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Two Dimensional Analysis of Chloroplast Proteins from Normal and Cytoplasmic Male Sterile *Brassica napus*

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Summary. Stromal and thylakoid proteins isolated from normal (N) and cytoplasmic male sterile (cms) lines of *Brassica napus* have been compared using a two dimensional gel separation. It has been shown that:
1) stromal compartments of the two lines were very similar;
2) although there was extensive homology between protein maps of thylakoids isolated from the two lines, these could be distinguished by the spots corresponding to the β subunits of the coupling factor CF_1 from the ATPase complex.

Key words: 2 D electrophoresis – Thylakoid proteins – Coupling factor – ATPase – *Brassica napus* – Male sterility

Introduction

Rape, *Brassica napus* L., is a natural amphidiploid (AACC genome, 2 n = 38) containing the chromosomal sets of *B. oleracea* (CC genome, 2 n = 18) and *B. campestris* (AA genome, 2 n = 20). Cytoplasmic male sterility of *B. napus* comes from intergeneric crosses involving a japanese line of cytoplasmic male sterile (cms) radish *Raphanus sativus* (Ogura 1968; Bannerot et al. 1974).

Chloroplast and mitochondrial DNAs of N and cms lines of *B. napus* have been characterized and compared using restriction enzyme analysis. Identical restriction patterns were found for chloroplastic DNAs from the cms *B. napus* lines and the cms lines of the japanese radish used to transfer the cms trait into *B. napus* (Vedel et al. 1983). More recently, physical maps of chloroplast DNAs from the two lines have been constructed and compared using the enzymes Sal I, Sma I, Bgl I and Kpn I (Vedel and Mathieu 1982).

In this study, chloroplast proteins from stroma and thylakoids of N and cms lines of *B. napus* have been characterized and compared using a two-dimensional polyacrylamide gel separation.

Materials and Methods

Plant Materials

The scheme of sexual crosses involved in the obtaining of cms *B. napus* has been previously published (Vedel et al. 1982). N and cms lines of *B. napus* were grown in a greenhouse of the phytotron in Gif-sur-Yvette at 22 °C and 16 h daylength.

Preparation of Chloroplast Proteins

After removal of the mid-ribs, about 50 g of rape leaves were homogenized at 4 °C with approximately 150 ml of cold 50 mM Tris-HCl buffer pH 8.2 containing 0.35 M sucrose in a Waring-blender at full speed, three times at 5 s each. The slurry was filtered through eight layers of cheesecloth plus one layer of nylon blutex (pore size 25 μ) and centrifuged at 1000 × g for 10 min.

The plastid pellet was osmotically disrupted in 50 mM Tris-HCl pH 8.2 containing 1 mM phenylmethylsulfonylfluoride using a teflon/glass homogenizer and recentrifuged at 20,000 × g for 15 min. The supernatant containing stromal proteins was collected and concentrated in an ultrafiltration cell Amicon fitted with a Diaflo membrane UM-10 and immediately stored in liquid nitrogen until use. The pellet containing insoluble materials was resuspended in the same buffer and purified on a discontinuous gradient made of three sucrose layers (1.5, 1, 0.5 M in 50 mM Tris) and centrifuged at 60,000 × g for 1 h. The purified thylakoid fraction was collected at the interphase between the 1 and 1.5 M sucrose layers and concentrated by centrifugation.

Solubilization of Chloroplast Proteins

Stromal and thylakoid fractions containing about 3 mg of proteins (Lowry et al. 1951) were precipitated by an ice-cold 80% acetone solution containing 35 mM mercaptoethanol. For thylakoid proteins, it was necessary to repeat this treatment two or three times in order to remove pigments and lipids which could interact with proteins and change their characteristics, a source of electrofocusing artifacts.

The protein pellets were solubilized in 200 µl of a mixture containing 0.0625 M Tris-HCl pH 6.8, 20% glycerol, 2% mercaptoethanol and 1.5% SDS. Nonidet P 40 was added to give a final Nonidet/SDS ratio of 8 : 1. After homogenization, the solubilization was completed by heating at 70 °C for 10 min. Then, after cooling, 10 µl of 40% ampholine (LKB, pH 5–8) were added to the sample. This solubilization technique (Rémy and Ambard-Bretteville, unpubl.) was a modification of two methods (Ames and Nikaido 1976; O'Farrel 1975).

