Nuclear Genes Associated With a Single Brassica CMS Restorer Locus Influence Transcripts of Three Different Mitochondrial Gene Regions

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Manuscript received November 8, 1995
Accepted for publication February 8, 1996

ABSTRACT

Previous studies have shown that the mitochondrial *orf224/atp6* gene region is correlated with the Polima (*pol*) cytoplasmic male sterility (CMS) of *Brassica napus*. We now extend this correlation by showing that the effects of nuclear fertility restoration on *orf224/atp6* transcripts cosegregate with the *pol* restorer gene *Rfp1* in genetic crosses. We also show, however, that the recessive *rfp1* allele, or a very tightly linked gene, acts as a dominant gene, designated *Mmt* (modifier of mitochondrial transcripts), in controlling the presence of additional smaller transcripts of the *nad4* gene and a gene possibly involved in cytochrome *c* biogenesis. A common sequence, TTGTGG, maps immediately downstream of the 5′ termini of both of the transcripts specific to plants with the *Mmt* gene and may serve as a recognition motif in generation of these transcripts. A similar sequence, TTGTTG, that may be recognized by the product of the alternate allele (or haplotype), *Rfp1*, is found within *orf224* just downstream of the major 5′ transcript terminus specific to fertility restored plants. Our results suggest that *Rfp1/Mmt* is a novel nuclear genetic locus that affects the expression of multiple mitochondrial gene regions, with different alleles or haplotypes exerting specific effects on different mitochondrial genes.

THE regulation of mitochondrial gene expression by nuclear genes is the key means by which the activities of the nuclear-cytoplasmic and mitochondrial genetic systems are coordinated. In yeast, a large number of nuclear genes that mediate the expression of specific mitochondrial genes have been identified (COSTANZO and FOX 1990; GRIVELL 1995). In most cases, individual nuclear genes influence the production of a single mitochondrial gene product. The regulation is generally postranscriptional and may affect mRNA processing, stability or translation. In several cases, more than one nuclear gene is specifically required for the production of a single mitochondrial-encoded protein.

In multicellular organisms, the mechanisms through which nuclear genes regulate mitochondrial gene expression are much less well understood. The trait of cytoplasmic male sterility (CMS) offers an opportunity for exploring this problem in flowering plants. CMS is a maternally inherited defect in pollen production that is thought to result from the expression of unusual or aberrant mitochondrial genes (HANSON 1991; HANSON and FOLKERTS 1992; BONEN and BROWN 1993). Many examples of nuclear genes that suppress CMS, called restorers of fertility (*Rf*), have been identified. In several of the cases that have been analyzed in detail, these restorer genes appear to act by inhibiting the expression of an aberrant CMS-associated mitochondrial gene, either by altering the production of specific mitochondrial transcripts or by inhibiting mRNA translation. In at least one case, the effects of restorer gene action on mitochondrial transcripts is tissue-specific (MONÉGER *et al.* 1994).

The Polima or *pol* CMS system of the oilseed rape species *Brassica napus* offers an attractive system for studying nuclear-mitochondrial interactions in plants. The Brassica mitochondrial genome is relatively small and simple in organization (PALMER and SHIELDS 1984), and the genetics of fertility restoration have been described (FANG and MCVETTY 1989); restoration can be achieved through the action of dominant alleles at either of two different nuclear loci. Previous studies have indicated that the mitochondrial *atp6* gene region may specify the *pol* CMS trait. It is the only region that is organized differently between the sterile *pol* and fertile *cam* mitochondrial genomes (L'HOMME and BROWN 1993) and the only mtDNA region that is genetically correlated with CMS in *B. napus/B. oleracea* somatic hybrids (WANG *et al.* 1995). Of 14 different mitochondrial gene regions investigated, only transcripts of the *atp6* region were found to differ between *pol* CMS, *pol* fertility-restored and fertile *nap* or *cam* cytoplasm plants (SINGH and BROWN 1991; WITT *et al.* 1991). Like several

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other CMS-associated mitochondrial gene regions (HANSON 1991; HANSON and FOLKERTS 1992; BONEN and BROWN 1993), the *pol atp6* region contains a chimeric gene, *orf224*, that is cotranscribed with *atp6* (SINGH and BROWN 1991). In the flowers of *pol* CMS plants, *orf224/atp6* transcripts are predominantly dicistronic. Expression of the region is modified in fertility restored plants: two additional transcripts that possess 5′ termini mapping within *orf224* appear, and as a result, predominantly monocistronic *atp6* transcripts are generated (SINGH and BROWN 1991, 1993).

There are several examples of nuclear gene influences on plant mitochondrial gene expression that appear unrelated to CMS or fertility restoration (KENNELL *et al.* 1987; COOPER and NEWTON 1989; COOPER *et al.* 1990; MAKAROFF *et al.* 1990; NEWTON *et al.* 1995), and it is not known to what extent the effects of restorer genes on mitochondrial gene expression are specific for CMS-associated regions. The initial goals of this study were to determine if the *orf224/atp6* transcript differences observed between CMS and restored plants were specifically due to the *pol* restorer gene *Rfp1* and to determine to what extent the effects of restoration on mitochondrial transcripts are limited to the *orf224/atp6* gene region. Our results indicate that effects of restoration on *orf224/atp6* transcripts are due to a single nuclear gene that cosegregates completely with *Rfp1*. Moreover, no effects of the dominant restorer allele of this gene on mitochondrial transcripts other than those of the *orf224/atp6* region were detected. Interestingly, however, we find that the alternative *rfp1* allele, which is recessive to *Rfp1* with respect to effects on fertility and *orf224/atp6* transcripts, is dominant to *Rfp1* with respect to effects on transcripts of the *nad4* gene and a gene possibly involved in cytochrome *c* biogenesis. Thus, a single nuclear locus appears to influence the transcript profiles of at least three different mitochondrial gene regions with different alleles influencing different sets of transcripts.

MATERIALS AND METHODS

Plant material: The phenotype and restorer genotype of the *B. napus* lines used in this study have been described previously (SINGH and BROWN 1991). Karat (*nap*) and Westar (*nap*) are male-fertile *nap* cytoplasm strains, while Westar (*pol*) and Karat (*pol*) are male-sterile cytoplasm lines. Westar-Rf and Italy are *pol* cytoplasm lines that are homozygous for the nuclear fertility restorer allele *Rfp1*, while UM2353 *pol* cytoplasm plants are homozygous for the nuclear fertility restorer allele *Rfp2*.

Crosses were performed using single individuals as parents. Pollen from flowers of the male parent was applied to the stigma of unopened buds of the female parent. Unpollinated buds from the branch were then removed, and the pollinated buds were covered with a glassine envelope. Seeds were allowed to mature for ~7 weeks following pollination. Progeny plants were classified male-sterile or male-fertile based on their ability/inability to produce both well developed stamens and functional pollen. Mitochondrial RNA was isolated from floral tissue of plants grown under normal growth conditions (day/night temperatures 22°/16°, 16 hr photoperiod).

Mitochondrial DNA probes: DNA clones, representing ~87% of the *B. campestris* mitochondrial genome (PALMER and SHIELDS 1984; PALMER and HERBON 1988), were used to analyze the mitochondrial transcripts of the *B. napus* lines Westar (*nap*), Westar (*pol*) and Westar-Rf, and are described in Table 1. Dr. CHRISTOPHER A. MAKAROFF (University of Miami, Oxford, OH) furnished all of the *PstI* and *SalI* *B. campestris* clones. The two *KpnI* clones, BC-51 and BC-52 (Table 1), were isolated by Dr. YVAN L'HOMME (L'HOMME and BROWN 1993).

Isolation of mtRNA and RNA gel blot analysis: Mitochondrial RNA was isolated as described earlier by SINGH and BROWN (1991). RNA was resolved on agarose-urea gels (FINNEGAN and BROWN 1986) or agarose-formaldehyde gels (MANIATIS *et al.* 1982), transferred to Gene Screen Plus (Dupont/NEN) hybridization membranes by capillary blotting with 1.5 M NaCl/0.5 M sodium citrate, or 1.5 M NaCl/0.1 M NaH_2PO_4/0.02 M EDTA, and hybridized to the radiolabeled probe as described (SINGH and BROWN 1991).

DNA labeling, cloning and sequencing: Double-stranded DNA probes were radiolabeled using the nick translation system of BRL Life Technologies Inc. The following oligonucleotides were synthesized at the Sheldon Biotechnology Centre, McGill University: E13, TATACTCTTTCCCATAACTTCTCATACCAG; E12, CGTATCGGTCGTATTCTTGA; E42, TTTCTTTGCTGATTCCTCTC; E43, CCTCCCTTTCGTTCTACTTA; ccl1-31, TACTGATCAAACGCTGTAGGGCGGACTGC. One hundred nanograms of each oligonucleotide were labeled by incubation for 45 min at 37° with 10 units of T4 polynucleotide kinase in buffer supplied by the manufacturer and 50–100 μCi of [γ-^{32}P] ATP. The enzyme was denatured 10 min at 65°, and the labeled oligonucleotides were purified from unincorporated nucleotides using QIAquick purification columns from QIAGEN.

