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CARBON MONOXIDE SENSITIVITY OF CYTOCHROME *c* OXIDASE IN MALE STERILE SEEDLINGS OF PEARL MILLET

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Key Word Index—*Pennisetum americanum*; Gramineae; pearl millet; cytoplasmic male sterility; cytochrome oxidase; CO effects; nuclear/cytoplasmic genes.

Abstract—The redox state of cytochrome a_3 during *in situ* respiration of leaves of young seedlings of pearl millet was assessed by *in vivo* aerobic assay of nitrate reductase, after one min exposure to carbon monoxide (CO). Cytoplasmic male sterile (CMS) line 81A derived from Tift 23 DB and its maintainer 81B were found to be completely insensitive to CO, indicating that cytochrome a_3 was in a highly oxidised state during steady-state respiration. Treatment of these leaves with DNP (2,4-dinitrophenol) however, rendered them responsive to CO. The uncoupler probably facilitated electron flow from cytochrome a to a_3. Four other CMS lines and their maintainers, restorers and hybrids were all found to be highly responsive to CO, indicating that cytochrome a_3 was largely reduced. Hybrid MH-179 obtained from CMS 81A and restorer ICMP-451, was found to be highly sensitive to CO, probably because of the influence of the male parent.

INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1), the terminal oxidase in the mitochondrial electron transfer chain, is a complex multiunit enzyme, containing cytochromes a and a_3 and associated CuA and CuB components. Carbon monoxide (CO) is a specific inhibitor of electron transfer to oxygen, since it forms an adduct with cytochrome a_3–CuB (binuclear component of cytochrome oxidase complex) [1–3]. Carbon monoxide reacts with reduced cytochrome a_3 only, and therefore, the steady-state redox condition of this terminal electron carrier influences the degree of CO inhibition, and the former in turn depends on the stoichiometry of cytochrome a_3 and other chain components, the rate of electron transport, as well as on the tightness of the coupling of oxidative phosphorylation. All these parameters are likely to be under the genetic control of both mitochondrial and nuclear genes and their interactions. Naik and Nicholas [4, 5] had shown that inhibition of cytochrome oxidase by CO in wheat leaves, results in the inhibition of oxidation of cellular NADH, which is then used by nitrate reductase to reduce nitrate to nitrite. Thus accumulation of nitrite in leaves under aerobic incubation after a brief exposure to CO is a consequence of the formation of cytochrome a_3 (reduced)–CO complex, which in turn reflects the redox state of cytochrome a_3 *in vivo*. This technique was used by Naik and Nicholas [6] to show that semi-dwarf cultivars of wheat (*Triticum aestivum* L.) containing the dwarfing *Rht* genes, did not respond to CO, while tall cultivars, lacking the *Rht* genes, readily reacted, as assessed by *in vivo* aerobic assay of nitrate reductase, after a brief exposure to CO. These results thus indicated striking differences in the redox state of cytochrome a_3 during *in situ* respiration of leaves from tall and semi-dwarf wheat seedlings which could be under genetic control.

Cytoplasmic male sterility (CMS) is a maternally-inherited defect in many higher plants including maize, sorghum and other millets. Earlier work on the identification of CMS in pearl millet, their sterility maintainers, fertility restorers and hybrids has been reviewed by Burton and Powell [7]. In India, where grain of pearl millet is predominantly used for human consumption, fertility restoration of CMS to produce agronomically desirable hybrids is necessary, unlike in the U.S.A. where it is largely used as a forage crop. Various nuclear/cytoplasmic interactions, including modifications in the genes coding for subunits of cytochrome *c* oxidase and ATPase are likely to be responsible for the expression of CMS character [8, 9]. The oxidation–reduction state of cytochrome a_3 during steady-state *in situ* respiration is also likely to be affected by the interactions of these nuclear/cytoplasmic genes. We have therefore used the CO-sensitivity of cytochrome *c* oxidase, as a probe to assess the steady-state redox condition of cytochrome a_3 in pearl millet seedlings as affected by CMS, maintainer and restorer genes and their interactions.