2 D-Gel Electrophoresis

Electrofocusing (1st dimension) was performed in glass tubes (100×3 mm inner diameter). For 8 gel tubes, 10 ml of the following gel solution were prepared: 5.5 g Urea, 2 ml glass-distilled water, 2 ml 10% (w/v) Nonidet P 40, 1.5 ml of a 26% acrylamide solution (25% acrylamide Serva 2× crystallised and 1% bisacrylamide Eastman chemical Co in water, 500 µl ampholine LKB pH 5–8, 20 µl of a fresh 10% persulfate solution, 15 µl N.N,N'N'-tetramethylene diamine. The tubes were filled with this solution and overlayed with water. After polymerization, the water was removed and the tubes were put into an acrylophor apparatus (Pleuger). The solubilized samples containing about 250 µg of proteins in 40 µl were loaded on the top of each gel and overlayed with 20 µl of a solution containing 10% glycerol and 1% ampholine. Electrofocusing was carried out at 400 V for 16 h with NaOH 0.02 M in the cathode chamber and 0.01 M H_3PO_4 in the anode chamber.

After running, the pH gradient was measured on 0.5 cm long gel pieces. Each gel piece was immersed for 3 h in 1 ml boiled water then the pH was taken and values were reported on a calibration curve.

The second dimension was essentially performed according to O'Farrel (1975), using the SDS-PAGE technique of Laemmli (1970), in a special apparatus made in the laboratory, which allows the running of two gels in parallel (Rémy and Ambard-Bretteville, unpubl.).

Staining of proteins was done with Coomassie brilliant blue R 250. In the 2 D-separations of N and cms thylakoid polypeptides, the identification of the α and β subunits of the

