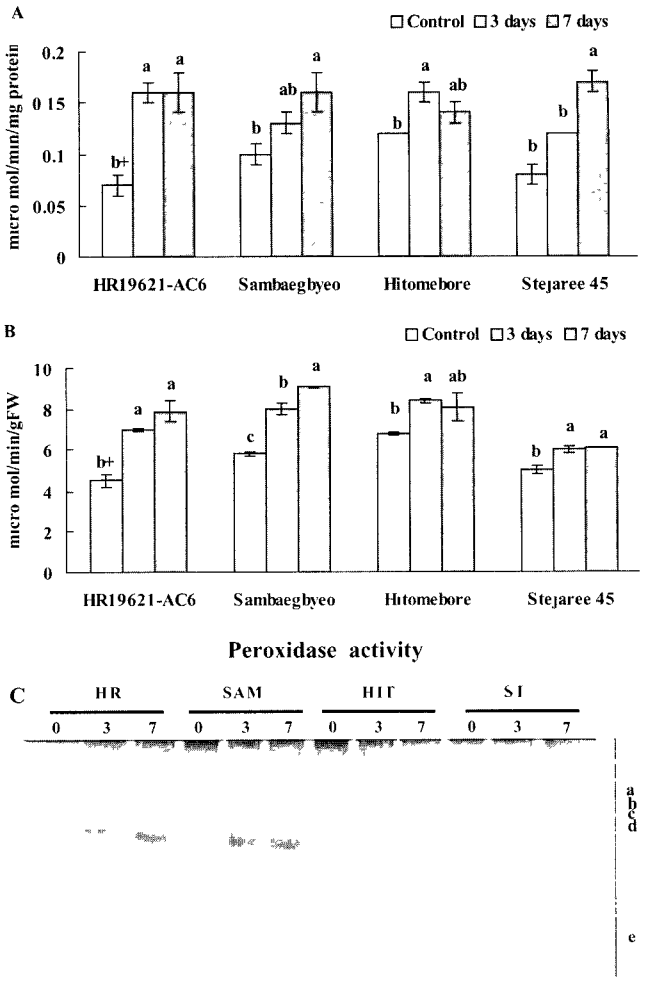

Fig. 3. Peroxidase in rice booting stage leaves by cold water treatments A. POX activities by the unit protein B POX activities by the fresh weight Values represent means ± SE of the two replicates C. POX isoforms in days after treatments of the four rice varieties, HR19621-AC6 (HR), Sambaegbyeo (SAM), Hitomebore (HIT), and Stejaree 45 (ST) Each letter indicates different isoforms The same letters are not significantly different among treatments within a variety(+) at the 5% level by DMRT

cells (Doulis *et al.*, 1997). Especially, GR is known to be an important factor limiting the degree of photodamage under chilling temperatures (Fryer *et al.*, 1998).

Total GR activities were higher in the treatments than control except Stejaree 45 on the fresh weight basis But, there was no difference for GR activities between chilling-tolerant and -susceptible varieties (Fig. 4A and 4B). Six isoforms were found for GR isozymes in this experiment. But, no difference were showed in all varieties (Fig. 4C).

Our results shows that antioxidative enzymes activities of total SOD, CAT, POX, and GR were found to be important factors related with cold water stress at the booting stage of japonica rice. Especially, SOD and CAT activities showed