To sequence the transcribed portions of certain clones, restriction fragments were first purified from agarose using the GeneClean II (Bio 101) system according to the manufacturer's protocol. Fragments were ligated into KS^+ Bluescript vectors digested to produce compatible ends, and plasmids were amplified in JM101 bacterial cells and purified using the QIAprep-spin Plasmid Kit from QIAGEN. The DNA was analyzed by double-stranded sequencing after denaturation with 2 M NaOH. Sequences complementary to the Bluescript T7 and T3 promoter regions were used to prime the polymerase reaction. Sequencing was done using the T7 Sequencing System from Pharmacia Biotech. The Genbank Database was searched using the program BLAST (ALTSCHUL *et al.* 1990).

Primer extension analysis: Fifty micrograms of RNA were combined with 5×10^4 cpm of an end-labeled complementary oligonucleotide. The nucleic acids were ethanol precipitated and dissolved in water. Samples were denatured for 5 min at 95° and annealed for 15 min at 42°. One-half of the annealed product was used for each primer extension reaction. The RNA was reverse transcribed in the presence of 50 mM Tris-HCl pH 8.0, 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 2.5 mM actinomycin D, 2.5 mM of each dNTP, 1 unit/ml RNAguard (Pharmacia) and 2.5 units of AMV reverse transcriptase (Pharmacia) for 90 min at 42°. Primer extension was followed by a 15-min digestion of the RNA by RNase A at 37° and subjected to a single phenol:chloroform extraction before being precipitated with ethanol. Primer extension products were separated on a 5% sequencing polyacrylamide urea gel alongside sequencing reactions primed by the same complementary oligonucleotide. Oligonucleotide E13 was used to map the 5′ termini of the *nad4* transcripts, while oligonucleotide ccl1-31 was

used to map the 5′ termini of transcripts of the *ccl1*-like open reading frame.

RESULTS

***Rfp1* or a tightly linked gene acts to modify *orf224/atp6* transcripts:** The view that the *orf224/atp6* transcript differences between *pol* CMS and restored plants are associated with the action of the *Rfp1* gene and not with unlinked nuclear genes is based primarily on the observation that such differences are observed between the nearly isogenic lines Westar (*pol*) and Westar-Rf (SINGH and BROWN 1991). On this basis alone, the possibility that the transcript modifications are conditioned by a gene unlinked to the restorer, fortuitously retained through the repeated backcrosses used in the creation of the Westar-Rf strain, cannot be ruled out. If the restored-specific transcript pattern were shown to cosegregate with the restorer gene in a genetic cross, however, the role of the restorer gene in conditioning the transcript modifications would be more firmly established. To address this, we analyzed the *orf224/atp6* transcripts in individuals of a BC_1 backcross (*rfp1/rfp1* × *Rfp1/rfp1*) population. We chose to use a cross of this type for this purpose because of the simple predicted outcome: only two progeny genotypes are expected, *rfp1/rfp1* (sterile) and *Rfp1/rfp1* (fertile), and these should occur in approximately equal numbers. All sterile plants would be expected to lack the restored specific transcript, while all fertile plants would be expected to possess these transcripts.

One hundred eleven individuals of a BC_1 population, formed by crossing a CMS Karat (*pol*) plant (*rfp1/rfp1*) by a Karat (*pol*) × Westar-Rf, *Rfp1/rfp1* heterozygote as male, were raised to maturity. Of these, 56 were male-fertile and 55 were male-sterile, consistent with the 1:1 ratio expected if restoration results from the action of a single gene (M. JEAN, G. BROWN and B. LANDRY, unpublished results). Northern analysis of mitochondrial transcripts of 15 male-sterile and 15 male-fertile backcross progeny using a Brassica *atp6* probe showed that all the male-sterile plants (*rfp1/rfp1*) had the CMS transcript pattern (absence of 1.3- and 1.4-kb restored-specific transcripts), while all the male-fertile individuals had the restored transcript pattern, *i.e.*, they had the 1.3- and 1.4-kb transcripts (Figure 1). This allows us to firmly reject the hypothesis that a gene unlinked to the restorer is responsible for the transcript modification ($\chi^2 = 30.0$, $P < 0.001$) and supports the view that the *Rfp1* gene itself acts as the modifier of *orf224/atp6* transcripts.

A gene associated with *rfp1* acts to modify transcripts of two additional mitochondrial gene regions: To search for effects of the *Rfp1* gene on transcripts of mtDNA regions other than the *pol orf224/atp6* locus, we probed floral mtRNA preparations with the set of cloned *B. campestris* mtDNA fragments shown in Table 1. The cloned DNA fragments cover all but ~28.5 kb of the Brassica mitochondrial genome and represent just under 90% of the master circle form of the molecule. We initially confined our analysis to Westar (*nap*), a fertile maintainer line, Westar (*pol*), an isogenic CMS line, and Westar-Rf (*pol*), a fertile restorer line that is homozygous for *Rfp1* but is otherwise nearly isogenic to Westar (*pol*) (SINGH and BROWN 1991). Only the *atp6*-containing clone detected transcript differences between the sterile Westar (*pol*) and fertile Westar (*nap*) lines. This, together with the findings indicating the modifications of *orf224/atp6* transcripts observed in restored plants are conditioned by the restorer gene *per se*, strengthens the view that this gene region is associated with the *pol* CMS trait. It should be noted, however, that some of the probes used in the transcript analysis were large, and in some cases these detected complex mixtures of both high and lower abundance transcripts. Some differences in low abundance transcripts between restored and CMS plants could have thereby been obscured.

Although no transcripts other than those of the *orf224/atp6* region were found to differ between the Westar (*pol*) and Westar (*nap*) lines, three clones, BC-10, BC-31 and BC-32, which contain 2.0-, 2.9- and 12.4-kb *Pst*I fragments, respectively, each detected a transcript that was absent in Westar-Rf (*pol*) but present in the other two lines. Because BC-31 and BC-32 are adjacent to one another on the *B. campetstris* mtDNA restriction map (PALMER and SHIELDS 1984) and detected similar transcript patterns, it seemed likely that the transcript differences detected by these clones originated from the same region. The transcripts missing in the restorer line that were detected by clones BC-10 and BC-32 were ~1.3 and 1.6 kb, respectively (Figure 2). Both clones hybridized to several transcripts, and, since the transcript sizes were different in each case, the two clones apparently detected different transcribed sequences.

Because Westar (*pol*) and Westar-Rf (*pol*) have the same cytoplasm, the transcript differences detected by these clones reflect nuclear genetic influences rather than differences in mitochondrial genotype. Because the nuclear genomes of these plants are nearly isogenic, the transcript differences are likely to result from the influence of genes at, or tightly linked to, the *Rfp1* locus and not from unlinked nuclear genes. It is unlikely that the transcript differences are related to *pol* CMS because the transcripts of the two regions that detect the differences are identical in both the sterile Westar (*pol*) and the fertile Westar (*nap*) maintainer line. Moreover, all the CMS-associated regions identified thus far are organized differently between the sterile and fertile mitochondrial genomes, and the regions represented by clones BC-10, BC-31 and BC-32 are organized identically in the *pol*, *nap* and *cam* mtDNAs (PALMER and HERBON 1988; L'HOMME and BROWN 1993).

