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Acclimation, Hydrogen Peroxide, and Abscisic Acid Protect Mitochondria against Irreversible Chilling Injury in Maize Seedlings[1]

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Our previous results indicated that 3-d-old dark-grown chilling-sensitive maize (*Zea mays* L.) seedlings did not survive 7 d of 4°C chilling stress, but 69% of them survived similar stress when the seedlings were either preexposed to 14°C for 3 d or pretreated with 0.1 mM H_2O_2 for 4 h at 27°C (T.K. Prasad, M.D. Anderson, B.A. Martin, C.R. Stewart [1994] Plant Cell 6: 65–74) or 1 mM abscisic acid (ABA) for 24 h at 27°C (M.D. Anderson, T.K. Prasad, B.A. Martin, C.R. Stewart [1994] Plant Physiol 105: 331–339). We discovered that chilling imposed oxidative stress on the seedlings. Since H_2O_2 accumulated during the periods of both acclimation and nonacclimation, we concluded that H_2O_2 had dual effects at low temperature: (a) During acclimation, its early transient accumulation signals the induction of antioxidant enzymes such as catalase 3 and peroxidase to scavenge H_2O_2; and (b) at 4°C in nonacclimated seedlings, it accumulates to damaging levels in the tissues because of low levels of these and perhaps other antioxidant enzymes. Three-day-old seedlings pretreated with H_2O_2 (a mild oxidative stress) or ABA showed induced chilling tolerance. In the present study, we investigated whether mitochondria are a target for chilling-induced oxidative stress and, if so, what differences do acclimation, H_2O_2, or ABA make to protect mitochondria from irreversible chilling injury. The results indicated that chilling, in general, impairs respiratory activity, the cytochrome pathway of electron transport, and ATPase activity regardless of the treatment. In pretreated seedlings, the activities of catalase 3 and peroxidase in the mitochondria increased severalfold compared with control and nonacclimated seedlings. The increases in these antioxidant enzymes imply that mitochondria are under oxidative stress and such increases could initiate a protective mechanism in the mitochondria. Mitochondrial respiration is partially cyanide resistant during chilling stress and also after the 1st d of recovery. Upon further recovery over 3 d, in contrast to nonacclimated seedlings, the mitochondria of acclimation-, H_2O_2-, and ABA-treated seedlings showed the following recovery features. (a) The mitochondrial respiration changed from a cyanide-resistant to a cyanide-sensitive cytochrome pathway, (b) cytochrome oxidase activity recovered to control levels, (c) the ability of mitochondria to generate ATP was regained, and (d) the antioxidant enzyme activities remained at or above control levels. Based on these results, we conclude that chilling impairs mitochondrial function and that chilling-induced oxidative stress seems to be a factor, at least in part, for causing possible irreversible damage to the mitochondrial membrane components. Acclimation, H_2O_2, and ABA provide a protective mechanism by inducing antioxidant enzymes to protect mitochondria from irreversible oxidative damage that is absent in nonacclimated seedlings. Therefore, we conclude that the ability of the seedlings to recover from chilling injury is, at least in part, due to the ability of the mitochondria to resume normal function.

Mitochondria are critical organelles in the metabolic production of energy in the cell. The competence and the stability of mitochondria are very important for the seedlings to survive low-temperature stress, especially during early seedling growth. It is known that germinating seeds and seedlings are sensitive to chilling stress. Therefore, a better understanding of molecular and biochemical mechanisms of chilling responses could provide clues for genetic manipulation of chilling-sensitive species for achieving better growth and higher crop yields (Purvis and Shewfelt, 1993).

Low temperature induces oxidative stress in the cell (Prasad et al., 1994). Under aerobic conditions, superoxide radicals and H_2O_2 are found to be normal metabolites of plant cells (Halliwell, 1987; Elstner, 1991) as well as animal cells (Chance et al., 1979; Halliwell and Gutteridge, 1986) and are kept at low, steady-state levels by the action of antioxidant enzymes such as superoxide dismutase, CAT, GSH peroxidase, and ascorbate peroxidase located in the organelles and cytosol (Jahnke et al., 1991; Cakmak et al., 1993; Walker and McKersie, 1993). Molecular O_2 is reduced to superoxide radical, which is subsequently enzymically or nonenzymically dismutased to H_2O_2. It was shown that mitochondria were a major source of superoxide and H_2O_2 production in heterotrophic tissue (Puntarulo et al., 1988), and they were suggested to be the most important source for cytosolic H_2O_2 (Puntarulo et al., 1991). In addition to being reduced to water by COX, molecular O_2 is partially reduced to superoxide in both cyanide-resistant and cyanide-sensitive electron transport systems in mitochondria (Rich et al., 1976; Huq and Palmer, 1978).

