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Control of superoxide production in mitochondria from maize mesocotyls

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Abstract To understand the biochemical events that control the generation of superoxide, the effect of inhibiting the respiratory complexes III and IV (C-III and C-IV) and alternative oxidase (AOX) on the rate of superoxide production was analyzed in mitochondria from maize seedlings. To increase superoxide production, it was required to inhibit C-III or C-IV by at least 30% or 50%, respectively. Below this inhibition threshold, AOX exerted the highest degree of control on superoxide production, whereas above it, the highest degree of control was exerted by C-IV. The contribution of C-III to control superoxide production became significant when AOX activity was modulated.

Keywords: Alternative oxidase; Flux-control coefficient; Electron transport complex; Metabolic control; Maize mitochondria; Superoxide

1. Introduction

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and hydroxyl radicals ($HO^{\cdot}$) are by-products of mitochondrial respiratory metabolism. ROS damage cellular macromolecules including DNA, proteins and lipids. To avoid this damage, accumulation of ROS is normally kept under control by various enzymatic and chemical scavenging systems [1,2].

Under environmental stress, germination and early seedling growth are often reduced and delayed [3] with subsequent effects on crop yield and quality [4]. The reduced ability to germinate and grow is accompanied by an increased production of ROS and impairment of the antioxidant mechanisms [2,5], with the consequent accumulation of oxidative damage [6]. Therefore, to reinforce germination and early seedling growth under stress, the mechanisms that induce an accelerated formation of ROS should be described and understood, at the levels of the respiratory chain and antioxidant defense system. To date, only the latter approach has been pursued. Several plant and animal systems have been transformed to express increased activities of anti-oxidative enzymes. Unfortunately, only a negligible or moderate increase in resistance to oxidative stress has been achieved with such strategy [7,8].

Since the cascade of reactions producing ROS begins with $O_2^{\cdot-}$, it may be hypothesized that strengthening the mechanisms that avoid the accelerated production of $O_2^{\cdot-}$ under stress should increase seedling stress resistance. An initial step in this direction is to establish the control of mitochondrial ROS production during germination and early growth, to identify the relevant enzymes that should be considered for further manipulation. The participant elements are the mitochondrial electron transport complexes (ETC [1]) and alternative oxidase (AOX) which limits the mitochondrial side-reaction of superoxide production [9,10]. Therefore, the aim of the present work was to examine the contribution of mitochondrial respiratory ETC and AOX to the control of superoxide generation in mitochondria isolated from mesocotyls of maize seedlings.

2. Materials and methods

2.1. Biological material

Seeds of maize "Tepecintle 100" (T_{100}) were from the Maize Germoplasm Bank of CIMMYT, Mexico. The seeds were kept at 4 °C until use; the moisture content was 8–10%. All manipulations were done aseptically. Before germination, seed samples were washed in 3% sodium hypochlorite for 2 min and rinsed with water. Seeds were germinated in the dark at 27 °C in wet rolled paper towels. After 4 days of germination mesocotyl tissue was obtained from the seedlings.

2.2. Isolation and purification of mitochondria

Mesocotyl tissue (40–50 g) was mixed with 0.6% (w/w) polyvinylpolypyrrolidone 40 and homogenized in two volumes (w/v) of cold isolation buffer (250 mM sucrose, 10 mM HEPES and 1 mM EGTA, pH 7.2, 0.004 mM cysteine and 5 mM pyruvate) that also contained 1% (w/v) fatty acid-free bovine serum albumin. The homogenate was centrifuged at $600 \times g$ for 10 min at 4 °C. The supernatant was centrifuged at $8500 \times g$ for 10 min at 4 °C. The mitochondrial pellet was carefully re-suspended in 500 μl of isolation buffer that contained 0.5% fatty acid-free bovine serum albumin and 1 mM ADP, and incubated for 10 min in ice with occasional agitation. The mitochondrial suspension was diluted with 30 vol of fresh isolation buffer and centrifuged at $7800 \times g$ for 10 min at 4 °C. The pellet was re-suspended in isolation buffer and was designated crude mitochondrial fraction (CMF). Mitochondria were further purified using a Percoll gradient [11]. The CMF was poured in a centrifuge tube containing 3 ml of 45% Percoll in isolation buffer added with 1% BSA; and centrifuged at $45\,000 \times g$ for 30 min. The purified mitochondrial fraction (PMF) was washed once. Protein was determined by the Lowry method [12]. The usual yield was 7 mg protein per 50 g wet weight.

