Theor Appl Genet (1997) 95:205–210

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Nuclear fertility restorer genes map to the same linkage group in cytoplasmic male-sterile bean

Received: 10 October 1996 / Accepted: 8 November 1996

Abstract Fertility restoration in cytoplasmic male-sterile plants (CMS) by nuclear restorer genes is one of the few useful systems for studying nuclear-mitochondrial interactions in higher eukaryotes. In CMS bean there exist multiple independently identified nuclear genes that restore fertility. Two restorer genes (*Fr* and Fr_2) have been characterized previously. We have genetically characterized two additional restorer genes; both restorers are single genes that behave similarly to Fr_2. We compared the linkage relationship of all four independently identified restorer genes to understand the relationship among these loci further. All four genes map to the same linkage group. It was not possible to distinguish between the two newly identified restorer genes and Fr_2. We suggest that they may be allelic.

Key words Mapping · CMS · Fertility restorers · Pollen · *Phaseolus vulgaris*

Introduction

In plants, mitochondrial mutations often give rise to a phenotype of male sterility (Hanson 1991). The association of mitochondrial dysfunction with abnormal pollen development has been observed in several plant species, though the cause-and-effect relationship has not been well defined in any case to date. For most cytoplasmic male-sterile (CMS) mutants, nuclear suppressor genes, termed fertility restorers, have been identified. Nuclear-induced fertility restoration represents one of the very few genetically approachable opportunities by which to investigate nuclear-mitochondrial interactions in higher eukaryotes.

In cytoplasmic male-sterile common bean (*Phaseolus vulgaris* L.), a sterility-associated mutation has been relatively well characterized. The *pvs-orf 239* mitochondrial sequence is contained within several lines of *P. vulgaris*, *P. coccineus*, and *P. polyanthus* (Hervieu et al. 1993, 1994) and encodes a polypeptide of 27 kDa in size (Johns et al. 1992). In the case of *P. vulgaris*, this sequence is expressed only in reproductive tissues of the male-sterile line (Abad et al. 1995). The association of *pvs-orf239* with cytoplasmic male sterility is further confirmed by the observation that this sequence causes male sterility in transgenic tobacco (He et al. 1996).

Several nuclear fertility restorers have been independently identified in bean (Mackenzie and Bassett 1987; Mackenzie 1991; Mackenzie unpublished data). This observation implies that it may be possible to select multiple means by which the nuclear genome can suppress *pvs-orf 239* expression or effect. We are currently investigating the mechanisms of these various suppressors to understand further their role in mitochondrial regulation. Toward this end we have, to date, genetically characterized two fertility restorer loci, *Fr* and Fr_2. Restorer *Fr* effects the loss of the mitochondrial sterility-associated sequence, *pvs*, from the genome (Mackenzie et al. 1988; Mackenzie and Chase 1990) to produce a permanent condition of fertility (Mackenzie and Bassett 1987). Nuclear restorer gene Fr_2, in contrast, appears to restore fertility by suppressing expression of *pvs-orf 239* posttranscriptionally (Chase 1994; Abad et al. 1995).

The map location of *Fr* has been determined recently (He et al. 1995b). In the investigation presented here we have characterized the genetics of restoration by two independently identified fertility restorers, $Fr_{PI207228}$ and Fr_{XR235}, and compared the genetic linkage relationships of Fr_2, $Fr_{PI207228}$, and Fr_{XR235} to *Fr*.

