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Genetics

Mitochondrial DNA rearrangement associated with fertility restoration and cytoplasmic reversion to fertility in cytoplasmic male sterile *Phaseolus vulgaris* L.

(common bean/cytoplasmic–nuclear interaction)

S. A. MACKENZIE*†, D. R. PRING‡, M. J. BASSETT*, AND C. D. CHASE*

*Vegetable Crops Department, University of Florida, Gainesville, FL 32611; and ‡U.S. Department of Agriculture–Agriculture Research Station, c/o Plant Pathology Department, University of Florida, Gainesville, FL 32611

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ABSTRACT **Restoration of pollen fertility to cytoplasmic male sterile (CMS) *Phaseolus vulgaris* by a nuclear restorer gene provides a system for studying nuclear–cytoplasmic interactions. Introduction of a nuclear restorer gene to this CMS line of *P. vulgaris* (CMS-Sprite) results in a mitochondrial genome rearrangement similar to that observed upon spontaneous cytoplasmic reversion to fertility. Three spontaneous heritable cytoplasmic revertants were derived from CMS-Sprite. Five fully fertile restored lines were also produced by using restorer line R-351 (BC_3F_3 populations). Comparison of the mitochondrial DNA restriction patterns of CMS-Sprite, the three fertile revertants, and the five restored lines revealed loss of a 6.0-kilobase (kb) *Pst* I fragment in all restored and revertant lines. Southern hybridizations with a 1.3-kb *Bam*HI clone, internal to the 6.0-kb *Pst* I fragment, as a probe revealed two configurations of 6.0-kb homologous sequences in the sterile cytoplasm; one of the configurations was lost upon reversion or restoration. Mitochondrial DNA rearrangement has thus been observed upon restoration by a nuclear restorer gene in this CMS system.**

The phenotype of cytoplasmic male sterility (CMS) is characterized by the inability of a plant to produce viable pollen. CMS is a maternally inherited trait, suggesting that the alteration giving rise to pollen sterility is encoded by the mitochondrial or chloroplast genome. Fertility may be regained either by a cytoplasmic reversion to fertility or by a nuclear restorer gene able to override the effects of the cytoplasm. CMS, therefore, serves as a useful phenotype in the study of direct nuclear–cytoplasmic interaction in plants.

A source of CMS in *Phaseolus vulgaris* L. (CMS-G08063) was derived from the accession line G08063 as a spontaneous event (1). A relatively stable maintainer line, "Sprite" snap bean, was identified (2) and used as recurrent pollen parent in a series of 10 backcrosses to CMS-G08063 producing the CMS line CMS-Sprite.

Restoration of fertility to CMS-Sprite is controlled by a single dominant gene (*Fr*) (3) that is expressed in the sporophyte. Fertility restoration by *Fr* has several aspects. An F_1 population derived from CMS-Sprite × restorer line R-351 is semisterile (a phenotype intermediate between male sterile and fully fertile). Fully fertile plants are obtained in the F_2 generation. In F_2 populations one observes ≈65:10:25 (fertile/semisterile/sterile) ratios with semisterile plants usually comprising <10% of the population. When semisterile and fertile classes are pooled, a reasonable fit to a 3:1 (fertile/sterile) ratio is obtained, indicating segregation for the *frfr* genotype is required for a sterile phenotype (3). Once fully fertile F_2 plants are obtained with restorer line R-351, no segregation for sterility or semisterility is observed in their testcross progeny (*Fr*− × *frfr*) or in self-pollinated progeny (F_3), although approximately two-thirds of the fertile F_2 plants should be *Frfr*. This observation of irreversible restoration led to the question of whether a cytoplasmic alteration accompanies the recovery of full fertility with this restorer gene.

