Unusual Mitochondrial Genome Organization in Cytoplasmic Male Sterile Common Bean and the Nature of Cytoplasmic Reversion to Fertility

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ABSTRACT

Spontaneous reversion to pollen fertility and fertility restoration by the nuclear gene *Fr* in cytoplasmic male sterile common bean (*Phaseolus vulgaris* L.) are associated with the loss of a large portion of the mitochondrial genome. To understand better the molecular events responsible for this DNA loss, we have constructed a physical map of the mitochondrial genome of a stable fertile revertant line, WPR-3, and the cytoplasmic male sterile line (CMS-Sprite) from which it was derived. This involved a cosmid clone walking strategy with comparative DNA gel blot hybridizations. Mapping data suggested that the simplest model for the structure of the CMS-Sprite genome consists of three autonomous chromosomes differing only in short, unique regions. The unique region contained on one of these chromosomes is the male sterility-associated 3-kb sequence designated *pvs*. Based on genomic environments surrounding repeated sequences, we predict that chromosomes can undergo intra- and intermolecular recombination. The mitochondrial genome of the revertant line appeared to contain only two of the three chromosomes; the region containing the *pvs* sequence was absent. Therefore, the process of spontaneous cytoplasmic reversion to fertility likely involves the disappearance of an entire mitochondrial chromosome. This model is supported by the fact that we detected no evidence of recombination, excision or deletion events within the revertant genome that could account for the loss of a large segment of mitochondrial DNA.

THE plant mitochondrial genome demonstrates a number of features unique to the plant kingdom. The organization of the genome has been the subject of investigation for a number of years and still remains somewhat controversial. It is not clear whether the mitochondrial genome exists predominantly as circular or linear DNA molecules (BENDICH and SMITH, 1990). The existence of a master chromosome, containing the entirety of the genetic information, has been proposed but not yet proven. Furthermore, little or no information is available regarding the role of nuclear genes in the maintenance of the mitochondrial genome organization.

The most generally accepted model of the mitochondrial genome in higher plants has consisted of a multipartite configuration, with a master chromosome giving rise to subgenomic circles (LONSDALE *et al.* 1988). These subgenomic molecules are presumably generated by recombination between pairs of repeated sequences found on the master chromosome. This model was derived primarily from the mapping of overlapping cosmid clones (LONSDALE, HODGE and FAURON 1984; PALMER and SHIELDS 1984). Efforts to test this model by observing intact mitochondrial DNA molecules using electron microscopy (KOLODNER and TEWARI 1972; SYNENKI, LEVINGS and SHAH 1978; BENDICH 1985) or various gel electrophoresis procedures (BENDICH and SMITH 1990; LEVY, ANDRE

and WALBOT 1991; NARAYANAN *et al.* 1993) have met with mixed results. Thus, the structure of the mitochondrial genome and the existence of a master molecule has not been clearly demonstrated.

Recent mapping data in petunia (FOLKERTS and HANSON 1991), rice (YAMATO *et al.* 1992; NARAYANAN *et al.* 1993) and maize (LEVY, ANDRE and WALBOT 1991) suggest that the structure of the plant mitochondrial genome in some species includes more than one autonomous molecule. The configuration of these molecules could not be predicted based on homologous recombinations; consequently, they could not be referred to as subgenomic molecules. These autonomous mitochondrial DNA molecules were designated as chromosomes (LEVY, ANDRE and WALBOT 1991; ANDRE and WALBOT 1992; NARAYANAN *et al.* 1993). Rare recombination events across small repeats, or site-specific recombination, are considered a mechanism for the generation of these molecules (MANNA and BRENNICKE 1986; ANDRE, LEVY and WALBOT 1992). If true, this is of considerable consequence to our understanding of nuclear-mitochondrial interactions in plants. A mitochondrial genome consisting of multiple autonomous chromosomes requires a number of essential functions by the nuclear genome to maintain a complete mitochondrial genetic complement for transmission to subsequent generations.

The plant mitochondrial genome is generally stable

as it is inherited generation to generation. One unusual exception is the process associated with spontaneous reversion and fertility restoration in the cytoplasmic male sterility system of common bean (*Phaseolus vulgaris* L.). Cytoplasmic male sterility (CMS) is a maternally inherited trait resulting in the inability to produce or shed viable pollen. CMS is caused by a mitochondrial lesion in nearly all cases examined (HANSON 1991). In CMS common bean, recovery of pollen fertility can occur spontaneously as the result of a low frequency cytoplasmic reversion event or by the introduction of the nuclear fertility restorer gene *Fr* (MACKENZIE *et al.* 1988). By either means, the recovery of pollen fertility is accompanied by the disappearance of at least 25 kb from the mitochondrial genome (MACKENZIE and CHASE 1990). This 25-kb region contains a 3-kb transcriptionally active sequence unique to the CMS line and designated *pvs* (JOHNS *et al.* 1992).

