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Molecular analysis of Polima cytoplasmic male sterility in *Brassica napus*

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December, 1992

A thesis submitted to the Faculty of Graduate Studies and
Research in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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My son, Rahul

From whom I stole the time he deserved most

ABSTRACT

To identify region(s) of the mitochondrial genome that might be involved in specifying the "Polima" (*pol*) cytoplasmic male sterility (CMS) of *Brassica napus*, transcripts corresponding to 14 mitochondrial genes and DNA clones representing >90% of the mitochondrial genome of *Brassica campestris* were analyzed in *nap* (male fertile), *pol* (male sterile) and nuclear fertility-restored *pol* cytoplasm plants. CMS-correlated transcriptional differences among these plants were detected only with the ATPase subunit 6 (*atp6*) gene. Sequence analysis of the *atp6* gene regions of *pol* and *nap* mitochondrial DNAs show that rearrangements in the *pol* mitochondrial genome upstream of *atp6* have generated a chimeric 224-codon open reading frame, designated *orf224*, that is cotranscribed with *atp6*. In male sterile plants, most transcripts of this region are dicistronic, comprising both *orf224* and *atp6* sequences. In fertility restored-plants, genes at either of two distinct nuclear restorer loci specifically alter this transcript pattern, resulting in predominantly monocistronic *atp6* transcripts. The effect of the restorer locus on *orf224/atp6* transcripts does not seem to be tissue or developmental stage specific. *orf224* comprises a portion of the mitochondrial gene, *orfB*, fused to sequence of unknown origin. The *pol* mitochondrial genome contains an apparently functional copy of *orfB*. The expression of the *atp6* region is developmentally regulated in *pol* plants such that levels of monocistronic *atp6* transcripts are increased in seedlings as compared to the floral tissue. Preliminary data indicate that the chimeric gene, *orf224*, is expressed at the protein level in *pol* plants.

Résumé

Dans le but d'identifier une ou plusieurs régions du génome mitochondriale pouvant jouer un rôle dans l'expression de la stérilité mâle cytoplasmique (SMC) chez *Brassica napus* ayant le cytoplasme stérile *pol*, l'ARN messager correspondant à 14 gènes mitochondriales ainsi qu' àdes clones représentant plus de 90% du génome mitochondriale de *Brassica campestris* a été analysé chez *nap* (fertile), *pol* (stérile) et chez des plantes ayant des gènes de restauration de la fertilité. Des différences au niveau de la transcription pouvant être corrélées avec la SMC n'ont été détectées parmi ces plantes qu' avec le gène *atp6*. Le séquençage de cette région chez *nap* et *pol* a révélé un réarrangement du génome mitochondriale de *pol* en amont du gène *atp6* de sorte qu'un nouveau gène chimérique appelé *orf224* se trouve cotranscrit avec *atp6*. Chez les plantes stériles, la majorité des ARN messagers provenant de cette région sont bicistroniques (*orf224-atp6*). Chez les plantes contenant un des deux gènes de restauration de la fertilité, présent dans deux régions differentes du génome, ce gène semble altérer la transcription de cette région de sorte que la forme monocistronique du gène *atp6* prédomine. L'effet des locus de restauration sur les ARN messagers *orf224/atp6* ne semble pas spécifique au stade de développement de la plante ou au tissu. Le gène chimérique *orf224* contient une partie du gène mitochondriale *orfB* fusionnée à une séquence d'origine inconnue. Le génome mitochondriale *pol* contient également une copie apparemment fonctionelle de *orfB*. La transcription du gène *atp6* semble liée au stade de développement de la plante *pol*, la forme monocistronique du gène *atp6* étant plus importante chez les très jeunes plantules que dans les tissus floraux. Des résultats préliminaires indiquent que le gène chimérique, *orf224*, est exprimé au niveau de protéine dans les plantes *pol*.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

- - Identification, cloning and structural characterization of the mitochondrial gene region (*orf224/atp6* region) associated with the "Polima" (*pol*) cytoplasmic male sterility in *Brassica napus*. Also, cloning and structural characterization of the corresponding region (*atp6* gene region) of the mitochondrial genome of male fertile cytoplasm (*nap*) of *Brassica napus*.

- - Provided first demonstration that mitochondrial *atp6* gene region is differentially expressed among *nap* (male fertile) , *pol* (male sterile) and nuclear fertility-restored *Brassica napus* plants, including near isonuclear lines.

- - For the first time screened mitochondrial DNA sequences covering >90% of the genome for their differential expression, at transcript level (RNA gel blot analysis), between male fertile and male sterile near isonuclear plants and demonstrated that *atp6* region is the only region of the screened genome whose altered expression is correlated with *pol* CMS.

- - Demonstrated for the first time by segregation analysis of the backcross progeny, with respect to the differential expression of *atp6* gene region in male sterile and male fertile plants, that altered expression of this region is very tightly linked to the fertility-restoration and CMS trait.

- - Demonstration that expression of the *atp6* gene region in *pol* plants is developmentally regulated.

- - Provided evidence that *pol* mitochondrial genome contains apparently functional copy of the *orfB* gene, indicating that *pol* CMS is not caused by the absence of an intact, expressed *orfB* gene.

- - Provided evidence that the complex transcript pattern of the *atp6* region in fertility restored *pol* plants appears to be generated by a combination of multiple transcription initiation and processing events and the nuclear restorer locus alters transcripts of this region most likely by affecting RNA processing.

- - Demonstration that the effect of the restorer locus on transcripts of *atp6* region is not tissue or developmental stage specific.

- - Demonstration of the presence of a chimeric 224-codon open reading frame, designated *orf224*, upstream of *atp6* gene. *Orf224* comprises a portion of the mitochondrial gene *orfB* fused to a sequence of unknown origin. Provided evidence that *orf224* is co-transcribed with *atp6*.

- - Developed a relatively fast procedure for isolating plant mitochondrial RNA, which is particularly suitable for isolating mitochondrial RNA from a large number of samples.

PREFACE

This thesis has been prepared as per the regulations of the Faculty of Graduate Studies and Research of McGill University. It consists of an abstract (in English and French), introduction and literature review, three chapters of experimental results presented in manuscript form, overall conclusion and appendices. All of the literature cited in this thesis has been combined and appears last.

Chapter II has been published in the scientific journal **The Plant Cell.** The complete reference of this paper is :

Singh, M., and Brown, G.G (1991). Suppression of cytoplasmic male sterility by nuclear genes alters expression of a novel mitochondrial gene region. **Plant Cell 3,** 1349-1362.

Chapter III has been submitted for publication. This manuscript is authored by **M. Singh and G.G. Brown.**

Chapter IV will be submitted soon for publication.

Preparation of manuscripts comprising various chapters was a collaborative endeavor undertaken with Dr. G. G. Brown. All the experimental results presented in this thesis, except otherwise stated in the acknowledgments of chapters III and IV, are solely the work of the candidate. Since this thesis has been written in manuscript format, some degree of redundancy is unavoidable.

Results presented in chapter II were considered interesting enough for oral presentation (presented by the candidate) at the following scientific meetings:

1. "Third International Congress of the International Society for Plant Molecular Biology", October 6-11, 1991, Tucson, Arizona.
2. "6th Canadian Society for Plant Molecular Biology Annual Meeting", May 26-29, 1991, Laval, Quebec.

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ACKNOWLEDGMENT

I extend my heartfelt gratitude to Dr. Gregory G. Brown, my thesis supervisor, for allowing me to pursue my research career in his laboratory after the departure of my previous supervisor, Dr. D.P.S. Verma, from McGill University. I always admire Greg's extraordinary power to be patient when things do not work for a long time. This thesis is the result of his patience, support and encouragement during times of failures. I must thank Greg for sending me to all those expensive national and international scientific gatherings. Greg has been very generous in supporting me financially from his research grants, which enabled me to avoid teaching assistantships during most of my stay at McGill.

I avail this opportunity to thank Drs. Paul Lasko, Stephen C. Gleddie and Adrian Tsang, members of my supervisory committee, for their time and help of every kind during the course of this study.

I sincerely endorse the assistance showered by my present and past colleagues and lab mates through different means and ways throughout the course of this investigation.

The financial assistance received in the form of a scholarship awarded by Govt. of India is gratefully acknowledged. I would also like to acknowledge the financial support I received from biology department in the form of a "Graduate Student Travel Grant".

I reserve my special thanks to my wife Dr. Krishna Singh, my parents, sister and brothers for their unconditional help, emotional support and love. This investigation would not have reached this stage without Krishna's cooperation and sacrifice. Krishna has tolerated the endless stresses of a scientific career during all these years.

I would like to thank Guy L'Heureux and Robert Lamarche for providing top quality photographic services.

I am grateful to those who provided seeds, mitochondrial DNA clones and manual help during the course of this work. These persons are acknowledged individually in the chapters of this thesis describing experimental results.

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ABBREVIATIONS

A	adenosine
ATP	adenosine triphosphate
atp (A,6,9)	plant mitochondrial genes for subunit α, 6 and 9 of the F1F0 ATP synthase, respectively
BmT	Toxin of fungus*Biopolaris maydis*, race T
bp	base pairs
BSA	bovine serum albumin
C	cytosine
°C	degrees centigrade
Ca^{++}	calcium ion
cam	*Brassica campestris* cytoplasm
CMS	cytoplasmic male sterility
cob	mitochondrial gene for apocytochrome *b*
cox (1,2,3)	plant mitochondrial genes for subunits 1,2 and 3 of cytochrome *c* oxidase, respectively
CsCl	cesium chloride
Da	dalton
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetracetic acid
G	guanosine
g	gravitational force
HCl	hydrochloride
HPLC	high pressure liquid chromatography
hr	hour
kb	kilobases

kDa	kilodalton
LiCl	lithium chloride
M	molar
min	minutes
mM	millimolar
mRNA	messenger ribonucleic acid
mt	mitochondrial
N	normal
NaCl	sodium chloride
nad (1,3,4,5)	plant mitochondrial genes for subunit 1,3,4 and 5 of NADH dehydrogenase, respectively
NADH	nicotinamide adenine dinucleotide (reduced form)
nap	napus, normal male fertile cytoplasm of *Brassica napus*
ogu	Ogura, male sterile cytoplasm of radish
ORF	open reading frame
pol	polima, male sterile cytoplasm of *Brassica napus*
Rf	plant nuclear gene for restoration of fertility
Rfp (1,2)	plant nuclear genes for restoration of fertility to Polima male sterile cytoplasm
RNA	ribonucleic acid
rps (3,12,13,14)	plant mitochondrial genes for ribosomal proteins S3, S12, S13 and S14, respectively
rrn (18,26)	plant mitochondrial genes for 18S and 26S ribosomal ribonucleic acids, respectively
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
T	thymidine

Tris	Tris-(hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
U	uridine
URF	unassigned reading frame
µg	microgram

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CHAPTER I

Introduction and Literature Review

Plants of the genus *Brassica* include some of the world's most important oilseed crops. *Brassica napus* (rape), grown in several parts of the world, is Canada's major oilseed crop and represents a significant contribution to Canadian revenue. Keeping in view the importance of *Brassica napus* to Canada's economy, appropriate strategies need to be developed for the improvement of this crop. Traditionally, crop improvement has been achieved through the development of hybrids of different inbred cultivated varieties which combine the desired traits of different parental lines. Such hybrids often yield more as a result of heterosis which arises from the complementation of deleterious alleles fixed in parental lines by inbreeding. The seed production from F_1 hybrids of rape produced by emasculation and cross pollination has been shown to exceed that of the parents by up to 60% (Sernyk and Stefansson, 1983; Fan et al., 1986). Thus, hybrid development would be highly useful for increasing seed yield and introducing desired characteristics into *Brassica* crops.

Since *Brassica napus* is a self pollinating crop, the large scale production of hybrid seed requires that a simple method be available for preventing the production of pollen by the seed parent of a cross. This can be done by incorporating a maternally transmitted inability to produce functional pollen, cytoplasmic male sterility (CMS), into the seed parent line. Plants possessing the CMS trait maintain female fertility and are unable to produce viable pollen unless they possess specific nuclear genes, termed restorers of fertility (*Rf*), which suppress the male sterile phenotype. The large scale production of hybrid seed is achieved by pollinating a CMS line with a variety that carries nuclear restorer gene(s). The CMS phenotype has been observed in more than 140 species (Laser and Lersten, 1972).

Polima (*pol*) CMS cytoplasm was chosen for the studies described in this thesis because this cytoplasm induces nearly complete male sterility in most of the *Brassica napus* lines and restorer lines have been developed by the plant breeders (Fan et al., 1986). However, hybrids produced using *pol* cytoplasm may show up to a 20% reduction in seed yield relative to the corresponding hybrids carrying normal *Brassica napus* cytoplasm (*nap*). In addition, *pol* male sterility breaks down at high temperatures (Fan and Stefansson, 1986). There are several other CMS systems (*ogu*, *nap*, Diplotaxis etc.) available in *Brassica* but they also have disadvantages; *e.g.* restorer lines for *ogu* cytoplasm in *Brassica napus* are not easily available and the introduction of restorer genes results in a decrease in seed set, and *nap* cytoplasm does not induce male sterility in most of the *Brassica napus* lines.

Obviously, there is a need to improve the CMS systems of *Brassica* in order to produce high yielding hybrids. Studies leading to identification of molecular lesions, responsible for CMS in Brassica, would help in finding out the ways and means to manipulate and improve the CMS system in well defined ways. Although the molecular analysis of CMS has been carried out in some plant species (Hanson, 1991; Braun et al., 1991), the molecular basis of the trait is not precisely understood in any system. Moreover, there is a need to study the molecular basis of CMS in more than very few plant species in order to allow generalization about the molecular principles underlying this agronomically important trait. For these reasons, I chose to analyze the *pol* CMS system in *Brassica napus* at the molecular level.

Considerable evidence strongly suggests that CMS is associated with mitochondrial dysfunction (Pelletier et al., 1983; Hanson and Conde, 1985; Barsby et al., 1987; Lonsdale, 1987). However, it is also clear that nuclear genes control the expression of the CMS phenotype. CMS can arise in one of the four ways (Edwardson, 1970) : (A) from intergeneric crosses; (B) from interspecific crosses ; (C) from intraspecific crosses ; (D) spontaneous occurrences. In addition, CMS can be transmitted by protoplast fusion (Pelletier et al., 1983; Barsby et al., 1987). The CMS phenotype is often observed when a nucleus from one species is combined with a foreign cytoplasm by backcrossing. This results in plants containing the cytoplasm of the original maternal parent and the nuclear genotype of the original male parent. In such plants, interaction between nuclear encoded and mitochondrial encoded gene products may be disturbed, resulting in alterations of mitochondrial function (Hanson and Conde, 1985). Incompatibility between nuclear and mitochondrial genomes could result in the alteration of mitochondrial gene expression (transcription, processing of RNAs, or translation). Alternatively, the enzymes of inner mitochondrial membrane, which contain subunits coded by both nuclear and mitochondrial genomes, may become partially dysfunctional as a result of aberrant interactions between various subunits.

Plant mitochondrial DNAs (mtDNAs) are very large in size and complex in sequence organization as compared to their animal or fungal counterparts. The smallest mtDNAs are those of multicellular animals; in most cases these occur as a single circular species and are about 16-18 kb in length (Sederoff, 1984). Several plant species, in addition to a high molecular weight component, contain low molecular weight linear and/or circular DNA or RNA molecules (Pring and Lonsdale, 1985; Brown and Finnegan, 1989; Lonsdale, 1989). These mini circular /linear species, in most cases, appear not to encode essential mitochondrial genes and their function in mitochondria is uncertain. The high molecular weight plant mtDNA varies in size from about 200 kb in the genus *Brassica* to 2500 kb in muskmelon (Palmer and Herbon, 1988; Ward et al., 1981). Physical maps of the mitochondrial genomes from several plant species have been generated in the past few years [Lonsdale et al., 1984 (maize); Palmer and shields, 1984 (*Brassica campestris*); Quetier et al., 1985 (wheat); Palmer and Herbon, 1986 (*Brassica nigra* and radish); Palmer and Herbon,1987 (*Brassica hirta*); Siculella and Palmer, 1988 (sunflower); Brears and Lonsdale, 1988 (sugarbeet); Folkerts and Hanson, 1989 (*Petunia*); Yamato et al., 1992 (rice)]. The simplest organization occurs in *Brassica hirta*, where the genome consists of a single circular species of 208 kb (Palmer and Herbon, 1987). The other *Brassica* species like *Brassica campestris*, *Brassica oleracea* and *Brassica nigra* all have similar size genomes, but have a relatively more complex organization than that of *Brassica hirta*. Each contains a pair of direct repeats. Homologous recombination between two copies of the repeated sequence results in the formation of two smaller subgenomic circles (Palmer and Shields, 1984; Palmer and Herbon, 1987).

Due to their larger size in comparison with those of other organisms, plant mitochondrial genomes have the capacity to encode genes in addition to those for transfer RNAs (tRNAs), ribosomal RNAs (rRNAs) and components of the respiratory chain that are normally found in other eukaryotes. The mitochondrial genes identified in higher plants include three rRNA genes (*rrn26*, *rrn18* and *rrn5*), genes for proteins of known function [*coxI*, *coxII*, *coxIII* (encode subunits of cytochrome oxidase); *cob* (cytochrome b); *atpA*, *atp6*, *atp9* (encode subunits of ATP synthase); *nad1*, *nad3*, *nad4*, *nad5* (NADH:CoQ reductase subunits); *rps3*, *rps12*, *rps13*, *rps14*, *rpl16* (encode ribosomal proteins)], about sixteen tRNA genes and some conserved open reading frames (ORFs) of unknown function (Brown, 1992). However, the total number of genes encoded by the mitochondrial genome of higher plants is not known. Recently, the complete mitochondrial genome of the liverwort *Marchantia polymorpha* (a bryophyte) has been sequenced (Oda et al., 1992). The genome (186 kb) encodes three species of ribosomal RNAs, 29 tRNAs genes and 30 genes for proteins of known function. It also encodes 32 unidentified open reading frames (Oda et al., 1992). This information will undoubtedly be useful in identifying more mitochondrial genes in higher plants.

This thesis has been written in manuscript-format and relevant literature is discussed in various sections of the chapters that follow. The following is a brief review of major findings on various CMS systems with an emphasis on molecular analyses. During the past few years, several reviews dealing with various aspects of plant mitochondrial genome, including CMS, have been published (Hanson and Conde, 1985; Lonsdale, 1987; Newton, 1988; Levings and Brown, 1989; Levings, 1990; Braun et al., 1991; Hanson, 1991; Brown, 1992; Gray et al., 1992). Although molecular analysis of

CMS has been carried out in several plant species, the maize *cms-T* (male sterile cytoplasm Texas or T) and *Petunia*-CMS are the most well characterized CMS-systems of higher plants.

Maize *cms-T*

Two dominant nuclear alleles, *Rf1* and *Rf2*, are required to restore fertility to male sterile *cms-T* maize plants. Plants carrying *cms-T* cytoplasm are susceptible to fungal pathogen, *Bipolaris maydis*, race T (Miller and Koeppe,1971). The toxin *BmT* produced by this fungus makes *cms-T* mitochondrial membranes permeable to small molecules such as Ca++ and causes swelling of the membrane and loss of membrane potential (Miller and Koepe 1971; Berville *et al.*, 1984; Holden and Sze 1984). However, the toxin does not affect the mitochondria from other maize cytoplasms.

Dewey et al. (1986) identified a novel mtDNA region specific to the *cms-T* cytoplasm by differential hybridization of mtRNA sequences from sterile and fertile mitochondria to mtDNA library from *cms-T* maize. The expression of this region at RNA level is affected by the nuclear restorer gene, *Rf1* (Kennell et al., 1987; Kennell and Pring, 1989). In fertile *cms-T* revertant plants, this region was either deleted or altered (Fauron et al., 1987; Rottman et al., 1987; Wise et al., 1987), providing additional evidence that this locus is associated with the cytoplasmic male sterility conferred by T-cytoplasm. The region contains a unique open reading frame (ORF) which is cotranscribed with a downstream normal mitochondrial gene, *orf25*, and has the potential to encode a 13 kDa protein. The ORF sequences are homologous to the coding and 3' flanking regions of *rrn26* and its expression is driven by the sequences which are homologous to a region upstream of *atp6* (Dewey et al., 1986; Kennell and Pring, 1989).

