Pollen Fertility Restoration by Nuclear Gene *Fr* in CMS Bean: Nuclear-Directed Alteration of a Mitochondrial Population

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ABSTRACT

Two nuclear genes, *Fr* and *Fr2*, have been identified that restore pollen fertility to cytoplasmic male sterile (CMS) common bean (*Phaseolus vulgaris L.*) by apparently distinct mechanisms. Whereas *Fr2* appears to suppress the expression of a male sterility associated mitochondrial sequence (designated *pvs*), *Fr* restores pollen fertility by causing the elimination of this unusual mitochondrial DNA segment. To further investigate the mechanism of *Fr* action, *Fr* and *Fr2* were cointroduced into the nucleus of a bean line containing the sterility inducing cytoplasm. When the effect of *pvs* was suppressed by *Fr2*, the presence of *Fr* no longer directed the elimination of the mitochondrial *pvs* sequence. This result suggests that the *Fr* function is dependent on proper expression of the *pvs* sequence. To evaluate the temporal and spatial patterns of *Fr* action, we undertook a polymerase chain reaction-based approach to trace the fate of the *pvs* sequence in different tissues of F_2 and F_3 fertile-restored plants derived from a genetic cross between a cytoplasmic male sterile line of common bean, CMS-Sprite (*frfr*), and fertility restorer line R351 (*FrFr*). We demonstrate that the *Fr*-directed disappearance of *pvs* sequence occurs during flower development. Elimination of the *pvs* sequence from developing megaspores results in permanent fertility restoration in the following generations. Genetic analysis demonstrated that permanent fertility restoration, that is, the complete elimination of *pvs* from reproductive tissues requires two doses of the *Fr* allele or the absence of *fr* in F_2 individuals. The effect of *Fr* was reversible until full fertility was achieved. On the basis of these results, we propose a model for the mechanism of *pvs* elimination by the *Fr* gene and discuss the dynamics of *pvs*-containing mitochondrial transmission in the presence of the *Fr* gene.

N UCLEAR genes play essential roles in mitochondrial biogenesis and function (TZAGALOFF and MYERS 1986; ATTARDI and SCHATZ 1988). Although much progress has been made in understanding how nuclear and mitochondrial genes interact in yeast and mammals (CHOMYN and ATTARDI 1992), little is known about the mechanism by which the nuclear genome regulates mitochondrial gene expression in higher plants. Because mitochondrial genes encode proteins that are required for essential cellular functions in every phase of plant growth and development, mutations in either the nuclear or mitochondrial genome that lead to mitochondrial dysfunction will likely be lethal. As a result, genetic approaches to investigating this complex intracellular communication are hindered due to the relatively small number of available mutants. Four promising plant nuclear-mitochondrial interaction systems have been investigated at a molecular level. They are cytoplasmic male sterility (CMS) and nuclear fertility restoration (HANSON and CONDE 1985), the "teosinte cytoplasmic-associated miniature" (tcm) resulting from nuclear-cytoplasmic incompatibility in maize (COOPER *et al.* 1990), nonchromosomal stripe mutations (NCS) of maize (NEWTON 1988; NEWTON *et al.* 1990) and a leaf variegation phenotype associated with mitochondrial genome rearrangements due to a nuclear mutation in Arabidopsis (MARTINEZ-ZAPATER *et al.* 1992). CMS, a maternally inherited trait characterized by the inability of the plant to produce viable pollen, has been studied extensively. In all cases investigated to date, it is associated with mitochondrial mutations (HANSON 1991). These mutations often create novel open reading frames that are expressed. Unusual mitochondrial sterility associated sequences apparently are derived by DNA recombinations, insertions, and rearrangements (DEWEY *et al.* 1986; YOUNG and HANSON 1987; SINGH and BROWN 1991; KOHLER *et al.* 1991; LAVER *et al.* 1991). In most of these cases, nuclear genes have been identified that apparently suppress expression of the mitochondrial sterility associated mutations. However, the mechanisms by which these nuclear restorer genes act to alter mitochondrial gene expression remain to be defined.