2.3. Oxygen uptake

Oxygen uptake of mitochondrial suspensions (0.5 mg protein in 1.9 ml) was measured at 30 °C with a Clark-type oxygen electrode. The

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Abbreviations: AOX, alternative oxidase; CMF, crude mitochondrial fraction; ETC, electron transport complexes; C-III, complex III; C-IV, complex IV; C_E, flux-control coefficient; PMF, purified mitochondrial fraction

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respiration medium contained 120 mM KCl, 20 mM MOPS, 1 mM EGTA, 5 mM K-phosphate and 1 mM $MgCl_2$, pH 7.5. The concentration of respiratory substrates was 1 mM NADH, 10 mM succinate or 2 mM malate. With succinate 5 μM rotenone and 150 μM ATP were also added. The O_2 concentration in air-saturated buffer at 30 °C and 2240 m altitude was taken to be 201.5 nmol O_2 ml^{-1}.

PMF showed a low respiratory control (1.4) with either of the oxidizable substrates. Respiration was unaffected by the addition of 50 μM reduced cytochrome *c*, indicating that the outer membrane was intact. PMF was able to generate an uncoupler-sensitive membrane potential (measured by the change in safranine O fluorescence [13]) similar to that attained by tightly coupled rat liver and heart mitochondria. The contamination of PMF by plasma membrane, tonoplast, endoplasmic reticulum and peroxisomes, detected by measurement of specific enzymatic markers [14–16], was 10%, 9%, 4% and 6%, respectively.

2.4. Superoxide radical assay

The production of superoxide was determined by measuring at 30 °C and 480 nm the superoxide dismutase-sensitive epinephrine oxidation rate to adrenochrome [17]. Measurements were made in respiration buffer (1 ml) that also contained 2 mM epinephrine, 1 mM NADH and 0.1 mg of PMF protein. The basal rate of superoxide production was similar with any of the substrates assayed (8.5 ± 0.8 nmol min^{-1} mg $protein^{-1}$; $n = 18$).

2.5. Enzyme activities

To measure cytochrome reductase activity (C-III), the reduction of cytochrome *c* was followed by the change in the absorbance difference at 550 minus 540 nm ($\varepsilon = 21.1$ mM^{-1} cm^{-1}). The reaction mixture (1 ml) contained respiration buffer in 0.01% Triton X-100, 0.03 mM cytochrome *c* and 0.1 mg PMF protein. The reaction was initiated by addition of 60 μM ubiquinol (prepared as described by Rieske [18]). In the absence of added ubiquinol, cytochrome *c* reduction was negligible; in the absence of mitochondria, ubiquinol-induced reduction of cytochrome *c* (non-enzymatic reaction) accounted for less than 5% of C-III activity.

Cytochrome oxidase activity (C-IV) was determined by measuring the KCN-sensitive oxidation of cytochrome *c* at 550 minus 540 nm [19]. The reaction mixture (1 ml) contained respiration buffer and 0.1 mg PMF protein. The reaction was started by the addition of 50 μM reduced bovine heart cytochrome *c*.

Alternative oxidase activity (AOX) was estimated from the rate of cyanide-insensitive respiration, which was totally inhibited by 100 μM *n*-propyl gallate (nPG). The assay medium described above for the respiration measurements also contained 2 mM pyruvate. AOX activity in Section 3 was measured using 1 mM NADH but it was also assayed with 2 mM malate or 5 mM succinate. Addition of 1 mM DTT did not modify the AOX activity. Western-blot analysis of maize seedling mitochondria with a monoclonal antibody raised against AOX from *Sauromatum guttatum* (kindly provided by T.E. Elthon, Michigan State University) revealed the presence of a protein of 38 kDa, presumably the monomeric AOX (data not shown) [20].

2.6. The model

The rate of mitochondrial superoxide formation from the coenzyme Q pool inversely depends on the activity of three components: the mitochondrial electron transport complexes C-III and C-IV and AOX. Thus, at high rates of the components of the pathway, the electrons are efficiently channeled towards C-IV and AOX, with the rate of the mitochondrial side reaction, i.e., superoxide radical production, maintained to a minimum or basal rate. On the other hand, when one of these respiratory enzymes is inhibited, the rate of mitochondrial superoxide production is expected to increase, due to a higher reduction of Q pool.

