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RFLP analysis of mitochondrial DNA in two cytoplasmic male sterility systems (CMS-D2 and CMS-D8) of cotton

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Abstract Cytoplasmic male sterility (CMS) in higher plants is a maternally inherited trait and CMS-associated genes are known to be located in the mitochondrial genome. However, CMS-inducing genes in CMS-D2 and CMS-D8 of Upland cotton (*Gossypium hirsutum* L., AD1) are currently unknown. The objective of this study was to identify potential candidate DNA or gene sequences for CMS-D2 and CMS-D8 through restriction fragment length polymorphism (RFLP) analysis. Seven mtDNA gene probes and five restriction enzymes were first used to compare D2 (from *G. harknessii* Brandegee) and AD1 cytoplasms. With *cox1*, *cox2*, and *atp1* as probes, RFLP polymorphisms were detected with one or more restriction enzyme digestions. The most notable difference was an additional fragment in the normal AD1 cytoplasm detected by *cox2* in digests of three enzymes, and by *cox1* and *atp1* in digests with *Pst*I. The RFLP analysis was then conducted among CMS-D2, CMS-D8 (from *G. trilobum* (DC.) Skovst.), and AD1 cytoplasms. Two probes from maize, *atp1* and *atp6*, detected polymorphism among the different cytoplasmic lines. However, no difference in RFLP patterns was noted between male sterile (A) and restorer (R) lines with the D2 or D8 cytoplasm, indicating that the presence of the D2 or D8 restorer gene does not affect mtDNA organization in Upland cotton. The results demonstrate that RFLP using *atp1* and *atp6* as probes can distinguish the three cytoplasms. The *atp1* and *atp6* in CMS-D8 and these two genes together with *cox1* and *cox2* in CMS-D2 could be the candidates of CMS-associated genes in the mitochondrial genome, providing information for further molecular studies and developing PCR-based markers for the CMS cytoplasms in breeding. This research represents the first work using RFLP to analyze the genetic basis of CMS in cotton.

Keywords Cytoplasmic male sterility · Cotton · CMS-D2 · CMS-D8

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Introduction

Mitochondria are the cellular sites for key metabolic pathways such as the tricarboxylic acid cycle, respiratory electron transfer, and ATP synthesis (Chase 2006). To date, 14 mitochondrial genomes have been sequenced in plants including: *Arabidopsis thaliana* L. (Unseld et al. 1997), sugar beet (*Beta vulgaris* L. subsp. *vulgaris*, Kubo et al. 2000), canola (*Brassica napus* L., Handa 2003), *Cycas taitungesis* C.F. Shen,

K.D. Hill, C.H. Tsou & C.J. Chen (Chaw et al. 2008), tobacco (*Nicotiana tobacco* L., Sugiyama et al. 2005), *Oryza sativa* L. subsp. *indica* (Tian et al. 2006), *Oryza sativa* L. subsp. *japonica* (Notsu et al. 2002), sorghum (*Sorghum bicolor* L., Allen et al. 2006), *Tripsacum dactyloides* L. (Allen et al. 2006), wheat (*Tritium aestivum* L., Ogihara et al. 2005), *Zea luxurians* L. (Allen et al. 2006), maize (*Z. mays* L. subsp. *mays*, Clifton et al. 2004), *Z. mays* L. subsp. *parviglumis* (Allen et al. 2006), and *Z. parennis* (Hitchc.) Reeves & Manglesdorf (Allen et al. 2006). The mitochondrial genome in plants encodes about 50–60 gene products, a small fraction of the gene products needed for mitochondrial functions. For example, the mitochondrial DNA of *A. thaliana*, has 366,924 nucleotides, 10% of which code for 57 genes, including those encoding for proteins that are components of the electron transport chain and the F_1F_0-ATPase, essential to produce ATP (Unseld et al. 1997).

