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A. Chahal · H. S. Sidhu · D. J. Wolyn

A fertile revertant from petaloid cytoplasmic male-sterile carrot has a rearranged mitochondrial genome

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Abstract A spontaneously derived fertile plant was recovered from a petaloid cytoplasmic male-sterile (CMS) carrot inbred line. Genetic analysis indicated a single nuclear gene was responsible for the restoration to fertility. Within a family segregating for the nuclear restorer in combination with the sterility-inducing cytoplasm, fertile plants were recovered that could not restore fertility when crossed to sterile genotypes. Genetic analysis indicated cytoplasmic reversion for fertility, and Southern analysis, comparing mtDNA organization of the fertile revertant and its CMS progenitor, identified mitochondrial genome rearrangements. Hybridization of cosmids representing a 108-kb subgenomic circle of the sterile line to DNA of a fertile maintainer and fertile revertant lines indicated a similar mtDNA organization for these genotypes that was distinct from that of the sterile line. Six restriction fragments totalling 43.2 kb were common to the fertile maintainer and revertant and absent in the sterile; other restriction fragments totalling 38.2 kb were present only for the sterile line. Unique fragments of low stoichiometry, two for the fertile maintainer and three for the revertant, distinguished these lines. The reversion to fertility in the sterile line could have resulted from the amplification of a mitochondrial submolar genome highly homologous to that found in the fertile maintainer line.

Key words Carrot CMS · Fertile revertant · Mitochondrial DNA · Subgenomic molecules · Cosmid mapping

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A. Chahal · H. S. Sidhu · D. J. Wolyn (✉)
Department of Horticultural Sciences, University of Guelph, Guelph, Ont., Canada N1G 2W1
Fax: +1 519-767-0755
E-mail: dwolyn@evbhort.uoguelph.ca

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait that prevents the production of viable pollen without affecting female fertility (Banga et al. 1964). In hybrid seed production of economically important plant species, CMS lines are used as female parents.

CMS-associated genes have been identified in mitochondrial (mt) genomes through the comparative mapping of sterility- and fertility-inducing genomes (L'Homme and Brown 1993), transcript analysis in sterile and restored lines (Singh and Brown 1991; Dewey et al.1986) and analysis of spontaneously derived fertile revertant plants (Fauron et al. 1990; Mackenzie et al. 1988; Gengenbach and Connelly 1981). From the latter, new insights into mitochondrial genome organization and reversion of CMS lines to fertility have been developed. For common bean CMS, fertility restoration by a dominant allele for the *Fr* gene was irreversible (Mackenzie and Bassett 1987) and associated with the loss of a mitochondrial chromosome containing a unique sequence, *pvs*, (Janska and Mackenzie 1993) encoding two open reading frames, ORF98 and ORF239 (Johns et al. 1992). The *Fr* nuclear gene appeared to promote cytoplasmic reversion to fertility. Deletion of the maize T-*urf13* gene has been reported in several fertile revertants from CMS-T cytoplasm (Rottmann et al. 1987). The physical map of the mitochondrial genome for a maize fertile revertant line revealed a 165-kb duplication and a 0.423-kb deletion (including the T-*urf13* gene) when compared with that of the male- sterile (cms-T) progenitor (Fauron et al. 1990). The reorganization resulted from recombination events among repeated mtDNA sequences.

In contrast to fertile revertants arising from altered mitochondrial genomes, fertility can be restored through the interaction of nuclear genes with a sterility-inducing cytoplasm. In maize, a nuclear restorer gene, *Rf1*, decreased the abundance of T-*urf13* transcripts

and URF13 protein by about 80%, and this reduction was attributed to RNA processing (Dewey et al. 1987; Kennell et al. 1987; Kennell and Pring 1989; Wise et al. 1996). Fertility restoration in CMS cytoplasms of rice and sorghum has also been correlated with the internal processing of CMS-specific mitochondrial transcripts (Iwabuchi et al. 1993; Tang et al. 1996) and in *Brassica napus*, alleles for a restorer gene of *pol* CMS cosegregated with two mitochondrial transcripts (Singh et al. 1996). In contrast, the *Fr2* restorer gene of bean appeared to affect mitochondrial CMS gene expression translationally or posttranslationally (Abad et al. 1995).