The process associated with fertility restoration and spontaneous cytoplasmic reversion provides a useful genetic system for the study of plant mitochondrial genome structure and its maintenance. We have constructed a physical map of the mitochondrial genome of cytoplasmic fertile revertant line WPR-3 and the predicted corresponding map of the cytoplasmic male sterile line CMS-Sprite to characterize mitochondrial events associated with the reversion phenomenon. Here we present the results of our mapping analysis as well as a model for the mitochondrial genome changes that occur during the fertility reversion process.

MATERIALS AND METHODS

Plant materials: The cytoplasmic male sterile line of *P. vulgaris* used in the study was derived from the cross of G08063 × Sprite, followed by 16 backcrosses using Sprite as recurrent pollinator. Fertile accession line G08063 contains a sterility-inducing cytoplasm (SINGH, WHITE and GUTIERREZ 1980; MACKENZIE 1991). The derived CMS line is designated CMS-Sprite. The fertile cytoplasmic revertant line WPR-3 was selected from a single seed-bearing pod on CMS-Sprite. The fully male-fertile line was then selfed over five generations to ensure phenotypic stability.

Mitochondrial DNA probes: Clones for mitochondrial ribosomal DNA genes *rrn26* from maize (4-kb *PvuII*; DALE, DUESING and KEENE 1984) and *rrn5/18* from *Zea diploperennis* (1.1-kb *HindIII/BglII*; GWYNN *et al.* 1987), ribosomal protein subunit *rps13*, ATPase subunit 9 (*atp9*) from tobacco (0.3-kb *EcoRI/BamHI*; BLAND, LEVINGS and MATZINGER 1986), maize *atpA* and NADH-ubiquinone oxidoreductase subunit 1 (*nad1*) from tobacco (1.5-kb *BamHI/PstI*; BLAND, LEVINGS and MATZINGER 1986) were supplied by C. S. LEVINGS; maize cytochrome *c* oxidase subunit III (*coxIII*) was supplied by W. HAUSWIRTH, and sorghum ATPase subunit 6 (*atp6*) and cytochrome *c* oxidase subunit II (*coxII*) were supplied by D. R. PRING. Probes for cytochrome *c* oxidase subunit I (*coxI*) and cytochrome *b* (*cob*) were derived by polymerase chain reaction amplifications using WPR-3 common bean mitochondrial DNA as template.

Primer sequences were derived from published maize sequences (DAWSON, JONES and LEAVER 1984; ISAAC, JONES and LEAVER 1985).

Other mitochondrial DNA probes used in the study were derived by restriction endonuclease digestion of cosmid DNA, gel electrophoresis, and extraction of the desired restriction fragment from the agarose. DNA extraction was accomplished by emulsification of the agarose band in phenol, freezing at -20° , thawing and refreezing, followed by high speed microfuge centrifugation, chloroform extraction, and ethanol precipitation.

Mitochondrial DNA preparation and cosmid library construction: Mitochondrial DNA was isolated as previously described (MACKENZIE *et al.* 1988) using differential centrifugation followed by DNase I treatment to purify mitochondria, an SDS buffer lysis of mitochondria, and CTAB precipitation of mitochondrial DNA. To construct a mitochondrial genomic library of WPR-3, purified mitochondrial DNA was partially digested with *Sau3A* and size-fractionated on a sucrose gradient (10–40%). Fractions above 30 kb were pooled, ethanol precipitated, and ligated into *BamHI*-digested cosmid vector pWE15 (Stratagene). The DNA ligation mixture was packaged *in vitro* using Gigapack II Gold (Stratagene) according to manufacturer's directions, and then transformed to *E. coli* strain NM 554. A total of 2112 colonies were picked and transferred to microtiter plates for storage. The cosmid library of mitochondrial clones from CMS-Sprite was developed previously in vector pHC79 (MACKENZIE and CHASE 1990).

Cosmid walking strategy: Identification of DNA cosmid clones encompassing the region associated with sterility was described previously (MACKENZIE and CHASE 1990). In CMS-Sprite, a 4.0-kb *PstI* fragment contains the point of divergence between the sterility-associated sequence and the sequences repeated elsewhere in the genomes of CMS-Sprite and revertant WPR-3. This fragment was used as a first probe to screen the revertant WPR-3 cosmid library. All selected clones, after digestion with *PstI*, showed one configuration (data not shown). From these initial clones, bidirectional cosmid walking was accomplished using end-specific RNA probes to select 14 overlapping cosmids at each step. The pWE15 vector allows the generation of end-specific RNA probes for each cosmid insert. These RNA probes initiate at the T3 and T7 promoters within the vector (procedure as per manufacturer's instructions). *PstI* restriction maps were constructed for all selected clones. This allowed the identification of 20 overlapping cosmid clones that cover the entire WPR-3 mitochondrial genome. To eliminate the possibility of recombinations during the cloning process, all selected cosmid clones were hybridized to blotted total mitochondrial DNA from WPR-3.