Abiotic stresses such as chilling inhibit the Cyt pathway of electron transport in various species (Purvis and Shewfelt, 1993). Several studies have documented the activation of the alternative respiratory pathway (Rich et al., 1976; Kiener and Bramlage, 1981; Elthon et al., 1986; Rybka, 1989; Stewart et

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Abbreviations: CAT, catalase; CAT3, catalase 3 isozyme; COX, cytochrome oxidase; KCN, potassium cyanide; POX, guaiacol peroxidase; SHAM, salicylhydroxamic acid.

al., 1990a; Purvis and Shewfelt, 1993; Stewart, 1993) as a compensating mechanism for the more labile Cyt pathway during chilling stress (Van de Venter, 1985; Purvis and Shewfelt, 1993). Lyons and Raison (1970) reported that an immediate response of chilling-sensitive tissues to low temperatures was to depress mitochondrial respiration. Their results also suggested that a phase change occurred in mitochondrial membrane components during low-temperature stress. Previous reports indicated that oxidative and phosphorylative activities of mitochondria isolated from chilling-sensitive sweet potato roots were impaired as a result of chilling treatment (Lieberman et al., 1958; Minamikawa et al., 1961) and factors such as the polyphenol content of parent tissue can markedly influence both oxidative rates and phosphorylative ability of isolated mitochondria (Lieberman and Biale, 1956).

Maize (*Zea mays* L.) is considered to be a chilling-sensitive crop. We have recently reported a chilling acclimation phenomenon in Pioneer maize inbred G50 seedlings (Prasad et al., 1994). Three-day-old dark-grown seedlings at 27°C did not survive 7 d of chilling stress at 4°C, but 69% of the seedlings survived 7 d of chilling stress when they were preexposed to acclimation at 14°C for 3 d. We also have molecular and biochemical evidence to indicate that chilling imposes oxidative stress on the seedlings. H_2O_2 was found to accumulate in the seedlings during both acclimation and nonacclimation. Exogenously applied H_2O_2 induced chilling tolerance in the seedlings and mimicked acclimation by inducing antioxidant enzymes such as CAT3, a mitochondrial isozyme, and POX in the mesocotyls of the seedlings, the region most sensitive to chilling damage. We concluded that H_2O_2 has dual effects at low temperature: mild oxidative stress during acclimation induces chilling tolerance by inducing an antioxidant system, and a severe oxidative stress at 4°C damages the nonacclimated tissues in the absence of increased antioxidant system.

Because of the location of CAT3 in mitochondria (Chandlee et al., 1983) and its increased activity by acclimation and oxidative stress agents, we hypothesized that mitochondria are also a target for chilling damage. We have also reported that 3-d-old dark-grown seedlings treated with 1 mM ABA for 24 h induced chilling tolerance in the seedlings (Anderson et al., 1994). Since acclimation, H_2O_2, and ABA treatments induced chilling tolerance in the seedlings, we envisioned their possible role in protecting the function of mitochondria from the otherwise injurious chilling stress. Therefore, the objective of this study was to investigate whether mitochondria are a target for chilling injury and, if so, what changes do acclimation, H_2O_2, and ABA treatments make to protect mitochondrial function against such chilling damage. In this report, two of the major enzymes involved in electron transport pathway and oxidative phosphorylation, COX and F_1-ATPase, respectively, were studied as marker proteins to reflect the membrane stability and functional competence of mitochondria during chilling stress. O_2 uptake was measured as a quantitative indication of mitochondrial function under various treatments. We also measured the activities of mitochondrial CAT3 and POX to indicate their role as antioxidant enzymes whose changes would imply the nature of oxidative stress on mitochondria during low temperatures. Based on our previous observations, the mesocotyl was determined to be the most sensitive region for chilling damage; therefore, in this study, all of the measurements were focused on the performance of mesocotyls during chilling-stress and recovery regimes.

MATERIALS AND METHODS

Chilling Acclimation and Nonacclimation Treatments

Seeds of *Zea mays* L. 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 d. The seedlings were then preexposed to either 14°C for 3 d in the dark (acclimation period), followed by 7 d in the dark at 4°C, or directly transferred to 4°C for 7 d in the dark (chilling period). After chilling, the acclimated and nonacclimated seedlings were transferred to the greenhouse and grown for 10 d. The survival data indicated that 2% of the nonacclimated seedlings and 69% of the acclimated seedlings survived 7 d of chilling stress (Prasad et al., 1994).

H_2O_2 or ABA Treatments

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 0.1 mM H_2O_2 for 4 h or 1 mM ABA for 24 h at 27°C in darkness. The treated seedlings were washed with distilled water, transplanted in peat moss, and further incubated at 27°C for an additional 12 h before transferring to 4°C. After the 7-d chilling treatment at 4°C, the chemically treated seedlings were transferred to the greenhouse for 10 d. The survival data indicated that 58% of the H_2O_2-treated seedlings (Prasad et al., 1994) and 60% of the ABA-treated seedlings (Anderson et al., 1994) survived 7 d of chilling stress. All of the material for mitochondrial isolations and O_2 uptake studies was harvested at various times as detailed in the respective figure captions and in Table I.