The objectives of the study presented here were to understand the genetic mechanism of reversion to fertility in a green-petaloid CMS carrot breeding line and to identify and map a sterility-associated region of the mitochondrial genome. Inheritance studies of the revertant indicated a genetic reversion in the cytoplasm. Partial mapping of the mitochondrial genome from the CMS line and comparative analysis with the revertant suggested complex organizational changes with reversion that could have arisen from the amplification of a substoichiometric genome in the sterile plant, a process common to fertile maintainer lines.

Materials and methods

Plant material

Four sources of petaloid CMS isolated independently from wild carrot populations were used: Cornell (Thompson 1961), Wisconsin (Morelock 1996), Guelph-202 and Guelph-301. An inbred carrot line with Cornell cytoplasm, W259A, and its isonuclear fertile maintainer line, W259B, were obtained from Dr. W.H. Gabelman, University of Wisconsin, USA. The 2566S petaloid line with Wisconsin cytoplasm was obtained from Dr. P.W. Simon, USDA-ARS/University of Wisconsin, USA. Guelph-202 and Guelph-301 petaloid accessions were found in wild carrot populations near Guelph, Ontario. A spontaneously derived restored genotype (W259A-R) and a fertile cytoplasmic revertant (W259A-RC) were derived from the stable CMS (W259A) line.

Roots were grown in the field at Cambridge, Ontario, harvested, and vernalized for 10 weeks at 4°C before planting in the greenhouse. Plants were grown at 18°/15°C (day/night) with a 16-h photoperiod until flower stalks appeared; then the temperature was increased to 22°/18°C.

Inheritance studies

Carrot umbels were bagged before anthesis to avoid cross-pollination. At anthesis, a cross between a CMS female parent and a fertile male parent was made by putting both plants together in a sealed cloth cage, and housefly pupae were added weekly to cages for 6 weeks. For phenotypic analysis, two to five umbellets from the centre of primary and secondary umbels of each plant were observed under a dissecting microscope. Flowers with anthers were scored as fertile (F), and those with petaloid flowers were scored as sterile (S).

DNA isolation and electrophoresis

A protocol of Thorsteinson and Wolyn (1994) was followed to isolate total DNA and mtDNA from carrot roots (200 g per sample), leaves or flowers (10 g per sample). Agarose gel (0.6%) electrophoresis of DNA was performed as described by Sambrook et al. (1989).

Southern analyses

Southern analyses were conducted with seven DNA probes for 19 fertile and 16 sterile plants from selected families segregating for a single restorer gene derived from W259A-R, in combination with the sterility-inducing cytoplasm, except for cross no. 9 (Table 1). DNA was isolated from carrot umbels as described above. Probes were maize *atp6* and *atp9* (Dewey et al. 1985a,b, supplied by Dr. C.S. Levings, North Carolina State University, USA), three distinct 6.7-kb *Bam*HI restriction fragments of carrot mtDNA (p1915, p1919 and p1403) a 5.1-kb carrot mtDNA *Bam*HI fragment (p1904) and total mtDNA. Umbel DNA from 3 fertile and 3 sterile progeny of cross no. 9 (Table 1), 4 CMS plants (W259A) and 4 fertile maintainer plants (W259B) were subjected to Southern analyses with the *atp6*, *atp9* and p1915 probes.

DNA fragments were labeled as probes for Southern blotting, either radioactively with [^{32}P]dCTP following the protocols of Sambrook et al. (1989), or nonradioactively with a DIG-labeled nucleotide (DIG-dUTP) according to the DIG System User's Guide for Filter Hybridization (Boehringer Mannheim 1993). Prehybridization and hybridization temperatures were 50°C for the heterologous probes from maize (*atp6* and *atp9*) or 65°C for the probes from carrot mtDNA.

Construction and screening of a mtDNA cosmid library

A root mtDNA cosmid library for the green petaloid CMS line (W259A) was constructed using a SuperCos 1 cosmid vector according to the vendor's protocol (SuperCos 1 cosmid vector kit, Instruction Manual, 1994, Stratagene). One microgram of ligated DNA was packaged into bacteriophage lambda particles using in vitro packaging lysates according to the protocol of the supplier (Lambda Packaging System, GIBCO BRL). The protocol of Sambrook et al. (1989) was followed for screening of the cosmid library by in situ colony hybridization except for the use of DIG-labeled probes.

