The Plant Cell, Vol. 4, 435–449, April 1992 © 1992 American Society of Plant Physiologists

A Mitochondrial DNA Sequence Is Associated with Abnormal Pollen Development in Cytoplasmic Male Sterile Bean Plants

Carol Johns, Meiqing Lu, Anna Lyznik, and Sally Mackenzie[1]

Department of Agronomy, Purdue University, West Lafayette, Indiana 47907

Cytoplasmic male sterility (CMS) in common bean is associated with the presence of a 3-kb unique mitochondrial sequence designated *pvs*. The *pvs* sequence encodes at least two open reading frames (297 and 720 bp in length) with portions derived from the chloroplast genome. Fertility restoration by the nuclear restorer gene *Fr* results in the loss of this transcriptionally active unique region. We examined the effect of CMS (*pvs* present) and fertility restoration by *Fr* (*pvs* absent) on the pattern of pollen development in bean. In the CMS line, pollen aborted in the tetrad stage late in microgametogenesis. Microspores maintained cytoplasmic connections throughout pollen development, indicating aberrant or incomplete cytokinesis. Pollen-specific events associated with pollen abortion and fertility restoration imply that a gametophytic factor or event may be involved in CMS. In situ hybridization experiments suggested that significant reduction or complete loss of the mitochondrial sterility-associated sequence occurred in fertile pollen of F_2 populations segregating for fertility. These observations support a model of fertility restoration by the loss of a mitochondrial DNA sequence prior to or during microsporogenesis/gametogenesis.

INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited trait that results in the inability of a plant to shed viable pollen. In most plant CMS systems, this male sterile phenotype can be masked by the presence of a nuclear fertility restorer genotype (for a review, see Hanson and Conde, 1985). CMS has been reported in at least one line of common bean to date (Bassett and Shuh, 1982). This sterility-inducing cytoplasm was derived from a fertile accession line designated G08063 (Singh et al., 1980). Line G08063 contains the sterility-inducing cytoplasm but remains fertile due to the presence of a fertility restoring nuclear genotype (Mackenzie, 1991). The cytoplasmic male sterile line is then derived by combining the G08063 cytoplasm with a nonrestoring nuclear genotype such as cv Sprite (CMS-Sprite). An unusual feature of this CMS system is the pattern in which normal pollen development is restored by the introduction of a nuclear gene designated *Fr*.

Introduction of the fertility restorer gene *Fr* to this CMS line results in loss of a portion of the mitochondrial genome in association with restoration of pollen fertility (Mackenzie et al., 1988b; Mackenzie and Chase, 1990). In this case, fertility restoration is a nonsegregating condition (Mackenzie and Bassett, 1987), and loss of a part of the mitochondrial genome suggests an association between this mitochondrial DNA region and pollen sterility. Restoration of fertility by *Fr* produces a condition of semisterility in the F_1 generation (CMS line × *Fr*), resulting in both parthenocarpic (seedless) and zygotic (seed-containing) pods on a single plant and full fertility in F_2 plants. This F_1 partial fertility implies that the process of restoration by *Fr* requires at least two generations and is incomplete in F_1 plants.

The association between a segment of the mitochondrial genome of the CMS line and fertility restoration by *Fr* suggests that the mitochondrial genome of accession line G08063 carries sequences that cause pollen abortion. In combination with particular nuclear genotypes, the sterility-inducing cytoplasm in bean undergoes spontaneous reversion to fertility. This is observed in the form of a single seed-bearing pod on an otherwise male sterile plant. These seed can give rise to both male sterile and fertile progeny. The frequency of reversion is influenced by nuclear genetic background (Mackenzie et al., 1988b), and each of five reversion events tested to date results in loss of the identical mitochondrial DNA region associated with *Fr* fertility restoration. This association between structural alterations and fertility restoration has led us to investigate the organization of this region of the G08063 mitochondrial genome.

Initial studies of the sterility-associated mitochondrial DNA region suggested that loss of >25 kb occurs upon fertility restoration (Mackenzie and Chase, 1990). This region carries at least

[1] To whom correspondence should be addressed.

one copy of the gene for the α subunit of mitochondrial ATPase (*atpA*). The *atpA* gene demonstrates no association with sterility, however. No *atpA* transcript differences are observed in association with sterility (Mackenzie and Chase, 1990), and antibodies against the *atpA* gene product cross-react with polypeptides of identical size in the CMS line (designated CMS-Sprite), fertile G08063, and Sprite (normal cytoplasm) mitochondrial protein preparations when tested by protein gel blot analysis (S. Mackenzie, unpublished results).

DNA gel blot analysis of the 25-kb SstII mitochondrial segment lost upon restoration or reversion indicates that the majority of this region is present elsewhere in the mitochondrial genome of G08063 (Mackenzie and Chase, 1990). Approximately 1 kb downstream (3′) to *atpA* is a 3-kb unique sequence. That this unique 3-kb sequence is not repeated in other regions of the mitochondrial genome is evident by the lack of hybridization to the mitochondrial genome of fertile *Fr*-restored lines (Mackenzie and Chase, 1990). This sequence is not found in the mitochondrial genome of other common bean lines (Mackenzie, 1991). The apparently complete loss of this 3-kb region upon restoration and the absence of this sequence from other common bean mitochondrial genomes indicate that its presence may cause the CMS phenotype. In this study, we have characterized the region encompassing this unique 3-kb mitochondrial sequence, and we present evidence that suggests its association with pollen abortion. We also discuss the effects of the G08063 cytoplasm on pollen development.

RESULTS

Map and Sequence of the *pvs* Region

We have mapped the mitochondrial DNA region associated with sterility. The map in Figure 1 contains the 3-kb unique region, now designated *pvs* (*Phaseolus vulgaris sterility* sequence), comprised mainly of the 1.5-kb SstI, 0.99-kb SstI/PstI, and 0.5-kb PstI/EcoRI fragments to the right of the map. These three fragments do not hybridize elsewhere in the mitochondrial genome. The exact left and right boundaries of the unique segment have not yet been determined. The approximate boundaries of the *pvs* region were defined based on differential hybridization to CMS and revertant lines. The 0.6-kb SstI fragment and the 0.7-kb PstI/SstI fragment immediately 3′ to *atpA* carry sequences that are present elsewhere in the mitochondrial genome on an 11.5-kb PstI fragment. This region is also present 3′ to the intact copy of *atpA* in the soybean mitochondrial genome (F. Chanut and R. Gesteland, personal communication). The region 3′ to the *pvs* sequence (not shown) is carried elsewhere in the genome on a 7.2-kb PstI fragment (Mackenzie and Chase, 1990).

Sequence analysis of the *pvs* region revealed some sequence similarity to known organellar sequences. Figure 1 indicates regions of most significant sequence homology to chloroplast or mitochondrial genes as well as the locations

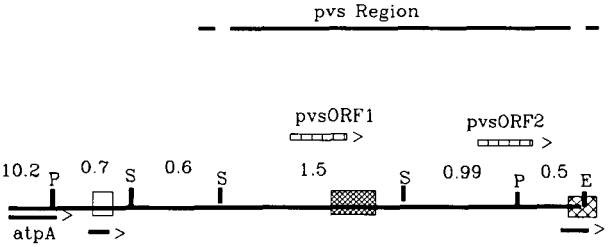


Figure 1. DNA Restriction Map of the Mitochondrial DNA Region Encompassing *pvs*.

The *pvs* unique sequence consists of the 1.5-kb SstI (s), 0.99-kb SstI/PstI (p), and the 0.5-kb PstI/EcoRI (e) fragments. The boundaries of the *pvs* region are not precisely defined; however, sequence analysis at both ends of the *pvs* sequence has extended into sequences repeated elsewhere within the mitochondrial genome. □□□ = open reading frames; ▨ = homology to chloroplast alanine tRNA intron; □ = homology to 3′ end of *coxII*, ▨ = homology to 3′ end of *cob*.

and direction of two open reading frames (ORFs) within the *pvs* region. The *pvs* DNA sequence is presented in Figure 2. Sequence data were derived by sequencing both strands of mitochondrial genomic clones using polymerase chain reaction (PCR) amplifications to derive the sequence across fragment junctions. Sequences of some regions were verified using cDNA as the template for PCR amplification.

At least two ORFs were detected within the *pvs* region. The first, designated pvsORF-1, is 297 bp in length and encodes a putative polypeptide of 10.9 kD. The second, pvsORF-2, is 720 bp in length and encodes a putative polypeptide of 26.7 kD. The direction of transcription (Figure 1) was determined using differential RNA blot hybridization with complementary single-stranded *pvs* clones within M13mp18 and mp19 phage vector (data not shown).

Origin of the *pvs* Sequence

We attempted to determine the origin of the *pvs* region by DNA gel blot hybridization of bean genomic and chloroplast DNA. Two large cytoplasmic double-stranded RNA molecules have been identified in a number of bean lines (Wakarchuk and Hamilton, 1985; Mackenzie et al., 1988a; S. Mackenzie, unpublished data). These RNAs were included in RNA gel blot hybridization experiments. We observed no hybridization to nuclear DNA (using film exposure periods of 10 to 14 days) or to the cytoplasmic RNAs (data not shown) but strong hybridization of chloroplast DNA, as demonstrated in Figure 3. This hybridization was likely due to the presence of a small (190 bp) region of homology (95.8%) within the *pvs* region to a chloroplast $tRNA^{ala}$ intron sequence (Figure 2) (Takaiwa and Sugiura, 1982). Beyond the unique *pvs* region, sequence analysis identified an 87-bp sequence homologous to the 3′ end of mitochondrial cytochrome b subunit (*cob*) (Dawson et al., 1984), and a 73-bp sequence homologous to the 3′ end of mitochondrial cytochrome oxidase subunit II (*coxII*) was carried

at the opposite end of the region encompassing *pvs* (Hensgen et al., 1984). These regions are designated in Figures 1 and 2. The origin of the remainder of the *pvs* sequence has not yet been determined. Database searches identified other small segments that demonstrated sequence similarity with genes of either mitochondrial or chloroplast origin (Figure 2), suggesting that multiple recombination events may have occurred within the region.