Isolation of Mitochondria

Mitochondria were isolated according to the method of Leaver et al. (1983). The mesocotyl tissue (10 g) was homogenized in grinding buffer (25 mM Mops, pH 7.8, 8 mM Cys hydrochloride, 0.1% BSA [w/v], and 1 mM EGTA) in a chilled mortar and pestle for 2 min at 4°C. Mitochondria were subsequently purified by two rounds of differential centrifugation. Further purification of mitochondria was done by using Suc density gradient centrifugation (Leaver et al., 1983). The mitochondria were recovered from the Suc gradient, pelleted, and suspended in resuspending buffer (250 mM Suc, 10 mM Tricine-KOH, pH 7.2, 1 mM $MgCl_2$, and 1 mM KH_2PO_4). The mitochondria were lysed by freeze-thaw cycles, and the lysate was used for the spectrophotometric analyses of enzyme activities. Total protein content in enzyme extracts was determined by the method of Lowry et al. (1951) using BSA as a standard.

Enzyme Assays

Submitochondrial membranes were prepared and F_1-ATPase activity was determined by the modified method of Hack and Leaver (1983). The reaction mixture containing 20 mM Tricine, pH 8.0, 4 mM $MgCl_2$, 4 mM ATP, and 50 μg of protein in a final volume of 300 μL was incubated at 37°C for 30 min, followed by addition of an equal volume of 10% (w/v) TCA to precipitate the proteins. After centrifugation for 5 min at 10,000*g*, 540 μL of supernatant was mixed with 50 μL of 70% (v/v)perchloric acid, 50 μL of 5% (w/v) ammonium molybdate, and 25 μL of fresh amino-naphthalic acid reagent (2 mg of amino-naphthalic acid mixed with 120 mg of sodium sulfite and 24 mg of sodium bisulfite in a final volume of 1 mL) in a final volume of 750 μL and incubated at room temperature for 10 min. *A* was determined at 660 nm.

COX was determined in a reaction mix containing 90 mM phosphate buffer, pH 7.0, 0.1% (v/v) Nonidet P-40, 50 μM Cyt *c* (reduced with 3 mg of sodium hydrosulfite), and 15 μg of protein in a final volume of 1 mL. Activity was determined as the rate of oxidation of the reduced Cyt *c* measured at A_{550}.

CAT activity was determined according to the method of Beers and Sizer (1952). To eliminate any contamination of peroxisomal CAT1 activity in our mitochondrial preparations, we included 10 mM aminotriazole, an irreversible CAT inhibitor, in our enzyme assays. As reported by Chandlee et al. (1983) and also as judged in our preliminary studies (data not shown), aminotriazole inhibits 93% of the CAT1 and 98% of the CAT2 activities but only inhibits 32% of the CAT3 activity. Peroxidase activity was determined by the method of Chance and Maehly (1955) using guaiacol as an electron donor.

For each of the four enzymes, the treatments were replicated twice and analyzed as a factorial design with treatment, duration of recovery, and replication as the main effects.

Immunoblotting

Mitochondrial proteins were separated by SDS-PAGE on 12.5% gels, and the proteins were transferred to nitrocellulose membranes. The filters were reacted with monoclonal antibodies raised against maize α and β subunits of F_1-ATPase or COXII subunit of yeast Cyt *c* oxidase. The second antibody was alkaline phosphatase-conjugated goat anti-mouse IgG antibody, and immunodetection was carried out as described by Hawkes et al. (1982).

Respiratory Activities

O_2 uptake in tissue segments was determined at 25°C with a Yellow Springs Instruments model 5300 biological O_2 monitor as previously described (Elthon et al., 1986). The electrode was first calibrated with water in the presence or absence of 1 mM KCN or 25 mM SHAM or in combination at 25°C and saturated with air. O_2 concentrations were calculated from the absorption coefficient for O_2 at 25°C. Rates were determined in the absence and presence of KCN and SHAM alone and in combination. These determinations were made for both sequences of adding inhibitors. Inhibition by KCN alone was taken as a measure of electron flow through COX. Inhibition by SHAM in the presence of KCN measures the capacity of the alternative pathway. Inhibition by SHAM alone measures electron flow through the alternative pathway. The presence of an hydroxamate-stimulated O_2 uptake alters this latter interpretation (see "Results" and "Discussion").

For the uninhibited rates and residual, treatments were replicated three to four times, and treatments for all others were replicated two times. Dependent variables were analyzed as a factorial design with treatment and duration of recovery as the main effects.

RESULTS

Respiratory Activity in Tissue Segments

Our previous results indicated that growth and survival of chilled seedlings after a 10-d greenhouse growth period were dramatically improved by pretreating the seedlings with either acclimation at 14°C for 3 d, H_2O_2 for 4 h at 27°C (Prasad et al., 1994), or ABA for 24 h at 27°C (Anderson et al., 1994). These pretreatments alleviated the chilling-induced oxidative stress in the seedlings. Since mitochondria are a possible source of oxidative stress, we determined the effect of chilling on mitochondrial function. Mitochondrial function as indicated by the O_2 uptake rates of tissue segments from seedlings of various treatments is presented in Table I.