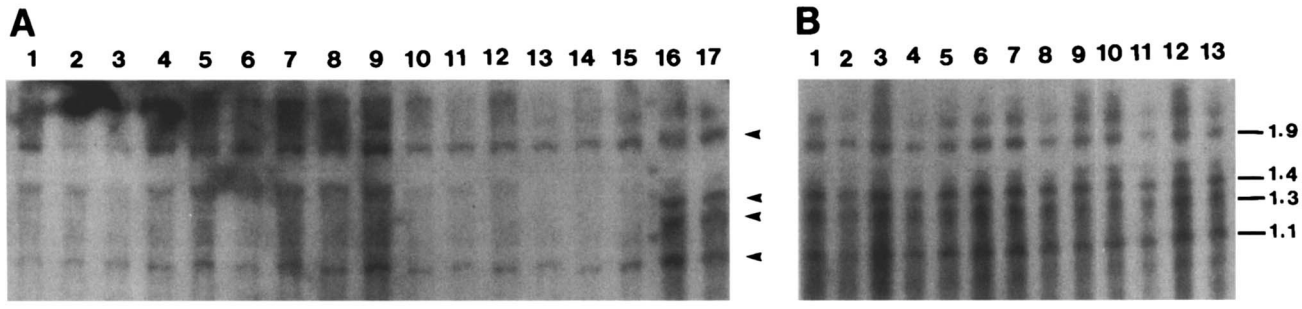

FIGURE 1.—RNA gel blot analysis of *orf224/atp6* transcripts in a *B. napus* (*pol*) backcross population segregating for the *Rfp1* restorer gene. MtRNA samples were isolated from individual progeny resulting from the cross of Karat (*pol*) by a Karat (*pol*) X Westar-Rf F_1 plant. These were subjected to electrophoresis on agarose-urea gels, blotted and probed with the Brassica *atp6* gene. (A) Lanes 1–15, male-sterile plants; lanes 16–17, male-fertile plants. (B) Lanes 1–13, male-fertile plants. Arrows indicate the location of the 1.1-, 1.3-, 1.4- and 1.9-kb transcripts.

To further analyze the nuclear influence on mitochondrial transcripts and its possible relationship to *pol* CMS, we probed gel blots of mtRNA from additional *B. napus* genotypes with BC-10 and BC-32 DNA segments (Figure 3). Fertile Karat (*nap*) and CMS Karat (*pol*) plants both expressed the 1.3-kb transcript seen in Westar (*nap*) and Westar (*pol*) but not in Westar-Rf (*pol*) plants (Figure 3A, lanes 1–3). We have shown previously

TABLE 1

***B. campestris* mtDNA probes used in transcript analysis**

Clone	Fragment	Genes, other features	Reference
BC-1	4.8-kb *Pst*I	*cox*II, recombination repeat	STERN and PALMER (1984) PALMER and SHIELDS (1984)
BC-3	7.7-kb *Pst*I		
BC-5	10.1-kb *Sal*I	*orfB*	BONHOMME *et al.* (1992) L'HOMME and BROWN (1993)
BC-9	10.2-kb *Pst*I		
BC-10	2.0-kb *Pst*I	*ccl1*-like sequence	This publication
BC-11	9.7-kb *Pst*I	*atp6*	MAKAROFF and PALMER (1987)
BC-12	7.5-kb *Pst*I	*coxI*	MAKAROFF and PALMER (1987)
BC-13	2.9-kb *Pst*I		
BC-14	1.3-kb *Pst*I		
BC-15	0.9-kb *Pst*I		
BC-17	5.7-kb *Pst*I	*rps3*	YE *et al.* (1993)
BC-21	21.1-kb *Pst*I	*rps3/rpl16, rpl5, rps14, cob, coxII, coxIII,* recombination repeat	YE *et al.* (1993) STERN and PALMER (1984) PALMER and SHIELDS (1984)
BC-22	3.6-kb *Pst*I		
BC-25	12.2-kb *Pst*I	*atp9, nad1*b,c	MAKAROFF and PALMER (1987)
BC-26	5.2-kb *Pst*I	*nad3/rps12*	L'HOMME and BROWN (1993)
BC-27	4.4-kb *Pst*I		
BC-28	10.1-kb *Pst*I	*nad1a*	L'HOMME and BROWN (1993)
BC-30	5.7-kb *Pst*I		
BC-31	3.1-kb *Pst*I	*nad6*	NUGENT and PALMER (1993)
BC-32	12.4-kb *Pst*I	*nad4*c,d	GASS *et al.* (1992), this publication
BC-33	2.9-kb *Pst*I	*nad4*b	GASS *et al.* (1992), this publication
BC-34	10.1-kb *Pst*I	*nad4*a	GASS *et al.* (1992), this publication
BC-36	5.0-kb *Pst*I	*atpA*	MAKAROFF and PALMER (1987)
BC-37	8.3-kb *Sal*I	*atpA*	MAKAROFF and PALMER (1987)
BC-38	6.2-kb *Sal*I		
BC-40	4.8-kb + 1.3-kb *Sal*I	*nad1*d,e, *matR*	L'HOMME and BROWN (1993)
BC-41	4.0-kb *Sal*I		
BC-51	8.8-kb *Kpn*I	*rrn5, rrn18*	L'HOMME and BROWN (1993)
BC-52	5.7-kb + 1.5-kb *Kpn*I		

The assignment of genes to cloned *B. campestris* mtDNA fragments is based on their hybridization to heterologous probes (STERN and PALMER 1984; MAKAROFF and PALMER 1987; L'HOMME and BROWN 1993) and/or on published sequence information, as cited.

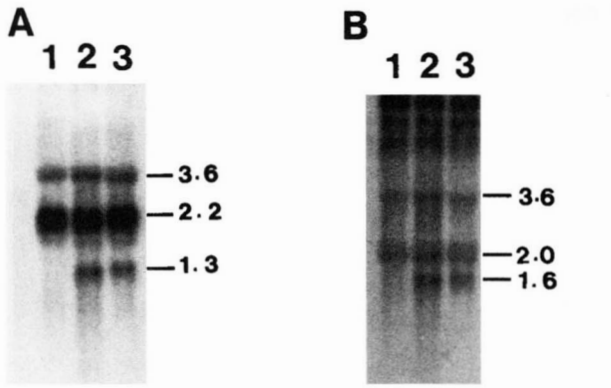

FIGURE 2.—Mitochondrial transcript analysis of near isogenic *B. napus* lines. Hybridization with 2.0-kb *Pst*I *B. campestris* clone BC-10 (A) and 12.4-kb *Pst*I *B. campestris* clone BC-32 (B). Lanes 1, Westar-*Rf* (*pol*) (restored to fertility); lanes 2, Westar (*pol*) (male-sterile); lanes 3, Westar (*nap*) (male-fertile). Molecular sizes are in kb.

that the *pol* restorer genes *Rfp1* and *Rfp2,* which originate in the cultivars Italy and UM2353, respectively, and are thought to reside at distinct chromosomal loci (FANG and MCVETTY 1989), have identical effects on *orf224/atp6* transcripts (SINGH and BROWN 1991). As shown in Figure 3A (lanes 6 and 7), the transcripts detected by clone BC-10 in the two lines are also identical, with neither line expressing the 1.3-kb RNA. The effects of nuclear genotype on the presence or absence of the 1.6-kb transcript detected by clone BC-32 were identical to those on the BC-10 1.3-kb transcript. BC-32 hybridizes to a 1.6-kb transcript in Karat (*nap*) and Karat (*pol*) plants (Figure 3B, lanes 1 and 2).

The *Rfp1* restorer gene is dominant to the maintainer *rfp1* allele with respect to the suppression of *pol* cytoplasm-induced male sterility. Since *orf224/atp6* transcripts of F_1 hybrids heterozygous for the restorer gene are identical to those of restorer lines and different

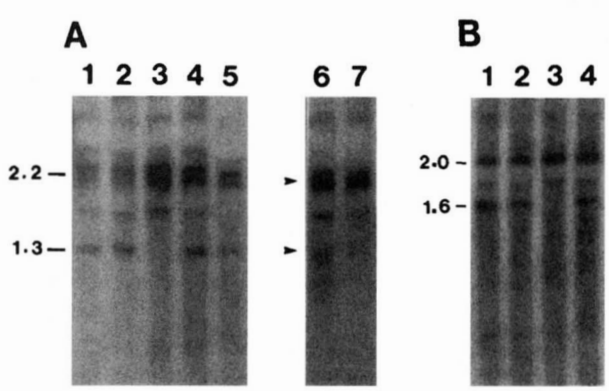

FIGURE 3.—Nuclear genotype effect on mitochondrial transcripts. Hybridization with 2.0 kb *Pst*I *B. campestris* clone BC-10 (A) and 12.4-kb *Pst*I *B. campestris* clone BC-32 (B). Lanes 1, Karat (*nap, rfp1/rfp1*); lanes 2, Karat (*pol, rfp1/rfp1*); lanes 3, Westar-Rf (*pol, Rfp1/Rfp1*); lanes 4, Karat (*pol*) × Westar-Rf (*pol*); lane 5, Karat (*nap*) × Westar-Rf (*pol*); lane 6, Italy (*pol, Rfp1/Rfp1*); lane 7, UM2353 (*pol, Rfp2/Rfp2*). Molecular sizes are in kb.

from those of CMS lines, *Rfp1* is also dominant to *rfp1* with respect to its effect on transcripts of the CMS-associated *orf224/atp6* region (SINGH and BROWN 1991). The finding that the male-fertile *Rfp1/rfp1* heterozygotes, obtained by crossing Karat (*pol*) CMS or fertile Karat (*nap*) plants (both *rfp1/rfp1*) with Westar-Rf plants, expressed the 1.3-kb BC-10 transcript at levels similar to those of the Karat (*pol*) or Karat (*nap*) plants (Figure 3A, lanes 4 and 5) was therefore unexpected. Similarly, the additional 1.6-kb BC-32 transcript that is absent in *Rfp1* homozygotes (Figure 3B, lane 3) was found to be expressed in *Rfp1/rfp1* heterozygotes (Figure 3B, lane 4) at levels comparable to those of *rfp1* homozygotes. Thus genotypes such as Westar and Karat that maintain the *pol* CMS and hence are homozyogous for the *rfp1* gene appear to possess a gene that conditions the appearance of additional transcripts detected by clones BC-10 and BC-32; this gene is dominant to the corresponding allele of restoring genotypes (Table 2).

