Comparative Macromolecular Analysis of the Cytoplasms of Normal and Cytoplasmic Male Sterile *Brassica napus*

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Summary. Chloroplast (cp) and mitochondrial (mt) compartments of normal (N) and cytoplasmic male sterile (cms) lines of *Brassica napus* have been characterized and compared on the basis of cp and mt DNA restriction enzyme analysis and in vitro protein synthesis by isolated mitochondria. Cytoplasmic male sterility of *B. napus* (rape) comes from cms *Raphanus sativus* (radish) through intergeneric crosses.

Cp DNAs isolated from N and cms lines had distinct restriction patterns with Sal I, Kpn I and Sma I enzymes. The size of the two cp DNAs measured from the restriction patterns was found to be identical and of about 95 x 10⁶ d. N and cms lines of *B. napus* were characterized by specific mt DNAs, as shown from Sal I, Kpn I, Pst I and Xho I cleavage patterns. The small number of well-separated restriction fragments obtained with Sal I enabled us to determine precisely mt DNA sizes. The values of 136.5 and 140.3 x 10⁶ d, obtained from restriction patterns with N and cms DNAs respectively, are smaller than any of those previously obtained from studies on other genera. With molecular hybridization experiments, it was possible to distinguish N and cms lines by the different locations of rRNA genes on the cp and mt DNAs.

Two lines of *B. napus* are characterized by specific mt translation products formed in isolated mitochondria.

Key words: Chloroplast DNAs – Mitochondrial DNAs – Cp and mt r RNA genes – In vitro mitochondrial translation – Maternal inheritance

Introduction

It is well known that the cytoplasm is an important source of heritable variability in eukaryotic organisms. In higher plants, one of the best examples of this type of inheritance is cytoplasmic male sterility, a trait that causes pollen abortion in the anthers. This trait has been extensively used in numerous higher plants for the production of commercial hybrids. There is some biochemical evidence that the mitochondrion, rather than the chloroplast, is the carrier of male-sterility factors in maize, wheat and tobacco. Restriction endonuclease analysis has revealed marked differences between the mitochondrial (mt) DNAs from normal and male-sterile cytoplasms in both maize and wheat, but the corresponding chloroplast (cp) DNAs were indistinguishable (Levings and Pring 1976; Quétié and Vedel 1977). More recently, it has been shown that mitochondria from lines carrying the T, S, C or normal (N) cytoplasms of maize can all be distinguished from one another on the basis of the polypeptides synthesized in vitro by isolated mitochondria (Forde et al. 1980) and low molecular weight mitochondrial DNAs (Kemble et al. 1980). Spontaneous reversion to fertility in S male-sterile cytoplasm of maize has been correlated with the disappearance of the mt plasmid-like DNA's, S-1 and S-2, and the appearance of new mt DNA restriction fragment patterns in the revertants (Levings et al. 1980). In the case of tobacco, the cms trait has been studied in cytoplasmic hybrids obtained from protoplast fusion between two varieties of *N. tabacum*. Restriction enzyme analysis of the cytoplasmic DNAs of different hybrids has shown that only one or the other parental cp DNA was present in the hybrids and their progeny (Belliard et al. 1978) regardless of the phenotype. However, the mt DNAs of hybrids were different from those of the parents and from the mixture of the two, indicating that mt DNA is the cytoplasmic support of cytoplasmic male sterility in tobacco (Belliard et al. 1979).

The rape *Brassica napus* is a natural amphidiploid (AACC genome, 2n=38) combining the chromosomal
basic sets of *B. oleracea* (CC genome, 2n = 18) and *B. campestris* (AA genome, 2n = 20). Cytoplasmic male sterility of *B. napus* comes from intergeneric crosses involving a Japanese line of *cms Raphanus sativus* (Ogura 1968; Bannerot et al. 1974). Unfortunately, the *cms* lines of *B. napus* are chlorophyll deficient preventing their use in agronomy. In this study, cp and mt compartments of N and *cms* lines of *B. napus* have been characterized and compared on the basis of cp and mt DNA restriction enzyme analysis and in vitro protein synthesis by isolated mitochondria.

**Materials and Methods**

**Species Examined**

N and *cms* lines of *B. napus* (Table 1) were grown in a greenhouse of the phytotron in Gif-sur Yvette at 22 °C and 16 h day-length. *Cms* lines of *B. napus* were obtained by sexual crosses involving a *cms* Japanese radish variety (Ogura 1968; Bannerot et al. 1974):

<table>
<thead>
<tr>
<th>female cms <em>R. sativus</em> × male <em>B. oleracea</em></th>
<th>(n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. oleracea</em></td>
<td>(n = 9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>female cms <em>B. oleracea</em> × male <em>B. napus</em></th>
<th>(n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. napus</em></td>
<td>(n = 19)</td>
</tr>
</tbody>
</table>

**Isolation of cp and mt DNAs**

cp and mt DNAs were isolated from well-expanded leaves as previously described by us using CsCl-ethidium bromide gradients (Herrman et al. 1975; Kolodner and Tewari 1975) with some modifications (Vedel et al. 1980). Furthermore, mitochondria were purified by centrifugation in discontinuous sucrose gradients (30%, 40%, 50%, 60% steps in buffer A from Herrman et al. 1975). Mitochondria were removed from the 40%–50% interface, slowly diluted with three volumes of homogenizing medium and recovered (20,000 g, 15 min).

