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Variant mitochondrial protein and DNA patterns associated with cytoplasmic male-sterile lines of *Nicotiana*

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Summary. Variation in mitochondrial protein synthesis and genome organization was investigated. Three different alloplasmic cytoplasmic male-sterile *Nicotiana tabacum* cultivars, carrying *N. repanda, N. suaveolens* or *N. debneyi* cytoplasm, were analysed together with corresponding male-fertile parental and restored material. Although several differences were detected in the proteins synthesized by isolated mitochondria from the male-sterile and male-fertile plants, most of these were related to the origin of the mitochondria. However, a 23 kD protein was synthesized in the male-sterile cultivar carrying *N. debneyi* mitochondria, but not in other lines containing this cytoplasm. This protein was also present in the male-fertile parent containing *N. tabacum* mitochondria. Only the enhanced production of a 30 kD protein in the lines carrying mitochondria from *N. repanda* or *N. debneyi* was exclusively correlated with CMS. This protein was not present in any of the corresponding male-fertile parental and restored lines. Restriction enzyme analysis of mitochondrial DNA revealed a difference in abundance of a 5.6 kb XhoI fragment between lines containing *N. debneyi* mitochondria. No rearrangements of mitochondrial DNA was found between male-fertile and male-sterile lines carrying *N. repanda* or *N. suaveolens* cytoplasm. These results might indicate that CMS in alloplasmic *Nicotiana* cultivars is caused by alterations in the expression of mitochondrial genes, rather than by induced changes in the genome.

Key words: Cytoplasmic male sterility – Mitochondria – Translation products – DNA – *Nicotiana*

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Introduction

In a CMS plant the inability to produce viable pollen is maternally inherited. This trait has been reported in many plant species (Edwardsson 1956, 1970; Laser and Lersten 1972) and is used by plant breeders as a very helpful tool in the production of hybrid seed. In a few species the basis for CMS has been more carefully studied (Hanson and Conde 1986). There is now strong evidence showing that the mitochondrion is the cytoplasmic factor involved in CMS (Leaver and Gray 1982). Differences in mitochondrial DNA restriction patterns and protein synthesis between CMS and male-fertile lines have been reported for several plant species (Forde and Leaver 1980; Boutry et al. 1984; Hanson and Conde 1986). These differences, however, have not yet led to the formulation of a unifying explanation for the developmental abnormalities found in CMS phenotypes.

In several of the most extensively studied plant species the CMS phenotype is associated with rearrangements of specific regions of mitochondrial DNA, resulting in the synthesis of altered proteins. In maize, for example, as many as seven recombination events in the mitochondrial DNA of the CMS-T type have been reported. These mainly involve the 26S rRNA gene and result in a chimeric gene (Dewey et al. 1986). A new open reading frame results that encodes a 13 kD protein specific for the CMS-T cytoplasm (Forde et al. 1978; Wise et al. 1987b). Reversion to fertility is associated with a deletion (Rottmann et al. 1987) or a small insertion (Wise et al. 1987a) in the chimeric gene, resulting in a loss of the 13 kD protein. In *Petunia,* CMS is associated with a chimeric gene involving ATPase 9, cytochrome oxidase II (coxII) and an unidentified reading frame (URF) (Young and Hanson

Table 1. Species origin of nuclei and mitochondria in sets of male-fertile, male-sterile and restored cultivars of *Nicotiana*

Type	Origin of nucleus	Fertility status[d]	Origin of mitochondria	Reference
Set 1				
A[a]	*N. tabacum*	MF	*N. tabacum*	
B	*N. repanda*	MF	*N. repanda*	
C[a]	*N. tabacum*	CMS	*N. repanda*	Burk (1967)
D[a]	*N. tabacum*	RestMF	*N. repanda*	Gerstel et al. (1978)
Set 2				
A[a]	*N. tabacum*	MF	*N. tabacum*	
B	*N. suaveolens*	MF	*N. suaveolens*	
C[a]	*N. tabacum*	CMS	*N. suaveolens*	Schweppenhauser and Mann (1968)
D[a]	*N. tabacum*	RestMF	*N. suaveolens*	Reed and Burns (1986)
Set 3				
A[b]	*N. tabacum*	MF	*N. tabacum*	
B	*N. debneyi*	MF	*N. debneyi*	
C[b]	*N. tabacum*	CMS	*N. debneyi*	Sand and Christoff (1973)
D[b]	*N. tabacum*	RestMF[c]	*N. debneyi*	Sand and Christoff (1973)

