

Cytoplasmic male sterility in sorghum: organization and expression of mitochondrial genes in Indian CMS cytoplasms

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Abstract. Cytoplasmic male sterility in sorghum has been reported in a number of varieties originating in different geographical regions (India, Africa and America). We have attempted to characterize three male sterile cytoplasms of Indian origin designated as Maldandi, Guntur and Vizianagaram by studying restriction fragment length polymorphisms (RFLPs) and expression patterns of 14 mitochondrial genes. Our results indicate that the cytoplasms, classified tentatively as Indian A₄ types, are distinct from the American A₄ and A₁ types. Although they are identical to each other with respect to the location of 10 of the mitochondrial genes selected, they can be distinguished from each other on the basis of RFLPs in *atp6*, *atp9* and *rrn18*. Further the three cytoplasms differ from their maintainers in the location of *nad3*, *rps12* and *atpA*. Differences are also observed in the pattern of expression of *atpA* between all the sterile lines and their respective maintainers.

Keywords. Cytoplasmic male sterility; sorghum; *atpA*; Indian CMS lines.

1. Introduction

Hybrid seed production in plants has, in the last few decades, been greatly facilitated by the discovery and development of cytoplasmic male sterile (CMS) lines. The CMS trait, characterized by the absence of functional pollen, is believed to be the outcome of incompatible nuclear–mitochondrial interactions (Hanson 1991). Restoration to fertility occurs through expression of the dominant restorer gene(s) resulting in a fertile hybrid plant. However, for successful exploitation of the male sterility trait it is necessary to understand the molecular mechanisms underlying male sterility and the factors involved in restoration to fertility. To some extent this has been done in maize (Dewey *et al.* 1986, 1987), *Petunia* (Young and Hanson 1987; Nivison and Hanson 1989), sunflower (Moneger *et al.* 1994; Smart *et al.* 1994) and *Phaseolus* (Abad *et al.* 1995). In most plants, however, the data are still inconclusive.

Sorghum is an important crop in which a number of male sterile cytoplasms have been identified and studied (Stephens and Holland 1954; Rao 1962; Hussaini and Rao 1964; Webster and Singh 1964; Nagur and Menon 1974; Schertz and Ritchey 1978; Pring *et al.* 1982; Lee *et al.* 1989; Xu *et al.* 1995). These cytoplasms are known to have originated in regions as geographically diverse as India, America and Africa. They are broadly classified into groups designated as A₁, A₂, A₃, A₄, etc. depending on their

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maintainer and restorer crosses, of which mainly the A₁ cytoplasm (milo) has so far been used for commercial hybrid seed production. However, reliance on only one type of cytoplasm, such as the milo, can be disastrous if the cytoplasm happens to be susceptible to attack by a particular pathogen. It is therefore necessary to look for alternative cytoplasm for hybrid seed production. In the last few decades Indian agriculturists and plant breeders under the All-India Coordinated Sorghum Improvement Project have developed several male sterile lines based on three Indian male sterile cytoplasm designated as Maldandi, Guntur and Vizianagaram. These cytoplasm are of Indian origin (race durra) and have been identified separately in the regions of Maldandi, Guntur and Vizianagaram. They have been tentatively grouped as Indian A₄ types. Restoration to fertility of these cytoplasm has been difficult and, so far, no restorer lines are known, which has prevented use of these cytoplasm for commercial hybrid seed production. Some genetic analysis of these cytoplasmic systems for seasonal variation in pollen sterility/fertility, seed setting, and their response to different fertility restorer lines have been carried out previously (Rao *et al.* 1984; Tripathi *et al.* 1985). But no studies on the molecular analysis of the mitochondrial DNA polymorphism and gene expression in these lines were initiated prior to this study. Since these CMS lines may play an important role in sorghum breeding, it is essential to have information on the molecular organization of mitochondrial DNA of these CMS lines.

In the work reported here, we have attempted to characterize these cytoplasm at the molecular level by Southern and Northern analysis of several mitochondrial genes to find out how the three sterile cytoplasm differ from each other and to explore if polymorphism in gene location and transcript patterns could be related to male sterility in these varieties. The data demonstrate that although the three cytoplasm have several similarities they differ in the location of *atp6*, *atp9* and *rrn18* genes. The observations reflect the cytoplasmic diversity that appears to be present among the Indian lines.

