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Large double-stranded RNA molecules in *Phaseolus vulgaris* L. are not associated with cytoplasmic male sterility*

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Summary. Two large double-stranded RNA molecules, 15 and 16 kilobases, were detected in cytoplasmic male sterile (CMS) *Phaseolus vulgaris* by agarose gel electrophoresis. A number of smaller RNA molecules were observed in 'Sprite', a maintainer line, and recurrent backcrossing of CMS *P. vulgaris* × 'Sprite' resulted in a combined electrophoretic pattern of the two large and numerous small RNA molecules. The large RNA molecules were seed- and pollen-transmissible, but were not transmitted by grafting. The RNAs were present in revertant and restored lines derived from CMS-Sprite and therefore were not associated with the cytoplasmic male sterile trait.

Key words: Common bean – Cryptic virus – CMS

Introduction

Several studies of cytoplasmic male sterility (CMS) have provided evidence that viral interaction may be involved, perhaps even causative, in some higher plants. Graft transmissibility of male sterility is reported in CMS alfalfa (Thompson and Axtell 1978), sunflower (LeClercq 1971) and sugar beet (Curtis 1967). In the case of sugar beet, meristem culture and heat treatment have been reported to effect restoration of fertility (Lichter 1978). Molecular analysis of CMS in a number of species, however, has associated the CMS phenotype with mitochondrial genome alterations (Galun et al. 1982; Boeshore et al. 1985; Schardl et al. 1985; Dewey et al. 1986). In CMS *Vicia faba* cytoplasm 447, perhaps the most convincing evidence of viral involvement in CMS is the observation of cytoplasmic "spherical bodies", of about 70 nm in diameter (Edwardson et al. 1976), containing double-stranded RNA (dsRNA) (Grill and Garger 1981; Scalla et al. 1981). Upon spontaneous reversion or nuclear restoration to fertility, the dsRNAs are no longer detectable (Grill and Garger 1981; Scalla et al. 1981) and restoration is no longer dependent upon the presence of the nuclear restorer gene (Bond et al. 1966).

Restoration of fertility to CMS *Phaseolus vulgaris* using restorer line R-351 appears to be controlled by a single dominant gene (designated *Fr*) (Mackenzie and Bassett 1987). Once full restoration is achieved in the F_2 generation, restoration appears to be irreversible. In other words, no segregation for sterility is observed in subsequent selfed or testcrossed (restored line × maintainer *frfr*) populations. The similarity between CMS *Vicia faba* and CMS *Phaseolus vulgaris* in the permanence of fertility restoration by a nuclear restorer gene suggested that virus-like particles may also be associated with CMS in *Phaseolus vulgaris*.

Materials and methods

Plant material

The CMS *Phaseolus vulgaris* line used in this study was derived from fertile accession line G08063 at Centro Internacional de Agricultura Tropical (Singh et al. 1980). The CMS line was backcrossed to 'Sprite' snap bean, a maintainer genotype, for ten generations and designated CMS-Sprite. Three spontaneous, heritable, cytoplasmic revertants were identified, and fertile restored BC_3F_3 lines, using the restorer line R-351, were produced as described previously (Mackenzie and Bassett 1987). Therefore, materials used in this study included fertile G08063, male sterile G08063, CMS-Sprite, fertile Sprite, fertile revertant lines 83-1, WPR-1 and WPR-5, and nonsegregating BC_3F_3 restored lines 24-13-7-12-2, 24-13-44-20-8 and 24-13-44-3-3.

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Isolation of mitochondrial nucleic acids

Isolation procedures were as described by McNay et al. (1983), utilizing differential centrifugation and deoxyribonuclease digestion of extramitochondrial DNA. Leaves from greenhouse grown plants, 2–4 weeks old, were used. Typically, 100 g tissue was homogenized in a Waring blender at maximum speed, using three 5 s grinding periods. Phenol extraction of nucleic acids was followed by ethanol precipitation prior to gel electrophoresis.

Isolation of chloroplast nucleic acids

Plants 2–4 weeks old were placed in the dark for 48–96 h to reduce starch content. Chloroplast isolation was as described by Kolodner and Tewari (1975) using Proteinase K at 20 µg/ml. CsCl ethidium bromide centrifugation was replaced by phenol extraction followed by ethanol precipitation as in mitochondrial preparations.

