Molecular genetic studies of
mitochondrial genome
in rapeseed (*Brassica napus* L.)
in relation to cytoplasmic male-sterility

Hirokazu Handa

1992

Contents

Introduction

At the present time, world production of seed from rapeseed (*Brassica napus* L.) amounts to about 22 million tons per year, in fourth place among oilseed crops after soybeans, cotton seed, and ground nuts. Between 1978 and 1988 world production of rapeseed was almost doubled from 10.5 million tons to 22 million tons. The annual average increase was 9.04 per cent, which was the highest value compared to other major oilseed crops (soybeans, 2.01%; cotton seed, 2.41%; ground nuts, 2.35%; sunflower seed, 4.97%). Furthermore, harvested area had also increased to 17,000,000 ha in 1988 from 11,500,000 ha in 1981 (FAO production year book, 1988). As mentioned above, rapeseed has become one of the most important oilseed crops at present.

Heterosis is defined as the increase in size, yield, and other characters of a hybrid over the average or mean of its parents. Utilization of heterosis in breeding necessitates the production of an F_1 progeny in sufficient quantity to be grown on a field scale. Usually, various means of controlling pollination to facilitate cross-pollination are required to produce the F_1 hybrid seeds. In *Brassica* crops, such as *B. campestris*, *B. oleracea*, and *Raphanus sativus*, F_1 hybrid seeds are now commercially produced using the self-incompatibility. However, the self-incompatibility cannot be applied for the F_1 seed production of rapeseed, because rapeseed is a self-compatible plant. Therefore, utilization of cytoplasmic male-sterility is expected to play an important role in F_1 hybrid seed production of rapeseed.

In plants with cytoplasmic male-sterility, viable pollen is not produced but female sex is normal. Thus, when viable pollen is provided, natural cross-pollination is permitted instead of self-pollination. Pollen fertility is restored in the hybrid by dominant fertility-restoring genes contributed by the pollen parent. Since both nuclear and cytoplasmic genes are required to produce male sterility, the sterility is due to nuclear and cytoplasmic gene interactions (Kaul 1988). Based on the origin of nucleus and cytoplasm, cytoplasmic

1

male-sterility is classified into autoplasmic and alloplasmic types. In rapeseed, the male-sterile plants arise following intraspecific crosses (autoplasmic type) (Shiga and Baba 1971, 1973; Thompson 1972; Fu 1981). In the alloplasmic types, male-sterile plants result after interspecific and intergeneric crosses of *B. napus* to *B. juncea* (Brar et al. 1980) and *R. sativus* (Bannerot et al. 1974), respectively.

The aim of this study

Cytoplasmic male-sterility is not only a very important trait for crop breeding but also a very interesting for basic plant genetics. It results from nuclear and cytoplasmic gene interactions. Little is known, however, about the molecular interactions between the nuclear and cytoplasmic genes in rapeseed as well as in the other species. Much circumstantial evidence, especially restriction endonuclease analysis, isolated *in-vitro* mitochondrial translation products and drug and fungal toxin sensitivity implicate mitochondria as site of the cytoplasmic male-sterility genes. However, no detailed information of mitochondrial genes which are assumed to be related to a molecular mechanism of cytoplasmic male-sterility is obtained for rapeseed. In this study, the author has intended to investigate the relationship between the cytoplasmic differentiation and mitochondrial genome variation in rapeseed, and the related molecular genetic mechanism of cytoplasmic male-sterility. Genetic diversity of the cytoplasm and mitochondrial genome in rapeseed is confirmed in Part I. Restriction endonuclease analysis of mitochondrial (mt) DNAs of 27 accessions of rapeseed was carried out to detect intraspecific variation of the mitochondrial genome (Chapter 1). Cytoplasmic types were reconfirmed in order to dissolve the discrepancies between both classifications of cytoplasm based on restriction endonuclease analysis of mitochondrial genome and the ability to induce male sterility (Chapter 2). Based on restriction fragment patterns and RFLP data of mtDNAs from rapeseed and related species, the origin and evolution of the

rapeseed cytoplasm are explored (Chapter 3). In Part II, the mechanism of the cytoplasmic male-sterility induction is approached by molecular genetic analyses. Physical organizations of several mitochondrial genes from male-sterile cytoplasm are examined and mitochondrial *atpA* gene is characterized (Chapter 4). Different organization and altered transcription of the mitochondrial *atp6* locus in the male-sterile cytoplasm are shown and a novel reading frame (*pol-urf*) related to the cytoplasmic male-sterility is found (Chapter 5). Origin and function of *pol-urf* gene are characterized and discussed (Chapter 6). The effect of RNA editing on the expression of the mitochondrial *atp6* genes from both normal and male-sterile cytoplasms had been studied, and it was found that there were no differences between the editing statuses of *atp6* transcripts from the two cytoplasms (Chapter 7). These studies provided the basic and valuable information on the cytoplasmic differentiation and the molecular genetic mechanism of cytoplasmic male-sterility in rapeseed.

Part I. Genetic diversity of the mitochondrial genome in rapeseed

Chapter 1. Intraspecific variation of mitochondrial DNA

Introduction

Cytoplasmic male-sterility (cms) was detected in F_2 progenies of intraspecific crosses between rapeseed cultivars by Shiga and Baba (1971, 1973) and Thompson (1972) (*nap* cytoplasm). Fu (1981) also observed that the cytoplasm of a Polish cultivar "Polima" induced male sterility (*pol* cytoplasm). Thus, the cytoplasms of rapeseed cultivars had been classified into three groups, namely, *nap*, *pol* and normal cytoplasm.

Recently a molecular genetic approach to organellar genomes such as chloroplast and mitochondrial genome has been well developed. Therefore it has become possible to elucidate the phylogenetic relationships between genera, species or lines of higher plants based on the analysis of organellar genomes.

In the genus *Brassica*, the phylogenetic relationship has been evaluated using the restriction endonuclease analysis of chloroplast (ct) DNA (Erickson et al. 1983; Palmer et al. 1983a) and mtDNA (Palmer 1988). Intraspecific variation of the chloroplast genome in rapeseed was reported by Erickson et al. (1983), Palmer et al. (1983a) and Ohkawa and Uchimiya (1985). Also mitochondrial genome variation among some cms-inducing cytoplasms in rapeseed was described by Erickson et al. (1986).

Though the cytoplasmic differentiation of the cms trait among rapeseed varieties can be clearly detected, studies on the relationship between the cytoplasmic differentiation and the mitochondrial genome variation in rapeseed has not been carried out systematically using a large number of rapeseed cultivars. The author carried out a restriction endonuclease analysis of mtDNAs of 27 rapeseed accessions in order to clarify: (1) the extent of mtDNA variation within rapeseed, and (2) the relationship between mtDNA variation and the cms trait.

Materials and Methods

Plant material

Twenty-seven accessions were selected from our rapeseed collection: (a) 14 cultivars carrying the *nap* cytoplasm, (b) one line with the *pol* cytoplasm, and (c) 12 cultivars with the normal cytoplasm (Table 1).

Table 1. Materials used as the source of mtDNA

Accession number	Cultivar	Origin
Euplasmic line (cultivar)		
1	Mali	Germany
2	Petranova=Lihonova	Germany
3	Erglu	Germany
4	Germany	Germany
5	Eckendorfer Mali	Sweden
6	Murasaki-natane	Japan
7	Isuzu-natane	Japan
8	Target	Canada
9	Nilla=1022	England
10	Szneszowichi	Poland
11	Niederarnbacher	Germany
12	Sv. Gulle	Sweden
13	Mlochowski	Poland
14	Gorczanski	Poland
15	Lembkes malchower	Germany
16	Ölquell	Germany
17	Rapol	Germany
18	R. Crésus	Sweden
19	Major	France
20	Mutsu-natane	Japan
21	Aomori 1	Japan
22	Asahi-natane	Japan
23	Norin 16	Japan
24	Wase-Chousen 618	Japan
25	Wase-Chousen 635	Japan
Alloplasmic line		
26	(*chi*)Isuzu-natane	
27	(*pol*)napus x Isuzu/2	

Extraction of mtDNA

Mitochondria were isolated from green leaves of 8-weeks-old plants by a modification of the procedure described by Kemble (1987) as follows: Green leaf tissue was homogenized in a Waring blender containing Buffer A (50 mM Tris-HCl pH 8.0, 3 mM EDTA, 0.1 % BSA, 1 mM Me-EtOH and 0.44 M sucrose). The homogenate was filtered through four layers of cheesecloth, two layers of Miracloth (Calbiochem Inc., CA, USA) and centrifuged at 2500 x g for 10 min. Mitochondria in the supernatant were collected by pelleting at 17000 x g for 20 min, resuspended in Buffer A and again centrifuged at 2500 x g for 10 min to remove remaining chloroplasts and other large cell components. One molar magnesium chloride and 20 mg/ml DNase I solution were added to the supernatant to give final concentrations of 10 mM and 40 µg/ml respectively. After incubation at room temperature for 1 hr, mitochondria were centrifuged through Buffer B (50 mM Tris-HCl pH 8.0, 20 mM EDTA and 0.6 M sucrose) at 17000 x g for 10 min. The pellet was resuspended in Buffer B, recentrifuged at 17000 x g for 10 min, and the final pellet lysed and incubated at 37°C for 2 hr in Buffer C (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 2 % Sarkosyl and 200 µg/ml Proteinase K).

MtDNA was purified using CsCl/EtBr buoyant density gradient centrifugation. The fractions containing mtDNA were pooled and then treated with CsCl-saturated isopropanol to remove EtBr. The DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol, then redissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at 4°C.

Restriction endonuclease digestion and gel electrophoresis

MtDNA samples were digested with the following ten enzymes; *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I and *Xho*I. Digestion of mtDNA with these enzymes was carried out according to the instructions given by the supplier.

Digests were separated by electrophoresis in 0.7 % agarose gel.

Results

Mitochondrial linear plasmid-like DNA

Mitochondria of many *Brassica* species contain, in addition to the main mitochondrial genome, a linear plasmid-like DNA of approximately 11.3 kb (Palmer et al. 1983b). MtDNAs of all the accessions used in this report were electrophoresed on agarose gels without prior restriction enzyme treatment. Fig. 1 shows the results of a representative experiment.

Of the 27 accessions tested, eight showed a fragment of 11.3 kb below the main genome band (Fig. 1, Table 2).

Restriction endonuclease analysis of mtDNA

Results are summarized in Table 2 and Fig. 2. In four accessions, only four endonucleases were used because of a limited amount of mtDNA available.

*Sma*I gave two types of restriction pattern. Of the 23 accessions analyzed, 17 showed pattern 1 and the remaining six pattern 2 (Fig. 2E). Among the accessions showing the *Sma*I-pattern 1, Asahi-natane (acc. no. 22) and Wase-chousen 635 (acc. no. 25) could be differentiated from the other accessions by showing the *Hind*III-pattern 1b and *Bam*HI-pattern 1b, respectively (Fig. 2C and A). Among the accessions with the *Sma*I-pattern 2, acc. no. 27 [(*pol*)napus x Isuzu/2] could be distinguished by its unique patterns caused by the digests of all nine enzymes except *Sma*I (Fig. 2). The digests of four enzymes, *Bgl*II, *Kpn*I, *Pst*I and *Pvu*II, produced the patterns that allowed distinction of acc. no. 2 (Petranova=Lihonova) from the other accessions with the same *Sma*I-pattern 2 (Fig. 2).

8
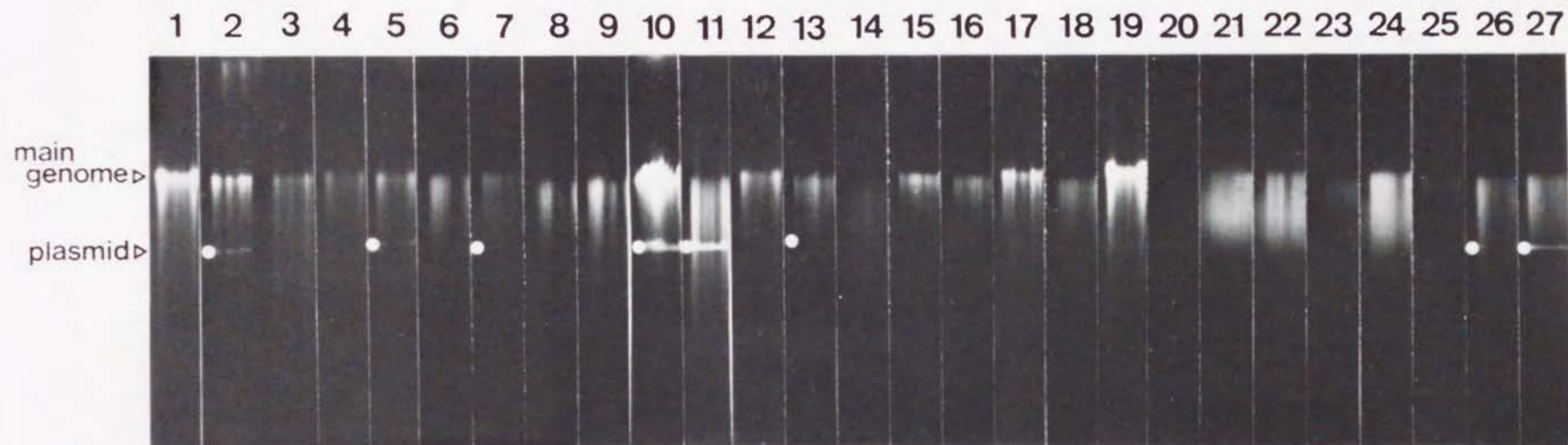
Fig. 1. Electrophoretic patterns of undigested rapeseed mtDNAs. Linear plasmid band of 11.3 kb in size appears below the main genome band, indicated by a *white dot*. *Accession number* is shown above each lane.

Table 2. Classification of the mitochondrial genome types based on fragment patterns of mtDNA treated with ten restriction enzymes

Accession number	Cultivar	Plasmid	Restriction endonuclease pattern							Mitochondrial genome type	Cytoplasm type	Chloroplast genome type
			BamHI	BglII	EcoRI	HindIII	KpnI PstI PvuII	SalI XhoI	SmaI		*)	**)
Euplasmic line (cultivar)												
1	Mali	-	2a	2a	2a	2a				IIa	N	II
2	Petranova=Lihonova	+	2a	2c	2a	2a	2c	2a	2	IIc	N	II
3	Erglu	-	2a	2a	2a	2a	2a	2a	2	IIa	N	II
4	Germany	-	2a	2a	2a	2a				IIa	N	II
5	Eckendorfer Mali	+	2a	2a	2a	2a	2a	2a	2	IIa	N	II
6	Murasaki-natane	-	2a	2a	2a	2a	2a	2a	2	IIa	N	II
7	Isuzu-natane	+	2a	2a	2a	2a	2a	2a	2	IIa	N	II
8	Target	-	1a	1	1	1a	1	1	1	Ia	N	I
9	Nilla=1022	-	1a	1	1	1a	1	1	1	Ia	N	I
10	Szneszowichi	+	1a	1	1	1a	1	1	1	Ia	N	I
11	Niederambacher	+	1a	1	1	1a	1	1	1	Ia	N	I
12	Sv. Gulle	-	1a	1	1	1a	1	1	1	Ia	N	I
13	Mlochowski	+	1a	1	1	1a	1	1	1	Ia	S(*nap*)	I
14	Gorczanski	-	1a	1	1	1a				Ia	S(*nap*)	I
15	Lembkes malchower	-	1a	1	1	1a	1	1	1	Ia	S(*nap*)	I
16	Ölquell	-	1a	1	1	1a	1	1	1	Ia	S(*nap*)	I
17	Rapol	-	1a	1	1	1a	1	1	1	Ia	S(*nap*)	I
18	R. Crésus	-	1a	1	1	1a	1	1	1	Ia	S(*nap*)	I
19	Major	-	1a	1	1	1a	1	1	1	Ia	S(*nap*)	I
20	Mutsu-natane	-	1a	1	1	1a	1	1	1	Ia	S(*nap*)	I
21	Aomori 1	-	1a	1	1	1a	1	1	1	Ia	S(*nap*)	I
22	Asahi-natane	-	1a	1	1	1b	1	1	1	Ib	S(*nap*)	I
23	Norin 16	-	1a	1	1	1a	1	1	1	Ia	S(*nap*)	?
24	Wase-Chousen 618	-	1a	1	1	1a				Ia	S(*nap*)	?
25	Wase-Chousen 635	-	1b	1	1	1a	1	1	1	Ic	S(*nap*)	?
Alloplasmic line												
26	(*chi*)Isuzu-natane	+	1a	1	1	1a	1	1	1	Ia	S(*nap*)	I
27	(*pol*)napus x Isuzu/2	+	2b	2b	2b	2b	2b	2b	2	IIb	S(*pol*)	?

*) S(*nap*), *nap* cms cytoplasm, S(*pol*), *pol* cms cytoplasm and N, normal cytoplasm cited from Shiga (1976), Shiga et al. (1983), and Ohkawa (unpublished data).

**) Cited from Ohkawa and Uchimiya (1985), and Ohkawa (unpublished data).
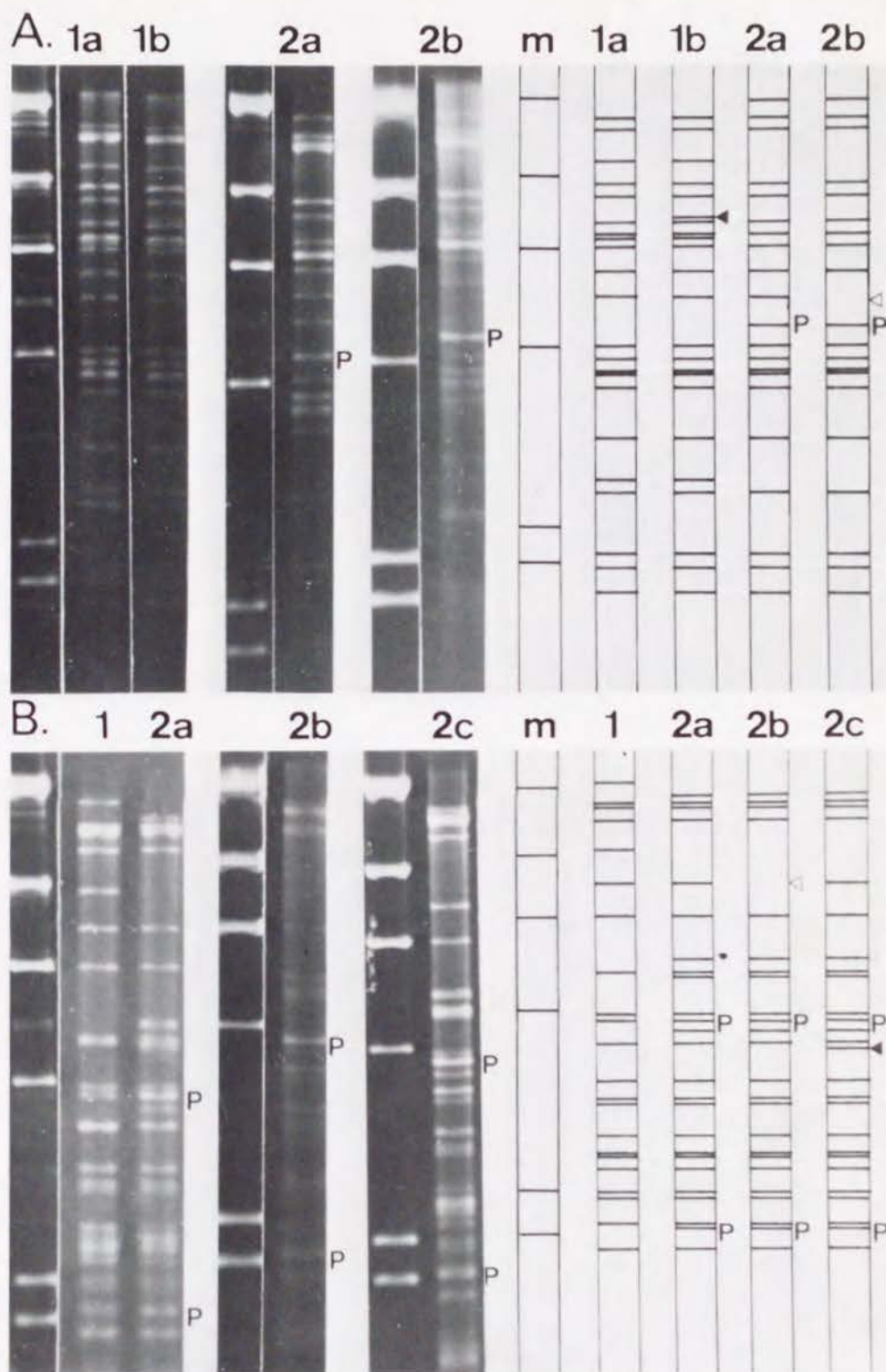
Fig. 2. Restriction fragment patterns of rapeseed mtDNAs and their schematic representations using ten restriction enzymes, *Bam*HI(A), *Bgl*II(B), *Hin*dIII(C), *Kpn*I(D), *Sma*I(E), *Eco*RI(F), *Pst*I(G), *Pvu*II(H), *Sal*I(I), and *Xho*I(J). ◁ and ◀, missing and differential fragment, respectively, as compared with the fragments of the subtype a in each major type. *P*, linear plasmid band. *m*, molecular size marker (phage lambda DNA fragments digested with *Hin*dIII).

11

Fig. 2 (continued).

Fig. 2 (continued).

13
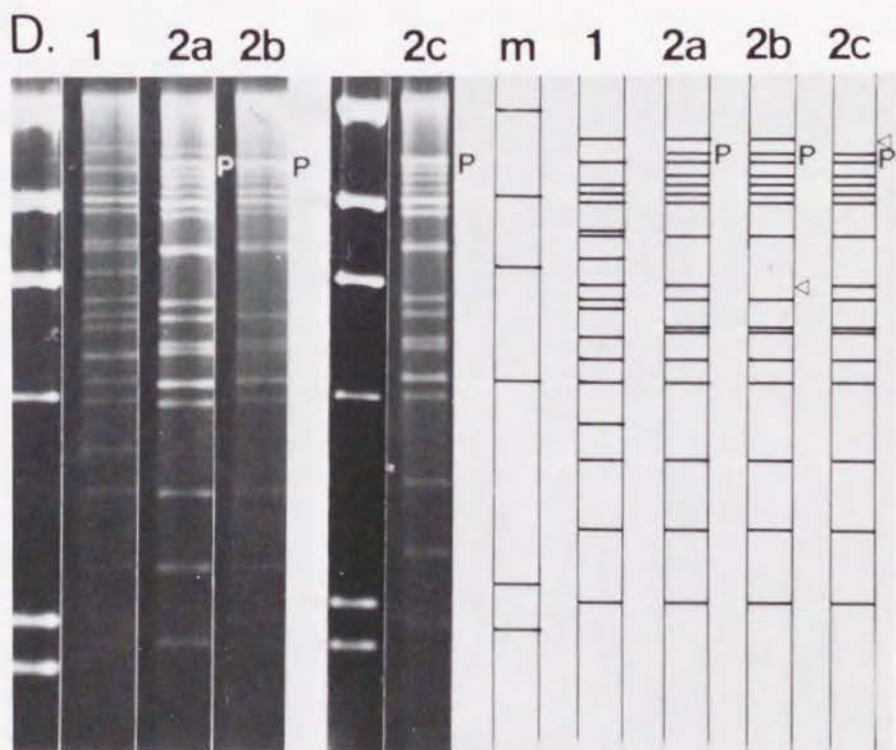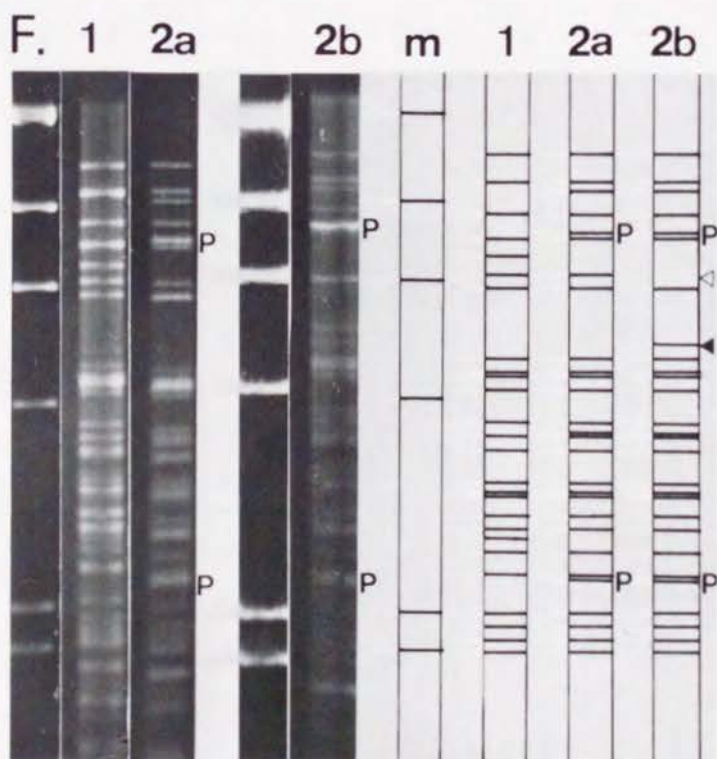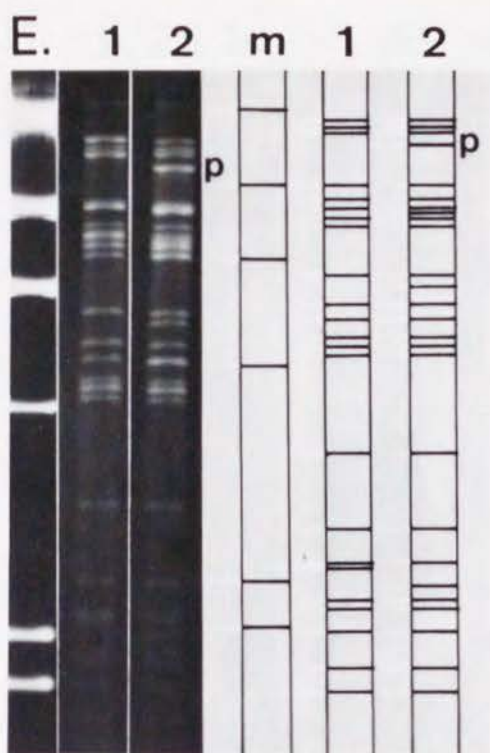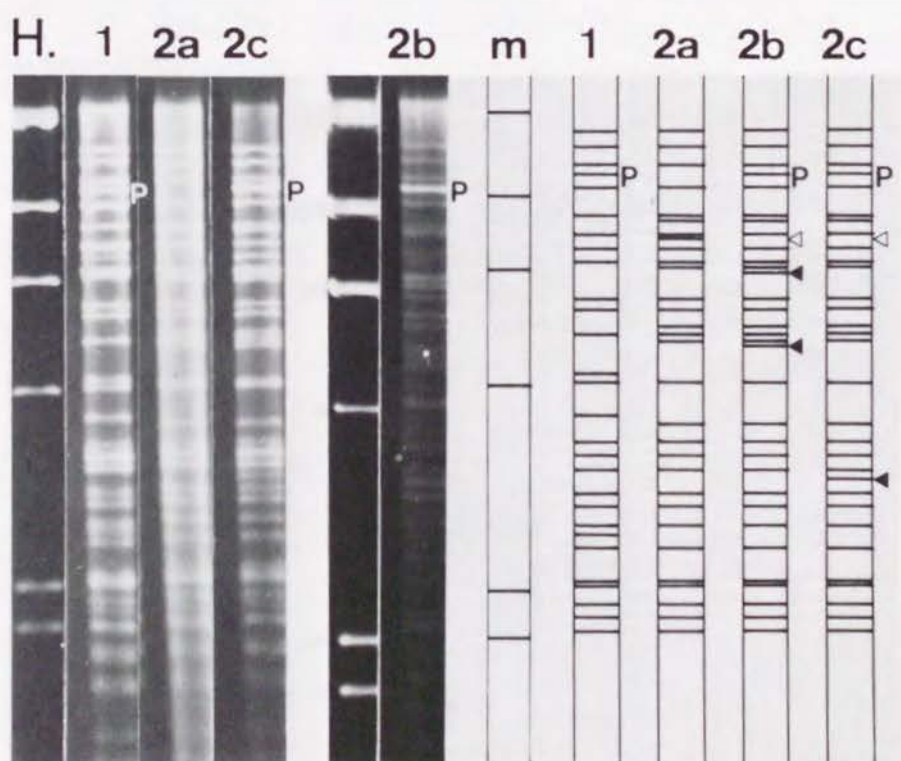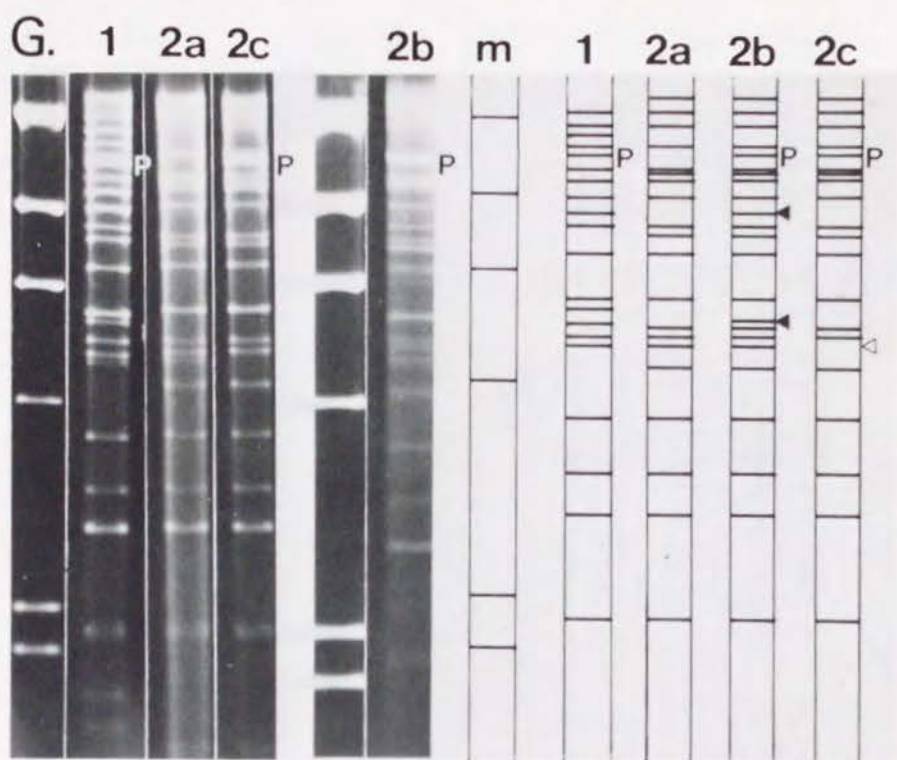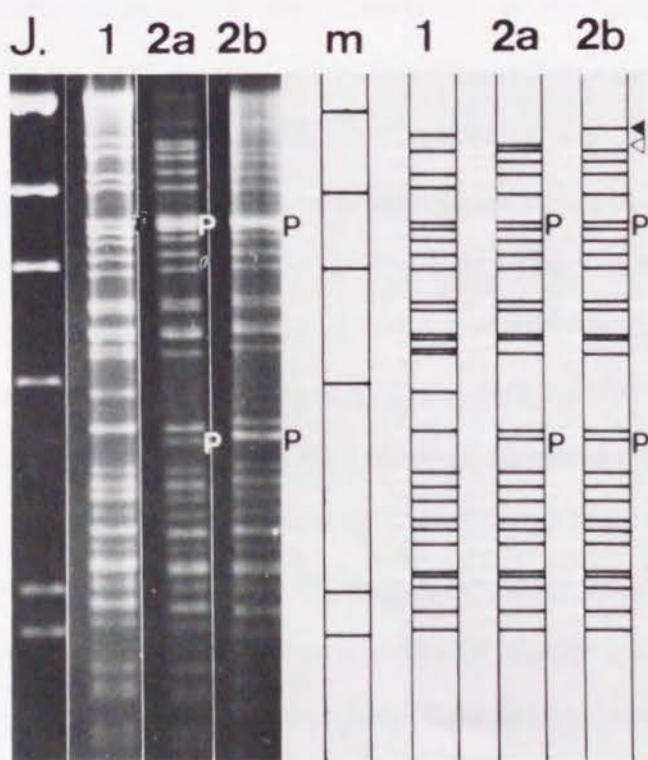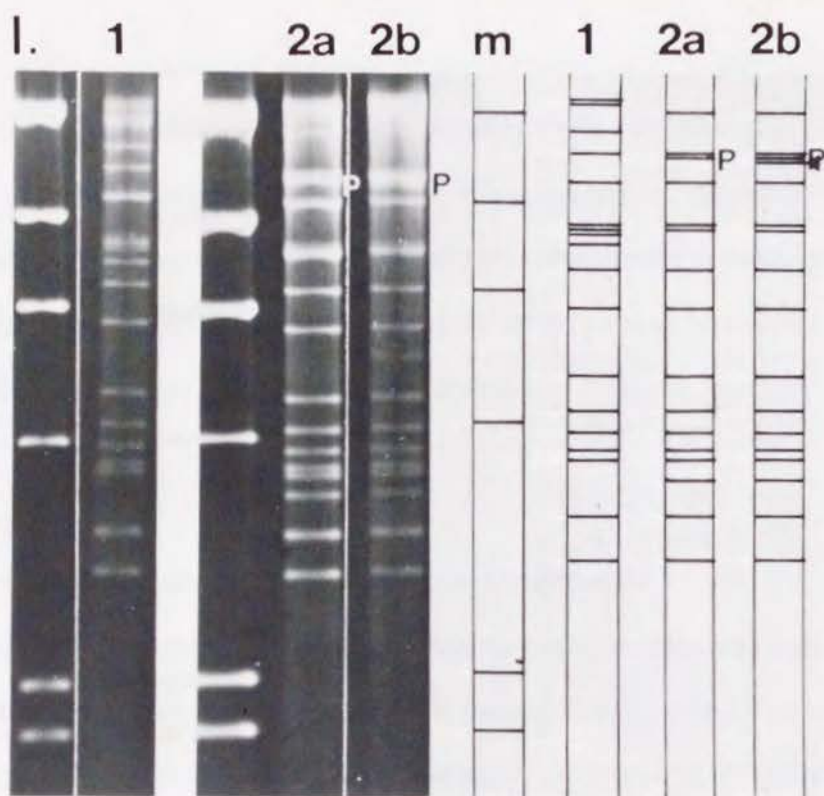
Fig. 2 (continued).

