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Changes in Isozyme Profiles of Catalase, Peroxidase, and Glutathione Reductase during Acclimation to Chilling in Mesocotyls of Maize Seedlings[1]

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The response of antioxidants to acclimation and chilling in various tissues of dark-grown maize (*Zea mays* L.) seedlings was examined in relation to chilling tolerance and protection from chilling-induced oxidative stress. Chilling caused an accumulation of H_2O_2 in both the coleoptile + leaf and the mesocotyl (but not roots), and acclimation prevented this accumulation. None of the antioxidant enzymes were significantly affected by acclimation or chilling in the coleoptile + leaf or root. However, elevated levels of glutathione in acclimated seedlings may contribute to an enhanced ability to scavenge H_2O_2 in the coleoptile + leaf. In the mesocotyl (visibly most susceptible to chilling), catalase3 was elevated in acclimated seedlings and may represent the first line of defense from mitochondria-generated H_2O_2. Nine of the most prominent peroxidase isozymes were induced by acclimation, two of which were located in the cell wall, suggesting a role in lignification. Lignin content was elevated in mesocotyls of acclimated seedlings, likely improving the mechanical strength of the mesocotyl. One cytosolic glutathione reductase isozyme was greatly decreased in acclimated seedlings, whereas two others were elevated, possibly resulting in improved effectiveness of the enzyme at low temperature. When taken together, these responses to acclimation illustrate the potential ways in which chilling tolerance may be improved in preemergent maize seedlings.

Stand establishment in maize (*Zea mays* L.) is greatly affected by exposure of seedlings to low temperature during germination and early seedling growth. Pioneer inbred G50 is particularly sensitive. By utilizing dark-grown seedlings to simulate a preemergent condition, Anderson et al. (1994) demonstrated that exposure of seedlings to a noninjurious low temperature induced a degree of chilling tolerance, allowing them to survive subsequent exposure to more severe low temperatures. This acclimation phenomenon provided an opportunity to examine the molecular basis for the improvement in chilling tolerance. The discovery that *cat3* (mitochondrial catalase) transcripts were up-regulated in response to acclimation led to investigation of the role of CAT and other antioxidant enzymes in alleviating chilling-induced oxidative stress.

There is increasing evidence that chilling causes elevated levels of active oxygen species (e.g. Omran, 1980; Wise and Naylor, 1987; Prasad et al., 1994a), which likely contribute significantly to chilling damage. The active oxygen species–H_2O_2 (hydrogen peroxide), O_2^- (superoxide), $OH^\cdot$ (hydroxyl radical), and $^1O_2$ (singlet oxygen)–are present in all plants to various degrees as a result of normal aerobic metabolism. Allowed to accumulate, these active oxygen species can cause damage to cellular components, severely disrupting metabolic function (Elstner, 1987). Under normal conditions, plants possess scavenging systems that keep active oxygen species below damaging levels (Larson, 1988). SOD scavenges O_2^- in the cytosol, chloroplasts, and mitochondria. CAT is the primary H_2O_2 scavenger in the peroxisomes and the mitochondria, at least in maize (Scandalios et al., 1980; Prasad et al., 1995). H_2O_2 generated in the chloroplasts is scavenged by an ascorbate-glutathione cycle (Foyer and Halliwell, 1976). In this system, APX utilizes H_2O_2 to oxidize AsA to monodehydroascorbate radical, which disproportionates to DHA nonenzymatically. Monodehydroascorbate reductase regenerates ascorbate at the expense of NAD(P)H, and DHAR regenerates ascorbate utilizing GSH to form GSSG. GSH is regenerated at the expense of NADPH by the action of GR, the rate-limiting step of the cycle (Jablonski and Anderson, 1981). Although this cycle is known to be responsible primarily for H_2O_2 scavenging in chloroplasts, its importance in the cytosol and in nonphotosynthetic tissues is also becoming apparent (Alscher, 1989). When a plant is stressed, the production of active oxygen can exceed the capacity of the scavenging systems, resulting in oxidative damage. Thus, the ability of a plant to improve its active-oxygen-scavenging capacity may be a key element in stress tolerance.

It has been suggested that in dark-grown seedlings or in nonphotosynthetic tissue, mitochondria are the primary source of active oxygen generation (Puntarulo et al., 1991;

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Abbreviations: Ac, acclimated; AcCh, acclimated and chilled; APX, ascorbate peroxidase; AsA, ascorbic acid; %C, percentage of bisacrylamide cross-linker; CAT, catalase; Ch, chilled; Cont, control; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; ϵ, extinction coefficient at wavelength x; GR, glutathione reductase; POX, guaiacol peroxidase; SOD, superoxide dismutase; %T, polyacrylamide gel concentration defined as percentage of total monomer (i.e. acrylamide + cross-linking agent, g/100 mL).

Prasad et al., 1994b). We have shown that chilling caused the accumulation of H_2O_2 in maize mesocotyls and that induction of a catalase isozyme (CAT3) in the mitochondria during acclimation prevented the elevation of H_2O_2 (Prasad et al., 1994a). Although we have previously focused on the role of CAT3 in protecting mitochondria from chilling-induced oxidative damage, it is likely that antioxidant systems throughout the cell also contribute to the protective effect. In maize, CAT is present as four isozymes (Scandalios et al., 1984), only two of which (CAT1 and CAT3) are present in dark-grown seedlings. Different isoforms also exist in maize for POX (Grison and Pilet, 1985), SOD (Scandalios, 1990), and APX (Mittler and Zilinskas, 1993), and for GR in pea (Edwards et al., 1990). The various antioxidant isozymes are differentially compartmentalized and, depending on the tissue, likely respond differently to acclimation and chilling. Thus, it became important to determine the status of the various antioxidants during acclimation and chilling so as to assess their contribution to chilling tolerance.

The goals of this study were (a) to examine acclimation-induced changes among isozymes of CAT, POX, SOD, APX, and GR to establish which antioxidant enzymes were most important in the protection of preemergent maize seedlings from oxidative stress, and (b) to demonstrate differences in the response of these antioxidant enzymes to acclimation among different tissues in relation to the susceptibility of the tissue to H_2O_2 accumulation and to chilling injury. Despite the fact that acclimation prevented the accumulation of H_2O_2 in both the coleoptile + leaf and the mesocotyl, antioxidant enzymes responded to acclimation only in the mesocotyl. Therefore, we measured the levels of ascorbate and glutathione to determine whether the oxidative status of these antioxidants was affected by acclimation in the absence of an effect on APX and GR in the coleoptile + leaf. By describing the acclimation-induced changes in antioxidant isozymes and the steady-state pools of ascorbate and glutathione in various tissues, we have suggested relationships among induction of antioxidants, H_2O_2 accumulation, and susceptibility of the tissue to chilling injury. In this way, we provided a more complete picture of the means by which preemergent maize seedlings utilize antioxidants to tolerate chilling stress.

