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Localization and Characterization of Peroxidases in the Mitochondria of Chilling-Acclimated Maize Seedlings[1]

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We present evidence of two peroxidases in maize (*Zea mays* L.) mitochondria. One of these uses guaiacol and the other uses cytochrome *c* as the electron donor. Treatments of fresh mitochondria with protease(s) indicate that ascorbate and glutathione peroxidases are likely bound to the mitochondria as cytosolic contaminants, whereas guaiacol and cytochrome peroxidases are localized within the mitochondria. These two mitochondrial peroxidases are distinct from contaminant peroxidases and mitochondrial electron transport enzymes. Cytochrome peroxidase is present within the mitochondrial membranes, whereas guaiacol peroxidase is loosely bound to the mitochondrial envelope. Unlike other cellular guaiacol peroxidases, mitochondrial guaiacol peroxidase is not glycosylated. Digestion of lysed mitochondria with trypsin activated mitochondrial guaiacol peroxidase but inhibited cytochrome peroxidase. Isoelectric focusing gel analysis indicated guaiacol peroxidase as a major isozyme (isoelectric point 6.8) that is also activated by trypsin. No change in the mobility of guaiacol peroxidase due to trypsin treatment on native polyacrylamide gel electrophoresis was observed. Although both peroxidases are induced by chilling acclimation treatments (14°C), only cytochrome peroxidase is also induced by chilling (4°C). Because chilling induces oxidative stress in the maize seedlings and the mitochondria are a target for oxidative stress injury, we suggest that mitochondrial peroxidases play a role similar to catalase in protecting mitochondria from oxidative damage.

Peroxidases are known to be present in both prokaryotes and eukaryotes. Mostly, they are heme-containing proteins that utilize H_2O_2 in the oxidation of various organic and inorganic substances. Although peroxidases are catalytically very active, they exhibit very little specificity for substrates and exist in various isozyme forms (Gaspar et al., 1991; Welinder, 1992; Siegel, 1993). There are two major classes of peroxidases based on function in plants (Asada, 1992). The peroxidases participating in lignification, degradation of IAA, cross-linking of cell wall polymers, ethylene biosynthesis, pathogen defense, and wound healing, all belong to a group of peroxidases that utilize guaiacol as the electron donor in vitro (Asada, 1992) and whose oxidative products have physiological roles in the cells. The other major group of plant peroxidases that utilize GSH, Cyt *c*, pyridine nucleotides, and ascorbate as the electron donors function in the scavenging of H_2O_2 in the cell (Asada, 1992). These two groups have distinct features in terms of their molecular structure, cellular location, and function (Asada, 1992; Chen et al., 1992). Ascorbate peroxidases are localized in chloroplasts and cytosol (Asada, 1992) and are not glycosylated. On the other hand, guaiacol peroxidases are glycoproteins that contain *N*-linked oligosaccharide chains and are located in cytosol, vacuole, cell wall, and extracellular space (Hu and van Huystee, 1989; Asada, 1992). The carbohydrate moieties have been shown to be necessary for the stabilization of the peroxidase molecules (Hu and van Huystee, 1989).

We recently reported the presence of guaiacol peroxidase activity in mitochondria of maize (*Zea mays* L.) seedlings (Prasad et al., 1994b). Because chilling induces oxidative stress in maize mitochondria (Prasad et al., 1994a, 1994b), we suggested that catalase and peroxidases could serve as antioxidant enzymes. Although the mitochondrial location of CAT3 has been previously reported (Scandalios et al., 1980), peroxidases have not been reported to be present within plant mitochondria. In light of the fact that all guaiacol peroxidases are proposed to be glycoproteins (Asada, 1992; Welinder, 1992; Siegel, 1993) and are suggested to be synthesized on RER and processed in the Golgi complex, they are not expected to be localized within the mitochondria. Therefore, the objective of this study was to localize and characterize the peroxidases that are associated with maize mitochondria. The results indicate that there are two peroxidases, mtPOX and Cyt peroxidase, that are localized within the mitochondria. They are distinct from mitochondrial contaminant peroxidases and mitochondrial electron transport enzymes because they respond differently to inhibitors. Unlike other cwPOX and cytosolic guaiacol peroxidase that are known to be glycosylated, mtPOX is not glycosylated. Since these two peroxidases are also induced by low temperature, we suggest that they could serve as antioxidant enzymes to protect mitochondria from chilling-induced oxidative stress.

MATERIALS AND METHODS

Low-Temperature Treatments

Seeds of *Zea mays* L. Pioneer inbred G50 were germinated in peat moss (Terra-lite Redi-earth; Grace Sierra Hor-

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Abbreviations: CAT3, catalase 3 isozyme; cwPOX, cell wall guaiacol peroxidase; ϵ_x, extinction coefficient at wavelength x; mtPOX, mitochondrial guaiacol peroxidase; SHAM, salicylhydroxamic acid.

ticultural Products Co., Milpitas, CA) at 27°C for 3 d in darkness. Three-day-old seedlings were acclimated at 14°C for 3 d in the dark and then chilled at 4°C for 4 d in the dark or directly chilled at 4°C for 4 d in the dark. At the end of the experiment, the mesocotyls were harvested for mitochondrial isolations.