To test the possibility that the *pvs* sequence is common to plant mitochondrial genomes, yet now lost from most common bean lines, we attempted to hybridize the *pvs* sequence to DNA gel blots of mitochondrial DNA from at least 12 plant species

Figure 2. DNA Sequence of the *pvs* Region.

DNA sequence (3004 bp total) was derived using cloned DNA fragments from the CMS-Sprite mitochondrial genome. Putative open reading frames pvsORF-1 and pvsORF-2 are indicated with capital letters and the predicted translation products. The regions of sequence homology are underlined with the sequence direction indicated by arrows (< >), and the percent sequence identity is included. Gene designations, in the order that they appear, are as follows: ct ORF1708, tobacco chloroplast ORF 1708 bases 93210–93225 (Shinozaki et al., 1986); ct tRNA-ala intron, chloroplast alanine tRNA intron (Takaiwa and Sugiura, 1982); maize mt 5-kb repeat, maize mitochondrial 5-kb repeat bases 7663–7675 (Houchins et al., 1986); ct psbD, chloroplast photosystem II D2 protein bases 9358–9374 (Hiratsuka et al., 1989); maize mt 5-kb repeat, maize mitochondrial 5-kb repeat bases 1524–1550 (Houchins et al., 1986); maize mt 5-kb repeat, maize mitochondrial 5-kb repeat bases 4506–4544 (Houchins et al., 1986); mt cob 3′, mitochondrial apocytochrome b bases 1064–1146 (Dawson et al., 1984). The DNA sequence of the *pvs* region has been assigned GenBank Accession No. M87062.

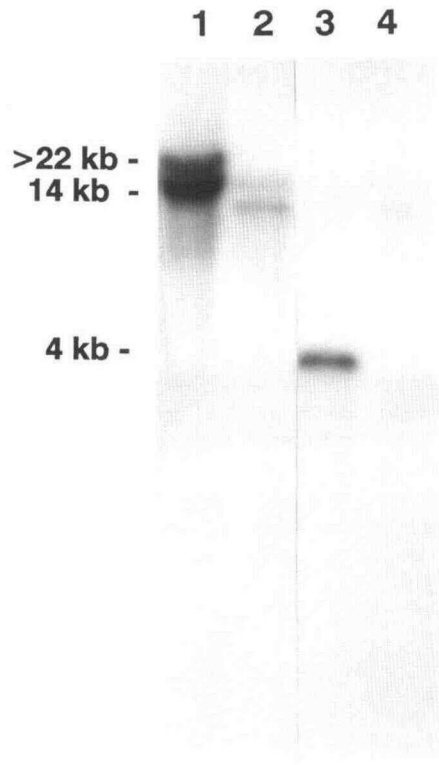


Figure 3. Chloroplast DNA Homology within *pvs*.

Preparations of chloroplast and mitochondrial DNA were digested with PstI, blotted to nylon, and hybridized with a ^{32}P-labeled insert derived from a 1.5-kb SstI clone within the *pvs* region (see Figure 1). Lane 1, fertile revertant 83-1 (lacks *pvs* from mitochondrial genome) chloroplast DNA; lane 2, accession G08063 (carries *pvs* within mitochondrial genome) chloroplast DNA; lane 3, accession G08063 mitochondrial DNA; lane 4, fertile *Fr*-restored line (CMS-Sprite × restorer R-351, BC_3F_4 population) mitochondrial DNA. Mitochondrial DNA contamination of chloroplast preparations is visible (lane 2) with chloroplast DNA contamination of mitochondrial preparations only slightly visible (lane 4). The slight difference in fragment migration observed between lanes 1 and 2 was due to the difference in DNA quantities loaded.

representing grasses, cucurbits, solanaceous species, brassica species, and legumes. We were unable to detect hybridization to any of the lines, indicating that the *pvs* sequence is poorly conserved within the mitochondrial genome or is of nonmitochondrial origin (data not shown).

We tested for the presence of intact copies of *cob* and *coxII* within the CMS-Sprite mitochondrial genome. This is because the observation of homology to the *cob* 3′ end adjacent to the pvsORF-2 sequence and homology to the 3′ end of *coxII* upstream to pvsORF1 suggested that the interruption of these essential mitochondrial genes might be the cause of sterility rather than the *pvs* sequence itself. Figure 4A demonstrates that two DNA bands were detected in EcoRI-digested and blotted mitochondrial DNA from CMS-Sprite when probed with the *cob* sequence. The *pvs* region is carried on a 3.1-kb EcoRI fragment that is not detected in Figure 4A because of the small

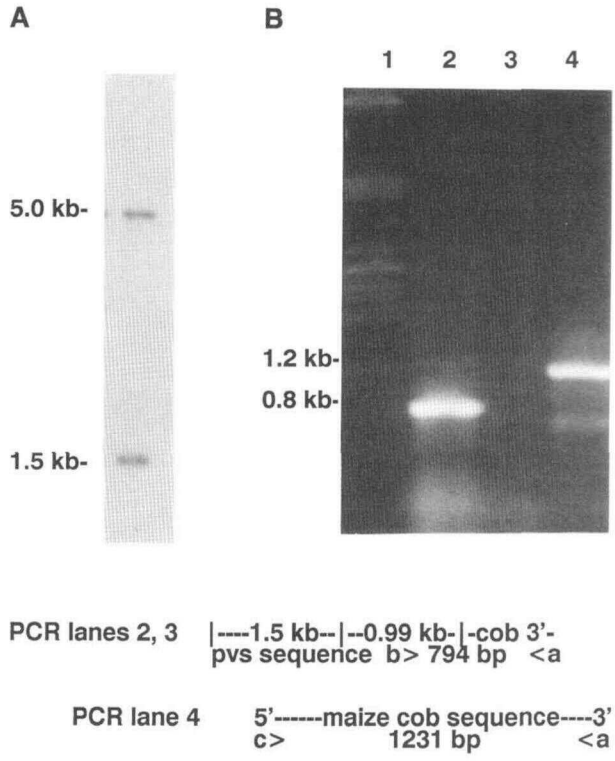


Figure 4. Evidence of an Additional Copy of *cob* within the G08063 Mitochondrial Genome.

(A) Total mitochondrial DNA from line CMS-Sprite digested with EcoRI, blotted to nylon, and hybridized with the cloned *cob* sequence from maize.

(B) PCR amplification products visualized on an ethidium bromide-stained agarose gel. Lane 1, PstI-digested λ molecular weight markers; lane 2, PCR-amplified product using CMS-Sprite mitochondrial DNA as template and oligonucleotide primers a and b specific for the *pvs* region; lane 3, PCR-amplified product using fertile revertant 83-1 mitochondrial DNA as template and primers a and b specific for the *pvs* region; lane 4, PCR-amplified product using CMS-Sprite mitochondrial DNA as template and primers a and c specific for the intact copy of cytochrome b. Identical results to lane 4 are obtained using revertant 83-1 mitochondrial DNA as template (data not shown). Sequence for primer c (5′-ATGACTATAAGGAACCAAC-3′) was derived from the maize *cob* sequence (position 1–19) (Dawson et al., 1984). Sequences for primer a (5′-TGGAATTCCTCTTCCAACT-3′) (position 2967–2986) and primer b (5′-CCATGGTCCTCCCATCAAC-3′) (position 2170–2189) were derived from the *pvs* region (Figure 2). Reaction conditions were 94°C, 1 min; 55°C, 1 min; 72°C, 1.5 min.

size of the region with homology to *cob* (87 bp) and the short film exposure period. The additional sites of hybridization should carry at least one intact copy of the *cob* gene. To further test this, PCR amplification was used. Oligonucleotide primers were synthesized using the strategy illustrated in Figure 4B. A PCR product of fragment size identical to the predicted intact copy of *cob* was distinguished from *cob* 3′ sequence present within the *pvs* region. Hybridization experiments using blotted PstI-digested mitochondrial DNA from normal Sprite and G08063 hybridized with *coxII* demonstrated that a second copy of *coxII* is present within the mitochondrial genome of line G08063. This second region of *coxII* homology is located on an 11-kb PstI fragment present in both G08063 and normal fertile Sprite (data not shown). This second site of hybridization is expected to carry an intact copy of *coxII* based on comigration of this DNA band in Sprite carrying a normal cytoplasm as well as similarity in PstI fragment size to that reported for common bean by Nugent and Palmer (1991).

Transcripts within *pvs*

To determine whether pvsORF-1 and pvsORF-2 were cotranscribed, we used RNA gel blot analysis and S1 nuclease protection experiments. In an earlier study, we determined that the region encompassing the *pvs* sequence is transcribed, with at least four transcripts, 7.0, 4.7, 3.3, and 2.8 kb in size, from this region disappearing upon restoration of fertility by *Fr* (Mackenzie and Chase, 1990). Figure 5A demonstrates that the 7.0-kb transcript hybridized faintly within the *atpA* region, indicating that the largest of the *pvs* transcripts initiates within

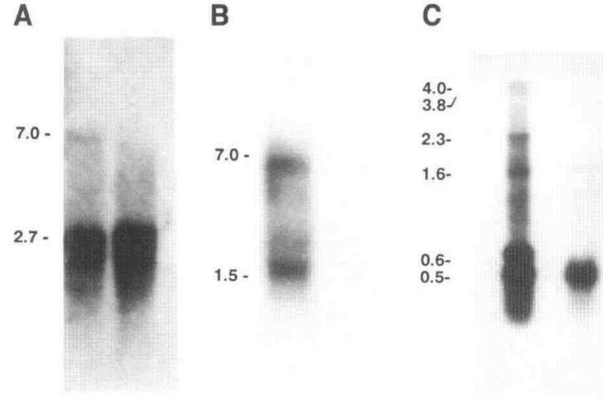


Figure 5. Transcripts within the *pvs* Region.