The uninhibited respiration rate slowed when the control, warm-grown seedlings were transferred to the greenhouse. Uninhibited rates measured at 25°C also slowed after seedlings were exposed to 4°C for 7 d. These lower rates were observed for all treatments, but rates of those seedlings that survived the grow-out appeared to decline more than those of the nonacclimated seedlings. The uninhibited rates of the surviving seedlings recovered to that of the warm-grown control after 1 and 3 d in the greenhouse, whereas those of nonacclimated seedlings recovered to a level higher than control seedlings.

The portion of O_2 uptake mediated by the Cyt pathway (i.e. the rate that was sensitive to KCN alone) declined from 62% in the warm-grown controls to 27 to 32% in the four treatments after 7 d of 4°C temperature. During the greenhouse grow-out period, this value declined further after the 1st d but recovered after 3 d in seedlings that survived the chilling temperature. In the nonacclimated seedlings that eventually died, the portion of O_2 uptake that was sensitive to KCN declined further at the 1- and 3-d measurements.

The capacity of the alternative pathway (that which was sensitive to SHAM in the presence of KCN) was about 23% of the O_2 uptake rate in the warm-grown seedlings and did not change upon transfer to the greenhouse. This proportion is similar to that previously observed for this inbred (Stewart et al., 1990a). The capacity of the alternative pathway increased slightly to 32 to 43% of the inhibited rate after 7 d of chilling in all treatments. During the grow-out period, this capacity continued to increase to about 62% in the seedlings that were dying. In seedlings that survived, this capacity increased at the 1st-d measurement but declined to the 17 to 28% level after 3 d. These changes in the surviving seedlings

Table I. *Oxygen uptake rates of mesocotyl tissue from seedlings grown under various conditions*

Three-day-old dark-grown seedlings (Cont) were pretreated with acclimation (Ac), nonacclimation (NAc), 0.1 mM H_2O_2, and 1 mM ABA as described in "Materials and Methods." The pretreated seedlings were chilled for 7 d at 4°C and recovered for 1 and 3 d in the greenhouse. Treatments for uninhibited rates and KCN and SHAM combined were replicated three to four times, and treatments for all others were replicated two times. SE values range as follows: uninhibited rates, 7.9 to 9.1%; KCN alone, 1.9 to 2.8%; SHAM in presence of KCN, 3.4 to 4.8%; SHAM alone, 3.0 to 4.2%; KCN in presence of SHAM, 4.3 to 6.0%; KCN and SHAM combined, 3.3 to 3.8%.

Treatments	Days of Recovery	Uninhibited Rates	Percentage of Uninhibited Rates Inhibited by				
			KCN alone	SHAM in presence of KCN	SHAM alone	KCN in presence of SHAM	KCN and SHAM combined[a]
		nmol O_2 min^{-1} g^{-1}					
Cont	0	272	62	23	19	66	15
	1	226	68	21	16	70	12
	3	184	63	26	16	74	11
NAc	0	179	27	43	30	32	35
	1	217	10	49	35	34	35
	3	214	8	62	49	22	29
Ac	0	157	31	32	29	31	38
	1	191	11	46	35	35	36
	3	186	34	25	9	52	41
H_2O_2	0	165	32	34	30	32	34
	1	190	11	43	34	37	38
	3	178	34	17	9	50	45
ABA	0	166	32	34	30	32	34
	1	186	15	52	36	36	30
	3	189	41	28	11	47	33

[a] Values represent average determinations made in the presence of KCN followed by SHAM and those made in the presence of SHAM followed by KCN.

were opposite to the changes that were observed in the Cyt pathway during the grow-out of the surviving seedlings. The alternative pathway capacity after 3 d of grow-out was similar to that of the warm-grown controls.

The residual rates (KCN and SHAM combined) were higher compared with the warm-grown controls in all measurements of O_2 uptake in seedlings exposed to the 7-d chilling treatment. These rates did not change during the greenhouse grow-out period and were the same proportion in the surviving seedlings as in the nonacclimated seedlings that did not survive.

The sensitivity of O_2 uptake to SHAM alone (activity of the alternative pathway) was greater in all of the chilled seedlings compared with the warm-grown controls. After 3 d in the greenhouse, the surviving seedlings were observed to have become less sensitive to SHAM alone, whereas the nonacclimated seedlings that died became more sensitive to SHAM alone.

The sensitivity of O_2 uptake to KCN in the presence of SHAM (capacity of the Cyt pathway) was not different from the sensitivity to KCN alone in the warm-grown seedlings and in seedlings from all treatments after the 7-d chilling treatment. However, in surviving seedlings, the measurement after 3 d in the greenhouse revealed that the sensitivity to KCN in the presence of SHAM had increased and was considerably greater than KCN alone. The opposite was true in the nonacclimated seedlings that died. This observation is consistent with the development of hydroxamate-stimulated, KCN-sensitive O_2 uptake during the grow-out period in seedlings that were treated so as to survive the 7-d chilling treatment. When hydroxamate-stimulated O_2 uptake is present, SHAM would have two opposing effects: stimulation of O_2 uptake and the inhibition of alternative oxidase.