RESULTS

When cytochrome *c* oxidase is inhibited by CO, it is possible that the oxidation of reducing equivalents by O_2 is still partially functioning via the cyanide–(and CO)–insensitive alternative oxidase pathway, which is inhibited by salicylhydroxamic acid (SHAM). However, Naik and Nicholas [10] had shown that the alternative oxidase is not involved in the regulation of NADH supply for nitrate reduction which is regulated only by inhibition of the cytochrome pathway. This could be due to the fact that the affinity of the alternative oxidase for oxygen is lower than that of cytochrome oxidase as

Table 1. Details of CMS cultivars of pearl millet

CMS	Details	Origin
732A	Derived from the cross between PT819 × PT732	Tamil Nadu Agricultural University, Coimbatore, India
834A	Derived from Serere 10 LA	ICRISAT Hyderabad, India
843A	Selection from Kansas State University line AKM 2068	ICRISAT Hyderabad, India
RHRB-1-A	Derived from a cross between 834B and Manmad local	Mahatma Phule Agricultural University, Rahuri, India
81A	Downy mildew resistant selection from Tift 23 DB	ICRISAT Hyderabad, India

shown by Azcon-Bieto [11]. Since the cytochrome pathway regulates the supply of NADH for nitrate reductase *in vivo*, it can reasonably be concluded that the extent of accumulation of nitrite in leaves during aerobic incubation after a brief exposure to CO, indicates the degree of inhibition of cytochrome oxidase by CO, which in turn is a reflection of the reduction state of cytochrome a_3 *in situ* at the time of contact with CO [6].

Carbon monoxide sensitivity of CMS lines

Details of CMS lines are given in Table 1. The *in vivo* nitrate reductase activity under anaerobic conditions, when oxidation of cellular NADH by oxygen is completely inhibited, represents the maximum activity possible in leaves under these experimental conditions. Out of the five CMS cultivars of pearl millet, four were highly sensitive to CO treatment (Table 2). The extent of cytochrome a_3 reduction as judged by the proportion of nitrite formation under CO-aerobic treatment and *in vivo* assay under anaerobic conditions was more than 90%. The only exception was cultivar 81A derived from Tift 23 DB which was completely insensitive to CO.

Table 2. Response of CMS cultivars of pearl millet to CO treatment

	In vivo nitrate reductase activity (μmol NO_2^- produced/hr/g fr.wt)		
CMS lines	A Anaerobic	B CO-aerobic	C % cytochrome a_3 reduced
732A	3.35	3.18	94.9
834A	0.51	0.63	> 100
843A	2.50	2.49	99.6
RHRB-1-A	1.22	1.10	90.2
81A	0.87	Nil	0

Leaves (0.2 g) of 10-day-old CMS seedlings were used for *in vivo* aerobic assay of nitrate reductase, after 1 min CO exposure (column B) as well as for *in vivo* anaerobic assay (column A). In column C percentage of cytochrome a_3 reduction is calculated as B/A × 100.

Table 3. CO response of leaves of maintainer seedlings

		In vivo nitrate reductase activity (μmol NO_2^- produced/hr/g fr.wt)		
CMS lines	Maintainers	Anaerobic	CO-aerobic	% cytochrome a_3 reduced
732A	732B	3.02	3.02	100
834A	834B	1.62	1.61	100
843A	843B	2.32	2.30	100
RHRB-1A	RHRB-1B	0.95	0.95	100
81A	81B	0.60	Nil	0

Experimental details and calculations of percent cytochrome a_3 reduction are as in Table 2.

CO-sensitivity of maintainer lines

It is interesting to note that the sterility maintainers of the CMS lines (the B types) also showed identical response to CO (Table 3). Thus only 81B derived from Tift 23 DB was completely insensitive to CO while the remaining four responded 100%.

CO-sensitivity of restorers of fertility and hybrids

All the restorers of fertility were found to be responsive to CO (Table 4), the extent of inhibition ranging from 50% in the case of RHRB-795-16 (restorer for 834A and 843A) to 100% in PNBM-83099 (restorer for 732A) and ICMP-451 (restorer for 81A). All the five hybrids obtained by crossing the CMS lines with the restorers were highly sensitive to CO, ranging from 81 to 100% (Table 4). CO-insensitive CMS line 81A when crossed with the highly CO-sensitive restorer, ICMP-451, produced a hybrid, MH-179, which readily responded to CO.

