CYTOPLASMIC-NUCLEAR MALE STERILITY IN COTTON: COMPARATIVE RFLP ANALYSIS OF MITOCHONDRIAL DNA

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Abstract

The mitochondrial (mt) genomes from D_2 (*Gossypium harknessii*) male sterile-inducing cytoplasm and normal upland cotton AD_1 (*G. hirsutum*) cytoplasm were compared by restriction fragment length polymorphism (RFLP) analysis. In the RFLP analysis, seven heterologous probes and 5 restriction enzymes were used. The results showed considerable differences in the overall mt genomic structure between the two cytoplasms. With coxI, coxII, atp6, atp9, and atpA as probes, minor differences in fragment size were detected with one or two restriction enzyme digestions. The most notable difference was an additional fragment in normal cytoplasm detected by coxII in digests of four enzymes, and by coxI and atpA in digests with PstI. However, no difference in RFLP patterns was noted between male sterile (A) and restorer (R) lines with the D_2 cytoplasm, indicating that the presence of the D2 restorer gene does not affect mtDNA organization.

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited characteristic, and fertility restoration is dependent on the presence of nuclear restorer gene(s). Such systems are excellent for studying nuclear-cytoplasmic interactions. In the case of a CMS and restoring system, the interaction between the male sterile gene(s) in the cytoplasm and the restorer gene(s) in the nucleus has received tremendous attention. During the past 20 years, CMS systems in many different plant species have been characterized at the morphological, genetical, histological, cytological, physiological, biochemical and molecular levels. With the advent of molecular technologies, great effort has been made to understand whether mitochondrial DNA (mtDNA) or chloroplast DNA (cpDNA) is responsible for CMS, because both organelle genomes are maternally inherited in virtually all the CMS systems identified so far. In many cases (more than 20 plant species), based on DNA markers, e.g. restriction fragment length polymorphisms (RFLPs), the CMS mtDNA can be easily distinguished from its normal mtDNA. On the contrary, CMS cpDNA has much fewer differences from normal cpDNA, even though the difference between alloplasmic CMS and normal cytoplasm is evident in cpDNA. To identify CMS-associated genes in mtDNA, comparative studies among CMS, normal fertile, restored fertile and revertant cytoplasm lines have been used with such techniques as comparative RFLP patterns and physical maps of mtDNA. In all the cases where data are available, a substantial reorganization of the mtDNA structure has occurred in CMS cytoplasms. Mitochondrial RNAs were isolated to compare transcript patterns between normal and CMS cytoplasms to identify mtDNA regions responsible for CMS, and at the same time to gain insight into how mtDNA rearrangements affect gene expression. Identified CMS-associated genes have been chimeric, derived from fusions between portions of normal genes and one or more unknown DNA sequences. Novel mtDNA-encoded polypeptides have been further isolated and characterized to establish the correlation between CMS-associated chimeric open-reading frames (orfs) and the novel proteins. In several plant species, CMS-associated orfs were isolated and used to generate transgenic plants for assaying their functions in CMS. Comprehensive reviews can be found in Schnable and Wise (1998), and Kempken and Pring, (1999).

In cotton, a CMS line HAMS277 was induced by transferring the cytoplasm of diploid *Gossypium harknessii* (D_2) into tetraploid *G. hirsutum* (AD_1) nuclear background, and its homologous fertility restorer line HAF277 and maintainer line HAB277 were developed simultaneously (Meyer, 1975). This system provided a promising way to utilize F_1 heterosis in cotton. The cytogenetic mechanism of the CMS was studied, but no report has been published on its molecular mechanism. The objective of this study is to compare the differences of mtDNA between the D_2 male sterile cytoplasm and the normal AD_1 cytoplasm by RFLP analysis.