Cytoplasmic male sterility (CMS) is defined as a maternally inherited trait that prevents the production of functional pollen or male gametes in plants (Schnable and Wise 1998). CMS has been widely used in plant breeding for the production of F1 hybrids, such as maize, sorghum, onions (*Allium cepa* L.), sugar beet, sunflower (*Helianthus annuus* L.), carrot (*Daucus carota* L.), canola, and rice (Budar and Pelletier 2001). CMS is associated with chimeric genes in the mitochondrial genome, which express novel open reading frames (ORFs) in most CMS plants (Schnable and Wise 1998). The mitochondrial genes associated with CMS are proposed to arise by aberrant recombination events or by intra-molecular rearrangement of the plant mitochondrial genome (Hanson and Bentolila 2004). The genes associated with CMS have been reported in numerous plant species: maize (Dewey et al. 1986), petunia (*Petunia hybrida* (Hook.) Vilm, Young and Hanson 1987), bean (*Phaseolus vulgaris* L., Johns et al. 1992), canola (Singh and Brown 1991; Bonhomme et al. 1991, 1992; Brown 1999; Grelon et al. 1994), radish (*Raphanus sativus* L., Makaroff et al. 1990), sunflower (Moneger et al. 1994), sorghum (Tang et al. 1996), rice (Akagi et al. 1995; Zhang et al. 2007), sugar beet (Yamamoto et al. 2005), and pepper (*Capium annuum* L., Kim et al. 2007). Most of these novel *orfs* are composed of fragments of genes for subunits of the ATP synthase complex, such as *atp1* (or *atpA*), *atp6*, *atp8*, and *atp9* (Hanson and Bentolila 2004; Chase 2006).

CMS systems have also been studied in cotton (*Gossypium hirsutum* L.). There are two internationally recognized cotton CMS systems, CMS-D2 and CMS-D8. CMS-D2 was developed by transferring the cytoplasm of wild *G. harknessii* Brandegee (D2) diploid cotton into tetraploid Upland cotton (*G. hirsutum*, AD1) (Meyer 1975). The alloplasmic CMS-D8 was derived from introducing *G. trilobum* (DC) Skovst (D8) cytoplasm into Upland cotton (Stewart 1992). Two different dominant genes, Rf_1 and Rf_2, restore the fertility for CMS-D2 and CMS-D8, respectively. Rf_1 can also recover fertility of CMS-D8, whereas Rf_2 only restores fertility of CMS-D8 (Zhang and Stewart 2001a, b). Zhang et al. (2004) designed 36 primer pairs based on *A. thaliana* mitochondrial DNA gene sequences. These mtDNA universal primers were used to amplify three alloplasmic lines, i.e., fertile AD1, CMS-D2, and CMS-D8. The absence of sequence tagged site (STS) polymorphisms among the three cytoplasms proved that the mitochondrial DNA genes in cotton are highly conserved in sequence length as in other plant species. Zhang et al. (2008) further compared gene expression between CMS-D8 restored plants (Rf_2rf_2) and normal non-restoring fertile plants (rf_2rf_2) in cotton by mRNA differential display. The results identified four genes that were up-regulated and 22 genes, including starch synthase (SS), which were down-regulated. The down-regulated SS explained the lack of starch accumulation in sterile rf_2 pollen grains in the heterozygous restored plants.

The mitochondrial CMS loci in the alloplasmic CMS-D2 and CMS-D8 are currently unknown. The objective of this study was to identify potential candidate mtDNA regions or gene sequences uniquely found in CMS-D2 and CMS-D8 through restriction fragment length polymorphism (RFLP) analysis. These novel mtDNA regions, not found in fertile cytoplasms would be candidate CMS loci for the D2 and D8 systems in cotton.

Materials and methods

Plant materials and DNA extraction

In Experiment 1, three lines (Meyer 1975), i.e., CMS-D2 line, HAMS277 (A line, $rf_1rf_1rf_2rf_2$ with D2 cytoplasm), its corresponding maintainer line, HAB277 (B line. $rf_1rf_1rf_2rf_2$ with AD1 cytoplasm), and fertility restorer

