MITOCHONDRIAL PROTEIN SYNTHESIS IN FERTILE AND MALE STERILE CANOLA

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INTRODUCTION

The recent interest in hybrid rapeseed (canola, Brassica napus and B. campestris) is based on substantial yield increases among F_1 hybrids and has led to the search for efficient pollination control methods. Cytoplasmic male sterility (CMS) is one such system known to function in canola. Several alien cytoplasms (nap, ogu, pol) are known to confer CMS in canola.

Polima (pol) cytoplasmic male sterility was first described by Fu (1981) in the rapeseed cultivar 'Polima' and is an attractive cytoplasm for use in the production of hybrid spring canola seed. All oilseed rape cultivars tested were shown to be maintainers or partial maintainers of the pol cytoplasm induced male sterility (Fan et al. 1986). Recently, two non-allelic male fertility restorer genes (Rfp1 and Rfp2) have been found in two winter-type rape cultivars (Fang and McVetty 1989).

Among the problems with this cytoplasm are the effect of elevated temperatures on the reversion of flowers from male-sterile to fertile, and the yield penalty associated with it.

The biochemical and molecular mechanisms of cytoplasmic male sterility in several crop species are currently under investigation (see review by Levings and Brown 1989). In an effort to conduct similar biochemical and molecular studies with canola mitochondria, we have developed 'in organello' mitochondrial protein synthesis and fractionation techniques. This technique will be described in relation to models of the causes of cytoplasmic male sterility.

MATERIALS AND METHODS

Mitochondria were extracted from aseptic seedlings, immature flower buds or cell suspension cultures. Seeds of Brassica napus cvs. Westar, Regent, or other fertile lines and sterile lines containing the Polima cytoplasm were surface sterilized in calcium hypochlorite (saturated solution) for 25 min. followed by three rinses in sterile distilled H_2O. Seeds were germinated in vitro on moist sterile filter paper in Magenta TM, containers and maintained in darkness at 25°C for 6-8 days as a source of aseptic seedlings.

Plants were grown in soil in growth chambers with 20°/15°C day/night temperatures under a 16 hr photoperiod supplied by fluorescent and incandescent lights. Buds were harvested from 6-8 week-old plants, extraneous materials (leaves, petals, etc.) were removed, and the flower buds were rinsed with distilled H_2O, 70% ethanol, and four times with cold, sterile distilled H_2O.

Cell suspensions of Brassica and Arabidopsis have been initiated and maintained as described previously (Gleddie 1989). Cells are routinely subcultured in MS medium containing 2% sucrose, and 2 mg/L 2,4-

dichlorophenoxyacetic acid (2,4-D) in 125 mL Erhlenmeyer flasks, at 25°C on a reciprocating shaker (150 rpm). Cells were subcultured into fresh medium 4 days prior to mitochondrial extractions.

In each of the three cases, aseptic seedlings, immature flower buds, or cell suspension cultures, the mitochondrial purification and labelling procedure was the same.

Extraction of mitochondria from plant tissue was performed by grinding with a mortar and pestle (cell suspensions) or by blending (seedlings, flower buds). The homoginization buffer of Kohmoto et al. (1986) was used with 4 g of washed XAD-7 (Sigma) resin per 1 g of tissue. The crude mitochondrial preparation obtained by differentiation centrifugation was further purified by ultracentrifugation on a discontinuous Percoll gradient (13.5/21/45%) in a Beckman SW-28 rotor at 15,000 rpm for 30' at 4°C. Purified mitochondria were collected from the 21/45% interface with a 21 gauge needle and washed in resuspension buffer (250 mM sorbitol, 2.5 mM HEPES pH7.2).