Restriction endonuclease analysis, agarose gel electrophoresis and DNA blotting: The cosmid mapping involved restriction endonuclease analysis using *PstI* (New England Biolabs), *XhoI* (Promega), and *SalI* (New England Biolabs) predominantly, with more refined mapping of some regions using *EcoRI* (Promega). All reaction conditions were according to manufacturer's instructions.

Agarose gel electrophoresis in 0.8% agarose, using 1× TPE buffer, and DNA gel blotting were as described by McNAY, CHOUREY and PRING (1984) except that Hybond-N nylon membrane (0.45 μ m, Amersham) was used and DNA was affixed to the membrane by baking for 2 hr (90°) under vacuum.

DNA probe preparation and gel blot hybridization analysis: DNA fragments were labeled using the random priming method (FEINBERG and VOGELSTEIN 1983). Labeled DNA probes were hybridized to DNA gel blots at 60° . Blots

FIGURE 1.—Genomic environments surrounding the repeat R1. (A) Mapping branch points around the repeat R1. Lowercase letters indicate different flanking regions, numbered arrows represent hybridization probes. Probe 1: 10.2-kb *Pst*I fragment derived from flanking sequence *c*. Probe 2: *atp9* coding region. The approximate locations of *atpA* and *atp9* are shown. (B) Restriction maps of products of recombination through the repeat R1. Numbers at the right of the map correspond to the sizes of *Pst*I fragments containing the repeat. (C) Hybridization experiment using probes 1 and 2 to *Pst*I-digested mitochondrial DNA from WPR-3 fertile revertant. The sizes of the hybridizing fragments are indicated at the right. The lowercase letters in parentheses represent the different combinations of flanking sequences.

were washed twice in $3\times$ SSC, 0.1% SDS at 65° and once in $0.3\times$ SSC at 65° for 15 min each wash. Autoradiography was carried out using X-Omat film (Kodak) with intensifying screens (Picker Intl.).

RESULTS

Sites of recombination within the mitochondrial genome: Two major families of recombinationally active repeats, R1 and R2, were identified in the mitochondrial genome of fertile revertant WPR-3. A repeat was defined as a sequence that mapped to more than one region of the genome, with recombinational activity inferred from the presence of multiple combinations of sequences flanking the repeat. Additional repeated sequences were detected, but with no evidence of active recombination.

Cosmid clones representing six configurations around repeat R1 were isolated using a DNA probe containing the repeat. These six identified genomic environments are diagrammed in Figure 1. The maximum fragment length shared among all is 5 kb, suggesting that the R1 repeat is 5 kb or smaller in size. However, the region of similarity is extended on one side of the repeat between two flanking sequences designated *b* and *c* in Figure 1. The homology between these sequences is demonstrated by DNA gel blot hybridization in Figure 1C. The internal segment of homology between *b* and *c* is less than 1.5 kb, based on restriction mapping. This additional divergence point results in three configurations to one side of R1

with only two to the other side. As a consequence, the repeat R1 is present in six genomic environments. The additional *b/c* branching point could be a product of a small recombination repeat close to R1, but we could not find cosmids containing the four environments predicted around this second small repeat. Figure 1C presents the results of hybridization to a gel blot of *Pst*I-digested mitochondrial DNA using part of the repeat R1 as probe. Restriction enzyme *Pst*I digests outside of the repeat but within the common *b/c* flanking region. Therefore, combinations *b-e* and *c-e* are not distinguished by *Pst*I and both give rise to a 23-kb fragment. Similarly *b-d* and *c-d* are not distinguished, and give rise to 7.2-kb fragments. Four hybridization bands are expected, but only three bands are observed in the figure because of co-migration of 25- and 23-kb fragments under these gel electrophoresis conditions.

Repeat R2 is also present in six genomic environments and is approximately 4.5 kb in size (Figure 2). Two different flanking sequences were identified on one end of the repeat. The other end of the repeat is flanked by three different configurations, with two of them sharing an additional 7.5 kb in common. The presence of a second point of divergence (*b/c*) to one side of R2 was verified by restriction mapping and hybridization experiments similar to those described for R1 (data not shown). These hybridization experiments also revealed that a part of the segment shared

FIGURE 2.—Genomic environments flanking the repeat R2. (A) Mapping branch points around the repeat R2. The lowercase letters indicate different flanking regions. The arrow represents the location of probe 3, a 10-kb *Pst*I fragment containing R2. (B) Restriction maps of the products of recombination through the repeat R2. Numbers at the right indicate the size of *Sal*I fragments containing the repeat. (C) Hybridization of probe 3, containing R2, to *Sal*I-digested mitochondrial DNA from revertant WPR-3. The sizes of the hybridizing restriction fragments are indicated to the right. The lowercase letters in parentheses represent the different combinations of flanking sequences.

between the R2 *b* and *c* flanking sequences is repeated at another site in the WPR-3 genome. This repeated segment is less than 1 kb in size; however, we have no evidence that this small repeat is recombinationally active.