Earlier work on *cms-T* has demonstrated synthesis of a 13 kDa protein specific to T-cytoplasm mitochondria. The levels of this protein were reduced by the presence of restorer gene, *Rf1* (Forde et al., 1978; Forde and Leaver, 1980). Antibodies raised against a peptide, synthesized on the basis of deduced amino acid sequence of *cms-T* specific ORF, detected a 13 kDa protein associated with the mitochondrial membrane fraction in T-cytoplasm plants, the levels of which were reduced by the *Rf1* allele (Dewey et al., 1987). These studies along with the genetic analysis (Wise et al., 1987) confirmed that *cms-T* specific ORF encodes the 13 kDa protein specific to the T-cytoplasm maize plants. This ORF, which represents a chimeric gene, was consequently designated as *T-urf13*. These observations also suggest that URF13 protein may be directly related to CMS trait. Since *Rf1* alone does not restore male fertility, reduction in the levels of URF13 protein is not enough for sterility suppression. The effects of the *Rf2* allele, at the molecular level, in suppressing male sterility are not known.

Interestingly, the effects of fungal toxin on *Escherichia coli* expressing the URF13 protein are similar to those observed in *cms-T* mitochondria (Dewey et al., 1988). Similarly, when this protein is expressed in yeast and targeted to mitochondria, the yeast cells become sensitive to the toxin when grown on a non-fermentable carbon source. However, the yeast cells expressing this protein without the target peptide remain insensitive to the toxin (Huang et al., 1990). These studies provide direct evidence that URF13 protein is involved in conferring sensitivity to fungal toxin in *cms-T* mitochondria. However, the role of this protein in male sterility remains unclear.

Petunia CMS

A mitochondrial DNA region associated with *Petunia* CMS was identified by analyzing mtDNA of the male sterile and male fertile somatic hybrid plants, produced by the protoplast fusion technique, for restriction fragment length polymorphisms (Boeshore et al., 1985). The region contains, like maize *cms-T*, a chimeric gene, designated *pcf* (*Petunia* CMS-associated fused), comprised of portions of *atp9* and *coxII* coding region sequences fused to an unidentified reading frame designated *urf-S* (Young and Hanson,1987). Again, like maize *T-urf13*, *pcf* is located upstream of and cotranscribed with the two normal mitochondrial genes *nad3* and *rps12* (Rasmussen and Hanson, 1989). In *Petunia*, the expression of the CMS-associated region at the RNA level is developmentally regulated and affected by the fertility restorer allele (Young and Hanson, 1987; Pruitt and Hanson, 1991). The levels of the CMS-associated mitochondrial transcripts were found significantly higher in anthers as compared to the vegetative tissue of male sterile plants. In presence of the restorer allele, the levels of the transcripts of one of the three 5' termini were reduced. However, nuclear genes, other than the fertility restorer gene, also affected the expression of the CMS locus at transcript level (Pruitt and Hanson, 1991). CMS cytoplasm-specific synthesis of a 25 kDa protein in mitochondria was demonstrated by Nivison and Hanson (1989) using antibodies raised against a synthetic peptide corresponding to the *urf-S* portion of the *pcf* gene. This protein was also observed in fertility restored lines but at significantly reduced levels. This protein was found in both the soluble and membrane fractions of mitochondria, suggesting its loose association with the mitochondrial membranes. At the physiological level, differences in the partitioning of the electron transport through the cytochrome oxidase and alternative pathway were reported in cell suspension cultures and immature anthers from near isonuclear male fertile and male sterile *Petunia* lines (Connett and Hanson 1990). Male sterile plants showed a significant reduction in the alternative oxidase activity as compared to the fertile plants. The fertility restoration of male sterile plants resulted in the increased activity of alternative oxidase. So there seems to be some correlation between these changes at the physiological level and CMS. However, how the *pcf* gene region is involved in these changes is not clear.

Sunflower, bean and rice CMS systems

In sunflower, the comparison of the physical maps of the mtDNAs from normal and male sterile cytoplasm plants, revealed a single region which was organized differently in the two types of plants (Siculella and Palmer, 1988). This rearranged region is located near the *atpA* gene and contains a 5 kb insertion of unknown origin. The inserted sequence possesses a 522 nucleotide open reading frame, *urfH522*, located downstream of *atpA* (Kohler et al., 1991). Analysis of transcripts from this region showed that *urfH522* is cotranscribed with *atpA* and that the transcript pattern is altered in some but not in all restored lines (Horn et al., 1991; Kohler et al., 1991). Since near isonuclear lines, differing in their alleles primarily at the locus of interest (restorer locus in the present case), were not used in these studies, it is difficult to judge whether the effects on transcripts of this region are specifically due to the restorer allele. Studies involving protein synthesis by isolated mitochondria from male sterile and normal sunflower lines showed that CMS plants synthesize a 16 kDa polypeptide which was not detected in male fertile mitochondria (Horn et al., 1991). Interestingly, the size of this protein is very close to the predicted size for the protein potentially encoded by *urfH522*. However, whether *urfH522* is actually translated *in vivo* and the role of its translated product in CMS remains to be demonstrated.

The bean (*Phaseolus vulgaris*) CMS system is unique in the sense that the nuclear restorer gene induces permanent change in the mtDNA of male sterile plants. Thus, once this has been effected, the plants remain male fertile even in the absence of the restorer allele. The permanent reversion of a male sterile to a male fertile cytoplasm involves the deletion of a 24 kb restriction fragment from the mtDNA (Mackenzie and Chase, 1990; Mackenzie et al., 1988). Sequence analysis of a 3 kb portion of the deleted mtDNA, which is not found in the mitochondrial genome of plants reverted to fertility, reveals the presence of two transcribed open reading frames. One of the ORFs contains a chloroplast sequence. These ORFs have the potential to encode polypeptides of 10.9- and 26.7 kDa (Johns et al., 1992).

In rice, the mitochondrial gene *atp6* shows differential expression between male fertile and male sterile near isonuclear plants at transcript level. Normal male fertile cytoplasm plants were found to contain a single copy of *atp6*-related sequences while the male sterile cytoplasm plants contain two. Sequence analysis showed that in male sterile plants one of the two *atp6* copies represents an intact *atp6* gene while the other represents a chimeric gene comprising 5' flanking and N-terminal *atp6* coding sequences fused to a sequence of unknown origin (Kadowaki et al., 1990). Since the nuclear restorer allele affects transcripts of this gene when near isonuclear lines were used, it appears to be associated with the CMS trait.

Radish Ogura CMS

In radish, Makaroff and Palmer (1988) have demonstrated mtDNA rearrangements and transcriptional alterations in the Ogura (*ogu*) male sterile cytoplasm as compared to the normal (male fertile) cytoplasm. The transcriptional patterns of a dozen known mitochondrial genes and rearranged radish mitochondrial gene regions were studied in fertile, CMS and fertility-restored radish lines. The transcription patterns of *atpA*, *atp6* and *coxI* genes were found to be different among these lines. Since the mtDNA rearrangements mapped near these genes, the rearrangements appeared to be responsible for their transcriptional alterations in CMS plants as compared to the normal cytoplasm plants. Nuclear fertility restoration does not affect the *atp6* and *coxI* transcript pattern, but it does affect the transcript pattern of *atpA* gene. Interestingly, after fertility-restoration the transcript pattern of *atpA* appears similar to that of normal cytoplasm plants. However, further analysis demonstrated that the *atpA* transcriptional differences were due to the non-specific effects of the nuclear genotype rather than the specific effect of the restorer gene (Makaroff et al., 1990). Thus, the differential expression of the *atpA* gene is unlikely to be involved in male sterility. In *ogu* plants, a 105 codon open reading frame, generated by mtDNA rearrangements, is located upstream of and cotranscribed with the *atp6* gene, whose coding region is disrupted at the N-terminal end (Makaroff et al., 1989). Since the expression of this region at transcript level is not affected by fertility restoration, its association with CMS is not clear. Recent studies involving analysis of mitochondrial DNAs of CMS and fertile *Brassica napus*/Ogura cybrids and somatic hybrids have led to the identification of another mtDNA region associated with *ogu* CMS (Bonhomme et al., 1991; Temple et al., 1992).

Brassica Polima CMS

Most of the earlier molecular work on CMS in *Brassica* centered around the 11.3 kb DNA plasmid which co-purifies with mitochondria and is observed in some CMS lines. Extensive genetic and molecular analyses showed that the presence or absence of this plasmid does not appear to be associated with *pol* CMS in *Brassica napus* (Kemble et al., 1986; Erickson et al.,1986). To identify regions of *pol* mtDNA which might be associated with *pol* CMS in *Brassica napus*, Witt et al. (1991) and Singh and Brown (1991) (chapter II of this thesis) screened 12-14 standard mitochondrial genes for their differential expression at RNA level among fertile, CMS and fertility restored *Brassica* plants. Only the *atp6* gene region showed differential expression among these plants and fertility restoration altered the *atp6* transcript pattern. Further studies using near isonuclear *Brassica napus* lines showed that the alteration in *atp6* transcript pattern is specific to the nuclear restorer locus and that two distinct restorer alleles, *Rfp1* and *Rfp2*, exert the same effect on transcripts of *atp6* gene region (Singh and Brown, 1991; Chapter II of this thesis). Sequence analysis of *atp6* gene region in *pol* plants revealed the presence of a chimeric gene comprising N-terminal region of *orfB* gene and a sequence of unknown origin. This chimeric gene is located upstream of and cotranscribed with the *atp6* gene (Singh and Brown, 1991; Handa and Nakajima, 1992a). No differences were observed by Handa and Nakajima (1992b) in RNA editing of *atp6* gene transcripts between male fertile (normal cytoplasm) and male sterile (*pol* cytoplasm) plants.

Possible mechanisms of CMS

Although in several cases studied, the rearrangements in the mitochondrial genome have been found associated with CMS, it is not clear how these changes in genome organization lead to the CMS phenotype and at the same time allow vegetative parts of the plant to remain unaffected. It is possible that CMS-related mutations cause mild mitochondrial dysfunction and that tissues which utilize energy at relatively slow rate, such as vegetative tissues, are able to tolerate this mild deficiency in mitochondrial function. However, the tissues that utilize energy at a faster rate, which conceivably could include those involved with pollen development, might be unable to cope with this deficiency in mitochondrial function and fail to carry out their physiological functions, thus resulting in the overt phenotype. In this regard, it is interesting to note that the number of mitochondria per cell increases about 20 times in sporogenous tissue and 40 times in tapetum, the inner layer of the pollen sac, indicating that the microsporogenesis is a particularly high energy demanding process (Warmke and Lee, 1978; Lee and Warmke, 1979). The tapetal cells are considered to perform a nutritive function for developing pollen. In CMS *Petunia* and maize *cms-T*, abnormalities in tapetal layer have been observed (Bino, 1985a; Bino, 1985b; Warmke and Lee, 1977). It is also possible that the CMS mitochondria become dysfunctional in anthers only as a result of interaction with anther-specific metabolic processes. For example, it has been hypothesized that T-type male sterility of maize is caused by mitochondrial dysfunction resulting from interaction of mitochondria with an anther-specific metabolite analogous to T-cytoplasm specific fungal toxin (Flavell, 1974).

In several cases, the CMS-associated mitochondrial gene regions contain chimeric genes. The protein product of these genes could interfere with the normal mitochondrial functions or it could compete with the corresponding normal protein(s) in interactions with other proteins as multisubunit complexes are assembled. Another possibility arises from the observations made in maize, *Petunia* and *Brassica* that the CMS-associated chimeric genes are located upstream of and cotranscribed with the normal mitochondrial genes. It has been suggested that the chimeric gene sequences might affect the translation of the downstream normal gene, leading to the deficiency of the normal gene protein and male sterility (Hanson et al., 1989; Singh and Brown, 1991). However, it is important to note that there are examples of chimeric genes being present in fertile lines and there are chimeric genes present in sterile lines in regions of mitochondrial genome that are not correlated with CMS (Hanson, 1991). It is also possible that the di- or polycistronic transcripts containing chimeric gene sequences are edited differentially than their counterparts in normal cytoplasm, resulting in abnormal protein which might interfere with normal mitochondrial function. Differential editing of *orf25* sequence located downstream of *urf13-T* has been observed in male sterile and male fertile maize plants, although no RNA editing was observed in *urf13-T* sequence (Ward and Levings, 1991).

Several laboratories are trying to demonstrate the role of these chimeric genes in CMS, through studies involving targeting of the protein product of chimeric genes to the mitochondria of normal cytoplasm plants. If male sterile transgenic plants are obtained as a result of such targeting, the involvement of these genes in CMS would become clear. On the other hand, if CMS is caused by the deficiency in expression of a normal mitochondrial gene, then targeting of the normal gene protein to mitochondria of male sterile plants should result in male fertile transgenic plants and the gene for the targeted protein can be used as a synthetic restorer gene.

The possible mechanisms underlying the Polima CMS in *Brassica napus* will be discussed in the following chapters.

CHAPTER II

Identification, isolation and characterisation of a mitochondrial gene region associated with Polima cytoplasmic male sterility in *Brassica napus*

SUMMARY

To identify regions of the mitochondrial genome that could potentially 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 ATP synthase 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 F1 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 (mt)DNAs (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, 1988). 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 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 ATP synthase, *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.

MATERIALS AND 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 Semyk, 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 fur 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 17000 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 X wash buffer at room temperature prior to autoradiography. For heterologous probes, the hybridization and high temperature washes were performed 5-8 °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 5 °C higher temperatures.

DNA fragments were purified from agarose gels for cloning and labeling purposes using a system from Bio-Rad. The Bluescript 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).

RESULTS

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

To determine regions of the *pol* mitochondrial genome that could potentially 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, coxII* [subunit 2 of cytochrome oxidase], *rrn18, rrn26* [18S and 26S ribosomal RNAs]), wheat (*cob* [cytochrome b], *coxI, nad5* [subunit 5 of NADH-ubiquinone reductase], *orf25*), *Oenothera* (*atp9, coxIII*), 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,

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*) x Westar-*Rf*	*Rfp1/rfp1*	*pol*	Fertile
Karat (*nap*) x 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.

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
coxI	Wheat	1.0-kb HindIII-PstI	Bonen et al. 1987
coxII	Maize	2.8-kb HindIII	Fox and Leaver 1981
coxIII	*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).

levels of this transcript appeared greatly reduced and two larger 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 further investigate the association between *atp6* gene expression and male sterility, the organization of *atp6* sequences in the *nap* and *pol* mtDNAs was examined. 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.

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*) x Westar-*Rf* (lane 9), and Karat (*nap*) x 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.

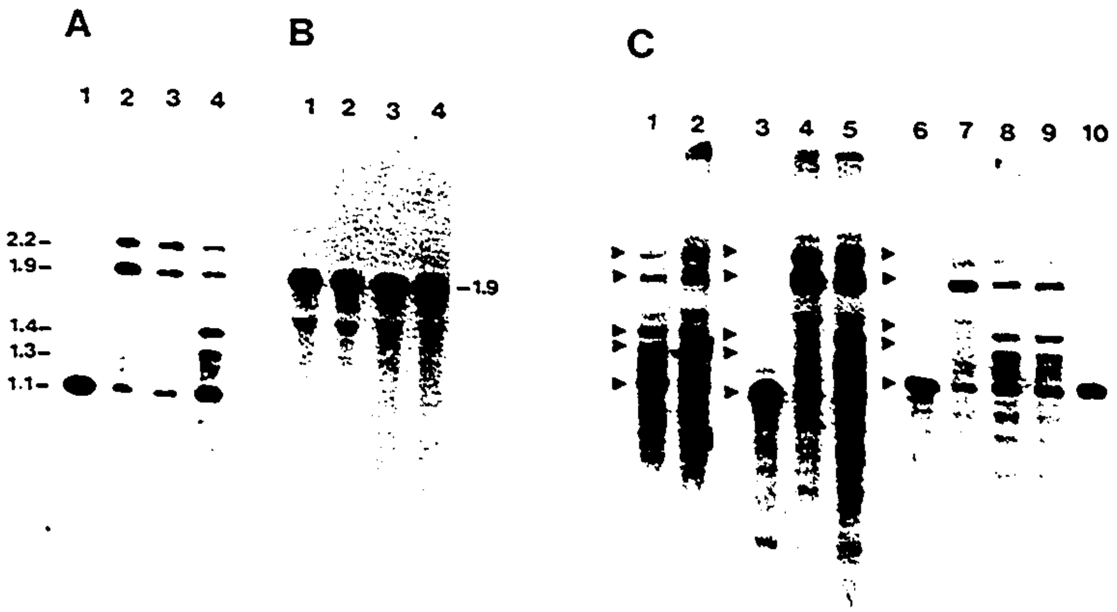

A
1 2 3 4
2.2–
1.9–
1.4–
1.3–
1.1–
B
1 2 3 4
–1.9
C
1 2 3 4 5 6 7 8 9 10

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.

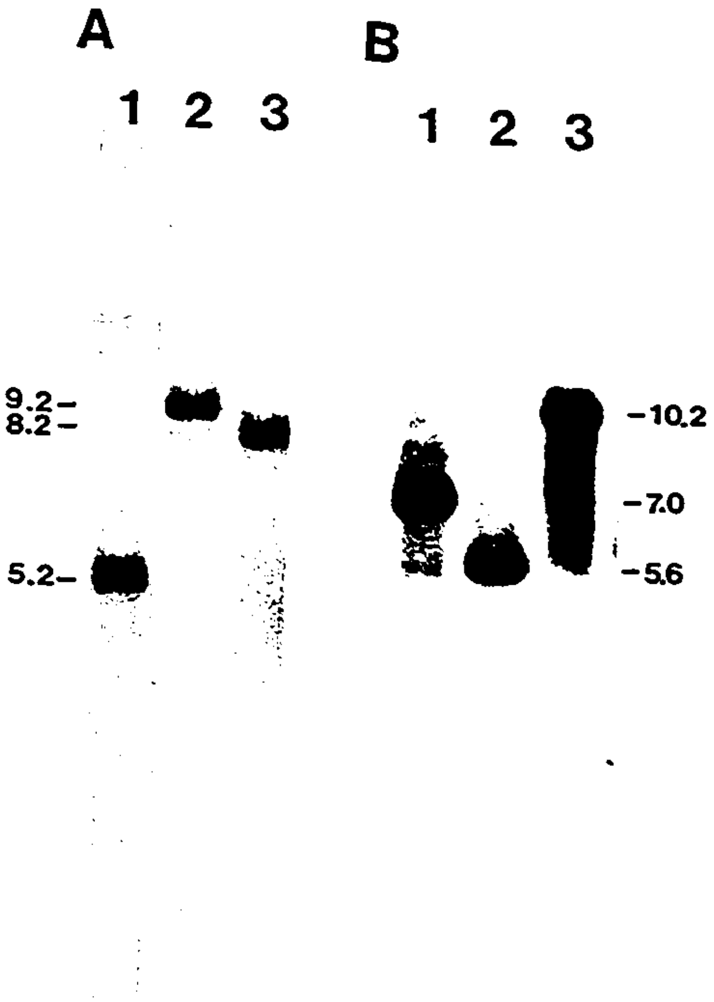
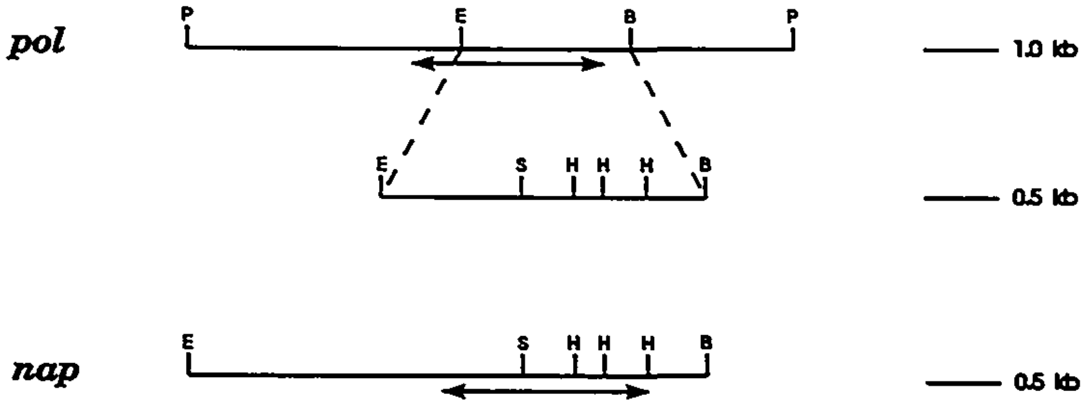

A
1
2
3
B
1
2
3
9.2
8.2
5.2
10.2
7.0
5.6

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; E, EcoRI; S, BstXI; H, HindIII; P, PstI.


pol
P
E
B
P
1.0 kb
E
S
H
H
H
B
0.5 kb
nap
E
S
H
H
H
B
0.5 kb

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 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 if 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 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, and 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 further investigate 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*) x 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 further investigate the association between the *pol atp6* region and CMS, the nucleotide sequences of regions 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 Da polypeptide. It is identical to the *atp6*

Figure 4. Nucleotide sequences of expressed regions of the *Brassica pol* and *nap atp6* gene loci.

