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Cytoplasmic-nuclear male sterility in pearl millet: comparative RFLP and transcript analyses of isonuclear male-sterile lines

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Abstract The identification of diagnostic cytoplasmic molecular markers is of prime interest to pearl millet breeders wishing to identify sources of cytoplasmic-nuclear male sterility (CMS) which can be used as an alternative to the single source currently used in the production of F_1 hybrid seed. Here, we report the classification of five pearl millet CMS sources based on RFLP analysis of isonuclear lines carried out using mitochondrial gene-specific DNA probes in combination with eight restriction endonucleases. On the basis of RFLP data, the five CMS cytoplasms can be distinguished from each other and from the isonuclear fertile cytoplasm. In addition, based on *cox1*, *cox3*, *atp6* and *atp9* polymorphisms, these lines can be classified into two major groups: one corresponds to A_5, A_{egp}, A_v and A_1 cytoplasms, and the other consists of the A_4 cytoplasm. Our results suggest that a rearrangement involving the *cox1* gene might be related to CMS in the first group (A_5, A_{egp}, A_v and A_1), whereas a rearrangement within the *atp6*/*cox3* cluster region might be related to CMS in the second group (A_4).

Key words Cytoplasmic-nuclear male sterility · Pearl millet · *Pennisetum glaucum* · RFLP

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Introduction

The ability to produce commercial hybrid seed has been of fundamental importance to modern agricultural practice for raising yields. However, the crossing of one plant with another is difficult in many agriculturally important crop species due to the intimate association of male and female reproductive organs in the same flower. To prevent self-fertilization, the pollen-producing organs, the anthers, must be removed mechanically or by hand unless the plant fails to produce pollen as the result of a genetic defect. A genetic approach to the production of F_1 hybrid seed was made possible by the exploitation of cytoplasmic male sterility (CMS). CMS is a maternally inherited phenotype characterized by an inability to produce viable pollen, while female fertility and vegetative development are unaffected. The widespread use of the CMS system in plant breeding programmes, while being cost-effective, is not without its drawbacks. Dependence on a single source of CMS in hybrid seed production has the inevitable consequence of conferring "cytoplasmic uniformity" in the hybrid. The genetic vulnerability associated with cytoplasmic uniformity has already been exemplified by the use of T-cytoplasm maize in the early 1970s, which led to the disastrous epidemic caused by the fungal pathogen, *Bipolaris maydis* (Levings 1990).

The exploitation of a single source of CMS (A_1 cytoplasm) in the production of commercial F_1 hybrids of pearl millet [*Pennisetum glaucum* (L.) R. Br.] predisposes this crop to potential devastation by disease and insect pest epidemics. Downy mildew, caused by *Sclerospora graminicola* (Sacc.) J. Schröt, is responsible for massive yield losses in pearl millet. To date, this cytoplasm has not been found to be associated with susceptibility to downy mildew (Yadav et al. 1993; Yadav 1996). Nor has this cytoplasm been found per se to be associated with the susceptibility to ergot (*Claviceps*

fusiformis Loveless) (Rai and Thakur 1995) and smut (*Tolyposporium penicillariae* Bref.) (Rai and Thakur 1996), the two important panicle diseases of pearl millet. However, there is a need to diversify the cytoplasmic bases of hybrids to reduce the potential hazards of vulnerability and also provide opportunities for greater genetic diversity of the male-sterile lines and their hybrids.

Although CMS can appear spontaneously, it is often produced in interspecific or intraspecific crosses that introduce a nucleus into an incompatible cytoplasm. The fact that male sterility appears as a result of an incompatibility between a particular cytoplasm and the nucleus suggests that it is critical to identify cytoplasm-specific variation independent of nuclear background. In many species, the CMS phenotype is associated with mutations in the mitochondrial genome (Hanson 1991), and rearranged mitochondrial genes are frequently co-transcribed with standard mitochondrial genes (Dewey et al. 1986; Laver et al. 1991; Pruit and Hanson 1991; Bonhomme et al. 1992; Chase 1994). In this study, we have carried out restriction fragment length polymorphism (RFLP) analysis using ten mitochondrial gene-specific DNA probes in combination with eight restriction endonucleases in order to characterize cytoplasmic diversity among five pearl millet isonuclear CMS lines as compared to the isonuclear fertile cytoplasm. We have investigated whether any polymorphism observed at the DNA level is correlated with gene-specific transcript variation. In addition, we report the classification of cytoplasms based on RFLP and transcript analyses. Our results also bring new insight into the rearrangement of mitochondrial genes which might potentially be related to the molecular basis of CMS.

