Plant Physiol. (1997) 114: 695–704

Changes in Activities of Antioxidant Enzymes and Their Relationship to Genetic and Paclobutrazol-Induced Chilling Tolerance of Maize Seedlings[1]

Reena Grittle Pinhero, Mulpuri V. Rao, Gopinadhan Paliyath*, Dennis P. Murr, and R. Austin Fletcher

Department of Horticultural Science (R.G.P., M.V.R., G.P., D.P.M.), and Department of Environmental Biology (R.A.F.), University of Guelph, Guelph, Ontario, Canada N1G 2W1

The potential role of antioxidant enzymes in protecting maize (*Zea mays* L.) seedlings from chilling injury was examined by analyzing enzyme activities and isozyme profiles of chilling-susceptible (CO 316) and chilling-tolerant (CO 328) inbreds. Leaf superoxide dismutase (SOD) activity in CO 316 was nearly one-half that of CO 328, in which the high activity was maintained during the chilling and postchilling periods. Activity of glutathione reductase (GR) was much higher in roots than in leaves. CO 328 also possessed a new GR isozyme that was absent in roots of CO 316. Ascorbate peroxidase (APX) activity was considerably lower in leaves of CO 328 than in CO 316, and nearly similar in roots. Paclobutrazol treatment of CO 316 induced several changes in the antioxidant enzyme profiles and enhanced their activities, especially those of SOD and APX, along with the induction of chilling tolerance. These results suggest that increased activities of SOD in leaves and GR in roots of CO 328, as well as SOD and APX in leaves and roots of paclobutrazol-treated CO 316, contribute to their enhanced chilling tolerance.

Many crop species of the temperate regions are subjected to chilling stress during early stages of their growth. Maize (*Zea mays* L.) seedlings are exposed to chilling temperatures especially during germination and early seedling growth. Sensitivity of maize to chilling varies depending on the cultivar (Miedema et al., 1987). Damage from exposure to chilling stress along with illumination may be mediated by AOS such as superoxide radicals, singlet oxygen, H_2O_2, and hydroxyl radicals (Wise and Naylor, 1987). The generation of AOS is a common event in growth and developmental processes (Thompson et al., 1987; Paliyath and Droillard, 1992; McKersie and Leshem, 1994). During normal conditions of growth and development plants are invariably exposed to several forms of stress, such as drought, heat, chilling, pollutants, and UV radiation. AOS are commonly generated under these conditions (Scandalios, 1993; Allen, 1995; Anderson et al., 1995; Rao et al., 1996). By virtue of their chemical properties, AOS are highly reactive and have the potential to damage membrane lipids, proteins, chlorophyll, and nucleic acids, thus disrupting the homeostasis of the organism (Shaaltiel and Gressel, 1986; Scandalios, 1993).

Plants have evolved several mechanisms to prevent or alleviate the damage from AOS. These mechanisms include scavenging the AOS by natural antioxidants such as ascorbate and α-tocopherol, and the use of an enzymatic antioxidant system that includes SOD, CAT, POX, APX, and GR, many of which act in tandem (Scandalios, 1993; Foyer et al., 1994; Allen, 1995; Anderson et al., 1995; Rao et al., 1996). The existence of multiple molecular forms of antioxidant enzymes, their location within tissues, cells, or organelles, and any changes they may undergo in response to various environmental signals or development imply potential roles for these isozymes in the detoxification of AOS. Several isozymes of SOD, CAT, GR, POX, and APX are present in plants and their relative compositions change during exposure to stress. Acclimation and chilling of maize (Scandalios, 1990; Anderson et al., 1995), air-adaptation of submerged rice seedlings (Ushimaru et al., 1995), and exposure of Arabidopsis to UV-B light (280–320 nm) or ozone (Rao et al., 1996) have been shown to result in changes in antioxidant isozyme composition. Additionally, increases in the activity of different antioxidant enzymes under stress appear to occur simultaneously. For example, in tobacco and maize subjected to paraquat treatment, an increase in SOD activity is accompanied by an increase in GR activity (Malan et al., 1990). Reports also suggest that overexpression of SOD results in improved oxidative stress tolerance (Bowler et al., 1991; McKersie et al., 1993; Sen Gupta et al., 1993; Pitcher and Zilinskas, 1996).

Paclobutrazol, which is a member of the triazole family of plant growth regulators, has been found to protect several crops from various environmental stresses, including drought, chilling, heat, and UV-B radiation (280–320 nm) (Davis and Curry, 1991; Lurie et al., 1994; Pinhero and Fletcher, 1994; Kraus et al., 1995; Paliyath and Fletcher,

[1] This research was supported by a Canadian Commonwealth Scholarship awarded to R.G.P. and research grants from the Natural Sciences and Engineering Research Council of Canada and from the Ontario Ministry of Agriculture, Food, and Rural Affairs to G.P. and D.P.M.

* Corresponding author; e-mail gpaliyat@evbhort.uoguelph.ca; fax 1–519–767–0755.

Abbreviations: AOS, active oxygen species; APX, ascorbate peroxidase; ASA, reduced ascorbate; CAT, catalase; DHA, dehydroascorbate; F_v/F_m, variable to maximal chlorophyll fluorescence ratio; GR, glutathione reductase; MDHA, monodehydroascorbate; P, paclobutrazol[(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazolyl) pentan-3-ol]; POX, guaiacol peroxidase; SOD, superoxide dismutase.

1995). Even though paclobutrazol-induced stress tolerance is reported to be due to increased antioxidant enzymes (Upadhyaya et al., 1989; Kraus and Fletcher, 1994; Lurie et al., 1994; Paliyath and Fletcher, 1995), no detailed studies have been conducted to delineate the isozyme profiles of antioxidant enzymes and their potential relation to stress tolerance. In a previous study evaluating the chilling tolerance of the maize inbred lines CO 328 and CO 316, it was observed that paclobutrazol treatment did not provide any added protection against chilling to the chilling-tolerant CO 328 compared with its untreated control (R.G. Pinhero, G. Paliyath, R.Y. Yada, and D.P. Murr, unpublished data). However, the same treatment protected the chilling-sensitive CO 316 from chilling injury. This result suggested that paclobutrazol treatment may cause biochemical changes, perhaps at the level of various antioxidant enzymes, thus affording chilling tolerance to the chilling-sensitive inbred.