Effect of uncoupler

The seedlings of two cultivars insensitive to CO, 81A and 81B, were allowed to absorb DNP before CO treatment. Both of them responded to CO exposure after absorbing the uncoupler (Table 5). In the absence of CO treatment, nitrite formation was not detected under aerobic conditions in these leaves. Naik and Nicholas [5] had similarly reported that wheat cultivars containing the

Table 4. CO response of leaves of seedlings of restorers and hybrids

CMS lines	Restorers: Name	Restorers: *In vivo* nitrate reductase activity (μmol NO_2^- produced/hr/g fr.wt) Anaerobic	Restorers: CO-aerobic	Restorers: % cytochrome a_3 reduced	Hybrids: Name	Hybrids: *In vivo* nitrate reductase activity (μmol NO_2^- produced/hr/g fr.wt) Anaerobic	Hybrids: CO-aerobic	Hybrids: % cytochrome a_3 reduced
732A	PNBM-83099	0.78	0.78	100.0	MH-182	0.88	0.88	100.0
834A	RHRB-795-16	0.60	0.30	50.0	RHRBH-8601	1.60	1.61	100.0
843A	RHRB-795-16	0.60	0.30	50.0	RHRBH-8602	1.32	1.32	100.0
843A	F_2S/86138-1	0.57	0.46	80.8	RHRBH-8605	2.10	2.10	100.0
RHRB-1A	F_2S/86138-1	0.57	0.46	80.8	RHRBH-8609	1.10	0.89	80.9
81A	ICMP-451	1.36	1.36	100.0	MH-179	0.57	0.48	84.2

Table 5. Effect of DNP on CO sensitivity of CMS 81A and its maintainer, 81B

DNP, mM	81A: *In vivo* nitrate reductase activity (μmol NO_2^- produced/hr/g fr.wt.) Anaerobic	81A: CO-aerobic	81A: % cytochrome a_3 reduced	81B: *In vivo* nitrate reductase activity (μmol NO_2^- produced/hr/g fr.wt.) Anaerobic	81B: CO-aerobic	81B: % cytochrome a_3 reduced
1	0.72	0.06	8.33	0.70	0.14	20.00
2	0.72	0.09	12.50	—	—	—
3	0.72	0.11	15.28	0.70	0.30	42.86

Ten-day-old seedlings of these two lines were allowed to absorb DNP as described in Experimental. Leaves of these seedlings were then examined for CO-aerobic and anaerobic *in vivo* nitrate reductase activity and percent cytochrome a_3 reduction was calculated as in Table 2.

Rht dwarfing genes, which were completely insensitive to CO, also responded to CO after they had absorbed uncouplers of oxidative phosphorylation in the leaves.

DISCUSSION

The biochemical and molecular basis for the observed dramatic differences in the redox states of cytochrome a_3 during *in situ* respiration in different CMS lines (Table 2) is unknown. Cytochrome a_3 is reduced by accepting electrons from cytochrome a and is subsequently oxidized by transferring these electrons to oxygen. During steady-state, difference between the rates of these two reactions would determine the extent of reduction of cytochrome a_3 and hence its capacity to complex with CO. Ducet [12] has shown that in potato mitochondria during anaerobiosis cytochrome a_3 remained oxidized while cytochrome a was reduced, indicating that electron transfer was inhibited between cytochromes a and a_3, namely site 3 of oxidative phosphorylation. On treatment of the mitochondria with uncouplers, cytochrome a_3 was reduced, because electron flow from a to a_3 was facilitated [12]. CMS 81A and its maintainer of sterility, 81B, which were completely insensitive to CO responded to the gas when treated with DNP (Table 5). It therefore, appears that tightness of coupling at the site of oxidative phosphorylation in the cytochrome oxidase complex determines the rate of flow of electrons from a to a_3 and influences the reduction state of a_3. Stitt *et al.* [13] have shown that mitochondrial energization is diminished by uncouplers and under these conditions cytochrome a_3 is likely to be more reduced compared with its redox state during electron transfer to oxygen in tightly coupled mitochondria. Thus the redox state of cytochrome a_3 is regulated by the rate of electron transport as well as by the tightness of coupling of oxidative phosphorylation and these factors may well vary between CMS cultivars as they are genetically determined and may explain the differential response to CO.