Communicated by K. Glimelius

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Materials and methods

Plant materials

Multiple populations segregating for Fr_2, $Fr_{PI207228}$, and Fr_{XR235} were used. The restorer $Fr_{PI207228}$, was identified in a cross between CMS-Sprite and accession line PI207228 of *P. vulgaris*: Populations derived from that cross included a BC_4F_2 population (CMS-Sprite X PI207228 with CMS-Sprite as the recurrent parent) of 70 individuals that was used for genetic analysis of this restorer and initial establishment of linkage, a BC_4F_2 population of 25 individuals that was used for further mapping, and BC_2F_3 (5 F_3 families), BC_3F_3 (4 F_3 families), and BC_4F_3 (10 F3 families) populations that were used to confirm the maintenance of *pvs*. Another restorer, Fr_{XR235} was identified in a cross between CMS-Sprite and line XR235: populations derived for this study included an F_2 population (CMS-Sprite X XR235) of 101 individuals that was used to characterize the genetics of restoration and linkage, another F_2 population of 22 individuals that was used to further map this restorer, and 13 F_3 families, ranging from 6 to 30 individuals, that were used for progeny testing. Four F_4 families derived from fertile F_3 individuals were used to confirm stability of *pvs*. To map restorer Fr_2, we developed an F_2 population of 32 individuals to establish linkage, a second F_2 population of 99 individuals to map this restorer, and a third F_2 population of 24 individuals to confirm map location due to the effects of environmental stress. Data obtained from all independently grown F_2 populations were combined for mapping and linkage analysis. Lines PI207228 and XR235 were supplied by the International Center for Tropical Agriculture (CIAT, Cali, Colombia).

Phenotypic classification

Plants were grown under standard greenhouse conditions and classified for pollen fertility using modified criteria described by He et al. (1995b). Pollen fertility was evaluated by three different criteria: stainability with I_2KI, pollen shed, and pod set. Plants were considered fertile if they contained more than 95% darkly stainable pollen, retained no microspores as tetrads, and produced normal seed-bearing pods without evidence of parthenocarpy. Semisterile plants produced both darkly stainable pollen and nonstainable pollen in tetrads, visible pollen shed was highly variable in amount, and both parthenocarpic and normal seed-bearing pods were produced at maturity. Sterile plants contained less than 5% stainable pollen, shed no pollen on the stigma, and produced no fertile seed-bearing pods.

DNA extraction

Genomic DNA was extracted using the procedure of Vallejos et al. (1992). About 2 g of tissue was ground in liquid nitrogen to a very fine powder and incubated with 16 ml of lysis buffer (133 m*M* Tris-HCl (pH 7.8), 6.7 m*M* Na_2EDTA, 0.95 *M* NaCl, 1.33% Na Sarkosyl and 1.33% β-mercaptoethanol) at 65°C for 1 h. The homogenate was chloroform-extracted once and then the aqueous phase separated by centrifugation. DNA was precipitated in a two-thirds volume isopropanol for 30 min. Precipitated DNA was then transferred with a glass hook to a new tube, washed once in 76% ethanol, 0.2 *M* sodium acetate for 30 min, once in 76% ethanol, 10 m*M* ammonium acetate for 2 mins, dried briefly, and dissolved in 200 ul TE buffer.

Random amplified polymorphic DNA (RAPD) marker analysis

RAPD marker analysis was conducted using the same amplification and electrophoresis conditions previously described by He et al. (1995b). An additional RAPD marker linked to *Fr* was identified using Bulked Segregant Analysis (Michelmore et al. 1991) wherein 5 fertile and 5 sterile individuals were separately bulked. We designated this primer *UBC190*. Its sequence was $^{5'}$AGAATCCGCC$^{3'}$.

Restriction fragment length polymorphism (RFLP) analysis

Genomic DNA from F_2, F_3, and F_4 populations was used for RFLP analysis. DNA samples purified from individual plants were digested with *Eco*RI, *Eco*RV, *Bam*HI, *Pst*I, and *Hin*dIII using reaction conditions recommended by the manufacturers, except that 8 m*M* spermidine was added to each sample. Electrophoretic separation, gel blotting, and hybridization were as described previously (He et al. 1995b). RFLP-cloned inserts were amplified using the conditions described by Vallejos et al. (1992), subjected to 0.6% agarose gel electrophoresis, and purified using the Geneclean kit (Biolabs 101, La Jolla Calif.). Mitochondrial RFLP genomic clones were amplified using specific primers and purified as described above. DNA fragments were radioactively labeled with ^{32}P-dCTP using the random priming method of Feinberg and Vogelstein (1983). Hybridization was performed at 60°C. Blots were washed twice in 3 × SSC, 0.1% SDS at 60°C and once in 0.3 × SSC at 60°C for 15 mins each wash. Fuji Medical X-Ray film was exposed to the membrane using intensifying screens (Pickett) for 5–7 days for nuclear DNA probes and 1–2 days for mitochondrial (mtDNA) probes.