Materials and methods

Plant material and mtDNA probes

The five near-isonuclear male-sterile lines (hereafter refered to as isonuclear A-lines) were developed by more than seven generations of backcrossing of the nuclear genome of 81B into five CMS sources (Table 1). The $81A_1$ derives its cytoplasm from Tift$23A_1$; $81A_v$ (ICMA88001) and $81A_4$ derive their cytoplasms from two different accessions of a wild relative of pearl millet, *P. Glaucum* subsp. *violaceum* (= *monodii*) from Senegal; and $81A_{egp}$ and $81A_5$ derive their cytoplasms from male-sterile segregants identified in two different gene pools (early gene pool = EGP and large-seeded gene pool = LSGP) constituted at ICRISAT Asia Center. Based on differential fertility restoration patterns, the $81A_1$, $81A_v$ and $81A_4$ CMS lines are different from each other (Rai et al. 1996), and the $81A_5$ line is distinct from the $81A_4$ line (Rai 1995). Unpublished data from Rai et al. also suggest that $81A_{egp}$ is different from the $81A_4$ and $81A_5$ lines. The maintainer line 81B (nuclear genome of all isonuclear A-lines) and two restored hybrids (81A1 × H77/833-2 designated as HA1 and 81A4 × ICMP85410-3 designated as HA4) were also included in the analysis. Ten specific mtDNA probes (see Table 2) corresponding to conserved coding regions were used in hybridization experiments.

Isolation and analysis of nucleic acids

RFLP analysis was performed using total DNA as its preparation is quicker and much simpler than mtDNA preparation and can be performed on much less plant material. Total DNA was isolated from frozen leaves of 2-week-old seedlings using the SDS method described by Draper et al. (1988). Restriction endonuclease-digested DNA was separated by electrophoresis on 0.8% (w/v) agarose gel and transfered to nylon filters (Hybond N, Amersham) in 20 × SSC (3 *M* sodium chloride, 0.3 *M* trisodium citrate, pH 7.0 with HCl).

Total RNA was isolated from leaves of 2-week-old seedlings using the following guanidinium extraction buffer: 8 *M* guanidinium chloride, 25 m*M* MOPS (pH 7.0), 20 m*M* EDTA, 50 m*M* 2-mercaptoethanol. Samples were phenol/chloroform extracted until no protein interface was visible and precipitated by adding 0.2 vol. 1 *M* acetic acid and 0.7 vol. absolute ethanol. The RNA pellet was washed twice with 3 *M* sodium acetate pH 5.5, once with 70% (v/v) ethanol and resuspended in sterile ultra-pure water. RNA was separated on 1.2% (w/v) agarose formaldehyde gels and transferred onto Hybond N filters (Amersham) in 10 × SSC. DNA probes were prepared using a random primed DNA labeling method (Draper et al. 1988). Hybridizations were carried out under standard conditions (Sambrook et al. 1989), and filters were washed at high stringency (0.1 × SSC, 0.1% (w/v) SDS, 50°C).

Analysis of data

The presence of specific restriction fragments on autoradiograms was scored in digital form for all lines and all fragment positions

Table 1 Isonuclear A-lines of pearl millet

Cytoplasm	Description	Reference
$81A_1$	Cytoplasm from Tift $23A_1$, in nuclear background of 81B	Anand Kumar et al. 1984
$81A_v$ = ICMA88001	Cytoplasm from *P. glaucum* subsp. *violaceum*, in nuclear background of 81B	Rai et al. 1996
$81A_4$	Cytoplasm from *P. glaucum* subsp. *violaceum*, (=*monodii*) on nuclear background of 81B	Hanna 1989
81 A_{egp}	EGP 261 cytoplasm in nuclear background of 81B	Sujata et al. 1994
81 A_5	LSGP66 cytoplasm in nuclear background of 81B	Rai 1995