We were unable to identify a restriction enzyme that would allow us to distinguish the six genomic environments around the repeat R2. Digestion with *Sal*I produces only four fragments spanning R2 because the restriction site is within the *b/c* divergence point (Figure 2). Combinations *b-d* and *c-d* are represented as 15-kb *Sal*I fragments and *b-e* and *c-e* are represented as 13-kb *Sal*I fragments. Restriction mapping data were verified by hybridization with probes encompassing the R2 repeat (Figure 2C) and flanking sequences (data not shown) to *Sal*I-digested total mitochondrial DNA.

A physical map of the WPR-3 mitochondrial genome: Using overlapping cosmid clone analysis, we have constructed a physical map of the mitochondrial genome of revertant line WPR-3 as described in MATERIALS AND METHODS. Figure 3 presents a linear representation of the physical map with an approximate length of 417 kb. The repeats R1 and R2 are each present at three sites. Two copies are in direct orientation and the third in inverted orientation.

Six environments surrounding a repeated sequence requires that two repeat configurations share one flanking environment. This is the case for both repeats R1 and R2. Therefore, to place these repeats onto a single circular map requires the introduction of a large duplication. Based on cosmid mapping data presented, the predicted master mitochondrial chromosome in WPR-3 would contain a 246-kb duplication and would produce a 673-kb molecule.

An alternate model of the WPR-3 mitochondrial genome would predict two autonomous chromosomes able to undergo intra- and inter-molecular recombinations (Figure 4A). The region that would be duplicated in a master chromosome model is represented on both chromosomes as the bold line. Each chromosome contains a unique region not present on the other (dotted line). Hybridization of WPR-3 total mitochondrial DNA with a radiolabelled clone spanning unique and duplicated regions of the 257-kb chromosome supports a model of both duplicated and unique regions within the genome (Figure 4B). Restriction fragments derived from the region unique to the 257-kb chromosome are present in lower copy number than those fragments that are duplicated.

Both inter- and intramolecular recombination is predicted between the chromosomes diagrammed in Figure 4A. The environments surrounding R1 and R2 repeats as presented in the diagram were chosen arbitrarily. All recombinational forms of the repeat R2 can be explained by intramolecular recombinations between R2 repeats that are in inverted orientation. This would produce an inversion of the intervening sequence. As a result, different forms of the 394- and 257-kb chromosomes are predicted to occur with respect to environments surrounding R2. Intramolecular recombinations between inverted forms of R1 account for five of the six identified configurations. The remaining configuration around R1 requires recombination between the two chromosomes. In this process, an intermediate of master chromosome size would be formed. Subsequent recombination at R2 repeats would then be predicted to produce two new circular molecules of 251 and 400 kb in size. The sequence present between R1 and R2 repeats on the 394-kb chromosome in Figure 4A (represented by a fine line) can be exchanged between chromosomes as a result of this intermolecular recombination.

FIGURE 3.—A physical map of the mitochondrial genome of fertile revertant WPR-3. Restriction sites are based on digestion with enzymes *Pst*I (P), *Sal*I (S) and *Xho*I (X). Boxes above the map indicate the locations of recombinationally active repeated sequences. The orientation of the recombination repeats is shown by an arrow. The ends of the map cannot be linked to one another but can be connected to corresponding regions within the map. The left flanking sequence of the repeat R1, located at the top of the map, can be connected to the left of the R1 repeat adjacent to *atpA* or to the right of the other R1 repeat. The repeat R2, at the bottom of the map, can be linked to the right flanking sequence of the first R2 repeat or to the left flank of the second R2 repeat. The stippled region contained between vertical dashed lines is present on a 210-kb chromosome in the CMS-Sprite line. The approximate locations of all mitochondrial genes mapped to date are indicated.

Placement of mitochondrial genes: The mitochondrial genes indicated on the map were placed by hybridization to *Pst*I digested DNA of cosmid clones spanning the entire mitochondrial genome. Most genes were present in only one location. However, an internal region (exon b) of *nad1* hybridized to two *Pst*I fragments separated by approximately 50 kb. We observed a difference in intensity of these two bands when the *nad1* probe was hybridized to total mitochondrial DNA, suggesting that at least a portion of the *nad1* gene may be present at two sites in the genome. The *atp9* gene was located within the R1 repeat, resulting in hybridization to three *Pst*I fragments in the genome (Figure 1). The influence, if any,

of the multiple *atp9* environments on gene expression is a question for future investigations.

