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Cytoplasmic male sterility in rapeseed (*Brassica napus* L.)

2. The role of a mitochondrial plasmid

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Summary. The linear mitochondrial (mt) plasmid in rapeseed occurs in normal, fertile plants, cytoplasmic male sterile (cms) lines (sterile and restored) and maintainers. To determine the role of plasmid genes in cytoplasmic male sterility the three internal Hind III fragments (78%) of the 11.3 kb plasmid were cloned, nick-translated and hybridized to electrophoretic blots of Pst I digests of chloroplast (cp) and mt DNA from normal and cms lines. There is no homology between plasmid DNA and the cp and mt genomes in any line examined nor do plasmid sequences appear to be directly involved in certain alterations of the mt genome. As well, loss of the free plasmid is not associated with integration of the plasmid DNA into the cp or mt genomes. Finally, plasmid copy number appears to be affected by nuclear genotype.

Key words: *Brassica napus* – Cytoplasmic male sterility – Mitochondrial DNA

Introduction

There are probably several molecular mechanisms which account for cytoplasmic male sterility in plants (Pearson 1981) and many believe that mitochondria are involved.

The presence (or absence) of mitochondrial (mt) plasmids in cytoplasmic male sterile (cms) lines in some species has reinforced this belief (Pring et al. 1977; Kemble and Bedbrook 1980; Powling 1981; Dixon and Leaver 1982; Pring et al. 1982; Boutry and Briquet 1982; Negruk et al. 1982; Goblet et al. 1983; Palmer et al. 1983). The integration and/or amplification of integrated S1 and S2 plasmid sequences in the mt DNA of fertile revertants in corn (Levings et al. 1980; Kemble and Mans 1983) have provided additional evidence.

Restriction patterns of organelle DNA in rapeseed (*Brassica napus, B. campestris* hereafter referred to as *napus* and *campestris* rapeseed) support an alloplasmic explanation for cms systems in this crop (Vedel et al. 1982; Vedël and Mathieu 1983; Erickson et al. 1986). Palmer et al. (1983) have suggested a correlation between the presence of a linear mitochondrial plasmid and sterility in two cms lines of *Brassica* – a *campestris* line with *Raphanus* cytoplasm and the other, a *napus* line identified only as being from Korea. The maintainer of the latter line did not have the plasmid, but the maintainer of the former did possess the plasmid although in a much lower concentration than in the cms line. However, the plasmid occurs in normal rapeseed cultivars and is most abundant in *Torch*, a *campestris* rapeseed cultivar which plays no known role in any cms system and was once widely grown in Western Canada.

Most of this mt plasmid has been cloned and the nick-translated clones used to probe chloroplast (cp) and mt DNA from five cms lines of rapeseed, their maintainers (where available) and normal fertile plants for homologous sequences. The clones were also used to probe for the plasmid and sequences homologous to the plasmid in mt DNA from populations segregating for sterility following crosses to cms lines, and from plants which have either lost the plasmid or undergone an alteration in the mt genome. The results of these investigations are the subject of this report.

Materials and methods

Plant material

The pedigrees and seed sources of the various lines and cms systems have been described (Erickson et al. 1986). The ogu cms line (*Raphanus* cytoplasm) from France had a winter growth habit and was designated ogu(W). An ogu cms line with spring habit, designated ogu(S), was developed by back-

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crossing to the Canadian spring cultivar *Regent* by Dr. L. Sernyk at the Plant Science Dept., University of Manitoba (presently at Allelix Inc., Mississauga, Ontario). Seed of *Diplotaxis muralis* was provided by Dr. K. Downy, Agriculture Canada, Saskatoon.

Extraction of chloroplast and mitochondrial DNA

Cp DNA was extracted from flowering plants or seedlings grown in flats as described (Erickson et al. 1983). Mt DNA was extracted from the same tissue as the cp DNA but the extraction included a proteinase K step to release the plasmid DNA from protein (Erickson et al. 1985).

Cloning of mitochondrial plasmid

Mt DNA, extracted from a *napus* line with the triazine resistant cytoplasm, was fractionated by electrophoresis on 0.6% gels (Seaplaque® Marine Colloids) (0.040 M Tris and acetate/0.001 M EDTA, pH 7.8) and the plasmid band excised and subjected to the freeze-thaw procedure of Vedel and Mathieu (1983). The aqueous supernatant was purified sequentially with phenol and chloroform before ethanol precipitation.