Table 2 Mitochondrial DNA probes used in analysis of pearl millet CMS lines

Probe		Origin	Restriction sites	Position (bp)	Reference
Cytochrome oxidase subunit 1	*cox1*	*Zea mays*	*Eco*RV/*Hae*III	427–1617	Isaac et al. 1985
Cytochrome oxidase subunit 2 exon 2	*cox2*	*Zea mays*	*Sau*3A/*Hin*dIII	1361–1712	Fox and Leaver 1981
Cytochrome oxidase subunit 3	*cox3*	*Zea mays*	*Sau*3A/*Sau*3A	880–1152	Muise and Hauswirth 1992
Cytochrome c reductase	*cob*	*Zea mays*	*Hin*dIII/*Eco*RI	642–1322	Muise and Hauswirth 1992
F1-ATPase subunit 1	*atp1*	*Zea mays*	*Sal*I/*Bam*HI	390–782	Muise and Hauswirth 1992
F0-ATPase subunit 6	*atp6*	*Zea mays*	*Eco*RI/*Hin*dIII	515–1559	Dewey et al. 1985
F0-ATPase subunit 9	*atp9*	*Sorghum bicolor*	*Eco*RI/*Bam*HI	251 bp	Salazar et al. 1991
NADH dehydrogenase subunit 2 exon b	*nad2*	*Oenothera berteriana*	*Eco*RI/*Eco*RV	1861–2091	Binder et al. 1992
NADH dehydrogenase subunit 9	*nad9*	*Arabidopsis thaliana*	*Hin*dIII/*Hin*dIII	2388–3187	Gift from Axel Brennicke
Ribsomal protein	*rps13*	*Oenothera berteriana*	*Eco*RI-*Hin*dIII	670 bp	Wissinger et al. 1990

checked. The data were analysed using the R package (Vaudor, University of Montréal, Quebec). The pairwise similitude coefficient (F-values) and the distance between all individual lines were calculated using the shared fragments method of Nei and Li (1979); the F-value being calculated from the RFLP data using $F(x,y) = 2n_{xy}/[n_x + n_y]$, where n_x was the number of DNA fragments in one cytoplasmic type, n_y the number of DNA fragments in a second cytoplasmic type and n_{xy} the number of fragments shared between the two cytoplasmic types. The dendrograms were obtained using the Unweighted Pair-Group Method Analysis (UPGMA) on matrices of distance values.

Results and discussion

A summary of the diagnostic combinations of probe and restriction enzyme and descriptions of the different RFLP patterns obtained are given in Table 3. No polymorphisms were detected among the cytoplasms using the *cox2*, *atp1*, *nad9* probes or 9 other probe and restriction enzyme combinations (*cox3* × *Bgl*II, *cob* × *Eco*RI, *Eco*RV or *Sst*I, *rps13* × *Bgl*II, *nad2* × *Eco*RI, *Bam*HI, *Hin*dIII or *Bgl*II) (data not shown). Cluster analysis was done on diagnostic RFLP data presented in the Table 3, and the dendrogram generated for CMS lines is presented in Fig. 1. As shown in the dendrogram, we have distinguished all millet CMS cytoplasms from each other and from the isonuclear fertile cytoplasm. In addition, based on *cox1*, *cox3*, *atp6* and *atp9* polymorphisms and potential rearrangement of mitochondrial genes, we have classified the millet cytoplasms into two major groups: one corresponds to A_1, A_v, A_5 and A_{egp} cytoplasms, and the other consists of the A_4 cytoplasm.

A_1, A_v, A_5 and A_{egp} cytoplasmic group

The *cox1* probe in combination with different restriction endonucleases allowed us to distinguish the A_5, A_{egp}, A_v, A_1 and A_4 cytoplasms from each other. The A_4 cytoplasm displayed the same RFLP pattern as the fertile maintainer line, 81B, which differed from the A_5, A_{egp}, A_v and A_1 cytoplasms. Southern blots of *Xho*I and *Pst*I digests hybridized with the *cox1* probe are shown in Fig. 2. The A_4 CMS and 81B fertile lines were identical in having three *Xho*I fragments: 8.4, 7.6 and 5.2 kbp. A_1 and A_v cytoplasms are closely related to A_4 and 81B in having the same 8.4 and 5.2 kbp fragments but are different from the A_4 and 81B in having an additional *Xho*I fragment (14.1 kbp for A_1 and 11.4 kbp for A_v). The *cox1* probe hybridized to a single *Xho*I fragment of 11.4 kbp in A_5 which is also present in A_v cytoplasm. The A_{egp} cytoplasm only shows hybridization to a single 14.1 kbp fragment which is also detected in the A_1 cytoplasm.

The A_4 CMS and 81B lines were identical in having three *Pst*I fragments: 12.8, 11.4 and 10.9 kbp. A_1 and A_v cytoplasms are closely related to A_4 and 81B in having the same 12.8 and 11.4 kbp *Pst*I fragments but are different as a 5.5 kbp fragment replaced the 10.9-kbp fragment. This 5.5 kbp *Pst*I fragment is the only fragment detected in the A_5 and A_{egp} cytoplasms. Whichever enzyme is used in combination with the *cox1* probe, no change in DNA polymorphism was observed upon restoration of A_1 and A_4 cytoplasms in the hybrids HA_1 and HA_4.