[a] Nuclear background: SC 58
[b] Nuclear background: Kupchunos
[c] This restoration is incomplete. The plants are still male sterile but the development of the corolla is normal and so is the development of stamen through meiosis of pollen mother cells
[d] MF: male fertile; CMS: cytoplasmic male sterile; RestMF: restored, male fertile

1987). This rearranged sequence is transcribed in CMS plants together with normal ATPase 9 and coxII genes. Antibodies raised against the URF sequence of the chimeric gene bind to a protein unique to the CMS cytoplasm (Nivison and Hanson 1986). Sorghum lines carrying the CMS-inducing 9E cytoplasm produce a 42 kD coxI subunit instead of the 38 kD subunit detected in other sorghum cytoplasms (Dixon and Leaver 1982). At least two rearrangements have occurred in the 9E mitochondrial DNA resulting in a 303 bp extension of the coxI gene and synthesis of the higher molecular weight protein (Bailey-Serres et al. 1986b).

According to Edwardsson (1970), a CMS plant can arise in one of four ways; i.e., through intergeneric, interspecific or intraspecific crosses, or by spontaneous origin. In the first three cases, the nucleus from one species or cultivar is combined with a foreign cytoplasm carrying organelles of a different origin. Since the was in which a CMS cultivar originated can be hard to determine, great care has to be taken in interpreting differences between CMS and male-fertile lines. In *Nicotiana,* numerous CMS cultivars of known background have been constructed (Gerstel 1980). These cultivars are, therefore, excellent materials to use for molecular analysis of CMS.

In this study, investigations are described of mitochondrial DNA and proteins in three different CMS cultivars of *Nicotiana.* Each male-sterile cultivar expresses abnormal stamen development of a different type. By selecting these materials, correlations between CMS and mitochondrial gene products can be sought. Furthermore, the possibility can be investigated that rearrangements of mitochondrial DNA, induced by nuclear-mitochondrial incompatibility, account for CMS.

Materials and methods

Plant material

Three sets of *Nicotiana* plant materials wer used (Table 1). The CMS (C) and restored (D) lines have all been produced from sexual crossings (see Table 1 for references). In each set the nuclear background for the male-fertile (A), male sterile (C) and restored (D) cultivars of tobacco is identical, except for the introduction of the genetic material that restores the CMS trait. In set 1, a restorer chromosome is present in homozygous condition, giving a somatic chromosome number of 50 (Gerstel et al. 1978). In set 2, the restored line has only 48 chromosomes, yet some material is incorporated in a homozygous condition in one pair of *N. tabacum* chromosomes (Reed and Burns 1986). In set 3, the restored line has one chromosome added to the *N. tabacum* genome, giving 49 chromosomes (Sand and Christoff 1973).

Tobacco seeds of the lines were kindly provided by Dr. D. S. Reed, North Carolina State University, Raleigh, USA (A, C and D, set 1), Dr. D. E. Wernsman, North Carolina State University (A, C and D, set 2) and Dr. S. S. Sand, Roswell Park Memorial Institute, New York, USA (A, C and D, set 3). Seeds of *N. repanda, N. suaveolens* and *N. debneyi* were obtained from Dr. V. Sisson, Tobacco Research Station, Oxford, North Carolina.

Plants were grown in greenhouse or climate chambers with 16 h light, 20 °C at day and 10 °C at night. The restored line (D) in set 3 was propagated by cuttings to preserve the unpaired restorer chromosome.

Isolation and purification of mitochondria for in organello protein synthesis

Isolation and purification of mitochondria from green leaves was caried out as described by Boutry et al. (1984), with some modifications. All procedures were performed under sterile conditions. After a dark treatment of 2–3 days the youngest leaves from 8–10 week old plants were used. The first slow spin centrifugation was performed for 2 min in 50 ml tubes. After pelleting, the mitochondria were resuspended in 0.5 ml of suspension medium and layered on a discontinous Percoll gradient containing 0.375 ml of a 50%, 0.50 ml of a 26% and 0.50 ml of a 13,5% Percoll/sucrose solution in 2.2 ml microtubes (Eppendorf). From each isolation 2 × 100 µl of the mitochondrial fraction were collected.

