The Plant Cell, Vol. 6, 65–74, January 1994 © 1994 American Society of Plant Physiologists

Evidence for Chilling-Induced Oxidative Stress in Maize Seedlings and a Regulatory Role for Hydrogen Peroxide

Tottempudi K. Prasad,[a] Marc D. Anderson,[a] Barry A. Martin,[b] and Cecil R. Stewart,[a,1]

[a] Department of Botany, Iowa State University, Ames, Iowa 50011
[b] Pioneer Hi-Bred International, Johnston, Iowa 50131

We have taken advantage of an acclimation phenomenon in a chilling-sensitive maize inbred to investigate the molecular, biochemical, and physiological responses to chilling in preemergent maize seedlings. Three-day-old seedlings were exposed to 4°C for 7 days and did not survive chilling stress unless they were preexposed to 14°C for 3 days. cDNAs representing three chilling acclimation-responsive (*CAR*) genes were isolated by subtraction hybridization and differential screening and found to be differentially expressed during acclimation. Identification of one of these *CAR* genes as *cat3*, which encodes the mitochondrial catalase3 isozyme, led us to hypothesize that chilling imposes oxidative stress in the seedlings. Hydrogen peroxide levels were elevated during both acclimation and chilling of nonacclimated seedlings. Further molecular and biochemical analyses indicated that whereas superoxide dismutase activity was not affected, the levels of *cat3* transcripts and the activities of catalase3 and guaiacol peroxidase were elevated in mesocotyls during acclimation. Accumulation of H_2O_2 following a short treatment with aminotriazole, a catalase inhibitor, indicated that catalase3 seems to be an important H_2O_2-scavenging enzyme in the seedlings. Control 3-day-old seedlings pretreated with H_2O_2 or menadione, a superoxide-generating compound, at 27°C induced chilling tolerance. Both of these chemical treatments also increased *cat3* transcripts and catalase3 and guaiacol peroxidase activities. We suggest that peroxide has dual effects at low temperatures. During acclimation, its early accumulation signals the production of antioxidant enzymes such as catalase3 and guaiacol peroxidase. At 4°C, in nonacclimated seedlings, it accumulates to damaging levels in the tissues due to low levels of these, and perhaps other, antioxidant enzymes.

INTRODUCTION

A number of tolerance mechanisms have been proposed based on the physiological and biochemical changes associated with chilling injury (for review, see Fridovich, 1978; Halliwell, 1981; Graham and Patterson, 1982; Wang, 1982; Markhart, 1986; Elstner, 1991; McKersie, 1991). Chilling injury causes changes in membrane properties (Lyons and Asmundson, 1965), solute leakage (Lieberman et al., 1958), reduced transport across the plasmalemma (Wright and Simon, 1973), malfunction of mitochondrial respiration (Lyons and Raison, 1970), and induction of peroxide and indoleacetic acid oxidase levels (Omran, 1980). Recently, chilling sensitivity has also been shown to be correlated with the extent of fatty acid unsaturation of the phosphatidyl glycerol of chloroplast membranes in genetically engineered tobacco plants (Murata et al., 1992). On the other hand, the damage that occurs during chilling stress accompanying illumination was thought to be mediated by an oxygen radical (Wise and Naylor, 1987). Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.16), and various peroxidases such as guaiacol peroxidase (POX, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11) constitute the cellular defense against oxidative stress. Overexpression of MnSOD or chloroplastic Cu/ZnSOD was reported to confer protection from light-mediated paraquat damage (Bowler et al., 1991) and from light-associated chilling damage (Gupta et al., 1993) in tobacco transformants. In these studies, greenhouse-grown mature plants were used. Photoinhibition and photooxidation were considered as the light-associated chilling damage in those transgenic plants.

Acclimation to chilling results in a lowering of the temperature at which the plant is damaged or killed by chilling temperature. We have characterized such a chilling acclimation or temperature-conditioning phenomenon in a chilling-sensitive Pioneer maize inbred, G50. Our system is unique in that the chilling damage and acclimation were observed in dark-grown seedlings rather than light-grown plants. The evaluation of chilling damage was based on growth and survival of the seedlings. Several studies have reported that chilling-resistant species or strains have more efficient antioxidant systems than the sensitive ones (Jahnke et al., 1991; Walker and McKersie, 1993). However, the advantage of working with one variety such as G50 is that we can study the molecular mechanisms involved in both sensitivity and resistance to chilling in the same genotype and thus eliminate the complexity of genetic differences. Although several biochemical and biophysical changes have

[1] To whom correspondence should be addressed.

been shown to be involved in the acclimation process (Graham and Patterson, 1982; Wang, 1982; Markhart, 1986), isolation and characterization of genes regulated during chilling acclimation when coupled with physiological and biochemical analyses will provide important new insights into chilling tolerance processes.

To identify the molecular mechanisms of chilling acclimation in maize inbred G50 seedlings, we isolated and characterized cDNAs representing three chilling acclimation-responsive (*CAR*) genes in this study. Identification of one of the *CAR* genes as *cat3* led us to hypothesize that chilling may be imposing oxidative stress in the seedlings. We present experimental evidence to indicate that chilling imposes severe oxidative stress that may be responsible for chilling-associated damage in nonacclimated seedlings. Furthermore, mild oxidative stress induced by either chemical treatment or by chilling acclimation appears to be beneficial to subsequently chilled seedlings.

RESULTS

Growth and Survival of Seedlings at Low, Nonfreezing Temperatures

The data in Table 1 illustrate an acclimation phenomenon in Pioneer maize inbred G50. Only 2% of the nonacclimated seedlings survived chilling at 4°C for 7 days compared to 69% of the acclimated seedlings that were exposed to 14°C for 3 days before chilling. Nonacclimated seedlings did not grow during 10 days in the greenhouse, whereas the fresh and dry weights in acclimated shoots increased nearly fourfold. The physical appearance of nonacclimated seedlings indicated that the mesocotyl seemed to be the most sensitive organ to chilling damage. In general, the symptoms of chilling damage include the inhibition of seedling growth, waterlogged appearance and browning of mesocotyls, and the browning and desiccation of coleoptile and undeveloped leaves.

Differentially Expressed Genes during Acclimation

Three putative cDNA clones representing chilling acclimation–responsive (*CAR*) genes, designated as *CAR30*, *CAR333*, and *CAR757*, reproducibly showed stronger hybridization signals with the first-strand cDNA probe from acclimated tissue compared to the control tissues and were further characterized. The RNA gel blot analysis shown in Figure 1 indicates that the cDNA inserts from *CAR30*, *CAR333*, and *CAR757* hybridized with single mRNA bands of ~1.6, 1.8, and 1.5 kb, respectively. Densitometric scans indicated that the levels of *CAR* gene transcripts increased threefold in acclimated seedlings. The cDNAs of *CAR30*, *CAR333*, and *CAR757* were sequenced (data not shown). A search of GenBank revealed no previously reported sequences with significant homology for *CAR30* and *CAR757*, but *CAR333* was identified as *cat3*, which was reported to encode for the maize mitochondrial catalase3 isozyme (Redinbaugh et al., 1988). However, no attempt was made to confirm the mitochondrial location of CAT3 in the present study.

