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Suppression of Cytoplasmic Male Sterility by Nuclear Genes Alters Expression of a Novel Mitochondrial Gene Region

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To identify regions of the mitochondrial genome that potentially could specify the "Polima" (*pol*) cytoplasmic male sterility (CMS) of *Brassica napus*, transcripts of 14 mitochondrial genes from *nap* (male fertile), *pol* (male sterile), and nuclear fertility-restored *pol* cytoplasm plants were analyzed. Transcriptional differences among these plants were detected only with the ATPase subunit 6 (*atp6*) gene. Structural analysis of the *atp6* gene regions of *pol* and *nap* mitochondrial DNAs showed that rearrangements in the *pol* mitochondrial genome occurring upstream of *atp6* have generated a chimeric 224-codon open reading frame, designated *orf224*, that is cotranscribed with *atp6*. In CMS plants, most transcripts of this region are dicistronic, comprising both *orf224* and *atp6* sequences. Nuclear restorer genes at either of two distinct loci appear to specifically alter this transcript pattern such that monocistronic *atp6* transcripts predominate. The differences in expression of this region appear to result, in part, from differential processing of a tRNA-like element comprising a tRNA pseudogene present immediately upstream of *atp6* in both the sterile and fertile mitochondrial DNAs. Possible mechanisms by which expression of the *orf224/atp6* locus and the Polima CMS trait may be specifically related are considered.

INTRODUCTION

Cytoplasmic male sterility (CMS) is a widespread trait of higher plants that is specified, in most cases, by the mitochondrial genome (Hanson et al., 1989; Levings, 1990; Braun et al., 1991). Although CMS is maternally inherited, in many cases specific nuclear genes, termed restorers of fertility (*Rf*), have been identified that can suppress the male sterile phenotype and restore fertility to F_1 hybrids. Although the regions of the mitochondrial genome that specify certain forms of CMS have been identified, the molecular basis of the trait is not precisely understood in any system.

Brassica napus, which is widely grown as the oilseed crop of rape or canola, offers several advantages as a system for the molecular analysis of CMS. The relatively simple organization of the mitochondrial genome in the *Brassica* and allied genera (Palmer and Shields, 1984) facilitates detailed analysis of structural differences between sterile and fertile mitochondrial DNAs (mtDNAs) (Makaroff and Palmer, 1988; Makaroff et al., 1989). In addition, the capability of producing Brassica somatic hybrids with recombinant mitochondrial genomes (Kemble and Barsby, 1988) potentially allows for direct genetic analysis of the cytoplasmic determinants of CMS. Because seed yield in *B. napus* hybrids may be enhanced by as much as 60% above that of parental lines, there is also considerable interest in applying Brassica CMS in the production of hybrid rapeseed.

Investigations of CMS in Brassica have focused on three male sterile cytoplasms: *ogu*, *nap*, and *pol*. The *nap* cytoplasm is the normal cytoplasm found in most *B. napus* cultivars; it is capable of conferring male sterility on only a few *B. napus* nuclear genotypes, and this male sterility is unstable under warmer growing conditions (Fan and Stefansson, 1986). The "Ogura" or *ogu* cytoplasm, which originated in radish, is associated with several disadvantageous traits in Brassica (Kemble and Barsby, 1988), and effective Brassica restorer lines are not available (Pellan-Delourme and Renard, 1989). Both because the "Polima" or *pol* cytoplasm confers a relatively temperature-stable male sterility (Fan and Stefansson, 1986) and because of the availability of restorer genotypes (Fang and McVetty, 1989), this system appears to be the most advantageous for hybrid rapeseed production. Despite the relative importance of the *pol* system, molecular analysis of CMS in crucifers has dealt primarily with the *ogu* system.

The mtDNA of *pol* cytoplasm can be distinguished from the mtDNAs of other Brassica cytoplasms by restriction analysis (Erickson et al., 1986). Analysis of cybrid lines has

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indicated that the determinants for *pol* CMS reside in the mitochondrial genome (Kemble and Barsby, 1988). To identify mitochondrial gene regions that might be associated with the *pol* CMS trait, we have searched for differences in expression among *nap* male fertile, *pol* CMS, and *pol* fertility-restored *B. napus* plants. Only a single region that is expressed differently in these plants was identified. In several respects, this region resembles the well-defined CMS-determining segments of the mitochondrial genomes of the T cytoplasm of maize and the S cytoplasm of petunia. It contains a novel open reading frame (ORF), created through mtDNA rearrangements, that is positioned upstream of, and cotranscribed with, a normal mitochondrial gene, in this case, the gene encoding subunit 6 of the mitochondrial ATPase (*atp6*). Suppression of the *pol* CMS phenotype by either of two distinct nuclear genes is associated with altered expression of the *pol* mitochondrial chimeric ORF and *atp6* gene.

RESULTS

Altered Organization and Expression of the *atp6* Gene Region in *pol* CMS Plants

To determine regions of the *pol* mitochondrial genome that potentially could specify the CMS trait, we attempted to identify mitochondrial gene regions that are expressed differently in *nap* cytoplasm, *pol* cytoplasm, and nuclear-restored *pol* cytoplasm plants. The characteristics of the *B. napus* strains used in this investigation are listed in Table 1. Initially, RNA gel blots of floral mtRNA from four lines, the male fertile cytoplasm line Regent (*nap*), the *pol* CMS lines Regent (*pol*) and 2007, and a nuclear restorer line, 4007, were analyzed using the probes (see Methods) of mitochondrial gene regions from maize (*atpA*, *atp6*, *cox2* [subunit 2 of cytochrome oxidase], *rrn18*, *rrn26* [18S and 26S ribosomal RNAs]), wheat (*cob* [cytochrome *b*], *cox1*, *nad5* [subunit 5 of NADH-ubiquinone reductase], *orf25*), Oenothera (*atp9*, *cox3*), watermelon (*nad1*), tobacco (*rps13* [encoding the ribosomal protein S12]), and broad bean (*rps14*). No qualitative or quantitative differences in transcript patterns were observed except with the probe for *atp6*.

As shown in Figure 1A, the *atp6* probe detected a single, 1.1-kb transcript in male fertile Regent (*nap*) cytoplasm plants. In the *pol* CMS plants Regent (*pol*) and 2007, levels of this transcript appeared greatly reduced and two longer transcripts of 2.2 and 1.9 kb were evident. In the nuclear restorer *pol* cytoplasm line 4007, the level of the 1.1-kb transcript appeared to be markedly enhanced relative to the *pol* CMS lines, levels of the 2.2- and 1.9-kb transcripts were slightly reduced, and two additional prominent transcripts of 1.4 and 1.3 kb were observed. Because at least 90% of the probe sequence was derived from the *atp6* coding region, it seemed likely that all the detected transcripts contained *atp6* coding sequences. When the same blot was stripped of *atp6* sequences and reprobed with the *atpA* gene, no differences among the lines were observed (Figure 1B), indicating that none of the observed differences arose from unequal loading of RNA samples on the gel or were otherwise artifactually generated.

To investigate further the association between *atp6* gene expression and male sterility, the organization of *atp6* sequences in the *nap* and *pol* mtDNAs was examined.