Isolation of Mitochondria and the Extraction of Cell Wall Proteins

Maize mitochondria were isolated according to the method of Leaver et al. (1983). The mesocotyl tissue (2–10 g) was homogenized in grinding buffer (400 mM mannitol, 25 mM Mops, pH 7.8, 8 mM Cys hydrochloride, 0.1% [w/v] BSA, and 1 mM EGTA). The crude mitochondrial preparations were obtained by two differential centrifugations and were further purified by Suc density gradient centrifugation. 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 fresh intact or lysed mitochondria (lysed by two freeze-thaw cycles followed by homogenization using a Teflon glass homogenizer) were used for the spectrophotometric measurements of various enzyme activities. To separate soluble and membrane proteins, mitochondria were lysed (as described above) and spun at 25,000 rpm for 30 min at 4°C. The supernatant was used as the soluble fraction. The pellet was resuspended in buffer and used as a membrane fraction. Alkaline carbonate treatment of mitochondrial membranes was performed according to the method of Fujiki et al. (1982). Mitochondrial membranes were resuspended in 0.1 mM Na_2CO_3, pH 11.3, and incubated on ice for 30 min. The membrane suspension was centrifuged at 25,000 rpm for 30 min, and the supernatant and pellet (membrane) fractions were collected.

Total cell wall proteins were extracted in 1 M NaCl for 1 h at 4°C from cell walls prepared from mesocotyl tissues, as previously described (Prasad and Cline, 1987). Total protein content was determined by the method of Lowry et al. (1951) using BSA as a standard.

Enzyme Analyses

Catalase activity was determined by measuring the disappearance of H_2O_2 (ϵ_{240} = 36 M^{-1} cm^{-1}), according to the method of Beers and Sizer (1952). Guaiacol peroxidase activity was determined by the method of Chance and Maehly (1955), using guaiacol (ϵ_{470} = 26.6 mM^{-1} cm^{-1}) as the electron donor. Cyt peroxidase activity was measured by monitoring the oxidation of reduced Cyt *c* (ϵ_{550} = 21 mM^{-1} cm^{-1}), according to the method of Verduyn et al. (1988). Cyt oxidase was determined (Prasad et al., 1994b) as the rate of oxidation of the reduced Cyt *c* (ϵ_{550} = 21 mM^{-1} cm^{-1}). GSH peroxidase was determined by measuring the oxidation of GSH (ϵ_{265} = 0.18 mM^{-1} cm^{-1}), as reported by Miyake et al. (1991). Ascorbate peroxidase activity was measured by the method of Nakano and Asada (1981), using ascorbate (ϵ_{290} = 2.8 mM^{-1} cm^{-1}) as the electron donor. Fumarase was determined (Racker, 1950) by measuring the conversion of malate to fumarate (ϵ_{240} = 2600 M^{-1} cm^{-1}).

Protease Treatments

Fresh intact or lysed mitochondria were incubated with trypsin at 25°C or proteinase K at 37°C at the ratio of 50:1 (milligrams of mitochondrial protein:milligrams of trypsin or proteinase K) in 0.1 M sodium phosphate buffer containing 250 mM Suc, pH 7.2, for 15 to 45 min. The protease activity was inhibited by soybean trypsin inhibitor at the ratio of 1:3 (milligrams of trypsin:milligrams of trypsin inhibitor), and the enzyme activities were determined. When fresh intact mitochondria were used, protease digests were also diluted severalfold with resuspending buffer. The treated mitochondria were repelleted and resuspended in buffer for measuring enzyme activities.

Periodate Oxidation of Guaiacol Peroxidases

Mitochondrial or total cell wall proteins were oxidized with 10 mM sodium periodate (meta) in 50 mM sodium acetate buffer, pH 4.5, at 4°C in the dark for various time intervals, according to the method of Biroc and Etzler (1978), and the enzyme activity was measured. The presence of periodate in the assay mix did not interfere with enzyme activity.

Con A Affinity Chromatography

Con A chromatography was performed according to the method of Toguri et al. (1986) in a 0.3-mL column packed with cyanogen bromide-cross-linked Con A-agarose. Mitochondrial or total cell wall proteins were applied to the Con A column at room temperature and flow-through fractions were collected. The Con A-bound proteins were eluted with 3 mL of equilibration buffer containing 0.5 M methyl α-D-mannopyranose.

