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Organizational differences between cytoplasmic male sterile and male fertile Brassica mitochondrial genomes are confined to a single transposed locus

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ABSTRACT

Comparison of the physical maps of male fertile (*cam*) and male sterile (*pol*) mitochondrial genomes of *Brassica napus* indicates that structural differences between the two mtDNAs are confined to a region immediately upstream of the *atp6* gene. Relative to *cam* mtDNA, *pol* mtDNA possesses a 4.5 kb segment at this locus that includes a chimeric gene that is co-transcribed with *atp6* and lacks an approximately 1kb region located upstream of the *cam atp6* gene. The 4.5 kb *pol* segment is present and similarly organized in the mitochondrial genome of the common *nap B.napus* cytoplasm; however, the *nap* and *pol* DNA regions flanking this segment are different and the *nap* sequences are not expressed. The 4.5 kb CMS-associated *pol* segment has thus apparently undergone transposition during the evolution of the *nap* and *pol* cytoplasms and has been lost in the *cam* genome subsequent to the *pol-cam* divergence. This 4.5 kb segment comprises the single DNA region that is expressed differently in fertile, *pol* CMS and fertility restored *pol* cytoplasm plants. The finding that this locus is part of the single mtDNA region organized differently in the fertile and male sterile mitochondrial genomes provides strong support for the view that it specifies the *pol* CMS trait.

INTRODUCTION

Cytoplasmic male sterility (CMS), the maternally transmitted failure to produce functional pollen, is a widespread trait in higher plants that results from rearrangements of the mitochondrial genome (1, 2). The CMS phenotype is a manifestation of nuclear-mitochondrial gene interactions, since, for many instances of the trait, specific nuclear genes termed restorers of fertility (*Rf*) have been identified which can suppress male sterility and allow for production of viable pollen. CMS has been the subject of extensive fundamental and applied studies because of its practical value in hybrid seed production and because it affords approaches both to the analysis of nuclear gene regulation of mitochondrial gene activity and to analysis of the developmental consequences of aberrant mitochondrial gene expression. However, substantial evidence implicating a particular mitochondrial gene region in the specification of the trait is available only in maize (3) and Petunia (4), and the molecular basis of the trait is not understood in any system.

Brassica napus (oilseed rape, canola) offers an attractive system for studying CMS. Investigations of mtDNA structure are facilitated by the small size and simple organization of *Brassica* mitochondrial genomes (5), and the amenability of *Brassica* to tissue culture allows for analysis of the trait through somatic hybridization approaches (6, 7). CMS in *B.napus* can be conferred by alien cytoplasms, such as the Ogura cytoplasm of radish, or by endogenous cytoplasms found within the cultivated varieties of the species (8). The endogenous systems involve an unusual and interesting set of nuclear-cytoplasmic interactions. The cytoplasm designated *nap*, found in most *B.napus* varieties, all normally male fertile, is capable of conferring male sterility on a limited number of *B.napus* nuclear genotypes that lack corresponding fertility restorer alleles, such as the cultivar 'Bronowski' (8, 9). Male fertile 'maintainer' forms of such genotypes possess a cytoplasm designated *cam* that appears to be derived from the related species, *Brassica campestris* (8, 10). A third cytoplasm, designated 'Polima' or *pol*, confers male sterility on most *B.napus* varieties (11). A few genotypes have been identified that restore *pol* sterility and restoration occurs through the action of dominant alleles at either of two different nuclear gene loci (12). Both the *nap* and *pol* cytoplasms, therefore, can induce male sterility in appropriate nuclear genetic backgrounds; the only cytoplasm identified in*Brassica napus* that has not been found to induce male sterility seems to have originated from *Brassica campestris*.

Studies in this (13) and another laboratory (14) have shown that as result of DNA rearrangements in the *pol* mitochondrial genome, the gene coding for subunit 6 of the mitochondrial ATPase, *atp6*, is preceded by and co-transcribed with a chimeric open reading frame (ORF) that we have designated *orf224*, the N-terminal coding region of which is derived from a standard plant mitochondrial gene, *orfB*. In floral tissue of *pol* CMS plants,

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transcripts of the region are predominantly dicistronic. Nuclear restorer genes specifically affect transcripts of the region such that the ratio of mono- to dicistronic transcripts is increased (13–15). In the *nap* mitochondrial genome no other genes in the immediate vicinity of *atp6* are evident and *atp6* transcripts are monocistronic. The *atp6* gene appears to be organized and expressed identically in the *nap* and *cam* cytoplasms, since *B. campestris* restriction fragments to which the *atp6* gene has been mapped (16) have not undergone rearrangement in *B.napus* (17), and since *nap* and *cam atp6* transcripts appear identical (15). To further investigate the possible role played by this chimeric gene region in specifying the CMS trait, we have constructed a physical map of the *pol* mitochondrial genome. We have found that the organizational differences between the sterile *pol* and fertile *cam* mitochondrial genomes are confined to an additional DNA segment in *pol* mtDNA that encompasses the previously described *orf224* rearrangement.