Segregation and linkage analysis

Allelic segregation data were scored manually from either ethidium bromide-stained agarose gels (RAPDs) or autoradiographic films (RFLPs) for each segregating individual. Linkage analysis was performed using the MAPMAKER MacIntosh program version 2.0.6800 (Lander et al. 1987; Du Pont) with a LOD of 3.0 and θ of 0.4 as default linkage criteria and χ^2 analysis. The Haldane mapping function was used to determine genetic distances. For the purpose of linkage analysis, semisterile and sterile phenotypic classes were combined to prevent misclassifications. Restorer genes were positioned on the linkage map using three-point analysis.

Results

Genetics of fertility restoration

Two additional restorer genes were identified in CMS bean. One, designated $Fr_{PI207228}$, was identified in a cross between CMS-Sprite and PI207228 and the other, designated Fr_{XR235}, was derived from a cross between CMS-Sprite and XR235. Both $Fr_{PI207228}$ and Fr_{XR235} acted in a semidominant fashion to produce three phenotypic classes, sterile, semisterile, and fertile, in a segregating F_2 population. Data for both genes were consistent with a single-gene model for fertility restoration (Tables 1 and 2). Table 1 shows backcross and F_2 segregation data to support a single gene model for restoration in line PI207228. Table 2 demonstrates that F_2 data from CMS-Sprite X XR235 were consistent with Fr_{XR235} acting as a single-gene trait. To confirm further a single-gene model for Fr_{XR235}, we analyzed 11 F_3 families derived from semisterile and fertile F_2 plants for segregation of fertility. Those F_3 families derived from fertile individuals did not

Table 1 Restorer $Fr_{PI207228}$, behaves as a single-gene trait

CMS-Sprite X PI207228 Population	Number of plants	Classification					
		Fertile	Semi-sterile	Sterile	χ^2 1:1	*P*	*pvs*[a]
BC_1	46		15	31	5.59	0.02	
BC_3	28		14	14	0.00	0.99	
BC_4	53		24	29	0.47	0.50	
BC_5	32		11	21	3.12	> 0.05	
					χ^2 *1:3*		
BC_4F_2	70	20	28	22	0.48	0.5	
BC_2F_3							+
31	29	29					+
35	5	5					+
14	3	3					+
8	3	3					+
13[b]	5	3	1	1			+
BC_3F_3							
15-12[c]	26	26					−
5-10	25	25					+
14-3	29	29					+
21-7[c]	29	29					−

[a] Presence of *pvs* sequence in 3–5 fertile F_3 individuals
[b] Derived from a semisterile individual
[c] Presumed to be a product of spontaneous reversion

Table 2 Restorer Fr_{XR235} acts a single-gene trait

Population CMS-Sprite X XR235	Number of plants	*Classification*					
		Fertile	Semi-sterile	Sterile	χ^2 (1:3)[a]	*P*	*pvs*[b]
F_1	17		16	1[c]			
F_2	101	29	42	30	0.67	0.4	
F_4							
9-15	30	30					+
4-2	26	22	4[c]				+
1-14	25	25					+

[a] 1:3 based on pooled sterile and semisterile individuals
[b] Presence of *pvs* in 2–3 individuals tested from each F_4 family
[c] Unexpected plant phenotype likely due to environmental conditions

segregate for fertility, while those derived from semisterile individuals did. The F_3 family data were also consistent with a segregation of 1:2 (nonsegregating:segregating), which would be expected for a single gene trait segregating 1 fertile:2 semisterile:1 sterile ($\chi^2 = 0.72$, $P = 0.3$). In chi-square analyses of F_2 populations for $Fr_{PI207228}$, and Fr_{XR235} segregation, we elected to combine the semisterile and sterile classes because, in these populations, environmental sensitivity was found to sometimes influence classifications (data not shown).