IEF, Nondenaturing PAGE, and Immunoblotting

IEF of mitochondrial proteins was performed with the LKB Multiphor 2119 (Pharmacia) IEF gel system. The proteins (30 μg) were focused on 5% polyacrylamide gel containing 10% glycerol and 3% Pharmalites, pH 3 to 10, for 5 h at 20 W. The gels were incubated in PBS buffer, pH 6.0, for 30 min and stained with 9.25 mM *p*-phenylenediamine in 0.1 M citrate buffer, pH 5.0, and 3.9 mM H_2O_2. Nondenaturing PAGE was performed as previously described (Hoefer Scientific Instruments, San Francisco, CA; 1992–1993 catalog). The proteins (30 μg) were resolved on 7% gels for 24 h at 150 V and 4°C. The gel was stained for peroxidase activity as described above. For immunoblotting, mitochondrial membrane proteins were resolved by SDS-PAGE on 12.5% gels, and the proteins were transferred to a nitrocellulose membrane. The proteins on the membrane were reacted with monoclonal antibodies directed against maize mitochondrial Cyt oxidase II subunit, α-subunit of F_1-ATPase, and hsp70 at 1:500 dilutions (a gift from Dr. Tom Elthon, University of Nebraska, Lincoln). The secondary antibody was alkaline phosphatase-conju-

gated goat anti-mouse IgG antibody at 1:1000 dilution, and immunodetection was carried out as described by Hawkes et al. (1982).

RESULTS

Association of Peroxidases with the Mitochondria

Mitochondria isolated from maize seedlings by differential centrifugation and partially purified on a Suc gradient contain measurable activities of ascorbate, GSH, and Cyt peroxidases and mtPOX and CAT3 activities in addition to mitochondrial enzymes, such as fumarase and Cyt oxidase. There was no change in Cyt peroxidase, mtPOX, or CAT3 activities when fresh intact mitochondria were treated with either trypsin or proteinase K (Table I). When these mitochondria were treated with trypsin, 49% of ascorbate peroxidase activity was lost and GSH peroxidase was activated by 90% in 45 min. When treated with proteinase K, almost all of ascorbate and GSH peroxidase activities were lost in 45 min.

Trypsin treatment of lysed mitochondria inhibited most of the Cyt peroxidase activity, activated mtPOX by 80% within 45 min, and had no effect on CAT3 activity (Table II). Proteinase K inhibited all of the enzyme activities in lysed mitochondria within 45 min. These results indicate that, whereas Cyt peroxidase, mtPOX, and CAT3 are localized within the mitochondria, ascorbate and GSH peroxidases seem to be associated with mitochondria as cytosolic contaminants.

Trypsin Activation of mtPOX Activity

Figure 1 shows the results of IEF and native PAGE of control and trypsin-treated proteins from lysed mitochondria. The IEF gel shows that untreated mitochondria contain at least two peroxidase isozymes having pIs of 6.8 and 8.0 (Fig. 1A). However, fresh intact mitochondria treated with proteinase K for 45 min contain a single major isozyme at pI 6.8, which suggests that the isozyme of pI 8.0 is bound to the mitochondria as a cytosolic contaminant. Confirming the spectrophotometric assays (Table II), the IEF gel also shows the trypsin activation of the major peroxidase isozyme at pI 6.8. To detect any change in protein mobility, trypsin-digested protein was resolved on native PAGE (Fig. 1B). Although trypsin-activated activity was observed, no change in mobility of the activated protein was observed.

Table II. *Proteolytic treatment of lysed mitochondria to localize the mitochondrial peroxidases*

The source of mitochondria and protease digestions are the same as described in Table I. Treatments were replicated three times. SEs are noted for each time.

Treatment and Duration (min)	CPX[a]	mtPOX[a]	CAT3[a]
Trypsin			
0	0.49 ± 0.05	0.39 ± 0.05	103.70 ± 4.90
15	0.23 ± 0.03	0.48 ± 0.03	102.78 ± 4.81
30	0.16 ± 0.02	0.62 ± 0.03	96.30 ± 1.85
45	0.07 ± 0.02	0.70 ± 0.06	93.52 ± 3.34
Proteinase K			
0	0.38 ± 0.04	0.38 ± 0.05	100.00 ± 6.41
15	0.10 ± 0.01	0.31 ± 0.02	64.82 ± 3.70
30	0.07 ± 0.01	0.21 ± 0.02	39.82 ± 2.45
45	0.03 ± 0.01	0.11 ± 0.02	25.93 ± 1.85

[a] Abbreviations and units for enzyme activities are as described in Table I.

Characterization of Mitochondrial Peroxidases Using Known Inhibitors

To distinguish mitochondrial peroxidases from the mitochondrial contaminant peroxidases and electron transport enzymes, we studied the effects of the known inhibitors of ascorbate peroxidase, hydroxylamine and DTT (Chen and Asada, 1990, 1992), and the known inhibitors of Cyt oxidase and alternative oxidase, KCN and SHAM, respectively (Day et al., 1980). Whereas GSH peroxidase

Table I. *Proteolytic treatment of fresh intact mitochondria to detect the association of the mitochondrial peroxidases*

Mitochondria from the mesocotyls of the acclimated seedlings were isolated and digested with trypsin or proteinase K for the indicated time intervals (min) and the enzyme activities were measured from the same sample digests. Treatments were replicated three times. SEs are noted for each time.