CMS common bean (*Phaseolus vulgaris L.*) provides a different genetic system for the study of plant nuclear-mitochondrial interaction. A 3.7-kb transcriptionally active CMS-associated sequence, designated *pvs*, was identified in the mitochondrial genome (CHASE and OR-

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TABLE 1

Genetic materials used in this study

Line	Nuclear genotype	pvs	Pollen phenotype	Reference
CMS-Sprite	frfrfr2fr2	+	Sterile	BASSETT and SHUH 1982
Sprite	frfrfr2fr2	−	Fertile	BASSETT and SHUH 1982
R351	FrFrfr2fr2	−	Fertile	MACKENZIE et al. 1988
GO8063	frfrFr2Fr2	+	Fertile	MACKENZIE 1991
CMS-GO8063	frfrfr2fr2	+	Sterile	This study

TEGA 1992; JOHNS et al. 1992). The unique sterility associated sequence is lost upon fertility restoration by the nuclear gene Fr (MACKENZIE et al. 1988; MACKENZIE and CHASE 1990). This unusual mitochondrial genome alteration can also occur spontaneously at low frequency as the result of spontaneous cytoplasmic reversion. Comparative cosmid mapping analysis of the mitochondrial genome of the CMS line and a fertile revertant suggests that the 3.7-kb pvs sequence is contained on an autonomous mitochondrial DNA molecule that is lost from the mitochondrial genome spontaneously during reversion or as the result of Fr gene action (JANSKA and MACKENZIE 1993).

Fertility in CMS bean also is restored by a second mechanism (MACKENZIE 1991). The nuclear restorer gene Fr2, contained in bean accession line GO8063, restores pollen fertility but, unlike Fr, does not appear to alter mitochondrial genome organization or the transcription pattern of pvs. Consequently, Fr2 is thought to restore fertility by suppressing the expression of pvs posttranscriptionally (MACKENZIE 1991; CHASE 1993).

Here we report our investigation of the function of the Fr fertility restorer gene. DNA marker-assisted and genetic segregation analyses indicate that Fr and Fr2 are nonallelic. We demonstrate that Fr becomes ineffective when Fr2 is present to suppress the effect of the pvs sequence. Results of polymerase chain reaction (PCR)-based experiments demonstrate that the loss of pvs reaches completion during flower development, and genetic tests show that one dose of Fr is insufficient to completely eliminate pvs from the mitochondrial population. These data, taken together, now allow us to begin to develop a model for the unusual role of Fr in effecting change of a plant mitochondrial population.

MATERIALS AND METHODS

Genetic crosses: The genetic resources utilized in this study are listed in Table 1. GO8063 is a fertile accession line with genotype frfrFr2Fr2. It contains the same sterility inducing mitochondrial genome as CMS-Sprite (frfrfr2fr2) (MACKENZIE 1991), a male sterile line derived by backcrossing the GO8063 cytoplasm to a "Sprite" nuclear background (BC_{16}) (BASSETT and SHUH 1982). Sprite is a fully fertile snap bean cultivar, which is isonuclear to CMS-Sprite but contains a normal fertile cytoplasm. Restorer line R351 is a fertile line

(FrFrfr2fr2) near-isogenic to CMS-Sprite and Sprite and containing a pvs⁻ mitochondrial genome. CMS-GO8063 is a sterile F_2 selection from the cross GO8063/Sprite. It contains the sterility inducing cytoplasm of GO8063 and a frfrfr2fr2 nuclear genotype. The genetic populations used in this study and their parentages are listed in Table 2.

Plants were grown in the greenhouse under light- and temperature-controlled conditions. All populations were developed using standard breeding methods. Fertility classification has been described in detail previously (MACKENZIE and BASSETT 1987). Briefly, fertile plants produce >90% viable pollen and produce normal seed-bearing pods with no evidence of parthenocarpy. Sterile plants produce microspores all aborted in tetrads; this results in no visible pollen shed and parthenocarpic pods bearing no seeds. Semisterile plants produce both fertile pollen and aberrant pollen in a single anther, giving rise to both seed-bearing and parthenocarpic pods on an individual plant.

Total genomic DNA preparation: High molecular weight genomic DNA was prepared from green tissues using the procedure described previously (HE et al. 1995). Genomic DNA samples were used for DNA gel blot and PCR analyses. Crude minipreparations of total genomic DNA were obtained from anther, pollen, ovary, ovule, funiculus, immature seed and seedlings for PCR assay using the following procedure. Different parts of male and female organs were dissected from buds under a dissecting microscope and ground to very fine powder in liquid N_2 in a microcentrifuge tube using a teflon pestle. The tissue then was lysed in 0.5 ml extraction buffer (100 mM Tris, 50 mM EDTA, 100 mM NaCl, 1% SDS) at 65° for 30 min. After incubation, 200 ul of 5 M potassium acetate was added to the tube, the sample was mixed vigorously and allowed to sit on ice for 1 hr. The solution was pelleted at 25,000 × g for 20 min, and the supernatant was transferred to a clean tube. DNA was precipitated from solution by the addition of 50 ul isopropanol and 5 μl of 5 M ammonium acetate. After centrifugation at 25,000 × g for 20 min, the

