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

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Key Word Index—*Sorghum bicolor*, Gramineae, CMS; cytochrome a_3, CO effects, nuclear/mitochondrial genes.

Abstract—In order to study the possible role of mitochondrial and nuclear genes in the regulation of redox condition of cytochrome a_3 during steady-state respiration, a large number of cytoplasmic male sterile (CMS) lines of sorghum, their maintainers and hybrids were examined for carbon monoxide (CO) sensitivity. Differences in the redox state of cytochrome a_3 were monitored by using the *in vivo* aerobic assay of nitrate reductase after one min exposure to CO. CMS lines obtained from A_1, A_2, A_3 and A_4 cytoplasms as well as their maintainers, BT × -398, 365B and 42B were found to be sensitive to CO, indicating that cytochrome a_3 was in a considerably reduced state during steady-state respiration A number of other indigenous CMS lines derived from Maldandi and other sources as well as their respective maintainers did not, however, react with CO Restorers of fertility, CS-3541 and SPV-475, responded to CO. Hybrids obtained from these restorers, when crossed with CO-insensitive CMS lines, 2219A and 296A, also readily responded to the gas Similarly CMS 365A derived from CO-resistant CMS 2219A was CO-sensitive because the maintainer 365B was CO-sensitive. Conversely CMS RSB-18A inherited CO-insensitivity from its male parent, RSB-18B; although the maternal parent CMS 2077A was CO-responsive. These results indicated that nuclear genes, contributed by the male parent, influence the redox state of cytochrome a_3 during steady-state respiration.

INTRODUCTION

Carbon monoxide sensitivity of cytochrome *c* oxidase was earlier used as a probe to assess the steady-state redox condition of cytochrome a_3 in seedlings of wheat [1] and pearl millet [2], during steady-state respiration and significant genetic differences were observed in different genotypes. We have now used this technique to examine genetic differences in the redox state of cytochrome a_3 in a number of CMS lines, maintainers and hybrids of sorghum. It was earlier suggested that CMS in sorghum can be linked to alterations in the mitochondrial genome as revealed by restriction endonuclease analysis of DNA and *in vitro* synthesis of ^{35}S-methionine-labelled polypeptides [3-6]. However, the relationship if any between these observed differences and the expression of CMS character is not clearly understood. Various nuclear/cytoplasmic interactions are likely to be involved. As far as the redox condition of cytochrome a_3 during steady-state respiration is concerned, we now suggest that this character is regulated by the nuclear genes contributed by the male parent.

RESULTS

CO-*sensitivity of different sorghum seedlings*

Details of sorghum CMS lines (A types), and their maintainers (B type) and also of restorers (R) of fertility and hybrids (H) are given in Table 1. Dramatic differences were observed in different CMS lines in their responses to CO. CMS lines and their maintainers which readily reacted with CO are listed in Table 2 while those which were completely resistant to the gas are listed in Table 3. It is interesting to note that CMS lines and their respective maintainers of sterility show identical responses to CO. Thus maintainers, viz., BTx-398, 365B and 42B were CO-sensitive. When these were used as male parents as maintainers of sterility in CMS line A_1 (milo), A_2 (IS-12662C), A_3 (IS-1112C) and A_4(IS-7920C) derived from different cytoplasms, all these CMS lines were also sensitive to CO (Table 2). On the other hand, a number of indigenous CMS lines derived from Maldandi and other local cytoplasms along with their respective maintainers were completely insensitive to CO (Table 4). The two maintainers, IS-1015 and IS-2014 of the recently discovered CMS line Jeur 2, were completely insensitive to CO, and so was CMS line Jeur 2 in these two nuclear background. RSB-18A, a local CMS line derived from CO-sensitive 2077A as a female parent by crossing with the CO-insensitive maintainer RSB-18B, was insensitive to CO (Tables 2 and 3). It therefore appears that RSB-18A inherited CO-insensitivity from the male parent, RSB-18B. CMS 365A derived from CO-insensitive CMS line 2219A inherited CO-sensitivity from the maintainer 365B (Table 2).

Restorers of fertility and hybrids sensitive to CO are listed in Table 4. Two restorers of fertility, viz., CS-3541 and SPV-475, were responsive to CO. It is interesting to note that when these were crossed with CO-resistant CMS lines 296A and 2219A, the resultant hybrids inherited the CO-sensitivity from their male parent.