2. Methods

Seeds of sorghum of the sterile (A) and maintainer (B) lines of varieties containing Maldandi cytoplasm (Ma1A, Ma1B), Guntur cytoplasm (G1A, G1B) and Vizianagaram cytoplasm (VzmA, VzmB), all in a CS3541 nuclear background (a converted form of IS3541) were obtained from Prof. A. R. Dabholkar, Sorghum Breeder, All India Coordinated Sorghum Improvement Project, Indore. Mitochondrial DNA and mitochondrial RNA from etiolated seedlings were isolated as described previously (Nath *et al.* 1993; Sane *et al.* 1994b). Restriction digestion of the mitochondrial DNA was carried out using endonuclease *Hind*III (Stratagene) following the manufacturer's instructions. Southern and Northern blots were prepared on a nylon membrane (Schleicher and Schuell) as described by Sambrook *et al.* (1989). Hybridization and washings were carried out as described previously (Sane *et al.* 1994b). Southern and Northern hybridizations for nonpolymorphic genes were carried out once while those where polymorphism was observed were repeated thrice.

The mitochondrial gene probes *cob* and *atpA* from maize were obtained from Prof. C. J. Leaver, UK, while *coxI*, *coxII*, *atp6* and *atp9* (also from maize) were obtained from Prof. C. S. Levings III, USA. Oligonucleotide probes for *coxIII*, *nad2*, *nad3*, *nad4*, *rps12*,

rps13, *rrn18* and *rrn26* (from wheat) were obtained from Prof. J. M. Grienenberger, France. Gene probes were radiolabelled by random priming using [α - 32 P]dATP, while oligonucleotides were end-labelled using [γ - 32 P]ATP as described by Sambrook *et al.* (1989).

3. Results

3.1 Analysis of mitochondrial DNA RFLPs

A Southern blot of the mitochondrial DNA from all the six lines, digested with *Hind*III, was probed with 13 mitochondrial gene probes covering most of the major multi-subunit complexes of the mitochondrial membrane and the translational machinery, viz. *nad2*, *nad3*, *nad4*, *coxI*, *coxII*, *coxIII*, *cob*, *atp6*, *atp9*, *atpA*, *rps12*, *rps13* and *rrn18*.

The results indicated lack of polymorphism in the location of seven of the 13 genes investigated using appropriate probes (table 1). The fragments hybridizing to the probes were identical in all the six lines. Significantly, the pattern obtained for *coxI* was different from that described for the 9E and IS7920 cytoplasms of the American A₄ group (Bailey-Serres *et al.* 1986a, b). Unlike in the American A₄ group, no polymorphism was seen. While hybridization to a 4.5-kbp *coxI*-specific fragment was observed in the Indian CMS lines, the *coxI* gene is known to be located on 10-kbp (9E) as well as 1.9-kbp and 1.8-kbp (IS7920C) *Hind*III fragments in the American A₄ cytoplasm.

RFLP patterns obtained using genes of ATP synthase, however, were able to distinguish not only the individual cytoplasms from each other but also the sterile and maintainer lines from one another. Such polymorphism has been described previously in other varieties (Bailey-Serres *et al.* 1986a; Mullen *et al.* 1992; Sane *et al.* 1994b).

In case of *atp6*, bands of 2.0 kbp and 3.3 kbp were observed in the sterile and maintainer lines of Maldandi and Guntur. The 3.3-kbp band, however, was missing in the sterile line of Vizianagaram cytoplasm (VzmA) even though it was present in its maintainer line (figure 1a).

When the blot was probed with *atp9*, hybridization to fragments of sizes 3.2 kbp and 3.6 kbp was observed in all the six lines. In addition, a 4.3-kbp band could be seen in the three maintainer lines. This 4.3-kbp band was completely absent in the MalA line. However, very faint signals for this band could be observed in the G1A and VzmA lines (figure 1b). The reason for the differences in relative intensity of the 4.3-kbp band are

Table 1. Restriction fragment sizes of the nonpolymorphic mitochondrial genes in the three Indian male sterile cytoplasms and their maintainer lines. DNA was digested with *Hind*III.

Gene	Size (kbp)
<i>nad2</i>	8.5
<i>nad4</i>	10.5
<i>coxI</i>	4.5
<i>coxII</i>	3.5
<i>coxIII</i>	4.8
<i>cob</i>	3.25
<i>rps13</i>	11.5