Fig. 2 (continued).

Classification of the mitochondrial genomes based on restriction fragment pattern

Based on the *Sma*I restriction fragment patterns, the mitochondrial genomes of the rapeseed accessions were classified into two major types, I and II. Each major type was further divided into three subtypes based on other restriction fragment patterns. As a whole, we could classify the mitochondrial genomes of the present materials into six types, Ia, Ib, Ic, IIa, IIb and IIc, as shown in Table 2.

Discussion

Intraspecific variation of the mitochondrial genome in rapeseed

Restriction endonuclease analysis of mtDNAs revealed that rapeseed contained two major mitochondrial genome types, I and II. The present classification of mitochondrial genome is in accord with that of chloroplast genome by Ohkawa and Uchimiya (1985) and Ohkawa (unpublished data). The accessions which carried the type I chloroplast genome possessed the type I mitochondrial genome, whereas the accessions with the type II chloroplast genome carried the type II mitochondrial genome. No accession with the type I chloroplast genome and type II mitochondrial genome, or type II chloroplast genome and type I mitochondrial genome was found. This association of a specific chloroplast genome with a specific mitochondrial genome in rapeseed strongly suggests that the two genomes might have been introduced into rapeseed together when or after rapeseed was formed. These results are in agreement with the findings of Palmer (1988).

Although Palmer (1988) classified mitochondrial genomes of four cultivars into two types corresponding to the present major types, the author observed small variations within each major mtDNA type, which was divided into three subtypes based on the mtDNA restriction patterns of 27 accessions. However, the level of the intraspecific variation of the mitochondrial genome was low, because 90% of the type I accessions carried the type Ia mitochondrial genome, and remaining 10 % carried type Ib and Ic

mitochondrial genomes. Both groups showed very small difference from it (see Fig. 2A and C).

Type II mitochondrial genome is similar to that of *B. campestris* reported by Palmer (1988). He reported that rapeseed lines with the cytoplasm identical to that of *B. campestris* had a common breeding history, in which either *B. campestris* or *B. juncea* was used as the maternal parent in hybridization. Shiga (1979) demonstrated that CN (*B. campestris* × *B. napus*) as well as NC crosses had been used in breeding of Japanese rapeseed cultivars. Based on the present data and the information obtained by Shiga (1979) and Palmer (1988), the cytoplasms with the type II mitochondrial genome can be assumed to have originated from *B. campestris*.

Relationship between the mitochondrial genome types and male-sterility induction

In many plants, recent results have suggested that the cms trait is associated with mitochondrial dysfunction (for a review, see Lonsdale 1987). Palmer et al. (1983b) reported that mitochondrial plasmid DNA was related to cms induction in rapeseed plants. Although we observed that eight of the 27 accessions studied harbored a 11.3 kb linear plasmid, there was no relationship between the presence of the plasmid DNA and cms induction, supporting the findings of Kemble et al. (1986), who did not find the exclusive association between them either. In this investigation, the author found that the *nap* cms cytoplasm carried any of Ia, Ib and Ic mitochondrial genomes and the *pol* cms cytoplasm carried only IIb mitochondrial genome. In earlier studies of crossing experiments (Fu 1981; Fan et al. 1986), the cms-inducing *pol* cytoplasm was maintained by pollinating, using the same maintainer as for *nap* cms system. Some of the restorers of this system restored male fertility under the presence of *pol* cytoplasm. Therefore, the *pol* cms seems to have a similar developmental bases to the *nap* cms, although their mitochondrial genome types were different. The *pol* cytoplasm was classified as type IIb that showed

17

only very small differences from type IIa, in which only male-fertile cytoplasms were included. It might be possible to find the mitochondrial gene controlling the cms trait in these fragments, such as the cms-related fusion gene, *T-urf13* found in *Xho* 6.6 kb fragment of T-type male-sterile maize (Umbeck and Gengenbach 1983; Dewey et al. 1986). To our knowledge, many mtDNAs of the cms cytoplasms exhibit altered restriction fragment patterns when compared with its normal counterpart (Dewey et al. 1986; Erickson et al. 1986; Lonsdale 1987). However, we could not detect the alterations of restriction patterns between the *nap* cms and normal cytoplasm with the Ia mitochondrial genome, although more than 200 restriction fragments were compared. Therefore the present results suggest that the expression of the *nap* cms depends upon the difference in the small part of the mitochondrial genome, probably one or a few genes. The following facts seem noteworthy: 1) The *nap* and *pol* cytoplasms carry a different mitochondrial genomes, although they are similar in the responses to the maintainer and the restorer, 2) the *pol* cms cytoplasm and its normal counterpart showed similar restriction patterns to each other except a few fragment differences by restriction endonuclease analysis of their mtDNAs. These facts suggest that the cms trait in rapeseed is controlled by a very small part of the mitochondrial genome, presumably one or a few genes.

Chapter 2. Reconfirmation of cytoplasmic types by backcross method

Introduction

Cytoplasmic male sterility (cms) is a maternally inherited trait which has provided an efficient way of producing commercial hybrid seeds by preventing self-fertilization of the seed parent. At present, the method employed to confirm the presence of a particular cms cytoplasm or to identify an unknown cms cytoplasm is a very labor-intensive backcrossing program involving many different fertility restorer and maintainer lines.

With the recent development of molecular genetic techniques, restriction endonuclease analysis can be used to determine the cytoplasmic organelle component of plant species instead of the conventional plant breeding techniques. Using this method, an unknown cytoplasm can be identified in a few days, which is a considerable saving in time and labor compared to the several years required by traditional methods.

In Chapter 1, the author carried out the restriction endonuclease analysis of mtDNAs of rapeseed cultivars in order to clarify the relationship between mtDNA variation and the cms trait. However, the alterations of restriction patterns between the *nap* cms and normal cytoplasm with the Ia mitochondrial genome were not detected although more than 200 restriction fragments were compared. This forms the basis of the first step toward analyzing once more the ability to induce cytoplasmic male-sterility in the cultivars which have "normal" cytoplasm and type I mitochondrial genome.

Materials and Methods

In this experiment, the degree of male sterility was expressed on the basis of the score for the relative position of the anther to the stigma because the number of pollen grains was closely correlated with this trait (Ohkawa 1984). Fig. 3 shows the criterion, i. e., 1 and 2 for male-sterile lines, 3 and 4 for partially male-sterile lines, and 5 and 6 for male-fertile
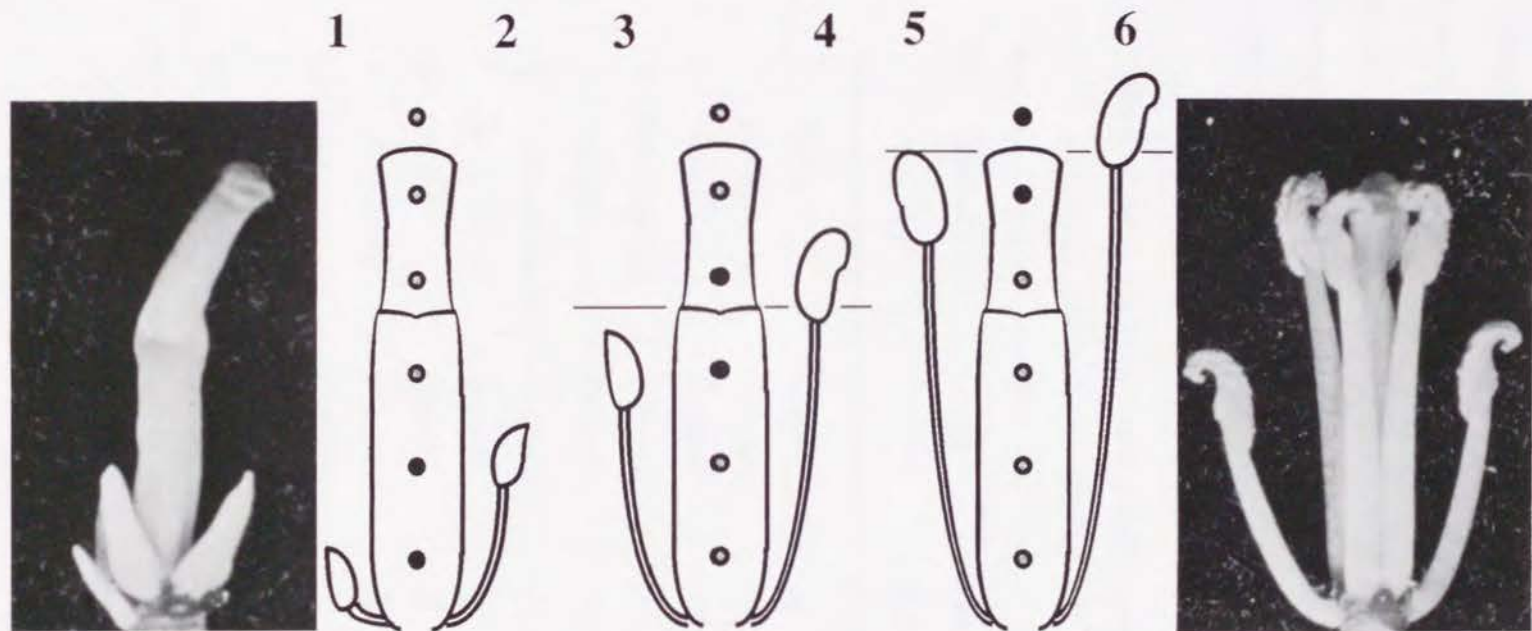
Fig. 3. Phenotypic criterion of male sterility based on the score for the relative position of the anther to the stigma.

lines.

Seven cultivars with a type I mitochondrial genome, previously determined as having normal cytoplasm (Shiga et al. 1983 and unpublished data) were used in this experiment. As a control, three cultivars with a type II mitochondrial genome and normal cytoplasm, and two cultivars with a type I mitochondrial genome and *nap* cms cytoplasm were also used. They are shown in Table 3.

Table 3. Rapeseed cultivars used in field experiments for
confirmation of male sterility trait

No.	Cultivar name	Origin	Cytoplasm type	Mt genome type
1	Target	Canada	N [*)	Ia
2	Komet	Germany	N	Ia
3	Nilla=1022	England	N	Ia
4	Wielkoposki	Poland	N	Ia
5	Szneszowichi	Poland	N	Ia
6	Niederarnbacher	Germany	N	Ia
7	Sv. Gulle	Sweden	N	Ia
8	Crésus	France	S	Ia
9	Mlochowski	Poland	S	Ia
10	Mali	Germany	N	IIa
11	Erglu	Germany	N	IIa
12	Eckendorfer Mali	Sweden	N	IIa

*) S, cms cytoplasm and N, normal cytoplasm cited from Shiga et al.
(1983 and unpublished data)

In spring 1989, 12 cultivars were crossed with the maintainer cultivar of *nap* cms system, Isuzu-natane which has the normal cytoplasm without the fertility-restoring gene. All F_1 seeds were sown in field plots, and their male fertility was checked. In spring 1990, the plants were self-pollinated using the bagging technique (except two cultivars with a type I mitochondrial genome and *nap* cms cytoplasm), or they were backcrossed with their pollen parent, Isuzu-natane. The degree of fertility of ten F_2 populations and 12 backcrossed (B_1F_1) populations was examined during the flowering season of 1991.

Results

Male sterility in F_2 and backcrossed (B_1F_1) populations

As shown in Table 4, there were male-sterile (ms) or partially male-sterile (pms) plants in the F_2 populations (no. 1 ~ 7) except (Szneszowichi x Isuzu-natane)F_2 (no. 5). All these F_2 populations originated from the seven cultivars with type I mitochondrial genome and have been assumed as having "normal" cytoplasms (Shiga et al. 1983). These seven cultivars were crossed with Isuzu-natane (pollen parent), maintainer cultivar of *nap* cms system. On the other hand, there were no ms or pms plants in the F_2 populations (no. 10 ~ 12) derived from the mother plants of three cultivars (female parent) with type II mitochondrial genome which were crossed with Isuzu-natane (pollen parent).

In the case of backcrossed (B_1F_1) populations, similar results were obtained (Table 5). In all B_1F_1 populations (no. 1 ~ 7) derived from seven cultivars with type I mitochondrial genome and "normal" cytoplasm, which had been backcrossed with Isuzu-natane, ms and pms plants were segregated. These results were not consistent with the previous findings. As expected, similar segregation of ms or pms plants was observed in B_1F_1 populations (no. 8 ~ 9) derived from two cultivars with type I mitochondrial genome and *nap* cms cytoplasm. No ms or pms plants were observed in the B_1F_1 populations (no. 10 ~ 12) derived from three cultivars with type II mitochondrial genome.

22

Table 4. Degree of male sterility observed in F_2 populations resulting from the cross between ten cultivars and Isuzu-natane

No.	F_2	No. of plants observed	Relative position of anther to stigma											Mean
			1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	
1	(Target x Isuzu-natane)F_2	55	-	-	-	-	-	-	1	2	2	10	40	5.78
2	(Komet x Isuzu-natane)F_2	59	-	-	2	1	3	-	-	1	3	17	32	5.43
3	(Nilla=1022 x Isuzu-natane)F_2	59	-	-	-	-	-	1	3	-	3	20	32	5.64
4	(Wielkoposki x Isuzu-natane)F_2	58	-	-	-	-	1	-	-	2	1	8	46	5.81
5	(Szneszowichi x Isuzu-natane)F_2	56	-	-	-	-	-	-	-	2	6	12	36	5.73
6	(Niederarnbacher x Isuzu-natane)F_2	54	-	-	-	2	1	2	1	2	6	25	15	5.29
7	(Sv. Gulle x Isuzu-natane)F_2	59	2	4	3	1	1	1	3	-	12	26	6	4.64
10	(Mali x Isuzu-natane)F_2	58	-	-	-	-	-	-	-	-	-	2	56	5.98
11	(Erglu x Isuzu-natane)F_2	48	-	-	-	-	-	-	-	-	-	8	40	5.92
12	(Eckendorfer Mali x Isuzu-natane)F_2	18	-	-	-	-	-	-	-	-	-	3	15	5.92

23

Table 5. Degree of male sterility observed in B_1F_1 populations resulting from the cross between 12 cultivars and Isuzu-natane

No.	B_1F_1	No. of plants observed	Relative position of anther to stigma											Mean
			1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	
1	(Target x Isuzu-natane)Isuzu-natane	58	-	3	4	1	11	5	1	2	3	12	16	4.41
2	(Komet x Isuzu-natane)Isuzu-natane	58	1	1	5	8	5	3	1	1	1	18	14	4.39
3	(Nilla=1022 x Isuzu-natane)Isuzu-natane	56	1	6	6	1	1	3	1	3	6	17	11	4.38
4	(Wielkoposki x Isuzu-natane)Isuzu-natane	38	-	-	1	-	-	-	3	3	6	7	18	5.37
5	(Szneszowichi x Isuzu-natane)Isuzu-natane	59	-	2	1	5	6	4	5	1	3	13	19	4.65
6	(Niederarnbacher x Isuzu-natane)Isuzu-natane	52	-	3	3	7	3	4	2	3	3	8	16	4.38
7	(Sv. Gulle x Isuzu-natane)Isuzu-natane	58	5	7	11	6	2	1	2	-	8	12	4	3.45
8	(Mlochowski x Isuzu-natane)Isuzu-natane	58	-	-	-	4	4	8	9	10	14	7	2	4.34
9	(Crésus x Isuzu-natane)Isuzu-natane	55	1	2	11	9	11	5	5	6	5	-	-	3.11
10	(Mali x Isuzu-natane)Isuzu-natane	60	-	-	-	-	-	-	-	-	-	6	54	5.95
11	(Erglu x Isuzu-natane)Isuzu-natane	55	-	-	-	-	-	-	-	-	2	20	33	5.78
12	(Eckendorfer Mali x Isuzu-natane)Isuzu-natane	56	-	-	-	-	-	-	-	-	-	21	35	5.81

24

Analyzing the data, it was concluded that all cultivars with type I mitochondrial genome had male-sterile (*nap* cms) cytoplasm, while all cultivars with type II mitochondrial genome had normal (male-fertile) cytoplasm. These results do not agree with the classification of Shiga et al. (1983), who classified these nine cultivars used in this investigation and grouped them into two cytoplasmic types: seven with normal and the remaining two with male-sterile cytoplasm.

Segregation on pollen fertility restoration in B_1F_1 populations

Segregation pattern of ms (score $1 \sim 2.5$), pms ($3 \sim 4$) and fertile ($4.5 \sim 6$) plants in the B_1F_1 populations derived from seven cultivars with type I mitochondrial genome and "normal" cytoplasm backcrossed with Isuzu-natane and Chi-square analysis for goodness of fit to expected ratios are shown in Table 6.

Segregation of male sterility in the B_1F_1 derived from five cultivars, Target, Komet, Szneszowichi, Niederarnbacher and Sv. Gulle (no. 1, 2, 5, 6, and 7, respectively) fitted very well to the expected ratio of 1:1. The results showed that these five cultivars had a single, completely dominant fertility-restoring gene. B_1F_1 segregation of Nilla=1022 (no. 3) was well fitted to the ratio, 1:3. This finding confirmed that Nilla=1022 had two dominant fertility-restoring genes. The observed frequency in the B_1F_1 of Wielkoposki (no. 4) fitted to the expected ratios of 1:1:6 and 1:7. Consequently, it was concluded that Wielkoposki had two dominant and one incompletely dominant, or three dominant fertility-restoring genes.

Discussion

Restriction endonuclease analysis classified mitochondrial genome of rapeseed into two types, type I and type II. In the group of cultivars with type II mitochondrial genome, mitochondrial genome type was further divided into three subtypes, IIa, IIb and IIc. As for

Table 6. Segregation pattern for sterile, partially sterile and fertile plants in seven B_1F_1 populations from cross between seven cultivars and Isuzu-natane, Chi-square analysis for goodness of fit to expected ratio and estimated number of fertility-restoring genes

No.	B_1F_1	No. of plants	Segregation [1]			Expected [2] ratio	Type of cytoplasm	Number [3] of genes
			ms	pms	f			
1	(Target x Isuzu-natane)Isuzu-natane	58	8	17	33	1 : 1	S	1
2	(Komet x Isuzu-natane)Isuzu-natane	58	15	9	34	1 : 1	S	1
3	(Nilla=1022 x Isuzu-natane)Isuzu-natane	56	14	5	37	1 : 3	S	2
4	(Wielkoposki x Isuzu-natane)Isuzu-natane	38	1	3	34	1 : 1 : 6 1 : 7	S	3
5	(Szneszowichi x Isuzu-natane)Isuzu-natane	59	8	15	36	1 : 1	S	1
6	(Niederarnbacher x Isuzu-natane)Isuzu-natane	52	13	9	30	1 : 1	S	1
7	(Sv. Gulle x Isuzu-natane)Isuzu-natane	58	29	5	24	1 : 1	S	1

1) Phenotype of male-sterile plant was classified into three groups according to the numerals indicating relative position of anther to stigma; ms plants: less than 3.0, pms plant : 3.0 to 4.0 and male-fertile plant : 4.5 to 6.0.

2) Probability for goodness of fit was all higher than 0.05.

3) Estimated number of genes from Chi-square analysis.

the type II group, this classification by restriction endonuclease analysis of mitochondrial genome was similar to that by cytoplasmic types, normal or cms inducing (Shiga 1976; Shiga et al. 1983); IIa and IIc were normal cytoplasm, and only IIb was *pol* cms cytoplasm. On the other hand, although the type I mitochondrial genome was also divided into three subtypes, Ia, Ib and Ic, the classification by restriction endonuclease analysis was not consistent with that by cytoplasmic types. In the group with the type Ia mitochondrial genome there were both cytoplasmic types, normal and cms inducing. Therefore, reconfirmation of cytoplasmic types in the group with type I mitochondrial genome was carried out in order to solve the discrepancies between the classification of cytoplasm by restriction endonuclease analysis of mitochondrial genome and by cytoplasmic types.

As was expected, two cultivars with type I mitochondrial genome and *nap* cms cytoplasm used as a control segregated ms or pms plants in the B_1F_1 populations. Another control, three cultivars with type II mitochondrial genome and normal cytoplasm did not segregate ms or pms plants in the B_1F_1 populations, as was also expected. These results reconfirmed that cytoplasms of the former cultivars were *nap* cms cytoplasms and those of the latter cultivars were normal ones as described previously. However, ms or pms plants were segregated in the B_1F_1 populations of seven cultivars with type I mitochondrial genome and "normal" cytoplasm. If the cytoplasms of these seven cultivars were normal cytoplasms, no segregations of ms or pms plants would have been observed in the B_1F_1, and all plants of the B_1F_1 should have had normal phenotype. Based on the results obtained in this experiment, it is certain that seven cultivars estimated as "normal" cytoplasmic type by Shiga et al. (1983), have in fact a male-sterile cytoplasm. It seems that wrong classification of cytoplasmic types of these cultivars has been resulted primarily from the differences of estimation methods. Shiga et al. (1983) classified the cytoplasmic types using the segregation pattern in F_1 populations derived from the reciprocal crosses,

27

while in this experiment segregation pattern in B_1F_1 populations was used. Additional reasons for the failure to find previously male sterility in these cultivars include the fact that these cultivars have one to three dominant fertility-restoring genes, and that in F_1 populations expression of male-sterile phenotype may be suppressed by the action of fertility-restoring genes.

The reconfirmed results for cytoplasmic types resolve the discrepancies between the classifications by restriction endonuclease analysis and by cytoplasmic types, and these two classifications become comparable. Cytoplasm with type I mitochondrial genome is male-sterile inducing (*nap* cms), and cytoplasm with type II mitochondrial genome (except type IIb, which is *pol* cms) is normal. Identical results derived from both classifications support the suggestion that mitochondrial genome may be a determinant of cytoplasmic male-sterility in rapeseed.

These results also demonstrate the usefulness of molecular genetic techniques, such as restriction endonuclease analysis for the identification of plant cytoplasm. It usually takes at least three years or more to confirm the cytoplasmic types using the conventional technique described in this chapter, while one week is sufficient to identify the mitochondrial genome type using restriction endonuclease analysis as the established routine screening procedure. It opens the possibility to employ this technique routinely for the identification of cytoplasm instead of conventional breeding method. However, it is also important to understand fully the relationship between the cytoplasmic types and mitochondrial genome types. Without this explanation, the classification based on the mitochondrial genome types could not be used for practical purposes.

Chapter 3. Phylogenetic relationships between the mitochondrial genomes of rapeseed and other related species

Introduction

In the genus *Brassica*, genetic diversity of the cytoplasms among species has been extensively studied by restriction endonuclease analysis of ctDNA (Erickson et al. 1983; Palmer et al. 1983a; Ohkawa and Uchimiya 1985). From these studies, a lot of information on interspecific relationships among *Brassica* species have been gained.

Recently, the phylogenetic studies for mitochondrial genomes in *Brassica* have also been carried out. Palmer and his coworkers reported that mtDNAs within species of *Brassica* were extremely homogeneous. MtDNA and ctDNA had been coinherited during the evolution of the genus, and mtDNA evolved rapidly in structure rather than in sequence (Palmer 1988; Palmer and Herbon 1988). The author has also showed using restriction endonuclease analysis that there were some intraspecific variations in mtDNAs of rapeseed, and classified mitochondrial genomes of rapeseed into six types based on restriction analysis data.

The recent development of restriction fragment length polymorphism (RFLP) technique has been useful for the genetic analysis of plant species (Helentjaris et al. 1985; Song et al. 1988). Both RFLP analysis and restriction endonuclease analysis of organellar genomes have considerable potential for exploring the evolutionary relationships among species.

Based on interspecific hybridization and cytogenetic evidence, it can be concluded that rapeseed is an amphidiploid species evolved from interspecific hybridization between *B. campestris* and *B. oleracea* (U 1935). However, the exact cytoplasm donor species of rapeseed remains unclear. Fraction I protein was analyzed to identify the female parent for rapeseed by Uchimiya and Wildman (1978). However, the female parent was not

identified because the large subunit of Fraction I protein from *B. campestris* and *B. oleracea* had the same isoelectric points. Ichikawa and Hirai (1983) using ctDNA restriction endonuclease analysis proposed that cytoplasm donor of rapeseed was *B. oleracea*. However, restriction pattern of ctDNA (Erickson et al. 1983; Palmer et al. 1983a) and that of mtDNA (Palmer 1988; Palmer and Herbon 1988) have provided the information that the cytoplasm of rapeseed was of a more complex origin.

In this chapter, the author explains the phylogenetic relationships between rapeseed and its related species based on the restriction endonuclease analysis and RFLPs of mtDNA, and discusses the origin of the cytoplasm of rapeseed.

Materials and Methods

Brassica species used for mtDNA extractions are listed in Table 7.

Preparation, and restriction endonuclease analysis of mtDNA

MtDNAs were isolated from green leaves of 8-weeks-old plants using the method of Handa et al. (1990), followed by CsCl/EtBr centrifugation, isopropyl alcohol extraction and dialysis. Digestions with restriction endonucleases and agarose gel electrophoresis of the digests were performed as previously described (Handa et al. 1990).

Detection of RFLPs

In order to detect RFLPs of mtDNA, DNA was transferred to a nylon membrane filter (Biodyne A, Pall Ultrafine Filtration Co., USA or Hybond N, Amersham, UK) by a modification of Southern (1980).