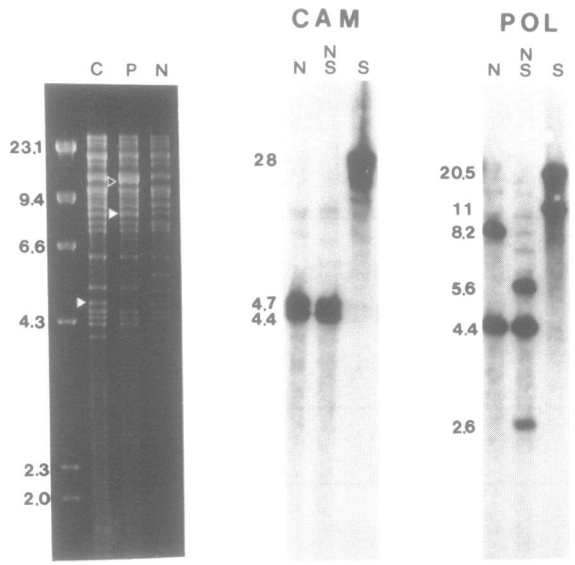

Figure 1. Left panel: *Nru*I digests of *Brassica napus* mtDNAs isolated from the fertile cultivar Bronowski (*cam* cytoplasm, C), the Polima (*pol*) CMS cultivar 2007 (P) and the fertile cultivar Regent (*nap* cytoplasm, N); closed arrows indicate fragments that differ in mobility between the *pol* and *cam* mtDNAs; the open arrow indicates the position of the linear mtDNA plasmid. Middle and left panel: hybridization of a *B.campestris Pst*I fragment spanning the *atp6* gene to fertile *B.napus* 'Bronowski' (CAM) or *pol* CMS 2007 (POL) mtDNAs digested with *Nru*I (N), *Sal*I (S) or *Nru*I + *Sal*I (NS). Numbers on the side of each panel refer to sizes of DNA fragments in kb.

MATERIALS AND METHODS

Plant material

According to the convention of Kemble and Barsby, *Brassica* cytoplasms are designated by parenthesized italics following the cultivar name. The *nap* CMS line Bronowski (*nap*) and its male fertile maintainer, Bronowski (*cam*), were obtained from Dr Z.Fan, Agriculture Canada, Ottawa. The sources of other *Brassica* lines have been described previously (13). Plants were grown to maturity in greenhouse growth rooms of the McGill Phytotron.

Mitochondrial probes

A set of *Pst*I and *Sal*I fragments cloned in pUC8, representing over 70% of the *B.campestris* mitochondrial genome, was provided by Dr C.Makaroff, Miami University, Oxford, Ohio. A second set of clones representing an additional 20% of the *B.campestris* mitochondrial genome was generated by cloning gel-purified *Kpn*I mtDNA fragments into the phagemid vector pBluescript SK– (Stratagene). Each of the cloned fragments was gel-purified, labeled with ^{32}P by nick translation, and hybridized to blots of single and selected double *Bgl*I, *Kpn*I, *Pst*I, *Nru*I, and *Sal*I mtDNA digests. Blots were also hybridized with the heterologous *atpA, atp9, cob, cox1, cox2, cox3, nad1, orf25, rrn26* and *rrn18* probes described previously (13), with an oligonucleotide corresponding to the *orfB* homologous region of *orf224* (13) and with Petunia probes for specific *nad1* exons (18), *mat-r* (18), *nad3* and *rps12* (19) (provided by Dr Maureen Hanson, Cornell University, Ithaca, New York).

Nucleic acid analysis

Mitochondrial DNA was extracted from approximately 50 g of young flower buds essentially by the procedure of Kemble (20). After precipitation with ethanol, the nucleic acid pellet was dissolved in 300 μl of water, reprecipitated, and the recovered mtDNA samples were digested to completion. The digested DNAs were subjected to electrophoresis on 0.7% agarose gels containing 2 μg/ml RNAse A for 16 hrs. Following staining with 0.5 μg/ml ethidium bromide and destaining in distilled water, the gels were photographed, treated twice with 0.25 M HCl for 15 min, once with 0.5 M NaOH/1.5 M NaCl for 30 min, and then transferred onto GeneScreen-Plus membranes (Dupont) by capillary blotting in 0.25 N NaOH/1.5 M NaCl for 6–12 hrs. Membranes were hybridized to radiolabelled probes in 1 M NaCl, 1% SDS, and 10% dextran sulfate at 65°C for 12–16 hrs. Membranes were subsequently washed twice for 5 min in 0.3 M NaCl/0.03 M Na citrate at room temperature before autoradiography. The sizes of the hybridizing fragments were estimated using *Hin*dIII digested phage λ and the BRL 1 kb ladder DNAs as standards. Mitochondrial RNA was isolated, size fractionated on 1.2% agarose/2.2 M formaldehyde gels (21), transferred to membranes, and hybridized with DNA probes as described by Singh and Brown (13).

