*Euphytica* **93:** 301–305, 1997.



# Characterization of the $A_4$ cytoplasmic male-sterile lines of sorghum using RFLP of mtDNA *

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Received 2 November 1995; accepted 29 July 1996

*Key words: Sorghum bicolor*, RFLP, cytoplasmic male sterility, sorghum, mitochondrial DNA

**Summary**

Three sorghum cytoplasmic male sterile lines CSV4 A(V), CSV4 A($G_1$) and CSV4 A(M), grouped as $A_4$, were compared with a milo ($A_1$) and two other non-milo ($A_2$ and $A_3$) cytoplasms for their RFLP patterns of mitochondrial DNA (mtDNA). A 9.7 kb clone from pearl millet mtDNA discriminated each of the three $A_4$ entries whereas other maize and pearl millet mtDNA clones used could not distinguish this group completely. The molecular differences within the $A_4$ cytoplasmic group offer some explanation for the inconsistency in the fertility restoration behaviour of these $A_4$ lines obtained with a definite set of testers in the field.

## Introduction

Cytoplasmic diversity is important in any commercial hybrid breeding program and it is necessary to have an efficient and rapid method to characterize the diversity of cytoplasm. The conventional method of using fertility restorer genes for the identification of different cytoplasms can be tedious and time-consuming and so alternate molecular approaches like isozymes, protein markers and restriction fragment length polymorphism (RFLP) have been employed for this purpose (Pring and Levings, 1978; Forde et al., 1980; Kemble et al., 1980). Cytoplasmic factors associated with male sterility have been shown to be encoded by the mitochondrial genome (Hanson and Conde, 1985), and RFLP of mitochondrial DNA (mtDNA) has been shown to provide a rapid procedure to classify male sterile cytoplasms in maize (Pring and Levings, 1978), sorghum (Pring et al., 1982; Bailey-Serres et al., 1986) and pearl millet (Smith and Chowdhury, 1989; Rajeshwari et al., 1994). Cytoplasms of sorghum have been distinguished based on RFLP of mtDNA, the presence of plasmid-like structures in mitochondrial DNA and the synthesis of variant mitochondrial translational products (Pring et al., 1982; Conde et al., 1982; Dixon and Leaver, 1982; Bailey-Serres et al., 1986). Xu et al. (1995) identified mitochondria-specific probes from the genomic library that could distinguish a range of cytoplasmic male-sterile lines.

Many of the sorghum male-sterile lines with different cytoplasms have been developed from germplasm collections. Sometimes, it is rather difficult to get testers that can differentiate the various cytoplasms based on consistent fertility restoration reaction across crop-growing seasons. Three male-sterile lines designated as CSV4 A (Maldandi), CSV4 A (Vizianagaram), and CSV4 A (Guntur) were grouped together as $A_4$ cytoplasm based on their similar fertility restoration reaction against the minimum testers, TAM 428 B, CK 60 B, IS 84 B(M) and IS 5757 R (Maldandi) during one crop-growing season. The testers could differentiate all the milo- and non-milo cytoplasms that are widely used at ICRISAT namely $A_1$, $A_2$ and $A_3$ but not within the $A_4$ cytoplasm. The lines within the $A_4$ group did not show a consistent fertility restoration reaction across all crop growing seasons for the same set of testers (Reddy and Rao, 1991). To understand this differential reaction of the cytoplasm in the field it was necessary to carry out the molecular characterization of these lines by RFLP of mtDNA. The

* Submitted as journal article No. 1818 by the International Crops Research Institute for the Semi-Arid Tropics.

main objective of this study is to compare the known cytoplasmic sources with the three lines in the $A_4$ cytoplasmic group for their RFLP of mtDNA using maize and pearl millet mtDNA specific clones.

## Materials and methods

### *Plant material*

We used one milo cytoplasm, $ICSA_1$, 26 and five non-milo cytoplasms, TAM 428 $A_2$, IS 1112C $A_3$ and three belonging to the $A_4$ type, CSV4 A (Maldandi), CSV4 A(VZM), CSV4 A($G_1$).

### *Clones used*

The maize *atp6* clone (ATPase subunit 6, Dewey et al., 1986), was supplied by C.S. Levings III, Genetics Department, North Carolina State University, Raleigh, NC, USA. Maize clones *cox*I (Cytochrome oxidase subunit I, Isaac et al., 1985), *cox*II (Cytochrome oxidase subunit II, Fox and Leaver, 1981) and *atp*$\alpha$(Isaac et al., 1985) were gifts from C.J. Leaver, Department of Plant Science, University of Oxford, Oxford, U.K. Pearl millet *Pst*I mtDNA fragments (10.9, 9.7, and 4.7 kb) were used as probes and two of these contained the *cox*I and *rrn18-rrn5* gene fragments and the other (9.7 kb) was associated with reversion from sterility to fertility in $A_1$ pearl millet cytoplasm (Smith et al., 1987).