Treatment and Duration (min)	APX[a]	GPX[b]	CPX[c]	mtPOX[d]	CAT3[e]
Trypsin					
0	1.44 ± 0.13	9.07 ± 0.98	0.51 ± 0.04	0.33 ± 0.01	103.70 ± 4.90
15	1.06 ± 0.07	11.67 ± 1.79	0.45 ± 0.05	0.37 ± 0.02	101.85 ± 4.90
30	0.87 ± 0.1	13.70 ± 1.88	0.45 ± 0.06	0.38 ± 0.02	103.70 ± 1.85
45	0.74 ± 0.14	17.22 ± 1.95	0.48 ± 0.06	0.41 ± 0.01	99.07 ± 3.34
Proteinase K					
0	0.96 ± 0.04	8.43 ± 0.76	0.53 ± 0.03	0.35 ± 0.01	98.15 ± 4.90
15	0.86 ± 0.16	6.85 ± 0.81	0.48 ± 0.03	0.36 ± 0.03	98.15 ± 4.90
30	0.31 ± 0.08	5.00 ± 0.85	0.49 ± 0.03	0.34 ± 0.03	96.30 ± 3.70
45	0.08 ± 0.02	1.94 ± 0.42	0.45 ± 0.04	0.30 ± 0.02	89.82 ± 4.04

[a] APX, ascorbate peroxidase, μmol ascorbate min^{-1} mg^{-1} protein. [b] GPX, GSH peroxidase, μmol GSH min^{-1} mg^{-1} protein. [c]CPX, cytochrome peroxidase, μmol Cyt c min^{-1} mg^{-1} protein. [d]mtPOX, μmol guaiacol min^{-1} mg^{-1} protein. [e]CAT3, μmol H_2O_2 $min^{-1}mg^{-1}$ protein.

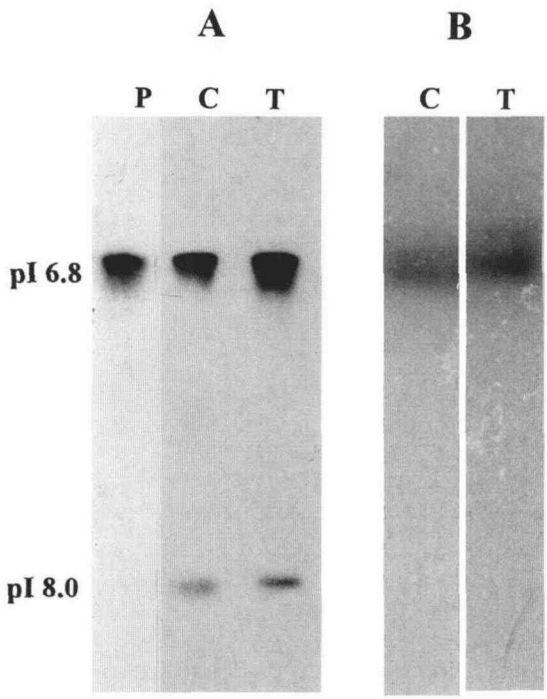

Figure 1. IEF gel (A) and nondenaturing PAGE (B) analysis of the pI and the molecular size of the trypsin-activated mtPOX. Mitochondrial protein (50 μg) was digested with 1 μg of trypsin for 45 min. The protein digests were focused on an IEF gel or resolved on native PAGE. Peroxidase activity was detected using *p*-phenylenediamine as a substrate. Control protein (C) and the trypsin-digested protein (T) lanes are labeled. For identifying the mitochondrial specific proteins, fresh intact mitochondria were treated with proteinase K for 45 min (P), as described in "Materials and Methods," and proteins were resolved on the IEF gel. The pIs of the marker proteins (Sigma) are labeled.

was resistant, hydroxylamine inhibited all of the other enzyme activities tested at various concentrations (Table III). CAT3 and mtPOX activities proved to be more sensitive to hydroxylamine than ascorbate and Cyt peroxidase enzyme activities.

Of all the enzymes tested, only ascorbate peroxidase and mtPOX showed sensitivity to DTT, with mtPOX being the more sensitive enzyme (Table III). However, depending on the concentration and the duration of the reaction, the mtPOX activity recovered to control levels after some time. Recovery time was proportional to DTT concentration (data not shown). These results indicate that the inhibitory effect of DTT on mtPOX activity was reversible, unlike that of ascorbate peroxidase activity.

The effects of KCN and SHAM on peroxidase activities are reported in Table IV. Although mtPOX was sensitive to both inhibitors, Cyt peroxidase was resistant to SHAM but sensitive to KCN. As expected, Cyt oxidase was very sensitive to KCN treatment. Although Cyt oxidase was resistant at 0.5 mM, its activity was inhibited by 30% in the presence of 1 mM SHAM.

Localization of Peroxidases in the Mitochondrial Compartments

To localize peroxidases in the mitochondrial compartments, the soluble and membrane (pellet) protein fractions of the lysed mitochondria were separated and analyzed (Fig. 2). The activities of Cyt oxidase and fumarase were used as membrane-specific and matrix-specific markers, respectively. Based on the marker enzyme activities, there was an estimated 10% contamination of the matrix protein with membrane protein and 13% contamination of the membrane proteins with matrix proteins. Similar to Cyt oxidase activity, Cyt peroxidase activity was also localized in the membrane fraction. Whereas CAT3 activity was located mostly in the soluble fraction, similar to fumarase, 45% of the mtPOX activity was localized in the soluble fraction and the remaining 55% was associated with the membrane fraction.