In CMS-S lines of maize, spontaneous cytoplasmic reversion events are associated with mitochondrial DNA (mtDNA) rearrangements (4). The frequency of these reversion events is influenced by nuclear genotype (5, 6), indicating that nuclear genes are involved in the determination of mitochondrial genome organization (7, 8). At least three other examples of CMS have also been associated with the mitochondrial genome (9–13). Here we examined the mtDNAs of CMS-Sprite, heritable cytoplasmic revertants, and nonsegregating fully restored CMS-Sprite lines (BC_3F_3) to determine whether genome rearrangements were associated with reversion events or with the condition of full restoration.

MATERIALS AND METHODS

Plant Materials. The male fertile line G08063 was provided by S. Singh (Centro Internacional de Agricultura Tropical, Cali, Colombia). The Sprite snap bean cultivar was obtained from Sun Seeds (Twin Falls, ID). The original CMS mutant was backcrossed 10 generations to Sprite to obtain a line designated CMS-Sprite. Five restored lines, 2-19-44-3-5, 2-19-44-3-3, 2-19-44-20-8, 2-24-13-7-12, and 2-19-44-20-11, were produced from CMS-Sprite × R-351 restorer as follows. Fertile F_2 plants were crossed to CMS-Sprite, and the progeny were evaluated for fertility with criteria described (3). Semisterile plants were backcrossed to CMS-Sprite (BC_2), the progeny were evaluated for fertility, and a third backcross was made with semisterile BC_2 segregants. Semisterile BC_3 plants were selected and allowed to set F_2 seed. Pedigrees were maintained throughout the backcrossing and self-pollinating process, and all plants were maintained in the greenhouse to prevent insect pollination. Fertile F_2 plants were allowed to self-pollinate, and five nonsegregating F_3 populations (derived from three BC_3 plants) were used in this study.

Estimation of Reversion Frequency. Frequency of reversion was estimated from a planting of 100 seeds of CMS-Sprite in the field in a single-row plot at a 10-cm spacing. The plants were maintained under netting to prevent insect pollination.

Seeds were collected from spontaneous seed-bearing pods and grown in the greenhouse, and the plants were evaluated

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Abbreviation: CMS, cytoplasmic male sterile.

†To whom reprint requests should be addressed.

for fertility. Male fertile plants were self-pollinated for three generations and testcrossed to CMS-Sprite.

mtDNA Isolation. Mitochondrial isolation procedures were as described by McNay *et al.* (14). Seven-day-old dark-grown hypocotyls and cotyledons (BC_3F_4) were used. The isolation procedure diverged from that of McNay *et al.* (14) at the point of mitochondrial lysis. Mitochondria were lysed in 5 ml of lysis buffer (100 mM Tris·HCl, pH 8/50 mM EDTA/100 mM NaCl/1% $NaDodSO_4$) and digested with proteinase K at 100 μg/ml for 30 min at 65°C. Following lysis the DNA isolation procedure was similar to that described by Dellaporta *et al.* (15) with cetyl trimethylammonium bromide to precipitate nucleic acids.

Chloroplast DNA Isolation. Approximately 100 g of green leaf tissue from 12-day-old seedlings was homogenized in homogenization buffer (50 mM Tris·HCl, pH 8/0.35 M sorbitol/5 mM EDTA/0.1% bovine serum albumin/5 mM 2-mercaptoethanol). The homogenate was filtered through four layers of cheesecloth and two layers of Miracloth and centrifuged at 1300 × *g* for 10 min. The pellet was resuspended in 250 ml of homogenization buffer, pelleted at 1300 × *g* for 10 min, and deoxyribonuclease-treated (0.01 M $MgCl_2$ and DNase I at 20 μg/ml; Sigma) in 10 ml of buffer G (0.3 M sucrose/50 mM Tris·HCl, pH 7.5) for 60 min at room temperature. The chloroplast suspension was brought to 250 ml with wash buffer (50 mM Tris·HCl, pH 8/0.35 M sorbitol/25 mM EDTA), and organelles were pelleted at 1300 × *g* for 10 min. The chloroplasts were washed twice by resuspending in 100 ml of wash buffer and pelleting at 1300 × *g* for 10 min. Lysis and DNA purification procedures were identical to those for mtDNA purification.