A nuclear-endcoded mitochondrial transcript modifier is linked to the *rfp1* maintainer allele of *Rfp1*: Analysis of the transcripts of the *Rfp1/rfp1* heterozygotes and the near isonuclear lines Westar (*pol*) and Westar-Rf suggested that the dominant gene that conditions the appearance of the additional BC-10 and BC-32 transcripts in genotypes that maintain the *pol* CMS is identical with, or tightly linked to, the maintainer *rfp1* allele of the *Rfp1* locus. This gene would therefore be expected to cosegregate with *rfp1* in crosses. If a dominant gene tightly linked to or identical with *rfp1* conditions the appearance of these transcripts, only plants homozygous for *Rfp1* will lack them. In the previously analyzed BC_1 cross, there were no *Rfp1* homozygotes and hence the character did not segregate in this population. To determine if the gene that controls the presence of the additional transcripts cosegregates with *rfp1,* it was therefore necessary to produce a genetic population that contained *Rfp1* homozgygotes. Such plants are expected to occur in the F_2 population (cross 1, Table 3) derived from self-pollination of the F_1 heterozygote (*Rfp1/rfp1*) of the cross Karat (*pol*) × Westar-Rf, the male parent of the BC_1 population described above.

Nine of the 39 plants of this F_2 population that were raised to maturity were male-sterile, consistent with the 1:3 ratio expected for a character controlled by a single Mendelian locus ($\chi^2 = 0.08$). If the dominant gene controlling the appearance of the additional transcript is identical with or tightly linked to *rfp1,* then all the sterile F_2 plants (*rfp1/rfp1*) and the approximately two-thirds of the fertile plants heterozygous for the restorer would be expected to express the additional transcript, while the transcript should be missing in the approximately one-third of the fertile plants homozygous for the restorer. Floral mtRNA was prepared from the individual F_2 plants and analyzed using the BC-10 and BC-32 probes. As indicated in Table 3, 100% (9/9) of the

TABLE 2

Effects of nuclear genotype on transcripts of three mitochondrial gene regions

	Cytoplasm	Restorer genotype	Phenotype	1.4- and 1.3-kb *orf224/atp6* transcripts	1.3-kb BC-10 transcript	1.6-kb BC-32 transcript
Westar (*nap*)	*nap*	*rfp1/rfp1*	Fertile	−	+	+
Westar (*pol*)	*pol*	*rfp1/rfp1*	Sterile	−	+	+
Westar-Rf	*pol*	*Rfp1/Rfp1*	Fertile	+	−	−
Karat (*nap*)	*nap*	*rfp1/rfp1*	Fertile	−	+	+
Karat (*pol*)	*pol*	*rfp1/rfp1*	Sterile	−	+	+
Italy	*pol*	*Rfp1/Rfp1*	Fertile	+	−	−
UM2353	*pol*	*Rfp2/Rfp2*	Fertile	+	−	−
Karat (*pol*) × Westar-Rf	*pol*	*Rfp1/rfp1*	Fertile	+	+	+
Westar (*pol*) × Westar-Rf	*pol*	*Rfp1/rfp1*	Fertile	+	+	+

sterile plants and 67.7% (21/30) of fertile plants possessed the additional BC-10 transcript, while nine of the fertile plants lacked the transcript. Analysis of the same mtRNA preparations with BC-32 showed that those individuals that lacked the 1.3-kb BC-10 transcript also lacked the 1.6-kb BC-32 transcript, while those that possessed the additional BC-10 transcript possessed the additional BC-32 transcript.

Analysis of this F_2 population strongly supported the hypothesis that a single gene controls the profile of transcripts detected by clones BC-10 and BC-32. Moreover, the perfect cosegregation of the additional BC-10 transcript with the additional BC-32 transcript in F_2 individuals further indicated that the same nuclear locus governs the expression of the two different mitochondrial gene regions. We subsequently refer to this locus as *Mmt* (for modifier of mitochondrial transcripts), by analogy to the *Mct* (modifier of *cox2* transcripts) gene of maize (COOPER *et al.* 1990). The finding that all sterile plants possessed the additional transcript detected by each clone is also consistent with the hypothesis that the gene governing their production (*Mmt*) is, or is linked to, *rfp1*. In this case, however, the number of sterile plants analyzed was not large enough to allow us to rule out the alternative hypothesis that a gene unlinked to *rfp1* is responsible for the appearance of the extra smaller transcripts of each mitochondrial gene region.

Two additional experiments were performed to obtain evidence for linkage between *rfp1* and the transcript modifier gene. The first of these was based on the premise that if *rfp1* and *Mmt* are linked, then we should also observe linkage between the recessive form, *mmt*, and the restorer gene, *Rfp1*. If *Rfp1* and *mmt* map to the same locus, all *mmt/mmt* individuals in an F_2 population (those that lack the smallest transcripts) should also be homozygous for *Rfp1* and give only male-fertile F_3 progeny upon self-pollination, whereas plants heterozygous at the *Mmt* locus should also be heterozygous for the restorer and should produce a mixture of fertile and sterile progeny upon selfing. To test this prediction, 43 F_3 individuals derived from self-pollination of six F_2 fertile plants lacking the smallest transcripts (*mmt/mmt* individuals) were raised to maturity, and all proved to be male-fertile, consistent with this expectation. By contrast, each of the six F_3 families derived from selfing fertile plants expressing the smallest transcripts segregated for male sterility.

To further test for linkage between *rfp1* and *Mmt*, we analyzed a larger number of sterile F_2 plants, in this case derived by selfing an *Rfp1/rfp1* heterozygote formed by crossing Westar (*pol*) by Westar-Rf (cross 2, Table 3). As in the case of crosses 1 and 2, the ratio of sterile to fertile (2.95) progeny was consistent with that expected of fertility restoration conditioned by a single dominant gene ($\chi^2 = 0.00$). All of the 20 sterile progeny expressed the transcript specific to plants possessing the *Mmt* gene. This result strongly (0.01 signifi-

TABLE 3

Segregation of male sterility and mitochondrial transcript patterns in F_2 generations

	Fertility Segregation			Plants with additional transcripts		
Cross	Fertile	Sterile	Proposed ratio	Fertile (%)	Sterile (%)	χ^2, assuming absence of linkage[a]
1. Karat (*pol*) × Westar-Rf ×	30	9	3:1 ($\chi^2 = 0.08$)	67.7	100	4.11^{ns}
2. Westar (*pol*) × Westar-Rf ×	59	20	3:1 ($\chi^2 = 0.00$)	ND	100	6.67**

[a] Values for closeness of fit to expected numbers, assuming complete absence of linkage between *rfp1* and a dominant gene that conditions the appearance of the additional transcripts; ns and **, respectively, indicate not significantly ($P = 0.05$, d.f. = 3) or significantly ($P = 0.01$, d.f. = 3) different from values expected if *Mmt* and *rfp1* are unlinked. ND, not determined.