TABLE 2

Genetic crosses used in this study

Cross	Generation
GO8063//[CMS-Sprite/R351]BC3	F_3
GO8063//[CMS-Sprite/R351]BC4F2	F_2, F_3
CMS-GO8063//[CMS-Sprite/R351]BC4F2	F_3
CMS-Sprite/GO8063	F_2
CMS-Sprite/R351BC4F2	F_2, F_3
[CMS-Sprite/R351]BC5//Sprite	F_1
[CMS-Sprite/R351]BC6//Sprite	F_1

TABLE 3

Genetic segregation data for populations used in this study

	Pollen fertility				
Cross and Generation	Fertile	Semisterile	Sterile	χ^2	P
GO8063//[CMS-Sprite/R351]BC4F2, F2	44	66	6		
CMS-Sprite/R351 BC4F2	13	36	15	(1:2:1)	
				1.12	0.55
[CMS-Sprite/R351]BC5//Sprite, F1	6[a]	11	30		
[CMS-Sprite/R351]BC6//Sprite, F1	0	8	38		

[a] The six fertile plants were derived from a single seed pod and are presumed to be the result of a spontaneous cytoplasmic reversion event.

DNA pellet was dissolved in 10 μl H_2O for subsequent PCR reactions.

DNA gel blot analysis and PCR assays: Total genomic DNA preparations were used to test for the presence of the mitochondrial *pvs* sequence. DNA was digested with restriction endonuclease *Pst*I using reaction conditions recommended by the manufacturer (Promega). Electrophoretic separation, gel blotting and hybridization were as described (HE *et al.* 1995). Random Amplified Polymorphic DNA (RAPD) marker analyses were conducted using primer *UBC487* with reaction conditions described elsewhere (HE *et al.* 1995). PCR assays for the presence of *pvs* in different plant tissues were as follows: DNA minipreparations from different tissues were used as template for PCR reactions. Three oligonucleotide primers were used in each reaction. Primer p76E (5'-ATGAC-TATAAGGAACCAAC-3') was derived from the maize *cob* sequence (position 1–19, DAWSON *et al.* 1984). Sequences for primer p75E (5'-TGGAATTCCTCTTCCAACT-3', position 2967–2986) and primer p5E (5'-CCATGGTCCTCCCAT-CAAC-3', position 2170–2189) were derived from the CMS-bean *pvs* region (JOHNS *et al.* 1992). Primers p5E and p75E together amplify a 0.8-kb *pvs* fragment, and primers p76E and p75E amplify the intact *cob* gene as an internal positive control (Figure 3B). Amplification reactions were in a total volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl, 2.0 mM $MgCl_2$, 0.1% Triton X-100, 125 μM each of dATP, dCTP, dGTP and dTTP, 0.2–0.4 ug genomic DNA and ~1 unit of *Taq* DNA polymerase (Promega). The concentrations for the three primers were 1 μM for p5E, 2 μM for p76E and 4 μM for p75E. Amplifications were performed in a Thermolyne Temp-Tronic Thermal Cycler (Barnstead/Thermolyne Co.) for 35 cycles. Each cycle consisted of 1 min at 94°, 1 min at 60° and 1.5 min at 72°, followed by 7 min at 72°. Amplifications were resolved by electrophoresis in a 1.2% agarose gel and visualized with ethidium bromide staining.

RESULTS

The effect of *Fr* action is reversible before completion of *pvs* elimination: When CMS-Sprite is crossed to restorer line R351 (Table 2), the first generation (F_1) is semisterile and the F_2 population segregates 1 fertile / 2 semisterile / 1 sterile in a Mendelian fashion (Table 3). An important observation in these F_2 populations is the occurrence of male sterile segregants, because the cytoplasm of these plants is derived from the semisterile, partially restored F_1 plants. A semisterile F_2 plant, in turn, gives rise to an F_3 family that, again,

segregates for the sterile phenotype (data not shown). These results suggest that *Fr*-directed restoration is reversible when interrupted before completion. To confirm the reversibility of *Fr* restoration before completion, we "removed" the nuclear gene *Fr* after F_1 semisterility was achieved. To do this, semisterile BCF$_1$ plants (*Frfr*) from CMS-Sprite/R351 were testcrossed as female parent to Sprite (*frfr*) (Table 2). Table 3 shows the fertility distribution of two independently derived populations. Two important observations are made in these populations. First, *Fr*-driven full fertility was not obtained and the semisterile segregants set fewer seed pods than the parent F_1 plants. This result indicates that the cytoplasmic effect of one dose of *Fr*, which initiates the process of *pvs* elimination, does not continue in the subsequent generation when two doses of *Fr* are not present. Second, more than half of the population appear fully sterile. This result demonstrates that the segregation of *Fr* before the completion of *pvs* elimination results in complete reversal to the original cytoplasmic state.