Chilling-Induced Oxidative Stress

The identification of one of the cDNAs representing *CAR* genes (*CAR333*) as *cat3* led us to hypothesize that chilling may be imposing oxidative stress in the seedlings. The results of RNA gel blot analysis shown in Figure 2A, using the *cat3* gene–specific probe, indicated that *cat3* was preferentially expressed in the mesocotyl and coleoptile plus leaf but that expression was undetectable in the root. Compared to the control seedlings, transcript levels increased fivefold in acclimated seedlings, sevenfold in acclimated and chilled seedlings, and less than threefold in nonacclimated chilled seedlings (Figure 2A). To further characterize the putative role of oxidative stress in chilling stress, the activities of antioxidant enzymes such as SOD, CAT3, and POX were measured in extracts of coleoptile plus leaf, mesocotyl, and root tissues, and APX was

Table 1. Effects of Acclimation, Nonacclimation, H_2O_2, and MD Treatments on the Growth of the Maize Seedlings

			Shoots[a]	
Treatments[b]	Conditions[c]	% Survival[d]	Fresh Weight (gm)	Dry Weight (gm)
NAc	I		0.74 ± 0.01	0.08 ± 0.0
	F	2.0 ± 0.0	0.53 ± 0.04	0.13 ± 0.01
Ac	I		1.08 ± 0.02	0.10 ± 0.01
	F	69.0 ± 6.1	3.02 ± 0.16	0.30 ± 0.04
H_2O_2	I		0.70 ± 0.01	0.07 ± 0.01
	F	58.0 ± 4.2	2.27 ± 0.12	0.24 ± 0.01
MD	I		0.72 ± 0.03	0.07 ± 0.01
	F	58.0 ± 9.7	2.71 ± 0.43	0.29 ± 0.02

[a] Values for fresh and dry weights are the means ± SD (in gm) of two replicates with five seedlings sampled in each replicate.
[b] Ac, acclimation; NAc, nonacclimation; see Methods for treatment conditions.
[c] I, at the end of 4°C treatment; F, at the end of 10 days in greenhouse.
[d] Values for percent survival are the means ± SD of 61 to 97 seedlings.

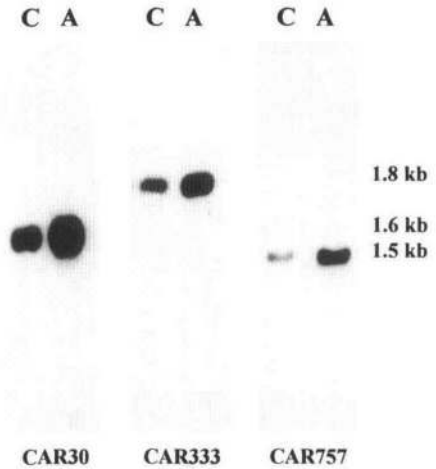

Figure 1. RNA Gel Blot Analyses of *CAR30*, *CAR333*, and *CAR757* Transcripts in Control and Acclimated Maize Seedlings.

Poly(A)$^+$ RNA (1 μg) isolated from control (C) and acclimated (A) whole seedlings was resolved on 1% formaldehyde–agarose gel, transferred to a nylon membrane, and then hybridized with ^{32}P-labeled *CAR30*, *CAR333*, and *CAR757* cDNAs as probes. The molecular weights of mRNA bands are given at right.

measured in extracts of mesocotyl tissues from seedlings exposed to acclimation, chilling, or chemical treatments. Total cellular SOD and APX activities were not affected by any temperature or chemical treatments in acclimated or nonacclimated seedlings (data not shown). Similarly, no changes in CAT3 and POX activities were observed in coleoptile plus leaf and roots between control and temperature or chemical treatments; therefore, no data were presented. Activities of CAT3 and POX increased three- to eightfold in acclimated and acclimated and chilled seedlings relative to nonacclimated and chilled and control seedlings (Figure 2B). Although coleoptiles also accumulated *cat3* transcripts fourfold during acclimation (Figure 2A), no increased CAT3 activity was observed (data not shown).

Because nonacclimated seedlings showed visual chilling damage and low levels of CAT3 and POX activities, we suspected that an increase in H_2O_2 levels caused by chilling stress could be partially responsible for damaging the tissue. As expected, nonacclimated seedlings contained higher levels of H_2O_2 compared to any other treatment (Figure 2B). This result clearly indicated that chilling induced an oxidative stress in the tissues.

Role of H_2O_2 in Acclimation to Chilling-Induced Oxidative Stress

To test our hypothesis that acclimation also imposes oxidative stress, a time-course analysis of *cat3* transcripts, CAT3 and POX activities, and H_2O_2 levels was determined during the early stages of acclimation. Figure 3B shows a dramatic, transient accumulation of H_2O_2 within 2 to 4 hr of the acclimation process when CAT3 and POX activities were at basal levels and then a slow decline over the next 32 hr of acclimation

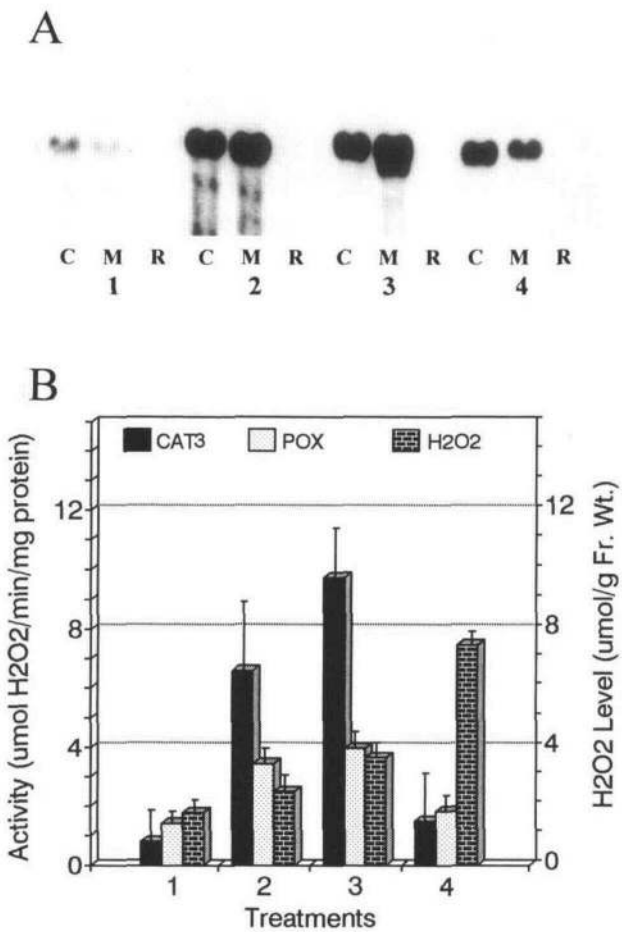

Figure 2. Analyses of *cat3* Transcripts, CAT3 and POX Activities, and H_2O_2 Levels during Acclimation and Nonacclimation in Maize Seedlings.