It is well known that nuclear and mitochondrial genes coordinate the synthesis of cytochrome oxidase and other respiratory enzymes which are assembled in the mitochondria [14]. Cytochrome oxidase is a complex of seven subunits, four synthesized on the cytoplasmic ribosomes coded by nuclear genes and three on mitochondrial ribosomes [9]. Fox and Leaver [15] identified the mitochondrial gene coding for cytochrome oxidase subunit II in maize. Musgrave *et al.* [16] showed that the majority of CMS lines, but not all, analysed by them lacked the cyanide-resistant alternative oxidase pathway. However, they did not study the tightness of coupling of oxidative phosphorylation via the cytochrome pathway. Watson *et al.* [17] reported that mitochondria from sterile anthers from CMS of maize showed deficiency of cytochrome oxidase and fewer isoenzyme bands as compared with mitochondria from fertile cytoplasm.

A report on a recent conference on the molecular basis of CMS in plants [18] concluded that certain proteins

located in the inner mitochondrial membrane cause some mitochondrial dysfunction that becomes limiting at a critical step in microsporogenesis. In various CMS lines different factors may be operating for the expression of CMS characters. It is not clear what factors regulate the redox state of cytochrome a_3 *in situ*.

MH-179, the hybrid obtained from the female parent CMS 81A, was found to be highly responsive to CO, probably because the male parent (ICMP-451) was 100% sensitive to CO (Table 4). This suggests that the electron transfer via cytochrome oxidase and the tightness of coupling are influenced to a large extent by the nuclear genes. In order to achieve a better understanding of the relative contributions of mitochondrial and nuclear genes in the regulation of redox state of cytochrome a_3, it appears necessary to obtain reciprocal crosses between the CO-insensitive maintainer 81B and other CO-sensitive CMS lines and also between other maintainers and CMS 81A.

EXPERIMENTAL

Plant materials: CMS lines of pearl millet (*Pennisetum americanum*), their maintainers, restorers and hybrids were obtained from the collections maintained at this University. Seedlings were grown in small pots in medium black soil in normal sunlight. After germination, the seedlings were daily irrigated with 15 mM KNO_3, so that sufficient nitrate accumulated in the leaves. Leaves from 10-day old seedlings were used for various experiments.

In vivo *nitrate reductase assay under aerobic conditions*. Whole leaves were cut into 2.5 cm fragments and *ca* 0.2 g of leaf material was placed in open tubes (1.5 × 15 cm) in the absence of aq. soln. The tubes were covered with a black plastic to eliminate light, because cytochrome *c* oxidase–CO complex is photolabile [19]. High purity CO from a cylinder obtained from Indian Oxygen Limited, Bombay was sprayed through the open tubes for 1 min. The tubes, which were open to the air, were then incubated in the dark at 30° for 45 min. The reaction was terminated by adding 5 ml H_2O at 100° to the leaves. In order to extract all the nitrite from the leaves, the tubes were kept at 100° for 10 min. Nitrite was then determined in suitable aliquots as described in ref. [19].

In vivo *nitrate reductase assay under anaerobic conditions*. About 0.2 g leaf segments (2.5 cm) were added to glass tubes (2 × 15 cm) containing 5 ml soln of 0.1 M Na-Pi buffer (pH 7.5), and *n*-propanol (4%). The tubes were placed in a vacuum desiccator and covered with black plastic. After rigorous evacuation to remove the last traces of air, the desiccator was closed. After incubation at 30° for 45 min in the dark, the desiccator was opened and the tubes were heated at 100° for 10 min. Nitrite formed was then determined in suitable aliquots as described above.

Absorption of DNP. Ten-day-old seedlings were excised just above the soil level and placed with their bases immersed in liquid in vials containing 2 ml aq. solns of different concns of DNP. The vials were placed in a vacuum desiccator and air was completely removed. The uncoupler was allowed to be absorbed by seedlings by vacuum infiltration for 10 min. At the end of this period, the desiccator was opened and leaves of the seedlings were tested for CO-sensitivity by the aerobic *in vivo* assay of nitrate reductase as described above.

Standard error. In various replicated experiments, s.e. varied from 5.75 to 16.01% with an average of 11.53%.

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