Cyt Pathway of Electron Transport in Mitochondria

Since chilling inhibited the rates of respiration and that of the Cyt pathway, we studied the protein levels and the activity of COX. Figure 1 indicates that COX activity was reduced by 15 to 20% in the mitochondria of seedlings pretreated with acclimation, H_2O_2, and ABA compared with control in the absence of chilling stress at 4°C. When the seedlings were transferred to 4°C for 7 d, the COX activity was decreased by 30 to 40% in mitochondria of all of the seedlings, irrespective of the treatment type. However, when the seedlings had recovered for 40 h, the COX activity recovered to control levels in all of the mitochondria of seedlings pretreated with acclimation, H_2O_2, or ABA, but the COX activity in the mitochondria of nonacclimated seedlings was further decreased to 25% of the control levels.

To understand whether the changes in COX activities were due to changes in protein levels, the COXII subunit protein

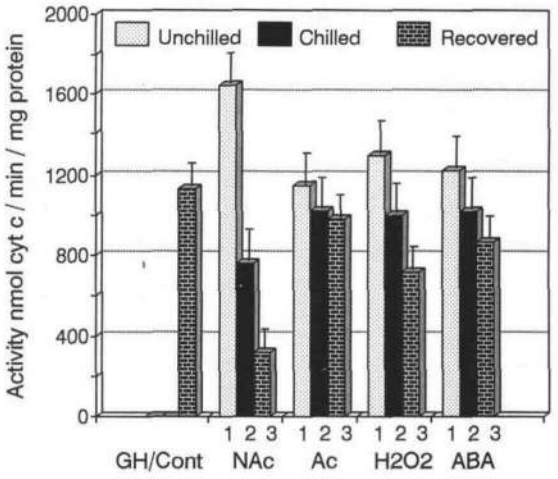

Figure 1. Activities of Cyt *c* oxidase in the mitochondria of the maize seedlings pretreated with acclimation (Ac), nonacclimation (NAc), H_2O_2, and ABA and subjected to various temperature regimes. Mitochondrial proteins were extracted from the mesocotyl tissue of the seedlings from the following treatments: GH/Cont, 3-d-old seedlings grown at 27°C in the dark and transferred to greenhouse for 40 h; NAc, 3-d-old seedlings grown at 27°C (lane 1), chilled for 7 d at 4°C (lane 2), and transferred to the greenhouse for 40 h of recovery (lane 3); Ac, seedlings acclimated for 3 d at 14°C (lane 1), chilled for 7 d at 4°C (lane 2), and transferred to the greenhouse for 40 h of recovery (lane 3); H_2O_2, seedlings treated with 0.1 mM H_2O_2 for 4 h, followed by incubation at 27°C for 12 h (lane 1), chilled for 7 d at 4°C (lane 2), and transferred to the greenhouse for 40 h of recovery (lane 3); ABA, seedlings treated with 1 mM ABA for 24 h at 27°C (lane 1), chilled for 7 d at 4°C (lane 2), and transferred to the greenhouse for 40 h of recovery (lane 3). The SE values are represented as vertical lines accompanying each bar.

levels were determined. Western blot analyses indicated that the COXII protein levels were lowered by pretreatment as well as by the chilling stress, irrespective of the treatment type (Fig. 2C), as also observed for COX activities. During recovery, the COXII protein levels returned to control levels only in the mitochondria of the seedlings that were pretreated with acclimation, H_2O_2, and ABA, which survive, but not in nonacclimated seedlings, which die, as observed for COX activities.

ATPase Activities in Mitochondria

Since the oxidative rates of mitochondria decreased upon incurring chilling stress, we were interested to know whether such a change in mitochondrial behavior had any influence on the capacity for mitochondrial ATP synthesis. To determine this, we studied the protein levels and the activity of F_1-ATPase during the chilling-stress phenomenon. Unlike respiration and COX activities, F_1-ATPase activity remained unchanged in the mitochondria of acclimation, H_2O_2, and ABA treatments compared with control, before transferring to 4°C treatment (Fig. 3). When the seedlings were transferred to 4°C for 7 d, ATPase activity was lost by 30 to 35%, irrespective of the treatment type. However, during 40 h of the recovery regime, these activities returned to the control levels in all of the pretreated seedlings. In contrast, mitochondria from nonacclimated seedlings further lost activity to 65 to 50% of the control levels during the same 40-h recovery period.

To further understand whether changes in F_1-ATPase activity were due to changes in protein levels, the levels of α and β subunits were determined. Western blot analyses indicated that both α and β subunit protein levels were lowered by chilling stress, but no significant change in their levels were observed with pretreatment alone, irrespective of the treatment type. The protein levels recovered to the control level only in the mitochondria of pretreated seedlings, which survive, but not in nonacclimated seedlings, which die, as observed for enzyme activities (Fig. 2, A and B).