Restriction mapping of mtDNA

About 50 cosmid clones, containing inserts in the size range from 25 to 45 kb, were selected for *Bam*HI and *Eco*RI restriction mapping by a partial digestion method (FLASH® nonradioactive gene mapping kit and instructions, Stratagene). The partially digested *Not*I cassette DNA (including the entire mtDNA insert flanked by T3 and T7 promoters) was hybridized separately with DIG-labeled oligonucleotides specific for T3 or T7 promoters. 'Genomic walking' was performed by generating T3/T7 end-specific RNA probes from isolated cosmids (DIG RNA labeling kit, Boehringer Mannheim) for subsequent hybridizations to the cosmid library.

Southern analysis of sterile, fertile maintainer and revertant lines

To determine the similarity of mitochondrial genomes from sterile, fertile maintainer and revertant lines, we used three cosmids

encompassing a 108-kb subgenomic circle as probes in Southern hybridizations to all three genotypes. Total DNA was digested for 12–16 h with 20 U *Bam*HI/μg DNA. *Bam*HI-digested total DNAs from each of 4 individual plants for W259A and W259B and 3 individual plants from revertant W259A-RC were assessed.

Results

Fertility in a revertant was cytoplasmically inherited

A partially fertile plant (W259A-R) was recovered from a petaloid CMS breeding line (W259A). Few seeds from self-pollination were recovered on the original plant, and 4 progeny, 3 fertile and 1 partially fertile, were observed. Genetic analyses initiated with these plants indicated that fertility resulted from a dominant allele for a nuclear restorer gene (Table 1, crosses 2–14). Mendelian inheritance, 3:1 and 1:1 segregation, was observed when plants were self-pollinated and backcrossed, respectively. Fertility was also restored with Mendelian segregation to CMS accessions isolated independently from wild carrot (crosses 11–14). In 13 crosses (nos. 2–14) over several generations, restoration was inherited predictably. Fertile progeny from 1 cross (no. 9), however, failed to restore fertility upon backcrossing to sterile cytoplasms (crosses 16–19), and no segregation was observed when a fertile plant, thought to be heterozygous for restorer alleles in combination with a sterility-inducing cytoplasm, was self-pollinated (cross 15). Fertile plants from the exceptional cross were considered revertant and hypothesized to have arisen from an altered cytoplasm.

Revertant cytoplasm (W259A-RC) had a rearranged mitochondrial genome

Hybridization of mtDNA probes (p1915, *atp6* and *atp9*) to *Bam*HI-digested total umbel DNA of 3 fertile revertant (W259A-RC) plants from cross no. 9 (Table 1) and 4 plants from each of the CMS (W259A) and fertile maintainer (W259B) breeding lines indicated that the mitochondrial genome organization of the fertile revertant differed from that of its progenitor CMS line but resembled that of the fertile maintainer. (Fig. 1). The p1915 probe detected a unique 2.6-kb fragment in the sterile line which was absent in the fertile maintainer and revertant lines. The atp6 probe detected an 8.0-kb fragment in both the fertile maintainer and revertant lines, and a 3.2-kb fragment only in the sterile line. The *atp9* gene hybridized to a 7.5-kb fragment in all lines, and a 6.1-kb fragment was prominent only in the sterile line. Restriction fragments profiles of 3 sterile plants from cross no. 9 were identical to those of the CMS breeding line (W259A) (data not shown).

Hybridization of seven mtDNA probes to DNA of 19 fertile and 16 sterile plants from several families segregating with Mendelian inheritance for restoration, except cross no. 9 (Table 1), indicated that all plants had restriction profiles identical to that of the CMS line

Table 1 Genetic analysis of male fertility for a spontaneous fertile revertant (W259A-R) from a green petaloid carrot (W259 A)