(A) RNA gel blot analysis of CMS-Sprite (left lane) and revertant 83-1 (right lane) total mitochondrial RNA hybridized with a ^{32}P-labeled clone of *atpA*. The transcript present in CMS-Sprite and absent from the revertant was derived from the *pvs* region. The transcripts present at ~2.7 kb in both lanes are normal *atpA* transcripts.

(B) Total mitochondrial RNA from fertile G08063 (left lane) and revertant 83.1 (right lane) hybridized with pvsORF-2. The pvsORF-2 sequence was derived by PCR amplification using primers 5′-CACGGAAATTGTGAGGCCTCAATGTGGTCA-3′ (position 2097–2126) and 5′-TAGTAAAGCTTGCCCCATTAGCGGGGATGC-3′ (position 2877–2906) (Figure 2).

(C) S1 nuclease analysis of the *pvs* region. An M13 single-stranded clone of 4.0-kb PstI fragment encompassing most of the *pvs* region (Figure 1) was incubated with total mitochondrial RNA from CMS-Sprite (left lane) and revertant (right lane) lines and S1 nuclease treated. The reaction was DNA gel blotted and probed with the ^{32}P-labeled 4.0-kb PstI clone. Increased intensity of the 0.6- and 0.5-kb protected fragments suggests that they result from protection of the 3′ ends of *atpA* transcripts that initiate 5′ to the *pvs* region.

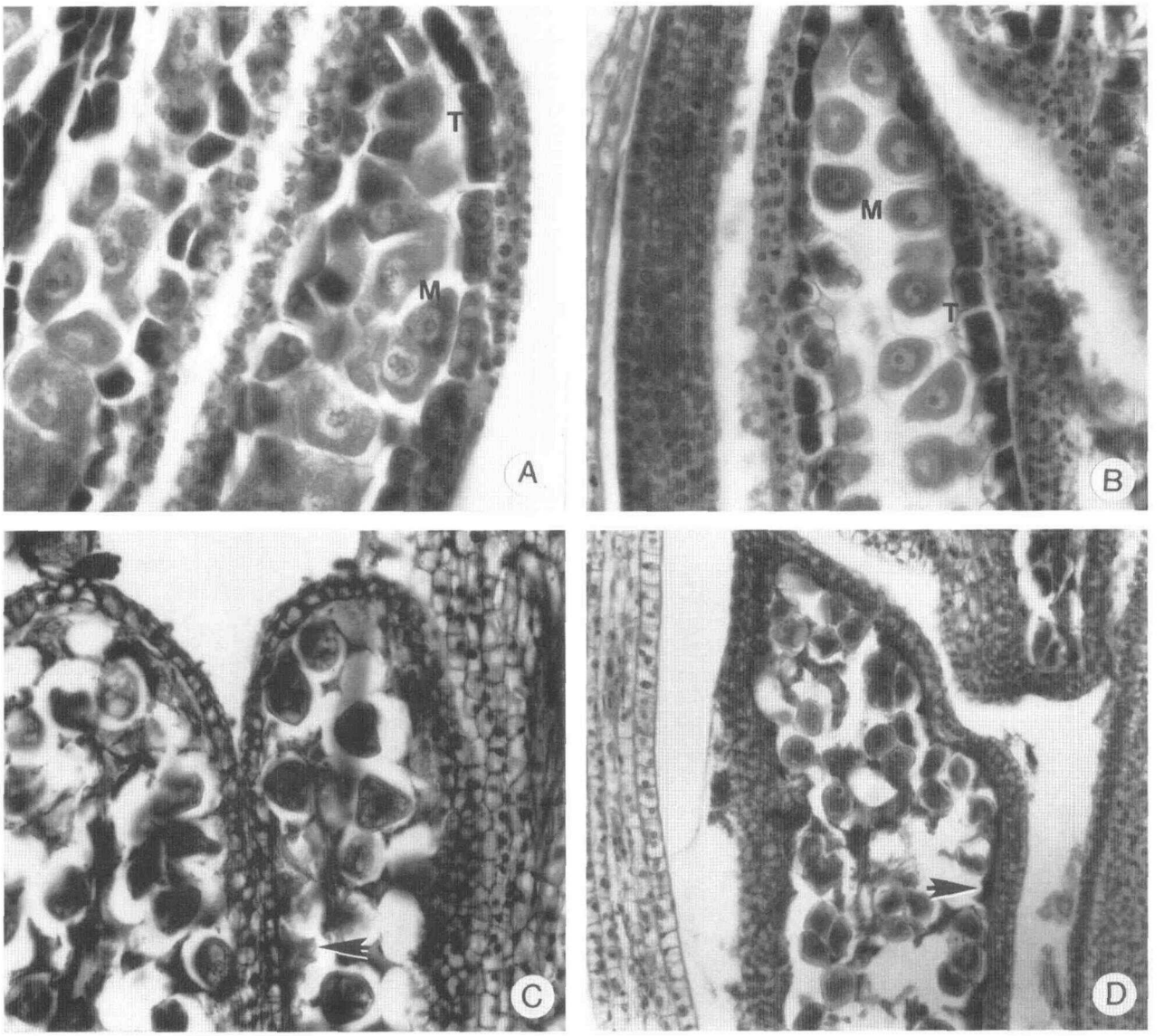


Figure 6. Tapetal Development in Fertile Sprite and CMS-Sprite Lines.

(A) Tapetum in fertile Sprite (*normal cytoplasm*). Meiocytes are undergoing prophase I of meiosis.
(B) Tapetum in CMS-Sprite (G08063 cytoplasm). Meiocytes are undergoing early prophase I of meiosis.
(C) Tapetal degeneration in fertile Sprite at late microspore stage.
(D) Tapetal degeneration in CMS-Sprite at late microspore stage.
T, tapetum; M, meiocytes; arrow, tapetal degeneration.

or immediately 3′ to the *atpA* gene. The intense bands around 2.7 kb represent normal *atpA* transcripts. S1 nuclease RNA protection experiments, using the 4.0-kb PstI clone encompassing most of the *pvs* region (Figure 1), resulted in RNA protection of the entire 4.0-kb fragment together with a number of smaller protected fragments (Figure 5C). The radioactively labeled probe used in this experiment was the 4.0-kb PstI clone. RNA protection of this entire 4.0-kb region, together with the observation of at least one transcript initiating 5′ to the *pvs* region (Figure 5A), suggests that both pvsORF-1 and pvsORF-2 are cotranscribed on at least one transcript. In addition to the 7.0-kb transcript, we observed two smaller transcripts, 3.3 kb and 2.8 kb in size, associated with the pvsORF-1 region using RNA gel blot analysis (data not shown) and at least one additional transcript, ~1.5 kb in size, associated with the pvsORF-2 region (Figure 5B). RNA protection of these smaller transcripts may account for the smaller protected fragments observed in the S1 nuclease protection experiment. However, one small

protected fragment of 500 bp was observed using either RNA from male sterile or fertile revertant lines (Figure 5C). This protected sequence mapped to the 0.7-kb PstI/SstI fragment present in both male sterile and fertile lines and outside of the *pvs* unique sequence (see map in Figure 1, data not shown).

Possible Association of Gametophytic Factors with Abnormal Pollen Development in CMS Bean

We compared the pattern of pollen and anther development in male sterile and fertile restored lines. This included comparison of the development of the tapetum in anthers of normal fertile Sprite and CMS-Sprite plants. These two lines are genetically isonuclear, suggesting that any differences observed in tapetal development would be the result of cytoplasmic effects. Figure 6 shows that no differences in tapetal development were observed at the light microscope level between these two lines. In both fertile and male sterile lines, the tapetal cells remained uninucleate throughout their development. They began to enlarge during meiosis, reached maximum development just after the young microspores were released from tetrads, and were nearly degenerated by the first microspore mitosis.

CMS-Sprite microspores underwent considerable postmeiotic development. Most microspores developed a thick pollen wall, became vacuolate, and underwent the first microspore mitosis to become binucleate pollen grains, as shown in Figure 7A. The pollen grains within CMS-Sprite did not engorge with starch and aborted late in pollen development. Prior to meiosis, microspore development appeared identical in fertile Sprite and CMS-Sprite. In fertile Sprite, the young microspores were released from their tetrad configuration as the callose broke down. This pattern of pollen development was similar to that described by Albertsen and Palmer (1979) in soybean. In CMS-Sprite, however, the microspores did not separate following meiosis but remained as tetrads throughout microgametogenesis.

We observed cytoplasmic connections between two or three microspores in many, but not all, tetrads, as shown in Figure 7B. The fact that all tetrads did not demonstrate these connections was likely due to different sectioning planes. Our observations suggested that cytokinesis following meiosis was often incomplete. Callose deposition during microsporogenesis appeared normal in CMS-Sprite. A normal callose wall was observed around the pollen mother cells, meiocytes, and tetrads (data not shown). Following meiosis, this callose wall dissolved, but the microspores retained their tetrahedral configuration. This appeared to be the result of the formation of a continuous pollen wall around the entire tetrad (Figure 7B).

Fertility restoration by restorer gene *Fr* results in a semisterile phenotype in F_1 progeny (Mackenzie and Bassett, 1987). Semisterility is characterized by the presence of fully fertile, sterile, and intermediate buds on a single plant, giving rise to both seedless and seed-bearing pods at maturity. Figure 8A demonstrates that in these semisterile plants buds carried both aborted and apparently viable pollen within a single anther locule. Of particular interest was the observation of both viable and aborted pollen within a single tetrad, as shown in Figure 8B. This observation suggests a gametophytic, or pollen-specific, factor in pollen abortion or fertility restoration.