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 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.

```
    TAAACCACCTCTCCCTCTTCCCTCCATCACCTACATAAATTAACTATATCTAATAACAATC 60
    AAAACTATGGCTTACCCTCTTGCATCAATCACTCATTCCTTTCATCTTGAATTAACTATA 120
    TACAAAACAACTCTTCTCTCCCCCAACTCTACAACTCCTACTATTACCGATCCTATCCAC 180
          ←  oligo A
    GGTCCTTATCGGGCTTCGCTGCTCGAATTTCAATAAACTCAACTTTCCTCAATCCCCTTTCT 240
    GTTCACTTCCCAACATTTCTCAATCCTTCTATTACTTCATCCTTTTGTTTATTCGAAACC 300
    TCCCCCGGCATGCTACTTCACCTACCCTACCCCCCCTCTATCATTGTCTCATTTTACCTTT 360
    CTCATCCCTACCCTCCCCCGGCTCCCCCCCCCATCAAACTATCAATCTCATAACAGAACAA 420
    ATCTCTATGCCCCCCTCTTCTTGCTTTTCTCCCATCCTTTTCTTCCTCAACAACCAACCAC 480
    AACTTTCTATACTTCTTCACTACTCCTACACCCTTCACCCACTCAACCTCTCTCCACCCA 540
                                orf224→
    ATCATTTTCTTGAAATCAATTAATCTAATCATGCCTCAACTGGATAAATTCACTTATTTT 600
                                  M  P  Q  L  D  K  F  T  Y  F
    TCACAATTCTTCTGGTTATGCCTTTTCTTCTTTACTTTCTATATTTTCATATGCAATGAT 660
     S  Q  F  F  W  L  C  L  F  F  F  T  F  Y  I  F  I  C  N  D
    CGACATCGACTACTTGCCATCACCACAATTCTAAAACTATGCAACCAACTCCTTTCACAC 720
     C  D  G  V  L  G  I  S  R  I  L  K  L  W  N  Q  L  L  S  H
    CCCCCCAACACCCTCCTCAGCAACCCAACCCTTCCAAAAAATCCTACTTCACATTCAACT 780
     R  C  K  T  L  L  S  K  G  R  L  C  K  N  R  S  S  D  S  S
    CGCTTCCACCTATCACCCTTCGCCCCCCATTATTTTATCATTTTCGTCCTCCCAAAATTC 840
     R  F  E  V  S  A  L  A  A  H  Y  F  I  I  F  V  V  P  K  L
    CGACCACTTTTCTACATTATATATATAATTTTTTTTTGTTTGTTCGCCTTGAAATGCCCCCTA 900
     C  P  V  F  Y  I  I  Y  N  F  F  C  L  L  G  L  K  W  C  V
    TTACCAAATCAAATTTTGTCATTTCGCCCGTCGGACCAGATCGCGTCGCCCCCCCAGCCCCTC 960
     L  G  N  E  I  C  H  F  G  V  G  P  D  G  V  A  P  P  A  L
    CATCTCAACCGACCCCCCCCCTCTCCATCTTTTTGTACCCCGCATGTTCACACTTCCCACTCT 1020
     D  L  N  E  R  P  P  L  H  L  L  Y  A  D  V  E  S  S  D  S
    CAACAAGCCCCAAATAATCACATGTACGCCCATCTTAGCCCCCTACACCACATCACCCAA 1080
     Q  Q  A  R  N  N  D  M  Y  A  H  L  R  R  V  Q  E  I  T  Q
              ← oligo B
    AAACTACACGGTCAGCCCCATATCCTGCCCCGTCAACCCCTCCTGGATATAATGAAATGG 1140
     K  L  E  C  E  R  D  I  V  R  R  Q  A  L  L  D  I  M  K  W

nap ACCAAACAACCACTCCCACTACAGTACACCAGCTTCGTACCTCCCAACCACCCAACCCTC
pol GAGCTCAGAAGCCCTTCACCACCACTTTTCCGATCTTTTCGCCCACCTTGATCGTCTCCCAGAT 1200
     E  V  R  S  L  Q  E  H  F  R  I  F  R  H  L  D  R  L  R  D
                   trnfM →
rad                            AGCCCGGTACAGCAATTCGTCAACTCATCAGCCTCATCA
nap CTTCACCAACCGGCAGAAGCCAACTCCCTTCCTCCACC** ************************
pol TCCCACAGACCCAAGCTCAACCAGATCCTTGATCTCTTTCGCCTCAACAACCTAGCTTCCT 1260
     S  Q  R  A  K  V  N  E  I  L  D  L  F  R  -

rad CCTGAAGATTAC******G*******G**C*******
nap *************************************—•
pol CCTTCATCTCCTAGCTTCAAATCCTATCTCCCGCAGTAACTAACCCTTTTCATTCTGCATCA 1320
    CTCTCCCCCGTCGTTCTCCACCTCGCAAGCTTTTTCAACCCCCCCAACCCCCAACTCACAA 1380
    TACCGCTTTTCTTCACCACATTTTCCATCATTTCACCCAAAACCCACTACAAACTTCACC 1440
            atp6→
    CTTTAAGCACCCTATGAATCAAATAGCCCTGCTGCCCGCACTCCCCACTTCACCAATTTCA 1500
                  M  N  Q  I  G  L  V  A  Q  S  P  L  D  Q  F  E
    CATTCTCCCATTCATTCCTATGAATATCGGAAACTTCTATTTCTCATTCACAAATCCATC 1560
     I  V  P  L  I  P  M  N  I  G  N  F  Y  F  S  F  T  N  P  S
    TTTCTTCATGCTCCTAACTCTCACTTTTTTCCTACTTCTCATTCATTTTATTACTAAAAA 1620
     L  F  M  L  L  T  L  S  F  F  L  L  L  I  H  F  I  T  K  K
    CGCAGCAGGAAACTTAGTCCCAAATGCTTGGCAATCCTTGGTAGAGCTTCTTTATGATTT 1680
     C  G  G  N  L  V  P  N  A  W  Q  S  L  V  E  L  L  Y  D  F
    CGTCCTCAACCTCGTAAAGCAACAAATAGGTGGTCTTTCCGGAAATGTCAAACAAATGTT 1740
     V  L  N  L  V  K  E  Q  I  G  G  L  S  G  N  V  K  Q  M  F
    TTTCCCTTCCATCTTGCTCACTTTTCTTTTTTTGTTATTTTCTAATCTTCAGCCTATGCAT 1800
     F  P  C  I  L  V  T  F  L  F  L  L  F  C  N  L  Q  C  M  I
    ACCTTATACCTTCACAGTCACAAGTCATTTTCTCATTACTTTGCCTCTCTCATTTTCTAT 1860
     P  Y  S  F  T  V  T  S  H  F  L  I  T  L  A  L  S  F  S  I
    TTTTATTGGCATTACTATAGTCGGATTTCAAAGACATGGCCTTCATTTTTTCAGCTTTTT 1920
     F  I  G  I  T  I  V  G  F  Q  R  H  G  L  H  F  F  S  F  L
    ATTACCCGCACGAGTCCCACTCCCGTTAGCACCTTTTTTACTAGTCCTTGAGCTAATTTC 1980
     L  P  A  C  V  P  L  P  L  A  P  F  L  V  L  L  E  L  I  S
    TTATTGTTTTCGCCGCATTAAGCTTAGCAATACGTTTATTTGCTAATATGATGGCCGCTCA 2040
     Y  C  F  R  A  L  S  L  C  I  R  L  F  A  N  M  M  A  C  H
    TACTTTAGTAAAGATTTTAAGTCGCTTCGCTTGGACTATGCTATGTATGAATGAGATTTT 2100
     S  L  V  K  I  L  S  G  F  A  W  T  M  L  C  M  N  E  I  F
    CTATTTTATAGGCGCTCTTGGTCCTTTATTTATAGTTCTTGCATTAACCGGTCTGGAATT 2160
     Y  F  I  G  A  L  G  P  L  F  I  V  L  A  L  T  G  L  E  L
    AGGTGTAGCTATATTACAAGCTTATGTTTTTACCATCTTAATCTGTATTTACTTGAATGA 2220
     G  V  A  I  L  Q  A  Y  V  F  T  I  L  I  C  I  Y  L  N  D
    TGCTATAAATCTCCATTAAAGTTCTTCTTTCTTTTATTTAGATTTATAATTGAACAAAAC 2280
     A  I  N  L  H  -
    CGAGCGATGACACTAGTCTTATTTAGAGCACTTACACAGCCCCTCTCCTTCCAGTCCAGT 2340
    GACTTCGCCCCTCAATGTCTTAGATACCTCTAAGTGAAACAACCGTACTAACTACGTCGC 2400
    AATCCCCCTAGCTAGTACTTACTTCTTTGTACTCCCCAGAAGCTCCAACCCTTAACTACA 2460
    ATCTTCTTCGCTCCTCTTTTTTTCTTTTTACAAACCTT                       2498
```

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 *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).

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 Da 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 *orfB* is a common protein coding sequence of plant mtDNAs. Over the first 58 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.

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.

```
Oenothera orfB          TTTTTCCCATGCAATGCATTC-CGTTGGTCAACAACCACAGCTCTCTATCAATAAAGATC
                        ::: ::::::::      ::   :::::::::::::::       :: :: :::  : ::
Brassica pol orf224     TTTCTCCCATGC------TTT-TGTTGGTCAACAACCAACCACAACTTTCTATA--GTTC
                        ::::::::::::      :::  ::::::::::::::::::: :    ::::::   :::
Tobacco atp9            TTTCTCCCATGC------TTTCCGTTGGTCAACAACCAACCAAAGTGCTCTATA--CTTC

Oenothera orfB          TTCACTACTCATACAGGCTTGACGAAGTAAAGCTGTCTGGAGGGAATTATTT----GATC
                        :::::::::: :: :::::::::: ::: :::::::::::::::::: ::::    ::
Brassica pol orf224     TTCACTACTCCTAGAGGCTTGACGGAGTGAAGCTGTCTGGAGGGAATCATTTTGTTGAAA
                        :::::::::: :: :::::::::::::: ::::::: : ::::::::: ::::::   ::
Tobacco atp9            TTCACTACTCGTACAGGCTTGACGGAGTTAAGCTGTATTGAGGGAATCGTTTTGTCTCAA

                                        M  P  Q  L  D  K  F  T  Y  F  T  Q  F  F  W
Oenothera orfB          TCAAT-----CGAGAATGCCTCAACTGGATAAATTTACTTATTTCACACAATTCTTCTGG
                        :::::       :  :::::::::::::::::::: ::::::::  ::::::::::::::
Brassica pol orf224     TCAATTAATCTAATCATGCCTCAACTGGATAAATTCACTTATTTTTCACAATTCTTCTGG
                                        M  P  Q  L  D  K  F  T  Y  F  S  Q  F  F  W

                         S  C  L  F  L  F  T  F  Y  I  P  I  C  N  D  G  D  G  V  L
Oenothera orfB          TCATGCCTTTTCCTCTTTACTTTCTATATTCCCATATGCAATGATGGAGATGGAGTACTT
                        : :::::::::: :::::::::::::::::  ::::::::::::::::::::::::::::
Brassica pol orf224     TTATGCCTTTTCTTCTTTACTTTCTATATTTTCATATGCAATGATGGAGATGGAGTACTT
                         L  C  L  F  F  F  T  F  Y  I  F  I  C  N  D  G  D  G  V  L

                         G  I  S  R  I  L  K  L  R  N  Q  L  L  S  H  R  T  K  N  I
Oenothera orfB          GGGATCAGCAGAATTCTCAAACTACGGAACCAACTGCTTTCACACCGGGGTAAGAACATC
                        ::::::::::::::::: :::::: ::::::::::::::::::::::::: :::: : ::
Brassica pol orf224     GGGATCAGCAGAATTCTAAAACTATGGAACCAACTGCTTTCACACCGGGGGAAGACCCTC
                         G  I  S  R  I  L  K  L  W  N  Q  L  L  S  H  R  G  K  T  L

                         L  R  K  D  P  N  S  L  E  E  L  L  R  K  G  F  S  T  G  V
Oenothera orfB          CTCCGCAAGGACCCAAACAGTTTGGAAGAACTCTTCAGAAAAGGTTTTAGCACCGGTGTA
                        ::  ::::::      :  : :::::: :: :: : ::   :: ::  ::       :
Brassica pol orf224     CTGAGCAAGG-----GAAGGCTTGGAAAAAATCGT-AGTTCAGATTCAAGTCGGTTCGAG
                         L  S  K       G  R  L  G  K  N  R   S  S  D  S  S  R  F  E
```

The remaining portion of the *Brassica* ORF does not show significant similarity to any sequence in the GenBank sequence library (GenBank release number 67.0; 1991).

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 *coxI* 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).

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

Figure 6. Mapping of *atp6* region transcripts from male fertile, male sterile, and fertility-restored plants.

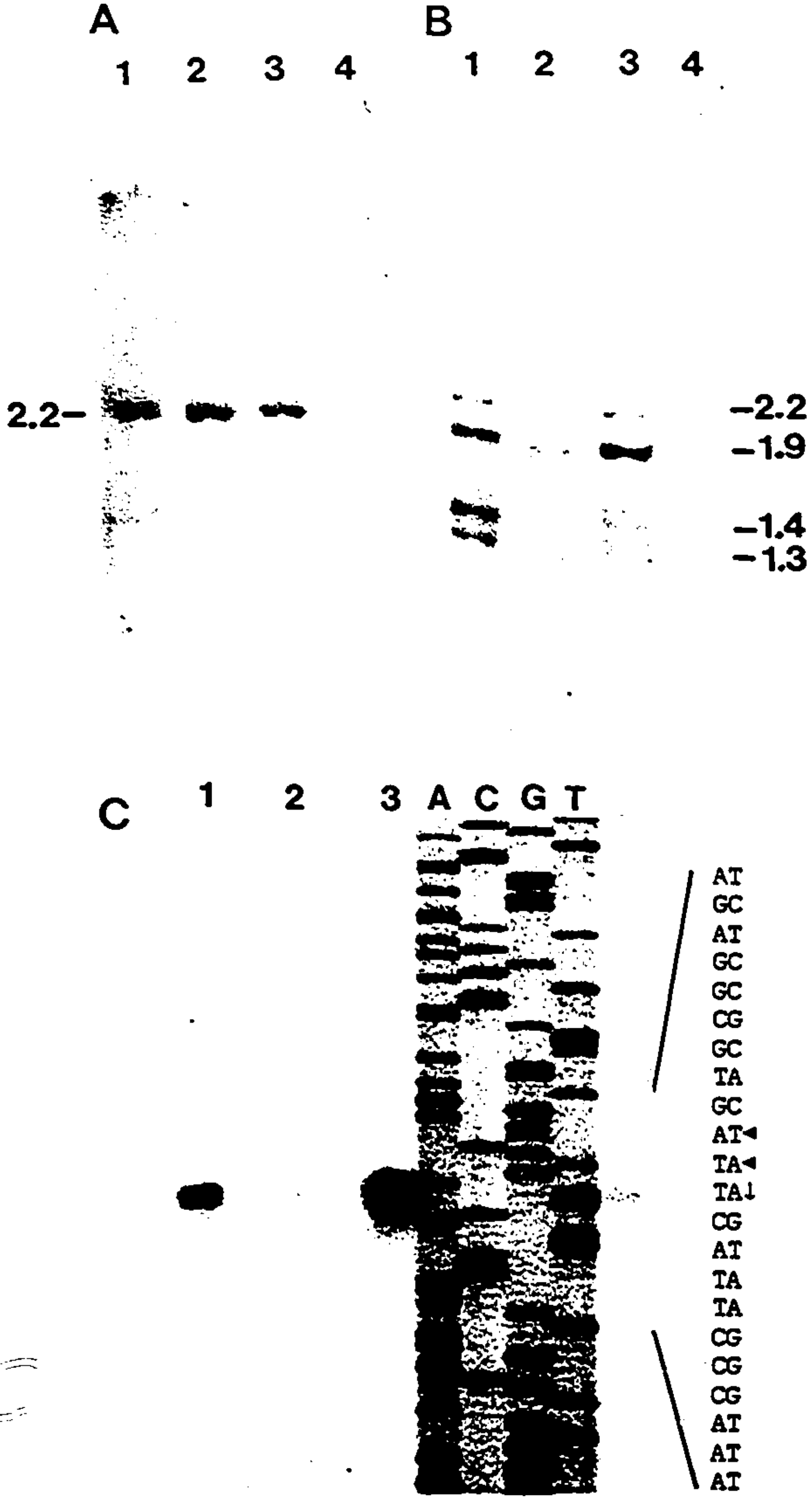
(A) oligo(A) probe.

(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 1466-1485 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.


A
1
2
3
4
2.2–
B
1
2
3
4
–2.2
–1.9
–1.4
–1.3
C
1
2
3
A
C
G
T
AT
GC
AT
GC
GC
CG
GC
TA
GC
AT◄
TA◄
TA↓
CG
AT
TA
TA
CG
CG
CG
AT
AT
AT

6A), whereas oligo(B), which corresponds to bases 1071 -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 1466-1485 of Figure 4 was used to prime cDNA synthesis off mtRNA from fertile *nap* 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 two 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.