Cross number	Phenotypes of parents[a]	Proposed genotypes of parents[b]	Segregation (F:S)	Ratio (F:S)	χ^2 *P*-value
1	$F^0_{\otimes}$	–	4:0	–	–
2	$F^{1a}_{\otimes}$	(*S*)*Rfrf*	15:4	3:1	0.9
3	$F^{1b}_{\otimes}$	(*S*)*Rfrf*	10:2	3:1	0.7–0.8
4	S(W259 A) × F^{1c}	(*S*)*rfrf* × (*S*)*Rfrf*	6:8	1:1	0.7–0.8
5	$F^{2a}_{\otimes}$	(*S*)*RfRf*	45:0	1:0	–
6	$F^{3a}_{\otimes}$	(*S*)*Rfrf*	32:13	3:1	0.5–0.7
7	S^{3b} × F^{3a}	(*S*)*rfrf* × (*S*)*Rfrf*	26:21	1:1	0.3–0.5
8	$F^{4a}_{\otimes}$	(*S*)*Rfrf*	41:17	3:1	0.3–0.5
9	S^{4b} × F^{4a}	(*S*)*rfrf* × (*S*)*Rfrf*	25:18	1:1	0.2–0.3
10	S^{8C} × F^5	(*S*)*rfrf* × (*S*)*RfRf*	18:0	1:0	–
11	S(2566S) × F^{2b}	(*S*)*rfrf* × (*S*)*Rfrf*	2:1	1:1	> 0.95
12	S(2566S) × F^{11}	(*S*)*rfrf* × (*S*)*Rfrf*	14:19	1:1	0.3–0.5
13	S(Guelph-301)F^{2c}	(*S*)*rfrf* × (*S*)*Rfrf*	12:18	1:1	0.2–0.3
14	S(Guelph-301)F^{13}	(*S*)*rfrf* × (*S*)*Rfrf*	8:7	1:1	> 0.95
15	$F^{9a}_{\otimes}$	(*R*)*rfrf*	12:0	1:0	–
16	S(2566 S) x F^{9a}	(S)*rfrf* × (R)*rfrf*	0:14	0:1	–
17	*S*(*Guleph*-301) × F^{9a}	(S)*rfrf* × (R)*rfrf*	0:13	0:1	–
18	*S*(*Guelph*-202) × F^{9b}	(S)*rfrf* × (R)*rfrf*	0:15	0:1	–
19	*S*(*W*259 *A*) × F^{9c}	(*S*)*rfrf* × (*R*)*rfrf*	0:28	0:1	–

[a] Superscripted numerals represent progeny group (cross number) from which parents were obtained; i.e. $F^1_{\otimes}$ indicates a fertile progeny from cross no. 1 was self-pollinated; letters (a, b, etc.) indicate different plants from same progeny group; F, fertile, S, sterile; CMS accession designation in parentheses; $^{\otimes}$, self-pollination; F^0, original revertant plant

[b] (Cytoplasmic designation)restorer genotype; S, sterile, R, revertant

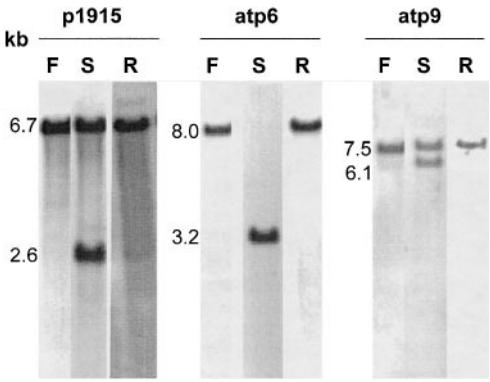

Fig. 1 DNA gel blot analysis of *Bam*HI-digested total carrot DNA hybridized to p1915 (*lanes 1–3*), *atp6* (*lanes 4–6*) and *atp9* (*lanes 7–9*) mtDNA clones. DNA was isolated from individual plants. *F* Fertile maintainer (W259B), *S* petaloid CMS (W259A), *R* fertile revertant (W259A-RC)