Deoxyribonuclease and ribonuclease digestion

Deoxyribonuclease digestion(Worthington Diagnostic Systems) was done at 1 µg enzyme/50 µl 0.05 M Tris, 0.01 M $MgCl_2$, 0.05 M NaCl, pH 8.0, for 60 min at 37 °C. Ribonuclease (Calbiochem) digestions were conducted with 1 ng enzyme in 50 µl 0.01 M Tris, 0.01 M NaCl, 0.001 M Na_2EDTA, pH 8.0, for 60 min at 37 °C. Ribonuclease digestions were also done under high salt (0.10 M NaCl) conditions. Organelles from 10–20 g of tissue were processed for each digestion.

Agarose gel electrophoresis

Nucleic acids from 10–20 g of tissue were electrophoresed in horizontal submarine agarose gels, 20×25 cm, in TPE (0.036 M Tris, 0.030 M NaH_2PO_4, 0.001 M Na_2EDTA). To resolve large dsRNAs, 0.4% agarose gels were run 24–48 h at 0.8 V/cm, with buffer changes every 16 h. Alternatively, 0.8% agarose gels were run 16 h at 1.6 V/cm. Bacteriophage lambda dsDNA, digested with restriction endonucleases, was used for molecular weight standards. Digestion procedures were those recommended by suppliers.

Pollen transmissibility

Fertile Sprite plants werer pollinated using pollen from line G08063. Ten F_1 plants were grown and allowed to set F_2 seed in an insect-proof greenhouse.

Graft transmissibility

Plants were selected at 14–16 days for grafting. Grafts of ten fertile Sprite scions to CMS-Sprite root stocks (Sprite/CMS-Sprite) and two CMS-Sprite scions to Sprite root stocks (CMS-Sprite/Sprite) were made using a razor blade to form a v-shaped cleft graft. The graft point was supported by wrapping in plastic wrap and masking tape. Grafts were then staked and misted 3–4 times daily for 2 weeks. The plants were also covered with small plastic bags to maintain high humidity for the first week after grafting. Grafts were grown under fluorescent and incandescent lights (16 h daylength) at room temperature.

When the graft unions set, the plants were transferred to the greenhouse and allowed to set selfed seed (S_1). The S_1 generation was grown in the greenhouse and allowed to set selfed (S_2) seed. S_3 progeny from four Sprite/CMS-Sprite grafts were tested for the presence of the dsRNAs.

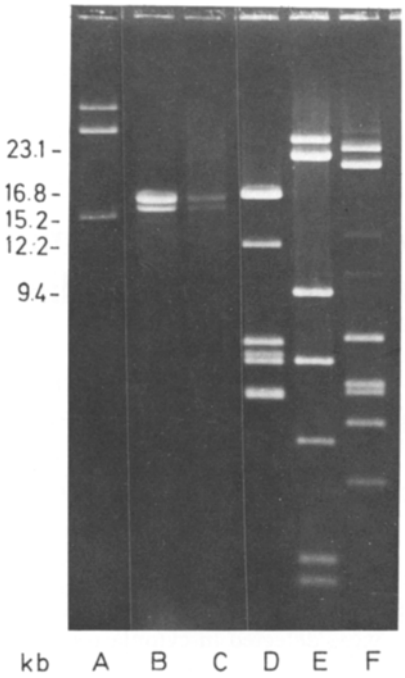

Fig. 1. Electrophoretic migration of dsRNAs in CMS-Sprite mitochondrial *B* and chloroplast *C* nucleic acid preparations. Markers were prepared using *Sal*I *A*, *Bam*HI *D*, *Hind*III *E*, and *Eco*RI *F* digests of bacteriophage lambda

Results

Agarose gel electrophoresis of nucleic acids from mitochondria and chloroplasts of male sterile G08063 revealed two high molecular weight molecules (Fig. 1 B, C). These molecules were also present in male fertile line G08063 from which the CMS line was derived. The molecules were present in high copy number; in some mitochondrial preparations mitochondrial DNA was just visible at dilutions required to resolve the two high molecular weight moecules (Fig. 1 B, C). Molecular size estimates were 15 and 16 kb. In contrast, mitochondrial preparations from 'Sprite' maintainer line (Fig. 2 B) showed no evidence of these molecules, but carried several smaller molecules (5.0, 4.9, 2.9, 1.2, 0.8, and 0.75 kb) not detectable in fertile G08063 (Fig. 2 C). CMS-Sprite exhibited the two large molecules, but also carried at least four of the smaller molecules characteristic of Sprite mitochondrial preparations (molecules migrating at 5.0 and 4.9 kb are apparently less stable and appear in only some mitochondrial preparations of both Sprite and CMS-Sprite) (Fig. 2 D). Spontaneous cytoplasmic revertants (Fig. 3 A) and fertile restored lines (Fig. 3 B) each exhibited patterns identical to CMS-Sprite.

