Stoichiometric Shifts in the Common Bean Mitochondrial Genome Leading to Male Sterility and Spontaneous Reversion to Fertility

Hanna Janska, Rodrigo Sarria, Magdalena Woloszynska, Maria Arrieta-Montiel, and Sally A. Mackenzie

a Institute of Biochemistry and Molecular Biology, University of Wroclaw, Tamka, 2, 50-137 Wroclaw, Poland
b Department of Agronomy, Lilly Hall, Purdue University, West Lafayette, Indiana 47907

The plant mitochondrial genome is characterized by a complex, multipartite structure. In cytoplasmic male-sterile (CMS) common bean, the sterility-inducing mitochondrial configuration maps as three autonomous DNA molecules, one containing the sterility-associated sequence pvs-orf239. We constructed a physical map of the mitochondrial genome from the direct progenitors to the CMS cytoplasm and have shown that it maps as a single, circular master configuration. With long-exposure autoradiography of DNA gel blots and polymerase chain reaction analysis, we demonstrate that the three-molecule CMS-associated configuration was present at unusually low copy number within the progenitor genome and that the progenitor form was present substoichiometrically within the genome of the CMS line. Furthermore, upon spontaneous reversion to fertility, the progenitor genomic configuration as well as the molecule containing the pvs-orf239 sterility-associated sequence were both maintained at substoichiometric levels within the revertant genome. In vitro mitochondrial incubation results demonstrated that the genomic shift of the pvs-orf239-containing molecule to substoichiometric levels upon spontaneous reversion was a reversible phenomenon. Moreover, we demonstrate that substoichiometric forms, apparently silent with regard to gene expression, are transcriptionally and translationally active once amplified. Thus, copy number suppression may serve as an effective means of regulating gene expression in plant mitochondria.

INTRODUCTION

The organizational features of the plant mitochondrial genome have been the subject of numerous investigations during the past several years. Unlike most other well-investigated eukaryotic systems, the plant kingdom has demonstrated striking variability in mitochondrial genome size and structure (reviewed in Wolstenholme and Fauron, 1995). Although it is unclear whether all plant species contain a mitochondrial genome maintained in both linear and circular conformations, this appears to be the case for several species (Bendich, 1993; Oldenburg and Bendich, 1996). Moreover, a high degree of recombinational activity at repeated sequences interspersed throughout the genome produces a population of molecules that contain only a portion of the mitochondrial genome; however, taken together, they give rise to a high degree of coding redundancy. These are referred to as subgenomic DNA molecules, and mapping evidence suggests that although able to undergo intermolecular and intramolecular recombination, at least some molecules may be replicated autonomously (Folkerts and Hanson, 1991; Yamato et al., 1992; Janska and Mackenzie, 1993).

The origin and mode of DNA replication in the plant mitochondrial genome have not yet been defined. Recent evidence suggests that a rolling-circle model might account for observations made using electron microscopy, pulsed-field gel electrophoresis, and various hybridization procedures (Backert et al., 1997). What is strikingly unusual about most plant systems, however, is the observation that subgenomic DNA molecules can be maintained at dramatically different relative copy numbers within the genome. In fact, it is possible to observe some genomic configurations only by using polymerase chain reaction (PCR) amplification or unusually long DNA gel blot exposure times (Small et al., 1987). These low-copy-number configurations, referred to as sublimons, have been suggested to account for one mechanism by which genetic variability is accumulated within the mitochondrion (Small et al., 1989). In this study, we suggest that the suppression of copy number provides an effective means of repressing dominant mutations within the mitochondrial genome. Furthermore, we provide evidence to suggest that...
this suppression of copy number is a function of nuclear genotype. This function can be relaxed by the incubation of purified mitochondria in vitro.

Cytoplasmic male sterility (CMS) in common bean has been associated with the expression of a mitochondrial sequence encoded within a 2.4-kb mitochondrial region designated pvs (for *Phaseolus vulgaris* sterility sequence). This region contains at least two unique open reading frames (ORFs) designated pvs-orf98, encoding a 98-amino acid ORF, and pvs-orf239, encoding a 239-amino acid ORF. We have detected translation of only pvs-orf239, which produces a 27-kD polypeptide that accumulates in reproductive tissues (Abad et al., 1995) but is rapidly degraded in vegetative tissues (Sarria et al., 1998). Evidence suggests that the ORF239 product may induce the sterility phenotype (Abad et al., 1995; He et al., 1996). Mapping of the CMS common bean mitochondrial genome by overlapping cosmid clone analysis indicates that the genome is most likely organized as three interrecombining and highly redundant mitochondrial molecules of 394, 257, and 210 kb (Janska and Mackenzie, 1993). The pvs mutation resides on the 210-kb form; this molecule disappears from the genome upon spontaneous cytoplasmic reversal to fertility (Mackenzie et al., 1988; Janska and Mackenzie, 1993) or the introduction of a nuclear gene designated Fr (Mackenzie and Chase, 1990). In this study, we identified the origin of the 210-kb molecular configuration within the progenitors of the CMS source and investigated the nature of CMS induction and its suppression upon spontaneous reversion.

**RESULTS**

**Confirmation of Progenitors to the CMS Common Bean Line**

The fertile progenitor of the CMS common bean line CMS-Sprite is designated accession number G08063 (Mackenzie, 1991; Centro Internacional de Agricultura Tropical, Cali, Colombia); its mitochondrial genomic configuration is indistinguishable from that of the CMS-Sprite line used in our studies, and it has been confirmed to contain a sterility-inducing cytoplasm. Accession line G08063 reportedly originated with the cross POP × NEP-2 (maternal parent not defined; Mackenzie, 1991; pedigree data obtained from Centro Internacional de Agricultura Tropical). To investigate the origin of the sterility-inducing mitochondrial configuration, it was necessary to confirm the pedigree giving rise to the CMS source. Therefore, random amplified polymorphic DNA (RAPD)-based fingerprinting was used to assess genetic distances separating NEP-2, its progenitor San Fernando, POP, its progenitors Opal, and the derived sister lines G08063, G08064, and G08065, using line CMS-Sprite as an outlier. In Figure 1, we present a phenogram based on the analysis of 68 scored bands derived from eight primer reactions. Our results are in clear agreement with the stated pedigree of line G08063. This conclusion is based on the high degree of relatedness among progeny, all intermediate between parental types, relative to the average genetic distances observed within com-

![Figure 1. Estimation of Genetic Distances between Putative Progenitors That Gave Rise to Sister Lines G08063, G08064, and G08065.](image)
mon bean germplasm (Tohme et al., 1996). Therefore, lines POP and NEP-2 were utilized for subsequent construction of a physical map of the progenitor mitochondrial genome.