Table 1. Genotype and Phenotype of *B. napus* Plants Used in This Study

Pedigree	Restorer Genotype[a]	Cytoplasm	Phenotype
Regent (*nap*)	*rfp1/rfp1*	*nap*	Fertile
Regent (*pol*)	*rfp1/rfp1*	*pol*	Sterile
Karat (*nap*)	*rfp1/rfp1*	*nap*	Fertile
Karat (*pol*)	*rfp1/rfp1*	*pol*	Sterile
Westar (*nap*)	*rfp1/rfp1*	*nap*	Fertile
Westar (*pol*)	*rfp1/rfp1*	*pol*	Sterile
Italy	*Rfp1/Rfp1*	*pol*	Fertile
UM2353	*rfp1/rfp1, Rfp2/Rfp2*	*pol*	Fertile
Westar-*Rf*	*Rfp1/Rfp1*[b]	*pol*	Fertile
2007	*rf/rf*	*pol*	Sterile
4007	*Rf/Rf*[c]	*pol*	Fertile
Karat (*pol*) × Westar-*Rf*	*Rfp1/rfp1*	*pol*	Fertile
Karat (*nap*) × Westar-*Rf*	*Rfp1/rfp1*	*nap*	Fertile

[a] All plants were homozygous for the *rfp2* allele unless otherwise indicated.
[b] Restorer allele from the *B. napus* cv Italy (Fang and McVetty, 1989) was introgressed into cultivar Westar through six backcross generations.
[c] Restorer locus in this line has not been characterized.

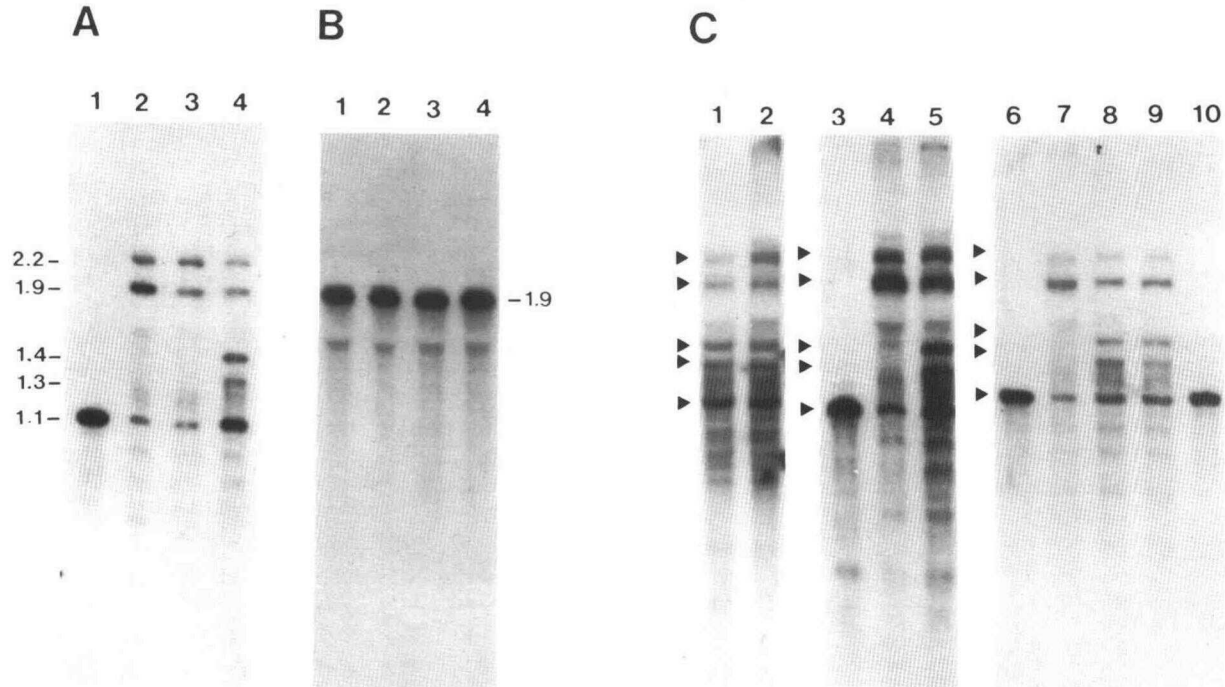

Figure 1. RNA Gel Blot Analysis of Mitochondrial Transcripts from Male Fertile, Male Sterile, and Fertility-Restored Brassica Plants.

(A) Maize *atp6* probe.
(B) Maize *atpA* probe.
(C) Brassica *atp6* probe.
In **(A)** and **(B)**, mtRNAs resolved on an agarose-urea gel were transferred to a hybridization membrane and probed with a gel-purified maize *atp6* coding region probe; after exposure and removal of the probe, the filter was rehybridized with the maize *atpA* gene probe. Lanes 1, Regent (*nap*) (male fertile); lanes 2, Regent (*pol*) (male sterile); lanes 3, 2007 (male sterile); lanes 4, 4007 (nuclear fertility restorer). Lengths in kilobases of the major discrete hybridizing transcripts are indicated.
In **(C)**, mtRNAs from the lines Italy (lane 1), UM2353 (lane 2), Westar (*nap*) (lane 3), Westar (*pol*) (lane 4), Westar-*Rf* (lane 5), Karat (*nap*) (lane 6), Karat (*pol*) (lane 7), Westar-*Rf* (lane 8), Karat (*pol*) × Westar-*Rf* (lane 9), and Karat (*nap*) × Westar-*Rf* (lane 10) were resolved on agarose-urea gels and probed with a Brassica *atp6* probe. Arrows indicate the locations of the 1.1-, 1.3-, 1.4-, 1.9-, and 2.2-kb transcripts.

EcoRI, BamHI, and PstI digests of both *nap* and *pol* mtDNAs, as shown in Figure 2, as well as SalI digests of *pol* mtDNA (data not shown) were probed with the maize *atp6* coding sequence. In each case, a single hybridizing fragment differing in size between the two DNAs was detected. Thus, the *atp6* gene is present in only a single copy that is organized differently in the *pol* and *nap* mitochondrial genomes. The *atp6* gene regions of *pol* and *nap* mtDNAs were cloned as 8.2-kb PstI and 3.5-kb EcoRI-BamHI fragments, respectively. Restriction maps indicated that sites were conserved at one end of the cloned fragments but that a point of divergence, apparently due to a sequence rearrangement, occurred between the EcoRI and BstXI sites of the two DNAs, as shown in Figure 3. Hybridization experiments using the maize probe indicated that the Brassica *atp6* coding region was located in the conserved portion of the two clones, and the failure of the 2.3-kb BamHI-PstI fragment at one end of the *pol* clone to detect transcripts in RNA gel blot analyses allowed the approximate boundaries of the expressed regions to be estimated.

Specific and Similar Alteration of *pol atp6* Transcripts by Either of Two Distinct *Rf* Genes

Fang and McVetty (1989) have shown that restorer alleles at either of two distinct genetic loci can suppress the *pol* CMS phenotype; the locus characteristic of the restorer genotype Italy has been designated *Rfp1* and that of the cultivar UM2353 has been designated *Rfp2*. Analysis of mtRNA from the cultivars Italy and UM2353, using the 2.2-kb EcoRI-BamHI fragment of *pol* mtDNA predicted to span the *atp6* coding sequence as a probe, showed that the transcript profiles of the two lines were similar and not

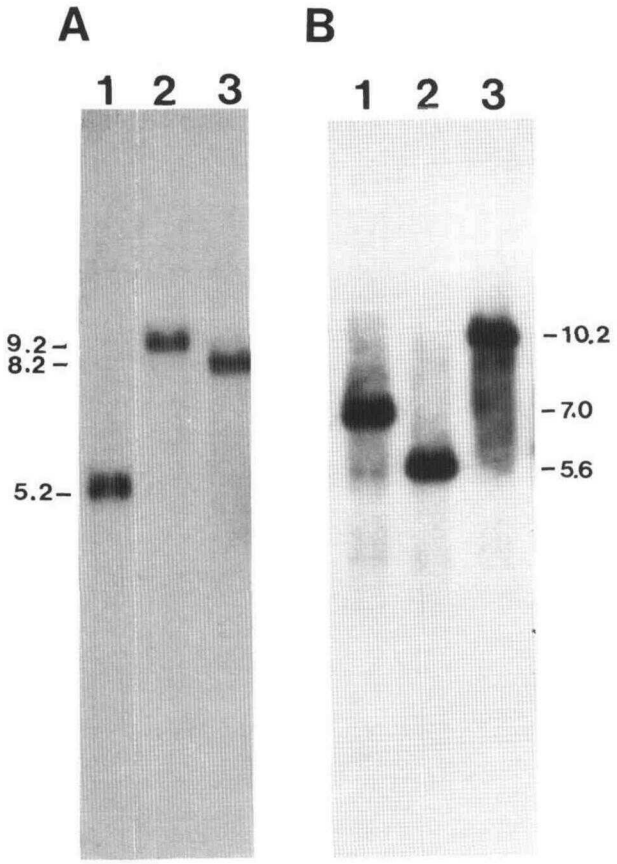

Figure 2. Analysis of *atp6* Gene Sequences Present in the *nap* and *pol* Mitochondrial Genomes.