The cloning vector was pDPL13 (courtesy of Dr. N. Straus, Botany Department, University of Toronto) which has a 79 bp polylinker containing restriction sites for 13 enzymes (Gendel et al. 1983). Vector DNA (10 µg in 40 µl) was digested with Hind III for 1 h after which the digest was heated at 65 °C for 10 min to inactivate the enzyme. 1.0 µl of 1.0 M Tris-Cl (pH 8.0) was added and the volume increased to 100 µl with water. Addition of 1.5 µl of calf intestinal alkaline phosphatase (Boehringer Mannheim) was followed by incubation at 75 °C for 30 min, addition of another 1.5 µl of phosphatase and incubation for another 30 min. The reaction was terminated with sequential extraction with phenol and chloroform (twice). Ethanol precipitated DNA was dissolved in 10 µl of water and checked on a gel for complete digestion.

Mt plasmid DNA was also digested with Hind III, extracted with phenol and chloroform, ethanol precipitated and dissolved in water. Vector DNA (1 µg) and mt plasmid DNA (0.5 µg) were incubated in 20 µl with 1 µl of ligase (New England Biolabs) at 15 °C for 1 h after which time the volume was increased with buffer to 100 µl and the reaction continued overnight. Transformation of HB101 was by the $CaCl_2$ method of Mandel and Higa (1970). Clones pBcmH1, pBcmH2 and pBcmH3 contain the internal Hind III fragments of 4.6, 2.6 and 1.7 kb, respectively (Fig. 1) and contain in total 78% of the 11.3 kb mitochondrial plasmid. An attempt to clone the 0.6 and 1.7 kb terminal Hind III fragments by treating the mt plasmid with S_1 nuclease followed by digestion with Hind III and ligation with vector DNA (digested with Sma 1 and Hind III, thereby creating a blunt and sticky end in the polylinker) met with failure. Subsequent experiments showed that the 5′ ends of the plasmid are probably not available for ligation because of the possible attachment of one or more amino acids from a terminal protein associated with the plasmid (Erickson et al. 1985).

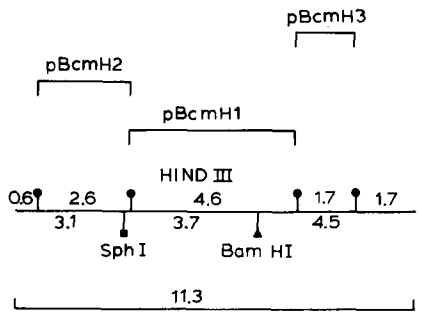

Fig. 1. Restriction map of linear mt plasmid in *Brassica* (from Palmer et al. 1983). Fragment lengths are in kilobase pairs and the total length is 11.3

Recombinant plasmid DNA was extracted from *E. coli* by alkaline lysis (Maniatis et al. 1982) and purified on $CsCl_2$.

Hybridizations

Gels (0.8% agarose) were acid-treated (0.25 M HCl) for 15 min, denatured (0.2 M NaOH, 0.6 M NaCl) for 1 h and washed for 1 h with three changes (0.025 M Na_2HPO_4/NaH_2PO_4, pH 6.5) all with gentle agitation at room temperature. DNA was electroblotted (Transblot® Bio-Rad) onto Gene Screen® (New England Nuclear) in 0.025 M Na_2HPO_4/NaH_2PO_4 at 4 °C on a magnetic stirrer at 250 mA overnight. The Gene Screen membrane was washed with the phosphate buffer, air-dried and baked at 85 °C for 2–4 h.