Effect of *Fr* depends on mitochondrial *pvs* expression: Genetic crosses were made to test whether *Fr* and *Fr2* interact in their effect. To address this question, it was first necessary to ascertain whether *Fr* and *Fr2* are allelic. When fertile line GO8063 (*frfrFr2Fr2*) was crossed to restorer line R351 (*FrFrfr2fr2*), six sterile segregants were obtained in the F_2 population of 116 plants (Table 3). Furthermore, an *Fr*-linked RAPD marker, *UBC487* (HE *et al.* 1995), was used to assay an F_2 population derived from the cross CMS-Sprite (*fr2fr2*)/GO8063 (*Fr2Fr2*) and segregating for *Fr2* (Table 2). The *Fr*-linked polymorphic DNA amplification pattern segregated from fertility (Figure 1). These results confirm that *Fr* and *Fr2* are nonallelic.

To investigate the effect of *Fr2* on the action of *Fr*, *Fr* was introduced to line GO8063 by genetic crossing (Table 2). GO8063 is a fertile accession line that contains the *Fr2* gene as well as the sterility inducing mitochondrial *pvs* sequence. This experiment addresses whether, in the presence of *Fr2*, *Fr* still functions to
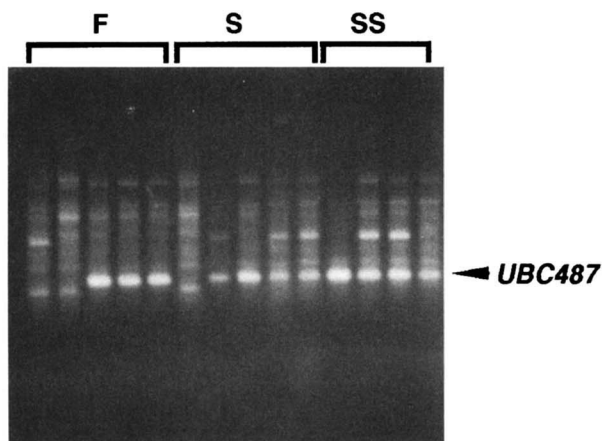
FIGURE 1.—Segregation of an *Fr*-linked marker (*UBC487*) in an F_2 population segregating for *Fr2*-directed fertility restoration. The population was derived from CMS-Sprite/ GO8063. F, fertile plants; S, sterile plants; SS, semisterile plants. The arrow indicates the RAPD marker linked to *Fr*.

eliminate *pvs*. Two independently derived F_3 populations were tested, one developed from GO8063// [CMS-Sprite/R351BC$_3$] and the other from GO8063// [CMS-Sprite/R351BC$_4$F$_2$] (Table 2). In the first cross, the pollen parent contributing the *Fr* gene was heterozygous at the *Fr* locus (semisterile) to avoid the possibility of mistakenly selecting a fertile spontaneous cytoplasmic revertant as parent. The *Fr* donor for the second population was a fertile BC$_4$F$_2$ (*FrFr*) selection from CMS-Sprite/R351. To ensure that the *Fr* allele was present in this population, we evaluated the derived F_2 population for segregation of the two restorer genes (Table 3). As a control for possible nuclear background effect in this cross, we selected a male sterile segregant (*fr2fr2*) from GO8063/Sprite. This F_2 selection, designated CMS-GO8063, contains the GO8063 sterility-inducing cytoplasm but no longer contains the restorer gene *Fr2*. The CMS-GO8063 line was used as the maternal parent in crosses to the restorer line CMS-Sprite/R351BC$_4$F$_2$ (*FrFr*) that was used above (Table 2), and a control F_3 population was developed. As is shown in Figure 2, A and B, the *pvs* sequence is retained in the F_3 populations derived from GO8063// [CMS-Sprite/R351BC$_3$] and GO8063//[CMS-Sprite/ R351BC$_4$F$_2$]. In contrast, F_3 plants from the control cross CMS-GO8063//[CMS-Sprite/R351BC$_4$F$_2$] demonstrated the loss of *pvs* (Figure 2C). These results imply that the presence of *Fr2* diminished the effect of *Fr*, so that *pvs* elimination no longer occurred. We suggest that the *Fr*-directed mitochondrial alteration is dependent on the proper expression of *pvs*, because the function of *Fr2* is presumably to suppress the expression of the *pvs* sequence.