The mechanism of restoration in crosses involving line PI207228 or line XR235 appeared to be distinct from that of *Fr*. In contrast to *Fr*, neither restorer $Fr_{PI207228}$ nor restorer Fr_{XR235} directed the loss of the sterility-associated *pvs* sequence (Fig. 1). Four BC_2F_3 families, 4 BC_3F_3 families, and 8 BC_4F_3 families derived from fertile individuals containing $Fr_{PI207228}$ were analyzed for the presence of *pvs*. If $Fr_{PI207228}$ acts similarly to *Fr*, we would expect to observe the loss of the *pvs* sequence in all of the F_3 families derived from fully fertile F_2 individuals (Mackenzie et al. 1988). However, the majority of these individuals contained *pvs* (Table 1 and Fig. 1). There were 3 F_3 families that did demonstrate the loss of *pvs*, but the low incidence of *pvs* loss suggested that this was the consequence of cytoplasmic reversion. Cytoplasmic reversion is the spontaneous loss of the *pvs* sequence; this phenomenon has been observed previously in common bean (Mackenzie et al. 1988) and is believed to occur due to the random segregation of *pvs*+ and *pvs*− mitochondria (He et al. 1995a). Three F_4 families and 3 F_3 families derived from fertile F_2 individuals containing Fr_{XR235} were analyzed for the presence of *pvs*. These individuals did not reveal the loss of *pvs* (Table 2). However, there were 2 F_3 families derived from semisterile F_2 individuals

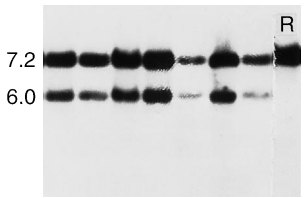

Fig. 1 DNA gel blot hybridization of total genomic DNA from $Fr_{PI207228}$, restored plants digested with *Pst*I and probed with a portion of the *pvs* sequence and an adjoining sequence (internal control) (Johns et al. 1992). The *pvs* sequence hybridizes to the 6.0-kb *Pst*-I fragments, and the adjacent sequence hybridizes to the 6.0- and 7.2-kb *Pst*I fragments. Only at low frequency was the 6.0-kb fragment lost, as evidenced in *lane R* (presumed reversion event)

that did lose the *pvs* sequence (data not shown); again, we assumed that this was the result of reversion. Based on these observations we concluded that the mechanism of restoration by loci $Fr_{PI207228}$ and Fr_{XR235} is distinct from that of restorer *Fr*. It is important to note, however, that the pattern of restoration by $Fr_{PI207228}$ and Fr_{XR235} could not be distinguished from that of restorer Fr_2 based on these criteria.

Linkage relationships among CMS bean fertility restorers

RFLP markers on linkage group K of the common bean RFLP map developed by Vallejos et al. (1992) were previously shown to cosegregate with *Fr* (He et al. 1995b). Consequently, RFLP markers from linkage group K were used as hybridization probes to determine whether Fr_2, $Fr_{PI207228}$, and Fr_{XR235} also reside within this same linkage group. Additionally, RAPD markers that cosegregated with *Fr* were used to screen populations segregating for the other three independently identified restorers. Marker analyses indicated that all four restorers mapped to linkage group K (Table 3, Table 4).