Prehybridization in 50% formamide, 10× Denhardt's solution, 0.05 M Tris-HCl (pH 7.5), 1.0 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulphate (MW 500,000, Sigma) and denatured salmon sperm DNA (100 µg/ml), lasted 24 h at 42 °C with agitation. Cloned DNA of the mt plasmid was nick-translated (Rigby et al. 1977) with [α^{32}P] dCTP (New England Nuclear, specific activity greater than 3200 Ci/mmol) and the probe DNA ($1–4\times10^8$ cpm/µg) was denatured by immersing in boiling water for 5 min and added to the prehybridization solution (final concentration of 10 ng/ml) in 15 ml of 50% formamide, 10× Denhardt's solution, 0.05 M Tris (pH 7.5), 0.1% sodium pyrophosphate, 1% SDS and 100 µg/ml of denatured salmon sperm DNA. Hybridization lasted at least 24 h at 42 °C with constant agitation followed by washing with two changes of 0.3 M NaCl, 0.06 M Tris-Cl (pH 8.0) 0.002 M EDTA for 15 min at room temperature with agitation. The membrane was then washed with the same solution and 1% SDS at 55 °C for 30 min followed by washing at room temperature in 0.03 M NaCl, 0.006 M HCl (pH 8.0) 0.0002 M EDTA all with agitation. The above protocol largely conforms to that recommended by the manufacturer for electroblotting and hybridization to Gene Screen. Autoradiography was at –70 °C with intensifying screens for 8–24 h.

Results and discussion

Presence of plasmic in mitochondrial DNA extracts – Shiga-Bronowski System

Our initial investigations revealed the widespread presence of the plasmid in cms lines when mt DNA from these lines was examined in agarose gels stained with ethidium bromide. *Torch* and *Westar* were included as normal fertile rapeseed cultivars, the former a *campestris* cultivar possessing the plasmid in abundance, the latter a *napus* cultivar with no detectable plasmid. Both Shiga's nap cms line and the maintainer (*Isuzu-natane*) possessed the plasmid in what appear to be similar concentrations (Fig. 2A) but much lower than in *Torch* (Fig. 3A). The presence of the plasmid appears to be variable in *Bronowski*, a cultivar which maintains sterility in Shiga's nap cms line and induces