**Construction of a Physical Map of the Mitochondrial Genome of Progenitor Lines POP and NEP-2**

To rapidly identify the differences in mitochondrial genome organization between our CMS line, designated CMS-Sprite (contains a Sprite nuclear genotype with the G08063 cytoplasm), and NEP-2 and POP (G08063 progenitors), we performed comparative hybridizations. Total genomic DNA preparations were digested with PstI, SalI, XhoI, and EcoRI. Nineteen overlapping mitochondrial cosmid clones, spanning the entire mitochondrial genome of CMS-Sprite, were used as probes against blots prepared with genomic DNA from the male-sterile line and both progenitor lines. The majority of clones revealed identical hybridization patterns for all lines, regardless of the restriction enzyme used. From this observation, we concluded that long stretches of the investigated genomes have a similar configuration. Figure 2 shows that comparative hybridizations helped to identify four regions, designated A, B, C, and D, in the CMS-Sprite mitochondrial genome. These regions were rearranged relative to the genomes of NEP-2 and POP. The exact left and right boundaries of these rearrangements have not yet been defined; in Figure 2, they are presented simply as PstI fragments for convenience. Only EcoRI digestion allowed discrimination between the mitochondrial genomes of NEP-2 and POP. With this enzyme, two restriction fragment length polymorphisms were detected; they are outside of regions A to D. Our preliminary investigations suggest that they result from point mutations rather than sequence rearrangements (data not shown).

Once the regions distinguishing the progenitor and sterility-inducing mitochondrial genomes were defined, it was possible to identify novel junction fragments within the progenitor genomes. Table 1 summarizes the results of comparative hybridization experiments in regions A to D. Note that no hybridization signals were detected on normally exposed autoradiograms for two PstI fragments: a 4.5-kb fragment from region A and a 4-kb fragment from region D. Also, four remaining PstI fragments used as probes hybridized with only two fragments in the NEP-2 and POP samples. A 7.2-kb fragment of region A and a 10.2-kb fragment from region C hybridized with one 12-kb fragment in the progenitors. Likewise, a 6.3-kb fragment in region A and a 4.8-kb fragment from region B hybridized with one 7-kb fragment in the progenitors. From these observations, we postulated that the 12- and 7-kb PstI fragments in the progenitor genomes represent distinct junctions in the mitochondrial genomes of NEP-2 and POP.

To test this hypothesis, we used long-range PCR conditions incorporating primers localized to each side of the putative junctions. In the case of the 12-kb fragment, the sequence of one primer was based on the cytochrome b (cob) gene sequence located within the cross-hybridizing CMS-Sprite 7.2-kb fragment. The second primer was derived from the gene encoding the α subunit of ATPase (atpA) known to be present on the cross-hybridizing CMS-Sprite 10.2-kb fragment. The positions of the primers are shown in Figure 3. Based on primer locations, we expected to obtain a PCR amplification product of ~10 kb. The results are illustrated in Figure 4A. As predicted, a 10-kb fragment was amplified only when DNAs isolated from NEP-2 and POP were used as templates. In accordance with our hypothesis, the amplification was unsuccessful for CMS-Sprite because copies of the cob and atpA genes in this genome are separated by ~200 kb. However, the particular long-range PCR conditions used for this experiment were not sufficiently sensitive to amplify substoichiometric forms. Only those forms present in the predominant genome could be amplified (data not shown).

A similar PCR-mediated approach was used to confirm the presence of a second junction specific to the NEP-2 and POP mitochondrial genomes. We designed two primers that allowed amplification of the 7-kb PstI fragment together with a short flanking sequence. One primer was derived from the cross-hybridizing CMS-Sprite 4.8-kb PstI fragment and the other from the 18S/5S rRNA (rrn18/rrn5) gene sequence known to reside close to the cross-hybridizing 6.3-kb CMS-Sprite PstI fragment. The results of the PCR amplifications again were consistent with those from DNA gel blot analysis (Figure 4B). The predicted fragment was amplified only in the progenitor lines but not in samples from CMS-Sprite. These results indicate that in the progenitor genomes of NEP-2 and POP, the left flanking sequence to region A is connected by the 12-kb junction to the right flanking sequence of region C. Likewise, the right flanking sequence of region B is linked by a 7-kb junction to the right flanking sequence of region A (see Figure 2).

Two families of recombinationally active repeats, R1 and R2, were identified previously in the mitochondrial genome of the male-sterile line CMS-Sprite (Janska and Mackenzie, 1993). Figure 5A presents the schematic diagram of the branch points around these two repeats. Both repeats have three flanking sequences designated a, b, and c at one end of the repeat and two designated d and e at the other. Flanking sequences b and c share a segment of homology forming an additional branched point. As is shown in Figure 2, the genomic regions designated B and C are located nearby to copies of repeats R2 and R1, respectively. Thus, we assumed that at least two genomic environments surrounding repeats R1 and R2 were rearranged in the mitochondrial genomes of NEP-2 and POP relative to the sterile line.

To examine environments surrounding the R1 and R2 repeats in the progenitors, we hybridized probes homologous to the R1 and R2 repeats, with DNA preparations from CMS-Sprite, NEP-2, and POP digested with four different restriction enzymes. Novel genomic environments were detected in the progenitors, but the loss of b–d and b–e environments around R1 could be observed, as demonstrated in Figure...
Hybridization with the repeat R2 resulted in the detection of like-sized fragments in both male-sterile and fertile progenitor lines, but the relative intensity of bands differed; the observed changes in intensity are consistent with the loss of R2-surrounding environments b–d and b–e (Figure 5D). We were forced to base this assumption on band intensity because a restriction enzyme distinguishing these configurations was not identified. The lack of two genomic environments surrounding each repeat (b–d and b–e for R1 and b–d and b–e for R2) in the progenitor genomes implies that R1 and R2 repeats are present in only four configurations (Figure 5B) rather than the six identified in CMS-Sprite. Results of comparative hybridizations, identification of distinctive junctions, and assessment of genomic environ-

Figure 2. A Physical Map of the CMS-Sprite Mitochondrial Genome Indicating Regions Rearranged Relative to Progenitors NEP-2 and POP.

The CMS-Sprite map previously was constructed with restriction enzymes PstI (P), SalI (S), and XhoI (X) (Janska and Mackenzie, 1993). The regions rearranged relative to POP and NEP-2 are indicated (A to D) and were localized based on comparative hybridizations. The small boxes above the map indicate the locations of recombination repeats R1 and R2, with arrows indicating their orientation. The vertical dashed lines designate the junction points between the genome and the pvs sequence. The region encompassing this junction is shown at the bottom. Genomic environments a–e surrounding repeats are indicated.
ments surrounding the recombinational repeats within the progenitor genomes were used to construct a physical map of the mitochondrial genome of lines NEP-2 and POP. This map is presented in Figure 3. We present only one map because as previously mentioned, no differences were detected between the NEP-2 and POP genomes in hybridization patterns with enzymes PstI, Sall, and Xhol. The approximate positions of the two aforementioned polymorphisms revealed by EcoRI digestion are indicated on the map by asterisks. The two families of repeats, R1 and R2, are indistinguishable between progenitor and male-sterile lines, although their surrounding environments are simplified in the progenitors. Based on the linear representations of the physical maps, the mitochondrial genomes of the male-sterile line and its progenitors are 420 and 393 kb, respectively. This difference results from the reduced copy number of the recombination repeats and the absence of particular segments from regions A to D within the progenitor genomes.