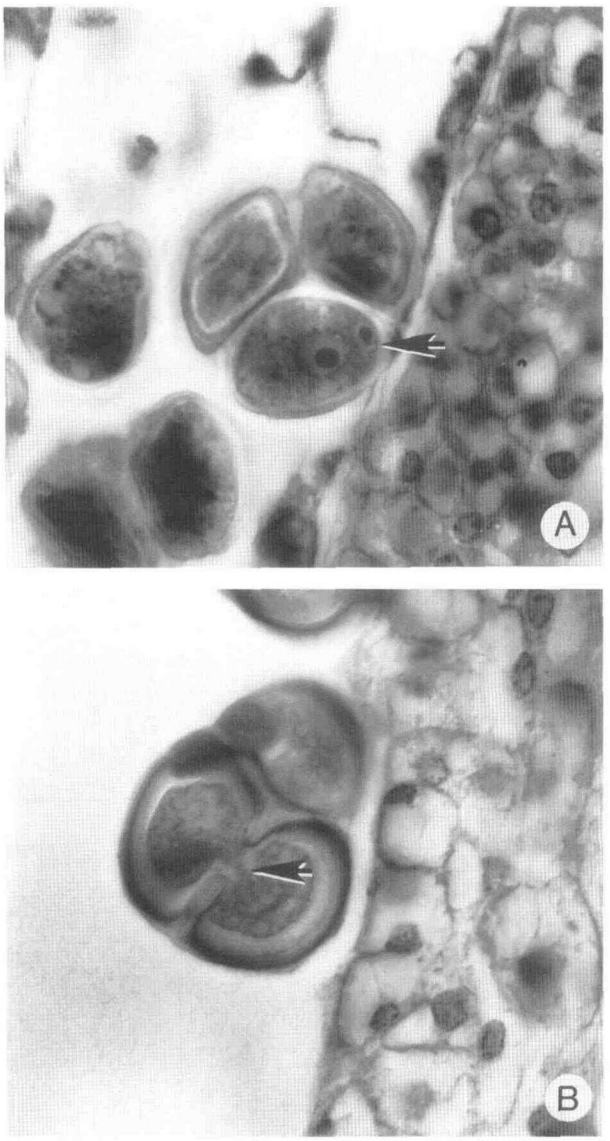


Figure 7. Microgametogenesis in CMS-Sprite.

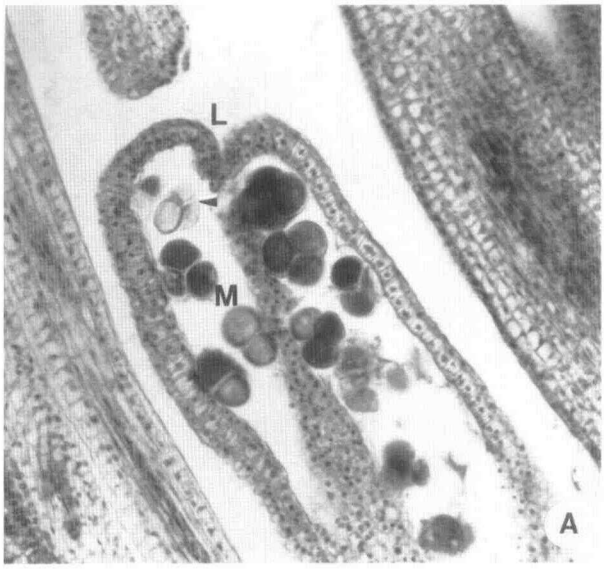
(A) Binucleate pollen grain. Arrow indicates presence of two nuclei within single pollen grain.

(B) Cytoplasmic connection between two microspores of a tetrad. Arrow indicates location of connection. A continuous developing wall is visible at the periphery of the entire tetrad.

Loss of the *pvs* Sequence during Development

Fertility restoration by *Fr* does not give rise to full fertility until the F_2 generation. Within F_2 populations, a range in fertility

exists, detected as the relative number of parthenocarpic versus seed pods per plant. Plants classified as fertile have fewer than three parthenocarpic pods and a large number of well-filled seed pods. The assumption that fertility restoration is associated with loss of a portion of the mitochondrial genome would predict that fertile F_2 plants carry mitochondria that have undergone this mitochondrial DNA alteration, with the mitochondrial genome of male sterile F_2 segregants structurally unchanged. Figure 9A indicates, however, that the vegetative tissue of fertile F_2 plants did not lose the sterility-associated sequence (present as a 6.0-kb PstI fragment), although stoichiometric differences of relevant mitochondrial DNA fragments were evident in some plants. The 7.2-kb band present in Figure 9A represents another region of the



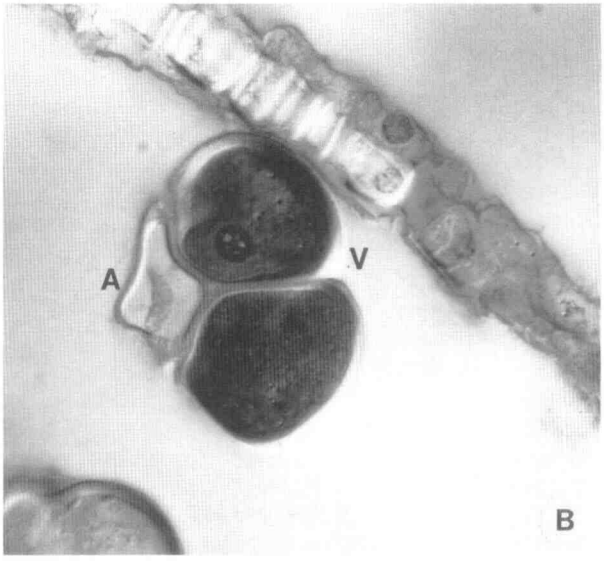


Figure 8. Pollen Viability in Semisterile F_1 Progeny (*Frfr*) (CMS-Sprite × R-351 Restorer).

(A) Aborted and viable microspores (M) within an anther locule (L). Arrow indicates the presence of aborted tetrad.
(B) Aborted (A) and viable (V) microspores within a single tetrad.

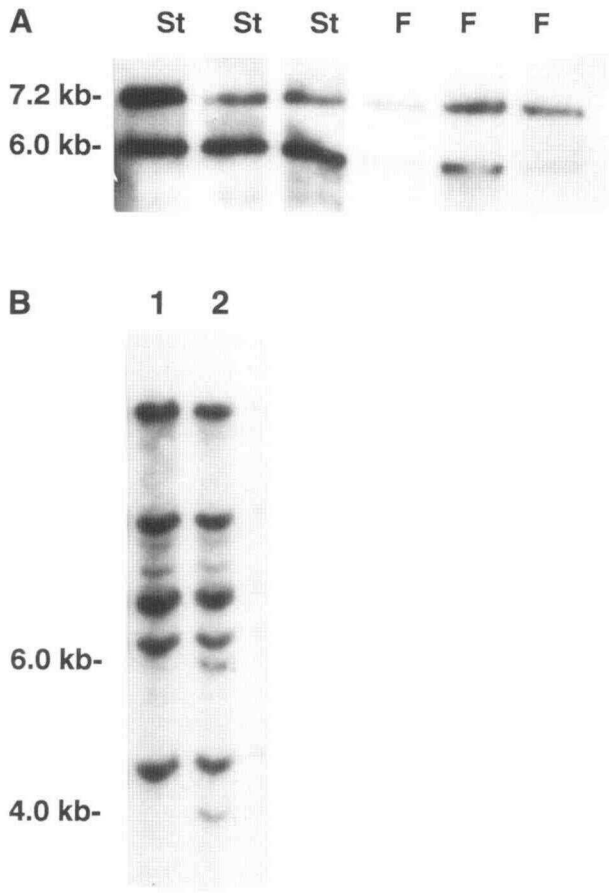


Figure 9. The Sterility-Associated Mitochondrial DNA Region Is Present in F_2 Vegetative Tissue but Absent from F_3 Vegetative Tissue in CMS-Sprite × R-351 Restorer Populations.

(A) Mitochondrial DNA gel blots from three sterile (St) and three fertile (F) F_2 plants from a segregating population. DNA was prepared from green vegetative tissue, digested with PstI, and the blot hybridized with a subclone (258-4) from the 6.0-kb PstI fragment associated with sterility (Mackenzie et al., 1988b).
(B) Mitochondrial DNA gel blot from green vegetative tissue of a fertile F_3 population (tissue bulked from four to five plants) derived from CMS-Sprite × R-351 restorer (lane 1). Mitochondrial DNA from fertile line G08063 was included as a control (lane 2). The DNA was digested with PstI and the blot hybridized with a mitochondrial DNA cosmid (16A4) that encompasses the mitochondrial sterility-associated region (Mackenzie and Chase, 1990). Identical results were obtained using DNA from three additional F_3 populations (data not shown).