Electrophoresis and Hybridization. Electrophoresis and restriction endonuclease digestion were performed as described by McNay *et al.* (14). DNA was immobilized on nitrocellulose filters as described by Southern (16). Nick-translation of probes and hybridization procedures were as described by Chase and Pring (17).

Molecular Cloning Procedures. The CMS-Sprite mtDNA preparation was digested with *Pst* I and *Sst* II and electrophoresed in 0.8% agarose. A 6.0-kilobase (kb) fragment was recovered from the NA45 DEAE-cellulose membrane (Schleicher & Schuell) as described by the supplier. The extracted fragments were digested with *Bam*HI, phenol-extracted, ethanol-precipitated, and ligated with *Bam*HI-digested pUC8 vector. The ligation was transformed into *Escherichia coli* as described (17). The mtDNA fragments were recovered from recombinant colonies as described by Lonsdale *et al.* (18).

RESULTS

Spontaneous cytoplasmic reversion of CMS-Sprite occurred in the form of single-seed-bearing pods on otherwise male sterile plants. Seeds from these exceptional pods gave rise to sterile and fertile plants. Spontaneous seed pods were observed on ≈16% of CMS-Sprite plants grown under spring field conditions in Florida. Of numerous reversion events observed, three revertant lines representing random independent heritable cytoplasmic reversion events were selected for further analysis on the basis of stable fertility over three generations of self-pollination and sterility of progeny from testcrosses (CMS-Sprite × revertant). These revertants were designated 83-1, WPR-1, and WPR-5.

Restriction endonuclease analysis of mtDNA from line G08063 and CMS-Sprite with six enzymes (*Sst* II, *Pst* I, *Sma* I, *Bam*HI, *Eco*RI, and *Hin*dIII) revealed no differences in restriction pattern (data not shown). However, differences in mtDNA restriction pattern were observed between CMS-Sprite and the male fertile Sprite maintainer line (Figs. 1 and 2). Because the male recurrent parent carried distinctive

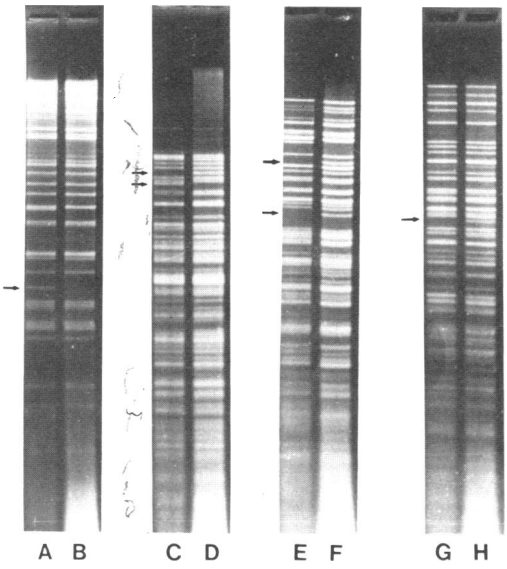

FIG. 1. mtDNA from lines CMS-Sprite (lanes A, C, E, and G) and Sprite (lanes B, D, F, and H) digested with restriction enzymes *Sst* II (lanes A and B), *Hin*dIII (lanes C and D), *Eco*RI (lanes E and F), and *Bam*HI (lanes G and H). Arrows indicate new fragments.

fragments that were not represented in CMS-Sprite, these results suggest that paternal inheritance of mtDNA cannot account for visible alterations in restriction pattern. This was relevant, since it has been reported that chloroplasts can be transmitted biparentally in *P. vulgaris* (19).

Restriction analysis of mtDNA from the three independent revertants revealed loss of a 6.0-kb fragment from a *Pst* I/*Sst* II double digest. This fragment was present in fertile G08063 and CMS-Sprite (Fig. 3 for revertant 83-1; data from others not shown). Digestion of mtDNA from five fertile BC_3F_3 lines restored by line R-351 also revealed loss of a 6.0-kb fragment in each line. The 6.0-kb fragment was present in submolar stoichiometry relative to the majority of the fragments in the digest.