(A) *B. napus* line Regent (*pol*).
(B) *B. napus* line Regent (*nap*).
mtDNAs were digested with EcoRI (lanes 1), BamHI (lanes 2), and PstI (lanes 3) and hybridized with the maize *atp6* coding region probe. Estimated lengths of individual hybridizing fragments are indicated in kilobases at the side of each panel.

obviously dissimilar from that detected for the restorer line 4007 using the maize probe (Figures 1A and 1C). This suggested that the two distinct restorer genes had an apparently identical effect on transcripts of the *pol atp6* gene region and indicated that no major features of the hybridization patterns detected in Figure 1A resulted from fortuitous homology with the maize probe.

Restorer lines may contain genes other than *Rf* genes that affect mitochondrial transcript profiles. This has been most dramatically demonstrated in radish, where, although *atpA* transcript differences have been observed between fertile, *ogu* CMS, and fertility-restored *ogu* plants (Makaroff and Palmer, 1988), subsequent analysis has indicated that nuclear influences on *ogu atpA* transcripts are unrelated to fertility restoration (Makaroff et al., 1990). To investigate whether the alterations in *atp6* region transcripts observed in the restored plants were specifically due to the corresponding *pol Rf* genes, we analyzed the transcripts of the near isogenic lines Westar (*pol*) and Westar-Rf (*pol*) and compared these with the corresponding Westar (*nap*) line. Westar-*Rf pol* is a restorer line derived from Westar (*pol*) by introgression of the *Rfp1* allele from the cultivar Italy through six backcross generations. The two lines are, therefore, expected to be isogenic at most of their loci; thus, any mitochondrial transcriptional differences between them are likely to be due to the introgressed *Rf* gene. As shown in Figure 1C, the *atp6* transcripts detected in the lines Westar (*nap*), Westar (*pol*), and Westar-*Rf* (*pol*) correspond to those of the other *nap* male fertile, *pol* CMS, and fertility-restored *pol* cytoplasm plants, respectively, described above. Thus, the nuclear gene responsible in the alteration of *pol atp6* region transcripts must reside at or be very tightly linked to the *Rfp1* locus.

Several additional lines and hybrids were analyzed to investigate further the effects of nuclear-cytoplasmic interactions on *atp6* transcripts. The *atp6* transcripts of the lines Karat (*nap*) and Karat (*pol*) were found to resemble those of their counterparts in the cultivar Regent (Figure 1). A restored F_1 hybrid formed by crossing Karat (*pol*) with Westar-*Rf* showed the same *atp6* transcript profile as the restorer lines, whereas no effects on *atp6* transcripts were evident in a Karat (*nap*) × Westar-*Rf* F_1 hybrid (Figure 1C). This suggested that the effects of the restorer on *atp6* transcripts were specific to *pol* cytoplasm and similar when the restorer is present in either homozygous or heterozygous condition.

The *atp6* Gene Regions of *nap* and *pol* mtDNAs

To investigate further the association between the *pol atp6* region and CMS, the nucleotide sequences of regions

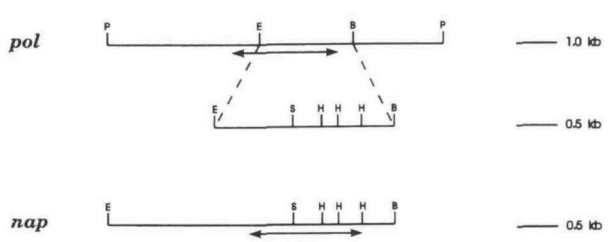

Figure 3. Physical Maps of the *atp6* Gene Regions of *nap* and *pol* mtDNAs.

Sequenced regions are indicated by arrows. Restriction sites are designated as follows: B, BamHI; S, BstXI; H, HindIII; P, PstI.

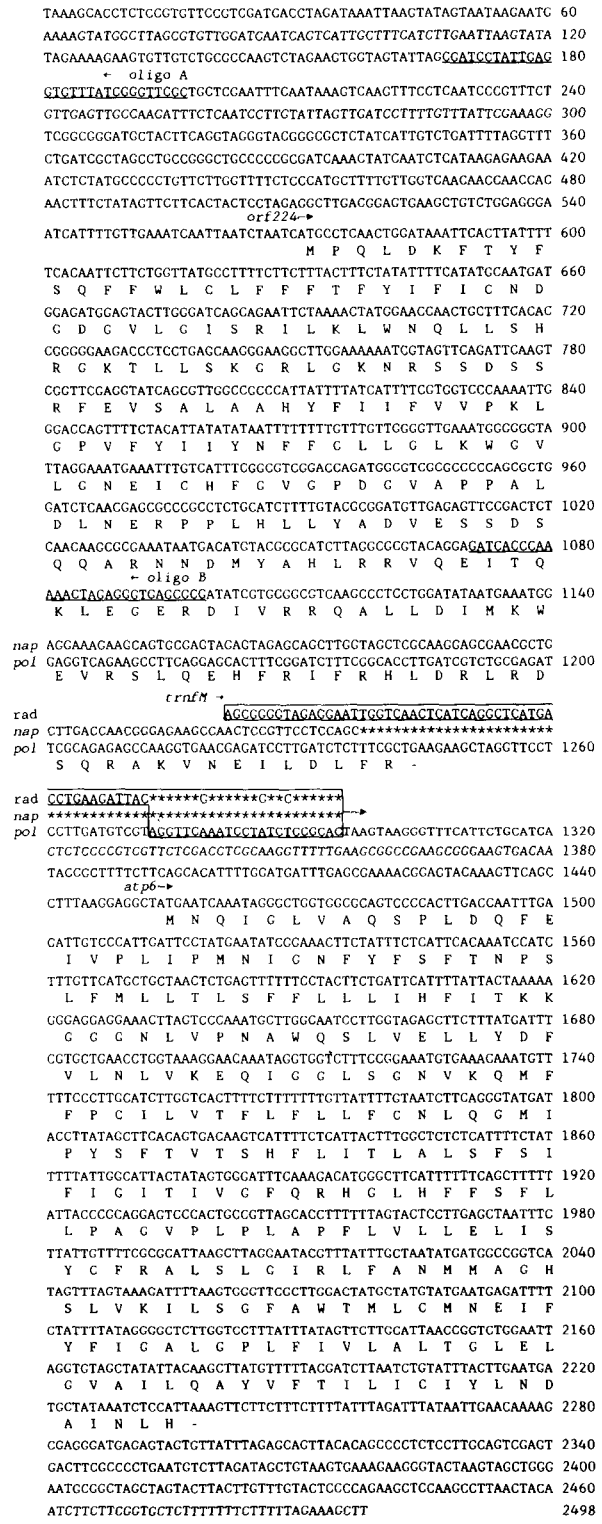

Figure 4. Nucleotide Sequences of Expressed Regions of the Brassica *pol* and *nap atp6* Gene Loci.

corresponding to the *atp6* transcripts of the *nap* and *pol* mtDNAs were determined. The boundaries of the sequenced regions are indicated in Figure 3. The derived DNA sequence of the *pol* mtDNA region is shown in Figure 4; the corresponding sequence of *nap* mtDNA, where it differs from *pol*, is indicated immediately above the *pol* sequence. The *nap* and *pol* sequences were found to be identical from one end of the sequenced regions up to the position indicated as nucleotide 1238; beyond this point the sequences diverge abruptly and no further similarity is evident.