### *DNA analysis*

Mitochondrial DNA was extracted from 5–6 day old seedlings grown in the dark according to Chase and Pring (1986). MtDNA was digested with several restriction enzymes according to the supplier's instructions in the presence of RNase I (50 $\mu$g/ml) and the fragments were separated on 0.8% agarose gel using TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 7.8) buffer. Southern transfer of DNA fragments onto Nylon membrane (Hybond N, Amersham, U.K.) was performed by capillary method. Blots were washed in 3×SSC (0.45 M Sodium chloride, 0.045 M Sodium citrate, pH 7.0) after transfer and UV-crosslinked as described by Smith et al. (1987).

The random primed labeling method of Feinberg and Vogelstein (1983) was used for the preparation of $^{32}$P-labeled probes. Southern blots were pre-hybridized with 30 mL of pre-hybridization solution containing 7% SDS, 1% BSA, 0.5 M $Na_2HPO_4$ and 20 $\mu$g/mL sheared and denatured salmon sperm DNA per two blots (20 × 15 cm) size. Hybridization was carried out by adding the labeled probe to the pre-hybridization solution and incubating for 16 h at 65 °C in standard bottles (30 × 3.5 cm, l × d) in a hybridization oven (Hybaid, U.K.). The blots were washed three times in 3×SSC containing 0.1% SDS at 65 °C for 30 min each. Autoradiography of the blots was conducted at - 70 °C for varying periods using X-AR (Kodak) films. The fragment sizes were determined using lambda DNA cut with *Hind*III and $\phi$X 174 cut with *Hae*III standard markers.

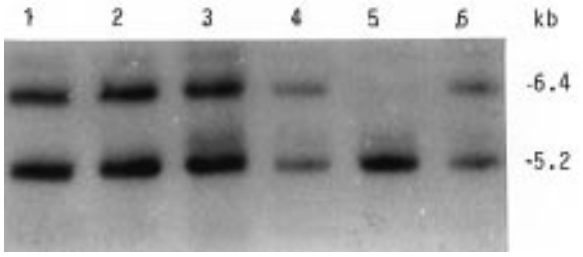


*Figure 1.* Autoradiogram of the maize *atp*6 gene clone hybridized to sorghum mtDNA. *BamH*I: Lanes 1. $ICSA_1$ 26: 2. TAM 428 $A_2$: 3. IS 1112C $A_3$: 4. CSV4 A(M): 5. CSV4 A(V): and 6. CSV4 A($G_1$).

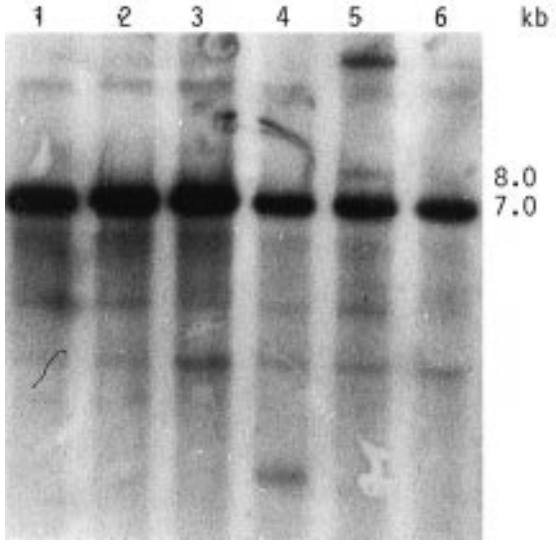


*Figure 2.* Autoradiogram of the maize *cox*I gene clone hybridized to *BamH*I digested sorghum mtDNA. Lanes 1. $ICSA_1$ 26: 2. TAM 428 $A_2$: 3. IS 1112C $A_3$: 4. CSV4 A(M): 5. CSV4 A(V): and 6. CSV4 A($G_1$).