A nonradioactive DNA labeling and detection kit, of Boehringer Mannheim, Germany, was used for labeling DNA fragments and detecting the hybrid bands. Mitochondrial genes were labeled by the random primed incorporation of digoxigenin-

Table 7. Plant materials used for mtDNA extraction

Euplasmic

No.	Accession	Species	Mt genome type*	Abbrev.
1	Norin 16 (oil seed)	*B. napus*	Ia	Ia
2	Asahi-natane (oil seed)	*B. napus*	Ib	Ib
3	Isuzu-natane (oil seed)	*B. napus*	IIa	IIa
5	Petranova=Lihonova (oil seed)	*B. napus*	IIc	IIc
6	Kinriki-kanran (cabbage)	*B. oleracea*	-	ole
7	Shiramizu-zairai (oil seed)	*B. campestris*	-	cam
10	Okura-daikon (radish)	*R. sativus*	-	rad

Alloplasmic

No.	Accession	Cytoplasm donor	Nucleus donor	Mt genome type*	Abbrev.
4	Polima-cms	*B. napus*	*B. napus*	IIb	IIb
8	Juncea-cms	*B. juncea*	*B. napus*	-	jun
9	Ogura-cms	*R. sativus*	*B. napus*	-	ogu

* Cited from Handa et al. (1990)

labeled dUTP (Feinberg and Vogelstein 1983) and were used as the hybridization probe. The filters were prehybridized with hybridization solution [5 x SSC, 0.1 % N-lauroyl sarcosine, 0.02 % SDS, and 0.5 % blocking reagent (Boehringer Mannheim, Germany)] at 68°C for 4 hr with gentle agitation. Hybridizations were carried out at 68°C for 16 hr after prehybridization. The filters were washed twice in 2 x SSC and 0.1 % SDS at room temperature for 5 min, and twice in 0.1 x SSC and 0.1 % SDS at 65°C for 15 min. After washing, the hybrid bands were detected by an enzyme-linked immunoassay using an anti-digoxigenin-alkaline phosphatase conjugate and a subsequent enzyme-catalyzed color

reaction with X-phosphate and NBT, or else the chemiluminescent reaction with AMPPD, following the manufacturers' instructions (Boehringer Mannheim, Germany; Tropix, Inc., Mass., USA).

Estimation of percent common fragments

For the estimation of the genetic similarity of mitochondrial genomes between accessions, percent common fragments was used. This parameter was calculated by $(2A/B) \times 100$, where A is the number of common fragments between the patterns of the two mtDNAs, and B is the sum of the numbers of all fragments found in them (Terachi and Tsunewaki 1986).

Results

Restriction fragment patterns of mtDNAs

The restriction endonuclease analysis of mtDNAs of ten *Brassica* accessions was carried out using seven restriction endonucleases (*Bam*HI, *Bgl*II, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, and *Sal*I). Fig. 4 shows restriction fragment patterns of the digested mtDNAs by seven enzymes from ten *Brassica* accessions.

The number of the common restriction fragments for seven endonuclease digests was summed for each pair of mitochondrial genomes, and the total fragment number for each genome was counted. Table 8 shows the numbers of total and common fragments, both pooled for all seven endonuclease digests, together with the percent common fragments. Based on the percent common fragments given in Table 8, phylogenetic relationship among mitochondrial genomes of *Brassica* species was drawn in the form of dendrogram by applying the UPGMA method (Sneath and Sokal 1973). The results are given in Fig. 5. This dendrogram shows that mitochondrial genomes of ten *Brassica* accessions were classified into three groups.
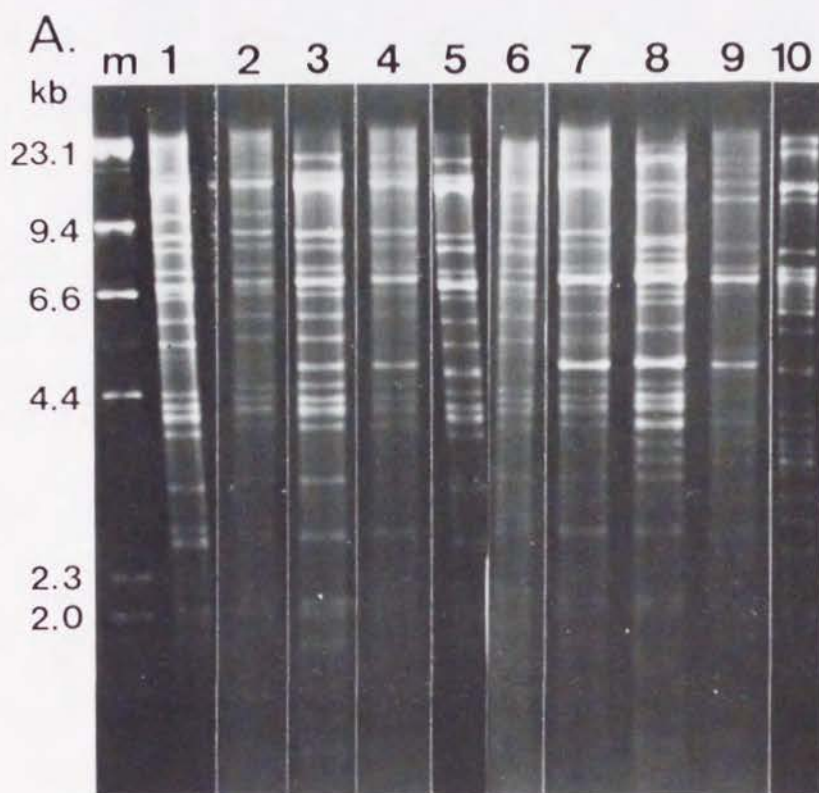
Fig. 4. Restriction fragment patterns of mtDNAs from ten *Brassica* accessions; *Bam*HI
(A), *Bgl*II (B), *Hin*dIII (C), *Kpn*I (D), *Pst*I (E), *Pvu*II (F), and *Sal*I (G).
Accessions are indicated by their *number* given in Table 7. *m* is *Hin*dIII-digested
lambda DNA, whose fragment sizes are given in kb.
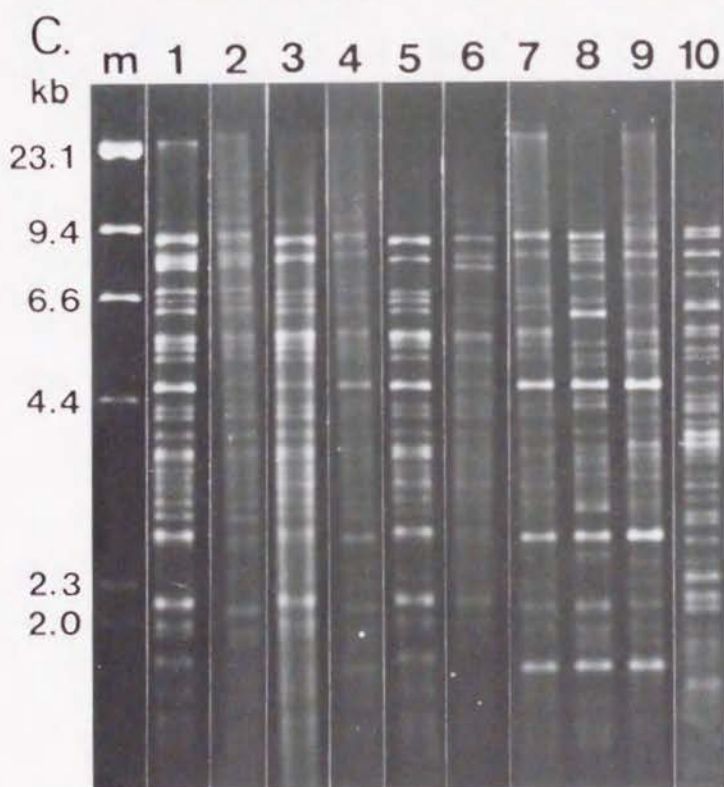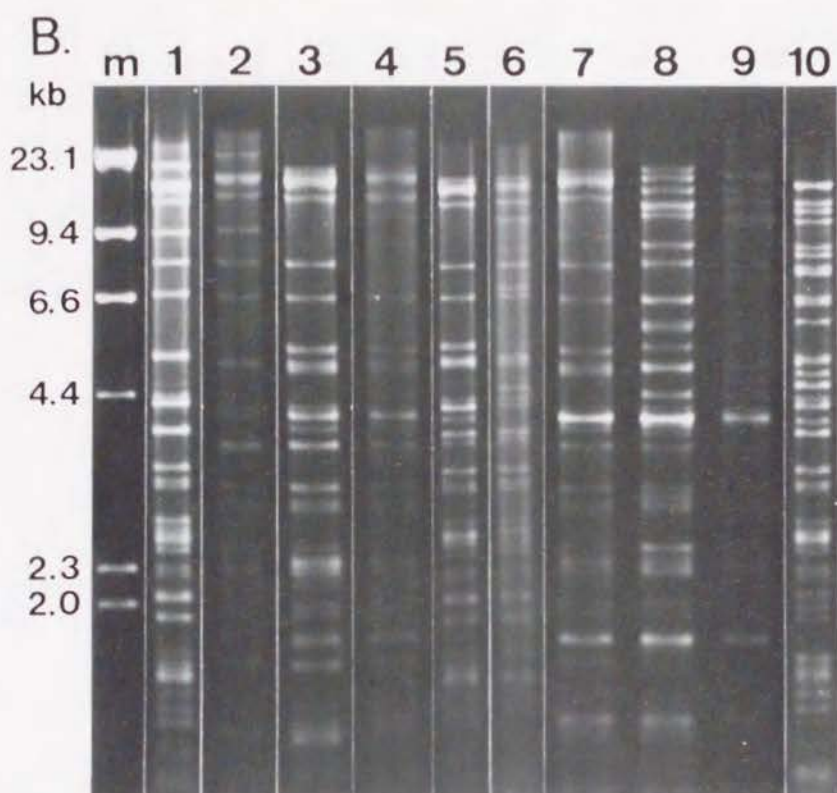
Fig. 4 (continued).
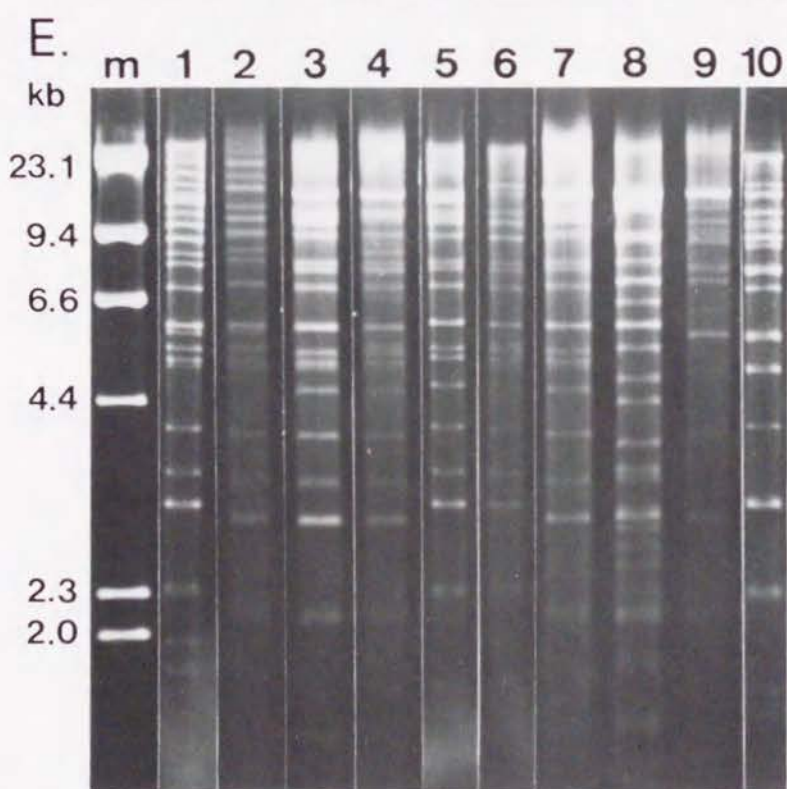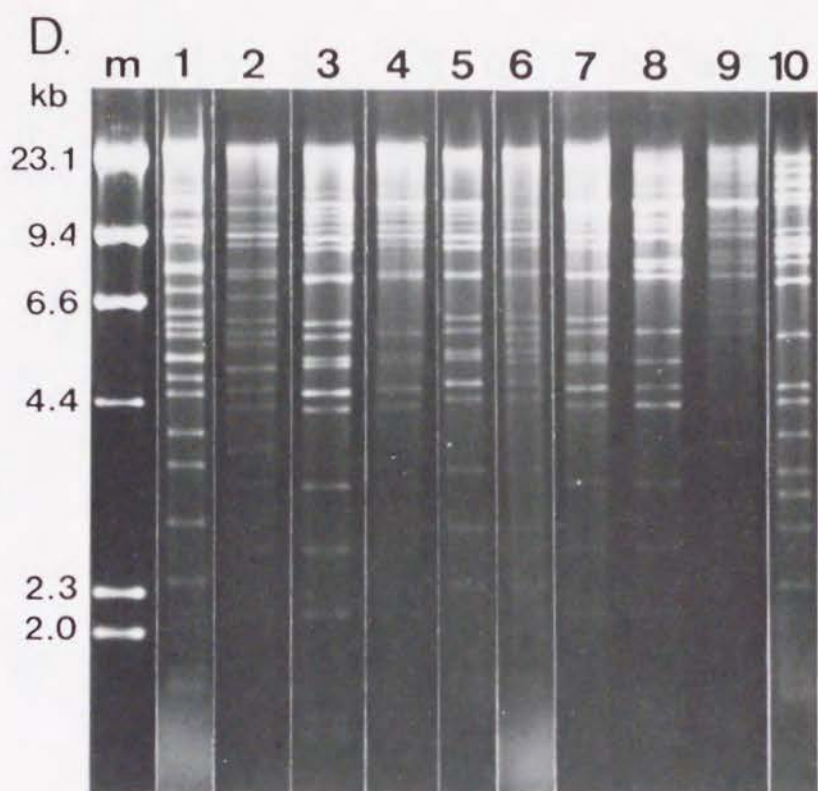
Fig. 4 (continued).

Fig. 4 (continued).

Table 8. Total number of the restriction fragments observed in each accession
(*italics*), number (upper right half of the table) and percent (its lower
left half) of common fragments observed between all pairs of accessions

Accession	Ia	Ib	IIa	IIb	IIc	ole	cam	jun	rad	ogu
Ia	*168*	167	141	139	139	146	141	109	116	109
Ib	99.4	*168*	140	139	138	145	140	108	115	108
IIa	84.7	84.1	*165*	161	162	152	165	115	121	114
IIb	83.0	83.0	97.0	*167*	159	150	161	113	120	115
IIc	83.7	83.1	98.5	96.1	*164*	151	162	113	119	112
ole	86.1	85.5	90.5	88.8	90.1	*171*	152	114	120	114
cam	84.4	83.8	99.7	96.7	98.2	90.2	*166*	115	121	114
jun	61.2	60.7	65.2	63.7	64.2	63.5	65.0	*188*	111	112
rad	67.1	66.5	70.6	69.6	69.6	68.8	70.3	60.7	*178*	146
ogu	64.5	63.9	68.1	68.2	67.1	66.9	67.9	62.6	83.9	*170*
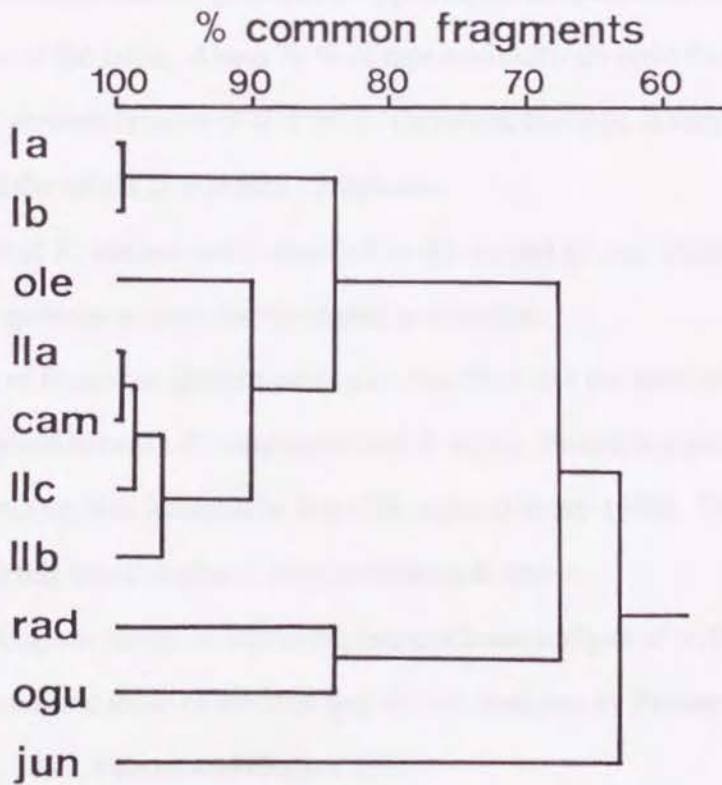
Fig. 5. The dendrogram showing the phylogenetic relationships among mitochondrial
genomes of ten *Brassica* accessions based on the restriction endonuclease analysis.

The first group included three species, i. e., *B. campestris*, *B. oleracea*, and rapeseed. Two species in this group, *B. campestris* and *B. oleracea*, are two of the three basic species according to the triangle of U (1935). On the other hand, rapeseed is an amphidiploid species derived from hybridization between the former two species. This group was further divided into two subgroups, 1) *B. campestris*, type II lines of rapeseed and *B. oleracea*, and 2) type I lines of rapeseed. Mitochondrial genomes of type II lines of rapeseed were similar to that of *B. campestris*. These results strongly indicated that their cytoplasms originated from *B. campestris*. The same results were reported on chloroplast genomes (Erickson et al. 1983; Palmer et al. 1983a). In the other subgroup, two subtypes of type I rapeseed were included. Although the cytoplasm of rapeseed has been considered to be introduced from one of its parental species, either *B. campestris* or *B. oleracea*, the mitochondrial genomes of type I rapeseed resembled neither that of the former nor that of the latter. About 70 % of rapeseed cultivars have this type I mitochondrial genome (Handa et al. 1990). Therefore, this type is very important for the clarification of the origin of rapeseed cytoplasms.

Two lines of *R. sativus* were classified as the second group. *Raphanus* mitochondrial genome is very closely related to *Brassica*.

Cms line of *B. juncea* (*juncea*-cms) was classified into the third group. *B. juncea* is an amphidiploid between *B. campestris* and *B. nigra*. Restriction pattern of this *juncea*-cms mtDNA was identical to that of *B. nigra* (Palmer 1988). Therefore, it was concluded that this cms cytoplasm originated from *B. nigra*.

The dendrogram based on restriction endonuclease analysis of mtDNAs of *Brassica* species was similar to those of mtDNA and ctDNA analyses by Palmer and coworkers (Palmer et. al. 1983; Palmer and Herbon 1988).

RFLPs of mtDNA

The RFLP analysis of mtDNAs from ten *Brassica* accessions was carried out using five mitochondrial specific genes, pea F_1ATPase α-subunit (*atpA*) (Morikami and Nakamura 1987), pea cytochrome oxidase subunit II (*coxII*) (Morikami, personal communication), rice apocytochrome b (*cob*) (Kadowaki et al. 1989), wheat 18S and 5S ribosomal RNA (*rrn18*), and wheat 26S ribosomal RNA (*rrn26*) (Falconet et al. 1984) as probes (Fig. 6). The data for the hybrid band formed by the combinations of five probes and seven restriction enzymes were compiled. Among 131 mitochondrial restriction fragments compiled, 40 fragments were unique among lines (30.5 % of total), 19 fragments were common to all lines (14.5 %) and the remaining 72 fragments were phylogenetically informative (55.0 %). Based on these data, the percent common fragments were also calculated (Table 9), and then the phylogenetic tree was constructed by the UPGMA method (Fig. 7).

Mitochondrial genomes of ten *Brassica* accessions were divided into two groups. The first group was further divided into two subgroups, 1) type I rapeseed and *B. oleracea*, and 2) type II rapeseed and *B. campestris*. Two lines of radish and cms line of *B. juncea* were included into the second group. These results of RFLP analysis were different from those of restriction endonuclease analysis of mt and ctDNAs (Palmer et al. 1983a; Palmer and Herbon 1988; present results stated above).

When comparing two dendrograms, two major different points were observed: 1) using restriction endonuclease analysis *B. oleracea* was related to *B. campestris* and type II rapeseed, but using RFLP analysis it was closely related to type I rapeseed, 2) using restriction endonuclease analysis two radish lines appeared at same branch, but using RFLP analysis they were separated.

To obtain more detailed relationships among rapeseed lines, *B. oleracea* and *B. campestris*, the RFLP analysis of them was further carried out using additional five
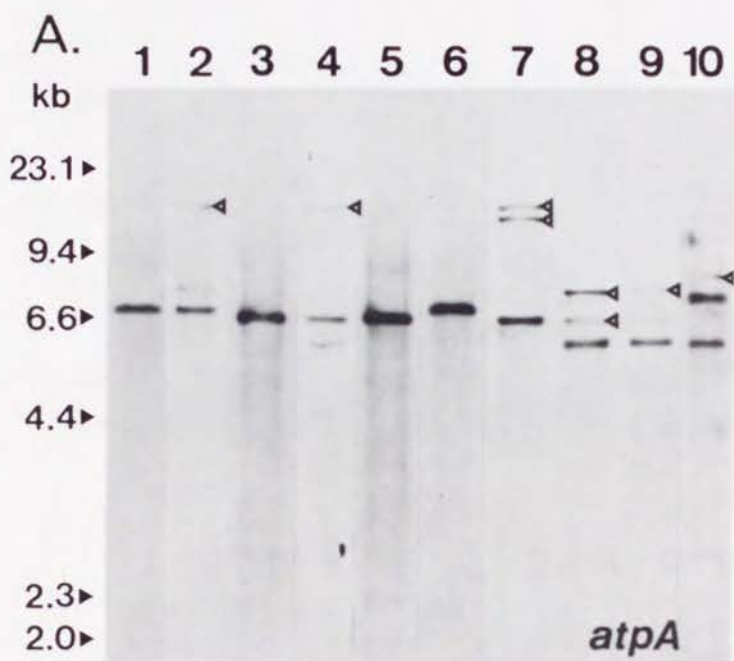
Fig. 6. Hybridization patterns of *Pvu*II-digested mtDNAs from ten *Brassica* accessions with the following gene probes: *atpA* (A), *cob* (B), *coxII* (C), *rrn18* (D), and *rrn26* (E). Accessions are indicated by their *number*. It should be noted that the bands indicated as ◁ are derived from residual color precipitates from the previous hybridization and do not represent the hybridized bands with the probes described in each panel.
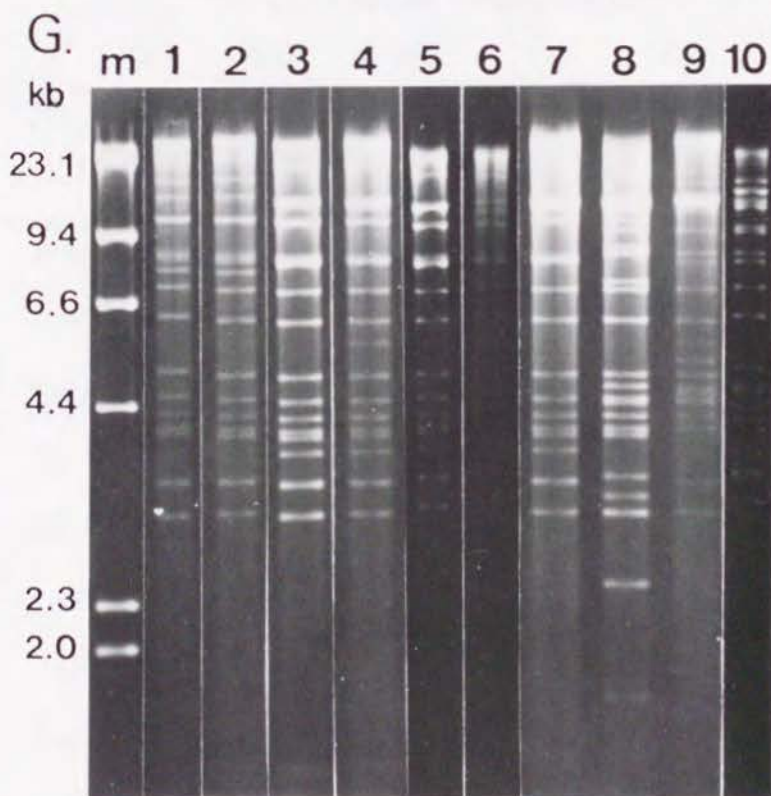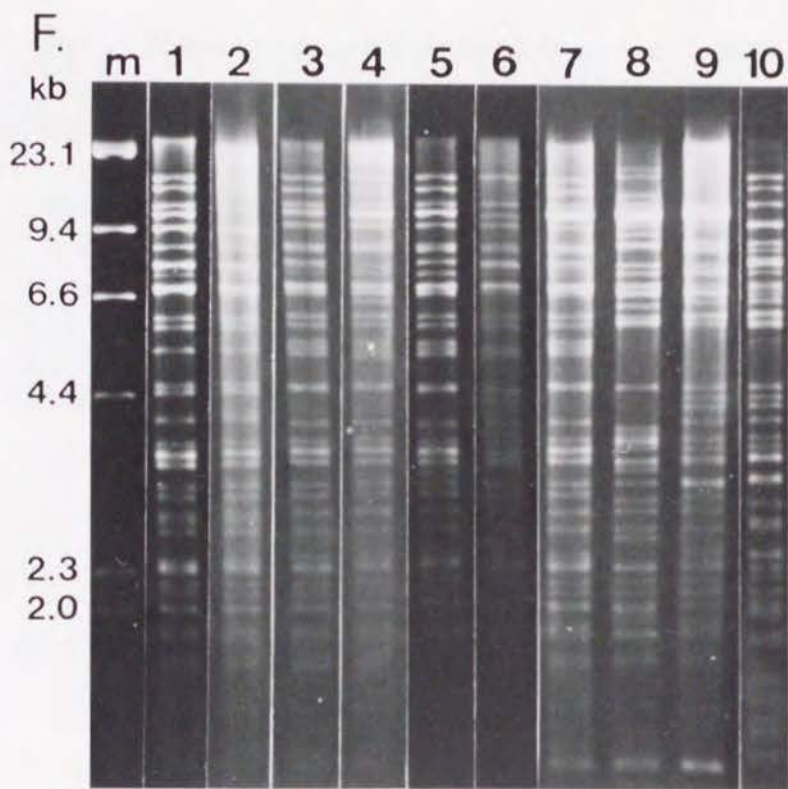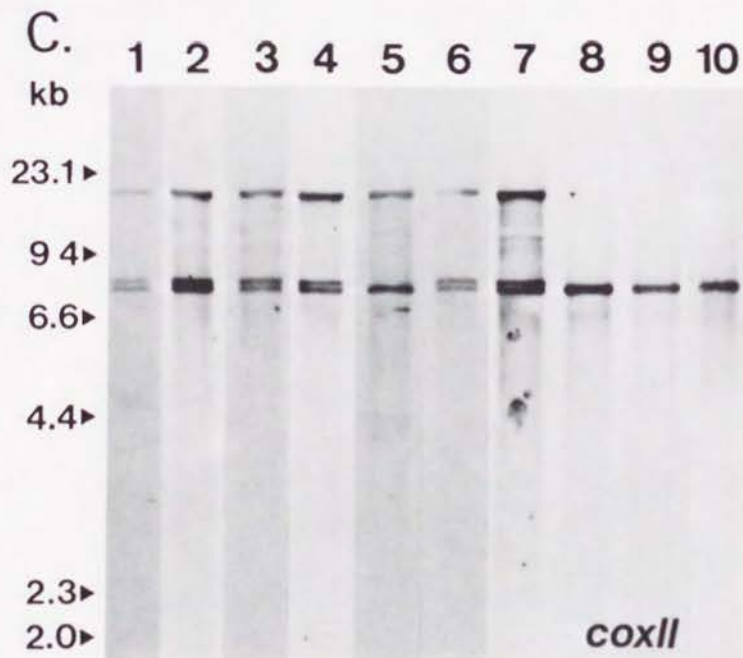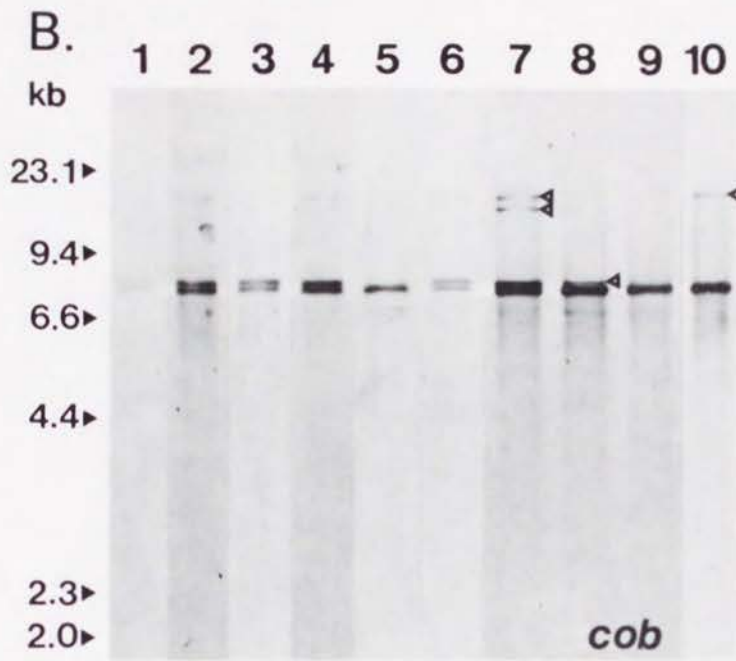
Fig. 6 (continued).

Fig. 6 (continued).