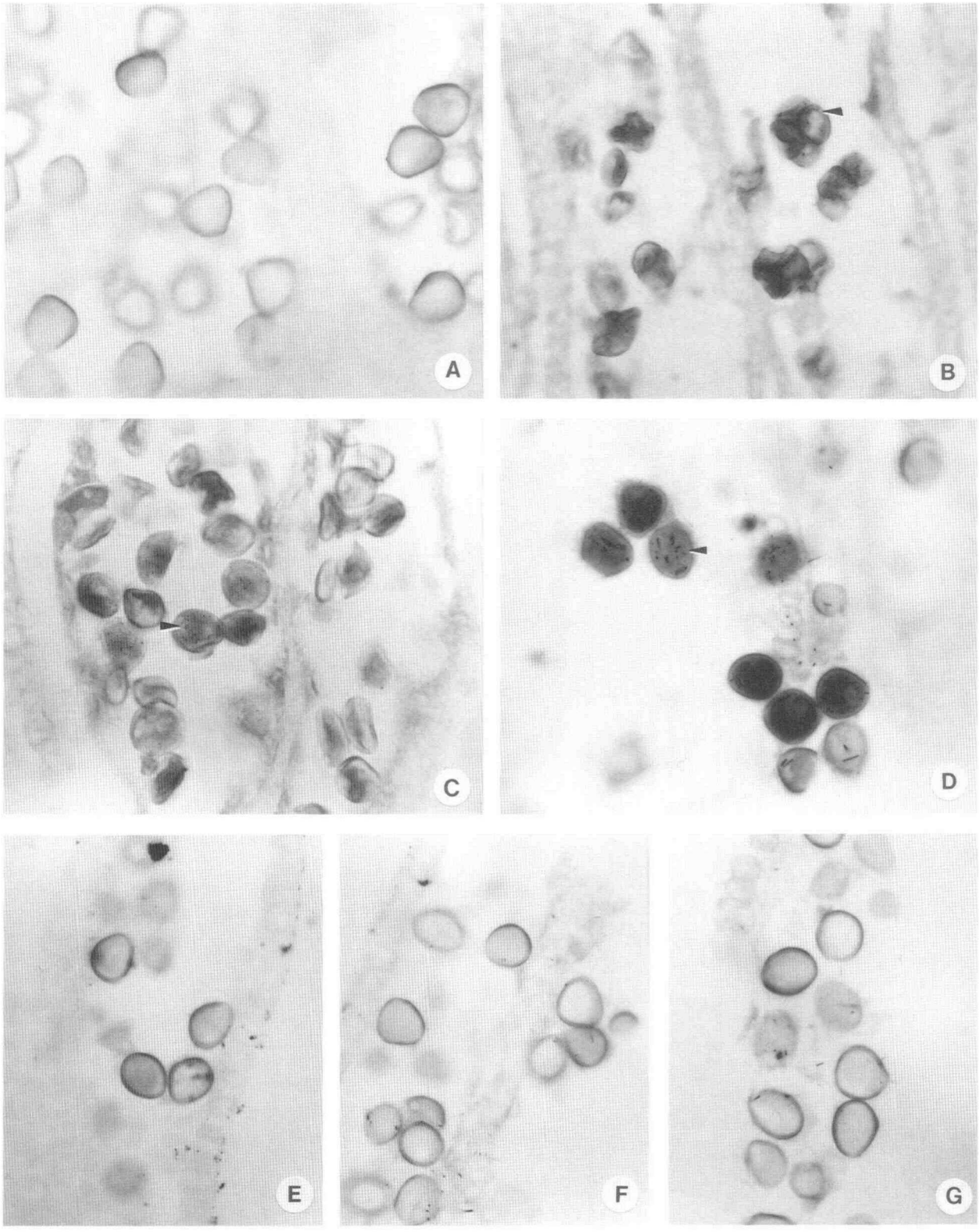


Figure 10. Control Experiments To Test in Situ Hybridization of Paraffin-Embedded and Sectioned Bud Tissues.

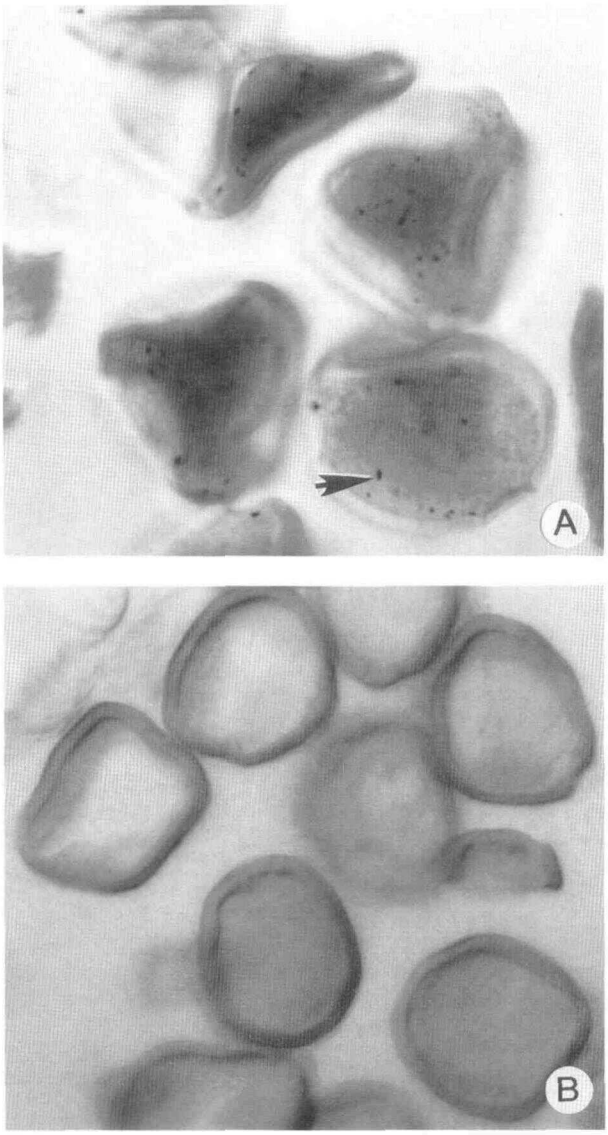


Figure 11. Enhanced Magnification of Bud Sections To Demonstrate in Situ Hybridization Results.

(A) Fertile G08063 bud section hybridized with the biotinylated 0.99-kb PstI/SstI *pvs* fragment. Positive hybridization signal is observed as black speckling within pollen (arrow). ×1000.
(B) Fertile F_2 plant (CMS-Sprite × R-351 restorer). Bud tissue was hybridized with the biotinylated 0.99-kb PstI/SstI *pvs* fragment. This panel serves as a negative control, and no hybridization signal is detected. ×1000.

mitochondrial genome containing homology to the region 3′ to the *pvs* sequence. Loss of the sterility-associated mitochondrial sequence, evident here as disappearance of a 6.0- and a 4.0-kb PstI fragment from the genome (Mackenzie and Chase, 1990), was not observed until the F_3 generation (Figure 9B). To date, we have tested green vegetative tissue of plants from four F_3 populations, and in each case loss of the sterility-associated region had occurred. Because full fertility was achieved within the F_2 generation, yet mitochondria in green vegetative tissue of fertile F_2 plants still carried the sterility-associated mitochondrial DNA region, we suggest that the mitochondrial genome change should be detectable during pollen development.

We used in situ hybridization of paraffin-embedded bud tissue to evaluate mitochondrial genome configuration in the *pvs* region in pollen of fertile restored F_2 plants. Postmeiotic buds were sectioned and hybridized with biotinylated DNA fragments carrying sequences unique to the sterility-associated mitochondrial DNA region (see Figure 1). Figure 10 shows the results of control hybridizations using tissue from fertile line G08063 and CMS-Sprite as positive controls and fertile Sprite as a negative control. Fertile line G08063 is known to carry the *pvs* region, a sterile cytoplasm, and a restorer nuclear genotype (*Fr2Fr2*) (Mackenzie, 1991). CMS-Sprite carries the G08063 cytoplasm but the Sprite maintainer nuclear genotype. Fertile Sprite contains a normal cytoplasm and therefore does not contain the *pvs* region (Mackenzie and Chase, 1990). The positive signal is observed as a black speckling over the microspore. This speckling is most pronounced in the G08063 (Figures 10C and 10D). Less pronounced signal is detected in CMS-Sprite (Figure 10B) due to the late stage in development and the onset of abortion. Figure 11 compares a positive (G08063) and negative (fertile restored) signal at higher

Figure 10. (continued).

(A) Fertile Sprite bud tissue hybridized with the 1.5-kb SstI *pvs* fragment (see Figure 1) plus biotin. Fertile Sprite does not contain the *pvs* sequence and serves as a negative control.
(B) CMS-Sprite bud tissue hybridized with the 1.5-kb SstI fragment plus biotin. CMS-Sprite contains the *pvs* sequence and serves as a positive control. Arrow indicates location of positive signal.
(C) Fertile G08063 bud tissue hybridized with the 0.99-kb PstI/SstI *pvs* sequence (see Figure 1) plus biotin. Fertile G08063 (*Fr2Fr2*) contains the *pvs* region in fertile pollen and serves as a positive control with a second *pvs* probe. Arrow indicates location of positive signal.
(D) Fertile G08063 bud tissue hybridized with the 1.5-kb SstI *pvs* fragment plus biotin. Arrow indicates example of positive signal.
(E) Fertile G08063 bud tissue hybridized with the 1.5-kb fragment and no biotin.
(F) Fertile G08063 bud tissue hybridized with a pHC79 nonspecific (nonhybridizing) subclone plus biotin.
(G) Fertile G08063 bud tissue plus biotin but no DNA included in probe preparation.

magnification. In the majority of experiments, background signal was minimal or not visible in negative controls using G08063 bud tissues hybridized with probe prepared without DNA, without biotin, or with nonspecific DNA (Figure 10). It should be mentioned that some experiments did result in a low level of observable background or nonspecific staining in these control hybridizations. We have therefore selected control samples with this low level of background for illustration purposes in Figures 10E, 10F, and 10G.

Using in situ hybridization, we observed hybridization to developing pollen from male sterile plants in a segregating F_2 population derived from CMS-Sprite × *Fr*, as demonstrated in Figure 12A. This indicates that the male sterile plants segregating in the F_2 population retained the *pvs* sequence throughout development. Pollen from fertile F_2 plants consistently demonstrated no hybridization to our mitochondrial DNA probe, as demonstrated in Figure 12D. Consequently, we conclude that the loss of the *pvs* region occurred prior to, or during,