The changes in metabolism of AOS in response to exposing roots to chilling stress have not been investigated in detail. Under chilling conditions both the plant canopy and roots located close to the soil surface are exposed to chilling temperatures. However, roots at the soil surface are exposed to chilling temperatures much longer than leaves because of the high heat capacity of water-saturated soil. Hence, the activities of various antioxidant enzymes such as SOD, POX, CAT, GR, and APX in leaves and roots of maize seedlings were compared between untreated CO 316, paclobutrazol-treated CO 316 (CO 316P), and CO 328. The activities observed were related to chilling tolerance. We also studied the changes in isozyme profiles of the antioxidant enzymes in leaves and roots to ascertain whether new isozymes that might provide an added protective role are synthesized in response to chilling.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of maize (*Zea mays* L.) chilling-susceptible (CO 316) and chilling-tolerant (CO 328) inbreds were allowed to imbibe for 18 h in 100 mL of aerated, distilled water or in 50 mg L^{-1} paclobutrazol, and air-dried for 4 d. Air-dried seeds were sown in a commercial potting medium (Promix PGX, Premier Brands, Red Hill, PA) in flats containing a total of 24 plastic cells, each having a dimension of 17.5 × 13.5 × 5.5 cm. Twenty seeds were sown in each cell. Seedlings were grown in a growth cabinet maintained at day/night temperatures of 23 ± 2°C/19 ± 2°C, 50 to 70% RH, and a 16-h photoperiod at 200 μE m^{-2} s^{-1} for 11 d. Eleven-day-old seedlings were exposed for 48 h to a cold temperature regime of 6°C/2°C (day/night), 50 to 70% RH, and a photoperiod of 16 h at 450 μE m^{-2} s^{-1} in an air-circulated cold room. Six cells containing 120 seedlings were sampled for each time period of treatment. The cells were randomly arranged in the growth cabinet and in the cold room. The experiments described were conducted twice, each with three to five replications. Data from nonchilled controls of CO 316, paclobutrazol-treated CO 316 (CO 316P), and CO 328 were statistically analyzed using analysis of variance as an independent set. Data were also analyzed within each group of seedlings between nonchilled controls, after 24 and 48 h of chilling, and 24 h postchilling. Means were separated using the Student-Newman-Keuls test.

Assessment of Chilling Injury

Chilling injury was assessed by measuring F_v/F_m and electrolyte leakage at different time periods as indicated. Loss of membrane integrity is expressed as percent leakage ([initial conductivity/total conductivity] × 100) of ions using the second true leaf. The leaves were cut into 2-cm segments, rinsed in distilled water, and placed in a test tube with 15 mL of distilled water at 24°C for 24 h. Initial conductivity of the solution and total ionic conductivity (after boiling for 20 min and cooling to room temperature) were measured using a conductance meter (model 32, Yellow Springs Instrument Co., Inc., Yellow Springs, OH) (Pinhero and Fletcher, 1994). F_v/F_m was measured in situ on the second leaf with a portable efficiency analyzer (Hansatech Instrument Ltd., King's Lynn, UK) after subjecting the seedlings to dark adaptation for 30 min (Pinhero and Fletcher, 1994).

Protein Extraction

Second true leaves (2 g), and the whole root system (4 g) were used for enzyme extraction and analyses. Plant tissues were homogenized in 2 mL of 100 mM sodium phosphate (pH 7.5) containing 1 mM EDTA, 1 mM PMSF, and 1% PVP-40. The homogenate was filtered through four layers of cheesecloth and centrifuged at 12,000 rpm (17,000*g*) for 20 min. The supernatant was collected and 1-mL aliquots of the extract were passed through a Sephadex G-25 column (PD-10, Pharmacia) equilibrated with 100 mM sodium phosphate (pH 7.5) at 4°C. Proteins were eluted with 100 mM sodium phosphate buffer (pH 7.5). Protein samples were stored at −80°C for further analyses. Protein extraction for determining APX activity was performed essentially as described above, except that the homogenization buffer contained 5 mM ascorbate. Protein content was determined using BSA as a standard, according to the method of Bradford (1976).

Enzyme Analyses

Activities of various antioxidant enzymes were determined as described in detail by Rao et al. (1996). Activity of SOD (EC 1.15.1.1) was determined by measuring ferricytochrome *c* reduction at 550 nm. One unit of SOD is defined as the amount of enzyme that inhibited the rate of ferricytochrome *c* reduction by 50%. CAT (EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 (extinction coefficient, 39.4 mM^{-1} cm^{-1}) at 240 nm for 2 min. POX (EC 1.11.1.7) activity was determined by the oxidation of guaiacol in the presence of H_2O_2 (extinction coefficient, 26.6 mM^{-1} cm^{-1}) at 470 nm. GR (EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient, 6.2 mM^{-1} cm^{-1}). APX (EC 1.11.1.11) activity was determined by following the decrease in A_{290} of an assay mixture containing 0.5 mM

ascorbate (extinction coefficient, 2.8 mM^{-1} cm^{-1}), and was expressed as nanomoles of ascorbate oxidized per milligram of protein per minute.

Native PAGE and Activity Staining

Proteins eluted from the Sephadex G-25 columns were concentrated further using ultraspin filters (10 K cutoff, Chromatography Specialities, Mississauga, Ontario, Canada). Equal amounts of protein from different treatments (in the linear range of increasing enzyme activity) were subjected to discontinuous PAGE under nondenaturing, nonreducing conditions, essentially as described by Rao et al. (1996). Electrophoretic separation was performed at 4°C for 4.5 h at a constant current of 35 mA per gel for SOD, POX, and GR. For separating CAT isozymes electrophoresis was performed for 18 h at a constant current of 12.5 mA per gel. SOD activity was localized by a reaction using nitroblue tetrazolium, riboflavin, and tetramethylethylenediamine (Rao et al., 1996). POX isozymes were stained by incubating the gels in sodium acetate buffer (pH 4.5) containing 2 mM benzidine dihydrochloride (dissolved in 50% [v/v] DMSO in water). CAT activity was localized by incubating the gel in a reaction mixture containing 1% potassium ferricyanide and 1% ferric chloride, based on the methods of Woodbury et al. (1971). GR isozymes were stained in a solution of 0.25 M Tris, pH 7.8, containing 0.24 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.4 mM NADPH, 0.34 mM 2,6-dichlorophenolindophenol, and 3.6 mM GSSG for 1 h in the dark, as described by Anderson et al. (1995). Duplicate gels were also stained in the absence of GSSG to distinguish GR from other nonspecific sources of reduction.

APX isozymes were separated by native PAGE (gels containing 10% glycerol) for 4.5 h at 4°C at a constant current of 35 mA per gel. Ascorbate (2 mM) was included in the carrier buffer. The gels were prerun for 30 min to allow ascorbate present in the carrier buffer to enter the gel prior to the application of samples, according to the method of Rao et al. (1996). Following electrophoretic separation, gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min. The gels were incubated in the above buffer containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. After a brief wash in the buffer for 1 min, the gels were submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM tetramethylethylenediamine and 2.45 mM nitroblue tetrazolium, and subjected to gentle agitation. The reaction was continued for 10 to 15 min and stopped by a brief wash in water. For a comparison between treatments gels were loaded with equal amounts of protein. Activity staining of the enzymes was repeated three times, and the photographs and gel-scanning data presented are from one representative experiment.

Intensities of isozymes were calculated with image analysis software (Northern Exposure, Empix Imaging Inc., Mississauga, Ontario, Canada) using the line-scan function. During line-scanning of photographs background subtraction was automatically adjusted to get the actual intensity of the bands. Data are presented only for SOD and APX isozymes of leaves and roots.