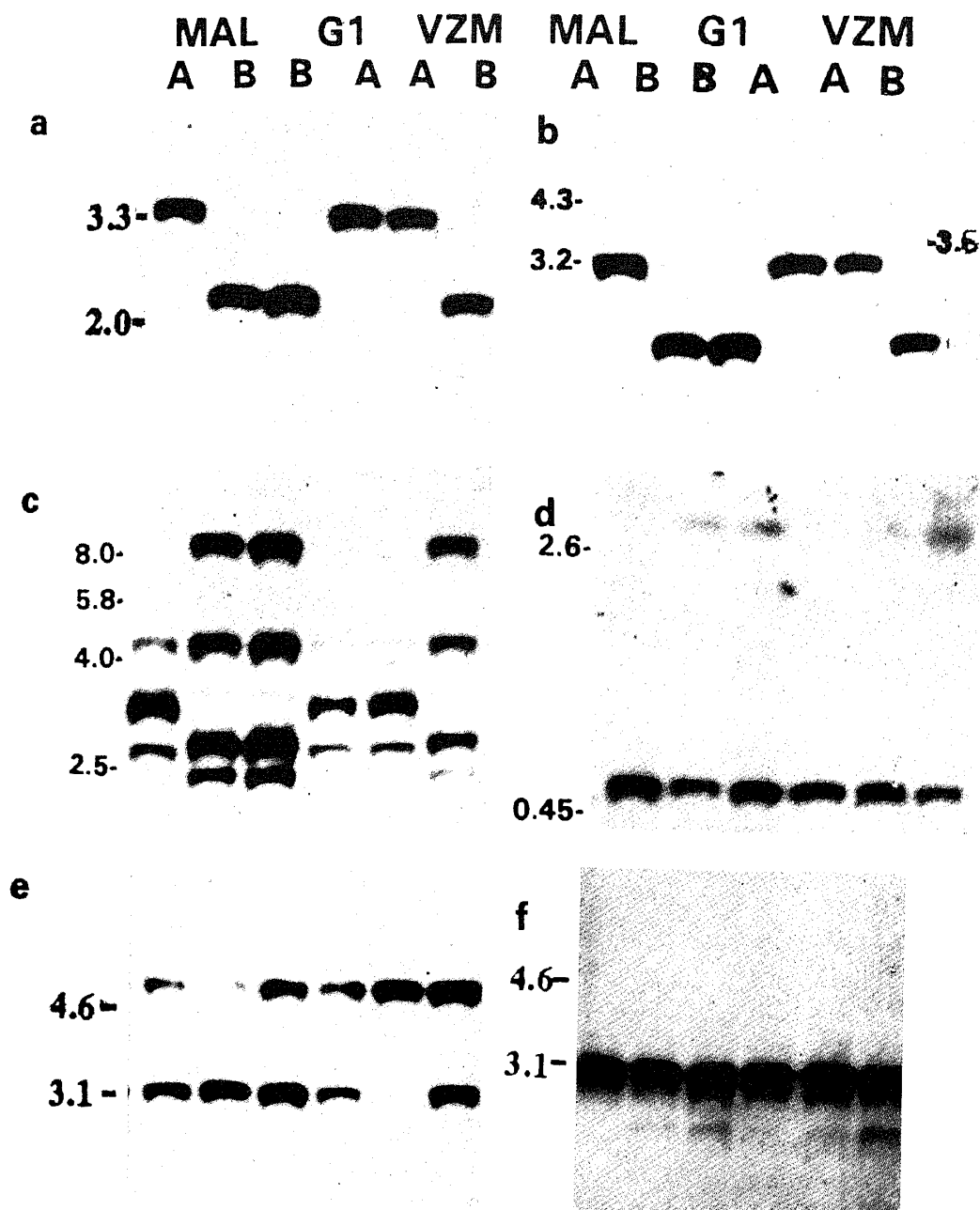


Figure 1. Southern blot hybridization of (a) *atp6*, (b) *atp9*, (c) *atpA*, (d) *rrn18*, (e) *nad3*, and (f) *rps12* probes to *Hind*III-digested mitochondrial DNA of various cytoplasms. A, male sterile lines; B, maintainer lines; MAL, Maldandi; G1, Guntur; VZM, Vizianagaram. The numbers on the left of the panels indicate size in kbp.

not known [equal amounts of DNA were loaded in each lane; this was also judged from the intensity of signals obtained in case of other gene probes (data not shown)]. When the experiment was repeated with a *Bam*HI-digest, similar differences in intensity of the polymorphic band were observed in G1A and VzmA compared to the maintainer lines (data not shown). While these stoichiometric differences in intensity may indicate the presence of the *atp9* on subgenomic circles (sublimons) in the Guntur and Vizianagaram lines, further studies will be required to confirm this.