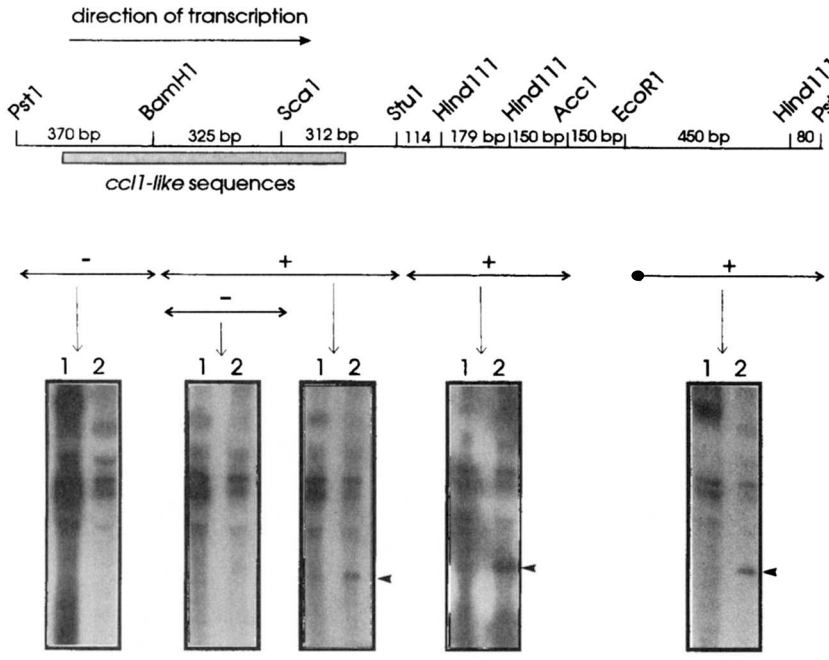

FIGURE 4.—Mitochondrial transcript analysis of near isogenic *B. napus* lines hybridizing to restriction fragments derived from clone BC10. Probes that detect the 1.3-kb transcript are marked with +; those that do not are marked with −. Sequences homologous to part of a gene involved in cytochrome *c* biogenesis are indicated by a shaded box. Lanes 1, Westar-*Rf (pol)* (restored to fertility); lanes 2, Westar *(pol)* (male-sterile).

cance level) supports the hypothesis that *Mmt* and *rfp1* are linked and is consistent with the possibility that they are the same gene.

***Mmt* is associated with modification of transcripts of a *ccl1*-like gene and *nad4*:** To more precisely characterize the transcript modifications conditioned by *Mmt*, we mapped the transcripts to specific regions of the three clones and determined from which genes they are derived. Restriction fragments from clones BC-10 and BC-32 were isolated, subcloned and used to probe gel blots of mtRNA isolated from Westar (*pol*) and Westar-Rf (*pol*). Fragments derived from a 0.7-kb region located at one end of clone BC-10 hybridized to the larger transcripts detected by the entire clone but not to the 1.3-kb *Mmt*-specific transcript (Figure 4). In contrast, fragments derived from the remaining portion of the clone hybridized to both the larger transcripts and the 1.3-kb transcript. This suggested that the 1.3-kb transcript was specifically derived from the larger of the clone's two *Bam*HI/*Pst*I fragments (Figure 4). DNA sequence analysis indicated that a 621-bp region of the clone surrounding the *Bam*HI site showed a high degree of similarity with sequences of carrot, wheat and Oenothera mtDNAs that encode a protein homologous to the product of a bacterial gene (*ccl1*) thought to be involved with cytochrome *c* biogenesis (BECKMAN *et al.* 1992; GONZALEZ *et al.* 1993; SCHUSTER *et al.* 1993). No open reading frame was found in the sequences of the downstream portion of the clone, and no other marked similarity with sequences available in the databases was found. The sequence of the *ccl1*-like region and studies related to its functional significance will be published separately; the entire sequence of clone BC-10 is available through Genbank (accession U40043). Analysis of the transcripts using strand-specific RNA probes indicated that all of the detected transcripts corresponded to the sense strand of the *ccl1*-like sequences. These observations suggest that transcripts detected by clone BC-10 are derived from the *ccl1*-like sequences, that these transcripts extend ≥1300 nucleotides beyond the termination codon of this putative gene, and that the 1.3-kb *Mmt*-specific transcript is derived largely from noncoding downstream sequences and hence cannot be translated to generate a functional gene product.

Similar analyses were performed on clones BC-31 and BC-32. A strong hybridization signal with mtRNA was obtained using a 4.5-kb *Pst*I/*Sna*BI fragment derived from the end of BC-32 that is adjacent to BC-31. This fragment hybridized to all the transcripts detected by the entire BC-32 clone, while fragments derived from other portions of the clone hybridized only poorly. Partial sequence analysis of this 4.5-kb fragment showed that it contained exons three and four, the adjoining introns and the downstream untranslated region of the *nad4* gene (GASS *et al.* 1992). Previous studies have shown that *nad4* exons one and two are located on the adjacent 10.1- and 2.9-kb mtDNA *Pst*I fragments, respectively (GASS *et al.* 1992). The 2.9-kb fragment corresponds to clone BC-31. An adjacent *Eco*RI/*Pst*I fragment spanning exon one that was derived from the 10.1-kb *Pst*I fragment hybridized to the full array of transcripts detected by clones BC-31 and BC-32. Oligonucleotide E42, which corresponds to a 20-bp sequence beginning within the *nad4* termination codon, also hybridized to all the transcripts detected by BC-31 and BC-32, whereas the oligonucleotide E43, corresponding to a sequence 135 bp further downstream, did not detect any discrete transcripts (Figure 5). This suggests that the 3′ termini of all the *nad4* transcripts map to the 135-bp interval between E42 and E43. An oligonu-

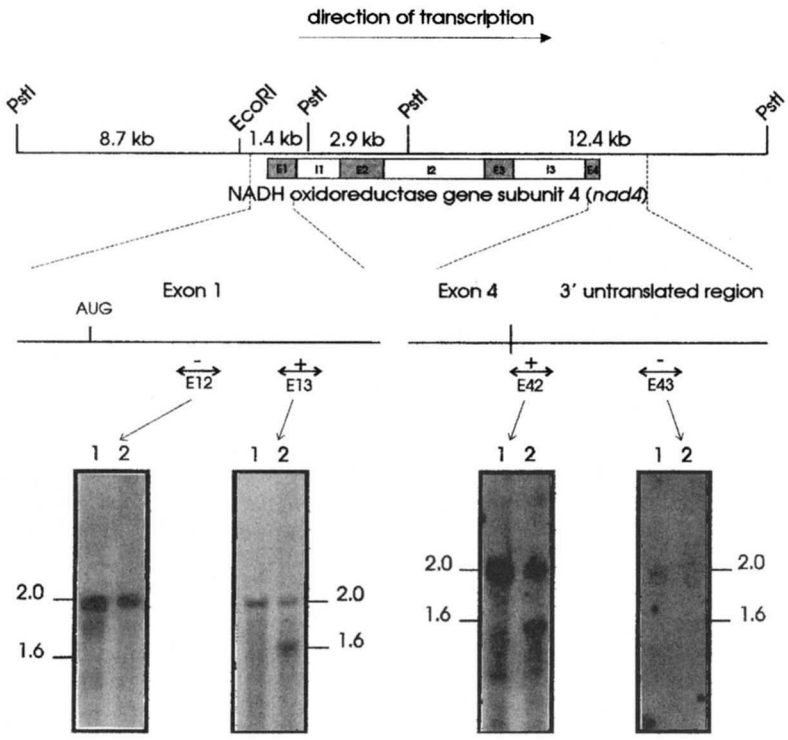

FIGURE 5.—Mitochondrial transcript analysis of near isogenic *B. napus* lines using oligonucleotides corresponding to portions of the *B. campestris nad4* mitochondrial gene. Probes that detect the 1.6-kb transcript are marked with +, while those that do not are marked with −. Exon sequences are represented by shaded boxes, and intron sequences by open boxes. Lanes 1, Westar-Rf (*pol*) (restored to fertility); lanes 2, Westar (*pol*) (male-sterile).

cleotide probe corresponding to a sequence in exon one, 329–359 nucleotides downstream of the initiation codon (E13, Figure 5), hybridized to all the BC-32 transcripts, but an oligonucleotide corresponding to bases 96–116 of the coding sequence (E12, Figure 5) hybridized only to the larger transcripts and not to the 1.6-kb *Mmt*-specific transcript. This indicated that the 5′ terminus of the *Mmt*-specific transcript mapped within exon one between bases 116 and 329. Thus transcripts derived from both mitochondrial gene loci that are specific to Mmt plants (plants possessing the *Mmt* allele), like *orf224/atp6* transcripts specific to plants with the *Rfp1* gene, cannot be translated to generate a normal full-length gene product. There are, however, four in-frame ATG codons in the remainder of exon one that, if used to initiate translation, would yield a truncated, presumably nonfunctional, gene product.

Of the three *nad4* introns, only intron 1 detects an abundant transcript in Northern blot hybridizations (GASS *et al.* 1992). Intron 1 sequences detect a 3.6-kb transcript that is also detected by probes derived from each of the four exons, suggesting that intron 1 is the last of the *nad4* introns to be removed during splicing. The *nad4* transcript mapping experiments suggest that the 1.6-kb transcript specific to Mmt plants lacks sequence of ~400 nucleotides present at the 5′ end of the 2.0-kb transcript found in both Mmt and *mmt/mmt* plants. If the action of the *Mmt* gene product on *nad4* transcripts precedes the removal of intron 1, we would expect to find a transcript of ~3.2 kb in Mmt plants. Such a transcript is not detected, suggesting that the *Mmt* gene product acts postranscriptionally, at a processing step occuring subsequent to the last splicing event.