(A) *CAR333* (*cat3*) transcripts during acclimation and nonacclimation. Total RNA (15 μg) isolated from coleoptile plus leaf (C), mesocotyl (M), and root (R) of control, acclimated, and nonacclimated seedlings was resolved and transferred as described in Figure 1. Filters were hybridized with the ^{32}P-labeled *CAR333* gene–specific cDNA as probe.

(B) Activities of CAT3, POX, and H_2O_2 levels in control, acclimated, and nonacclimated seedlings. Dark-grown 3-day-old seedlings were treated as described below, and total proteins were extracted from mesocotyl for enzyme analyses.

The seedlings were treated in both **(A)** and **(B)** as follows: lanes 1, control 3-day-old seedlings; lanes 2, seedlings acclimated for 3 days at 14°C; lanes 3, acclimated seedlings transferred to 4°C for 4 days; lanes 4, nonacclimated, control seedlings transferred to 4°C for 4 days. CAT3 and POX activities and H_2O_2 levels were replicated two to nine times and analyzed as a completely randomized design. Standard errors are represented as vertical lines accompanying each bar. H_2O_2 levels are given in micromoles per gram fresh weight of tissue.

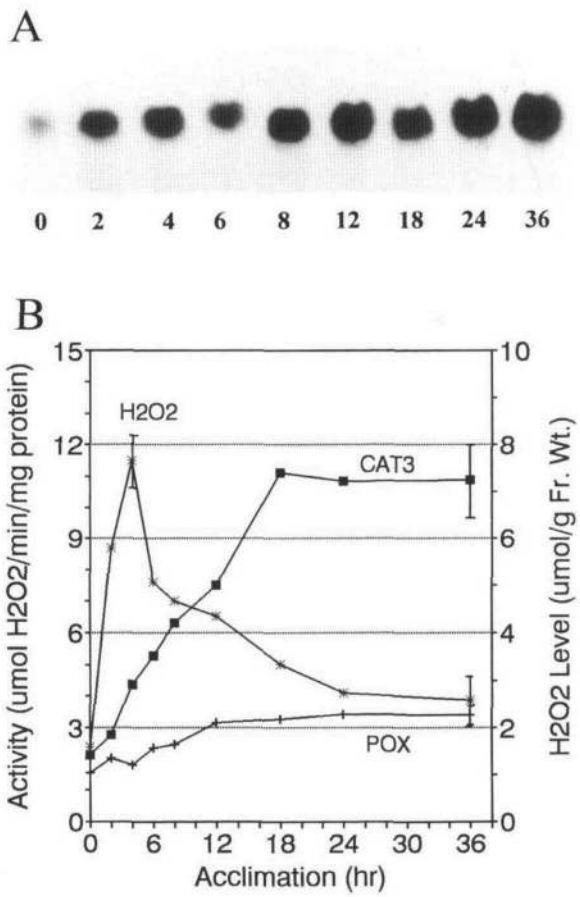

Figure 3. Time-Course Analysis of *cat3* Transcripts, CAT3 and POX Activities, and H_2O_2 Levels during the Early Stages of Acclimation.

(A) RNA gel blot analysis of *cat3* transcripts. Total RNA (15 μg) was isolated from mesocotyls of control and acclimated seedlings (at 0, 2, 4, 6, 8, 12, 18, 24, and 36 hr), and RNA gel blot analysis was performed as described in Figure 1 using the *cat3* gene–specific probe.
(B) Activities of CAT3 and POX and H_2O_2 levels in mesocotyls at 0, 2, 4, 6, 8, 12, 18, 24, and 36 hr of acclimation. CAT3 and POX activities were analyzed using a quadratic regression model. The first three time points of the H_2O_2 level curve were analyzed by linear regression and the third through the seventh time points were analyzed by a quadratic regression model. Error bars representing two standard errors are given on the last time point in each analysis. Each data point represents two replicates. H_2O_2 levels are given in micromoles per gram fresh weight of tissue.

treatment when CAT3 and POX activities increased severalfold. RNA gel blot analysis (Figure 3A) indicated that *cat3* transcripts increased fourfold at 2 hr of acclimation treatment and increased further to sevenfold by 36 hr. Interestingly, both CAT3 and POX activities did not increase during the first 2 hr of acclimation treatment but later increased slowly to the maximum at 18 hr and remained high until the end of the acclimation period (Figure 3B).

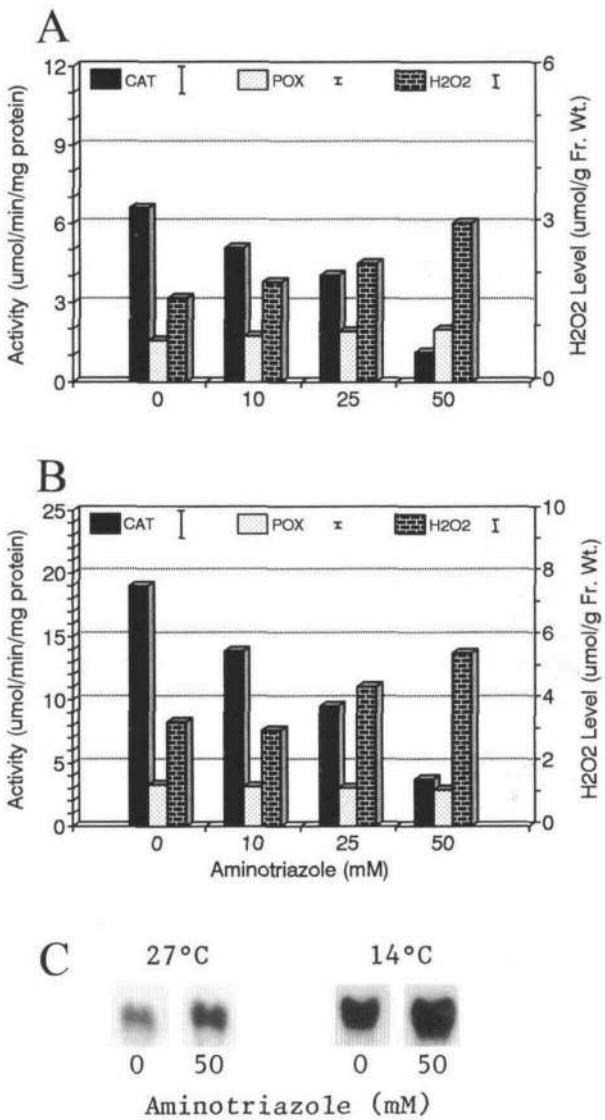

Figure 4. Effects of Aminotriozole on *cat3* Transcripts and the Activities of CAT and POX and H_2O_2 Levels in the Mesocotyls of Maize Seedlings.