Unlike the construction of the CMS-Sprite mitochondrial map, the ends of a linear representation of the physical map of NEP-2 and POP could be directly connected to produce a single master circular form; this is because the same R1 repeated sequence is located at both map ends. Thus, the entire configuration of the fertile progenitor mitochondrial genome is contained in a circular map of 393 kb, as diagrammed in Figure 6. In contrast, a model involving a single master chromosome in the CMS-Sprite genome would require the triplication of a segment of >200 kb. Thus, the corresponding model for the structure of the CMS-Sprite genome consists of three autonomous chromosomes (394, 257, and 210 kb) differing only in short, unique regions. Figure 6 presents the two proposed models, with the corresponding regions being designated. The genomic environments flanking the repeats R1 and R2 were chosen arbitrarily. To date, we have identified no sequences in the POP or NEP-2 genome that were not also present in the CMS-Sprite genome; however, sequences unique to the CMS-Sprite genome are indicated. The sequence of particular interest to our study is the sterility-associated sequence designated pvs.

The G08063 Sterility-Inducing Cytoplasm Arose in Part as a Consequence of a Mitochondrial Genomic Shift

With the identification of regions A to D unique to the sterility-inducing cytoplasm, it was possible to determine whether the G08063 genomic configuration was present at substoichiometric levels within the progenitor lines. To test this possibility, gel-blotted POP and NEP-2 total DNA preparations were hybridized with clones containing regions A to D, and autoradiograms were developed after extended exposure times. As shown in Figures 7A to 7D, low- to medium-intensity bands of expected sizes reflecting all four regions could be detected in POP, but only region B was detectable in NEP-2 (Figure 7B). In Figure 7D, a pvs probe was used to investigate the presence of the sterility-associated region within progenitor lines. The lower band observed in CMS-Sprite and POP represents an EcoRI fragment of 3.1 kb encompassing the pvs sequence. These results strongly suggest that mitochondrial DNA regions specific to the sterility-inducing cytoplasm preexist in the progenitor POP mitochondrial genome at substoichiometric levels. This result also implies that POP was likely the maternal parent in the cross that gives rise to G08063. We are investigating this possibility further.

A similar approach was used to determine whether the sequences distinguishing the progenitor POP genome are maintained at substoichiometric levels within the G08063 cytoplasm. In Figures 8A and 8B, autoradiography was used to demonstrate that the configurations characteristic of the fertile progenitor genome, represented as 7- and 12-kb PstI fragments described earlier, are present in CMS-Sprite. As expected, cross-hybridization of the probes to other previously defined regions was also observed. Thus, we conclude from these observations that the transition from progenitor POP mitochondrial configuration to that of the sterility-inducing configuration involved the amplification of the G08063 tripartite form concomitant with the suppression of the POP form.

Evidence That Spontaneous Reversion to Fertility Occurs as a Consequence of a Distinct Mitochondrial Genomic Shift

One unusual feature of the G08063-derived mitochondrial configuration is its propensity to undergo spontaneous cyttoplasmic reversion to fertility. These reversion events, influenced in frequency by nuclear genotype (Mackenzie et al., 1988), are associated with the disappearance of the pvs-containing mitochondrial molecule from the genome (Janska and Mackenzie, 1993). We examined the possibility that the reversion phenomenon also could be due to mitochondrial

---

Table 1. Sizes of NEP-2 and POP Mitochondrial DNA Fragments Hybridizing to Regions A to D of the CMS-Sprite Mitochondrial Genome

<table>
<thead>
<tr>
<th>Region</th>
<th>CMS-Sprite PstI Fragments Used as a Probe (kb)</th>
<th>NEP-2/POP PstI Cross-Hybridizing Fragments (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.2</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>— a</td>
</tr>
<tr>
<td></td>
<td>6.3</td>
<td>7.0</td>
</tr>
<tr>
<td>B</td>
<td>4.8</td>
<td>7.0</td>
</tr>
<tr>
<td>C</td>
<td>10.2</td>
<td>12.0</td>
</tr>
<tr>
<td>D</td>
<td>4.0 (pvs)</td>
<td>—</td>
</tr>
</tbody>
</table>

*The (—) indicates that no hybridization signal was detected. Hybridizing fragments that were common to both progenitor and CMS-Sprite genomes are not included in this table.*
To study this reversion event, we used long-distance PCR amplification, using as primers a sequence located at the 3' end of the atpA gene (residing 5' to pvs) and a sequence derived from the 3' end of the pvs-orf239 gene. These primers produce a fragment of 4437 bp unique to the pvs configuration. PCR-amplified fragments from revertant line WPR-3 were almost invisible when stained with ethidium bromide after gel electrophoresis (data not shown), but their identity and size were confirmed by subsequent DNA gel blot hybridization, as demonstrated in Figure 9A. These results indicate that the pvs sequence is not lost from the mitochondrial genome of the CMS line upon reversion but is maintained substoichiometrically.

These observations led to the prediction that the POP progenitor genome configuration also should be present substoichiometrically in the WPR-3 revertant line. Figure 9B shows the result of DNA gel blot hybridization to a mitochondrial DNA preparation of revertant WPR-3, using as a probe the PCR-amplified 7-kb PstI fragment specific to the POP genome. Although we were able to observe evidence of the presence of the progenitor configuration within WPR-3, our results suggest that the progenitor form was present in lower...
amounts within the revertant genome than in the male-sterile genome, although it was not feasible to quantify this difference accurately.

The Genomic Shift Associated with Reversion May Arise from Active Copy Number Suppression of the pvs-Containing Mitochondrial Molecule

Because the pvs-containing mitochondrial DNA molecule expresses a novel sequence, namely, pvs-orf239, it is possible to monitor its presence not only by DNA analysis but by expressional analysis as well. During this investigation, we posed the hypothesis that nuclear-directed reduction in relative copy number of a mitochondrial molecule should be reversible and might then provide one means of suppressing gene expression. To examine this possibility further, we purified mitochondria from lines G08063, CMS-Sprite, and two independently derived revertants, WPR-3 and 83-1, by fractionation over Percoll gradients. An aliquot of these mitochondria was lysed and tested by PCR and reverse transcription (RT)-PCR amplification for the presence of the pvs-orf239 sequence and its transcript. ELISA and immunoprecipitation with anti-ORF239 polyclonal antibodies were used to test for the presence of the ORF239 protein. The remaining intact, purified mitochondria were incubated in the presence of 35S-cysteine and an energy source to allow for in organello translations. Previous studies have indicated that the orf239 gene product is highly susceptible to proteolysis (Sarria et al., 1998), so mitochondrial incubations were in the presence of the protease inhibitor vanadate to allow for the accumulation of ORF239 protein. The 83-1 revertant sample was not included for all treatments.