MATERIALS AND METHODS

Plant Material

Chilling-sensitive maize (*Zea mays* L.) inbred G50 (Pioneer Hi-Bred International Inc., Johnston, IA) was used for all experiments. Seeds were planted in Redi-Earth Peat-Lite Mix (Grace Sierra Horticultural Products Co., Milpitas, CA) and grown in darkness at 27°C for 3 d. Seedlings exposed to the AcCh treatment were then exposed to an acclimation temperature of 14°C for 3 d in darkness, followed by a chilling temperature of 4°C for 4 d in darkness. Seedlings of the Ch treatment were transferred directly to the chilling temperature without acclimation. Seedlings of the Ac treatment were acclimated but not chilled. Cont seedlings received neither the Ac nor the Ch treatments.

Protein Extraction

Cell-free extracts of the various low-temperature treatments were analyzed for CAT, POX, SOD, GR, and APX both spectrophotometrically and on nondenaturing gels. Plant material was harvested directly into liquid nitrogen and 0.5 g of frozen tissue was ground in 0.75 mL of 0.1 M sodium phosphate, pH 7.8, containing 1 mM EDTA, 1 mM PMSF, and 20 mg of polyvinylpolypyrrolidone. For analysis of APX, the extraction buffer also contained 5 mM ascorbate. Insoluble material was removed by centrifugation at 16,000*g* for 15 min at 4°C, and the supernatant was immediately made to 40% (v/v) glycerol. Since maintenance of consistent CAT electrophoretic mobility and maintenance of GR activity was found to require the presence of DTT, an aliquot of each sample was made to 10 mM DTT to be used for CAT and GR zymograms and GR spectrophotometric assays (Guy and Carter, 1984; protected GR with 5 mM β-mercaptoethanol). Addition of DTT was within 0.5 h from the time of maceration. The remainder of each extract was used for POX and SOD zymograms, for CAT and POX spectrophotometric assays, and for determination of total protein content (Lowry et al., 1951) with BSA as the standard. For the spectrophotometric assay of SOD, extracts were passed through Sephadex G-25 columns (1-mL bed volume) at 4°C using 0.1 M sodium phosphate, pH 7.8, as the elution buffer to remove low-molecular-weight compounds that interfere with the assay. Extracts were then made to 40% (v/v) glycerol. All samples were stored at −20°C until enzyme analysis. Routinely, CAT and APX gels were run immediately after extraction; the stability of POX, SOD, and GR during storage was sufficient so that immediate analysis was not necessary. All spectrophotometric assays were run on the day after extraction.

To determine which POX isozymes were localized in the cell wall and therefore potentially involved in lignification, protoplast and ionically bound cell-wall proteins from Ac and AcCh mesocotyls were extracted according to the method of Prasad and Cline (1987). The cell-wall fraction was desalted and concentrated on a Centricon microconcentrator (Amicon, Danvers, MA) with a 10,000 molecular weight exclusion limit, according to the manufacturer's instructions.

Cell fractionation was conducted for the purpose of localizing GR isozymes. Mesocotyls (10 g) from Ac seedlings were harvested and ground in 20 mL of a solution of 0.4 M Suc, 165 mM Tricine, 10 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA, and 10 mM DTT, pH 7.5. Macerated material was passed through four layers of cheesecloth and centrifuged at 2,000*g* for 5 min. The plastid-containing pellet was resuspended in 0.4 M Suc, 10 mM Tricine, 1 mM EDTA, 10 mM DTT, pH 7.2. The supernatant was centrifuged at 12,000*g* for 10 min and the supernatant was retained as the cytosolic fraction. The mitochondria-containing pellet was resuspended in 0.4 M Suc and mitochondria were further purified on a 0.6 M Suc cushion, centrifuging at 10,000*g* for 20 min. The mitochondrial pellet was then resuspended in resuspending medium. Mitochondria and plastids were

lysed by repeated freeze/thaw cycles and samples were centrifuged at 10,000*g* to remove particulates.

Enzyme Analyses

Total CAT activity was determined spectrophotometrically by following the decline in A_{240} as H_2O_2 (ϵ = 36 M^{-1} cm^{-1}) was catabolized, according to the method of Beers and Sizer (1952). CAT isozymes were separated on nondenaturing polyacrylamide gels (7% T, 3% C, notation of Righetti, 1983) at 80 V for 22 h at 4°C using the procedures of Laemmli (1970). The 3× loading buffer contained no β-mercaptoethanol (which was found to inhibit CAT3) but was made to 60 mM DTT. Gels were then soaked in 3.27 mM H_2O_2 for 25 min, rinsed in water, and stained in a solution of 1% (w/v) potassium ferricyanide, 1% (w/v) ferric chloride (equal volumes of 2% [w/v] solutions of each component, added sequentially) in a method similar to that of Woodbury et al. (1971).

Total POX activity was determined spectrophotometrically by monitoring the formation of tetraguaiacol (ϵ = 26.6 mM^{-1} cm^{-1}) from guaiacol at A_{470} in the presence of H_2O_2 by the method of Chance and Maehly (1955). POX isozymes were separated by IEF on a flat-bed electrophoresis apparatus (LKB [Bromma, Sweden] Multiphor 2117). The 2-mm gel (5% T, 3% C) contained 10% (v/v) glycerol, 3% (v/v) Pharmalytes (Sigma) pH 3–10, 1% (v/v) Pharmalytes pH 5–8, and 1% (v/v) Pharmalytes pH 8–10.5 and was supported by GelBond PAG film (Pharmacia). The anode strip was saturated with 1 M phosphoric acid and the cathode strip with 1 N sodium hydroxide. Gels were run at 20 W constant power for 5 h at 10°C, with sample applicators removed after 45 min. After electrophoresis, the gel was soaked in PBS (10 mM sodium phosphate, pH 6.0, 150 mM sodium chloride) for 45 min to equalize pH throughout the gel. The gel was then stained with *p*-phenylenediamine (0.1 M sodium citrate, pH 5.0, 9.25 mM *p*-phenylenediamine, and 3.92 mM H_2O_2 for 10–15 min; Olson and Varner, 1993) or with guaiacol (0.1 M potassium phosphate, pH 6.4, 20 mM guaiacol, and 5.55 mM H_2O_2 for approximately 30 min). Since considerable POX activity was observed at the cathode strip, a gel was sectioned and run for various lengths of time ranging from 1 to 2 h. Bands were underfocused but isozymes with pI values greater than the pH range of the gel were visible, allowing comparison of activity levels among treatments.