(A) Activities of CAT and POX and H_2O_2 levels in control seedlings treated with aminotriazole (AT).
(B) Activities of CAT and POX and H_2O_2 levels in preacclimated seedlings treated with AT.
(C) RNA gel blot analysis of *cat3* transcripts. Total RNA (15 μg) was isolated from the mesocotyls of control 3-day-old seedlings grown at 27°C and seedlings that were preacclimated at 14°C for 15 hr treated with or without 50 mM AT. RNA gel blot analysis was performed as described in Figure 1; the *cat3* gene–specific probe was used. Seedlings were treated with 10, 25, and 50 mM concentrations of AT (on all panels) for 2 hr and were then transplanted in peat moss and further incubated at 27° or 14°C for an additional 1 hr. H_2O_2 levels are given in micromoles per gram fresh weight of tissue. CAT and POX activities and H_2O_2 levels were replicated two times and analyzed using a linear regression model.

To determine the role of the CAT enzyme, CAT3 in particular, in scavenging H_2O_2, control 3-day-old seedlings were treated with aminotriazole (AT) and analyzed for H_2O_2 accumulation. Figure 4A shows the accumulation of H_2O_2 with increasing inhibition of CAT activity, and in response a twofold increase in *cat3* transcripts (Figure 4C) was observed. This result is consistent with the idea that endogenously accumulated H_2O_2 induces *cat3* transcription. Similarly, when seedlings that were preacclimated for 15 hr were treated with AT, there was also a significant accumulation of H_2O_2 as a result of the inhibition of CAT activity (Figure 4B) and in response an increase of nearly twofold in *cat3* transcripts (Figure 4C) compared to the corresponding 18-hr acclimation levels. As expected, AT did not have any effect on POX activity at 27° or 14°C (Figures 4A and 4B). These results clearly indicated that CAT3 is an important H_2O_2-scavenging enzyme in maize seedlings and that maintenance of H_2O_2 at steady state levels in acclimated seedlings is to some extent due to increased CAT3 activity.

H_2O_2- and Menadione-Induced Chilling Tolerance

The results of time-course analysis of acclimation and also the preliminary experiments with the exogenous application of H_2O_2 to the seedlings suggested that mild oxidative stress at 27°C in the seedlings might induce chilling tolerance. To further document the regulatory role of H_2O_2, the 3-day-old dark-grown seedlings were treated for 4 hr with H_2O_2 and menadione (MD), a superoxide-generating compound, transplanted in peat moss, and transferred to 4°C for 7 days followed by greenhouse grow out. Both H_2O_2- and MD-pretreated seedlings exhibited chilling tolerance as indicated by survival and growth (Table 1). As Figure 5 indicates, the *cat3* transcripts increased fivefold by H_2O_2 and sevenfold by MD at 27°C (Figures 5A and 5B). Upon further incubations at 27°C for 12 hr followed by 4°C treatment for 4 days, the levels of *cat3* transcripts increased eightfold with H_2O_2 treatments and eight- to tenfold with MD treatments compared to controls (Figures 5A and 5B). Similarly, CAT3 and POX activities also increased with both treatments at 27°C (Figures 6A and 6B). Upon further incubations at 27°C for 12 hr followed by 4°C treatment for 4 days, CAT3 and POX (Figures 6A and 6B) activities remained high in seedlings receiving both of these chemical treatments. Total cellular SOD and APX activities remained unchanged (data not shown). We interpret these results to mean that higher levels of CAT3 and POX are necessary to scavenge H_2O_2, which otherwise accumulates to potentially damaging levels. The results from experiments in which seedlings were treated with H_2O_2 and MD were similar to the results obtained from the acclimated and nonacclimated treatments.

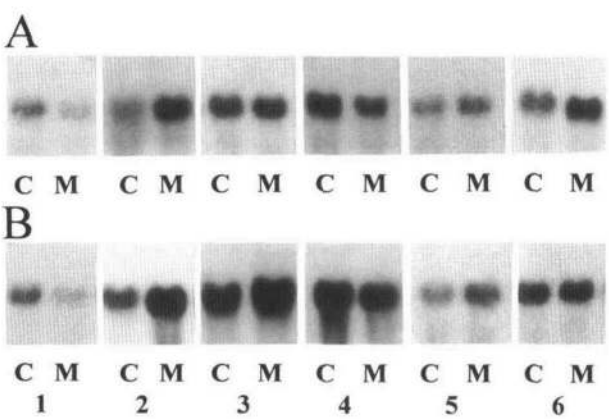

Figure 5. Effects of H_2O_2 and MD on *cat3* Transcript Levels.

(A) Total RNA treated with 0.1 mM H_2O_2.

(B) Total RNA treated with 0.1 mM MD.

Total RNA (15 μg) was isolated from coleoptile plus leaf (C) and mesocotyl (M) of seedlings treated with 0.1 mM H_2O_2 or MD, and RNA gel blot analysis was performed with the *cat3* gene–specific probe as described in Figure 1. The seedlings were treated in panels of both **(A)** and **(B)** as follows: lanes 1, control 3-day-old seedlings; lanes 2, seedlings treated with 0.1 mM H_2O_2 or MD for 4 hr at 27°C; lanes 3, treated seedlings further incubated at 27°C for 12 hr; lanes 4, treated seedlings, after 12-hr recovery, transferred to 4°C for 4 days; lanes 5, seedlings prechilled at 4°C for 4 days and treated with 0.1 mM H_2O_2 or MD for 4 hr at 4°C; lanes 6, seedlings treated with H_2O_2 or MD at 14°C for 4 hr.