Antioxidant Enzymes in Mitochondria

We have previously reported that low temperature induced oxidative stress during acclimation and nonacclimation processes and that mitochondria were also a target for oxidative stress. Consistent with our results, other reports have indi-

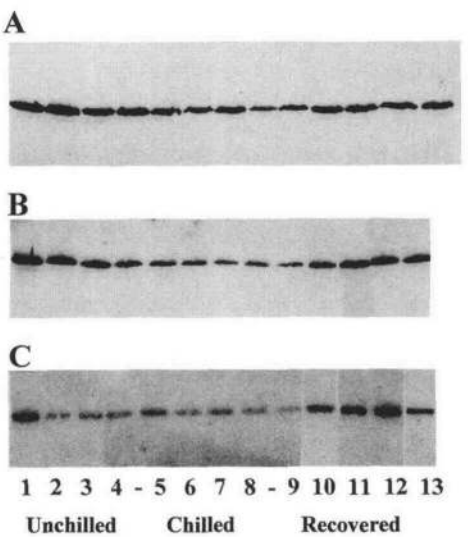

Figure 2. Western blot analysis showing the levels of α and β subunits of F_1-ATPase (A and B, respectively) and COXII subunits of Cyt *c* oxidase (C) in the mitochondria of the seedlings treated with acclimation, nonacclimation, H_2O_2, and ABA and subjected to various temperature regimes. The mitochondrial proteins (20 μg/lane) were resolved on SDS-PAGE using 12.5% gels and transferred to nitrocellulose filters. The filters were reacted with monoclonal antibodies raised against α and β subunits of maize F_1-ATPase and COXII subunits of yeast Cyt *c* oxidase as described in "Materials and Methods." The mitochondrial proteins were extracted from the mesocotyl tissues of the seedlings treated as follows: Lane 1, Three-day-old seedlings grown at 27°C in the dark; lane 2, seedlings acclimated for 3 d at 14°C; lane 3, seedlings treated with 0.1 mM H_2O_2 for 4 h and further incubated at 27°C for 12 h; lane 4, seedlings treated with 1 mM ABA for 24 h at 27°C; lanes 5 to 8, treatments of lanes 1 to 4, respectively, transferred to 4°C for 7 d; lanes 9 to 12, chilled seedlings in lanes 5 to 8, respectively, transferred to greenhouse for 40 h of recovery; lane 13, seedlings in lane 1 transferred to the greenhouse for 40 h.

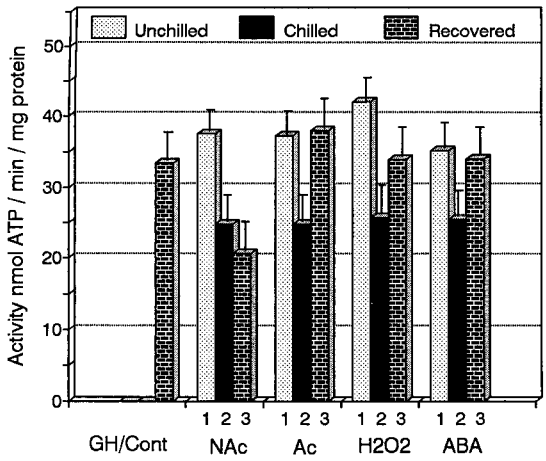

Figure 3. Activities of F_1-ATPase in the mitochondria of the maize seedlings pretreated with acclimation (Ac), nonacclimation (NAc), H_2O_2, and ABA and subjected to various temperature regimes. Treatments to the seedlings and mitochondrial protein extractions were done as described in Figure 1. The SE values are represented as vertical lines accompanying each bar.

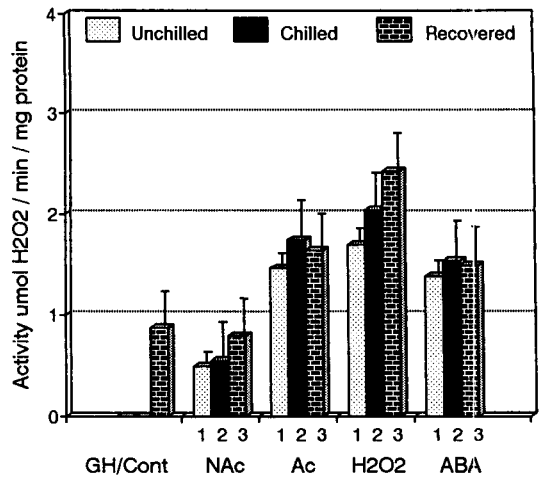

Figure 5. Activities of peroxidase in the mitochondria of the maize seedlings pretreated with acclimation (Ac), nonacclimation (NAc), H_2O_2, and ABA and subjected to various temperature regimes. Treatments to the seedlings and mitochondrial protein extractions were done as described in Figure 1. The SE values are represented as vertical lines accompanying each bar.

cated that mitochondria are the major source of superoxide and H_2O_2 production (Puntarulo et al., 1988, 1991; Purvis and Shewfelt, 1993). Therefore, we investigated the activities of the antioxidant enzymes such as CAT3 and POX in mitochondria. Figures 4 and 5 indicated that CAT3 (Fig. 4) and POX (Fig. 5) activities were elevated more than 3-fold in the mitochondria of the seedlings pretreated with acclimation, H_2O_2, and ABA compared with control before transferring to 4°C treatment. These activities remained high in the mitochondria of all of the pretreated seedlings but were slightly inhibited in nonacclimated seedlings after 7 d of 4°C treatment, compared with control seedlings at 27°C. Upon 40 h of recovery from chilling stress, the mitochondria from all of the pretreated seedlings maintained CAT3 and POX activities at or above the control levels. In contrast, the CAT3 activity was 70% lower and POX activity was slightly higher in the mitochondria of nonacclimated seedlings during the same recovery period.