To further investigate the nature of the membrane association of mtPOX, mitochondrial membranes were extracted with alkaline carbonate, a treatment that would remove peripherally bound proteins. After the sample was centrifuged, the resulting pellet and supernatant fractions were assayed for specific proteins by IEF or immunoblotting (Fig. 3). Antisera raised against known mitochondrial proteins were used to monitor the submitochondrial proteins. The integral membrane protein subunit II of Cyt oxidase was recovered in the initial membrane fraction and remained in the membrane fraction following the alkaline carbonate treatment. On the other hand, the α-subunit of F_1-ATPase and also mitochondrial hsp70 (Vidal et al., 1993), the two known peripherally bound mitochondrial

Table III. *Effects of hydroxylamine and DTT on peroxidase activities associated with the fresh mitochondria*

Inhibitors were added to the reaction mix during the assay. Treatments were replicated three times. Treatment effects on enzyme activities are represented by I_{50} values (amount of inhibitor required to inhibit 50% of the enzyme activity). SEs are noted for each treatment. N, No inhibition.

Treatment	APX[a]	GPX[a]	CPX[a]	mtPOX[a]	CAT3[a]
Hydroxylamine					
Control rate	1.35 ± 0.03	9.35 ± 0.13	0.48 ± 0.01	0.39 ± 0.01	103.70 ± 1.76
I_{50} (mM)	0.57	N	1.35	0.15	0.001
DTT					
Control rate	1.35 ± 0.03	9.35 ± 0.37	0.48 ± 0.01	0.39 ± 0.01	103.70 ± 1.07
I_{50} (mM)	0.06	N	N	0.015	N

[a] Abbreviations and units for enzyme activities are as described in Table I.

Table IV. *Effect of KCN and SHAM on mtPOX, Cyt peroxidase, and Cyt oxidase activities*

Inhibitors were added to the mitochondrial proteins during the assays for enzyme activities. Treatments were replicated three times. SEs are noted accordingly.

Treatment and Concentration (mM)	mtPOX[a]	CPX[a]	COX[a]
Control	0.32 ± 0.03	0.37 ± 0.03	1.48 ± 0.11
KCN			
0.01	0.03 ± 0.01	0.13 ± 0.01	0.08 ± 0.01
0.05	0.01 ± 0.01	0.05 ± 0.01	0.02 ± 0.01
SHAM			
0.01	0.26 ± 0.02	0.36 ± 0.04	1.53 ± 0.05
0.05	0.16 ± 0.01	0.37 ± 0.03	1.54 ± 0.08
0.1	0.14 ± 0.01	0.37 ± 0.04	1.50 ± 0.10
0.25	0.09 ± 0.01	0.37 ± 0.02	1.46 ± 0.06
0.5	0.04 ± 0.01	0.36 ± 0.03	1.31 ± 0.14
1.0	0.02 ± 0.01	0.33 ± 0.01	0.98 ± 0.03
2.0	–	0.31 ± 0.01	–

[a] Abbreviations and units for enzyme activities are as described in Table I.

membrane proteins, were recovered from the initial membrane fraction and extracted to the supernatant fraction following alkaline carbonate treatment. Whereas extensive washing of mitochondrial membranes with buffer containing high salt concentrations did not release mtPOX activity from the membranes (data not shown), the alkaline carbonate treatment of membranes completely released mtPOX activity into the supernatant fraction, similar to α-subunit of F_1-ATPase and hsp70 proteins. Based on these results, we conclude that mtPOX is loosely bound to the mitochondrial envelope.

Testing for the Glycosylation of mtPOX Protein

Because guaiacol peroxidases are thought to be glycosylated (Asada, 1992; Welinder, 1992), we tested whether mtPOX was glycosylated. Mitochondrial or total cell wall (positive glycosylated control) proteins were treated with 10 mM sodium periodate, which oxidizes *vic*-hydroxy groups of carbohydrates (Dyer, 1956) that are required to stabilize the peroxidase molecules (Hu and van Huystee, 1989). Periodate had no effect on the mtPOX activity, but 50% of the total cwPOX activity was lost during 20 h of treatment (Table V). To further test for glycosylation, mitochondrial or total cell wall proteins were subjected to Con A chromatography (glycosylated proteins are ex-

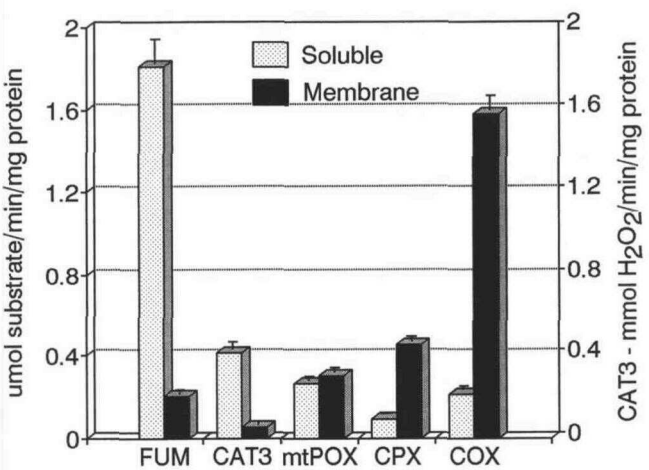

Figure 2. Localization of peroxidases in mitochondrial compartments. Mitochondrial soluble and membrane fractions were collected, as described in "Materials and Methods," and used for spectrophotometric analyses. Treatments were replicated three times. SEs are designated as vertical lines. COX, Cyt oxidase; CPX, Cyt peroxidase; FUM, fumarase.