2.7. Estimation of the flux control coefficient C_E

The flux control coefficient (*C*) exerted by an enzyme (Eo) or block of enzymes on the production of superoxide radical (SOX) is $C_{Eo}^{SOX} = (\partial SOX/\partial E)$ (Eo/SOX) [21]. Since the rate of superoxide formation increases as the activity of ETC and AOX decreases, the value of C_{Eo}^{SOX} is negative. Then, by plotting in percentage values the rate of superoxide production against the inhibition (instead of activity) of anyone of the enzymes accepting electrons from the coenzyme Q pool, the negative C_E^{SOX} value might be estimated directly from the positive slope ($\partial SOX/\partial E$), and the corresponding scalar factor (Eo/SOX). Therefore, the experimental method to determine $-C_E^{SOX}$ was to diminish the activity of ETC or AOX with specific inhibitors.

3. Results

3.1. A fraction of mitochondrial electron transport activities was resistant to inhibition

To establish the influence of C-III, C-IV and AOX on the production of superoxide, progressive inhibition of their activities on the rate of superoxide production was analyzed in isolated mitochondria. The ETC or AOX activities diminished as the inhibitor concentration was increased (Fig. 1), although significant inhibitor-resistant activity was apparent. For C-III, 26% of activity was not inhibited by antimycin A; the maximal inhibition was achieved at around 0.38 nmol antimycin mg^{-1} protein. With 0.15 μM stigmatellin, the rate of cytochrome reduction was 83% inhibited. Activity of C-IV was 68% inhibited by 0.25 μM cyanide, and totally inhibited by 1 μM. Inhibition of AOX by nPG was higher than 93%. In agreement with the cytochrome *c* measurements (Fig. 1), mitochondrial respiration in the presence of nPG was fully blocked by 2 mM cyanide, whereas 1.5 μM antimycin inhibited by 88%. These results suggested that there was a significant heterogeneity in the population of C-III, whereas that of AOX was more homogeneous regarding inhibitor sensitivity.

3.2. In stressed mitochondria the control of superoxide production was at the level of production whereas in the unstressed condition, avoidance exerted the flux-control

Stimulation of superoxide production by diminution of C-IV or C-III activity was apparent after 50% or 30% inhibition, respectively. Once reached that inhibition threshold, kinetics of superoxide production proceeded with a different slope. In contrast, enhancement of superoxide production increased as AOX was inhibited (Fig. 2). The control that ETC and AOX exerted on superoxide production was estimated at 0% inhibition (100% activity). At high oxidoreductase activity, the highest $-C_E^{SOX}$ value was for AOX, indicating that this oxidoreductase was the most important in controlling superoxide production (Table 1).

Under environmental stress, there might be a high oxidoreductase inhibition [1]. For this reason, $-C_E^{SOX}$ values were also estimated at high levels of enzyme inhibition (Fig. 2). Under such conditions, the main control of superoxide production was now exerted by C-IV (Table 1).

3.3. AOX modulates the level of ETC inhibition required to increase the rate of superoxide production

Some environmental stresses induce impairment in oxygen uptake indicating that the cytochrome pathway has been damage [22,23]. Therefore, to establish whether participation of AOX in controlling superoxide production is modified under stress conditions, the rate of superoxide production was measured in mitochondria exhibiting different levels of AOX activity when C-III was progressively inhibited (Fig. 3). The absolute value of superoxide production increased from 8.5 to 14 nmol min^{-1} mg^{-1} protein when AOX was inhibited by 93% (Fig. 3). A more remarkable increase

