Theor Appl Genet (1989) 77:135–141



# Correction of chlorophyll-defective male-sterile winter oilseed rape (*Brassica napus*) through organelle exchange: molecular analysis of the cytoplasm of parental lines and corrected progeny

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Received February 2, 1988; Accepted June 2, 1988
Communicated by Y.Y. Gleba

**Summary.** Cytoplasmic differences between male-fertile and male-sterile *Brassica napus* as well as *Raphanus sativus* were investigated. Plastids of the male-fertile *B. napus* were found to differ from those of male-sterile *B. napus* and *R. sativus* with respect to DNA restriction enzyme patterns. Differences between male-fertile and male-sterile *B. napus* mitochondria were detected not only in the restriction fragment patterns of their DNA, but also at the level of expression by in organello translation of mitochondrial polypeptides.

The chlorophyll deficiency obtained upon transferral of the male-sterility-conferring radish cytoplasm to a winter variety of *B. napus* had been corrected earlier through protoplast fusion. The cytoplasmic composition of the corrected lines was analysed using DNA restriction analysis and in organello translation. The stability of the recombined cytoplasm in the corrected lines was confirmed by analysis of the subsequent seed-derived generation.

**Key words:** Chloroplast DNA – Cybrids – *In organello* mitochondrial translation – Mitochondrial DNA

## Introduction

Hybrid breeding is facilitated if one of the parental lines in the breeding programme carries a male-sterile cytoplasm. One source of cytoplasmic male-sterility (cms) usable in *B. napus* has been found in a Japanese radish variety (Ogura 1968): the radish cytoplasm was backcrossed into *B. napus*, resulting in alloplasmic *B. napus* plants that expressed radish cms. The introduction of radish cytoplasm into *B. napus*, however, results in a chlorophyll deficiency in the cms plants (Bannerot et al. 1977). This is probably caused by an incompatibility between the two unrelated genomes, namely the rapeseed nucleus and the radish chloroplast (Jarl et al. 1988). Pelletier et al. (1983) corrected this deficiency in spring varieties of rapeseed using protoplast fusion, a strategy also used by Jarl and Bornman (1988) in correcting a winter variety of rapeseed. In the latter study, fusion experiments were carried out between a male-sterile, deficient line and a normal, male-fertile line (both of a winter type). Of 95 plants regenerated from such fusions, 2 were selected to have the combined traits of radish mitochondria and rapeseed chloroplasts, as judged visually from their display of male-sterile flowers and green phenotype.

The organellar genomes of male-fertile and male-sterile oilseed rape and radish were compared at the molecular level. The differences obtained between male-fertile rapeseed versus male-sterile rapeseed were used for characterizing the organelles of the two plant lines visually selected for the desired organellar composition. From one of these lines, two lines from the second seed generation were also examined for the stability of the cytoplasmic rearrangements that resulted in male-sterile flowers combined with a green phenotype.

A major difficulty with the molecular investigation of plants produced by protoplast fusion is the limited mass of plant material available for destructive analysis. In this study, micromethods requiring 0.5–1.0 g leaf material for the analysis of organellar DNA and in organello translation of mitochondrial proteins were used.

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## Material and methods

*Plant material*

Two-month-old seedlings were used as leaf material. The plants were grown under normal greenhouse conditions at 24±5 °C and then placed under dark conditions for 4 days prior to use. One line of radish, three different lines of male-fertile oilseed rape and three different lines of male-sterile oilseed rape were used. The three male-sterile lines all had the same cytoplasm – one transferred from radish – but had been backcrossed to different oilseed rape varieties. One of the male-fertile lines and one of the male-sterile lines had been used for protoplast fusions in previous experiments (Jarl and Bornman 1988). These latter two lines, as well as the radish line, were used for DNA analysis, while the four other lines were used in a comparison of the in organello synthesis of mitochondrial protein.