A 1.3-kb *Bam*HI fragment internal to the 6.0-kb fragment from CMS-Sprite was inserted into the vector pUC8; this recombinant clone was designated 258-4. The clone 258-4 hybridized to fragments of 7.2 kb and 6.0 kb in *Pst* I- and *Sst* II-digested mtDNA from fertile line G08063 and CMS-Sprite (Fig. 2). However, hybridization of clone 258-4 to blots of three revertant and five restored lines occurred at a 7.2-kb band but not at a 6.0-kb band, confirming loss of the 6.0-kb fragment upon reversion and nuclear restoration. The 6.0-kb

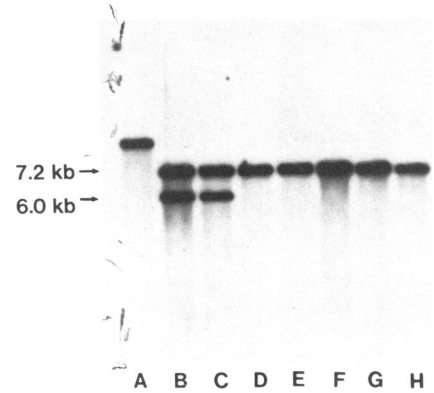

FIG. 2. Southern blot of mtDNA digested with *Pst* I and *Sst* II from Sprite (lane A), fertile G08063 (lane B), CMS-Sprite (lane C), revertant 83-1 (lane D), revertant WPR-1 (lane E), revertant WPR-5 (lane F), restored line 2-24-13-7-12 (lane G), and restored line 2-19-44-20-11 (lane H) probed with radiolabeled clone 258-4.

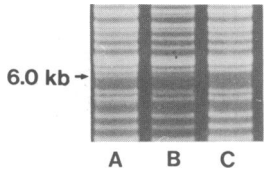

FIG. 3. Enlargement of *Pst* I- and *Sst* II-digested mtDNA from fertile G08063 (lane A), CMS-Sprite (lane B), and revertant 83-1 (lane C).

and 7.2-kb fragments were determined to be *Pst* I fragments and were cloned, eliminating the possibility that the restriction pattern difference was due to methylation. *Pst* I/*Sst* II double digestion of chloroplast DNA from the lines G08063, CMS-Sprite, and WPR-1 revertant produced no restriction fragments of 6.0 kb (Fig. 4 for CMS-Sprite; data from others not shown), indicating the substoichiometric 6.0-kb *Pst* I fragment was of mitochondrial origin rather than due to chloroplast contamination.

Autoradiographs of Southern blots of mtDNA were scanned with a laser densitometer (LKB 2202 Ultroscan). When clone 258-4 was used to probe mitochondrial DNA digested with *Pst* I and *Sst* II, a 3:1 ratio of the 7.2-kb to the 6.0-kb *Pst* I fragments was observed in CMS-Sprite. When six mtDNA preparations from green tissue of three fertile and three sterile F_2 plants were scanned, no significant reduction in the 6.0-kb fragment was detected in fertile F_2 plants. This suggests that in restoration, loss of the 6.0-kb fragment is not complete in the F_2 plant until seed formation. When mtDNA from two first-generation fertile revertant plants was similarly analyzed, the 6.0-kb fragment was absent from green tissue of one plant, but in the second plant a 4:1 ratio of 7.2-kb to 6.0-kb fragments existed in green tissue. Therefore, in reversion the 6.0-kb fragment may be present in vegetative tissue in the first generation following the reversion event.