42
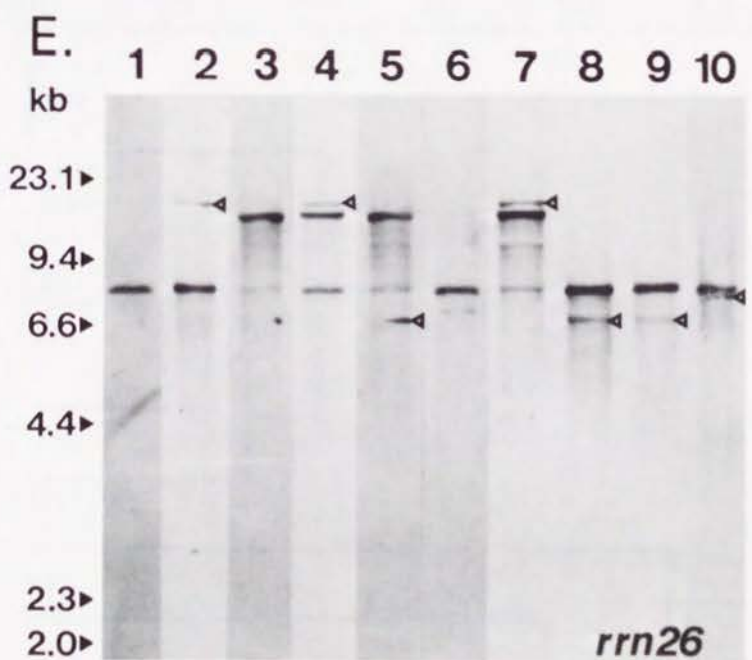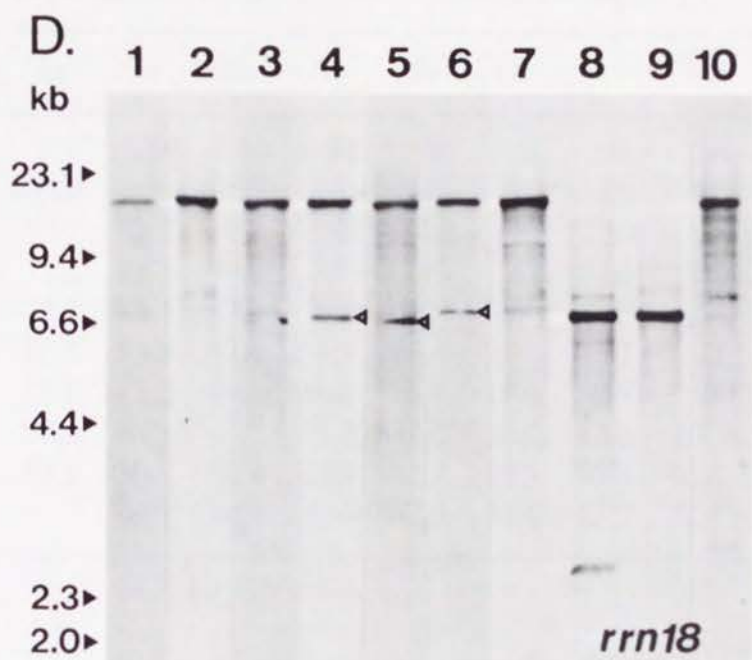
Table 9. Total number of the hybrid bands detected in each accession (*italics*), number (upper right half of the table) and percent (its lower left half) of common bands detected between all pairs of accessions

| Accession	Ia	Ib	IIa	IIb	IIc	ole	cam	jun	rad	ogu
Ia	*59*	59	51	51	40	58	51	26	28	22
Ib	100.0	*59*	51	51	40	58	51	26	28	22
IIa	85.7	85.7	*60*	60	49	51	60	26	26	23
IIb	77.3	77.3	90.2	*73*	49	51	60	29	26	31
IIc	72.1	72.1	87.5	78.4	*52*	40	49	24	27	23
ole	93.5	93.5	81.6	73.9	68.4	*65*	51	22	27	22
cam	85.0	85.0	99.2	89.6	86.7	81.0	*61*	26	26	23
jun	48.1	48.1	47.7	47.5	47.5	38.6	47.3	*49*	23	29
rad	50.9	50.9	46.8	41.9	52.4	46.6	46.4	46.0	*51*	29
ogu	42.7	42.7	44.2	53.0	47.9	40.4	43.8	62.4	61.1	*44*
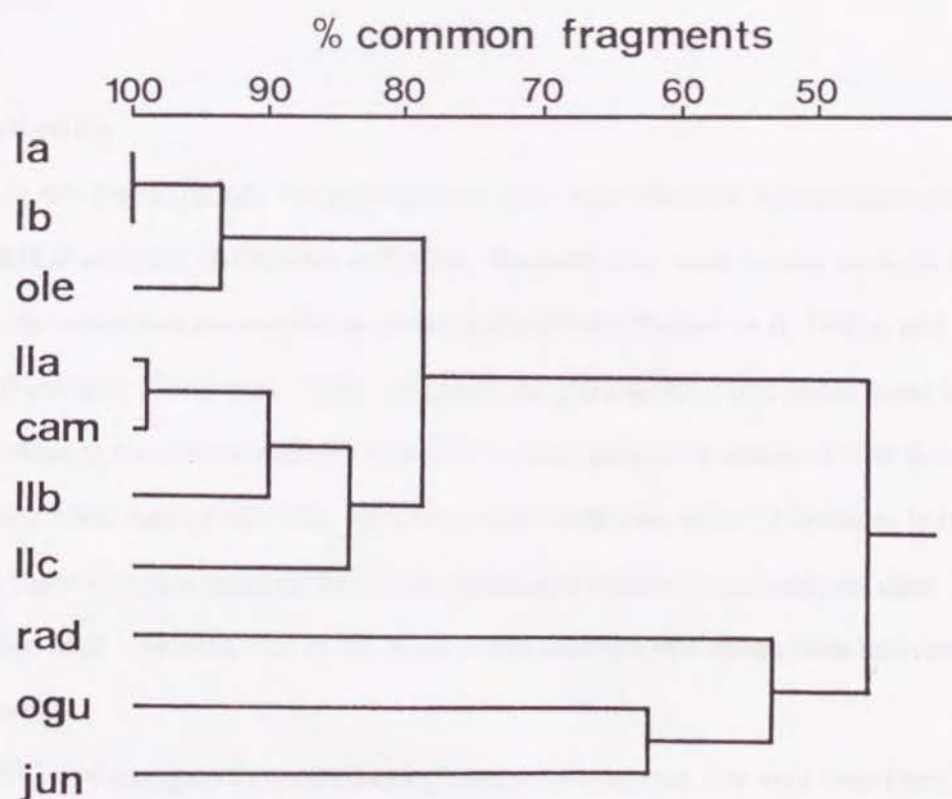
Fig. 7. The dendrogram showing the phylogenetic relationships among mitochondrial genomes of ten *Brassica* accessions based on the RFLP data.

43

mitochondrial gene probes, rice F_0-ATPase subunit 6 (*atp6*) (Kadowaki et al. 1990), tomato F_0-ATPase subunit 9 (*atp9*) (Kazama et al. 1990), rice cytochrome oxidase subunit I (*coxI*) (Kadowaki, personal communication), *Aegilops columnaris* cytochrome oxidase subunit III (*coxIII*) (Ikeda et al. 1990), and rice NADH dehydrogenase subunit 3 and ribosomal protein S12 (*nad3/rps12*) (Suzuki et al. 1991) on three restriction digests (*Bam*HI, *Eco*RI, and *Pst*I) of four *Brassica* accessions (rapeseed type I and II, *B. oleracea* and *B. campestris*) (Fig. 8). The additional data for the hybrid band were added to the former data of RFLP analysis. Based on these combined data, the percent common fragments were calculated again (Table 10), and the phylogenetic tree was constructed (Fig. 9). This second phylogenetic tree was identical with the first one of RFLP analysis, except for slightly longer distance between type I rapeseed and *B. oleracea*.

Discussion

In this investigation, two phylogenetic trees were obtained by restriction endonuclease and RFLP analyses of *Brassica* mtDNAs. Basically they were similar to those derived from the restriction endonuclease analysis of ctDNAs (Palmer et al. 1983a) and nuclear RFLP analysis (Song et al. 1988). Notably, the phylogenetic tree constructed by the restriction endonuclease analysis of mtDNAs was completely identical with that based on the restriction data of ctDNAs. However, there were two major differences between the trees based on mitochondrial RFLP and restriction endonuclease analyses data: 1) the location of *B. oleracea*, and 2) the relationship between two radish lines and cms line of *B. juncea*.

When the origin of rapeseed cytoplasm is investigated, it is very important to clarify the relationships between rapeseed, *B. oleracea* and *B. campestris* on the phylogenetic tree. The latter two species are considered to be parental species of rapeseed. Based on
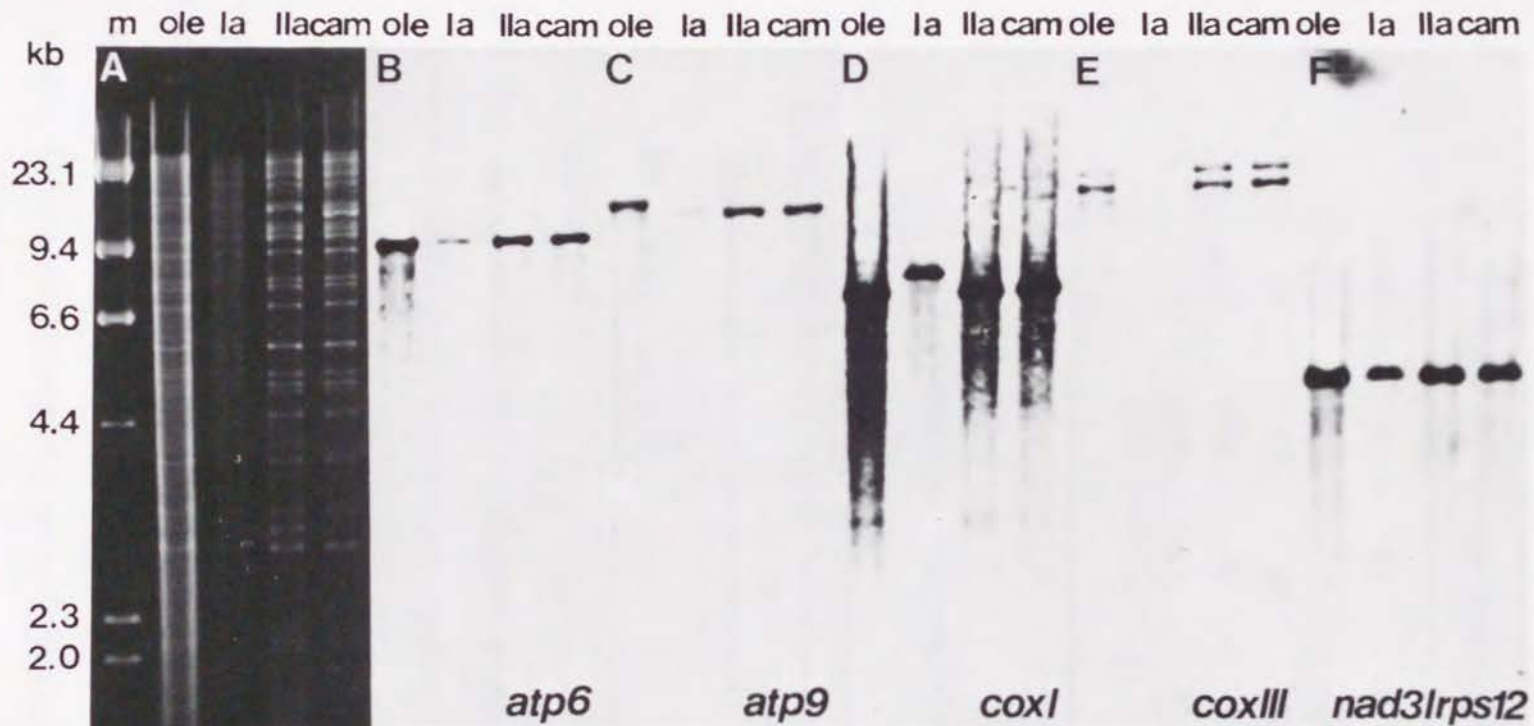
Fig. 8. Restriction fragment patterns of *Pst*I-digested mtDNAs from four *Brassica*

accessions (A). Accessions are indicated by their *abbreviation*. *m* is *Hind*III-

digested lambda DNA, whose fragment sizes are given in kb. Hybridizations of

panel A with the following gene probes: *atp6* (B), *atp9* (C), *coxI* (D), *coxIII* (E),

and *nad3/rps12* (F).

Table 10. Total number of the hybrid bands detected in
each accession (*italics*), number (upper right half of
the table) and percent (its lower left half) of common
bands detected between all pairs of four accessions.

Accession	Ia	IIa	ole	cam
Ia	*78*	64	70	64
IIa	79.0	*84*	69	81
ole	84.9	80.7	*87*	69
cam	80.0	97.6	81.7	*82*

% common fragments
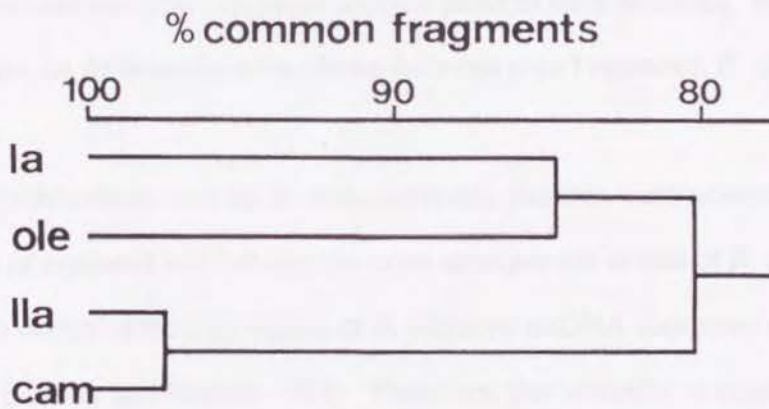
Fig. 9. The dendrogram showing the phylogenetic relationships among mitochondrial
genomes of three *Brassica* species, *napus*, *campestris* and *oleracea* based on the
RFLP data.

the phylogenetic tree using the data of the restriction endonuclease analysis of mtDNA,
mitochondrial genome of type II rapeseed is closely related to that of *B. campestris*.
Therefore, this type of cytoplasm is considered to be introduced to rapeseed from *B.
campestris*. However, mitochondrial genome of type I rapeseed differs from those of

both diploid parents, *B. campestris* and *B. oleracea*. Thus, its origin cannot be traced using the result based on the restriction endonuclease analysis of mtDNAs. These results are in accord with those of Palmer (1988). He proposed that the cytoplasm of rapeseed was derived by introgression of some foreign cytoplasms, which are distantly related to those of both parental species. However, the second phylogenetic tree based on the mitochondrial RFLP data clearly revealed that the mitochondrial genomes of type I rapeseed were closely related to that of *B. oleracea*.

Fifty probe-enzyme combinations for RFLP analysis of mtDNAs were chosen, five probes and seven enzymes in the first experiment, and five probes and three enzymes in the second experiment. From total 50 combinations, type I rapeseed showed the identical hybridized pattern as *B. oleracea* in seven and as *B. campestris* in two combinations. In eight combinations type I rapeseed specific patterns were obtained. In the remaining combinations, no differences were shown between type I rapeseed, *B. oleracea* and *B. campestris*.

Two combinations with the *B. campestris*-like patterns were related to *atp9*. The *atp9* region of rapeseed mtDNA has the same arrangement as that of *B. campestris*, but an inversion occurs at the *atp9* region of *B. oleracea* mtDNA compared to that of *B. campestris* (Palmer and Herbon 1988). Therefore, this inversion is considered to occur in the *B. oleracea* mitochondrial genome after divergence of *B. oleracea* cytoplasm from ancestral cytoplasm.

Four of seven combinations with the *B. oleracea*-like patterns were related to *atpA*, and the other three to *rrn26*. These two genes, *atpA* and *rrn26*, were closely located on the unrearranged region of mitochondrial genome of all three species (Palmer and Herbon 1988). Therefore, there should be no differences among the Southern hybridization patterns of these three species when *atpA* or *rrn26* is used as a probe. However, in this experiment the polymorphisms were detected between rapeseed, *B. campestris* and *B. oleracea*. These results indicated that sequential changes or small rearrangements

47

occurred in this region including two genes, *atpA* and *rrn26*, of rapeseed and *B. oleracea*. Furthermore, on the basis of these results, it can be suggested that type I rapeseed is more closely related to *B. oleracea* than *B. campestris*.

Palmer and his coworkers reported that most of the *Brassica* mitochondrial genome changes at the interspecific level were structural, consisting of deletions, insertions, duplications, and especially inversions, and such DNA rearrangements are considered to be a major driving power for the evolution of *Brassica* mitochondrial genomes. They proposed the model of genome rearrangements throughout the evolution of three species, rapeseed, *B. campestris* and *B. oleracea* (Palmer and Herbon 1987, 1988; Makaroff and Palmer 1988; Palmer 1988).

Based on their model, it is probable that sequential changes have occurred in the region including two genes, *atpA* and *rrn26* of rapeseed and *B. oleracea* mitochondrial genomes, however, in the same region no rearrangements were observed. Furthermore, it is more likely that these changes occurred in the common ancestor of rapeseed and *B. oleracea* before their divergence, rather than occurring independently in rapeseed and *B. oleracea*. Based on these results, the origin of type I rapeseed cytoplasm can be discussed as follows: type I rapeseed originated from the hybridization between ancestral *B. oleracea*-like species as female parent and ancestral *B. campestris*-like species as pollen parent. Afterwards, extensive rearrangements occurred on the mitochondrial and/or chloroplast genome of newly synthesized rapeseed and its parent species. As a result, mitochondrial genomes of type I rapeseed seem to be different from those of either *B. oleracea* or *B. campestris* if the method which detects the changes on the whole genome, such as a restriction endonuclease analysis is used, because the phylogenetic information might be disturbed by the extensive rearrangements of the genome. However, when RFLP analysis is used, which detects the changes within the specific regions, the rapeseed mitochondrial genome has some sequential remains which are common to those of *B.*

oleracea.

This hypothesis on the origin of rapeseed cytoplasms makes a step further compared to that of Palmer and Herbon (1988), who proposed that the mitochondrial genomes of three related species, rapeseed, *B. campestris* and *B. oleracea* were evolved from common ancestral mitochondrial genome through large inversions. *B. campestris* and *B. oleracea* were confirmed as parental species of rapeseed, with no other ancestral parent, by Morinaga (1934) and U (1935). Their hypothesis supports the results cited in this chapter.

However, some ambiguities still remain concerning the phylogeny of individual species. Additional work is required to characterize in detail the phylogenetic relationships among *Brassica* species. To obtain the full explanation of the intergeneric relationship, it will be necessary to study not only the structural rearrangements but also the sequential differentiation of mitochondrial genomes.

Introduction

Part II. Molecular genetic approach to the mechanism of the cytoplasmic male-sterility induction

50

Chapter 4. Examination of physical organizations of several mitochondrial genes from male-sterile cytoplasm

Introduction

Cytoplasmic male-sterility (cms) is a maternally inherited trait which prevents the production of functional pollen. Male-sterile cytoplasm has long been of interest for its usefulness in the production of hybrid seeds. In many crops, the cms phenotype is expressed in alloplasmic lines arising from interspecific or intergeneric crosses. It is thought to be due to incompatibility between the nucleus and the cytoplasm. Several lines of evidence suggest that the cms determinants reside on the mitochondrial genome (reviewed in Hanson and Conde 1985; Lonsdale 1987). There are two types of cms cytoplasm in rapeseed, *nap* cms (Shiga and Baba 1971, 1973; Thompson 1972) and *pol* cms (Fu 1981).

The mitochondrial genome of *Brassica* is the smallest and the best-characterized among higher plant mitochondrial genomes (Palmer 1988). A complete restriction map showed the genome size of rapeseed mitochondria to be 221 kb (Palmer and Herbon 1988). The mitochondrial genome of rapeseed was previously classified into two major types, each of which was further divided into three subtypes based on length differences in the restriction fragments of mtDNAs. The mitochondrial genome of *nap* cms cytoplasm was classified as type I, whereas the *pol* cms cytoplasm carried the type II mitochondrial genome. To localize the differences in the mitochondrial genome of rapeseed with normal and male-sterile cytoplasms, and to determine the molecular basis of cms in rapeseed, the physical organization of several mitochondrial genes from *pol* cms cytoplasm, type IIb mitochondrial genome, and its normal counterpart, type IIa was examined by Southern blot analysis using heterologous mitochondrial genes as probes.

In addition, altered transcript patterns have been identified for three genes, *atpA*,

51

atp6 and *coxI* in the mitochondrial genome of Ogura cms radish, which is a closely related species to rapeseed (Makaroff and Palmer 1988). From these genes, *atpA* genes were cloned from *pol* cms and normal cytoplasms of rapeseed, and characterized in order to clarify the states of *atpA* genes in *pol* cms and normal cytoplasms.

Materials and Methods

Plant materials

B. napus L. cv. Isuzu-natane was the source of the normal (fertile) cytoplasm. A cytoplasmic male-sterile line, which has the "Polima" cms cytoplasm under the nuclear background of Isuzu-natane, was also used.

Isolation of nucleic acids

Mitochondria were isolated from 8-week-old plants. MtDNA was extracted as described previously (Handa et al. 1990). Mitochondrial (mt) RNA was isolated by the procedure of Stern and Newton (1986), except that no sucrose step gradient was used for the purification of mitochondria.

Southern and northern blot analyses

Restricted mtDNAs separated by agarose gel electrophoresis were transferred to a nylon membrane filter (Hybond N, Amersham, UK) by a modification of Southern (1980). Isolated mtRNA (10µg/lane) was electrophoresed in a 1.2 % agarose gel containing 0.6 M formaldehyde, 20 mM MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA, and blotted to Hybond N filters with 20 X SSC. Ten mitochondrial specific genes [pea *atpA*, *coxII* (Morikami and Nakamura 1987, and unpublished data), rice *atp6*, *cob*, *coxI*, *nad3/rps12* (Kadowaki et al. 1989, 1990, unpublished data, and Suzuki et al. 1991), tomato *atp9* (Kazama et al. 1990), *Aegilops columnaris coxIII* (Ikeda et al.

1990), and wheat *rrn18*, *rrn26* (Falconet et al. 1984)] were labeled by the random primer method incorporating digoxigenin-labeled dUTP (Feinberg and Vogelstein 1983) and were used as hybridization probes. Hybridizations were carried out at 68°C (for DNA blots without formamide), and at 42°C (for RNA blots with 50% formamide) for 16 hr after prehybridization. Following hybridization, the hybrid bands were detected by an enzyme-linked immunoassay using an anti-digoxigenin-alkaline phosphatase conjugate and a subsequent enzyme-catalyzed color reaction with X-phosphate and NBT, or the chemiluminescent reaction with AMPPD, following the manufacturer's instructions (Boehringer Mannheim, Germany; Tropix, Inc., Mass., USA).

Results and Discussion

Southern blot analysis of restricted mtDNA

The mtDNAs from *pol* cms and normal cytoplasms were compared by restriction digests using *Eco*RI, *Hind*III, and *Kpn*I (Fig. 10). The mtDNAs from both cytoplasms showed different fragment patterns as mentioned in the previous chapter.

To understand the nature of the mtDNA alterations distinguishing the two rapeseed cytoplasms, Southern blot analysis was used and determined the gene organizations of mtDNA from *pol* cms and normal cytoplasms. Ten mitochondrial genes, *atpA*, *atp6*, *atp9*, *cob*, *coxI*, *coxII*, *coxIII*, *nad3/rps12*, *rrn18* and *rrn26* were used to probe Southern blots containing *Eco*RI, *Hind*III, and *Kpn*I-digests of *pol* cms and normal mtDNAs. The mtDNA from *pol* cms and normal cytoplasms had the same hybridization signals when probes for the *atp9*, *cob*, *coxI*, *coxII*, *coxIII*, *nad3/rps12*, *rrn18* and *rrn26* genes were used (Fig. 11).

When the *atpA* probe was used, two bands were identified in restricted fragments from *pol* cms cytoplasm, and only one band in those from normal cytoplasm (Fig. 11). However, the extra band found only in *pol* cms mtDNA was not reproducible, because

53
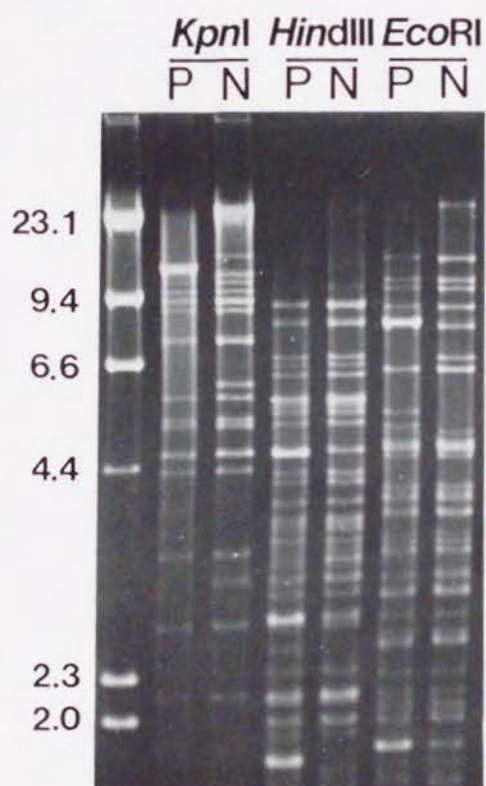
Fig. 10. Restriction patterns of mtDNAs from *pol* cms (P) and normal (N) cytoplasms.
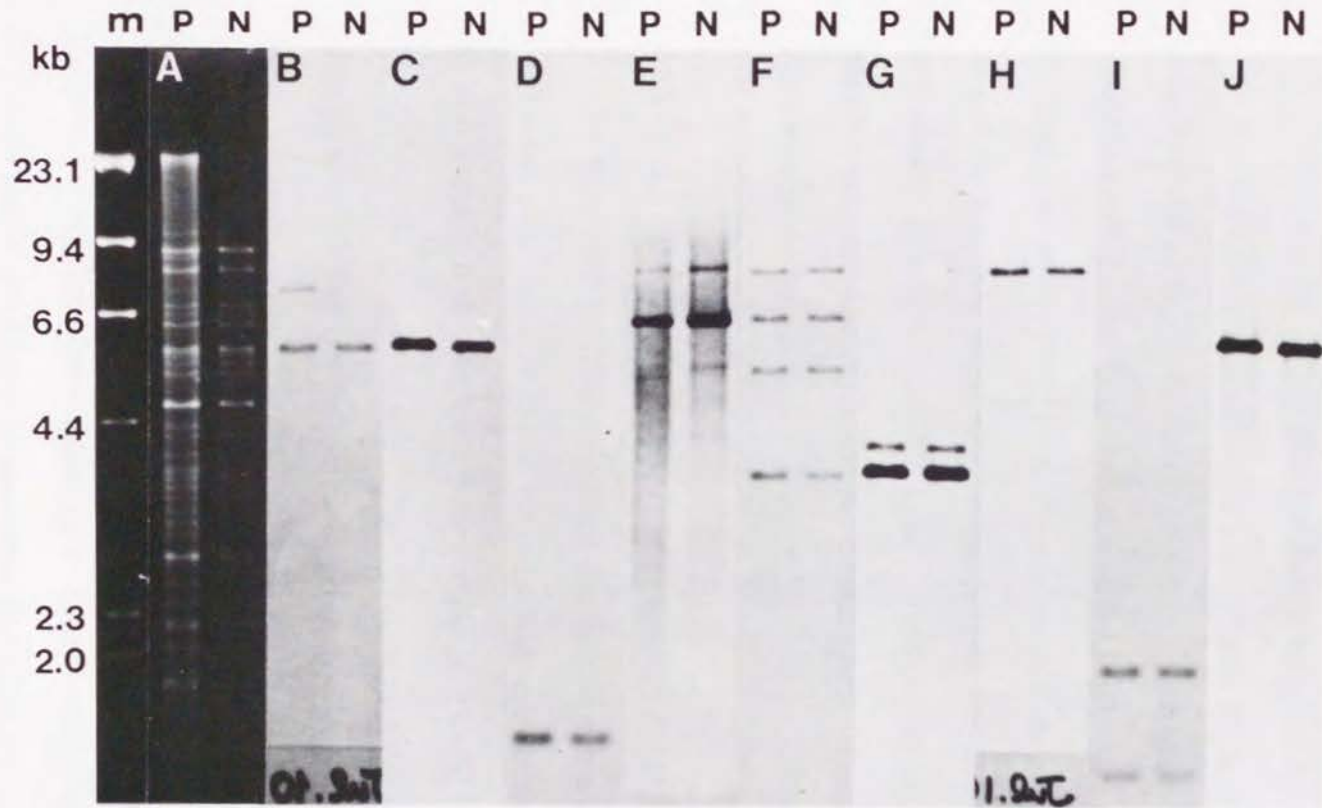
Fig. 11. Southern blot analysis of *Hind*III-digested mtDNAs from *pol* cms (P) and
normal (N) cytoplasms. Agarose gel profile (A). Hybridizations of panel A with the
following gene probes; *atpA* (B), *atp9* (C), *cob* (D), *coxI* (E), *coxII* (F), *coxIII*
(G), *rrn18* (H), *rrn26* (I), and *nad3/rps12* (J).

this band disappeared in other preparations of *pol* cms mtDNA. Therefore, it may be an unstable product of rare recombination of mitochondrial genome, which was reported in *atpA* gene of maize (Small et al. 1987), or an experimental artifact, and therefore it should not be associated with *pol* cms cytoplasm.

Different patterns were observed only if the probe for the *atp6* gene was used (Fig. 12). Restriction fragments encoding the *atp6* gene of *pol* cms and of the normal mitochondrial genome were determined to be 4.9 kb and 6.5 kb, respectively, after restriction with *Eco*RI. These results from the Southern blot experiments suggested a different gene organization around the *atp6* locus in the two mtDNAs. Recently, Witt et al. (1991), using Southern hybridization analysis, also detected a different gene organization around the *atp6* loci in *pol* cms and normal mitochondrial genomes. Their results are consistent with the above mentioned data, although their estimated fragment sizes are slightly larger.