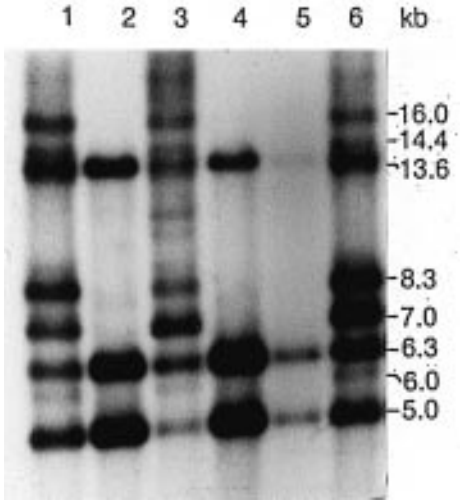


*Figure 3*. Southern blot hybridization of sorghum mtDNA digested with *Pst*I and probed with pearl millet 10.9 clone. Lanes 1. $ICSA_1$ 26: 2. TAM 428 $A_2$: 3. IS 1112C $A_3$: 4. CSV4 A(M): 5. CSV4(V) and 6. CSV4 A($G_1$).

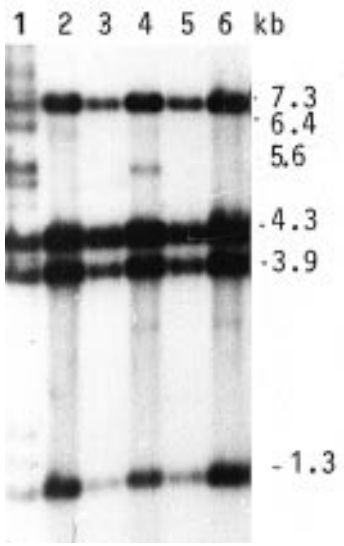


*Figure 4*. Southern blot hybridization of sorghum mtDNA digested with *BamH*I and probed with pearl millet 10.9 kb clone. Lanes 1. $ICSA_1$ 26: 2. TAM 428 $A_2$: 3. IS 1112C $A_3$: 4. CSV4 A(M): 5. CSV4 A(V) and 6. CSV4 A($G_1$).

## Results and discussion

Southern blots of mtDNA from male-sterile lines of sorghum digested with *BamH*I and probed with maize *atp*6 clone identified only two groups of cytoplasms based on their hybridization pattern (Figure 1). All sorghum lines gave two fragments of 6.4 and 5.2 kb except CSV4 A(V) which showed only the 5.2 kb fragment (Figure 1, lane 5). Southern blots of *Xho*I and *Pst*I digested mtDNA were not able to distinguish the lines in the $A_4$ group when probed with *atp*6 (data not shown). Maize mitochondrial gene probe, *atp*6 has been known to distinguish the various cytoplasmic systems in pearl millet in several restriction enzyme combinations (Rajeshwari et al., 1994).

Southern blots of mtDNA digested with *Bam*HI and probed with maize *cox*I gene probe showed two hybridizing fragments of 8 and 7.0 kb in CSV4 A(V) (Figure 2, lane 5) whereas all other lines showed only a strong hybridizing band of 7.0 kb. Our results are similar to those of Bailey-Serres (1986) who reported that *cox*I could distinguish only the 9E cytoplasm from the others in *EcoRI* digested mtDNA where only a strongly hybridizing band was seen with all other cytoplasms. In the case of pearl millet, *cox*I could only distinguish mainly the $A_1$ group of cytoplasms from others (Sujata et al., 1994).

Since pearl millet 10.9 and 4.7 kb probes gave identical results, data is given only for the 10.9 kb probe. *Pst*I digested mtDNA showed two distinct hybridization patterns when probed with the 10.9 kb pearl millet clone (Figure 3). ICSA 26, IS 1112C A and CSV4 A($G_1$) (Figure 3, lanes 1, 3 and 6) showed eight hybridizing bands of 16, 14.4, 13.6, 8.3, 7.0, 6.3, 6.0 and 5.0 kb whereas TAM 428 A, CSV A(M) and CSV4 A(V) (Figure 3, lanes 2, 4 and 5) had only three fragments of 13.6, 6.3 and 5.0 kb. With *BamH*I digested mtDNA, the pearl millet 10.9 kb probe showed three different hybridization patterns (Figure 4). ICSA 26 with $A_1$ cytoplasm showed a unique hybridization pattern with six hybridizing fragments of 7.3, 6.4, 5.6, 4.3, 3.9 and 1.3 kb (Figure 4, lane 1). CSV4 A(V) and CSV4 A($G_1$) showed hybridization patterns similar to $A_2$ and $A_3$ cytoplasms with four hybridization fragments of 7.3, 4.3, 3.9 and 1.3 kb (Figure 4, lanes 5, 6, 2 and 3 respectively) whereas CSV4 A(M) was distinguished by a faintly hybridizing band of 5.6 kb (Figure 4, lane 4) that was also present in the $A_1$ cytoplasm (Figure 4, lane 1).