RESULTS

Changes in Ionic Conductivity and F_v/F_m

Leakage of ions, as measured by increases in conductivity and F_v/F_m, showed major changes in response to chilling and during the recovery period. Leakage of ions from leaf tissue was nearly the same in all plants prior to chilling, whereas the percent leakage was higher in CO 316 compared with CO 316P and CO 328 at the end of the chilling period and during postchilling periods (Fig. 1A). Photosynthetic efficiency as measured by the F_v/F_m ratio decreased in CO 316, CO 316P, and CO 328 after 48 h of chilling, with maximal decline (80%) observed in CO 316 (Fig. 1B). However, the F_v/F_m ratio in CO 316P and CO 328 recovered from a chilling-induced decline, attaining near normal levels within 48 h of postchilling, whereas CO 316 showed only a 40% recovery.

SOD

Different patterns of changes were observed when SOD activity was measured by spectrophotometry and when activity estimation was conducted through nondenaturing PAGE, activity staining, and band-intensity measurements.

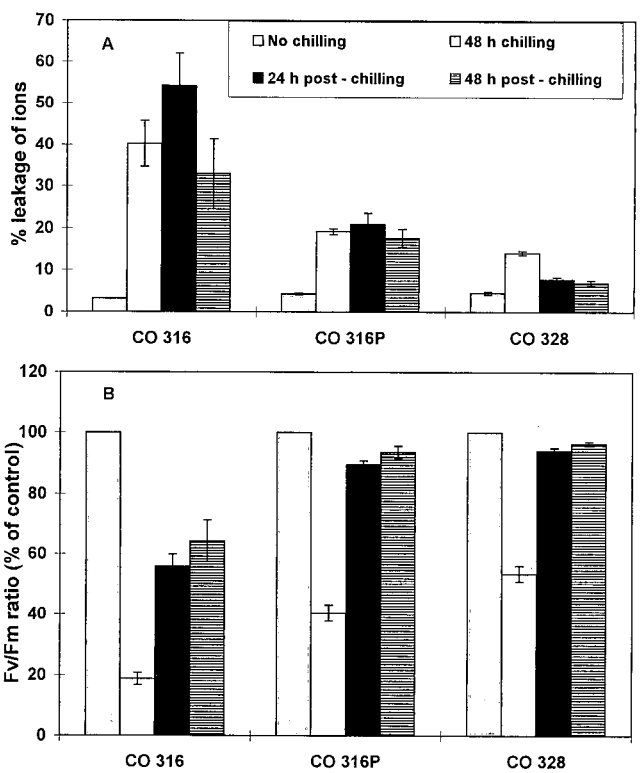

Figure 1. Percent leakage of ions (A) and percent change of F_v/F_m (B) in maize leaves. Eleven-day-old seedlings were exposed for 48 h to a low-temperature regime of 6°C/2°C (day/night) and a photoperiod of 16 h, and returned to growth cabinets, where the seedlings were raised. F_v/F_m and leakage of ions were measured before chilling, after 48 h of chilling, and after 24 and 48 h of postchilling recovery, respectively. Results are from means of eight replications. Values of F_v/F_m before chilling stress are: CO 316, 0.74; CO 316P, 0.73; and CO 328, 0.81; $P \leq 0.05$.

Significant differences were observed in SOD activities in leaves of CO 328, CO 316P, and CO 316 prior to and following chilling when assayed by spectrophotometry (Table I). The activity of SOD was lower in the leaves of CO 328 compared with CO 316. Paclobutrazol treatment increased SOD activity in leaves of CO 316. During chilling and subsequently, leaf SOD activity decreased in CO 316 and CO 316P, while the activity in CO 328 remained nearly the same compared with initial levels. During the postchilling period a significant increase in leaf SOD activity was observed in CO 328. Unlike leaves, no significant differences were noticed in SOD activities in roots between CO 328, CO 316P, and CO 316 prior to chilling (Table I). SOD activity of roots increased significantly in CO 316 and CO 316P during 24 h of chilling. A significant decrease in SOD activity was noticed during 48 h of chilling in CO 328.

Nondenaturing PAGE coupled with activity localization revealed four SOD isozymes in leaves of CO 316, CO 316P, and CO 328 (Fig. 2). Incubation of gels in 2 mM potassium cyanide or 5 mM H_2O_2 before staining for SOD activity indicated isozymes SOD-1 and SOD-2 to be Mn-SOD, and isozymes SOD-3 and SOD-4 to be Cu,Zn-SOD (data not shown). No Fe-SOD isozymes were observed. SOD-2 and SOD-3 were more prominent in CO 316P compared with CO 316 and CO 328 prior to chilling (Fig. 2, lanes a, e, and i, and Fig. 3). SOD-4 was the most prominent in all three treatments. Quantification of the band intensities by line-scanning showed that the total intensity due to SOD bands was nearly double in CO 316P and CO 328 compared with that in CO 316 prior to chilling, and remained at similar levels during chilling and postchilling periods (Fig. 3). By contrast, the intensity of SOD-4 in CO 316 increased from 3.35 before chilling to 7.80 during the 24 h postchilling. The intensities of other isozymes also increased in CO 316 during 48 h of chilling and 24 h postchilling, attaining the prechilling levels of SOD activity shown by CO 328 and CO 316P.

Table I. *Total activity of SOD in partially purified leaf and root tissue homogenates of maize seedlings*

One unit of SOD is defined as the quantity of enzyme required to inhibit the reduction of Cyt *c* by 50% in a 3-mL reaction volume per minute. Treatments were replicated six times.

Treatment	Total Activity of SOD (±SE)	
	Leaf	Root
	units	
CO 316		
Control	42.33 ± 1.90	47.79 ± 3.09
24 h of Chilling	36.63 ± 0.71[a]	51.34 ± 1.56[a]
48 h of Chilling	34.99 ± 1.40[a]	47.14 ± 3.45
24 h Postchilling	41.37 ± 1.41	46.49 ± 2.27
CO 316P		
Control	50.05 ± 1.13[b]	49.77 ± 3.83
24 h of Chilling	42.49 ± 3.14[a]	60.03 ± 4.26[a]
48 h of Chilling	42.33 ± 1.79[a]	47.18 ± 3.78
24 h Postchilling	46.25 ± 2.57[a]	53.72 ± 1.42
CO 328		
Control	33.81 ± 1.21[b]	45.97 ± 2.01
24 h of Chilling	30.72 ± 1.27	46.27 ± 2.98
48 h of Chilling	32.91 ± 0.90	40.55 ± 2.75[a]
24 h Postchilling	39.34 ± 2.97[a]	43.82 ± 0.94

[a] Significantly different compared with control values within the treatment group ($P \leq 0.01$). [b] Significantly different between the control values of CO 316, CO 316P, and CO 328.

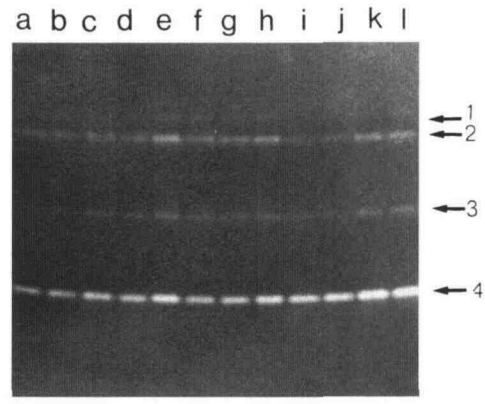

Figure 2. Native gels stained for the activity of SOD in leaves of maize inbreds before, during, and after a chilling exposure for 48 h at 6°C/2°C (day/night) and a photoperiod of 16 h. Equal amounts of protein (100 μg) from leaves of seedlings subjected to different treatments were loaded on the gel. Lanes a to d, CO 316 unchilled control, 24 h of chilling, 48 h of chilling, and 24 h postchilling, respectively; lanes e to h, CO 316P unchilled control, 24 h of chilling, 48 h of chilling, and 24 h postchilling, respectively; lanes i to l, CO 328 unchilled control, 24 h of chilling, 48 and 24 h postchilling, respectively.