Comparison of the mitochondrial genome configurations in CMS-Sprite and WPR-3: A general map of the region encompassing the sterility-associated *pvs* sequence in CMS-Sprite was presented previously (MACKENZIE and CHASE 1990). The *pvs* sequence is located between sequences that are repeated elsewhere in the CMS-Sprite mitochondrial genome. These repeated sequences are also present in the WPR-3 revertant line. The precise junctions between *pvs* unique and repeated sequences were reported by CHASE and ORTEGA (1992).

To test for other differences in mitochondrial genome organization between CMS-Sprite and WPR-3,

FIGURE 4.—(A) Dicircular model of the mitochondrial genome in fertile revertant WPR-3. Boxes represent recombinationally active repeats, with arrows indicating repeat orientation. The region shared by both chromosomes is indicated by the bold line. The dotted line on each circle designates the regions unique to each chromosome. The sequence represented by a fine line can be exchanged between chromosomes as a result of intermolecular recombination. Approximate locations of mitochondrial genes are indicated. (B) *Pst*I-digested mitochondrial DNA from revertant WPR-3 hybridized with cosmid 1H5. This clone overlaps the unique and duplicated regions flanking repeat R1 on the 257-kb chromosome. The 5.2-kb band represents a fragment that is repeated on both chromosomes while the 8.8-kb fragment is unique to the 257-kb chromosome.

clones spanning the entire mitochondrial genome of WPR-3 were hybridized to total mitochondrial DNA preparations from the CMS and revertant lines. No differences were detected between the two lines except at the region encompassing the *pvs* sequence. Furthermore, no regions unique to the WPR-3 revertant line were found. These results were obtained using comparative digestions with *Pst*I, *Sal*I, and *Xho*I. They suggest that the mitochondrial genomes of CMS-Sprite and WPR-3 are colinear, with CMS-Sprite containing only a single addition of the unique 3-kb *pvs* sequence.

The genomic environments flanking the *pvs* sequence: The sequences flanking the sterility-associated *pvs* region are present in both CMS-Sprite and WPR-3. Vertical dashed lines in Figure 3 indicate the junction points between the *pvs* region and the map of WPR-3. The *pvs* sequence lies immediately adjacent to the *atpA* gene and close to repeat R1. Figure 5A indicates the position of *pvs* (flanking region f) relative to the other branching points surrounding repeat R1. The region encompassing *pvs* forms an additional branch point around R1 in CMS-Sprite that is not present in WPR-3. A DNA probe containing this extra divergence point (probe 4) was hybridized to *Eco*RI-digested mitochondrial DNA from CMS-Sprite and WPR-3. The resulting hybridization pattern verifies the presence of this extra configuration in the CMS-Sprite genome (Figure 5B).

We screened the CMS-Sprite and WPR-3 cosmid libraries with the probe 4 (Figure 5) and a fragment spanning repeat R1 plus flanking sequence d (probe 5, Figure 6). Four classes of clones which hybridized to both probes were obtained from the CMS-Sprite

FIGURE 5.—Extra mapping branch point close to the R1 repeat in the CMS-Sprite genome. (A) Mapping branch points around the repeat R1 in CMS-Sprite. The lowercase letters indicate different flanking regions and are the same as Figure 1 with the exception of the f branch unique to the CMS line. Probe 4 represents a 2.8-kb *Eco*RI fragment from CMS-Sprite. (B) Hybridization of probe 4 to *Eco*RI-digested mitochondrial DNA from CMS-Sprite and revertant WPR-3.

library but only two from the WPR-3 library. Figure 6A illustrates the relationship between these configurations. The sequence common to all configurations is approximately 19 kb and includes R1. The four configurations detected using probes 4 and 5 are presented in Figure 6B. Results of these experiments indicate that the *pvs* sequence is present in two genomic environments in the sterile line, demonstrated in Figures 6 and 7, with both lost upon spontaneous reversion to fertility.

FIGURE 6.—The genomic environments flanking the *pvs* sequence. (A) Restriction map of four cosmid clones encompassing the repeat R1. All of these configurations are present in the CMS-Sprite line, but only two are present in WPR-3 (*c-d*, *c-e*). Lowercase letters designate the different flanking sequences. The numbered arrows represent probes. Probe 4: 2.8-kb *EcoRI* fragment from CMS-Sprite; probe 5: 20-kb *PstI* fragment spanning the repeat R1 and flanking sequence *e*. (B) Identification of four cosmid configurations that hybridized to both probes 4 and 5. Lane 1 represents the *c-d* configuration, lane 2 (*c-e*), lane 3 (*f-e*) and lane 4 (*f-d*). *EcoRI* profiles fractionated through 0.7% agarose are shown on the left with corresponding autoradiographs from DNA gel blot hybridization experiments at right. Hybridization with probe 4 produced two bands corresponding to flanking sequences *c* and *f*. Hybridization with probe 5 produced three bands corresponding to flanking sequences *e*, with one additional band corresponding to region *d*. Differences in hybridization intensities reflect gel loading differences.