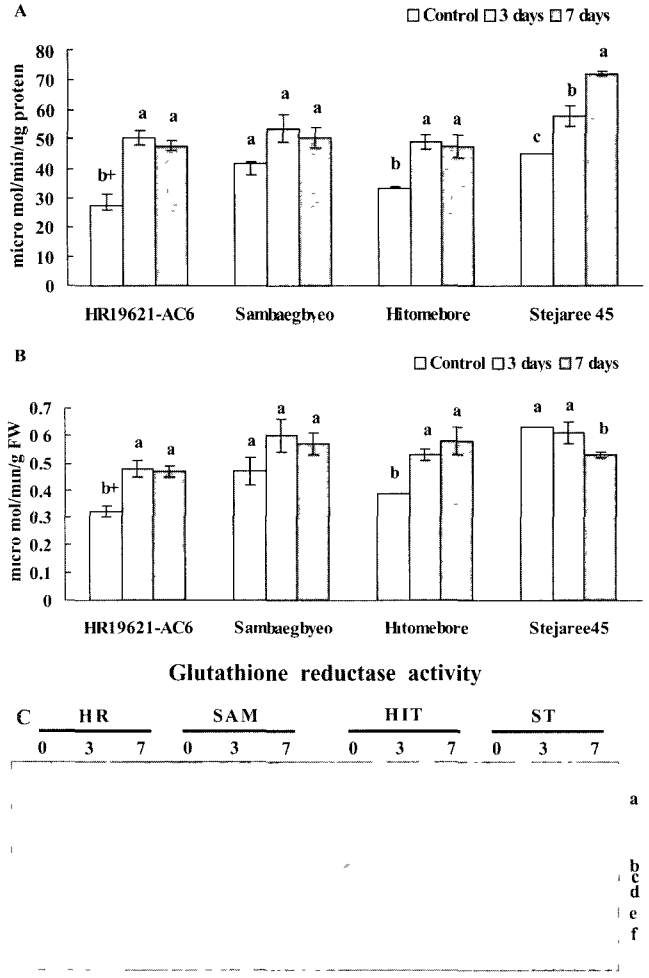

Fig. 4. Glutathione reductase in rice booting stage leaves by cold water treatments. A GR activities by the unit protein B GR activities by the fresh weight Values represent means ± SE of the two replicates C GR isoforms in days after treatments of the four rice varieties, HR19621-AC6 (HR), Sambaegbyeo (SAM), Hitomebore (HIT), and Stejaree 45 (ST). Each letter indicates different isoforms The same letters are not significantly different among treatments within a variety (+) at the 5% level by DMRT

clearly differences between chilling-tolerant and -susceptible varieties. Though isozymes of these antioxidative enzymes were not showed the difference between chilling-tolerant and -susceptible varieties in this experiment, we found the inducible isozyme polymorphisms after cold stress treatments.

REFERENCES

Alscher, R G and Hess J L 1993 Antioxidants in higher plants CRC Press, Boca Raton pp. 1-174

Anderson, M. D., T. K. Prasad, and C R. Stewart 1995 Changes in isozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings Plant Physiol. 109 1247-1257

Baum, J. A and J G. Scandalios 1979 Developmental expression and intracellular localization of superoxide dismutases in maize. Differentiation 13 . 133-140

Baum, J. A and J. G. Scandalios 1982. Multiple genes controlling superoxide dismutase expression in maize J Hered. 73 : 95-100

Beers, R. F. and I W Sizer. 1952 A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biolochem 195 : 133-140

Blokhina, O , E. Virolainen, and K. V. Fagerstedt 2003 Antioxidants, oxidative damage and oxygen deprivation stress Annals of Botany 91 . 179-194

Bradford, M M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Anal. Biochem 72 248-254

Chance, B and A C Maehly. 1955. Assay of catalase and peroxidases. Methods Enzymol. 2 764-775

Doulis, A G., N. Debian, A H Kingston-Smith, and C H Foyer 1997 Differential localization of antioxidants in maize leaves Plant Physiol. 114 1031-1037

Fryer, M. J., J. R. Andrews, K. Oxborough, D. A Blowers, and N. R. Baker. 1998 Relationships between CO_2 assimilation, photosynthetic electron transport and active O_2 metabolism in leaves of maize in the field during periods of low temperature Plant Physiol. 116 . 571-580.