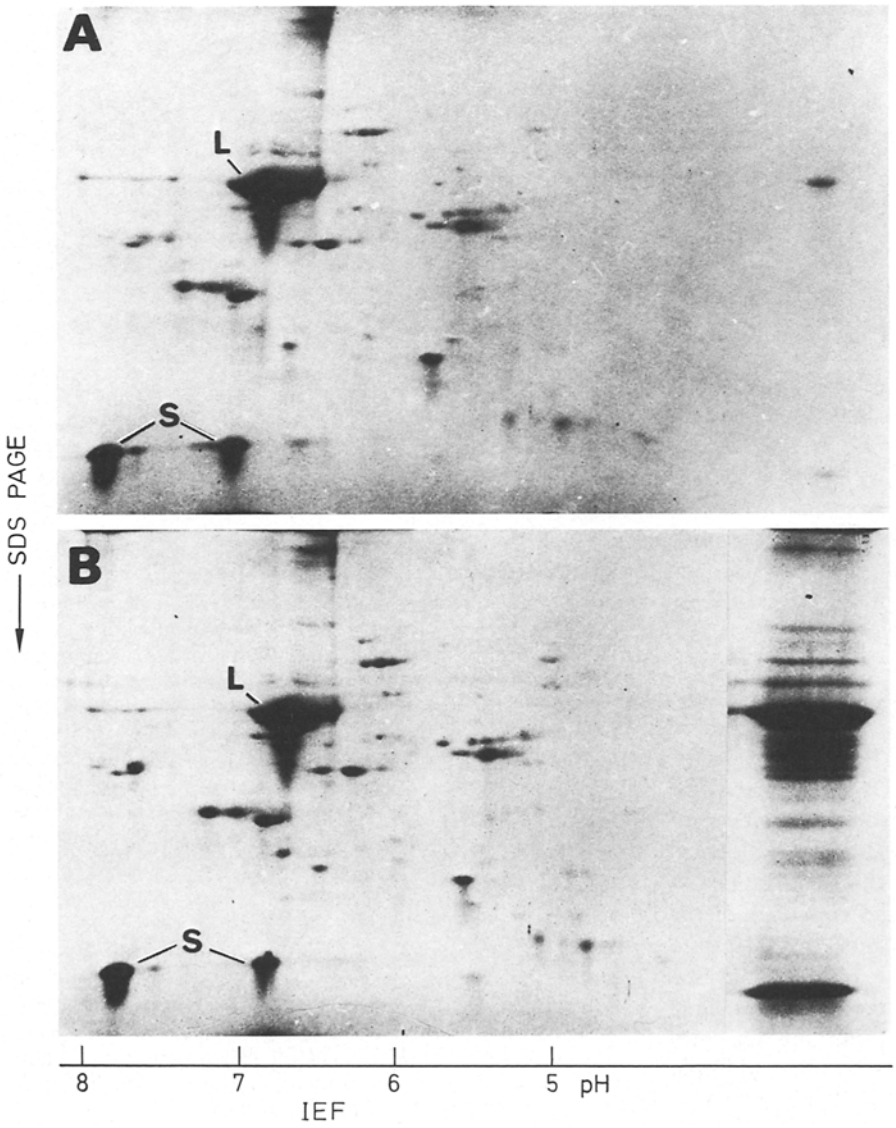

Fig. 1 a and b. Chloroplast stromal polypeptides separated on 2 D-gel electrophoresis and stained with Coomassie blue. **a** normal *B. napus;* **b** cms *B. napus. L* and *S,* large and small subunits of RuBPcase

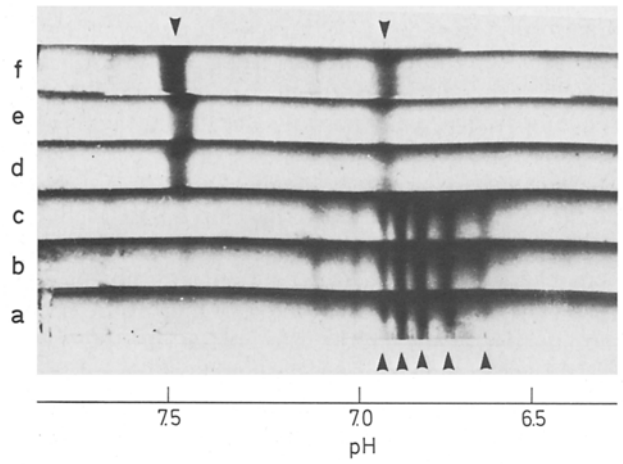

Fig. 2. Electrofocusing on cylindrical gels of RuBPcase subunits. **a** large subunits of *a* N *B. napus; b* cms *B. napus; c* mixture of N and cms *B. napus.* **b** small subunits of *d* N *B. napus; e* cms *B. napus; f* mixture of N and cms *B. napus.* The different types of subunits are marked with arrows