Five isozymes of SOD were observed in roots (Fig. 4), of which SOD-1, SOD-2, and SOD-3 were identified to be Mn-SODs and SOD-4 and SOD-5 as Cu,Zn-SOD (data not shown). Isozyme composition was qualitatively similar in all treatments. Total intensity due to SOD isozyme activity in the root was much higher in CO 316P and CO 316 than in CO 328 (Fig. 5). The intensities of these isozymes declined during 48 h of exposure to chilling and during

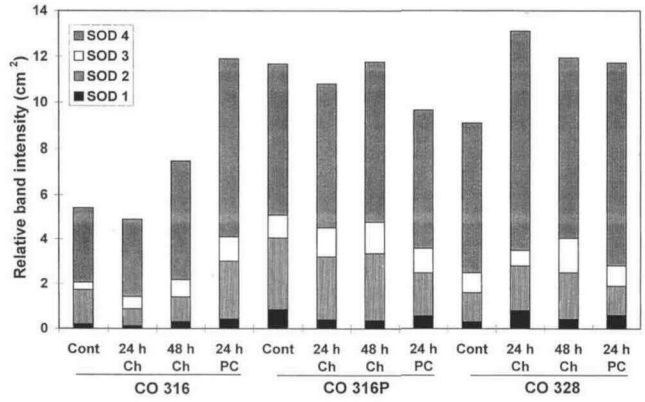

Figure 3. Changes in leaf SOD isozyme activities as estimated by native PAGE, activity staining, and determination of band intensities by line-scanning. A photograph (Fig. 2) depicting SOD isozyme activities of a leaf was scanned using the line-scanning function of a computer program. Cont, Unchilled control; 24 h Ch, 24 h of chilling; 48 h Ch, 48 h of chilling; and 24 h PC, 24 h postchilling. The data are representative of two separate experiments showing similar results.

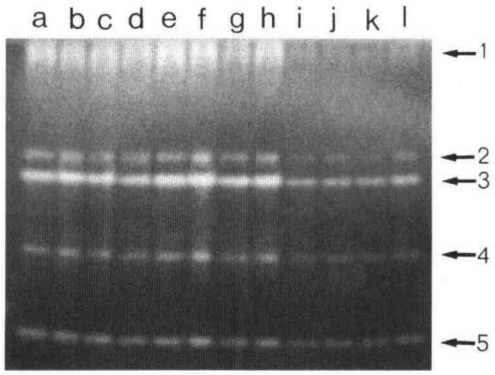

Figure 4. Native gels stained for the activity of SOD in roots of maize inbreds. Conditions are the same as described in Figure 2.

postchilling periods in CO 316. However, the activities remained similar during chilling and postchilling periods in CO 316P, and showed slight increases in CO 328. Major changes were noticed in SOD-3, SOD-4, and SOD-5.

GR

Spectrophotometric determination of GR activity showed that maize seedlings derived from paclobutrazol-treated seeds possessed significantly higher GR activity in leaves compared with CO 328 and CO 316 before chilling (Table II). There was an increase in GR activity in CO 316, CO 316P, and CO 328 during chilling and postchilling periods compared with their respective prechilling levels. In contrast to GR activity in leaves, GR activity in roots was significantly higher in CO 328 than in CO 316 and CO 316P prior to chilling (Table II). No significant difference was noticed in root GR activity in CO 316, whereas a decline in the activity was observed during chilling in CO 316P and CO 328. GR activity was nearly three times higher initially

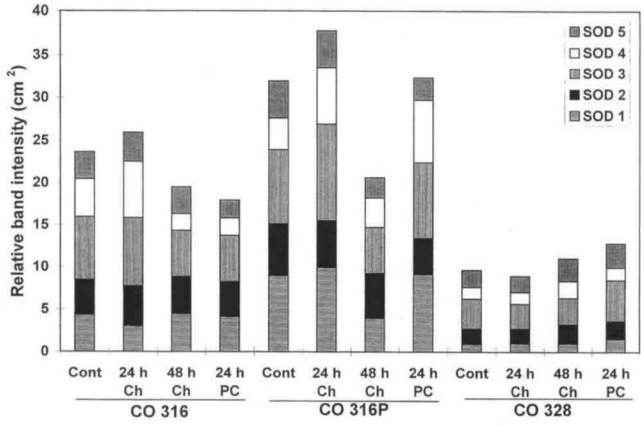

Figure 5. Changes in root SOD isozyme activities as estimated by native PAGE, activity staining, and determination of band intensities by line-scanning. A photograph (Fig. 4) depicting the SOD isozyme activities of a root was scanned using the line-scanning function of a computer program. Cont, Unchilled control; 24 h Ch, 24 h of chilling; 48 h Ch, 48 h of chilling; and 24 h PC, 24 h postchilling. The data are representative of two separate experiments showing similar results.

Table II. *Total activity of GR in leaf and root tissue preparations of maize seedlings*

Treatments were replicated six times. GR activity was monitored following the decrease in NADPH at A_{340} nm.

Treatment	Total Activity of GR (±SE)	
	Leaf	Root
	nmol NADPH	
CO 316		
Control	37.83 ± 1.57	131.17 ± 13.62
24 h of Chilling	52.67 ± 1.62[a]	128.33 ± 7.38
48 h of Chilling	63.50 ± 3.11[a]	127.50 ± 11.24
24 h Postchilling	95.33 ± 4.65[a]	121.67 ± 2.55
CO 316P		
Control	51.50 ± 1.36[b]	140.83 ± 7.21
24 h of Chilling	67.17 ± 2.86[a]	127.67 ± 8.10
48 h of Chilling	64.50 ± 2.01[a]	97.00 ± 7.42[a]
24 h Postchilling	69.67 ± 3.21[a]	116.33 ± 8.69
CO 328		
Control	33.83 ± 1.30	240.17 ± 18.38[b]
24 h of Chilling	50.33 ± 2.67[a]	145.50 ± 4.43[a]
48 h of Chilling	54.33 ± 3.71[a]	127.67 ± 16.16[a]
24 h Postchilling	48.83 ± 3.22[a]	201.83 ± 11.25

[a] Significantly different compared with control values within the treatment group ($P \leq 0.01$). [b] Significantly different between the control values of CO 316, CO 316P, and CO 328.

in roots of CO 316 and CO 316P compared with their respective activities in leaves (Table II). GR activity was 7-fold higher in roots of CO 328 compared with the activity in leaves (Table II).