In organello protein synthesis

Protein synthesis was performed according to Boutry and Briquet (1982) with slight modifications. Each aliquot of the purified mitochondria was incubated in 100 µl of synthesis medium [0.4 *M* mannitol, 60 m*M* KCl, 50 m*M* HEPES, 10 m*M* $MgCl_2$, 10 m*M* malic acid, 5 m*M* KH_2PO_4, 4 m*M* ADP, 2 m*M* Na_2GTP, 2 m*M* dithiothreitol, 1 m*M* Na pyruvate, 0.1% BSA, 25 µ*M* of each of 19 amino acids (no methionine), pH 7.0] with 30 µCi ^{35}S-methionine (Amersham). Incubation was carried out for 90 min in 20 ml tubes on a shaker. After incubation 5 µl of each sample was used to test for bacterial contamination. Labeled mitochondria were lysed in 30 µl of sample buffer [2% SDS (BDH Chemicals), 62.5 m*M* Tris-HCl pH 6.8, 10% glycerol, 1% dithiothreitol and 0.01% bromophenol blue] by the use of a whirl mixer and then stored at −20 °C.

SDS-polyacrylamide gel electrophoresis

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli 1970) in 12.5% or 14%, 20 cm long, vertical slab gels. Samples containing 100 000 cpm of labeled proteins were loaded in each lane. The proteins were solubilized for 3–5 min at 100 °C. Molecular weight standard proteins (MW 14 000–70 000; Sigma) were used as size markers. After electrophoresis, gels were stained with Coomassie brilliant blue, destained, treated with Enlightning (New England Nuclear) and dried. The proteins were visualized by exposing the gels to Kodak X-Omat S film for 4–7 days at −80 °C.

Isoalation of organelle DNA

For isolation of mitochondrial and chloroplast DNA 12–19 weeks old plants were used. Mitochondrial DNA was isolated according to the method of Bland et al. (1985), with some modifications. Young leaves, 30–40 g, were ground in several batches in a mortar and pestle together with homogenizing buffer (0.3 *M* mannitol, 3 m*M* EDTA, 50 m*M* Tris-HCl pH 7.5, 0.1% BSA, 10 m*M* β-mercaptoethanol, 15 m*M* $Na_2S_2O_5$ and 10 mg/ml PVP-40). Large contaminating materials were removed by 2–3 centrifugations at 1000 × *g*, 10 min each. Mitochondria were collected and then treated with DNase. The mitochondria were then lysed by incubation with sarkosyl/proteinase-K at 60 °C for 1 h and mitochondrial DNA was purified in a CsCl gradient. The DNA was precipitated twice with ethanol and dissolved in Tris-EDTA buffer.

Chloroplast DNA was isolated as previously described (Sundberg et al. 1987).

Organelle DNA digestion and agarose gel electrophoresis

Mitochondrial and chloroplast DNA was digested with XhoI, BamHI, PvuII or EcoRI (Pharmacia) and restriction fragments were separated by electrophoresis in 0.4% or 0.5% agarose slab gels (20 × 20 × 0,5 cm) with the addition of 0.5 mg/l of EtBr. Lambda DNA digested with HindIII together with EcoRI was used as molecular weight markers. The electrophoresis was run at a constant current starting at 80 mA for 10 min and then run for 15 h at 40–45 mA. Gels were photographed with Polaroid 665 film under UV light.

Results

In organello protein synthesis by isolated mitochondria

for all the *Nicotiana*-species examined, the size and number of proteins synthesized by their mitochondria were highly conserved (Fig. 1). About 15 proteins within the size range of 10–55 kD were found. A few differences were apparent, some of which were related to the species of origin of the mitochondria. For example, the 15 kD protein synthesized by *N. tabacum* mitochondria was slightly smaller (about 14.5 kD) in mitochondria of *N. repanda* origin (Fig. 1 a). Similarly, the 17.5 kD protein synthesized by *N. tabacum, N. repanda* and *N. suaveolens* mitochondria was found as an 18 kD protein in *N. debneyi* (Fig. 1 c). *Nicotiana debneyi* mitochondria also synthesized a 17 kD protein not seen in the other species (Fig. 1 d). Finally, a 16 kD protein synthesized by mitochondria from *N. tabacum* does not appear in any of the other species. These differences were present irrespective of whether the mitochondria were isolated from parental species, CMS or restored lines.