Figure 4. PCR Amplification Results Demonstrating Two Distinct Junctions Present within the Progenitor Mitochondrial Genomes of NEP-2 and POP.

(A) Primers P2 and P8757 (locations designated in Figure 3) amplified a 10-kb fragment when mitochondrial DNA templates from NEP-2 and POP were used. Under the same conditions, no amplification product was detected with CMS-Sprite. A PstI-digested λ size standard (MW) is included for size estimation as well as the results of a reaction run without added template DNA (Control).

(B) Primers P18/5r and P4.8 (locations designated in Figure 3) amplified a 7-kb fragment when samples of mitochondrial DNA from NEP-2 and POP were used as templates. When CMS-Sprite mitochondrial DNA was used as a template, nonspecific products were detected; these products did not hybridize with the amplified 7-kb fragment used as probe in DNA gel blot experiments (data not shown). A size marker of HindIII-digested λ is included (MW) as well as a control reaction minus template (Control). Numbers at right indicate fragment lengths in kilobases.

Figure 5. Genomic Environments Surrounding Recombinationally Active Repeats R1 and R2 in the CMS-Sprite (G08063 Cytoplasm) Genome versus Progenitor Lines POP and NEP-2.

(A) and (B) Schematic diagrams show the branch points surrounding repeats R1 and R2 within the mitochondrial genomes of the CMS-Sprite (A) and progenitor (B) lines. Lowercase letters indicate different flanking sequences.

(C) DNA gel blot hybridization with blotted mitochondrial DNA digested with EcoRI and probed with repeat R1. Lowercase letters indicate the respective combinations of the flanking sequences.

(D) DNA preparations were digested with SalI and probed with repeat R2.
From these experiments, we obtained the results that are illustrated in Figures 10 and 11. PCR amplification experiments demonstrated a marked increase in the concentration of the \( pvs-orf239 \) sequence after 90-min incubations in all lines, suggesting that DNA replication occurs in vitro under the conditions used in our experiment. In the case of revertant WPR-3, the PCR product was undetectable at time zero with both ethidium bromide staining of the agarose gel after electrophoresis and DNA gel blot analysis. After 90-min incubations, the \( pvs-orf239 \) sequence was difficult to detect in WPR-3 samples by using ethidium bromide staining of agarose gels, but it was readily detected by subsequent DNA gel blot hybridization, as indicated in Figures 10A to 10F. These results suggest that the \( pvs-orf239 \) sequence is not only present in substoichiometric amounts within the WPR-3 line, as demonstrated, but that it is replicatively active. The difficulty encountered in detecting the sequence was presumably due to the relatively low concentration of mitochondria in our incubation sample as well as the lack of added exogenous nucleotides to our reaction, but the increase over time was dramatic and highly reproducible nonetheless. Of additional interest was our observation with primers specific for the \( cob \) gene. In the revertant samples, amplification with these primers also demonstrated an increase in intensity after incubation; however, incubation in the G08063 and CMS-Sprite samples demonstrated a detectable and reproducible decrease in amplification of the \( cob \) sequence. These results imply that incubation conditions led not only to amplification of some genomic regions but also to a possible decline in copy number of others.

To confirm that these results reflect de novo DNA synthesis, we performed similar incubations in the presence of a labeled nucleotide with and without the addition of ddTTP as a mitochondrial DNA replication inhibitor. Figures 10G and 10H demonstrate good incorporation of label into mitochondrial DNA fractions, markedly lowered with the addition of ddTTP to the incubation. As expected, the addition of the nuclear DNA replication inhibitor aphidicolin showed no effect. Our analysis of counts-per-minute values indicated that after the 90-min in organello incubation, 600 cpm was incorporated into 200 ng of mitochondrial DNA in the treatments with no inhibitor or added aphidicolin, and 250 cpm was incorporated into the ddTTP treatment.

Mitochondrial RNA preparations derived from purified CMS-Sprite and WPR-3 were used for RT-PCR, in vitro translation, and immunoprecipitation experiments to test for the presence and translational competence of the \( pvs-orf239 \) transcript in the revertant line after a 90-min incubation. Figure 11A shows the results of these experiments. RT-PCR amplification experiments, using primers specific for

---

**Figure 6.** Comparison of the Mitochondrial Genome Organization of CMS-Sprite (G08063 Cytoplasm) and Its Fertile Progenitors NEP-2 and POP.

Regions showing the same restriction pattern are indicated by the same color. Sequences of the CMS-Sprite genome rearranged relative to the genomes of NEP-2 and POP are designated in red, with the letters A to D corresponding to Figure 2. Two distinctive junctions exist within the POP and NEP-2 genomes and are shown as rectangles marked J-7 and J-12. The positions of recombinationally active repeats are represented by boxes with arrows to indicate relative orientations. The filled box shows the location of the \( pvs \) sequence.
the pvs-orf239 transcript, demonstrated that the transcript was undetectable in mitochondrial RNA preparations from the revertant before incubation but readily detectable after incubation. Although, admittedly, these experiments were not quantitative, they allow us to conclude that a marked increase in concentration of the pvs-orf239 transcript occurred over the 90-min incubation period. Similar in organello transcription activity in the absence of added exogenous ribonucleotides was reported previously in rat mitochondria by Enriquez et al. (1993). Immunoprecipitation of the in vitro translation product with anti-ORF239 antibodies confirmed that the pvs-orf239 transcript was present and translationally competent in both the CMS-Sprite and revertant WPR-3 samples after incubation, although significantly less product was obtained from the WPR-3 preparation.

These results suggest that the pvs-orf239 sequence is transcriptionally active within the revertant once DNA copy number is increased. This is important, because no pvs-orf239 transcript is detectable in mitochondria purified from whole plant tissues of a revertant line (Chase, 1994), suggesting that the substoichiometric form of pvs-orf239 is present in sufficiently low copy numbers to render it effectively silent. It is not possible, based on these experiments, to state that the pvs-orf239 sequence is fully silent in the revertant, because it is possible that transcripts are produced at levels below detection.