Probing with *atpA* revealed no differences between the Maldandi, Guntur and Vizianagaram cytoplasms. However, the restriction fragment location of *atpA* in the sterile lines differed from their respective maintainer lines. In sterile lines fragments of sizes 4.0 kbp, 5.8 kbp and 8.0 kbp hybridized to the probe while in the maintainer lines the probe hybridized to bands of sizes 2.5, 4.0, 7.5 and 9.5 kbp (figure 1c). The pattern obtained here was opposite to that obtained for milo cytoplasm and its maintainers in the 2077 and 296 varieties (Sane *et al.* 1994a). With each of the three genes of the ATP synthase complex hybridization to multiple bands was observed, indicating the possible existence of more than one copy of these genes. This has been previously reported for *atp6* and *atpA* in the other varieties of sorghum (Bailey-Serres *et al.* 1986a; Mullen *et al.* 1992) and also appears to be the case in the Indian cytoplasms.

Polymorphism was also observed using the *rrn18* probe. In this case the gene was localized on fragments of sizes 2.6 kbp and 0.45 kbp in all the maintainer lines and also the sterile Vizianagaram line. In the sterile lines of Maldandi and Guntur, however, there was no hybridization to the 0.45-kbp band (figure 1d).

Besides these, differences were observed when blots were probed with *nad3* and *rps12*. In both cases, the probe hybridized to a fragment of size 3.1 kbp in all the sterile lines, while in the maintainer lines hybridization to a 4.6-kbp fragment was observed (figure 1e and figure 1f). Thus in all the three CMS cytoplasms the genes appeared to be located on a single fragment that was different from that in the respective maintainer lines.

3.2 Transcript analysis

A Northern analysis of these lines was performed to look for differences in gene expression pattern between the sterile and maintainer lines and between the individual cytoplasms. Results of probing with *nad3*, *nad4*, *coxI*, *cob*, *atp6*, *rps12* and *rrn26* are shown in table 2. No differences were observed in transcript pattern between the sterile and maintainer lines and between the three sterile cytoplasms in spite of the polymorphism seen in the Southern patterns. But in the Northern analysis with *atpA*, polymorphism was associated with major differences in the transcript patterns of the sterile and maintainer lines. All the sterile lines (Ma1A, G1A and Vzma) showed a single transcript of 2.1 kb. The respective maintainer lines, however, showed two transcripts:

Table 2. Sizes of various mitochondrial gene transcripts in the three Indian male sterile cytoplasms and their maintainer lines.

Gene	Size (kb)
<i>nad3</i>	1.8, 0.95, 0.6
<i>nad4</i>	1.85, 1.55, 2.5
<i>coxI</i>	1.8, 2.1
<i>cob</i>	1.8, 2.3
<i>atp6</i>	1.45
<i>rps12</i>	3.2, 1.8, 0.95
<i>rrn26</i>	3.2, 1.45

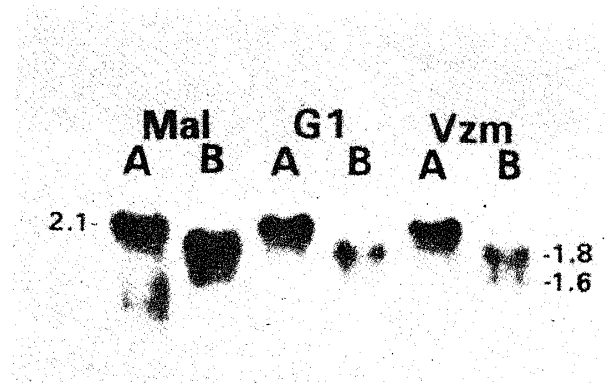


Figure 2. Northern blot hybridization of *atpA* gene probe to mitochondrial RNA from the Indian cytoplasms. A, male sterile lines; B, maintainer lines; MAL, Maldandi; G1, Guntur; VZM, Vizianagaram. The numbers on the panel indicate size in kb.

a major transcript of 1.8 kb and a minor transcript of 1.6 kb (figure 2). On longer exposure, an additional transcript of 2.5 kb was observed in the maintainer lines but not in the male sterile lines (data not shown). The differences observed with *atpA* were opposite to those observed in the 2077 and 296 milo varieties, where the longer transcript (2.1 kb) was present in the maintainer line and the shorter transcript (1.8 kb) was present in the male sterile line (Sane *et al.* 1994b). The difference of 300 nucleotides in the *atpA* transcripts of sterile and maintainer lines, however, appears to be a consistent feature of the various sorghum lines with the exception of the A₃ group of cytoplasm (P. Jaiswal, unpublished).

4. Discussion

In the present study we have attempted molecular analysis of three male sterile cytoplasms of sorghum, reportedly originating in three different places in India. Through studies on the organization and expression of various mitochondrial genes, we have attempted to determine their relations with each other and with their respective maintainers. To our knowledge this is the first report that describes the three Indian CMS cytoplasms and their maintainers in such detail at the molecular level. In a previous study, we reported that a characteristic feature of these male sterile cytoplasms of Indian origin was their possession of plasmids of sizes 5.8, 5.3, 2.3, 1.7 and 1.3 kb (Sane *et al.* 1994a).