line (R line, $Rf_1Rf_1rf_2rf_2$ with D2 cytoplasm) were used. In Experiment 2, five Upland cotton lines were used. (1) CMS-D8-8518 (Stewart, unpublished), a CMS line, carries CMS-D8 cytoplasm from American diploid wild species *G. trilobum* (D8 genome) and has the non-restoring $rf_1rf_1rf_2rf_2$ nuclear background of 8518. The breeding line, 8518 (Bourland 1996), is a normal male fertile line with fertile AD1 cytoplasm and non-restoring $rf_1rf_1rf_2rf_2$ nuclear background. (2) Stoneville 474 (ST474) is a normal male fertile commercial cultivar with fertile AD1 cytoplasm and non-restoring $rf_1rf_1rf_2rf_2$ nuclear background. (3) D8R-8518 (Stewart, unpublished) is a restorer line with CMS-D8 cytoplasm and the restorer genotype $rf_1rf_1Rf_2Rf_2$ in the 8518 background. (4) D8R-ST474 (Stewart, unpublished) is a restorer line with the CMS-D8 cytoplasm and the restoring genotype $rf_1rf_1Rf_2Rf_2$ in ST474 background. (5) D2R-B418R (Cook and Namken 1995) is a restorer line with CMS-D2 cytoplasm from American diploid wild species *G. harknessii* (D2 genome) and the restorer genotype $Rf_1Rf_1rf_2rf_2$. Total DNA from the above genotypes were extracted using a cTAB method with (Zhang and Stewart 2000) or without modifications (Paterson et al. 1993).

Southern blot analysis

Genomic DNA (10 or 20 μg) was digested separately with *Ava*I, *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I or *Xba*I; blots were prepared, hybridized and washed as described (Rodriguez-Uribe and O'Connell 2006). Probes for hybridization were prepared using cloned mtDNA genes: wheat for *cox1*, cotton for *cox2* and *cox3* (Wang 2008), maize for *atp1* (*atpA*) (Braun and Levings 1985), *atp6* (Dewey et al. 1986), *cob* and *rrn26* (26S rRNA) (Stern et al. 1982), and petunia for *atp9* (Young and Hanson 1987).

Results

Comparison between CMS-D2 and AD1 mtDNA

The CMS-D2 cytoplasm had the same mtDNA genome organization in the A or R nuclear background for all seven genes tested (Fig. 1). Four genes, *atp6*, *atp9*, *cob*, and *rrn26* (Fig. 1d–g) showed no polymorphism in mtDNA from CMS-D2 line and the fertile Upland cotton (AD1) cytoplasm or the maintainer B line. With *cox1* as a probe, a novel *Pst*I fragment was observed in the AD1 cytoplasm (Fig. 1a). When *cox2* was the probe, polymorphisms were detected in at least three digests from three of the five restriction enzymes (*Eco*RV, *Hin*dIII, and *Pst*I). As compared with the CMS-D2 cytoplasm, the AD1 mtDNA had an extra fragment of various sizes