In organello translation experiments using mitochondria purified from CMS-Sprite and revertant WPR-3 likewise demonstrated an accumulation of the ORF239 protein after incubation (Figure 11B). Again, lower levels were detected in

Figure 7. Overexposed Autoradiograms Reveal G08063-Specific Mitochondrial Sequences at Substoichiometric Levels in the Progenitors POP and NEP-2.

Total genomic DNAs prepared from CMS-Sprite, NEP-2, and POP were blotted and hybridized with probes representing regions A to D from the CMS-Sprite mitochondrial genome (see Figure 2). For (A) to (D), the CMS-Sprite sample was underloaded intentionally by approximately one-fourth relative to the progenitor samples, and numbers denote molecular weights of fragments indicated by arrowheads.

(A) DNA samples were digested with EcoRI and hybridized with a cosmid clone that overlaps region A and its right flanking sequence. Arrowheads indicate the EcoRI fragments derived from the region.

(B) Samples were digested with PstI and hybridized with a cloned 4.8-kb PstI fragment that itself represents region B. Other bands evident are known to correspond to homologous regions within the genomes.

(C) Samples were digested with EcoRI and hybridized with a cosmid clone that overlaps region C and its flanking sequences. The 2.8-kb fragment represents the junction between region C and pvs.

(D) Samples were digested with EcoRI and hybridized with a PCR-amplified pvs sequence; the band indicated by an arrowhead represents the mitochondrial fragment encompassing pvs. The upper, more intensely hybridizing band represents a chloroplast fragment that hybridizes by virtue of a small region of chloroplast trRNA^{A^B} intron homology contained within the pvs region (Chase and Ortega, 1992; Johns et al., 1992). All mitochondrial DNA fragments present in the CMS-Sprite genome were detected in the genome of fertile progenitor POP.

In (A) to (D), numbers at right indicate fragment lengths in kilobases.
of the G08063-associated configuration then would involve the amplification of the tripartite form and the suppression of the POP progenitor form to substoichiometric levels. Upon spontaneous reversion, a second suppression event is postulated, now involving only the pvs-containing mitochondrial molecule to substoichiometric concentrations.

Only with the availability of progenitors to accession line G08063 has investigation into the origin of the CMS-associated mitochondrial configuration become feasible. Although construction of a physical map of the progenitor genome in lines POP and NEP-2 produced a model predicting a single circular master chromosome, the greatest proportion of the genome is similar to that found in the male-sterile line. Direct comparison of the progenitor and G08063 tripartite mitochondrial configurations does not allow us to deduce the series of events that mediated the formation of one from another over evolutionary time; most likely, the two forms have evolved independently for some period. From mapping data, we postulate that several sequential events have occurred in the derivation of the tripartite form, with one important event involving the creation of the pvs region. This would be consistent with the means of generating mitochondrial genome

**DISCUSSION**

Based on the observations presented here, we propose the following model to account for the mitochondrial genome changes observed between progenitor line POP, derived accession line G08063, and the fertile revertants WPR-3 and 83-1. Our model is outlined schematically in Figure 12. We propose that the present-day mitochondrial genome of progenitor line POP contains not only the predominant form, represented as a single master chromosome, but also the sterility-associated tripartite configuration characteristic of derived line G08063 at substoichiometric levels. Generation

**Figure 8.** Overexposed Autoradiograms Demonstrating the Presence of Progenitor (NEP-2 and POP)-Specific DNA Fragments in CMS-Sprite Mitochondrial DNA Preparations.

Mitochondrial DNA preparations were digested with PstI and hybridized with probes derived from amplified junction fragments unique to the NEP-2 and POP genomes. Probes were PCR amplified from the POP mitochondrial genome. Other bands correspond to homologous regions identified in the genomes.

(A) Probed with the 7-kb junction fragment.

(B) Probed with the 12-kb junction fragment.

**Figure 9.** Evidence for the Presence of the pvs Sequence and the POP and NEP-2 Progenitor Genome Forms within Revertant Line WPR-3.

(A) PCR amplification of the pvs region (4.4 kb) from the mitochondrial genome of revertant line WPR-3 by using long-distance PCR amplification with primers ATP-A5 and orf239-3′ (see Methods). The PCR product from revertant samples is barely visible by ethidium bromide staining of an agarose gel (data not shown). The identity of the PCR product is confirmed by DNA gel blot hybridization, using a cloned orf239 sequence as a probe.

(B) Overexposed autoradiogram demonstrating the presence of the 7-kb junction fragment specific to NEP-2 and POP progenitor genomes within the genome of fertile revertant WPR-3. The mitochondrial DNA samples were digested with PstI, blotted, and hybridized with the PCR probe encompassing the 7-kb junction fragment. The position of this fragment is indicated.
The observed amplification and expression of the pvs-containing mitochondrial molecule in vitro in mitochondria purified from the revertant lines suggest that the reversion phenomenon is reversible and that the male-fertile phenotype of the revertant is a direct consequence of reduced pvs copy number. Moreover, our results imply that the genomic shift occurring upon reversion involves differential replication as opposed to mitochondrial population shifts or intercellular competition, as have also been postulated by others (Albert et al., 1996). This conclusion is based on the fact that we were able, with a 90-min incubation of a defined population of mitochondria isolated from revertant seedling tissues, to observe a reversal of the reversion phenomenon in the form of amplification and expression of the pvs-orf239 sequence. Because we have, in previous studies, associated expression of the pvs-orf239 sequence with the sterility phenotype (Abad et al., 1995; He et al., 1996), we assume that the changes observed in vitro would have led to the reestablishment of the male-sterile phenotype in vivo. We are currently conducting experiments to test for the reversibility in vivo of the fertile revertant to the male-sterile phenotype.

The in organello experiments to test for reversibility of pvs-orf239 suppression were intentionally conducted for DNA amplification, RNA amplification, and protein expression simultaneously, using identical incubation conditions optimized for in organello protein synthesis. This is because our observation of in organello ORF239 protein accumulation in mitochondria from revertant lines over a 90-min incubation directly implied concomitant DNA and RNA amplification under these conditions. However, it can be assumed that the addition of exogenous nucleotides to the incubation mixture would have enhanced dramatically the rate of DNA and RNA synthesis in vitro. Enriquez et al. (1994) showed incorporation of $^{32}$P-dCTP in the absence of added dTTP, dATP, and dGTP in in organello incubations of rat mitochondria, using 1 mg of protein per incubation, and then compared these results with those obtained after adding the four $^{32}$P-labeled deoxynucleotide triphosphates. They obtained up to 100,000 cpm over a 10-hr in organello incubation period, when all four labeled nucleotides were added, and a 75% reduction in this value in the absence of the added nucleotides; however, it was not clear how much mitochondrial DNA was used to determine these values. In the same study, it was shown that a concentration of mitochondria equivalent to 2 mg/mL of protein represents an optimal amount to achieve good incorporation. In our study, the amount of mitochondria included per incubation was equivalent to 0.2 mg of protein, representing 20% of the amount used by Enriquez et al. (1994), with the incubation lasting only 90 min. Because of the striking differences in reaction conditions as well as the organisms from which mitochondria were prepared, it is difficult to compare directly the efficiency of our reaction with that reported previously. However, the results reported by Enriquez et al. do support our conclusion that mitochondrial DNA replication occurs under the conditions used in our experiment.
Figure 10. Evidence of Amplification of the pvs-orf239 DNA Sequence within the Mitochondrial Genome of Revertant Lines after in Organello Incubation.