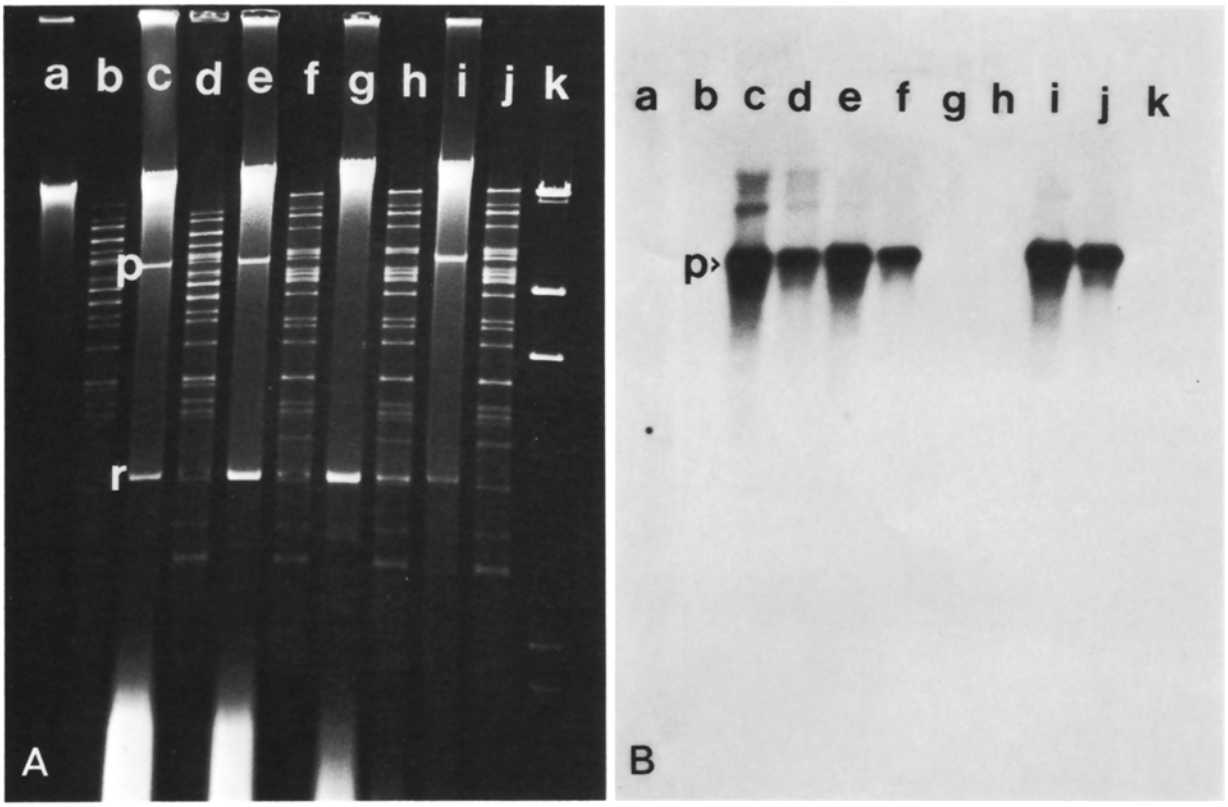

Fig. 2. **A** Paired undigested and digested (Pst I) samples of mt DNA from *Westar* (*a, b*), Shiga's nap cms line (*c, d*), *Isuzu-natane* (*e, f*), *Bronowski* (*g,h*) and tr (*i, j*). *Lane k* contains a Hind III digest of lambda DNA. The position of the plasmid band (marked p in *lane c*) is the same in digested and undigested lanes since Pst I does not digest the plasmid. The largest Pst fragment present in both *campestris* and *napus* lines is very faint in Shiga's nap cms line (*lane d*). **B** Autoradiograph of blot of gel in **A** probed with nick-translated clones (pBcmH1, -H2, -H3) of the three central Hind III fragments of the mt plasmid. Note that the cloned plasmid DNA hybridized only to the free plasmid band (marked p) in lanes with undigested DNA (*c, e, i*) as well as in lanes containing mt DNA digested with Pst I (*d, f, j*) since this enzyme does not digest the plasmid DNA

and maintains sterility in *napus* cytoplasms (Shiga 1980). In our earlier mt extractions from *Bronowski* the plasmid was visible on gels but was considerably less intense than in Shiga's nap cms line or *Isuzu-natane*. Palmer et al. (1983) also found the plasmid in *Bronowski*. However, the plasmid was not detectable in more recent extractions whether on a gel (Fig. 2A) or in an autoradiograph of a blot of the gel (Fig. 2B). Seed for our early extractions came from Dr. K. Downey in Saskatoon whereas seed for the recent extractions came directly from Poland. Since *Bronowski* is known to be very heterogeneous for a variety of traits, it is perhaps also heterogeneous for plasmid presence.

Dr. L. Sernyk has succeeded in transferring the recessive nuclear genes conferring sterility from Shiga's nap cms line to the cultivar *Regent* (Erickson et al. 1986) thereby creating a new cms line in *napus*. As might be expected, this line does not have the mt plasmid (R. Kemble, personal communication). Therefore, in the Shiga-*Bronowski* system presence of the plasmid does not necessarily induce male sterility (for example in the maintainer) and absence of the plasmid does not necessarily confer fertility (for example, in Dr. Sernyk's new nap cms lines).

Triazine resistant cytoplasm

The plasmid is also evident in mt DNA from the *napus* rapeseed line with triazine resistant (tr) cytoplasm at approximately similar intermediate concentrations to Shiga's nap cms line and *Isuzu-natane* (Fig. 2A), as visually estimated by gel fluorescence. *Bronowski* has been used as a male in crosses with this cytoplasm in an attempt to combine cytoplasmic male sterility and triazine resistance (Grant and Beversdorf 1985). Both sterile and fertile F_2's from this cross have the plasmid (data not shown) as well as sterile and fertile F_1's from a backcross of these sterile F_2's to *Bronowski* (Fig. 4A, ctr/Bronowski). In this cytoplasm male sterile lines without the mt plasmid have been generated in tissue culture.