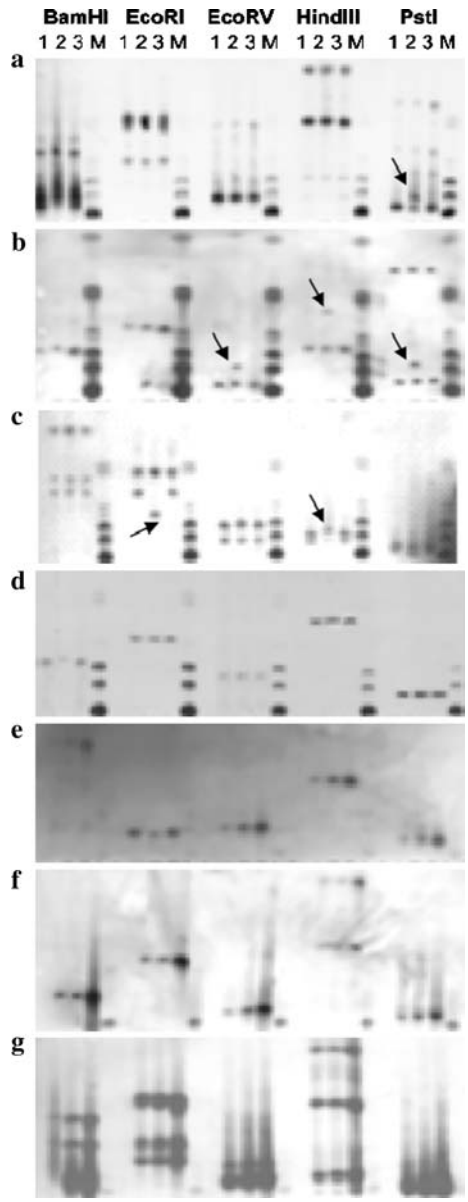

Fig. 1 RFLP analysis of mtDNA from CMS A (1), maintainer B (2) and restorer R (3) lines. M-DNA ladder. Blots probed with **a** *cox1* b *cox2* **c** *atp1* **d** *atp6* **e** *atp9* **f** *cob* **g** *rrn26*. Fragment sizes of polymorphic fragments are indicated in kb and marked by *arrowheads*

(Fig. 1b). With the *atp1* as the probe, the CMS-D2 cytoplasm had a larger *Eco*R1 fragment, while the AD1 cytoplasm had a smaller fragment. In the *Hin*dIII digest, the AD1 cytoplasm fragment was slightly larger (Fig 1c).

Comparison between CMS-D8 and AD1 mtDNA

To evaluate the possible effect of nuclear backgrounds on mtDNA organization RFLP analyses were performed on a B line, three alloplasmic lines with the CMS-D8 cytoplasm, and a D2 restorer line containing the D2 cytoplasm for a comparison. Probes for *atp9*, *cox1*, *cox2*, *cox3*, and *rrn26* did not reveal polymorphic mtDNA RFLPs among the five lines containing three different cytoplasms; the results for *cox3* are presented as an example in Fig 2. The maize *atp1* probe revealed polymorphic RFLP among CMS-D8, CMS-D2, and AD1 cytoplasms (Fig. 2). Specifically, the *atp1* probe detected RFLP polymorphisms with the *Eco*RI digestion. All the cytoplasms shared a common fragment of 3.0 kb. The 4.5 kb fragments were only present in CMS-D2 cytoplasm, while the 5.5 kb fragment was unique in CMS-D8 cytoplasm. The 6.0 kb fragment was present in AD1 cytoplasm but absent in both CMS-D8 and CMS-D2 cytoplasm.

The maize *atp6* probe also revealed RFLP polymorphisms among the three cytoplasms (Fig. 2). For *Eco*RI digested cotton genomic DNA, the *atp6* probe detected polymorphism among the three cytoplasms. A 1.5 kb fragment was only present in CMS-D8 cytoplasm (Lane 2–4), but absent in AD1 cytoplasm. The fragment in CMS-D2 cytoplasm was larger (Lane 5). Both CMS-D8 and AD1 cytoplasm had two common fragments, i.e., 3.0 and 4.0 kb, while the respective fragments in CMS-D2 cytoplasm were larger.

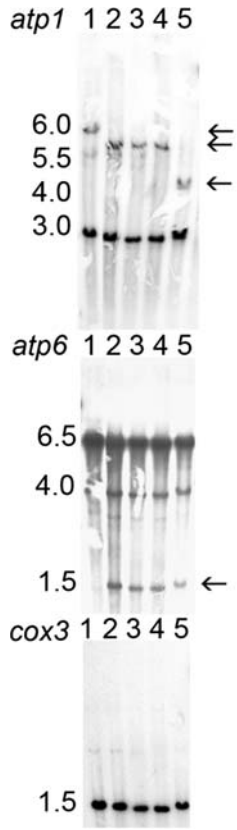

Fig. 2 Autoradiograph of *Eco*RI digested cotton genomic DNA, probed with mitochondrial genes. *Lane 1*. Stoneville 474 (ST474), a male fertile line with AD1 cytoplasm. *Lane 2*. D8R-ST474, a restorer line with CMS-D8 cytoplasm. *Lane 3*. D8R-8518, a restorer line with CMS-D8 cytoplasm. *Lane 4*. CMS-D8-8518, a CMS line, with CMS-D8 cytoplasm. *Lane 5*. D2R-B418R, a restorer line with CMS-D2-2 cytoplasm. Sizes of hybridizing fragments are indicated, polymorphic bands are marked with arrowheads. Fragment sizes of polymorphic fragments are indicated in kb