Sequences surrounding *Mmt*-specific transcript termini: Because a single gene, *Mmt,* appears to be responsible for the appearance of additional transcripts of both the *ccl1*-like sequences and *nad4,* we sought to identify structural elements held in common by these two genes and/or their derived transcripts that could potentially serve as recognition sites for the *Mmt* gene product. Precise mapping of the 5′ termini of the *Mmt*-specific transcripts of each of the two genes within the regions identified from the Northern analyses was accomplished by primer extension. As shown in Figure 6, when *nad4* exon 1 oligonucleotide E13 (Figure 5) was used to prime reverse transcriptase reactions, two extension products, differing in size by approximately five nucleotides, were observed when Mmt mtRNA, but not when *mmt/mmt* mtRNA, was used as a template. These sites map to nucleotides located 194 and 199 bases downstream of the *nad4* initiation codon. Similarly, when the *ccl1*-like sequence oligonucleotide *ccl1*-31 was used as a primer, a single major primer extension product was observed using Mmt mtRNA that was not present when *mmt/mmt* mtRNA was used (Figure 6). This terminus is located 86 bp upstream of the putative termination codon for the gene.

Additional approaches were used to confirm the locations of the *Mmt*-specific transcript termini. In the case of *nad4,* we used PCR to generate a labeled, single-stranded DNA fragment extending from oligo E13 to the *Eco*RI site immediately 5′ to *nad4,* annealed this to mtRNA from Mmt and *mmt/mmt* plants and analyzed

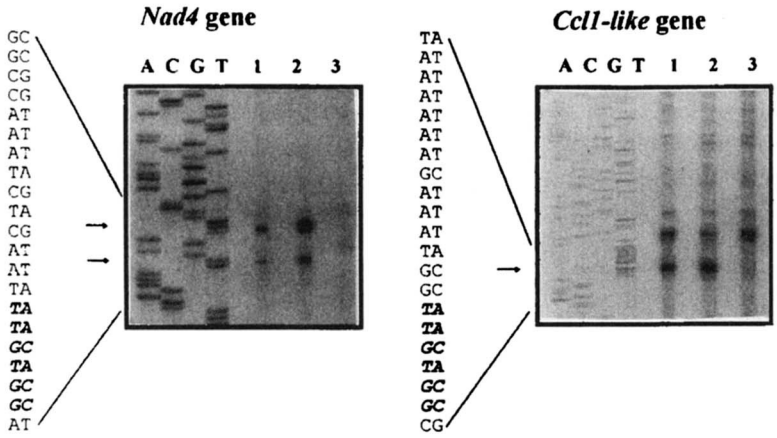

FIGURE 6.—Primer extension analysis of mitochondrial transcripts of near isogenic *B. napus* lines. Exon 1 sequences of the *nad4* gene were primed by the E13 oligonucleotide, while BC10 sequences were primed by the ccl1-31 oligonucleotide. Lanes 1, Westar (*nap*) (male-fertile); lanes 2, Westar (*pol*) (male-sterile); lanes 3, Westar-Rf (*pol*) (restored to fertility). The cDNA bands mapping to the 5′ ends of the *Mmt*-specific transcripts are indicated by arrows. The TTGTGG putative recognition motif is shown in bold. The alignment of the sequences surrounding the *Mmt*-specific *nad4* and *ccl1*-like transcript termini and of the *Rfp1*-specific *atp6*-4 transcript terminus (SINGH and BROWN 1993) is shown below with the putative recognition motifs in bold.

nad4 GGCCAAAATCTCAATTTGTGGAAAGCCTTC
ccl1-like TAAAAAAGAAATGGTTGTGGCGCGAAGTA
atp6-4 TAAATTTTTTTTGTTTGTTGGGGTTGAAA

the labeled products formed following S1 nuclease digestion; the size of the *Mmt*-specific protected segments were precisely those predicted for termini mapping to the position determined by primer extension (data not shown). In the case of the *ccl1*-like sequence, oligonucleotides mapping immediately up and downstream of the site of the *Mmt*-specific terminus were used to probe gel blots mtRNA from Mmt and *mmt/mmt* plants. As expected, the downstream oligonucleotide detected the *Mmt*-specific transcript while the upstream oligonucleotide did not (data not shown).

Figure 6 shows a comparison of the sequences surrounding the *Mmt*-specific termini of the *nad4* and *ccl1*-like genes. Because *Mmt* may be identical to *rfp1*, we have also included the sequences surrounding *atp6*-4, the major *Rfp1*-specific transcript terminus mapping within the CMS-associated *orf224* gene. Comparison of the *Mmt*-specific termini of the *nad4* and *ccl1*-like transcripts shows that the same hexanucleotide sequence, TTGTGG, is positioned eight and two nucleotides downstream of the two termini mapping within *nad4* and six nucleotides downstream of the terminus mapping within the *ccl1*-like putative gene. Interestingly, five of the six nucleotides are present in a sequence positioned two nucleotides downstream of the *Rfp1*-specific *atp6*-4 transcript terminus (TTGTTG, SINGH and BROWN 1993).

DISCUSSION

While several studies have found that transcripts of mitochondrial CMS-associated genes differ in CMS- and fertility-restored plants (reviewed in HANSON 1991; HANSON and FOLKERTS 1992; BONEN and BROWN 1993), in this study we demonstrate these transcript differences are specifically associated with the restorer gene by showing that the differences cosegregate with the restorer in genetic crosses. COOPER *et al.* (1990) have similarly used genetic segregation to show that the profile of *Zea diploperennis* and *Z. perennis* mitochondrial *cox2* transcripts in certain *Z. mays* nuclear genotypes is controlled by a single nuclear gene, *Mct*. The effects of *Mct* resemble those of *Mmt* in that the dominant allele conditions the appearance of additional shorter transcripts.

In addition to *Mmt* and *Mct*, other examples of plant nuclear genes that are not restorers of fertility and that influence size and number of mitochondrial transcripts have been described (KENNELL *et al.* 1987; MAKAROFF *et al.* 1990). Two features distinguish *Mmt* from these genes and *Mct*. First, *Mmt* affects transcripts of two different mitochondrial gene regions; in other cases, modifications of transcripts of only a single mitochondrial genetic locus have been reported. Second, we have been unable to separate *Mmt*, through genetic recombination, with the alternate, maintainer *rfp1* allele of the nuclear restorer gene *Rfp1*. Thus, it seems possible that *Mmt* is simply the alternate allele of *Rfp1*, with *Mmt (rfp1)* influencing *nad4* and *ccl1*-like transcripts, and *Rfp1(mmt)* influencing *orf224/atp6* transcripts. In the cases of *Mct* and other similar genes, linkage to a restorer locus has not been observed. In fact, we know of no other examples in which two tightly linked genes influence transcripts of different mitochondrial genes or in which both alleles of a nuclear locus exert distinct effects on mitochondrial transcript expression.

Two complementary pieces of evidence support the view that *Mmt* maps very close to, or is identical with, *rfp1*. First, the transcript differences conditioned by *Mmt* are observed between the near isogenic lines Westar and Westar-Rf. Westar-Rf is the product of an introgression breeding program aimed at introducing

the *Rfp1* gene from the cultivar Italy (*Rfp1, mmt/Rfp1, mmt*) through six backcross generations into the cultivar Westar (*rfp1, Mmt/rfp1, Mmt*). Westar and Westar-Rf are therefore expected to be isogenic at nearly all chromosomal loci except for those in the immediate vicinity of *Rfp1,* where Westar-Rf will resemble the donor of the *Rfp1* gene, Italy. As Westar-Rf and Italy are both homozygous for *mmt,* the analysis of near isogenic lines alone makes it likely that this gene lies close to, or is identical with *Rfp1.* Since Westar-Rf and Westar may still differ at loci unlinked to *Rfp1,* the analysis of near isogenic lines alone does not unambiguously demonstrate linkage between *Rfp1* and *mmt.* Since we also find that *mmt* and *Rfp1* cosegregate in genetic crosses, however, it is extremely likely that the genes are linked.