Examination of GR isozyme profiles in leaves revealed three GR isozymes (Fig. 6). Most of the activity appeared to be due to GR-3, which showed much more intense activity than GR-1 and GR-2 (Fig. 6, lanes a–l). Additionally, higher staining activity was observed in CO 316P (due to isozyme 3) compared with that in CO 316 and CO 328 (Fig. 6, lanes a–l).

Paclobutrazol treatment did not cause any major changes in isozyme profiles or intensities of GR in roots of CO 316 (Fig. 7). Three isozymes (GR-1, GR-2, and GR-4) were revealed by the activity staining in root preparations of CO 316 and CO 316P before chilling (Fig. 7, lanes a and e). Activities due to GR isozymes were considerably lower in CO 316 and CO 316P. Substantially higher GR-staining activity could be deciphered due to GR-2 and GR-3 in CO 328. GR-3 was predominant prior to, during, and after chilling in CO 328. Isozyme profiles did not undergo major changes during chilling (24 or 48 h) or after chilling in CO 316. Additionally, staining activity due to GR-3 could be

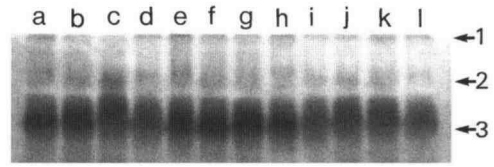

Figure 6. Native gels stained for the activity of GR in leaves of maize inbreds. Equal amounts of protein (200 μg) from leaves of seedlings subjected to different treatments were loaded on the gel. The order of lanes is the same as described in Figure 2.

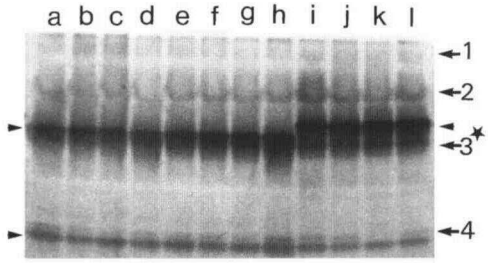

Figure 7. Native gels stained for the activity of GR in roots of maize inbreds. Equal amounts of protein (100 μg) from roots of seedlings subjected to different treatments were loaded on the gel. The order of lanes is the same as described in Figure 2. Note that isozyme 3, marked by ★ is noticeable only in CO 328 and in CO 316P during the 24-h postchilling period.

observed in CO 316P during the postchilling period. However, this activity could be masked by a nonspecific band located adjacent to it (marked by an arrowhead) during pre- and postchilling periods (Fig. 7, lanes, e, f, g, and h). Because of higher background staining and nonspecific bands, it was difficult to estimate the band intensities that corresponded to specific GR activity by scanning the gel photographs accurately.

APX

Estimation of APX activity by spectrophotometry also showed a varying pattern compared with the respective activity-stained preparations on gels. Spectrophotometric analyses showed that APX activity in leaves of CO 316P was high compared with that of CO 316 and CO 328 before chilling (Table III). APX activity increased during and after chilling in CO 316, CO 316P, and CO 328. Paclobutrazol treatment resulted in increased APX activity in roots of CO 316 compared with that of CO 328 and untreated CO 316 (Table III). An increase in APX activity was noticed in CO 316P exposed to 48 h of chilling. But APX activity declined in CO 316 and CO 328 during and after chilling (Table III). Nearly 7- to 12-fold higher activity was observed in APX activity of roots prior to chilling compared with the activity in leaves of CO 316, CO 316P, and CO 328.

Table III. *APX activities of leaf and root tissue preparations of maize seedlings*

Treatments were replicated four times. APX activities were estimated by following the oxidation of ascorbate and monitored as the decrease in A_{290}.

Treatment	Total Activity of APX (±SE)	
	Leaf	Root
	nmol of ascorbate	
CO 316		
Control	169.0 ± 7.1[a]	1307.7 ± 64.8
24 h of Chilling	220.6 ± 30.2[b]	1017.0 ± 97.0[b]
48 h of Chilling	239.9 ± 2.9[b]	1021.7 ± 7.5[b]
24 h Postchilling	202.3 ± 2.4[b]	702.5 ± 30.5[b]
CO 316P		
Control	203.2 ± 21.2[a]	1475.7 ± 4.6[a]
24 h of Chilling	249.9 ± 2.4[b]	1439.0 ± 90.6
48 h of Chilling	275.9 ± 32.8[b]	1625.6 ± 26.2[b]
24 h Postchilling	278.5 ± 11.9[b]	1390.7 ± 71.5
CO 328		
Control	106.2 ± 7.1[a]	1359.0 ± 60.9
24 h of Chilling	139.3 ± 13.3[b]	700.0 ± 10.0[b]
48 h of Chilling	123.8 ± 8.3[b]	947.0 ± 90.5[b]
24 h Postchilling	152.2 ± 3.0[b]	872.2 ± 19.1[b]

[a] Significantly different between the control values of CO 316, CO 316P, and CO 328. [b] Significantly different compared with control values within the treatment group ($P \leq 0.01$).

Examination of APX isozymes revealed four isozymes in leaves of CO 316 and CO 316P before chilling exposure (Fig. 8, lanes a and e), whereas only APX-1 was observed in CO 328 before chilling (Fig. 8, lane i). APX activity as revealed by the staining intensity was generally high in CO 316P. Considerable increases in the staining intensities of all of the isozymes (APX-1, APX-2, APX-3, and APX-4) were observed during and after chilling in CO 316P; the highest increase was observed in APX-1, APX-3, and APX-4 (Fig. 8, lanes e, f, g, and h, and Fig. 9). By contrast, CO 316 showed only a marginal increase in activity due to APX isozymes. An increase in staining intensity due to APX-1 was noticed in leaves of CO 328 during chilling (Fig. 8, lanes, j, k, and l, and Fig. 9). APX-2, APX-3, and APX-4 appeared during chilling and increased during postchilling in CO 328 (Fig. 8, lanes, i, j, k, and l, and Fig. 9).

Zymograms of roots of CO 316 and CO 316P revealed four isozymes that appeared to be similar (Fig. 10, 2–5) before chilling (Fig. 10, lanes a and e), whereas in CO 328 five bands were observed before chilling (Fig. 10, lane i, 1–5). In general, activity due to APX-2, APX-3, APX-4, and APX-5 increased during and after chilling in CO 316P (Fig. 10, lanes e, f, g, and h, and Fig. 11). APX-5 appeared to increase in intensity during the postchilling period in CO 328 (Fig. 10, lane l, and Fig. 11). No major changes were observed in the isozyme composition of CO 316. CO 316 and CO 316P also possessed staining activity, presumably due to several slow-moving isozymes (Fig. 10, indicated by broad, open arrow).