It was not possible to precisely position Fr_2, $Fr_{PI207228}$, and Fr_{XR235} relative to one another on the map. To distinguish between tight linkage versus allelism in the relationship of Fr_2, $Fr_{PI207228}$, and Fr_{XR235} was not feasible in the current study due to the following complications. Absolutely unambiguous discrimination between a semisterile and fully sterile phenotype would have been necessary to identify the rare recombinant in the event of distinct loci. Such resolution was not possible given the general sensitivity of pollen development to environment and the variable nature of the semisterile phenotype. It is not uncommon to observe a range of 20% to 80% stainable pollen on a single semisterile plant (data not shown). More importantly, however, recombination suppression was observed in the Fr_2 and Fr_{XR235} populations. This was detected as significantly different mapping distances arising between known RFLP markers from the K linkage group in these populations relative to the *Fr*

Table 3 All three independently isolated restorer genes map to linkage group K using marker *Bng228* as a genetic point of reference[a]

Genotype and Population[b]	Number of plants	Plants cosegregating with *Bng 228* polymorphism associated with sterility	χ^2 Independence (1:3:3:9)	*P*	χ^2 1:3 F:S	*P*	χ^2 1:2:1 *Bng228* AA:Aa:aa	*P*
Fr_2								
F	24	0	94.22	< 0.001	2.67	0.10	6.75	< 0.05[c]
S	104	104						
$Fr_{PI207228}$								
F	23	1	94.90	< 0.001	1.84	0.15	1.20	0.5
S	49	49						
Fr_{XR235}								
F	24	0	114.03	< 0.001	2.93	> 0.05	3.83	0.1
S	47	47						

[a] A small portion (about 5%) of these populations could not be used because they could not be unambiguously classified
[b] F designates fully fertile class and S designates pooled sterile and semisterile class
[c] Only a subgroup of this population was used for DNA marker analysis, thus accounting for the deviation from a 1:2:1 expectation

Table 4 RFLP (*Bng*) and RAPD (*UBC*) markers on linkage group K showing polymorphism with the enzymes indicated in populations segregating for individual restorer genes

Restorer gene	*Bng-228*	*Bng-102*	*Bng-64*	*Bng-14*	*Bng-149*	*Bng-134*	*Bng-198*	*UBC-487*	*UBC-190*
Fr_2	*Eco*RI	*Eco*RI	*Eco*RI	*Eco*RI	*Eco*RI	*Eco*RV	−	+	+
$Fr_{PI207228}$	*Eco*RI	−	−	−	−	−	−	+	+
Fr_{XR235}	*Eco*RI	*Hin*dIII	*Eco*RI	*Eco*RV	*Eco*RI	*Eco*RV	*Eco*RI	−	+