We further investigated whether the *cox1* polymorphisms observed at the DNA level in A_5, A_{egp}, A_v and A_1 cytoplasms were correlated with any transcript variation. Total RNA blot analysis of leaf and spikelet samples was carried out using the *cox1* probe (Fig. 3). The *cox1* probe detected two transcripts of 2.3 and 2.4 kb present in the 81B, A_v, A_1 and A_4 lines and an additional 2.8-kb transcript in the A_v and A_1 CMS lines. This 2.8-kb transcript was the only one detected in A_5 and A_{egp}. In maize, the *cox1* gene was reported to consist of a continuous open reading frame of 1584 bp and two major transcripts of 2.3 and 2.4 kb were detected (Isaac et al. 1985). In comparison with maize, the 2.3-and 2.4-kb transcripts detected in the A_1 and A_v cytoplasms and also in the A_4 and fertile cytoplasms correspond to the normal *cox1* transcripts. Therefore,

Table 3 Summary of RFLP patterns obtained with total DNA from five pearl millet isonuclear CMS lines, the maintainer line and two restored hybrids. DNA was digested with eight restriction endonucleases and probed with seven mtDNA gene sequence probes. Numbers in bold indicate the presence of patterns type nos. 1, 2 . . . for each probe and restriction enzyme combination. Description of each pattern type is indicated in kilobasepairs. For example, all cytoplasms scored **1** were identical and displayed the same RFLP pattern no. 1

Probe × enzyme	Isonuclear A-lines					Maintainer line	Restored hybrids	
	A_5	A_{egp}	A_v	A_1	A_4	81B	HA_1	HA_4
cox1 × *Eco*RI	**1**	**1**	**2**	**2**	**3**	**3**	**2**	**3**
kbp	no1, 12.1; no2, 12.1 + 11.5 + 9.6; no3, 11.5 + 9.6							
*Bam*HI	**1**	**1**	**2**	**2**	**3**	**3**	**2**	**3**
kbp	no1, 5.0; no2, 6.7 + 5.0; no3, 6.7 + 6.3 + 5.0							
*Hin*dIII	**1**	**1**	**2**	**2**	**3**	**3**	**2**	**3**
kbp	no1, 3.7; no2, 3.7 + 2.2 + 1.7; no3, 2.2 + 1.7							
*Bgl*II	**1**	**2**	**3**	**4**	**5**	**5**	**4**	**5**
kbp	no1, 9.0; no2, 12.2; no3, 10.8 + 4.4 + 3.6; no4, 12.2 + 4.4 + 3.6; no5, 4.4 + 3.6							
*Eco*RV	**1**	**2**	**3**	**4**	**5**	**5**	**4**	**5**
kbp	no1, 6.8; no2, 8.9; no3, 12.3 + 5.5 + 2.6; no4, 8.9 + 5.5 + 2.6; no5, 6.8 + 5.5 + 2.6							
*Sst*I	**1**	**2**	**3**	**3**	**4**	**4**	**3**	**4**
kbp	no1, 12.3; no2, 8.4; no3, 9.8 + 9.6 + 8.4; no4, 9.8 + 9.6 + 5.9							
*Pst*I	**1**	**1**	**2**	**2**	**3**	**3**	**2**	**3**
kbp	no1, 5.5; no2, 12.8 + 11.4 + 5.5; no3, 12.8 + 11.4 + 10.9							
*Xho*I	**1**	**2**	**3**	**4**	**5**	**5**	**4**	**5**
kbp	no1, 11.4; no2, 14.1; no3, 11.4 + 8.4 + 5.2; no4, 14.1 + 8.4 + 5.2; no5, 8.4 + 7.6 + 5.2							
cox3 × *Eco*RI	**1**	**1**	**2**	**1**	**3**	**1**	**1**	**3**
kbp	no1, 5.5; no2, 9.7; no3, 11.8							
*Bam*HI	**1**	**1**	**2**	**1**	**3**	**1**	**1**	**nd**[a]
kbp	no1, 13.0; no2, 13.0 + 12.7; no3, 7.9							
*Hin*dIII	**1**	**1**	**2**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 4.3; no2, 3.5							
*Eco*RV	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 8.3; no2, 6.3							
*Sst*I	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 4.3; no2, 8.4							
*Xho*I	**1**	**1**	**2**	**1**	**3**	**1**	**1**	**3**
kbp	no1, 14.1; no2, 15.5; no3, 12.2							
cob × *Bam*HI	**2**	**2**	**1**	**2**	**2**	**2**	**2**	**2**
kbp	no1, 12.1; no2, 11.3							
*Hin*dIII	**2**	**2**	**1**	**2**	**2**	**2**	**2**	**2**
kbp	no1, 6.5 + 4.9; no2, 4.9							
*Bgl*II	**2**	**2**	**1**	**2**	**2**	**2**	**2**	**2**
kbp	no1, 9.7; no2, 10.2							
*Pst*I	**nd**	**nd**	**1**	**2**	**1**	**3**	**nd**	**nd**
kbp	no1, 14.8; no2, 13.3; no3, 15.6							
*Xho*I	**2**	**2**	**1**	**2**	**2**	**2**	**2**	**2**
kbp	no1, 15.6; no2, 14.8							
atp6 × *Eco*RI	**1**	**1**	**2**	**1**	**3**	**1**	**1**	**3**
kbp	no1, 7.7 + 5.5; no2, 9.7 + 7.7; no3, 11.8 + 10.4							
*Bam*HI	**1**	**1**	**2**	**1**	**3**	**1**	**1**	**3**
kbp	no1, 13.0; no2, 13.0 + 12.7; no3, 7.9 + 3.3							
*Hin*dIII	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 2.3; no2, 1.4							
*Bgl*II	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 7.1; no2, 1.4							
*Eco*RV	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 13.3 + 8.3; no2, 11.3 + 6.3							
*Sst*I	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 4.3 + 3.4; no2, 8.4 + 7.5							
*Pst*I	**nd**	**nd**	**1**	**2**	**3**	**2**	**nd**	**nd**
kbp	no1, 16.4 + 14.4 + 5.5; no2, 14.4 + 5.5; no3, 14.4 + 2.6							
*Xho*I	**1**	**1**	**2**	**1**	**3**	**1**	**1**	**3**
kbp	no1, 15.5 + 14.1; no2, 15.5; no3, 12.2 + 8.8							