Ninety-five plants were regenerated from the protoplast fusions, from which two were selected as having combined the male-sterility-conferring mitochondria of radish with oilseed rape chloroplasts. These two plants were hand-pollinated, each one with a different rapeseed line. From the lines produced from this first seed generation (designated Lines 1 and 2), plants were selected for analysis. For comparison, plants regenerated from the protoplast fusions but which did not show any rearrangements of the organelles, were also analysed (designated Lines Ms and Mf). For Line 1, a second generation of plants was also obtained by hand-pollinating plants of the first seed generation with different pollinators. Two plant lines of these crossings (designated Lines 1A and 1B) were also analysed.

For the in organello synthesis of mitochondrial proteins, callus produced from the corrected Lines 1 and 2, as well as from Lines 1A and 1B was analysed. The callus was induced from hypocotyl segments and grown through at least two passages on a $B_5$ medium (Gamborg 1968).

*Isolation of chloroplast DNA of parental lines*

Chloroplast DNA was isolated essentially as described by Overbeeke et al. (1984), with the following modifications. A 50 g sample of leaf material was taken from each of the parental lines: the radish parent, the male-sterile oilseed rape line and the male-fertile oilseed rape line. Leaves were deribbed and washed first in water, then in 20 m*M* KCl and 5 m*M* EDTA, and finally in 20 m*M* KCl. The leaf material was ground 4 times for 5 s at high speed in a Waring blender in 300 ml sterile grinding buffer consisting of 330 m*M* sorbitol, 50 m*M* TrisHCl, 5 m*M* EDTA, 0.1% bovine serum albumin and 5 m*M* β-mercaptoethanol, pH 8.0. The homogenate was filtered through 4 layers of 200 µm nylon mesh followed by 2 layers of 30 µm mesh, prior to a 2 min centrifugation at 500 *g*, whereby the filtrate was depleted of nuclei. The supernatant was collected and centrifuged for 5 min at 2000 *g* in order to pellet the chloroplasts. The chloroplast pellet was gently resuspended using an artist's brush, and lysed for 30 min in 50 m*M* Tris, 20 m*M* EDTA, pH 8.0, with a final concentration of 1% *N*-lauroylsarcosine, at room temperature. Covalently closed circular chloroplast (cp) DNA was isolated by centrifugation (40 h, 150,000 *g*, 15 °C) on a cesium chloride/ethidium bromide density gradient (final density 1.55 g/ml, final concentration 500 µg/ml EtBr). Finally, the purified DNA was extracted by ethanol precipitation. All procedures were performed at 4 °C unless stated otherwise.

*Restriction enzyme analyses of cpDNA of parental lines*

Differences among male-fertile versus male-sterile rapeseed and radish with respect to the restriction enzyme patterns of their chloroplast DNAs were screened with the aid of 13 different restriction enzymes: BamHI, EcoRI, HaeIII, HhaI, HindIII, HpaII, KpnI, PstI, PvuI, SacI, SalI, XbaI and XhoI (Boehringer-Mannheim). Reactions were carried out as recommended by the supplier with 3 µg of cpDNA being digested by 10 units of enzyme. The resulting DNA fragments were separated on a 0.7% agarose gel in 0.5 X TAE-buffer (Maniatis et al. 1982) and 0.5 µg/ml ethidium bromide, and electrophoresed overnight at 80 mA. Restriction fragments were visualized on a 302 nm UV transilluminator.

*Analyses of organellar DNA*

From all plant lines, total DNA was isolated by the minipreparation procedure of Dellaporta et al. (1983), in which 0.5 g leaf material yields 3 µg DNA. Restriction enzyme patterns of the total DNA were made using BamHI and EcoRI. Digestion of the total DNA was performed for a 3 h period according to the supplier's recommendation. Horizontal 0.7% agarose gels were prepared as described above and electrophoresed at 80 mA overnight.

After denaturation of the gels, the DNA fragments were transferred to Sleicher and Schüll nitrocellulose membranes (Southern 1975) by a bidirectional procedure (Maniatis et al. 1982). The blots were hybridized with either male-fertile rapeseed chloroplast DNA fragments or plasmid pUC9 derivative containing the 5S/18S ribosomal mitochondrial RNA (kindly provided by Dr. C. S. Levings III, North Carolina State Univ., USA). The probes were nick-translated with $\alpha[^{32}P]$dATP as described in Maniatis et al. (1982). After prehybridization of the blots for 2h at 65 °C, hybridization with the $\alpha[^{32}P]$dATP-labelled probes was carried out overnight at 65 °C. The blots were washed with 2XSSC (300 m*M* NaCl, 30 m*M* Na-citrate), 0.1% SDS for 15 min at 65 °C, followed by 15 min at room temperature, prior to autoradiographing with Kodak Xomat-S film for varying lengths of time.