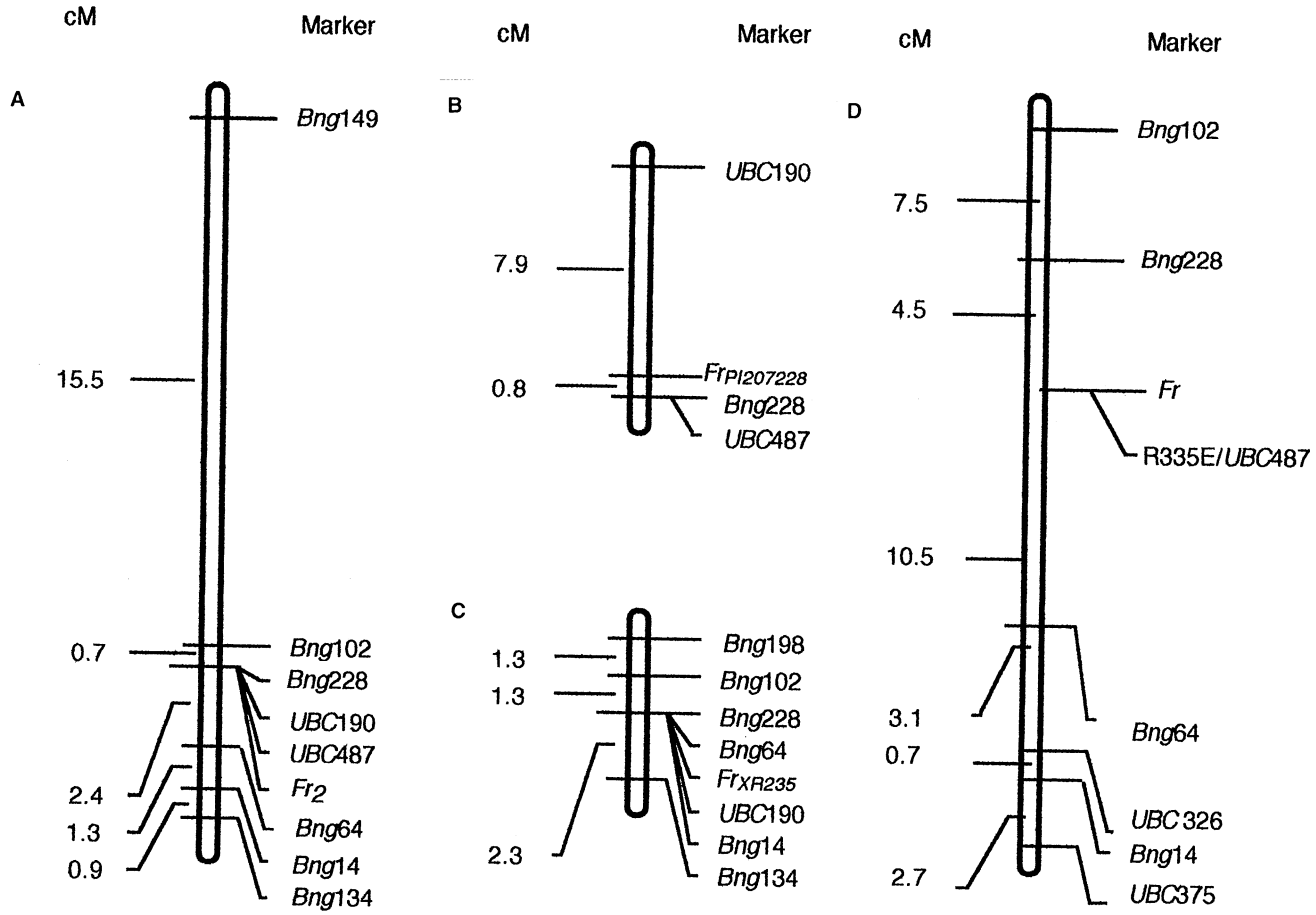

Fig. 2A–D Fr_2 (A), $Fr_{PI207228}$ (B), Fr_{XR235} (C), and *Fr* (D) genetic maps showing the location of linked RFLP and RAPD markers that were known to reside on linkage group K

and $Fr_{PI207228}$ populations (Fig 2). Such crossover suppression artificially compresses the distance between loci in the region. Thus, we propose that Fr_2, Fr_{XR235} and $Fr_{PI207228}$, represent allelic forms of a single locus based on phenotype conferred and map position, but without the benefit of a formal allelism test.