DISCUSSION

The process of spontaneous cytoplasmic reversion was associated with loss of a 6.0-kb *Pst* I mtDNA restriction fragment in the three independent revertants tested. Because a new fragment was not detected in revertants, either in ethidium bromide-stained gels or by probing with clone 258-4, this mtDNA rearrangement probably involves a deletion. The rearrangement appeared to involve a repeated sequence, since the 1.3-kb clone hybridized to two fragments in the *Pst* I and *Sst* II digestion. Repeated sequences have been associated with sites of recombination in the mitochondrial genome in other plant species (20, 21). Such recombination events can result in subgenomic circles that contain portions of the complete mitochondrial genome (22). The submolar stoichiometry of the 6.0-kb *Pst* I fragment suggests that it may reside on a molecule distinct from the master mitochondrial genome. Submolar mtDNA molecules have also been observed in maize (23). Although the means used to detect the rearrangement would not be sensitive enough to detect small differences in the rearrangement events among the three revertants, it did appear that each reversion event involved a very similar mtDNA alteration (Figs. 2 and 3).

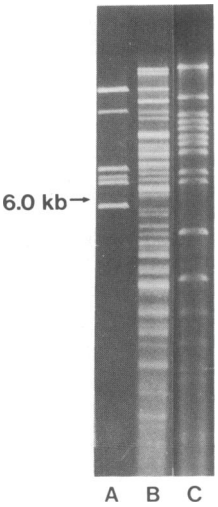

FIG. 4. Double-stranded λ DNA digested with *Bam*HI (lane A), CMS-Sprite mtDNA (lane B), and chloroplast DNA (lane C) digested with *Pst* I and *Sst* II.

Of fundamental importance was the observation that the same region of the mtDNA was affected by introduction of a nuclear restorer gene from line R-351. These data suggest we have identified a single nuclear gene that interacts with the mitochondrial genome to alter its conformation. Detection of a mitochondrial genome rearrangement in the five restored lines was not entirely unexpected since genetic analysis of restorer gene action suggests restoration of full fertility with line R-351 is a permanent condition accompanied by a change in the cytoplasm (3). To date, the only other observation of irreversible restoration, or fertility restoration that is not dependent on the continued presence of the nuclear restorer gene in subsequent generations, was made in CMS *Vicia faba* (24). However, full fertility is observed in F_1 populations (CMS *V. faba* × restorer) (24) and appears to be associated with loss of large double-stranded RNA molecules from the cytoplasm (25, 26).

Spontaneous reversion to fertility in CMS-S maize involves alterations of the mitochondrial genome (4). The frequency of spontaneous reversion is influenced by nuclear genotype (5, 6), showing that nuclear genes may affect mitochondrial genome organization. In fact, recurrent backcrossing to different nuclear genotypes not only results in changes in spontaneous reversion frequency, but also influences the copy number of the S1 and S2 mitochondrial plasmids (7). This alteration in S1 and S2 copy number is observed after only two generations, suggesting involvement of very few genes.

Nonchromosomal stripe mutations of maize (which occur in plants with the WF9 nucleus in combination with the CMS-T cytoplasm) also involve mtDNA rearrangements (27). Although the WF9 nuclear genotype may result in altered mtDNA conformation at a more sporadic frequency than we observe in our restored lines, it is clear that nuclear genes carried by WF9 can influence organization of the mitochondrial genome.

The genetics of restoration with line R-351 resemble reports of the maize nuclear gene *iojap*. *iojap* produces an altered phenotype that is then independent of nuclear genotype in testcrosses (28). To date, however, the effects of *iojap* have not been associated with a detectable organelle genome alteration (29).

The similarity in mtDNA restriction patterns of revertant and restored lines suggests the nuclear restorer gene from line R-351 promotes an mtDNA rearrangement that already occurs spontaneously at much lower frequency. This could occur through a nuclear gene effect on mtDNA replication or recombination. Such a model would not explain why fertile G08063 and CMS-Sprite are indistinguishable in mtDNA conformation, assuming the region including the 6.0-kb *Pst* I fragment is involved in fertility expression. The difference between fertile G08063 and CMS-Sprite may lie in the expression of this region of the mitochondrial genome. This can only be determined with more extensive molecular

analysis of the region encompassing the 6.0-kb *Pst* I fragment.

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