While our results are consistent with the possibility that *Mmt* is identical with *rfp1,* we cannot rule out the alternative hypothesis that the two genes are simply tightly linked. Indeed, the modifications of transcripts of the *ccl1*-like and *nad4* genes influenced by the *Mmt* locus could also conceivably result from the actions of two or more tightly linked but distinct genes, one affecting transcripts of the *ccl1*-like sequence, the other the *nad4* transcripts. Recently, we have found, through nuclear DNA polymorphism analysis of the Westar, Westar-Rf and Italy lines, that a chromosomal region of ~18–24 cM surrounding *Rfp1* has been retained from Italy during the introgression procedure used for the construction of Westar-Rf (M. JEAN, G. BROWN and B. LANDRY, unpublished results). Thus if *Rfp1* and *mmt* are distinct genes, they reside no more than 24 cM apart on the same chromosome. Similarly, *rfp1* and *Mmt* must be confined to the corresponding interval on the chromosome of the complementary haplotype. If *Rfp1* and *mmt* are different genes, it is possible that *Rfp1/Mmt* is a complex genetic locus, resembling several recently described plant disease resistance loci (DAGL 1995). In these cases, distinct genes conferring resistance to different pathogens have been found clustered in the same chromosomal region. By analogy, *Mmt* and *Rfp1* may be components of a nuclear gene cluster involved in the regulation of expression of different mitochondrial genes.

While CMS appears to result from mtDNA rearrangements that give rise to novel mitochondrial genes, the evolutionary origin of restorer genes is unclear. Restorer genes are specific for particular CMS cytoplasms and many act by modifying the expression of novel CMS-associated genes (HANSON 1991; HANSON and FOLKERTS 1992; BONEN and BROWN 1993). For example, the maize restorer gene *Rf1* specifically restores the *cms-T* cytoplasm by altering expression of the CMS-associated T-*urf13* gene; *Rf1* does not restore male sterility induced by the maize male-sterile cytoplasms S and C. Regardless of whether *Rfp1* and *Mmt* are alleles of the same gene or represent haplotype-specific forms of a more complex locus, our findings suggest they encode related products that affect the expression of, in one case, a CMS-associated gene and, in the other case, two normal mitochondrial genes. This in turn suggests that restorer genes such as *Rfp1* may originate as variant forms of genes involved with the modification of the transcripts of normal mitochondrial genes.

We have previously suggested that the *Rfp1*-controlled transcript modifications may be achieved postranscriptionally, through RNA processing events, because the sequences at which the *Rfp1*-specific transcript termini map do not resemble motifs characteristic of plant mitochondrial promoter sequences (SINGH and BROWN 1993). NEWTON *et al.* (1995), however, have recently reported that the maize *Mct* gene acts by allowing transcription to be initiated at a novel site, downstream of other, non-*Mct* dependent *Z. diploperennis cox2* transcription initiation sites. This novel site does not possess the minimal sequence required for accurate mitochondrial transcription initiation *in vitro* (RAPP *et al.* 1993), suggesting that the *Mct* gene product allows the utilization of an alternative, possibly gene-specific promoter. While it is conceivable that *Rfp1* and *Mmt* act in a similar manner, the *in vitro* capping analysis of *orf224/atp6* transcripts supports the view that *Rfp1* acts to affect transcript processing rather than initiation. The 5′ termini of transcripts mapping upstream of the *orf224* open reading frame can be labeled using guanylyltranferase, suggesting that they originate from transcript initiation, while the labeling of termini of the *Rfp1*-specific transcripts, which map within *orf224,* cannot be detected (R. MENASSA, unpublished results). Moreover, the absence of a 3.2-kb *Mmt*-specific *nad4* transcript suggests that the *Mmt* gene product also acts posttranscriptionally. Since both *Rfp1* and *Mmt* map to the same chromosomal locus, both probably act through similar biochemical mechanisms. It seems likely, therefore, that *Rfp1* and *Mmt,* like the maize *Rf1* restorer gene (KENNELL and PRING 1989), act to affect RNA processing rather than initiation events. Further analysis of this issue may be warranted, however, as comparison of the relative levels of the different *nad4* and *ccl1*-like transcripts in plants possessing and lacking the *Mmt* gene has not allowed us to identify possible precursors for the *Mmt*-specific transcripts.

If *Mmt* and *Rfp1* do act by affecting RNA processing, the biochemical mechanisms involved in their action may be complex, as the products of both genes would allow selective destabilization of the 5′ ends of longer transcripts, while allowing the 3′ ends of these transcripts to remain intact, similar to the action of the sunflower nuclear restorer gene on transcripts of CMS-associated *orf522* gene region (MONÉGER *et al.* 1994). The presence of the same sequence near the 5′ termini of both the *Mmt*-specific transcripts suggests that a key aspect of this mechanism may be the utilization of UUGUGG sequences as a component of a recognition site for processing/stabilization of transcripts of the *ccl1*-

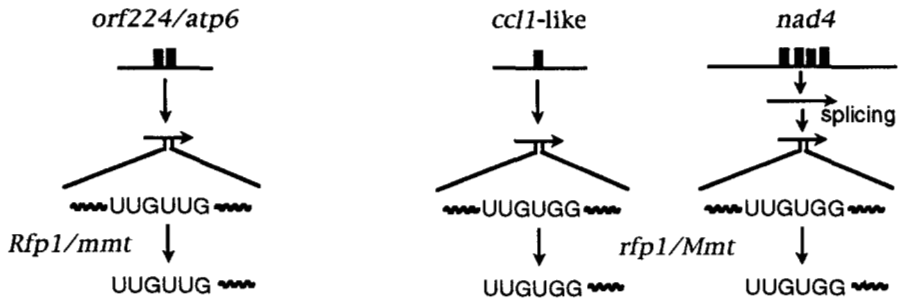

FIGURE 7.—Model for the reciprocal actions of the *Mmt* and *Rfp1* genes on different mitochondrial transcripts. The *ccl1*-like sequence and the *orf224/atp6* genes and individual exons of the *nad4* gene are indicated by closed boxes. Horizontal arrows above the line indicate the locations of the 2-kb repeat, while arrows below the line indicate the direction of transcription. Wavy lines indicate transcripts and processing events are indicated by the vertical arrows. According to the model, the *rfp1/Mmt* nuclear genotype encodes a product that conditions specific processing of *ccl1*-like and *nad4* transcripts at UUGUGG sequences, while the reciprocal *Rfp1/mmt* nuclear genotype encodes a product that conditions specfic processing of *orf224/atp6* transcripts at the UUGUUG sequence.

like sequence and the *nad4* gene. Similarly, UUGUUG may function in the recognition motif for the specific processing of *orf224/atp6* transcripts conditioned by *Rfp1*. In both cases, it seems unlikely that the hexanucleotide sequences alone are sufficient to account for the observed specificity of the transcript modifications. Conceivably, the function of the UUGU(U/G)G motifs may be analogous to that of a dodecamer sequence found at the 3′ termini of yeast mitochondrial transcripts, which is thought to serve as a site for processing of primary transcripts as well as for the binding of protein complex that protects transcripts from exonuclease action (HOFMANN *et al.* 1993; MIN and ZASSENHAUS 1993). The yeast and mammalian MRP RNases (KARWAN *et al.* 1991; STOHL and CLAYTON 1992) are additional examples of mitochondrial endoribonucleases with a high degree of cleavage site specificity conferred through the recognition of primary structural motifs.

A model for the complementary action of *Rfp1* on *orf224/atp6* transcripts and *Mmt* on *ccl1*-like and *nad4* transcripts is presented in Figure 7. The model assumes that *Rfp1* and *Mmt* act by influencing processing events but could be easily adapted to account for their effects through modification of transcription initiation sites. It is based on the view that *Rfp1 (mmt)* and *Mmt (rfp1)* are alternate alleles of the same locus. In the model, *Rfp1 (mmt)* encodes a factor that allows processing of *orf224/atp6* but not *ccl1*-like or *nad4* transcripts, while *Mmt (rfp1)* encodes a factor that allows processing of the the *ccl1*-like and *nad4* but not *orf224/atp6* transcripts. The complementary action of the different alleles could be accounted for if each allele recognizes a different processing motif, *e.g.*, if the UUGUGG sequence (as well as other recognition elements) served as a signal for *Mmt* processing while UUGUUG provided a similar recognition site for *Rfp1* processing. Plants homozygous for *Rfp1 (mmt)* would lack the *Mmt* allele and hence be unable to process transcripts of the the *ccl1*-like gene and *nad4*, while plants homozygous for *Mmt (rfp1)* would be unable to process *orf224/atp6* transcripts. Heterozygotes would, of course, be capable of processing both classes of transcripts.

In summary, we have shown here that a single nuclear chromosomal locus can exert an effect on the transcripts of at least three different mitochondrial genes with different alleles or haplotypes of the nuclear locus controlling different sets of mitochondrial transcripts. The cloning and molecular characterization of the *Mmt* and *Rfp1* genes will be critical for clarifying key features of this novel system of organelle gene regulation, such as the biochemical mechanisms through which *Mmt* and *Rfp1* act and the molecular structure of the *Mmt/Rfp1* locus.