Total SOD activity was measured spectrophotometrically by measuring the inhibition of the O_2^--dependent reduction of Cyt *c* at A_{550}, according to the method of McCord and Fridovich (1969). One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of Cyt *c* by 50% in a 1-mL reaction volume. Isozymes of SOD were separated on nondenaturing polyacrylamide gels (10% T, 3% C) at 80 V for 19.5 h at 4°C according to the method of Laemmli (1970). β-Mercaptoethanol was withheld from the loading buffer. After electrophoresis, gels were stained with a method similar to that of Beauchamp and Fridovich (1971). Gels were soaked in 50 mM sodium phosphate, pH 7.5, containing 2.45 mM 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)ditetrazolium chloride in darkness for 20 min, followed by soaking in 50 mM sodium phosphate, pH 7.5, containing 26.5 mM *N,N,N′,N′*-tetramethylethylenediamine and 26.5 μM riboflavin in darkness for 40 min. Gels were then exposed to low light (9 μmol m^{-2} s^{-1}) for approximately 50 min and transferred to 1% (v/v) acetic acid to stop the reaction.

Total GR activity was measured spectrophotometrically by measuring the decline in A_{340} as NADPH (ϵ = 6.22 mM^{-1} cm^{-1}) was oxidized, as described by Edwards et al. (1990). The DTT present in the crude extracts, which was required to maintain GR activity, was not found to interfere with the assay. Instead, it eliminated the majority of the GSSG-independent oxidation of NADPH, thereby improving the accuracy of the assay. GR isozymes were separated on nondenaturing polyacrylamide gels (7% T, 3% C) at 80 V for 17 h and 4°C using the procedures of Laemmli (1970). Gels 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 in darkness for 1 h (similar to the procedure of Anderson et al., 1990). Duplicate gels were also stained in the absence of GSSG to distinguish GR from other sources of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction.

Total APX activity was measured spectrophotometrically by monitoring the decline in A_{290} as ascorbate (ϵ = 2.8 mM^{-1} cm^{-1}) was oxidized, using the method of Nakano and Asada (1981). APX isozymes were separated on nondenaturing polyacrylamide gels (10% T, 3% C) supported by 10% (v/v) glycerol and stained according to Mittler and Zilinskas (1993).

H_2O_2, Ascorbate, Glutathione, and Lignin Analyses

Fresh tissue from coleoptile + leaf, mesocotyl, and roots from Cont, Ch, Ac, and AcCh treatments were analyzed for H_2O_2 levels by the method of Brennan and Frenkel (1977). For measurement of ascorbate and glutathione, coleoptile + leaf, mesocotyl, and root from Cont, Ch, Ac, and AcCh seedlings were harvested into liquid nitrogen and 1 g of each sample was extracted according to Walker and McKersie (1993). AsA and DHA were measured according to Law et al. (1983) and GSH and GSSG were measured according to Hissin and Hilf (1976). Lignin was quantified by a modification of the method of Iiyama and Wallis (1990). Fresh mesocotyls (approximately 0.15 g) were sectioned into approximately 3-mm segments, weighed, and placed in test tubes with 3 mL of 80% (v/v) ethanol. Segments were extracted with three successive 1.5-h extractions in 3 mL of 80% (v/v) ethanol at 80°C, followed by a 1-h extraction in 3 mL of chloroform at 62°C. Segments were then dried for at least 2 d at 50°C. Dried segments were digested in 2.6 mL of a solution of 25% (v/v) acetyl bromide in acetic acid containing 2.7% (v/v) perchloric acid. After exactly 1 h, 100 μL of each sample was added to 580 μL of a solution of 17.24% (v/v) 2 N sodium hydroxide, 82.76% (v/v) acetic acid, and 20 μL of 7.5 M hydroxylamine hydrochloride was added to ensure termination of the reaction. The volume was made to 2 mL with acetic acid

and A_{280} was measured. Lignin was quantified with a standard curve using milled Douglas fir (containing 31.6% lignin; Musha and Goring, 1974) as the standard.

Statistical Analyses

H_2O_2 content was analyzed as a completely randomized design using an unweighted means analysis to account for unequal replication. Lignin, AsA, DHA, total ascorbate, GSH, GSSG, and total glutathione contents, as well as total activities of CAT, POX, SOD, APX, and GR, were all analyzed as a randomized complete block design. Analyses were conducted separately for each tissue type. Means were separated with Tukey's Studentized range test. All reported SE values were calculated using the mean square of the error term from each analysis.

RESULTS

H_2O_2 Levels

Our previous results indicated that chilling caused an elevation of H_2O_2 in mesocotyls but acclimation brought about changes that prevented H_2O_2 accumulation (Prasad et al., 1994a). Therefore, we measured H_2O_2 levels in the coleoptile + leaf and root to determine the oxidative status of these organs (Table I). In the coleoptile + leaf, the same pattern was observed as in the mesocotyl, where H_2O_2 levels were elevated nearly 4-fold in the Ch treatment but were maintained at lower levels in the AcCh treatment. In the root, H_2O_2 levels were elevated to a lesser extent by all low-temperature treatments, although these differences were not statistically significant. Either chilling does not cause as much active oxygen generation or an adequate antioxidant system is already in place in the roots.

Region of CAT and POX Induction in the Mesocotyl

Total CAT and POX activities were examined in various regions of the mesocotyl (Fig. 1) to more precisely locate the primary site of induction. In Cont seedlings, CAT activity was similar throughout the mesocotyl. However, although acclimation induced CAT to some extent in the apical three segments of the mesocotyl, the site of the greatest induction was in the middle to the basipetal region (segments 5 and 8). Compared to apical segments, POX activity was higher in the basipetal portion of Cont mesocotyls, possibly as a function of tissue maturity. Like CAT, the site of the greatest induction of POX by acclimation was in the middle to basipetal portion of the mesocotyl. Coincidentally, this was the region of the mesocotyl that visibly was most sensitive to chilling damage. In accordance with these results, the apical 1 cm of the mesocotyl (mesocotyl top) and the region 1.5 to 2.5 cm from the coleoptilar node (mesocotyl bottom) were sampled separately in all subsequent experiments.