RESULTS

Physical mapping

Initial comparisons of restriction digests of different *B.napus* mtDNAs indicated that the mitochondrial genomes of *pol* CMS lines and of the fertile variety 'Bronowski', which is thought to contain the *cam* cytoplasm, were very similar. Only one or two fragment polymorphisms were detected between the *pol* and the Bronowski *(cam)* mtDNAs for any of the five restriction enzymes employed. The similarity of the *Nru*I digests of the two mtDNAs is shown in Fig 1A. Apart from the occurrence in the *pol* mtDNA sample of an 11.3 kb linear plasmid DNA, previously been shown to be unrelated to CMS (22), the only apparent differences are the absence in *pol* mtDNA of a 4.7 kb Bronowski fragment and the presence of an additional 8.2 kb fragment in *pol* mtDNA. Both DNAs share several fragment differences with the common *B.napus nap* mitochondrial genome (Fig. 1A). This suggested that there were few organizational differences between the sterile *pol* and fertile Bronowski *(cam)* mtDNAs and therefore that the number of rearranged regions representing potential sites for determinants of the *pol* male sterility might be relatively few.

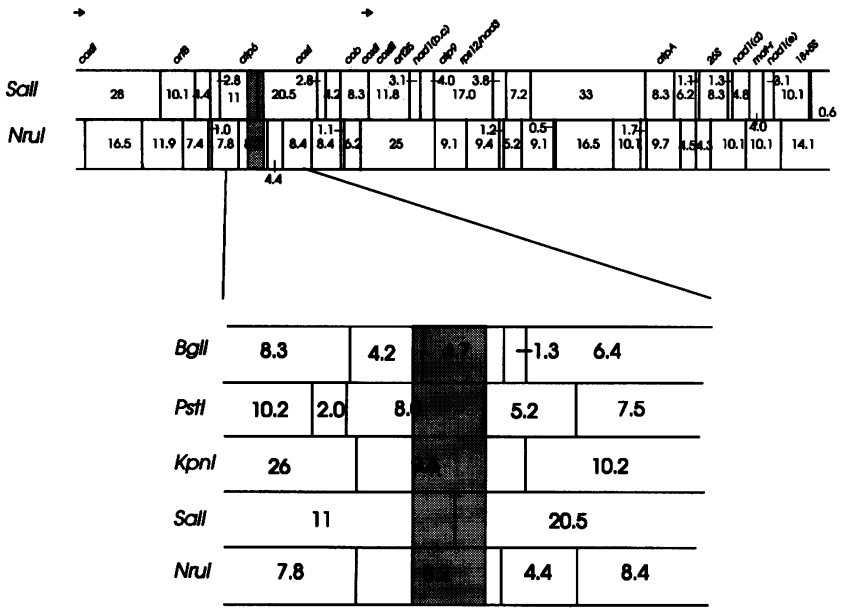

Figure 2. Physical and genetic maps of the Polima mitochondrial genome deduced by probing comparative digests of the fertile Bronowski line and the *pol* CMS line 2007 with probes representing 90% of the *B.campestris* mitochondrial genome (Makaroff and Palmer, 1987) as well as various heterologous gene probes. Arrows above the top map indicate the position of repeated sequences active in recombination. The lower portion presents a more detailed map of the region surrounding the *atp6* gene; shaded portions indicate the inserted region not present in *cam* mtDNA; the unshaded portions of both maps indicate regions that are identical in organization in the *cam* and *pol* mtDNAs.

To further characterize the structural differences between the *pol* and *cam* mtDNAs, a set of clones covering 90% of the *B.campestris* genome, as well as various heterologous mitochondrial gene probes (see material and methods) were hybridized to membranes containing single and selected double *BglI*, *KpnI*, *PstI*, *NruI*, and *SalI* digests of the *pol* and Bronowski mtDNAs. In every case, the sizes of the hybridizing fragments from Bronowski mtDNA corresponded precisely with those predicted from the physical map of the *B.campestris* turnip variety analyzed by Palmer and Shields (5) and all of the fragments predicted from the turnip map were detected with at least one probe in the Bronowski mtDNA sample. This indicated that the Bronowski map was identical to that of *B.campestris* and confirmed that the cytoplasm of the *B.napus* variety Bronowski was derived from *B.campestris*. Only one probe, the *PstI* fragment carrying the ATPase subunit 6 (*atp6*) gene detected differences between the two mtDNAs (Fig. 1, middle and right hand panel), indicating that organizational differences between the *pol* and *cam* mitochondrial genomes are restricted to the *atp6* region. This finding is consistent with mitochondrial transcript analyses, which indicate that the *atp6* region is expressed differently among fertile, *pol* CMS and fertility restored *pol* cytoplasm plants (13–15).