The proximity of a recombinationally active repeat to a unique sterility-associated sequence has been observed in other CMS systems (FAURON, HAVLIK and BRETTELL 1990; FOLKERTS and HANSON 1991; FAURON *et al.* 1992). In CMS-T maize, it has been suggested that recombination by two families of repeats, followed by the selective elimination of recombination products containing the *T-urf13* sequence, would account for the observed loss of the sterility sequence in tissue culture-induced revertants to fertility (FAURON, HAVLIK and BRETTELL 1990). In CMS-Sprite, we found that a copy of the R1 repeat resides to one side

FIGURE 7.—Regions surrounding the *pvs* sterility-associated sequence in CMS-Sprite. The *pvs* segment lies between sequences that are repeated elsewhere in the genome of CMS-Sprite. To one side of *pvs* resides a recombinationally active R1 repeat.

of the *pvs* sequence (Figure 7); we examined the region flanking the *pvs* sequence to the opposite side for evidence of a second repeat. If such a repeat were present, we would expect to observe a novel junction fragment in the revertant WPR-3. Similarly, if the *pvs* sequence were lost as the result of a site-specific deletion or excision event, we should also observe such a novel fragment unique to the WPR-3 line. CMS-Sprite cosmid clones spanning over 37 kb on the side of *pvs* opposite to R1 were hybridized to total mitochondrial DNA from CMS-Sprite and WPR-3 revertant lines. Individual DNA samples were digested with *PstI*, *Sall*, *XhoI*, *EcoRI*, *DraI*, *KpnI* and *SmaI*. Comparison of hybridization patterns for the seven different restriction patterns revealed no DNA polymorphism specific to WPR-3 within 37 kb of the *pvs* sequence.

Putative structure of the CMS-Sprite mitochondrial genome: DNA gel blot hybridization experiments demonstrated that all restriction fragments detected in WPR-3, using *PstI*, *XhoI* and *Sall* digestions, were also present within the CMS-Sprite mitochondrial genome. In addition, the CMS-Sprite mitochondrial genome contained fragments not present within the WPR-3 revertant. The simplest model to account for these data would propose that the CMS-Sprite mitochondrial genome contains the two mitochondrial chromosomes present within WPR-3 plus an additional mitochondrial chromosome containing the *pvs* sequence. This extra chromosome in CMS-Sprite would be colinear with the region presented between vertical dashed lines in Figure 3. These map breakpoints are linked by the *pvs* sequence. The size of the predicted chromosome is 210 kb (Figure 8). Repeats R1 and R2 contained on the *pvs* chromosome can undergo intermolecular recombinations with corresponding repeats on the 394- and 257-kb chromosomes. As a consequence of intermolecular recombination between the 394- and 210-kb chromosomes, the *pvs* sequence could be present on an alternative 204-kb molecule (Figure 9). These 204- and 210-kb *pvs*-containing molecules account for the two genomic environments of *pvs* found in the CMS line.

To evaluate the relative copy number of molecules containing the *pvs* sequence, we compared copy number of a fragment that encompasses the breakpoint between common (*atpA* region) and unique sequences

FIGURE 8.—Proposed model of the CMS-Sprite mitochondrial genome. The CMS-Sprite genome contains two chromosomes identical to those mapped within revertant WPR-3 plus one additional chromosome containing the sterility-associated *pvs* sequence. This extra chromosome is colinear with a part of the 394-kb molecule (stippled region), with breakpoints linked together by the *pvs* segment. These breakpoints are indicated in Figure 3 by vertical lines. Boxes represent recombination repeats with arrows indicating orientation. Approximate locations of mitochondrial genes are indicated.

on the 210- and 394-kb chromosomes (see Figure 8). The probe used was internal to both segments and produced a 2.8-kb *EcoRI* fragment unique to the *pvs* region and a 3.6-kb *EcoRI* fragment unique to the 394-kb molecule. Densitometry scans of multiple autoradiographs produced by hybridization indicated a ratio of 1:1.2 (2.8:3.6 kb) (data not shown). Because of the predicted complexity of the genome due to recombination, hybridization analysis does not indicate the ratio of one predicted molecule relative to another but rather, indicates the relative copy number of the two sequences monitored. These results show that the sterility-associated *pvs* sequence is not contained on a substoichiometric molecule.