Effect of uncoupler

Some of the CMS lines and their maintainers which were insensitive to CO were allowed to absorb 2,4-

Table 1 Details of CMS lines (A type), their maintainers (B type), restorers (R) and hybrids (H) of sorghum

CMS lines (A), maintainers (B), restorers (R), hybrids (H)	Details	Reference
A_1	Milo cytoplasm from Texas, U S A	[7]
A_2	Isolated from IS12662C, a race caudatum type from Ethiopia, Texas, U S A	[8]
A_3	Isolated from IS1112C or converted Nilwa, a race durra (*Durra bicolor*) type from India-Texas, U S A	[9]
A_4	Female with cytoplasm from IS7920C, a race guinea type from Nigeria, Texas, U S A	[10]
BT × -398	Common maintainer for A_1, A_2, A_3 and A_4 obtained from kafir Texas, U S A	[11]
365A	2219A × 365B from Agril University, Rahuri, India	
365B	Maintainer for 365A and A_1, A_2, A_3 and A_4, from Agril. University, Rahuri, India	
42A	CK60A × 42B from Agril University, Rahuri India	
42B	Maintainer for 42A and A_1, A_2, A_3 and A_4 1258B × SPV42 derivative from Agril University, Rahuri, India	
CK60A	Milo cytoplasm originally from U S A	[12]
CK60B	Maintainer for CK60A, combined kafir type Texas, originally from U S A	[12]
2077A	IS-2077 yellow endosperm from Sorghum Improvement Project, Hyderabad, India	
2077B		
2219A	IS2219 yellow endosperm type (6323, Nebrasca, U S A, Welaster) from Sorghum Improvement Project, Hyderabad, India	
2219B		
296A	IS-3922 × Karad Local from Sorghum Improvement Project, Hyderabad, India	
296B		
RSB-18A	2077A × RSB18B from Agril University, Rahuri, India	
RSB-18B	SPV1 × SPV303 derivative from Agril University, Rahuri, India	
Jeur-2A × 2014	CMS cytoplasm from local cultivar (Bedari) from Agril University, Rahuri India	
Jeur-2A × 1015	CMS cytoplasm from local cultivar (Bedari) from Agril University, Rahuri, India	
IS-2014	SA 8293 2 (U S A), maintainer for Jeur 2A from U S A	
IS-1015	Sirsa, Punjab, India, maintainer for Jeur 2A	
VZM-2A	Unidentified cytoplasmic source from Vizianagaram, Andhra Pradesh, India	[12]
VZM-2B		
G-1A	Unidentified cytoplasmic source from Guntur, Andhra Pradesh, India	[12]
G-1B		
M-35-1A	Unidentified cytoplasmic source from Maharashtra, India	[12]
M-35-1B		
M-31-2A	Unidentified cytoplasmic source from Raichur, Karnataka, India	[12]
M-31-2B		
AKMS-3A	148 × IS11167 derivative from Akola, Maharashtra, India	
AKMS-3B		
SPV-346 (R)	SB-1066 × CS-3541	
SPV-472 (R)	(SPV-35 × E 35-1) × (CS-3541-8-1) (ICRISAT)	
SPV-475 (R)	(IS-12622 C × 555) × (IS-3612 C × E 35-1-52) (ICRISAT)	
SSV-84 (R)	PAB-84, Zera-Zera, Ethiopia	
SSV-7073 (R)	SBI-100-(Sudan)	
CS-3541 (R)	IS-3675 × IS-3541-Hybrid derivative of IS-3675 × IS-3541 (SA 887 × Nyithin)	
No 168 (R)	IS-368 × Aispuri (India) (CSV-5)	
IS-84 (R)	Selection from yellow endosperm Felarita of hybrid origin U S A (SA-7529)	
Swarna	Selection 3924 (India) (CSV-1)	
Hybrids (H)		
2077A × CS-3541		
2219A × CS-3541		
296A × CS-3541		
365A × CS-3541		
2077A × SPV-475		
296A × SPV-475		
42A × No 168		

Table 2. CMS lines and their maintainers responsive to CO treatment

CMS lines	Maintainers	*In vivo* nitrate reductase activity (μmol NO_2^- produced/hr/g fr. wt.)	
		Anaerobic	CO-aerobic
A_1 × BT × -398	—	1.55	0.62
A_1 × 365B	—	1 78	0.90
A_1 × 42B	—	2.21	1 00
A_2 × BT × -398	—	1.32	0 91
A_2 × 365B	—	2.86	0.75
A_2 × 42B	—	2.36	1.06
A_3 × BT × -398	—	2.55	1.18
A_3 × 365B	—	3.08	0.99
A_3 × 42B	—	2.27	0.51
A_4 × BT × -398	—	1 93	1.37
A_4 × 365B	—	1 63	0.28
A_4 × 42B	—	2.34	0.79
—	BT × -398	1.05	0.62
365A × 365B	—	1.66	0.71
—	365B	2 09	1.19
42A × 42B	—	1.59	0.48
—	42B	1 42	0.34
CK60A × CK60B	—	1.81	1.81
—	CK60B	1.32	0.70
VZM-2A × VZM-2B	—	1.44	0.67
—	VZM-2B	1.29	0.52
AKMS-3A × AKMS-3B	—	1.53	0.53
—	AKMS-3B	1.82	1.06
2077A × 2077B	—	1.65	0.41
—	2077B	0.99	0.22