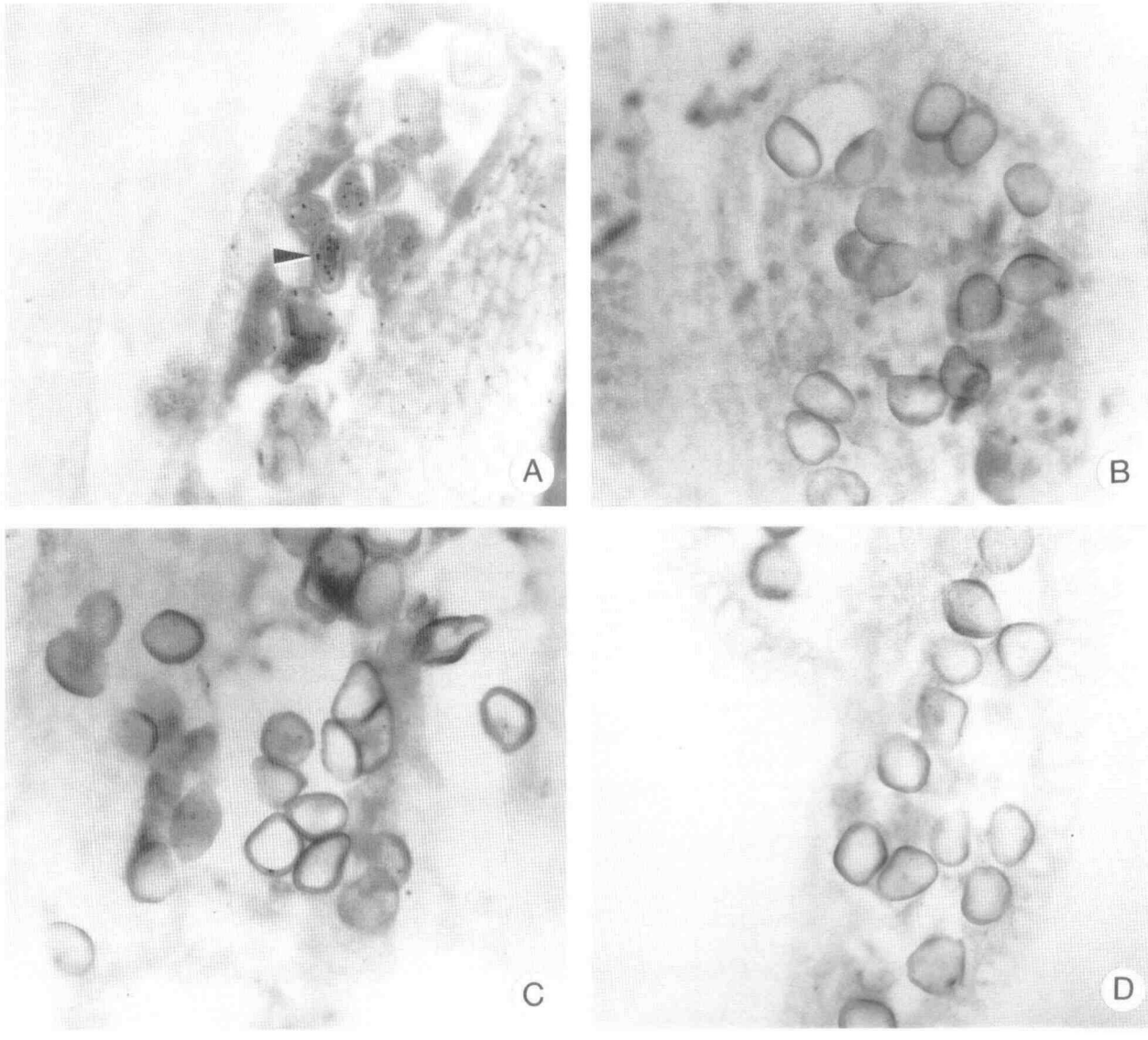


Figure 12. In Situ Hybridization Experiments Using Buds from F_2 Plants (CMS-Sprite × R-351 Restorer) Segregating for Fertility.

(A) Male sterile F_2 plant. Bud tissue hybridized with a biotinylated 0.99-kb PstI/SstI *pvs* fragment (see Figure 1). Arrow indicates location of positive signal.

(B) Male sterile F_2 plant. Bud tissue hybridized with a biotinylated probe using nonhybridizing pHC79 DNA as control.

(C) Male sterile F_2 plant. Probe preparation included biotin but omitted DNA as control.

(D) Fertile F_2 plant. Bud tissue hybridized with biotinylated 0.99-kb PstI/SstI *pvs* fragment.

pollen development. Five sterile and five fertile plants were tested (10 buds each). This does not imply that the mitochondrial DNA alteration is limited to the developing microspores. This study evaluates pollen development apart from any changes in the mitochondrial genome during megagametogenesis. Because we have developed these lines in a determinate bush-type growth habit, our study does not allow us to distinguish between gradual loss of the *pvs* sequence over development and more rapid, stage-specific loss in pollen. We have included control hybridization results (Figures 12B and 12C) to demonstrate that the observed hybridization was specific for the presence of the *pvs* sequence.

DISCUSSION

We have identified and sequenced a unique mitochondrial DNA region carried within the accession line G08063 and associated with cytoplasmic male sterility. The apparent absence from other common bean lines suggests that line G08063 has undergone a relatively recent mitochondrial alteration. Observations reported here indicate that portions of the *pvs* sequence were derived from the chloroplast genome. The remainder of this region is of unknown origin. The presence of chloroplast sequence within the mitochondrial genome of bean is not necessarily surprising; a number of cases of apparent chloroplast DNA transfer to the plant mitochondrial genome have been observed (Lonsdale, 1985; Schuster and Brennicke, 1988).

The alteration that has given rise to the G08063 mitochondrial configuration appeared to involve DNA insertion and, perhaps, multiple recombinations within the region 3′ to *atpA*. This is also the case in CMS sunflower, where inversion and DNA insertion 3′ to *atpA* results in cotranscription of *atpA* with a novel CMS-associated ORF of unknown origin (Kohler et al., 1991). The similarities between these two CMS systems may provide important information about the role of the region 3′ to *atpA* in mitochondrial genome stability.

The association of a mitochondrial sequence with CMS has been described extensively in two plant systems, the TURF 2H3 region of maize T-cytoplasm (Dewey et al., 1986; Rottman et al., 1987; Wise et al., 1987) and the *pcf* region in CMS petunia (Young and Hanson, 1987). In both cases, male sterility-associated mitochondrial ORFs were apparently derived from multiple recombination events within a limited region. Further analysis of these two highly rearranged regions indicates that they reside in close proximity to a recombinationally active repeated sequence (Rottmann et al., 1987; Fauron et al., 1990; Folkerts and Hanson, 1991). This association between a CMS-associated mitochondrial sequence and a recombinationally active mitochondrial repeat also exists in bean, where the *pvs* sequence lies immediately adjacent to *atpA*, contained within an 18-kb repeat (H. Janska, A. Lyznik, and S. Mackenzie, manuscript in preparation). Further investigation of this feature in these three CMS plant systems will likely provide information relevant to the origin of these novel rearranged sequences.

At least two ORFs have been identified within the *pvs* region. Because the transcripts from the region are present both in CMS-Sprite and fertile accession G08063, we cannot yet conclude that expression of *pvs* is associated with or causative in pollen sterility. It will be necessary to evaluate translational activity of the *pvs* sequence by developing antibody against the putative products of pvsORF1 and pvsORF2 to pursue these questions. The identification of a second fertility restorer system in bean, *Fr2*, that does not result in mitochondrial DNA loss (Mackenzie, 1991) allows us to compare translational activity of the *pvs* ORFs in male sterile versus *Fr2*-restored material.

Two interesting features were observed during the process of cloning and sequence analysis of the *pvs* region. DNA clones that contained the entire 0.5-kb PstI/EcoRI, 0.99-kb PstI/SstI, and a portion of the 1.5-kb SstI fragments (Figure 1) within either pUC18 plasmid or M13 phage vectors were highly unstable and susceptible to rearrangement within *Escherichia coli* hosts (strains TB-1 for pUC18 plasmid; JM107 or JM101 for M13 phage vector). However, DNA clones that contained the 0.99-kb fragment alone, or the 0.99-, 1.5-, 0.7-, and 0.6-kb fragments together, were highly stable and demonstrated no evidence of rearrangement. An obvious difference between these two cloning strategies is the interruption of pvsORF-2 by those clones that were stable. This clone instability might be an effect of the pvsORF-2 intact sequence or expression within *E. coli*, although this has not been determined. A second feature of pvsORF-2 is the presence of a hydrophobic domain at the amino terminus of the predicted translation product, based on Chou and Fasman (1978) hydropathy predictions (data not shown). This suggests the possibility of a membrane spanning domain within the predicted product.

Computer analysis of all possible ORFs indicated that, aside from the two ORFs described here, all other possible ORFs would require transcript editing to introduce either a stop or a start codon. We have sequenced cDNA from only portions of the *pvs* region (with no evidence of transcript editing observed); consequently, we cannot rule out the possibility of editing within the region. Analysis of the predicted translation products from these additional incomplete ORFs revealed no significant pattern of similarity to translation products in the EMBL and GenBank databases.

In nearly all cases of CMS, with the exception of CMS-S maize (Lee et al., 1980), microspore abortion is preceded by abnormal tapetal development or premature tapetal breakdown (Overman and Warmke, 1972; Horner and Rogers, 1974; Horner, 1977; Warmke and Lee, 1977; Lee et al., 1979; Bino, 1985). Light microscope analysis of anther development in male sterile, partially restored, and fully restored bean lines suggested that pollen abortion in this CMS source was not the result of abnormal sporophytic (tapetal) development. Moreover, the observation of both viable and inviable pollen within a single locule suggested that sterility and fertility restoration might involve gametophytic factors. The difference in pollen development within a single locule might, however, indicate a limiting sporophytically expressed component in pollen maturation. This could involve a threshold effect in restoration, with only

a proportion of the developing microspores allowed to continue normal development within the F_1 generation due to a limiting factor necessary for pollen development.

The loss of a unique mitochondrial DNA sequence upon restoration of pollen fertility provides a useful means to monitor this mitochondrial change throughout plant development. This mitochondrial genomic alteration apparently begins within the F_1 generation (CMS-Sprite × *Fr*), resulting in partial pollen fertility. This condition of incomplete restoration in semisterile plants is likely a result of heteroplasmy, or a mixture of altered and unaltered mitochondria. Full fertility, as well as detectable loss of the mitochondrial segment, was not achieved until anthesis in the F_2 generation. Presence of the mitochondrial sterility-associated DNA region within green tissue of fertile restored F_2 plants and absence of the sequence from restored F_2 pollen suggest that the mitochondrial DNA loss must occur prior to or during the process of meiosis and microgametogenesis. The observation of semisterility in F_1 plants, as well as a small proportion of F_2 plants, supports a model of gradual mitochondrial change throughout plant development over two generations. From our observations, we propose that the presence of the *pvs* sequence within individual pollen resulted in abnormal pollen development of those tetrads carrying the *pvs* mitochondrial sequence. The action of nuclear gene *Fr* in effecting mitochondrial DNA alteration was apparently not limited to a single developmental stage and resulted in heteroplasmy or semisterility in the F_1 generation.