*In organello synthesis of mitochondrial proteins*

For analysis of in organello translated mitochondrial proteins, a modified micromethod (Boutry et al. 1984) was used. All procedures were carried out under sterile conditions. One g leaf material was used from each plant line. The extraction and purification of the mitochondria were performed according to Boutry et al. (1984). The incubation time of the mitochondrial protein synthesis was increased to 90 min at 25 °C. The synthesis medium was a modification of that published by Boutry et al. (1982): 0.4 *M* mannitol, 5 m*M* $KH_2PO_4$, 2 m*M* $Na_2GTP$, 60 m*M* KCl, 2 m*M* dithiothreitol, 50 m*M* *HEPES*, 10 m*M* $MgCl_2$, 25 m*M* total concentration of the 19 *L*-amino acids (without methionine), 10 m*M* malic acid, 1 m*M* Na pyruvate, 4 m*M* ADP (potassium salt), 0.1% bovine serum albumin, pH 7.0. From each mitochondrial isolation, two incubations in 100 µl of synthesis medium were performed routinely. To 100 µl synthesis medium, 2 µl $^{35}S$-methionine (specific activity 800Ci mmol$^{-1}$, Amersham) having a radioactive concentration of 20 µCi/µl were added.

The samples were solubilized and analysed on a 13% polyacrylamide gel according to Laemmli (1978). The gels were treated with Enhancer (New England Nuclear), dried and fluorographed.

## Results

*Chloroplast DNA*

In order to test which of the restriction enzymes gave the most distinct differences in digestion patterns between