Table 3 Continued

Probe × enzyme	Isonuclear A-lines					Maintainer line	Restored hybrids	
	A_5	A_{egp}	A_v	A_1	A_4	81B	HA_1	HA_4
atp9 × *Eco*RI	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 6.8; no2, 6.8 + 3.3							
*Bam*HI	**nd**	**nd**	**1**	**1**	**2**	**1**	**nd**	**nd**
kbp	no1, 5.8 + 3.8; no2, 9.4 + 5.8 + 3.8							
*Hin*dIII	**nd**	**nd**	**1**	**1**	**2**	**1**	**nd**	**nd**
kbp	no1, 9.4 + 6.5; no2, 9.4 + 6.5 + 2.5							
Bgl II	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 6.6; no2, 9.5 + 6.6							
*Eco*RV	**1**	**1**	**2**	**1**	**3**	**1**	**1**	**3**
kbp	no1, 16.0 + 10.9 + 10.1; no2, 15.2 + 10.9 + 10.1; no3, 15.2 + 14.0 + 10.9 + 10.1							
*Sst*I	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 9.6; no2, 13.4 + 11.4 + 9.6							
*Pst*I	**nd**	**nd**	**1**	**2**	**3**	**2**	**nd**	**nd**
kbp	no1, 13.0 + 4.4; no2, 15.2 + 4.4; no3, 13.0 + 11.4 + 4.4							
*Xho*I	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 4.8; no2, 8.6 + 4.8							
rps13 × *Eco*RI	**1**	**1**	**2**	**1**	**3**	**1**	**1**	**3**
kbp	no1, 5.1; no2, 9.0; no3, 11.0							
*Bam*HI	**1**	**1**	**2**	**1**	**3**	**1**	**1**	**3**
kbp	no1, 13.6; no2, 14.1; no3, 7.5							
*Hin*dIII	**1**	**1**	**2**	**1**	**2**	**1**	**1**	**2**
kpb	no1, 3.3 ; no2, 2.6							
*Eco*RV	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 8.2; no2, 6.7							
*Sst*I	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**
kbp	no1, 3.6; no2, 7.2							
*Xho*I	**1**	**1**	**2**	**1**	**3**	**1**	**1**	**3**
kbp	no1, 12.1; no2, 13.8; no3, 9.2							
nad2 × *Eco*RV	**1**	**2**	**2**	**2**	**2**	**1**	**2**	**2**
kbp	no1, 8.0 + 4.7; no2, 8.0							
*Sst*I	**1**	**2**	**2**	**2**	**2**	**1**	**2**	**2**
kbp	no1, 12.1 + 11.5; no2, 11.5							
*Pst*I	**1**	**2**	**2**	**2**	**2**	**1**	**2**	**2**
kbp	no1, 16.8 + 15.6; no2, 16.8							
*Xho*I	**1**	**2**	**2**	**2**	**2**	**1**	**2**	**2**
kbp	no1, 5.5 + 3.5; no2, 3.5							