**DNA Restriction and Agarose Gel Electrophoresis**

Two to 4 µg of cp or mt DNA were digested in 30 µl reactions with sufficient enzyme to give complete digestion. The restriction enzymes used were *Sal I*, *Xho I*, *Pst I*, prepared as indicated by Vedel et al. (1980), *Sma I* (Boehringer Mannheim) and *Kpn I* (Bethesda Research Laboratories). The restriction fragments were separated by electrophoresis in 0.7% agarose vertical slab gels, 20 or 40 cm long. The procedures of gel staining and ultra violet fluorescence photography have previously been described (Quétier and Vedel 1977).

A mixture of DNA fragments generated from *λ* DNA by Hind III and from *λ* DNA by Hind III + EcoRI (Boehringer Mannheim) was used as a molecular weight standard. Molecular weights of *B. napus* cp and mt DNAs and band multiplicity were determined by the method of Vedel et al. (1980).

**DNA/RNA Hybridization**

After denaturation, the DNA fragments in gels were transferred to nitrocellulose membrane strips (Schleicher and Schüll BA 85) according to the standard Southern procedure (1975) modified to allow overnight elution from slab gels. For hybridization, DNA strips (0.4 x 20 cm) were soaked with 150 µl of hybridization medium containing 100–500 ng (32P) RNA in 5 x SSC (0.75 M NaCl – 0.075 M sodium citrate), 50% formamide. Strips were dipped in a paraffin oil bath at 42 °C for 24 h. After hybridization, strips were washed in 2 x SSC successively for 30 min at 30 °C, 30 min at 45 °C, 30 min at 65 °C and 2 h at 65 °C, before being dried and autoradiographed for 1 to 3 days using Ilford X-ray films (Rapid R, type S).

**Protein Synthesis by Isolated Mitochondria**

Seeds were surface sterilized with sodium hypochlorite and germinated in darkness at 22 °C on sterilized gauze moistened with sterile distilled water. Mitochondria were isolated as mentioned above for mt DNA preparation except that the 4-step sucrose gradients did not contain bovine serum albumin (normally present in buffer A). The band at the 40% /50% sucrose interface was removed and diluted to an osmolarity of 0.6 M.

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**Table 1. B. napus lines used in the experiments**

<table>
<thead>
<tr>
<th>Lines</th>
<th>Cytoplasms</th>
<th>Floral phenotype</th>
<th>Agronomic type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jet 9</td>
<td><em>B. napus</em></td>
<td>normal</td>
<td>winter oil-seed</td>
</tr>
<tr>
<td>R 48</td>
<td><em>B. napus</em></td>
<td>normal</td>
<td>winter oil-seed</td>
</tr>
<tr>
<td>Br Br</td>
<td><em>R. sativus</em></td>
<td>cms</td>
<td>spring oil-seed</td>
</tr>
<tr>
<td>S 82</td>
<td><em>R. sativus</em></td>
<td>cms</td>
<td>winter oil-seed</td>
</tr>
<tr>
<td>S 109</td>
<td><em>R. sativus</em></td>
<td>cms</td>
<td>winter forage</td>
</tr>
</tbody>
</table>

Jet 9 was provided by Coopérative du Hurepoix, Limours, France; other lines were produced by Station d’Amélioration des Plantes, INRA, Rennes-Le Rheu, France.

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**Isolation and Labeling of cp and mt rRNAs**

Organelles, organelle ribosomes and rRNAs were successively isolated as previously described (Whitfeld et al. 1978; Bonen and Gray 1980). Purified rRNAs were labeled with (32P) ATP in vitro following the technique of Maizels (1976) with the following modifications: rRNAs in a solution of 100 mM Tris-HCl pH 9.5 (0.5 µg/µl), were partially hydrolyzed by heating in sealed 20 µl-microproperts at 95 °C, 3 min - 50 µl 10 mM Tris-HCl (pH 7.4), 1 mM spermidine, 0.1 mM EDTA were added to 10 µl of the heated RNA solution and the mixture held at 50 °C for 3 min. Then, to label the 5' ends, the following was added: 10 µl 500 mM Tris-HCl (pH 9.5), 50 mM dithiothreitol, 100 mM MglyCl2, 100 p moles (32P)ATP (Amersham ref. PB 10168, specific activity >2000 Ci/m mol), and several units of T4 polynucleotide kinase (Boehringer Mannheim) to make a final volume of 100 µl. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 200 µl 2 M ammonium acetate and 50 µg E. coli tRNA. The rRNAs were precipitated three times with cold ethanol to remove unreacted (32P)ATP. The specific activity of the labeled rRNAs was greater than 106 cpm/µg.

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**Protein Synthesis by Isolated Mitochondria**

Seeds were surface sterilized with sodium hypochlorite and germinated in darkness at 22 °C on sterilized gauze moistened with sterile distilled water. Mitochondria were isolated as mentioned above for mt DNA preparation except that the 4-step sucrose gradients did not contain bovine serum albumin (normally present in buffer A). The band at the 40% /50% sucrose interface was removed and diluted to an osmolarity of 0.6 M.