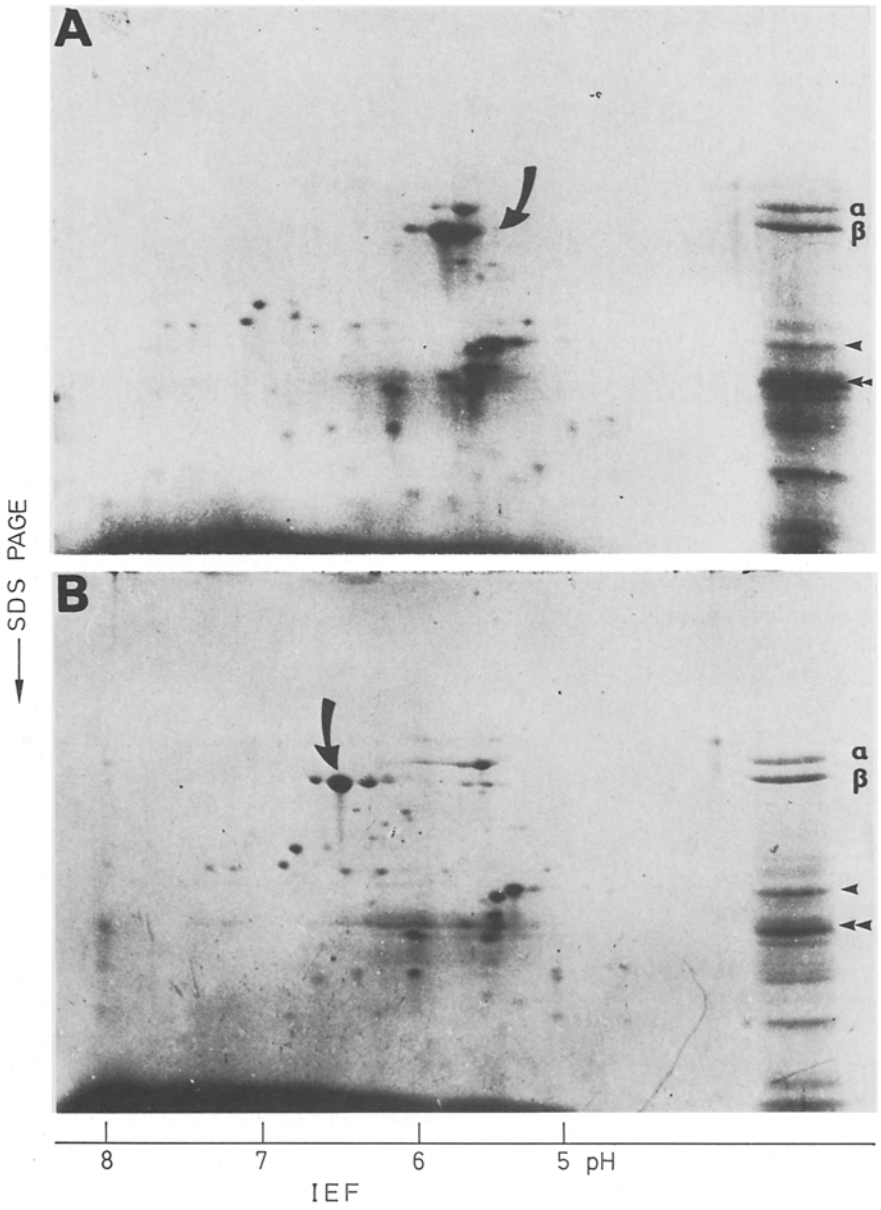

Fig. 3a and b. Thylakoid polypeptides separated on 2 D-gel electrophoresis and stained with Coomassie blue. **a** normal *B. napus;* **b** cms *B. napus.* The big arrows indicate the position of β subunits of coupling factor. On the right side of the figure are one dimensional SDS-PAGE of thylakoid polypeptides. Single and double arrows indicate respectively the position of a 32 Kd polypeptide and apoproteins of light harvesting chl a/b protein (25 Kd)

chloroplast ATPase was performed by comparison with the respective position of these subunits in 2 D-separations realized with coupling factors purified according to Lien and Racker (1971).

Results and Discussion

Two-dimensional Separation of Stromal Proteins from Chloroplasts of N and cms B. napus

Figure 1 a and b show these separations. A close homology is observed between the protein maps of the two lines. Only some minor qualitative or quantitative differences are observed.

More than 50% of the total protein is represented by the large (L) and small (S) subunits of the enzyme ribulose-1.5-bisphosphate carboxylase, each focusing at several positions in the gel. Since the enzyme is known as a good nuclear and chloroplast marker (review; Von Wettstein et al. 1978), it is surprising that no differences are detected between the two lines. RuBPcase subunits have indeed been extensively used:

1) to determine the origin and evolution of some higher plants (Gray et al. 1974; Kung et al. 1975; Chen et al. 1975; Strøbaek et al. 1976; Gatenby and Cocking 1977, 1978).

2) to characterize the different chloroplast genomes present in *Oenothera* (Holder 1976, 1978).

3) to confirm some somatic hybrid plants (Kung et al. 1975; Melchers et al. 1978; Poulsen et al. 1980).

4) to investigate some aspects of cytoplasmic male sterility (Chen et al. 1975).

Consequently, more detailed analyses of N and cms *B. napus* RuBPcase were undertaken. RuBPcase from the two rape lines was purified by preparative electrophoresis. In the same way, their subunits were purified by SDS-PAGE and then analysed by a single isoelectric focusing. It is clear in Fig. 2 that no difference is observed between the two lines. Each of them is composed of 5 similar large subunit bands (three majors and two minors) and two comparable small subunits bands.

Two-dimensional Separation of Thylakoid Proteins from N and cms B. napus

As seen on the right side in Fig. 3 a and b, N and cms thylakoid polypeptides could not be distinguished by one dimensional SDS-PAGE analysis. However 2 D-analysis reveals a striking difference in the isoelectric focusing position of the cms β subunit of the chloroplast ATPase.