Gupta, A S , R. P. Webb, A. S Holaday, and R. D. Allen. 1993. Overexpression of superoxide dismutase protects plants from oxidative stress · Induction of ascorbate peroxidase in superoxide dismutase-overexpressing plants Plant Physiol 103 1067-1073

Hiraga, S , K Sasaki, H Ito, Y. Ohashi, and H Matsui 2001 A large family of class plant peroxidases. Plant Cell Physiol 42 462-468

Jimenez, A., J A Hernandez, G. Pastori, L A del Rio, and F. Sevilla 1998 Role of the ascorbate-glutathione cycle of mitochondria and peroxisomes in the senescence of pea leaves Plant Physiol. 118 . 1327-1335.

Kawano, T 2003 Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. Plant Cell Rep 21 · 829-837

Kuk, Y I , J S Shin, T E. Whang, and J O Guh 2002 Mechanisms of chilling tolerance in relation to antioxidative enzymes in rice Korean J Crop Sci. 47(5) . 341-351

May, M J , T. Vernoux, C Leaver, M Van Montagu, and D. Inze 1998 Glutathione homeostasis in plants · implications for environmental sensing and plant development Journal of Experimental Botany 49. 649-667

Mckersie, B D., S R Bowley, and K S Jones 1999. Winter survival of transgenic alfalfa overexpressing superoxide dismutase. Plant physiol. 119 . 839-848

Mckersie, B. D , Y Chen, M. Beus, S R Bowley, C. Bowler, D. Inze, K. D'Hallum, and J. Botterman. 1993. Superoxide dismutase enhances tolerance of freezing stress in transgenic Alfalfa(*Medicago sativa* L). Plant physiol 103 : 1155-1163

Noctor, G. and C. Foyer. 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 : 249-279.

Oberley, L. W. and D. R. Spitz. 1984. Assay of superoxide dismutase activity in tumor tissue. Methods Enzymol. 105: 457-464.

O'Kane, D., V. Gill, P. Boyd, and R. Burdon. 1996. Chilling, oxidative stress and antioxidant responses in Arabidopsis thaliana calluus. Planta 198 : 371-377.

Prasad, T. K. 1997. Role of catalase in inducing chilling tolerance in preemergent maize seedlings. Plant Physiol. 114 : 1319-1376.

Rao, M. V., G. Paliyath, and D. P. Ormrod. 1996. Ultraviolet-B- and ozone-induced biochemical change in antioxidant enzymes of Arabidopsis thaliana. Plant Physiol. 110 : 125-136.

Rennenberg, H. 1982. Glutathione metabolism and possible biological riles in higher plants. Phytochemistry 21 : 2771-2781.

Saruyama, H. and M. Tanida. 1995. Effect of chilling on activated oxygen-scavenging enzymes in low temperature-sensitive and -tolerant cultivars of rice(*Oryza sativa* L.). Plant Physiol. 110 : 125-136.

Scandalios, J. G. 1993. Oxygen stress and superoxide dismutases. Plant Physiol. 101 : 7-12.

Spychalla, J. P. and S. L. Desborough. 1990. Superoxide dismutase, catalase, and *a*-tocopherol content of stored potato tuber. Plant Physiol. 94 : 1214-1218.

Steponkus, P. L. 1984. Role of the plasma membrane in freezing injury and cold acclimation. Annu. Rev. Plant Physiol. 35 : 543-84.

Takahashi, M. A. and K. Asada. 1983. Superoxide anion permeability of phospholipid membranes and chloroplast tylakoids. Archives of Biochemistry and Biophysics 226 : 558-566.

Thomashow, M. F. 1999. Plant cold acclimation : Freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 : 571-599.

Willekens, H., D. Inze, and C. W. Van. 1995. Catalase in plants. Molecular Breeding 1 : 207-208.

Woodbury, W., A. K. Spencer, and M. A. Stahmann. 1971. An improved procedure using ferricyanide for detecting catalase isozymes. Anal. Biochem. 44 : 301-305.

Xiong, L., S. K. S. Schumaker, and J. K. Zhu. 2002. Cell signaling during cold, drought, and salt stress. The plant cell 165-183.

Yun, S. J. and W .C. Lee. 1994. Characteristics of superoxide dismutases of mulberry leaf. Kor. J. Breed. 26 : 389-393.