[a] Not determined

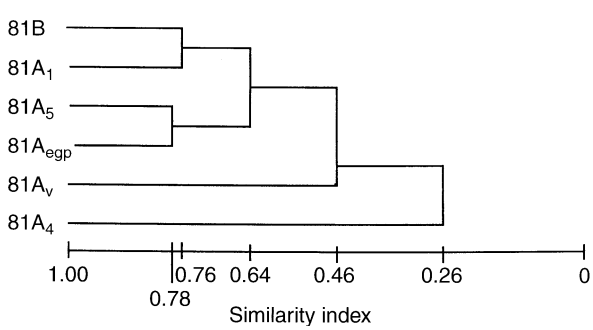

Fig. 1 UPGMA (Unweighted Pair-Group Method Analysis) tree of pearl millet CMS lines based on cluster analysis of diagnostic RFLP data presented in Table 3

the *cox1* polymorphism detected at the DNA level in A_5, A_{egp}, A_v and A_1 cytoplasms is correlated with the presence of a variant transcript of 2.8 kb. No transcript variation was observed either in the leaves or spikelets or upon restoration of A_1 and A_4 cytoplasms (HA_1 and HA_4).

Smith et al. (1987) reported identifying and cloning fragments that were rearranged upon reversion of the A_1 CMS cytoplasm to fertility. Four *Pst*I fragments were cloned: a 4.7 kbp fragment found only in the A_1 CMS line, a 9.7 kbp fragment found in 81B and in eight fertile revertants of the A_1 cytoplasm, a 13.6 kbp fragment found in $81A_1$, 81B and one revertant and a 10.9 kbp fragment found in all cytoplasms. The gene content of those fragments was determined by hybridization to known maize mitochondrial gene probes, and the presence of *cox1*, *rrn18* and *rrn5* genes was detected (Smith and Chowdhury 1991). Despite the difference in size, it is likely that the 4.7 kbp *Pst*I fragment they have cloned and the 5.5 kbp *Pst*I fragment we have identified are the same. Smith and Chowdhury (1991) have identified three *cox1* transcripts of 2.2, 2.3 and 1.7 kb on northern blots that were

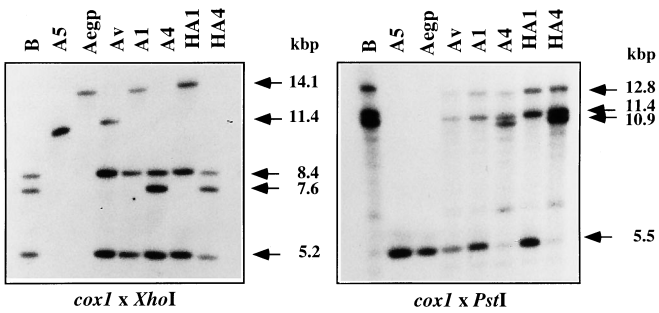

Fig. 2 Southern blot hybridization analysis of total DNA from pearl millet CMS lines, the common maintainer line and two restored hybrids. *Lanes B*: 81B, fertile maintainer line; A_5, A_{egp}, A_v, A_1 and A_4 CMS lines; HA_1 and HA_4: fertile restored hybrids of the A_1 and A_4 CMS lines, respectively. Five micrograms of total DNA was digested with *Xho*I and *Pst*I and separated on 0.8% (w/v) agarose gels. Both blots were hybridized with the maize *cox1* probe. The autoradiographs shown were exposed for 2 days at −80°C. *Arrows* indicate sizes of mtDNA fragments in kilobasepairs

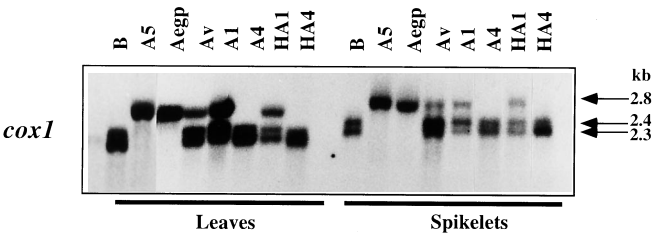

Fig. 3 Northern blot analysis of *cox1* transcripts in leaves and spikelets from pearl millet CMS lines, the common maintainer line and two restored hybrids. *Lanes B*: 81B, fertile maintainer line; A_5, A_{egp}, A_v, A_1 and A_4 CMS lines; HA_1 and HA_4 fertile restored hybrids of the A_1 and A_4 CMS lines, respectively. Ten micrograms of total RNA was separated on 1.2% (w/v) agarose formaldehyde gels of 20 cm in length and run overnight at 3–4 V/cm. The blot was hybridized with the maize *cox1* probe. The autoradiograph was exposed overnight at −80°C. *Arrows* indicate sizes of mtRNA transcripts in kilobases