Additional differences in positions of polypeptides with unknown function can also be observed. No difference is observed in the 32 Kd region (single arrow) corresponding to cytochrome f and two other polypeptides which are extensively studied at present. It is noteworthy that the 25 Kd region corresponding to apoproteins of the light harvesting chlorophyll a/b protein, generally gives a bad resolution in 2 D-gels, resulting from streaks possibly due to the hydrophobicity of these polypeptides.

Since it is known that subunit β as well as α and ε of the ATPase are encoded by chloroplast DNA in spinach (Mendiola-Morgenthaler et al. 1976), it has been checked if the characteristics of the subunit β of the ATPase in the cms of rape originates from the introduction of the radish chloroplasts into the rape. Fig. 4 a and b show that the β subunits of the cms rape and radish indeed have the same isoelectric points. Moreover, it can be observed that some polypeptides

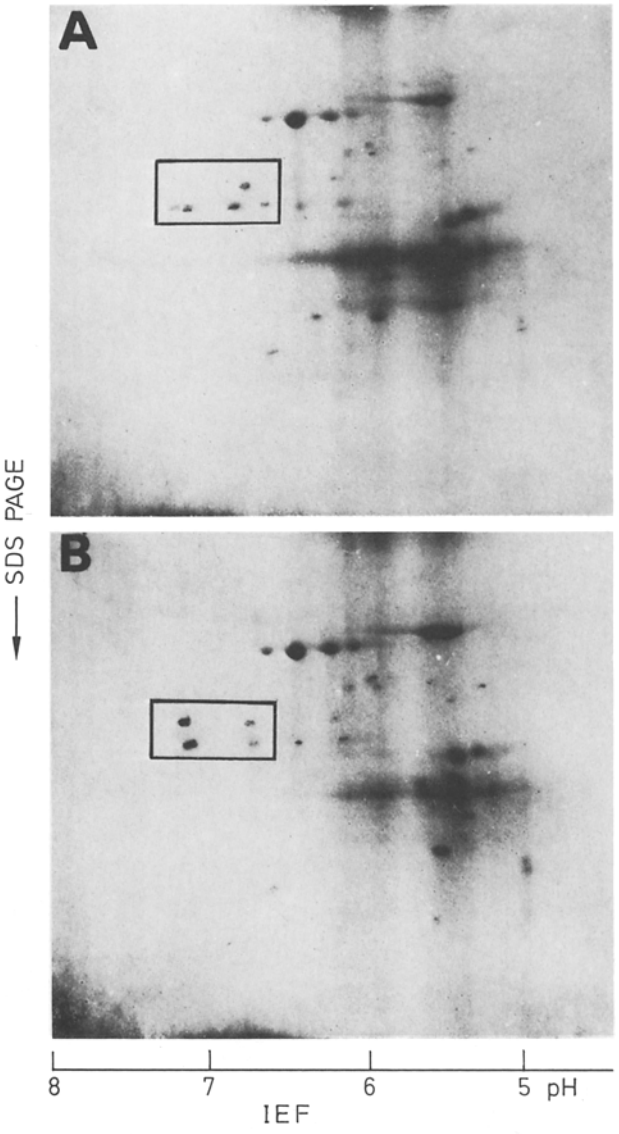

Fig. 4 a and b. Thylakoid polypeptides separated on 2 D-gel electrophoresis and stained with Coomassie blue. **a** cms *B. napus;* **b** *Raphanus sativus.* The area outlined indicates polypeptides which differ in **a** and **b**

with unknown function in the area outlined of Fig. 4b (radish) differ in position from those in Fig. 4a (cms rape). It is possible that these are encoded by nuclear genes since they appear at similar positions in the N and cms rape.

Conclusions

The highly sensitive and reproducible 2 D-gel technique used here to analyse the composition of chloroplast polypeptides allowed us to distinguish the N and cms chloroplast proteins. The technique is a good tool to screen for differences in proteins before proceeding with more detailed investigations. The striking difference observed here for the β subunits of chloroplast ATPase which could not be detected with the one dimensional SDS-PAGE analysis is a good example. In this case, the coupling factor (ATPase) subunits could serve as chloroplast markers in the 2 D-gel technique while the large subunit of RuBPcase was not distinguishable by isoelectric focusing.

The question whether the difference observed in the isoelectric focusing position of the cms and N β subunits of the chloroplast ATPase is due to different conformational states of the same polypeptide chain or represent polypeptides with different primary structure makes new investigations necessary.

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