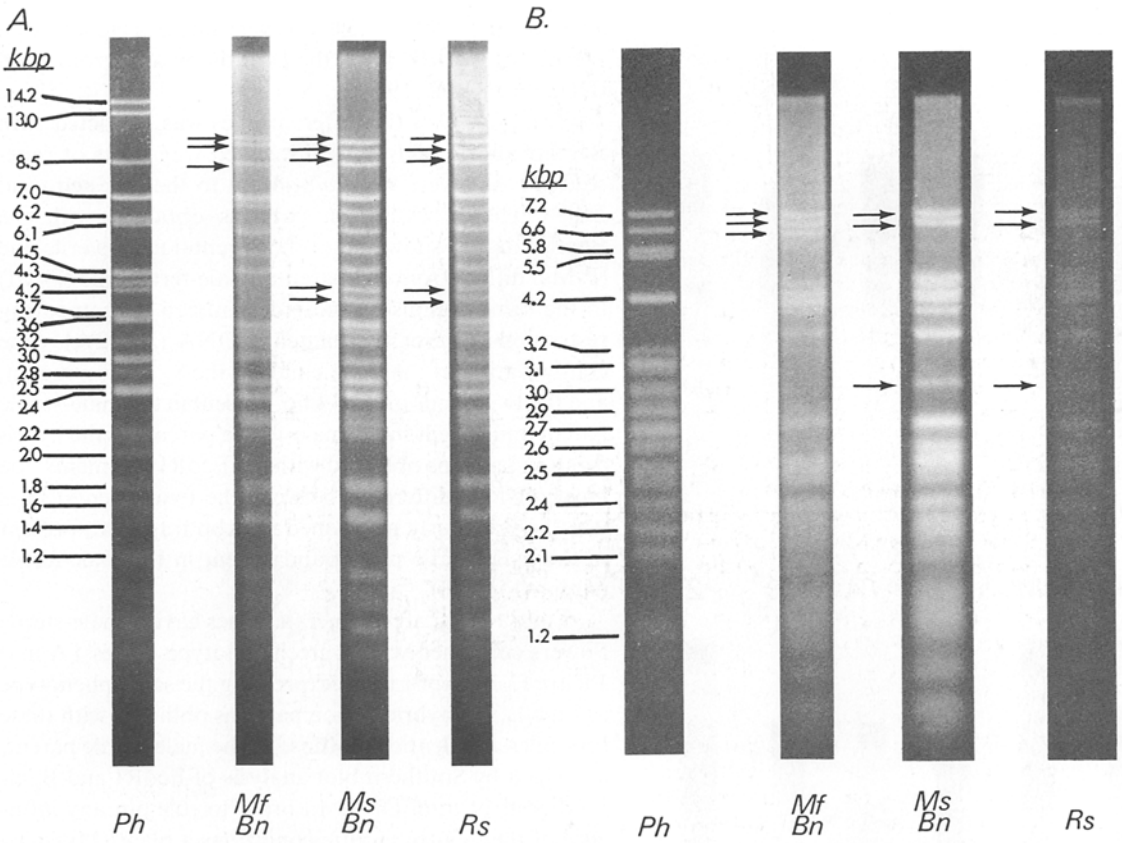


**Fig. 1 A and B.** Chloroplast DNA digested with **A** BamHI and **B** EcoRI. CpDNA was isolated from *Petunia hybrida, Ph*; male-fertile *Brassica napus, Mf Bn*; male-sterile *B. napus, Ms Bn*; and *Raphanus sativus, Rs*. Restriction fragment lengths are given in kilobase pairs (kbp) for *Ph. Arrows* indicate differences in banding patterns, later used for plastid characterization

the male-fertile and the male-sterile rapeseed lines, 13 different enzymes were employed to restrict isolated cpDNA of the two rapeseed types. As the cpDNA of radish was included, the relationships between the three cytoplasms – the rapeseed cytoplasm in the male-fertile line, the cms-conferring radish cytoplasm introduced into rapeseed in the male-sterile line, and the normal radish cytoplasm in the radish – could also be deduced from the obtained results. Of the restriction enzymes, 11 gave differences in digestion patterns between the male-fertile rapeseed line, on one hand, and the radish and the male-sterile rapeseed line on the other. In all cases, DNA of radish chloroplasts exhibited identical digestion patterns, regardless of whether the chloroplasts were in normal radish or had been transferred into rapeseed. To our knowledge, this has not previously been shown.

Two of the restriction enzymes, BamHI and EcoRI, gave a reasonable number of bands as well as showing very distinct differences between the rapeseed chloroplasts of the male-sterile line and those of the male-fertile rapeseed line (Fig. 1 A and B). By comparing the polymorphic fragments with fragments of equal length obtained from digested cpDNA of *Petunia hybrida* R27 (W. A. Bovenberg, personal communication), the molecular weight of the differing fragments could be estimated. The digestion pattern obtained by BamHI (Fig. 1 A) showed the following differences: the two bands at about 8–10 kbp differed in mobility between the lines, while the two bands at 4 kbp present in the chloroplasts of the radish and male-sterile rapeseed were absent in the male-fertile line. With EcoRI (Fig. 1 B), there is a 6.6 kbp band specific for the male-fertile line, while there is a 3.0 kbp fragment that is only present in the male-sterile line and in the radish. By digesting cpDNA with BamHI as well as with EcoRI, the chloroplasts of the male-fertile line could be easily distinguished from those of the male-sterile line. Therefore, further analysis of the chloroplast genomes of the cybrids were performed using BamHI and EcoRI.

In the case of the cybrids, the method used for analysing the plastid genomes of the parental lines, the male-sterile and the male-fertile rapeseed lines, was not applicable. This method requires a large mass of young leaf material (40 g), and the amount of plant material