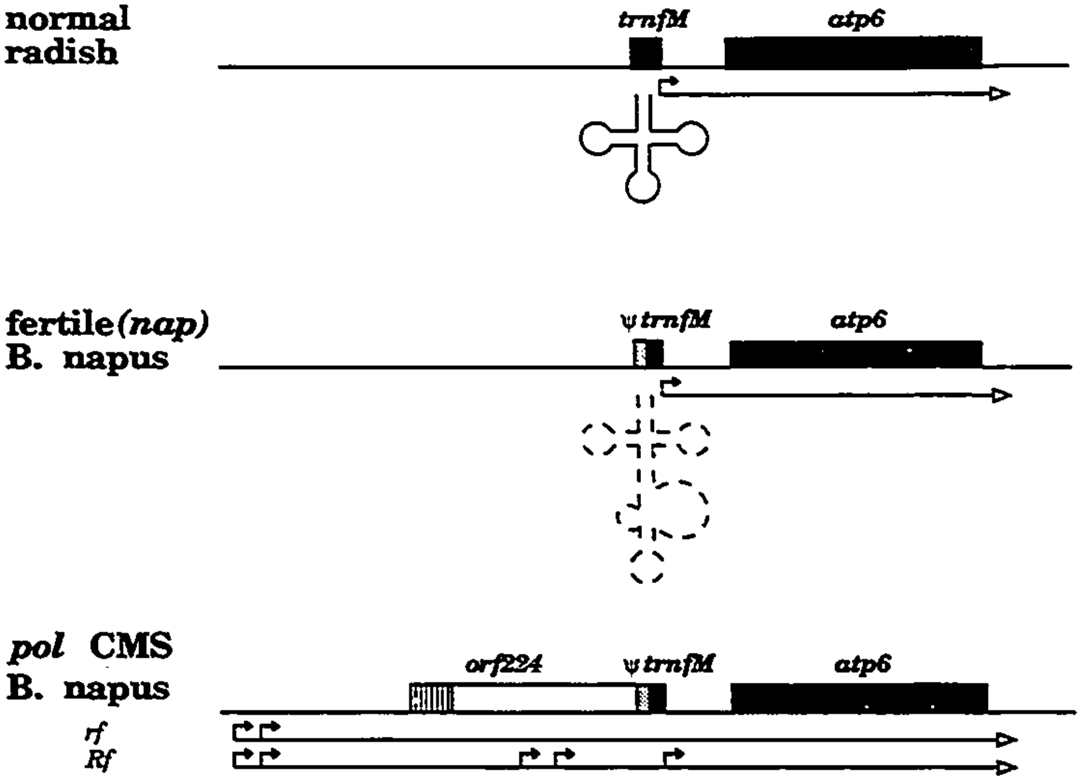
DISCUSSION

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

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.


normal
radish
trnfM
atp6
fertile (nap)
B. napus
ψ trnfM
atp6
pol CMS
B. napus
orf224
ψ trnfM
atp6
rf
Rf

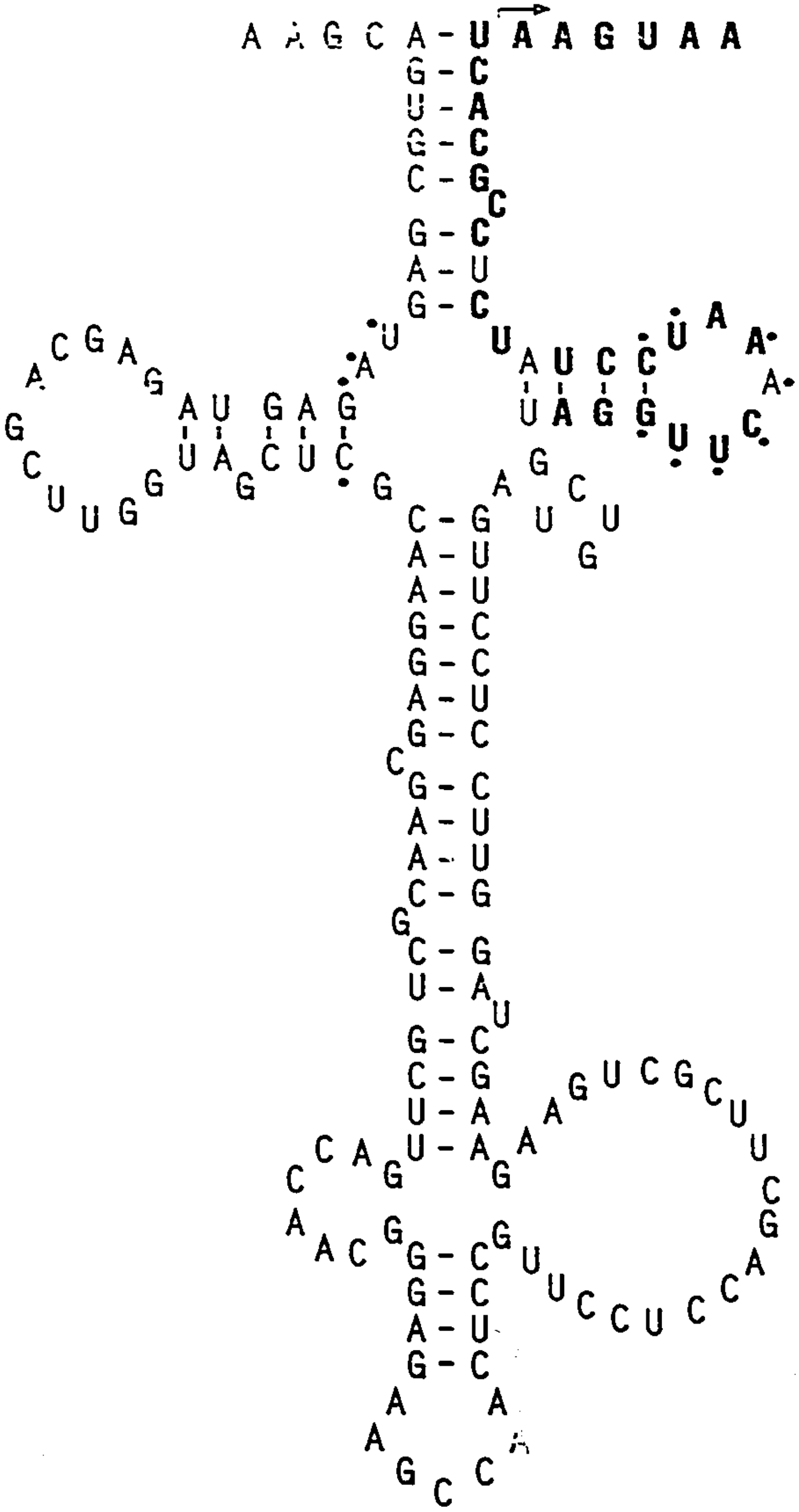
messages through tRNA processing (Attardi and Schatz, 1988). Recent analysis of tRNA processing activity in plant mitochondria indicates that, as in animal mitochondria, both the 5' and 3' termini of tRNAs are formed through precise endonucleolytic cleavage of a precursor species (Hanic-Joyce and Gray, 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 semiinvariant among all tRNAs. Several of these invariant nucleotides occur in 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

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 A G C A - U A A G U A A
G - C
U - A
G - C
C - G C
G - C
A - U
G - C
U U U A
A C G A A U A U C C A
A G G A U G A G U A G G A
G U A C U C U U C
C G A G G C
U U G G A U C U
C - G U G
A - U
A - U
G - C
G - C
A - U
G - C
C
G - C
A - U
A - U
C - G
G
C - G
U - A U
G - C
C - G A G U C G C
U - A A U
C C A U - A A U
C G G A C
A G
A A C G G - C G U A
G - C U C C
A - U C C U C
G - C
A A
A A
G C C

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 which 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, 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; Gualaberto 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 previously shown that the restorer genes present in the lines Italy and UM2353 reside at two distinct, independently segregating loci, and the finding that both 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 *coxIII* 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 (chapter III of this thesis), 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 (chapter III of this thesis).

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 12-27) and one derived from the downstream portion of the ORF (residues 82-97). The ORF224 protein is predicted to be an integral membrane protein.

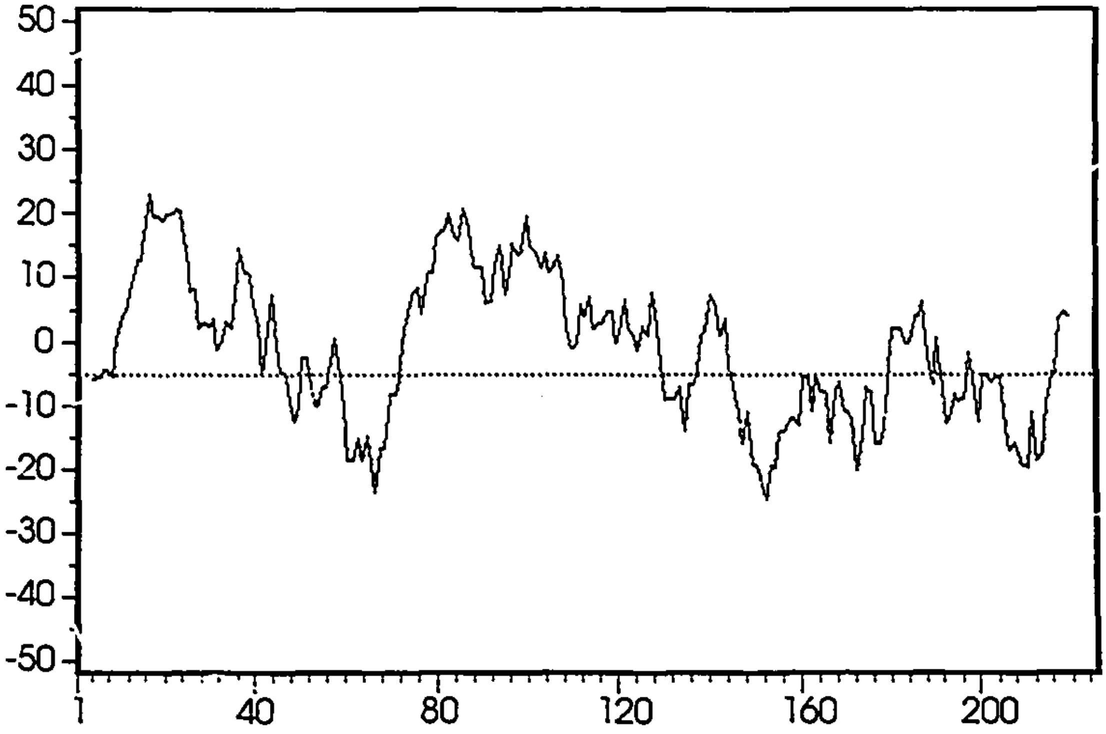
Possible Role of the *orf224/atp6* Gene Region in *Brassica pol* CMS

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

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.


50
40
30
20
10
0
-10
-20
-30
-40
-50
1
40
80
120
160
200

(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 a 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 exert 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 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 ATP synthase 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 was directly due to the presence of the ORF224 protein, then it might be expected that conditions which suppress male sterility would also suppress expression of the *orf224* gene. While 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 ATP synthase 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 ATP synthase 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 extensively employed 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 since 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 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 ATP synthase 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.

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CHAPTER III

Relationship between expression of the mitochondrial *orf224/atp6* gene region and Polima cytoplasmic male sterility in *Brassica napus*

In chapter II, I have shown that the *orf224/atp6* gene region of the mitochondrial genome of male sterile Polima cytoplasm is associated with cytoplasmic male sterility trait. In this chapter, I have characterized further the expression of *orf224/atp6* gene region at the RNA level with respect to the Polima CMS in *Brassica napus*. Experimental data will be presented to demonstrate that the expression of *orf224/atp6* region is developmentally regulated in *pol* cytoplasm plants. Results presented in this chapter also suggest that the *pol* CMS is not caused by the absence of an intact, expressed *orfB* gene.

SUMMARY

The mitochondrial genome of the Polima (*pol*) male sterile cytoplasm of *Brassica napus* contains a chimeric 224-codon open reading frame (*orf224*) that is located upstream of and co-transcribed with the *atp6* gene. The N-terminal coding region of *orf224* is derived from a conventional mitochondrial gene, *orfB*, while the origin of the remainder of the sequence is unknown. We show that an apparently functional copy of *orfB* is present in the *pol* mitochondrial genome, indicating that the *pol* CMS is not caused by the absence of an intact, expressed *orfB* gene. The 5' termini of *orf224/atp6* transcripts present in both sterile and fertility-restored (*Rf*) *pol* cytoplasm plants are shown to map to sequences resembling mitochondrial transcription initiation sites, whereas the 5' termini of two transcripts specific to restored lines map to sequences which resemble neither one another nor mitochondrial promoter motifs. It is suggested that the complex *orf224/atp6* transcript pattern of *Rf* plants is generated by a combination of multiple transcription initiation and processing events and that the nuclear restorer gene acts to specifically alter *orf224/atp6* transcripts by affecting RNA processing. Northern analyses demonstrate that the effect of the restorer gene on *orf224/atp6* transcripts is not tissue or developmental stage specific. However, the expression of the *atp6* region is developmentally regulated in *pol* plants, resulting in increased levels of monocistronic *atp6* transcripts in seedlings relative to floral tissue. It is suggested that this developmental regulation may be related to the absence of overt phenotypic effects of the CMS mutation in vegetative tissues

INTRODUCTION

Cytoplasmic male sterility (CMS), a maternally inherited inability of plants to produce functional pollen, is a widespread trait of higher plants that is encoded by the mitochondrial genome (Edwardson 1970; Hanson and Conde 1985; Lonsdale 1987). Although the regions of the mitochondrial genome associated with CMS have been identified in some plant species (Hanson 1991; Braun et al. 1991; Levings 1990), the molecular basis of the trait is not precisely understood. Because in many cases, specific nuclear genes termed restorers of fertility (*Rf*) have been identified which suppress CMS and restore fertility to F1 hybrids, the CMS trait can be viewed as a manifestation of nuclear-mitochondrial gene interactions.

The "Polima" or *pol* CMS system of *Brassica napus* has several features which have facilitated its analysis at the molecular level. These include the relatively small size of the *Brassica* mitochondrial genomes (Palmer and Herbon 1988), which has simplified mitochondrial DNA (mtDNA) structural studies, and the system's well-defined genetics of fertility restoration: dominant alleles at either of two unlinked nuclear gene loci can restore fertility to *pol* cytoplasm F1 hybrids (Fang and McVetty 1989). Previous studies on the molecular basis of the *pol* CMS have demonstrated the association of altered expression of the mitochondrial *atp6* gene region with this trait (Singh and Brown 1991; chapter II of this thesis; Witt et al. 1991; Handa and Nakajima 1992). While in male fertile *nap* cytoplasm plants there is only one abundant *atp6* transcript of 1.1 kb, *pol* cytoplasm plants exhibit a complex transcript pattern which is affected by the fertility restoring nuclear background. Sequence analysis of the *atp6* gene regions of the *nap* and *pol* mtDNAs has shown 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* (Singh and Brown 1991; chapter II). In sterile plants, the predominant *orf224/atp6* transcripts are 2.2 and 1.9 kb, in fertility restored plants additional transcripts of 1.4 and 1.3 kb are detected. In addition, in *pol* CMS plants levels of the 1.1 kb *atp6* transcript of fertile *nap* cytoplasm plants are greatly reduced; levels of this transcript are increased upon nuclear fertility restoration (Singh and Brown 1991; chapter II). The first 58 codons of *orf224* show very high similarity with the amino- terminal coding region of the *orfB* gene of Oenothera and sunflower (Hiesel et al. 1987; Quagliariello et al. 1990).

Mitochondrial gene regions which are thought to govern the CMS trait have been identified in a number of other species (Hanson 1991). These regions have also been found to contain chimeric genes, and it has been suggested that the expression of these genes results, directly or indirectly, in partially dysfunctional mitochondria and, consequently, male sterility. CMS mutations generally affect only the male reproductive organs and it is unclear why mitochondrial dysfunction would not have an effect on vegetative plant growth. To gain insight into the mechanistic basis for *pol* CMS and its suppression by nuclear restorer genes, we have further characterized the expression of the *orf224/atp6* region of *pol* cytoplasm *Brassica napus* plants. We show that its expression is developmentally regulated and that *pol* CMS does not appear to result from the absence of an intact, expressed *orfB* gene.

MATERIALS AND METHODS

Plant Material

The genotype and phenotype of the *B. napus* strains used in this study have been described earlier (Singh and Brown 1991; chapter II). Cytoplasms are designated as per the convention of Kemble and Barsby (1988), the parenthetical italicized cytoplasm designations following the cultivar name. Regent (*nap*) and Karat (*nap*) are male fertile *nap* cytoplasm strains while Regent (*pol*), Karat (*pol*), and 2007 (*pol*) are male sterile *pol* cytoplasm strains. Westar-Rf and 4007 are male fertile *pol* cytoplasm restorer lines. The sterile and fertile (nuclear-restored) plants possessing the *pol* cytoplasm were differentiated by their ability to produce well developed stamens and functional pollen. Floral tissue from plants grown under normal growth conditions (day/night temperatures of 22/16 °C, 16 hours photoperiod) was used, unless otherwise stated, for the isolation of mitochondrial nucleic acids.

Isolation of nucleic acids

The procedure of Kemble (1987) was followed for the isolation of mtDNA, except that the mtDNA was further purified by equilibrium centrifugation in CsCl gradients (Brown and Simpson 1981) to improve its digestion by restriction endonucleases. Mitochondrial RNA (mtRNA) was isolated essentially as described by Singh and Brown (1991) (chapter II).

Northern and Southern hybridizations

The mitochondrial RNA was size fractionated on agarose-urea gels (Finnegan and Brown 1986), transferred to Genescreen Plus (Dupont/NEN) hybridization membrane by overnight capillary blotting with 1.5M NaCl/0.15M sodium citrate, and hybridized to the radiolabeled probe as described earlier (Singh and Brown 1991; chapter II). Restriction endonuclease-digested mtDNA was size fractionated on 0.7% agarose gels. Gels were successively treated with 0.4 N NaOH/0.6 M NaCl and1.5 M NaCl/0.5 M Tris-HCl (pH 7.5), for 30 min each; DNA was then transferred to the hybridization membrane (GeneScreen-Plus) and hybridized with the labeled probe as described by Singh and Brown (1991) (chapter II). The hybridization and high-temperature washes were performed at 60 °C.

Labeling of nucleic acids

The 2.2 kb *EcoRI-BamHI* fragment (spanning *atp6* coding region) of an 8.2 kb *PstI* clone from *pol* mtDNA (Singh and Brown 1991; chapter II) was purified from agarose gels using the Prep-A-Gene system from Bio-Rad, and the purified DNA fragments were labeled using the nick translation system of BRL Life Technologies Inc.. The oligonucleotides were end labeled by phosphorylation with bacteriophage T4 polynucleotide kinase as described by Sambrook *et al.* (1989).

Sequence determination and primer extension analysis

The double stranded DNA was alkali-denatured, annealed with the appropriate primer and sequenced with the Sequenase system of U.S. Biochemical Corporation by following the vendor's protocol. Primer extension analysis was carried out as described in Brown et al. (1991).

RESULTS

A copy of *orfB* is similarly organized and expressed in male sterile and male fertile plants

We have shown previously (Singh and Brown 1991; chapter II) that transcripts of mitochondrial *atp6* gene region, associated with the Polima CMS in *Brassica napus*, span two open reading frames, one coding for subunit 6 of the ATPase complex and the other, upstream of *atp6* sequence and designated *orf224*, coding for a chimeric protein, the first 58 codons of which are derived from the amino-terminal coding region of the mitochondrial *orfB* gene (Hiesel et al. 1987). This gene has been found in the mitochondria of several plant species and shown to be transcribed and translated (Quagliariello et al. 1990; Gualberto et al. 1991), thus is apparently an essential gene.

It seemed possible that the Polima CMS might be caused by the absence of an intact *orfB* gene in the mitochondrial genome of *pol* cytoplasm plants rather than the *orf224/atp6* rearranged gene region *per se*. To explore this possibility, the Southern blots of *nap* and *pol* mtDNA, digested with *SalI*, *EcoRI*, *BamHI* and *PstI*, were probed with an end labelled synthetic oligonucleotide (29-mer) specific to the *orfB* portion of *orf224* (Figs. 1 and 4). The presence of two hybridizing fragments in each lane of figure 1A and 1B shows that the *orfB* sequence is present in two copies in both *pol* and *nap* cytoplasm plants. One of the copies in *pol* cytoplasm plants represents the *orf224* sequence; we have previously shown that this region is organized differently in *nap* and *pol* mtDNAs (Singh and Brown 1991; chapter II). The other copy, which is the same size (10.5 kb) in both mtDNAs (Fig. 1C), likely represents the intact *orfB* gene in *pol* plants. To determine if the copy that is organized similarly in *nap* and *pol* mtDNAs is expressed in *pol* plants, a gel blot of mtRNAs isolated from *nap*, *pol* and fertility restored *pol* plants was probed with the end labelled *orfB* specific oligonucleotide. Transcripts of 1.2-, 0.9- and 0.8 kb were common to all three types of plants (Fig. 2). As expected, the *orfB* specific probe also hybridized to 2.2- and 1.9 kb transcripts in *pol* cytoplasm plants (Fig. 2, Lanes 2, 3 and 4), corresponding to the dicistronic *atp6* transcripts containing the chimeric *orfB* gene sequence (Singh and Brown 1991; chapter II). As we have previously shown that sequences specific to *orf224* are not expressed in *nap* mitochondria, we infer from these results that the transcripts common to both the *nap* and *pol* cytoplasm plants are derived from intact *orfB* and that a functional copy of *orfB* is present in *pol* plants. It is thus more probable that the Polima CMS results from the rearrangement upstream of *atp6*, and not from the absence of a functional *orfB* gene.

The Complex *atp6* transcript pattern in *pol* plants appears to be generated by a combination of multiple initiation and processing events.

The *atp6* gene is present in single copy in both *pol* and *nap* mitochondrial genomes.

Figure 1. Southern analysis of *orfB* sequences of *nap* and *pol* mtDNAs.