The *atp6* coding sequence spans 261 codons in the region conserved between the two DNAs and is capable of encoding a 29,126-D polypeptide. It is identical to the *atp6* coding sequence of normal radish mtDNA (Makaroff et al., 1989). The similarity between the radish and Brassica mtDNAs extends from 181 nucleotides upstream of the *atp6* initiation codon to 101 nucleotides downstream of the *atp6* termination codon and encompasses a putative ribosome binding motif (Makaroff et al., 1989). The upstream boundary of this sequence similarity falls within the initiator methionine tRNA gene (*trnfM*) of the normal radish mtDNA. There are three nucleotide differences between the radish and Brassica DNAs (indicated above the Brassica sequences in Figure 4) in the conserved upstream region, all of which fall within the 23-bp *trnfM* region of similarity. Interestingly, the normal radish and Brassica mtDNAs are more similar in both the coding and 5′ flanking regions of the *atp6* gene than are the normal radish and CMS Ogura radish mtDNAs (Makaroff et al., 1989).

The nucleotide sequence of *pol* mtDNA extending from a position 685 bp beyond the EcoRI site of the *pol* PstI clone to the most distal HindIII site indicated in Figure 3 is shown. The *nap* sequence extending from a position approximately 500 bp upstream of the conserved BstXI site to the distal HindIII site indicated in Figure 3 was determined; the sequence upstream of the point of divergence with *pol* mtDNA is indicated immediately above the *pol* sequence. The amino acid sequences of the proteins predicted to be encoded by the *orf224* ORF, which extends from positions 571 to 1242, and *atp6* gene, which extends from positions 1454 to 2236, are indicated immediately below the *pol* DNA sequence. Nucleotides enclosed in boxes indicate the *trnfM* gene of normal radish mtDNA (Makaroff et al., 1989) and the corresponding sequences of *nap* and *pol* mtDNAs; positions of sequence identity between the *nap* and radish sequences and the *pol* sequences are indicated by asterisks. The *nap* and *pol* sequences are identical from position 1238 through the end of the analyzed region. With the exception of two adjacent nucleotide substitutions in the noncoding region 3′ to the *atp6* gene, the Brassica sequences are identical to the normal radish sequence from the 3′ end of the *trnfM* gene to a point of sequence divergence located 101 bp downstream of the *atp6* termination codon. Regions corresponding to the oligonucleotides oligo(A) and oligo(B) used in the hybridization analysis of Figure 6 are underlined.

An ORF Encoding a Fusion Protein Located Upstream of the *pol atp6* Gene

A second ORF terminating 208 nucleotides upstream of the *atp6* initiation codon is found in the *pol* mtDNA sequence. This ORF is capable of encoding a 224-amino acid protein with a predicted molecular mass of 26,218 D and has been designated *orf224*. The first 58 codons of *orf224* are highly similar to the amino-terminal coding region of an Oenothera and sunflower mtDNA sequence designated *orfB*, as shown in Figure 5 (Hiesel et al., 1987; Quagliariello et al., 1990). The *orfB* coding sequence is transcribed in both Oenothera and sunflower mitochondria (Hiesel et al., 1987; Quagliariello et al., 1990), and filter hybridization with the sunflower *orfB* coding sequence has detected homologous regions in a number of monocot and dicot mtDNAs (Quagliariello et al., 1990), suggesting that *orfB* is a common protein coding sequence of plant mtDNAs. Over the first 53 codons, the sequence similarities between the Brassica *orf224* and Oenothera *orfB* are 96% and 90% at the nucleotide and amino acid sequence levels, respectively. A 5-nucleotide deletion relative to the Oenothera sequence occurs after codon 58, and only a short stretch of limited identity between the two sequences is apparent beyond this point. The remaining portion of the Brassica ORF does not show significant similarity to any sequence in the GenBank sequence library.

Similarity between the Brassica and Oenothera sequences is maintained over approximately 120 nucleotides in the noncoding region upstream of *orf224*. This region falls within a 657-bp repeated sequence of Oenothera mtDNA that also occurs upstream of the *cox1* gene (Hiesel et al., 1987). The 657-bp Oenothera repeat spans a second ORF and a putative promoter element that are not included in the region homologous to the Brassica mtDNAs. The noncoding region conserved between the Brassica and Oenothera mtDNAs, however, represents a general plant mitochondrial expression element that includes a putative ribosome binding site and is positioned upstream of several other plant mitochondrial genes, including tobacco *atp6* and *atp9* (Bland et al., 1987; Figure 5).

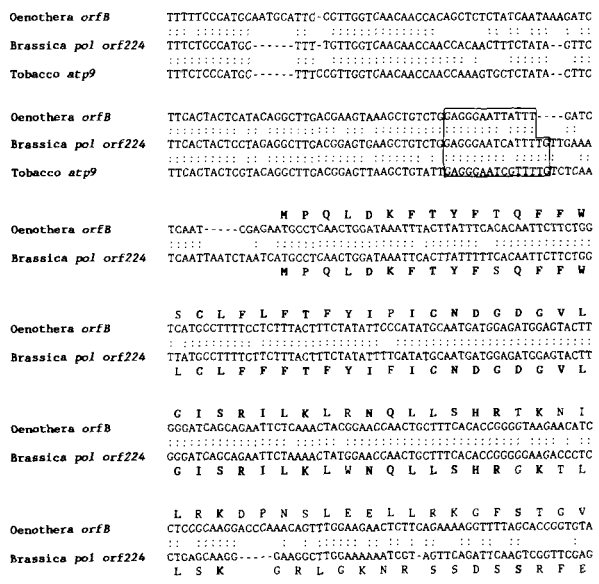

Figure 5. Nucleotide and Amino Acid Sequence Similarities between 5′ Upstream and the N-Terminal Coding Region of the *Brassica pol orf224* and Corresponding Regions of the Oenothera *orfB* (Hiesel et al., 1987) and Tobacco *atp9* (Bland et al., 1986) Genes.

Conserved amino acid residues are indicated in bold type; dashes are used to indicate deletions; the boxed region corresponds to a putative ribosome binding site.

Expression of Brassica *atp6* Regions

Transcription of the Brassica *atp6* regions was investigated in greater detail by probing membrane blots of mtRNAs from the lines Regent (*nap*), Regent (*pol*), 2007, and 4007 with sequences derived from different segments of the *pol atp6* clone, as shown in Figure 6. A subclone extending from a HindIII site 253-bp downstream of the *atp6* gene (nucleotides 2493 to 2498 of Figure 4) to the conserved BamHI site (Figure 3) failed to detect transcripts in any of the lines, indicating that the 3′ termini of both the *pol* and *nap* transcripts are located within 250 nucleotides downstream of the *atp6* termination codon. This further indicated that the 5′ terminus of the 1.1-kb transcript, which constitutes the major *nap atp6* transcript and which is elevated in *pol* mitochondria by the nuclear restorer gene, corresponds to a site positioned in the vicinity of the *pol/nap* homology breakpoint and the truncated tRNA pseudogene.