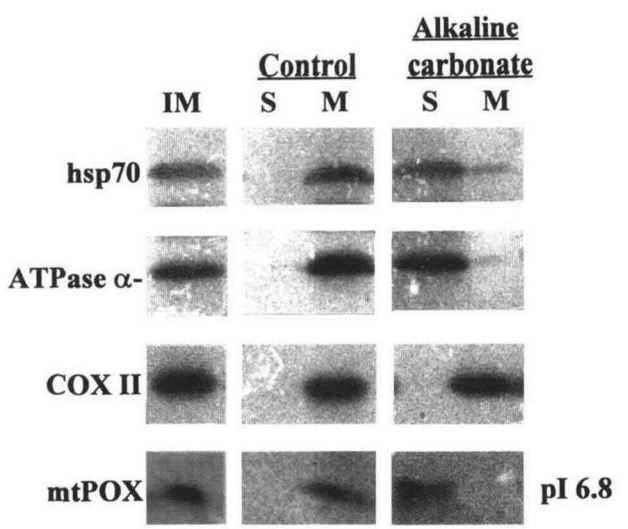

Figure 3. Localization of mtPOX and marker proteins in submitochondrial fractions. Mitochondrial membranes (25 μg) were extracted with or without (Control) alkaline carbonate. After extractions, the membranes were centrifuged, and the membrane and supernatant fractions were collected. The membrane fraction was resuspended in buffer. Both supernatant and membrane fractions were made to an equal final volume. Equal proportions of each fraction were analyzed by IEF (for mtPOX activity) or by SDS-PAGE followed by immunoblotting (using monoclonal antibodies raised against maize subunit II of Cyt oxidase, α-subunit of F_1-ATPase, and mitochondrial hsp70). IM, Initial mitochondrial membrane proteins (before extraction with or without alkaline carbonate); S, supernatant fraction after extraction; M, membrane fraction after extraction.

Table V. *Periodate oxidation and Con A chromatography for testing the glycosylation of mtPOX protein*

Mitochondrial or total cell wall proteins (50 μg) in mitochondrial resuspending medium was mixed with an equal volume of 20 mM sodium periodate and incubated at 4°C in the dark for the indicated time intervals and assayed for mtPOX activity. Mitochondrial or total cell wall proteins were subjected to Con A chromatography. Flow-through and Con A-bound protein fractions were collected, and peroxidase activity was measured. Treatments were replicated three times. SEs are noted accordingly.

Treatments	mtPOX	cwPOX
	μmol guaiacol min^{-1} mg^{-1} protein	
Control at 4°C	0.36 ± 0.03	0.93 ± 0.03
Periodate (10 mM)		
0 h	0.35 ± 0.03	1.06 ± 0.07
2 h	0.36 ± 0.02	–
4 h	0.37 ± 0.01	0.69 ± 0.04
8 h	0.40 ± 0.02	0.53 ± 0.03
20 h	0.41 ± 0.01	0.42 ± 0.04
Con A		
Control at 22°C	0.36 ± 0.03	1.06 ± 0.07
Flow-through	0.39 ± 0.03	0.31 ± 0.02
Con A-bound	0.04 ± 0.01	0.69 ± 0.04

pected to bind to the Con A column), and the flow-through and Con A-bound fractions were analyzed for peroxidase activity (Table V). Whereas all of the mtPOX activity was localized in the flow-through fraction, most of the cwPOX activity was localized in the Con A-bound fraction. These results suggest that mtPOX was not glycosylated.

Effects of Low Temperature on Mitochondrial Peroxidase Activities

Mitochondrial proteins extracted from the mesocotyl tissues of low-temperature-treated seedlings were assayed for peroxidase activities. Although reported previously (Prasad et al., 1994b), mtPOX and Cyt oxidase activities were assayed in the present study for comparative studies. As expected, mtPOX activity was induced by acclimation and acclimation/chilling treatments by 2- to 4-fold (Fig. 4A) but not by the chilling treatment. Confirming the spectrophotometric assays, the IEF gel also showed the induction of mtPOX (pI 6.8) only by the acclimation treatments (Fig. 4B). Whereas Cyt oxidase activity progressively declined, Cyt peroxidase activity was induced by 70 to 100% in all of the low-temperature treatments. Although Cyt oxidase activity was inhibited by low-temperature treatments, the benefit of acclimation was manifested as the ability to recover Cyt oxidase activity during rewarming (Prasad et al., 1994b).