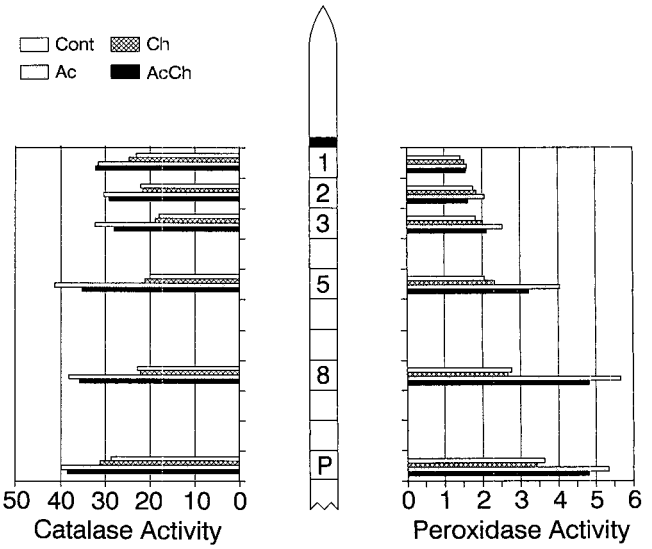


Figure 1. CAT and POX activities in various regions of the maize mesocotyl. Mesocotyls from Cont, Ch, Ac, and AcCh treatments were sectioned into 3-mm segments and segments 1, 2, 3, 5, 8, and the penultimate segment (P) were assayed for CAT and POX. Units for both enzymes were μmol H_2O_2 min^{-1} mg^{-1} protein. Treatments were replicated three times. CAT and POX activities were analyzed as a randomized complete block design within each segment. For CAT: segment 1, SE = 0.68; segment 2, SE = 1.36; segment 3, SE = 1.48; segment 5, SE = 2.09; segment 8, SE = 1.85; penultimate segment, SE = 2.12. For POX: segment 1, SE = 0.15; segment 2, SE = 0.22; segment 3, SE = 0.22; segment 5, SE = 0.10; segment 8, SE = 0.25; penultimate segment, SE = 0.26.

Table I. *H_2O_2 contents in various tissues of maize seedlings exposed to acclimation and chilling*

Seedlings were grown in potting medium at 27°C for 3 d in darkness. AcCh seedlings were then exposed to an Ac treatment of 14°C for 3 d, followed by a Ch treatment of 4°C for 4 d in darkness. Ac seedlings did not receive the Ch treatment; Ch seedlings did not receive the Ac treatment; Cont seedlings did not receive either the Ch or the Ac treatment. Treatments were replicated two to four times and the SE values were derived using a harmonic mean of 2.5263. H_2O_2 contents are expressed as μmol H_2O_2/g fresh weight. H_2O_2 contents in mesocotyls were previously reported by Prasad et al. (1994a).

Treatment	Coleoptile + Leaf	Mesocotyl	Root
Cont	2.38	1.77	1.79
Ch	8.01	7.45	3.51
Ac	2.64	2.50	3.13
AcCh	4.70	3.64	3.43
SE	0.62	0.38	0.79

CAT Isozymes

In shoots and roots of dark-grown maize seedlings, CAT is present as two isozymes, CAT1 and CAT3. During the separation of these isozymes on nondenaturing polyacrylamide gels, we observed that CAT1 but not CAT3 was very susceptible to oxidation on the gel. Inclusion of β-mercaptoethanol in the loading buffer protected CAT1 from oxidation but caused significant inactivation of CAT3 at concentrations of 10 mM (30 mM in the 3× loading buffer) and above. Lower concentrations did not fully protect CAT1. DTT (60 mM in the 3× loading buffer) was found to protect

CAT1 with no deleterious effect on CAT3 activity. In addition to requiring DTT in the loading buffer, it was found that even though CAT1 activity was maintained in extracts (as long as glycerol was present), it was still susceptible to oxidation (presumably oxidation of sulfhydryl groups; Morikofer-Zwez et al., 1969), manifested as an increase in electrophoretic mobility. Addition of DTT (10 mM final concentration) to samples no more than 30 min from the time of tissue maceration was routinely used to maintain consistent electrophoretic mobility among treatments, as has been done by other workers (e.g. Kunce and Trelease, 1986; Mullen and Gifford, 1993).

We observed no significant differences in total CAT activity (Table II) among treatments in either the coleoptile + leaf or the root. CAT activity was significantly higher than Cont in the Ac and AcCh treatments in both the top and bottom of the mesocotyl. In contrast to data previously reported (Anderson et al., 1994), we saw a small but significant decline in total CAT activity in the Ch treatment. The contributions of CAT1 and CAT3 closely reflect the total activity data (Fig. 2). We observed that both CAT1 and CAT3 contribute prominently to the total activity in coleoptile + leaf and mesocotyls, whereas CAT1 dominates in roots. In the mesocotyl, the increased total CAT activity in the Ac and AcCh treatments appeared to be primarily due to an increase in CAT3. Induction of CAT3 by acclimation was visible in both the top and bottom portions of the mesocotyl, with the bottom having generally greater activities than the top for both CAT1 and CAT3. The small decline in total CAT activity in the Ch treatment appeared to result from a decline in both CAT1 and CAT3, perhaps more so from CAT1. This decline was consistent among replicate gels.

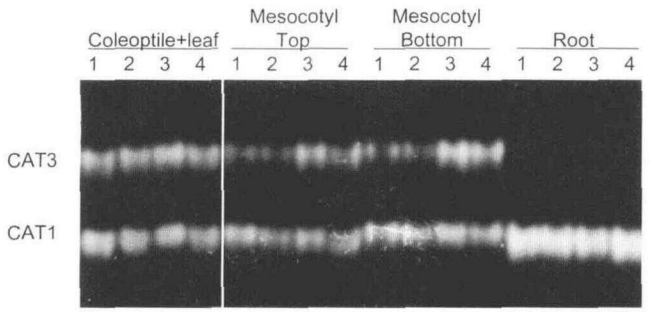


Figure 2. Separation of CAT isozymes from seedlings exposed to acclimation and chilling. Cell-free extracts from coleoptile + leaf, mesocotyl top, mesocotyl bottom, and root of Cont (lanes 1), Ch (lanes 2), Ac (lanes 3), and AcCh (lanes 4) treatments (see Table I for treatment descriptions) were loaded at a 30-μg total protein level and stained for CAT activity.

Peroxidase Isozymes

Total POX activity (Table II) was generally lowest in the coleoptile + leaf, higher in the mesocotyl, and highest in the root. Chilling caused a significant reduction of POX activity in both the mesocotyl and the root. Significant induction of total POX activity by acclimation occurred only in the mesocotyl, in the bottom more so than in the top. When Ac seedlings were subsequently chilled, POX activity was lower than when seedlings were acclimated

Table II. *Lignin content and total activities of CAT, POX, SOD, GR, and APX in various tissues of maize seedlings exposed to acclimation and chilling*

Lignin is expressed as mg lignin/g dry weight. Enzyme units are: CAT and POX, μmol H_2O_2 min^{-1} mg^{-1} protein; GR, nmol NADPH min^{-1} mg^{-1} protein; APX, μmol ascorbate min^{-1} mg^{-1} protein; SOD, the quantity of enzyme required to inhibit the reduction of Cyt *c* by 50% in a 1-mL reaction volume. CAT and POX were replicated nine times; SOD, GR, APX, and lignin were replicated three times. See Table I for treatment descriptions. Mesocotyl top = a 1-cm segment immediately below the coleoptilar node. Mesocotyl bottom = a 1-cm segment 1.5 to 2.5 cm below the coleoptilar node.