When compared on Southern blots, for all 5 enzymes used, the altered *pol* fragments associated with the *atp6* gene were found to be 3.5 kb larger than the corresponding *cam* fragments (*NruI* 8.2 vs 4.7 kb, *KpnI* 9.6 vs 6.1 kb, *PstI* 8.0+5.2 vs 9.7 kb, *SalI* 20.5+11 vs 28 kb and *BglI* 4.2+4.7 vs 5.4 kb). Because no other structural differences could be detected, this indicated that an additional 3.5 kb *pol* mtDNA segment flanking the *atp6* gene represented the single organizational difference between the *pol* and *cam* mitochondrial genomes. The physical map of the *pol* mitochondrial genome deduced from this analysis is shown in Fig. 2. The shaded region of the map corresponds to the additional *pol* mtDNA segment; the approximate locations of different mitochondrial genes, positioned by hybridizing various digests with heterologous gene probes, are indicated. The locations of some of these genes have been previously determined (7, 16) and the genetic map is largely consistent with a *B.campestris* genetic map independently constructed by Nugent and Palmer (personal communication). This analysis also indicated that the different mtDNA fragments were present at approximately the same stoichiometries in both the *cam* and *pol* mitochondrial genomes, and that the *pol* mitochondrial genome contains two recombinationally active copies of the 2 kb *B.campestris* mtDNA repeat (5, data not shown). The *pol* mitochondrial genome therefore has the tripartite structure of most *Brassica* mtDNAs (5, 17).

Characterization of the *pol* rearrangement

To further characterize the *pol* mtDNA alteration, the region represented by the *pol* 8.2 kb *NruI* fragment (Fig. 1) was cloned and compared by more detailed restriction mapping to the corresponding *cam* 4.7 kb *NruI* fragment (Fig. 3). The presence of conserved restriction sites on either side of the additional *pol* DNA segment indicated the limits of the *pol* mtDNA rearrangement. One rearrangement endpoint, situated immediately upstream of *atp6*, has previously been characterized at the nucleotide sequence level (13, 14). The map distances from restriction sites located within the sequenced *pol* region to the *PstI* site situated near the opposite end of the *pol* insert indicated that the endpoints proximal and distal to *atp6* are situated approximately 4.5 kb apart. Since the rougher mapping analysis indicated a net 3.5 kb of additional *pol* DNA, this suggested that the *pol* rearrangement involved the loss of *cam* sequences from the region upstream of *atp6* as well as a 4.5 kb DNA

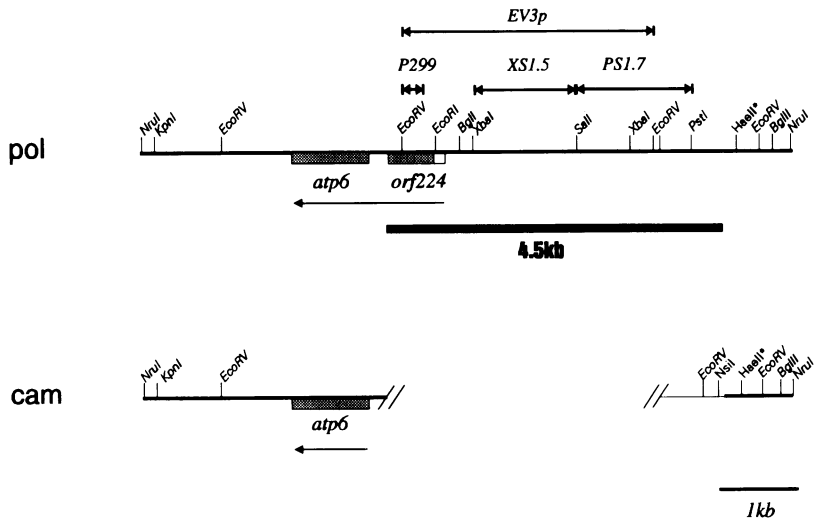

Figure 3. Detailed map of the *Nru*I fragments spanning the *atp6* gene in *pol* and *cam* mtDNAs. The bar between the two maps indicates the limits of the additional 4.5 kb *pol* segment relative to *cam* mtDNA. Hatched regions indicate the locations of the *atp6* and *orf224* open reading frames. The blank region of the *orf224* region represents *orfB* homologous sequences. The regions referred to as EV3p, P299, XS1.5 and PS1.7 were used as probes in gene organization and expression studies. The asterisk (*) next to the *Hae*II site is used to indicate that other unmapped *Hae*II sites are also present in the regions shown. The thinner portion of the line in the region of the *cam* genome distal to *atp6* represents sequences that are deleted from the *pol* mitochondrial genome. The arrows indicate the direction of transcription.

insertion/deletion. This 4.5 kb region will subsequently be referred to as the '4.5kb segment'.

The presence of *Eco*RV and *Nsi*I sites that are absent from *pol* mtDNA in the region of *cam* mtDNA upstream of *atp6* suggested that the DNA segment spanning these sites represented the region deleted from the *pol* mitochondrial genome. When this *Eco*RV−*Nsi*I fragment was subcloned and used to probe digests of the *cam* and *pol* mtDNAs, a strong hybridization signal was generated from *cam* fragments carrying the *atp6* gene, as expected, but only faint signals, apparently due to a short repeat element (see below) were detected from the *pol* mtDNA digests (data not shown), confirming that most of the sequences within the *cam* *Eco*RV−*Nsi*I segment were missing from the *pol* mitochondrial genome.