Restoration to fertility of these cytoplasms has been rather difficult and, with the exception of Maldandi which shows partial restoration (Tripathi *et al.* 1985), none of the cytoplasms were known to have any restorer until recently. In fact the A₁ restorer line CS3541 acts as a maintainer for these lines. The three cytoplasms were included in the Indian A₄ group owing to many similarities among themselves (A.R. Dabholkar, personal communication). However, the correspondence of the Indian A₄ group with the American A₄ group has not been established. An important conclusion of our study is that the Indian A₄ group is different from the American A₄ group. This is evident from the restriction-fragment location and transcript pattern of *coxI* in the Indian

cytoplasms and the American A₄ and A₁ cytoplasms. A distinct polymorphism and a change in the transcript resulting in an extended *coxI* polypeptide has been reported in sorghum varieties of the American A₄ group but not in the A₁ (milo) group (Bailey-Serres *et al.* 1986a, b; Sane *et al.* 1994b). The Indian lines are identical to other groups of sorghum varieties in that they are nonpolymorphic with respect to *coxI*. Our studies also bring out the similarity with respect to the organization/localization of the *nad2*, *nad3*, *nad4*, *coxII*, *coxIII*, *cob*, *atpA*, *rps12* and *rps13* genes.

Differences between the individual cytoplasms, can, however, be seen when probed with *atp6* and *rrn18*. The studies reveal that the Vizianagaram cytoplasm is different from the Maldandi and Guntur types (figure 1, a and d) in lacking the additional *atp6* copy but possessing an additional *rrn18*-specific fragment. Differences among the cytoplasms are also apparent as RFLP with respect to *atp9*. While a clear distinction between the sterile and maintainer lines of Maldandi is observed with respect to presence of a 4.3-kbp band, the differences are not so clear between G1A/VzmA and G1B/VzmB. Stoichiometric differences in the intensity of this polymorphic band between A and B lines might be due to presence of this copy on sublimons, as reported for *atpA* in maize (Small *et al.* 1987). However, further studies will be required to confirm this. The comparison of RFLP patterns thus indicates that the Guntur cytoplasm is closer to the Maldandi cytoplasm than the Vizianagaram cytoplasm. This is interesting because earlier studies on pollen sterility, pollen shedding and seed setting indicated that the Guntur cytoplasm was closer to the Vizianagaram cytoplasm compared to the Maldandi cytoplasm (Rao *et al.* 1984). Subsequent studies on fertility restoration also indicated that while the Maldandi cytoplasm can undergo partial restoration, neither the Vizianagaram nor the Guntur cytoplasm were restored by any line (Tripathi *et al.* 1985). Taken together, this would mean that the similarity/differences in the mtDNA RFLP patterns of Mal/G1 and Vzm cytoplasms might be localized differences and incidental and may not be involved in the CMS phenotype.

Polymorphism only between the sterile and maintainer lines (but not between the three cytoplasms) could be observed with respect to *nad3*, *rps12* and *atpA* genes. Both *nad3* and *rps12* appear to be linked and cotranscribed as evident from Southern patterns and the fact that at least two transcripts, viz. 1.8 kb and 0.95 kb, hybridize to both. Cotranscription of *nad3* and *rps12* has been reported earlier in *Petunia* (Rasmussen and Hanson 1989), wheat and maize (Gualberto *et al.* 1988), and rice (Nakazono *et al.* 1995). In both *nad3* and *rps12*, the Indian cytoplasms showed a pattern that was opposite to that obtained for the milo cytoplasm (data not shown). However, the differences between the sterile and maintainer cytoplasms did not lead to any visible change in transcript pattern. Hence they are not likely to be implicated in male sterility.

The RFLP pattern obtained with *atpA* is interesting and indicates a possible rearrangement of gene locus between A and B lines. The polymorphism was accompanied by a change of 300 nucleotides in the transcript sizes of the Indian male sterile and maintainer lines. The differences could arise from differential transcription (either upstream or downstream of the gene) or differential processing in the sterile and maintainer lines, or, alternatively, from differences in the coding region of the gene. We are carrying out further characterization of the mitochondrial *atpA* gene and mitochondrial F₁ ATPase to see if it has any role in the CMS phenomenon in sorghum.

In conclusion, our work suggests that the tentatively designated A₄ Indian male sterile cytoplasms differ at the molecular level from the established American A₄ and A₁ cytotypes, and that they differ among themselves and can be differentiated on the basis of Southern and Northern analysis.

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