Organ	Treatment	CAT	POX	Lignin	SOD	APX	GR
Coleoptile + leaf	Cont	35.50	1.43		4.17	1.43	50.97
	Ch	32.36	1.39		4.85	1.36	51.80
	Ac	33.69	1.23		3.21	1.23	45.45
	AcCh	34.37	1.23		4.04	1.23	48.90
	SE	0.93	0.03		1.06	0.04	1.70
Mesocotyl top	Cont	26.46	1.85	55.41	6.25	1.29	35.20
	Ch	21.77	1.60	51.31	6.61	1.24	37.19
	Ac	34.16	2.23	72.05	6.14	1.41	26.13
	AcCh	34.25	1.93	67.65	5.36	1.38	32.96
	SE	0.97	0.06	3.23	0.34	0.03	2.36
Mesocotyl bottom	Cont	28.82	2.80	64.34	5.37	1.13	41.77
	Ch	25.84	2.42	56.99	5.65	1.06	43.29
	Ac	40.43	4.67	81.85	6.75	1.18	51.10
	AcCh	41.53	3.78	84.74	6.67	1.20	52.26
	SE	0.70	0.12	3.51	0.67	0.05	2.12
Root	Cont	23.69	6.47		2.93	1.37	54.44
	Ch	23.52	5.49		4.63	1.32	46.84
	Ac	23.26	6.58		4.33	1.38	50.08
	AcCh	24.77	6.03		4.55	1.54	45.69
	SE	0.81	0.20		0.94	0.05	2.89

without chilling. Examination of the POX isozyme profiles by IEF revealed a number of changes that occurred in response to acclimation and chilling (Fig. 3). All major isozymes observed using *p*-phenylenediamine as the substrate were also responsive to guaiacol, indicating that these results should be comparable to the total activities in Table II. Conservatively, we observed a total of 10 distinct bands in Figure 3. Even though the band at pH 8.4 appeared to be a doublet in Ac and AcCh treatments in the mesocotyl bottom and in the root, we have questioned whether these were separate isozymes, since it occurred as a doublet only in samples with very high activity. At a lower loading level, only one band appeared. Until we obtain evidence to the contrary, we consider the doublet to be one isozyme and discuss its intensity here in terms of both bands. There was also considerable POX activity present at the cathode, where peroxidases showed pI values higher than the pH range of the gel. When gels were underfocused (Fig. 4), we were able to visualize at least three additional bands. In the 1.5-h gel section, the uppermost arrow indicates an isozyme that was both induced by acclimation and reduced by chilling, and the lower arrow indicates an isozyme that was unaffected by treatment. In the 1.33-h section, the arrow indicates a band that was strongly induced by acclimation.

In the coleoptile + leaf, POX activity was very low but there were two isozymes, focusing at pH 8.0 and 8.4, that were higher in both the Ac and AcCh treatments. In the mesocotyl top we saw induction of nearly all of the major bands in the Ac and AcCh treatments; bands at pH 5.1, 6.7, 7.6, 8.0, 8.4, 8.8, and 9.0 were most notable. The band at pH 6.7 was found to be a mitochondrial peroxidase (Prasad et al., 1995) and is likely responsible for the induction of POX by acclimation in mitochondrial preparations (Prasad et al., 1994b). These bands were also induced by acclimation from a higher Cont level (when compared to mesocotyl top) in the bottom of the mesocotyl, except those at pH 6.7, 7.6, and 8.0. No notable induction of any POX isozyme was observed from the constitutively high levels present in the root. Several bands appeared to decline somewhat in response to chilling. In the mesocotyl bottom, bands focusing at pH 7.6 and 8.8 were most notably reduced. One band, focusing at pH 7.0, was elevated in only the AcCh treatment in coleoptile + leaf and in the mesocotyl top and in both Ch and AcCh treatments in the bottom of the mesocotyl, indicating that it may be an isozyme that was induced, not by acclimation, but by chilling.

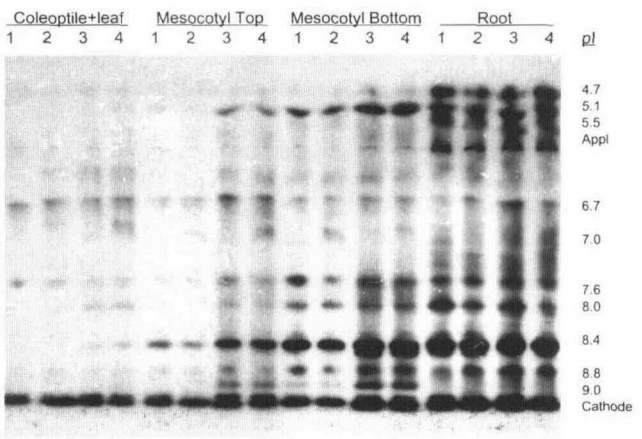


Figure 3. Separation of POX isozymes on a 5% IEF gel, pH 3 to 10, from seedlings exposed to acclimation and chilling. Cell-free extracts from coleoptile + leaf, mesocotyl top, mesocotyl bottom, and root of Cont (lanes 1), Ch (lanes 2), Ac (lanes 3), and AcCh (lanes 4) treatments were loaded at a 75-μg total protein level and stained for POX activity. pI values were determined using an IEF marker kit, pI range 3.5 to 9.3, and stained according to the manufacturer's instructions (Sigma, catalog No. I-3018). Precipitation at the applicator is indicated as "Appl."

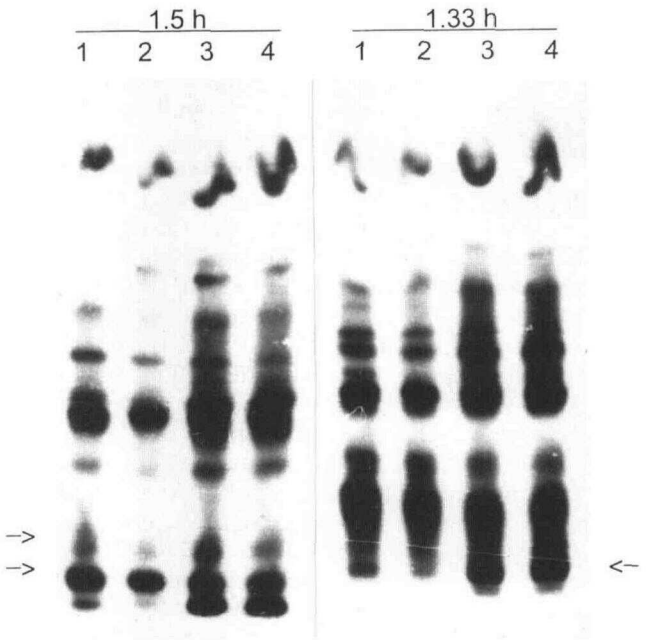


Figure 4. Underfocusing peroxidase isozymes so that those with pI > 9.3 could be visualized. Cell-free extracts from mesocotyl bottom of Cont (lanes 1), Ch (lanes 2), Ac (lanes 3), and AcCh (lanes 4) treatments were loaded at a 75-μg total protein level and run at 20 W constant power for various lengths of time. Isozymes were most visible in the gel sections run for 1.33 and 1.5 h. Three additional isozymes not observed in Figure 3 are indicated by arrows. The lowest band on the 1.5-h gel section had reached the cathode and was better represented on the 1.33-h section.