Developmental timing of the *pvs* loss: To determine the developmental stages of *Fr*-directed *pvs* elimination, fully fertile plants were selected from a BC$_4$F$_2$ and a
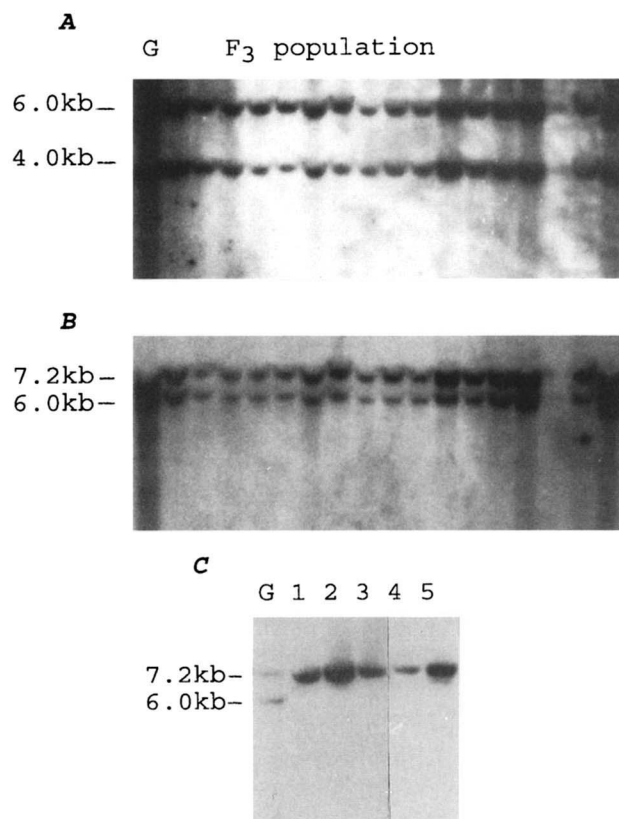

FIGURE 2.—*Fr* does not effect loss of *pvs* in the presence of *Fr2*. (A) DNA gel blot hybridization of total genomic DNA showing the persistence of the *pvs* sequence in fertile F_3 plants derived from GO8063/R351. Total genomic DNA was prepared from green leaf tissue and digested with *Pst*I, and the blot was hybridized with PCR-amplified *pvs* orf239 (CHASE and ORTEGA 1992; JOHNS *et al.* 1992). The *orf239* sequence spans 6.0- and 4.0-kb mitochondrial *Pst*I fragments (JOHNS *et al.* 1992). (B) The same gel blot from the above experiment was used for hybridization with a mitochondrial DNA clone (258–1) that is internal to the 6.0-kb *Pst*I fragment of the *pvs* sequence and a homologous 7.2-kb fragment residing elsewhere in the mitochondrial genome (MACKENZIE *et al.* 1988). (C) The genomic DNA was prepared from the F_3 fertile plants of CMS-GO8063/R351, in which *Fr2* has been eliminated. Same procedures were used for DNA gel blot hybridization using mitochondrial clone 258–1 as probe. Loss of the 6.0-kb *Pst*I fragment of the *pvs* sequence from the F3 plants is evident. G, GO8063.