expressed uniformly across A_1 CMS, revertant and normal 81B cytoplasms. We did not detect the 1.7 kb transcript but the 2.2/2.3 kb transcripts they identified and the 2.3/2.4 kb transcripts we detected are probably the same. The perfect correlation of fragment rearrangement (the loss of the 4.7 kbp fragment and the gain of the 9.7 kbp fragment) with reversion from CMS to fertility in eight independent spontaneous fertile revertants of the A_1 cytoplasm suggested that a gene or altered gene responsible for the expression of CMS may be located on those rearranged fragments. Consequently, the single 5.5 kbp *Pst*I mtDNA fragment present in A_5 and A_{egp} cytoplasms and also present in A_1 and A_v cytoplasms together with other *Pst*I fragments may represent a good candidate for the source of the rearrangement involving the *cox1* gene which may be causally related to CMS in these cytoplasms.

The A_4 cytoplasm

The *cox3* probe in combination with *Bam*HI, *Eco*RV and *Sst*I, the *atp6* probe in combination with *Hin*dIII, *Bgl*II, *Eco*RV, *Sst*I and the *atp9* probe in combination with *Eco*RI, *Bam*HI, *Hin*dIII, *Bgl*II, *Sst*I, *Xho*I allowed us to identify polymorphisms specific to the A_4 cytoplasm. The results we obtained with the same blot of *Eco*RV digests hybridized with *cox3* and *atp6* probes and for the blot of *Xho*I digests hybridized with the *atp9* probe are shown in Fig. 4.

With the *cox3* probe, A_1, A_v, A_5, A_{egp} cytoplasms and the isonuclear fertile cytoplasm (81B) were identical in having an 8.3 kbp *Eco*RV fragment, whereas A_4 differed and contained a 6.3 kbp *Eco*RV fragment. Whichever enzyme is used, only one fragment hybridized, suggesting that the *cox3* gene is present in one copy in the pearl millet mitochondrial genome.

The *atp6* probe revealed that the A_1, A_v, A_5, A_{egp} cytoplasms and the isonuclear fertile cytoplasm (81B) were identical in having two *Eco*RV fragments of 12.3 and 8.3 kbp, whereas A_4 was different in having 11.3 and 6.3 kbp *Eco*RV fragments. It was interesting to note that the 8.3 and 6.3 kbp *Eco*RV fragments were detected with both probes, suggesting that the *cox3* and *atp6* genes are probably clustered in the millet mitochondrial genome.

The *atp9* probe revealed that the A_1, A_v, A_5, A_{egp} cytoplasms and the isonuclear fertile cytoplasm (81B) were identical in having a 4.8 kbp *Xho*I fragment, whereas A_4 differed and contained an additional 8.6 kbp *Xho*I fragment.

Whichever probe and restriction enzyme combination was used, no change in DNA polymorphism was observed upon restoration of A_1 and A_4 cytoplasms (HA_1 and HA_4). Our results agree with the data previously reported by Rajeshwari et al. (1994) which showed that the maize *atp9* probe differentiated the A4 CMS line from the A_1 and A_v CMS lines. They have also reported distinguishing all three cytoplasms using the maize *atp6* probe.

We further investigated whether the polymorphisms obtained with *cox3*, *atp6* and *atp9* probes specific to the A_4 cytoplasm were also correlated with any transcript variation. Total RNA blot analysis of leaf and spikelet samples was carried out using the three probes and the same northern blot (Fig. 5). The *cox3* probe revealed two transcripts of 3.4 and 3.2 kb in leaves of the A4 cytoplasm, whereas the other CMS cytoplasms and the fertile 81B line were characterized by the presence of a 2.9 kb transcript. Additional transcripts (1.2 and 1.0 kb for A_4 and 1.4 and 0.9 kb for the other cytoplasms and 81B) were detected in spikelets. These transcripts were also detected in leaf samples after 3 days of exposure (data not shown). The *atp6* probe revealed three transcripts of 3.4, 3.2 and 1.6 kb in the A_4 cytoplasm, whereas two transcripts of 2.9 and 1.4 kb were detected in A_5, A_{egp}, A_v, A_1 cytoplasms and in