Since peroxidase involvement in lignification is well established (Gaspar et al., 1991; Siegel, 1993), it was of interest to determine which of these POX isozymes was cell-wall localized. The POX isozyme profiles in cell-wall and protoplast fractions are shown in Figure 5. Bands at pH 4.7 and 8.0 were clearly cell-wall peroxidases. The band at pH 8.4 appeared most abundantly in the cell wall but was also prominent in the protoplast fraction. It may be a cell-wall peroxidase that was loosely associated with the cell wall so that a significant portion was extracted into the protoplast fraction. The band appearing at approximately pH 7.5 in the cell-wall fraction did not have the same appearance as the other bands, but was reminiscent of protein precipitation on the gel. At a lower loading level, this band was not present. Thus, it was likely not a true isozyme. All other POX isozymes were localized in the protoplast.

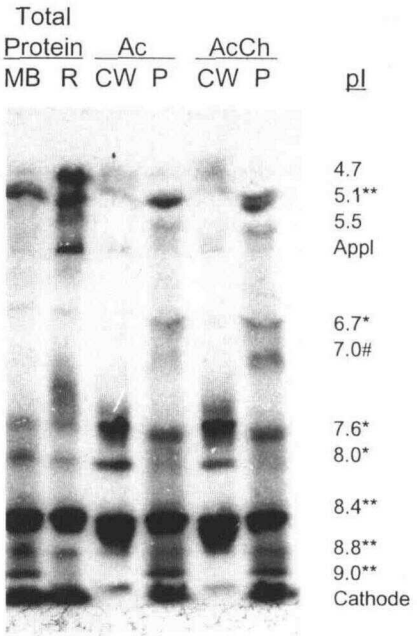


Figure 5. Separation of POX isozymes in cell wall (CW) and protoplasmic (P) fractions from the bottom of the mesocotyl of Ac and AcCh seedlings. Lanes 1 and 2 are total protein extracts (75 μg total protein) from the mesocotyl bottom and root of the AcCh treatment, respectively. Lanes 3 and 4 are cell-wall (15 μg total protein) and protoplasmic (15 μg total protein) fractions from Ac and lanes 5 and 6 are cell-wall and protoplasmic fractions from AcCh, all taken from the bottom of the mesocotyl. Those bands that were induced by acclimation in both the top and bottom of the mesocotyl (Fig. 3) are marked with double asterisks (**). Bands induced in the top but not the bottom of the mesocotyl are marked with single asterisks (*). The band induced by chilling is marked #. Precipitation at the applicator is indicated as "Appl." The band at approximately pH 7.5 in the cell-wall fractions did not have the same appearance as other bands and was considered an artifact.

Lignin Content

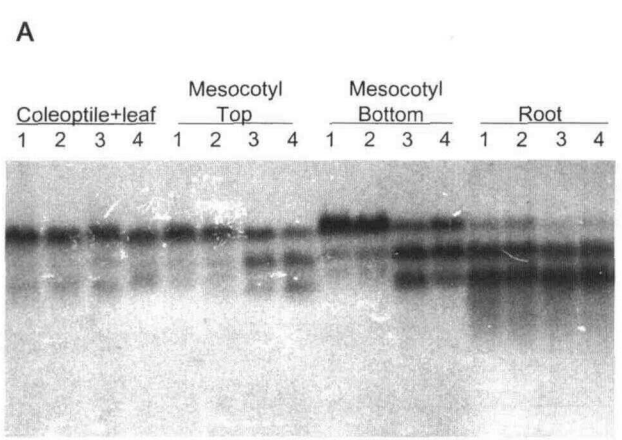
The fact that of the three POX isozymes localized in the cell wall, two were induced by acclimation (the band at pH 8.0 was induced in the mesocotyl top and the band at pH 8.4 was induced in both the top and the bottom of the mesocotyl) suggested that lignification may be a process affected by acclimation. Lignin contents are reported in Table II and, indeed, lignin content was significantly higher in both the top and the bottom of the mesocotyl in the Ac and AcCh treatments when compared to Cont and Ch treatments.

SOD and APX Isozymes

We have previously reported, without showing data, that total SOD and APX activities were unaffected by acclimation and/or chilling in mesocotyls of dark-grown maize seedlings (Prasad et al., 1994a). Total SOD activities are given in Table II and, although there appeared to be a small induction of SOD by acclimation in the bottom of the mesocotyl, there were no significant differences in SOD activity among treatments in any of the tissues. We observed four SOD isozymes on nondenaturing polyacrylamide gels, but none of them were strikingly affected by acclimation or chilling (data not shown). APX activity is also reported in Table II and, like SOD, there were no meaningful differences among treatments in any of the tissues tested. Zymograms revealed four APX isozymes; two were prominent and two were very faint. None of the bands were affected by treatment. With the lack of response of SOD and APX isozymes to acclimation or chilling, no effort was made to further characterize these enzymes.

GR Isozymes

Only minor changes in total GR activity (Table II) were observed in response to acclimation and chilling. GR activity was increased slightly in the Ac and AcCh treatments in the bottom of the mesocotyl. No other significant differences among treatments were observed in the other tissues tested. However, examination of GR isozyme profiles (Fig. 6A) revealed three isozymes that were greatly affected by acclimation in the mesocotyl. Activity in the three bands was dependent on the presence of GSSG during the staining reaction, verifying their identities as GR. In Cont seedlings, the top band was most prominent in the coleoptile + leaf and the mesocotyl and the bottom two bands were most prominent in the roots. No significant changes in the



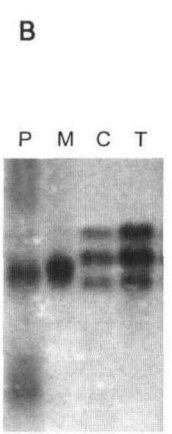


Figure 6. A, Separation of GR isozymes from seedlings exposed to acclimation and chilling. The same cell-free extracts used for CAT (Fig. 2) were loaded at a 100-μg total protein level. The three bands observed did not stain in the absence of GSSG, verifying their identity as GRs. B, Separation of GR isozymes from plastid (P), mitochondria (M), and cytosol (C) fractions (all loaded at 100-μg total protein) from the mesocotyl of Ac seedlings. Plastid and mitochondrial samples contained 1% (v/v) Nonidet P-40 to disrupt membranes. A total protein extract (T, 75 μg total protein) containing all three bands in high quantities was run for comparison.