In some sets, differences in the protein pattern were dependent on the nuclear environment of the mitochondria. For example, a protein of 23 kD was synthesized in set 3, lines A and C (Fig. 1 c), but not in lines B or D. Mitochondria from *N. suaveolens* (lane B, Fig. 1 b) synthesized a 14.5 kD instead of the 15 kD protein synthesized by all other lines in this set.

In addition to the differences already described, an enhanced production of a 30 kD protein was found in the CMS lines in sets 1 and 3 (lane C, Fig. 1 a and c). After long exposure a weaker 30 kD band could be seen in the restored lines of both set 1 (data not shown) and set 3 (Fig. 1 d), and a very faint band also appeared in lanes A and B of the two sets (data not shown). However, there was always a higher intensity of the 30 kD protein band in the CMS lines.

Of all the differences recorded including presence, position and intensity of the protein bands, only the changes in the 30 kD protein found in sets 1 and 3 was exclusively correlated with the appearance of male sterility and restoration of fertility.

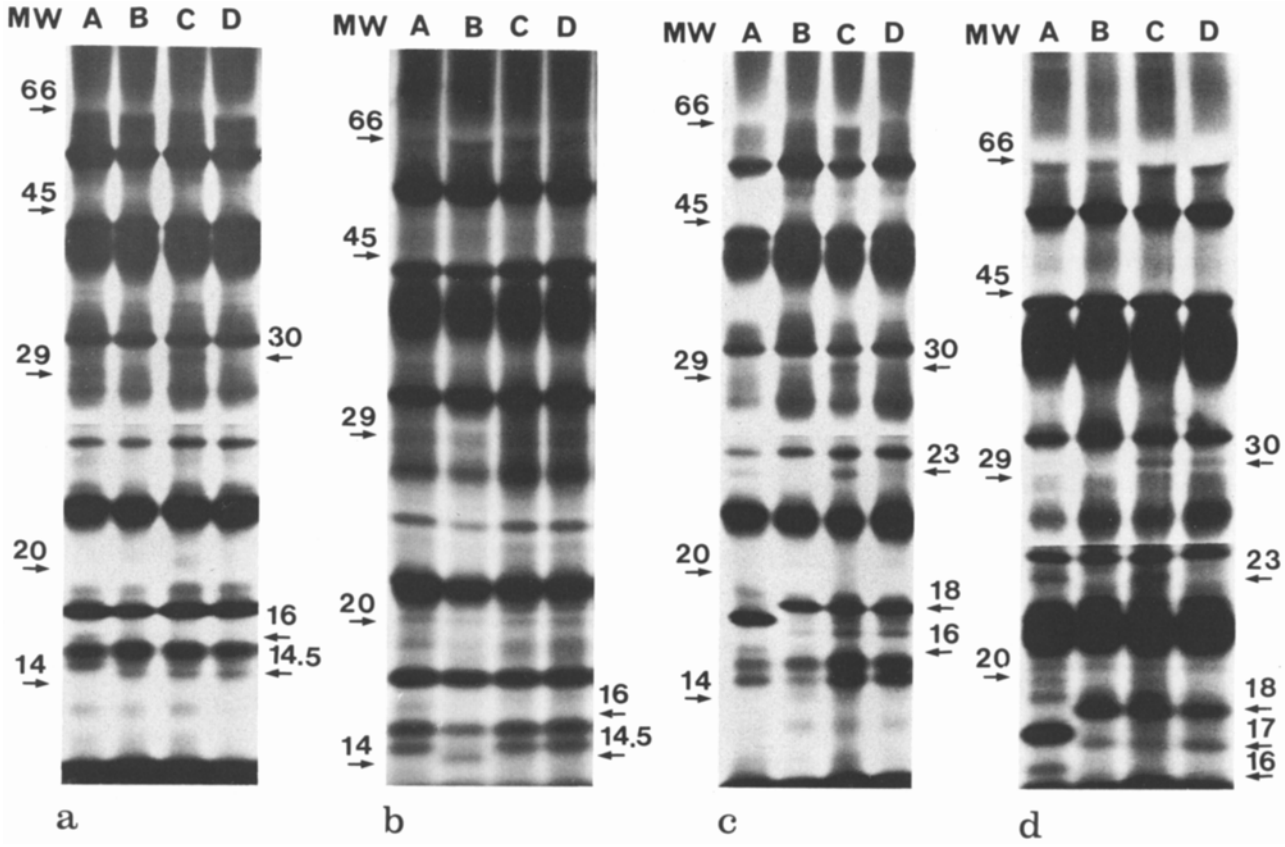

Fig. 1 a–d. Polypeptides synthesized by isolated mitochondria from male-fertile and male-sterile lines of *Nicotiana* (see Table 1 for more detailed description of the lines). The molecular weights of marker proteins are indicated (kD). Variable proteins are indicated with estimated molecular weights (kD). **a** *A: N. tabacum,* MF; *B: N. repanda,* MF; *C: N. rep,* CMS; *D: N. rep,* RestMF. **b** *A: N. tab* MF; *B: N. suaveolens,* MF; *C: N. suav,* CMS; *D: N. suav,* RestMF. **c** and **d** *A: N. tab,* MF; *B: N. debneyi,* MF; *C: N. debn,* CMS; *D: N. debn,* RestMF. In order to visualize weaker bands in **a, c** and **d,** the exposure time of the lower half of the gel is 2.5 times that of the upper half

Mitochondrial DNA analysis