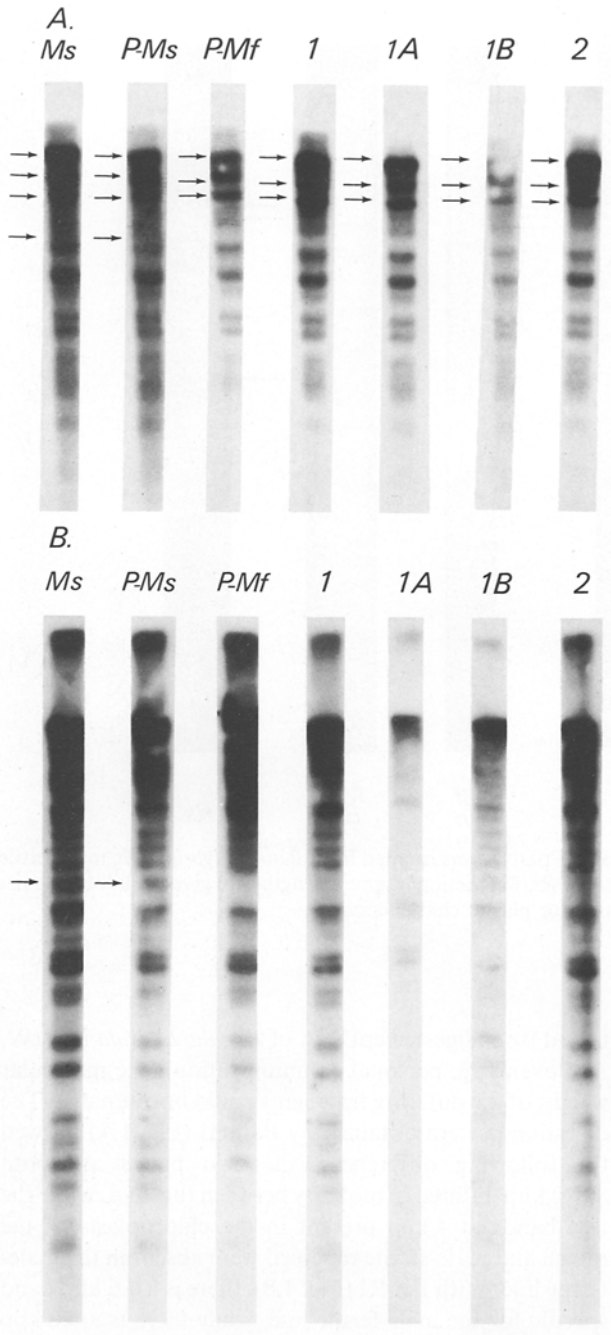


**Fig. 2 A and B.** Hybridization of *B. napus* chloroplast DNA fragments to total DNA digested with **A** BamHI and **B** EcoRI. Total DNA was isolated from male-sterile and male-fertile *Brassica napus* fusion parents (*P-Ms* and *P-Mf*); 1st seed-generation of fusion products (*1* and *2*, male-sterile, green); 1st seed-generation of fusion product (*Ms*, male sterile, chlorophyll defective); and 2nd seed-generation of line 1 (*1A* and *1B*). Lanes have been arranged to aid comparison. *Arrows* indicate characteristic polymorphic fragments

available from the cybrids was very limited. Hence, total DNA was isolated from the cybrids by a micromethod (Dellaporta et al. 1983).

Total DNA of the different lines was restricted with BamHI and EcoRI. Nick-translated fragments of rapeseed cpDNA were then hybridized to the digested total DNA. The hybridization patterns obtained with the BamHI digests showed that the parental male-sterile line (P-Ms) differs from the parental male-fertile line (P-Mf) in the same regions as those determined by comparing restriction patterns of isolated cpDNA (Fig. 2A) – the configuration of the three bands in the 8–10 kbp region, and the two bands in the 4 kbp, present in the male-sterile parent and absent in the male-fertile parent. In the hybridization patterns obtained with the EcoRI fragments, the most obvious difference between the two parental lines was the previously mentioned 3.0 kbp fragment, present in the male-sterile parent and absent in the male-fertile parent (Fig. 2B).

Lines 1 and 2 are regenerated lines having male-sterile flowers combined with a green genotype. Lines 1A and 1B are $F_2$ lines of Line 1, expressing the same phenotype as Line 1. The hybridization patterns obtained with these four lines are identical to those of the male-fertile parent, as judged by Southern blot analysis of EcoRI and BamHI digests of total DNA. In order to rule out any influence of the in vitro culture conditions a plant (Ms), also regenerated after protoplast fusion but having male-sterile flowers and a deficient phenotype, was analysed for comparison. Both with BamHI and EcoRI, the patterns obtained for this plant were identical to those of the male-sterile parent. Thus, the regenerated lines, Lines 1 and 2, did contain the chloroplasts of rapeseed; in addition, the transmission of those plastids to the second generation was stable, at least in Line 1.