DISCUSSION

We demonstrated that (a) maize mitochondria contain two peroxidases, one that uses guaiacol and another that uses Cyt *c* as the electron donor, and both are also induced by low-temperature treatments; (b) whereas Cyt peroxidase activity fractionates with the mitochondrial membrane, mtPOX is partially localized in both soluble and membrane fractions; (c) CAT3, which also has peroxidative activity, is mostly localized in the soluble fraction; (d) contrary to the notion that all guaiacol peroxidases are glycosylated, the mitochondrial form is not glycosylated and is activated by trypsin treatment; and (e) treatments with various known inhibitors indicate that these two new mitochondrial peroxidases are distinct from mitochondrial contaminant peroxidases and mitochondrial electron transport enzymes.

Localization of the Peroxidases Associated with Maize Mitochondria

It was reported that ascorbate peroxidase activity was localized in chloroplasts and cytosol in many plants (Asada, 1992) and was associated with mitochondria only as a cytosolic contaminant in bean hypocotyls (Plesnicar et al., 1967). Although cDNA clones (Criqui et al., 1992) and enzyme activities for GSH peroxidase were reported in

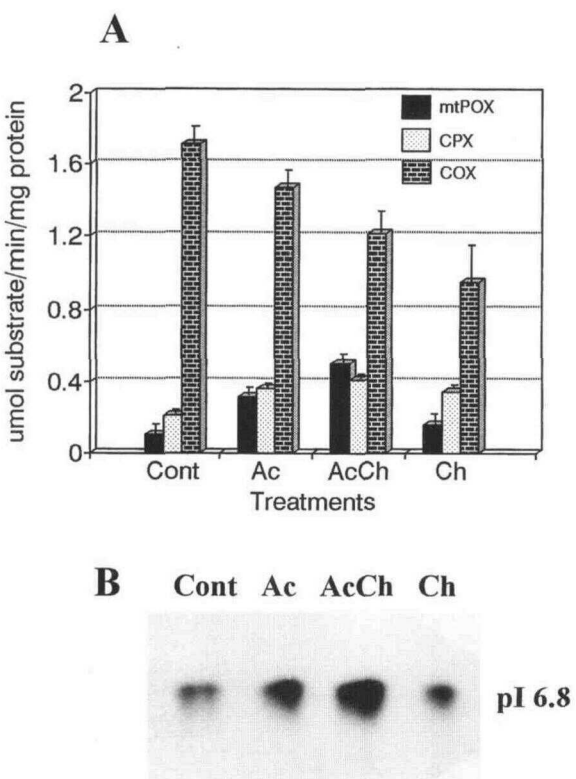

Figure 4. Effects of low-temperature stress on peroxidase activities. Three-day-old dark-grown maize seedlings (Cont) were treated with acclimation at 14°C (Ac), acclimation followed by chilling at 4°C for 4 d (AcCh), and chilling alone at 4°C for 4 d (Ch). Mitochondria from the mesocotyls were isolated, and the mitochondrial proteins were used for spectrophotometric (A) and IEF gel (B) analyses. The results of the spectrophotometric analyses (A) are the means of triplicate samples. SEs (as derived by a randomized complete block design) are designated as vertical lines. Mitochondrial proteins (30 μg) were focused on an IEF gel. Peroxidase activity was detected using *p*-phenylenediamine as a substrate. The pI of the marker proteins (B) are labeled.

plants (Badiani et al., 1993), its cellular location is not yet known. Cyt peroxidase activity has not yet been identified in plants. In the present study, mitochondria isolated by differential and Suc gradient centrifugation contained the activities of ascorbate, GSH, and Cyt peroxidases and mtPOX and CAT3. Using protease treatments, we demonstrated that ascorbate and GSH peroxidases are cytosolic contaminants but Cyt peroxidase, mtPOX, and CAT3 are localized within the mitochondria. This conclusion is drawn from three lines of evidence: (a) when fresh intact mitochondria are digested with proteinase K, only the activities of ascorbate and GSH peroxidases are removed by the protease but not the other peroxidase enzyme activities; (b) the mitochondria are expected to be intact after protease treatment because all of the CAT3 activity, a soluble enzyme like fumarase, and all of the membrane-associated Cyt peroxidase activity are recovered after protease digestion; and (c) if the mitochondria were not intact, at least part of the soluble and membrane proteins would have been lost during the repelleting and resuspending procedure following protease digestions. They were not lost.

When mitochondrial proteins were fractionated, CAT3 and fumarase activities were localized in the soluble fraction. Cyt peroxidase and Cyt oxidase activities were found in the membrane fraction. Although partial activities of mtPOX were recovered in both soluble and membrane fractions, the results of alkaline carbonate extraction of mitochondrial membranes indicate that this enzyme is loosely bound to the mitochondrial membrane.