Two oligonucleotides, designated as oligo(A) and oligo(B), were used to map the longer *pol* transcripts. Oligo(A), which corresponds to bases 168 to 197 on the *pol* sequence of Figure 4, detected only the 2.2-kb transcript of *pol* cytoplasm plants (Figure 6A), whereas oligo(B), which corresponds to bases 1071 to 1099, also detected the 1.9-kb transcript and the 1.4- and 1.3-kb transcripts specific to fertility-restored plants (Figure 6B). Neither probe detected transcripts in the male fertile *nap* cytoplasm line. These results indicated that the various *atp6* transcripts have different 5′ termini and one or a few closely spaced 3′ termini mapping approximately 200 nucleotides downstream of the gene.

The 5′ transcript termini mapping in the vicinity of the tRNA pseudogene were more precisely positioned by primer extension analysis (Figure 6C). An oligonucleotide corresponding to positions 1466 to 1485 of Figure 4 was used to prime cDNA synthesis off mtRNA from fertile *nap*

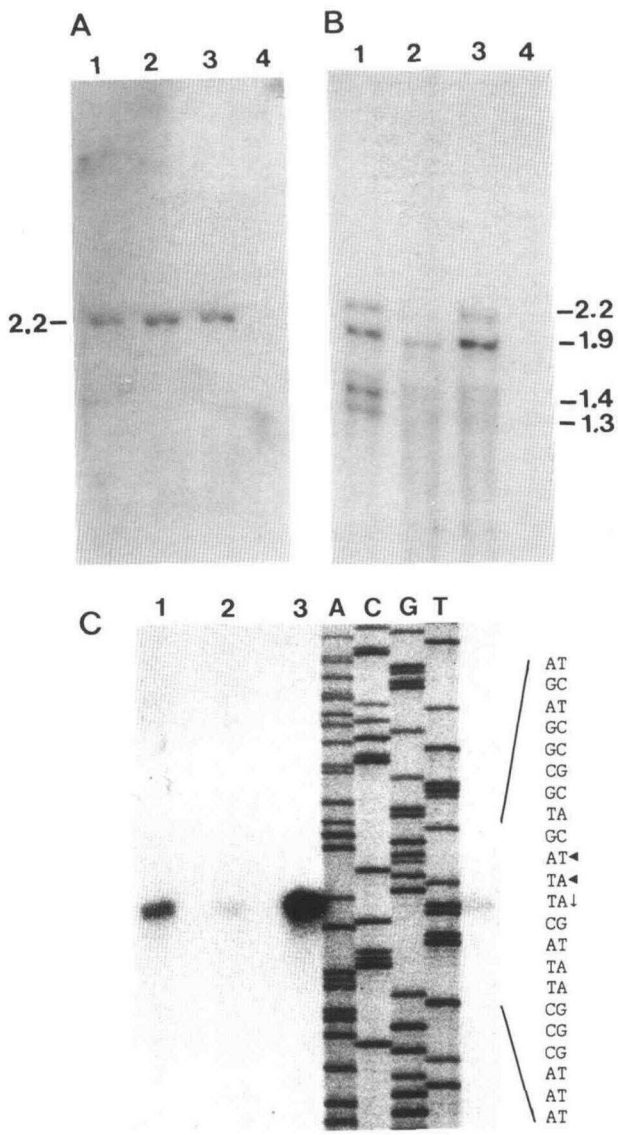

Figure 6. Mapping of *atp6* Region Transcripts from Male Fertile, Male Sterile, and Fertility-Restored Plants.

(A) Oligo(A) probe.
(B) Oligo(B) probe.
(C) Primer extension analysis.
In **(A)** and **(B)**, RNA gel blots of mtRNAs isolated from 4007 (lanes 1), 2007 (lanes 2), Regent (*pol*) (lanes 3), or Regent (*nap*) (lanes 4) plants were probed with oligonucleotides corresponding to the regions indicated as oligo(A) or oligo(B) of Figure 4.
In **(C)**, primer extension products were obtained using an oligonucleotide complementary to bases 1466 to 1485 of Figure 4 and 5 μg of mtRNA from 4007 (lane 1), 2007 (lane 2), or Regent (*nap*) (lane 3) plants. One-fifth of the product of each reaction was run alongside DNA sequencing reactions primed with the same oligonucleotide; approximately one-twentieth of the amount of the product loaded in lane 3 was run on the opposite side of the gel. Horizontal arrowheads indicate positions of 5′ transcript termini; the vertical arrow indicates direction of transcription.

cytoplasm, *pol* CMS, and fertility-restored *pol* cytoplasm plants. Major transcript termini were identified in *nap* cytoplasm and fertility-restored *pol* cytoplasm plants that mapped to 2 adjacent nucleotides positioned at the precise 3′ terminus of the truncated tRNA pseudogene. These transcript termini were present at reduced levels in *pol* CMS plants, consistent with the lower observed levels of the 1.1-kb transcript.

DISCUSSION

Role of a tRNA-Like Element in Formation of the 5′ Termini of Brassica *atp6* mRNAs

The organization and transcription of the *atp6* mitochondrial gene regions of normal radish cytoplasm (Makaroff et al., 1989) and the Brassica *nap* and *pol* cytoplasms are summarized in Figure 7. In radish, the major 5′ *atp6* transcript termini map to two sites positioned very near the 3′ end of the *trnfM* gene. Makaroff et al. (1989) have suggested that the 5′ end of the normal radish *atp6* message may be generated as a result of endonucleolytic cleavage of the upstream initiator-methionine tRNA from a polycistronic precursor RNA, analogous to the formation of mature mammalian mitochondrial messages through tRNA processing (Attardi and Schatz, 1988). Recent analysis of tRNA processing activity in plant mitochondria indicates that, as in animal mitochondria, both the 5′ and 3′ termini of tRNAs are formed through precise endonucleolytic cleavage of a precursor species (Hanic-Joyce and Gray, 1990; Marchfelder et al., 1990), thus providing support for this view.

Because the 5′ *atp6* mRNA terminus of *nap* mitochondria maps precisely to the 3′ end of the truncated *trnfM* pseudogene (indicated as ψ*trnfM* in Figure 7) corresponding to the intact radish tRNA gene, it seems probable that it too is formed through a tRNA processing mechanism. Thus, the sequence similarity between the radish and Brassica mtDNAs over the 23 bp corresponding to the 3′ end of the tRNA sequence appears to allow for maintenance of efficient processing in *nap* but not in *pol* mitochondria. Processing in CMS *pol* mitochondria is apparently limited by the rearrangement occurring 58 bp upstream of the putative processing site.

The finding that extracts of wheat mitochondria will process not only bona fide tRNA precursors, but also transcripts of a class of short wheat mtDNA repeats termed "t-elements" (Hanic-Joyce et al., 1990) provides a possible explanation for these observations. T-element transcripts are potentially capable of folding into tRNA-like structures that possess analogs of the amino acceptor, TΨC, and anticodon arms, as well the appropriate nucleotides at positions that are invariant or semi-invariant among all tRNAs. Several of these invariant nucleotides occur in

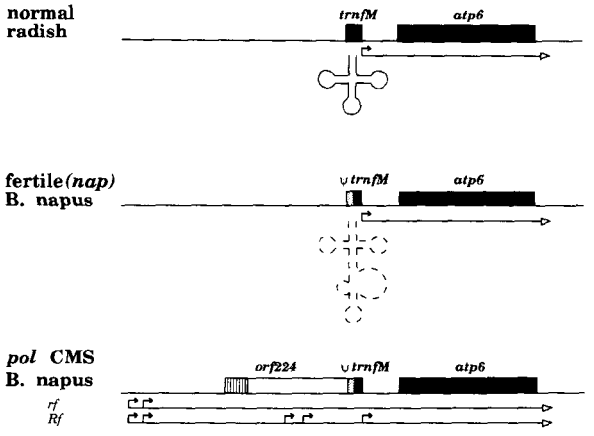

Figure 7. Organization and Expression of the *atp6* Gene Regions of Fertile Radish, Fertile (*nap*) *B. napus*, and *pol* CMS *B. napus* mtDNAs.