To determine if sequences of the additional 4.5 kb *pol* segment were present elsewhere in the *pol* and *cam* mitochondrial genomes, 1.5 kb *Xba*I−*Sal*I and 1.7 kb *Pst*I−*Sal*I fragments (Fig. 3) were subcloned (designated clones XS1.5 and PS1.7, respectively) and used to probe gel blots of *Nru*I and *Sal*I single and double digests of *B.napus pol* and *cam* mtDNAs. Both clones detected only a single major copy in *pol* mtDNA. Clone PS1.7, in addition to the main copy, detected 5−8 other weakly hybridizing fragments, which were also present in *cam* mtDNA (data not shown). Similar results were obtained using a probe derived from the opposite end of the *pol* insertion (clone P299, Fig. 3). These observations suggest that short repeats such as those which serve as sites for mitochondrial genome rearrangements (23−25) may be present at each end of the *pol* 4.5kb segment.

The additional *pol* 4.5 DNA segment is present in *nap* mtDNA

Clones PS1.7 and XS1.5 were also used to determine if the sequences of the additional 4.5kb segment were present in the *nap* mitochondrial genome; both clones detected single major hybridizing *Nru*I, *Sal*I and *Nru*I+*Sal*I fragments (Fig. 4). Clones XS1.5 and PS1.7 detected *Sal*I fragments of 18.4 kb and 7.6

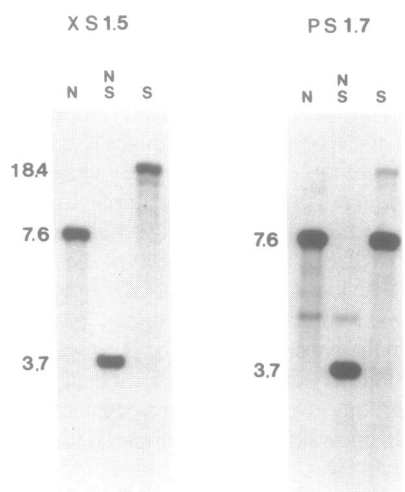

Figure 4. Organization of the *pol* insert sequences in the *nap* mitochondrial genome. Westar (*nap*) mtDNA, digested with *Nru*I (N), *Sal*I (S), or a combination of the two enzymes (N/S) was probed with nick-translated clone XS1.5 or PS1.7. The sizes, in kb, of the hybridizing fragments are indicated on the left of each panel.

kb respectively, suggesting that these regions adjoining the *pol* segment *Sal*I site were detecting a segment of *nap* mtDNA containing adjacent 18.4 and and 7.6 *Sal*I fragments. Both clones detected *Nru*I and *Nru*I−*Sal* I fragments of 7.6kb and 3.7kb respectively. The sizes of the combination digest fragments as well as the analysis of other single and double digests, allowed the hybridizing region to be localized on the *B.napus* map constructed by Palmer and Herbon (17) (Fig. 5). Although the major hybridizing region in *nap* mtDNA also maps close to the *atp6* gene, it is located within a region that has undergone rearrangement during the evolution of the *nap* and *cam* cytoplasms (17), and the sequences surrounding the *nap* segment