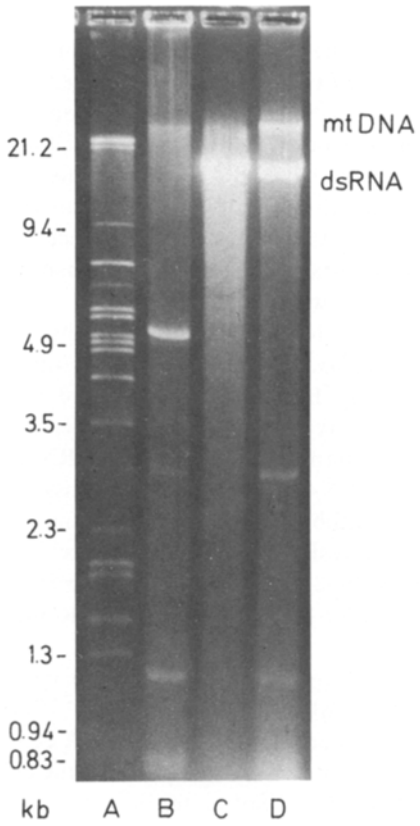

Fig. 2. 'Sprite' *B*, G08063 *C* and CMS-Spite *D* mitochondrial nucleic acid preparations. Markers *A* were prepared by combining *Eco*RI and *Hind*III digests of bacteriophage lambda

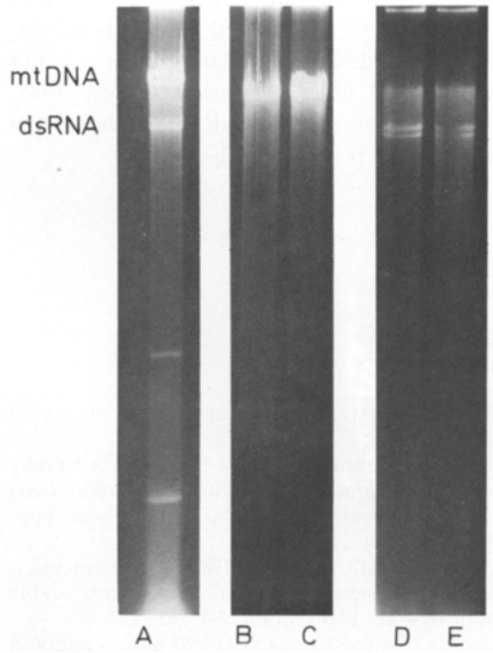

Fig. 3. Agarose gel electrophoresis of nucleic acids from *A* cytoplasmic revertant WPR-1, *B* restored BC_3F_3 line 24-13-44-3-3, *C* F_2 generation from Sprite × fertile G08063, and *D*, *E* S_3 generations from two grafts (Sprite/CMS-Sprite)

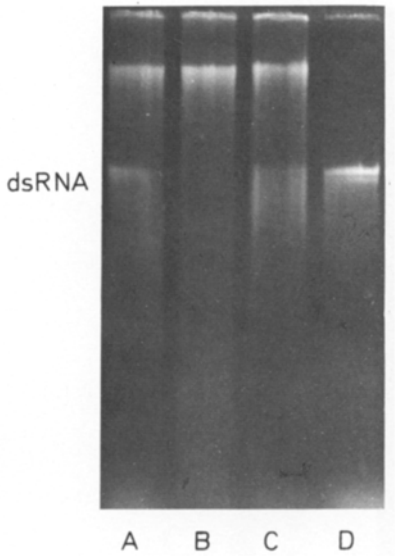

Fig. 4. Agarose gel electrophoresis of CMS-Sprite nucleic acids *A* digested with ribonuclease (10 m*M* NaCl) *B*, ribonuclease (100 m*M* NaCl) *C*, and deoxyribonuclease *D* digests of CMS-Sprite. Some degradation of the control *A* occurred, apparently due to ribonuclease migration from the adjoining well

Digestions of these preparations with deoxyribonuclease and ribonuclease indicated that the molecules were RNA. Deoxyribonuclease digestion eliminated only the principal mitochondrial DNA (mtDNA) (Fig. 4 D), leaving the smaller molecules intact. Digestion with ribonuclease in 100 m*M* NaCl left the 16 and 15 kb molecules partially intact (Fig. 4 C), while digestion in 10 m*M* NaCl resulted in their disappearance (Fig. 4 B). This suggests that the 16 and 15 kb molecules are double-stranded. Agarose gel electrophoresis of CMS-Sprite chloroplast nucleic acids and post-mitochondrial supernatants (pelleted at $2.2 \times 10^5 \times g$ for 3 h and phenol extracted) also showed the presence of the RNA molecules (data not shown).