Discussion

Our results demonstrated that four independently isolated fertility restorers each behaved as single-gene loci to restore fertility in CMS common bean. In all four cases, only partial fertility was achieved in the F_1 generation, with full fertility segregating in the F_2 generation. More importantly, all four fertility restorers mapped to the same linkage group in common bean, with Fr_2, $Fr_{PI207228}$, and Fr_{XR235} genetically indistinguishable. These three fertility restorers may represent independent, tightly linked loci; our study did not rule out this possibility. Yet the similarity in their pattern of restoration with each conditioning only partial fertility restoration in the F_1 generation, a highly similar semisterile phenotype (data not shown), and a nonpermanent condition of fertility with the *pvs* sequence retained, implied that they are allelic. A formal allelism test to distinguish Fr_2, $Fr_{PI207228}$, and Fr_{XR235} was not feasible in this system; the degree of genetic linkage coupled with the observed recombination suppression in the region and inherent classification difficulties would render such an approach inconclusive. Previously, He et al. (1995a) performed an allelism test between Fr_2 and *Fr*. Although in that case it was possible to identify recombinants between the two loci, the genetic distance between *Fr* and Fr_2 is predicted to be much greater than between Fr_2, $Fr_{PI207228}$, and Fr_{XR235} based on marker data. Whereas Fig. 2 demonstrates the tight compression existing between *Bng228* with Fr_2, $Fr_{PI207228}$, and Fr_{XR235}, a genetic distance of approximately 4.5 cM was observed between the *Bng228* marker and the *Fr* locus. Consequently, we will attempt to distinguish these fertility restorers based on mechanism of action. At present we have arrived at a model for Fr_2 action involving posttranscriptional regulation of *pvs-orf 239* expression (Abad et al. 1995). Once we have further resolved this regulatory mechanism, it will be possible to evaluate the effects of $Fr_{PI207228}$, and Fr_{XR235} in comparison.

It would not be altogether suprising to find Fr_2, $Fr_{PI207228}$, and Fr_{XR235} representing alleles of a single locus; each of these was identified from *P. vulgaris* lines of Mesoamerican origin. It is also not surprising that none of these is allelic to *Fr*; we have reason to believe that the *Fr* allele was derived by mutation and is not commonly found in natural populations (Mackenzie, unpublished). With these results we will now begin to survey *P. vulgaris* lines of Andean origin, as well as *P. coccineus* and *P. polyanthus* materials, for novel mechanisms of fertility restoration.

The recombination suppression observed when mapping Fr_2 and Fr_{XR235} was likely a consequence of the particular crosses used. In the case of Fr_2, a Mesoamerican line (G08063) was crossed to a snap bean genotype, CMS-Sprite. Because of the diverse background of these genotypes, there may be a translocation or an inversion in this chromosomal region. Other indications of genetic incompatibility in these crosses were evident, including stunting and leaf disfiguration in a small proportion of the population (data not shown). Additionally, XR235 was developed as an introgression of *P. coccineus* to *P. vulgaris* (Freytag et al. 1982), likely an additional factor contributing to the unusually low crossover frequency in the region we tested. Similar suppression was previously observed using XR235 in the development of the common bean RFLP map (He et al. 1995b: Vallejos et al. 1992). A comparison of marker distribution on linkage group K reported by Vallejos et al. (1992) and He et al. (1995b) shows that when XR235 was used as a parent recombination was suppressed in the region encompassing *Fr*. Map compression was not observed previously in crosses between CMS-Sprite and CMS-Sprite-*Fr*, near-isolines developed for the mapping of the *Fr* locus (He et al. 1995b and Fig. 2D). For mapping $Fr_{PI207228}$, backcrosses to CMS-Sprite prior to genetic analysis allowed us to introgress the region of interest into a Sprite genetic background. Sufficient recombination occurred in this process to eliminate DNA polymorphisms for several of the markers distal to the target loci. Consequently, the degree of recombination suppression in the region could not be examined in this particular mapping population.

The various mechanisms that we will be able to identify for the nuclear suppression of *pvs-orf239* expression or effect should prove invaluable in our studies of mitochondrial regulation. To date, it is not clear to what extent gene regulation and mitochondrial selection strategies are employed by the nucleus to influence mitochondrial gene expression in plants. With the vast amount of recombination and genomic rearrangement possible in the plant mitochondrial genome (Wolstenholme and Fauron 1995), relative to other eukaryotic systems, the coevolution of several unique mutation suppression mechanisms would not be unexpected.

Acknowledgements The authors wish to thank I. Dweikat for his critical review of the manuscript. This work was supported, in part, by grants from NSF (9316342-MCB) and USDA (94-37300-0451) to S.M.

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