GR profile were observed in the coleoptile + leaf or the roots in response to acclimation and chilling. In the mesocotyl (both top and bottom), the upper band was greatly diminished in the Ac and AcCh treatments, whereas the lower two bands were greatly increased. The net effect was the absence of a significant change in total GR activity (Table II).

Examination of GR isozyme profiles in plastidic, mitochondrial, and cytosolic fractions (Fig. 6B) revealed that all three bands observed in the cell-free extracts were cytosolic. Apparently, the plastidic and mitochondrial isozymes were too dilute to be observed in the cell-free extracts. Plastid and mitochondrial isolates each had one GR band that migrated differently than any of the cytosolic bands. A rapidly migrating band also appeared in the plastids; it was found to stain at equal intensity in the absence of GSSG and thus was not GR. Preliminary results from mesocotyl tissue revealed no differences in plastidic and mitochondrial GR activity among low-temperature treatments (data not shown).

Ascorbate and Glutathione Levels

Since H_2O_2 levels in Ch coleoptile + leaf were elevated but were maintained at low levels in the AcCh treatment, some antioxidant mechanism must be induced by acclimation in these tissues. However, none of the antioxidant enzymes examined in this study (CAT, POX, SOD, APX, or GR) were affected by acclimation in the coleoptile + leaf. Thus, the levels of ascorbate and glutathione were measured to determine if there were changes in the pools of these antioxidants that could account for the low H_2O_2 levels in the AcCh coleoptile + leaf. In both coleoptile + leaf and in mesocotyl, we generally saw a decline in AsA, no significant change in DHA (although Cont DHA appears slightly higher in the coleoptile + leaf, it was not statistically higher), and a decline in the total ascorbate pool in response to chilling (Table III). Neither AsA nor DHA was significantly different in the Ac and AcCh treatments when compared to Cont. In the root, AsA was significantly lower in both Ch and AcCh treatments than Cont, but DHA was unaffected by treatment. Like AsA, GSH was lower in both coleoptile + leaf and mesocotyl in the Ch treatment compared to Cont, and GSSG was correspondingly higher, resulting in no change in the total glutathione pool. However, in the Ac and AcCh treatments, GSH remained near Cont levels while GSSG was near Ch levels, resulting in an increase in the total glutathione pool. This effect was more pronounced in the coleoptile + leaf than in the mesocotyl. In the root, there was no change in the total glutathione pool among treatments, and there was lower GSH and correspondingly higher GSSG in the Ch and AcCh treatments than in Cont.

DISCUSSION

Response of Antioxidant Enzymes to Acclimation in the Mesocotyl

Consistent with our previous contention that protection from oxidative stress is an important aspect of chilling tolerance, we have provided correlative evidence that the enhanced chilling tolerance of Ac seedlings was associated with changes in antioxidant enzymes and the maintenance of low H_2O_2 levels. Although SOD and APX activities were unchanged, activities of CAT3, at least nine POX isozymes, and three cytosolic GR isozymes were all affected in the Ac and AcCh treatments. These responses were observed primarily in the mesocotyl and few changes were observed in the coleoptile + leaf or the root. As previously reported, we feel that induction of CAT3 during acclimation is of primary importance in protecting mitochondrial components from oxidative damage and is therefore a key element in chilling tolerance (Prasad et al., 1994a, 1994b). Although acclimation did not appear to significantly induce CAT1 (Fig. 2), we observed that CAT1 activity declined in response to chilling. A number of studies have shown that CAT activity rapidly declines in response to chilling in

Table III. *Ascorbate and glutathione levels in various tissues of maize seedlings exposed to acclimation and chilling*

AsA and DHA levels are expressed as μmol/g dry weight. GSH and GSSG are expressed as mg/g dry weight. Treatments were replicated three times. See Table I for treatment descriptions.

Organ	Treatment	AsA	DHA	Total	GSH	GSSG	Total
Coleoptile + leaf	Cont	21.91	7.09	29.00	2.49	1.07	3.56
	Ch	14.97	4.49	19.46	2.19	1.54	3.73
	Ac	21.09	4.24	25.33	2.70	1.70	4.41
	AcCh	19.06	3.98	23.03	2.53	1.98	4.51
	SE	0.77	0.69	0.91	0.08	0.08	0.15
Mesocotyl	Cont	12.80	4.75	17.56	2.72	1.57	4.29
	Ch	9.70	4.98	14.68	2.43	1.94	4.37
	Ac	14.00	5.08	19.08	2.70	1.95	4.65
	AcCh	12.05	3.74	15.79	2.69	2.22	4.92
	SE	0.56	0.60	1.05	0.07	0.06	0.12
Root	Cont	8.79	4.02	12.93	1.94	0.88	2.82
	Ch	6.35	3.38	9.73	1.46	1.23	2.69
	Ac	8.93	3.01	12.10	1.84	1.01	2.85
	AcCh	6.26	3.09	9.35	1.56	1.23	2.79
	SE	0.44	0.33	0.82	0.08	0.05	0.12

combination with high light (MacRea and Ferguson, 1985; Volk and Feierabend, 1989; Feierabend et al., 1992; Mishra et al., 1993), likely a result of inactivation of CAT by active oxygen (Feierabend and Engel, 1986). We have observed that CAT1, and to a lesser extent CAT3, were susceptible to oxidative inactivation in vitro (data not shown). Although we cannot rule out a chilling-induced down-regulation of *cat* gene expression, we have shown that H_2O_2 levels are elevated in response to chilling, and it is possible that one of the effects of chilling is oxidative inactivation of CAT. It is noteworthy that CAT1 activity was maintained in the AcCh treatment, since prevention of a loss of activity may be as important to chilling tolerance as induction of activity. However, whether acclimation directly affects CAT1 or indirectly prevents its inactivation through maintenance of low H_2O_2 levels is unknown.

We observed one plastidic, one mitochondrial, and three cytosolic isozymes of GR in maize (Fig. 6B), although we do not discount the possibility of other unresolved isozymes that require two-dimensional PAGE (Edwards et al., 1990). Only the cytosolic isozymes were observed in cell-free extracts (Fig. 6A). A shift in the intensity from the upper band to the lower two bands was observed in the Ac and AcCh treatments, but only in the mesocotyl. It is unknown whether this is due to differential expression of distinct GR genes or a posttranslational modification of one or more of the GR subunits. The increased intensity of the two lower bands in Ac mesocotyls is suggestive of an involvement in chilling tolerance. Similar to our results, exposure to low temperature has resulted in altered GR isozyme profiles in pea (Edwards et al., 1994) and in spinach (Guy and Carter, 1984). In both cases, the altered isozyme profile was associated with an increased affinity of GR for its substrates. If such is also the case with the GR isozymes in our system, it would enhance the ability of Ac seedlings to maintain GSH in the reduced form in the cytosol, contributing to the ability to tolerate chilling-induced oxidative stress.