Mitochondrial samples (200 μg of protein) taken from Percoll gradient–purified mitochondria were used for isolation of DNA template to test for changes in stoichiometry within the mitochondrial genome after a 90-min in organello incubation (two time points: 0 and 90 min). A 5-ng sample of mitochondrial DNA was used for the PCR amplification reactions, and amplified samples subsequently were subjected to electrophoresis in a 1% agarose gel.

(A) PCR amplification results, using the orf5’ and orf3’ primers, are shown on an ethidium bromide–stained gel. Numbers at left indicate fragment size in kilobases.
It becomes increasingly clear that substoichiometric forms are prevalent within the mitochondrial genomes of many plant species (Small et al., 1987; Bonhomme et al., 1992; Pla et al., 1995; Yesodi et al., 1995; Suzuki et al., 1996; Gutierrez et al., 1997) and that changes in their relative stoichiometry can be reversed (Kanazawa et al., 1994). Although the tracking of genomic shifts over generations has not previously been reported to the extent that we describe here, it would not be surprising to find that these phenomena occur in several plant lineages. In fact, such spontaneous genomic shifts are predicted to account for much of the heterogeneity found in plant mitochondrial populations (Lonsdale et al., 1988). What is relevant about our observations in common bean is that they demonstrate that these shifts can occur in a very short time span within a single plant lineage under natural growth conditions.

Evidence suggests that some genomic shifts are under the control of specific nuclear loci. For instance, introduction by crossing of the fertility restorer Fr in common bean produces a phenomenon indistinguishable from reversion (Mackenzie and Chase, 1990), and in fact, the pvs-orf239 sequence is present substoichiometrically in fertile Fr-restored lines (W. Van Houten and S.A. Mackenzie, unpublished data). It is conceivable that our in vitro mitochondrial incubations to amplify the pvs molecule simply have allowed the relaxation of control by the Fr locus in the experiments reported here. Moreover, in Arabidopsis, mutation at the chloroplast mutator (CHM) locus results in the selective amplification of specific substoichiometric forms, depending on the cytoplasm contained in the line to which the chm mutation is introduced (Sakamoto et al., 1996). These examples provide the most compelling evidence to date that nuclear genotype directly influences the stoichiometric relationships among mitochondrial molecules.

One of the distinguishing features of plants with regard to mitochondrial biology is the subdividing of their genome into a complex, recombinationally active organization. The evolutionary rationale for such a complex structure has not been immediately obvious. However, our observations in this study suggest that such a structure may provide one means of modulating expression of particular regions of the genome. In plant mitochondria, where the roles of transcriptional, post-transcriptional, and translational mechanisms in gene inactivation are not altogether clear and where dominant mitochondrial mutations are prevalent (Hanson, 1991), the ability to dramatically and reversibly suppress copy number of a subsegment of the genome most likely provides an important mechanism for maintaining proper mitochondrial function while retaining tremendous adaptive genetic diversity.

METHODS

Plant Materials

Common bean (Phaseolus vulgaris) accession lines G04459 (NEP-2) and G14067 (POP), G08063, G08064, and G08065, and line San Fernando were obtained from the Centro Internacional de Agricultura Tropical Germplasm Collection Center (Cali, Colombia). The cultivar Opal was provided by Rogers Brothers Seed Co. (Twin Falls, ID). CMS-Sprite was derived from recurrent backcrossing (20 generations) of the G08063 cytoplasm to a cultivar Sprite nuclear genotype. Spontaneous reversions were selected as single seed-bearing pods.

Figure 10. (continued).

(B) Primers orf5’ and cob3’ were used for PCR amplification, and the products are shown on an ethidium bromide-stained gel.

(C) An autoradiogram of the gel shown in (A) hybridized with the orf239 probe.

(D) The corresponding autoradiogram from the gel shown in (B), using the orf239 sequence as probe.

(E) Primers cob5’ and cob3’ were used for PCR amplifications. These primers were selected as controls to monitor an unrelated region of the mitochondrial genome. Revertant lines consistently showed an increase in amplification product after the in organello incubation, whereas CMS-Sprite and G08063 showed a slight decrease in amplification product. We assume that changes in concentration of amplified product are most likely reflective of the changes in the relative amount of template after the in organello incubations because equal amounts of mitochondrial DNA template were included in each reaction. Numbers at left indicate fragment size in kilobases.

(F) Diagram depicting the relative location of the primers used in the regions containing the pvs-orf239 and cob genes, respectively.

(G) and (H) Results of 32P-dCTP incorporation experiments to demonstrate de novo DNA synthesis by purified bean mitochondria in the absence of added deoxynucleotides during in organello incubations. In organello incubations (200 μg of mitochondrial protein) were performed in the presence of 32P-dCTP, with or without the addition of the replication inhibitors ddTTP or aphidicolin. Samples for three time points (0, 30, and 90 min) in each of the treatments described above were taken during the incubation to test for 32P-dCTP incorporation into newly synthesized mitochondrial DNA. DNA samples of 200 ng each were subjected to electrophoresis in 0.8% agarose DNA gel blotted, counted, and exposed to x-ray film. Because of the lack of identified specific mitochondrial γ DNA polymerase inhibitors, the nonspecific replication inhibitor ddTTP (20 μM) was used to demonstrate that the observed incorporation of 32P-dCTP was dependent on mitochondrial DNA replicative activity. The count-per-minute values obtained for each of the bands show that a 45% inhibition of 32P-dCTP incorporation occurs in the presence of ddTTP when compared with the aphidicolin and no-inhibitor treatments after 90 min. Subsequent hybridization of the resulting DNA gel blot with a chloroplast DNA probe (ATP synthase subunit I) indicated that chloroplast DNA contamination of the prepared mitochondrial DNA sample was undetectable (data not shown).
served at time zero of an in organello incubation or when ORF239 fraction only) were used. These larger forms never have been ob-