Polima cytoplasm

The cms line polima also has the plasmid in intermediate concentration (Fig. 3A). Since most *napus*

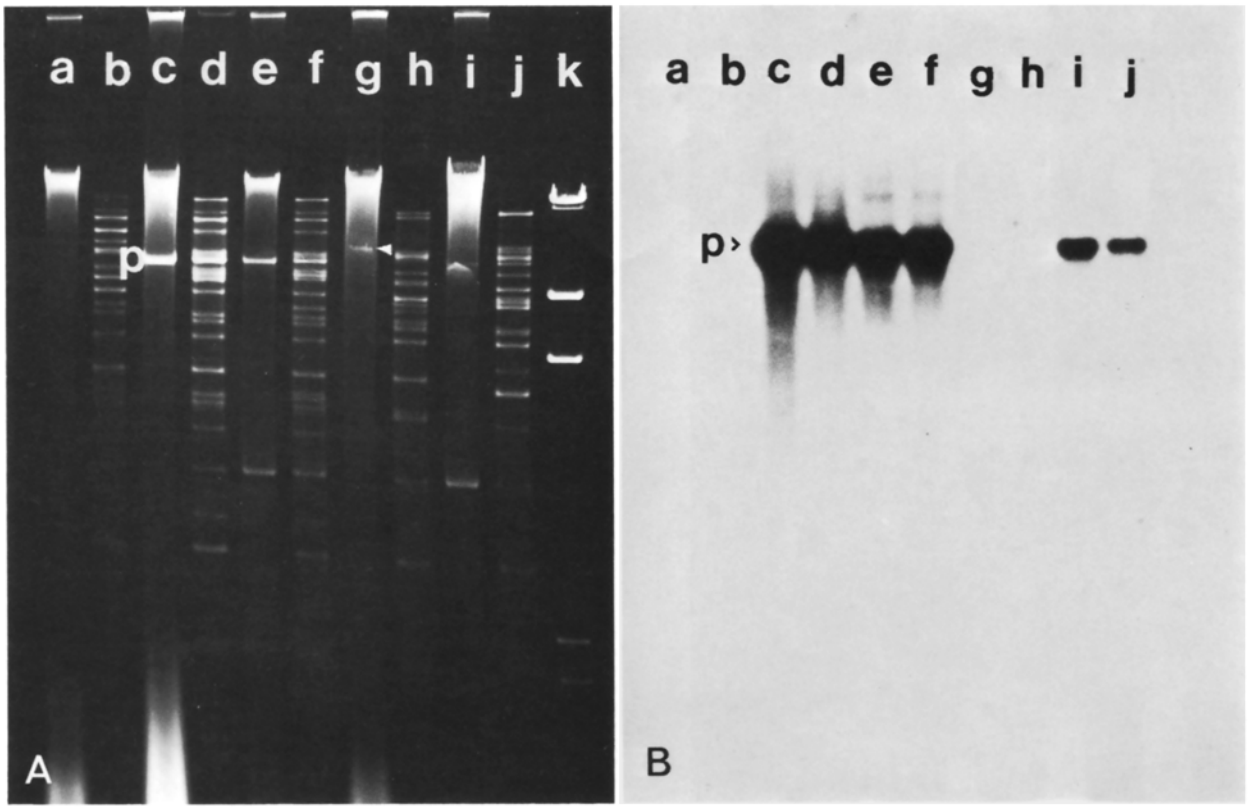

Fig. 3. A Paired undigested and digested (Pst I) samples of mt DNA from *Westar* (*a, b*), *Torch* (*c, d*), polima (*e, f*), *Diplotaxis* (st) (*g, h*) and ogu(W) (*i, j*). *Lane k* contains a Hind III digest of lambda DNA. *Westar* and *Torch* are normal, fertile *napus* and *campestris* cultivars respectively. The position of the plasmid band is marked p (*lane c*). Fluorescence in region of plasmid band in lane g (marked with *arrowhead*) and *lane i* was not seen on any other gel with these samples. Autoradiography (Fig. 2B) confirms that the fluorescence in *lane g* is not related to the plasmid. **B** Autoradiograph of gel in **A** probed as in Fig. 2B. Only the free plasmid band (marked p) hybridized to probe DNA

lines tested to date maintain polima male sterility, maintainers may or may not have the plasmid. There is some evidence that the polima cytoplasm is from *B. juncea* (Erickson et al. 1986), but its origin and route to *napus* are unknown. Palmer et al. (1983) found no plasmid in the three *juncea* lines they examined. Recently the correlation between male sterility and the mt plasmid was broken in this cytoplasm as well when a polima cms line without the plasmid was generated by sexual hybridization.