Cloning and transcription analysis of mitochondrial F$_1$-ATPase α-subunit gene

To understand the relationship between the cms trait and mitochondrial *atpA* gene, *atpA* genes were cloned from *pol* cms and normal cytoplasms and transcriptional patterns of *atpA* genes in both cytoplasms were compared. *Hind*III libraries of mtDNAs of *pol* cms and normal cytoplasms were screened for the presence of clones containing the *atpA* gene by colony hybridization with digoxigenin-labelled pea *atpA* clone as a probe. Positive clones were isolated from these two mtDNA libraries, and they contained a *Hind*III mtDNA fragment of a size (5.6 kb) as an insertion. Detailed physical mapping and further Southern analysis of 5.6 kb *Hind*III fragments from both mtDNAs were performed to localize *atpA* coding region. Two physical maps showed these two fragments shared common restriction sites. Therefore, the *atpA* loci from *pol* cms and normal cytoplasms were completely identical (Fig. 13). The sizes of the isolated

56
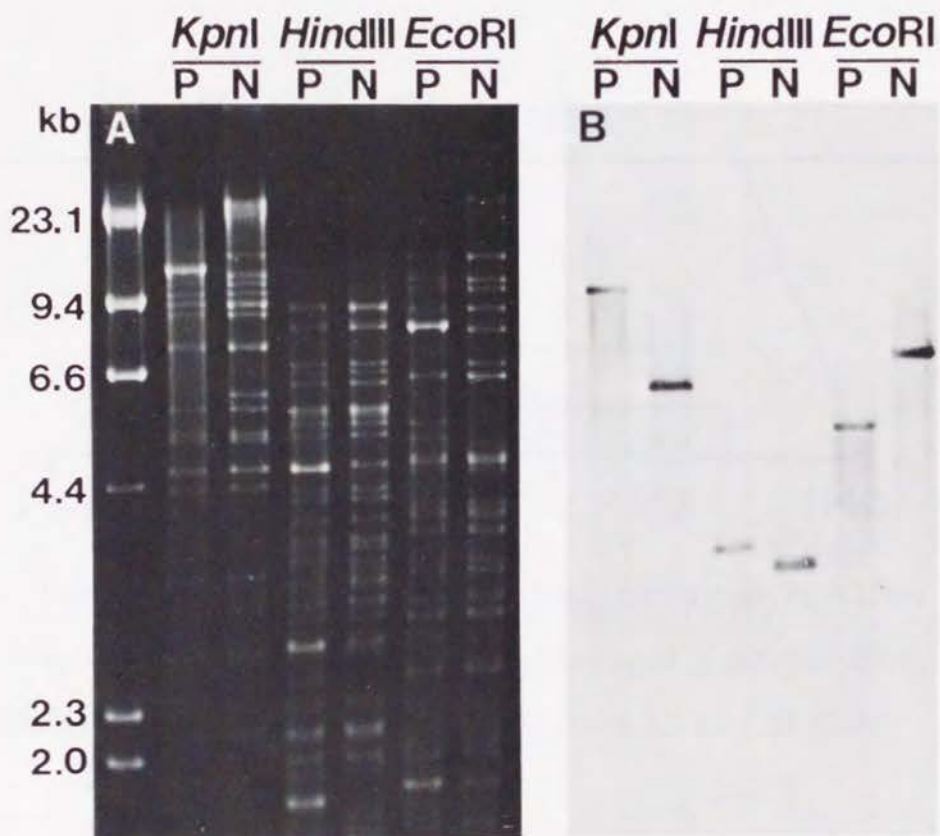
Fig. 12. Southern blot analysis of mtDNAs from *pol* cms (P) and normal (N) cytoplasms. A, agarose gel profile; B, hybridization patterns using the rice *atp6* gene as a probe.

fragments were also consistent with those which are expected to be derived from Southern blot analysis (Fig. 11).
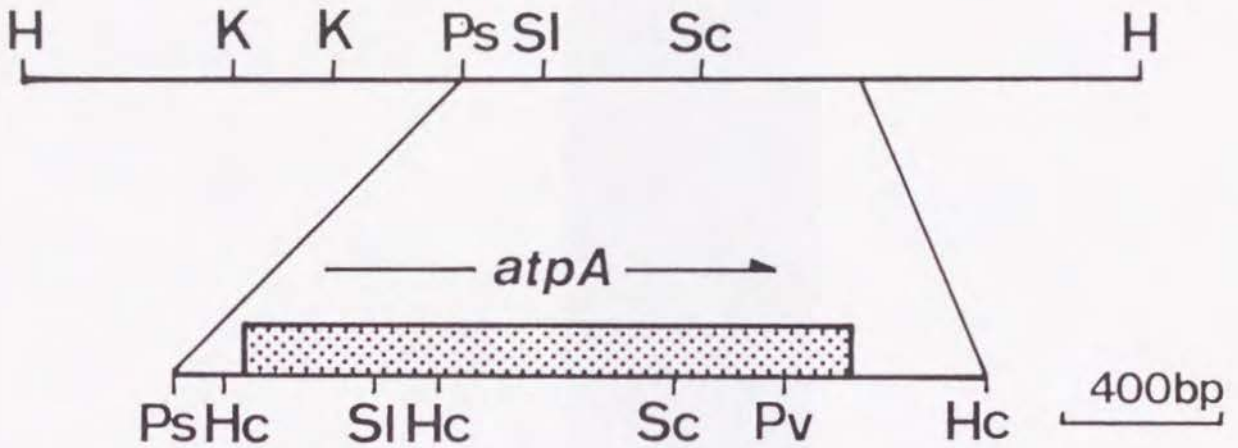
Fig. 13. Physical map of a rapeseed mtDNA segment carrying the F_1-ATPase α-subunit coding sequence. The *atpA* coding region is indicated by the *shaded box*. H: *Hind*III, Hc: *Hinc*II, K: *Kpn*I, Ps: *Pst*I, Pv: *Pvu*II, Sc: *Sac*I, Sl: *Sal*I.

In order to determine the relationship between the cms trait and *atpA* gene, the patterns of transcription were observed for the *atpA* gene using mtRNA isolated from normal and *pol* cms rapeseed, as shown in Fig. 14. The rapeseed pattern consists of a single abundant transcript of approximately 1900 nucleotides, differing from pea where multiple *atpA* transcripts have been observed (Morikami and Nakamura 1987). Identical transcriptional patterns were observed for mtRNAs of *pol* cms and normal cytoplasms.

The *atpA* loci of normal and *pol* cms cytoplasms were characterized in order to investigate the relationship between the cms trait and *atpA* gene, which was suggested to be related with the cms trait in the cases of Ogura cms radish (Makaroff and Palmer 1988; Makaroff et al. 1990) and sunflower cms line (Siculella and Palmer 1988; Köhler et al.
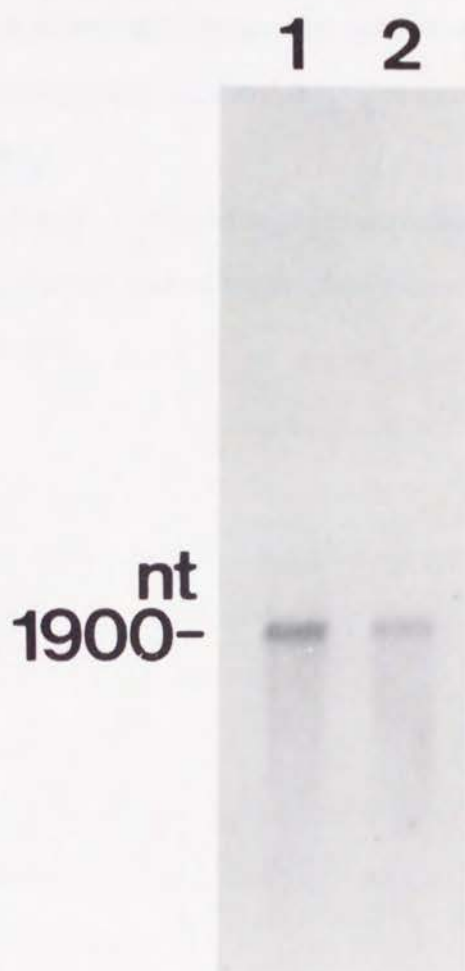
Fig. 14. Northern analysis of the rapeseed *atpA* transcription patterns. Each lane
containing 10 μg of mtRNA was probed with a 580 bp *Hinc*II-*Sac*I fragment (see
physical map in Fig. 13). Lane 1: mtRNA from *pol* cms cytoplasm, Lane 2: mtRNA
from normal cytoplasm.

1991). Several lines of evidence indicate that the *atpA* locus may not be involved in male sterility: 1) The same 5.6 kb *Hind*III fragment of mtDNA from both normal and *pol* cms cytoplasms was detected by Southern analysis. 2) Physical maps of two 5.6 kb *Hind*III fragments showed the *atpA* loci from normal and *pol* cms rapeseed were identical. 3) Analysis of *atpA* transcript patterns indicated that there were no differences between normal and *pol* cms plants.

Different organization of *atp6* found in *pol* cms mitochondria was described, using Southern blot analysis. Further studies, which clarify the molecular basis of cms trait, are presented in the next chapter.

Chapter 5. Different organization and altered transcription of the mitochondrial *atp6* gene in the male-sterile cytoplasm

Introduction

Polima cytoplasm of rapeseed can be used as an example for the description and explanation of cms in this species (Fu 1981). Rapeseed plants containing the Polima cytoplasm develop normally except the undeveloped anthers, short stamen length, and narrow petals.

The mitochondrial genomes of Polima (*pol*) cytoplasm was classified as type IIb mitochondrial genome by restriction endonuclease analysis, slightly rearranged if compared to its normal (i. e. male-fertile) counterpart, type IIa mitochondrial genome. In addition, a different organization has been identified specifically for *atp6* gene by Southern blot analysis in Chapter 4. These findings implicate DNA rearrangements located around the mitochondrial *atp6* locus of *pol* cms cytoplasm as being responsible for the male-sterile phenotype.

In order to identify these alterations, *pol* mitochondrial genome was further characterized, and a detailed analysis of the *atp6* locus from both *pol* cms and normal cytoplasms is described. Nucleotide sequences of the two *atp6* loci were determined, and the *pol* cms-specific *atp6* locus was found to be associated with a novel 105-amino acid ORF (*pol-urf*) which may be co-transcribed with *atp6*. The possible role of this alteration in *pol* cms cytoplasm is discussed.

Materials and Methods

Plant materials

B. napus L. cv. Isuzu-natane was the source of the normal (fertile) cytoplasm. A cytoplasmic male-sterile line, which has the "Polima" cms cytoplasm under the nuclear

61

background of Isuzu-natane, was also used.

Isolation of nucleic acids

MtDNA was extracted as described previously (Handa et al. 1990). MtRNA was isolated by the modification procedure of Stern and Newton (1986) as described previously.

Southern and northern blot analyses

Restricted mtDNAs or mtRNAs separated by agarose gel electrophoresis were transferred to a nylon membrane filter (Hybond N, Amersham, UK) by a modification of Southern (1980) as previously described. Rice mitochondrial *atp6* gene (Kadowaki et al. 1990) was used as a hybridization probe. Hybridizations were carried out at 68°C (for DNA blots without formamide), and at 42°C (for RNA blots with 50% formamide) for 16 hr after prehybridization. Following hybridization, the hybridized bands were detected as described previously. Strand-specific RNA probes were synthesized from the 1.35 kb *Hind*III-*Eco*RI fragment cloned into pBluescriptII vector (Stratagene, CA, USA) (Fig. 15, probes F and F') using T3 or T7 RNA polymerase.

Sequence determination and analysis

The dideoxynucleotide chain-termination method of Sanger et al. (1977) was used for DNA sequencing. This sequencing strategy was supplemented with nested deletion methods using exonuclease III and subsequent mung bean nuclease digestions, following the manufacturer's procedures (Takara Shuzo, Japan). Sequence data were compiled and analyzed with the aid of GENETYX computer software programs.

Results and Discussion

Cloning of rapeseed atp6 genes from pol cms and normal cytoplasms

The heterologous *atp6* gene from rice (Kadowaki et al. 1990) was used as a probe to isolate the rapeseed *atp6* gene. An 11 kb *Sal*I fragment of clone pBNP3 was isolated from *pol* cms, and a 6.5 kb *Eco*RI fragment of clone pBNI54 was obtained from normal cytoplasm. Detailed physical mapping and further Southern analysis of plasmids pBNP3 and pBNI54 were performed (Fig. 15) to specify the difference between the two *atp6* loci. The sizes of the isolated fragments were identical with those which are expected to be
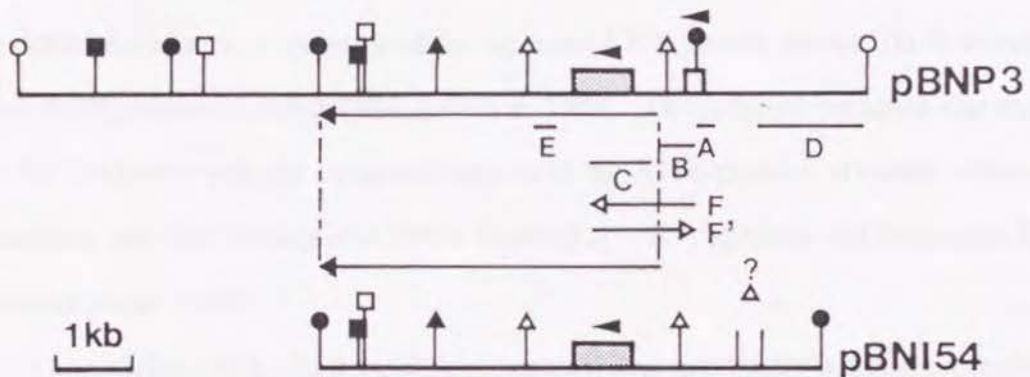
Fig. 15. Physical maps of the *atp6* loci from *pol* cms (top) and normal mitochondria (bottom). Clone pBNP3 was isolated from *pol* cms cytoplasm, and clone pBNI54 from normal cytoplasm. *Shaded* and *open boxes*, respectively, represent the *atp6* coding region and *pol-urf*. *Horizontal arrows with a solid head* indicate the conserved sequences between the two clones. *Arrow heads* indicate the transcriptional direction of the *atp6* and *pol-urf* genes. *Horizontal lines* indicate the position of the probes (A, B, C, D, and E) used in this study. *Horizontal arrows with open heads* also indicate the position of the probes (F and F') used and their directions. Restriction sites are indicated as follows: ●, *Eco*RI; △, *Eco*RV; ▲, *Kpn*I; □ , *Pst*I; ○, *Sal*I; ■, *Xho*I.

derived from Southern blot analysis (Fig. 12) and the two clones shared a common 2.2 kb

fragment including the *atp6* coding sequence (Fig. 15). However, the two fragments

diverged upstream of the *atp6* coding sequence, indicating that a rearrangement existed in

the 5'-flanking region of the *pol* cms *atp6* gene. To characterize these differences

further, both *atp6* genes and their flanking regions were sequenced.

Sequence analysis of the normal atp6 gene

The nucleotide sequence of the *atp6* locus on pBNI54 from normal cytoplasm was

determined (Fig. 16). This sequence contains a 783 bp open reading frame corresponding

to a 261-amino acid polypeptide that was identified as *atp6* by DNA sequence homology.

The deduced amino acid sequence of the rapeseed ATP6 protein shows 100 % homology

to the ATP6 protein of radish (Makaroff et al. 1989). The deduced sequence also exhibits

81 ~ 86 % identity with the conserved regions of the ATP6 proteins of maize, tobacco,

Oenothera, and rice (Dewey et al. 1985; Bland et al. 1987; Schuster and Brennicke 1987;

Kadowaki et al. 1990).

A comparison of the flanking regions between *Brassica* family members revealed a

number of differences. The 5'-flanking region of normal radish contains a gene for

tRNAfMet (*trnfM*) located on the same strand and situated between -232 bp and -159 bp

upstream of the *atp6* gene (Makaroff et al. 1989). A 98.3 % sequence homology between

the 5'-flanking region of normal rapeseed and radish *atp6* genes was observed from -181

bp to the ATG initiation codon (Fig. 16), but upstream from -182 the two genes diverge

extensively. Therefore, in normal rapeseed, the *trnfM* was truncated, leaving only 23 bp

of the 3' end. Located 152 bp 5' of the *atp6* initiation ATG site was a 7-bp sequence,

TAAGTAA (Fig. 16), which is nearly identical to part of the consensus sequence of the

putative plant mitochondrial promoter (Young et al. 1986).

In the 3'-flanking region, the 104 bp (from position +784 to +887) after the

```
                             pol:GATCGCTACGCTGCCGGGCTGCCCCCGCGGATCAAACTAT  -1052

        pol: C AATCTCATAAGAGA AGAAATCTCTATGCCCCCTGTTCTTGGTTTTCTCCCATGCTTTTG  -992

        pol: TTGGTCAACAACCAACCACAACTTTCTATAGTTCTTCACTACTCCTAGAGGCTTGACGGA
                                        normal: GTTTAGCGGTAATGGCA  -932
                                                   -882
                                        pol-urf: M  P  Q  L        4
        pol: GTGAAGCTGTCTGGAGGGAATCATTTTGTTGAAATCAATTAATCTAATCATGCCTCAACT
     normal: TAAGCCAAAGCTAGATCACCGGAGTTTAGGCAAATGGGTACGAGAGAAAAAGGTTTTGAA  -872

             D  K  F  T  Y  F  S  Q  F  F  W  L  C  L  F  F  F  T  F  Y   24
             GGATAAAATTCACTTATTTTTCACAATTCTTCTGGTTATGCCTTTTCTTCTTTACTTTCTA
             ATGCATCTTCCGAAACCAGATAATCAACCGAAGGAGATTCCCCTGCAAAAAGGAGATGGC  -812

             I  F  I  C  N  D  G  D  G  V  L  G  I  S  R  I  L  K  L  W   44
             TATTTTCATATGCAATGATGGAGATGGAGTACTTGGGATCAGCAGAATTCTAAAACTATG
             TCTAGTTTCATACTAATCTTCGATCTTCGAGATTCCCCCACGGAGCGGTAAGGCATCCCA  -752

             N  Q  L  L  S  H  R  G  K  T  L  L  S  K  G  R  L  G  K  N   64
             GAACCAACTGCTTTCACACCGGGGGAAGACCCTCCTGAGCAAGGGAAGGCTTGGAAAAAA
             AGGAGCGAAGCGGCTCAAAGGATTGGCAATGAAGCTCAGTCTGGTCTGAGGGGATGGGCT  -692

             R  S  S  D  S  S  R  F  E  V  S  A  L  A  A  H  Y  F  I  I   84
             TCGTAGTTCAGATTCAAGTCGGTTCGAGGTATCAGCGTTGGCCGCCCATTATTTTATCAT
             AGGCTACTCTCCCCATACATAGGAAGAGGGGGAAGTCAAGTACCTAGATCCAAGATCACG  -632

             F  V  V  P  K  L  G  Q  F  S  T  L  Y  I  I  F  F  V  C  W   104
             TTTCGTGGTCCCAAAATTGGGACAGTTTTCTACATTATATATAATTTTTTTTGTTTGTTG
             ATATCCGCTTTGGTGGTAATATCCGGCATAAAAGAAAGCACCAAAGCAAACAAAGTCGAT  -572
             -565
             G  *                                                          105
             GGGTTGAAATGGGGGGGTATTAGGAAATGAAATTTGTCATTTCGGCGTCGGACCAGATGGC
             TCTTTGTTGAGTCAACTGGCAACTGGGATTGCAGCGGATCTAGTGGTTCTCCTATTCTAT  -512

             GTTCCGGCCCCCAGCGCTGGATCTCAACGAGCGCCCGCCTCTGCATCTTTTGTACGCGGA
             GGTCTTGCTGCTGAGAGAGTAAGAATAGAAAGCAGAAGCTCGCCCGCGGGCAAAGACAAG  -452

             TGTTGAGAGTTCCGACTCTCAACAAGCGCGAAATAATGACATGTACGCGCATCTTAGGCG
             GGATGATTCTTCTAAGATACGAACTTGCGGAAGGAATGAAAAGGTATTCGACTAAGACGG  -392
```

Fig. 16. Nucleotide and deduced-amino acid sequences of the *atp6* loci from the *pol* cms and the normal mitochondrial genome. The *pol* cms sequence (top row) is aligned with that from normal cytoplasm (bottom row). Where the two are identical, only the normal sequence is shown. The first base of the *atp6* initiation codon ATG is numbered as +1. Amino acids are shown above (*pol-urf*) and below (*atp6*) the nucleotide sequence and are numbered relative to the translation initiation codon. The homologous region to normal radish *atp6* locus is represented by *arrows* (below the sequence for normal cytoplasm). The truncated *trnfM* gene is *underlined with a dashed line*. The sequences nearly identical to the conserved plant mitochondrial promoter are *boxed*.

```
CGTACAGGAGATCACCCAAAAACTAGAGGGTGAGCGCGATATCGTGCGGCGTCAAGCCTC
GGACGGGTATTCCCGAAGATGCTTGACCTAAGAGATAGAGACGAGAACGAGACTAAGAAA    -332

CTGGATATAATGAAATGGGAGGTCAGAAGCCTTCAGGAGCACTTTCGGATCTTTCGGCAC
ATCAGATATTGGAGAGGAAAGAAGCAGTGCAAGTAGAGTAGAGCAGCTTGGTAGCTCGCA    -272

CTTGATCGTCTGCGAGATTCGCGAAGAGCCAAGGTGAACGAGATCCTTGATCTCTTTCGC
AGGAGCGAAACGGCTGCTTGACCAACGGGAAGAAGCCAACTCCGTTCCTCCAGCTTCGCG    -212

GA                               -181
TAGAGAAGCTAGGTTCCTCCTTGATGTCGTAGGTTCAAATCCTATCTCCGCACTAAGTAA    -152

GGGTTTCATTCTGCATCACTCTCCCCGTCGTTCTCGACCTCGCAAGGTTTTTGAAGCGGC    -92

                              A
CGAAGCGGGAAGTGACAATACCGCTTTTCTTCAGCACATTTTGGATGATTTGAGCGAAAA    -32
                            1
CGGAGTACAAAGTTCAGCCTTTAAGGAGGCTATGAATCAAATAGGGCTGGTGGCGCAGTC     29
                      atp6: M  N  Q  I  G  L  V  A  Q  S          10

CCCACTTGACCAATTTGAGATTGTCCCATTGATTCCTATGAATATCGGAAACTTCTATTT     89
 P  L  D  Q  F  E  I  V  P  L  I  P  M  N  I  G  N  F  Y  F       30

CTCATTCACAAATCCATCTTTGTTCATGCTGCTAACTCTGAGTTTTTTCCTACTTCTGAT    149
 S  F  T  N  P  S  L  F  M  L  L  T  L  S  F  F  L  L  L  I       50

TCATTTTATTACTAAAAAGGGAGGAGGAAACTTAGTCCCAAATGCTTGGCAATCCTTGGT    209
 H  F  I  T  K  K  G  G  G  N  L  V  P  N  A  W  Q  S  L  V       70

AGAGCTTCTTTATGATTTCGTGCTGAACCTGGTAAAGGAACAAATAGGTGGTCTTTCCGG    269
 E  L  L  Y  D  F  V  L  N  L  V  K  E  Q  I  G  G  L  S  G       90

AAATGTGAAACAAATGTTTTTTCCCTTGCATCTTGGTCACTTTTCTTTTTTTGTTATTTTG    329
 N  V  K  Q  M  F  F  P  C  I  L  V  T  F  L  F  L  L  F  C      110

TAATCTTCAGGGTATGATACCTTATAGCTTCACAGTGACAAGTCATTTTCTCATTACTTT    389
 N  L  Q  G  M  I  P  Y  S  F  T  V  T  S  H  F  L  I  T  L      130

GGCTCTCTCATTTTCTATTTTTATTGGCATTACTATAGTGGGATTTCAAAGACATGGGCT    449
 A  L  S  F  S  I  F  I  G  I  T  I  V  G  F  Q  R  H  G  L      150

TCATTTTTTCAGCTTTTTATTACCCGCAGGAGTCCCACTGCCGTTAGCACCTTTTTTAGT    509
 H  F  F  S  F  L  L  P  A  G  V  P  L  P  L  A  P  F  L  V      170

ACTCCTTGAGCTAATTTCTTATTGTTTTCGCGCATTAAGCTTAGGAATACGTTTATTTGC    569
 L  L  E  L  I  S  Y  C  F  R  A  L  S  L  G  I  R  L  F  A      190

TAATATGATGGCCGGTCATAGTTTAGTAAAGATTTTAAGTGGGTTCGCTTGGACTATGCT    629
 N  M  M  A  G  H  S  L  V  K  I  L  S  G  F  A  W  T  M  L      210

ATGTATGAATGAGATTTTCTATTTTATAGGGGCTCTTGGTCCTTTATTTATAGTTCTTGC    689
 C  M  N  E  I  F  Y  F  I  G  A  L  G  P  L  F  I  V  L  A      230

ATTAACCGGTCTGGAATTAGGTGTAGCTATATTACAAGCTTATGTTTTTACGATCTTAAT    749
 L  T  G  L  E  L  G  V  A  I  L  Q  A  Y  V  F  T  I  L  I      250
```

Fig. 16 (continued).

```
                                  786
CTGTATTTACTTGAATGATGCTATAAATCTCCATTAAAGTTCTTCTTTCTTTTATTTAGA  809
    C  I  Y  L  N  D  A  I  N  L  H  *                         261

TTTATAATTGAACAAAAGCGAGGGATGAGAGTAGTGTTATTTAGAGCAGTTACACAGCCC  869
                   887
CTCTCCTTGCAGTCGAGTGACTTCGCCCCTGAATGTCTTAGATAGCTGTAAGTGAAAGAA  929
              ◄───┘
GGGTACTAAGTAGCTGGGAATGCGGCTAGCTAGTACTTACTTGTTTGTACTCCCCAGAAG  989

CTCCAAGCCTTAACTACAATCTTCTTCGGTGCTCTTTTTTTCTTTTTAGAAAGCTTACCG 1049

GGGCTTGAGAGTCTTTATATTTATAAATGGATACACGGGTTAGACCTTCTTCTTTTCATT 1109

CTTGGTCTTATTGTTTTCGTTGAGAATGAGAAGTCATAGAAAAAAGGTCTTAGTCCTAAC 1169

TCGCACAGAGAAGGATTCTAAAAAGCAAAAGAAGCGTGAGTCAAATTAATTGATATGGAG 1229

TTGATTTCCGAGGGAGGGAAAGGCAAGTAGTCAGAAGCAGGAAGCATAGGAAGCAGCAGT 1289

AAAAGCAGTCCGAAAAGCAGTGAAGGATGGCAAGGGGAGAATAAAGACTATGAATGAATG 1349

TTGAATGGG 1358
```

Fig. 16 (continued).

termination codon are highly conserved (98 % identical) between the normal rapeseed and normal radish *atp6* genes. Downstream from +888 there is no further sequence homology. This lack of homology differed from what was observed in comparison with the *atp6* gene from Ogura cms radish. Compared to the Ogura cms *atp6* gene, a 575 bp sequence beginning from the termination codon in normal rapeseed *atp6* (from position +784 to +1358) has 99.6 % homology. In the normal radish mitochondrial genome, the homologous sequence is located on another part of the genome 60 kb from the *atp6* locus (Makaroff et al. 1989). These results indicate that extensive rearrangements have occurred at the *atp6* loci of *Brassica*. Unexpectedly, the mitochondrial genome of Ogura cms radish might be more closely related to that of normal rapeseed than to that of normal radish, because it is more likely that the 575 bp sequence shared by Ogura cms radish and rapeseed in the 3'-flanking region of the *atp6* locus was present in the common ancestor of the genus and was subsequently rearranged in normal radish; rather than presumption that the same rearrangements have occurred independently in rapeseed and Ogura cms

radish. These results also support the findings that plant mtDNA has evolved rapidly in structure, but slowly in sequence, as described previously by Palmer and Herbon (1988).

Sequence analysis of the pol cms atp6 gene and an associated 105-amino acid ORF

Nucleotide sequence analysis of pBNP3 revealed a complete *atp6* gene having an ORF of 783 nucleotides which could encode a polypeptide of 261 amino acids (Fig. 16). The *atp6* coding region (783 bp), 3'-flanking sequence (575 bp), and part of the 5'-flanking sequence (211 bp) are highly conserved (99.7 % identical) between the *pol* cms and normal *atp6* genes. These results indicated that the *pol* cms *atp6* gene was intact and probably functional.

The differences in the *atp6* loci observed in the physical mapping of the two genomic fragments were detected in the upstream region beginning from nucleotide -212, after which the sequences diverge completely. The region spanning from -882 to -565 in *pol* cms *atp6* harbors an open reading frame that has the capacity to encode 105-amino acid polypeptide (12270-Da). This ORF was named *pol-urf* (unidentified reading frame of *pol* cms mitochondria). This novel ORF showed a partial sequence homology to the ORFB gene associated with the *Oenothera* mitochondrial *coxIII* gene (Hiesel et al. 1987), beginning in *pol-urf* at position -118 in the 5'-flanking region and ending at position +175 bp in the open reading frame (Fig. 17). However, the following downstream coding sequence showed no homology with the *Oenothera* ORFB gene, or any other reported sequence. Neither ORFB nor *pol-urf* show a preference for U in the third position of their codons, a common feature of plant mitochondrial genes; U is found in 33 % of all codons in the third position of the *pol-urf* gene (40.5 % in *atp6*). The absence of a typical plant mitochondrial codon usage suggested that ORFB and *pol-urf* are not originally mitochondrial genes despite their mitochondrial location.