DISCUSSION

Spontaneous cytoplasmic reversion to fertility in CMS common bean is associated with the loss of at least 25 kb encompassing the sterility-associated *pvs* sequence (MACKENZIE and CHASE 1990). To characterize better the molecular events associated with reversion, we have constructed physical maps of the mitochondrial genomes of a fertile revertant line, WPR-3, and the CMS line from which it was derived. Based on cosmid mapping and mitochondrial DNA gel blot hybridization data, the simplest model for the structure of the CMS-Sprite mitochondrial genome consists of three chromosomes able to undergo intra- and intermolecular recombination. The *pvs* sequence is present in two different genomic environments, suggesting that the *pvs* sequence is contained on at least two mitochondrial DNA molecules, one gener-

FIGURE 9.—Two different *pvs*-containing circles are predicted within the CMS-Sprite genome based on restriction mapping data. The 204-kb molecule is predicted to arise by intermolecular recombination between the 210- and 394-kb chromosomes diagrammed in Figure 8. As a consequence, the sequence between the R1 and R2 repeats, represented by a fine line, is exchanged between chromosomes.

ated by recombination. Analysis of the mitochondrial genome configuration of revertant WPR-3 indicates that the process of reversion likely involves the loss of the *pvs*-containing chromosomes from the CMS genome (Figure 10). This model is supported by the fact that we detected no evidence near the *pvs* sequence of recombination, excision or deletion events that would be necessary if less than an entire chromosome were deleted from the genome.

In predicted mitochondrial genome configurations of CMS and revertant lines, a large portion of each autonomous molecule is shared. Therefore, the molecules are distinguished by only short regions unique to each. Homologous recombination events are assumed to account for the various arrangements surrounding repeats R1 and R2. It is also possible that homologous recombination occurs across the long stretches of sequence shared by each chromosome, although our study could not test for this. Consequently, the data presented here cannot exclude the possibility that the predicted mitochondrial molecules interconvert to form a master circle. However, proposal of a master chromosome would necessitate the triplication of a segment over 200 kb in size onto a single molecule in the CMS genome.

The possibility of more than one autonomously replicating form within the plant mitochondrial genome has been raised previously. Coexistence of two distinct mitochondrial chromosomes based on cosmid mapping data was first proposed in CMS petunia (FOLKERTS and HANSON 1991). In the maize BMS cultivar, use of pulsed field gel electrophoresis allowed the mapping of a 120-kb chromosome that does not contain any large repeats, suggesting that it cannot be generated by recombination from a master circle and must be maintained autonomously (LEVY, ANDRE and WALBOT 1991). The genome organization of multiple autonomous chromosomes has also been suggested in the development of a physical map of the rice mitochondrial genome (YAMATO *et al.* 1992; NARAYANAN *et al.* 1993). Therefore, the predicted configuration

FIGURE 10.—Proposed model of reversion to fertility in CMS *P. vulgaris*. Based on mapping data, we propose that spontaneous reversion to fertility involves the loss of the *pvs*-containing chromosomes from the CMS-Sprite mitochondrial genome.

of the bean mitochondrial genome was not altogether surprising.

The proposed model of the genome in common bean is consistent with six genomic environments surrounding repeats R1 and R2. Analysis of cosmid clones containing repeat sequences and hybridization of these cosmids to gel blots of total mitochondrial DNA preparations showed that there is an unequal number of flanking sequences to each side of the repeats. Similar observations were made in petunia (FOLKERTS and HANSON 1991) and rice (YAMATO *et al.* 1992). In both cases, it was suggested that the missing environments around a repeat were lost from the genome. In rice, one sequence flanking repeat R1 was missing in the tissue cultured A-58CMS line while it was present in the cultured Chinsurah Boro II line. Similarly, nine genomic environments surrounding a repeat were found in the petunia fertile line 3704, but only six flanking environments were identified in the sterile line 3688. Investigation of the progenitor lines to CMS-Sprite may allow us to characterize the events leading to the unusual genome organization observed in bean.

Analysis of the progenitors to CMS-Sprite might also answer questions about the nature of additional branching points identified to one side of both R1 and R2 repeats. At least two interpretations of these branch points are possible. These divergence points could be the products of recombination at small repeats nearby to the larger, more active repeats R1 and R2. Alternatively, R1 and R2 may exist in both full length and truncated forms. We are currently investigating the structure and locations of repeats R1 and R2 in the reported progenitor lines NEP-2 and POP to better understand the origins of this unusual genome configuration.

Studies of the mitochondrial genome configuration

of common bean have been reported previously and were taken into account during the interpretation of our mapping data. Although small supercoiled DNA molecules have been observed in mitochondrial preparations from common bean (DALE, DUESING and KEENE 1981), we did not observe these molecules in mitochondrial DNA preparations from CMS-Sprite or WPR-3. This could reflect differences in mitochondrial DNA isolation procedures or differences in plant genotypes. Consequently we were unable to account for the genetic organization of these supercoiled molecules.

Using three restriction enzyme digestion profiles, Khairallah, Adams and Sears (1991) estimated the mitochondrial genome size of *P. vulgaris* to be close to 450 kb. Based on the linear representations of physical mapping data, the mitochondrial genomes of WPR-3 and CMS-Sprite are 417 and 420 kb in size, respectively. These estimates agree with the earlier study. However, if we sum fragments from both chromosomes in WPR-3 or the three chromosomes in CMS-Sprite in our calculations, the size of the genomes would increase substantially. This discrepancy between earlier estimates and the size of the genomes based on physical mapping data can be explained by the fact that determination of genome size using restriction patterns prevents accurate determination of fragment copy number. According to our multiple chromosome model, regions present at one copy number per genome would appear in digestion profiles as submolar bands.