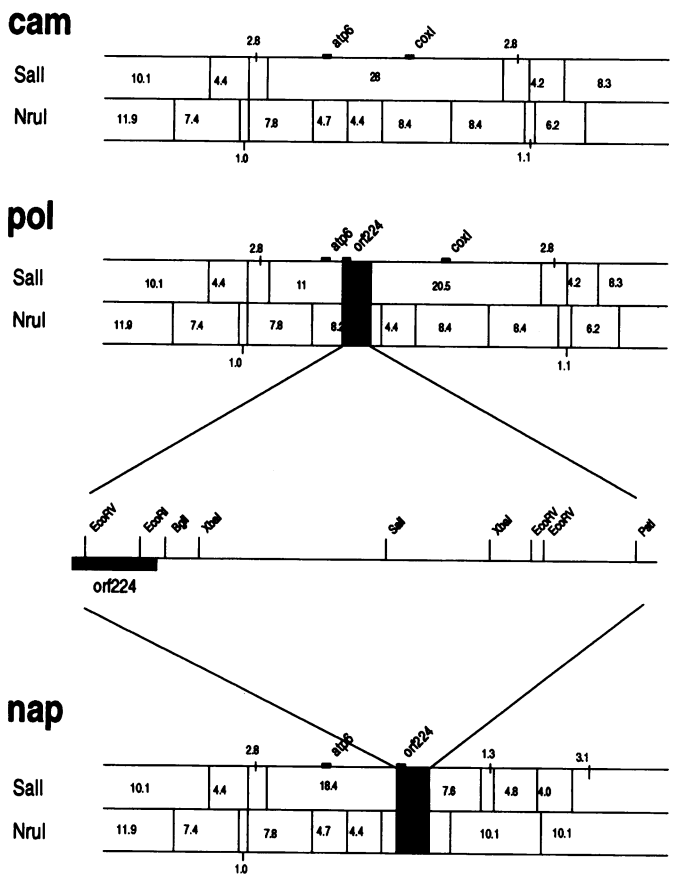

Figure 5. Comparisons of physical map locations of the sequences of the *pol* insertion in *pol*, *nap* and *cam* mtDNAs. The shaded regions indicate the position of the 4.5 kb *pol* mtDNA segment. The expanded region shows a detailed map of the *nap* and *pol* segments; all restriction sites shown map to similar positions in the *pol* and *nap* 4.5 kb segments. Although sequences homologous to the *pol orf224* gene are present in *nap* mtDNA, the presence of an intact *nap* open reading frame at this position has not been confirmed.

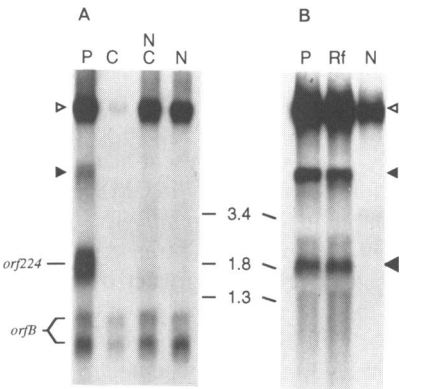

Figure 6. Transcripts of the *pol* 4.5kb segment in *pol*, *cam* and *nap* mitochondria. **A:** a blot of a formaldehyde agarose gel of mitochondrial nucleic acid from Westar *pol* (CMS, P), Bronowski *cam* (fertile, lane C), Bronowski *nap* (CMS, NC) and Westar *nap* (N, fertile) floral tissue was hybridized with labeled probe EV3p (Fig. 3). **B:** blot of formaldehyde agarose gel of mtRNA from Westar *pol* (CMS, P), Westar *nap* (N) and Westar-Rf *pol* (fertility restored, Rf) was hybridized with labeled probe PS1.7. Open arrows indicate locations of mtDNA, and the smaller and larger closed arrows the 6 kb and 2 kb transcripts of *pol* cytoplasm, respectively; the positions of *orf224*, *orfB* and marker RNAs are indicated. The greater intensity of the 6 kb transcript in B results from the relative increase in film exposure, as evidence by the increased signal from mtDNA.

are therefore distinct from those flanking the *pol* segment. The hybridizing fragments map to the site of a *nap* DNA segment (insertion E of Fig 1 of ref. 17) that has not previously been found in other *Brassica* mtDNAs. The similarity in the size of the *nap* and *pol* segments, as well as the similarity in position of *Bgl*I, *Pst*I and *Sal*I restriction sites within these DNA regions, suggested that the sequences within the two segments are organized in a similar manner. A second hybridizing region possessing a more limited homology to clone PS1.7 was also detected in *nap* mtDNA (Fig. 4). Interestingly, in this case the sizes of the hybridizing fragments were the same as those of the *atp6* gene in *nap* mtDNA, suggesting the presence of a sequence repeated at both the *nap* segment and *atp6* loci.

To further characterize the relationship between the *nap* and *pol* 4.5kb segments, the *nap* mtDNA region was cloned and a more detailed map was constructed. As shown in Fig. 5, the eight mapped restriction sites span nearly the full length of the 4.5kb segment, extending from an *Eco*RV site located in the *atp6* proximal region of *orf224* to the *Pst*I site in the *atp6* distal portion. All eight of the mapped sites were found to be located at positions corresponding to those in the *pol* 4.5kb segment (Fig. 5). This indicated that virtually all of the *pol* 4.5kb segment, including a region possessing similarity to the CMS-associated *orf224* gene, was present in *nap* mtDNA and confirmed that the sequences of the *nap* and *pol* 4.5kb segments were similarly organized. It should be emphasized, however, that the presence of an intact *orf224* open reading frame in the *nap* segment has not yet been demonstrated.

Transcriptional analysis of the 4.5 kb segment

It has been previously shown that the region of the 4.5 kb segment immediately upstream of *atp6* contains an open reading frame, *orf224*, that is co-transcribed with *atp6* (13). The *orf224* gene is chimeric, consisting of sequences derived from a standard mitochondrial gene, *orfB*, and sequences of unknown origin (13). Probes containing sequences derived from the *orfB* homolgous region of *orf224* detect both *orf224* and *orfB* transcripts in *pol* mitochondria, but only *orfB* transcripts in *nap* cytoplasm plants (M.Singh and G.Brown, unpublished results); probes containing sequences derived only from the remaining part of *orf224*, which does not possess sequence similarity to *orfB* or any other known sequence, detect only *orf224* transcripts in *pol* plants and do not detect transcripts in *nap* mitochondria (13). Only the *orf224* transcripts are affected by nuclear fertility restoration (13, M.Singh and G.Brown, unpublished results).