Lack of Response to H_2O_2 during Chilling

Although the exogenous H_2O_2 treatment increased CAT3 and POX activities at 27°C, we were puzzled by the fact that the endogenously accumulated peroxide failed to increase these enzyme activities at 4°C. To determine whether chilling interferes with the effects of H_2O_2 and MD on CAT3 and POX activities, control seedlings and seedlings prechilled at 4°C for 4 days were treated with H_2O_2 or MD for 4 hr at 14° and 4°C, respectively. Although both H_2O_2 and MD treatments induced *cat3* transcripts by five- to sevenfold (Figures 5A and 5B) and the CAT and POX activities induced by two- to fourfold at 14°C were similar to the levels seen at 27°C (Figures 6A and 6B), no significant change in *cat3* transcript levels or enzyme activity levels was observed in prechilled seedlings treated at 4°C. This finding indicated that the mechanism by which H_2O_2 and MD induces CAT3 and POX gene expression is indeed sensitive to cool temperature. However, we cannot rule out the possibility that endogenous H_2O_2 might also serve a signal function to some extent because *cat3* transcripts also increased more than twofold at 4°C in nonacclimated seedlings (Figure 2A) compared to control seedlings. For an unknown reason, however, there was no increase of CAT3 activity in these nonacclimated seedlings (Figure 2B), suggesting that CAT3 activity is post-transcriptionally or post-translationally controlled. On the other hand, 14°C is apparently not cool enough to greatly affect the mechanism.

In spite of the increased CAT3 and POX activities, higher levels of peroxide were observed in mesocotyl tissues treated

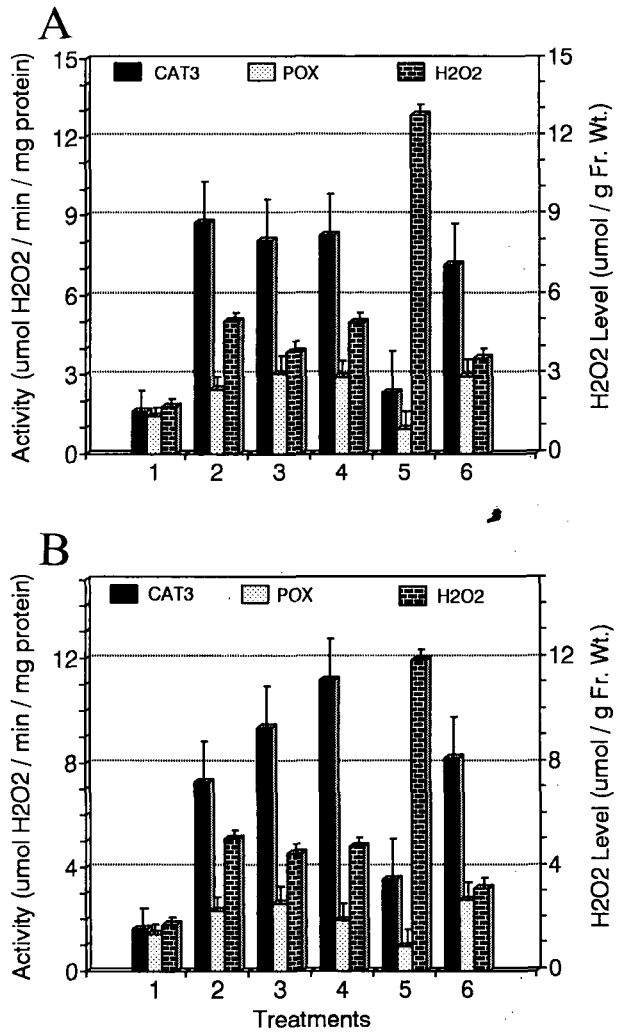

Figure 6. Activities of CAT3 and POX and H_2O_2 Levels in the Mesocotyls of the Seedlings Treated with H_2O_2 or MD.

(A) CAT3 and POX and H_2O_2 levels in the seedlings treated with 0.1 mM H_2O_2.

(B) CAT3 and POX and H_2O_2 levels in the seedlings treated with 0.1 mM MD.

The seedlings were treated with H_2O_2 or MD as described in Figure 5 (lanes 1 to 6 in both panels of **[A]** and **[B]**) and the mesocotyls were harvested for protein extractions. CAT3 and POX activities and H_2O_2 levels were replicated two to nine times and analyzed as a completely randomized design. Standard errors are represented as vertical lines accompanying each bar. H_2O_2 levels are given in micromoles per gram fresh weight of tissue.

with H_2O_2 and MD at 27° and 14°C (Figures 6A and 6B). These chemicals are expected to increase peroxide levels in the tissues more rapidly than observed in acclimation treatments. H_2O_2- and MD-pretreated seedlings transferred to 4°C also contained moderately increased peroxide levels. Peroxide levels were almost twofold higher in prechilled seedlings treated with H_2O_2 and MD at 4°C compared to the levels in nonacclimated seedlings. This result indicated that the lack of an effect on CAT3 and POX activities by these treatments in prechilled seedlings in not due to the lack of the absorption of these compounds by the tissues during the 4°C treatment.

DISCUSSION

Regulatory Role for H_2O_2 in Chilling-Induced Oxidative Stress

Consistent with the previous hypothesis that free oxygen radicals are involved in light-associated chilling stress (Wise and Naylor, 1987), we have presented molecular and biochemical evidence to indicate that low temperature also imposes oxidative stress in dark-grown seedlings during acclimation and chilling treatments. We have also shown that endogenously and exogenously generated H_2O_2 induced similar metabolic events that led to the chilling tolerance process in the seedlings. Although acclimation is considered a complex phenomenon, our results indicated that whereas severe oxidative stress could be detrimental to nonacclimated seedlings during chilling, a mild oxidative stress during acclimation could be responsible for inducing chilling tolerance. A high H_2O_2 concentration combined with other reactive oxygen species could increase lipid peroxidation and other damaging reactions related to oxyradicals (Leshem, 1984; Markhart, 1986; Asada and Takahashi, 1987). The accumulation of H_2O_2 after a short treatment with AT indicated that CAT appears to be an important antioxidant enzyme in overcoming acclimation-imposed oxidative stress. Evidently, CAT, POX, and APX are reported to be the most predominant H_2O_2-utilizing enzymes in plant systems (Asada and Badger, 1984; Puntarulo et al., 1988, 1991).

A novel circadian regulation of the *cat3* gene expression was described previously in light- and dark-grown developing maize seedlings (Acevedo et al., 1991). However, when seedlings were grown continuously in light or dark, *cat3* transcripts were present at steady state levels in the leaves throughout the period and did not show any diurnal variation (Acevedo et al., 1991). Because the seedlings in the present study were grown continuously in the dark at various temperatures, we do not suspect circadian regulation of *cat3* gene expression in our model system.

Even though our results indicated an important role for the CAT3 isozyme in this acclimation phenomenon, we cannot rule out the role of other antioxidant enzymes such as SOD. Superoxide radicals, the toxic byproducts of oxidative metabolism, can interact with H_2O_2 to form highly reactive hydroxyl radicals that are thought to be primarily responsible for oxygen toxicity in the cell. Therefore, the dismutation of superoxide radicals into H_2O_2 and oxygen is an important step in protecting the cell, and in that conversion SOD is considered a key

enzyme. Although our studies indicated no increases in total SOD activity between acclimated and nonacclimated treatments (data not shown), it is possible that alterations in the relative contributions of MnSOD or Cu/ZnSOD isoforms to the total activity might change, and such a change in isoforms could contribute to chilling tolerance. Different SOD isoforms have been shown to be regulated differently upon exposure to various environmental stresses (Tsang et al., 1991). On the other hand, increased total SOD activity may not necessarily be required for acclimation. Thus, constitutive levels of SOD may be sufficient for inducing the chilling tolerance mechanism in maize seedlings.