What is most unusual about our observations in the CMS bean system is suggestion of the loss of an entire mitochondrial chromosome upon reversion to fertility. We have detected no differences between the structure of the mitochondrial genome of spontaneous revertant lines and fertile lines that have been

restored by the nuclear gene *Fr* (MACKENZIE *et al.* 1988; MACKENZIE and CHASE 1990). This result suggests that the mitochondrial alterations associated with the process of reversion are identical to those effected by the nuclear restorer gene. We selected fertile revertant WPR-3 for this study, rather than a fertile restored line, because WPR-3 and CMS-Sprite are genetically isonuclear, eliminating the possibility of unrelated mitochondrial changes resulting from variation in nuclear genotype that may accompany the introduction of *Fr*.

In maize CMS-T, tissue culture-induced cytoplasmic reversion to fertility is correlated with the rearrangement of the mitochondrial genome by recombination (FAURON, HAVLIK and BRETTELL 1990; FAURON *et al.* 1992). We have no evidence of recombination in the mitochondrial alteration associated with reversion in CMS bean, based on the fact that we detected no products of recombination in the revertant line. Comparison of DNA hybridization patterns between the WPR-3 and CMS-Sprite genomes, using three restriction enzymes for the entire genome plus four additional enzymes in the region encompassing *pvs*, revealed no fragments unique to the revertant. We have not eliminated the possibility that both products of recombination are undetectable using this approach. Interestingly, the model accounting for mitochondrial rearrangements in revertants of the maize CMS-T system assumes that the intramolecular recombination events are followed by the spontaneous loss or elimination of two of the four recombination products. The process by which this DNA loss occurs may be similar to that involved in the CMS bean system.

The mitochondrial genome of CMS petunia bears important similarities to that of CMS bean. The CMS petunia line 3688 consists of two circular DNA molecules differing at only one site. One of the chromosomes contains the CMS-associated *pcf* sequence. The elimination of this molecule would presumably result in recovery of fertility. However, spontaneous reversion to fertility is not observed. This is likely due to genetic linkage between the *pcf* sequence and the only copy of *nad3* and *rps12* genes (FOLKERTS and HANSON 1991). The elimination of the *pcf*-containing chromosome would result in loss of not only the sterility-associated sequence but two genes essential for mitochondrial function. In the case of CMS bean, however, the *pvs*-containing chromosome appears to be dispensable, with all sequences linked to *pvs* repeated elsewhere in the genome.

The loss of a mitochondrial chromosome might occur by at least two mechanisms. One possibility is by differential rates of replication of individual mitochondrial chromosomes. This model is feasible when speculating on a role for nuclear fertility restorer gene

Fr as a factor in mitochondrial chromosome copy number control. Introduction of *Fr* would then lead to a reduction in replication of the *pvs*-containing chromosome relative to the remainder of the genome. However, it is more difficult to visualize the role of replication rate in effecting a spontaneous event such as cytoplasmic reversion, particularly when the chromosome deleted is not substoichiometric.

An alternative model for the reversion and fertility restoration process would involve the unequal sorting of mitochondria leading to the random loss or selective elimination of those mitochondria containing the *pvs* sequence. This model would presuppose the existence of a heterogeneous population of mitochondria in the CMS line, those containing the *pvs* chromosome and those with *pvs* absent. Aberrant mitochondrial transmission during mitosis and zygote formation has been observed in a number of mutants of *Saccharomyces cerevisiae* (DUCHER 1982; MCCONNELL *et al.* 1990; DIFFLEY and STILLMAN 1991; ZWEIFEL and FANGMAN 1991; JONES and FANGMAN 1992; AZPIROZ and BUTOW 1993; CHEN and CLARK-WALKER 1993). In nearly all cases, mitochondrial sorting and selection are under nuclear gene control. In *Physarum*, heteroplasmy is also associated with changes in frequency of mitochondrial fusion. The phenomenon of mitochondrial fusion in the *Physarum* system is regulated by nuclear genotype (KAWANO *et al.* 1993).

A mechanism of unequal mitochondrial sorting or selection would account for the spontaneous nature of cytoplasmic reversion events; one could envision an unequal partitioning of mitochondria during gamete formation leading to pollen and/or egg cells with few or no *pvs*-containing mitochondria. A similar result by the introduction of nuclear fertility restorer gene *Fr* would suggest that *Fr* participates as a nuclear factor in the direction of the cytoplasmic sorting process. We are currently using both genetic and molecular approaches to test these alternative models.

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