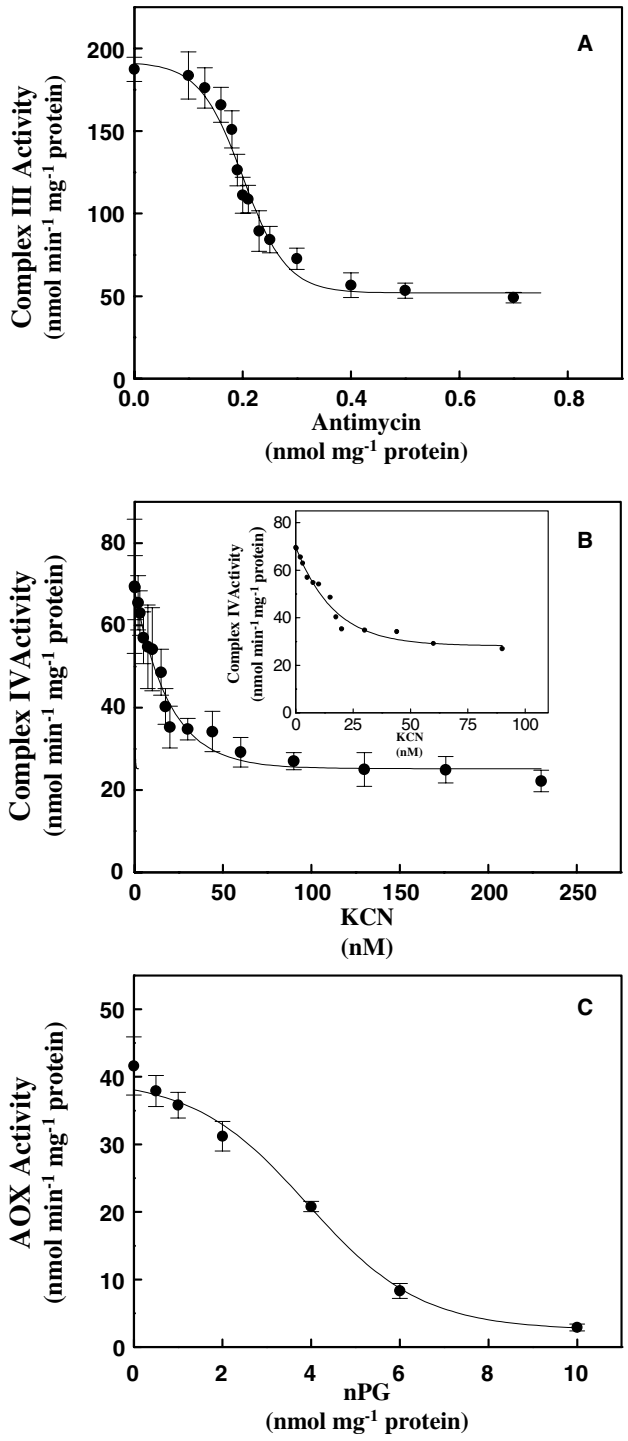

Fig. 1. Effect of specific inhibitors on the activity of ETC and AOX. Inhibition of C-III (A), C-IV (B) and AOX (C) activities was achieved by titrating with antimycin, cyanide (KCN) and nPG respectively, in mitochondria from maize mesocotyls. Data shown represent the means of three different assays ± standard deviation (S.D.) from two independent preparations. Inset shows an expansion of the C-IV titration from 1 to 100 nM cyanide. The initial activities using 1 mM NADH as substrate were 187 ± 7 nmol cytochrome *c* min^{-1} mg^{-1} protein for C-III; 69.5 ± 16 nmol cytochrome *c* min^{-1} mg^{-1} protein for C-IV and 41.6 ± 4 nmol O_2 min^{-1} mg^{-1} protein for AOX. C-IV activity determined by the 2 mM cyanide-sensitive oxygen uptake induced by 5 mM ascorbate plus 1 mM TMPD was 899 nmol O_2 min^{-1} mg^{-1} protein. The difference in activity between the spectral and polarographic assays has been previously documented [31].