erred at time zero of an in organello incubation or when ORF239 because they were present in both immunoprecipitated products are thought to represent aggregation or multimeric forms shown in the fifth and sixth lanes. These higher molecular weight revealed the presence of two higher molecular weight products (not dicted size was obtained (30 kD). Longer exposure of IVT samples using the anti-ORF239 polyclonal antibody. The product of the pre- precipitate the in vitro–transcribed and –translated (IVT) ORF239 pep-
tide. In all remaining lanes, the products were immunoprecipitated using the anti-ORF239 polyclonal antibodies. Lanes labeled Anti-ORF239 and Preim show the results of experiments with anti-ORF239 polyclonal antibody and preimmune serum, respectively, used to immunopre-

cipitate the in vitro–transcribed and –translated (IVT) ORF239 peptide. In all remaining lanes, the products were immunoprecipitated using the anti-ORF239 polyclonal antibody. The product of the predicted size was obtained (30 kD). Longer exposure of IVT samples revealed the presence of two higher molecular weight products (not shown; also presented in Sarria et al., 1998) of identical size to those shown in the fifth and sixth lanes. These higher molecular weight products are thought to represent aggregation or multimeric forms of ORF239 because they were present in both immunoprecipitated IVT and in organello samples when ORF239 antibodies (purified IgG fraction only) were used. These larger forms never have been ob-

erved at time zero of an in organello incubation or when ORF239 on otherwise male-sterile CMS-Sprite plants. They were confirmed to be cytoplasmic events, as described by Mackenzie et al. (1988).

DNA Isolation, Gel Blotting, and Hybridization Procedures

Mitochondrial DNA was purified from 7-day-old etiolated seedlings, according to the procedure described by Mackenzie et al. (1988). Total genomic DNA was extracted from leaves of greenhouse-grown plants, as described by He et al. (1995). Cosmid or plasmid DNAs were prepared as minipreparations by the method described by Sambrook et al. (1989). DNAs were digested with restriction enzymes according to the manufacturers’ instructions (PstI and EcoRI from Gibco BRL; XhoI and Sall from Promega). Agarose gel electrophoresis, DNA transfer to Hybond-N nylon membrane (0.45 μm; Amersham), extraction of DNA from the membrane, labeling of DNA, and filter hybridizations were con-
ducted according to the procedures described by J. Anoka and Mackenzie (1993). Autoradiography was performed using an Instant-imager (Packard, Meriden, CT) and exposure to x-ray film (Fuji, Stamford, CT) with intensifying screens at ~70°C. Short exposure times were generally overnight, with long exposure times to visualize substoichiometric forms extended to ~1 week.

DNA Fingerprint Analysis

Oligonucleotide primers (10mers) were purchased from Operon Technologies (Alameda, CA). The polymerase chain reaction (PCR) conditions were optimized for this study. Reaction mixtures (20 μL total volume) consisted of 10 mM Tris-HCl, pH 9.0, at 25°C, 50 mM KCl, 2.25 mM MgCl₂, nucleotides dATP, dTTP, dCTP, and dGTP (125 μM each), 0.2 μM primer, 60 ng of template DNA, and 1.25 units of Taq DNA polymerase (Promega). The amplifications were performed in a thermal cycler (model PTC100; MJ Research, Water-
town, CT) programmed for 1 min at 94°C followed by 35 cycles of 35 sec at 94°C, 50 sec at 36°C, 50 sec at 72°C, and ending with 5 min at 72°C. The primers were used individually.

Rapid amplified polymorphic DNA (RAPD) profiles were scored visually. Variation among samples was evaluated from pairwise comparisons of the proportion of shared fragments among samples, that is, two times the number of shared fragments divided by the total number in the profiles being compared. This method was equivalent to calculating Nei and Li’s index (1979) for genetic similarity (Sxy) for restriction fragment length polymorphism comparisons. Relationships among samples were evaluated with a phenetic cluster analysis, using the unweighted pair group method for arithmetic averages, and plotted in a phenogram. All computations and statistical analyses were performed using SAS (version 6; Cary, NC) programs.

was absent (e.g., in the absence of added protease inhibitor). Lanes designated CMS (CMS-Sprite) and WPR-3 IVT-mRNA show the re-
cov of ORF239 peptide by immunoprecipitation with anti-ORF239 antibodies after in vitro translation of mitochondrial RNA (1 μg per reaction) isolated after a 90-min in organello incubation. Lanes des-

ignated in organello show recovery by immunoprecipitation of the ORF239 protein product after in organello translation reactions with purified CMS-Sprite and WPR-3 mitochondria in the presence of the protease inhibitor vanadate (4 mM).
Mitochondrial DNA Probes and DNA Cloning

Cosmid clones used in comparative DNA gel blot hybridizations were derived from previously developed mitochondrial DNA libraries of CMS-Sprite (Mackenzie and Chase, 1990) and WPR-3 (Janska and Mackenzie, 1993). Other mitochondrial probes were derived by restriction endonuclease digestion of cosmid DNA and fragment purification from agarose, as described by Janska and Mackenzie (1993). Probes used to identify regions unique to the progenitor NEP-2 and POP genomes were derived by PCR amplification, using total DNA as a template.

The 4.8-kb PstI restriction fragment derived from cosmid clone 12G4 (WPR-3 library) was purified and ligated to vector pBluescript II KS+ (Stratagene, La Jolla, CA) for transformation to Escherichia coli strain XL-1 Blue MRF9 (Stratagene). The orientation was deduced by restriction pattern, and the appropriate fragment end was sequenced (Sequenase 2.0; Amersham) to identify a suitable primer site for subsequent PCR amplification experiments.

Long-Distance PCR

To amplify two distinctive junctions present in the NEP-2 and POP mitochondrial genomes, we performed reactions in a Thermocycler Poly-Chain II (PolyGen, Kiev, Ukraine). Amplification between atpA and cob utilized the following primers: primer P2 (5'-ATAGCAGGTCTAATTCCGCG-3') is homologous to the atpA gene from common bean at positions 1084 to 1103 (Chase and Ortega, 1992), and primer P8757 (5'-AGCTATGCATTACACCTC-3') was derived from the maize cob gene at positions 168 to 187 (Dawson et al., 1984). These
primers allowed amplification of the 10-kb fragment. The second specific junction, ~7 kb in length, utilized primers P4.8 (5'-GTGCTATAGTGCCCTTTGAC-3') derived from sequencing the 4.8-kb PstI fragment at the junction site and P18/5r (5'-CATGCTCCAACCGCTTGT-3') derived from the soybean m18 gene sequence at positions 929 to 945 (Grabau, 1985). The locations of all primers are shown in Figure 3.