In addition to SOD, GR, and APX, the changes in activities and isozyme profiles of POX and CAT in CO 316, CO 316P, and CO 328 were studied. In general, POX activities

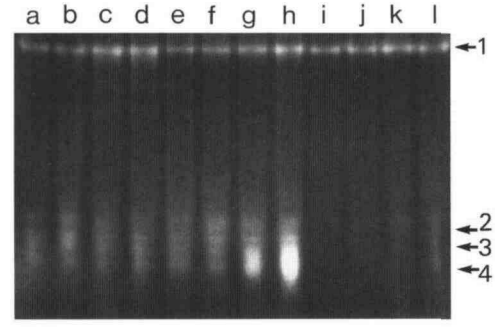

Figure 8. Native gels stained for the activity of APX in leaves of maize inbreds. Equal amounts of protein (200 μg) from leaves of seedlings subjected to different treatments were loaded on the gel. The order of lanes are the same as described in Figure 2. Note that APX-2, APX-3, and APX-4 are absent in the CO 328 unchilled control and that they appear during chilling.

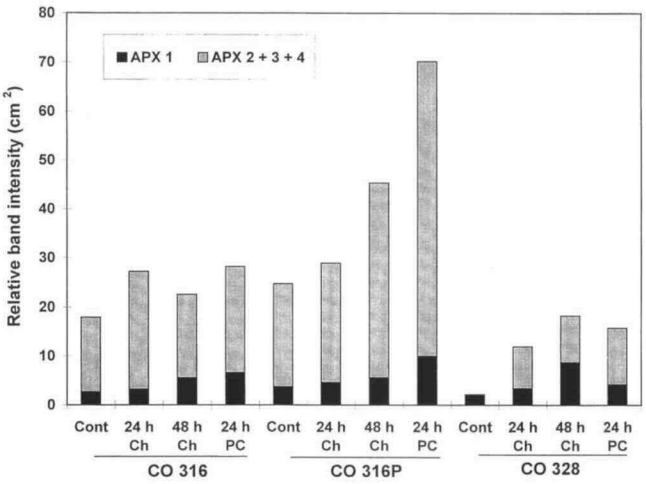

Figure 9. Changes in leaf APX activities as determined by native PAGE, activity staining, and determination of band intensities by line-scanning. A photograph (Fig. 8) of the native gel showing activities of a leaf was scanned using the line-scanning function of a computer program. APX-2, APX-3, and APX-4 were analyzed together, since it was difficult to separate activities of individual isozymes in this cluster. Cont, Unchilled control; 24 h Ch, 24 h of chilling; 48 h Ch, 48 h of chilling; and 24 h PC, 24 h postchilling. The data are representative of two separate experiments showing similar results.

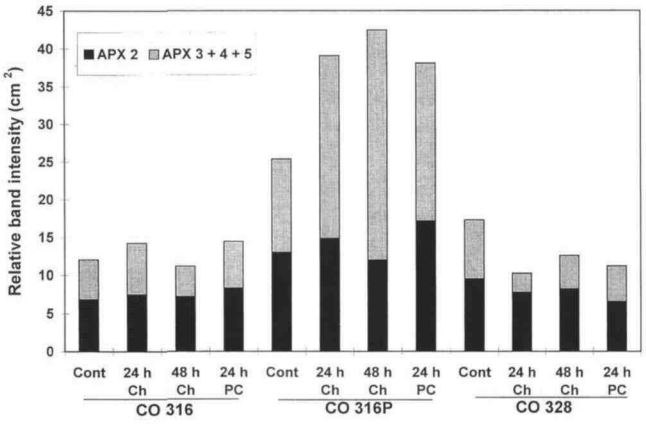

Figure 11. Changes in root APX activities as determined by native PAGE, activity staining, and determination of band intensities by line-scanning. A photograph (Fig. 10) of the native gel showing activities of a root was scanned using the line-scanning function of a computer program. APX-3, APX-4, and APX-5 were analyzed together, since it was difficult to separate activities of individual isozymes in this cluster in the later stages of sampling. Cont, Unchilled control; 24 h Ch, 24 h of chilling; 48 h Ch, 48 h of chilling; and 24 h PC, 24 h postchilling. The data are representative of two separate experiments showing similar results.

were similar in CO 316, CO 328, and CO 316P. CAT activity was nearly equal in CO 316 and CO 316P and higher compared with CO 328. There were no marked changes in the activities of POX and CAT during chilling or postchilling periods. Additionally, the isozyme profiles of these enzymes also remained nearly the same during and after chilling (data not shown).

DISCUSSION

It has been suggested that a tolerant plant might use three different mechanisms to survive or even grow at chilling temperatures (Steffen, 1991): (a) it may avoid the production of AOS, (b) it may protect itself from the deleterious, degradative reactions associated with AOS generation by efficiently scavenging them, or (c) it may repair the injury after the degradation has occurred. The chilling-tolerant tomato (*Lycopersicon hirsutum*) does not possess an inherently higher antioxidant potential than the chilling-susceptible *Lycopersicon esculentum*, except in the activities of GR; this chilling tolerance has been attributed to the control of electron transport during chilling (Walker et al., 1991). This suggests that chilling tolerance could be achieved through several adaptive mechanisms, and the modulation of antioxidant enzyme levels could be only a part of the whole mechanism.

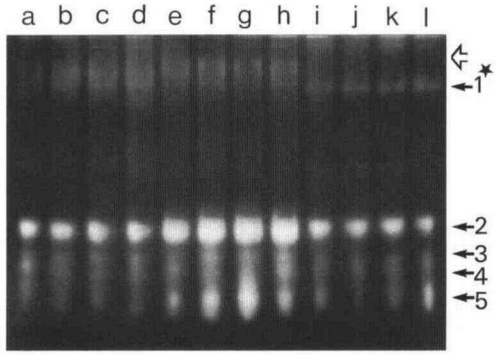

Figure 10. Native gels stained for the activity of APX in roots of maize inbreds. Equal amounts of protein (100 μg) from roots of seedlings subjected to different treatments were loaded on the gel. The order of lanes is the same as described in Figure 2. Note that isozyme 1, marked by ★, is present only in CO 328. The broad, open arrow indicates the position of several slow-moving isozymes of APX in CO 316 and CO 316P.

F_v/F_m has been widely used as a good criterion to assess chilling sensitivity in various crops (Wilson and Greaves, 1990). Analysis of chilling damage and subsequent recovery as shown by F_v/F_m and ion leakage indicates that CO 316P and untreated CO 328 sustained a lower degree of chilling damage compared with untreated CO 316. Our results show interesting differences in the manner in which antioxidant enzymes undergo changes in response to chilling in these three experimental systems studied. Estimation of SOD activity in partially purified enzyme extracts of CO 316 showed a significant decrease in leaf SOD activity during the chilling period; root SOD activity did not undergo major changes during chilling. However, the estimation of SOD activity in crude extracts is subject to a higher degree of variability due to interfering reactions such as Cyt oxidase and Cyt peroxidase (Beauchamp and Fridovich, 1971). Also, assay of SOD activity in crude leaf extracts of rice, papaya, and tobacco results in an overestimation of activity compared with gel electrophoresis of proteins, activity staining, and densitometric quantitation (Chen and

Pan, 1996). Therefore, we relied more on the estimation of activity by gel electrophoresis and activity staining than on spectrophotometric estimation of the activity of crude preparations in our interpretation of results. By this method total leaf SOD activity in CO 316 was estimated to be nearly one-half that of CO 328 and CO 316P prior to chilling (Fig. 3). The increase in leaf SOD activity during the latter part of the chilling and postchilling periods in CO 316 appears to be too late to afford any protective role. Higher levels of leaf SOD activity observed in CO 316P and CO 328 throughout the chilling and postchilling periods may contribute to their chilling tolerance. Additionally, root SOD activity showed a decline (Fig. 5) in CO 316 during the chilling and postchilling periods. Root SOD activity was inherently high in CO 316P, which enhanced its chilling tolerance capacity. These results are consistent with previous observations of increased SOD activity and tolerance to oxidative stresses (Bowler et al., 1991; McKersie et al., 1993; Pitcher and Zilinskas, 1996).