Southern blot hybridization of *BamH*I digested mtDNA could distinguish the three lines in the $A_4$ cytoplasmic group when probed with the pearl millet 9.7 kb clone. This enzyme-probe combination gave five distinct hybridization patterns in which each of the $A_4$ cytoplasms showed a characteristic pattern (Figure 5). ICSA 26 and TAM 428 A, representing the $A_1$ and $A_2$ cytoplasms respectively, showed identical RFLP

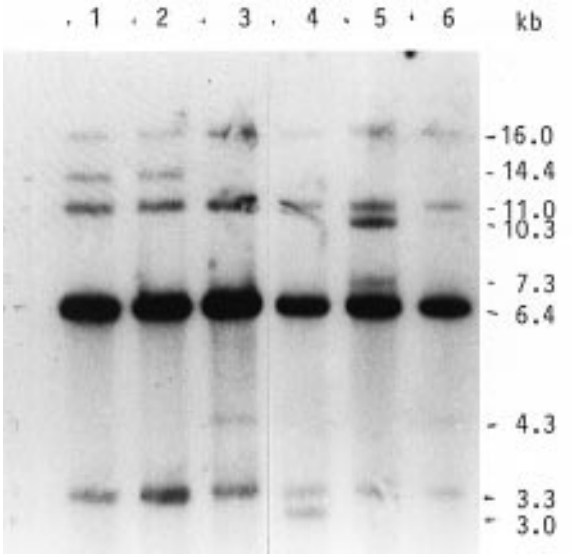


*Figure 5.* Southern blot hybridization of sorghum mtDNA digested with *Eco*RI and probed with pearl millet 9.7 kb clone. Lanes 1. $ICSA_1$ 26: 2. TAM 428 $A_2$: 3. IS 1112C $A_3$: 4. CSV4 A(M): 5. CSV4 A (V) and 6. CSV4 A($G_1$).

patterns (Figure 5, lanes 1 and 2) with five hybridization fragments of 16, 14.4, 11, 6.4 and 3.3 kb. The 14.4 kb fragment was present only in these two cytoplasms. IS 1112C A and CSV4 A ($G_1$) showed similar hybridization patterns (Figure 5, lanes 3 and 6) except for a minor 4.3 kb fragment that was present only in the former CSV4 A(M) (Figure 5, lane 4) was different from $A_3$ and CSV4 A($G_1$) (Figure 5, lanes 3 and 6) by the absence of the 4.3 kb fragment and presence of a 3.0 kb fragment. CSV4 A(V) (Figure 5, lane 5) was unique compared to the other cytoplasm in the $A_4$ group by the presence of the 10.3 kb and 7.3 kb size fragments which were absent in all other sorghum lines. The 9.7 pearl millet clone thus separated the $A_4$ cytoplasms into three separate groups. The $A_1$ and $A_2$ cytoplasms formed a single group with identical hybridization patterns.

Our results show that $A_4$ group indeed consists of cytoplasms with differing mtDNA characteristics as seen from the hybridization data. In the present study we have not identified a single enzyme-probe combination that could distinguish all the cytoplasms. The differentiation of cytoplasms within the $A_4$ group was possible only with *BamH*I digested mtDNA suggesting that there is a high degree of homology among the lines within the $A_4$ group for the fragments hybridizing with pearl millet 9.7 kb clone. The present study suggests that among the $A_4$ lines studied CSV4 A($G_1$) could possibly be included in the $A_3$ cytoplasmic group with IS 1112C A where as CSV4 A(M) and CSV4 (V) could be considered as distinct cytoplasmic groups. The inconsistent fertility restoration reaction observed in the field with the three differential testers (Reddy and Rao, 1991) can be explained on the basis of the differential hybridization data obtained with the three lines in the $A_4$ group. Xu et al. (1995) could distinguish five cytoplasmic groups using their organellar probes. However, one of the groups included the A1, A2, A5, and A6 cytoplasms suggesting that a unique enzyme-probe combination may not be available to distinguish all the cytoplasmic groups. The only $A_4$ line used by Xu et al. (1995) was from Nigeria whereas those used in the present study are from India. It is important to have group specific probes so that cytoplasms can be distinguished by simpler methods like RAPDs from a germplasm collection.

Our studies on pearl millet indicated the involvement of *cox*I and *atp*6 in the mitochondrial rearrangements in the different cytoplasmic male sterile systems (Sujata et al., 1994). In sorghum, the rearrangements in the *cox*I gene resulted in altered gene product in the 9E cytoplasm (Leaver et al., 1988). further study is needed to detect the mitochondrial genomic rearrangements that may be determining the different male sterility systems in sorghum.