Mitochondrial protein synthesis "in organello" was performed essentially as described by Leaver et al. (1983). The reaction mixture contained 250 mM manitol, 90 mM KCl, 10 mM, $MgCl_2$, 10 mM tricine, 5 mM KH_2PO_4 and 1 mM EGTA pH 7.2. To 150 uL of this reaction mixture was added 50 uL of mitochondria, 40 uCi of ^{35}S methionine, 2.5 uL of 100 mM GTP, 2.5 uL of 200 mM DTT, 25 uL of a 250 uM solution of unlabelled amino acids (minus methionine), and 50 uL of energy generating solution (40 mM phosphocreatine, 30 mM ATP, 125 ug creatine phosphokinase). Mitochondria were incubated for 85 min. at 22°C with constant shaking at 120 rpm and then they were chased with unlabelled methionine (20 mM) for 5 min.

Mitochondria were lysed, and proteins were precipitated in cold acetone at -20°C overnight. The pellet was resuspended in lysis buffer containing PMSF and leupeptine and an aliquote was removed for scintillation counting.

Gel electrophoresis was performed on a 12-17% linear gradient polyacrylamide gel with a 5% stacking gel for the sepration of polypeptides. Samples were diluted to equivalent numbers of radioactive counts, loaded and run at 5mA constant current overnight.

Gels were stained with Coomassie Blue and then incubated in 'Enhance' (NEN) prior to drying. Dry gels were exposed to Kodak XAR or Amersham Hyperfilm in Kodak cassettes for 3-10 days.

Mitochondrial ATP synthase subunits (F_o complex) were extracted from the isolated mitochondria using an organic solvent mixture as described by Michon et al. (1987). Mitochondria were suspended in resuspension buffer (250 mM sorbitol, 2.5 mM HEPES, pH 7.2 + PMSF + leupeptine). Extraction was performed with ten volumes of chloroform:methanol (1:1) for 18 hr with shaking at 25°. The suspension was centrifuged at 3000 xg, 5 min, and the supernatant was filtered. The organic extract was washed with chloroform and water, mixed and centrifuged at 3000 rpm 10 min. Proteins were dried under vacuum at 40°C. The dry pellet was dissolved in chloroform:methanol (2:1) and stored at -20°C for 10 min. Proteins were precipitated with 4 volumes of cold diethyl ether at -20°C for 2 hr. The precipitate was dissolved in chloroform:methanol (2:1).

Fractionation of hydrophobic polypeptides was performed on a Phenomenex W-POREX C4 column (4.6 x 250 mm) with a flow rate of 1 mL/min. Solvent A consisted of 1% methanol, 0.1% Tri fluoroacetic acid (TFA) in water and solvent B consisted of 1% methanol, 0.1% TFA in acetonitrile.

RESULTS

Morphological

Male sterile canola plants with the Polima cytoplasm appear vegetatively very normal although this cytoplasm has dramatic effects on anther development (Fig. 1). These effects of the pol cytoplasm on Brassica appear to be exerted prior to pollen mother cell formation. Tapetal degeneration is not apparently responsible for the lack of microsporogenesis within the young anthers of male sterile plants. Rather, it appears that a general lack of differentiation occurs very early in development, resulting in predominantly dedifferentiated cells within the locules of the anthers (Figs. 2, 3). Further histological work is required to precisely locate the causes for this lack of differentiation, although our investigations have not indicated any ultrastructural differences between the mitochondria of sterile or fertile anthers.

Biochemical

The procedure of 'in organello' protein synthesis is a suitable method of comparing the mitochondrial and cytosolic polypeptides which may be associated with cytoplasmic male sterility (Boutry et al. 1984). Highly purified mitochondrial preparations free of plastid and cytosolic contamination are essential for studies of mitochondrial protein synthesis. The use of etiolated seedlings for mitochondrial extractions has proven advantageous since chloroplast contamination can often be avoided. It is essential that bacterial and fungal contamination be avoided, and for this reason the use of aseptically maintained shoots or cell suspension cultures is very effective. By the use of selective antibiotics which inhibit either cytosolic or plastid ribosomes, it is possible to eliminate any background contamination from the preparation and to verify that the protein synthesis is occurring on mitochondrial ribosomes (Fig. 4).