### *Mitochondrial DNA*

In order to investigate mitochondrial origin in the regenerated plants, the EcoRI digestions of total DNA were hybridized with a mitochondrial probe, 18S/5S rRNA. Here also, hybridization patterns distinguished between the male-sterile (P-Ms) parent and the male-fertile (P-Mf) parent, as shown in Fig. 3. The regenerated plants, Lines 1 and 2, and the progenital lines of Line 1, all exhibited a hybridization pattern identical to that of the male-sterile parent. For comparison, a regenerated plant with male-fertile flowers and green phenotype was used. This plant showed the same digestion pattern as the male-fertile line (P-Mf). We thus concluded that the male-sterile lines, Lines 1 and 2, contained the mitochondria of the male-sterile parent. The fact that Lines 1A and 1B also showed the pattern of the male-sterile parent proved that the mitochondrial genomes had been transmitted through meiosis to a second generation.

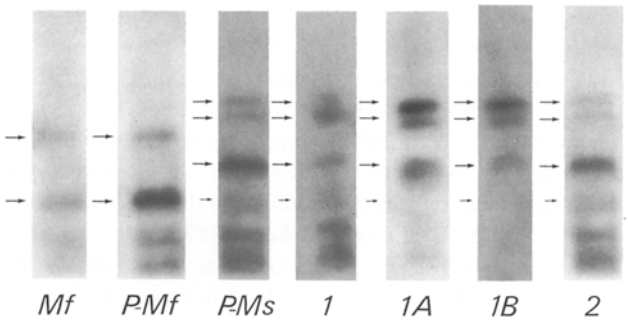


**Fig. 3.** Hybridization of the plasmid pUC9 derivative containing 5S/18S ribosomal mitochondrial RNA to EcoRI digests of total DNA of male-sterile and male-fertile *Brassica napus* fusion parents (*P-Ms* and *P-Mf*); 1st seed generation of fusion products (*1* and *2*, male-sterile, green); 1st seed-generation of fusion products (*Mf*, male-fertile, green); and 2nd seed-generation of Line 1 (*1A* and *1B*). *Arrows* indicate characteristic polymorphic fragments

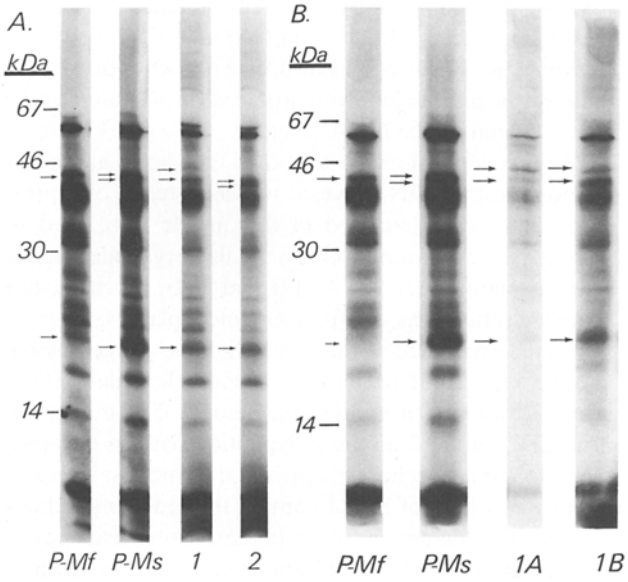


**Fig. 4 A and B.** Polypeptides synthesized by mitochondria isolated from leaf material of male-fertile and male-sterile *Brassica napus*, *P-Mf* and *P-Ms* (fusion parents); **A** 1st seed-generation of fusion products, *1* and *2*; and **B** 2nd seed-generation of Line 1, *1A* and *1B*. Molecular weights in kilodaltons (kDa). *Arrows* indicate polypeptides not common to male-sterile and male-fertile lines