The 177 bp sequence block adjacent to the 5'-end of the *pol-urf* also has sequence

```
A pol-urf:  GATCGCTACGCTGCCGGGCTGCCCCCGCGGGATCAAACTATCAATCTCATAAGA..GAAG  -1035
   ORFB:  -TAT-TGTTC-GCGTC-TTCTATGTTTGTCGC---G-A----G-TA-TACG--CC-GGC
 ORF105:  TC--T---AC--A-AACT--TA-TAAA-TA-A----G-.----------------..----

          AAATCTCTATGC.CCCCTGTTCTTGGTTTTCTCCCATGC.TTTTGTTGGTCAACAACCAA   -977
          G--GG-T-G---AT-AAA-G----TT-CCCA-G-A----A--CC--------------C
          ------------.-------------------------.--------------------

          CCACAACTTTCTAT..AGTTCTTCACTACTCCTAGAGGCTTGACGGAGTGAAGCTGTCTG   -919
          AGCTCT--A--A--AAG-A-----------A--C----------A---A----------
          ---------------..----------------------.T-CT-A------------
                                      -882
          GAGGGAATCATTTTGTTGAAATCAATTAATCTAATCATGCCTCAACTGGATAAATTCACT   -859
                                       pol-urf: M  P  Q  L  D  K  F  T        8
          --------T--.....----TC----.....--G-GA--------------------T---
                                         ORFB: *  *  *  *  *  *  *  *
          -----.....A--------------------C--.A----GA-G-A-CTT-C----TGA-
                                       ORF105: *  R  R  I  F  G  *  D

          TATTTTTCACAATTCTTCTGGTTATGCCTTTTCTTCTTTACTTTCTATATTTTCATATGC   -799
             Y  F  S  Q  F  F  W  L  C  L  F  F  F  T  F  Y  I  F  I  C     28
          -----CA----------------C----------C----------------CC-------
             *  *  T  *  *  *  *  S  *  *  *  L  *  *  *  *  *  P  *  *
          G-AAC-AGC-TTAAT-CAA-T-CCA-TGA-ACA-AT-C--GAACTGCG-G-CAATCCACG
             E  T  *  L  N  S  S  S  S  D  T  Y  S  R  T  A  S  Q  S  T

          AATGATGGAGATGGAGTACTTGGGATCAGCAGAATTCTAAAACTATGGAACCAACTGCTT   -739
             N  D  G  D  G  V  L  G  I  S  R  I  L  K  L  W  N  Q  L  L     48
          ----------------------------------C------C--------------
             *  *  *  *  *  *  *  *  *  *  *  *  *  *  R  *  *  *  *
          -CGAC-ATTA-C-ATTATAG-TT--AATCA--TTCCAGTG-T-C--ATTCTAG-ACTGCG
             T  T  I  N  D  Y  S  L  K  *  S  S  S  D  P  Y  S  R  T  A
                                      -708
          TCACACCGGGGGAAGACCCTCCTGAGCAAGGGAAGGCTTGGAAAAAATCGTAGTTCAGAT   -679
             S  H  R  G  K  T  L  L  S  K  G  R  L  G  K  N  R  S  S  D     68
          -----------T----A-A----CC------ACCCAAACA-TTTGG-AGAACTC-TGAGA
             *  *  *  *  *  N  I  *  R  *  D  P  N  S  L  E  E  L  L  R
          AGT--ATCCACT--CTTTTCTAGTG-TC-AC-TCC-GAATAC-GGATG-T--TGCT--TG
             *  Q  S  T  N  F  S  S  G  Q  R  P  E  Y  R  M  L  M  L  V
```

Fig. 17. **A** An alignment of the nucleotide sequences around the *pol-urf* gene. The nucleotide sequence around the *pol-urf* gene (top) is shown aligned with the *Oenothera* ORFB (middle) and Ogura radish ORF105 (bottom) sequences. Nucleotides of *pol-urf* are numbered as in Fig. 16; identical bases are shown as a -. Gaps (indicated by •) have been introduced only in the 5'-flanking region of the *pol-urf* gene in order to achieve the best alignment. The deduced amino acids are shown below each respective nucleotide sequence; identical amino acids are represented by an *asterisk*. The 14-nucleotide sequence nearly identical to the consensus sequence of the putative plant mitochondrial promoter is *boxed*. **B** A schematic drawing to indicate the alignment of nucleotide sequences around the *pol-urf* gene. Open reading frames are represented by a *box*; *open boxes* represent homologous regions between *pol-urf* and ORFB, *other boxes* represent non-homologous regions. Homologous 5'-flanking regions are shown as a *filled bar*.

```
TCAAGTCGGTTCGAGGTATCAGCGTTGGCCGCCCATTATTTTATCATTTTCGTGGTCCCA    -619
 S  S  R  F  E  V  S  A  L  A  A  H  Y  F  I  I  F  V  V  P     88
AA-G--TTTAG-ACC-GTGT-T-C-ATATGTA-TC-AG---AT--GAGG-ATCCCAATGG
 K  G  F  S  T  G  V  S  Y  M  Y  S  S  L  F  E  V  S  Q  W
GTC-AG--TCCG-TTC-CAGGAT-C-TATGCTAGTGGTCAAGCGTCCGG-AC-CAG-ATG
 V  K  R  P  V  L  R  M  *  M  L  V  V  K  R  P  V  L  S  M
                                 -565
AAATTGGGACAGTTTTCTACATTATATATAATTTTTTTTGTTTGTTGGGGTTGAAATGGG    -559
 K  L  G  Q  F  S  T  L  Y  I  I  F  F  V  C  W  G  +++         105
TGTAA--CTGTCGAC-TATTGGG-A-A-GG-AGAAAA-CAC--TGATATC---TTTC--A
 C  K  A  V  D  L  L  G  K  R  K  K  I  T  L  I  S  C  F  G
CTTA--CT-GT-GGA-T-TTGAAGA--CA-TCCAGG-C--AA-CC-ACC-A---GCGA-T
 L  M  L  V  G  F  L  K  N  Q  S  R  S  E  S  Y  R  +++
```


B

pol-urf —177bp / 1 / 53 / 105 a.a.

ORFB —118bp / 1 / 53 / 159 a.a.

ORF105 —177bp / 1 / 105 a.a.
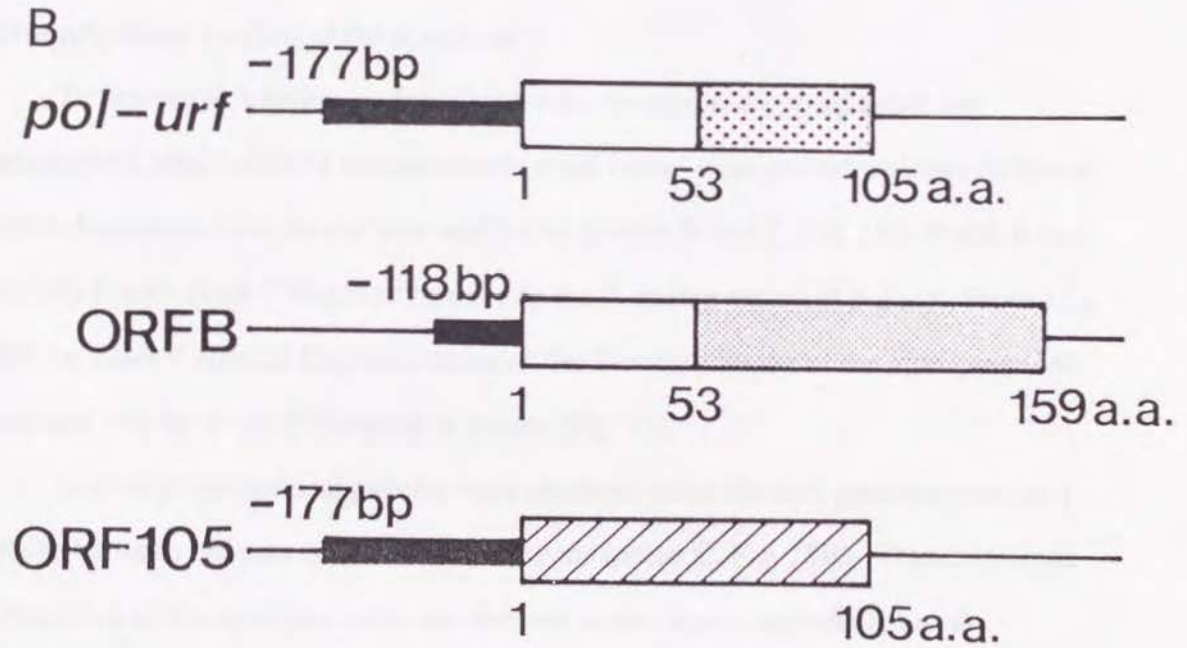
Fig. 17 (continued).

homology (91 %) with the 5'-flanking region of the radish ORF105 sequence which is

associated with the *atp6* gene in Ogura cms radish (Makaroff et al. 1989). The 14 bp se-

quence AATCTCATAAGAGA located at the 5'-end of this sequence block (from -1050 to

-1037, Fig. 17) is nearly identical to the consensus sequence of the putative plant

mitochondrial promoter (Young et al. 1986). If this 14 bp sequence works as a promoter,

then ORF105 and *pol-urf* may both be transcribed in the same manner, for example specifically in male-sterile cytoplasm. Much (118 bp) of this sequence block is homologous to the 5'-flanking region of *Oenothera* ORFB (described above). Further work is necessary to determine what role this 177 bp sequence block plays in plant mtRNA structure and/or function. The coding sequence showed no homology with the radish ORF105 gene (Fig. 17), and the structure of a truncated gene fused to an unidentified sequence suggested that *pol-urf* might have been generated through a series of duplication and rearrangement events.

Transcriptional analysis of the atp6 locus

To determine whether, and to what levels, the *atp6* and *pol-urf* genes are transcribed, total mtRNAs extracted from green leaves were probed with two different DNA fragments from the *pol* cms *atp6* locus (probes B and C, Fig. 18). Probe B is a 415 bp *Eco*RI-*Eco*RV fragment containing the 3'-coding region of *pol-urf*. Probe C, a 897 bp *Eco*RV-*Hin*dIII fragment, contains the 5'-coding region of the *atp6* gene (546 bp) and 351 bp of the 5'-flanking sequence (Fig. 15).

Altered transcriptional patterns were observed when the *atp6* gene was used as a probe between *pol* cms and normal cytoplasms (probe C, Fig. 18B). Transcriptional alterations of the *atp6* gene were also detected in the Ogura male-sterile radish mitochondria (Makaroff and Palmer 1988). In rapeseed, one highly abundant transcript [1100 nucleotides (nt)] was observed in mtRNA from normal cytoplasm, while two abundant transcripts (2100 and 1100 nt) were evident in *pol* cms cytoplasm. These results of northern hybridizations are similar to those of Witt et al. (1991), although several minor transcripts were detected in their results in addition to the major transcripts. Witt et al. (1991) carried out the northern analysis of *pol* cms and normal mtRNA using 12 mitochondrial gene probes, and showed that only hybridization to *atp6* led to different

71
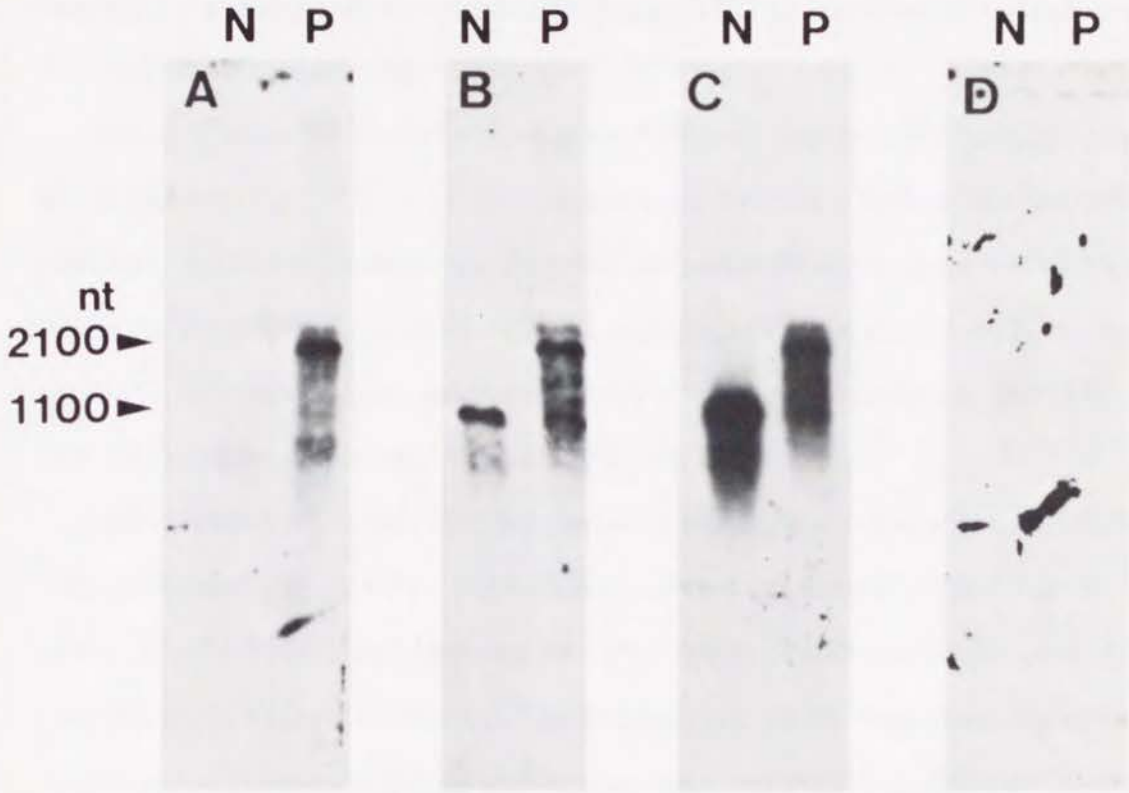
Fig. 18. Transcriptional patterns of the *atp6* and *pol-urf* genes. Transcriptional patterns observed when mtRNAs from *pol* cms (P) and normal (N) cytoplasm were probed with probes B and C (Fig. 15), in panels A and B, respectively. Filters were probed with strand-specific probe F', corresponding to the anti-sense RNA (panel C) and probe F, the sense RNA, which is the complement of the probe in panel C (panel D). Panel D was obtained by a five times longer exposure than panel C.

patterns between *pol* cms and normal lines. They also reported that the large major transcript (2100 nt in our data, 2000 nt in theirs) is influenced by the nuclear background, indicating the presence of restorer genes. When the 3'-portion of the *pol-urf* (probe B) was used as a probe, only one abundant transcript (2100 nt) was detected and then only from *pol* cms cytoplasm (Fig. 18A).

Probe F', corresponding to the anti-sense RNA, revealed the same transcript patterns as did probe C (Fig. 18C). A 1100 nt transcript was detected in both *pol* cms and normal mtRNAs. A 2100 nt transcript was present in *pol* cms mtRNA, not in normal mtRNA. When the sense RNA from the same region was used as a probe in northern hybridizations (probe F), no hybridization signals were observed for any of *pol* cms and normal mtRNAs even upon prolonged exposure (Fig. 18D).

No hybridization signals were detected when probing *pol* and normal mtRNA blots with a *Xba*I-*Sal*I fragment covering a further upstream sequence of *pol-urf* (Fig. 15, probe D) and a *Hin*dIII-*Sac*I fragment covering a further downstream sequence of *atp6* (Fig. 15, probe E) (data not shown). These results, and the presence of sequences similar to the putative promoter sequence of other plant mitochondrial genes, indicated that the abundant 1100 nt RNA represents the transcript of the *atp6* gene, while the 2100 nt transcript from *pol* cms mitochondria may be a co-transcribed RNA consisting of both *pol-urf* and *atp6* sequences.

The cms trait has been associated with a number of mitochondrial chimeric genes and pseudogenes, including maize *urf-13T* (Dewey et al. 1986), sorghum *coxI* (Bailey-Serres et al. 1986), petunia *Pcf* (Young and Hanson 1987), and rice *urf-rmc* (Kadowaki et al. 1990). The *pol-urf* reading frame also has the structure of a chimeric gene (Fig. 17). Moreover, it is associated with one of only three or four unique restriction fragments noted when comparing the *pol* cms mtDNA with that of normal rapeseed (Fig. 12), and its postulated transcript is altered by nuclear restoring-genes (Witt et al. 1991).

Therefore, *pol-urf* is a candidate that contributes to the defect in male fertility. Its possible translation product and observed transcription are consistent with two postulated models for an explanation of chimeric genes and their role in the cms trait. First, though no translation product for *pol-urf* has yet been identified, it would be expected to have an amino-terminus homologous to the *Oenothera* ORFB protein, since the first 53 amino acids of the two peptides are 91 % homologous (Fig. 17). Because ORFB is well conserved among sunflower and several other plant mitochondrial genomes (Quagliariello et al. 1990), it would appear to play a vital role in mitochondrial function. The possible *pol-urf* protein, a fusion of a homologue to ORFB and an unknown peptide, might thus act as an antagonist to the functional ORFB in the mitochondria. Second, the presence of the 2100 nt transcript may interfere with normal *atp6* mRNA function either by limiting the availability of the 1100 nt *atp6* mRNA by necessitating a processing step or interfering with normal transcription. Makaroff and Palmer (1988) also reported transcriptional differences for the *atpA* locus between sterile and restored Ogura radish. But their further work demonstrated that transcriptional alteration of *atpA* was not associated with sterile or restored states (Makaroff et al. 1990). Therefore, additional work is required for more detailed characteristic of the structure of 2100 nt transcript and the relationship between the cms trait and the presence of this transcript. These and other studies of *pol-urf* could help in understanding the differences between *pol* cms and normal cytoplasm, and so provide certain clues necessary for the clarification of the cms-inducing mechanism.

Chapter 6. Origin of *pol-urf* gene associated with *pol* cms mitochondrial genome

Introduction

Recombination event, involving the repeated DNA elements dispersed in the mitochondrial genome, is considered to be common contributor to the gene mutations seen among higher plants. Mutant genes are often found in the mitochondria of cms plants.

Among these are cases where recombination events have resulted in (1) the alteration of regulatory sequences [e. g., *atpA* in cms sunflower (Siculella and Palmer 1988), *atpA* and *coxI* in cms radish (Makaroff et al. 1990, 1991)], (2) the alteration of existing mitochondrial genes, leading to the synthesis of a modified protein [e. g., *coxI* in cms sorghum (Bailey-Serres et al. 1986), *atp6* in cms rice (Kadowaki et al. 1990), *coxII* in cms sugar beet (Senda et al. 1991)], and (3) the generation of novel chimeric ORFs encoding variant polypeptides [e. g., *T-urf13* in cms T-maize (Dewey et al. 1986), *Pcf* in cms petunia (Young and Hanson 1987), ORF105 in cms radish (Makaroff et al. 1989), *orf256* in cms wheat (Rathburn and Hedgooth 1991)]. These genes are postulated to be associated with the cms trait, although no sequence homology exists among them. However, all these genes have a similar chimeric gene structure constructed by the intra- and/or intermolecular recombination.

The author has also found the novel chimeric gene, *pol-urf* associated with *pol* cms cytoplasm of rapeseed and co-transcribed with the *atp6* gene. *Pol-urf* might have originated by recombinations between portions of the flanking and/or coding regions of the ORFB gene associated with *Oenothera* mitochondrial *coxIII* gene (Hiesel et al. 1987) and unidentified sequence. To investigate this possibility, the *pol-urf* gene was further examined and its origin determined.

Materials and Methods

Isolation of nucleic acids and Southern and northern blot analyses

MtDNA was extracted as described previously. MtRNA was isolated by the modification procedure of Stern and Newton (1986) as described previously. Restricted mtDNAs or mtRNAs separated by agarose gel electrophoresis were transferred to a nylon membrane filter (Hybond N, Amersham, UK) by a modification of Southern (1980) as previously described. Hybridization was carried out as described previously.

Sequence determination and analysis

The dideoxynucleotide chain-termination method of Sanger et al. (1977) was used for DNA sequencing as described previously.

Results and Discussion

Identification of homologous sequences to pol-urf

At first, in order to determine where the *pol-urf* sequence resides on the normal mitochondrial genome, Southern blot analysis was performed. Two DNA fragments containing the 5'-half or 3'-half of the *pol-urf* sequence (probes A, a 219 bp *Hinc*II-*Eco*RI fragment, and B, a 415 bp *Eco*RI-*Eco*RV fragment, respectively, Fig. 15) were used to probe Southern blots containing *Eco*RI, *Hind*III, and *Kpn*I-digests of *pol* cms and normal mtDNA (Fig. 19). The 3'-half, probe B, hybridized only to the same fragments containing the *atp6* gene from both *pol* cms and normal mtDNAs, indicating that this sequence is not repeated in the mitochondrial genome.

However, hybridization experiments with the 5'-half (probe A) showed more complicated patterns for both *pol* cms and normal mtDNAs. As shown in Fig. 19, three fragments, 3.6, 3.1 and 1.3 kb, were found in *Hind*III-digested *pol* mtDNA and two fragments, 3.1 and 1.3 kb, in *Hind*III-digested normal mtDNA. *Pol-urf* sequence was
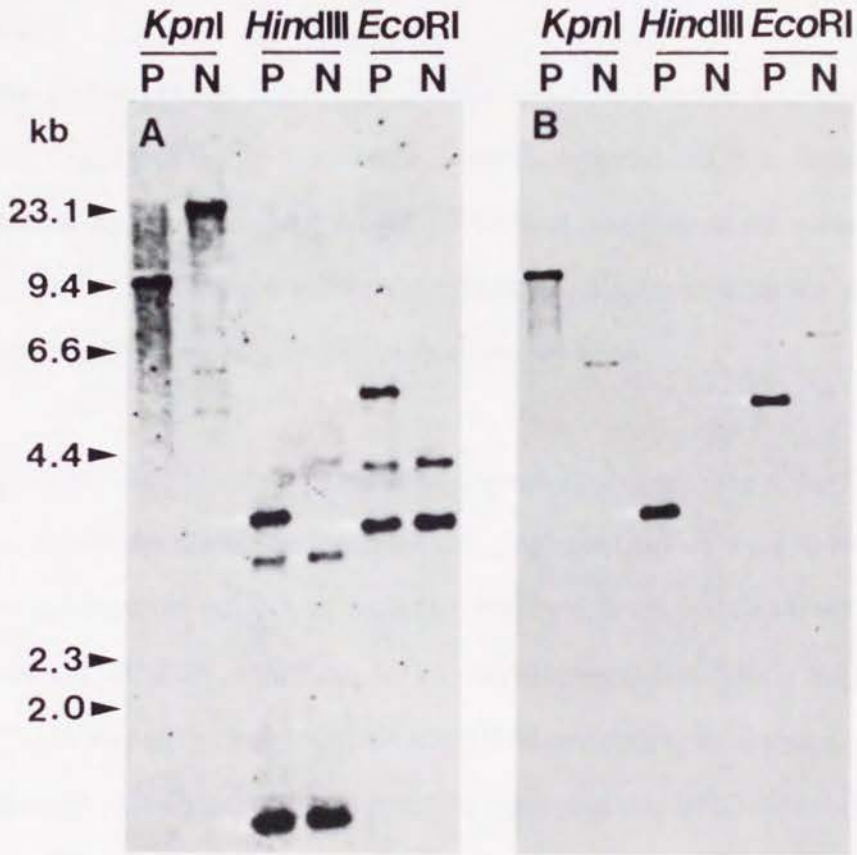
Fig. 19. Detection of homologies with the *pol-urf* gene in *pol* cms (P) and normal (N) mtDNA. Probes used for hybridizations to the filters shown in panels A and B were probe A and probe B (Fig. 15), respectively.

located in the 3.6 kb *Hind*III fragment of *pol* mtDNA. Two fragments, 3.1 and 1.3 kb, were common to both mtDNAs. There was the difference in stoichiometry of these two fragments, hybridization signal of 3.1 kb fragment was faint compared with that of 1.3 kb fragment.

These data suggested that the homologous sequence to probe A was present about three times in *pol* cms mtDNA and twice in normal rapeseed mtDNA. Because plant mitochondrial genomes are characterized by frequent homologous recombination events (Lonsdale et al. 1984; Palmer and Shields 1984), this dispersed sequence may be involved in such events, and may explain the origin of *pol-urf* gene.

Cloning and sequence analysis of the homologous sequences to the pol-urf

Two clones containing the sequence homologous to *pol-urf* were isolated by colony hybridization from the mtDNA library of normal cytoplasm; pBNI214, which has 6.2 kb *Pst*I insert and pBNI309, which has 1.3 kb *Hind*III insert. Restriction mapping revealed that pBNI214 was equivalent to 3.1 kb *Hind*III fragment described above.

The nucleotide sequence of the homologous region on pBNI214 was determined (Fig. 20). This sequence contains a 132 bp sequence which shows perfect homology to 5'-flanking region of *pol-urf* (nucleotides -170 to -39). This 132 bp sequence is also present in 5'-flanking region of ORF105 of Ogura cms radish mitochondria (Makaroff et al. 1989) as described in Chapter 5. Much of this sequence (85 bp) is homologous to common sequence to the 5'- and 3'-flanking regions of tobacco *atp6* and *atp9*, respectively (Bland et al. 1986, 1987), the 5'-flanking regions of *Oenothera atp6*, *coxI* and *coxIII* (Schuster and Brennicke 1987; Hiesel et al. 1987), and the 5'-flanking regions of cms radish *atpA* (Makaroff et al. 1990) (Fig. 20). The *Oenothera coxI*- and *coxIII*-linked copies of the repeat are part of a 657 bp sequence that has been implicated in both promoter function and mtDNA rearrangements (Hiesel et al. 1987). Further work is

necessary to determine what role, if any, this sequence, which is found in flanking regions of several mitochondrial genes, actually plays in the transcription of plant mitochondrial gene, and mtRNA structure and its function.

```
1) CGGGCTGCCC CCGCGGATCA AACTATCAAT CTCATAAGAG AAGAAATCTC TATGCCCCCT -139
2) AGCCTTGGCT TTGGCATTTC CTCGC----- ---------- ---------- ----------

3) CAACTCTTAC TAAACTAAAC AAAGT----- ---------- ---------- ----------


1) GTTCTTGGTT TTCTCCCATG CTTT.TGTTG GTCAACAACC AACCACAACT TTCTAT..AG -79
2) ---------- ---------- ----.----- ---------- ---------- ------..--

3) ---------- ---------- ----.----- ---------- ---------- ------..--
4) CCATCTTT-- ---------- ----CC---- ---------- -----A-GTG C-----..-C
5) AAAAAAAAGG CCGC------ ----C----- ---------- -CAGCTCT-- A--A--AAG-
6) GGTCTTTTTC CCATG-A--- -A--CC---- ---------- -CAGCTCT-- A--A--AAG-


1) TTCTTCACTA CTCCTAGAGG CTTGACGGAG TGAAGCTGTC TGGAGGGAAT CATTTTGTTG -19
2) ---------- ---------- ---------- ---------- CCCTGTCATT CAGTGAAAAT

3) ---------- ---------- ---TC-T.-A ---------- TGGAGGGATT TGTTGAAATC
4) ---------- ---G--C--- ---------- -T-------A TTGAGGGAAT CTTTTTATCT
5) A--------- ---A--C--- -------A-- -A-------- TGGAGGGAAT TATTTGATCT
6) A--------- ---A--C--- -------A-- -A-------- TGGAGGGAAT TATTTGATCT
```

Fig. 20. Comparison of homologous nucleotide sequences to the 5'-flanking region of *pol-urf* gene. The nucleotide sequences from the homologous sequence of pBNI214 (2), the 5'-flanking of Ogura ORF105 (3), the 5'-flank of tobacco *atp6* and 3'-flank of *atp9* (4), the 5'-flank of *Oenothera atp6* (5), and ORFB and *coxI* (6) are shown aligned with the 5'-flank of *pol-urf* (1). The first base of *pol-urf* ATG codon is numbered as +1; identical bases are shown as -, missing bases as •.

ORFB sequence present in the mitochondrial genome of rapeseed

The nucleotide sequence of a 1.3 kb segment of normal rapeseed mtDNA cloned into pBNI309 is shown in Fig. 21. Two open reading frames of 474 bp (ORF474) and 159 bp (ORF159) respectively were observed in this sequence. ORF159 shows no homology to sequences in the EMBL sequence database.

```
AAGCTTCCCGGCAGTCAACCAAGAAAGACTCTTTCTCCCCTACTGAAGAAATGGAAGTCA      60

GATCTTTTTCCATACCATAACGTATATAGAATCGATTTTCTTTTCTGATCGCTAGCCTGC     120

CGGGCCGCCCCCGCGATCAAACTATCAATCTCATAAGAGAAGAAATCTCTATGCCCCCTG     180

TTCTTGGTTTTCTCCCATGCTTTTGTTGGTCAACAACCAACCACAACTTTCTATAGTTCT     240

TCACTACTCCTAGAGGCTTGACGGAGTGAAGCTGTCTGGAGGGAATCATTTTGTTGAAAT     300

CAATTAATCTAATCATGCCTCAACTGGATAAATTCACTTATTTTTCACAATTCTTCTGGT     360
               M   P   Q   L   D   K   F   T   Y   F   S   Q   F   F   W    15

TATGCCTTTTCTTCTTTACTTTCTATATTTTCATATGCAATGATGGAGATGGAGTACTTG     420
L   C   L   F   F   F   T   F   Y   I   F   I   C   N   D   G   D   G   V   L    35

GGATCAGCAGAATTCTAAAACTACGGAACCAACTGCTTTCACACCGGGGGAAGACCATCC     480
G   I   S   R   I   L   K   L   R   N   Q   L   L   S   H   R   G   K   T   I    55
                              ORF474
AGAGCAAGGACCCCAACAGTTTGGAAGATCTCTTGAGAAAAGGTTTTAGCACTGGTGTAT     540
Q   S   K   D   P   N   S   L   E   D   L   L   R   K   G   F   S   T   G   V    75

CCTATATGTATGCTAGTTTATTCGAAGTATCCCAATGGTGTAAGGCCGTCGACTTATTGG     600
S   Y   M   Y   A   S   L   F   E   V   S   Q   W   C   K   A   V   D   L   L    95

GAAAAAGGAGGAAAAATCACTTTGATCTCTTGTTTCGGAGAAATAAGTGGCTCACGAGGAA     660
G   K   R   R   K   I   T   L   I   S   C   F   G   E   I   S   G   S   R   G    115

TGGAAAGAAACATATTATATAATATATCGAAGTCCTCTCCTTCAAATACTGGAAGGTGGA     720
M   E   R   N   I   L   Y   N   I   S   K   S   S   P   S   N   T   G   R   W    135

TCACTTGTAGGAATTGTAGGAATGACATAATGCTAATCCATGTTGTACAGTGCCAAGGAA     780
I   T   C   R   N   C   R   N   D   I   M   L   I   H   V   V   Q   C   Q   G    155

GCATAAAATGATTCTTTCATTCTATAGATACCTCTGGTAGGTAAAGCACTCTACTGTGCT     840
S   I   K   *                                                             158

TTATTGAAAGTTCCCATCGCGGGGGCGAGGATACTTGCCTTCGCGGTTCGACTTTCTTTT     900

CAGGCTTGACTCATTATTTTCCGGTCCTCTCACACCCCTTTAGAGCTCTTTATGATGCCC     960

ACTGAGTAAGATTCGGGGGGCTTCCCGGGGCAGAAGCTCATTCTGAACCGCGGGAACCTTC    1020

GTCTCTTCGACACAAACGTTTTATGAAGAGGCTGATGGTGATGAGGATCCATGCGCACTC    1080
                                                         M   R   T    3

AAATAAAAGTAGCTTGCGTATTGGGTTATCACGGTGTTAGTGCTCCATTGCACCCTCATG    1140
Q   I   K   V   A   C   V   L   G   Y   H   G   V   S   A   P   L   H   P   H    23
                              ORF159
TGGAGGGTAGGATAATTTGGACTCTTTTGGAGCACTATACTCCGAAAGATCCTATCCTTC    1200
V   E   G   R   I   I   W   T   L   L   E   H   Y   T   P   K   D   P   I   L    43

CTCCTCTTCTTTCAGTCGAGACTTCCTCCTAGTGAGGTGTTGCCTATCCAGGTATGGAAG    1260
P   P   L   L   S   V   E   T   S   S   *                                  53

TATTCAATGAATACACTCTGTACCATGGGTGGATGAAGCTT                       1301
```

Fig. 21. The nucleotide and deduced amino acid sequences of the 1.3 kb *Hin*dIII fragment of pBNI309. The open reading frames are *boxed*. Deduced amino acids are shown below the nucleotide sequence. The homologous region to *pol-urf* is *underlined*.