Materials and Methods

Plant Materials and DNA Extraction

Cotton CMS line, HAMS277 (A line), and its corresponding maintainer line, HAB277 (B line) and fertility restorer line (R line) were planted on the Experimental Farm, Huazhong Agricultural University. Newly expanded young leaves less than 7 days of age were frozen at –80ºC or with liquid nitrogen and ground with a mortar and pestle chilled at -20 ºC. For each genotype five grams of the fine powder were placed in a 50 ml centrifuge tube. To each tube was added 200 µl β-mercaptoethanol and 20 ml lysis buffer [0.1M Tris-HCl, pH8.0, 1.4 M NaCl, 0.02 M Na_2-EDTA pH8.0, 0.2% (W/V) cTAB, 0.1% (W/V) DIECA, 2% (W/V) PVP]. The tubes were preheated at 90ºC to re-suspend the powder quickly and then incubated at 65 ºC for 30 minutes. The suspension was extracted twice with 20 ml chloroform: isoamyl alcohol (24:1). The supernatant was then mixed with

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2/3 volume of chilled isopropanol, and stored at -20°C for 2 hours. The DNA was hooked out, washed with 76% ethanol three times, air dried, and dissolved in 2 ml TE buffer. After addition of 20 µg RNase, and incubation at 37°C for 1 hour, the DNA solution was extracted with chloroform:isoamyl alcohol (24:1) once. The DNA supernatant was pipetted out, and mixed with 1/10 volume of 3 M NaAc, and 2 volume of absolute ethanol. The cleaned DNA was hooked out, air dried, dissolved in 500 µl of TE buffer, and stored at -20°C for future use.

Southern Blot Analysis

Five 20 µg DNA factions were digested separately with BamHI, EcoRI, EcoRV, HindIII, and PstI. The digested DNA was electrophoresed in a 0.8% agarose gel, and transferred to nylon membranes following Sambrook et al (1989).

Southern hybridization was as follows. Seven heterologous mtDNA probes, coxI, atp6 (from maize), coxII, apt9, atpA (from rice), and cob, rrn26 (from wheat) were labeled with ^{32}P. For prehybridization, each blot (200 cm^2) was incubated at 65°C for 5 to 6 hours in 25 ml of prehybridization buffer consisting of 17 ml ddH_2O, 0.5 ml 50X denharts, 0.5 ml 10% SDS, 6.25 ml P/H stock, and 0.25 ml ssDNA. For hybridization, the blot was incubated at 65°C for 12-15 hrs in buffer with 1 g dextran, 6.8 ml ddH_2O, 0.2 ml 50X denharts, 0.2 ml 10% SDS, 2.7 ml P/H stock, and 0.1 ml ssDNA. Membranes were washed in 2X SSC, 0.1% SDS twice at room temperature, 5 minutes each time; 1X SSC, 0.1% SDS once at 42°C for 15 minutes; and 0.1X SSC, 0.1% SDS at 65°C four times for 5 minutes each. The membranes were exposed to Fuji film.

Results and Discussion

The A line of the CMS-D2 system was male sterile because it carried the *G. harknessi*i (D_2) male sterile-inducing cytoplasm with upland cotton nuclear background. The B line, the maintainer line for the A line, had the same nuclear background as the A line but normal upland cotton cytoplasm. The R line was male fertile even though it also had the *harknessii* cytoplasm, because the dominant restorer gene (Rf_1) inhibited the male sterile cytoplasmic effect. Theoretically, the A line and R line should have the same mtDNA genome since both of them carry the same D_2 cytoplasm, whereas the B line is expected to have some differences in mtDNA structure since it carries the normal upland cotton cytoplasm. The mtDNA from the three lines was digested with five restriction enzymes, transferred to nylon membranes, and hybridized to seven heterologous mtDNA probes. The numbers of major bands present in different enzyme/probe combinations were summarized in Table 1.

With coxI as a probe, 3-5 fragments were produced. As shown in Figure 1a, the digestion by BamHI, EcoRI, and EcoRV gave similar RFLP banding patterns among A, B, and R lines. However, compared with B line with upland cotton cytoplasm, A and R line with D_2 cytoplasm had an extra fragment when the mtDNA was digested with HindIII, while they lack one PstI fragment that was present in the B line.

When the mtDNA was hybridized with a coxII probe, a significantly different banding pattern was observed with respect to digestion by all five restriction enzymes (Figure 1b). Even though all three lines showed only one BamHI-digested fragment, while the B line had a slightly smaller fragment. With the other four restriction enzymes, the B line had two fragments, while the A and R line only had one fragment with the same size depending on enzymes. The result indicates that the copy number of the coxII gene could be different between the D_2 and AD_1 cytoplasms.