In situ hybridization techniques are not adequately sensitive to detect sequences at very low copy number per cell. We cannot conclude, therefore, that the loss of the *pvs* sequence in F_2 fertile pollen is complete. We have attempted to use PCR amplification to test for heteroplasmy in F_2 pollen. Results from our pollen PCR experiments were not included here because of the technical difficulty in devising appropriate control experiments to exclude the possibility of contamination and artifact due to inconsistent tissue preparation. Preliminary results from these experiments suggested that a few fertile F_2 buds contained pollen that carried the *pvs* sequence at a level undetectable using in situ hybridization, but most did not.

We have observed spontaneous cytoplasmic reversion to occur in CMS-Sprite in the form of a single seed-bearing pod on an otherwise male sterile plant. The frequency of these reversion events is influenced by nuclear genotype (Mackenzie et al., 1988b). The seed taken from these revertant pods will give rise to both male sterile and male fertile progeny, the male fertile having undergone an apparently identical mitochondrial DNA alteration to that of restoration by *Fr* (Mackenzie et al., 1988b). The observation of both fertile and sterile progeny from a single pod suggests that events that cause the loss of the *pvs* sequence do not involve coordinated loss from both pollen and egg. The presence of *pvs* apparently affects only the phenotype of the pollen. The mitochondrial population within the egg cell determines fertility in the next generation.

From our studies of CMS bean, we conclude that the *pvs* sequence unique to this cytoplasm likely includes DNA insertion(s). Presence of the *pvs* sequence appears to cause abnormal development of individual microspores. Introduction of nuclear gene *Fr* results in restoration of fertility to only some of the pollen within an anther locule, suggesting changes that are gradual over development and, again, affect individual microspores. Full restoration in a segregating F_2 population (CMS-Sprite × *Fr*) results in loss of *pvs* from pollen of fertile F_2 segregants and presence of *pvs* in male sterile F_2 segregants. This genetic system will allow us to study the effect of *Fr* on mitochondrial genome structure and the relative role of nuclear genotype versus cytoplasmic sorting during plant development in the process of fertility restoration of CMS bean.

METHODS

Plant Materials

Fertile accession line G08063 was provided by Centro Internacional de Agricultura Tropical (Cali, Colombia). The cytoplasmic male sterility (CMS) *Phaseolus vulgaris* line used in this study (designated CMS-Sprite) was derived from line G08063 (Singh et al., 1980) and was backcrossed to Sprite snap bean, a sterility maintainer nuclear genotype, over 15 generations. The *Fr*-restored populations, F_1, F_2, and F_3, were derived from BC_3 populations (CMS-Sprite × R-351) using restorer line R-351 (Mackenzie and Bassett, 1987). Revertant line 83-1 was selected as a spontaneous fertility reversion event from CMS-Sprite and has been genetically characterized to be a stable cytoplasmic event, as previously described (Mackenzie et al., 1988b).

Mitochondrial DNA Clones

pK9ECOB (Maize *cob*) was supplied by Dr. C. S. Levings (North Carolina State University, Raleigh, NC), and a sorghum cosmid carrying *coxII* was supplied by Dr. D. R Pring (University of Florida, Gainesville, FL). Clones for the *pvs* region were obtained as described previously (Mackenzie and Chase, 1990).

Preparation of Mitochondrial DNA

Mitochondrial DNA was prepared as described previously (Mackenzie et al., 1988b). The procedure of McNay et al. (1984) was used to isolate mitochondria. The procedure of Dellaporta et al. (1983) was then used for mitochondrial lysis and mitochondrial DNA purification. Cetyl trimethylammonium bromide was used to precipitate nucleic acids. Most preparations used 7-day-old etiolated hypocotyl tissues. Mitochondrial preparations from F_2 and F_3 plants involved greenhouse grown tissues from 25-day-old plants.

Preparation of Mitochondrial RNA

Mitochondria were prepared as above, without DNase or proteinase K. The mitochondria were lysed in 6 M guanidinium thiocyanate (Maniatis et al., 1982), and phenol/chloroform was extracted. The nucleic acid suspension was brought to 2.5 mL, combined with 1 g of CsCl, and centrifuged for 12 hr at 32,000*g* over a 3.5-mL cushion of 5.7 M CsCl/0.1

M Na_2EDTA at 20°C in a rotor (model No. SW 40.1, Beckman Instruments; Wise et al., 1987).

DNA/RNA Electrophoresis, Transfer, and Hybridization

DNA restriction endonuclease digestion, electrophoresis, blotting, and hybridization were performed as described by McNay et al. (1984). Mitochondrial RNA was denatured in glyoxyl, subjected to electrophoresis through 0.6% agarose gels in 10 mM Na_2HPO_4 (pH 7.0), and transferred to nitrocellulose membranes (Thomas, 1980). Standard hybridization and wash conditions were as described by Wise et al. (1987).

S1 *Nuclease Protection*

S1 nuclease RNA protection experiments were conducted using the reaction conditions provided in Ausubel et al. (1988). Single-stranded DNA was prepared from a 4.0-kb PstI clone encompassing the *pvs* region in M13 phage vector. The entire single-stranded unlabeled DNA clone plus vector was used in the annealing with total mitochondrial RNA (20 μg) overnight at 55°C. The annealed products were then digested with 100 to 300 U/mL of S1 nuclease. Agarose gel electrophoresis of the S1 digestion products was followed by DNA gel blot hybridization. The same 4.0-kb PstI DNA clone was then used as probe to detect digestion products.

Chloroplast and Genomic DNA Preparation

Chloroplasts were prepared from 7- to 10-day-old greenhouse grown seedlings. The seedlings were ground in a blender in 0.5 M sucrose, 5 mM Na_2EDTA, 0.1% BSA, and 50 mM Tris, pH 7.5. The solution was filtered and centrifuged 10 min at 800*g*. If the pellet was not significant, the suspension was recentrifuged at 1000*g*. The pellets were resuspended in 0.3 M sucrose, 0.05 M Tris, pH 7.5, and brought to 10 mM $MgCl_2$ and 20 μg/mL DNase. After 60 min of incubation at 4°C, the suspension was centrifuged twice in 0.6 M sucrose, 20 mM Na_2EDTA, 10 mM Tris, pH 7.5, for 10 min at 2200*g*. The resulting pellet was resuspended in 100 mM Tris, pH 8.0, 50 mM Na_2EDTA, 100 mM NaCl, and 1% SDS and incubated 15 min at 65°C for lysis. Chloroplast DNA preparation followed the procedure of Dellaporta et al. (1983) using precipitation in cetyl trimethylammonium bromide for further DNA purification. Preparation of genomic DNA followed the same protocol described above with the exception that the pellets were collected from centrifugation at 1240*g* after two centrifugations at 860*g* to remove chloroplasts.

DNA Sequencing and Analysis

DNA fragments to be sequenced were cloned into M13 phage vector according to the procedure provided by Bethesda Research Laboratories. Single-stranded DNA template was sequenced using the procedure of Sanger (1981) using Sequenase II (United States Biochemical Corp.). Sequencing extension was facilitated using synthetic DNA oligonucleotide primers synthesized by the Purdue University Department of Biochemistry (West Lafayette, IN). Both strands of the *pvs* region were sequenced. Sequence analysis included a sequence and peptide homology search of GenBank, EMBL, VecBase, NBRF, and the Protein Sequence Databases available through the Genetics Computer Group (University of Wisconsin, Madison, WI).

Flower Bud Preparation

Flower buds of all developmental stages were collected from greenhouse grown plants of line G08063, CMS-Sprite, Sprite, an F_1 population (CMS-Sprite × R-351 restorer line [*Frfr*]) segregating 1:1 semisterile/sterile, and the derived F_2 population segregating for fertile, semisterile, and sterile progeny. All flower buds, whether used for developmental studies or in situ hybridizations, were processed using the method of Berlyn and Mischke (1976). Flower buds were fixed immediately in either FAA for 24 hr or 4% paraformaldehyde/2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2, overnight. A gentle vacuum was applied, when necessary, for fixative penetration. Following fixation, buds fixed in FAA were rinsed three times in 70% ethanol. Buds fixed in 4% paraformaldehyde/25% glutaraldehyde were rinsed in three changes in 0.2 M phosphate buffer following fixation and three changes of distilled water, and were moved through 25 and 50% ethanol. All buds were dehydrated in a graduated tertiary butyl alcohol series and infiltrated with paraffin over a period of 3 days. Flower buds used for the developmental study were sectioned longitudinally at 14 μm, mounted as serial sections with Fink's (1987) adhesive, stained with Mayer's hemalum, dehydrated, and mounted in Permount. Callose deposition during microsporogenesis was examined in sectioned flower buds stained with aniline blue (Jensen, 1962) and observed with fluorescence microscopy.

In Situ Hybridization

Flower buds processed for in situ hybridization were sectioned longitudinally at 8 to 10 μm and affixed to glass slides with Fink's adhesive. All slides processed for hybridizations were pretreated, hybridized, and washed according to the method outlined by Brigati et al. (1983) for paraffin-embedded tissue. The slides were incubated in a Streptavidin–alkaline phosphate complex and washed as directed by Boehringer Mannheim. The color was developed enzymatically with BCIP/NBT as directed by Bethesda Research Laboratories. The slides were then dehydrated and mounted in Permount.

The biotinylated (biotin-14-dATP) DNA probes used in the in situ hybridizations were prepared using random priming. The templates used were purified inserts derived from either a 0.99-kb SstI/PstI or a 1.5-kb SstI subclone of the unique sterility-associated mitochondrial sequence described in Mackenzie and Chase (1990). The three negative control probes were also prepared using random priming and included (1) the same mitochondrial 0.99-kb or 1.5-kb mitochondrial DNA segments without biotin (dATP added); (2) biotin-14-dATP added, DNA template omitted; and (3) a nonspecific template (1.0-kb segment of cosmid vector pHC79) with biotin-14-dATP added. All random primed reactions were spun through sephadex G50 and dot blotted to nylon to test incorporation of biotin.