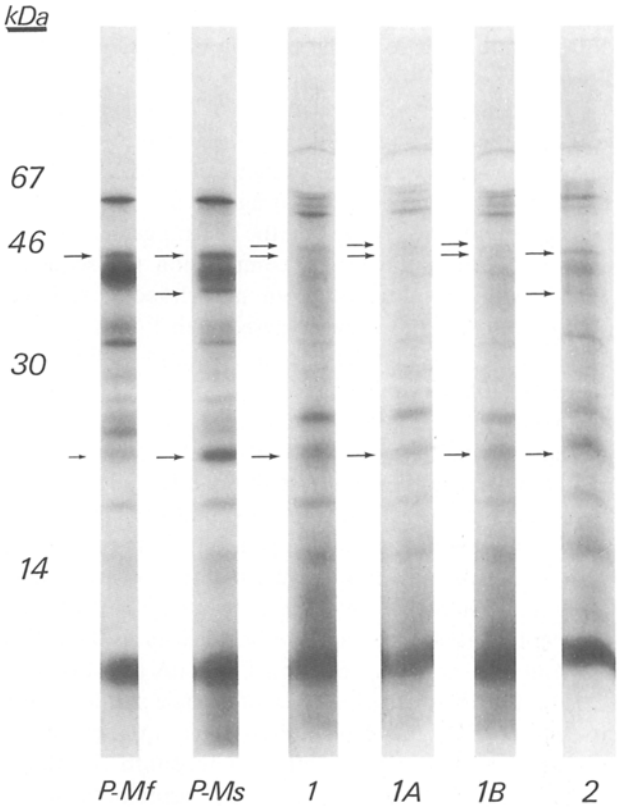


**Fig. 5.** Polypeptides synthesized by mitochondria isolated from callus material of male-fertile and male-sterile *Brassica napus*, *P-Mf* and *P-Ms* (fusion parents); 1st seed-generation of fusion products, *1* and *2*; 2nd seed-generation of Line 1, *1A* and *1B*. Molecular weights of markers in kilodaltons (kDa). *Arrows* indicate polypeptides not common to male-sterile and male-fertile lines

*In organello translation of mitochondrial proteins*

Differences in the expression of the mitochondrial genomes of the male-sterile and male-fertile rapeseed were investigated by comparing the polypeptide patterns of proteins synthesized by isolated mitochondria. In Fig. 4, the patterns of the two parental lines can be compared, and distinct differences can be observed. In the 40 kDa region, the cms line shows a double band, whereas the male-fertile line has only a single band. The polypeptide band at 25 kDa is much more intense in the male-sterile line than in the male-fertile line. Occasionally, this polypeptide was observed to occupy a slightly higher position in the male-fertile line than in the male-sterile line. To further investigate the background of those differences, the two parental lines, which are of winter types, were compared to two other male-sterile and two other male-fertile varieties of spring rapeseed. No difference in the biochemical expression of the mitochondria could be detected between winter or spring varieties. In addition, the different nuclear complements obtained from the different pollinators of line 1, 2, 1 A and 1 B appeared to exert no influence (Fig. 4). The two lines, Lines 1 and 2 (Fig. 4 A), regenerated from protoplast fusion, and the two $F_2$ lines of Lines 1, 1 A and 1 B (Fig. 4 B) were compared with the parental lines, P-Ms and P-Mf. Lines 1, 1 A, 1 B and 2 all showed the green phenotype combined with male-sterile flowers. The banding pattern of Line 2 was identical to that of the male-sterile parent. Line 1 also exhibited the pattern of the male-sterile parent, ex-

cept at the 40 kDa region. Here the upper band of the double band specific to the male-sterile lines showed a slower migration.

This rearrangement is evidently of a stable nature. It is transmitted through meiosis, as the same positional change was observed in the progeny of Lines 1, 1 A and 1 B (Fig. 4 B). Since the pollinating parents of the 3 lines were different, the reason for the rearrangement is unlikely to be within the nuclear composition. Consequently, polypeptides obtained from mitochondria isolated from callus material were analysed with PAGE on gels having a 14% polyacrylamide concentration – the lower concentration previously used gave poorer resolution of the bands. Material obtained from callus differs from leaf-isolated material in the configurational pattern of mitochondrial polypeptides at the 55 kDa and 25 kDa regions. As shown in Fig. 5, the positions of the polypeptides at 40 kDa have now changed, but in this case also, Lines 1, 2, 1 A and 1 B all gave a pattern similar to that of the male-sterile parent. The positional change of the polypeptide at 40 kDa can also be detected in the callus material of Line 1, as well as in Lines 1 A and 1 B.