In crosses between Sprite and G08063 (pollen donor) pooled F_2 progeny from 10 F_1 plants showed a high concentration of the large dsRNAs suggesting pollen transmissibility (Fig. 3 C). Graft experiments indicated that neither CMS nor dsRNAs were graft transmissible. Sixty S_3 plants (ten per graft) from six Sprite/CMS-Sprite grafts were grown and all were fully fertile. S_3 progeny from the Sprite/CMS-Sprite grafts were tested and showed no evidence of the RNAs (Fig. 3 D, E).

Using the same isolation and electrophoretic procedure with 'Black Turtle Soup' two large dsRNAs were observed, presumably the same as those described by Wakarchuk and Hamilton (1985). These comigrated with the dsRNAs in line G08063 (Fig. 5 A, B).

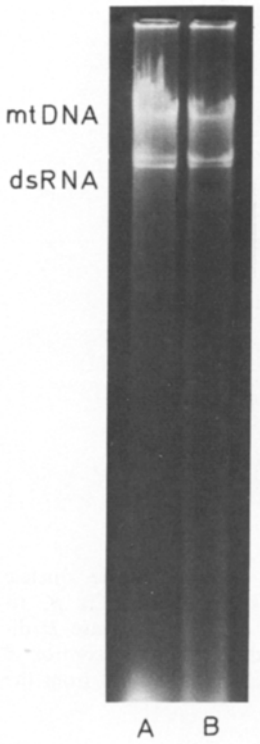

Fig. 5. Agarose gel electrophoresis of mitochondrial nucleic acids from fertile G08063 *A* and Black Turtle Soup *B*

Discussion

The 15 and 16 kb dsRNA molecules observed in line G08063 appeared to be similar,, if not identical, to those described by Wakarchuk and Hamilton (1985). Comparisons of 'Sprite', a line that does not carry these dsRNAs, and CMS-Sprite, an isonuclear line carrying the dsRNAs, revealed no foliar or growth abnormalities associated with presence of the RNAs.

The dsRNA molecules did not appear to be organellar since they fractionated with chloroplast, mitochondrial and post-mitochondrial supernatant preparations. Although the dsRNAs were present in high copy number and could be detected in nucleic acid preparations from less than 1 g tissue, the highest concentrations of the molecules were observed in mitochondrial and post-mitochondrial supernatant fractions. In addition, ribonuclease treatment of mitochondrial preparations prior to lysis left the large RNAs intact (data not shown). Therefore, the RNA molecules may be associated with a nucleoprotein complex or are membrane-bound.

The data suggest the dsRNA molecules may be viral, although the absence of virus-like symptoms would suggest that they are unusual, non-pathogenic agents (for review see Boccardo et al. 1987). The dsRNAs appeared to be pollen transmissible which, again, would suggest the molecules were not organellar. Amplification of the large dsRNAs, otherwise undetected in Sprite, as a result of pollination by G08063 presents another possible explanation for their appearance in (Sprite × G08063) F_2 progeny. This explanation would imply that Sprite nuclear genotype suppresses replication of the dsRNAs to undetectable levels. Nucleic acid preparations from the cytoplasm of CMS-Sprite indicated this was not the case.

Wakarchuk and Hamilton (1985) did not observe the dsRNAs in *Phaseolus vulgaris* lines tested other than Black Turtle Soup. Black Turtle Soup and line G08063, a white-seeded type, may have been derived from a common population containing the RNAs. However, since these RNAs are pollen transmissible and common bean, although highly self-pollinated, does have a low outcrossing frequency (Mackie and Smith 1935; Barrons 1938), the mechanisms exist to create wide distribution of these cytoplasmic molecules over time.

The large dsRNAs were not associated with cytoplasmic male sterility. Unlike the case of CMS *Vicia faba* cytoplasm 447 (Grill and Garger 1981; Scalla et al. 1981), the dsRNAs were present in cytoplasmic nucleic acid preparations from CMS-Sprite fertile revertants 83-1, WPR-1 and WPR-5. Restoration of fertility in CMS-Sprite, using restorer line R-351, resembles restoration in CMS *Vicia faba* cytoplasm 447 in the permanence of fertility, once achieved, and lack of segregation for sterility in subsequent generations (Makkenzie and Bassett 1987; Bond et al. 1966). However, the dsRNAs associated with CMS in *V. faba* are not detectable in restored lines (Grill and Garger 1981, Scalla et al. 1981), whereas these dsRNAs remained in the cytoplasm of fertile restored CMS-Sprite.

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