Raphanus cytoplasm

Although partially obstructed by a fluorescent anomaly, a faint plasmid band was evident in mt DNA from ogu (W) (Fig. 3A); this observation has been repeated on other gels with other mt DNA extracts from ogu(W). In contrast, extracts of ogu(S) revealed a much more conspicuous plasmid band approximately equivalent to that in Shiga's nap cms line (data not shown). Ogu(S) has been produced by backcrossing an ogu(W) line to the spring *napus* rapeseed cultivar *Regent.* In the winter nuclear background the mt plasmid in this cytoplasm does not seem to be as abundant as when this cytoplasm has the spring nuclear background of *Regent,* indicating the possible role of nuclear genes in the presence and/or abundance of the plasmid. It should be noted that these differences in plasmid intensity, although only visually assessed from a gel, were consistent from extraction to extraction and from gel to gel.

Diplotaxis cytoplasm

Pst I digests of mt DNA from fertile and sterile F_1 plants with the *Diplotaxis* cytoplasm showed no differences in banding patterns nor was the plasmid present in either case (Fig. 4A). Earlier extracts of mt DNA from *Diplotaxis muralis* plants (from Dr. K. Downey) revealed the presence of the plasmid. Either the *Diplotaxis muralis* plants used initially to establish this F_1 population did not have the plasmid or subsequent backcrossing has possibly eliminated nuclear genes required for plasmid replication. Dr. Sernyk's