BC$_4$F$_3$ population of CMS-Sprite/R351, using CMS-Sprite as the recurrent parent (Table 2). Green leaf (immediately adjacent to floral bud initiation), anther, ovary, ovule, funiculus, immature seed and embryo tissues were collected for PCR analysis. As shown in Figure 3A, the *pvs* sequence was present in the leaf tissues of all assayed fertile F_2 plants. In different parts of the female organs, however, *pvs* loss was observed. Of the 16 buds assayed, ∼80% had lost *pvs* from ovule, funiculus and ovary, as well as immature F_3 seed. The *pvs* sequence was undetectable in all 10 germinating F_3 embryos (data not shown), F_3 seedlings or F_3 leaf tissue
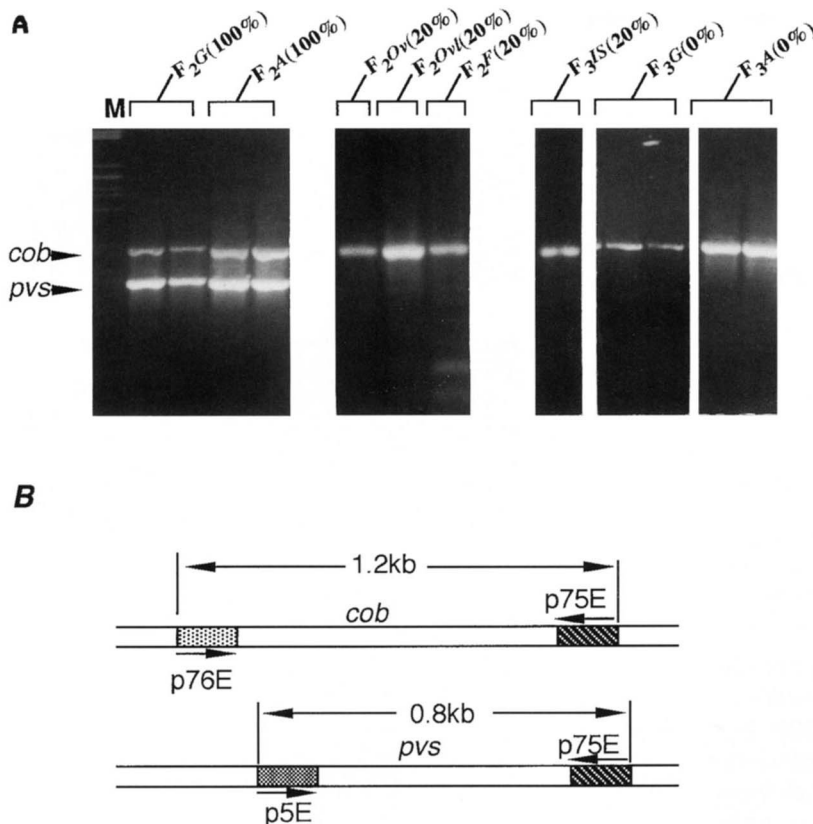
A

B

FIGURE 3.—The temporal and spatial pattern of *Fr*-directed *pvs* disappearance. (A) PCR amplification of *pvs* in various tissues from plants derived from CMS-Sprite/R351. F₂G, F₂ green leaf; F₂A, F₂ anther; F₂Ov, F₂ ovary; F₂Ovl, F₂ ovule; F₂F, F₂ funiculus; F₃IS, F₃ immature seed; F₃G, F₃ green leaf; and F₃A, F₃ anther. The percentage represents the occurrence of *pvs* in all samples assayed. The amplification of *cob* serves as an internal control. M, λ*Pst*I molecular weight marker. (B) Position of the three primers used in the above PCR amplification. Sequence of these primers are described in MATERIALS AND METHODS.

samples assayed. These results suggest that permanent *Fr*-directed fertility restoration is due to the loss of *pvs* from the F₂ female gametes, a process that begins in young bud tissues. However, our PCR assay revealed the presence of *pvs* in all the 18 whole anthers (Figure 3A) and 16 pollen samples (data not shown) tested from fertile F₂ plants.

DISCUSSION

A mechanism of *Fr* action: Based on the genetic analysis presented in this study, we have made three important observations about the behavior of the *Fr* restorer gene. First, the presence of two doses of *Fr*, or the absence of the *fr* allele, is apparently essential in completing the *pvs* elimination process. Two generations of *Fr* in the heterozygous condition are not sufficient to achieve full fertility. Second, *Fr* action to eliminate *pvs* appears to depend on *pvs* expression. Finally, the *Fr*-directed loss of *pvs* occurs in female tissues during flower development. These results, together with observations made in this system during the past several years, support a working hypothesis for the mechanism by which *Fr* influences mitochondrial genome structure. The model that appears to be consistent with all experimental observations to date involves interaction of the *Fr* product, directly or indirectly, with the mitochondrial population to direct mitochondrial segregation or

selective elimination of those organelles containing the *pvs* sequence. Experiments presented here have tested the validity of some assumptions inherent in this mitochondrial "phenotypic selection" model.

Selective elimination of mitochondria containing the *pvs* sequence relies on three important cellular conditions. (1) To restore a normal mitochondrial population by eliminating dysfunctional *pvs*⁺ mitochondria, the population in CMS-Sprite must be heteroplasmic; in other words, a mitochondrial population composed of organelles containing the *pvs* sequence (exhibiting a dysfunctional phenotype) together with organelles devoid of the *pvs* sequence (exhibiting a normal phenotype). (2) There must exist phenotypic distinctions between mitochondria containing the *pvs* sequence and those that do not. (3) The driving force, that is, the product of nuclear gene *Fr*, must somehow recognize these mitochondrial phenotypic distinctions.