In order to ascertain if any of the changes in protein patterns associated with CMS could result from changes in the mitochondrial genome, mitochondrial DNA was isolated from the parental species, the CMS lines and the restored lines. The DNA was restricted with the enzymes BamHI, EcoRI, PvuII and XhoI. The pattern of DNA fragments for each of the four species of *Nicotiana* could easily be discriminated with all four enzymes. No differences in the restriction patterns between the lines containing mitochondria of *N. repanda* or *N. suaveolens* origin were found (Fig. 2a and b). However, with XhoI, a difference between the lines containing *N. debneyi* mitochondria was observed. A fragment of 5.6 kb was found in male-fertile *N. debneyi* (Fig. 2c, lane B). This band was much fainter in the restored line (D) and extremely faint in the CMS line (C). Chloroplast contamination can be excluded as an explanation for this band since chloroplast DNA from *N. debneyi* does not show any fragment of this size (chl). Besides the 5.6 kb band, two barely detectable sub-stoichometric fragments (about 10 and 6 kb) were detected in male-fertile *N. debneyi* (data not shown).

Discussion

In all three CMS lines of *Nicotiana* studied in this investigation, male sterility was associated with a disturbance in development of the stamens that occurs at different developmental stages and results in different phenotypic expression of CMS. In male-sterile material with *N. suaveolens* mitochondria, CMS is manifested at a very early stage, due to arrest of stamen primordia shortly after they are initiated (Rosenberg and Bonnett 1983). The *N. repanda* cytoplasm causes shortening of the filament and shrivelling of the anther, frequently producing a terminal stigma. In tobacco with *N. debneyi* cytoplasm, the disturbance results in stigmatoid structures on shortened filaments. The corolla is not modified