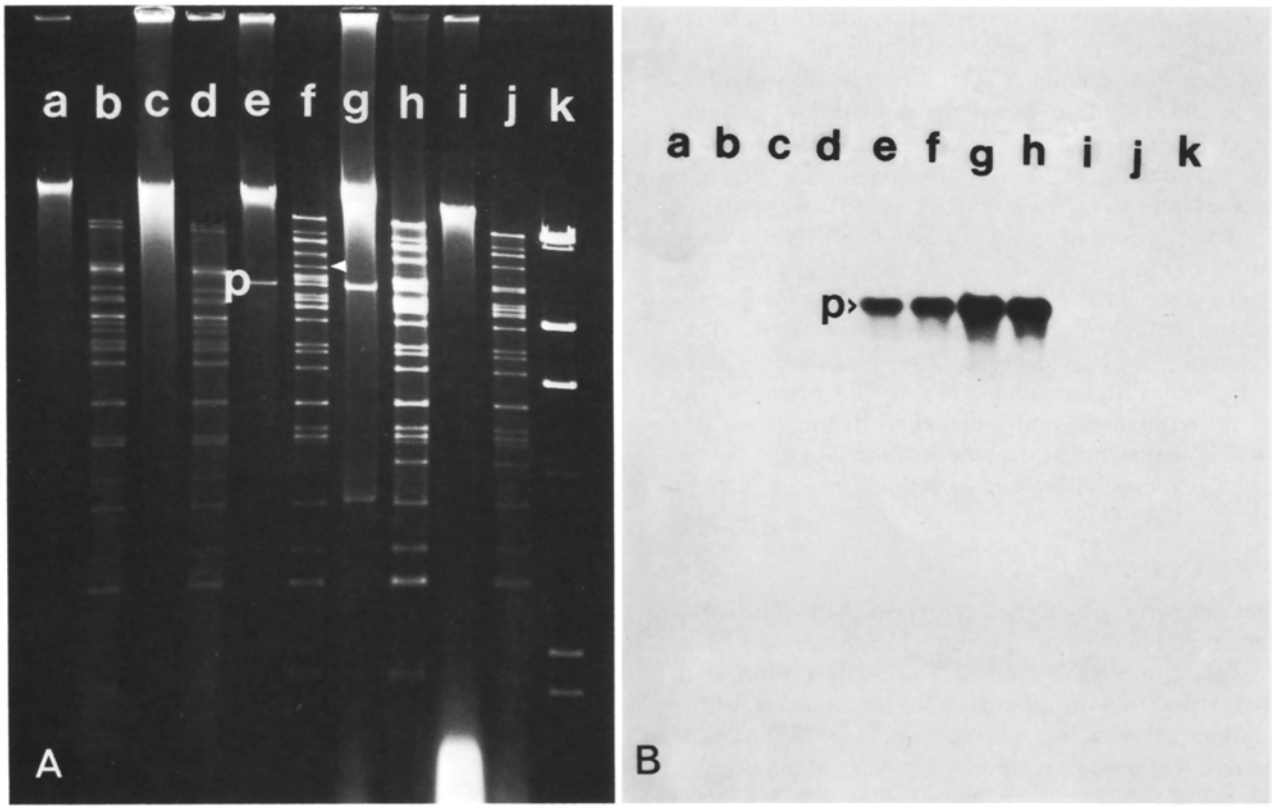

Fig. 4. **A** Paired undigested and digested (Pst I) samples of mt DNA from sterile (*a, b*), and fertile (*c, d*) plants with *Diplotaxis* cytoplasm, ctr/Bron (st) (*e, f*), ctr/Bron (*f*) (*g, h*) and trp$^-$ (*i, j*). *Lane k* contains a Hind III digest of lambda DNA and the plasmid band position is marked p. **B** Autoradiograph of gel in **A** probed as in Fig. 2 B. Only the free plasmid band showed hybridization in lanes with undigested (*e, g*) and digested (*f, h*) mt DNA. Note the absence of plasmid DNA in a particular line with the triazine cytoplasm (trp$^-$, *lanes i, j*)

population with the *Diplotaxis* cytoplasm and segregating for fertility, was not initiated from *Diplotaxis muralis* seed obtained from Dr. K. Downey (Dr. L. Sernyk, personal communication).

Recent cytogenetic studies of this population in the Plant Science Department at the University of Manitoba indicate that sterile plants have an extra chromosome (Fan et al. 1985). This observation presents the possibility that the sterility in this cross may not need a cytoplasmic component.

Hybridization of plasmid clones to mitochondrial DNA

To probe for sequences homologous to the plasmid in the mt genome, mt DNA was digested with Pst I, fractionated electrophoretically and electroblotted to hybridization membranes. Pst I gives fairly simple but distinctive patterns for the various cytoplasms and does not cut the plasmid (Palmer et al. 1983). Undigested mt DNA was run in a lane adjacent to the corresponding digested DNA to provide a marker for the free plasmid band in the lane with digested DNA (Figs. 2 A–4 A, 2 B–4 B). All other bands in the digest represent mt genomic fragments and hybridization to any of these bands would indicate the presence of plasmid-like sequences in the mt genome.

Hybridization data confirmed the gel data and revealed an absence of the plasmid in mt DNA extracts from *Westar*, the fertile and sterile *Diplotaxis* plants and *Bronowski* (Fig. 2 B and 4 B). As mentioned above, *Bronowski* extracts usually possess the plasmid. One cannot conclude that the plasmid is totally absent from these cytoplasms, but if it is present, it must be in very low copy number. Hybridization experiments with decreasing concentrations of cloned mt plasmid DNA indicated a lower detection limit of approximately 10 pg. Since the 11.3 kb plasmid is 5.2×10^{-2} as large as the mt genome (Palmer and Shields 1984), a typical restriction digest with 1.0 μg of mt DNA would contain 5.2×10^{-8} g of plasmid DNA if there were one full-length copy of the plasmid per mt genome. This amount of DNA is 5,200× the minimum detection level and, therefore, these hybridization conditions should be sufficient to detect as little as one full-length plasmid per 5,000 mt genomes.

More significantly, no sequence homology to the cloned plasmid DNA is present in the mt genomes of any cytoplasm examined (Figs. 2 B–4 B) since hybridization did not occur to any band of the mt chromosomal DNA. A similar lack of homology between the plasmid and mt DNA was observed in the rapeseed lines examined by Palmer et al. (1983). As well, the alterations observed in the mt genomes of Shiga's nap cms line (Fig. 2 A, B) and ctr/*Bronowski* (st) (Fig. 4 A, B) (Erickson et al. 1986) do not directly involve sequences homologous to cloned plasmid DNA. However, since the cloned probe DNA included the three central Hind III fragments (78% of the plasmid), but not the terminal 0.6 and 1.7 kb Hind III fragments, it is possible that terminal sequences of the plasmid exist in the mt genome and escaped detection.