80

On the other hand, ORF474 shows significant homology with ORFB sequences from *Oenothera* and sunflower mitochondrial genomes (Hiesel et al. 1987; Quagliariello et al. 1990, respectively). Alignment of the rapeseed ORF474 and *Oenothera* and sunflower ORFB nucleotide sequences and of their derived proteins is shown in Fig. 22. ORF474 sequence shares 88.1 % homology to both ORFB sequence, while its derived protein sequence consists of 158 amino acid residues, which is one amino acid shorter than those of *Oenothera* and sunflower. ORF474 protein sequence shows 82.8 % amino acid identity to *Oenothera* ORFB, and 81.5 % to sunflower ORFB. About a half of amino acid replacements are confined within the C-terminal 30 amino acid residues. The hydropathy profile of ORF474 polypeptide is similar to those of both ORFB polypeptides (data not shown).

Presence of ORFB homologous sequence was revealed in several plant mitochondrial genomes, including *B. oleracea* which is a closely related species of rapeseed (Qualiariello et al. 1990). The author does not have a conclusive data for judging whether ORF474 of rapeseed is actually ORFB or not, but it is possible to presume that ORF474 is ORFB in rapeseed mitochondria because ORF474 is an only homologous reading frame to ORFB. If ORFB is conserved commonly to many plant mitochondrial genomes and presumably expressed, the ORFB-encoded polypeptide appears to be of important biological function in plant mitochondria. Whether ORFB is indeed coding for a functional polypeptide needs to be shown by *in vivo* identification of the encoded polypeptide in mitochondria and by an analysis of its function.

Origin of pol-urf gene

A computer search was performed on the DNA sequence database of rapeseed mitochondrial genome, which compiled sequence data analyzed by the author, for the 3'-half of *pol-urf*. As described previously in this chapter, this sequence was not repeated in the mitochondrial genome. The computer search identified a short sequence (43 bp)

81

```
rapeseed ORF474  ATGCCTCAACTGGATAAATTCACTTATTTTTCACAATTCTTCTGGTTATGCCTTTTCTTC  60
                 M  P  Q  L  D  K  F  T  Y  F  S  Q  F  F  W  L  C  L  F  F    20
Oenothera ORFB   ........................T........CA............C..........C...
                 .  .  .  .  .  .  .  .  .  T  .  .  .  .  S  .  .  .  L
sunflower ORFB   .........................CA............C..........C..
                 .  .  .  .  .  .  .  .  .  T  .  .  .  .  S  .  .  .  L

rapeseed ORF474  TTTACTTTCTATATTTTTCATATGCAATGATGGAGATGGAGTACTTGGGATCAGCAGAATT  120
                 F  T  F  Y  I  F  I  C  N  D  G  D  G  V  L  G  I  S  R  I    40
Oenothera ORFB   ...............CC...........................................
                 .  .  .  .  .  P  .  .  .  .  .  .  .  .  .  .  .  .  .  .
sunflower ORFB   ...............GC...........................C................
                 .  .  .  .  .  A  .  .  .  .  .  .  .  .  L  .  .  .  .  .

rapeseed ORF474  CTAAAACTACGGAACCAACTGCTTTCACACCGGGGGAAGACCATCCAGAGCAAGGACCCC  180
                 L  K  L  R  N  Q  L  L  S  H  R  G  K  T  I  Q  S  K  D  P    60
Oenothera ORFB   ..C.............................T.....A......TCC...........A
                 .  .  .  .  .  .  .  .  .  .  .  .  N  .  L  R  .  .  .
sunflower ORFB   ...............T....................TAC...C.A.....G...........
                 .  .  .  .  .  .  .  .  .  .  .  T  N  N  .  R  .  .  .  .

rapeseed ORF474  AACAGTTTGGAAGATCTCTTGAGAAAAGGTTTTAGCACTGGTGTATCCTATATGTATGCT  240
                 N  S  L  E  D  L  L  R  K  G  F  S  T  G  V  S  Y  M  Y  A    80
Oenothera ORFB   ................A..................C..................CT..
                 .  .  .  .  E  .  .  .  .  .  .  .  .  .  .  .  .  .  S
sunflower ORFB   ................A..................C...C.............CT.C
                 .  .  .  .  .  I  .  .  .  .  .  .  .  .  L  .  .  .  S

rapeseed ORF474  AGTTTATTCGAAGTATCCCAATGGTGTAAGGCCGTCGACTTATTGGGAAAAAGGAGGAAA  300
                 S  L  F  E  V  S  Q  W  C  K  A  V  D  L  L  G  K  R  R  K   100
Oenothera ORFB   ............G.................T.................A....
                 .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  K  .
sunflower ORFB   ............AC..............................................G
                 .  .  .  .  D  .  .  .  .  .  .  .  .  .  .  .  .  .  .

rapeseed ORF474  ATCACTTTGATCTCTTGTTTCGGAGAAATAAGTGGCTCACGAGGAATGGAAAGAAACATA  360
                 I  T  L  I  S  C  F  G  E  I  S  G  S  R  G  M  E  R  N  I   120
Oenothera ORFB   ............A...................A.......................
                 .  .  .  .  .  .  .  .  .  .  S  .  .  .  .  .  .  .  .
sunflower ORFB   ...............................C..................G......
                 .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

rapeseed ORF474  TTATATAATATATCGAAGTCCTCTCCTTCAAATACTGGAAGGTGGATCACTTGTAGGAAT  420
                 L  Y  N  I  S  K  S  S  P  S  N  T  G  R  W  I  T  C  R  N   140
Oenothera ORFB   ..C...TTG..C..A.........ATA.AGC.C.T..TCT.ATCTTGGATGGG.GGTC.C.
                 F  .  L  .  .  .  .  .  Y  .  T  S  S  N  L  G  W  G  V  T
sunflower ORFB   ..C...TTG..C............ATA.AGC.C.T..TCC.ATCCTGGATGGG.G.TC.C.
                 F  .  L  .  .  .  .  .  Y  .  T  S  S  N  P  G  W  G  I  T

rapeseed ORF474  TGTAGGAATGACATAATGCTAATCCATGTTGTACAGTGCCAAGGAAGCATAAAATGA      477
                 C  R  N  D  I  M  L  I  H  V  V  Q  C  Q  G  S  I  K  *      158
Oenothera ORFB   ............................CC...CG...........CGTT.TTTAA
                 .  .  .  .  .  .  .  .  .  P  H  G  .  .  .  .  V  F  *
sunflower ORFB   ............................CC...CG...........CGGT.TTTAA
                 .  .  .  .  .  .  .  .  .  P  H  G  .  .  .  G  F  *
```

Fig. 22. Alignment of the nucleotide and deduced amino acid sequences of the rapeseed

ORF474 (*top*) with those of *Oenothera* (*middle*) and sunflower ORFB (*bottom*).

Identical bases and amino acid residues are represented by *dots*.

located downstream of the gene for tRNALys (*trnK*, Handa and Nakajima 1992). This
sequence showed a high homology to a part of 3'-half of *pol-urf* (Fig. 23). The computer
search also identified that this 43 bp sequence was a part of homologous sequence to

```
                          ▼
pol-urf TTGTCTGATTTTAGGTTTCTGATCGCTACGCTGCCGGGCTGCCCCCGCGGATCAAACTAT -170
        *  *   *   ***   *************  ********* ******** ***********
ORF474  TAGAATCGATTTTCTTTTCTGATCGCTAGCCTGCCGGGCCGCCCCGC-GATCAAACTAT

pol-urf CAATCTCATAAGAGAAGAAATCTCTATGCCCCCTGTTCTTGGTTTTCTCCCATGCTTTTG -110
        ************************************************************
ORF474  CAATCTCATAAGAGAAGAAATCTCTATGCCCCCTGTTCTTGGTTTTCTCCCATGCTTTTG

pol-urf TTGGTCAACAACCAACCACAACTTTCTATAGTTCTTCACTACTCCTAGAGGCTTGACGGA  -50
        ************************************************************
ORF474  TTGGTCAACAACCAACCACAACTTTCTATAGTTCTTCACTACTCCTAGAGGCTTGACGGA
                                                              1
pol-urf GTGAAGCTGTCTGGAGGGAATCATTTTGTTGAAATCAATTAATCTAATCATGCCTCAACT   11
        **************************************************  *********
ORF474  GTGAAGCTGTCTGGAGGGAATCATTTTGTTGAAATCAATTAATCTAATCATGCCTCAACT

pol-urf GGATAAATTCACTTATTTTTCACAATTCTTCTGGTTATGCCTTTTCTTCTTTACTTTCTA   71
        ************************************************************
ORF474  GGATAAATTCACTTATTTTTCACAATTCTTCTGGTTATGCCTTTTCTTCTTTACTTTCTA

pol-urf TATTTTCATATGCAATGATGGAGATGGAGTACTTGGGATCAGCAGAATTCTAAAACTATG  131
        *********************************************************** *
ORF474  TATTTTCATATGCAATGATGGAGATGGAGTACTTGGGATCAGCAGAATTCTAAAACTACG

rps3            TATGGCA---CGAAAAGGAAATCC-GATTTCGG-----TAAGACTTGGA
               *  *  **    *   ***    *** **     **     *** ******
                                                      ▼
pol-urf GAACCAACTGCTTTCACACCGGGGGAAGACCCTCCTGAGCAAGG-----GAAGGCTTGGA  186
        ***************************** *** ********    *   *  *****
ORF474  GAACCAACTGCTTTCACACCGGGGGAAGACCATCCAGAGCAAGGACCCCAACAGTTTGGA

rps3    AAAAATCGT-AGTTCAGATTCAAGTCGGTTCAGTGAGGGCGACCGCGAAAGTCACCGAAT
        ********* ********************    *      *      *      **    *
pol-urf AAAAATCGT-AGTTCAGATTCAAGTCGGTTCGAGGTATCAGCGTTGGCCGCCCATTATTT  244
        * *  ** * **      ** **      ▲
ORF474  AGATCTCTTGAGAAAAGGTT

pol-urf TATCATTTTCGTGGTCCCAAAATTGGGACAGTTTTCTACATTATATATAATTTTTTTTGT  304
                  318
pol-urf TTGTTGGGGTTGAAATGGGGGGTATTAGGAAATGAAATTTGTCATTTCGGCGTC
```

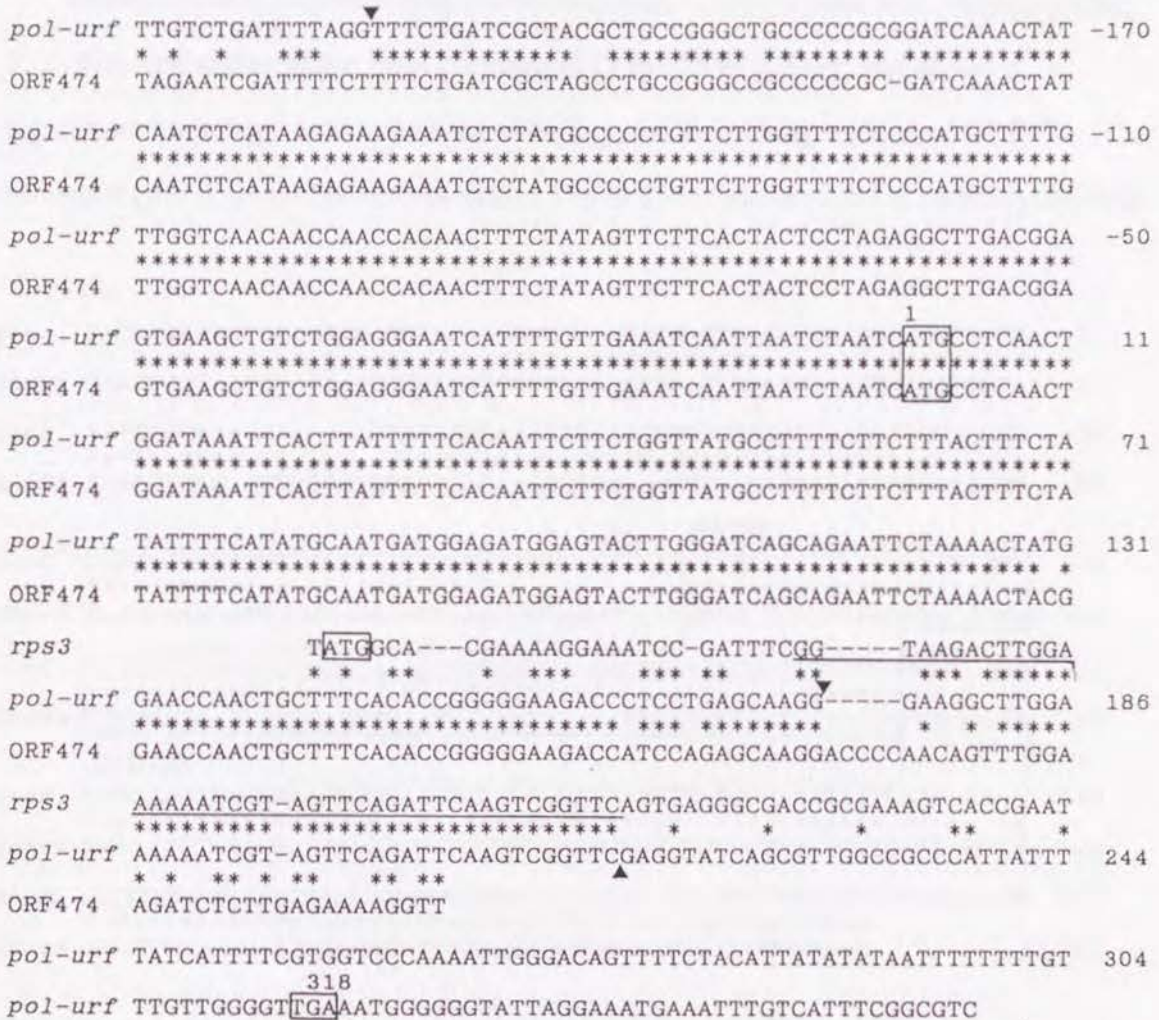Fig. 23. Sequence alignment of the *pol-urf* gene with ORF474 and *rps3* genes. The
first base of the *pol-urf* initiation codon ATG is numbered as +1. *Asterisks* indicate
matching bases. Initiation codons and termination codon are *boxed*. Putative
recombination sites are indicated by *arrowheads*. Gaps (indicated by -) have been
introduced in order to achieve the best alignment. A 43 bp sequence is *underlined*.

Oenothera mitochondrial *rps3* (ribosomal protein S3) gene (Schuster and Brennicke 1991a) (Fig. 24). This sequence homology continues for 118 bp into first intron sequence downstream of exon 1 of *rps3*. Therefore, it is certain that the region including 43 bp sequence is a part of *rps3* gene in rapeseed mitochondria.

Based on these sequence data, *pol-urf* sequence is divided into three segments (Fig. 23). The first region spans from positions -215 to 175 of *pol-urf*, and shows a significant homology with sequences of coding and 5'-flanking regions of ORF474. The second region is found between positions 176 to 216 of *pol-urf*, and is homologous with

```
rapeseed   CACTATATAGTAAAGATGCGTAGTAGCGTTCTAAGATCACTGAGGGGAAGGTTGGTCATA   -91
           *  *  **  *  *    *  *   *      ****  ************ *  *************
Oenothera  GAATTTACAATGGAAAAACTCCTGTTCGTTGTAAGATCACTGAAGAAAAGGTTGGTCATA   -97

rapeseed   AATTTGGAGA---------GTACACGGAAACTCAGACTTTCGAGAACAAATATTGGACCGG   -39
           *****  ****          ***********   ****  ******************** ***
Oenothera  AATTTTGAGAGTTTGCTTTTACACGGAAACGAAGACGTTCGAGAACAAATATTGGATCGG   -37
```

exon1

```
rapeseed   GAAGAAAAAAGGGACAGAAATCCATAAAGTCAAAAAATATGGCACGAAAAGGAAATCCGA    22
           ****  ********    *  ***     *  *       *  *    **********************************
Oenothera  GAAG-AAAAAGGGGGA-AAAAGTAAAGTCTAAGCGCATATGGCACGAAAAGGAAATCCGA    22

rapeseed   TTTCGGTAAGACTTGGAAAAAAATCGTAGTTCAGATTCAAGTCGGTTCAGTGAGGGCGACC    82
           ***************       ********************       **********  *********
Oenothera  TTTCGGTAAGACTTGATCTGAATCGTAGTTCAGATTCAAGTTGGTTCAGTGAGGGCGACC    82
```

intron

```
rapeseed   GCGAAAGTCACCGAATGGGTAAGTCTTTTCCTTCAGTTTATCCTATATTTTCAA--AAAA   140
           *********************  ******************  ***********  **       *
Oenothera  GCGAAAGTCACCGAATGGGTCAGTCTTTTCCTTCAGTTTGTCCTATATTTTGAATAGTTA   142

rapeseed   AGCGTGGGAGGACGTTGCAGATGTCCGGGACGGACACGACTTCTTCTAACGCTAGAAGAC   200
           ***********************************  *  ***************
Oenothera  AGCGTGGGAGGACGTTGCAGATGTCCGGGACGCA-ACGACTTCTTCTAAC           191
```

Fig. 24. Comparison of the nucleotide sequences of the *rps3* locus between rapeseed (*top line*) and *Oenothera* (*bottom line*). The numbering of the nucleotides starts from the predicted translation start of the *rps3* gene. Sequence homologies between the two lines are indicated by *asterisks*. A 43 bp sequence homologous to *pol-urf* is *underlined*. The *bold framed box* marks exon 1 sequence of the *rps3* gene. The *light framed* region represents the intron sequence of *rps3*.

the exon 1 coding sequence of *rps3* gene. The third region is located downstream from position 217. No significant homology to the third region can be found in any of the EMBL database sequences.

The extensive sequence homologies between *pol-urf* and the 5'-flanking and coding region of ORF474, and the exon 1 of *rps3* indicate that several recombinational events have occurred to form this fragment. Assuming the *pol-urf* sequence originated via recombination, a minimum of three recombination points (positions -215, 175 and 216 of Fig. 23) are identified.

Intragenomic rearrangements mediated by recombination repeats to generate subgenomic molecules have been observed in several higher plant mitochondrial genomes (Palmer and Shields 1984; Lonsdale et al. 1984). In addition to these regularly occurring recombination events, several gene structures indicating past rearrangements have been reported (Dewey et al. 1986; Bailey-Serres et al. 1986; Young and Hanson 1987). For example, in rice a homologous recombination event occurred within *atp6* coding region mediated by seven nucleotide sequence, and resulted in *urf-rmc* gene associated with cms-Bo cytoplasm (Kadowaki et al. 1990).

However, such a short repeat sequence cannot be found between the *pol-urf* sequence and original normal counterpart sequences. Rearrangements involving the ORF474 and *rps3* genes have most likely arisen either by nonhomologous recombination or by homologous recombination involving small areas of nucleotide homology, which cannot be found in this sequence study. The author cannot rule out either of the two alternatives based on the present data. Further studies are required to determine the molecular mechanism of intra- and/or intermolecular recombinations which caused the fusions to make the *pol-urf* sequence.

Chapter 7. RNA editing of *atp6* genes from male-sterile and normal cytoplasms

Introduction

The mRNAs of plant mitochondria have recently been shown to be modified; at specific locations, cytidine residues on the genomic sequence are converted to uridines on the mRNA, resulting in polypeptides different from those predicted by the genomic DNA (Gualberto et al. 1989; Covello and Gray 1989; Hiesel et al. 1989). The number of nucleotides altered differs, however, between plant species and also between individual genes within one mitochondrion.

This RNA editing event is essential for the correct expression of plant mitochondrial protein genes. The reasons for the existence of RNA editing in plant mitochondria is not clear, but it appears that RNA editing in plant mitochondria plays a role in the conservation of protein sequences during evolution. Therefore, it is likely that RNA editing confers functional advantages as a modulator of gene expression. The basis for the specificity of the editing system is still not clear, but recent results in the study of the editing process occurring in the mitochondria of *Leshmania tarentolae* (Blum et al. 1990) suggest that anti-sense RNAs (guide RNAs) might be involved in the RNA editing recognition process.

RNA editing could regulate gene expression, and it could be both tissue-specific and dependent on the developmental stages. The mechanism of cytoplasmic male-sterility is of agronomic importance, and the role of RNA editing is yet to be established. To investigate a correlation between RNA editing and expression of cytoplasmic male-sterility, cDNAs of rapeseed *atp6* transcripts were sequenced and the extent of RNA editing was determined in the *atp6* transcripts from both normal and *pol* cms mitochondria.

Materials and Methods

cDNA synthesis

MtDNA and mtRNA were prepared from 8-week-old plants as previously described. Total mtRNA (30 µg) was treated with 30 units of RNase-free DNase (Stratagene, CA, USA) during 30 min at 37°C. After phenol extraction and ethanol precipitation, mtRNA was mixed with 12.5 pmole of primer ATP6REV (a 27-mer oligonucleotide complementary to the 3'-end of the *atp6* mRNA, which has the sequence GGGCTGTGTAACTGCAGTAAATAACAC) in 10 µl of 0.4 M NaCl, 10 mM Pipes pH 6.4, 1 mM EDTA. After denaturation at 80°C for 5 min, the reaction mixture was incubated for 2 hr at 50°C for primer hybridization. First strand synthesis was initiated by addition of 90 µl of 20 mM Tris-HCl pH 6.9, 100 mM KCl, 5 mM $MgCl_2$, 166 µM β–NAD, 11 mM $(NH_4)_2SO_4$, 11 mM DTT, 0.55 mM of each dNTP, 36 units of RNAguard (Pharmacia, Sweden), and 200 units of M-MLV reverse transcriptase (GIBCO BRL, MD, USA).

PCR amplification

The resulting cDNAs were amplified by PCR using primers ATP6REV and ATP6FOR (a 25-mer oligonucleotide whose sequence was derived from the 5'-end of the mRNA, and which has the sequence CGGGAAGTGAGAATTCCGCTTTTCT). The primers ATP6REV and ATP6FOR contained mismatches as compared to the DNA sequence, in order to create *Pst*I and *Eco*RI sites, respectively, at the ends of the DNA fragments amplified by PCR (Fig. 25). The amplification mix contained in 50 µl, 50 mM KCl, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 20 µM of each dNTP, 10 pmole of each oligonucleotide primer, and 1.25 units of *Taq* DNA polymerase (Promega, WI, USA). Amplification was obtained by 60 cycles of the following steps: denaturation (92°C; 2 min), annealing (47°C; 2 min) and DNA polymerization (72°C; 6

87

min), followed by a final incubation at 72°C for 6 min. Amplification products were purified on CHROMA SPIN-100 Columns (Clontech, CA, USA). After double digestion with *Pst*I and *Eco*RI, amplified cDNAs were cloned into pBluescript II and sequenced. Primers ATP6INT1 and ATP6INT2 (17-mer oligonucleotides which have the sequences, ACTAAAAAGGGAGGAGG corresponding to positions 246 to 262, and TAGTCCAAGCGAACCCA complementary to positions 711 to 695, respectively) were used for internal sequence priming.

Results

Cloning of atp6 cDNAs

To synthesize and amplify *atp6* cDNA sequences, two oligonucleotides (ATP6REV and ATP6FOR) were prepared, with sequences in opposite orientation, corresponding to the extremities of the rapeseed *atp6* mRNA. Before cDNA synthesis, mtDNA had been eliminated by DNase treatment, since it acts as a contaminant of mtRNA preparations and it would be also amplified during the PCR reaction. ATP6REV, complimentary to the 3'-end of the *atp6* mRNA was used to prime cDNA synthesis. The primer extension products were subsequently amplified by PCR. The 1 kb amplified fragment was cloned into *Pst*I and *Eco*RI sites of pBluescript II vector.

The analysis of the cDNA sequence was performed in several clones, as the high error frequency of *Taq* DNA polymerase results in misincorporations of nucleotide in the PCR products. In the 25 clones sequenced, several modifications were found which were attributed to errors introduced by the *Taq* DNA polymerase. None of those modifications was found at the same position in more than one of the clones. Therefore, the author only considered the nucleotide different from that in the genomic DNA sequence which was found in more than one cDNA clone.

Editing sites of the rapeseed atp6 cDNAs

Sequencing of cDNA clones revealed only one C to U transcript editing event within the *atp6* open reading frame. The edited site and the corresponding genomic sequence with the amino acid sequence is shown in Fig. 25. This C to U editing at nucleotide 189 within the coding region resulted in Proline > Serine change (Fig. 26). The frequency of this editing event that resulted in a changed amino acid was very high; eight cDNA clones derived from normal cytoplasm were examined for this RNA editing site, and seven of the eight cDNA clones sequenced had an edited transcript (87.5 %). No modification was found in the untranslated flanking region.

Comparison of the amino acid sequence deduced from the rapeseed cDNA sequence with respective polypeptides from *Oenothera* and sorghum cDNA sequences (Schuster and Brennicke 1991b; Kempken et al. 1991, respectively) reveals strong sequence homologies between three amino acid sequences (Fig. 27). These data show that the resultant polypeptide was very similar in all three species, as has been generally observed after the editing process. As an example, homology between the core sequences of the rapeseed ATP6 protein and the *Oenothera,* and rapeseed and sorghum ATP6 proteins would increase from 85.4 % to 92.5 % and from 83.4 % to 89.7 %, respectively.