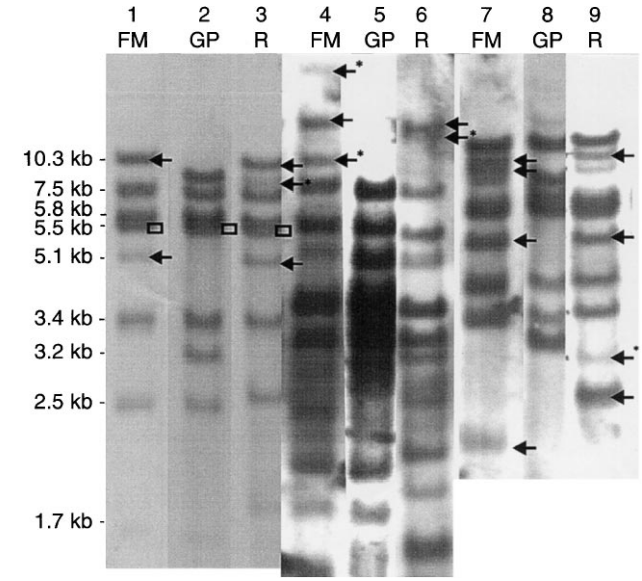

Fig. 2 DNA gel blot analysis of *Bam*HI-digested total DNA from different carrot accessions using three cosmids (C148 for *lanes 1–3*, C227 for *lanes 4–6* and C229 for *lanes 7–9*) spanning the 108-kb subgenomic circle as probes. *FM* Fertile maintainer (W259B), *GP* petaloid CMS (W259 A), *R* fertile revertant (W259A-RC). *Arrows* show extra *Bam*HI fragments detected in *FM* and *R* cytoplasms as compared with CMS cytoplasm. *Arrows* with *stars* indicate unique fragments either to *FM* or *R* cytoplasms. *Open squares* indicate a 5.5 kb fragment, containing a repeat sequence, not corresponding to any complete *Bam*HI fragment of the subgenome. Fragments of identical sizes in *R*, *FM* and *GP* lanes do not correspond exactly because data are combined from two different gels. Different membranes, however, were hybridized together for each probe

(data not shown). Thus, cytoplasmic reversion to fertility could only be detected in progeny of cross no. 9, and reversion was not a common event in the presence of the restorer allele.

A subgenomic circle from the sterile line was not intact in the revertant

Three mtDNA probes which distinguished sterile and revertant lines, p1915, *atp6* and *atp9*, identified cosmids in a mtDNA library for the CMS line which mapped to a 108-kb subgenomic circle (data not shown). The mtDNA *Bam*HI fragments that were unique to the CMS line from Southern blots of total DNA, 2.6 kb, 3.2 kb and 6.1 kb (Fig. 1), were recognized by p1915, *atp6* and *atp9* probes on this subgenomic circle, respectively. The fragments common to the CMS line, fertile maintainer and revertant, 6.7 kb and 7.5 kb, or present only in the fertile maintainer and revertant, 8.0 kb, when total DNA was probed with the three genes, were not present on the subgenomic circle. When three cosmids encompassing the 108-kb subgenomic circle were used as probes in Southern analysis to DNA of the sterile genotype, a 5.5 kb fragment was detected in addition to the predicted fragments on the circle, and may represent a repeated sequence elsewhere in the mitochondrial genome (Fig. 2).

Fertile maintainer and revertant lines had similar restriction patterns

When comparing all *Bam*HI fragments on the 108-kb subgenome of the CMS line with those detected in Southern hybridization of cosmids representing the subgenome to DNA of the fertile maintainer and revertant, we observed that fragments totaling 38.2 kb were absent in both fertile lines (Fig. 2 and Table 2A). Hybridization did reveal additional fragments for the fertile maintainer and revertant not present for the CMS line that could be classified into three categories: fragments unique to the revertant, 9.8 kb, 8.7 kb and 2.8 kb, (arrows with stars in Fig. 2 and Table 2B), fragments unique to the fertile maintainer, 21.3 kb and 8.2 kb, (arrows with stars in Fig. 2 and Table 2B), and fragments common to the fertile maintainer and revertant, 11.4 kb, 10.3 kb, 9.0 kb, 5.2 kb, 5.1 kb and 2.2 kb, (arrows in Fig. 2 and Table 2B). The fragments unique to either the fertile maintainer or revertant were present at low stoichiometries (arrows with stars in Fig. 2).