We observed the induction of at least nine major POX isozymes in Ac seedlings. Although the significance of CAT and GR as H_2O_2 scavengers is clear, the role of POX as an H_2O_2 scavenger is not well established. We have previously suggested that the mitochondrial peroxidase (pI 6.7) may contribute to the maintenance of low H_2O_2 levels in the mitochondria (Prasad et al., 1995), although the utilization of H_2O_2 may be incidental to its true function. At this point, we have made no effort to identify the substrates most reactive with individual isozymes and cannot suggest possible functions. However, the fact that these isozymes were elevated in Ac seedlings suggested a role in chilling tolerance. Since two of the induced isozymes were localized in the cell wall (Fig. 5) and lignin content was elevated in mesocotyls from Ac seedlings (Table II), it is possible that lignification may be a component of acclimation-induced chilling tolerance. In this way, Ac mesocotyls would have an increased rigidity, allowing them to remain intact while unacclimated mesocotyls become desiccated and mechanically weak in response to chilling.

Differences in the Antioxidant Responses among Tissues

We have observed that the effect of acclimation on antioxidant enzymes differs depending on the tissue. The mesocotyl is visibly the most susceptible to chilling damage, and it is in this organ that we observe the induction of antioxidant enzymes. It is understandable that if the mesocotyl is the most sensitive to chilling damage, chilling tolerance mechanisms would be focused on this organ. We also noted that the mature cells of the mesocotyl appeared to be more susceptible to chilling damage than the cells in the apical region. Total CAT and POX activity (Fig. 1) were induced in Ac seedlings more strongly in the mature cells, supporting the correlation of chilling sensitivity of the tissue with induction of antioxidant enzymes during acclimation. These responses were confirmed by examination of isozyme profiles. Although there were dramatic changes in CAT3, POX, and GR in the top of the mesocotyl of Ac seedlings (Figs. 2, 3, and 6A), the changes were more pronounced in the bottom.

In contrast to the mesocotyl, none of the antioxidant enzymes were affected in the coleoptile + leaf of Ac seedlings. This is despite the fact that H_2O_2 in the AcCh treatment was maintained at low levels, just as in the mesocotyl. Thus, some mechanism of antioxidant protection must exist in these tissues. Examination of the ascorbate and glutathione pools in Table III may provide some insight. Among other functions, ascorbate and glutathione are key elements in the scavenging of H_2O_2 through their participation in the ascorbate-glutathione cycle (Rennenberg, 1982; Foyer et al., 1991). The H_2O_2-scavenging capacity of the cycle is dependent on the maintenance of reduced ascorbate and glutathione pools by the action of DHAR and GR. Jahnke et al. (1991) reported that the activities of both DHAR and GR in maize were severely inhibited when measured at 5°C; thus, we would expect a depletion of AsA and GSH and a corresponding increase in DHA and GSSG in Ch seedlings. This is essentially what we see in the coleoptile + leaf of the Ch treatment (Table III). The lack of an increase in DHA may be the result of oxidation to tartaric acid and oxalic acid (Loewus, 1988), a process that may be enhanced under conditions of oxidative stress. Most importantly, we observed that the total glutathione pool increased in the coleoptile + leaf of the Ac and AcCh treatments. In the absence of sufficient GR activity or NADPH, this may provide sufficient GSH to reduce DHA so that the AsA level can be maintained when Ac seedlings are subsequently chilled. The lower level of DHA in the AcCh treatment (although the difference was not statistically significant) may be interpreted as the combined effects of DHA oxidation and DHA reduction to AsA. Our results are similar to those of Walker and McKersie (1993), who observed an increased synthesis of total glutathione and a maintenance of the reduced form in chilling-resistant tomato while the susceptible tomato species exhibited only an oxidation of GSH with no increase in the total glutathione pool. We acknowledge that caution should be exercised when drawing conclusions about the efficacy of the ascorbate-glutathione cycle in scavenging H_2O_2 based solely on metabolite pool sizes, since the capacity of the

cycle is also dependent on the kinetic parameters of the associated enzymes at the treatment temperature. However, the importance of maintaining reduced glutathione as a factor in low-temperature tolerance has been suggested by Alscher (1989), and our results are consistent with this view. Thus, we suggest that the maintenance of GSH in Ac seedlings near that of unchilled controls, possibly through increased synthesis of GSH, may contribute to the prevention of H_2O_2 accumulation in the coleoptile + leaf.

The response of ascorbate and glutathione in mesocotyls to acclimation and chilling was generally the same as that in the coleoptile + leaf, although the increase in the total glutathione pools in response to acclimation was not as extensive. Induction of other antioxidants in the mesocotyl may reduce the need for GSH synthesis. In the roots, there was no effect of acclimation or chilling on any of the antioxidants tested. However, the fact that H_2O_2 levels in the roots were not greatly increased by chilling suggests that a modification of antioxidants was unnecessary to prevent oxidative stress.

The Antioxidant System in Preemergent Maize Seedlings

The importance of antioxidants in the protection of light-grown plants from chilling-induced oxidative damage has been well established (Halliwell, 1987; Jahnke et al., 1991; Mishra et al., 1993). Restricted electron flow through the photosystems in the presence of excess light leads to an increased generation of superoxide in the chloroplasts and places a greater demand on the active oxygen-scavenging system of the chloroplast. However, in dark-grown maize seedlings exposed to a chilling stress, the primary source of active oxygen is likely the mitochondria (Prasad et al., 1994b). Mitochondria have also been implicated as a major source of extramitochondrial H_2O_2 in nonphotosynthetic tissue (Puntarulo et al., 1991), and thus, induction of CAT3 and mitochondrial peroxidases by acclimation may be the first line of defense against oxidative damage throughout the cell. The catabolism of cytosolic H_2O_2 is likely dependent on the maintenance of reduced AsA and GSH. Thus, the increased synthesis of GSH, or the conversion of GR to isoforms that are more efficient at low temperature, would contribute significantly to the ability to prevent oxidative stress in Ac seedlings. Peroxidases located in the vacuole may also contribute to the scavenging of cytosolic H_2O_2 (Takahama, 1991), although we have not determined whether any of the peroxidases induced by acclimation in our study were vacuole localized. In this study, we have noted numerous differences in antioxidant enzymes between Ac and Cont seedlings. Although we cannot definitively tie all changes to acclimation, our data are consistent with the idea that the acclimation process involves enhancement of antioxidant systems throughout the cell. Although mitochondrial antioxidants may be key in preemergent maize seedlings, we conclude that antioxidants in other compartments likely also contribute to the protection from chilling-induced oxidative stress.

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