Regent (*pol*) (A) and Regent (*nap*) (B) mtDNAs were digested with *SalI* (lanes 1), *EcoRI* (lanes 2), *BamHI* (lanes 3) and *PstI* (lanes 4) and hybridized with the end labeled 29-mer *orfB*-specific oligonucleotide (5' CTGCTGATCCCAAGTACTCCATCTCCATC 3'). *SalI* digested mtDNAs (C) from Regent (*pol*) (lane 1) and Regent (*nap*) (lane 2) plants were hybridized with the labeled *orfB*-specific oligonucleotide. The 20.1 kb band in lane 2 is faint due to the poor transfer of the larger DNA fragments from the gel. Estimated lengths of the hybridizing fragments are indicated in kilobases at the side of each panel.

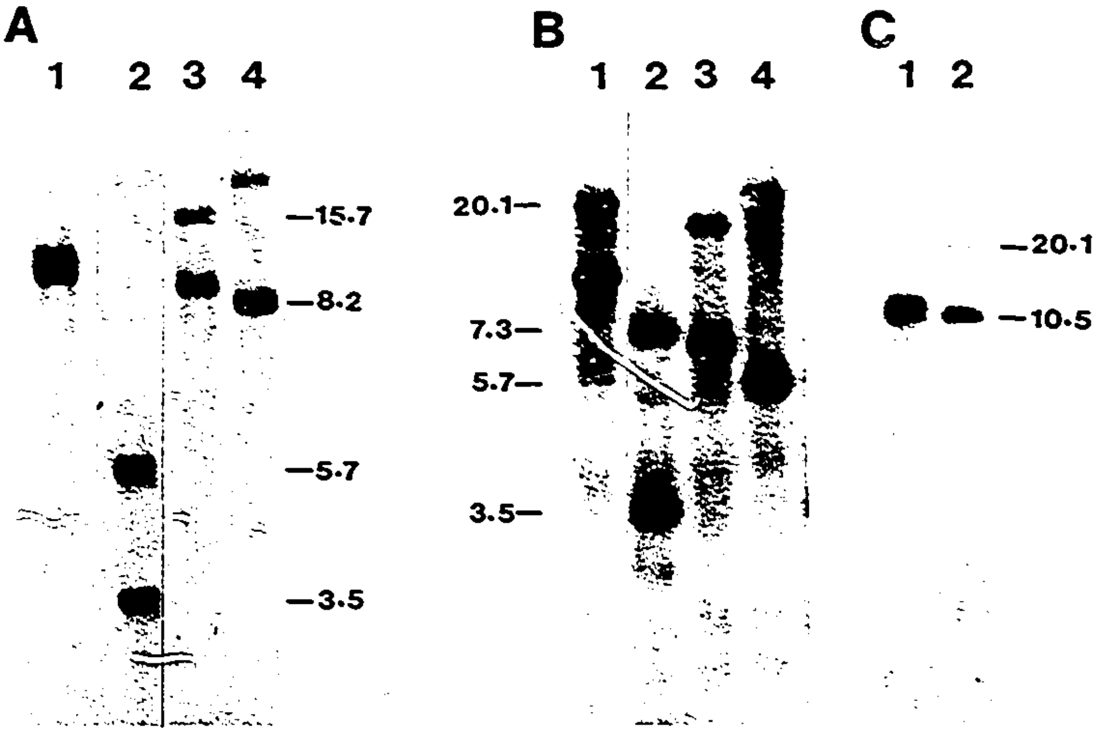
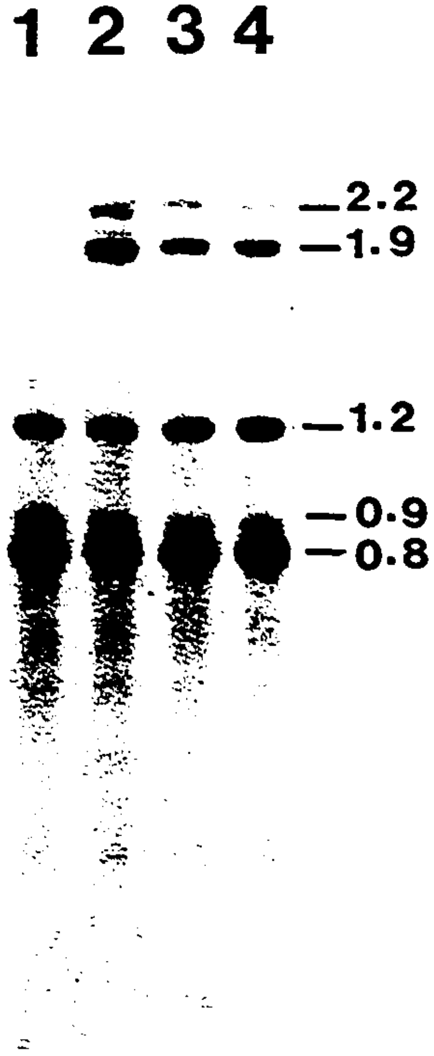

A
1
2
3
4
—15·7
—8·2
—5·7
—3·5
B
1
2
3
4
20.1—
7.3—
5.7—
3.5—
C
1
2
—20·1
—10·5

Figure 2. RNA gel blot analysis of *orfB* transcripts of male fertile, male sterile and fertility restored *B. napus* plants.

Mitochondrial RNAs resolved on an agarose-urea gel were transferred to a hybridization membrane and probed with the end labeled *orfB*-specific oligonucleotide. Lane 1, Karat (*nap*) (male fertile); lane 2, Karat (*pol*) (male sterile); lane 3, Westar-*Rf* (*pol* cytoplasm, nuclear fertility restored); lane 4, Karat (*pol*) x Westar-*Rf*. Lengths of the transcripts are indicated in kilobases.

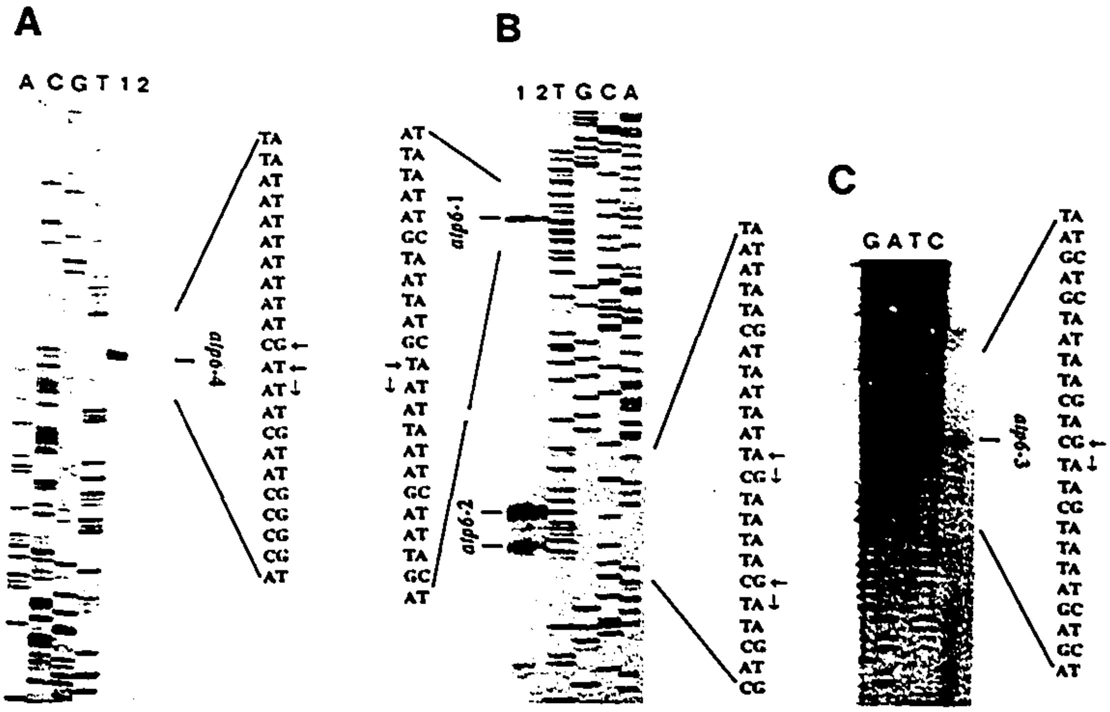

1
2
3
4
2.2
1.9
1.2
0.9
0.8

While a single abundant transcript is observed in *nap* plants, male sterile and fertility restored *pol* cytoplasm plants display a relatively complex pattern (Singh and Brown 1991; chapter II). To understand the basis of this complex transcript pattern, the 5' ends of the 2.2- and 1.9 kb *atp6* transcripts, found in both sterile and restored *pol* plants, as well as the 1.4- and 1.3 kb transcripts, specific to restored *pol* plants, were mapped by primer extension analysis (Figs. 3 and 4; primer extension data for 1.3 kb transcript shown in Figure 1 of appendix II).

The largest *pol* transcripts, all approximately 2.2 kb in size, mapped to sites located 443, 449 and 521 nucleotides upstream of the *orf224* initiation codon. These sites comprised two similar sequence motifs (*atp6*-1 and *atp6*-2; Figs. 3B and 5A) which closely resemble (8 of 9 nucleotide identity) the yeast mitochondrial promoter (Fig. 5A) (Biswas et al. 1985; Schinkel et al. 1986). The finding of two 5' mitochondrial transcript termini mapping to a single sequence motif is unusual, but has been observed previously (Covello and Gray 1991). In addition, several other plant mitochondrial transcript termini have been found to map to sequences similar to the yeast promoter (Issac et al. 1985; Young and Hanson 1986). The 5' end of the 1.9 kb transcript maps to a sequence motif (Figs. 3C and 5B) which has previously been shown by sequencing *in vitro* capped transcripts and primer extension analysis to be associated with the transcription initiation in soybean mitochondria (Brown et al. 1991). Eighteen of the twenty one nucleotides at positions -13 to +8 are identical in the *Brassica* (*atp6*-3) and soybean sequences surrounding the 5' transcript termini (Fig. 5B). Similar regions have also been found surrounding 5' transcript termini in *Petunia* (Fig. 5B) and other dicot mitochondria (Young et al. 1986; Moon et al. 1985; Schuster et al. 1987). The 1.4- and 1.3 kb transcripts, specific to the fertility restored *pol* plants, map to sequence regions which show no significant similarity with any of the known mitochondrial promoter-like elements (Figs. 3A and 5C), and thus RNA processing seems more likely to be responsible for generating these

Figure 3. Mapping of 5' termini of mitochondrial *atp6* transcripts of male sterile and fertility restored *pol* cytoplasm plants.

Primer extension products were obtained using an appropriate oligonucleotide as primer with 5 µg of mtRNA. The extension-reaction product was run alongside DNA sequencing reactions primed with the same oligonucleotide. The positions of 5' transcript termini and direction of transcription are indicated by horizontal and vertical arrows, respectively. (A) Primer extension with oligonucleotide (5' CCAGGAGGGCTTGACGCCG 3') complementary to bases -328 to -346 upstream of the *atp6* initiation codon of *pol* mtDNA. Lane 1 represents 4007 plants (fertility restored) and lane 2 represents 2007 plants (*pol* cytoplasm, male sterile). (B) Primer extension with oligonucleotide (5' GCAACTCAACAGAAACGG 3') complementary to bases -1204 to -1221 upstream of the *atp6* initiation codon of *pol* mtDNA. Lane 1, 2007 (*pol*); lane 2, 4007. (C) Primer extension with oligonucleotide (5' AGTTGTGGTTGGTTGTTG 3') complementary to bases -970 to -987 upstream of the *atp6* initiation codon of *pol* mtDNA. mtRNA from 2007 (*pol*) plants was used for primer extension reaction.


A
A C G T 1 2
TA
TA
AT
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AT
AT
AT
AT
AT
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CG ←
AT ←
AT ↓
AT
CG
AT
AT
CG
CG
CG
CG
AT
atp6-4
B
1 2 T G C A
AT
TA
TA
AT
AT
GC
TA
AT
TA
AT
GC
→ TA
↓ AT
AT
TA
AT
AT
GC
AT
AT
TA
GC
AT
atp6-1
atp6-2
TA
AT
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TA
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CG
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CG ↓
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G A T C
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GC
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GC
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atp6-3

Figure 4. Graphical representation of the *pol atp6* region and mapping of 5' transcript termini.

Vertical lines at the right end of lines with arrows on the left indicate the position of the primers used for the primer extension reactions; the position of the arrows indicate the mapped 5' transcript termini. The 5' termini of the 2.2 kb *atp6* transcript observed in RNA gel blots correspond to *atp6*-1 and *atp6*-2, while the 5' termini of the 1.9-, 1.4-, 1.3- and 1.1 kb *atp6* transcripts correspond to *atp6*-3, *atp6*-4, *atp6*-5 and *atp6*-6, respectively. Asterisks indicate the 5' termini of transcripts specific to fertility-restored plants. The unfilled box represents the region of *orf224* which corresponds to the *orfB* coding sequence. The position of the 29-mer *orfB*-specific oligonucleotide, used for the Northern and Southern analyses (Figs. 1 and 2), is shown by the vertical bar below the *orfB* coding region. The filled inverted triangle represents the approximate position of the 3' *atp6* transcript terminus.

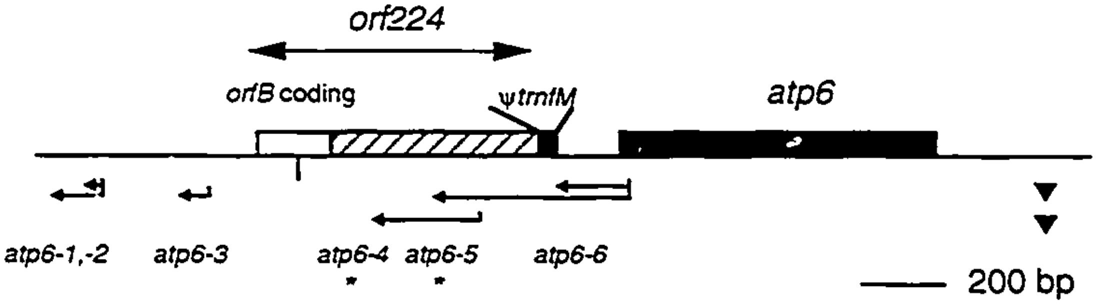
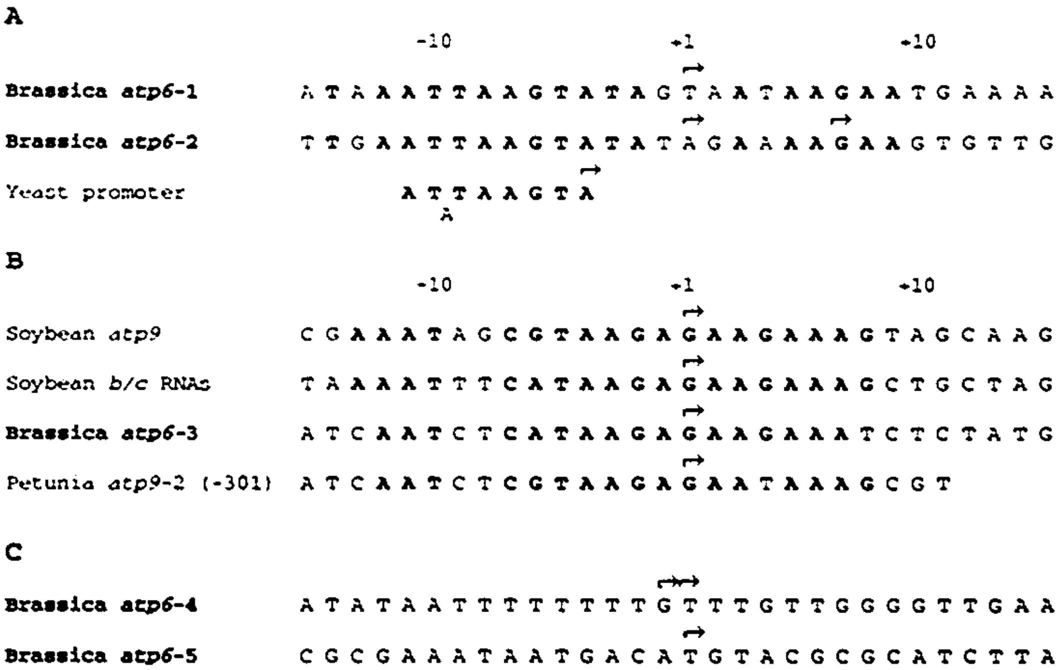

orf224
orfB coding
ψtrnfM
atp6
atp6-1,-2
atp6-3
atp6-4
*
atp6-5
*
atp6-6
200 bp

Figure 5. Comparison of nucleotide sequences surrounding the 5' termini of *pol atp6* transcripts with the yeast mitochondrial promoter and dicot mitochondrial initiation sequences.

(A) Alignment of nucleotide sequences surrounding the 5' transcript termini representing the 2.2 kb *atp6* transcript and their similarity with the yeast mitochondrial promoter sequence. The identical nucleotides are shown by bold letters. (B) Comparison of 5' terminal region of the 1.9 kb *atp6* transcript with mitochondrial initiation sequences of soybean and the 5' terminal region of the *Petunia atp9-2* (-301) transcript. Nucleotides corresponding to the derived soybean identity (Brown et al., 1991) are indicated by bold letters. (C) 5' terminal regions of 1.4- (*atp6*-4) and 1.3 kb (*atp6*-5) *atp6* transcripts of fertility restored *pol* cytoplasm plants. The initiating nucleotide or the mapped 5' transcript end is indicated by arrow. Nucleotide +1 corresponds to either the mapped 5' transcript end or the first nucleotide of the soybean primary transcripts.


A
-10
+1
+10
Brassica atp6-1
A T A A A T T A A G T A T A G T A A T A A G A A T G A A A A
Brassica atp6-2
T T G A A T T A A G T A T A T A G A A A A G A A G T G T T G
Yeast promoter
A T T A A G T A
A
B
-10
+1
+10
Soybean atp9
C G A A A T A G C G T A A G A G A A G A A A G T A G C A A G
Soybean b/c RNAs
T A A A A T T T C A T A A G A G A A G A A A G C T G C T A G
Brassica atp6-3
A T C A A T C T C A T A A G A G A A G A A A T C T C T A T G
Petunia atp9-2 (-301)
A T C A A T C T C G T A A G A G A A T A A A G C G T
C
Brassica atp6-4
A T A T A A T T T T T T T T G T T T G T T G G G G T T G A A
Brassica atp6-5
C G C G A A A T A A T G A C A T G T A C G C G C A T C T T A

transcript termini. We have already shown that the 5' end of 1.1 kb *atp6* transcript maps to the 3' end of a tRNA pseudo gene (Fig. 4) and suggested that it is most likely generated by processing of a tRNA-like element (Singh and Brown 1991). Taken together, these observations suggest that the complex *atp6* transcript pattern observed in *pol* cytoplasm plants is a result of multiple transcript initiation and processing events.

Expression of the *pol atp6* gene region is developmentally regulated

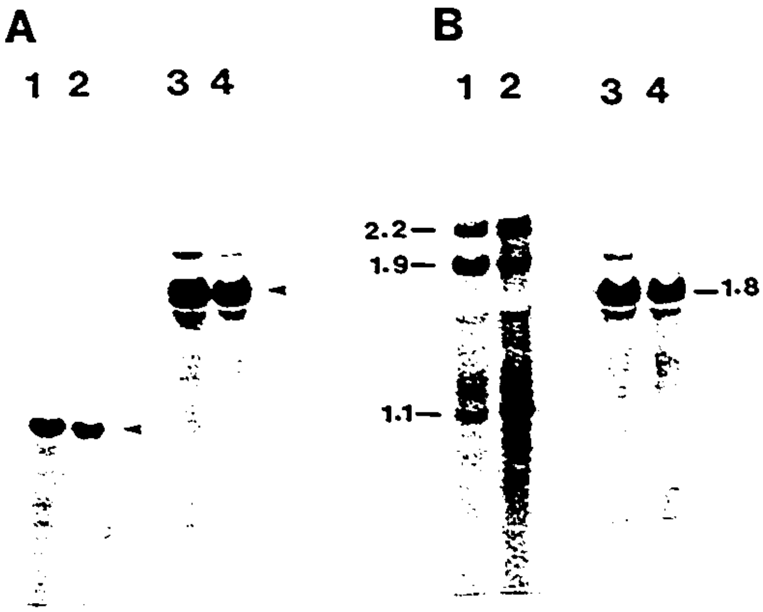
Young and Hanson (1987) observed anther-specific quantitative changes in CMS-associated transcripts in *Petunia*, and suggested that this developmental regulation might account, in part, for the failure of CMS mutations to affect tissues other than those involved in pollen production. To determine if expression of the *pol orf224/atp6* region is developmentally regulated, transcripts from floral tissue and dark-grown seedlings, were probed with *Brassica atp6* sequences (Fig. 6). The levels of monocistronic 1.1 kb *atp6* transcripts were found higher in seedlings as compared to the floral tissue in *pol* plants (Figs. 6b and 6c) and this increase in the seedlings was observed irrespective of the presence or absence of the restorer gene. However, no significant differences in the levels of *atp6* transcripts were observed between seedlings and floral tissue in *nap* cytoplasm plants (Fig. 6A), nor between the *rrn18* transcripts of seedlings and floral tissue of any of the plants examined. Smaller shifts in the relative amounts of the 2.2- and 1.9 kb *pol* specific transcripts were also observed (Fig. 6).