To determine if regions of the *nap/pol* 4.5kb segments other than *orf224* are transcribed, an *Eco*RV fragment extending from the C terminal coding region of *orf224* to a site located approximately 600 bp from the *atp6* distal insertion endpoint (fragment EV3p, Fig. 3) was used to probe gel blots of floral mtRNA from Westar *nap* (fertile), Westar *pol* (CMS), and fertile (*cam*) and *nap* CMS Bronowski plants (Fig. 6A). As expected, this probe detected abundant 1.8−2.0 kb *orf224/atp6* transcripts as well as smaller 0.9 and 1.2 kb transcripts derived from the intact *orfB* gene in *pol* CMS plants; only *orfB* transcripts were detected in *nap* and *cam* cytoplasm plants. The EV3p probe also detected a large (approx. 6 kb), relatively low-abundance transcript in *pol* CMS mtRNA (closed arrow, Fig. 6A). To determine if this transcript was also present in fertility restored

pol cytoplasm plants and to determine which region of the 4.5kb segment it was derived from, subclones XS1.5 and PS1.7 were hybridized to gel blots of mtRNAs from the near isonuclear fertile Westar (*nap*), sterile Westar (*pol*) and Westar -*Rf* (*pol* cytoplasm, nuclear fertility restored) lines. As seen in Fig. 6B, transcripts of approximately 6 and 2 kb, present at roughly equal levels, were detected with subclone PS1.7 in CMS and nuclear-restored *pol* mtRNAs, but not in fertile *nap* mtRNA [the greater intensity of the signals generated by the 6 kb transcript in Fig. 6B relative to 6A is the result of relatively greater overall film exposure, as evidenced by the increased signal generated by mtDNA (open arrows)]. As nearly all of the sequences present in clone PS 1.7 are also present in EV3p, it is likely homology also exists between the 2 kb transcript and the EV3p probe, but that this signal was obscured by the more abundant *orf224/atp6* transcripts of the same size. No transcripts were detected in any of the samples with either the XS1.5 clone, or with the *Eco*RV−*Nsi*I clone representing the region of *cam* mtDNA that is deleted from the *pol* mitochondrial genome (not shown).

We conclude from this analysis that abundant transcripts of the insert are derived only from the *orf224* region although the lower abundance 6 and 2 kb transcripts observed in *pol* cytoplasm plants appear to be derived in part from the *atp6* distal region of the insert. As the *atp6* distal transcripts are identical in *pol* CMS and fertility-restored plants, nuclear restoration affects only the *atp6* proximal transcripts that span the co-transcribed *orf224* gene. No stable transcripts are derived from the 4.5kb segment in either CMS or fertile *nap* cytoplasm plants.

DISCUSSION

Transposition of a DNA region associated with CMS

The observation that sequences within the 4.5 kb segment from *pol* are similarly organized but situated in a different context in the *nap* mitochondrial genome suggests that this DNA region underwent transposition during the evolution of the *pol* and *nap* cytoplasms. This is to our knowledge, the first example of a rearrangement of this type to be found in plant mtDNA. Rearrangements in plant mitochondrial genomes are thought to be generated through infrequent recombination events across relatively short repeated sequences (23−25). The finding of regions with limited homology to sequences at both endpoints of the 4.5 kb segment in various *Brassica* mitochondrial genomes suggests the transposed/deleted segment is flanked by such short repeats. Recombination across these short repeats could have led to the excision of the segment as a circular DNA, with retention of the repeat on the excised circle as well as at the site of the deletion. Transposition of the sequence could have been achieved through recombination between the repeat on the excised circle and the repeat at another mtDNA site. Similar double recombination models have been used to explain other mtDNA rearrangements (23, 25) and analysis of the sequences surrounding the transposition endpoints may clarify the mechanisms involved.

Although *orf224* or a closely related sequence is present in *nap* mtDNA, this sequence is not expressed. The failure to detect these transcripts is somewhat surprising, since the corresponding sequence of the transposed segment, in the context of different surrounding sequences, is expressed as stable transcripts in isonuclear *pol* cytoplasm plants (13). Recent data obtained in this laboratory suggest that the 5′ termini of the *orf224/atp6* transcripts of *pol* mitochondria mapping upstream of *orf224* are generated by *in vivo* initiation events. The absence of these transcripts in *nap* mitochondria could therefore result from instability of the *nap* transcripts, or from a lack of activity in the corresponding promoters, due either to mutation or possibly to the influence of remote sequences outside of the borders of the inserted segment, between 1 and 3 kb away. It should be possible to distinguish between these alternatives experimentally, and the results may provide insight into processes mediating plant mitochondrial gene expression.