Microscopy and Photography

All slides were observed with a photomicroscope (model Vanox; Olympus Corporation of America, New Hyde Park, NY) with bright-field or Nomarski optics. Photomicrographs were taken using either

black and white (Technical Pan 2415; Kodak) or color (EPY-135; Kodak) film.

ACKNOWLEDGMENTS

We would like to thank Dr. Charles Bracker for the use of the Electron Microscopy Facility of Purdue University and Debra Sherman for her valuable technical advice. We thank Dr. Sam Levings III and Dr. Daryl Pring for providing mitochondrial DNA clones, and Dr. Hanna Janska for critical reading of the manuscript. This work was supported in part by a United States Department of Agriculture grant to S.M. This is journal paper No. 12898 from the Indiana Agricultural Experiment Station.

Received September 23, 1991; accepted January 27, 1992.

REFERENCES

Albertsen, M.C., and Palmer, R.G. (1979). A comparative light- and electron-microscopic study of microsporogenesis in male sterile (Ms_1) and male fertile soybeans (*Glycine max* (L) Merr.). Am. J. Bot. **66,** 253–265.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, K., eds (1988). Preparation and analysis of RNA. In Current Protocols in Molecular Biology (New York: John Wiley & Sons), pp. 4.6.1–4.6.11.

Bassett, M.J., and Shuh, D.M. (1982). Cytoplasmic male sterility in common bean. J. Am. Soc. Hort. Sci. **107,** 791–793.

Berlyn, G.P., and Mischke, J.P. (1976). Fixation and storage. In Botanical Microtechnique and Cytochemistry (Ames, IA: State University Press), pp. 24–34.

Bino, R.J. (1985). Histological aspects of microsporogenesis in fertile, cytoplasmic male sterile and restored fertile *Petunia hybrida*. Theor. Appl. Genet. **69,** 423–428.

Brigati, D.J., Myerson, D., Leary, J.J., Spalholz, B., Travis, S.J., Fong, C.K.Y., Hsiung, G.D., and Ward, D.C. (1983). Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. Virology **126,** 32–50.

Chou, P.Y., and Fasman, G.D. (1978). Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47,** 45–147.

Dawson, A.J., Jones, V.P., and Leaver, C.J. (1984). The apocytochrome b gene in maize mitochondria does not contain introns and is preceded by a potential ribosome binding site. EMBO J. **3,** 2107–2113.

Dellaporta, S.L., Wood, J., and Hick, J.B. (1983). A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. **1,** 19–21.

Dewey, R.E., Levings, C.S., III, and Timothy, D.H. (1986). Novel recombination in the maize mitochondrial genome produces a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell **44,** 439–449.

Fauron, C., Havlik, M., and Brettell, R. (1990). The mitochondrial genome organization of a maize fertile cmsT revertant line is generated through recombination between two sets of repeats. Genetics **124,** 423–428.

Fink, S. (1987). Some new methods for affixing sections to glass slides. I. Aqueous adhesives. Stain Tech. **62,** 27–33.

Folkerts, O., and Hanson, M. (1991). The male sterility-associated *pcf* gene and the normal *atp9-1* gene in petunia are located on different mitochondrial DNA molecules. Genetics **129,** 885–895.

Hanson, M.R., and Conde, M.F. (1985). Functioning and variation of cytoplasmic genomes: Lessons from cytoplasmic nuclear interactions affecting male fertility in plants. Int. Rev. Cytol. **94,** 213–267.

Hensgen, L.A., Brakenhoff, J., De Vries, B.F., Sloof, P., Tromp, M.C., Van Boom, J.H., and Benne, R. (1984). The sequence of the gene for cytochrome c oxidase subunit I, a frameshift containing gene for cytochrome c oxidase subunit II and seven unassigned reading frames in *Trypanosoma brucei* mitochondrial maxi-circle DNA. Nucl. Acids Res. **12,** 7327–7344.

Hiratsuka, J., Shimada, H., Whittier, R., Ishibashi, T., Sakamoto, M., Mori, M., Kondo, C., Honji, Y., Sun, C.R., Meng, B.Y., Li, Y.Q., Kanno, A., Nishizawa, Y., Hirai, A., Shinozaki, K., and Sugiura, M. (1989). The complete sequence of the rice (*Oryza sativa*) chloroplast genome: Intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. Mol. Gen. Genet. **217,** 185–194.

Horner, H.T. (1977). A comparative light and electron microscopic study of microsporogenesis in male fertile and cytoplasmic male sterile sunflower (*Helianthus anuus*). Am. J. Bot. **64,** 745–759.

Horner, H.T., and Rogers, M.A. (1974). A comparative light and electron microscopic study of microsporogenesis in male fertile and cytoplasmic male sterile pepper (*Capsicum anuum*). Can. J. Bot. **52,** 435–441.

Houchins, J.P., Ginsburg, H., Rohrbaugh, M., Dale, R.M., Schardl, C.J., Hodge, T.P., and Lonsdale, D.M. (1986). DNA sequence analysis of a 5.27-kb direct repeat occurring adjacent to the regions of S-episome homology in maize mitochondria. EMBO J **5,** 2781–2788.

Jensen, W.A. (1962). Preparation of tissue. In Botanical Histochemistry (San Francisco, CA: W.H. Freeman and Co.), pp. 55–144.

Kohler, R.H., Horn, R., Lossl, A., and Zetsche, K. (1991). Cytoplasmic male sterility in sunflower is correlated with the co-transcription of a new open reading frame with the *atpA* gene. Mol. Gen. Genet. **227,** 369–376.

Lee, S.L.J., Gracen, V.E., and Earle, E.D. (1979). The cytology of pollen abortion in C-cytoplasmic male sterile corn anthers. Am. J. Bot. **66,** 656–667.

Lee, S.L.J., Earle, E.D., and Gracen, V.E. (1980). The cytology of pollen abortion in S cytoplasmic male sterile corn anthers. Am. J. Bot. **67,** 237–245.

Lonsdale, D.M. (1985). Movement of genetic material between the chloroplast and mitochondrion in higher plants. In Genetic Flux in Plants, B. Hohn, and E. Dennis, eds (New York: Springer-Verlag), pp. 51–60.

Mackenzie, S. (1991). Identification of a sterility-inducing cytoplasm in a fertile accession line of *Phaseolus vulgaris* L. Genetics **127,** 411–416.

Mackenzie, S., and Bassett, M. (1987). Genetics of restoration in cytoplasmic male sterile *Phaseolus vulgaris* L. Theor. Appl. Genet. **74,** 642–645.

Mackenzie, S., and Chase, C. (1990). Fertility restoration is associated with loss of a portion of the mitochondrial genome in cytoplasmic male-sterile common bean. Plant Cell **2,** 905–912.

Mackenzie, S., Pring, D., and Bassett, M. (1988a). Large double-stranded RNA molecules in *Phaseolus vulgaris* L. are not associated with cytoplasmic male sterility. Theor. Appl. Genet. **76,** 59–63.

Mackenzie, S., Pring, D., Bassett, M., and Chase, C. (1988b). Mitochondrial DNA rearrangement associated with fertility restoration and reversion to fertility in cytoplasmic male sterile *Phaseolus vulgaris* L. Proc. Natl. Acad. Sci. USA. **85,** 2714–2717.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Extraction, purification, and analysis of mRNA from eukaryotic cells. Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), p. 196.

McNay, J.W., Chourey, P.S., and Pring, D.R. (1984). Molecular analysis of genomic stability of mitochondrial DNA in tissue cultured cells. Theor. Appl. Genet. **67,** 433–437.

Nugent, J., and Palmer, J. (1991). RNA-mediated transfer of the gene *coxII* from the mitochondrion to the nucleus during flowering plant evolution. Cell **66,** 473–481.

Overman, M.A., and Warmke, H.E. (1972). Cytoplasmic male sterility in sorghum. II. Tapetal behavior in fertile and sterile anthers. J. Hered. **63,** 227–234.

Rottmann, W., Brears, T., Hodge, T., and Lonsdale, D. (1987). Mitochondrial gene is lost via homologous recombination during reversion of cmsT maize to fertility. EMBO J. **6,** 1541–1546.

Sanger, F. (1981). Determination of nucleotide sequences in DNA. Science **214,** 1205–1207.

Schuster, W., and Brennicke, A. (1988). Interorganellar sequence transfer: Plant mitochondrial DNA is nuclear, is plastid, is mitochondrial. Plant Sci. **54,** 1–10.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H., and Sugiura, M. (1986). The complete nucleotide sequence of the tobacco chloroplast genome: Its gene organization and expression. EMBO J. **5,** 2043–2049.

Singh, S., White, J., and Gutierrez, J. (1980). Male sterility in dry beans. Annu. Rep. Bean Improv. Coop. **23,** 55–57.

Takaiwa, F., and Sugiura, M. (1982). Nucleotide sequence of the 16S–23S spacer region in an rRNA gene cluster from tobacco chloroplast DNA. Nucl. Acids Res. **10,** 2665–2676.

Thomas, P.S. (1980). Hybridization of RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77,** 5201–5202.

Wakarchuk, D.A., and Hamilton, R.I. (1985). Cellular double-stranded RNA in *Phaseolus vulgaris.* Plant Mol. Biol. **5,** 55–63.

Warmke, H.E., and Lee, S.-L. (1977). Mitochondrial degeneration in Texas cytoplasmic male-sterile corn anthers. J. Hered. **68,** 213–222.

Wise, R., Pring, D.R., and Gengenbach, B.G. (1987). Mutation to male fertility and toxin insensitivity in Texas cytoplasm maize is associated with a frameshift in a mitochondrial open reading frame. Proc. Natl. Acad. Sci. USA **84,** 2858–2862.

Young, E.G., and Hanson, M.R. (1987). A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell **50,** 41–49.