## Acknowledgements

We thank Mr. K.D.V. Prasad for cluster analysis and Mr. R. Luke for technical assistance.

## References

Bailey-Serres, J., L.K. Dixon, A.D. Liddel & C.J. Leaver, 1986. Nuclear-mitochondrial interactions in cytoplasmic male-sterile sorghum. Theor Appl Genet 73: 252–260.

Bailey-Serres, J., D.K. Hanson, T.D. Fox & C.J. Leaver, 1986a. Mitochondrial genome rearrangement leads to extension and relocation of the cytochrome c oxidase subunit I gene in sorghum. Cell 47: 567–576.

Belum, V.S., Reddy & K.E.P. Rao, 1992. Cereals Program. ICRISAT. Annual Report 1991. Patancheru, A.P. 502 324, India: Cereals Program, International Crops Research Institute for the Semi-Arid Tropics, pp. 59–64.

Conde, M.F., D.R. Pring, K.F. Schertz & W.M. Ross, 1982. Correlation of mitochondrial DNA restriction endonuclease patterns with sterility expression in six male-sterile sorghum cytoplasms. Crop Sci 22: 536–539.

Dewey, R.E., C.S. Levings III & D.H. Timothy, 1986. Novel recombinations in the maize mitochondrial genome produce a unique

transcriptional unit in the Texas male sterile cytoplasm. Cell 44: 439–449.

Dixon, L.K. & C.J. Leaver, 1982. Mitochondrial gene expression and CMS in sorghum. Plant Mol Biol 1: 89–102.

Forde, B.G. & C.J. Leaver, 1980. Nuclear and cytoplasmic genes controlling synthesis of variant mitochondrial polypeptides in male-sterile maize. Proc Natl Acad Sci (USA) 77: 418–422.

Fox, T.D. & C.J. Leaver, 1981. The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. Cell 26: 315–323.

Hanson, M.R. & M.F. Conde, 1985. Functioning and variation of cytoplasmic genomes: Lessons from cytoplasmic-nuclear interactions affecting male sterility in plant. Int Rev Cytol 9: 213–267.

Isaac, P.G., V.P. Jones & C.J. Leaver, 1985. The maize cytochrome c oxidase subunit I gene: sequence, expression and rearrangement in cytoplasmic male sterile plants. The EMBO Jour 4: 1617–1623.

Kemble, R.J., R.E. Gunn & R.B. Flavall, 1980. Classification of normal and male-sterile cytoplasms in maize. Genetics 95: 454–458.

Leaver, C.J., P.G. Isaac, I.D. Small, J. Bailey-Serres, A.D. Liddell & M.J. Hawkesford, 1988. Mitochondrial genome diversity and cytoplasmic male sterility in higher plants. Phil Trans R Soc Lond B 319: 165–176.

Mann, V., L. McIntosh & T.C. Hirschberg, 1989. A new cytoplasmic male sterile genotype in sugar beet *Beta vulgaris* L.: a molecular analysis. Theor Appl Genet 78: 293–297.

Pring, D.R. & C.S. Levings III, 1978. Heterogeneity of maize cytoplasmic genomes among male-sterile cytoplasms. Genetics 89: 121–136.

Pring, D.R., M.F. Conde & K.F. Schertz, 1982. Organelle genome diversity in sorghum: male-sterile cytoplasm. Crop Sci 22: 414–421.

Rajeshwari, R., S. Sivaramakrishnan, R.L. Smith & N.C. Subrahmanyam, 1994. RFLP analysis of mitochondrial DNA from cytoplasmic male-sterile lines of pearl millet. Theor Appl Genet 88: 441–448.

Sane, A.P., P. Jaiswal, P. Nath, A.R. Dabholkar & P.V. Sane, 1994. Mitochondrial plasmids in sorghum: presence of linear plasmids in Indian male sterile and milo 296 lines. J Plant Biochem Biotech 3: 121–124.

Smith, R.L. & M.K.U. Chowdhury, 1989. Mitochondrial DNA rearrangements in *Pennisetum* associated with reversion from cytoplasmic male sterility to fertility. 9: 277–286.

Smith, R.L. & M.K.U. Chowdhury, 1989. Mitochondrial DNA polymorphism in male-sterile and fertile cytoplasms of pearl millet. 1989. Crop Sci 29: 809–814.

Sujata, V., S. Sivaramakrishnan, K.R. Rai & K. Seetha, 1994. A new source of cytoplasmic male sterility in pearl millet: RFLP analysis of mitochondrial DNA. Genome 37: 482–486.

Young, E.G. & M.R. Hanson, 1987. A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell 50: 41–49.