Peroxidases such as APX and POX are important antioxidant enzymes in scavenging or utilizing H_2O_2. Whereas APX is localized mainly in chloroplasts and partly in the cytosol, POX is localized in the vacuole, cell wall, and cytosol (Asada, 1992). The ratio of APX to POX is low in nonphotosynthetic tissues (Asada, 1992). Our results indicated that, unlike POX, the levels of total APX are not significantly different between the mesocotyls of acclimated and nonacclimated seedlings (data not shown). As argued for SOD isoforms, although total APX activity might remain stable, the relative contributions of its isoforms to the total activity might change and such a change in isoforms could contribute to chilling tolerance.

H_2O_2 as a Component of Oxidative Stress

The location of CAT3 in the mitochondria (Redinbaugh et al., 1988) and its increased activity during acclimation focus attention on this organelle. In these heterotrophic maize seedlings, mitochondria are very active in supporting growth and are also a major source of superoxide and H_2O_2 production in the cell (Puntarulo et al., 1988, 1991). We found that the amounts and activities of mitochondrial α and β subunits of F_1-ATPase, the enzyme involved in oxidative phosphorylation, and cytochrome oxidase II (COX II) subunits decrease at low temperatures (T.K. Prasad, M.D. Anderson, and C.R. Stewart, unpublished results). This possible disruption of normal mitochondrial electron flow at low temperatures could result in a greater production of active oxygen species including H_2O_2. Therefore, the mitochondria might also be the target of damaging reactions resulting from the oxidative stress.

H_2O_2 as an Inducer of Protection Against Oxidative Stress

We also showed that both in vivo–generated oxidative stress and exogenously supplied H_2O_2 increase the same antioxidant enzymes. The time-course analysis of acclimation clearly indicated that H_2O_2 accumulation occurs first and then, probably in response, CAT3 and POX activities increase. The evidence that H_2O_2 is the inducer of an antioxidant mechanism is as follows: (1) exogenously supplied H_2O_2 and MD induced *cat3* transcription and chilling tolerance; (2) a short treatment with AT inhibited CAT3 activity with simultaneous accumulation of H_2O_2 and the elevation of *cat3* transcripts. In this context, one can interpret the results of time-course analysis of acclimation to indicate that acclimation-imposed oxidative stress is involved in inducing CAT3 and POX gene expression during acclimation.

In spite of the observed accumulation of peroxide in nonacclimated and prechilled seedlings treated with H_2O_2 and MD, these chemicals could increase *cat3* transcripts and CAT3 and POX activities only at 27°C and 14°C but not at 4°C. This result indicated that H_2O_2 is probably acting through some intermediate, temperature-sensitive signal transduction mechanism. A DNA binding protein such as the transcription activator NF-*k*B in human cells (Baeuerle, 1991; Schreck et al., 1991; Naumann et al., 1993) or OxyR in bacteria (Storz et al., 1990), which are also activated by oxidative stress, could be a possible intermediate responding to H_2O_2 in our system. It was suggested that H_2O_2 can act either directly or indirectly through a metabolite or through any oxidative free radicals after interacting with superoxides (Schreck et al., 1991; Naumann et al., 1993). Although it seems unlikely, highly reactive compounds like H_2O_2 or oxidative free radicals might function as secondary messengers. Oxidative free radicals are the undesirable side products of cellular electron transfer reactions, but there are ubiquitous enzyme systems that produce and control the toxic levels of oxygen radicals and H_2O_2 (Jahnke et al., 1991; Cakmak et al., 1993; Walker and McKersie, 1993). Reduced glutathione was proposed to be acting directly as an antioxidant and simultaneously activating cytosolic Cu/ZnSOD gene during oxidative stress (Herouart et al., 1993). Our results indicated that peroxide has dual effects in the chilling stress process because a short treatment with low concentrations of H_2O_2 or MD at 27°C induced tolerance, but toxic levels of H_2O_2 accumulated continuously in the absence of increased CAT3 and POX activities in nonacclimated seedlings at 4°C. Therefore, there might be a narrow concentration range in which H_2O_2 can function exclusively as a secondary messenger but still be cytotoxic at higher concentrations (Christman et al., 1985; Greenberg et al., 1990).

Speculation About a Mechanism of Chilling Tolerance

In view of our results with MD, it is tempting to speculate that the induced tolerance against light accompanied chilling damage (Gupta et al., 1993) and that the tolerance against paraquat (Bowler et al., 1991) produced by SOD overexpression in plants could actually be the result of a peroxide-induced antioxidant mechanism. None of those studies determined the levels of peroxide or APX or any other H_2O_2-scavenging enzymes in their transformants. We suspect that SOD overexpressed in those transformants could be inducing increased levels of peroxide that can diffuse freely between organelles and cytosol. Thus, the newly accumulated peroxide in turn may be triggering a mechanism that increases the activities of several enzymes such as CAT or APX. Because the transformants

would be expected to have moderately higher levels of peroxide, the peroxide-inducible protective mechanism must be turned on constantly in the absence of any stress. This hypothesis is consistent with our results in which peroxide-adapted maize seedlings developed chilling tolerance.

It is possible that different signaling pathways can converge to activate a single signal transduction mechanism. A fungal pathogen, chilling, cold, and oxidative stresses will induce a similar kind of gene expression. Our results that show the adaptation of plants to H_2O_2, as has been observed in bacteria (Christman et al., 1985; Greenberg et al., 1990), constitute compelling evidence to search for a common transcription factor(s) involved in various plant signal transduction mechanisms.

METHODS

Chilling Acclimation Analysis

Seeds of maize (*Zea mays*) Pioneer inbred G50 were germinated in peat moss (Terra-lite Redi-earth; Grace Sierra Horticultural Products Co., Milpitas, CA) at 27°C in darkness for 3 days. The seedlings were then preexposed at either 14°C for 3 days in the dark (acclimation period) followed by 7 days in the dark at 4°C or directly transferred to 4°C for 7 days in the dark (chilling period). The final growth analysis was done after the acclimated and nonacclimated seedlings were transferred to the greenhouse and grown for 10 days. Initial and final fresh and dry weights of the seedlings were determined. The seedlings were evaluated for their percent survival based on the observations that actively growing seedlings were determined to be survivors and the nongrowing and wilted seedlings were determined to be nonsurvivors. All of the experiments were repeated at least twice.