Transcripts are represented by the lines immediately below each depicted gene region. Small and open arrowheads indicate 5′ and 3′ termini, respectively. Black boxes indicate regions corresponding to or showing sequence similarity with *atp6* and *trnfM* genes. Lightly shaded boxes indicate the region upstream of the Brassica/radish divergence that is conserved in the *nap* and *pol* mtDNAs; the regions of the Brassica DNAs showing sequence similarity to the radish *trnfM* gene are designated as Ψ*trnfM*. The region of *orf224* that is derived from the *orfB* sequence is indicated by the vertically striped box, the remainder by the unfilled box. The positions of the radish initiator-methionine tRNA and corresponding tRNA-like element of the *nap* mtDNA transcript (see text and Figure 8) are depicted to show their postulated roles in the formation of the 5′ termini of *atp6* transcripts; dashed lines indicate hypothesized unstable transcripts. Transcripts of fertility-restored and male sterile *pol* cytoplasm plants are indicated by the symbols *Rf* and *rf*, respectively.

the TΨC loop that, together with the 3′ end of the anticodon arm, is represented in the Brassica ψ*trnfM* pseudogene. Computer-aided secondary structure modeling indicates that in the most stable predicted conformation, sequences of the *nap* pseudogene transcript are positioned in the amino acceptor arm-TΨC arm configuration of an intact tRNA, as shown in Figure 8. In addition, the *nap* upstream sequences are predicted to adopt stem-loop structures at the positions of the dihydrouridine and anticodon arms, and the derived structure maintains most of the invariant nucleotides of a conventional tRNA. It is likely, therefore, that this tRNA-like element is recognized and cleaved by the activity normally responsible for 3′ end processing of mitochondrial tRNA precursors to generate a 5′ terminus for the *atp6* message that corresponds to that postulated to be formed in normal radish by the processing of the intact tRNA.

Potential Modes of Action of Restorer Genes

As a result of the *pol* mtDNA rearrangement, the sequences of the *nap* tRNA-like element that form the dihydrouridine and anticodon arms and form base pairs with the amino acceptor stem component of the pseudogene are replaced with unrelated sequences. As a result,

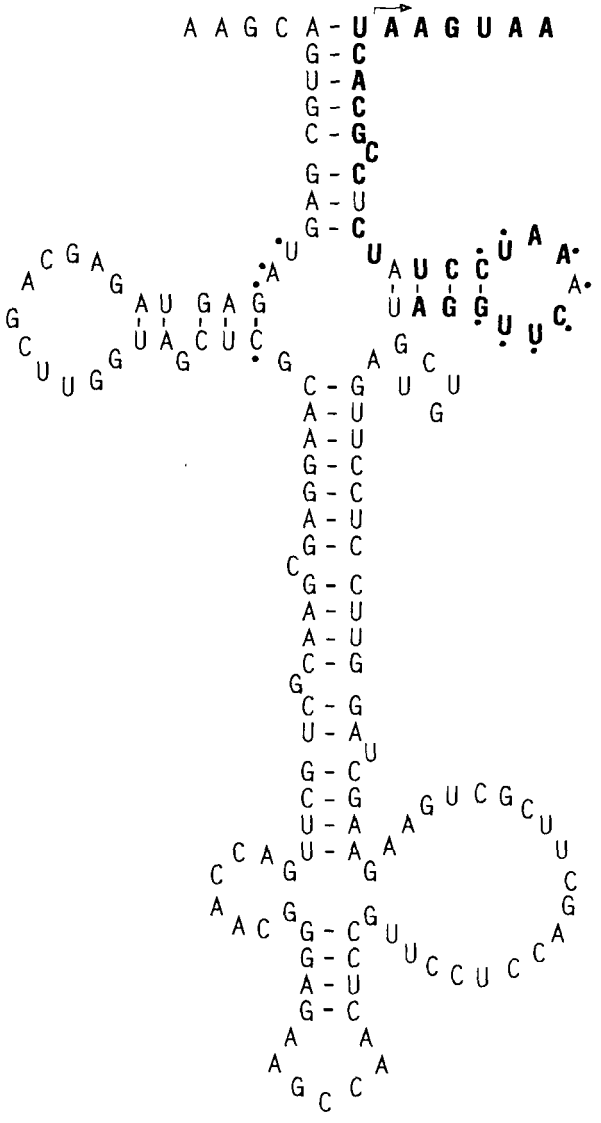

Figure 8. Predicted Secondary Structure of a tRNA-Like Element in a Transcript from the *nap atp6* Upstream Region.

Sequence similarity with the radish *trnfM* gene and flanking region is indicated by boldface type. Closed circles designate bases corresponding to nucleotides that are invariant or semi-invariant in all tRNAs. Arrow indicates the 5′ terminus of the *nap atp6* transcript terminus determined by primer extension analysis.

a stable tRNA-like element is not predicted to form in the *pol* transcript, and processing at the 3′ end of the pseudogene would be expected to be reduced, consistent with the observed reduction in the level of the 1.1-kb transcript. Nuclear fertility restoration leads to the occurrence of additional upstream *atp6* 5′ transcript termini and to a noticeable increase in transcripts mapping to the 3′ end of the pseudogene. Thus, one consequence of restorer gene action appears to be enhanced processing at the 3′ pseudogene site. Conceivably, the restorers could either cause a subtle alteration in the specificity of the processing machinery such that the *pol* transcript is more efficiently recognized as a substrate or cause the *pol* transcript to adopt a configuration more resembling that of a conventional tRNA precursor.

A number of possible mechanisms by which the restorer genes might act to alter the folding of the *pol* transcript can be envisioned. For example, the genes might act to promote transcription at sites corresponding to the termini of the 1.3- and 1.4-kb transcripts specific to fertility-restored plants; increased processing at the 3′ end of the pseudogene might occur if these transcripts adopted a secondary structure different from that of the 2.2- and 1.9-kb transcripts found in both CMS and restored *pol* cytoplasm plants. Altered folding could also occur through a specific interaction between the restorer gene product and the *pol* transcript. Proteins that assist in the processing of specific fungal mtRNAs are thought to act by facilitating formation of correct RNA structures (Lambowitz and Perlman, 1990), and the yeast nuclear gene *NAM2*, which encodes one such protein, is analogous to the *pol Rf* genes in that it can suppress mtDNA alterations leading to processing defects (Labouesse et al., 1987). The occurrence of RNA editing in plant mitochondria (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989) provides another potential mechanism for restorer gene action because differential editing in restored plants would result in transcripts with altered primary and hence secondary structures. We are currently attempting to distinguish among some of these possibilities experimentally.

Fang and McVetty (1989) have shown previously that the restorer genes present in the lines Italy and UM2353 reside at two distinct, independently segregating loci, and the finding that both of these genes had an apparently identical effect on *pol* CMS transcripts was, therefore, somewhat unexpected. One possible explanation may lie in the fact that *B. napus* is an amphidiploid, with one set of its chromosomes derived from *B. oleracea* (the c genome), the other from *B. campestris* (the a genome). Conceivably, *Rfp1* and *Rfp2* are allelic forms of homologous genes, one derived from the a genome and the other from the c genome.