Leaves (0.2 g) of 10-day-old sorghum seedlings were used for *in vivo* aerobic assay of nitrate reductase after one min exposure to CO as well as for *in vivo* anaerobic assay as described in Experimental.

Table 3. CMS lines and their maintainers non-responsive to CO treatment

CMS Lines	Maintainers	*In vivo* nitrate reductase activity (μmol NO_2^- produced/hr/g fr.wt)
		Anaerobic
2219A × 2219B	—	3 30
—	2219B	3.06
296A × 296B	—	1.37
—	296B	0.89
RSB-18A × RSB-18B	—	0.81
—	RSB-18B	0.37
Jeur 2 × IS-2014	—	0.60
—	IS-2014	1.47
Jeur 2 × IS-1015	—	0.53
—	IS-1015	1.92
G-1A × G-1B	—	1.86
—	G-1B	1 56
M-35-1A × M-35-1B	—	2.14
—	M-35-1B	2.16
M-31-2A × M-31-2B	—	1.63
—	M-31-2B	1.48

Experimental details as in Table 2. *In vivo* nitrate reductase activity under CO—aerobic conditions was 'Nil' in all these genotypes.

Table 4 Response of restorers of fertility and hybrids of sorghum to CO treatment

Restorers, hybrids	*In vivo* nitrate reductase activity (μmol NO_2^- produced/hr/g fr. wt)	
	Anaerobic	CO-aerobic
Restorers		
SPV-346	1 60	1 04
SPV-472	0.96	0 35
SPV-475	1 47	0 98
SPV-84	1 53	1 32
SSV-7073	1.27	0 81
CS-3541	3 90	2 82
No 168	3 94	1 26
IS-84	3 26	1 12
Swarna, IS-3691	2 76	2 24
Hybrids		
2077A × CS-3541	1 40	0 46
2219A × CS-3541	1.25	0 28
296A × CS-3541	1 49	0 23
2077A × SPV-475	1 39	0 21
296A × SPV-475	1 20	0 18
365A × CS-3541	1.48	0.68

Experimental details as in Table 2

dinitrophenol (DNP) before treatment. The uncoupler promoted a ready response to CO in these seedlings (Table 5) It was earlier similarly reported that wheat [1] and pearl millet [2] seedlings which were completely insensitive to CO also responded to the gas 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 (Tables 2 and 3) 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 A large number of seedlings which were completely insensitive to CO responded to the gas when treated with DNP (Table 5) As suggested by Ducet [13] and Stitt *et al.* [14], 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 Pring *et al* [4] classified A_1, A_2, A_3 and A_4 cytoplasms in different groups on the basis of restriction endonuclease patterns, but as far as CO response is concerned, all these along with their maintainers belonged to one group (Table 2)

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 [15, 16] All the CMS lines and their respective maintainers showed identical responses to CO (Tables 2 and 3) This could be due to the influence of nuclear genes contributed by the maintainers as indicated by the facts that CMS line RSB-18A inherited CO-insensitivity from its male parent, RSB-18B and conversely CMS 365A derived CO-sensitivity from its maintainer 365B (Tables 2 and 3). Similarly results in Table 4 showed that hybrids obtained from two restorers of fertility, namely, CS-3541 and SPV-475, when crossed with CO-insensitive

Table 5. Effect of DNP on CO sensitivity of CMS lines and maintainers insensitive to CO

CMS lines	Maintainers	DNP concentration, mM	*In vivo* nitrate reductase activity (μmol NO_2^- produced/hr/g fr wt)
			CO-aerobic
2219A × 2219B	—	3	0 44
RSB-18A × RSB-18B	—	1	0 27
Jeur-2 × IS-2014	—	3	0.58
Jeur-2 × IS-1015	—	3	0 42
G-1A × G-1B	—	1	0 31
—	G-1B	1	0 45
		2	0 91
		3	1 55
M-35-1A × M-35-1B	—	1	0 31
		2	0 91
		3	1 00
—	M-31-2B	1	0 27
		2	0 32
		3	1 00