It is also essential that the isolated mitochondria be metabolically active which usually means that they be intact. Coupled oxidative phosphorylation is essential for the generation of ATP in the energy-requiring polypeptide synthesis reactions. Since many Brassicaceae are known to produce phenolic compounds upon homogenation, the use of the nonionic polyacrylate adsorbent resin (XAD-7, Sigma) is recommended. This helps to prevent damage to the mitochondrial membranes by the release of phenolics, oils, and organic acids.

In general we have found that protein synthesis in isolated mitochondria of Brassica proceeds rapidly in organello and that after 90 min of labelling the mitochondria can be lysed and examined (Figs. 4, 5). The separation of the mitochondrial polypeptides may be by polyacrylamide gel electrophoresis (PAGE) followed by antoradiography to differentiate cytosolic from mitochondrially-encoded proteins. The few mitochondrially-encoded proteins lend themselves to 1 dimensional separation, whereas 2-D separation would be required to separate and identify those polypeptides which are synthesized cytosolically and transported into the mitochondria.

There are both qualitative and quantitative differences between sterile and fertile mitochondrial proteins, (Fig. 5) however, high pressure liquid chromatography (HPLC) is an alternative method of polypeptide separation with increased sensitivity. This may allow the identification of polypeptides that are under- or over-represented in the

mitochondria of male sterile plants. We have used this approach to identify the apparent under-representation of a component of the Fo complex of ATPase among Polima mitochondria.

CONCLUSIONS

In organello protein synthesis is a method which has been employed for the identification of variant polypeptides among the mitochondria of male sterile plants of several species (Boutry et al.). We have also employed this technique for the biochemical analysis of the Pol cytoplasm of canola. In addition, a useful technique has been the coupling of in organello protein synthesis with HPLC separation of specific oxidative reaction complexes. We have used this approach to identify apparent irregularities between the Fo ATPase complexes of fertile and sterile mitochondria. Further molecular and biochemical data will be required to determine the precise molecular interactions between pol mitochondria and canola nuclei that result in CMS.

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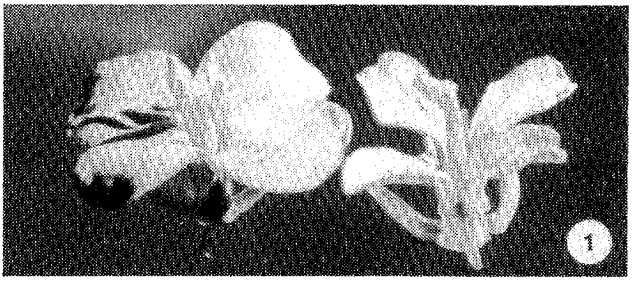


Fig. 1. Flowers of B. napus fertile line (left) and CMS line (right).

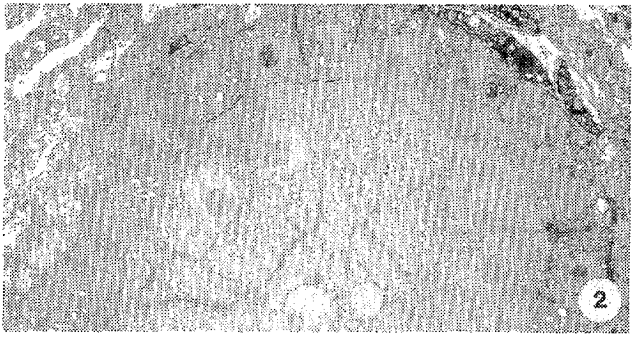


Fig. 2. Electron micrograph (EM) of an immature anther of a fertile Regent plant, displaying well developed pollen mother cells surrounded by the tapetal cell layer.