With atp6 as the probe, both A and R lines had a slightly larger BamHI fragment, but no difference could be seen with the digestion from other enzymes (Figure 1c). However, with atp9, the EcoRI fragment in the A and R lines was slightly smaller than that in the B line (Figure 1d).

With the atpA probe, RFLP were notable among the three lines when the restriction enzymes EcoRI, HindIII and PstI were used for the mtDNA digestion (Figure 1e). Compared with the B line, both A and R lines had a significantly smaller EcoRI fragment and a slightly larger HindIII fragment, but they had one less PstI fragment.

The probes cob and rrn26 did not detect any differences among the three lines or five restriction digests (Figure 1f and 1g).

In summary, RFLP among the three lines reflect the mt genomic differences between the male sterile D_2 cytoplasm and normal upland cotton cytoplasm. The most striking differences involve coxII and atpA genes. The differences detected could be due to recombination between non-coding intergenic regions, variations in the copy numbers of mt genes and sequence variations within or around the coding regions. At present, we are not certain that these differences are related to expression of the CMS-D2. However, it is clear that the D_2 restorer gene does not change the mt genome organization since both A and R lines showed the same RFLP patterns with the seven probes and five restriction enzymes used. The present investigation represents the first attempt in exploring the mechanisms of CMS in cotton. Further studies will be needed to obtain a clearer picture regarding mechanisms of CMS and its restoration in cotton.

References

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Table 1. Number of bands for each of enzyme/probe combination

Probe	BamHI	EcoRI	EcoRV	HindIII	PstI
coxI	5	3	3	5	5
coxII	1	1	2	2	3
atp6	1	1	1	1	1
atp9	1	1	1	1	1
atpA	3	3	2	2	3
cob	1	1	1	2	1
rrn26	7	7	3	5	3

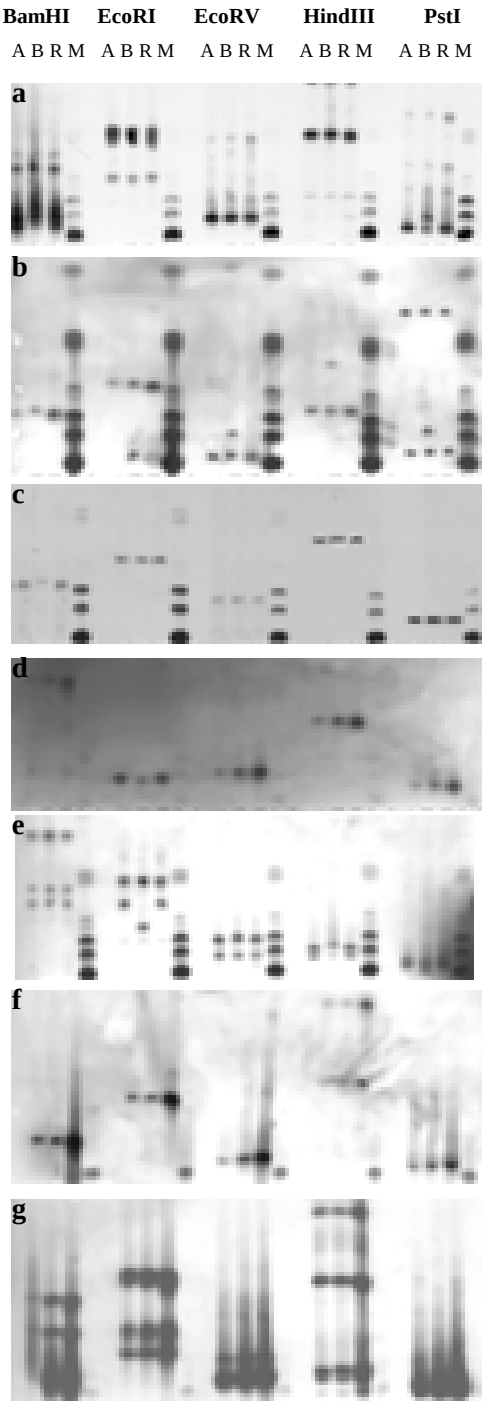

Figure 1. RFLP analysis of mtDNA from A, B and R lines. M-DNA ladder. a. DNA probed with coxI. b. DNA probed with coxII. c. DNA probed with atp6. d. DNA probed with atp9. e. DNA probed with atpA. f. DNA probed with cob. g. DNA probed with rrn26.