Ten-day-old seedlings of CMS lines and maintainers were allowed to absorb DNP as described in Experimental. Leaves of these seedlings were then examined for CO-aerobic *in vivo* nitrate reductase activity. *In vivo* nitrate reductase activity under anaerobic conditions of these genotypes is shown in Table 3

CMS lines, 2219A and 296A, were readily responsive to CO. Since these two restorers were CO-sensitive, it appears that nuclear genes from the male parent do influence the redox state of cytochrome a_3. The differential CO response of various CMS lines may, therefore, be a result of the influence of the maintainer male parents. In the case of pearl millet also [2] it was similarly observed that the male parent, ICMP-451, was probably involved in the observed CO-sensitivity of the hybrid, MH-179, obtained from CMS 81A which was completely insensitive to CO The possible role of nuclear genes in the regulation of mitochondrial respiratory activity is not yet clearly understood. That the nuclear context can be important also in the expression of CMS trait appears to be evident from studies with sorghum reported in a recent conference [17]. A variant cytochrome oxidase subunit I gene was identified by Bailey-Serres in a CMS line of sorghum which resulted in the synthesis of a long form of the protein The precise relationship if any of this large cytochrome oxidase subunit I polypeptide to CMS is not yet known. In different CMS lines, different factors, nuclear or cytoplasmic, may be operating for the expression of CMS character However, as far as the redox condition of cytochrome a_3 during steady-state respiration is concerned, it appears that the nuclear genes have an important influence.

EXPERIMENTAL

Plant materials. CMS lines of sorghum [*Sorghum bicolor* (L.) Moench], 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 irrigated daily with 15 mM KNO_3, so that sufficient nitrate accumulated in the leaves. Leaves from 10-day-old seedlings were used for various experiments.

CO-aerobic assay of nitrate reductase 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 [18]. High purity CO from a cylinder obtained from Indian Oxygen Limited, Bombay was sparged 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. [18].

In vivo *nitrate reductase assay under anaerobic conditions Ca* 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 which was covered with a black plastic After rigorous evacuation with a vacuum pump 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 concentrations 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. was calculated, the average s e being 8 84%.

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REFERENCES

1. Naik, M S. and Nicholas, D J D. (1986) *Theor Appl. Genet* **72**, 735.
2. Munjal, S. V., Desai, B. B, Ugale, S. D, Daftardar, S. Y., Bapat, D. R. and Naik, M. S. (1988) *Phytochemistry* **27** (in press).
3. Lonsdale, D. M. (1984) *Plant Mol. Biol.* **3**, 201.
4. Pring, D. R., Conde, M. F. and Schertz, K. F (1982) *Crop Sci.* **22**, 414.
5 Chase, C D. and Pring, D. R (1986) *Plant Mol. Biol.* **6**, 53.
6. Dixon, L. K. and Leaver, C. J. (1982) *Plant Mol Biol.* **1**, 89.
7 Stephens, J C and Holland, P. F. (1954) *Agron. J.* **46**, 20.
8. Schertz, K. F. (1977) *Crop Sci.* **17**, 983.
9. Quinby, J. R. (1980) in *Proceedings 35th Corn and Sorghum Research Conference*, American Seed Trade Association, Chicago, Illinois, U.S.A.
10 Worstell, J V., Kidd, H J and Schertz, K. F. (1984) *Crop Sci.* **24**, 186
11. Schertz, K. F. and Pring, D. R. (1982) Cytoplasmic sterility systems in sorghum In *Sorghum in the Eighties* **1**, 373. ICRISAT, Hyderabad, India
12. Tripathi, D. P., Mehta, S L., Rana, B. S. and Rao, N.G.P. (1980) *Sorghum Newsletter* **23**, 107.
13. Ducet, G (1985) in *Higher Plant Cell Respiration, Encyclopaedia of Plant Physiology, New Series* (Douce, R. and Day, D A., eds) Vol 18, p 72 Springer, Berlin
14. Stitt, M., McLilley, R and Heldt, H. W. (1982) *Plant Physiol.* **70**, 971.
15. Butow, R A and Strausberg, R. L. (1979) *Trends Biochem. Sci* **4**, 110
16 McDaniel, R. G. (1986) *CRC Critical Rev. Plant Sci.* **4**, 227.
17 Butow, R. A. (1986) *Nature* **324**, 620.
18. Sawhney, S K , Naik, M S. and Nicholas, D. J. D. (1978) *Nature* **272**, 647