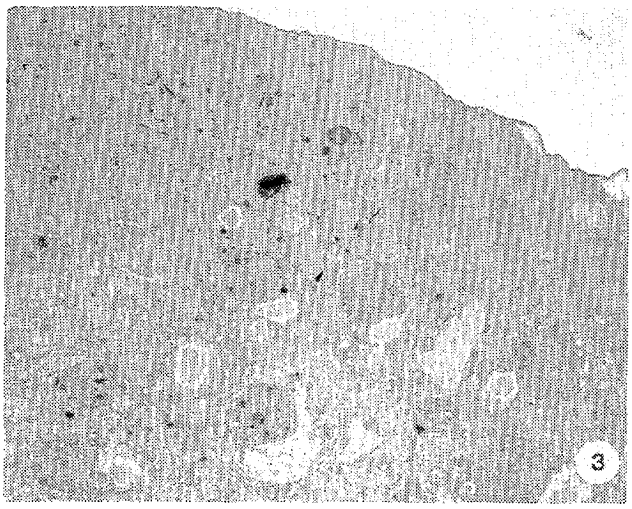
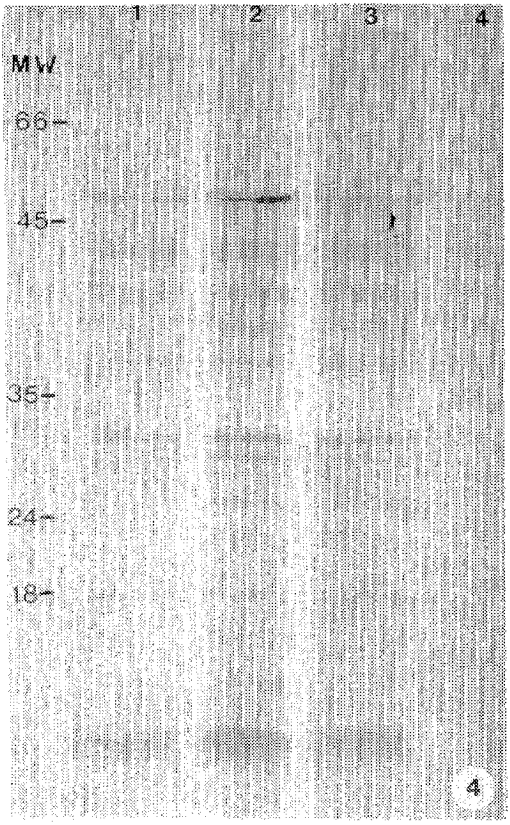


Fig. 3. EM of immature anther of a sterile Regent pol plant (right), showing the failure of PMCs to develop.



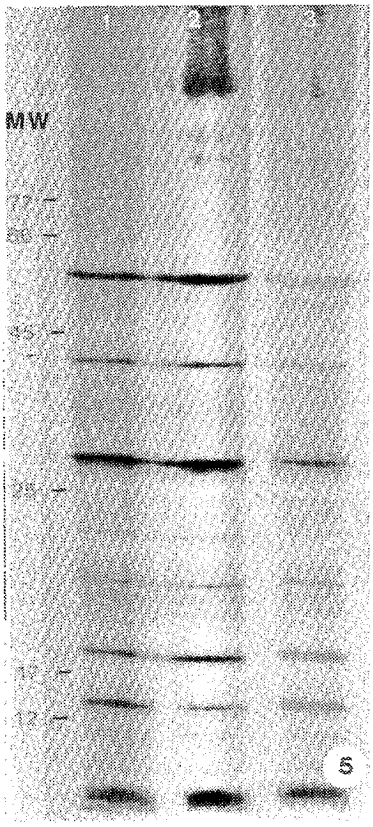


Fig. 4. Polypeptides synthesized "in organello" isolated mitochondria of canola cv. Westar. Lane 1 - protein synthesis is not sensitive to cycloheximide. Lanes 2, 3, protein synthesis "in organello" in the absence of antibiotics. Lane 4 - protein synthesis in mitochondria is sensitive to chloramphenicol. MW markers in Kilodaltons.

Fig. 5. Polypeptides synthesized 'in organello' by mitochondria isolated from seedlings of fertile and sterile cv. Regent. Lane 1 pol Regent, lane 2 pol Regent, lane 3 fertile Regent.