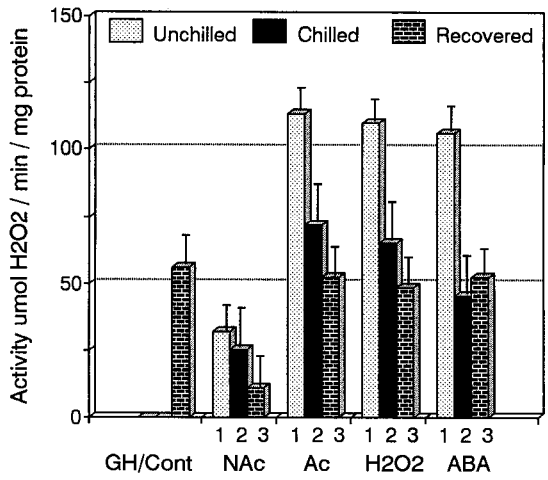

Figure 4. Activities of CAT3 in the mitochondria of the maize seedlings pretreated with acclimation (Ac), nonacclimation (NAc), H_2O_2, and ABA and subjected to various temperature regimes. Treatments to the seedlings and mitochondrial protein extractions were done as described in Figure 1. The SE values are represented as vertical lines accompanying each bar.

DISCUSSION

Effects of Chilling and Recovery on Mitochondrial Function

Our results clearly demonstrate that mitochondria are indeed a target for chilling damage in maize seedlings. We conclude that (a) the O_2 uptake ability, Cyt pathway of electron transport, and ATPase activity of mitochondria are depressed by chilling stress, irrespective of the ability of the seedling to survive the stress; (b) during recovery the only seedlings that survive the chilling stress regain the cyanide-sensitive Cyt pathway, and nonsurviving seedlings continue to exhibit the SHAM-sensitive alternative pathway; (c) the ability to recover COX and F_1-ATPase at both protein and activity levels in the mitochondria of pretreated seedlings correlates with the ability of the seedlings to survive chilling stress during the recovery regime; (d) the increase in the activities of CAT3 and POX in the mitochondria of acclimation and chemically treated seedlings implicate the imposition of oxidative stress in mitochondria at low temperature; and (e) the ability of mitochondria to recover from chilling stress only in acclimation, H_2O_2, and ABA treatments but not from nonacclimation treatment indicates the possibility of irreversible chilling-induced oxidative stress injury to the membrane structure in nonacclimated mitochondria.

Chilling Impairs Respiratory Activity

We demonstrated that the mitochondrial respiration is significantly reduced at low temperatures, which indicates that the immediate response of the tissue to chilling is a depression of respiratory activity. The decline in O_2 uptake rates is apparently a result of damage to the more sensitive Cyt pathway. The increases in alternative pathway during low-temperature stress was thought to be limiting the production of O_2 free radicals during such a stress (Purvis and Shewfelt, 1993). Although it may play such a role in chilling tolerance, the increase in the capacity of the alternative pathway, which has been observed in several studies, was clearly not sufficient to effect survival of these chilling-damaged seedlings. From our previous studies (Prasad et al., 1994), we hypothesize that the recovery of mitochondrial function, which contributes to the survival of the seedling, is likely due to the induction of enzymic mechanisms to protect them against oxidative stress in the acclimation, H_2O_2, and ABA treatments.

The lesser sensitivity of O_2 uptake to SHAM alone compared to the sensitivity to SHAM in the presence of KCN and the sensitivity of this uptake to KCN is consistent with the presence of hydroxamate-stimulated O_2 uptake, which is apparently due to peroxidases (Spreen Brouwer et al., 1986; Van der Plas et al., 1987). The SHAM concentration in our experiments would have been sufficient to inhibit some of these peroxidases (Spreen Brouwer et al., 1986) but not others (Van der Plas et al., 1987). Thus, if this SHAM-stimulated, cyanide-sensitive O_2 uptake is due to peroxidases, which we know are elevated at the low temperatures (Prasad et al., 1994), then these peroxidases must not be sensitive to the 25 mM SHAM used in our experiments.