Discussion

Progeny analysis of a spontaneously derived fertile plant (W259A-R) from a stable petaloid CMS line (W259A) suggested that the presence of a dominant restorer allele (*Rf*) was responsible for its fertility. Hybridization data based on selected carrot and maize mtDNA probes revealed that there was no cosegregation of any mtDNA restriction fragment with flower

Table 2 Occurrence of *Bam*HI restriction fragments in petaloid CMS (GP), revertant (R) and fertile maintainer (FM) carrot lines when cosmids representing a 108-kb mitochondrial subgenomic circle were used as a probes in Southern analysis to total carrot DNA

Fragment size (kb)	GP (W259A)	R (W259A-RC)	FM (W259B)
A Linear order of *Bam*HI fragments present in 108-kb subgenomic circle			
3.0	+	−	−
1.6	+	+	+
1.4	+	−	−
3.5	+	+	+
4.9	+	+	+
6.1*atp9*[a]	+	+	+
4.2	+	+	+
2.7	+	+	+
2.6p1915	+	−	−
1.5	+	+	+
2.4	+	+	+
1.3	+	+	+
2.3	+	+	+
10.6	+	−	−
3.1	+	−	−
3.4	+	+	+
4.0	+	+	+
6.3	+	−	−
9.3	+	+	+
7.5	+	+	+
3.2*atp6*	+	−	−
5.8	+	+	+
1.7	+	+	+
8.0	+	−	−
3.4	+	+	+
2.5	+	+	+
1.8	+	+	+
5.5 (putative repeat)	+	+	+
B Fragments not found on 108-kb subgenomic circle			
21.3	−	−	+
11.4	−	+	+
10.3	−	+	+
9.8	−	+	−
9.0	−	+	+
8.7	−	+	−
8.2	−	−	+
5.2	−	+	+
5.1	−	+	+
2.8	−	+	−
2.2	−	+	+

[a] *atp6*, p1915 and *atp9*: mtDNA fragments which have homology to these mtDNA probes

phenotypes or restorer alleles except for the fertile revertant plants (designated as W259A-RC cytoplasm) from one exceptional cross. With Mendelian segregation observed over generations and stable CMS restriction patterns for progeny of all crosses except one, the restorer gene did not likely induce reversion as was observed in bean (Johns et al. 1992; Janska and Mackenzie 1993); consequently, the reversion was a spontaneous event in some progeny of a single sterile plant.

Upon hybridization of cosmids representing a mapped 108-kb subgenomic circle from the original sterile line to DNA of the fertile maintainer and revertant lines, 8 restriction fragments (totaling 38 kb) from the sterile line were not observed in the fertile lines. Unique restriction fragments totalling 42.3 kb were present at high stoichiometry in both fertile and revertant lines, and 3 submolar mtDNA fragments were unique to the fertile maintainer and 2 were unique to the revertant (Fig. 2, Table 2B). Submolar mtDNA restriction fragments were consistently found in replicate plants of fertile maintainer and revertant carrot lines, suggesting that they were not likely artifactual and could be used to distinguish the two sources of fertility-inducing cytoplasms. The potential for the revertant to arise as a seed contaminating the sterile line is possible if the mitochondrial genome of a fertile maintainer plant underwent rearrangements and stoichiometric changes to produce unique submolar bands, or if the revertant was a contaminant from an unknown cytoplasm.

Wright and coworkers (1996) have reported that the mtDNA restriction pattern of a sterile regenerant from carrot cell cultures originating from a fertile breeding line was nearly identical to that of the common petaloid sterile line. Since we observed the opposite reversion, sterile to fertile, with the mtDNA organization of the fertile revertant similar to that of the fertile maintainer line, carrot cytoplasms could be heteroplasmic, containing both fertile and sterile mitochondrial genome organizations, with one predominant in sterile or fertile plants.

The identification of submolar mtDNA fragments in plants (Small et al. 1987) suggested the existence of substoichiometric genomes capable of accumulating mutations over time which might change the mitochondrial genotype through their amplification and replacement of the prominent genome. Amplification of submolar mtDNA restriction fragments with plant tissue culture (Vitart et al. 1992; Kane et al. 1992) supports the premise of spontaneous complex changes in genome organization. Additional analyses in carrot mtDNA are necessary to determine if the revertant originated through the amplification of a submolar genome in the sterile line. The reversion reported here could also have resulted from a rare paternal inheritance of the fertility- inducing genome followed by amplification since a strict maternal inheritance of mitochondria does not always occur in plants (Reboud and Zeyl 1994).

In conclusion, inheritance studies and mtDNA restriction analysis led to the identification of a cytoplasmic revertant from a stable petaloid CMS line. The mitochondrial genome organization of the revertant resembles that of the fertile maintainer, however, the two lines contained some unique restriction fragments and were distinguishable. The reversion could have arisen by the amplification of a submolar genome in the CMS breeding line.

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