A Chimeric Protein Gene Associated with *pol* CMS

Chimeric genes, formed by rearrangement of coding and noncoding segments of mtDNA, have been found to be associated with CMS in a number of species, including maize (Dewey et al., 1986; Braun et al., 1991), sorghum (Bailey-Serres et al., 1986), petunia (Young and Hanson, 1987), and rice (Kadowaki et al., 1990). The *orf224* gene of *pol* mitochondria also has the characteristics of a chimeric gene. The 58 N-terminal codons appear to be derived from a conventional mitochondrial gene of unknown function, designated *orfB*, that is positioned upstream of, and cotranscribed with, the *cox3* gene in Oenothera and sunflower mtDNAs. The source of the sequences comprising the remainder of the *orf224* gene is not known. Because an oligonucleotide corresponding to the *orfB* homologous region of *orf224* detects three transcripts in both *pol* and *nap* mitochondria in addition to those detected by *atp6* probes (M. Singh and G. Brown, unpublished observations), it is likely that an expressed, intact *orfB* gene resides elsewhere on Brassica mitochondrial genomes. The finding that the *orfB* probe detects two restriction fragments in BamHI, EcoRI, and PstI digests of both *nap* and *pol* mtDNAs is consistent with this possibility (M. Singh and G. Brown, unpublished observations).

Figure 9 shows the relative hydropathy profile of the predicted *orf224* gene product. The ORF224 protein is predicted (Klein et al., 1985) to contain two membrane-spanning domains, one derived from the *orfB* homologous region (amino acid residues 12 to 27) and one derived from the downstream portion of the ORF (residues 82 to 97). The ORF224 protein is predicted to be an integral membrane protein.

Possible Role of the *orf224/atp6* Gene Region in Brassica *pol* CMS

Of 14 mitochondrial gene regions surveyed, only *atp6* showed differential expression at the RNA level between

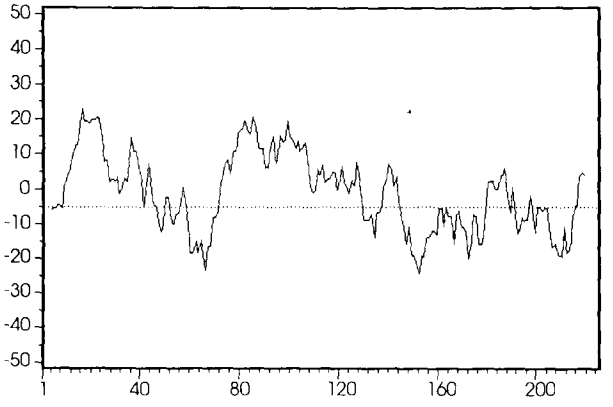

Figure 9. Hydropathy Plot of ORF224.

Values for hydropathic index (*y* axis) calculated according to Kyte and Doolittle (1982) are plotted against amino acid position. Hydrophobic domains are represented by positive values.

pol and *nap* cytoplasm plants; of these 14 regions, which represent approximately one-half of the probable protein coding regions of Brassica mtDNA (Makaroff and Palmer, 1987), only *atp6* transcripts were found to be affected by nuclear restoration. Witt et al. (1991) have recently described the use of a similar approach to compare the transcripts of *B. campestris* to those of *pol* cytoplasm *B. napus*. Their results are similar to those described here, although they do not report a specific increase in the level of the 1.1-kb *pol* transcript upon nuclear fertility restoration. The collective results of these two surveys, both previously reported in abstract form (Hansen et al., 1990; Singh and Brown, 1990), suggest that the pattern of expression of the Brassica *pol atp6* region is tightly associated with male sterility.

Previously, only in the cases of *cms*-T maize, CMS rice, and CMS petunia have specific nuclear restorer genes been shown to exert an effect on mitochondrial transcripts (Dewey et al., 1986; Kennell and Pring, 1989; Kadowaki et al., 1990; Pruitt and Hanson, 1991). In the case of *cms*-T cytoplasm maize, there is substantial genetic evidence correlating an mtDNA region that is affected by the restorer, the T-*urf13* region, with the CMS trait. Our finding that either of two independently segregating restorer genes exerts specific and similar effects on transcripts of the *pol orf224/atp6* locus, the only region found to be expressed differently between *pol* and *nap* cytoplasm plants, provides the strongest support for the view that the locus may specify male sterility.

Although rearranged genes have been found on several CMS mitochondrial genomes, only two chimeric gene regions, the maize T-*urf13* locus and the petunia S-*pcf* locus, have been implicated in specifying the trait by genetic analysis (Hanson et al., 1989; Levings, 1990; Braun et al., 1991). These regions share certain features of their organization and expression with one another and with the Brassica *pol orf224/atp6* locus. In all three cases, the chimeric gene is cotranscribed with conventional downstream mitochondrial genes to form a polycistronic mRNA, and in each case, nuclear restorer genes exert specific effects on the expression of the region. In the case of T-*urf13*, the effect of the restorer gene *Rf1* appears to resemble that of the *pol* restorers in that processing of the transcript is affected (Dewey et al., 1986; Kennell and Pring, 1989). These similarities also suggest that the *pol orf224/atp6* region may be involved in specifying male sterility.

It has been suggested that the CMS trait could result directly from the presence of the aberrant proteins encoded by T-*urf13* and *pcf* genes or from the indirect effect that translation of these genes might have in inhibiting translation of the proteins encoded on the downstream mRNA regions (Hanson et al., 1989; Braun et al., 1991). In each case, only partially dysfunctional mitochondria would result. Such partial mitochondrial dysfunction may be manifested at the gross phenotypic level as male sterility (Levings, 1990; Braun et al., 1991); more severe mitochondrial defects might lead to a loss in cell viability in vegetative organs, as expressed in nonchromosomal stripe mutants of maize (Newton et al., 1990).

By similar reasoning, the *pol* CMS could result either from a partial impairment of mitochondrial function as a result of the presence of the putative ORF224 protein or from a deficiency in ATPase subunit 6 due to a limitation in translation imposed by the cotranscribed upstream *orf224* gene. The effects of restorer gene action on *orf224/atp6* transcripts, however, are more consistent with the latter alternative. If CMS were directly due to the presence of the ORF224 protein, then it might be expected that conditions that suppress male sterility would also suppress expression of the *orf224* gene. Although nuclear restoration leads to a slight decrease in the levels of *orf224/atp6* dicistronic transcripts, this, pending unforeseen specific effects on *orf224* translation, would not be expected to lead to markedly reduced levels of the putative ORF224 protein. The major effect of the restorer genes is to elevate the levels of monocistronic *atp6* transcripts. Because translation of *atp6* on such messages would not be affected by the upstream ORF, it is anticipated that a specific increase in the rate of translation of the ATPase subunit 6 protein would result and, thus, the possible deficiency in the subunit would be compensated. It is also possible that the combined effects of an ATPase subunit 6 deficiency and the presence of the aberrant *orf224*-encoded protein contribute to the CMS phenotype.

Stronger support for a role for the *orf224/atp6* locus in specifying CMS could be obtained if the region could be shown to be genetically correlated with male sterility. Analysis of recombinant mtDNAs formed by somatic hybridization of male-sterile and fertile lines has allowed for genetic correlation of the petunia S-*pcf* locus with CMS (Boeshore et al., 1985; Hanson et al., 1989). Although somatic hybridization has been employed extensively in Brassica to obtain novel organelle combinations, no recombination between *pol* and male fertile mtDNAs has been reported (Kemble and Barsby, 1988). There are many examples of mtDNA recombination between the *ogu* and male fertile mitochondrial genomes in Brassica, however (Kemble and Barsby, 1988), and because a somatic hybrid in which the *pol* CMS and male fertile mtDNAs have apparently recombined has recently been identified in this laboratory (H.M. Kao and G.G. Brown, unpublished observations), this approach seems likely to prove useful in further analyzing the *pol* CMS determinant(s).