DISCUSSION

We have previously shown that the *atp6* and flanking sequences of the *pol* and *nap* mtDNAs are identical to a point situated 34 bp upstream of a truncated gene (ψ*trnfM*) that lies within the conserved region and encodes only the sixteen 3'

Figure 6. RNA gel blot analysis of atp6 transcripts of seedlings and floral tissue of *B. napus* plants.

Mitochondrial RNAs resolved on an agarose-urea gel were transferred to a hybridization membrane and hybridized with the *Brassica atp6* probe (2.2 kb EcoRI-BamHI fragment of *pol* mtDNA; Singh and Brown 1991; chapter II) (lanes 1 and 2). After removal of the *atp6* probe, the filter was rehybridized with the maize *rrn18* probe (2.6 kb HindIII fragment; Finnegan and Brown 1990) (lanes 3 and 4). **(A)** Regent (*nap*) mtRNAs; **(B)** 2007 (*pol*) mtRNAs; **(C)** 4007 mtRNAs. Lanes 1 and 3 represent mtRNAs isolated from floral tissue; lanes 2 and 4 represent mtRNAs isolated from seedlings. Lengths of the transcripts are indicated in kilobases. Arrows indicate the locations of the 1.1-, 1.8-, 1.9- and 2.2 kb transcripts.


A
1 2 3 4
B
1 2 3 4
2.2
1.9
1.1
1.8
C
1 2 3 4
1.4
1.3

terminal nucleotides of $tRNA^{fMet}$; no similarity between the *pol* and *nap* sequences further upstream of this point is evident. We have suggested that the single 1.1 kb *atp6* transcript found in *nap* cytoplasm plants, which maps precisely to the 3' end of the pseudo gene, is generated by the tRNA processing machinery, since the *nap* sequences upstream of *ψtrnfM* are capable of base pairing with the remnant tRNA sequence such that the transcript would be expected to adopt a tRNA-like structural configuration (Brown and Singh 1991; chapter II). Larger *nap atp6* transcripts of approximately 2 kb observed upon longer exposure of the autoradiograph of figure 6A (shown in Figure 2 of appendix II) are likely candidates for precursors to this 1.1 kb transcript. The *pol* sequences upstream of *ψtrnfM* do not allow for the formation of a tRNA-like element, and processing at the 3' end of the pseudo gene is thereby expected to be markedly reduced, resulting in the predominance of long dicistronic *orf224/atp6* transcripts observed in floral tissue of CMS plants.

Densitometric analysis (not shown) indicates that the ratio of the 1.1 kb monocistronic to dicistronic *atp6* transcripts is approximately four times higher in seedlings than in floral tissues of male sterile *pol* plants and it seems likely that this relative increase in 1.1 kb *atp6* transcripts results from enhanced transcript processing at the 3' end of the pseudo gene. In floral tissue of nuclear fertility restored plants, the ratio of monocistronic to dicistronic transcripts is increased, in part as a result of generation of the restored-specific 1.4- and 1.3 kb transcripts and in part as a result of enhanced processing at the 3' end of the tRNA pseudo gene. Because of the close correlation of monocistronic transcripts with male fertility, we have suggested that the *pol* CMS may be caused by partial mitochondrial dysfunction that results from inefficient translation of the ATP6 protein off dicistronic messages. The relatively higher levels of monocistronic transcripts in seedling tissues might circumvent this possible deficiency in the ATP6 protein and could account, at least partially, for the failure of the *pol* cytoplasm to induce overt phenotypic effects on vegetative tissues in male sterile plants. The activity responsible for enhanced levels of the 1.1 kb *pol* transcript in seedlings is apparently different from the restorer gene product, since this increase is observed in both CMS and restored plants and since 1.4- and 1.3 kb transcripts specific to restored plants are not seen in seedlings of CMS plants. The 1.1 kb transcript is apparently generated by tRNA processing activity and it seems likely that there is differential expression of this activity in seedlings and flowers. Developmental regulation of plant mitochondrial transcript levels has previously been reported by Young and Hanson (1987); these transcripts were also derived from a CMS-associated gene region.

Since the 1.4- and 1.3 kb *atp6* transcripts specific to fertility restored *pol* cytoplasm plants were observed in seedlings and flowers (Fig. 6C) as well as leaves (shown in Figure 2 of appendix II), the effect of the restorer gene is not specific to floral tissue. We have shown (chapter IV of this thesis), by analyzing individual progeny of a backcross between CMS and fertility restored *pol* cytoplasm plants, that transcript alterations of restored lines co-segregate with, and hence are very likely to be due to, the *Rfp1* restorer allele. Primer extension analysis maps the 5' ends of the 1.4 and 1.3 kb *atp6* transcripts, specific to restored lines, to regions of mtDNA which show no similarity either to one another or to suspected plant mitochondrial promoter elements (Young and Hanson 1986; Schuster et al. 1987; Mulligan et al. 1991; Covello and Gray 1991; Brown et al. 1991). It seems unlikely that the product of a single nuclear gene could create new transcription initiation sites at two unrelated sequences which bear no resemblance to mitochondrial promoters, and we therefore infer that an RNA processing event is likely to be involved in the generation of the restored-specific transcripts. Hence, the restorer gene probably encodes a factor involved, directly or indirectly, in transcript processing. However, the sequences to which these transcripts map also show no significant similarity to putative mitochondrial transcript processing signals (Maloney et al. 1989; Schuster and Brennicke 1989) or to sequences corresponding to the processing sites of transcripts specific to fertility restored *cms-T* maize (Kennel and Pring 1989); we, therefore, cannot rule out the possibility that the regions surrounding the 5' ends of 1.4- and 1.3 kb transcripts are recognized as highly unusual promoters specific to fertility restored *pol* plants by an unusual mechanism. Since the regions surrounding 5' termini of the two transcripts specific to the restored plants are not similar to one another, it is possible that the specificity of the nuclear restorer activity does not involve primary structure recognition and that the restorer gene product enhances *orf224/atp6* transcript processing by recognizing or altering mtRNA secondary structure.

We have previously suggested that the *pol* CMS could result from partial mitochondrial dysfunction, due either to the presence of the aberrant ORF224 protein or to a deficiency in ATPase subunit 6 resulting from a limitation in translation imposed by the co-transcribed *orf224* gene (Singh and Brown 1991; chapter II). However, fertility restoration leads to only a slight decrease in *orf224/atp6* dicistronic transcripts, and would not therefore be expected to result in appreciably lower amounts of the ORF224 protein. Since fertility restoration increases the levels of monocistronic *atp6* transcripts, it may thus be more likely that mitochondrial dysfunction in sterile plants results from a deficiency in the ATP6 protein. In this sense, it is interesting that seedling tissues of sterile plants also have increased levels of monocistronic *atp6* transcripts, while the levels of dicistronic transcripts do not change significantly. This would reduce the impediment imposed on vegetative growth by mitochondrial dysfunction arising from ATP6 deficiency but not from the presence of the ORF224 protein. Considered together, the alterations in the *pol orf224/atp6* region transcripts observed both during developmental and upon fertility restoration suggest that mitochondrial dysfunction in floral tissues of sterile plants is more likely to result from ATP6 deficiency.

Recent studies in this laboratory provide further evidence in support of the possible involvement of the *orf224/atp6* gene region in *pol* CMS. A comparison of the physical maps of mitochondrial genomes of the sterile *pol* and fertile *cam* cytoplasms shows that the only difference detected between the two genomes is an insertion upstream of *atp6* in the *pol* mtDNA that encompasses the previously described rearrangement upstream of *atp6* . In addition, the *orf224/atp6* region appears to be the only region whose transcripts are affected by nuclear fertility restoration (Y. L'Homme and G. G. Brown, unpublished observations; chapter IV of this thesis).

ACKNOWLEDGMENTS

We thank Drs. Peter B.E. McVetty, Dave Hutcheson and Larry Sernyk for gifts of seed; Barbara Young for her help in performing one of the primer extension experiments (Figure 3C); and Martine Jean for useful comments on the manuscript and for providing floral tissue for F_1 hybrid plants used in this study. This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada. Plant growth facilities of McGill University Phytotron were used for this study. Mahipal Singh is the recipient of a Government of India scholarship.

CHAPTER IV

Nuclear gene influences on expression of the *Brassica* mitochondrial genome and Polima cytoplasmic male sterility

In chapters II and III, I have presented experimental results regarding identification and detailed characterization at the structural and expression levels of the *pol* mitochondrial gene region, *orf224/atp6*, associated with CMS in *Brassica napus*. Since only sequences representing 14 known mitochondrial genes were screened in these studies, it was not clear whether *orf224/atp6* is the only differentially expressed region among fertile, sterile and fertility-restored plants. To determine if region(s) of the *pol* mitochondrial genome, other than *orf224/atp6*, might be differentially expressed at RNA level among these plants, *Brassica campestris* clones representing more than 90% of the mitochondrial genome were used as probes. The results of this screening are presented in this chapter. Also, the tight correlation between the altered expression of *orf224/atp6* transcripts in *pol* plants and CMS will be demonstrated through the analysis of backcross progeny.

SUMMARY

The association of altered expression of the *atp6* gene region with Polima (*pol*) cytoplasmic male sterility (CMS) in *Brassica napus* has previously been demonstrated by screening fourteen of the known mitochondrial genes for their differential expression among male fertile *nap*, male sterile *pol* and fertility restored *pol* cytoplasm plants at the RNA level. To explore the possibility of other *pol* mitochondrial DNA (mtDNA) region(s) being associated with the *pol* CMS, we screened *Brassica campestris* clones, representing more than 90% of the mitochondrial genome, for their differential expression among these plants. Two clones, BC-10 and BC-32, representing 2.0- and 12.4 kb *PstI* fragments, respectively, were found to show differential expression. In each case, a complex pattern of transcripts was obtained and only the smallest of the hybridizing transcripts showed differential expression, suggesting that a transcript processing event might be involved in the generation of the transcript differences. The smallest of the transcripts detected by clone BC-10 was 1.3 kb while the smallest transcript detected by clone BC-32 was 1.6 kb; both transcripts were detected in male fertile *nap* and male sterile *pol* cytoplasm plants but not in male fertile *pol* cytoplasm plants homozygous for the fertility restorer nuclear gene *Rfp1*. Further analysis showed that both these transcripts were present in the male fertile *pol* cytoplasm plants heterozygous for the *Rfp1* gene, suggesting that *Rfp1* is recessive to *rfp1* with respect to effects on transcripts of these mitochondrial gene loci. Interestingly, these transcripts were not detected in male sterile *nap* cytoplasm plants (*nap* cytoplasm induces male sterility in Bronowski line of *Brassica napus*) and F_1 hybrid male fertile plants obtained from the cross Bronowski (*nap*) X Westar-*Rf* (Westar-*Rf* line is homozygous for the *Rfp1* gene), suggesting that Bronowski nuclear genome is homozygous for the recessive allele affecting the expression of these transcripts. Since these transcripts are present in male fertile *nap*, male sterile *pol* and male fertile *pol* cytoplasm plants heterozygous for *Rfp1* gene and absent in male fertile *pol* plants homozygous for the *Rfp1* gene and male sterile

and fertility restored Bronowski (*nap*) plants, their differential expression does not show any correlation with the cytoplasmic male sterility in *Brassica napus*. However, the nuclear gene involved in their expression might reside at or be very tightly linked to the *Rfp1* locus. On the other hand, analysis of the backcross progeny shows that the altered expression of the *pol atp6* transcripts in male fertile plants cosegregates with the fertility restoration , demonstrating its very tight association with the *pol* CMS and *Rfp1* gene.

INTRODUCTION

The nuclear influences on mitochondrial gene expression with respect to CMS have been examined in several cases. In Texas male sterile (T) cytoplasm maize, the abundance of *T-urf13* (a mitochondrial gene associated with CMS) transcripts is slightly reduced and a new transcript is generated as a result of fertility restoration by the dominant nuclear restorer gene, *Rf1*. The 5' terminus of the transcript specific to the fertility restored plants mapped internal to *T-urf13* (Kennel et al., 1987 : Kennel and Pring, 1989). The nuclear restorer gene, *Rf*, influences the relative abundance of the three 5' transcript termini of *Petunia* CMS-associated *S-pcf* locus (Pruitt and Hanson, 1991). In addition, other nuclear genotypes which lack the *Rf* allele also influence the total abundance of *pcf* transcripts (Pruitt and Hanson, 1991).

Alterations in mitochondrial gene expression have been observed when teosinte (wild relative of maize) cytoplasms are introduced into certain maize inbred nuclear backgrounds (Cooper et al.,1990). *Zea perennis* and *Zea diploperennis* (teosintes) mitochondrial *coxII* transcript patterns were found to be dependent upon the maize nuclear genotype. Genetic analysis showed that a single nuclear gene, designated *Mct*, is responsible for altering the *coxII* transcript pattern. The action of this gene was found to be specific for *coxII*, as the transcript pattern of other two mitochondrial *cox* genes (*coxI* and *coxIII*) remained unaffected. However, no direct correlation between the expression of CMS and the altered *coxII* transcript pattern was found (Cooper et al.,1990). In *Nicotiana*, nuclear influences on mitochondrial gene expression at RNA level were analyzed in six different male-fertile and male-sterile alloplasmic lines. Changes in transcript pattern, which showed correlation with male sterility were detected for three genes (*atpA*, *orf25* and *coxII*). However, the changes were not found to be consistent for all the male-sterile materials (Hakansson and Glimelius, 1991). Transcripts of the mitochondrial gene *atpA* are affected by nuclear genotype in radish and the alterations in transcript pattern were found not to be strictly correlated with cytoplasmic male sterility (Makaroff et al., 1990).

The differential expression of the mitochondrial *atp6* gene region at RNA level among male fertile *nap* cytoplasm, male sterile and fertility restored *pol* cytoplasm plants and its association with the *pol* CMS has been demonstrated (Singh and Brown, 1991; chapter II of this thesis; Handa and Nakajima, 1992). Since only sequences representing relatively small portion of the mitochondrial genome (sequences representing 14 known mitochondrial genes) were screened in these studies, it was not clear whether or not *atp6* is the only differentially expressed region. To determine if region(s) of the *pol* mitochondrial genome, other than *atp6*, might be differentially expressed among *nap, pol* CMS and fertility-restored cytoplasm plants, *Brassica campestris* clones representing more than 90% of the mitochondrial genome were used as probes to analyze the mtRNA gel blots of fertile, CMS and restored plants. *Brassica napus* and *Brassica campestris* mitochondrial genomes are similar in size (approximately 220 kb) (Palmer and Herbon, 1988). Two clones, carrying 2.0- and 12.4 kb *PstI* fragments of the *Brassica campestris* mitochondrial genome, were expressed differently in these plants. However, unlike the *atp6* region, the differential expression of these regions is not correlated with the Polima CMS. On the other hand, the analysis of the progeny of the backcross, involving *pol* male sterile (*rf/rf*) plants and *pol* male fertile F_1 hybrid plants (*Rf/rf*), demonstrates a very tight association between altered expression of the *atp6* region and the Polima CMS in *Brassica napus*.

MATERIALS AND METHODS

Plant material

The phenotype and restorer genotype of the *Brassica napus* lines used in the present study have already been described (Singh and Brown, 1991; chapter II of this thesis), except for the line Bronowski (*nap*), which is male sterile and lacks both of the nuclear restorer alleles *Rfp1* and *Rfp2* (restorers of *pol* CMS) (Brown et al., unpublished observations). Conventions of Kemble and Barsby (1988) were followed for designating cytoplasms (parenthetical italicized cytoplasm designations following the cultivar/strain name). 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 which are homozygous for the nuclear fertility restorer allele *Rfp1* while UM2353 *pol* cytoplasm plants are homozygous for the nuclear fertility restorer allele *Rfp2*. Plants were classified male sterile or male fertile based on their ability to produce 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 °C, 16 hr photoperiod).

Mitochondrial DNA clones

Mitochondrial DNA clones, representing more than 90% of the *Brassica campestris* genome (Palmer and Shields, 1984; Palmer and Herbon, 1988), used in the analysis of *Brassica napus* mitochondrial transcripts are described in Table 1. Dr. Christopher A. Makaroff (University of Ohio, Oxford, USA) furnished all of the *PstI* and *SalI Brassica campestris* clones used in this study. The two *KpnI* clones (Table 1), BC-51 and BC-52, were generated in this laboratory (Yvan L'Homme and Gregory G. Brown, unpublished

Table 1. Mitochondrial DNA Clones From *B. campestris* Used in Transcript Analysis

Clone	Fragment	Clone	Fragment
BC-1	4.8-kb *PstI*	BC-27	4.4-kb *PstI*
BC-3	7.7-kb *PstI*	BC-28	10 1-kb *PstI*
BC-5	10.1-kb *SalI*[a]	BC-30	5.7-kb *PstI*
BC-9	10.2-kb *PstI*	BC-31	3.1-kb *PstI*
BC-10	2.0-kb *PstI*[b]	BC-32	12.4-kb *PstI*
BC-11	9.7-kb *PstI*	BC-33	2.9-kb *PstI*
BC-12	7.5-kb *PstI*	BC-34	10.1-kb *PstI*
BC-13	2.9-kb *PstI*	BC-36	5.0-kb *PstI*
BC-14	1.3-kb *PstI*	BC-37	8.3-kb *SalI*[c]
BC-15	0.9-kb *PstI*	BC-38	6.2-kb *SalI*
BC-17	5.7-kb *PstI*	BC-40	4.8- and 1.3-kb *SalI*
BC-21	21.1-kb *PstI*	BC-41	4.0-kb *SalI*[d]
BC-22	3.6-kb *PstI*	BC-43	6.9-kb *PstI*
BC-25	12.2-kb *PstI*	BC-51	8.8-kb *KpnI*
BC-26	5.2-kb *PstI*	BC-52	5.7- and 1.5-kb *KpnI*[e]

[a] Adjacent to 4.4-kb *SalI* fragment of the *B. campestris* physical map (Palmer and Herbon, 1988).

[b] Adjacent to 9.7-kb *PstI* fragment of the *B. campestris* physical map.

[c] Adjacent to 33-kb *SalI* fragment of the *B. campestris* physical map.

[d] Adjacent to 4.8-kb *SalI* fragment of the *B. campestris* physical map.

[e] Adjacent to 5.7-kb *KpnI* fragment of the *B. campestris* physical map.

data).

Isolation of mtRNA and RNA gel blot analysis

Mitochondrial RNA was isolated as described earlier by Singh and Brown (1991) (chapter II). RNA was resolved on agarose-urea gels (Finnegan and Brown, 1986) or agarose-formaldehyde gels (Maniatis, 1982), transferred to gene screen plus (Dupont/NEN) hybridization membrane by capillary blotting with 1.5M NaCl/0.5M sodium citrate, and hybridized to the radiolabelled probe as described earlier (Singh and Brown, 1991) (chapter II).

Labelling of nucleic acids

DNA probes were radiolabelled using the nick translation system of BRL Life Technologies Inc., as per the protocol supplied by the vendor.