Synthesis of new isozymes of antioxidant enzymes with altered kinetic properties may be more beneficial for the metabolism of AOS than mere enhancement of the activities of existing antioxidant enzymes (Edwards et al., 1994; Rao et al., 1996). Isozyme profiles of SOD varied in CO 316, CO 316P, and CO 328 in response to chilling exposure and during the postchilling period. Constitutive levels of Mn-SOD (SOD-1 and SOD-2) were higher in the leaves of CO 316P (Figs. 2 and 3) compared with those of CO 316 and CO 328. Additionally, SOD-2 increased in CO 328 during the chilling and postchilling periods. In maize Mn-SODs are reported to be localized in mitochondria, whereas Cu,Zn-SODs are associated with the cytosol or chloroplast (Baum and Scandalios, 1979). It has been suggested that during exposure to low temperatures the normal mitochondrial electron transport might be disrupted, causing the production of AOS (Prasad et al., 1994; Purvis et al., 1995). Therefore, higher levels of Mn-SOD activity in CO 316P and CO 328 are related to the protection of mitochondria during chilling exposure. Similarly, the greater induction of SOD-3 and SOD-4 (Cu,Zn-SOD) in CO 328 during the chilling and postchilling periods could have helped CO 328 to prevent damages due to any AOS formed in chloroplast.

GR is an important enzyme involved in the enzymatic detoxification of AOS (Fig. 12) and contributes to the maintenance of a higher GSH to GSSG ratio. GSH is an efficient scavenger of AOS (Alscher, 1989). Inherently higher levels of GR activity were observed in the roots of CO 328. GR activity was much lower in CO 316, CO 316P, and CO 328 leaves compared with roots. Even though leaf GR activity increased during the chilling and postchilling periods in CO 316 (Table II), this does not appear to have contributed to the chilling tolerance. This is perhaps due to the limiting SOD activity in the leaves, which showed an increase only during the postchilling period. Also, it is likely that higher levels of GR activity in roots of CO 328 lead to an increased translocation of GSH from the roots to the leaves, which could contribute to enhanced scavenging of AOS and to providing chilling tolerance (Rennenberg, 1982). It has also been suggested that increased

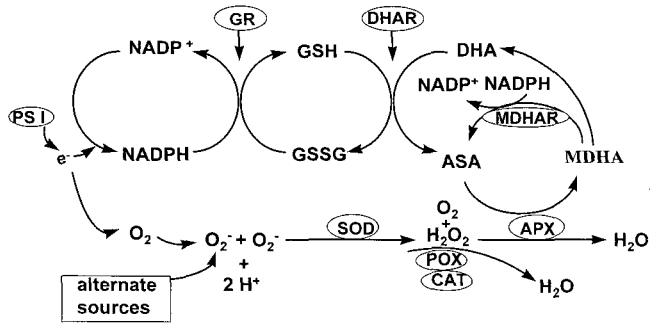

Figure 12. Schematic diagram showing the scavenging pathway of AOS by antioxidant enzymes. The superoxide anion radical (O_2^-) is dismutated to H_2O_2 by SOD. APX, POX, and CAT metabolize H_2O_2 to water. APX metabolizes H_2O_2 to water by oxidizing ASA to MDHA and/or DHA. MDHA reductase reduces MDHA to ASA, or MDHA is spontaneously converted to DHA. DHA reductase converts DHA to ASA, along with the oxidation of GSH to GSSG. GSH is regenerated by GR, utilizing NADPH as the reductant. Alternate sources of O_2^- include the mitochondrial electron transport system and NADP/NADPH oxidase activity.

synthesis of GR isozymes with higher affinities toward GSSG could be an alternate mechanism to keep higher GSH levels in the tissue (Edwards et al., 1994; Anderson et al., 1995). Potentially, higher levels of GR activity in leaves of CO 316P (GR-3) and the presence of an additional isozyme (GR-3) in roots of CO 328 and CO 316P will enhance the adaptive mechanisms that result in chilling tolerance in these systems.

APXs are chloroplastic or cytosolic enzymes that act in tandem with SOD to scavenge H_2O_2 generated through SOD action (Fig. 12). APX activities were higher in roots than in leaves of CO 316, CO 316P, and CO 328. Changes in isozyme composition of APX occurred in a cluster of fast-moving isozymes numbered APX-2, APX-3, and APX-4 in leaves and APX-2, APX-3, APX-4, and APX-5 in roots. The large increase in APX activity in leaves and roots in CO 316P contributed to its chilling tolerance. Surprisingly, leaf APX activity due to APX-2, APX-3, and APX-4 were not detectable in CO 328 before chilling. These isozymes appeared only during the chilling and postchilling periods. Thus, increases in APX activity in conjunction with higher GR activity of roots appear to contribute to the chilling tolerance exhibited by CO 328. CAT and peroxidase activities were generally high in CO 316 and CO 316P and do not appear to be limiting factors in the scavenging of AOS.

Among the leaves and roots tested we found inherently higher activities of SOD, GR, and APX in roots, irrespective of treatments (Tables I, II, and III). Like leaves, roots are exposed to environmental stresses such as drought, chilling, flooding, and pathogen attack, which cause the generation of AOS. It has been suggested that in dark-grown seedlings or in nonphotosynthetic tissues, mitochondria are the primary source of AOS generation (Puntarulo et al., 1991). Even though the potential contribution of the roots toward chilling tolerance of the whole plant is not fully understood, roots may influence the chilling

tolerance of aerial portions by supplying chemical antioxidants such as GSH through increased GR activity (Rennenberg, 1982). Increased activities of GR in roots, especially in CO 328, is in agreement with this contention.

Differential modulation of antioxidant isozymes and their activity are important features that contribute to the chilling tolerance of maize seedlings. It appears that the mechanism of chilling tolerance in the genetically chilling-tolerant CO 328 and chilling-sensitive CO 316P involve the use of similar antioxidant enzyme systems, but in differing proportions. A combination of the GSH cycle and the SOD-APX pathway thus contributes to the chilling-tolerance mechanism of CO 328. The predominance of the SOD and APX activities in CO 316P suggests that the SOD-APX pathway contributes much toward the chilling tolerance exhibited by these seedlings.

It has also been suggested that the cyanide-insensitive alternative pathway of respiration reduces the potential production of AOS in mitochondria. Cold-resistant cultivars and tissues generally develop a greater potential of electron flux through alternative pathways than the cold-sensitive cultivars and tissues (Purvis, 1985; Purvis et al., 1995). In addition, it is possible that other mechanisms responsible for stabilizing the membranes may contribute to the chilling tolerance of CO 328.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Bob Hamilton of Plant Research Centre, Agriculture and Agri-Food Canada, Ottawa, for kindly supplying the seeds of the two inbreds; Brad Smith of Zeneca Corp., Stony Creek, Ontario, Canada, for a generous supply of paclobutrazol; and Dr. Bryan McKersie, Department of Crop Science, University of Guelph, for providing facilities to scan the gel photographs.