Chilling Tolerance in H_2O_2- and Menadione-Treated Seedlings

Three-day-old dark-grown seedlings were used for the treatment analysis. The root portions of the whole seedlings were immersed in 400 mL of either 0.1 mM H_2O_2 or 0.1 mM menadione (MD) (initially dissolved in 0.5 mL of ethanol), a redox-cycling quinone that generates superoxide, for 4 hr at 27°C in darkness. Preliminary studies with various concentrations of H_2O_2 or MD at 0.01, 0.1, and 0.5 mM indicated that 0.1 mM was closer to the optimum concentration to induce tolerance in the seedlings. The treated seedlings were washed with distilled water, transplanted in peat moss, and further incubated at 27°C for an additional 12 hr, which was considered as a recovery period from oxidative shock, before transfer to 4°C. Preliminary observations also indicated that this recovery from oxidative shock for at least 12 hr was necessary for the seedlings to survive before transfer to 4°C. After 7 days of chilling treatment at 4°C, the seedlings were transferred to the greenhouse for 10 days. When prechilled seedlings were used for chemical treatments, control 3-day-old seedlings were chilled for 4 days at 4°C and then treated with 0.1 mM H_2O_2 or 0.1 mM MD for 4 hr at 4°C in darkness, as described for treatments at 27°C.

Aminotriazole Treatment

Control 3-day-old seedlings or seedlings preacclimated for 15 hr were treated with 10, 25, or 50 mM aminotriazole (AT), an irreversible catalase inhibitor. Roots of the whole seedlings were immersed in solutions (100 mL) for 2 hr. The treated seedlings were then transplanted in peat moss and further incubated for 1 hr at 27° or 14°C.

Construction and Screening of the cDNA Library

A maize cDNA library was constructed in ZAPII using poly(A)$^+$ RNA isolated from acclimated (14°C) seedlings of inbred G50. The cDNA was synthesized with cDNA Synthesis System Plus (Amersham Corp.), according to the manufacturer's instructions. Blunt-ended, double-stranded cDNA was ligated to EcoRI adapters, cloned into ZAPII vector, and packaged in vitro using packaging extract (Gigapack Gold; Stratagene). A subtraction probe was prepared by hybridizing biotinylated poly(A)$^+$ RNA extracted from control seedlings grown at 27°C with first-strand–labeled cDNA synthesized from poly(A)$^+$ RNA that was extracted from acclimated seedlings. The cDNA library was screened with subtraction probe, and 65 crude first-screen positive clones were isolated. These clones were further screened with differential first-strand cDNA probes prepared from control and induced poly(A)$^+$ RNAs. Three putative positive cDNA clones representing chilling acclimation–responsive (*CAR*) genes were isolated. The plasmids were rescued in vivo and the plasmid DNA was made. The DNA sequence analysis was done by the dideoxynucleotide chain termination method (Sanger et al., 1977), and the sequences of both strands were determined.

RNA Gel Blot Analysis

Total RNAs from control, acclimated, chilled, and chemically treated seedlings were isolated according to the method of Chomczynski and Sacchi (1987). Equal amounts of poly(A)$^+$ RNA (1 μg) or total RNA (15 μg) were electrophoresed on 1% formaldehyde–agarose gels and transferred to nylon membranes (GenScreen; Du Pont). Stained gels were observed to confirm the spectrophotometric quantitations that equal amounts of total RNAs were contained in each sample (data not shown). The respective gel-purified cDNA inserts were labeled with ^{32}P-dCTP (3000 Ci/mmol) by the random hexamer priming technique and used as probes to hybridize filters. Relative levels of *cat3* transcripts on autoradiograms were measured using a scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA).

Enzyme Analyses

The tissue (0.5 to 1 gm) was homogenized in 0.2 M sodium phosphate buffer, pH 7.8, containing 2 mM Na_2-EDTA. The extracts were centrifuged at 10,000*g* for 30 min, and parts of the supernatants were stored overnight at −70°C. These frozen extracts were used for the spectrophotometric analyses of catalase (CAT) and guaiacol peroxidase (POX) activities. CAT activity was determined according to the method of Beers and Sizer (1952). Because we do not know the nature of the regulation of various CAT isozymes in the acclimation phenomenon, reporting total CAT activities rather than CAT3 activity would not be meaningful. Therefore, to be consistent with *cat3* gene expression, we reported here only the CAT3 activities. CAT3 was determined by including 5 mM AT, an irreversible CAT inhibitor, in our enzyme assay. As reported by Chandlee et al. (1983) and also as determined in our studies (data not shown), AT inhibits 98% of the CAT1 and CAT2 activities but only inhibits 32% of the CAT3 activity. POX was determined by the method of Chance and Maehly (1955) using guaiacol as an

electron donor. Ascorbate peroxidase (APX) was determined in fresh protein extracts by the method of Asada and Badger (1984) using ascorbate as an electron donor. Total superoxide dismutase (SOD) activity was measured according to the method of Beauchamp and Fridovich (1971). The fresh protein extracts were first passed through a column of Sephadex G-25, and the proteins were eluted with 0.1 M sodium phosphate, pH 7.8, containing 1 mM Na_2-EDTA. These desalted proteins were stored overnight at −70°C. SOD activity in 1-mL reaction volume was expressed as relative units defined as the inverse of the amount of protein required to inhibit by 50% cytochrome *c* reduction. Total protein content in enzyme extracts was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Peroxide content was determined using the technique described by Brennan and Frenkel (1977). The concentration of peroxide in the samples was determined using a standard curve representing titanium–H_2O_2 complex ranging from 0.1 to 1 mM.

ACKNOWLEDGMENTS

We thank Dr. James Colbert for providing the laboratory facilities in the initial stages of this project. This research was supported by grants from Pioneer Hi-Bred International to C.R.S. and U.S. Department of Agriculture National Research Initiative Competitive Grants Program No. 92-37100-7646 to C.R.S. and T.K.P.

Received September 1, 1993; accepted November 3, 1993.

REFERENCES

Acevedo, A., Williamson, J.D., and Scandalios, J.G. (1991). Photoregulation of the *cat2* and *cat3* catalase genes in pigmented and pigment-deficient maize: The circadian regulation of *cat3* is superimposed on its quasi-constitutive expression in maize leaves. Genetics **127,** 601–607.

Asada, K. (1992). Ascorbate peroxidase—a hydrogen peroxide-scavenging enzyme in plants. Physiol. Plant. **85,** 235–241.

Asada, K., and Badger, M.R. (1984). Photoreduction of $^{18}O_2$ and $H_2{}^{18}O_2$ with concomitant evolution of $^{18}O_2$ in intact spinach chloroplasts: Evidence for scavenging of hydrogen peroxide by peroxidase. Plant Cell Physiol. **25,** 1169–1179.