The presence of the 4.5 kb DNA segment in both the *nap* and *pol* mitochondrial genomes suggests that this sequence was present in the most recent common ancestor of the *nap* and *cam* cytoplasms. The *pol* and *cam* mitochondrial genomes are clearly more closely related to one another than either is to *nap* mtDNA, and it is therefore likely that loss of the 4.5 kb segment occurred during the evolution of the *cam* cytoplasm after the *pol*−*cam* divergence. The association of the loss of a specific DNA segment with the conversion of a sterile to a fertile cytoplasm is reminiscent of the mtDNA alterations observed upon reversion of *cms*-T maize to male fertility (25−27). The consistent loss or alteration of the T-*urf13* gene in these revertants has provided powerful evidence for the association of this chimeric mitochondrial gene with CMS (1, 2).

Mitochondrial DNA rearrangements and *pol* male sterility

Several approaches have proven useful in identifying mtDNA regions potentially capable of specifying CMS, including the analysis of transcripts in fertile, sterile and restored lines (3, 13, 15), and the characterization of the mitochondrial genomes of somatic revertants and hybrids (4, 6, 25−27). In general, the structural comparison of the mitochondrial genomes of fertile and male sterile cytoplasms has been of only limited use in this regard, since in most cases, fertile and CMS mtDNAs are differentiated by multiple rearrangements and it is not possible, in the absence of other information, to clearly associate a specific rearrangement with the male sterile trait. In a few cases, however, such as in the sunflower *Helianthus annuus* (28, 29), the fertile and sterile mtDNAs are sufficiently similar that the number of rearrangements potentially responsible for CMS can be limited to only a few possibilities. This is clearly the case for the *Brassica* mitochondrial genomes analyzed here.

We have shown by comparative physical mapping analysis that structural differences between the fertile *cam* and sterile *pol* mitochondrial genomes, and hence the determinants for the *pol* CMS, are confined to the additional DNA situated immediately upstream of the *pol atp6* gene. Detailed analysis indicates that within the delimiting endpoints of this insertion there is an approximately 4.5 kb *pol* DNA segment that is not found in the *cam* mitochondrial genome, and that approximately 1 kb of *cam* mtDNA situated immediately upstream of *atp6* is missing in *pol* mtDNA. Consideration of mtDNA structural differences alone, therefore, would suggest that the *pol* CMS could result from either the additional sequences present in *pol* mtDNAs or from the segment of *cam* mtDNA that is missing in the *pol* genome.

Gene structure and expression analysis, however, suggests that it is more likely that *pol* male sterility results from alterations at the endpoint of the 4.5kb segment proximal to *atp6*. This region is the only one of 13 analyzed mitochondrial gene regions found to be expressed differently between sterile *pol* and fertile *nap* or *cam* cytoplasm plants (13, 15) and also the only one of these regions whose transcripts were found to be affected by fertility restoration (13, 15). The *atp6* proximal region of the insertion

contains a chimeric open reading frame, *orf224*, that is co-transcribed with *atp6*, and co-transcription of such chimeric genes with standard mitochondrial genes is characteristic of other CMS associated gene regions (2). No transcripts of the *pol* insertion region immediately upstream of *orf224*, nor of the *cam* sequences upstream of *atp6* that are absent from *pol* mtDNA, could be detected, and it is therefore unlikely that either region is involved with CMS. Although transcripts with homology to the region of the pol 4.5kb segment most distal to the *atp6* gene were detected, because of the relatively low abundance of these transcripts, their significance is uncertain. In any case, since nuclear fertility restoration had no effect on these transcripts, the region seems less likely than the *orf224/atp6* region to be directly related to the *pol* CMS trait. The failure to detect transcripts of the 4.5kb segment in either sterile or fertile *nap* cytoplasm plants indicates that the region is not associated with the *nap* CMS trait.

Several independent lines of evidence suggest that the *pol orf224/atp6* region may specify the *pol* CMS trait of *B.napus*. The same set of *B.campestris* probes used in the mapping analysis reported here has been used to perform a more extensive and potentially complete analysis of the transcripts of near isonuclear fertile *nap*, *pol* CMS and fertility-restored *pol* cytoplasm plants; no transcript differences between isonuclear *nap* and *pol* plants other than those reported for the *orf224/atp6* region were detected and only transcripts of the *orf224/atp6* region were found to be affected by nuclear fertility restorer allele (M.Singh, unpublished observations). In addition, this region has been found to be consistently present in sterile *Brassica* somatic hybrids containing recombinant *pol/ole* mitochondrial genomes (T.Ketela and G.Brown, unpublished observations). In this respect, the present finding that this locus falls within the single region that is organized differently in the sterile *pol* and fertile *cam* mitochondrial genomes, complements parallel studies on mitochondrial gene expression and provides strong support for view that this chimeric mitochondrial gene region specifies the *pol* CMS trait. Although the precise mechanism by which expression of this region influences male sterility remains unknown, the amenability of *Brassica* to transformation and regeneration makes this system highly suitable for developing transgenic approaches that should allow this problem to be rigorously addressed.

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