Received December 9, 1996; accepted March 4, 1997.

LITERATURE CITED

Allen RD (1995) Dissection of oxidative stress tolerance using transgenic plants. Plant Physiol **107:** 1049–1054

Alscher RG (1989) Biosynthesis and antioxidant function of glutathione in plants. Physiol Plant **77:** 457–464

Anderson MD, Prasad TK, Stewart CR (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 JA, Scandalios JG (1979) Developmental expression and intracellular localization of superoxide dismutases in maize. Differentiation **13:** 133–140

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem **44:** 276–287

Bowler C, Slooten L, Vandenbranden S, De Rycke R, Botterman J, Sybesma C, Van Montagu M, Inze D (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic tobacco. Plant Physiol **97:** 452–457

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Chen CN, Pan SM (1996) Assay of superoxide dismutase activity by combining electrophoresis and densitometry. Bot Bull Acad Sin **37:** 107–111

Davis TD, Curry EA (1991) Chemical regulation of vegetative growth. Crit Rev Plant Sci **10:** 204–216

Edwards EA, Enard C, Creissen GP, Mullineaux PM (1994) Synthesis and properties of glutathione reductase in stressed peas. Planta **192:** 137–143

Foyer CH, Descourvieres P, Kunert KJ (1994) Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. Plant Cell Environ **17:** 507–523

Kraus TE, Evans RC, Fletcher RA, Pauls KP (1995) Paclobutrazol enhances tolerance to increased levels of UV-B radiation in soybean (*Glycine max*) seedlings. Can J Bot **73:** 797–806

Kraus TE, Fletcher RA (1994) Paclobutrazol protects wheat seedlings from heat and paraquat injury: is detoxification of active oxygen involved? Plant Cell Physiol **35:** 45–52

Lurie S, Lipsker RZ, Aloni B (1994) Effects of paclobutrazol and chilling temperatures on lipids, antioxidants and ATPase activity of plasma membrane isolated from green bell pepper fruits. Physiol Plant **91:** 593–598

Malan C, Greyling MM, Gressel J (1990) Correlation between Cu,Zn superoxide dismutase and glutathione reductase and environmental and xenobiotic stress tolerance in maize inbreds. Plant Sci **69:** 157–166

McKersie BD, Chen Y, Debeus M, Bowley SR, Bowler C, Inze D, D'Halluin K, Botterman, K (1993) Superoxide dismutase enhances tolerance to freezing stress in transgenic alfalfa (*Medicago sativa* L.). Plant Physiol **103:** 1155–1163

McKersie BD, Leshem YY (1994) Stress and stress coping in cultivated plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 79–100

Miedema P, Post J, Groot, PJ (1987) The Effects of Low Temperature on Seedling Growth of Maize Genotypes. Centre for Agricultural Publishing and Documentation, Wageningen, The Netherlands, pp 30–68

Paliyath G, Droillard MJ (1992) The mechanism of membrane deterioration and disassembly during senescence. Plant Physiol Biochem **30:** 789–812

Paliyath G, Fletcher RA (1995) Paclobutrazol treatment alters peroxidase and catalase activities in heat-stressed maize coleoptiles. Physiol Mol Biol Plants **1:** 171–178

Pinhero RG, Fletcher RA (1994) Paclobutrazol and ancymidol protect corn seedlings from high and low temperature stresses. Plant Growth Reg **15:** 47–53

Pitcher LH, Zilinskas BA (1996) Overexpression of copper/zinc superoxide dismutase in the cytosol of transgenic tobacco confers partial resistance to ozone-induced foliar necrosis. Plant Physiol **110:** 583–588

Prasad TK, Anderson MD, Martin BA, Stewart CR (1994) Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell **6:** 65–74

Puntarulo S, Galleano M, Sanchez RA, Boveris A (1991) Superoxide anion and hydrogen peroxide metabolism in soybean embryonic axes during germination. Biochim Biophys Acta **1074:** 277–283

Purvis AC (1985) Low temperature induced azide-insensitive oxygen uptake in grapefruit flavedo tissue. J Am Soc Hortic Sci **110:** 782–785

Purvis AC, Shewfelt RL, Gegogeine JW (1995) Superoxide production by mitochondria isolated from green bell pepper fruit. Physiol Plant **94:** 743–749

Rao MV, Paliyath G, Ormrod DP (1996) Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol **110:** 125–136

Rennenberg H (1982) Glutathione metabolism and possible biological roles in higher plants. Phytochemistry **21:** 2771–2781

Scandalios JG (1990) Response of plant antioxidant defence genes to environmental stress. Adv Genet **28:** 1–41

Scandalios JG (1993) Oxygen stress and superoxide dismutases. Plant Physiol **101:** 7–12

Sen Gupta A, Heinen JL, Holaday AS, Burke JJ, Allen RD (1993)

Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. Proc Natl Acad Sci USA **90:** 1629–1633

Shaaltiel Y, Gressel J (1986) Multienzyme oxygen radical detoxifying system correlated with paraquat resistance in *Conyza bonariensis*. Pestic Biochem Physiol **26:** 22–28

Steffen KL (1991) Avoidance of photooxidative stress: balancing energy flux within the chloroplast. *In* E Pell, KL Steffen, eds, Active Oxygen/Oxidative Stress and Plant Metabolism. Current Topics in Plant Physiology, Vol 6. American Society of Plant Physiologists, Rockville, MD, pp 119–130

Thompson JE, Legge RL, Barber RF (1987) The role of free radicals in senescence and wounding. New Phytol **105:** 317–344

Upadhyaya A, Davis TD, Walser RH, Galbraith AB, Sankhla N (1989) Uniconazole-induced alleviation of low temperature damage in relation to antioxidant activity. Hortic Sci **24:** 955–957

Ushimaru T, Ogawa K, Ishida N, Shibasaka M, Kanematsu S, Asada K, Tsuji H (1995) Changes in organelle superoxide dismutase isozymes during air adaptation of submerged rice seedlings: differential behaviour of isozymes in plastids and mitochondria. Planta **196:** 606–613

Walker MA, McKersie BD, Pauls KP (1991) Effects of chilling on the biochemical and functional properties of thylakoid membranes. Plant Physiol **97:** 663–669

Wilson JM, Greaves JA (1990) Assessment of chilling sensitivity by chlorophyll fluorescence analysis. *In* CY Wang, ed, Chilling Injury of Horticultural Crops. CRC Press, Boca Raton, FL, pp 129–141

Wise RR, Naylor AW (1987) Chilling-enhanced photooxidation. The peroxidative destruction of lipids during chilling injury to photosynthesis and ultrastructure. Plant Physiol **83:** 272–277

Woodbury W, Spencer AK, Stahmann MA (1971) An improved procedure using ferricyanide for detecting catalase isozymes. Anal Biochem **44:** 301–305