Characterization of Peroxidases Using Known Inhibitors

In an attempt to distinguish the mitochondrial peroxidases from mitochondrial contaminant peroxidases and electron transport enzymes, we found that hydroxylamine and DTT are not specific to ascorbate peroxidase but also inhibit other peroxidases. CAT3, which also has peroxidase activity (Scandalios et al., 1984; Havir and McHale, 1989), proved to be even more sensitive to hydroxylamine but was unaffected by DTT. On the other hand, mtPOX is inhibited by both inhibitors at much lower concentrations compared to ascorbate peroxidase activity. DTT-inhibited mtPOX activity is recovered when the reaction is allowed to proceed, and this recovery time is dependent on the amount of DTT added to the reaction. As reported by Yamazaki et al. (1960), during the peroxidative catalytic reaction, peroxidase combines with H_2O_2 to form compound I, which univalently oxidizes the electron donor to produce compound II, which is in turn reduced back to the native enzyme complex by the donor substrate. So, the initial inhibition of mtPOX could be due to the interference with the formation of compound I, in a concentration-dependent manner. As DTT is oxidized by hydrogen peroxide (Chen and Asada, 1992), compound I will be formed and the enzyme activity is eventually restored.

Whereas mtPOX is inhibited by both KCN and SHAM, Cyt peroxidase is sensitive only to KCN and resistant to SHAM. In addition to KCN, Cyt oxidase also seems to be sensitive to 1 mM SHAM. Although both peroxidases seem to be localized in mitochondrial membranes, no conclusions could be drawn from these inhibitor studies to suggest that they participate in the electron transport pathway. However, the results do suggest that these two peroxidases are distinct from the known mitochondrial electron transport enzymes.

Glycosylation and Trypsin Activation of mtPOX Activity

All guaiacol peroxidases are localized in the vacuole, cytosol, and cell wall and are thought to be glycosylated (Asada, 1992). They are suggested to be synthesized on RER and processed in the Golgi complex and, therefore, are not expected to be localized in mitochondria. Even though mtPOX is localized in mitochondria, in the present study, it is not expected to be glycosylated. If it is, it would raise an intriguing question of whether it is glycosylated before or after entering mitochondria and, if so, how. We have three lines of evidence to suggest that it is not glycosylated: (a) the enzyme activity is not inhibited by periodate treatment, because periodate is expected to oxidize the *vic*-hydroxyl groups of carbohydrates and thus inactivate the enzyme (Dyer, 1956; Hu and van Huystee, 1989); (b) the enzyme does not bind to a Con A column, to which typical glycosylated proteins are expected to bind; and (c) the enzyme is activated by trypsin, although glycosylated peroxidases are insensitive to trypsin treatment (Stegemann et al., 1973).

We have shown that mtPOX is activated by trypsin, although the biological significance of this activation is not yet known. It was previously reported that some protein complexes from prokaryotes and eukaryotes are activated by trypsin. For example, the activation of CF_1-ATPase was shown to be due to the cleavage of the β and γ subunits of the complex (Moroney and McCarty, 1982) and the activation of plasma membrane H^+-ATPase was shown to be due to the cleavage of the C-terminal inhibitory domain of the protein (Palmgren et al., 1991). Our results from native PAGE analysis did not confirm that the trypsin activation is due to the cleavage of any part of the protein. Although no change in protein mobility is observed, possibly because a change in charge density could mask a change in protein size, the trypsin activation could still be due to the cleavage of an autoinhibitory domain of the enzyme.

Possible Functions of Mitochondrial Peroxidases during Low-Temperature Regimes

We have previously demonstrated that low temperature induces oxidative stress in maize seedlings (Prasad et al., 1994a), and the mitochondria are a potential target for chilling-induced oxidative stress injury (Prasad et al., 1994b). It was also reported that mitochondria generate oxidative stress and are the major source of the cytosolic H_2O_2 (Puntarulo et al., 1991) during soybean seed germination. Because CAT3 and mtPOX were induced by acclimation but not by chilling alone in the mitochondria (Prasad et al., 1994b), we suggest that both CAT3 and mtPOX could function as H_2O_2-scavenging enzymes in mitochondria. Because yeast Cyt peroxidase is a known H_2O_2-scavenging enzyme in the mitochondria (Il'chenko et

al., 1991), we assume that plant Cyt peroxidase should be playing a similar functional role in the maize mitochondria. Although CAT3 is abundant, the role of peroxidases in scavenging H_2O_2 could also be significant in the mitochondria, in addition to their participation in any other, unknown functional roles. We also found the presence of GSH reductase, a known antioxidant enzyme, within the maize mitochondria (M.D. Anderson, T.K. Prasad, and C.R. Stewart, unpublished data). This suggests that multiple antioxidant enzymes are required to protect mitochondria from oxidative damage.

Evidence from yeast indicates that Cyt peroxidase is also involved in the mitochondrial electron transport, since like Cyt oxidase it can accept electrons from Cyt *c* (Hahm et al., 1992; Stemp and Hoffman, 1993). Because Cyt peroxidase is also induced by low-temperature treatments, it raises an intriguing possibility that Cyt peroxidase might supplement the chilling-sensitive Cyt oxidase function (Prasad et al., 1994b) during low-temperature treatments, in addition to functioning as an antioxidant enzyme, to protect the mitochondria from oxidative stress injury.

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