RESULTS

Mitochondrial DNA regions other than *atp6* show differential expression between male sterile and male fertile plants

Previous studies have shown that the differential expression of the mitochondrial *atp6* region is associated with the Polima CMS in *Brassica napus* (Singh and Brown, 1991; chapter II; Handa and Nakajima, 1992); no differences in the expression of the other thirteen mitochondrial gene regions analyzed were observed. To explore the possibility of association of some other region(s) of the *pol* mitochondrial genome with CMS, we searched for differences in expression among the near isogenic lines male fertile Westar (*nap*), male sterile Westar (*pol*) and fertility-restored Westar (*pol*), using a set of *Brassica campestris* clones (Table 1), representing >90% of the mitochondrial genome. Two clones, BC-10 and BC-32 containing 2.0- and 12.4 kb *PstI* fragments, respectively, showed differential expression among these three types of plants (Fig. 1). Both clones hybridized to more than one transcript and since the transcript sizes are different in each case, these two clones most likely represent two different transcribed sequences. Interestingly, clone BC-10 detects a 1.3 kb transcript in male fertile *nap* and male sterile *pol* plants but not in fertility restored *pol* plants which are homozygous with respect to the fertility restorer nuclear gene *Rfp1* (Fig. 1A). Similarly, the clone BC-32 detects a 1.6 kb transcript in *nap* and male sterile *pol* plants but not in *pol* (*Rfp1/Rfp1*) plants (Fig. 1B). This suggests that a nuclear gene located at or near the *Rfp1* restorer locus is influencing the transcripts of these regions. In case of both clones, it is of interest to note that the differentially expressed transcript is the smallest of the hybridizing transcripts, suggesting that it is most likely generated by a transcript processing event, though the possibility of involvement of transcript initiation or termination event cannot be ruled out.

Mitochondrial DNA regions showing differential expression do not appear to be involved in cytoplasmic male sterility

To further study the nature of the nuclear gene(s) influencing this differential expression and its relationship with the *pol* CMS, we probed northern blots of mtRNA isolated from various *Brassica napus* lines of different nuclear backgrounds with the clone BC-10 (Fig. 2). The Karat (*nap*) and Karat (*pol*) CMS plants showed the expression of 1.3 kb transcript similar to the Westar (*nap*) and Westar (*pol*) CMS plants (Fig. 2, lanes 1 and 2). The nuclear restorer gene is dominant with respect to its effect on both the suppression of *pol* cytoplasm induced male sterility and transcripts of the *pol* mitochondrial *atp6* locus. The finding that the male fertile F_1 hybrid plants (*Rfp1/rfp1*; *pol* cytoplasm), obtained from a cross between Karat (*pol*) CMS (*rf/rf*) plants and the Westar-*Rf* (*pol*) male fertile plants (*Rfp1/Rfp1*), also expressed the 1.3 kb transcript at levels similar to that of the Karat (*pol*) CMS plants (Fig. 2, lane 4), was therefore

Figure 1. RNA gel blot analysis of mitochondrial transcripts of near isogenic *Brassica napus* lines, hybridizing to 2.0 - and 12.4 kb *PstI* fragments of *Brassica campestris* mtDNA.

Mitochondrial RNAs resolved on an agarose-formaldehyde gel were transferred to a hybridization membrane and probed with (A) 2.0 kb *PstI Brassica campestris* clone, BC-10 and (B) 12.4 kb *PstI Brassica campestris* clone, BC-32. Lanes 1, Westar-*Rf* (*pol* cytoplasm, nuclear fertility restored plants); lanes 2, Westar (*pol*) (male sterile); lanes 3, Westar (*nap*) (male fertile). Molecular sizes are in kilobases.

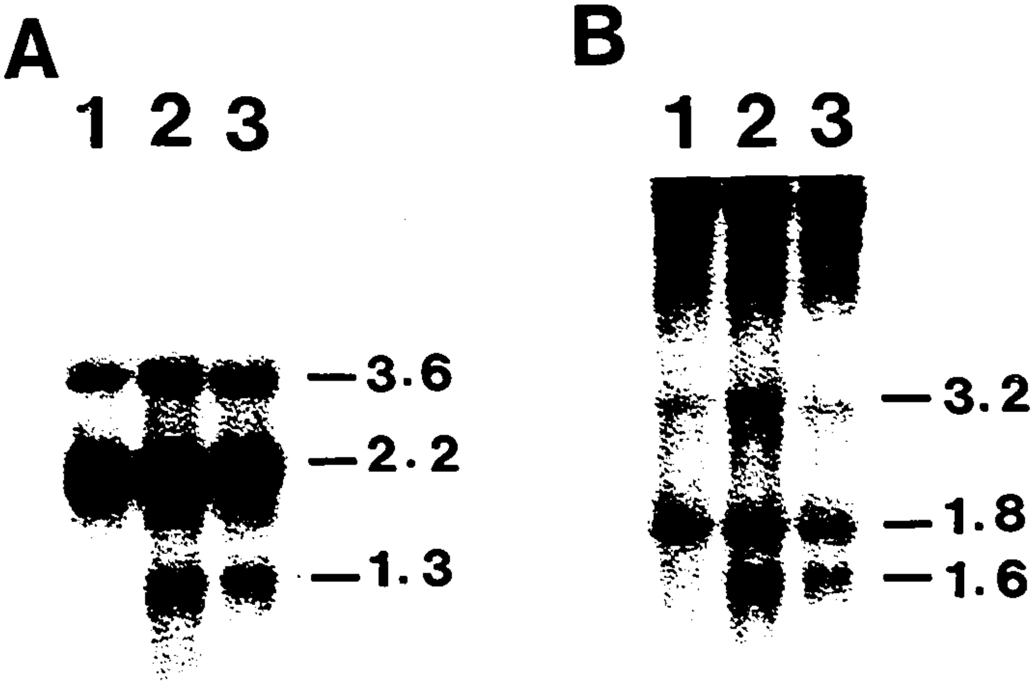

A
1 2 3
3.6
2.2
1.3
B
1 2 3
3.2
1.8
1.6

Figure 2. RNA gel blot analysis of mitochondrial transcripts of *Brassica napus* lines with different nuclear genotypes, using a 2.0 kb *PstI Brassica campestris* clone.

Mitochondrial RNAs resolved on agarose-urea gels were transferred to a hybridization membrane and probed with the labeled 2.0 kb *PstI* clone, BC-10.

Lane 1, Karat (*nap*) (male fertile); Lane 2, Karat (*pol*) (male sterile); Lane 3, Westar-*Rf* (*pol* cytoplasm, male fertile, *Rfp1/Rfp1*); Lane 4, Karat (*pol*) X Westar-*Rf*; Lane 5, Karat (*nap*) X Westar-*Rf*; Lane 6, Italy (*pol* cytoplasm, male fertile, *Rfp1/Rfp1*) Lane 7, UM2353 (*pol* cytoplasm, male fertile, *Rfp2/Rfp2*); Lane 8, Karat (*nap*) (male fertile); Lane 9, Bronowski (*nap*) (male sterile); lane 10, Bronowski (*nap*) X Westar-*Rf*. Molecular sizes are in kilobases. Arrows indicate the locations of 1.3- and 2.2 kb transcripts.

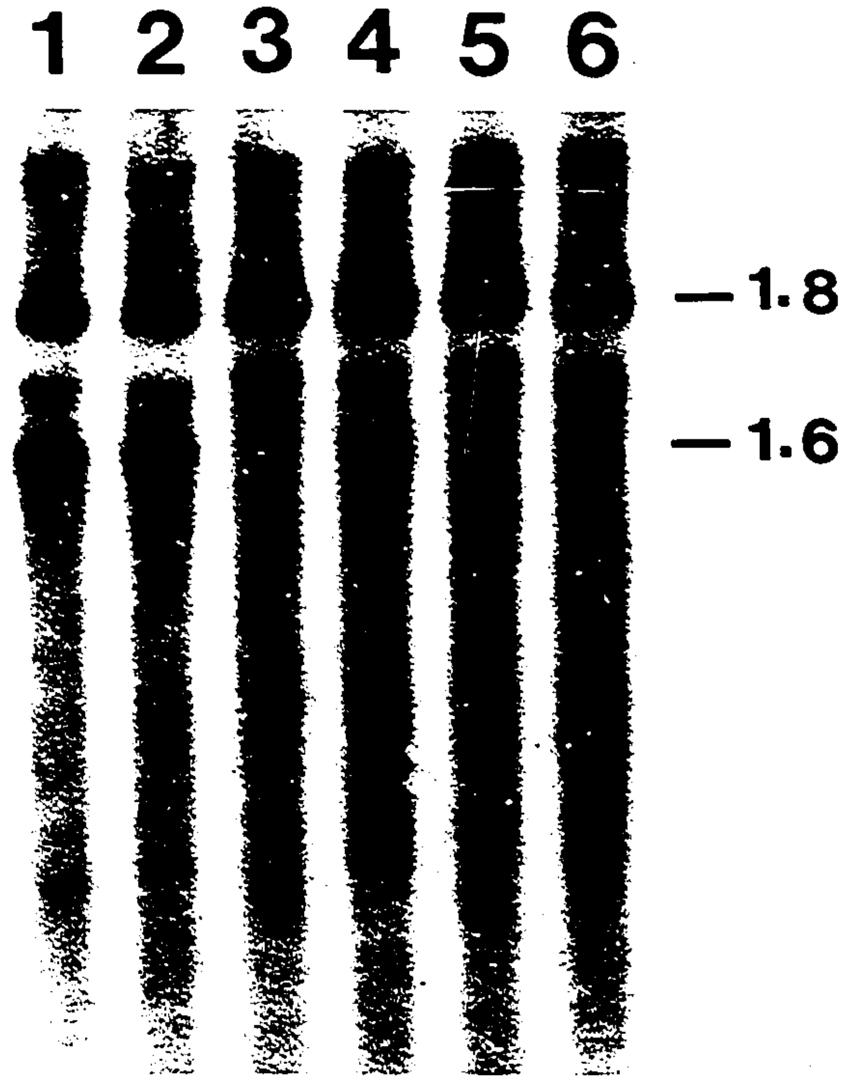

1
2
3
4
5
6
7
8
9
10
2.2
1.3

unexpected. Similarly, the F_1 hybrid plants obtained from the cross Karat (*nap*) X Westar-*Rf* (*pol*; *Rfp1/Rfp1*) showed the presence of this transcript (Fig. 2, Lane 5). We have shown earlier that the two nuclear restorer genes *Rfp1* and *Rfp2*, which are located at two distinct chromosomal loci and segregate independently of one another (Fang and McVetty, 1989), have similar effects on expression of the *atp6* gene region (Singh and Brown, 1991; chapter II). It was, therefore, of interest to examine the effects of these two restoring nuclear genotypes on the BC-10 1.3 kb transcript. As with the *atp6* transcripts, both nuclear backgrounds have similar effects on expression of the 1.3 kb transcript; this transcript was not detected in either of the two strains (Fig. 2, Lanes 6 and 7). The *nap* cytoplasm induces male sterility in certain *Brassica napus* lines such as the cultivar Bronowski (Fan et al., 1986). Again surprisingly, the combination of *nap* cytoplasm with the Bronowski nuclear background results in the disappearance of the 1.3 kb transcript (Fig. 2, Lane 9), and as expected the F_1 hybrid plants (male fertile) from the cross Bronowski (*nap*) CMS X Westar-*Rf* do not show the detectable levels of this transcript (Fig. 2, Lane 10).

The effects of nuclear genotype on the presence or absence of the 1.6 kb transcript detected by clone BC-32 were similar to those on the 1.3 kb transcript detected by clone BC-10. BC-32 hybridizes to a 1.6 kb transcript in Karat (*nap*), Karat (*pol*) and F_1 hybrid plants from the cross Karat (*pol*) CMS X Westar-*Rf* (Fig. 3, Lanes 1,2 and 4). Again, the 1.6 kb transcript was not detected in Bronowski (*nap*) CMS plants and F_1 hybrids obtained from the cross Bronowski (*nap*) CMS X Westar-*Rf* (Fig. 3, Lanes 5 and 6). The male fertile F_1 hybrid plants (*Rfp1/rfp1*) showed a transcript pattern similar to that of fertile *nap* and male sterile *pol* plants (both *rfp1/rfp1*). The results suggest that nuclear restoring genotypes (*Rfp/Rfp*) possess an allele which is recessive to that of genotypes which maintain *pol* sterility (*rfp/rfp*) with respect to effects on transcripts detected by clones BC-10 and BC-32. The nuclear genotype Bronowski, which maintains *nap* cytoplasm induced male sterility, also possesses this or a related recessive

Figure 3. RNA gel blot analysis of mitochondrial transcripts of *Brassica napus* plants with different nuclear backgrounds hybridizing to 12.4 kb *PstI* fragment of *Brassica campestris* mtDNA.

Mitochondrial RNAs resolved on an agarose-urea gel were transferred to a hybridization membrane and probed with the labelled 12.4 kb *PstI* clone. Lane 1, Karat (*nap*) (male fertile); Lane 2, Karat (*pol*) (male sterile); Lane 3, Westar-*Rf*; Lane 4, Karat (*pol*) X Westar-*Rf*; Lane 5, Bronowski (*nap*) (male sterile); Lane 6, Bronowski (*nap*) X Westar-*Rf*. Molecular sizes are in kilobases.


1
2
3
4
5
6
1.8
1.6

allele.

Nuclear gene effects on expression of the *pol atp6* gene region cosegregate with fertility restoration

We have previously shown (Singh and Brown, 1991; chapter II) that the altered expression of the mitochondrial *atp6* gene region is associated with the *pol* CMS. To further confirm this association, we analyzed the backcross population of male sterile and male fertile *pol* plants with respect to the expression of *atp6* region. The male sterile Karat (*pol*) plant was crossed as the female to a male fertile F_1 hybrid plant (*Rfp1/rfp1*) obtained from the cross Karat (*pol*) CMS (*rf/rf*) X Westar-*Rf* (*Rfp1/Rfp1*). Northern analysis of mitochondrial transcripts of fifteen male sterile and fifteen male fertile *pol* individual backcross progeny, using *atp6* as a probe, showed that all the sterile plants (*rf/rf*) tested exhibit male sterile-specific transcript pattern (absence of 1.3- and 1.4 kb *atp6* transcripts specific to fertility restored *pol* cytoplasm plants) and all the male fertile individuals show transcript pattern that is specific to the fertility restored *pol* plants, i.e., the presence of 1.3- and 1.4 kb transcripts (Fig. 4). This gives further support to the view that the *atp6* region of the *pol* mitochondrial genome is involved in CMS in *Brassica napus*.

Discussion

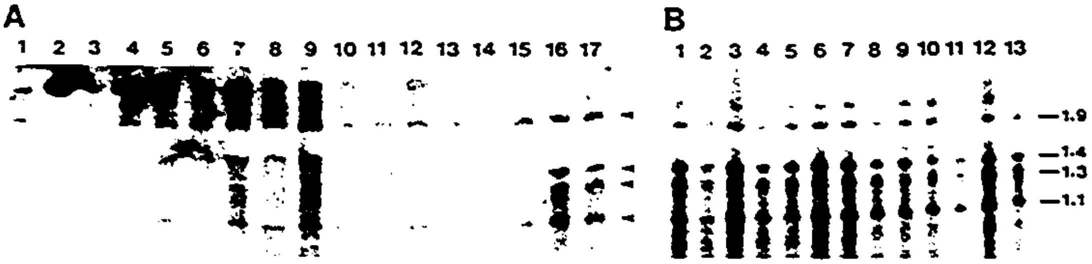
It has been shown previously that the *pol* mitochondrial *atp6* gene region is differentially expressed between male sterile and fertility restored *pol* cytoplasm *Brassica napus* plants (Singh and Brown, 1991; chapter II). The nuclear restorer gene *Rfp1* is dominant with respect to its effects on both male fertility and the atp6 region transcript pattern. The studies involving mapping of the 5' ends of the restored plant specific *atp6* transcripts (1.3- and 1.4 kb) by primer extension analysis suggest that the product of the restorer gene is a nuclear encoded factor involved in transcript processing event (chapter III of

Figure 4. RNA gel blot analysis of *atp6* transcripts of segregating backcross progeny of *pol* cytoplasm *Brassica napus* plants.

Mitochondrial RNAS size fractionated on agarose-urea gels were transferred to hybridization membranes and hybridized with the *Brassica napus atp6* probe (2.2 kb *EcoR1-BamH1* fragment of *pol* mtDNA; Singh and Brown 1991; chapter II).

(A) Lanes 1-15 male sterile plants of the backcross progeny; Lanes 16-17, male fertile plants of the backcross progeny

(B) Lanes 1-13, male fertile plants of the backcross progeny. Arrows represent the 1.1-, 1.3-, 1.4- and 1.9 kb transcripts. Molecular sizes are in kilo bases. Arrows indicate the locations of the 1.1-, 1.3-, 1.4- and 1.9 kb transcripts.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
B
1 2 3 4 5 6 7 8 9 10 11 12 13
1.9
1.4
1.3
1.1

this thesis).

Present studies identify two additional mtDNA regions which are differentially expressed between male sterile and male fertile *pol* plants homozygous for the nuclear fertility restorer gene *Rfp1*. Surprisingly, however, these two mtDNA regions do not show differential expression at the RNA level between male sterile *pol* plants (*rfp1/rfp1*) and male fertile F_1 hybrids *pol* plants (*Rfp1/rfp1*). Since these differences are observed in near isogenic lines, it appears that the *Rfp1* gene behaves as a recessive allele in affecting the expression of these two mtDNA regions or that there is a recessive allele(s), which is recessive with respect to effects on transcripts of these regions, that is tightly linked to *Rfp1*. Interestingly, the nuclear gene(s) affecting the expression of the mtDNA regions corresponding to clones BC-10 and BC-32, appears to be some factor(s) involved in the transcript processing event since it is the smallest hybridizing transcript which is differentially expressed and more likely to be generated by processing event. If this assumption is true, then it raises an interesting possibility that the restorer gene *Rfp1* and the gene(s) affecting the expression of 1.3- and 1.6 kb transcripts are two allelic forms of the same gene. These different alleles could conceivably allow for processing of transcripts of different mitochondrial gene regions by recognizing different structural motifs in RNA.

Obviously, the presence or absence of the 1.3- and 1.6 kb transcripts is not involved in the *pol* CMS, since they are present in male sterile and male fertile (F_1 hybrids) *pol* plants and absent in male fertile *pol* plants homozygous for the fertility restorer nuclear gene *Rfp1*. Also, they are absent in *nap* CMS plants (Bronowski) and fertility restored Bronowski (*nap*) plants, and thus show no correlation with the CMS phenotype. On the other hand, a very tight correlation between the altered expression of *atp6* region transcripts in fertility restored *pol* plants and CMS is demonstrated through the analysis of backcross progeny with respect to expression of *atp6* region.

This study was initiated with the intent of determining if there were any regions of the mitochondrial genome other than *atp6* whose expression differed among *nap* male fertile, *pol* CMS and *pol* fertility restored plants. Only the *atp6* region was found to be expressed differentially between *pol* CMS and male fertile *nap* plants. Although the expression of mtDNA regions other than *atp6* were found to be affected by alleles at the restorer locus, only the *pol atp6* region expression was found to be affected by the restorer allele *per se*.

ACKNOWLEDGMENTS

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Overall Conclusions

The following conclusions emerge from research data presented in this thesis:

The mitochondrial *atp6* gene region is differentially expressed at the RNA level in male fertile (*nap*) and male sterile (*pol*) cytoplasm plants of *Brassica napus*. In fertility restored *pol* plants, the transcript pattern of the *atp6* gene region is altered as compared to male sterile *pol* plants. Since near isonuclear lines were used in these studies, the alteration in transcript pattern seems to be due to the presence of the restorer locus (Chapter II). Segregation analysis of the backcross progeny with respect to the expression of *atp6* region also demonstrates the co-segregation of the restorer locus and the transcript alteration (Chapter IV). The *atp6* gene region seems to be the only region of the *pol* mitochondrial genome whose altered expression at RNA level is correlated with the CMS trait (Chapter IV). The two distinct restorer loci have similar effects on the expression of *atp6* region at RNA level (Chapter II). The effect of the restorer locus on transcripts of *atp6* gene region does not appear to be tissue or developmental stage specific (Chapter III).