Loss of the plasmid

The triazine resistant (tr) cytoplasm was transferred from a wild biotype of *campestris* to *napus* by the backcrossing to the *napus* cultivar *Tower* (Beversdorf et al. 1980). Mt DNA from various stages of backcrossing continue to show the plasmid (eg. tr, Fig. 2 A). The plasmid was probably present originally in the *campestris* biotype. It is not known whether *Tower* has the plasmid, but since the plasmid remained in the tr cytoplasm after several crosses with *Tower, Tower* must at least possess the nuclear genes required to perpetuate the plasmid. However, mt DNA from plants from a backcross of triazine resistant *Tower* to *Westar* did not have the plasmid (trp^-, Fig. 4 A). Absence of the plasmid was verified by hybridization (Fig. 4 B). The loss of the plasmid was not associated with any alteration of the Pst I restriction pattern of mt DNA (Fig. 4 A) nor were any plasmid sequences apparently integrated into the mt genome (Fig. 4 B).

The possibility that specific nuclear genes affect the presence and/or abundance of the plasmid is thus suggested by three observations: 1. increased plasmid concentration in the *Raphanus* cytoplasm upon crossing a winter line with the cytoplasm to the spring cultivar Regent; 2. presence of the plasmid in one seed source of *Bronowski* and absence in another; 3. loss of the plasmid in the triazine resistant cytoplasm in crosses to the cultivar *Westar*. Control of plasmid number could be exercised, for example, by a nuclear-encoded DNA polymerase in the mitochondria. Replication of plasmid DNA could conceivably require interaction between the DNA polymerase and the protein(s) attached to the termini of the plasmid (Erickson et al. 1985). The nuclear gene for this polymerase could undergo a mutation which would prevent such interaction but which would not impair its polymerization function or its capacity to replicate the mt genome.

Presence of RNA band in mitochondrial extracts

Because of the extraction procedure, the mt DNA extracts contain mt RNA as well. If an aliquot of undigested mt DNA is run on a gel, a conspicuous band runs ahead of the plasmid (eg. Fig. 2 A, lane c, marked r). This band is sensitive to RNAse digestion. Initially this band was observed only when the plasmid was present and only in lines which played a role in cms systems. Notably the band was absent in every extract of *Torch,* a cultivar which contains the plasmid in abundance but plays no role in cms systems. This led to the belief that the RNA band was a transcript of the plasmid and that expression of some plasmid gene differed between cms cytoplasms and that of *Torch.* However, the appearance of the band in recent *Bronowski* extracts where no plasmid was detected (Fig. 2 A, B) suggests that this RNA is not transcribed from the plasmid. Although the molecular weight of the RNA molecule cannot be accurately determined from these gels, it would appear to be much larger than the few hundred bases typical of viroids although viroid replicative multimers close to 2,000 b have been observed (Branch et al. 1981). Evidence supporting the viral nature of cms in *Vicia faba* includes the occurrence of a high molecular weight ds RNA molecule in the cytosol (Grill and Garger 1981). The presence of high molecular weight RNA has also been reported in extracts of nucleic acids from mitochondria of cms plants in sugarbeet (Powling 1981).

Examination of chloroplast and nuclear DNA

Hybridization to blots of Pst I digests of cp DNA from *Westar, Torch,* Shiga's nap cms line, *Isuzu-natane, Bronowski,* tr, polima, sterile *Diplotaxis* plants and ogu(S) revealed no homology with plasmid DNA.

Preliminary investigations of nuclear DNA from normal and cms lines also showed the absence of sequences homologous to the plasmid (unpublished data). These studies included nuclear DNA from the plants which had lost the plasmid.

Thus, the *Brassica* plasmid appears to be a true plasmid in that there is no evidence of homologous sequences in all three genomes examined from several lines. However, no correlation was found in this study between the simple presence of the plasmid and the cms trait. The plasmid is found in both male-sterile cytoplasms, maintainer cytoplasms and cytoplasms which play no role in any cms system of rapeseed. Any role it might play in the sterility of cms lines in rapeseed may depend on differential gene expression in the cytoplasms of sterile and fertile plants.

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