Chilling Inhibits Cyt Pathway of Electron Transport

We have demonstrated that chilling stress decreases the COX activity, its protein level, and cyanide sensitivity of O_2 uptake. These results are consistent with a possible leakage of electrons from impaired electron transport chain to reduce molecular O_2 to superoxide and H_2O_2. Low temperature also increases electron flow via the alternative pathway. It was speculated that a broader role for the alternative pathway could be one way of protection against extremes (Kiener and Bramlage, 1981; Van de Venter et al., 1985; Stewart et al., 1990a) and, therefore, considered as a reserve for the failure of the more sensitive Cyt pathway during chilling. Chilling sensitivity has also been attributed to critical changes in membrane fluidity (Lyons et al., 1964; Lyons and Raison, 1970). Any phase changes in the inner mitochondrial membrane would have drastic effects on the orientation of the respiratory chain components with a concomitant reduction of electron flow through the Cyt chain (Creencia and Bramlage, 1971). These data support the hypothesis that the ability of the tissue to recover from these damaging temperature regimes depends on restoring electron flow to the normal Cyt pathway during recovery from chilling stress, which is evident from our recovery data.

Chilling Impairs the Ability of Mitochondria to Generate ATP

Our results indicate that chilling, in general, inhibits not only the F_1-ATPase activity but also its protein level, similar to COX. It is likely that efficient oxidative phosphorylation may be important for full recovery from chilling stress. Lyons et al. (1964) proposed that mitochondria of chilling-sensitive plants do not possess dynamic properties at low temperature and that phosphorylation is therefore disrupted. The changes in mitochondrial behavior might also be a result of sharply reduced energy generation, since oxidation rates of mitochondria from chilling-sensitive tissues decrease as chilling temperatures are incurred. Consistent with this observation, our results also indicate that F_1-ATPase activity was decreased by chilling, irrespective of the treatment type. However, the real difference between the mitochondria of pretreated and nonacclimated seedlings was observed during the recovery period. Whereas the mitochondria of pretreated seedlings regained ATPase activity, mitochondria of nonacclimated seedlings failed to do so. Thus, the recovery from chilling injury must also depend on whether or not the mitochondria can quickly and fully regain their capacity to generate ATP. Similar conclusions were drawn by Creencia and Bramlage (1971) for maize seedlings in which the mitochondria of chilled seedlings failed to synthesize ATP upon recovery.

Chilling Imposes Oxidative Stress in Mitochondria

Oxidative free radicals can be highly reactive toward cell components, and therefore, the ability of the cell to remove these undesirable species might be viewed as an important feature in improved resistance to chilling stress. We assume that the increases in the activities of CAT3 and POX provide evidence for the increased production of superoxide and H_2O_2 in mitochondria of maize seedlings. Increases in superoxide and H_2O_2 can be expected in cases in which there is either high O_2 uptake or decreased ability of the electron transport pathway, which increases potential for higher electron leakage to O_2 for subsequent production of superoxide and H_2O_2 (Chance et al., 1979; Halliwell, 1987; Elstner, 1991). Because we reported previously that chilling imposes oxidative stress on the seedlings, the increased activities of mitochondrial CAT3 and POX would justify the conclusion that acclimated or H_2O_2- or ABA-treated seedlings respond to oxidative stress by generating antioxidant enzymes to scavenge or utilize H_2O_2 (Scandalios, 1993), whereas nonacclimated seedlings do not. Such a consequence of imbalance between the activities of CAT3 and POX and H_2O_2 levels would result in the elevated production and low destruction of H_2O_2. Similarly, Leprince et al. (1990) interpreted the loss of desiccation tolerance as a consequence of increased formation of one or more forms of activated oxyen with much decreased activities of POX and superoxide dismutase in germinating maize seedlings. This assumption is consistent with our previous evidence that a short treatment with aminotriazole, an irreversible CAT inhibitor, resulted in the inhibition of CAT activity with concomitant accumulation of H_2O_2 in the maize seedlings (Prasad et al., 1994). Although the relative impor-

tance of CAT3 versus POX in mitochondria is unknown, we previously concluded that CAT3 appeared to be an important H_2O_2-scavenging enzyme in the seedlings (Prasad et al., 1994). Since we report here the increased mitochondrial CAT3 and POX activities only in acclimation-, H_2O_2- and ABA-treated but not in nonacclimated mitochondria, we conclude that the inability of mitochondria to recover from chilling injury in nonacclimated seedlings is probably due, at least in part, to the irreversible damage to the membrane stability caused by the chilling-induced oxidative stress. High levels of H_2O_2 could promote lipid peroxidation in the membranes (Leshem, 1984; Senaratna et al., 1987) in the absence of increased antioxidant mechanism.

CONCLUSIONS

Our results demonstrate that mitochondria are also a target for chilling stress. Chilling impairs mitochondrial function by depressing the respiratory activity, Cyt pathway of electron transport, and ATPase activity. Changes in these processes seem to be, at least in part, under the control of translation. Increases in mitochondrial CAT3 and POX activities during stress indicate that mitochondria are a source of H_2O_2 production, potentially generating oxidative stress in the mitochondria during chilling. Such an oxidative stress seems to be at least partially responsible for irreversible damage to the mitochondrial membrane. Whereas acclimation, H_2O_2, and ABA treatments provide a mechanism to protect mitochondria from such oxidative damage by inducing antioxidant enzymes or other factors involved in membrane stability, the nonacclimation fails to induce a similar protection mechanism.

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