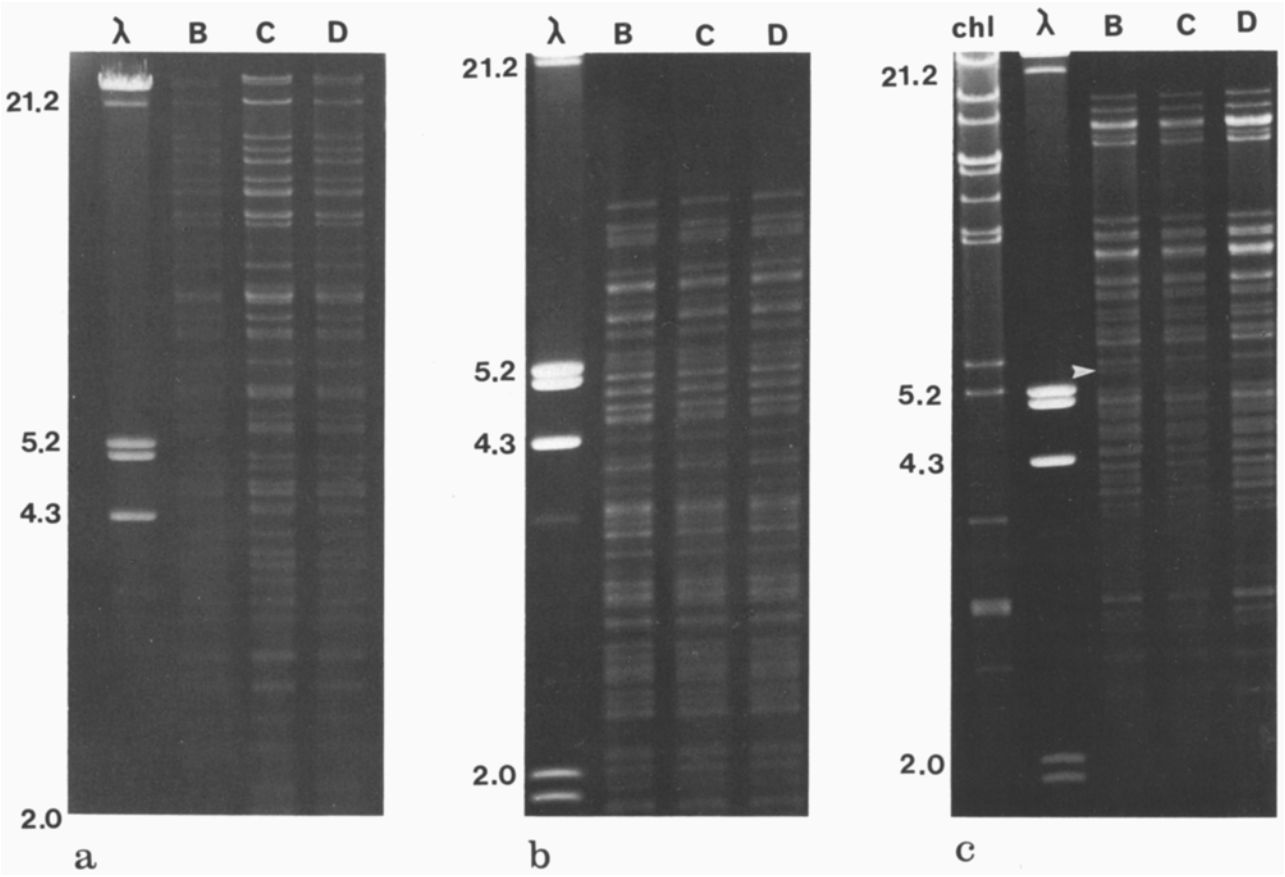

Fig. 2a–c. Restriction pattern of mitochondrial DNA from male fertile and male-sterile lines of *Nicotiana* (see Table 1 for more detailed description of the lines). λ: molecular weight standard lambda-DNA cut with EcoRI/HindIII (kb). **a** Enzyme – XhoI. *B: N. repanda,* MF; *C: N. rep,* CMS; *D: N. rep,* RestMF. **b** Enzyme – EcoRI. *B: N. suaveolens,* MF; *C: N. suav,* CMS; *D: N. suav;* RestMF. **c** Enzyme – XhoI. chl: *N. debneyi* chloroplast DNA; *B: N. debn,* MF; *C: N. debn,* CMS; *D: N. debn,* RestMF

in the CMS material with *N. suaveolens* or *N. repanda* mitochondria, but it is divided into five narrow petals in the CMS material with *N. debneyi* mitochondria.

These differences in the expression of CMS might be due to the involvement of different mitochondrial genes (Rosenberg and Bonnett 1983). However, analysis of the mitochondrial proteins showed that even though several differences can be detected in the proteins synthesized by isolated mitochondria from male-sterile and male-fertile plants, only the enhanced production of a 30 kD protein was exclusively correlated with CMS in the lines carrying mitochondria from *N. repanda* or *N. debneyi.* No protein associated with CMS was detected in the lines with *N. suaveolens* mitochondria. If this 30 kD protein is causally related to male sterility, it results in the manifestation of CMS at different developmental stages depending on the genotype in which it is expressed.

When the *N. tabacum* nucleus is associated with a foreign cytoplasm, as in the CMS lines of sets 1 and 3, the production of the 30 kD protein is greatly enhanced. However, in the male-fertile parental *Nicotiana* lines the 30 kD protein could also be found, although in extremely low amounts. This is in accordance with the results obtained by Bailey-Serres et al. (1986a) investigating alloplasmic CMS lines in sorghum. In sets 1 and 3, incorporation in the male-sterile line of nuclear material from the same species as the CMS-inducing cytoplasm results in suppressed synthesis of the 30 kD polypeptide and restoration of fertility. Similarly, the 13 kD protein associated with CMS-T lines in maize is never fully suppressed in restored plant material (Forde and Leaver 1980).