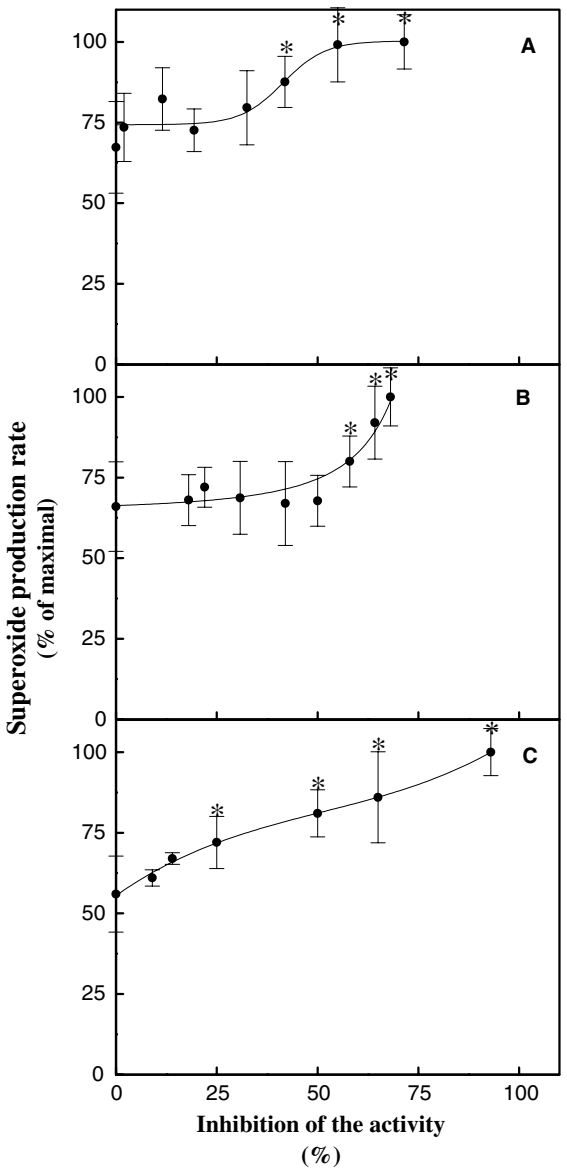

Fig. 2. Effect of the inhibition of ETC and AOX on the superoxide production rate. Superoxide production for different levels of C-III (A), C-IV (B) and AOX (C) were measured as described under materials and methods. Data represent the means of three different assays ± S.D. from two independent preparations. Rates showed in this figure were expressed as percentage of the superoxide production rate at the maximal inhibition of each respiratory complexes. *Values significantly different from 0% inhibition ($P < 0.001$).

in superoxide production was attained when C-III was also diminished; with 50% inhibition of C-III, superoxide production increased from 10 at zero AOX inhibition to 42 nmol min^{-1} mg^{-1} protein at 93% AOX inhibition (Fig. 3). These observations suggested that one AOX role in maize seedling mitochondria was to avoid accumulation of quinol thus limiting superoxide production.

The contribution of C-III to control of superoxide production became significant when AOX activity was modulated (Fig. 3). With an uninhibited AOX, $-C_{CIII}^{SOX}$ was 0.05 (Table 1); with almost full AOX inhibition (7% remaining activity), the degree of control exerted by C-III elevated to 4 (Fig. 3, inset).

Table 1
Flux-control coefficients of the rate of superoxide production in maize mitochondria

Oxidoreductase	High activity			Low activity		
	Jo (%)	Eo (%)	$-C_E^{SOX}$	Jo (%)	Eo (%)[2]	$-C_E^{SOX}$
C-III	74	100	0.05	80	68	0.70
C-IV	68	100	0.06	80	42	1.05
AOX	56	100	1.14	81	50	0.27

$-C_E^{SOX}$, was calculated for each oxidoreductase at high and low activity (according to Fig. 2) by $C_{Eo}^{SOX} = (\partial SOX/\partial E)\ (Eo/SOX)$ [21].

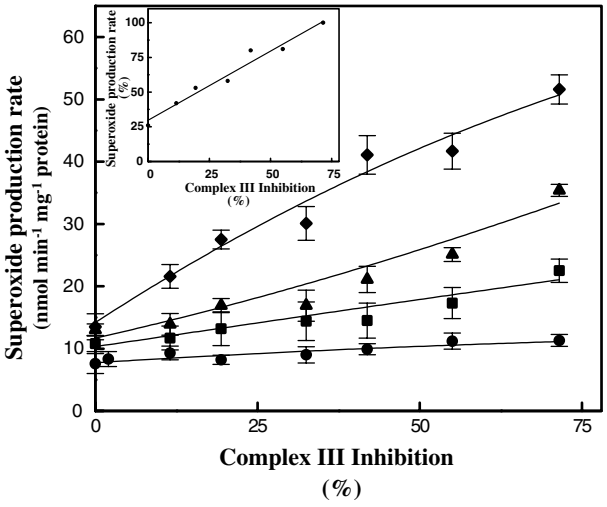

Fig. 3. Role of AOX on the control of superoxide production rate. Different symbols represent variations in the level of AOX: -●- 100% (0 nmol nPG mg^{-1} protein), -■- 50% (4 nmol nPG mg^{-1} protein), -▲- 20% (6 nmol nPG mg^{-1} protein) and -◆- 7% (10 nmol nPG mg^{-1} protein). Data represent the means of three different assays ± S.D. from two independent preparations. *Inset*: The superoxide production rate was expressed as percentage of maximal superoxide produced by the inhibition of C-III (51.6 ± 2.3 nmol $O_2^{\cdot -}$ min^{-1} mg^{-1} protein). AOX activity was 7% of the maximal value detected in mesocotyl mitochondria.