PCR amplifications involved a 50-μL reaction mixture of 50 mM Tris-HCl, pH 9.0, 10 mM (NH4)2SO4, 0.1% Triton X-100, 2 mM MgCl2, 400 μM of each deoxynucleotide triphosphate, 800 nM of each primer, and 100 ng of template mitochondrial DNA. The samples were overlaid with 50 μL of mineral oil. After an initial denaturation step of 2 min at 92°C, the reaction was initiated by adding 2.5 units of PrimeZyme DNA polymerase (Biometra, Gottingen, Germany). Amplification of the 10-kb fragment was performed for 30 cycles with denaturation at 92°C for 10 sec, annealing at 62°C for 30 sec, and extension at 68°C for 12 min. During the last 20 cycles, 100 sec was added to the extension time after each fifth cycle. The reaction was followed by a 25-min incubation at 68°C. To amplify the 7-kb fragment, we lowered the annealing temperature to 58°C, and the extension times were as follows: 10 cycles of 7 min in duration, with the following 20 cycles lengthened each fifth cycle by 60 sec. The final elongation was 15 min. In the CMS-Sprite sample, nonspecific bands of various sizes amplified using these conditions; none of these amplification products demonstrated hybridization with the 4.8-kb PstI fragment. PCR products were agarose gel fractionated.

For long-distance PCR amplification of the pvs region in revertant WPR-3 and 83-1 mitochondrial DNA preparations, primers ATP-A5 (5'-GGTGAACGCTATTGCAGA-3') from the 3' end of the atpA gene (Chase and Ortega, 1992) and orf239 (5'-CCATTACGGGGATGCCTT-3') derived from the 3' end of the pvs-orf239 sequence were used. The Expand long-template PCR system was used (Boehringer Mannheim). Reaction conditions involved a 30-min synthesis reaction. For subsequent PCR reactions, 5 μL of cDNA first-strand synthesis was used. PCR amplification of pvs-orf239 was as described below.

### Mitochondrial Preparation for in Organello Incubations

Dark-grown 10-day-old seedlings of common bean lines CMS-Sprite and WPR-3 were used for mitochondria isolation, according to Forde et al. (1978), and purified over a 26% Percoll gradient. Purified mitochondria were resuspended in 10 times the final pellet volume, which gives an approximate protein concentration of 40 μg/μL.

### In Organello Incubations and Nucleic Acid and Protein Isolations

Mitochondrial in organello incubations were conducted without modifications, according to the procedure of Forde et al. (1978). After a 90-min incubation, samples were used for mitochondrial RNA, DNA, or protein isolation. In organello reactions to demonstrate de novo DNA synthesis in the absence of added deoxynucleotide triphosphates were conducted in the presence of 4 μL of Redivue-32P-dCTP (Amersham), with a specific activity of 3000 Ci/mmol (0.05 μM) with and without 20 μM ddTTP (Sigma) and 20 μM/mL aphidicolin (Fluka, Milwaukee, WI). For RNA and DNA isolation, mitochondria were lysed in lysis buffer (150 mM NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 2% SDS). Samples were incubated at 65°C for 10 min, after which potassium acetate (0.5 M) was added. Samples were then centrifuged for 5 min at 14,000 rpm and precipitated overnight with 2 volumes of absolute ethanol. Preparations were resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA). For the purification of RNA, samples were treated with RNase-free DNase, and for the isolation of DNA, with DNase-free RNase. In organello translations, 35S-cysteine and the protease inhibitor vanadate (4 mM) were included to recover labeled ORF239 product. For protein isolation, mitochondria were treated as described by Ragan et al. (1987), without fractionation. Counts per minute in 32P-dCTP incorporation experiments were determined using a Packard InstantImager.

### Reverse Transcription–PCR Reactions

Reverse transcription (RT) and RT-PCR reactions for detection of pvs-orf239 were performed in the presence of 1 μL of avian myeloblastosis virus reverse transcriptase (25 units/μL; Promega), 4 μL of 5' x avian myeloblastosis virus reverse transcriptase buffer, 2 μL of deoxynucleotide triphosphates (10 mM each), 1 μL of Rnasin (40 units/μL), 1 μL of orf3 primer (12.5 μM), and 400 ng of template mitochondrial RNA in a total volume of 20 μL. The cDNA first-strand synthesis reaction was incubated at 55°C for 1 hr. Samples subsequently were incubated at 65°C for 15 min to stop the reverse transcription reaction. For subsequent PCR reactions, 5 μL of cDNA first-strand synthesis was used. PCR amplification of pvs-orf239 was as described below.

### In Vitro Translation and Immunoprecipitation

The clone orf239 (1 μg) was transcribed and translated using the TNT-coupled transcription/translation reticulocyte lysate system (Promega). Transcription was performed using T7 RNA polymerase. Reactions were incubated for 90 min. The rabbit reticulocyte system (Promega) was used to translate purified mitochondrial RNA samples. Reactions were incubated for 90 min. Immunoprecipitations of both in organello translations as well as in vitro translations were performed according to Sarris et al. (1998). Anti-ORF239 polyclonal antibodies were used as described by Abad et al. (1995). Protein samples were run in an SDS-PAGE discontinuous system, as described by Laemmli (1970).

### PCRs and DNA Gel Blotting from in Organello Incubation Samples

Primers orf5 (5'-CTGTTAAATTTCGCGAGGAC-3'), orf3 (5'-CTGTTAAATTTCGCGAGGAC-3'), and cob3 (5'-TTGAAATTTTCGCGAGGAC-3') were used for the PCR reactions. Primers orf5 and orf3 amplify a 720-bp fragment that comprises the pvs-orf239 gene. Primers orf5 and cob3 generate a 783-bp fragment that contains the pvs-orf239 gene and the flanking 83 bp that have 100% homology with the 3' end of the cob gene. Primers cob5 and cob3 generate a 1148-bp fragment.
that corresponds to most of the coding region of the cob gene. A 5-ng sample of mitochondrial DNA obtained before and after in organello incubations was used per PCR reaction. PCR cycling conditions were an initial denaturation step of 94°C for 1 min, 35 cycles of 92°C for 30 sec, 56°C for 1 min, and 72°C for 1 min and 30 sec, with a final elongation step of 72°C for 5 min. DNA gel blots were prepared and hybridized as described by Sambrook et al. (1989).

ACKNOWLEDGMENTS

We thank the Biological Illustrations Department at Purdue University for their assistance in the preparation of illustrations. This work was supported by Grant No. 9630252 MCB from the National Science Foundation to S.A.M. and Grant No. 6P20302307 from the Polish State Committee for Scientific Research (KBN) to H.J. .

Received January 20, 1998; accepted April 28, 1998.

REFERENCES


Stoichiometric Shifts in the Common Bean Mitochondrial Genome Leading to Male Sterility and Spontaneous Reversion to Fertility
Hanna Janska, Rodrigo Sarria, Magdalena Woloszynska, Maria Arrieta-Montiel and Sally A. Mackenzie

*Plant Cell* 1998;10;1163-1180
DOI 10.1105/tpc.10.7.1163

This information is current as of September 7, 2017