Gerstel and his associates have demonstrated that restoration of fertility in *Nicotiana* CMS lines caused by *N. repanda, N. debneyi, N. undulata* or *N. bigelovii* cytoplasms is associated with the introduction of new nucleolar organizers that suppress *N. tabacum* nucleolar organizers to a variable extent (Gerstel et al. 1978; Burns et al. 1978; Burns and Gerstel 1981; Gerstel and Burns 1983). However, at this time no conclusion can be made regarding a connection between nucleolar

organizers, production of the 30 kD protein and restoration of male fertility in *Nicotiana* CMS material.

In addition to the 30 kD CMS correlated protein, several other changes in protein pattern exist between lines in the investigated material. Most of these variations in proteins can be explained by the fact that the mitochondria are of different species origin. A 17.5 kD polypeptide in mitochondria of *N. tabacum* origin, estimated to 17 kD by Boutry et al. (1984), is replaced by a protein of 18 kD in *N. debneyi* mitochondria, independent of the origin of the nucleus (B, C and D, set 3). This 18 kD polypeptide has been referred to as a CMS-correlated protein by Charbonnier et al. (1985), but here we show that the polypeptide is also found in the male-fertile and restored lines carrying *N. debneyi* cytoplasm. This protein could be associated with male sterility, but there is no reason to correlate that, or the other polypeptides in the gels, to CMS. Bailey-Serres et al. (1986 b) suggested that even though an altered protein of 42 kD was found in both male-fertile and alloplasmic CMS lines of sorghum carrying the 9E cytoplasm, the absence of nuclear genes compensating for the variant protein could cause CMS in the alloplasmic line.

The expression of the 23 kD protein seen in set 3 (lanes A and C) is not correlated with the disappearance of any other protein band. This shows that the *N. tabacum* nucleus can induce the synthesis of what seems to be a new protein in *N. debneyi* mitochondria, but is readily found in *N. tabacum* mitochondria. Of further interest is the finding that the 23 kD polypeptide is synthesized in the *N. tabacum* variety Kupchunos, but not in the variety SC 58. The introduction of *N. debneyi* nuclear material in the CMS line results in the disappearance of the 23 kD band as well as restoration of fertility (line D). This protein is not, as the 30 kD protein, only associated with the CMS phenotype but can also be found in the male-fertile line A. However, in lines with *N. debneyi* cytoplasm the 23 kD protein is correlated with CMS, suggesting an involvement in manifestation of the male-fertile phenotype, together with the 30 kD protein. Since the CMS line in set 3 has a highly altered flower morphology, the involvement of more than one mitochondrial gene seems logical.

Restriction enzyme patterns of mitochondrial DNA were analyzed for the male fertile (B), CMS (C) and restored (D) lines in all three sets, in order to reveal if nuclear-mitochondrial interaction could cause mitochondrial rearrangements that account for CMS. In set 3 the 5.6 kb XhoI band found in male fertile *N. debneyi* (B, set 3) was seen as a very weak band in the restored line (D) and was hardly detectable in the CMS (C) material. These results agree with the observation that the additional 30 kD band in set 3 was synthesized in relatively high quantities in the CMS line, much less in the restored line and was barely detectable in the male-fertile *N. debneyi* line. Nuclear influence on the organization of the mitochondrial genome could be a possible explanation. The mitochondrial genome in plants is known to consist of a population of different sized molecules (Leaver and Gray 1982; Pring and Lonsdale 1985). Selection for a subpopulation of molecules, as suggested by Borck and Walbot (1982), could explain the differences in abundance of the 5.6 kb XhoI fragment, as well as the two very faint 10 kb and 6 kb bands, in set 3 materials. In sorghum, Bailey-Serres et al. (1986 a) found that the organization of mitochondrial DNA in one sorghum cytoplasm was influenced by the nuclear genotype.

Even though there are differences in the expression of the CMS phenotype between the three sets of *Nicotiana* material studied, as association between two of the CMS types and the synthesis of an additional mitochondrial protein has been revealed. Set 3, involving *N. debneyi* mitochondria, shows a minor alteration in mitochondrial DNA while no rearrangements of mitochondrial DNA was detected in the other two sets of material, even though several restriction enzymes were used. Therefore, the mechanisms by which nuclear-mitochondrial incompatibilities in the genus *Nicotiana* leads to CMS does not appear to rely on the induction of changes in the mitochondrial genome, but rather seems to rely on alterations in the expression of the genome.

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