Nucleotide sequence and RNA gel blot analyses of the *atp6* region show that a chimeric gene, capable of encoding a polypeptide of 224 amino acids, is located upstream of and cotranscribed with the normal mitochondrial gene, *atp6*. This chimeric gene comprises a portion of the *orfB* gene fused to a sequence of unknown origin (Chapter II). Thus, the *pol* CMS-associated gene region resembles the CMS-associated regions of *Petunia* and maize (*cms-T*). An apparently functional copy of *orfB* (a normal mitochondrial gene) is present in *pol* mitochondrial genome, indicating that the *pol* CMS is not caused by the absence of an intact, expressed *orfB* gene (Chapter III).

Rearrangements in the *pol* mitochondrial genome upstream of the *atp6* gene which apparently affect the folding of transcripts of a pseudo tRNA gene, present immediately upstream of *atp6*, into a tRNA-like structure, appear to be responsible for the differential expression of this region in male fertile (*nap*) and male sterile (*pol*) *Brassica napus* plants (Chapter II). In male fertile normal cytoplasm (*nap*) plants, sequence in the region of pseudo tRNA gene could potentially fold into a tRNA-like structure which could allow for efficient processing of *atp6* transcripts (Chapter II).

In fertility-restored *pol* plants, the levels of monocistronic *atp6* transcripts are increased as compared to the male-sterile *pol* plants which contain predominantly dicistronic *atp6* transcripts (Chapter II). The expression of *atp6* gene region is developmentally regulated in *pol* plants, resulting in increased levels of monocistronic *atp6* transcripts in seedlings relative to floral tissue (Chapter III). Together, these observations suggest that the deficiency in ATP6 protein might be responsible, at least partly, for causing the *pol* CMS in *Brassica napus*.

The 5' termini of dicistronic *atp6* transcripts (2.2- and 1.9 kb) present in both sterile and fertility restored *pol* plants map to sequences resembling mitochondrial transcript initiation sites, whereas the 5' termini of *atp6* transcripts (1.3- and 1.4 kb) specific to fertility-restored *pol* plants map to sequences which resemble neither one another nor mitochondrial promoter motifs (Chapter III). Thus, it appears that the complex *atp6* transcript pattern of fertility-restored plants is generated by a combination of multiple transcription initiation and processing events and that the nuclear restorer locus alters transcript pattern of *atp6* region by affecting RNA processing.

In summary, this study was undertaken with the aim of identifying the region of the *pol* mitochondrial genome that is responsible for the CMS trait. The following lines of evidence presented in this thesis indicate that I have been successful in identifying the *atp6* region as the locus that specifies *pol* cytoplasmic male sterility: (i) the *atp6* gene region is the only mitochondrial gene region found to be expressed differently in *pol* CMS and male fertile *nap* cytoplasm plants; (ii) the *atp6* region is the only region whose expression is affected by the nuclear restorer allele, *Rfp1*; (iii) the *atp6* region resembles the well characterized CMS-encoding gene regions of maize and *Petunia* with respect to both its organization and expression; (iv) the pattern of developmental regulation of *atp6* region transcripts, is consistent with a role for this locus in specifying the CMS trait.

Appendix I

Detection of *pol orf224* encoded protein

SUMMARY

The mitochondrial *atp6* gene region, associated with polima (*pol*) cytoplasmic male sterility (CMS) in *Brassica napus*, contains a chimeric 224-codon open reading frame, designated *orf224*, that is located upstream of and cotranscribed with *atp6* gene. To determine whether *orf224* is expressed at the protein level, we used antibodies raised against a fourteen amino acid-long synthetic peptide corresponding to the deduced amino acid sequence from the portion of *orf224* containing sequence of unknown origin. The antibodies recognize a protein with an apparent molecular mass of approximately 21 kDa in both male sterile and fertility restored *pol* cytoplasm near isonuclear plants. Although the protein is present in fertility restored plants, its levels are somewhat reduced in comparison to *pol* CMS plants.

INTRODUCTION

The experimental results in chapters II, III, and IV demonstrate a very strong correlation between the expression of *atp6* gene region of the *pol* mitochondrial genome and CMS. This gene region has structural and expression features which are similar to those CMS-associated mitochondrial gene regions of maize and *Petunia* (chapter I). In each case, there is a chimeric gene which is located upstream of and cotranscribed with standard mitochondrial gene(s) (chapter I). In maize *cms-T*, the chimeric gene *T-urf13* encodes a 13 kDa protein and its levels are reduced by the presence of fertility restorer allele *Rf1* (Dewey et al., 1987). Similarly in *Petunia*, levels of the CMS-associated 25 kDa protein, encoded by the *pcf* gene, were significantly reduced in fertility restored plants as compared to sterile plants (Nivison and Hanson, 1989). So, it was of interest to examine the expression of *orf224* at the protein level. Antibodies raised against a synthetic peptide derived from the portion of *orf224* containing sequence of unknown origin were used to study the expression of *orf224* at the protein level. Preliminary data indicate that this chimeric gene is expressed at the protein level.

MATERIALS AND METHODS

Isolation of mitochondria and protein extraction

Mitochondria from the floral tissue were isolated by the modified high ionic strength medium procedure of Perez et al. (1990), as described in chapter II of this thesis. Isolated mitochondria were washed twice with acetone, centrifuged for 10 min and the pellet was dried. The pellet was suspended in 2X sample buffer (0.14M Tris-HCl (pH 6.8), 6% SDS (sodium dodecyl sulfate), 10% β-mercaptoethanol and 20% glycerol), boiled for 5min and 1 microlitre of each sample was dot blotted on a nitrocellulose membrane. The membrane was stained with amido black. Amount of protein in each sample was calculated by densitometeric tracing using UltroScan XL (LKB). Bovine serum albumin was used as a standard.

Generation of antibodies

Rabbit antisera to a synthetic oligopeptide [RLGKNRSSDSSRFE; encoded by nucleotides 747-789 of Figure 4 (chapter II)] derived from the predicted ORF224 amino acid sequence were generated by Assay Research Inc. (College Park, MD) after subcutaneous administration of peptide in rabbits. The rabbits were bled after seven days of boost. The peptide was furnished by the Sheldon Biotechnology Center, McGill University, Montreal.

Protein gel electrophoresis, Western blotting and immunodetection

Approximately 150 microgram of each protein sample was resolved by 16% polyacrylamide gel electrophoresis under denaturing condition according to Laemmli,

1970. Proteins were transferred to nitrocellulose sheets as described by Towbin et al., (1979), using a Bio-Rad transblot apparatus. The membranes were incubated overnight at 4 °C in a blocking buffer containing 1% BSA (bovine serum albumin) in PBST (10 mM sodium phosphate, 150 mM NaCl, 0.05% Tween 20). The blots were then reacted for 2 h with anti-ORF224 peptide rabbit antisera diluted 1:1000 in PBST containing 0.5% BSA at room temperature. The membranes were then washed three times (15 min each) in PBST and incubated for 1 h with a 1:4000 dilution of an affinity purified horseradish peroxidase-conjugated goat anti-rabbit IgG in PBST containing 0.5% BSA. The blots were then washed extensively with PBS (10 mM Sodium phosphate, 150 mM NaCl) and incubated with 4-Chloro-1-naphthol developing solution (prepared according to Maniatis et al., 1989). The immunostained membranes were washed with PBS, dried and photographed.

RESULTS AND DISCUSSION

Western blots, containing total mitochondrial proteins from male fertile (*nap*), male sterile (*pol*) and fertility restored (*pol*) near isonuclear Westar lines of *Brassica napus*, were probed with the ORF224 -specific antibodies. The antibodies recognized a protein with an apparent molecular mass of approximately 21 kDa, which is close to the predicted molecular mass of 26 kDa, in *pol* plants (figure 1A, lanes 2 and 3). The levels of this proteins were slightly reduced by the presence of the restorer gene *Rfp1* (figure 1A, lane 3) as compared to the levels in the male sterile plants (figure 1A, lane2). The male fertile *nap* plants do not appear to synthesize this protein (figure 1A, lane 1), although there seems to be a very faint band present in *nap* plants. One of the possibilities is that there is some leakage of the *pol* protein from lane 2. It is interesting to note that Nivison and Hanson (1989) also observed the presence of a faint band corresponding to 25 kDa protein, specific to CMS-cytoplasm, in *Petunia* plants

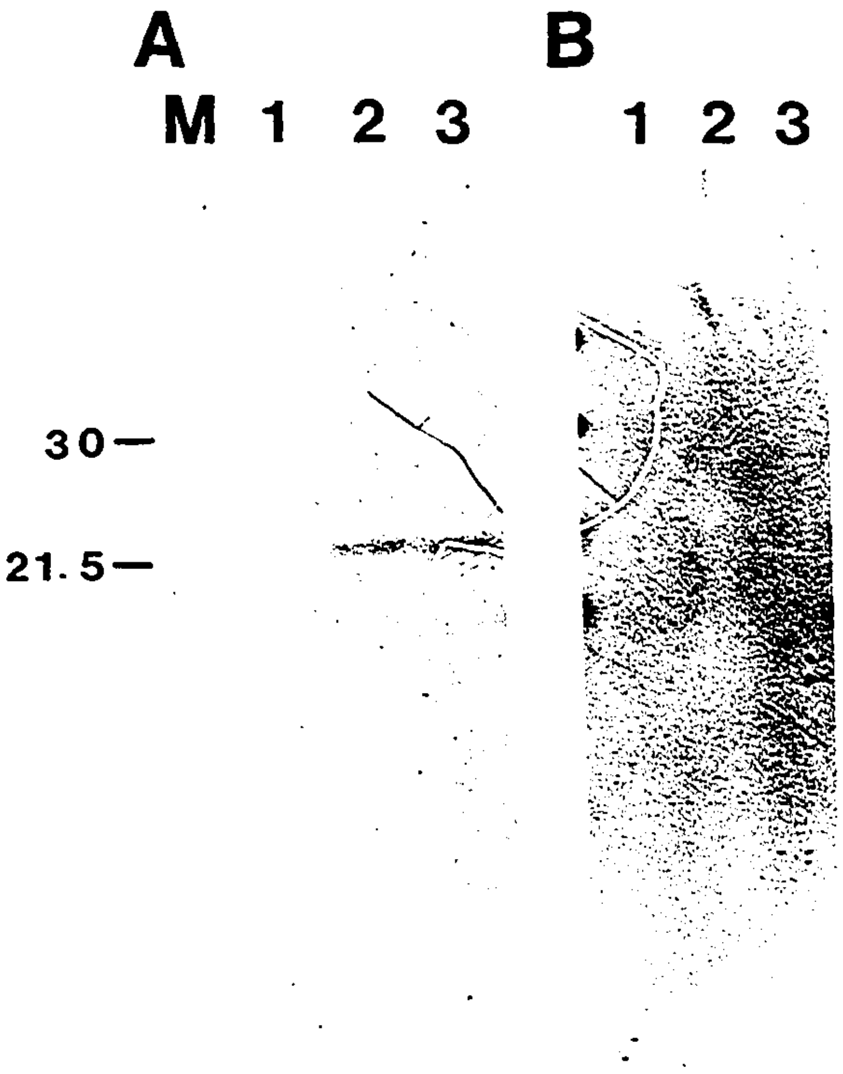
Figure 1. Protein blot analysis of mitochondrial proteins of *Brassica napus* with anti-ORF224 antiserum.

The total mitochondrial proteins isolated from male fertile Westar (*nap*) (lane 1), male sterile Westar (*pol*) (lane 2) and fertility-restored Westar (*pol*) (lane 3) plants were size fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to nitrocellulose membrane. The protein blot was cut to make replicate blots. Equal amounts of protein (150 micrograms) were loaded in each lane.

(A) Protein blot probed with anti-ORF224 peptide antiserum.

(B) Protein blot probed with anti-ORF224 peptide antiserum that had been preincubated with an excess of ORF224 peptide.

Lane M represents prestained molecular mass standards (in kilodaltons).


A
B
M
1
2
3
1
2
3
30
21.5

containing male fertile cytoplasm. They believed that it was an artifact caused by bleeding of protein, or possibly the immunoblot developing reagent, from the adjacent lanes containing protein from CMS lines. They did not detect any signal at 25 kDa in male fertile lines, when adjacent lane does not carry protein from a CMS line (Nivison and Hanson, 1989). Another possibility is that the seed stock for *nap* cytoplasm plants had a low level contamination of seeds from fertility restored *pol* plants.

The specificity of the 21 kDa signal to the anti-peptide antibody was demonstrated by the competition experiment (figure 1B). A replicate protein blot was probed with anti-peptide antibody that had been pre-incubated with an excess of ORF224 peptide. Figure 1B shows that the antibody does not bind to 21 kDa protein in the presence of competitive peptide, showing specificity of the anti-peptide antibody for the 21 kDa protein. These preliminary results indicate that the *pol* CMS-associated chimeric gene *orf224* is expressed at the protein level.

Since the levels of the 21 kDa protein are not greatly reduced on fertility restoration, it may be more likely that the *pol* CMS in *Brassica napus* is caused by a deficiency of the ATP6 protein, which is also indicated by the results presented in chapters II and III of this thesis. If this turns out to be true, then the *atp6* gene has the potential to be used as a synthetic restorer gene for the *pol* CMS system. Appropriate *atp6* constructs can be designed, using mitochondrial targeting presequences, to introduce an ATP6 protein encoded by a nuclear *atp6* gene (generated through plant transformation) into *pol* mitochondria. In this way, one could convert any maintainer line of interest into a restorer line relatively quickly as compared to the conventional methods of plant breeding, which usually take more than 5 years to transfer a restorer gene from one line to another. Moreover, by using a synthetic restorer gene, one can avoid the deleterious alleles which might be linked to the natural restorer genes and get transferred along with it when conventional methods are used. Hence, the results presented in this thesis may eventually be applied in the production of hybrid Brassica crops.

Appendix II

Figure 1. Mapping of 5' terminus of mitochondrial 1.3 kb *atp6* transcript of fertility restored *pol* cytoplasm plants.

Primer extension products were obtained using an oligonucleotide complementary to bases 1466-1485 of Figure 4 of chapter II of this thesis and 5 µg of mtRNA. The extension-reaction product was run alongside DNA sequencing reactions primed with the same oligonucleotide. The position of 5' transcript terminus and direction of transcription are indicated by horizontal and vertical arrows, respectively. Lane 1 represents Regent (*nap*) plants (male fertile); lane 2 represents 2007 (*pol*) plants (male sterile); Lane 3 represents 4007 (*pol*) plants (fertility-restored).

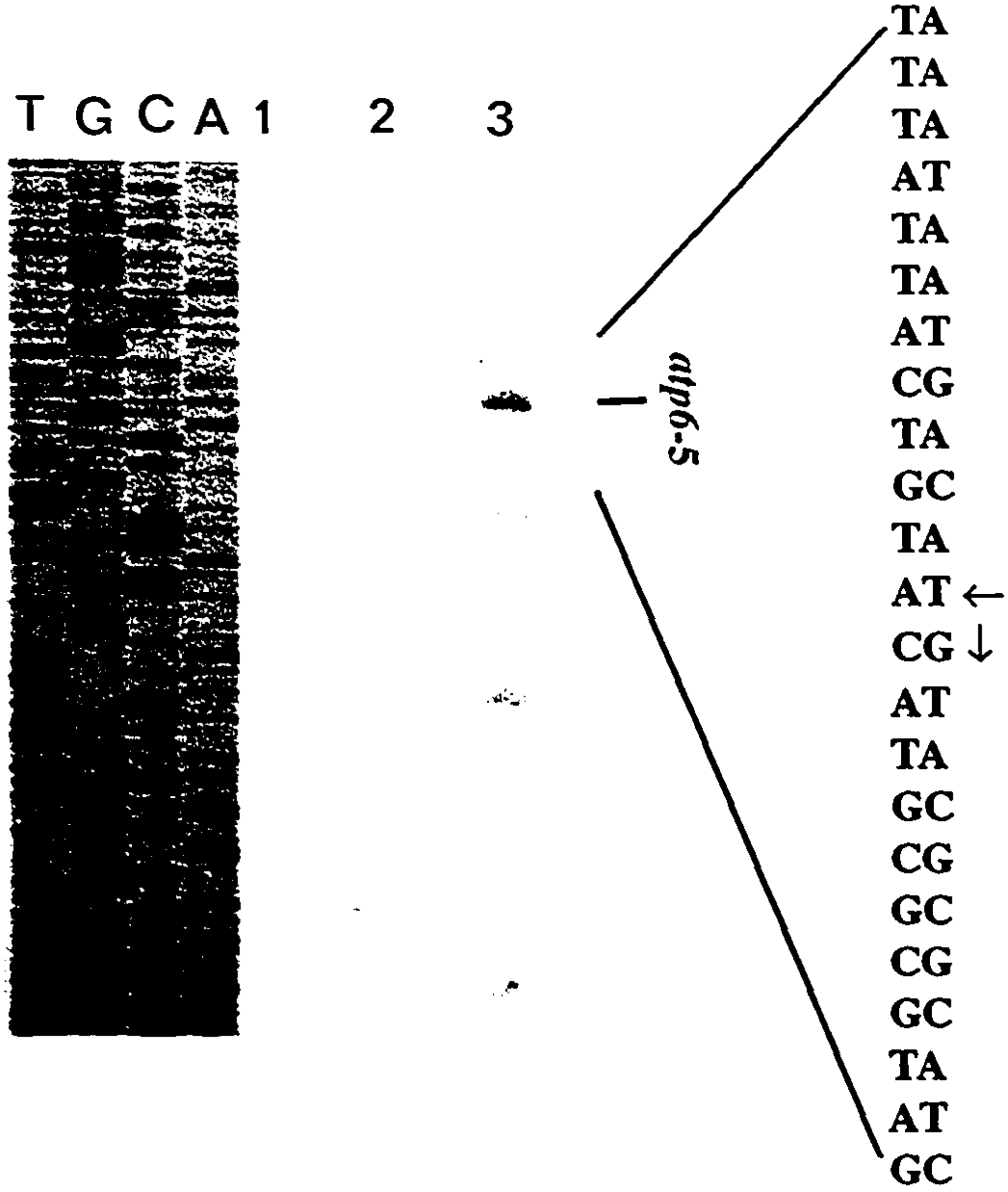
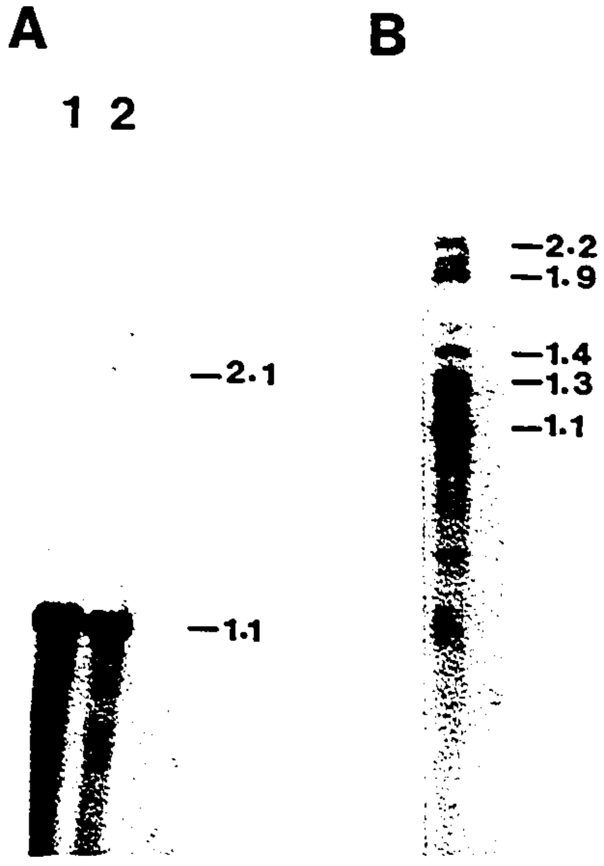

T
G
C
A
1
2
3
atp6-5
TA
TA
TA
AT
TA
TA
AT
CG
TA
GC
TA
AT ←
CG ↓
AT
TA
GC
CG
GC
CG
GC
TA
AT
GC

Figure 2. RNA gel blot analysis of *atp6* transcripts of *B. napus* plants.

(A) mtRNAs isolated from Regent (*nap*) seedlings (lane 1) and floral tissue (lane 2). **(B)** mtRNAs isolated from leaves of Westar-*Rf* plants. The RNA gel blots were hybridized with the *Brassica atp6* probe.


A
B
1
2
—2.1
—1.1
—2.2
—1.9
—1.4
—1.3
—1.1

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