We thank Dr. CHRISTOPHER MAKAROFF for the *B. campestris* mtDNA clones and Dr. SIEGFRIED HEKIMI for helpful comments on the manuscript. This work was supported by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada. M.S. was the recipient of a Government of India Fellowship, N.H. and M.J. the recipients of fellowships from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (Quebec) and M.J. the recipient of an NSERC Postgraduate Fellowship.

LITERATURE CITED

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS and D. J. LIPMAN, 1990 Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

BECKMAN, D. L., D. R. TRAWICK and R. G. KRANZ, 1992 Bacterial cytochromes *c* biogenesis. Genes Dev. **6:** 268–283.

BONEN, L., and G. G. BROWN, 1993 Genetic plasticity and its consequences: perspectives on gene organization and expression in plant mitochondria. Can. J. Bot. **71:** 645–660.

BONHOMME, S., F. BUDAR, D. LANCELIN, I. SMALL, M.-C. DEFRANCE *et al.*, 1992 Sequence and transcript analysis of the *Nco2.5* Ogura specific fragment correlated with cytoplasmic male sterility in *Brassica* cybrids. Mol. Gen. Genet. **235:** 340–348.

COOPER, P., and K. J. NEWTON, 1989 Maize nuclear background regulates the synthesis of a 22-kDa polypeptide in *Zea luxurians* mitochondria. Proc. Nat. Acad. Sci. USA **86:** 7423–7426.

COOPER, P., E. BUTLER and K. J. NEWTON, 1990 Identification of a maize nuclear gene which influences the size and number of *cox2* transcripts in mitochondria of perennial teosintes. Genetics **126:** 461–467.

COSTANZO, M. C., and T. D. FOX, 1990 Control of mitochondrial gene expression in *Saccharomyces cerevisiae*. Annu. Rev. Genet. **24:** 91–113.

DAGL, J. L., 1995 Pièce de résistance: novel classes of plant disease resistance genes. Cell **80:** 363–366.

FANG, G. H., and P. B. E. MCVETTY, 1989 Inheritance of male fertility restoration and allelism of restorer genes for the Polima cytoplasmic male sterility system in oilseed rape. Genome **32:** 1044–1047.

FINNEGAN, P. M., and G. G. BROWN, 1986 Autonomously replicating RNA in mitochondria of maize plants with S-type cytoplasm. Proc. Natl. Acad. Sci. USA **83:** 5175–5179.

GASS, D. A., C. A. MAKAROFF and J. D. PALMER, 1992 Variable intron content of the NADH dehydrogenase subunit 4 gene of plant mitochondria. Curr. Genet. **21:** 423–430.

GONZALEZ, D. H., G. BONNARD and J.-M. GRIENENBERGER, 1993 A gene involved in the biogenesis of cytochromes is co-transcribed with a ribosomal protein gene in wheat mitochondria. Curr. Genet. **24:** 248–255.

GRIVELL, L. A., 1995 Nucleo-mitochondrial interactions in mitochondrial gene expression. Crit. Rev. Biochem. Mol. Biol. **30:** 121–164.

HANDA, H., and K. NAKAJIMA, 1991 Different organization and altered transcription of the mitochondrial *atp6* gene in the male-sterile cytoplasm of rapeseed (*Brassica napus* L). Curr. Genet. **21:** 153–159.

HANSON, M. R., 1991 Plant mitochondrial mutations and cytoplasmic male sterility. Annu. Rev. Genet. **25:** 461–486.

HANSON, M. R., and O. FOLKERTS, 1992 Structure and function of the higher plant mitochondrial genome. Int. Rev. Cytol. **141:** 129–172.

HOFMANN, T. J., J. MIN and H. P. ZASSENHAUS, 1993 Formation of the 3′ end of yeast mitochondrial mRNAs occurs by site-specific cleavage two bases downstream of a conserved dodecamer sequence. Yeast **9:** 1319–1330.

KARWAN, R., J. L. BENNETT and D. A. CLAYTON, 1991 Nuclear RNase MRP processes RNA at multiple discrete sites: interaction with an upstream G box is required for subsequent downstream cleavages. Genes Dev. **5:** 1264–1276.

KENNELL, J. C., and D. R. PRING, 1989 Initiation and processing of *atp6*, T-*urf13* and ORF221 transcripts from mitochondria of T cytoplasm maize. Mol. Gen. Genet. **216:** 16–24.

KENNELL, J. C., R. P. WISE and D. R. PRING, 1987 Influence of nuclear background on transcription of a maize mitochondrial gene region associated with Texas male-sterile cytoplasm. Mol. Gen. Genet. **210:** 399–406.

L'HOMME, Y., and G. G. BROWN, 1993 Organizational differences between cytoplasmic male sterile and male fertile *Brassica* mitochondrial genomes are confined to a single transposed locus. Nucleic Acids Res. **21:** 1903–1909.

MAKAROFF, C. A., and J. D. PALMER, 1987 Extensive mitochondrial specific transcription of the Brassica campestris mitochondrial genome. Nucleic Acids Res. **15:** 5141–5156.

MAKAROFF, C. A., I. J. APEL and J. D. PALMER, 1990 Characterization of radish mitochondrial *atpA:* influence of nuclear background on transcription of *atpA*-associated sequences and relationship with male sterility. Plant Mol. Biol. **15:** 735–746.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

MIN, J., and H. P. ZASSENHAUS, 1993 Identification of a protein complex that binds to a dodecamer sequence found at the 3′ ends of yeast mitochondrial mRNAs. Mol. Cell. Biol. **13:** 4167–4173.

MONÉGER, F., C. J. SMART and C. J. LEAVER, 1994 Nuclear restoration of cytoplasmic male sterility in sunflower is associated with the tissue-specific regulation of a novel mitochondrial gene. EMBO J. **13:** 8–17.

NEWTON, K. J., B. WINBERG, K. YAMATO, S. LUPOLD and D. B. STERN, 1995 Evidence for a novel mitochondrial promoter preceding the *cox 2* gene of perennial teosintes. EMBO J. **14:** 585–593.

NUGENT, J. M., and J. D. PALMER, 1993 Characterization of the *Brassica campestris* mitochondrial gene for subunit six of NADH dehydrogenase: *nad6* is present in the mitochondrion of a wide range of flowering plants. Curr. Genet. **23:** 148–153.

PALMER, J. D., and L. A. HERBON, 1988 Plant mitochondrial DNA evolves rapidly in structure, but slowly in sequence. J. Mol. Evol. **28:** 87–97.

PALMER, J. D., and C. R. SHIELDS, 1984 Tripartite structure of the *Brassica campestris* mitochondrial genome. Nature **307:** 437–440.

RAPP, W. D., D. S. LUPOLD, S. MACK and D. B. STERN, 1993 Architecture of the maize mitochondrial *atp1* promoter as determined by linker-scanning and point mutagenesis. Mol. Cell. Biol. **13:** 7232–7238.

SCHUSTER, W., B. COMBETTES, K. FLIEGER and A. BRENNICKE, 1993 A plant mitochondrial gene encodes a protein involved in cytochrome *c* biogenesis. Mol. Gen. Genet. **239:** 49–57.

SINGH, M., and G. G. BROWN, 1991 Suppression of cytoplasmic male sterility by nuclear genes alters expression of a novel mitochondrial gene region. Plant Cell **3:** 1349–1362.

SINGH, M., and G. G. BROWN, 1993 Characterization of expression of a mitochondrial gene region associated with the *Brassica* "Polima" CMS: developmental influences. Curr. Genet. **24:** 316–322.

STERN D. B., and J. D. PALMER, 1984 Recombination sequences in plant mitochondrial genomes: diversity and homologies to known mitochondrial genes. Nucleic Acids Res. **15:** 6141–6157.

STOHL, L. L., and D. A. CLAYTON, 1992 *Saccharomyces cerevisiae* contains an RNase MRP that cleaves at a conserved mitochondrial RNA sequence implicated in replication priming. Mol. Cell. Biol. **12:** 2561–2569.

WANG, H. M., T. KETELA, W. A. KELLER, S. C. GLEDDIE and G. G. BROWN, 1995 Genetic correlation of the *orf224/atp6* gene region with Polima CMS in Brassica somatic hybrids. Plant Mol. Biol. **27:** 801–807.

WITT, U., S. HANSEN, M. ALBAUM and W. O. ABEL, 1991 Molecular analyses of the CMS-inducing 'Polima' cytoplasm in *Brassica napus* L. Curr. Genet. **19:** 323–327.

YE, F., J. BERNHARDT and W. O. ABEL, 1993 Genes for ribosomal proteins S3, L16, L5 and S14 are clustered in the mitochondrial genome of *Brassica napus* L. Curr. Genet. **24:** 323–329.

Communicating editor: M. HANSON