Asada, K., and Takahashi, M. (1987). Production and scavenging of active oxygen in photosynthesis. In Photoinhibition, D.J. Kyle, C.B. Osmond, and C.J. Arntzen, eds (Amsterdam: Elsevier Science Publishers), pp. 227–289.

Baeuerle, P.A. (1991). The inducible transcription activator NF-*k*B: Regulation by distinct protein subunits. Biochim. Biophys. Acta. **1072,** 63–80.

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

Beers, R.F., and Sizer, I.W. (1952). A spectrophotometric method for measuring breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195,** 133–140.

Bowler, C., Slooten, L., Vandenbranden, S., De Ryke, R., Botterman, J., Sybesma, C., Van Montagu, M., and Inzé, D. (1991). The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbaginifolia.* EMBO J. **10,** 1723–1732.

Brennan, T., and Frenkel, C. (1977). Involvement of hydrogen peroxide in the regulation of senescence in pear. Plant Physiol. **59,** 411–416.

Cakmak, I., Strbac, D., and Marschner, H. (1993). Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. J. Exp. Bot. **44,** 127–132.

Chance, B., and Maehly, A.C. (1955). Assay of catalase and peroxidases. Methods Enzymol. **11,** 764–775.

Chandlee, J.M., Tsaftaris, A.S., and Scandalios, J.G. (1983). Purification and partial characterization of three genetically defined catalases of maize. Plant Sci. Lett. **29,** 117–131.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. Anal. Biochem. **162,** 156–159.

Christman, M.F., Morgan, R.W., Jacobson, F.S., and Ames, B.N. (1985). Positive control of a regulon for defenses against oxidative stress and some heat shock proteins in *Salmonella typhimurium.* Cell **41,** 753–762.

Elstner, E.F. (1991). Mechanisms of oxygen activation in different compartments of plant cells. In Active Oxygen/Oxidative Stress and Plant Metabolism, E.J. Pell and K. L. Steffen, eds (Rockville, MD: American Society of Plant Physiologists), pp. 13–25.

Fridovich, I. (1978). The biology of oxygen radicals. Science **201,** 875–880.

Graham, D., and Patterson, B.D. (1982). Responses of plants to low, non-freezing temperatures: Proteins, metabolism, and acclimation. Annu. Rev. Plant Physiol. **33,** 347–372.

Greenberg, J.T., Monach, P., Chou, J.H., Josephy, P.D., and Demple, B. (1990). Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli.* Proc. Natl. Acad. Sci. USA **87,** 6181–6185.

Gupta, A.S., Heinen, J.L., Holaday, A.S., Burke, J.J., and Allen, R.D. (1993). Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. Proc. Natl. Acad. Sci. USA **90,** 1629–1633.

Halliwell, B. (1981). Free radicals, oxygen toxicity and aging. In Age Pigments, R.S. Sohal, ed (Amsterdam: Elsevier), pp. 1–62.

Herouart, D., Van Montagu, M., and Inzé, D. (1993). Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in *Nicotiana.* Proc. Natl. Acad. Sci. USA. **90,** 3108–3112.

Jahnke, L.S., Hull, M.R., and Long, S.P. (1991). Chilling stress and oxygen metabolizing enzymes in *Zea mays* and *Zea diploperennis.* Plant Cell Environ. **14,** 97–104.

Leshem, Y.Y. (1984). Interaction of cytokinins with lipid-associated oxy free radicals during senescence: A prospective mode of cytokinin action. Can. J. Bot. **62,** 2943–2949.

Lieberman, M., Craft, C.C., Audia, W.A., and Wilcox, M.S. (1958). Biochemical studies of chilling injury in sweet potatoes. Plant Physiol. **33,** 307–311.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.L. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193,** 265–275.

Lyons, J.M., and Asmundson, C.M. (1965). Solidification of saturated/unsaturated fatty acid mixtures and its relationship to chilling sensitivity in plants. J. Am. Oil Chem. Soc. **42,** 1056–1058.

Lyons, J.M., and Raison, J.K. (1970). Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. Plant Physiol. **45,** 386–389.

Markhart, A.H., III. (1986). Chilling injury: A review of possible causes. HortScience **21,** 1329–1333.

McKersie, B.D. (1991). The role of oxygen free radicals in mediating freezing and desiccation stress in plants. In Active Oxygen/Oxidative Stress and Plant Metabolism, E.J. Pell and K.L. Steffen, eds (Rockville, MD: American Society of Plant Physiologists), pp. 107–118.

Murata, N., Ishizaki-Nishizawa, O., Higashi, S., Hayashi, H., Tasaka, Y., and Nishida, I. (1992). Genetically engineered alteration in the chilling sensitivity of plants. Nature **356,** 710–713.

Naumann, M., Wulczyn, F.G., and Scheiderelt, C.S. (1993). The NF-kB precursor p105 and the proto-oncogene product Bcl-3 are I*k*B molecules and control nuclear translocation of NF-*k*B. EMBO J. **12,** 213–222.

Omran, R.J. (1980). Peroxide levels and the activities of catalase, peroxidase, and indoleacetic acid oxidase during and after chilling cucumber seedlings. Plant Physiol. **65,** 407–408.

Puntarulo, S., Sanchez, R.A., and Boveris, A. (1988). Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. Plant Physiol. **86,** 626–630.

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

Redinbaugh, M.G., Wadsworth, G.J., and Scandalios, J.G. (1988). Characterization of catalase transcripts and their differential expression in maize. Biochim. Biophys. Acta **951,** 104–111.

Sanger, F., Nicklen, S., and Coulson, A.C. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Schreck, R., Rieber, P., and Baeuerle, P.A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-*k*B transcription factor and HIV-1. EMBO J. **10,** 2247–2258.

Storz, G., Tartaglia, L.A., and Ames, B.N. (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science **248,** 189–194.

Tsang, E.W.T., Bowler, C., Hérouart, D., Van Camp, W., Villarroel, R., Genetello, C., Van Montagu, M., and Inzé, D. (1991). Differential regulation of superoxide dismutases in plants exposed to environmental stress. Plant Cell **3,** 783–792.

Walker, M.A., and McKersie, B.D. (1993). Role of ascorbate-glutathione antioxidant system in chilling resistance of tomato. J. Plant Physiol. **141,** 234–239.

Wang, C.Y. (1982). Physiological and biochemical responses of plants to chilling stress. HortScience **17,** 173–186.

Wise, R.R., and Naylor, A.W. (1987). Chilling-enhanced peroxidation. The peroxidative destruction of lipids during chilling injury to photosynthesis and ultrastructure. Plant Physiol. **83,** 272–277.

Wright, M., and Simon, E.W. (1973). Chilling injury in cucumber leaves. J. Exp. Bot. **79,** 400–411.