Discussion

Since the CMS cytoplasms studied here are alloplasmic from two wild diploid D-genome cotton species, it is expected that both chloroplast and mitochondrial genomes in the CMS cytoplasms are different from one another and from that of tetraploid Upland cotton. Indeed, Wendel and Albert (1992) used chloroplast DNA RFLP analysis to construct a phylogenetic tree of 40 species in *Gossypium*. Both chloroplast and mitochondrial DNA RFLP analyses also confirmed that tetraploid cotton contains the A genome-like cytoplasm (Galau and Wilkins 1989; Wendel 1989; Small and Wendel 1999). Galau and Wilkins (1989) furthered demonstrated that the D2 chloroplast DNA was maintained in the alloplasmic CMS-D2 lines of Upland cotton. Our current mtDNA RFLP analysis indicates that both CMS-D2 and CMS-D8 have different mitochondrial genomes from that of Upland cotton. In all cases where polymorphic mtDNA RFLP was detected, the Upland cotton B lines had the same RFLPs, which were different from the D2 restorer line containing the D2 cytoplasm or the three lines (one A line and two R lines) containing the CMS-D8 cytoplasm which had the same RFLPs. Therefore, the present study provides evidence that the exotic mtDNAs have been maintained in the CMS-D2 and CMS-D8 cytoplasms. By including both CMS lines and restorer line(s) with the CMS-D2 or CMS-D8 cytoplasms, our study also clearly demonstrates that, as with most other CMS systems, the D2 or D8 restorer gene does not change the mitochondrial genome organization, since both A and

R lines showed the same RFLP patterns when various probe and restriction enzyme combinations were used. It has been documented that tissue culture may induce structural mutations of mitochondrial genes in plants (Sadoch et al. 2000). The presence of the restorer gene (*Fr*) results in the permanent deletion of the mitochondrial CMS-inducing sequence (*pvs*) in common bean (Mackenzie and Chase 1990).

The current study represents the first attempt to employ mtDNA RFLP to analyze mitochondrial genomes of CMS cotton towards a better understanding of male sterility mechanism conditioned by exotic cytoplasms in cotton. Clear mtDNA RFLPs between CMS-D2 cytoplasm and AD1 cytoplasm were seen in *cox1*, *cox2*, and *atp1*, while no apparent polymorphism was observed in *atp6* and *atp9*. Further analyses, included CMS D8, presented in Fig. 2 did confirm the results for *atp1* and *atp9*; however, polymorphisms for *cox1* and *cox2* were not detected in CMS-D8, while polymorphisms for *atp6* were seen. The discrepancies may be due to the use of different probes and enzymes in the two experiments. Taken together, CMS-D2 showed different mtDNA RFLPs from the AD1 cytoplasm in *atp1*, *atp6*, *cox1*, and *cox2*; while CMS-D8 showed mtDNA RFLPs in *atp1* and *atp6*. The present study demonstrated that mtDNA RFLP is a reliable method to distinguish the two different alloplasmic CMS lines in cotton using *atp1* and *atp6* as probes. This provides useful information for developing more convenient and portable PCR-based markers to assay CMS cytoplasms for hybrid cotton breeding.

At present, it is still not understood as to how these mtDNA differences are related to the expression of the male sterility in CMS-D2 or CMS-D8. With the completion of sequencing of two chloroplast cotton genomes (Ibrahim et al. 2006; Lee et al. 2006) and a number of other plant mitochondrial genomes (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=33090&opt=organelle), the 700-kb cotton mitochondrial genome (Hsu and Mullin 1989) should be sequenced in comparison with the CMS-D2 and CMS-D8 mtDNA genomes. This will allow a mitochondrial genome-wide comparative analysis, but tremendous sequence variations are expected between cytoplasms from different species. This will undoubtedly complicate the identification of CMS-causative factors, as exemplified in sugar beet and maize (Satoh et al. 2004, 2006; Allen et al. 2007). Other avenues will be needed to obtain a clearer picture regarding the mechanism of CMS and its restoration in cotton.

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