No variation of RNA editing observed between normal and pol cms cytoplasms

To explore the extent of RNA editing within *atp6* coding sequence of *pol* cms mitochondria, analogous cDNA clones were constructed from *pol* cms mtRNA. Only one RNA editing event occurred in *pol* cms transcript at the same position as in the normal transcript. The frequency of this editing event was also very high, similar as in the normal transcript: 16 of the 17 clones analyzed had an edited transcript (94.1 %). C to U editing event at position 189 is a common phenomenon in both normal and *pol* cms mitochondrial *atp6* transcripts and its frequency is similar in both cases.

```
                     •   •
                    GAATTC
  1  CGGGAAGTGACAATACCGCTTTTCTTCAGCACATTTTGGATGATTTGAGCGAAAACGGAG   60

 61  TACAAAGTTCAGCCTTTAAGGAGGCTATGAATCAAATAGGGCTGGTGGCGCAGTCCCCAC  120
                             M  N  Q  I  G  L  V  A  Q  S  P

121  TTGACCAATTTGAGATTGTCCCATTGATTCCTATGAATATCGGAAACTTCTATTTCTCAT  180
     L  D  Q  F  E  I  V  P  L  I  P  M  N  I  G  N  F  Y  F  S

                  ┌──┐
                  │ S│
                  ┌──┐
                  │TCA│
181  TCACAAAT CCA TCTTTGTTCATGCTGCTAACTCTGAGTTTTTTCCTACTTCTGATTCATT  240
     F  T  N │P│ S  L  F  M  L  L  T  L  S  F  F  L  L  I  H

241  TTATTACTAAAAAGGGAGGAGGAAACTTAGTCCCAAATGCTTGGCAATCCTTGGTAGAGC  300
     F  I  T  K  K  G  G  G  N  L  V  P  N  A  W  Q  S  L  V  E

301  TTCTTTATGATTTCGTGCTGAACCTGGTAAAGGAACAAATAGGTGGTCTTTCCGGAAATG  360
     L  L  Y  D  F  V  L  N  L  V  K  E  Q  I  G  G  L  S  G  N

361  TGAAACAAATGTTTTTCCCTTGCATCTTGGTCACTTTTCTTTTTTTGTTATTTGTAATC  420
     V  K  Q  M  F  F  P  C  I  L  V  T  F  L  F  L  L  F  C  N

421  TTCAGGGTATGATACCTTATAGCTTCACAGTGACAAGTCATTTTCTCATTACTTTGGCTC  480
     L  Q  G  M  I  P  Y  S  F  T  V  T  S  H  F  L  I  T  L  A

481  TCTCATTTTCTATTTTTATTGGCATTACTATAGTGGGATTTCAAAGACATGGGCTTCATT  540
     L  S  F  S  I  F  I  G  I  T  I  V  G  F  Q  R  H  G  L  H

541  TTTTCAGCTTTTTATTACCCGCAGGAGTCCCACTGCCGTTAGCACCTTTTTTAGTACTCC  600
     F  F  S  F  L  L  P  A  G  V  P  L  P  L  A  P  F  L  V  L

601  TTGAGCTAATTTCTTATTGTTTTCGCGCATTAAGCTTAGGAATACGTTTATTTGCTAATA  660
     L  E  L  I  S  Y  C  F  R  A  L  S  L  G  I  R  L  F  A  N

661  TGATGGCCGGTCATAGTTTAGTAAAGATTTTAAGTGGGTTCGCTTGGACTATGCTATGTA  720
     M  M  A  G  H  S  L  V  K  I  L  S  G  F  A  W  T  M  L  C

721  TGAATGAGATTTTCTATTTTATAGGGGCTCTTGGTCCTTTATTTATAGTTCTTGCATTAA  780
     M  N  E  I  F  Y  F  I  G  A  L  G  P  L  F  I  V  L  A  L

781  CCGGTCTGGAATTAGGTGTAGCTATATTACAAGCTTATGTTTTTACGATCTTAATCTGTA  840
     T  G  L  E  L  G  V  A  I  L  Q  A  Y  V  F  T  I  L  I  C

841  TTTACTTGAATGATGCTATAAATCTCCATTAAAGTTCTTCTTTCTTTTATTTAGATTTAT  900
     I  Y  L  N  D  A  I  N  L  H  *
                                      ••
                                     CTGCAG
901  AATTGAACAAAAGCGAGGGATGAGAGTAGTGTTATTTAGAGCAGTTACACAGCCC       955
```

Fig. 25. Comparison between rapeseed genomic and cDNA *atp6* sequences. The upper sequence is obtained from *atp6* cDNA clones, while the lower sequence is the corresponding genomic DNA sequence. Where the two are identical only the genomic sequence is shown. The sequences corresponding to oligonucleotides ATP6REV, ATP6FOR, ATP6INT1 and ATP6INT2 used for cDNA amplification by PCR and internal sequence priming are *underlined*. The amino acid sequence deduced from the genomic sequence using the universal genetic code is presented. Codon modified by editing is *boxed*, with the corresponding amino acid modification indicated above the cDNA sequence. *Dots* indicate nucleotide mismatches introduced in the oligonucleotide sequences in order to create *Pst*I and *Eco*RI sites.
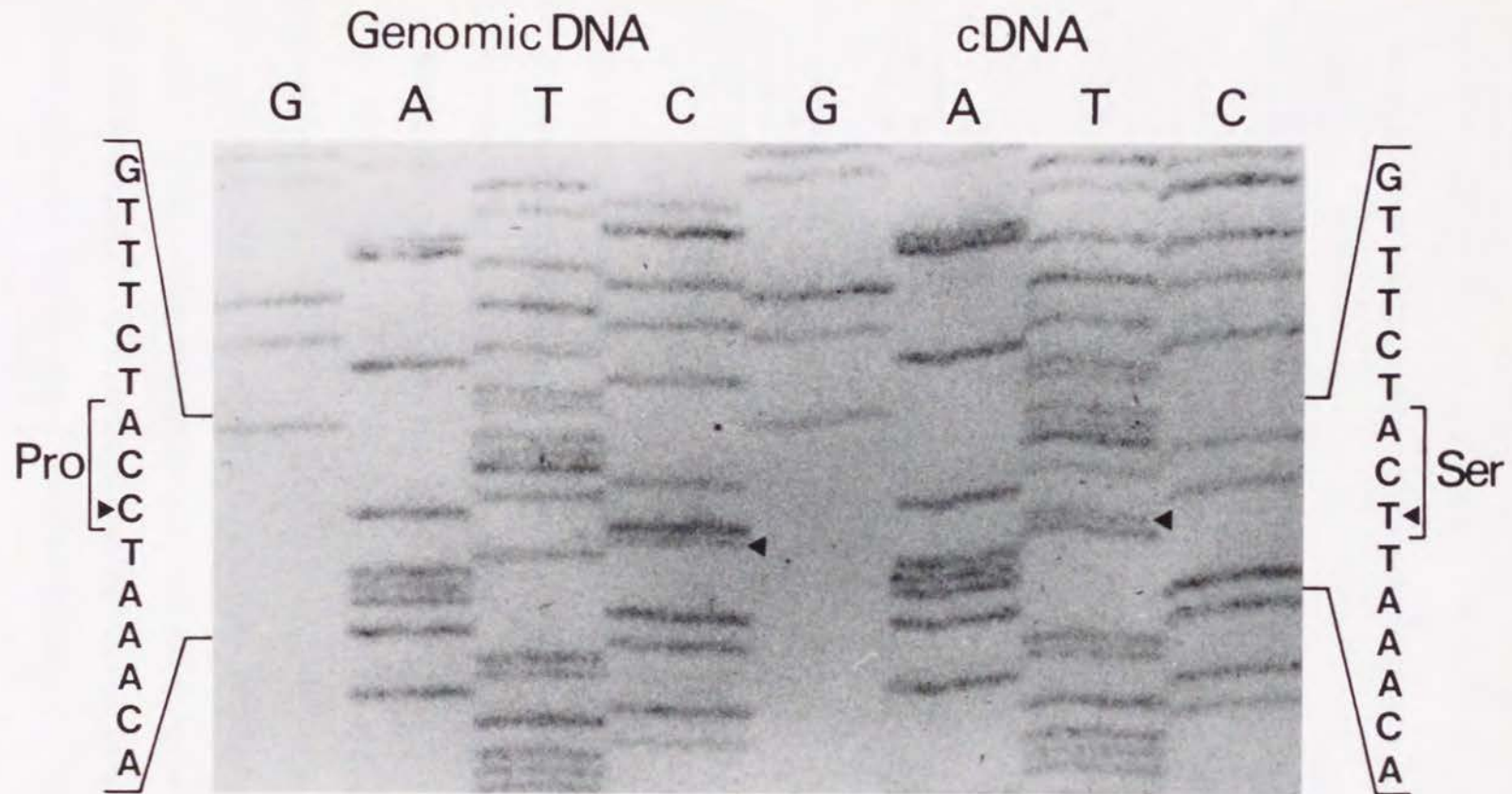
90

Fig. 26. Comparison of *atp6* cDNA and the corresponding genomic DNA sequences. Only part of the sequences are presented. Position where C to U modification is found is indicated by *arrowhead*.

```
                      10         20         30         40         50         60
        ┌rapeseed  MNQIGLVAQSPLDQFEIVPLIPMNIGNFYFSFTNSSLFMLLTLSFFLLLIHFITKKGGGN
cDNA    │Oenothera FWADTMSSH...E..S.L.........L...................LV...VN.V.......
        └sorghum   ERTGE.LIP......G.H.ILDL......L....L........GLV...VFVV......K
        ┌rapeseed  ...........................................P.................
genomic │Oenothera FWADTMSSH...E..S.L.........L......P.........LV...VN.V.......
        └sorghum   ERTGE.LIP......G.H.ILDL......L....L..S.....GLV...VFVV......K

                      70         80         90        100        110        120
        ┌rapeseed  LVPNAWQSLVELLYDFVLNLVKEQIGGLSGNVKQMFFPCILVTFLFLLFCNLQGMIPYSF
cDNA    │Oenothera .............I........N...........K.......T.................
        └sorghum   .............I........N...........K.......T.............F..
        ┌rapeseed  ...........................................................
genomic │Oenothera S............I....P.P.N...........K...R.S...T.S..R.P........
        └sorghum   S............I....P...N...........K.....S...T.S..R.P.....F..

                     130        140        150        160        170        180
        ┌rapeseed  TVTSHFLITLALSFSIFIGITIVGFQRHGLHFFSFLLPAGVPLPLAPFLVLLELISYCFR
cDNA    │Oenothera ..........G..................N....L.........................
        └sorghum   ...........................................................
        ┌rapeseed  ...........................................................
genomic │Oenothera ..........G..................N....L..S..............PH...
        └sorghum   ..........................................................H...

                     190        200        210        220        230        240
        ┌rapeseed  ALSLGIRLFANMMAGHSLVKILSGFAWTMLCMNEIFYFIGALGPLFIVLALTGLELGVAI
cDNA    │Oenothera ............................DL.....D.......................
        └sorghum   ............................FL.N.....D.....................
        ┌rapeseed  ...........................................................
genomic │Oenothera ...S...........S.........DL.....D...........P......
        └sorghum   ...S...........S.........FL.N.....D.....................

                     250        260        270        280        290
        ┌rapeseed  LQAYVFTILICIYLNDAINLH*
cDNA    │Oenothera ....................*
        └sorghum   ....................*
        ┌rapeseed  ....................*
genomic │Oenothera S....S..S........T...QSGWFFFIIEQKRKNQKIKEHRRERALHKPRRELL*
        └sorghum   S..H.S..S........T...QNE*
```

Fig. 27. Amino acid alignment of the ATP6 polypeptides deduced from genomic and
cDNA sequences of rapeseed and other plant species. The amino terminal extensions
of *Oenothera* and sorghum are not shown in their complete length. Identical amino
acid residues are indicated by *dots*.

Discussion

The investigation of RNA editing in the *atp6* mRNAs of rapeseed reported here
shows that only one editing event occurs in the *atp6* coding region. Comparative analyses
of the extent of RNA editing in plant *atp6* transcripts indicate substantial variation in the
extent of editing for *atp6* transcripts. *Oenothera* (Schuster and Brennicke 1991b) and

sorghum (Kempken et al. 1991) represent extreme examples, compared to rapeseed, the former two species having 21 and 19 editing events within the core polypeptide, while rapeseed having only one editing. In rapeseed, most of the amino acids which are specified only after editing in mitochondria of other two species, are already encoded by the mitochondrial genomic sequence. As defined for *atp6*, rapeseed seems to have already "edited" sequence on its mitochondrial genome, while *Oenothera* and sorghum have "pre-editing" sequence which needs extensive RNA editing for mature mRNA. Such an extreme divergence of editing patterns between different higher plant species is very interesting, considering the requirements of RNA editing in plant mitochondria for the conservation of protein sequences. "Edited" genomic sequence found in rapeseed mitochondria implies that, during evolution, edited sequences have been integrated in the mitochondrial genome of rapeseed which no longer exhibit editing at these positions. There is also another alternative, namely that genomic sequence of rapeseed remains in its primitive and original form, and does not show the mutations which can be seen in the other species.

Araya et al. (1991) observed the differences in the editing patterns of *atp9* and *cob* transcripts from normal and cms wheat lines. However, no variation of the extent and frequency of RNA editing between *atp6* transcripts from normal and *pol* cms cytoplasms can be observed, which suggests that RNA editing is not primary determinant for male-sterility induction in rapeseed.

A correlation between RNA editing and RNA maturation has been found in wheat *nad3-rps12* transcript (Gualberto et al. 1991); RNA editing in plant mitochondria is a process with a temporal development correlating with transcript maturation. The *atp6* and *pol-urf* genes of *pol* cms cytoplasm are co-transcribed into a same precursor RNA, and then mature transcripts might be made from precursor RNA by RNA processing. Therefore, it is possible that RNA editing patterns are different between precursor RNA

(*atp6-pol-urf* cotranscript) and mature RNA, and RNA maturation and RNA editing both take part in the induction of male sterility . Additional work is required to clarify the relationship between RNA editing and regulation of gene expression such as male-sterility induction, and to explain the role of RNA editing in the conservation of protein sequences.

94

Summary

Chapter 1. Intraspecific variation of mitochondrial DNA

Restriction endonuclease analysis was carried out for mtDNAs of 27 accessions of rapeseed to detect intraspecific variation of the mitochondrial genome. MtDNA isolated from green leaves was digested with ten endonucleases and was electrophoresed using agarose slab gel. Mitochondrial genomes of the 27 accessions were classified into two major types, each of which was further divided into three subtypes based on the length differences in the restriction fragments of mtDNAs. In total, six mitochondrial genome types (type Ia, Ib, Ic, IIa, IIb and IIc) could be identified. To analyze the relationship be-tween the mitochondrial genome types and cms induction, restriction fragment patterns of two male-sterile cytoplasms, namely, *nap* cms and *pol* cms, were compared with those of the normal cytoplasm. The mitochondrial genome of the *nap* cytoplasm was found to be either of Ia, Ib and Ic types, whereas the *pol* cytoplasm carried the type IIb mitochondrial genome. Mitochondrial linear plasmid DNA was also observed in several accessions, but the relationship between its presence and the cms trait could not be established.

Chapter 2. Reconfirmation of cytoplasmic types by backcross method

Reconfirmation of cytoplasmic types in the group with type I mitochondrial genome was carried out in order to solve the discrepancies between both classifications of cytoplasm based on restriction endonuclease analysis of mitochondrial genome and the ability to induce male sterility. In the B_1F_1 populations derived from seven cultivars with type I mitochondrial genome and "normal" cytoplasm, which had been backcrossed with maintainer cultivar, Isuzu-natane, male-sterile plants were segregated contrary to the expectation. Therefore, it is certain that these cultivars have a male-sterile cytoplasm.

These findings contradict the previous estimation. The reconfirmed results for cytoplasmic types dissolve the discrepancies between both classifications, and demonstrate the usefulness of molecular genetic techniques, such as restriction endonuclease analysis for the identification of plant cytoplasm.

Chapter 3. Phylogenetic relationships between the mitochondrial genomes of rapeseed and other related species

Restriction fragment patterns and restriction fragment length polymorphisms (RFLPs) of mtDNAs have been examined to explore the origin and evolution of the five *Brassica* species, especially rapeseed. Based on restriction fragment patterns and RFLP data of mtDNAs from rapeseed and related species, two different phylogenetic trees were obtained, which enabled a detailed analysis of the relationship among *Brassica* species. The results obtained from the data of restriction endonuclease and RFLP analyses suggested that 1) two organellar genomes, mitochondrial and chloroplast which has been extensively studied by Erickson et al. (1983) and Palmer et al. (1983a), were inherited in the same manner, 2) rearrangements of mitochondrial genomes occurred during the evolution of rapeseed and its parental species, 3) type I rapeseed cytoplasm might be originated from *B. oleracea*, and type II rapeseed cytoplasm from *B. campestris*.

Chapter 4. Examination of physical organizations of several mitochondrial genes from male-sterile cytoplasm

Southern blot analysis was carried out to understand the nature of the mtDNA alterations distinguishing between *pol* cms and normal cytoplasms and to determine the gene organizations of the two mtDNAs using ten mitochondrial gene probes. Different hybridization patterns were observed only when the *atp6* gene was used as a probe. These results suggested a different organization around the *atp6* locus in the two

96

mtDNAs. Furthermore, to understand the relationship between the cms trait and mitochondrial *atpA* gene, *atpA* gene was cloned and its transcriptional patterns in *pol* cms and normal cytoplasms were compared. Detailed physical mapping showed these two *atpA* loci was completely identical. Analysis of *atpA* transcript patterns indicated that there were no differences between normal and *pol* cms cytoplasms. These results indicated that the *atpA* locus may not be involved in male sterility.

Chapter 5. Different organization and altered transcription of the mitochondrial *atp6* region in the male-sterile cytoplasm

The F_O-ATPase subunit 6 gene (*atp6*) of rapeseed mitochondria has been isolated from both *pol* cms and normal (fertile) cytoplasms in order to determine whether the rearrangements around the *atp6* locus in *pol* cms cytoplasm play a role in the cms induction. The *pol* cms and normal *atp6* genes are identical and encode a 261-amino acid polypeptide. As a result of extensive rearrangement, a novel reading frame (*pol-urf*) was generated upstream of the *atp6* gene in *pol* cms mitochondria only, which encoded 105 amino acids and might be co-transcribed with *atp6*. A 5'-portion of *pol-urf* shows sequence homology to the *Oenothera* ORFB gene associated with *coxIII*. A 5'-flanking region of the *pol-urf* also shows homology to that of ORF105 in Ogura cms radish mitochondria. These DNA rearrangements which give rise to *pol-urf* in the vicinity of the *atp6* locus may be responsible for cms in rapeseed.

Chapter 6. Origin of *pol-urf* gene associated with *pol* cms mitochondrial genome

Pol-urf was a novel reading frame generated upstream of the *atp6* gene only in *pol* cms mitochondria. In order to determine the origin of *pol-urf* and the mechanism of its generation, the sequences homologous to the *pol-urf* sequence were isolated and

characterized. Sequence analysis indicated that *pol-urf* originated by recombinations among the 5'-flanking region and the amino-terminal segment of coding region of ORF474 which was homologous to ORFB, a part of exon 1 of ribosomal protein S3 (*rps3*) gene, and an unidentified sequence. However, the mechanism of the generation of *pol-urf* is still unknown, although it has likely arisen either by nonhomologous recombination or by homologous recombination involving small areas of nucleotide homology.

Chapter 7. RNA editing of *atp6* genes from male-sterile and normal cytoplasms

The complete cDNA sequence corresponding to the rapeseed *atp6* gene transcript (coding for subunit 6 of F_O-ATPase) has been determined by a method involving cDNA synthesis using specific oligonucleotides as primers followed by PCR amplification, cloning and sequencing of the amplification products. Only one modification, C to U conversion, has been found as compared to the genomic DNA sequence. Comparison of the extent and frequency of RNA editing of *pol* cms *atp6* transcript with those of normal *atp6* transcript indicates that there is no variation between the editing status of *pol* cms and normal *atp6* transcripts.

Acknowledgements

References

Araya A, Begu D, Graves PV, Mouras A, Hernould M, Gobin P, Litvak S (1991) *In vivo* and *in vitro* studies of mRNA editing in wheat mitochondria. Abst 3rd Int Con Plant Mol Biol No. 2045

Bailey-Serres J, Hanson DK, Fox TD, Leaver CJ (1986) Mitochondrial genome rearrangement leads to extension and relocation of the cytochrome *c* oxidase subunit I gene in sorghum. Cell 47:567-576

Bannerot H, Boulidard L, Cauderon Y, Tempe J (1974) Transfer of cytoplasmic male sterility from *Raphanus sativus* to *Brassica oleracea*. Proc Eucarpia Meet Cruciferae Crop Sect 25:52-54

Bland MM, Levings III CS, Matzinger DF (1986) The tobacco mitochondrial ATPase subunit 9 gene is closely linked to an open reading frame for a ribosomal protein. Mol Gen Genet 204:8-16

Bland MM, Levings III CS, Matzinger DF (1987) The ATPase subunit 6 gene of tobacco mitochondria contains an unusual sequence. Curr Genet 12:475-481

Blum B, Bakalara N, Simpson L (1990) A model for RNA editing in kinetoplastid mitochondria: "Guide" RNA molecules transcribed from maxicircle DNA provide the edited information. Cell 60:189-198

Brar GS, Singh S, Labana KS, Chopra S (1980) Identification of male sterile lines in Indian mustard (*Brassica juncea*). Agron Abstr, p 50

Covello PS, Gray MW (1989) RNA editing in plant mitochondria. Nature 341:662-666

Dewey RE, Levings III CS, Timothy DH (1985) Nucleotide sequence of ATPase subunit 6 gene of maize mitochondria. Plant Physiol 79:914-919

Dewey RE, Levings III CS, Timothy DH (1986) Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell 44:439-449

100

Erickson L, Grant I, Beversdorf W (1986) Cytoplasmic male sterility in rapeseed
(*Brassica napus* L.). 1. Restriction patterns of chloroplast and mitochondrial DNA.
Theor Appl Genet 72:145-150

Erickson LR, Straus NA, Beversdorf WD (1983) Restriction patterns reveal origins of
chloroplast genomes in *Brassica* amphidiploids. Theor Appl Genet 65:201-206

Falconet D, Lejeune B, Quetier F, Gray MW (1984) Evidence for homologous
recombination between repeated sequences containing 18S and 5S ribosomal RNA
genes in wheat mitochondrial DNA. EMBO J 3:297-302

Fan Z, Stefansson BR, Sernyk JL (1986) Maintainers and restorers for three male-
sterility-inducing cytoplasms in rape (*Brassica napus* L.). Can J Plant Sci 66:229-
234

FAO production year book (1988) vol. 42

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction
endonuclease fragments to high specific activity. Anal Biochem 132:6-13

Fu TD (1981) Production and research of rapeseed in the People's Republic of China.
Eucarp Crucif Newslett 6:6-7

Gualberto JM, Bonnard G, Lamattina L, Grienenberger JM (1991) Expression of the
wheat mitochondrial *nad3-rps12* transcription unit: Correlation between editing and
mRNA maturation. Plant Cell 3:1109-1120

Gualberto JM, Lamattina L, Bonnard G, Weil JH, Grienenberger JM (1989) RNA editing
in wheat mitochondria results in the conservation of protein sequences. Nature
341:660-662

Handa H, Nakajima K (1992) Gene for tRNA^{Lys} is encoded in the rapeseed (*Brassica
napus* L.) mitochondrial DNA. Biophys Biochim Acta 1130:117-119

Handa H, Ohkawa Y, Nakajima K (1990) Mitochondrial genome of rapeseed (*Brassica
napus* L.). 1. Intraspecific variation of mitochondrial DNA. Jpn J Genet

65:17-24.

Hanson MR, Conde MF (1985) Functioning and variation of cytoplasmic genomes: Lessons from cytoplasmic-nuclear interactions affecting male fertility in plants. Int Rev Cytol 94:213-267

Helentjaris T, King G, Slocum M, Siedenstrang C, Wegman S (1985) Restriction fragment polymorphisms as probes for genetic diversity and their development as tools for applied plant breeding. Plant Mol Biol 5:109-118

Hiesel R, Schobel W, Schuster W, Brennicke A (1987) The cytochrome oxidase subunit I and subunit III genes in *Oenothera* mitochondria are transcribed from identical promoter sequences. EMBO J 6:29-34

Hiesel R, Wissinger B, Schuster W, Brennicke A (1989) RNA editing in plant mitochondria. Science 246:1632-1634

Ichikawa H, Hirai A (1983) Search for the female parent in the genesis of *Brassica napus* by chloroplast DNA restriction patterns. Jpn J Genet 58:419-424

Ikeda TM, Ishii K, Terachi T, Tsunewaki K (1990) Structure analysis of mitochondrial *atpA* and *cox3* genes and their flanking regions in common wheat with *Ae. columnaris* cytoplasm. Jpn J Genet 65:435

Kadowaki K, Suzuki T, Kazama S, Oh-fuchi T, Sakamoto W (1989) Nucleotide sequence of the cytochrome oxidase subunit I gene from rice mitochondria. Nucleic Acids Res 17:7519

Kadowaki K, Suzuki T, Kazama S (1990) A chimeric gene containing the 5' portion of *atp6* is associated with cytoplasmic male-sterility of rice. Mol Gen Genet 224:10-16

Kaul MLH (1988) Monographs on Theoretical and Applied Genetics 10. Male Sterility in Higher Plants, Springer-Verlag, Berlin, Heidelberg, Germany, p 1005

Kazama S, Suzuki T, Kadowaki K, Akihama T (1990) Nucleotide sequence of the F_O-ATPase subunit 9 gene from tomato mitochondria. Nucleic Acids Res 18:5879

Kemble RJ (1987) A rapid, single leaf, nucleic acid assay for determining the
cytoplasmic organelle complement of rapeseed and related *Brassica* species. Theor
Appl Genet 73:364-370

Kemble RJ, Carlson JE, Erickson LR, Sernyk JL, Thompson DJ (1986) The *Brassica*
mitochondrial DNA plasmid and large RNAs are not exclusively associated with
cytoplasmic male sterility. Mol Gen Genet 205:183-185

Kempken F, Mullen JA, Pring DR, Tang HV (1991) RNA editing of sorghum
mitochondrial *atp6* transcripts changes 15 amino acids and generates a carboxy-
terminus identical to yeast. Curr Genet 20:417-422

Köhler RH, Horn R, Lössel A, Zetsche K (1991) Cytoplasmic male sterility in sunflower
is correlated with the co-transcription of a new open reading frame with the *atpA*
gene. Mol Gen Genet 227:369-376

Lonsdale DM (1987) Cytoplasmic male sterility: a molecular perspective. Plant Physiol
Biochem 25:265-271

Lonsdale DM, Hodge TP, Fauron CMR (1984) The physical map and organization of the
mitochondrial genome from the fertile cytoplasm of maize. Nucleic Acids Res
12:9249-9261

Makaroff CA, Apel IJ, Palmer JD (1989) The *atp6* coding region has been disrupted and
a novel reading frame generated in the mitochondrial genome of cytoplasmic male-
sterile radish. J Biol Chem 264:11706-11713

Makaroff CA, Apel IJ, Palmer JD (1990) Characterization of radish mitochondrial *atpA*:
influence of nuclear background on transcription of *atpA*-associated sequences and
relationship with male sterility. Plant Mol Biol 15:735-746

Makaroff CA, Apel IJ, Palmer JD (1991) The role of *coxI*-associated repeated sequences
in plant mitochondrial DNA rearrangements and radish cytoplasmic male sterility.
Curr Genet 19:183-190

Makaroff CA, Palmer JD (1988) Mitochondrial DNA rearrangements and transcriptional

alterations in the male-sterile cytoplasm of Ogura radish. Mol Cell Biol 8:1474-1480

Morikami A, Nakamura K (1987) Structure and expression of pea mitochondrial
F$_1$ATPase α-subunit gene and its pseudogene involved in homologous
recombination. J Biochem 101:967-976

Morinaga T (1934) Interspecific hybridization in *Brassica* VI. The cytology of F$_1$
hybrids of *B. juncea* and *B. nigra*. Cytologia 6:62-67

Ohkawa Y (1984) Cytoplasmic male sterility in *Brassica campestris* ssp. *rapifera* L.
Japan J Breed 34:285-294

Ohkawa Y, Uchimiya H (1985) Classification of chloroplast DNA in *Brassica napus*
possessing normal and male sterile cytoplasms. Jpn J Genet 60:249-253

Palmer JD (1988) Intraspecific variation and multicircularity in *Brassica* mitochondrial
DNAs. Genetics 118:341-351

Palmer JD, Herbon LA (1987) Unicircular structure of the *Brassica hirta* mitochondrial
genome. Curr Genet 11:565-570

Palmer JD, Herbon LA (1988) Plant mitochondrial DNA evolves rapidly in structure, but
slowly in sequence. J Mol Evol 28:87-97

Palmer JD, Shields CR (1984) Tripartite structure of the *Brassica campestris*
mitochondrial genome. Nature 307:437-440

Palmer JD, Shields CR, Cohen DB, Orton TJ (1983a) Chloroplast DNA evolution and the
origin of amphidiploid *Brassica* species. Theor Appl Genet 65:181-189

Palmer JD, Shields CR, Cohen DB, Orton TJ (1983b) An unusual mitochondrial DNA
plasmid in the genus *Brassica*. Nature 301:725-728

Quagliariello C, Saiardi A, Gallerani R (1990) The cytochrome oxidase subunit III gene in
sunflower mitochondria is cotranscribed with an open reading frame conserved in
higher plants. Curr Genet 18:355-363

Rathburn HB, Hedgcoth C (1991) A chimeric open reading frame in the 5' flanking

region of *coxI* mitochondrial DNA from cytoplasmic male-sterile wheat. Plant Mol Biol 16:909-912

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467

Schuster W, Brennicke A (1987) Nucleotide sequence of the *Oenothera* ATPase subunit 6 gene. Nucleic Acids Res 15:9092

Schuster W, Brennicke A (1991a) RNA editing makes mistakes in plant mitochondria: editing loses sense in transcripts of a *rps19* pseudogene and in creating stop codons in *coxI* and *rps3* mRNAs of *Oenothera*. Nucleic Acids Res 15:9092

Schuster W, Brennicke A (1991b) RNA editing in ATPase subunit 6 mRNAs in *Oenothera* mitochondria. A new termination codon shortens the reading frame by 35 amino acids. FEBS Lett 295:97-101

Senda M, Harada T, Mikami T, Sugiura M, Kinoshita T (1991) Genomic organization and sequence analysis of the cytochrome oxidase subunit II gene from normal and male-sterile mitochondria in sugar beet. Curr Genet 19:175-181

Shiga T (1976) Studies on heterosis breeding using cytoplasmic male sterility in rapeseed, *Brassica napus* L. Bull Natl Inst Agric Sci D27:1-101

Shiga T (1979) Cytoplasmic, genic male sterility and cytoplasmic differentiation. Ikushugaku Saikin no Shinpo 20:55-62, (in Japanese)

Shiga T, Baba S (1971) Cytoplasmic male sterility in rape plant (*Brassica napus* L.). Japan J Breed 21 Suppl 2:16-17, (in Japanese)

Shiga T, Baba S (1973) Cytoplasmic male sterility in oil rape, *Brassica napus* L., and its utilization to breeding. Japan J Breed 23:187-197, (in Japanese with English summary)

Shiga T, Ohkawa Y, Takayanagi K (1983) Cytoplasmic types of European rapeseed (*Brassica napus* L.) cultivars and their ability to restore fertility in cytoplasmic male

sterile lines. Bull Natl Inst Agric Sci D35:103-124

Siculella L, Palmer JD (1988) Physical and gene organization of mitochondrial DNA in fertile and male sterile sunflower. CMS-associated alterations in structure and transcription of the *atpA* gene. Nucleic Acids Res 16:3787-3799

Small ID, Isaac PG, Leaver CJ (1987) Stoichiometric differences in DNA molecules containing the *atpA* gene suggest mechanisms for the generation of mitochondrial genome diversity in maize. EMBO J 6:865-869

Sneath PHA, Sokal RR (1973) Numerical Taxonomy. p 573. Freeman & Company, San Francisco

Song KM, Osborn TC, Williams PH (1988) *Brassica* taxonomy based on nuclear restriction fragment polymorphisms (RFLPs) 1. Genome evolution of diploid and amphidiploid species. Theor Appl Genet 75:784-794

Southern E (1980) Gel electrophoresis of restriction fragments. Methods Enzymol 68:152-176

Stern DB, Newton KJ (1986) Isolation of plant mitochondrial RNA. Methods Enzymol 118:488-496

Suzuki T, Kazama S, Hirai A, Akihama T, Kadowaki K (1991) The rice mitochondrial *nad3* gene has an extended reading frame at its 5' end: nucleotide sequence analysis of rice *trnS*, *nad3*, and *rps12* genes. Curr Genet 20:331-337

Terachi T, Tsunewaki K (1986) The molecular basis of genetic diversity among cytoplasms of *Triticum* and *Aegilops*. 5. Mitochondrial genome diversity among *Aegilops* species having identical chloroplast genomes. Theor Appl Genet 73:175-181

Thompson KF (1972) Cytoplasmic male-sterility in oil seed rape. Heredity 29:253-257

U N (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jpn J Bot 7:389-452

Uchimiya H, Wildman SG (1978) Evolution of fraction I protein in relation to origin of
amphidiploid *Brassica* species and other members of the Cruciferae. J Heredity
69:299-303

Umbeck PF, Gengenbach BG (1983) Reversion of male-sterile T-cytoplasm maize to
male fertility in tissue culture. Crop Sci 23:584-588

Witt U, Hansen M, Albaum M, Abel WO (1991) Molecular analyses of the CMS-
inducing 'Polima' cytoplasm in *Brassica napus* L. Curr Genet 19:323-327

Young EG, Hanson MR (1987) A fused mitochondrial gene associated with cytoplasmic
male sterility is developmentally regulated. Cell 50:41-49

Young EG, Hanson MR, Dierks PM (1986) Sequence and transcription analysis of the
Petunia mitochondrial gene for the ATP synthase proteolipid subunit. Nucleic Acids
Res 14:7995-8006