At least two important observations support the assumption that a heteroplasmic mitochondrial population exists in CMS-Sprite. The occurrence of spontaneous cytoplasmic reversion in this system (MACKENZIE *et al.* 1988) presumably results from the random sorting of a heteroplasmic mitochondrial population. In an earlier study, our laboratory constructed a physical map of the common bean mitochondrial genome using overlapping cosmid clone analysis (JANSKA and MACKENZIE 1993). Results from this study indicated that the mito-

chondrial genome of CMS-Sprite likely exists as three interrecombining molecules, each having the potential to be maintained autonomously. The *pvs* sequence resides on only one of these molecules, with the remainder of this molecule composed of sequences repeated elsewhere in the genome. This genome configuration allows for the dispensability of the *pvs*-containing molecule. Consequently, a mitochondrial population in equilibrium would be expected to contain both pvs^+ and pvs^- mitochondrial forms.

The *pvs* sequence has been shown to contain at least two open reading frames and is transcriptionally active (CHASE and ORTEGA 1992; JOHNS *et al.* 1992). Expression of *pvs* would, in a heteroplasmic mitochondrial population, allow the distinction between dysfunctional mitochondria (pvs^+) and normal mitochondria that do not contain *pvs*. We have used polyclonal antibodies raised against a putative product of *pvs* to demonstrate that the largest *pvs* open reading frame is expressed only in CMS-Sprite reproductive tissues. These results are presented elsewhere (ABAD *et al.* 1995).

To test whether the effect of *Fr* is dependent on a mitochondrial phenotypic distinction between pvs^+ and pvs^- mitochondria, we introduced nuclear gene *Fr* to a fertile line (GO8063) that contains *pvs* but demonstrates no mitochondrial dysfunction (full male fertility). Results from this experiment indicate that the role of *Fr* in elimination of the *pvs* sequence was diminished under circumstances when a second fertility restorer was present to suppress the *pvs* effect (Figure 2, A and B). The control experiment, in which removal of *Fr2* from GO8063 returned the ability of *Fr* to eliminate *pvs* (Figure 2C), supports the assumption that the loss of *Fr* action was because of the presence of *Fr2*. The dependence of *Fr* action on the effect of *pvs* offers a plausible means for *Fr*-directed *pvs* elimination to occur at the mitochondrial population level.

Although all data to date have provided support to a model of *Fr*-directed mitochondrial segregation or selective elimination in a population containing the *pvs* mutation, at least two additional possibilities must also be considered. One possible explanation for the loss of *pvs* in response to the introduction of *Fr* involves the directed excision of *pvs* from the mitochondrial genome. Based on physical mapping data, we have no evidence of a novel mitochondrial junction fragment that would be required to join the two segments flanking the excision site (JANSKA and MACKENZIE 1993). In addition, ≥30 kb of the region encompassing the *pvs* sequence is lost during the restoration/reversion process (MACKENZIE and CHASE 1990). Physical mapping of the mitochondrial genome in maize cmsT lines that undergo reversion to fertility indicates that deletion of a mitochondrial DNA segment may occur via recombination involving two pairs of repeated sequences flanking the target site (FAURON *et al.* 1990a,b). This mechanism of two-point

recombination is predicted to result in not only deletion of the segment of the genome separating the two different repeat sequences, but large duplicated segments of the genome as well. We were unable to identify two repeats flanking the *pvs* region of interest and were, based on the mitochondrial genome organization of CMS-Sprite, unable to find evidence that such a recombination model might account for the loss of *pvs* (discussed further in JANSKA and MACKENZIE 1993).

Another possibility also must be considered in studying *Fr-pvs* interaction. Because mitochondrial physical mapping indicates that the *pvs* sequence likely resides on a dispensable molecule in the genome, the possibility exists that *Fr* causes the loss of *pvs* by suppressing DNA replication of the *pvs*-containing molecule, resulting in the selective elimination of *pvs*. We have not yet fully tested a model for differential mitochondrial DNA replication because of the inherent technical difficulties. Assuming the validity of a model implicating a role of *Fr* in differential replication, it is difficult to account for the observation that *Fr* loses its effect in the presence of restorer *Fr2*. Also, because all sequences present on the *pvs*-containing mitochondrial molecule, except the 3.7-kb *pvs* sequence, are present elsewhere in the genome, one must presume that differential replication of *pvs* would be regulated by *Fr* via sequences present within *pvs*. This possibility appears remote but merits further testing.