A role for the *orf224/atp6* region in specifying the *pol* CMS is supported by two additional observations. First, we note that restriction analysis indicates a high degree of overall similarity between the mtDNAs of the *pol* and fertile *B. campestris* (*cam*) cytoplasms (Erickson et al., 1986), the latter of which acts as a male fertile cytoplasm in both *B. campestris* and *B. napus* (Kemble and Barsby, 1988; Braun et al., 1991); the *atp6* locus is one of only a very

Table 2. Mitochondrial Gene Probes Used in Transcript Analysis

Gene	Species	Fragment	Reference
atpA	Maize	4.2-kb HindIII	Braun and Levings (1985)
atp6[a]	Maize	1.0-kb TaqI	P. Finnegan and G. Brown, unpublished data
atp9	Oenothera	6.2-kb BamHI	Schuster and Brennicke (1989)
cob	Wheat	0.7-kb BamHI-HindIII	Boer et al. (1985
cox1	Wheat	1.0-kb HindIII-PstI	Bonen et al. (1987)
cox2	Maize	2.8-kb HindIII	Fox and Leaver (1981)
cox3	Oenothera	1.1-kb EcoRI-PstI	Hiesel et al. (1987)
nad1	Watermelon	2.2-kb BamHI	Stern et al. (1986)
nad5	Wheat	1.1-kb BamHI	L. Bonen, unpublished data
rps13[b]	Tobacco	2.9-kb PstI	Bland et al. (1986)
rps14	Bean	178-bp deletion	Wahleithner and Wolstenholme (1988)
orf25	Wheat	2.0-kb BamHI	Bonen et al. (1990)
rrn26[c]	Maize	2.5-kb HindIII-SmaI	Finnegan and Brown (1990)
rrn18[d]	Maize	2.6-kb HindIII	Finnegan and Brown (1990)

[a] Derived from clone T25H (Dewey et al., 1985).
[b] Also includes *atp9* and 5′ *nad1* sequences.
[c] Derived from pB406 (Iams and Sinclair, 1982).
[d] Derived from pB401 (Iams and Sinclair, 1982).

few regions that appear to be arranged differently in these two mitochondrial genomes (Y. L'Homme and G.G. Brown, unpublished observations). Second, HPLC analysis of F_0 ATPase preparations indicates that the amount of subunit 6 relative to other subunits is about 40% lower in *pol* CMS plants than in fertility-restored plants (S. Gleddie, unpublished observations); this is consistent with the hypothesis that the *pol* CMS may result from a deficiency in the *atp6* gene product. Our results clearly indicate that nuclear genes that suppress *pol* cytoplasm-induced male sterility specifically alter expression of this region. More directed experiments addressing the possible role of the *orf224/atp6* locus in *pol* CMS and the mechanisms of restorer gene action should now be possible.

METHODS

Plant Material

Brassica napus cytoplasms are designated according to the convention of Kemble and Barsby (1988) and are indicated by the parenthetical italicized designations following the cultivar name. The strains Regent (*nap*), Regent (*pol*), Italy, and UM2353 were obtained from Dr. P.B.E. McVetty, University of Manitoba, Winnipeg; 2007 and 4007 were from Dr. Larry Sernyk, Conti Seeds, Winnipeg, Manitoba; all other strains were provided by Dr. D. Hutcheson, Agriculture Canada, Saskatoon, Saskatchewan. Floral tissue from plants grown in the McGill Phytotron growth chambers under normal growth conditions (day/night temperatures of 22/16°C, 16-hr photoperiod) was used for the isolation of mitochondrial nucleic acids.

Mitochondrial Genes

The mitochondrial gene regions used in the analysis of Brassica transcripts are described in Table 2. Sources of the maize probes have been described previously (Finnegan and Brown, 1990). Drs. Linda Bonen (University of Ottawa, Ottawa), C.S. Levings III (North Carolina State University, Raleigh), and David Wolstenholme (University of Utah, Salt Lake City) furnished the wheat, tobacco, and bean probes, respectively; the Oenothera and watermelon probes were supplied by Dr. Axel Brennicke (Institut für Genbiologische Forschung, Berlin).

Isolation of Nucleic Acids

mtDNA was isolated essentially as described by Kemble (1987), except that further purification by equilibrium centrifugation in CsCl gradients was occasionally used to improve its susceptibility to digestion by restriction endonucleases. mtRNA was isolated by a modification of the high ionic strength medium procedure of Perez et al. (1990). All steps were performed at 4°C unless otherwise stated. Plant tissue was homogenized in 3 to 5 volumes of high ionic strength buffer (50 mM Tris-HCl, pH 8.0, 25 mM EDTA, 1.3 M NaCl, 0.1% BSA, and 56 mM mercaptoethanol). The homogenate was filtered through the two layers of Miracloth (Calbiochem Inc.), and the filtrate was centrifuged twice at 2600*g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 17,000*g* for 20 min to sediment mitochondria. Mitochondria were lysed in the presence of aurintricarboxilic acid, and mtRNA was obtained by LiCl precipitation as described by Stern and Newton (1986).

Nucleic Acid Analysis

mtRNA was size fractionated on agarose-urea gels (Finnegan and Brown, 1986), transferred to GeneScreen-Plus (Dupont-New

England Nuclear) hybridization membranes by overnight capillary blotting with 1.5 M NaCl/0.15 M sodium citrate, and hybridized to radiolabeled probes in the presence of 1 M sodium chloride, 1% SDS, and 10% dextran sulfate for 24 hr. For homologous probes, membranes were hybridized at 60°C according to the instructions of the supplier (Dupont-New England Nuclear). Membranes were subsequently washed two times for 5 min with wash buffer (0.3 M NaCl/0.03 M sodium citrate) at room temperature, twice for 30 min with wash buffer/1% SDS at 60°C, and twice for 30 min with 0.05 × wash buffer at room temperature before autoradiography. For heterologous probes, the hybridization and high-temperature washes were performed 5 to 8°C lower than the corresponding temperatures for homologous probes.

mtDNA was digested with restriction endonucleases and size fractionated on 0.7% agarose gels. Gels were treated with 0.4 N NaOH/0.6 M NaCl for 30 min at room temperature, and neutralized by incubation in 1.5 M NaCl/0.5 M Tris-HCl, pH 7.5, for 30 min; DNA was then transferred to the GeneScreen-Plus membranes by overnight capillary blotting and hybridized with the labeled probe as described above for RNA gel blot hybridization, except that hybridization and high-temperature wash steps were conducted at temperatures 5°C higher.

DNA fragments were purified from agarose gels for cloning and labeling purposes using a system from Bio-Rad. The pBluescript II phagemid vectors SK+ and SK− (Stratagene) were used for cloning. Recombinant DNA fragments were labeled using the nick translation system of Bethesda Research Laboratories Life Technologies Inc., according to the manufacturer's instructions. Oligonucleotides were end labeled and primer extension analysis was conducted as described in Brown et al. (1991).

DNA was sequenced with the Sequenase system (U.S. Biochemical Corporation). To obtain the *pol* mtDNA sequence, individual HindIII, HindIII-EcoRI, and HindIII-BamHI fragments were first gel purified from digests of the 8.2-kb PstI clone and subcloned in pBluescript II vectors. Sequencing runs were primed either with T3 and T7 promoter primers or with oligonucleotides designed on the basis of obtained DNA sequence that were furnished by the Sheldon Biotechnology Centre, McGill University, Montreal. RNA secondary structure was predicted using the FOLD algorithm of Zuker and Stiegler (1982), and GenBank data base searches were conducted using the FASTA program of Pearson and Lipman (1988).

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