The phenotypical character of a male-sterile flower associated with the radish cytoplasm appears to be related to a specific polypeptide composition. The linking of specific mitochondrial gene expression of the mitochondria isolated from Lines 1, 1 A, 1 B and 2 to the specific gene expression of the male-sterile fusion parent's mitochondria is taken as proof for the stable inheritance of the cms-conferring radish mitochondria in those lines, namely Lines 1, 1 A, 1 B and 2.

## Discussion

Out of the 95 plants regenerated from protoplast fusions, 2 were selected visually to have combined the green phenotype conferred by rapeseed chloroplasts of the male-fertile fusion partner, on the one hand, with the cytoplasmic male-sterility of the radish mitochondria from the male-sterile fusion partner, on the other. The cytoplasm of the male-sterile fusion partner had been introduced from radish. By restriction enzyme analysis of the plastid DNA of male-fertile and male-sterile rapeseed and radish, the male-fertile line was shown to differ from both the male-sterile line and radish (Figs. 1 and 2). The two latter gave identical digestion patterns with all the restriction enzymes used.

The chlorophyll deficiency of the male-sterile rapeseed (Bannerot et al. 1977; Jarl et al. 1988) could be related to the observed polymorphism of the rapeseed and radish chloroplast genomes. In male-sterile rapeseed, the rapeseed nucleus and the radish chloroplasts are involved in many processes in the plastids that require the coexpression of both nuclear and plastid genomes.

The cytoplasms of male-fertile and male-sterile rapeseed were also distinguished by characteristic polypeptide patterns produced by isolated mitochondria in an in organello translation system (Figs. 4 and 5). The specificity of expression of the mitochondria was influenced by the nuclear environment, but it was always associated with the phenotypical character of male-sterility. In another translation system, Vedel et al. (1982) also detected differences between male-fertile and male-sterile *B. napus*. Under their conditions, the differing polypeptides were located in the 40 and 15 kDa regions. Male-sterility has also been found in other species to be associated with specific polypeptide patterns, although the polypeptides specific for the male-sterile lines were of varying molecular weight: *Nicotiana tabacum,* 18 kDa (Boutry et al. 1984); *Vicia faba,* 52, 25 and 19 kDa (Boutry and Briquet 1982; Boutry et al. 1984); *Sorghum bicolor,* 82, 65, 42 and 12 kDa (Bailey-Serres et al. 1986); *Beta vulgaris,* 32 kDa and 21 kDa (Boutry et al. 1984); as well as in different male-sterility-conferring cytoplasms of maize, 58–84, 42, 21, 17.5, 16, 15.5, 13 kDa (Forde et al. 1978; Forde and Leaver 1980; Forde et al. 1980). The mitochondria of the male-sterile and male-fertile rapeseed lines could also be characterized at the DNA level.

The specific chloroplast (at the DNA level) and mitochondrial (at the DNA level as well as in terms of expression) characters described in this article were used to distinguish the parental origin of the organelles in the selected plant Lines 1, 1 A, 1 B and 2. In all cases, our visual selection was confirmed. Chloroplasts of the regenerated lines were of rapeseed origin, as judged by the specific restriction patterns of their chloroplast DNA after digestion with EcoRI and BamHI. The mitochondrial DNA as well as the polypeptide pattern obtained from isolated mitochondria proved that the two lines and the progeny lines of Line 1 contain the radish mitochondria of the male-sterile fusion parent. Thus, visual selection for the green phenotype and male-sterile flowers also truly selected for rapeseed chloroplasts and radish mitochondria.

*Acknowledgements.* C. I. Jarl was supported by the Royal Physiographic Society in Lund, Sweden and by Hilleshög Research AB, Landskrona, Sweden. We also thank Prof. C. H. Bornman for critical reading of the manuscript and Dr. A. J. Kool for valuable discussions and suggestions.

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