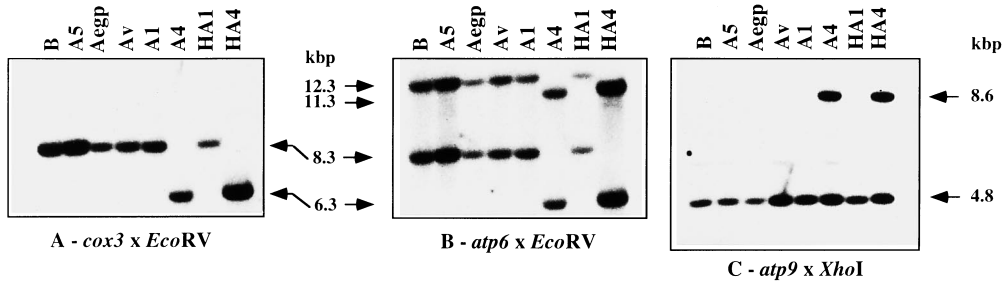

Fig. 4A–C Southern blot hybridization analysis of total DNA from millet CMS lines, the common maintainer line and two restored hybrids. *Lanes B*: 81B, fertile maintainer line; A_5, A_{egp}, A_v, A_1 and A_4 CMS lines; HA_1 and HA_4 fertile restored hybrids of the A_1 and A_4 CMS lines, respectively. Five micrograms of total DNA was digested with *Eco*RV (**A** and **B**) or with *Xho*I (**C**) and separated on 0.8% (w/v) agarose gels. The same blot of *Eco*RV digests was hybridized with the maize *cox3* (**A**) and *atp6* (**B**) probes. The blot of *Xho*I digests was hybridized with the sorghum *atp9* probe (**C**). The autoradiographs shown were exposed for 3 days at −80°C. *Arrows* indicate sizes of mtDNA fragments in kilobasepairs

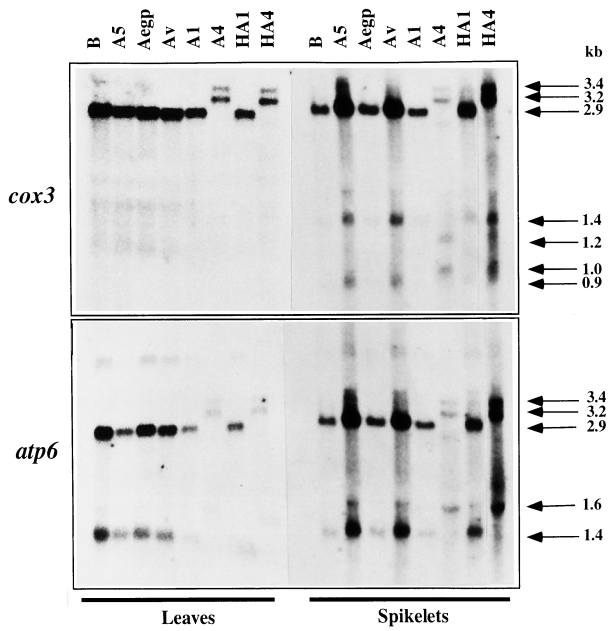

Fig. 5 Northern blot analysis of *cox3* and *atp6* transcripts in leaves and spikelets from millet CMS lines, the common maintainer line and two restored hybrids. *Lanes B*: 81B, fertile maintainer line; A_5, A_{egp}, A_v, A_1 and A_4 CMS lines; HA_1 and HA_4 fertile restored hybrids of the A_1 and A_4 CMS lines, respectively. Ten micrograms of total RNA was separated on 1.2% (w/v) agarose formaldehyde gels of 20 cm in length and run overnight at 3–4 V/cm. The same blot was hybridized with maize *cox3* and *atp6* probes. The autoradiographs were exposed overnight at −80°C. *Arrows* indicate sizes of mtRNA transcripts in kilobases

81B. A 420 nucleotide transcript was detected in all the lines using the sorghum *atp9* probe (data not shown). Interestingly, the two major transcripts of 3.4 and 3.2 kb detected in A_4 and the 2.9-kb transcript detected in the other cytoplasms were detected with both *cox3* and *atp6* probes. These results suggest that the *cox3* and *atp6* genes are not only clustered in the millet mitochondrial genome but are also cotranscribed.

Because of the difficulty in obtaining immature anthers we isolated RNA from spikelets, which include vegetative tissues and from leaves, and found no transcript variation whichever of the three probes is used. In addition, we found no effect on transcript patterns in these tissues upon restoration of A_1 and A_4 cytoplasms (HA_1 and HA_4).

In conclusion our results suggest that a rearrangement involving the *cox1* gene might be related to CMS in the A_5, A_{egp}, A_v and A_1 cytoplasmic group, whereas a rearrangement within the *atp6*/*cox3* cluster region might be related to CMS in the A_4 cytoplasm.

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