4. Discussion

Under environmental stress, mitochondria from germinated seeds or seedlings show enhanced rate of superoxide production [5,6]. The magnitude of these changes depends on the stress severity. One way to mimic different levels of stress over the mitochondrial quinol oxidizing pathways was to modulate ETC and AOX activities with specific inhibitors and analyze, for the first time, the superoxide production using the metabolic control theory. This theory allows the identification of controlling steps through the estimation of the flux-control coefficients (C_E^{SOX}) which reflected, quantitatively, the sensitivity of the pathway flux to changes in the activity of each pathway element (enzyme) [21].

In this work, the value of C_{Eo}^{SOX} was negative, since the rate of mitochondrial superoxide formation inversely depends on the activity of C-III, C-IV and AOX. Thus, the higher value for $-C_{AOX}^{SOX}$ in control mitochondria indicated that, principally, AOX controlled superoxide production (Table 1). In stressed mitochondria (significant inhibition of the respiratory complexes), C-IV exerted the higher flux control (Table 1). Under stress, mitochondria (and cells) might be switched from redox homeostasis to redox-signaling, through a moderate increment in the rate of H_2O_2 production, which is recognized as a signaling molecule in the adjustment of metabolism [24]. These changes (shift in flux-control distribution, redox signaling) may lead to a successful response of the seedling against the environmental stress. A similar behavior has been described during chilling acclimation of maize, cucumber and rice [6,25].

The AOX activity attenuated the ETC-dependent superoxide production. Thus, with an AOX decrease, the C-III inhibition required to accelerate superoxide production also decreased. The above suggests that under stressed conditions AOX plays a preponderant role in limiting superoxide production by competing for electrons from the quinol pool. In consequence, tissues bearing mitochondria with low AOX activity may show proclivity to experience oxidative stress even with a small inhibition of ETC activity. Furthermore, in mitochondria from germinating tissues the enhanced AOX activity results from a change in the monomer/dimer equilibrium toward the isoform with the maximal activity, the monomer [20]. However, such an AOX transition seemed unlikely in maize mesocotyls since AOX activity was insensitive to DTT, indicating that AOX was fully active.

Titration of ETC and AOX with specific inhibitors evidenced two important features of mitochondria from maize mesocotyls. One was the existence of an inhibition threshold to induce an enhanced contribution of the quinol oxidizing pathways to superoxide production. For C-III and C-IV, the inhibition threshold was 30% and 50%, respectively. Based on ETC sigmoidal inhibition curves (see Fig. 1), a threshold concept describing the metabolic effect of enzyme deficiencies has been developed [26]. This concept predicts the amount of enzyme that has to be deleted to bring about a significant perturbation of the metabolic flux. Threshold effects, usually with values above 80% of deleted enzyme, have been observed in mitochondrial-dependent muscle [27] and neuro-degenerative pathologies [28]. The occurrence of a threshold for the superoxide production enhancement indicates the plasticity or buffering capacity of the respiratory enzymes of maize mitochondria to efficiently deal with oxidative stress.

The other important feature of maize seedling mitochondria was that a fraction of the C-III activity was resistant to its specific inhibitor whereas only a fraction of C-IV was inhibited at low concentrations of cyanide (Fig. 1). Since C-III inhibitors bind directly to cytochrome *b* [29], significant changes in its substrate and inhibitor affinity may arise from variations in the primary protein structure. This is the molecular explanation for natural resistance of some protozoa and fungus to inhibitors that bind to cytochrome *b* [29, and references therein]. Titration of C-IV with cyanide expressed in a Dixon plot ([I] versus $1/v$) showed two slopes (data not shown). This suggested the presence of either two components, one of high and another of low affinity for cyanide, or alternatively a single component with different affinity for cyanide depending on the redox state, high affinity in the reduced form and low affinity in the oxidized form [30]. The fraction of C-III resistant to

antimycin and the two possible components of C-IV may also be explained in terms of heterogeneity in the native ETC structure. This might be due to a variation in their primary structure as consequence of nuclear and mitochondrial DNA heterogeneity in maize seedlings [29].

In conclusion, the present results suggest that to reinforce seedling establishment under stress, by avoiding an accelerated mitochondrial superoxide production, is required to maintain high levels of AOX activity.

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