Dynamics of the mitochondrial population during *Fr*-directed restoration: Although the *Fr*-directed mitochondrial genome alteration in CMS bean is phenotypically detected as pollen fertility, the mitochondrial genome is maternally inherited (KHAIRALLAH *et al.* 1990; MACKENZIE 1991). Our results from PCR experiments indicate that the effect of *Fr* is most pronounced within female reproductive tissues. Anther tissues that give rise to fertile pollen still retained the *pvs* sequence. A substantial reduction of *pvs* in developing F_2 pollen was observed previously using *in situ* hybridization analysis (JOHNS *et al.* 1992). The restoration of pollen fertility serves as a phenotypic indication of *pvs* loss from the male gametes. Because pollen sampling in this experiment involved pooling all pollen collected from individual flowers, the detection of *pvs* in pollen samples likely reflects remaining *pvs* in a small proportion of F_2 pollen due to the high level sensitivity of PCR amplification.

Genetic experiments indicate that *Fr* acts sporophytically, demonstrating a normal Mendelian segregation in the F_2 generation of CMS-Sprite/R351. These results imply that *Fr* functions premeiotically, perhaps during pollen mother cell development. In the female reproductive tissues, we detected the loss of *pvs* from 80% of the F_2 ovaries, funiculi and ovules assayed and from all the F_3 embryos assayed. From this result we suggest that the loss of *pvs* is probably initiated at early stages of female tissue differentiation and is completed at ga-

metogenesis. The loss of *pvs* from megaspores conditions permanent restoration in subsequent generations, as reflected in the loss of *pvs* from all F_3 tissues tested.

The proposed model, together with results presented here, suggests that *Fr* and *fr* behave as codominant alleles. A 1:2:1 (fertile:semisterile:sterile) segregation pattern is observed in the F_2 population of CMS-Sprite / R351BC$_4$F$_2$ (Table 3). A heterozygous condition at the *Fr* locus results in semisterility, that is, the presence of both fertile (pvs^-) and sterile (presumably heteroplasmic) pollen in the same anther. Assuming that CMS-Sprite is heteroplasmic, the very low rate (0.5–5%) of spontaneous reversion in this line (HE and MACKENZIE, unpublished data) and the reversibility of the *Fr/fr*-directed mitochondrial population shift (Table 3) imply that the alternative allelic form of *Fr* (designated *fr*) may play a role in stabilizing the pvs^+ / pvs^- mitochondrial population. The introduction of one dose of *Fr* (*Fr/fr*) appears to initiate the *pvs* elimination process in both male and female reproductive tissues, resulting in a mitochondrial population reduced in the frequency of *pvs* (semisterility). The phenotype of semisterility, producing both fertile and sterile pollen, is a logical consequence of stochastic partitioning in the segregation of a heteroplasmic mitochondrial population. A heteroplasmic population is generally unstable once nuclear control is relaxed (BIRKY 1983). With a nuclear genotype of *Fr/fr*, the predominant mitochondrial type (pvs^-) may sort to homoplasmy in a proportion of cells, giving rise to fertile pollen. The degree of semisterility varies among plants, producing from 1 to >50 seeds per individual plant (data not shown). This phenotypic variation reflects the predicted random segregation process of the mitochondrial population.

In the F2 generation, because *Fr* does not have a detectable effect on the mitochondrial population in vegetative tissues, vegetative tissues from all the plants, regardless of the nuclear genotype, retain the heteroplasmic mitochondrial population. In the reproductive tissues, three phenotypes result. The model predicts that plants of *fr/fr* genotype contain a mitochondrial population reversed to the original pvs^+ / pvs^- state in all tissues. Plants of *Fr/fr* genotype maintain an unstable mitochondrial population similar to that of the first generation (F_1), demonstrating that one dose of *Fr* over multiple generations is insufficient to complete *pvs* elimination. Plants of *Fr/Fr* genotype are fully fertile; two copies of *Fr*, or the removal of *fr*, are required to achieve a homogeneous *pvs*$^-$ mitochondrial population.

It is becoming increasingly evident that nuclear genes play important roles in controlling the mitochondrial genome structure in all eukaryotic organisms. One possible role of nuclear genes is to monitor and relieve the mitochondrial population of detrimental mutations. For example, nuclear gene defects have been associated with the accumulation of a number of human mitochondrial

DNA deletions, resulting in the phenomena of mitochondrial myopathies (SCHAPIRA 1993). Whether or not these mitochondrial mutations occur generally and should normally be eliminated from the mitochondrial population by a nuclear-directed process has not been determined. Essentially nothing is known about how nuclear genes might influence the mitochondrial genetic composition. The *Fr* gene in common bean has been shown to alter the mitochondrial genome structure by directing loss of the abnormal *pvs* sequence. Elucidation of the mechanism of *Fr* action should provide valuable insight not only into the molecular basis of pollen fertility restoration but also to our understanding of intergenomic regulation in other higher eukaryotes.

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